Advances in Experimental Medicine and Biology 817
Microbial Endocrinology

Mark Lyte
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Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease

Springer

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Volume 817

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
ISBN 978-1-4939-0896-7 ISBN 978-1-4939-0897-4 (eBook)
DOI 10.1007/978-1-4939-0897-4
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2014942372

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Prof. Mark Lyte: "To my loving wife and my two remarkable sons who are my pillars of strength"
Prof. John F. Cryan: "To Colleen, Oisín & Alannah: For constant support and patience"

Foreword

This book is the second volume of a continuing series. The first volume published by Springer in 2010, "Microbial Endocrinology: Interkingdom Signaling in Infectious Disease and Health", contained little in regard to brain and behavior, but instead focused almost exclusively on aspects of infectious disease. Health consequences as such were mainly concerned with the role that stress could play in altering the interface between host and microbiota. The present volume is therefore a testament to the great strides during the intervening years which have illuminated the myriad ways in which microbiota interfaces with the host. It is anticipated that future volumes in this series will reflect the ever increasing acceleration of research into the microbiota–gut–brain axis.

Abilene, TX, USA
January 2014

Mark Lyte
Series Editor

Preface

If one was to ask whether a book dealing with the ability of the microbiota to influence the brain, and ultimately cognition and behavior, would have been possible just a few short years ago, the answer would most likely be no. A simple search of PubMed using the index words "microbiota AND gut AND brain" reveals only 134 publications as of 16th January 2014. However, this would not be an accurate reflection of the work that has been ongoing for many decades, but yet remained on the outer fringes of the disciplines that constitute the study of the mechanisms by which the microbiota and the brain communicate with each other. A comprehensive series of articles by Bested and colleagues [1] catalog the numerous studies going back over a century which amply demonstrate that the investigation of the role of the microbiota in brain function, and by extension mental health, has a long and varied (some may say checkered) scientific history. During this time it remained, for large measure, outside mainstream scientific inquiry following an initial burst of enthusiasm both in the scientific and public arenas at the turn of the twentieth century. That such scientific skepticism remained, and in many cases became entrenched, in the very scientific disciplines that form the basis of the microbiota–gut–brain axis is owed to a number of factors. One of these is surely the increasing specialization that occurred within each discipline over the years and the inherent lack of interdisciplinary thought that accompanied such specialization. With the advent of the concerted research into the microbiota and the microbiome, as best evidenced by the tremendous strides that the Human Microbiome Project has made over the last decade in cataloging the incredible diversity in the microbiota in health and disease, the realization that the microbiota has a role to play in the development and function of the nervous system and hence behavior and cognition, has once again entered into mainstream scientific and medical thought. However, old beliefs die hard. The recent experience of one of us (ML) as described in the prologue to Chap. 1 is but one example of the resistance that is still being encountered today for a role of the microbiota in the functioning of the brain. In many conservative Learned Societies the concept that the gut and indeed the gut microbiota can have such an influence on brain & behavior is still looked upon with incredulity. However, this is changing.

This book represents the realization that any attempt to understand the ability of the microbiota to interface with the brain (and by association any part of the host's neurophysiology) must attempt to address multiple disciplines, such as microbiology, anatomic neuropathology, and endocrinology to name but a few, that while on the first examination appear to be rather disparate from each other but on further examination are in fact highly interconnected as evidenced, for example, by the development of the field of microbial endocrinology itself. As described in Chap. 1, as well as detailed in a chapter in the first book of this series [2], the field of microbial endocrinology developed out of need to understand the paradox in which stress resulted in increased death from a bacterial challenge at the same time greatly increasing the phagocytic activity of the immune system. In considering the microbiota as an interactive player in the host that can both respond to signals from the host and influence the host through the provision of the very same host signaling molecules (i.e., neurochemicals) that are more commonly associated only with vertebrates, but in fact have a long evolutionary history involving the prokaryotes, the potential role of the microbiota in brain functioning and its potential for treatment of mental disorders becomes apparent.

As such, the book is organized along three thematic lines which will provide the reader not only a fuller understanding of the capabilities of the microbiota to interface with the brain and form the microbiota–gut–brain axis, but will also provide detailed examination of the consequences of the microbiota-driven gut-to-brain communication for both health and disease. The first four chapters cover the "Basic Concepts Underlying the Microbiota–Gut–Brain Axis"; the next eight chapters examine the "Mechanistic Factors Influencing the Microbiota–Gut–Brain Axis" and the concluding seven chapters address the "Microbiota–Gut–Brain Axis in Health and Disease".

We have assembled a group of contributors who are recognized to be at the front of their respective fields to review the state of the art of this growing field. As the chapters in this book amply demonstrate, the field of microbiota–gut–brain axis is still in its infancy although its origins are now over a century old. With the advent of modern techniques ranging from deep pyrosequencing of the microbiota to brain imaging, the tools are in place to address those questions which were raised many decades ago. Given our evolving understanding of the complexity of the microbiota which when one couples that to the complexity of the brain and nervous system, this book represents only one more chapter in what promises to be a long and challenging story.

Abilene, TX, USA — Mark Lyte
Cork, Ireland — John F. Cryan
January 2014

References

1. Bested AC, Logan AC, Selhub EM (2013) Intestinal microbiota, probiotics and mental health: from Metchnikoff to modern advances: Part I – autointoxication revisited. Gut Pathog 5(1):5
2. Lyte M (2010) Microbial endocrinology: a personal journey. In: Lyte M, Freestone PPE (eds) Microbial endocrinology: interkingdom signaling in infectious disease and health. Springer, New York, pp 1–16

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Part I
Basic Concepts Underlying the Microbiota-Gut-Brain Axis

Chapter 1
Microbial Endocrinology and the Microbiota-Gut-Brain Axis

Mark Lyte

Abstract Microbial endocrinology is defined as the study of the ability of microorganisms to both produce and recognize neurochemicals that originate either within the microorganisms themselves or within the host they inhabit. As such, microbial endocrinology represents the intersection of the fields of microbiology and neurobiology. The acquisition of neurochemical-based cell-to-cell signaling mechanisms in eukaryotic organisms is believed to have been acquired due to late horizontal gene transfer from prokaryotic microorganisms. When considered in the context of the microbiota's ability to influence host behavior, microbial endocrinology with its theoretical basis rooted in shared neuroendocrine signaling mechanisms provides for testable experiments with which to understand the role of the microbiota in host behavior and as importantly the ability of the host to influence the microbiota through neuroendocrine-based mechanisms.

Abbreviations

CNS	Central nervous system
ENS	Enteric nervous system
GABA	Gamma aminobutyric acid

Prologue

"*If you are right that the bacteria in the gut can communicate with the brain and induce cognitive behavioral changes such as anxiety, then why aren't all the patients we give*

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_1,

antibiotics to in the hospital running around the floors crazy?" NIH Director's Pioneer Award Study Section Member—July, 2008

In July 2008 I found myself as a finalist for the coveted NIH Director's Pioneer Award being asked that very question following my PowerPoint presentation by a study section member in front of not only the other assembled study section members but also the representatives of all the NIH Institutes and the Director's office. Earlier that year I had submitted an application for the Pioneer Award entitled "The Microbial Organ in the Gut" where I proposed that bacteria in the gut were not only able to communicate with the brain and influence behavior, but also that the brain could likewise communicate with the gut bacteria to achieve regulation of microbial populations that would benefit the host. The mechanism by which this bi-directional communication was governed was proposed to be that of microbial endocrinology—the ability of bacteria to respond to as well as produce the same neurohormones found in the host. The study section member's question of why people weren't "running around crazy" was the first one asked following a short presentation to all present. I had anticipated that questions during the 15 min following my presentation would be probing given that from hundreds of applicants for the first round, only 25, including myself, had been selected for a live presentation to a completely new panel of experts at the Lawton Chiles International House on the NIH campus. I also knew that the presentation would meet with some skepticism but hadn't been prepared for the very same study section member spitting water in a veritable geyser after taking a drink and hearing me say, not 2 min into my talk, that bacteria can communicate with the brain and change behavior (the incident was witnessed by all in the room for which I did receive a telephone apology for the member's behavior weeks later from the Director's office). So, the sarcastic, condescending nature of the question came as no surprise. And it was no surprise that my answer (which in many ways forms the basis of this chapter) satisfied neither the member nor the rest of the panel and I did not receive one of the Pioneer Awards that year. But, as they say, times change and science marches on.

Microbial Endocrinology: Conceptual Framework

Microbial endocrinology represents the intersection of two seemingly disparate fields, microbiology and neurobiology. The field of microbial endocrinology was founded in 1993 when the term was first coined by Lyte [1, 2] based on experimental data obtained the prior year [3, 4]. As will be seen in this chapter, although the concept of microbial endocrinology was founded just two decades ago [1, 3–5], there has been published evidence by numerous investigators over the preceding six decades going back to 1930 [6] that demonstrate the validity of uniting the fields of microbiology and neurobiology as a conceptual framework with which to understand interactions between the microbiota and the host in homeostasis and disease.

That these two fields should intersect and play a role in not only infectious disease, but also microbiota-gut-brain communication can be best understood when one considers how the two fields are similar to one another. The presence of neuroendocrine hormones that are exactly the same in structure, as well as share the same biosynthetic pathways, to that found in mammalian systems has been recognized for decades (for review see [7]). Prominent examples include members of the catecholamine family that have been found not only in bacteria [8], but in fish [9], plants [10] and insects [11]. The complete biosynthetic pathway including co-factors for catecholamines, from tyrosine through epinephrine, is found in *Escherichia coli* as well as other bacterial species [12]. Acetylcholine [13], histamine [14], serotonin [15, 16], and even more newly described neurotransmitters such as agmatine [17–19] have all been shown to be produced by microorganisms. The spectrum of neuroactive compounds produced by bacteria that can potentially interact with the host also includes a number of neuropeptides [20]. *That many of the described neurohormones produced by bacteria also function in mammals as part of the neurophysiological system suggests, as will be discussed in the succeeding sections, that their production within the mammalian host can impact the neurophysiological aspects of the host including cognition.*

The ubiquitous presence of neuroendocrine hormones in non-mammalian systems means that the presence of the very same neuroendocrine hormones in mammalian systems has a long evolutionary shared history. Iyer et al. [12] proposed that acquisition of cell-to-cell signaling systems, such as those that characterize neuroendocrine pathways in mammalian systems, are due to late horizontal gene transfer from bacteria. The theory that neurochemical signaling in mammalian cell systems is due to bacterial gene transfer has been bolstered by recent results from the human microbiome project. Riley et al. [21] have shown that such bacterial-mammalian cell lateral gene transfer of bacterial DNA into the human somatic genome occurs via integration of a RNA intermediate and is more common than previously recognized.

In non-mammalian systems the presence of neuroendocrine hormones often serves in a similar capacity to that seen in mammals. For example, tomato plants exposed to various stressors such as cold temperatures can produce large amounts of stress-related catecholamines. As in mammals [22], stress and the production of stress-related hormones such as norepinephrine and epinephrine in tomato plants are also associated with increased susceptibility to infectious agents such as the plant fungal and bacterial pathogens [23, 24]. Interestingly, in response to an infectious insult during periods of stress and increased production of catecholamines tomato plants produce antimicrobial compounds that use as their backbone the complete structure of catecholamines such as norepinephrine and dopamine [23, 24]. Whether evolution has afforded other non-plant-based systems a similar way to deal with stress-induced susceptibility to infectious challenge by constructing antimicrobial compounds based on neurochemical structures has not yet been fully examined.

What is still incompletely understood for the majority of bacteria from which neuroendocrine hormones have been isolated is the simple question of "why". Why

do bacteria produce neuroendocrine hormones? In large part, most reports of neurochemical production by bacteria are mainly descriptive and the "why" aspect is too often left unanswered. However, for some bacterial species which are known to produce certain neurochemicals via the same mechanism found in animals, such as gamma aminobutyric acid (GABA) which utilizes α-decarboxylation of L-glutamic acid catalyzed by glutamate decarboxylase, a reason for its production has been reported. For example, production of GABA can confer resistance to acidic pH for a number of *Lactobacilli* species such as *Lactobacillus reuteri* [25] as well as have a role in the germination of bacterial spores [26]. As an acid-protective mechanism, the GAD system employed by *Lactobacilli* may offer a sound explanation concerning survival of the bacterium following ingestion and subsequent transfer through the acidic conditions within the stomach and into the intestine, but falls short to explain from an evolutionary perspective why *Lactobacilli* that normally reside in the gut should possess the biosynthetic pathway to produce GABA. Nor for the reports that other commensal microbiota such as those belonging to the *Clostridia* also possess the ability to decarboxylate glutamic acid and produce GABA [27]. Can it instead be proposed that the production of GABA by bacteria can also serve as a mechanism by which such bacteria can not only influence the host through interaction with host cell receptors for GABA that can be found in the intestinal tract both in neuronal cells that belong to the enteric nervous system (ENS) [28] as well as immune cells [29], but additionally as a way by which one bacterial species can communicate with another within the microbiota that also possesses receptors for GABA? In fact, the presence of a high affinity receptor for GABA in *Pseudomonas* spp. had formed the basis for the use of a bacterial-based system to quantify nanomolar concentrations of GABA in clinical fluids such as cerebrospinal fluid [30]. The isolation and characterization of the high affinity receptor for GABA in *Pseudomonas* was reported a few years later [31].

The concept that the production of neuroactive chemicals by members of the microbiota can not only serve in the capacity of interacting with the host, but also as a means of signaling among other members of the microbiota, has been proposed [32]. Such neurochemical-signaling mechanisms between members of the microbiota would constitute a type of primitive nervous system and satisfy the requirements contained with any definition of an organ—namely, that the cellular elements which comprise the organ can be influenced, and in turn influence, the host. From a microbial endocrinology-based standpoint the microbiota contained within the gut can therefore be termed as a microbial organ [32].

Origins of Microbial Endocrinology: Evidence from the 1930s to Present

Over the last decade the number of reports which have demonstrated the ability of bacteria to respond to neuroendocrine hormones produced by the host, especially

during times of stress, have steadily increased. The first report that a stress-related neurochemical could influence bacterial growth appeared in the early 1930s due to an unfortunate set of occurrences. Epinephrine (adrenaline) as the first hormone purified to homogeneity was beginning to find increasing use in the clinical arena. One of those uses was for the treatment of urticaria. Reports began to appear almost immediately following its use in the clinic of patients dying from fulminating sepsis within hours after administration of epinephrine [6]. The cause was traced to the glass syringes and metal needles that pre-dated the modern use of disposable syringes and needles [33]. Although glass syringes and needles were cleaned with various agents between patients, it was quickly discovered that such cleaning of a needle and syringe set used to drain infected abscesses of patients with infections such as the spore-forming *Clostridium perfringens* was inadequate. The combination of epinephrine and the very small number of spores or injured bacteria left in the syringe and needle proved to be a dangerous combination. Since all patients who died from epinephrine injections were traced back to syringes and needles that had been used to drain bacterial abscesses it became standard medical practice for decades that a syringe and needle set could not be used for epinephrine injections if it had been recently used to drain a bacterial abscess. Although this association has been largely lost to history, it should be noted that on occasion such associations have proved beneficial for the evaluation of drugs to treat infectious bacteria such as *C. perfringens*. Traub et al. [34] demonstrated that in order to get *C. perfringens* to infect a mouse it was necessary to co-inject fresh, non-oxidized, adrenaline and that by utilizing such a neuroendocrine hormone-based model system one could evaluate the efficacy of antimicrobial candidate drugs to treat gas gangrene infections.

The majority of reports that have dealt with various aspects of neuroendocrine hormone production by bacteria or their recognition of host-produced hormones have done so in the context of infectious disease. This is not surprising given the fact that the first reports of hormones having a role in host health started in the 1930s with the reports of gas gangrene following injection of epinephrine. The first report that described a *direct* interaction of bacteria and neuroendocrine hormones and ascribed a role in infectious disease was the demonstration 60 years later in 1992 that the stress-related neurohormones norepinephrine and dopamine could increase the growth of human intestinal bacterial pathogens by over six orders of magnitude within hours [3, 4]. Importantly, intestinal pathogens which are not commonly associated with extra-intestinal infection, such as *Yersinia enterocolitica*, do not respond to the stress hormone epinephrine. This is a critical observation as it indicates that bacteria may have developed the ability to recognize host hormones based on evolutionary association with specific anatomical regions of the host. Reports, such as Sperandio et al. [35], which have subsequently appeared and suggest that epinephrine plays a key role in the pathogenesis of bacteria within the gut critically have not recognized (or even ignored) the fact that epinephrine does not exist in appreciable amounts within the gastrointestinal tract. This is due to the fact that neurons contained with the enteric nervous system (ENS) that innervates the entire length of the gut do not possess the enzyme

phenylethanolamine-*N*-methyltransferase which is needed for conversion of norepinephrine to epinephrine in the catecholamine biosynthetic pathway [36].

As can be expected, the more one digs into the literature to find instances of where neurochemicals and bacteria have been examined the more one finds papers which provided tantalizing clues that these two systems, one the neurophysiological and the other microbial, could interact in totally unexpected ways. For example, *Campylobacter jejuni* is a highly prevalent food-borne pathogen that requires a microaerophilic environment in the laboratory for its propagation. However, the addition of norepinephrine to the microbiological growth medium was shown by Bowdre et al. [37] to result in tolerance to and growth of *C. jejuni* in an aerobic environment. The mechanisms to account for this have not been elucidated but further highlight the ability of neuroendocrine hormones to affect bacterial physiology. Along these lines, in the succeeding years since the demonstration of catecholamine-induced growth of bacteria and increased production of virulence-associated factors [38, 39], numerous reports have appeared that further document the ability of neuroendocrine hormones, chiefly the catecholamines, to influence bacteria. For example, stress-related hormones have been shown to increase conjugative transfer of antibiotic resistant genes between enteric bacteria thereby contributing to the increased prevalence of antibiotic-resistant food borne bacterial pathogens in the food supply [40]. Additionally, the ability of monoamines such as norepinephrine and dopamine to alter gene expression has now been shown for a number of pathogenic microorganisms including *Mycoplasma hyopneumoniae* [41], *Salmonella enterica* serovar Typhimurium [42] and *Vibrio parahaemolyticus* [43].

Evolution of Current Microbial Endocrinology-Based Perspective of Microbiota-Gut-Brain Axis

Of specific relevance to the current study of the subject of microbiota-gut-brain axis was the dominating scientific view of the time that sought to explain the mechanisms by which stress neurohormones could influence the pathogenesis of infectious disease. Miles and colleagues undertook a series of experiments starting in the late 1940s and continuing into the 1950s in which they co-injected stress hormones with a wide range of bacterial species into animals [33, 44, 45]. Their findings corroborated earlier studies that showed that epinephrine had the ability to increase the growth rate of bacteria, such as *C. perfringens* (referred to in this series of papers by the former name *C. welchii*) and *E. coli*, while decreasing the dose needed to cause infection by up to one-million fold [6, 46]. However, all attempts to identify the involved mechanism(s) had been centered on the host side as it was not conceived that the bacterium itself could be as active a player in the infectious disease process as the host and most critically could utilize the host's own neuroendocrine hormone production during stress to identify where it was and initiate

processes to ensure its own survival. The most prevalent reason given by the researchers during this time to account for the ability of epinephrine to increase bacterial numbers was that it was due to an inhibition of phagocyte migration into the area where the bacteria were actively growing thereby allowing them to grow in an unrestricted manner [33, 44]. However, these researchers had also observed that epinephrine was principally effective during the early stages of infection when bacteria were low in number and that the injection of epinephrine later in the infective process did not appreciably inhibit the response of phagocytic cells. This seeming contradiction was resolved decades later when it was shown that the response of bacteria to catecholamines is highest when bacteria are in low concentration [47, 48] and that as the bacteria increase in density their need for catecholamines decreases at the same time a catecholamine-induced autoinducer of growth is produced [48, 49]. The critical distinction between these two research periods separated by nearly 40 years is the examination of the site of action of neuroendocrine hormones in a biological system containing both prokaryotic and eukaryotic cells, wherein during the former period researchers considered that since neuroendocrine hormones were of mammalian origin they would naturally influence mammalian, and not prokaryotic, cells as part of the infective process. That bacteria were known even at that time to produce neurochemicals such as acetylcholine [13] did not seem to enter into the infectious disease equation. *That there still is today a similar view that two systems*, *host and microbial*, *are separate and distinct as far as behavior can be regarded is best exemplified by the skepticism discussed in this chapter's prologue*.

As already partly discussed, there have been numerous reports since the 1930s regarding the ability of specific bacterial species to produce and/or recognize through specific receptors neuroendocrine hormones many of which are involved in key aspects of neurotransmission. One of the most prominent, GABA, has been extensively described for members of the *Lactobacilli* family as already discussed in a previous section as well as for *Bifidobacteria* [50] and characterization of a high affinity receptor in *Pseudomonas* spp. [31]. In the presence of the same substrates and coenzymes that are found in mammalian cells involved in the production of GABA, bacterial strains isolated from the human gastrointestinal tract have been shown to produce over 20,000 μg ml^{-1} of GABA [50]. Acetylcholine [13], dopamine [8, 51], norepinephrine [8, 51], histamine [14] and even precursors of benzodiazepine ligands [52, 53] are just a few of the examples that can be found in the literature. Roshchina [7] has authored the most extensive review to date regarding the capacity of bacteria to produce a wide panoply of neuroactive compounds. Further, while the interaction of neuroendocrine hormones such as the catecholamines has most often been examined in bacteria, there have been reports which demonstrate the utilization of catecholamines by other microorganisms such as the pathogenic yeast *Cryptococcus neoformans* [54, 55].

In Vivo Veritas

As noted above, the demonstration that the microbiota itself is capable of producing neuroendocrine hormones is the crucial first step in evaluating the feasibility of microbial endocrinology-based mechanisms in gut-to-brain interactions. Although there have been reports which have concluded that increased neurochemicals found in the circulation of the host, for example serotonin [56], are due to the presence of neurochemical secreting bacteria, it has only been very recently that a comprehensive study has conclusively demonstrated the production of physiological levels of neuroendocrine hormones by bacteria within the intestinal lumen. In this study by Asano et al. [51], levels of the catecholamines norepinephrine and dopamine were quantitated in the gastrointestinal lumen in three microbiota-distinct types of mice: specific pathogen-free, germ-free and gnotobiotic mice reconstituted with a mixture of various bacterial species. Appreciable physiological amounts of both catecholamines were only found in specific pathogen-free mice while substantially lower amounts were detected in luminal contents of germ-free animals. Critically, whereas the majority of catecholamines in pathogen-free animals were structurally determined to be free and biologically active, those found in germ-free animals were present in a biologically inactive, conjugated form. Inoculation of germ-free animals with the microbiota from specific pathogen-free mice resulted in the production of free, biologically active, catecholamines within the gut lumen. As such, this report [51] clearly established that in vivo the microbiota is capable of producing neuroendocrine hormones that are commonly only associated with host production. That these substances also are intimately involved in host neurophysiology provides solid evidence that the fields of microbiology and neurophysiology do intersect with attendant consequences for both host and microbiota as further discussed below.

Microbiota and Behavior: Does Microbial Endocrinology Have a Role to Play?

The ability of microbes to influence behavior has been shown in a large number of studies, many of which are discussed in length in other chapters in this book. What is at question, however, is whether the ability of microorganisms to produce neuroactive compounds provide for a mechanism(s) by which such microbial-induced changes in behavior can be accounted for.

In many of the studies which have addressed mechanisms by which microbes can influence behavior they have often concluded that such mechanisms involve to some degree immune system involvement. This is not surprising given that such studies often involve the administration of a microorganism in a manner that nearly guarantees an immune system response. Further, microorganisms are often given in such large doses that do not reflect actual “real-life” scenarios where infective doses

tend to be very low. Following such administration, the development of immune-related sequelae involving the production and release of cytokines and inflammatory mediators result in the interaction with well-characterized neuronal targets both within the central nervous system (CNS) and the ENS [57]. These CNS and ENS targets then communicate to the brain, via vagal afferents for example, and result in altered behavioral responses.

While the sequence of pathogen infection resulting in immune activation that then ultimately results in an alteration of behavior is well recognized, it is perhaps somewhat surprising to learn that increasingly studies are reporting the direct, *non-immune*, *non-infectious*, related ability of microbes to influence behavior. The first study which demonstrated the ability of a bacterium within the gut to influence behavior in the absence of any detectable immune response was shown in a series of studies utilizing *C. jejuni* in mice [58]. Although an important human food-borne pathogen, *C. jejuni* in mice, unlike in humans, does not cause diarrhea. In this series of studies, a low per oral dose of *C. jejuni* was employed to introduce a novel, replicating organism into the microbiota and examine whether this new member could be "seen" by the brain. As reported in this series of studies, *C. jejuni* was able to induce anxiety-like behavior in mice through a vagal-mediated pathway in the absence of any immune activation [59]. Further, it was shown that within hours following the introduction of *C. jejuni* into the microbiota that neuronal activation in specific brain regions occurred as detected by expression of the neuronal activation marker c-Fos. It is therefore evident that a mechanism exists whereby changes in the microbiota can be "seen" by the brain and these changes can result in modification of behavior. To date, the mechanism(s) by which this non-immune mediated neuronal activation within the brain occurs has not been identified and awaits to be explored.

Given that bacteria are prolific producers of neuroendocrine hormones, as well as other neuroactive compounds [20], it would seem reasonable to conclude that such bacterial production of neuroactive compounds within the gut lumen could influence either host-specific neural receptors within the gut or extra-intestinal neuronal sites following luminal uptake into the portal circulation. There are a number of reports that provide support that neurochemical production by bacteria within the gut can influence behavior in both humans and animal model systems [60–62]. Most often, these reports employ probiotic bacteria, such as *Lactobacillus* or *Bifidobacterium*, many of which species belonging to these two genera are prolific producers of neurochemicals for which well-defined neural mechanisms are known by which behavior may be modulated. Of particular interest, Bravo et al. [61] observed reduced anxiety-like and depressive-like behavior in mice fed the probiotic strain *L. rhamnosus* (JB-1). Following probiotic administration they were able to demonstrate changes in the levels of $GABA_{A\alpha 2}$ mRNA in those brain regions associated with the specific behavior [61]. Although they did not quantify the amount of GABA produced by the administered *L. rhamnosus* (JB-1) strain, the demonstration of a mechanism, such as that mediated via central GABA receptor expression, provides evidence that the ability of bacteria to influence behavior can occur through a neurochemical-mediated route.

And as to whether bacteria are capable of producing enough quantities of neurochemicals to affect behavior, a recent study which employed the GABA-producing *Lactobacillus brevis* FPA 3709 amply demonstrates that ability. In this functional food study, *L. brevis* was used to enrich black soybean milk with GABA which was then fed to rats subjected to a forced swim behavioral test [63]. The forced swim test, in which animals are placed in a water-containing glass cylinder and the duration of immobility before the animals begin to swim is measured, is a well-recognized test of depressive-like behavior. In this study, it was shown that GABA-enriched soybean milk significantly reduced the immobility time before rats began to swim and was as effective as the selective serotonin reuptake inhibitor fluoxetine as an antidepressant [63].

Experimental Challenges

While the studies described above do provide tantalizing evidence that microbial endocrinology does indeed play a role in microbiota-gut-brain interactions that ultimately culminate in changes in behavior, a number of experimental challenges have yet to be addressed. To date, substantial direct cause and effect evidence to support such a microbial endocrinology-based mechanism is still lacking. The reasons for this are many-fold and include the only recent development of the necessary analytical tools both on the microbiome as well as neuroimaging sides to examine such interactions. However, the larger reason may be due to the experimental rigor that must be employed to unequivocally demonstrate that it is the actual production of a neurochemical in vivo by a specific microorganism, and not a non-neurochemical aspect of the microorganism such as a cell wall component interacting with immune cells in the gut, that is responsible for a specific change in behavior. Further, receptor specific binding within the gut or extra-intestinal site must be demonstrated for the specific neurochemical produced by the microorganism. These are only two, of a number of requirements that must be fulfilled for one to conclude that a microbial endocrinology-based mechanism can be responsible for a specific change in host behavior. Recently, a step-by-step experimental approach was introduced to guide the experimental design for probiotics which seek to examine such microbial endocrinology-based mechanisms [64]. As shown in Table 1.1, a sequential research plan is proposed which combines in vitro and in vivo methodologies to specifically demonstrate that a specific neurochemical produced in vivo by the microorganism binds to a specific host receptor which ultimately results in an alteration of behavior/cognition in the host. The use of microorganisms that only produce one type of neurochemical is preferred as a number of bacterial strains have been shown to produce more than one neurochemical. For example, production of acetylcholine and GABA by certain *Lactobacillus* has been reported [7]. Other considerations, which are more extensively covered in hypothetical papers addressing the role of the microbiota in nutrition and appetite [65, 66], cover aspects such as ensuring that the diet contains the neurochemical

Table 1.1 Sequential design to evaluate ability of neurochemical-producing probiotics to influence disease pathobiology

Step	Comments
Identify neurochemical of interest to be produced by probiotic based on desired physiological and/or behavioral effect in host.	Physiological and/or behavioral measures should be readily quantifiable. Measures that are receptor-based with known antagonists readily available are preferred as can subsequently be employed at in vivo steps involving animal models.
Screen candidate probiotic in vitro for neurochemical production using robust assay to determine if neurochemical of interest as well as other neurochemicals are produced.	An example of a metabolomics-based screen is given in [64]. More than one microbiological growth medium should be used. Preferably a medium that reflects the gut environment should also be employed.
Define kinetics (i.e. time dependent achievable intra- and extra-cellular concentrations) of neurochemical production.	Identify in vitro growth conditions which result in sustained levels of neurochemical production throughout growth period.
Obtain non-producer mutant (either through in vitro screening or site-directed mutagenesis procedure).	A mutant that does not produce the neurochemical will provide critical control for in vivo experiments.
Conduct time and dose-dependent per oral administration of neurochemical-producing probiotic to normal animals to determine ability of probiotic to produce neurochemical in vivo. Employ vehicle—only animals as control.	Measure levels of neurochemical of interest in intestinal luminal fluid and plasma. Determine time-dependent colonization of gut tissue using quantitative PCR. Perform gross pathology and immunohistopathology of relevant tissue and compare to control (vehicle only) animals.
Perform per oral administration of probiotic in an animal model which involves a neurochemical-responsive element.	Animal models of specific disease pathology or behavior are suitable candidates. Select dosage of neurochemical-secreting probiotic from prior step that is found to result in high and sustainable levels of neurochemical within the gut. If known receptor antagonists are available, give antagonist to block neurochemical-responsive element of disease or behavioral process.
Perform control experiments utilizing per oral administration of mutant (non-neurochemical-secreting) probiotic.	Quantifiable changes in animal model that are obtained by administration of neurochemical-secreting probiotic in above step should not be present (or at lower levels) with mutant strain.

From Lyte M. Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. Bioessays. 2011;33 (8):574–581. Reprinted with permission from John Wiley & Sons.

precursors that are needed as substrates in the synthesis of the specific neurochemical.

The steps outlined in Table 1.1 present a sizable research hurdle to overcome to unequivocally demonstrate the validity of microbial endocrinology-based mechanisms in the microbiota-gut-brain axis. While the research task of identifying

microbial endocrinology-based mechanisms as regards a known neurochemical-producing microorganism is rather straightforward, unequivocally demonstrating such changes within the microbiota itself (i.e. identifying members that may be responsible for such production and the neurochemicals themselves) presents another level of difficulty. Such an approach would undoubtedly involve the use of metabolomics to demonstrate that the microbiota is capable of producing neuroactive compounds during various physiological conditions whether it be stress or changes in diet that may or may not possess the necessary substrates for members of the microbiota to synthesize the candidate neurochemicals.

Differentiation of host versus microbiota produced neurochemicals will, of course, be an essential first step. Recently, Matsumoto et al. [67] have published an elegant study of the mouse intestinal metabolome in which they analyzed the metabolome from the intestine, food and host compartments thereby providing a roadmap by which to differentiate the contribution of each compartment to the overall metabolome within the gut. The finding of neurochemical production exclusively within the microbiota raises the question of which member(s) are responsible. It will be necessary, then, to employ a functional genomics approach to sift through the genomic data obtained from the microbiota community analyzed from the same sample that the metabolome was obtained from to identify those members that possess the genes necessary for the biosynthetic pathways required for the production of the neurochemical of interest. Such a functional metagenomics-based approach has found increasing utility in applications such as human nutrition where elucidating the roles that the human and microbial genomes play in nutrition, starting with microbial biotransformation of food specific to the microbiome of a particular individual and the end products that are produced that impact human physiology for that particular individual, have been examined [68–70]. Once a metagenomics-based approach can identify the bacterial populations that do account for production of a particular neurochemical, it will then be necessary to isolate that population and attempt to culture in vitro to determine if the neurochemical in culture is in fact produced and the relative capacity for production under varying substrate conditions. The design and use of physiologically-relevant intestinal medium complete with the same substrates that are present in the diet of the individual from which the bacterial population was isolated will be crucial to the evaluation. Utilization of standard microbiological medium without requisite concern regarding physiological relevance nor the contribution of food components for substrates and co-enzymes needed for production of a particular neurochemical will most likely lead to non-relevant results. As shown in Fig. 1.1, food itself can contain both the substrates for neurochemicals as well as neurochemicals themselves (such as histamine) and thus plays a critical role in the ability of the microbiota to function in a microbial endocrinology-based manner. Once the identification of the neurochemical-producing population has been achieved, the remaining steps to demonstrate microbial endocrinology-based mechanisms(s) mediating gut-to-brain changes in behavior in the normal host microbiota would then closely follow the steps outlined in Table 1.1.

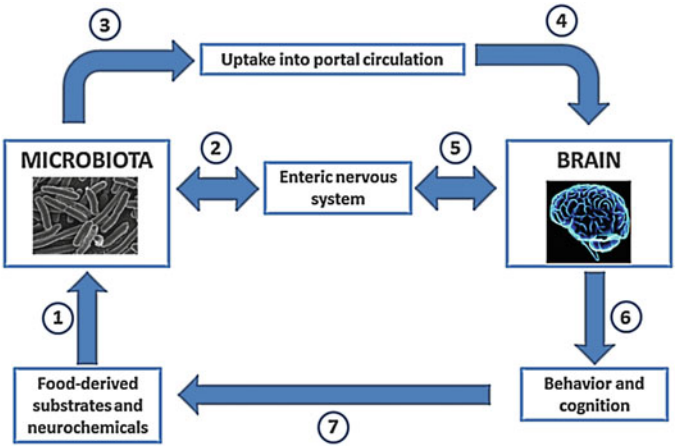

Fig. 1.1 The microbial endocrinology-based pathways by which neuroactive compounds produced by both the host and the microbiota can serve as a mechanism by which the brain and behavior can be modulated within the microbiota-gut-brain axis. Food ingested by the host contains both the substrates needed for neurochemical production by the host and the microbiota as well as fully functional neuroactive components ①. The microbiota in the gut is capable of either forming neurochemicals from the substrates present in the ingested food; or responding to the neuroactive food components themselves; or responding to neurochemicals secreted into the gut by components of the host enteric nervous system ②. Neurochemicals produced by the microbiota in the gut have two pathways by which to influence the host; they can either be taken up from the gut into the portal circulation ③ or they can directly interact with receptors found on components of the enteric nervous system which innervates the complete length of the gastrointestinal tract ②. Once in the portal circulation, microbiota-derived neurochemicals can influence components of the nervous system and ultimately the brain ④. Microbiota-derived neurochemicals can also influence components of the nervous system such as the brain through enteric nervous system-central nervous system communication ⑤. The result of either pathway ④ or ⑤ on the brain may result in an alteration of behavior or cognition ⑥ as well as food preferences and appetite ⑦. As described in the text, this should not be viewed as a one-way direction of only gut-to-brain since the brain may influence the composition of the microbiota through the specific release of neurochemicals into the gut lumen ②. From Lyte M. Microbial endocrinology: Host-microbiota neuroendocrine interactions influencing brain and behavior. Gut Microbes. 2014. Reprinted with permission [87]

Methodological Approach to Examining Putative Neurochemical-Microbiota Interactions

Experimental design employed in both past and present studies involving the examination of neurochemicals and bacteria, whether to investigate the possible production of neuroactive compounds or test the effects of neurochemicals on various aspects of bacterial physiology, have done so in medium that is not reflective of the in vivo environment from which the bacteria were originally isolated. For example, a study by Parr et al. [71] which evaluated the ability of epinephrine to influence the growth of a number of bacterial pathogens responsible

for nosocomial wound infections utilized the standard rich microbiological medium such as Mueller-Hinton and not surprisingly concluded that there was no effect on growth. When one starts from the point that the bacterium can already achieve maximal growth in the test medium, then addition of other substances such as neurochemicals can only affect growth in one direction, negatively. The use of a medium more relative to the tissue environment present in wound infections with its array of inhibiting substances such as transferrin and other specific and non-specific immune components would have been more appropriate. Other studies which have used more relevant medium have in fact shown that *Staphylococci* can dramatically increase their rate of growth in the presence of catecholamines and catecholamine inotropes used clinically in the maintenance of cardiac and kidney function such as dopamine and dobutamine [72, 73]. As such, these findings are more in agreement with the historical clinical-based literature discussed previously on the association of catecholamines and infection.

In designing an experiment to test the ability of a bacterium to produce a putative neuroactive substance in vivo that may then have a role in modulation of host behavior or even immune function within the gut, the use of a medium which incorporates in elements of the diet that the host consumes is essential. It is well recognized (as already discussed) that the exact same biochemical pathway utilized by eukaryotic cells to synthesis neuroactive compounds are also used by a number of microorganisms. For the microbiota then, as well as for the host cells, the substrates that are required are often dietary components. With that said, it is easily recognized that the diets of most individuals can vary greatly thereby confounding efforts to understand what neuroactive compounds may be produced in the microbiota at any given time. Use of laboratory animals with standard diets only offers a modicum of more control since such diets are not routinely analyzed for substrates that may be used in the production of neurochemicals.

Impact of Diet on Determining a Role for Microbial Endocrinology in Gut-to-Brain Communication

As discussed above, the role of diet in the ability of members of the microbiota to produce and respond to neurochemicals is one that is often overlooked in experimental design. Further complicating experimental design in animals such as rodents is the fact that most diets are composed of plant-based materials. Since plants themselves can produce a wide spectrum of neurochemicals as part of their own intercellular communication and known to have neuronal function in animals [74], it should be expected that both substrates for any putative bacterial produced neurochemical within the microbiota may also be contained within the diet itself being of plant origin. According to standard laboratory practice in the use of neurochemicals, this should not be a problem since exposure to air and heating during the preparation of diets and their formation into pellets would result in the

oxidation of most neurochemicals present in the diet. However, that would be an incorrect assumption since neurochemicals found in high concentrations in some plants, such as L-dopa, that are easily rendered inactive by oxidation and heating, are hardly altered following treatment in harsh conditions such as autoclaving as long as they are associated within a food matrix [75]. As discussed above, Matsumoto et al. [67] have provided a technical roadmap by which the chemical composition of the diet or diet metabolome and the metabolome of the lumen can be simultaneously analyzed and those neurochemicals which are specific to the diet versus from the host or microbial origin can be separated out.

As noted above, nutrition plays a significant role in shaping the microbiota [68–70]. One of the first studies which examined the ability of diet-induced changes to influence the microbiota and in turn influence cognition involved the feeding of a meat-based diet to rodents [76]. In this study, mice were fed either standard rodent chow or chow containing 50 % lean ground beef for up to 3 months. Diet-induced changes in the microbiota revealed higher bacterial diversity in the animals which consumed a beef-supplemented diet. Interestingly, assessment of animals for memory and learning using a hole-board open field apparatus demonstrated that increased bacterial diversity in beef-fed animals correlated in a positive manner with improved working and reference memory [76]. While this study provided the first, albeit correlational, data to suggest that the composition of the microbiota may have a role to play in memory and learning, a potential confounder for this study [76], as well as for any other diet-based study, is the presence of nutritive or non-nutritive elements within any diet that may also influence cognition irrespective of any effects on the microbiota. While the two diets in the Li et al. [76] study were balanced for a large number of these factors besides simple calories, it still remains a potential confounder that needs to be recognized and addressed.

Location, Location, Location

In proposing that a microbial endocrinology-based mechanism is involved in the ability of the microbiota to influence behavior the issue of the spatial juxtaposition of the microbiota with the host neuroanatomy presents itself. The innervation by the CNS and the ENS is extensive along the gastrointestinal tract [36, 77]. What is often not fully appreciated it that the ENS does not uniformly innervate the intestinal tract. Anatomical sections of the gut are differentially innervated by CNS and ENS components with direct gut-to-brain neural-based communication dependent on the specific anatomical region of the gut. In a similar fashion the microbiota is also not uniform throughout the length of the gastrointestinal tract and as such it cannot be assumed that one anatomical region of the gut possesses the same capacity to produce neuroactive compounds as another region that even may be immediately adjacent to one another. As such, understanding the location of the neuroactive-producing members of the microbiota in relation to the neuronal elements that can

communicate to the brain will be critically important if the microbiota can be shown to have a role in determining behavior through gut-to-brain communication. For example, could microbiota-induced neuronal activation within the brain resulting in a quantifiable behavior be traced to a specific bacterial species that inhabits a mucus layer immediately adjacent to a specific part of the gut from which sensory information obtained by ENS elements travels to the CNS via extrinsic primary afferent neurons that track along either vagal or spinal afferent routes? Can we distinguish that from bacteria that specifically inhabit the proximal gut instead of the distal gut where communication to the brain in that region occurs instead via the vagus nerve? Use of multiple techniques, such as MALDI-MS image analysis of tissue sections to demonstrate specific production of the neuroactive compounds by the microbiota in specific anatomical regions [78], will be needed to definitively demonstrate that microbial endocrinology-based mechanisms account for the ability of the microbiota to influence behavior.

And, it should be noted that question of location doesn't necessarily diminish in any way the ability of the microbiota to directly interact with extra-intestinal neuronal elements of the CNS (effectively bypassing the ENS) and influence behavior through the direct uptake by the host of microbially-produced neurochemicals within the microbiota into the systemic circulation.

Two-Way Street

The phrase "microbiota-gut-brain axis" is often mistakenly interpreted as a one-way street—that is, communication principally in the direction of gut to brain. While numerous reviews have emphasized the bi-directional nature of gut-to-brain communication [79–83], the consideration of microorganisms as neurochemical producers that also possess cognate high-affinity receptors, means that the microbiota is responsive to signals from the brain to the gut and as such can alter its function and composition in response to host-originated neurochemical signals.

One of the first demonstrations that host derived neuroendocrine hormones could radically alter the composition of the microbiota was the observation that the systemic wide release of catecholamines following the administration of the adrenergic neurotoxin 6-hydroxydopamine resulted in the shifting of the gut bacterial populations from predominantly Gram-positive to Gram-negative with a nearly 7 log-fold increase in numbers of *E. coli* within 24 h following neurotoxin administration [84]. That signaling from the host to the microbiota is a determining factor in the composition of the microbiota was further observed as the adrenergic nerves within the gut re-healed over the ensuing 14 day post-neurotoxin administration, the distribution of Gram-positive and Gram-negative bacteria within the gut returned to the normal pre-neurotoxin distribution [84]. More recent work by Bailey et al. [85] have shown that the application of social stressor altered the bacterial population in the intestine with decreased amounts of bacteria in the genus

Bacteroides while at the same time numbers of bacteria in the genus *Clostridia* increased.

The concept of a two-way street whereby elaboration of neuroendocrine hormones by the host affects the community structure of the microbiota can be applied to the clinical arena. For example, antibiotic associated diarrhea is a well-recognized complication following the administration of wide-spectrum antimicrobials [86]. An unanswered question in gastroenterology is the identity of the mechanism(s) by which the microbiota is able to reconstitute itself following the cessation of antimicrobial treatment to the same community structure that existed prior to the administration of antimicrobials [86]. Can release of neuroactive compounds (hormones, peptides, etc.) by host elements within the gastrointestinal tract, such as enterochromaffin cells which secrete serotonin or release of other substances from neural elements that innervate the villi and crypts along the intestinal wall, specifically stimulate those populations of microbes to grow that are the same populations that are most beneficial to the host? And can this brain-gut-microbiota direction essentially re-populate the gut with the pre-antimicrobial microbial community structure? The long evolutionary symbiosis between host and the microbial inhabitants in the gastrointestinal tract necessitate that the host's nervous system must have developed the means by which to not only monitor, but also influence the composition of the microorganisms within [32]. This recognition of such active monitoring by the host also implies that certain gastrointestinal-related clinical conditions, in which the microbiota is intimately involved such as antibiotic-associated diarrhea, can be viewed anew and hopefully lead to new therapeutic approaches.

Concluding Thoughts: Speculation into the Unknown of the Gut-Microbiota-Brain Axis

The unequivocal demonstration that microbial endocrinology-based mechanisms are prime mediators of microbiota-gut-brain interactions has not yet been achieved. While there are a number of studies which provide indirect evidence that such mechanisms are indeed operative in the ability of the microbiota to influence behavior, mechanisms based on the production of neuroactive compounds by the microbiota will still need to fulfill all the steps outlined in Table 1.1. The highly interactive (and complex) network of interactions with which the microbiota can interface with the host as shown in Fig. 1.1 provides for a number of varied research approaches. With that said the microbiota contains the capacity to both produce and recognize neuroactive compounds that are recognized by most researchers to be solely associated with a mammalian nervous system. Evolution has insured that the microbiota possesses such neuroactive capacity and if the increasing demonstration of the role of such microbial endocrinology-based mechanisms in the pathogenesis

of infectious disease is any indication, a role in microbiota-gut-brain communication will also be demonstrated.

References

1. Lyte M (1993) The role of microbial endocrinology in infectious disease. J Endocrinol 137(3): 343–345
2. Lyte M (2010) Microbial endocrinology: a personal journey. In: Lyte M, Freestone PPE (eds) Microbial endocrinology: interkingdom signaling in infectious disease and health. Springer, New York, pp 1–16
3. Lyte M (1992) The role of catecholamines in gram-negative sepsis. Med Hypotheses 37(4): 255–258
4. Lyte M, Ernst S (1992) Catecholamine induced growth of gram negative bacteria. Life Sci 50 (3):203–212
5. Lyte M (2004) Microbial endocrinology and infectious disease in the 21st century. Trends Microbiol 12(1):14–20
6. Renaud M, Miget A (1930) Role favorisant des perturbations locales causees par l' adrenaline sur le developpement des infections microbiennes. C R Seances Soc Biol Fil 103:1052–1054
7. Roshchina VV (2010) Evolutionary considerations of neurotransmitters in microbial, plant and animal cells. In: Lyte M, Freestone PP (eds) Microbial endocrinology: interkingdom signaling in infectious disease and health. Springer, New York, pp 17–52
8. Tsavkelova EA, Botvinko IV, Kudrin VS, Oleskin AV (2000) Detection of neurotransmitter amines in microorganisms with the use of high-performance liquid chromatography. Dokl Biochem 372(1–6):115–117
9. Guerrero HY, Caceres G, Paiva CL, Marcano D (1990) Hypothalamic and telencephalic catecholamine content in the brain of the teleost fish, *Pygocentrus notatus*, during the annual reproductive cycle. Gen Comp Endocrinol 80:257–263
10. Kulma A, Szopa J (2007) Catecholamines are active compounds in plants. Plant Sci 172(3): 433–440
11. Pitman RM (1971) Transmitter substances in insects: a review. Comp Gen Pharmacol 2: 347–371
12. Iyer LM, Aravind L, Coon SL, Klein DC, Koonin EV (2004) Evolution of cell–cell signaling in animals: did late horizontal gene transfer from bacteria have a role? Trends Genet 20(7): 292–299
13. Stephenson M, Rowatt E (1947) The production of acetylcholine by a strain of *Lactobacillus plantarum*. J Gen Microbiol 1(3):279–298
14. Devalia JL, Grady D, Harmanyeri Y, Tabaqchali S, Davies RJ (1989) Histamine synthesis by respiratory tract micro-organisms: possible role in pathogenicity. J Clin Pathol 42(5):516–522
15. Hsu SC, Johansson KR, Donahue MJ (1986) The bacterial flora of the intestine of *Ascaris suum* and 5-hydroxytryptamine production. J Parasitol 72(4):545–549
16. Shahkolahi AM, Donahue MJ (1993) Bacterial flora, a possible source of serotonin in the intestine of adult female *Ascaris suum*. J Parasitol 79(1):17–22
17. Uzbay TI (2012) The pharmacological importance of agmatine in the brain. Neurosci Biobehav Rev 36(1):502–519
18. Arena ME, Manca de Nadra MC (2001) Biogenic amine production by *Lactobacillus*. J Appl Microbiol 90(2):158–162
19. Gale EF (1940) The production of amines by bacteria: the decarboxylation of amino-acids by strains of Bacterium coli. Biochem J 34(3):392–413

20. Holzer P, Farzi A (2014) Neuropeptides and the microbiota-gut-brain axis. In: Lyte M, Cryan JF (eds) Microbial endocrinology: the microbiota-gut-brain axis in health and disease. Springer, New York (in this volume)
21. Riley DR, Sieber KB, Robinson KM, White JR, Ganesan A, Nourbakhsh S et al (2013) Bacteria-human somatic cell lateral gene transfer is enriched in cancer samples. PLoS Comput Biol 9(6):e1003107
22. Freestone PP, Sandrini SM, Haigh RD, Lyte M (2008) Microbial endocrinology: how stress influences susceptibility to infection. Trends Microbiol 16(2):55–64
23. Von Roepenack-Lahaye E, Newman M, Schornack S, Hammond-Kosack K, Lahaye T, Jones J et al (2003) p-Coumaroylnoradrenaline, a novel plant metabolite implicated in tomato defense against pathogens. J Biol Chem 278(44):43373–43383
24. Zacares L, Lopez-Gresa MP, Fayos J, Primo J, Belles JM, Conejero V (2007) Induction of p-coumaroyldopamine and feruloyldopamine, two novel metabolites, in tomato by the bacterial pathogen *Pseudomonas syringae*. Mol Plant Microbe Interact 20(11):1439–1448
25. Su MS, Schlicht S, Ganzle MG (2011) Contribution of glutamate decarboxylase in *Lactobacillus reuteri* to acid resistance and persistence in sourdough fermentation. Microb Cell Fact 10(Suppl 1):S8
26. Foerster CW, Foerster HF (1973) Glutamic acid decarboxylase in spores of *Bacillus megaterium* and its possible involvement in spore germination. J Bacteriol 114(3):1090–1098
27. Gale EF (1941) Production of amines by bacteria: the decarboxylation of amino-acids by organisms of the groups Clostridium and Proteus With an addendum by G. L. Brown, F. C. MacIntosh and P. Bruce White. Biochem J 35(1–2):66–80
28. Krantis A (2000) GABA in the mammalian enteric nervous system. News Physiol Sci 15: 284–290
29. Bjurstom H, Wang J, Ericsson I, Bengtsson M, Liu Y, Kumar-Mendu S et al (2008) GABA, a natural immunomodulator of T lymphocytes. J Neuroimmunol 205(1–2):44–50
30. Nicholson-Guthrie CS, Guthrie GD, Daly EC, Shuck CS (1995) Determination of gamma-aminobutyric acid levels in human cerebrospinal fluid using *Pseudomonas*. Anal Biochem 225 (2):286–290
31. Guthrie GD, Nicholson-Guthrie CS, Leary HL Jr (2000) A bacterial high-affinity GABA binding protein: isolation and characterization. Biochem Biophys Res Commun 268(1):65–68
32. Lyte M (2010) The microbial organ in the gut as a driver of homeostasis and disease. Med Hypotheses 74(4):634–638
33. Evans DG, Miles AA, Niven JS (1948) The enhancement of bacterial infections by adrenaline. Br J Exp Pathol 29(1):20–39
34. Traub WH, Bauer D, Wolf U (1991) Virulence of clinical and fecal isolates of *Clostridium perfringens* type A for outbred NMRI mice. Chemotherapy 37(6):426–435
35. Sperandio V, Torres AG, Jarvis B, Nataro JP, Kaper JB (2003) Bacteria-host communication: the language of hormones. Proc Natl Acad Sci U S A 100(15):8951–8956
36. Furness JB (2006) The enteric nervous system, Blackwell, Malden, MA, xiii, 274 pp
37. Bowdre JH, Krieg NR, Hoffman PS, Smibert RM (1976) Stimulatory effect of dihydroxyphenyl compounds on the aerotolerance of *Spirillum volutans* and *Campylobacter fetus* subspecies jejuni. Appl Environ Microbiol 31(1):127–133
38. Lyte M, Erickson AK, Arulanandam BP, Frank CD, Crawford MA, Francis DH (1997) Norepinephrine-induced expression of the K99 pilus adhesin of enterotoxigenic *Escherichia coli*. Biochem Biophys Res Commun 232(3):682–686
39. Lyte M, Arulanandam B, Nguyen K, Frank C, Erickson A, Francis D (1997) Norepinephrine induced growth and expression of virulence associated factors in enterotoxigenic and enterohemorrhagic strains of *Escherichia coli*. Adv Exp Med Biol 412:331–339
40. Peterson G, Kumar A, Gart E, Narayanan S (2011) Catecholamines increase conjugative gene transfer between enteric bacteria. Microb Pathog 51(1–2):1–8

41. Oneal MJ, Schafer ER, Madsen ML, Minion FC (2008) Global transcriptional analysis of *Mycoplasma hyopneumoniae* following exposure to norepinephrine. Microbiology 154(Pt 9): 2581–2588
42. Bearson BL, Bearson SM, Uthe JJ, Dowd SE, Houghton JO, Lee I et al (2008) Iron regulated genes of *Salmonella enterica* serovar Typhimurium in response to norepinephrine and the requirement of fepDGC for norepinephrine-enhanced growth. Microbes Infect 10(7):807–816
43. Nakano M, Takahashi A, Sakai Y, Nakaya Y (2007) Modulation of pathogenicity with norepinephrine related to the type III secretion system of *Vibrio parahaemolyticus*. J Infect Dis 195(9):1353–1360
44. Miles AA, Niven JS (1950) The enhancement of infection during shock produced by bacterial toxins and other agents. Br J Exp Pathol 31(1):73–95
45. Miles AA, Miles EM, Burke J (1957) The value and duration of defence reactions of the skin to the primary lodgement of bacteria. Br J Exp Pathol 38(1):79–96
46. Cooper EV (1946) Gas-gangrene following injection of adrenaline. Lancet 1(6396):459–461
47. O'Donnell PM, Aviles H, Lyte M, Sonnenfeld G (2006) Enhancement of in vitro growth of pathogenic bacteria by norepinephrine: importance of inoculum density and role of transferrin. Appl Environ Microbiol 72(7):5097–5099
48. Lyte M, Frank CD, Green BT (1996) Production of an autoinducer of growth by norepinephrine cultured *Escherichia coli* O157:H7. FEMS Microbiol Lett 139(2–3):155–159
49. Freestone PP, Haigh RD, Williams PH, Lyte M (1999) Stimulation of bacterial growth by heat-stable, norepinephrine-induced autoinducers. FEMS Microbiol Lett 172(1):53–60
50. Barrett E, Ross RP, O'Toole PW, Fitzgerald GF, Stanton C (2012) Gamma-aminobutyric acid production by culturable bacteria from the human intestine. J Appl Microbiol 113(2):411–417
51. Asano Y, Hiramoto T, Nishino R, Aiba Y, Kimura T, Yoshihara K et al (2012) Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. Am J Physiol Gastrointest Liver Physiol 303(11):G1288–G1295
52. Yurdaydin C, Walsh TJ, Engler HD, Ha JH, Li Y, Jones EA et al (1995) Gut bacteria provide precursors of benzodiazepine receptor ligands in a rat model of hepatic encephalopathy. Brain Res 679(1):42–48
53. Hu Y, Phelan V, Ntai I, Farnet CM, Zazopoulos E, Bachmann BO (2007) Benzodiazepine biosynthesis in *Streptomyces refuineus*. Chem Biol 14(6):691–701
54. Polacheck I, Platt Y, Aronovitch J (1990) Catecholamines and virulence of *Cryptococcus neoformans*. Infect Immun 58(9):2919–2922
55. Liu L, Wakamatsu K, Ito S, Williamson PR (1999) Catecholamine oxidative products, but not melanin, are produced by *Cryptococcus neoformans* during neuropathogenesis in mice. Infect Immun 67(1):108–112
56. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC et al (2009) Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A 106(10):3698–3703
57. Wood JD (2004) Enteric neuroimmunophysiology and pathophysiology. Gastroenterology 127(2):635–657
58. Lyte M, Varcoe JJ, Bailey MT (1998) Anxiogenic effect of subclinical bacterial infection in mice in the absence of overt immune activation. Physiol Behav 65(1):63–68
59. Goehler LE, Gaykema RPA, Opitz N, Reddaway R, Badr N, Lyte M (2005) Activation in vagal afferents and central autonomic pathways: early responses to intestinal infection with *Campylobacter jejuni*. Brain Behav Immun 19(4):334–344
60. Messaoudi M, Lalonde R, Violle N, Javelot H, Desor D, Nejdi A et al (2011) Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. Br J Nutr 105(5):755–764
61. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG et al (2011) Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 108(38):16050–16055

62. Collins SM, Kassam Z, Bercik P (2013) The adoptive transfer of behavioral phenotype via the intestinal microbiota: experimental evidence and clinical implications. Curr Opin Microbiol 16 (3):240–245
63. Ko CY, Lin HTV, Tsai GJ (2013) Gamma-aminobutyric acid production in black soybean milk by *Lactobacillus brevis* FPA 3709 and the antidepressant effect of the fermented product on a forced swimming rat model. Process Biochem 48(4):559–568
64. Lyte M (2011) Probiotics function mechanistically as delivery vehicles for neuroactive compounds: microbial endocrinology in the design and use of probiotics. Bioessays 33(8): 574–581
65. Lyte M (2013) Microbial endocrinology and nutrition: a perspective on new mechanisms by which diet can influence gut-to-brain communication. PharmaNutrition 1(1):35–39
66. Norris V, Molina F, Gewirtz AT (2013) Hypothesis: bacteria control host appetites. J Bacteriol 195(3):411–416
67. Matsumoto M, Kibe R, Ooga T, Aiba Y, Kurihara S, Sawaki E et al (2012) Impact of intestinal microbiota on intestinal luminal metabolome. Sci Rep 2:233
68. Flint HJ (2012) The impact of nutrition on the human microbiome. Nutr Rev 70(Suppl 1): S10–S13
69. Mischke M, Plosch T (2013) More than just a gut instinct-the potential interplay between a baby's nutrition, its gut microbiome, and the epigenome. Am J Physiol Regul Integr Comp Physiol 304(12):R1065–R1069
70. Kovatcheva-Datchary P, Arora T (2013) Nutrition, the gut microbiome and the metabolic syndrome. Best Pract Res Clin Gastroenterol 27(1):59–72
71. Parr AM, Zoutman DE, Davidson JS (1999) Antimicrobial activity of lidocaine against bacteria associated with nosocomial wound infection. Ann Plast Surg 43(3):239–245
72. Lyte M, Freestone PP, Neal CP, Olson BA, Haigh RD, Bayston R et al (2003) Stimulation of *Staphylococcus epidermidis* growth and biofilm formation by catecholamine inotropes. Lancet 361(9352):130–135
73. Neal CP, Freestone PP, Maggs AF, Haigh RD, Williams PH, Lyte M (2001) Catecholamine inotropes as growth factors for *Staphylococcus epidermidis* and other coagulase-negative *Staphylococci*. FEMS Microbiol Lett 194(2):163–169
74. Brenner ED, Stahlberg R, Mancuso S, Vivanco J, Baluska F, Van Volkenburgh E (2006) Plant neurobiology: an integrated view of plant signaling. Trends Plant Sci 11(8):413–419
75. Vijayakumari K, Siddhuraju P, Janardhanan K (1996) Effect of different post-harvest treatments on antinutritional factors in seeds of the tribal pulse, *Mucuna pruriens* (L.) DC. Int J Food Sci Nutr 47(3):263–272
76. Li W, Dowd SE, Scurlock B, Acosta-Martinez V, Lyte M (2009) Memory and learning behavior in mice is temporally associated with diet-induced alterations in gut bacteria. Physiol Behav 96(4–5):557–567
77. Furness JB (2012) The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol 9(5):286–294
78. Hanrieder J, Ljungdahl A, Andersson M (2012) MALDI imaging mass spectrometry of neuropeptides in Parkinson's disease. J Vis Exp (60)
79. Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 13(10):701–712
80. Al-Asmakh M, Anuar F, Zadjali F, Rafter J, Pettersson S (2012) Gut microbial communities modulating brain development and function. Gut Microbes 3(4):366–373
81. Forsythe P, Kunze WA (2013) Voices from within: gut microbes and the CNS. Cell Mol Life Sci 70(1):55–69
82. Forsythe P, Kunze WA, Bienenstock J (2012) On communication between gut microbes and the brain. Curr Opin Gastroenterol 28(6):557–562
83. Douglas-Escobar M, Elliott E, Neu J (2013) Effect of intestinal microbial ecology on the developing brain. JAMA Pediatr 167(4):374–379

84. Lyte M, Bailey MT (1997) Neuroendocrine-bacterial interactions in a neurotoxin-induced model of trauma. J Surg Res 70(2):195–201
85. Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M (2011) Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain Behav Immun 25(3):397–407
86. McFarland LV (2008) Antibiotic-associated diarrhea: epidemiology, trends and treatment. Future Microbiol 3(5):563–578
87. Lyte M (2014) Microbial endocrinology: host-microbiota neuroendocrine interactions influencing brain and behavior. Gut Microbes 5(3); PMID: 24690573; http://dx.doi.org/10.4161/gmic.28682

Chapter 2
Utilizing "Omics" Tools to Study the Complex Gut Ecosystem

Anthony Fodor

Abstract In a healthy gut, the immune system tolerates a diverse microbial commensal community avoiding inappropriate inflammation responses and minimizing the presence of pathogens. When the balance between host and microbes is disrupted, risk for disease increases. There is mounting evidence that microbial dysbiosis is a substantial risk factor for common gut diseases including IBS, IBD and colorectal cancer. Understanding this dysbiosis is challenging because of the extraordinary complexity of the gut ecosystem and the tremendous variability between healthy individuals in the taxa that make up the human microbiome. Advances in technology, especially sequencing technology, are beginning to allow for a full description of this complexity. In this review, we consider how new "omics" technology can be applied to the study of the gut ecosystem in human and animal models with special consideration given to factors that should be considered in the design of experiments and clinical trials.

Abbreviations

ATP	Adenosine triphosphate
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
FDR	False Discovery Rate
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_2, © Springer New York 2014

OUT	Operational Taxonomic Units
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid

Introduction

In the healthy gut, host immune processes tolerate a diverse commensal population avoiding excessive inflammation responses and minimizing the presence of pathogens. However, there is compelling evidence that microbial dysbiosis—an imbalance in the microbial community—plays a formative role in many diseases of the gut including IBD [1], IBS [2, 3] and colorectal cancer [4, 5]. All gut microbes are acquired from the external environment and for the first 3 years of life the diversity and complexity of the gut microbial community steadily increases [6]. By the 3rd year of life, the microbial community is more stable but numerous studies have repeatedly shown that there is a high degree of individual variation in the microbial community between different people [7–13]. The factors that determine why different people end up with such different microbial communities are poorly understood, although twin studies suggest that host genetics does not exert substantial control over the composition of the microbial community [14].

If we are to understand how host and microbes together produce the full spectrum of health and disease phenotypes, we will need to determine which alleles are represented and expressed in the host, which microbes are present and where in the gut microenvironment the microbes are found and, for both host and microbes, how genes are expressed to produce metabolites within activated pathways. To understand the state of the human and microbial ecosystem in the gut, therefore, requires an accounting of an ecosystem of phenomenal complexity. There are on the order of three billion base pairs in the human genome [15], but there are ~10 times more bacterial cells within the human body than bacterial cells [16] and encoded within the genomes of those microbial cells is likely more than 100 times more distinct genes than are encoded within the human genome [17]. And, of course, only knowing the genome sequence of either host or microbes by itself does not tell us which genes are expressed or where or when or how epigenetic changes to genomes influence pathway structure and function. Within the last decade, there has been explosive growth in "omics" technologies that are allowing us to begin to approach an initial accounting of this tremendous complexity. Development of these technologies have primarily, but not exclusively, been driven by the stunning drop in the cost of DNA sequencing. Only 10 years ago, the cost of sequencing a megabase of DNA was well over $1,000. Today, it is less than $0.10 and there is every reason to think that this greater than exponential drop in cost of sequencing will continue into the future (http://www.genome.gov/sequencingcosts/). Newly armed with ever more affordable sequencing technology, biologists have begun to characterize in detail the complex microbial gut environment. In this

review, we will discuss the technologies that are making this exploration possible together with the experimental and bioinformatics challenges inherent to performing studies that try to link the state of the microbial community to host disease phenotypes.

16S Sequencing Is an Economical Way to Ask “Who Is There” for Both Common and Rare Taxa

For nearly 30 years [18], microbial ecologists have been using sequencing of the 16S rRNA gene to ask which microbes are present in complex microbial environments. The 16S rRNA gene is among the most conserved genes in bacterial genomes. It is especially useful for phylogenetic characterization because it consists of a number of “variable regions”, which tend to be different in different bacteria, separated by “conserved regions”, which tend to be the same across a wide phylogenetic space. The conserved regions can be used to place PCR primers that sequence across the variable regions, yielding a surprisingly informative degree of phylogenetic information from minimal sequencing effort. Before the advent of next-generation sequencing, capillary-based Sanger sequencing was often performed on clone-libraries created from the 16S gene. With a read length on the order of 1,000 basepairs, a paired-end Sanger sequencing strategy could sequence the entire 16S rRNA gene. This approach has been widely utilized and successfully generated descriptions of microbial communities both associated with the human microbiome [19, 20] and external environmental microbial communities such as soil and ocean.

Despite these successes, the cloning approach suffers from several limitations. Because sequences generated from clone libraries are relatively difficult and expensive to generate, studies that characterized microbial communities via sequencing of clone libraries generally could only achieve on the order of 100 16S sequences per sample, and only then with a great deal of expense and effort. Next generation sequencing eliminated the need for the laborious cloning step even as it offered nucleotide base costs that were orders of magnitude cheaper than Sanger sequencing. Next generation sequencing platforms exploit massively parallel chemistry in which numerous sequencing reactions are run at the same time and the results captured with a computer camera. Because many sequencing reactions are run in parallel, next generation sequencing platforms such as Illumina and 454 generate sequences much more quickly than older dye-termination based technologies. In 2005, the year in which the 454 sequencing platform was described in a Nature paper [21], there were ~136,000 16S sequences cataloged in the Ribosomal Database Project (http://rdp.cme.msu.edu/download/posters/ASM2005.pdf). Today, using the Illumina HiSeq platform, we can routinely generate 100 million 16S sequences for a cost of only a few thousand dollars [4, 22, 23].

This ability to generate with next generation sequencing in a single experiment more sequences than had been accumulated world-wide in decades of dye

termination sequencing provides an enormous opportunity to interrogate complex ecosystems, such as the human gut, while maintaining a sensitivity to detect even rare taxa. But it brings with it significant bioinformatics challenges. Some of these challenges involve finding the hard-disk space and network capacity to handle these large volumes of sequence data. Without proper planning for these mundane considerations, it is not uncommon for the initial analysis of metagenomics projects to be severely impacted. There has been considerable recent interest in developing cloud computing capacity to handle these challenges [24] and investigators considering generation of large sequence datasets may wish to explore storing and analyzing their data in the cloud [25].

Bioinformatics challenges can also arise from the short read length inherent to the currently popular next-generation platforms. The early 454 platforms had a read length of only ~100 basepairs [26] and the initial 454 pyrosequencing characterizations of ocean microbial communities therefore utilized this read-length [27, 28]. Recent Illumina platforms, while many orders of magnitude cheaper than 454 sequencing, also have a read length of only 100 basepairs, but 16S sequences of this length can clearly distinguish the microbial community in inflamed and non-inflamed mammalian guts [4] showing the utility of even such short reads. Bioinformatics simulation studies have shown that the information that is available in short reads can be reasonably close to the information available in full length sequences [29], with the V1–V3 and V3–V5 regions of the 16S rRNA considered to be especially appropriate targets given read-lengths of a few hundred base pairs [30, 31] such as are now achievable on 454 and Illumina platforms [26].

The ability to use 16S rRNA sequencing to characterize in-depth the microbial community from cohorts of interest allows for the intersection of phylogeny and traditional hypothesis testing in ways that can yield interesting insights into how the microbial community might impact disease. As an example, a recent study used 454 sequencing of 16S rRNA amplicons to compare the microbial community in punch biopsies taken from 33 subjects with colorectal adenomas and 38 control subjects [12]. In total, slightly more than half a million 16S rRNA sequences with an average length of just over 300 basepairs were generated from these 71 subjects. In order to place the information in these sequences into a phylogenetic context, we can build a tree that shows the relationship of the sequences to one another (Fig. 2.1). Each node of the tree represents a cluster of sequences that have on average 97 % identity to one another. Nodes of the tree that are close to one another have sequences that are more similar while nodes that are further from each other. As we would expect, most of the bacteria that we see in the human gut can be assigned to the phyla Bacteroidetes or Firmicutes, although other phyla, notably Proteobacteria which harbors many known pathogens, are also present. For each taxa in the tree, we can form a null hypothesis that the relative abundance of that taxa is not different in the case and control subjects. *P*-values can be generated for each null hypothesis using a non-parametric Wilcoxon test. In setting thresholds for significance, we must be careful to correct for testing multiple hypotheses. Rather than using a simple-threshold of $p < 0.05$, we instead set a threshold based on a 10 % false discovery rate (FDR), where we expect 10 % of the taxa that we call

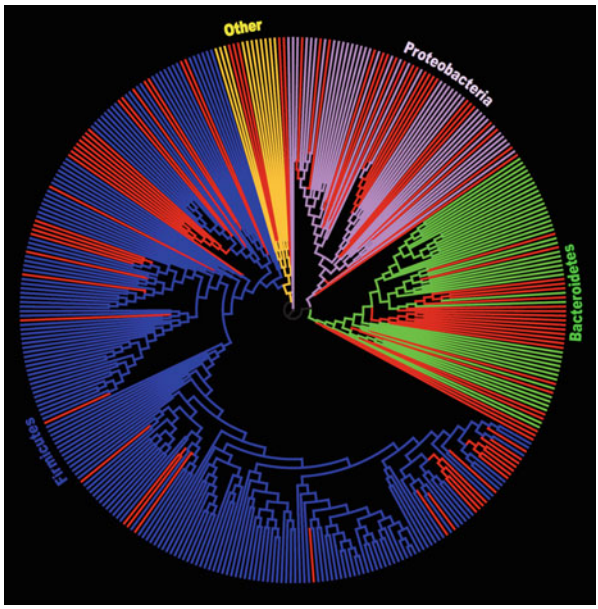

Fig. 2.1 Phylogenetic tree generated from Operational Taxonomic Units (OTUs) representing clusters of sequences with an average 97 % identity from a study of colorectal adenomas in humans [12]. Branches that are colored *red* represent taxa that are significantly different between cases and controls at a 10 % False Discovery Rate (see [12] for methodological details)

significantly different to be false positives. Each taxa that was found to be significantly different between case (adenomas) and control at this threshold is colored red in Fig. 2.1. We see that many of the taxa that are different between case and control are in the phyla Proteobacteria. By creating a visualization that merges phylogeny with canonical hypothesis testing, we are therefore able to begin to implicate specific groups of taxa in disease (see [12] for more information).

Given that early 16S sequencing experiments based on clone libraries could be performed generating less than a hundred reads per sample, it may seem foolish to plan 16S experiments with read depths of over a million sequences per sample. But a simple thought experiment shows that such sequence depths are not inappropriate. Consider *E. coli*, which is a possible driver of colorectal cancer in mouse and human studies [4] but in fecal samples can represent less than 1 % of all sequences collected. On average 100 sequences must be obtained to observe 1 sequence that represents such a rare taxon. If one wishes to study a population with a 1,000-fold range in such a taxon, one must utilize an additional 1,000-fold sequencing depth in order to maintain the full dynamic, quantitative range of sensitivity across people with different relative abundances of the taxon. Finally, in utilizing 454 and Illumina sequences, a barcode method is used in which many samples are put together on the same sequencing run [32, 33]. This procedure can easily introduce a tenfold variation in how many sequences are collected per sample. Putting this together—two orders of magnitude to detect a taxa at average 1 % abundance times three orders of magnitude variation in that taxa between people of different phenotypes times one order of magnitude technical variation in the number of sequences collected per sample—we see that it is not unreasonable to produce and analyze one million 16S sequences per sample.

As technology continues to develop, both read length and read depth will improve allowing for more information to be generated from each sample but also increasing the challenges associated with managing and interpreting so much data. Besides taxonomical considerations, there are many other challenges to the analysis of 16S sequence data including quality assurance steps [34], choosing appropriate clustering algorithms [35] and chimera detection [36]. The setting up of analysis pipelines for 16S sequences has been reviewed elsewhere [37].

Individual Variation Is a Primary Challenge for Studies in the Gut Micobiome

While there are millions of SNP variations between any two non-twin individuals, human genomes have many essential common features that all healthy individuals must share. Every person has to have a working copy of an actin gene, for example, or survival will be impossible. By contrast, the structure of the microbiome does not appear to be essential in the same way. As we will discuss below, mice can be raised in a sterile environment with no gut microbes whatsoever, and while these mice have a great range of phenotypic differences from control mice, they are able to survive [38]. The mammalian gut, therefore, appears to have a certain amount of flexibility with regards to the microbiome. This may explain why, at least at the taxa level as measured by 16S rRNA, a high degree of variability is tolerated in the human microbiome. Perhaps the most dramatic example of microbiome variation was demonstrated by the Human Microbiome Project, which recruited 242 healthy patients and characterized the microbiome by 16S sequencing at 18 distinct body sites [8]. At all the measured body sites, there were tremendous individual differences in this healthy cohort [7, 10]. Moreover, within this cohort, associations between individual taxa and host phenotypes were generally modest. While there were taxa with reasonably strong associations with ethnicity and, as previously observed [39] vaginal pH, associations with phenotypes such as BMI, gender, temperature and blood pressure were moderate at best [10]. It is currently not well understood to what extent the differences in the microbiome associated with ethnicity are driven by genetic or cultural differences, but the possibility of microbial variability produced by ethnicity should be explicitly considered in recruiting cohorts for and powering clinical studies. In general, the modest correlations between healthy human phenotypic variation and microbiome variation suggest that many non-pathological phenotypes are not directly controlled by which taxa are present in the microbiome.

The complexity, individual variation and weak association with phenotypes of the healthy human microbiome represent a substantial challenge for studies that hope to link the state of the microbiome to human phenotypes. If we each have our own unique relationship to the microbiome that defines our own individualized healthy or dysbiotic state, then cross-sectional studies that look across people will

have substantial difficulty in coming to any consistent conclusion. One intriguing idea that has been proposed as a framework to deal with this complexity is enterotypes [40, 41]; it has been argued that much of the complexity of the gut microbiome could be summarized by two or three types of categories dominated by distinct taxa. This hypothesis is enormously appealing as clinical studies could dramatically reduce complexity (and hence improve power) by assigning each participant to one of these pre-defined types before attempting to associate the state of the microbial community to disease phenotypes. Unfortunately, subsequent studies have demonstrated that the presence of enterotypes appears to rely on particular methods of analyzing 16S rRNA data and does not therefore appear to be robust and reproducible in new cohorts [10, 13, 42, 43]. The idea of distinct microbial types likely makes sense for the low-diversity vaginal microbiome [10, 39], but for the more complex gut microenvironment, there appears to be more evidence for a continuum of microbes rather that distinct types.

The variety of gut microbes that will be encountered, and the possibility of only weak associations of taxa with phenotype, must be explicitly considered when powering clinical studies of the human gut microbiome. One approach that may help ease power concerns is to design studies around longitudinal sampling. In a longitudinal sample, each patient in some sense can serve as their own control, which has the potential to reduce variance and hence increase power. Ideally, a longitudinal sampling scheme would recruit a cohort before disease developed and then follow the cohort as some individuals developed disease and others remained healthy. The analysis can then ask both whether the initial state of the microbial community predicted disease and whether changes to the microbial community differ between those who remain healthy and those who develop disease. While this approach is often optimal from the perspective of experimental design, it can be difficult to achieve in practice, especially if the time required to follow a cohort is longer that the length of grant support from funding agencies interested in gut disease.

The Fecal and Mucosal Microbiomes Are Distinct

One great challenge of surveying the gut microbiome, as opposed to more external microbiota such as skin, is that often the microbes that we are most interested in are not the easiest to sample. Fecal samples, obviously, are relatively easy to obtain, but their handling and storage can provide challenging from an operations point of view. Fortunately, it has been demonstrated that issues with how fecal samples are handled, for example how quickly they are frozen, does not appear to have a large effect on the measured 16S community [44]. As an alternative to fecal samples, there has been some interest in utilizing fecal swabs [45], which are easier to collect and store and in the future may be a standard implementation for large clinical studies. No matter how they are collected, however, fecal samples may be inappropriate for studies that evaluate hypotheses regarding the mucosal microbiota.

For example, a recent paper has suggested that microbial DNA may be more present in cancer samples than in non-cancer [46]. Presumably, the microbial invasion that would explain this observation is more likely to occur in the tight contact of host and microbial cells in mucosal material than in the luminal gut. In both human and mouse microbiomes, mucosal and fecal microbiomes generally cluster separately [20] suggesting that there are very distinct luminal and mucosal microbial communities. Obviously, with humans, directly sampling the mucosal microbiota requires an invasive sampling scheme and produces additional IRB requirements, although this collection of internal gut samples can be incorporated into normal colonoscopies. In designing studies, thought should be given to the specific questions being asked and sampling schemes designed accordingly in order to maximize observation of the microbial community most likely to be involved in the phenotype under study.

Mouse Models Have Great Utility but Results Must Be Interpreted with Great Caution

While human association studies are crucial, ultimate evaluation of mechanistic hypotheses about how host-microbe interactions impact disease must be tested in animal models. Because mice can be raised sterile, and then inoculated with a pre-defined microbiome consisting of either cultured [4] or mixed microbial samples [47, 48], gnotobiotic mice allow for testing of hypotheses about how microbes directly cause phenotypes such as cancer [4] or obesity [48]. Despite their power, a number of caveats must be observed when designing and performing mouse microbiome experiments. In particular, once the gavage has been performed, a number of factors not related to the contents of the initial gavage can substantially alter the microbial community. These factors include the cage the mice are housed in [49], the facilities the animals are housed in [50], the amount of time that has elapsed since exposure to microbes [51] and (in animals not raised sterile) the line of maternal transmission [52]. If these factors are not accounted for, they may induce variations in the microbial community that may confound interpretation of experimental design. In a recent study comparing animals gavaged to animals allowed to acquire their microbial community from the environment of the animal facility, it was found that while the initial gavage had an effect on the microbial community, most of the composition of the microbial community was driven by the amount of time that had elapsed since animals were removed from germ-free conditions and the cage in which the animals were kept [53]. Clearly, experimental designs that do not explicitly consider these factors are likely to lead to flawed conclusions and in powering mouse studies, the number of cages, in addition to the number of animals, must be explicitly considered.

Whole-Genome Metagenome Sequencing and RNA-Seq Can Be Used to Interrogate Genome Function

As outlined above, small regions of the 16S rRNA sequence can be surprisingly informative, but there are limits to how much information can be generated by measuring a single gene. The drop in the cost of sequencing has made much more feasible experiments which measure all the genes present in microbial genomes (whole genome metagenome-shotgun sequencing) and experiments which measure microbial transcripts from mixed microbial communities (metagenomic RNA-seq experiments). As is the case for 16S sequencing, initial sequencing effort using Sanger sequencing for whole-genome metagenome experiments required substantial investments of time and expense. An early whole-genome metagenome shotgun sequencing experiment [54] using clone libraries and Sanger sequencing produced ~78 million bases of unique sequence from fecal samples of two human subjects, producing our first look at the genome content of the gut microbiome. Today, through the use of Illumina HiSeq, it is not uncommon to produce ~2 gigabases of sequences per sample, with per sample costs in the hundreds of dollars. As is the case for 16S sequences, therefore, we can now produce in a single experiment more sequences than were produced by multiple labs over years of experiments using Sanger sequencing.

To be of any utility, whole-genome metagenome sequencing generally requires many more sequences per sample than 16S sequencing. This translates both into more expense and a more difficult analysis path. Not only does hard-disk and network capacity need to be found for the large numbers of sequences that will be generated by these methods, but the mapping of individual reads to reference gene databases can require substantial computational times. Investigators wishing to perform whole-genome or RNA-seq on microbial communities must therefore ensure they have adequate computational resources or risk project paralysis in attempting to sift the data once the sequences have been obtained.

Despite the increased overhead and expense of whole-genome sequencing approaches, these experiments can yield great insights into the gut microbial community. An intriguing result from the Human Microbiome Project found that while across body sites and individuals there was great variability in taxonomy (as defined by 16S sequences), if one looks at the fraction of reads assigned to gene functions, they was much more consistency [7]. This result suggests the intriguing hypothesis that while taxa vary substantially in the human microbiome, the gene functions encoded in those taxa are much more constant. Of course, this interpretation of these results is very dependent on the accuracy of functions that are in gene function databases and there has been some question as to how biased these databases may be [55]. Moreover, it is perhaps not surprising that across samples and subjects, the fraction of genes assigned to broad categories such as “ATP synthesis” and “central carbohydrate metabolism” is reasonably constant. It remains an open question how much this high-level consistency is reflected in consistency in specific metabolic pathways. It will be fascinating to watch

resolution of the question as to the best way to biologically interpret gene function annotations as the technologies and approaches that power the study of the human microbiome continue to mature.

If instead of whole-genome sequencing of DNA, RNA is isolated, largely the same informatics pipelines can be used to assign gene functions at the transcript level. Because RNA is much less stable than DNA, these experiments are often more difficult to perform than whole-genome shotgun sequencing, but since message is being measured, rather than just genomic potential for message, the biological insights generated from these experiments can be considerable. In addition to the usual difficulties associated with any RNA preparation, RNA-seq on microbial and metagenomic populations has its own set of challenges. These arise from the fact that unlike eukaryotic mRNA, prokaryotic mRNA does not have a poly-A tail. Message and ribosomal RNA therefore cannot be easily separated by the use of poly-T primers during transcription of cDNA. Strategies that utilize beads that preferentially bind to, or enzymes that preferentially cleave, rRNA have been developed to separate mRNA from rRNA, although these strategies have been found to vary substantially in effectiveness [56]. One strategy that becomes more attractive as sequencing costs drop is to not attempt to separate rRNA from message RNA and simply rely on sequencing depth to characterize the mRNA that may be present in a sample. This strategy has the appeal of simplicity and will also generate a complete rRNA profile, that can itself be useful in taxonomic assignment. Its successful application, however, depends on sequencing being inexpensive enough that sufficient sampling depth can be generated to characterize the small fraction of reads that are message.

For both whole-genome metagenome sequencing and RNA-seq from mixed microbial communities within the human microbiome, there is also the problem of host contamination. The bulk of nucleotides in fecal samples is microbial, but in other tissues the fraction of microbial vs. host DNA and RNA can vary substantially. Again, as sequencing becomes ever cheaper, the strategy of simply applying more sequences and computationally removing human contaminant becomes more attractive, assuming that sufficient computational resources are available to achieve an initial parse of sequence data.

Future Studies Will Integrate Multiple "Omics" Techniques to Generate a Complete Picture of Host and Microbial Pathways

In parallel to the decrease in the cost of nucleotide sequencing, metabolomic and proteomic platforms are continuing to increase in power, robustness and accessibility. In proteomics, a major challenge is identifying spectra and this challenge is only increased in the case of mixed metagenomic communities where the genome sequences that give rise to proteins are not necessarily known [57]. Despite this,

recent efforts have demonstrated not only that proteomics on metagenomics samples is feasible [58] but that the combination of metagenomics and metaproteomics approaches can pinpoint particular host and microbial pathways that are associated with disease [59]. Further integration of these techniques with metabolomics will undoubtedly yield additional insights [60]. The principle challenge of performing these types of studies is the integration of diverse genomics datasets, but this is an area of active research in bioinformatics [61]. We will unquestionably see more and more studies in the future that will combine nucleotide sequencing with proteimic and metabolomic techniques.

While the new world of “omics” and its associated bioinformatics tools are often thought of as the “microscope” through which we can understand the gut ecosystem in all its complexity, the tools of traditional microbiology, having been continuously refined over the last century, are powerful and should not be overlooked. It is often stated that most gut microbes are not cultivable, but a recent study that attempted to systematically cultivate gut microbes from fecal metagenomic samples found that a substantial proportion of microbes that were detectable with 16S sequencing could be cultivated with high-throughput anaerobic techniques [62]. Because these organisms can be introduced into sterile mice, creation of these biobanks of cultivated organisms will allow for explicit testing of hypotheses about which taxa and groups of taxa are associated with disease phenotypes. Moreover, with newly affordable high-throughput sequencing, whole-genome sequences can be easily obtained for these cultivated organisms, which will allow for delineation of which genes and genome regions drive health and disease associations in humans and produce measurable phenotypes in mice. This marriage of classical microbiology with gnotobiotic and sequencing technology will likely prove a powerful tool in the next decade’s attempt to understand how specific pathways are implicated in disease phenotypes.

Conclusion

The gut ecosystem is very complex, but there has been substantial and exciting recent progress in development of genomic and bioinformatics tools that can allow for delineation of that complexity. The initial phase of the Human Microbiome Project focused on utilizing sequencing to characterize variation in healthy adults. As we move into the next phase of the study of the human microbiome, a central focus will be on determining which microbial taxa, genes and pathways are implicated in disease. Careful design of clinical trials and experiments in animal models will be required to overcome the substantial background variation in the gut microbiome and separate confounding variables that are often closely related to the disease categories of interest. A central challenge will be the integration of different types of “omics” data to produce mechanistic descriptions of how host and microbe together produce phenotype.

References

1. Manichanh C, Borruel N, Casellas F, Guarner F (2012) The gut microbiota in IBD. Nat Rev Gastroenterol Hepatol 9:599–608
2. Öhman L, Simrén M (2013) Intestinal microbiota and its role in irritable bowel syndrome (IBS). Curr Gastroenterol Rep 15:1–7
3. Talley NJ, Fodor AA (2011) Bugs, stool, and the irritable bowel syndrome: too much is as bad as too little? Gastroenterology 141:1555–1559
4. Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM et al (2012) Intestinal inflammation targets cancer-inducing activity of the microbiota. Science 338:120–123
5. Zhu Q, Gao R, Wu W, Qin H (2013) The role of gut microbiota in the pathogenesis of colorectal cancer. Tumour Biol 34:1285–1300
6. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG et al (2012) Human gut microbiome viewed across age and geography. Nature 486:222–227
7. Human Microbiome Project Consortium (2012) Structure, function and diversity of the healthy human microbiome. Nature 486:207–214
8. Human Microbiome Project Consortium (2012) A framework for human microbiome research. Nature 486:215–221
9. Spencer MD, Hamp TJ, Reid RW, Fischer LM, Zeisel SH et al (2011) Association between composition of the human gastrointestinal microbiome and development of fatty liver with choline deficiency. Gastroenterology 140:976–986
10. Huse SM, Ye Y, Zhou Y, Fodor AA (2012) A core human microbiome as viewed through 16S rRNA sequence clusters. PLoS One 7:e34242
11. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R (2012) Diversity, stability and resilience of the human gut microbiota. Nature 489:220–230
12. Sanapareddy N, Legge RM, Jovov B, McCoy A, Burcal L et al (2012) Increased rectal microbial richness is associated with the presence of colorectal adenomas in humans. ISME J 6:1858–1868
13. Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM et al (2012) Gut microbiota composition correlates with diet and health in the elderly. Nature 488:178–184
14. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A et al (2009) A core gut microbiome in obese and lean twins. Nature 457:480–484
15. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ et al (2001) The sequence of the human genome. Science 291:1304–1351
16. Savage DC (1977) Microbial ecology of the gastrointestinal tract. Annu Rev Microbiol 31:107–133
17. Yang X, Xie L, Li Y, Wei C (2009) More than 9,000,000 unique genes in human gut bacterial community: estimating gene numbers inside a human body. PLoS One 4:e6074
18. Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML et al (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci 82:6955–6959
19. Hayashi H, Sakamoto M, Benno Y (2002) Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. Microbiol Immunol 46:535–548
20. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L et al (2005) Diversity of the human intestinal microbial flora. Science 308:1635–1638
21. Margulies M, Egholm M, Altman WE, Attiya S, Bader JS et al (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437:376–380
22. Bartram AK, Lynch MDJ, Stearns JC, Moreno-Hagelsieb G, Neufeld JD (2011) Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. Appl Environ Microbiol 77:3846–3852
23. Gloor GB, Hummelen R, Macklaim JM, Dickson RJ, Fernandes AD et al (2010) Microbiome profiling by Illumina sequencing of combinatorial sequence-tagged PCR products. PLoS One 5:e15406

24. Schatz MC, Langmead B, Salzberg SL (2010) Cloud computing and the DNA data race. Nat Biotechnol 28:691–693
25. Dai L, Gao X, Guo Y, Xiao J, Zhang Z (2012) Bioinformatics clouds for big data manipulation. Biol Direct 7:43
26. Glenn TC (2011) Field guide to next-generation DNA sequencers. Mol Ecol Resour 11:759–769
27. Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM et al (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". Proc Natl Acad Sci 103:12115–12120
28. Huber JA, Mark Welch DB, Morrison HG, Huse SM, Neal PR et al (2007) Microbial population structures in the deep marine biosphere. Science 318:97–100
29. Liu Z, DeSantis TZ, Andersen GL, Knight R (2008) Accurate taxonomy assignments from 16S rRNA sequences produced by highly parallel pyrosequencers. Nucleic Acids Res 36:e120
30. Kim M, Morrison M, Yu Z (2011) Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. J Microbiol Methods 84:81–87
31. Hamp TJ, Jones WJ, Fodor AA (2009) Effects of experimental choices and analysis noise on surveys of the "Rare Biosphere". Appl Environ Microbiol 75:3263–3270
32. Parameswaran P, Jalili R, Tao L, Shokralla S, Gharizadeh B et al (2007) A pyrosequencing-tailored nucleotide barcode design unveils opportunities for large-scale sample multiplexing. Nucleic Acids Res 35:e130
33. Hamady M, Walker JJ, Harris JK, Gold NJ, Knight R (2008) Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex. Nat Methods 5:235–237
34. Huse S, Huber J, Morrison H, Sogin M, Welch D (2007) Accuracy and quality of massively parallel DNA pyrosequencing. Genome Biol 8:R143
35. Huse SM, Welch DM, Morrison HG, Sogin ML (2010) Ironing out the wrinkles in the rare biosphere through improved OTU clustering. Environ Microbiol 12:1889–1898
36. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200
37. McCafferty J, Fodor A (2013) Human microbiome analysis via the 16s rRNA gene. In: Bishop OT (ed) Bioinformatics and data analysis in microbiology. Caister Academic Press, Norfolk
38. Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI (2007) Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci 104:979–984
39. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK et al (2010) Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A 108(Suppl 1):4680–4687
40. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T et al (2011) Enterotypes of the human gut microbiome. Nature 473:174–180
41. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y et al (2011) Linking long-term dietary patterns with gut microbial enterotypes. Science 334:105–108
42. Jeffery IB, Claesson MJ, O'Toole PW, Shanahan F (2012) Categorization of the gut microbiota: enterotypes or gradients? Nat Rev Microbiol 10:591–592
43. Koren O, Knights D, Gonzalez A, Waldron L, Segata N et al (2013) A guide to enterotypes across the human body: meta-analysis of microbial community structures in human microbiome datasets. PLoS Comput Biol 9:e1002863
44. Wu G, Lewis J, Hoffmann C, Chen Y-Y, Knight R et al (2010) Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiol 10:206
45. Lautenbach E, Harris AD, Perencevich EN, Nachamkin I, Tolomeo P et al (2005) Test characteristics of perirectal and rectal swab compared to stool sample for detection of fluoroquinolone-resistant *Escherichia coli* in the gastrointestinal tract. Antimicrob Agents Chemother 49:798–800
46. Riley DR, Sieber KB, Robinson KM, White JR, Ganesan A et al (2013) Bacteria-human somatic cell lateral gene transfer is enriched in cancer samples. PLoS Comput Biol 9:e1003107
47. Faith JJ, McNulty NP, Rey FE, Gordon JI (2011) Predicting a human gut microbiota's response to diet in gnotobiotic mice. Science 333:101–104

48. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER et al (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444:1027–1131
49. Hildebrand F, Nguyen TLA, Brinkman B, Yunta R, Cauwe B et al (2013) Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome Biol 14:R4
50. Ma BW, Bokulich NA, Castillo PA, Kananurak A, Underwood MA et al (2012) Routine habitat change: a source of unrecognized transient alteration of intestinal microbiota in laboratory mice. PLoS One 7:e47416
51. Gillilland MG, Erb-Downward JR, Bassis CM, Shen MC, Toews GB et al (2012) Ecological succession of bacterial communities during conventionalization of germ-free mice. Appl Environ Microbiol 78:2359–2366
52. Ubeda C, Lipuma L, Gobourne A, Viale A, Leiner I et al (2012) Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. J Exp Med 209:1445–1456
53. McCafferty J, Marcus M, Gharaibeh RZ, Arthur JC, Perez-Chanona E et al (2013) Stochastic changes over time and not founder effects drive cage effects in microbial community assembly in a mouse model. ISME J 7:2116–2125
54. Gill SR, Pop M, DeBoy RT, Eckburg PB, Turnbaugh PJ et al (2006) Metagenomic analysis of the human distal gut microbiome. Science 312:1355–1359
55. Schnoes AM, Ream DC, Thorman AW, Babbitt PC, Friedberg I (2013) Biases in the experimental annotations of protein function and their effect on our understanding of protein function space. PLoS Comput Biol 9:e1003063
56. Giannoukos G, Ciulla D, Huang K, Haas B, Izard J et al (2012) Efficient and robust RNA-seq process for cultured bacteria and complex community transcriptomes. Genome Biol 13:r23
57. Cantarel BL, Erickson AR, VerBerkmoes NC, Erickson BK, Carey PA et al (2011) Strategies for metagenomic-guided whole-community proteomics of complex microbial environments. PLoS One 6:e27173
58. Verberkmoes NC, Russell AL, Shah M, Godzik A, Rosenquist M et al (2008) Shotgun metaproteomics of the human distal gut microbiota. ISME J 3:179–189
59. Erickson AR, Cantarel BL, Lamendella R, Darzi Y, Mongodin EF et al (2012) Integrated metagenomics/metaproteomics reveals human host-microbiota signatures of Crohn's disease. PLoS One 7:e49138
60. Turnbaugh PJ, Gordon JI (2008) An invitation to the marriage of metagenomics and metabolomics. Cell 134:708–713
61. Joyce AR, Palsson BO (2006) The model organism as a system: integrating 'omics' data sets. Nat Rev Mol Cell Biol 7:198–210
62. Goodman AL, Kallstrom G, Faith JJ, Reyes A, Moore A et al (2011) Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. Proc Natl Acad Sci U S A 108:6252–6257

Chapter 3
The Enteric Nervous System and Gastrointestinal Innervation: Integrated Local and Central Control

John B. Furness, Brid P. Callaghan, Leni R. Rivera, and Hyun-Jung Cho

Abstract The digestive system is innervated through its connections with the central nervous system (CNS) and by the enteric nervous system (ENS) within the wall of the gastrointestinal tract. The ENS works in concert with CNS reflex and command centers and with neural pathways that pass through sympathetic ganglia to control digestive function. There is bidirectional information flow between the ENS and CNS and between the ENS and sympathetic prevertebral ganglia.

The ENS in human contains 200–600 million neurons, distributed in many thousands of small ganglia, the great majority of which are found in two plexuses, the myenteric and submucosal plexuses. The myenteric plexus forms a continuous network that extends from the upper esophagus to the internal anal sphincter. Submucosal ganglia and connecting fiber bundles form plexuses in the small and large intestines, but not in the stomach and esophagus. The connections between the ENS and CNS are carried by the vagus and pelvic nerves and sympathetic pathways. Neurons also project from the ENS to prevertebral ganglia, the gallbladder, pancreas and trachea.

The relative roles of the ENS and CNS differ considerably along the digestive tract. Movements of the striated muscle esophagus are determined by neural pattern generators in the CNS. Likewise the CNS has a major role in monitoring the state of the stomach and, in turn, controlling its contractile activity and acid secretion, through vago-vagal reflexes. In contrast, the ENS in the small intestine and colon contains full reflex circuits, including sensory neurons, interneurons and several classes of motor neuron, through which muscle activity, transmucosal fluid fluxes, local blood flow and other functions are controlled. The CNS has control of defecation, via the defecation centers in the lumbosacral spinal cord. The importance of the ENS is emphasized by the life-threatening effects of some ENS neuropathies. By contrast, removal of vagal or sympathetic connections with the

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_3, © Springer New York 2014

gastrointestinal tract has minor effects on GI function. Voluntary control of defecation is exerted through pelvic connections, but cutting these connections is not life-threatening and other functions are little affected.

Abbreviations

5HT	5-Hydroxytryptamine
ATP	Adenosine triphosphate
CA	Cervical afferents
CGRP	Calcitonin gene related peptide
CM	Circular muscle
CNS	Central nervous system
DRG	Dorsal root ganglia
EEC cell	Enteroendocrine cell
ENS	Enteric nervous system
EPSP	Excitatory Postsynaptic Potential
GALT	Gut associated lymphoid tissue
GEP	Gastroenteropancreatic
GLP-2	Glucagon-like peptide 2
ICCs	Interstitial cells of Cajal
IF	Intestinofugal neurons
IGLEs	Intraganglionic laminar endings
IMAs	Intramuscular arrays
IPANs	Intrinsic Sensory Neurons (or intrinsic primary afferent neurons)
LES	Lower esophageal sphincter
LM	Longitudinal muscle
MMC	Migrating myoelectric complexes
MP	Myenteric plexus
Muc	Mucosa
NHMRC	National Health and Medical Research Council of Australia
NO	Nitric oxide
NPY	Neuropeptide Y
PVG	Prevertebral ganglia
SCG	Sympathetic chain ganglia
SGLT	Sodium/glucose linked transporter
SMP	Submucosal plexus
TRH	Thyrotropin-releasing hormone
TRPV1	Transient receptor potential cation channel subfamily V member 1
VIP	Vasoactive Intestinal Peptide
VMR	Visceromotor Reflex

Introduction

The innervation of the digestive tract is involved in determining the patterns of its movements, in the control of gastric acid secretion, in regulating movement of fluid between the gut lumen and body fluid compartments, in changing local blood flow, in release of gut hormones, in modifying nutrient handling and interacting with the gut immune system.

The gastrointestinal tract differs from all other peripheral organs in that it has an extensive intrinsic nervous system, the enteric nervous system (ENS), that can control functions of the small and large intestines even when they are completely separated from the central nervous system (CNS). But in reality the ENS is not autonomous. The neuronal control of gastrointestinal function is an integrated system in which local enteric reflexes, reflexes that pass through sympathetic ganglia, reflexes that pass from the gut and back through the CNS and central control systems interact (Fig. 3.1).

This review is confined to discussion of monogastric mammals, in which most investigations have been done and which are arguably most relevant to human.

The Extrinsic Innervation of the Gastrointestinal Tract

Connections between the gut and the central nervous system can be conveniently classified as vagal, spinal thoracolumbar and spinal lumbosacral. Each of these includes afferent (sensory) innervation and efferent (motor innervation). The efferent pathways contain pre-enteric neurons that end within enteric ganglia and control or modify the activities of enteric neurons. Pathways from the CNS also contain neurons that directly innervate a restricted number of gastrointestinal effectors, such as striated muscle of the esophagus (vagal innervation), sphincters (sympathetic innervation) and intrinsic blood vessels (also sympathetic innervation).

Vagal Innervation

The human abdominal vagus contains about 40,000–50,000 axons [1]. These fibers provide a sensory innervation and efferent (motor) control pathways for the upper gastrointestinal tract and digestive organs (Fig. 3.1). The afferents include mucosal mechanoreceptors, chemoreceptors and tension receptors in the esophagus, stomach and proximal small intestine, and sensory endings in the liver and pancreas. There is a less prominent vagal afferent innervation of the distal small intestine and proximal colon. Sensory information concerning luminal contents is detected by EEC cells which release hormones that act on vagal afferent nerve endings [2]. This indirect chemoreceptor activation is important for the detection of nutrients and

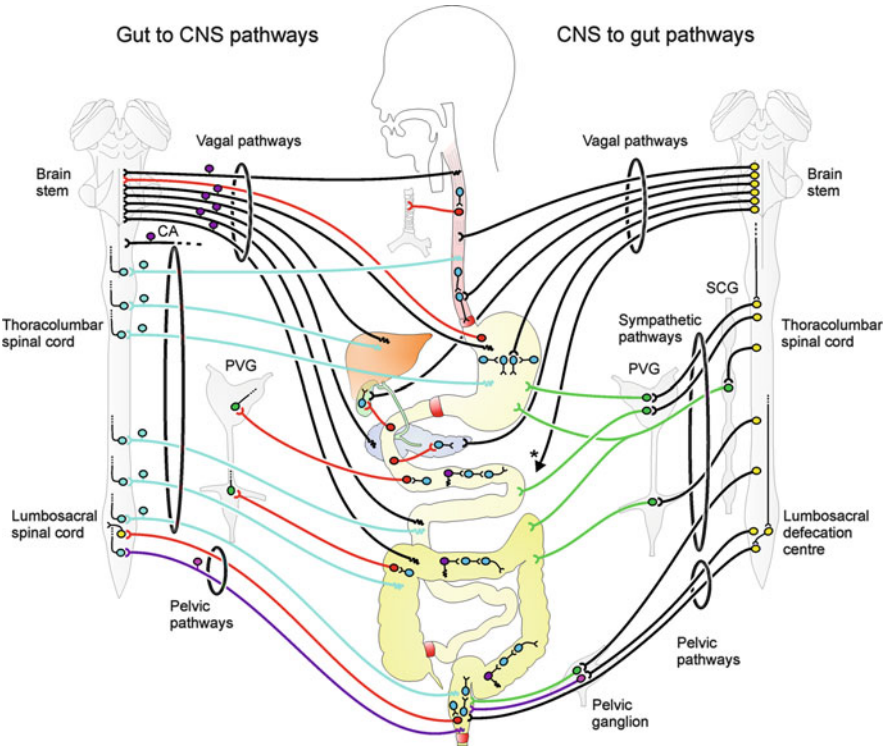

Fig. 3.1 The innervation of the gastrointestinal tract. The neural connections between the enteric nervous system (ENS), the central nervous system (CNS) and sympathetic ganglia, and neural connections between gastrointestinal organs are illustrated. Connections from the ENS to other organs and the CNS are at the *left*, and connections from the CNS are at the *right*. The small and large intestines (*middle* of figure) contain full ENS reflex circuits (motor neurons and interneurons in *blue*, sensory neurons in *purple*). Pathways from the gastrointestinal tract (*left*) project outwards, via intestinofugal neurons (*red*), to the CNS, sympathetic ganglia, gallbladder, pancreas and trachea. Some neurons in sympathetic prevertebral ganglia (PVG, *green* neurons) receive both CNS and ENS inputs. Sensory information goes both to the ENS, via intrinsic primary afferent (sensory) neurons (*purple*) and to the CNS via extrinsic primary afferent neurons (*left* of figure) that follow spinal and vagal nerve connections. Cervical afferents (CA) connect the esophagus to the cervical spinal cord. Pathways from the CNS reach the ENS and gastrointestinal effector tissues through vagal, sympathetic and pelvic pathways (*right* of figure). Vagal medullary and pelvic spinal outflows include pre-enteric neurons (ending in enteric ganglia) and most gut-projecting sympathetic neurons with cell bodies in PVG are also pre-enteric neurons. *SCG* sympathetic chain ganglia

also potentially noxious agents in the gut contents [3]. The functions that are regulated by the vagal sensory innervation include appetite and satiety, esophageal propulsion, gastric volume, contractile activity and acid secretion, contraction of the gallbladder and secretion of pancreatic enzymes.

Structural and Functional Characteristics of Vagal Afferent Pathways

Three distinct types of vagal afferent ending occur in the gastrointestinal tract, intraganglionic laminar endings (IGLEs), intramuscular arrays (IMAs) and mucosal varicose nerve endings (Fig. 3.2) [4]. IGLEs are complex branching nerve endings that give rise to flat (laminar) expansions within myenteric ganglia. They were originally described in the esophagus and shown to be of vagal origin [5], and were subsequently demonstrated throughout the gastrointestinal tract [6]. IGLEs in the rectum, and some of those in the distal colon, arise from pelvic nerves [7]. IGLEs that were identified by anterograde filling responded promptly to probing with a von Frey hair [8]. Firing rates diminished within the first 2–3 s, but were maintained above the background level for the duration of the stimulus, thus these are partially adapting mechanoreceptors. IGLEs that responded to direct probing also responded to stretching the stomach wall, which provides a direct proof that IGLEs are stretch receptors [7, 8]. They almost certainly correspond to the low threshold tension receptors that have been known for a long time, and, in the case of the stomach, probably signal filling [9, 10].

IMAs are formed by single afferent axons that branch within the circular muscle layer to form arrays of varicose fibers that run parallel to muscle bundles [11]. They form synapse-like complexes with interstitial cells of Cajal (ICCs) and it has been suggested that IMAs, ICCs and smooth muscle work cooperatively or synergistically to transduce specific stretch or muscle length information [12]. Close approaches of IMAs to ICC include lamella structures, which have some similarities to the lamellae of IGLEs [12].

Three types of vagal mucosal afferent have been identified: gastric mucosal afferent endings, afferents supplying villi in the small intestine (villus afferents) and afferents supplying intestinal crypts (crypt afferents) [13]. The axons of gastric mucosal afferents branch extensively in the mucosa to provide an innervation that lies close beneath the epithelium; there are commonly flattened structures (lamellae) near the endings of these branches [13]. These are reminiscent of the mechanosensitive lamellae of IGLEs and IMAs. Gastric mucosal receptors are responsive to low intensity stroking of the mucosa, but not to muscle stretch or contraction, and are also sensitive to chemical stimuli, such as acid in the lumen [14–16]. Solid food is titurated in the stomach into smaller particles that are able to pass through the pylorus [17]. Experiments in which the antral mucosa was separated from the underlying muscle, a procedure that abolishes vago-vagal reflexes, suggest that mucosal mechanoreceptors may discriminate particles by size and regulate their passage into the duodenum [18]. Mucosal afferents may also be involved in the control of satiety, as their mechanosensitivity is enhanced by the satiety hormone, leptin, and reduced by the feeding hormone, ghrelin, both of which are released from gastric enteroendocrine cells that are in close proximity to the gastric mucosal afferent endings [19, 20]. In humans, ghrelin signalling to hypothalamic feeding centers is via the vagus [21].

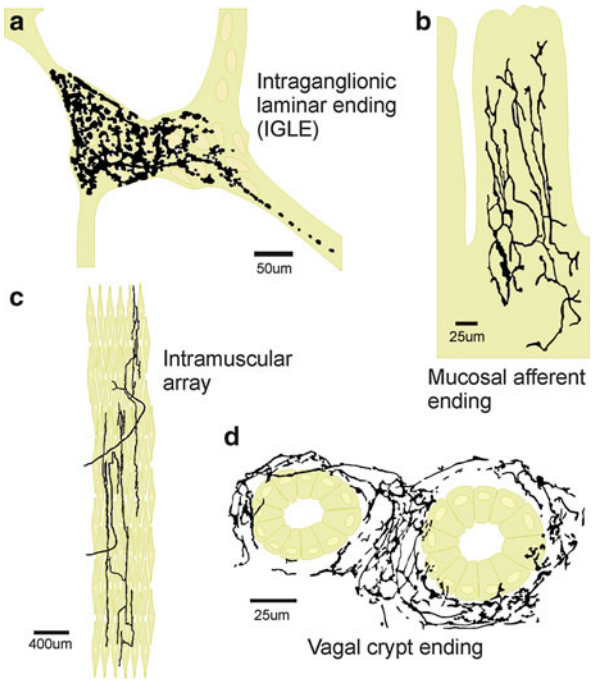

Fig. 3.2 Sensory nerve endings in the gastrointestinal tract. Different types of sensory endings in the intestine: (**a**) intraganglionic laminar endings (IGLEs); (**b**) mucosal varicose nerve endings that supply the villi; (**c**) intramuscular arrays (IMAs); (**d**) sensory endings around crypts in the small intestine. IGLEs branch extensively and provide laminar endings on the surfaces of myenteric ganglia (*green*). Perivascular sensory endings are not illustrated. (**a**) From Castelucci P, Robbins HL, Furness JB. $P2X_2$ purine receptor immunoreactivity of intraganglionic laminar endings in the mouse gastrointestinal tract. Cell Tissue Res. 2003;312:167–174 [169]. Reprinted with permission from Springer Science + Business Media. (**b**) From Powley TL, Spaulding RA, Haglof SA. Vagal afferent innervation of the proximal gastrointestinal tract mucosa: Chemoreceptor and mechanoreceptor architecture. J Comp Neurol. 2011;519:644–660. Reprinted with permission from John Wiley and Sons. (**c**) From Berthoud HR, Kressel M, Raybould HE, Neuhuber WL. Vagal sensors in the rat duodenal mucosa: distribution and structure as revealed by in vivo DiI tracing. Anat Embryol. 1995;191:203–212 [170]. Reprinted with permission from Springer-Verlag. (**d**) From Berthoud HR, Powley TL. Vagal afferent innervation of the rat Fundic stomach: morphological characterization of the gastric tension receptor. J Comp Neurol. 1992;319:261–276. Reprinted with permission from John Wiley and Sons

Separate villus and crypt afferents innervate the mucosa of the small intestine [13]. Villus afferents have axons that project toward the villus tip, where they branch extensively. The branches have irregular flat expansions that tend to be close to the internal surface of the villus epithelium. Each villus afferent fiber typically innervates a cluster of two or more neighboring villi. The villus afferents are ideally positioned to detect substances released from the epithelium, including local hormones such as CCK and 5HT that are known to activate vagal nerve endings [2, 3]. The crypt afferents form subepithelial rings of varicose processes below the

crypt-villus junction (Fig. 3.2). Assessment of single fibers filled by anterograde transport indicates that the villus and crypt afferents are independent endings of different vagal sensory neurons [13].

Vagal Efferent Pathways

The vagal efferent pathways arise from the dorsal motor nucleus of the vagus and the nucleus ambiguus. Most of these neurons are pre-enteric, that is, they form synapses with neurons in enteric ganglia, but some run directly to the striated muscle cells of the esophagus. The major roles of the vagal innervation are to control esophageal propulsion, to relax the lower esophageal sphincter for swallowed food to pass, to increase gastric capacity, to facilitate antral contractions, to relax the pylorus, to increase gastric acid secretion, to contract the gallbladder and to promote pancreatic exocrine secretion (Fig. 3.1). Intracellular microelectrode recordings from individual gastric enteric neurons indicate that the majority, at least 2/3, of gastric myenteric neurons receive direct cholinergic excitatory synaptic inputs from pre-enteric vagal neurons [22]. These experiments were done by stimulating a vagal branch connected to an isolated region of gastric corpus. It is possible that not all inputs to each neuron were retained or effectively stimulated, so the data might underestimate the numbers of neurons receiving direct excitatory inputs from the vagus. Structural studies also indicate that the majority of gastric neurons receive vagal input, and even suggest that the vagal inputs outnumber those that arise from intrinsic gastric neurons [23–26]. Surprisingly, only about 10 % of myenteric ganglia in the striated muscle part of the esophagus receive vagal efferent inputs [26].

Comparable analyses of projections of vagal pre-enteric neurons to the small intestine do not appear to have been made. However, tracing studies indicate that there is a sparse vagal innervation of myenteric and submucosal ganglia in the small intestine [25]. Consistent with a minor vagal influence, structural and functional investigations of nerve circuits in the small intestine indicate that there is a predominance of local connections made with enteric neurons [27]. In contrast, vagal pre-enteric neurons innervate all intrinsic neurons in the bladder [28]. The exocrine pancreas has a strong reliance on vagal control [29], suggesting that here also there are pre-enteric inputs to a high proportion of pancreatic neurons.

Thoracolumbar Innervation

The thoracolumbar spinal cord connects with the gastrointestinal tract through spinal afferent neurons with cell bodies in dorsal root ganglia (DRG) and through sympathetic efferent pathways (Fig. 3.1). Thoracolumbar afferent axons are almost all unmyelinated C-fibers. Fiber numbers have been counted in the cat. The greater splanchnic nerve that supplies the upper abdomen contains about 3,000–4,000 afferent fibers and the lumbar splanchnic nerves contain about 4,000–5,000 afferent

axons [1]. A high proportion is immunoreactive for CGRP and tachykinins, and they are commonly immunoreactive for the TRPV1 channel, which is associated with pain afferents [30, 31]. Deletion of TRPV1 results in diminished afferent responses to distension and to acid in the lumen [32]. A high proportion of the afferent neuron endings is around arterioles in the gut wall [7]. The axons of spinal afferent neurons also provide a sparse network of varicose axons in the myenteric ganglia [30, 33]. Thoracolumbar afferent endings also branch within the lamina propria of the mucosa throughout the gastrointestinal tract, although their branching patterns have not been defined [7]. Rare thoracolumbar afferent fibers are found in the muscle layers. As they pass through sympathetic prevertebral ganglia, the axons of spinal afferent neurons provide collaterals that form synapses with cell bodies of postganglionic neurons [34].

There is little evidence that pain comes from the healthy gastrointestinal tract. In fact, it seems remarkably insensitive to stimuli, such as cutting, that would cause pain elsewhere. Gastrointestinal pain is associated with inflammation, and post-inflammatory disorders [35, 36]. Experimental studies indicate that inflammation causes long term changes in the properties of spinal afferents, that causes unresponsive neurons to become sensitive and responsive neurons to become hypersensitive [37, 38].

The sympathetic efferent pathways have four primary targets: myenteric ganglia, submucosal ganglia, blood vessels and sphincter muscle (Fig. 3.3). The preganglionic sympathetic neurons have their cell bodies in the intermediolateral columns of the spinal cord. Postganglionic neurons of vasoconstrictor pathways are in sympathetic chain and prevertebral ganglia. Postganglionic (pre-enteric) neurons with cell bodies in prevertebral ganglia provide a dense innervation of myenteric and submucosal ganglia. In both cases these are inhibitory; the sympathetic innervation of myenteric ganglia inhibits excitatory effects of enteric neurons on the muscle of the stomach and intestine, thus slowing passage of the contents of the gastrointestinal tract [39]. The innervation of submucosal ganglia inhibits secretomotor neuron activity (see later section “Neural Control of Fluid Movement: Secretomotor and Vasomotor Reflexes”). Sympathetic post-ganglionic neurons contract the sphincters of the gastrointestinal tract, which, like the innervation of myenteric ganglia, inhibits transit of contents.

Pelvic Innervation

The distal colon and rectum are provided with afferent and efferent innervation via the pelvic nerves and sacral plexuses. The pelvic nerves are commonly regarded as providing an innervation to the distal gut similar to that provided by the vagus to the proximal gut. However, unlike the vagal afferent nerves, the pelvic afferents include pain fibers [40]. Colorectal distension causes a visceromotor reflex (VMR) contraction of abdominal muscles in rats, a response that is deduced to be a consequence of stimulating pain pathways [41, 42]. The VMR was not affected by

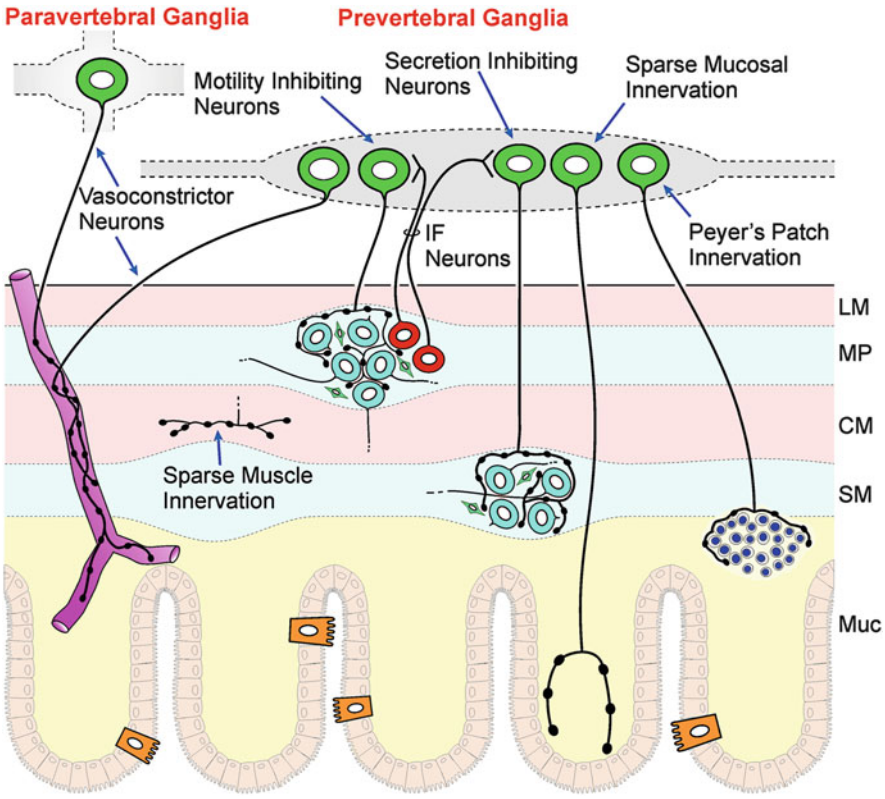

Fig. 3.3 Sympathetic innervation of the gastrointestinal tract. This diagram illustrates the innervation pathways for the non-sphincter regions of the stomach, small and large intestines. The densest innervation is of the myenteric ganglia throughout these regions, the submucosal ganglia of the small and large intestines, and intramural arteries. Few sympathetic fibers innervate the muscle of non-sphincter regions, whereas the sphincter muscle is densely innervated. The postganglionic neurons that innervate gut effectors have noradrenaline as their primary transmitter. Intestinofugal neurons (IF) synapse with sympathetic neurons in prevertebral ganglia. Modified from Lomax AE, Sharkey KA, Furness JB. The participation of the sympathetic innervation of the gastrointestinal tract in disease states. Neurogastroenterol Motil. 2010;22:7–18 [171]

cutting the lumbar colonic or hypogastric nerves, but was abolished when the pelvic (rectal) nerves were cut [40]. It is established that the pelvic nerves carry afferent information from low threshold mechanoreceptors. These have been identified as IGLEs, similar to those in the esophagus and stomach [43]. Action potential firing in the preterminal axons of IGLEs was evoked by direct probing, or by stretching the wall of the rectum. Rectal IGLEs detect stretch over a wide range, including into the level for pain [44]. Mucosal mechanoreceptors in the large intestine are similar to those in the stomach and proximal small intestine, in that they respond to mild stroking of the mucosa, but not to distension or contraction of the colon [45]. There are about 3,500 afferent axons in the pelvic nerves of the cat [1].

The efferent pathways in pelvic nerves provide innervation to enteric ganglia of the distal colon and rectum [46]. Retrograde tracing indicates that nerve cells in the sacral spinal cord project directly to the colon, and that there are also nerve cells that project from the pelvic ganglia to the colon [47], suggesting that pre-enteric neurons are in both the spinal cord and in pelvic ganglia (Fig. 3.1). For motility control, the innervation of enteric ganglia comes from the defecation centers that are in the lumbosacral spinal cord, between L5 and S3 (the levels being slightly different between species) [48]. In the rat the center is located primarily at L6-S1 [49–51] and in the guinea-pig at S1–S2 [47]. Reflexes through this center can be initiated by irritation or distension of the rectum; they persist after transection of the more rostral spinal cord, but are eliminated by section of the sacral outflows or the pelvic nerves [48, 52, 53]. In healthy individuals, the propulsive reflexes of the distal colon and rectum are kept in check to maintain fecal continence by central control centers that relay in the spinal defection center, and when defecation is appropriate it is triggered by central commands that impinge on the defecation center. Direct stimulation of the defecation center causes co-ordinated emptying of the colon, via the ENS [54]. Voluntary control of defecation (both inhibition and facilitation) is lost if cortico-spinal connections to the defecation centers are severed by spinal injury [55]. Nevertheless, if the defecation center remains intact after spinal injury it can be stimulated to command the ENS pathways for bowel emptying [56]. The pelvic pathways also carry pathways that cause vasodilation in the colorectum [57].

Cervical Spinal Afferents

Although the gut does not receive efferent inputs from the cervical spinal cord, afferent neurons that supply the upper, striated muscle, part of the esophagus do make connections at this level [58]. It is probable that these pathways carry esophageal pain signals.

Essential Nature of the ENS, in Contrast to Innervation from the CNS

In Hirschsprung's disease, the ganglia of the ENS fail to develop in the distal bowel, but all other tissue components are intact and functional [59]. Under these circumstances, no propulsive activity occurs in the aganglionic bowel, and the newborn child will die if this region is not removed. Similar absence of enteric neurons in the distal bowel is also lethal in other species, including horse (lethal white syndrome), rats and mice [60]. Degeneration of colonic enteric neurons in Chagas' disease, precipitated by infection with the protozoan *Trypanosoma cruzi*, causes colorectal propulsion to fail and megacolon to develop in the adult, similar to the problems

associated with Hirschsprung's disease in the child [61]. Other enteric neuropathies that have significant effects on the motor functions of the digestive tract include esophageal achalasia, gastroparesis and hypertrophic pyloric stenosis [62]. These diseases illustrate essential roles of the ENS.

The control of fluid movement between the intestinal lumen and body fluid compartments (discussed below) is also subject to pathological, life threatening, influences. The fluid movement is controlled by enteric secretomotor neurons that are abnormally activated by certain infective agents or their products. These pathogens, including cholera toxin and rotavirus, act directly on the secretomotor neurons and on the mucosal epithelial cells to cause life-threatening fluid loss [63].

In contrast to the severe, even life-threatening, effects of enteric neuron loss or dysfunction, severing connections with the CNS has relatively minor effects. Pavlov achieved a complete vagal denervation of the abdominal organs in dogs: these animals showed no evidence of ill-health, although responses to sham feeding, which are vagally mediated, were lost [64]. In humans, total abdominal or selective vagotomy has been used as a treatment for tens of thousands of peptic ulcer patients, without any indication of significant morbidity due to the vagotomy itself [65]. Minimal effects are also observed after sympathectomy. Complete removal of the sympathetic chains in cats left the animals in good health for many months after the surgery, although they became very sensitive to a cold environment [66]. Likewise, in humans in which sympathetic innervation of the gastrointestinal tract is removed for vascular disease or pain, there is no significant morbidity [67, 68]. Denervation of the gut by destructive lesions of the pelvic nerves or sacral plexus does not significantly disturb colorectal function, but it does compromise voluntary control of defecation and it can cause fecal incontinence [55, 69].

Structure of the ENS and Its Constituent Neurons

The enteric nervous system is composed of thousands of small ganglia that lie within the walls of the esophagus, stomach, small and large intestines, pancreas, gallbladder and biliary tree, the nerve fibers that connect these ganglia, and nerve fibers that supply the muscle of the gut wall, the mucosal epithelium, intramural arteries and other effector tissues (Fig. 3.4). Large numbers of neurons are contained in the enteric nervous system, about 200–600 million in human [27]. This is more than the total numbers of neurons of all sympathetic and parasympathetic ganglia combined and about the same number of neurons that are in the spinal cord. The enteric nervous system originates from neural crest cells that colonise the gut during intra-uterine life. It becomes functional in the last third of gestation in human, and continues to develop following birth.

Figure 3.4 is representative of the ENS of the mammalian small intestine. Enteric ganglia contain neurons and glial cells, but not connective tissue elements, and in many respects they are similar in structure to the CNS, except that there is no

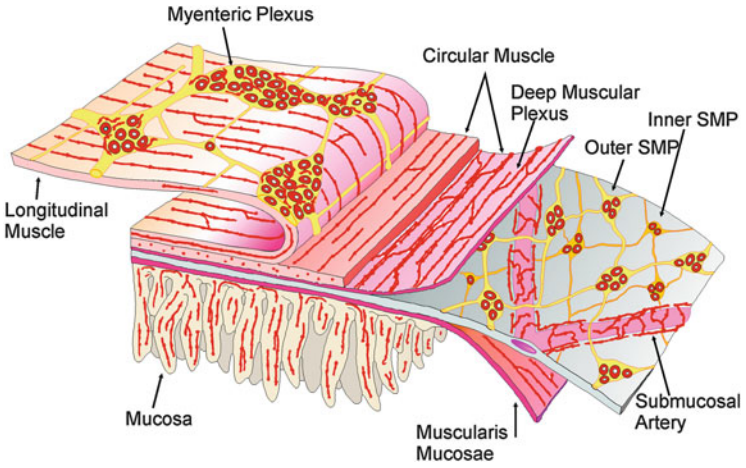

Fig. 3.4 The organisation of the ENS. This diagram illustrates the ENS of the small intestine of human and medium sized to large mammals. It has two ganglionated plexuses, the myenteric plexus between the longitudinal and circular layers of the external musculature and the submucosal plexus (SMP), that has outer and inner components. Nerve fiber bundles connect the ganglia and form plexuses innervating the longitudinal muscle, circular muscle, muscularis mucosae, intrinsic arteries and the mucosa. Axons of extrinsic origin also run in these nerve fiber bundles. There are also innervations of gastroenteropancreatic (GEP) endocrine cells and gut associated lymphoid tissue (GALT) that are not illustrated here. From Furness JB. The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol. 2012;9:286–294. Reprinted with permission from Nature Publishing Group

significant blood-enteric nervous system barrier. Two major sets of ganglia are found, the myenteric ganglia between the external muscle layers, and the submucosal ganglia (Fig. 3.4). The myenteric plexus forms a continuous network, around the circumference of the gut and extending from the upper esophagus to the internal anal sphincter. A ganglionated submucosal plexus is present in the small and large intestines. It is absent from the esophagus and almost no submucosal ganglia occur in the stomach. These organs also lack the large fluid fluxes across the mucosal epithelium that occur in the small and large intestines. Nerve fiber bundles within the enteric nervous system consist of the axons of enteric neurons, axons of extrinsic neurons that project to the gut wall, and glial cells.

There are some differences in structure and organisation between regions and species that are reviewed elsewhere [27, 70, 71]. There is a single layer of ganglia in the intestinal submucosa of small mammals. This is in contrast to large mammals that have two layers of submucosal ganglia, and sometimes have an intermediate layer, and in which there are structural and functional differences between the inner and outer submucosal plexuses [27, 72].

The gastrointestinal tract also harbors an extensive endocrine signaling system, and many gastrointestinal functions are under dual neuronal and endocrine control. Enteric neurons also interact with the extensive intrinsic immune system of the gastrointestinal tract.

Types of Enteric Neurons

Approximately 20 types of enteric neurons can be defined, the numbers differing slightly between regions [27, 73]. Combinations of features (morphology, neurochemical properties, cell physiology, projections to targets and functional roles) help to define each type. Amongst the 20 types, three classes can be identified, intrinsic primary afferent neurons (IPANs, also referred to as intrinsic sensory neurons), interneurons and motor neurons (Fig. 3.5). IPANs detect the physical state of the organs (for example, tension in the gut wall) and chemical features of the luminal contents [74]. They react to these signals to initiate appropriate reflex control of functions including motility, secretion and blood flow. IPANs connect with each other, with interneurons and directly with motor neurons. Interneurons connect with other interneurons and with motor neurons. Amongst the motor neurons are muscle motor neurons, secretomotor neurons, secretomotor/vasodilator neurons, motor neurons to enteroendocrine cells, and an innervation of lymphoid follicles (Fig. 3.5).

Intrinsic Sensory Neurons (IPANs)

The intrinsic sensory neurons (or intrinsic primary afferent neurons, IPANs) were first identified as large multi-axonal neurons (type II morphology) that respond to changes in luminal chemistry, mechanical distortion of the mucosa, and direct mechanical distortion of their processes in the external musculature [75–78]. It has been more recently discovered that distortion also excites other neurons, for example interneurons, in the enteric nerve circuits [79–81], indicating that reflexes are not uniquely initiated or modulated through type II neurons. Cell bodies of multi-axonal IPANs are 10–30 % of neurons in the submucosal and myenteric ganglia of the small and large intestines. Consistent with the motor functions of the esophagus being controlled from or via the brain stem, type II neurons are not found in the esophagus [27]. They are rare in the stomach, where motility is primarily controlled by vagal efferent pathways that originate in the medulla oblongata.

Motor Neurons

Muscle Motor Neurons

Excitatory and inhibitory neurons innervate the longitudinal and circular smooth muscle and the muscularis mucosae throughout the digestive tract. These are uni-axonal neurons that receive prominent fast excitatory synaptic potentials. The primary transmitters of the excitatory neurons are acetylcholine and tachykinins.

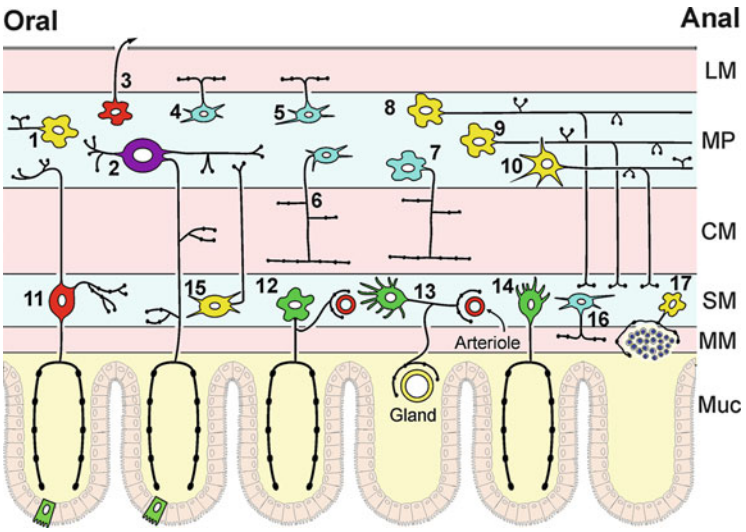

Fig. 3.5 Neuron types in the ENS. The types of neurons in the small intestine, that have been defined by their functions, cell body morphologies, chemistries, key transmitters and projections to targets. *LM* longitudinal muscle, *MP* myenteric plexus, *CM* circular muscle, *SM* submucosal plexus, *Muc* mucosa. Neuron Types: Ascending interneurons (*1*); Myenteric intrinsic primary afferent neurons (IPANs) (*2*); Intestinofugal neurons (*3*); Excitatory longitudinal muscle motor neurons (*4*); Inhibitory longitudinal muscle motor neurons (*5*); Excitatory circular muscle motor neurons (*6*); Inhibitory circular muscle motor neurons (*7*); Descending interneurons (local reflex) (*8*); Descending interneurons (secretomotor and motility reflex) (*9*); Descending interneurons (migrating myoelectric complex) (*10*); Submucosal IPANs (11); Non-cholinergic secretomotor/vasodilator neurons (*12*); Cholinergic secretomotor/vasodilator neuron (*13*); Cholinergic secretomotor (non-vasodilator) neurons (*14*); Uni-axonal neurons projecting to the myenteric plexus (*15*); motor neuron to the muscularis mucosa (*16*); innervation of Peyer's patches (*17*). Not illustrated, motor neurons to enteroendocrine cells. Modified from Furness JB. The Enteric Nervous System. Oxford: Blackwell 2006

The inhibitory neurons have multiple transmitters, including nitric oxide (NO), VIP and ATP-like transmitters [27, 82]. The primary transmitter of the neurons appears to be NO, and deficits in transmission are observed if NO synthase is knocked out [83].

The majority of neurons that innervate the circular muscle have their cell bodies in the myenteric ganglia. In fact, they are almost all in myenteric ganglia in small mammals, such as mice, rats and guinea-pigs. In larger mammals, including dog [84, 85], pig [86], and probably human, a component of circular muscle innervation comes from submucosal ganglia. The cell bodies of motor neurons that supply the longitudinal muscle are in the myenteric plexus of small animals. In the pig, and probably in other large mammals, the majority of the cell bodies are in the myenteric plexus, but some longitudinal muscle motor neurons have cell bodies in the outer submucosal plexus [72].

Similar to other smooth muscle of the wall of the gastrointestinal tract, the muscularis mucosae is innervated by excitatory and inhibitory motor neurons. In the small intestine and colon of the dog, removal of myenteric ganglia, allowing time for axon degeneration, did not change the innervation of the muscularis mucosae, which indicates that the innervation derives from nerve cells in submucosal ganglia [85]. In the esophagus, where there are no submucosal nerve cells, and the stomach, where there are few, the innervation must arise from nerve cells in myenteric ganglia.

The endings of vagal motor neurons, with their cell bodies in the nucleus ambiguus of the brain-stem, form conventional motor end-plates on the striated muscle cells [87, 88]. However, about a third of the endplates have an additional innervation from myenteric neurons, through which vagal excitation is modulated (see below section "Neural Control of Gastrointestinal Muscle Activity", "Esophagus"). The distal, smooth muscle part of the esophagus and the lower esophageal sphincter are innervated by enteric neurons.

Secretomotor and Secretomotor/Vasodilator Neurons Controlling Fluid Exchange

Exocrine fluid secretion, such as that from the salivary glands, sweat glands and pancreas, relies on supply of water and electrolytes from the blood. Because of this, exocrine secretion is coupled to vasodilation. Coupling also occurs in the intestine, where secretion and vasodilation are controlled together [89]. Analysis of transport of water and electrolytes across the intestinal mucosa shows that neurally evoked fluid transport is mediated by the active secretion of chloride ion, which is accompanied by sodium and water secretion [90, 91]. Pharmacological analysis of responses to nerve stimulation indicates that there are two components of transmission to the mucosa, a cholinergic component and a non-cholinergic component [92, 93]. Consistent with this, immunohistochemical analysis of neurons projecting to the mucosa identifies VIP-containing neurons that lack synthesizing enzymes for acetylcholine, and other neurons that contain choline acetytransferase [94, 95].

VIP is found in neurons innervating the mucosa throughout the small and large intestines and in the gallbladder of all mammalian species, including human. VIP both causes fluid secretion and increases blood flow [96, 97], and there is evidence that collaterals from the VIP containing secretomotor neurons innervate arterioles in the submucosa [98]. In human, overproduction of VIP causes the watery diarrhea syndrome [99]. The chemical markers of the cholinergic neurons differ between species. In the guinea-pig there are two groups, one containing NPY and the other immunoreactive for calretinin [94, 95]. Acetylcholine is both a stimulant of mucosal secretion and a vasodilator.

Motor Neuron Influence on the Glucose Transporter

There is emerging, but incomplete, evidence that enteric neurons influence the transport of glucose across the mucosa of the small intestine. Glucose is detected by receptors on enteroendocrine cells that release several gut hormones when stimulated, including glucagon-like peptide 2 (GLP-2) [100–102]. In turn, there is an induction and functional activation of the glucose transporter SGLT1 [100]. Although the induction of SGLT1 is mediated through GLP-2, the GLP-2 receptor is on submucosal neurons, not on the epithelium, which implies that the increased glucose transport is a nerve-mediated effect [103]. GLP-2 excites submucosal neurons [104]. There is also evidence that vago-vagal reflexes contribute to induction of SGLT1 in the small intestine [105]. The afferent component of the vago-vagal reflex was blocked by capsaicin application to the abdominal vagus [105]. The efferent pathway probably involves vagal pre-enteric neurons and enteric final motor neurons.

Gastric Secretomotor Neurons That Stimulate Acid Output

Some secretomotor neurons govern gastric acid secretion [106]. These neurons are cholinergic and act on the parietal cells through muscarinic receptors. Projection studies indicate that the secretomotor neurons have cell bodies in the myenteric plexus close to the regions of mucosa that they innervate [107].

Gastric Vasodilator Neurons

Gastric acid secretion and blood flow are enhanced when the vagus nerve is stimulated and these effects are reduced by muscarinic antagonists. In most experiments, it is not possible to determine whether vasodilation is due to a direct vascular action of cholinergic neurons in addition to a functional hyperemia consequent on the increased secretion [108]. However, centrally administered thyrotropin-releasing hormone (TRH) stimulates a vagal pathway in the rat that causes gastric vasodilation after acid secretion is blocked by omeprazole, suggesting a direct vasodilator pathway [109]. The blood flow increase in the absence of secretory change was antagonized by atropine. There is also evidence for non-cholinergic gastric vasodilator neurons that use VIP as a transmitter [110], but whether these are vasodilator alone or secretomotor/vasodilator neurons (as in the intestine) has not been determined.

Motor Neurons to Enteric Endocrine Cells

Twelve or more classes of endocrine cells reside in the mucosa of the gastrointestinal tract [3], and because the mucosa is densely innervated, these cells have nerve fibers in close proximity, but it is not clear in all cases whether the endocrine cells are functionally innervated. The best documented motor neurons innervating enteric endocrine cells are those controlling release of gastrin, which is under the influence of vagal and of intrinsic gastric pathways [27]. Transmission from the final secretomotor neurons is mediated at least in part by gastrin-releasing peptide [111]. Hormone release from other entero-endocrine cells is also likely to be under neural control. Peptide YY is released from the distal small intestine by vagal stimulation, and there is evidence of vagal reflex control of its release [112]. The release is attenuated by the muscarinic antagonist, atropine. The basal release of motilin is reduced by atropine and by tetrodotoxin, and stimulated by muscarinic agonists, suggesting that motilin cells receive an excitatory cholinergic input [113].

Innervation of Lymphoid Tissue (Peyer's Patches), Lymphocytes and Mast Cells

Lymphoid aggregations of the gastrointestinal tract, the most prominent being Peyer's patches, have surrounding nerve fibers, but it is difficult to trace the fibers into the follicles [114, 115]. However, careful examination does reveal an innervation of the suprafollicular dome region, but not an innervation of the germinal centers, in porcine jejunal lymphoid aggregations [116, 117], human ileal Peyer's patches [117] and follicles in the lamb small intestine [118]. Retrograde tracing from follicles reveals that they are innervated from submucosal ganglia [118].

In addition, receptors for transmitters of enteric neurons occur on lymphocytes that are scattered in the connective tissue (lamina propria) of the mucosa, and there are close approaches that suggest functional innervation of isolated lymphocytes within the connective tissue of the mucosa [119]. There are also close appositions between axons and mast cells in the mucosa [120].

Enteric Interneurons

Studies of the projections of neurons within the gut wall have identified several types of interneurons. However, these are more difficult to investigate physiologically than other neurons, because they can only be definitively studied by direct recording techniques, even though elegant divided organ bath methods have provided insights into the properties of enteric interneurons [121]. Because of the

inherent difficulties in studying the neurons, knowledge of their properties, connections and roles have been obtained from limited numbers of species and regions.

Within the myenteric plexus, the interneurons form chains of like neurons that run both orally and anally [122–124]. In the guinea-pig small intestine, three classes of descending interneurons and one class of ascending interneuron have been identified. Detailed studies of synaptic connections indicate that the chains formed by two of the types of descending interneuron interconnect [125]. The ascending interneurons appear to be involved in local motility reflexes, as are two types of descending cholinergic neurons, those which contain NOS and those containing 5HT [121]. Another type of descending interneuron, the ACh/SOM interneurons, might be involved in the passage of the migrating myoelectric complexes (MMC) along the intestine. The somatostatin containing neurons have numerous branching, tapering, filamentous dendrites [123]. Recent evidence suggests that some classes of interneurons in the colon are mechanoceptive and that reflexes can be initiated when they are activated by stretch [126].

Neural Control of Gastrointestinal Muscle Activity

The muscle layers of the gastrointestinal tract direct propulsion, mixing of contents, reservoir capacity (notably in the stomach) and expulsion of pathogens and noxious chemicals. The degree to which the ENS is essential for coordinated muscle function, and the extent to which nerve pathways that originate outside the alimentary tract are necessary for adequate control vary with the region of the gastrointestinal tract and also with the physiological circumstance. In broad terms, the body of the esophagus is controlled through brain stem circuits located in the medulla oblongata and the stomach is controlled through the brain stem and vago-vagal reflexes. Small intestine motility is primarily controlled through the ENS, as is large bowel motility, except for the essential role of the CNS in defecation [27].

The Esophagus

The nerve circuits for motor programs of propulsive activity in the upper, striated muscle, part of the esophagus are in the medulla oblongata of the CNS. These circuits relay through the nucleus ambiguous, which contains the cell bodies of the motor neurons that innervate the striated muscle [127, 128]. Although there are numerous ganglia that form an ENS of conventional appearance in the striated muscle esophagus, the ENS has little influence on the pattern of propulsive activity, and esophageal propulsion fails and never recovers its function if the vagal innervation is severed [129]. Nevertheless, myenteric neurons do supply an innervation to about a third of the end-plates and thus, unlike motor endplates elsewhere,

individual endplates in the esophagus receive dual innervation, one axon being from a vagal motor neuron and the other originating from a cell body in the myenteric plexus [130–133]. The endings of myenteric origin have NOS immunoreactivity, implying that transmission from enteric neurons is nitrergic. The myenteric neurons exert a presynaptic inhibition of vagal excitatory transmission, that has been demonstrated by experiments in which enteric NOS neurons were stimulated indirectly [134]. Thus the enteric nervous system seems to have a role in modulating peristalsis in the upper esophagus. The enteric innervation may have a greater role in young animals, because all motor endplates receive an enteric innervation at days 4–10 postnatal, after which there is partial withdrawal of innervation [135].

The nerve fibers that innervate the smooth muscle of the lower esophagus have their cell bodies in enteric ganglia. Nevertheless, peristalsis in this region is also coordinated from the CNS. The enteric ganglia of the smooth muscle esophagus are directly innervated by pre-enteric neurons of the dorsal motor nucleus of the vagus, and lesion of this nucleus impairs the motility patterns of the smooth muscle esophagus [128]. The vagus is involved in relaxing the lower esophageal sphincter (LES), to allow passage of food, through a descending inhibitory reflex that relaxes the sphincter when a bolus of food enters the last part of the esophageal body and its intraluminal pressure is raised. The reflex relaxation is inhibited by cooling the vagus nerve [136]. However, sphincter relaxation still occurs in response to distension following vagal block, indicating that a local reflex can be elicited [136].

Peak pressures during gastric mixing contractions exceed resting pressures in the body of the esophagus and the LES has an important role in limiting reflux of the corrosive contents of the stomach into the esophageal body. This role is apparent when pressure in the stomach is increased and a reflex constriction of the LES is initiated [137, 138]. This sphincter contraction is mediated by a vago-vagal reflex pathway that passes through the brain stem. Failure of this guarding results in reflux esophagitis and esophageal mucosal damage.

Stomach

A well-developed ganglionated myenteric plexus is found in the stomach, whose activity is significantly controlled through the vagus (see also above section "Vagal Efferent Pathways").

The stomach has a reservoir function; it increases volume as it fills, and relaxes prior to food arriving. It also has a function to mix the food with gastric juices and to push the liquefied products of gastric digestion into the duodenum. The fundus (proximal stomach) is primarily associated with the gastric reservoir function and the corpus-antrum (distal stomach) is associated with gastric mixing and antral propulsion [139]. Each antral contraction propels a small amount of liquid into the duodenum, while solid material is retained in the stomach [17].

Gastric Reservoir Function

The pressure in the stomach does not increase as it is filled [140], implying that the muscle of the proximal stomach relaxes to accommodate the meal. In fact, relaxation occurs before the food arrives, a phenomenon called receptive relaxation [141]. The relaxation that occurs when the pharynx or esophagus is distended occurs even when the esophagus is severed and no food reaches the stomach [142]. The reflex is prevented if the vagus nerves are cut. Relaxation of the proximal stomach also occurs if the gastric volume is increased, for example by distension with an intragastric balloon. This accommodation reflex is substantially reduced after vagotomy [143, 144]. A vagally mediated gastro-gastric reflex relaxation is also be elicited when distension is confined to the antrum [145]. In addition, there appears to be a small residual component of accommodation that is due to an intrinsic reflex [146]. As the volume in the stomach reduces, the fundus contracts. This also appears to be a vagally mediated effect [144]. Thus the stomach adjusts its volume both by relaxation and contraction, via vago-vagal reflexes.

Gastric Peristalsis and Mixing (the Distal Stomach: Corpus and Antrum)

Gastric peristalsis, which occurs in the body and antrum, is not prevented when the myenteric plexus is cut through or nicotine is given in a dose that blocks peristalsis in the intestine [147, 148]. Moreover, the frequency of peristalsis corresponds to the frequency of gastric slow waves in the muscle, indicating that gastric peristalsis is generated by the slow waves and, unlike peristalsis in the small intestine and colon, it does not require activity of excitatory neurons to be observed.

The augmentation of the gastric contractions when the stomach is artificially distended with fluid is almost entirely through vago-vagal reflexes [149]. When the antrum, or the whole stomach, is extrinsically denervated, antral peristaltic contractions are smaller and emptying times are prolonged [149–151]. Moreover, the strengths of the antral contractions are sequentially reduced when the vagal branches entering the antrum are successively cut, from proximal to distal [152].

Nevertheless, a number of studies indicate that there is intrinsic activity of excitatory cholinergic neurons, even in the completely isolated stomach. Intracellular microelectrodes have demonstrated the spontaneous occurrence of fast EPSPs in some enteric neurons in the isolated stomach [153], and other investigations have demonstrated an excitatory tone that is reduced by tetrodotoxin, or by antagonists of muscarinic or nicotinic receptors [154–156]. The amplitudes, but not the frequencies of occurrence of contractile waves are reduced when transmission from excitatory neurons to the muscle is prevented by tetrodotoxin [156]. The effectiveness of the excitatory neurons is enhanced when the stomach is distended [156], presumably because their rates of firing are increased.

There is little evidence for a gastric intrinsic reflex that is organised like that in the small intestine and which is necessary for intestinal peristalsis. After vagotomy, gastric distension causes very much weaker phasic contractions than are seen in the vagally innervated stomach [149]. The residual responses to distension are reduced by hexamethonium, indicating that there is a component of the enhancement of gastric peristaltic waves that is due to intrinsic reflexes. Furthermore, if the muscarinic receptor agonist, carbachol, is applied to the isolated stomach in which all nerve-mediated events have been prevented by tetrodotoxin, gastric peristaltic waves are restored [156]. This suggests that neuronal circuits are not required to co-ordinate peristaltic movement, direct excitation of the muscle being sufficient. IPANs, the types of neurons through which reflexes in the intestine are initiated are absent or very rare in the stomach [27].

It is concluded that gastric peristalsis is a consequence of contractions that are induced in the muscle by slow waves that are themselves generated by the pacemaker activity of ICC [157].

The Small Intestine and Colon

These regions rely on the ENS to direct various patterns of movement. In the small intestine, these patterns are rapid orthograde propulsion of contents (peristalsis), mixing movements (segmentation), slow orthograde propulsion (the migrating myoelectric complex, MMC) and retropulsion (expulsion of noxious substances associated with vomiting). In the large intestine there are mixing and propulsive movements, including the colonic MMC [46]. To orchestrate these movement patterns, the state of the intestine is sensed and appropriate motor patterns are generated through ENS circuits. The structural organisation of the circuits that detect the state of the small intestine, integrate the information and direct the activities of motor neurons is known (Fig. 3.6) and the colonic circuits appear to be similar [126, 158], but the mechanisms, within the integrative circuitry, through which one pattern of activity is converted to another are not known. Signals that trigger changes in patterns of movement in the small intestine have been identified. For example, fatty acids added to the luminal surface convert propulsive contractile activity to mixing movements, through a neural mechanism [159]. Conversion from one pattern to another can also be achieved with some drugs that target enteric neurons [160].

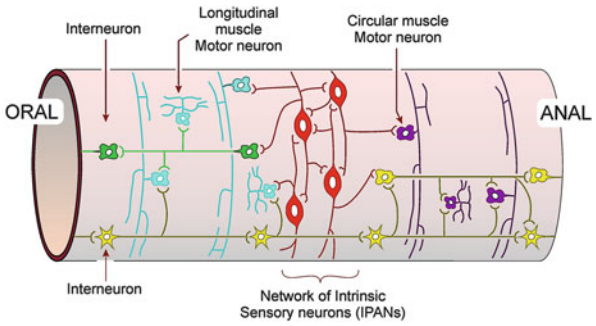

Fig. 3.6 Nerve circuits for control of motility in the small intestine. This diagram is based on studies in the guinea-pig small intestine. Similar component neurons have been identified in the small intestine of other species, including human, and in the large intestine. This is a simplified circuit diagram showing the major circuit features that have been identified. Networks of interconnected intrinsic sensory neurons (IPANs, *red*) detect mechanical distortion and luminal chemistry. These synapse with descending (*yellow*) and ascending (*green*) interneurons, and connect with excitatory muscle motor neurons (*blue*) and inhibitory muscle motor neurons (*purple*) directly and via interneurons. Based on Furness JB. The Enteric Nervous System. Oxford: Blackwell 2006

Neural Control of Fluid Movement: Secretomotor and Vasomotor Reflexes

It is essential that the movement of fluid between the lumen of the intestine and the body fluid compartments is regulated. More than two blood volumes cross the mucosal epithelial surface each day, and disruption of fluid transport regulation, such as occurs in cholera intoxication, is life-threatening.

One reason for the large flux is that the absorption of sugars (monosaccharides) and amino acids is through cation-coupled transporters. Thus the absorption of a glucose molecule through the sodium/glucose linked transporter (SGLT) brings with it a sodium ion together with counter ions, mainly chloride. It is calculated that 100 g of absorbed glucose takes with it 1.8 L of water [27, 161]. Enteric reflexes, through activation of secretomotor neurons, return water and electrolyte to the lumen (Fig. 3.7). This fluid is drawn from the circulation and from the absorbed fluid. Enteric secretomotor reflexes cannot act in isolation, they must be modulated to take into account whole body fluid balance. This control is exerted through blood volume and blood pressure detectors that change the activity of two sympathetic pathways, vasoconstrictor pathways and secretomotor inhibitory pathways (Fig. 3.7) [27, 162].

The fine control of fluid balance through local (ENS) and systemic (sympathetic) reflexes is thrown into chaos when there is an excessive luminal content of certain pathogens or their toxins. These agents, including cholera toxin, rotavirus and pathogenic *E. coli*, activate enteric secretomotor neurons. In mild cases, this stimulates diarrhea that helps expel the pathogens and their toxic products.

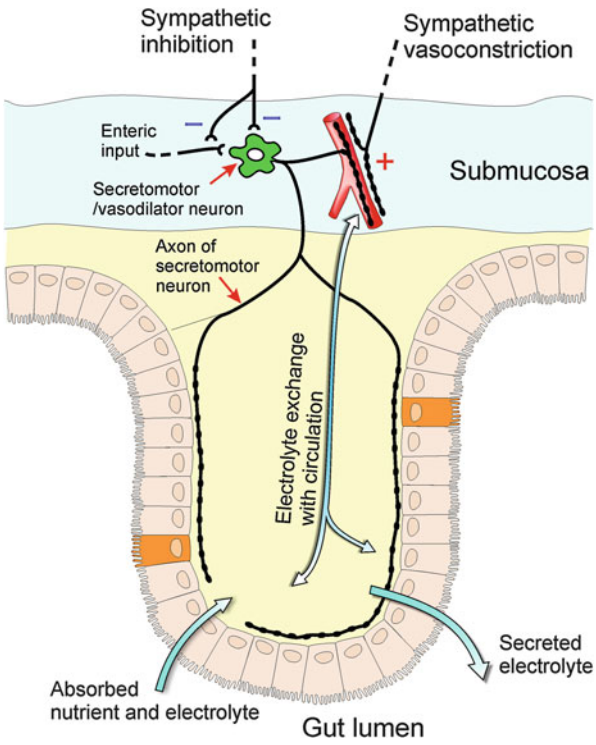

Fig. 3.7 Neural control of transmucosal water and electrolyte movement in the small intestine. The final secretomotor neuron of reflexes that play an essential role in balancing local fluid fluxes and in whole body water and electrolyte balance is illustrated. Large volumes of fluid are absorbed from the lumen with nutrients, such as glucose. These fluids are returned through secretomotor reflexes. The absorption of nutrients with fluid activates enteric secretomotor reflex pathways that impinge on the secretomotor neurons. It is important that the balance of this fluid exchange is modulated by sympathetic vasoconstrictor and secretomotor inhibitory pathways. Activity in these sympathetic pathways, which inhibit secretion and reduce local blood flow, is determined by whole body fluid status, which includes sensory detection through blood volume detectors, baroreceptors and osmoreceptors. Modified from Furness JB. The Enteric Nervous System. Oxford: Blackwell 2006

However, when there are high levels of pathogens or toxins, the intestine is overwhelmed and a pathological, life-threatening hypersecretion can ensue. The hypersecretion results in copious diarrhea. Infectious diarrhea causes about 1.5 million deaths a year, primarily in underdeveloped tropical countries [163].

Entero-Enteric Reflexes

Figure 3.1 shows the intestinofugal neurons that have cell bodies in the digestive tract and project to sympathetic ganglia, other organs and to the CNS. Only the roles of those projecting to the sympathetic ganglia are known. These intestinofugal neurons are in the afferent limbs of entero-enteric reflexes, that pass from distal to proximal regions through sympathetic ganglia, where intestinofugal neurons form synapses [27, 164, 165]. The reflex pathways bypass the CNS. Distension of segments of intestine activates the reflex pathways, causing sympathetic inhibition of motility in more proximal regions. In the case of the stomach, acid or hypertonic solution in the lumen of the upper small intestine causes inhibition of gastric motility and emptying into the duodenum through entero-enteric reflexes [166, 167]. The entero-enteric reflex that is initiated by fat in the distal intestine and slows transit in the proximal small intestine is referred to as the ileal brake [168]. Thus the reflexes arise in distal regions and regulate more proximal regions, so that luminal contents that arrive at more distal regions are adequately processed proximally.

Summary and Conclusions

Neural control of the gastrointestinal tract is exerted by integration of signals that originate in the CNS and ENS. Gastrointestinal function is maintained in the absence of influence from the CNS, but if ENS control of the intestine is lost, propulsion of content in the affected region is ineffective, which is life-threatening. Three major regions of the CNS connect with the gastrointestinal tract, the brain stem through the vagus nerve, the thoracolumbar spinal cord through spinal afferent and sympathetic efferent pathways, and the lumbosacral spinal cord through pelvic nerve afferent and efferent pathways. Vagal afferents carry mechanoreceptive and chemoceptive information from the esophagus, stomach and intestine to the CNS, but do not signal pain. Thoracolumbar and lumbosacral afferents both signal pain of gut origin. In addition, there is cervical afferent innervation of the upper esophagus. The primary control centers for the smooth muscle esophagus, lower esophageal sphincter, stomach, gallbladder and pancreas are in the CNS; they exert control through vagal efferent pathways. The vagal neurons that control gastric motility, acid secretion and hormone release form synapses in the ENS. The major efferent connections of sympathetic pathways are to myenteric ganglia, through which gastrointestinal movements are inhibited, to submucosal ganglia, through which fluid movement into the lumen is inhibited, and to intramural arteries that are constricted by sympathetic nerve activity. The efferent pelvic nerves convey the outputs of the lumbosacral defecation centers.

The enteric nervous system consists of many thousands of interconnected ganglia that extend from the upper esophagus to the internal anal sphincter. These

ganglia in human contain in total about 200–600 million neurons. Motor neurons in the enteric ganglia supply all major effectors in the gastrointestinal tract. In the small and large intestines, the ENS contains full reflex pathways that are essential to direct the movements of these parts of the digestive tract. Another critical role of the ENS, in concert with signals from the CNS, is whole body fluid balance. This is necessary because of the very large fluid load that is contributed to by water and electrolyte movement that is associated with nutrient digestion and absorption. The ENS contains a type of neuron not found anywhere else in the periphery. These are intestinofugal neurons, with cell bodies in enteric ganglia, that send their axons to sympathetic ganglia, to other organs (the pancreas, gallbladder and trachea), and to the CNS via the vagus and pelvic nerves. Thus the digestive tract is controlled through integrating centers in the brainstem, spinal cord, sympathetic ganglia and gut wall that are extensively interconnected through conventional afferent and efferent pathways and via the intestinofugal neurons.

Acknowledgements Research in the authors' laboratories is supported by the National Health and Medical Research Council of Australia (NHMRC). LRR is supported by a NHMRC post-doctoral fellowship.

References

1. Sengupta JN, Gebhart GF (1994) Gastrointestinal afferent fibers and sensation. In: Johnson LR (ed) Physiology of the gastrointestinal tract, 3rd edn. Raven Press, New York, pp 483–519
2. Raybould HE (2010) Gut chemosensing: interactions between gut endocrine cells and visceral afferents. Auton Neurosci 153:41–46
3. Furness JB, Rivera LR, Cho H-J, Bravo DM, Callaghan B (2013) The gut as a sensory organ. Nat Rev Gastroenterol Hepatol 10:729–740
4. Berthoud HR, Blackshaw LA, Brookes JH, Grundy D (2004) Neuroanatomy of extrinsic afferents supplying the gastrointestinal tract. Neurogastroenterol Motil 16:28–33
5. Rodrigo J, De Felipe J, Robles Chillida EM, Pérez Antón JA, Mayo I, Gómez A (1982) Sensory vagal nature and anatomical access paths to esophagus laminar nerve endings in myenteric ganglia. Determination by surgical degeneration methods. Acta Anat 112:47–57
6. Wang FB, Powley TL (2000) Topographic inventories of vagal afferents in gastrointestinal muscle. J Comp Neurol 421:302–324
7. Brookes SJH, Spencer NJ, Costa M, Zagorodnyuk VP (2013) Extrinsic primary afferent signalling in the gut. Nat Rev Gastroenterol Hepatol 10:286–296
8. Zagorodnyuk VP, Chen BN, Brookes SJH (2001) Intraganglionic laminar endings are mechano-transduction sites of vagal tension receptors in the guinea-pig stomach. J Physiol 534:255–268
9. Paintal AS (1954) A study of gastric stretch receptors. Their role in the peripheral mechanism of satiation of hunger and thirst. J Physiol 126:255–270
10. Iggo A (1955) Tension receptors in the stomach and the urinary bladder. J Physiol 128: 593–607
11. Berthoud HR, Powley TL (1992) Vagal afferent innervation of the rat Fundic stomach: morphological characterization of the gastric tension receptor. J Comp Neurol 319:261–276

12. Powley TL, Phillips RJ (2011) Vagal intramuscular array afferents form complexes with interstitial cells of Cajal in gastrointestinal smooth muscle: analogues of muscle spindle organs? Neuroscience 186:188–200
13. Powley TL, Spaulding RA, Haglof SA (2011) Vagal afferent innervation of the proximal gastrointestinal tract mucosa: chemoreceptor and mechanoreceptor architecture. J Comp Neurol 519:644–660
14. Clarke GD, Davison JS (1978) Mucosal receptors in the gastric antrum and small intestine of the rat with afferent fibres in the cervical vagus. J Physiol 284:55–67
15. Leek BF (1977) Abdominal and pelvic visceral receptors. Br Med Bull 33:163–168
16. Page AJ, Martin CM, Blackshaw LA (2002) Vagal mechanoreceptors and chemoreceptors in mouse stomach and esophagus. J Neurophysiol 87:2095–2103
17. Kelly KA (1980) Gastric emptying of liquids and solids: roles of proximal and distal stomach. Am J Physiol 239:G71–G76
18. Becker JM, Kelly KA (1983) Antral control of canine gastric emptying of solids. Am J Physiol 8:G334–G338
19. Page AJ, Slattery JA, Milte C, Laker R, O'Donnell T, Dorian C et al (2007) Ghrelin selectively reduces mechanosensitivity of upper gastrointestinal vagal afferents. Am J Physiol 292:G1376–G1384
20. Kentish SJ, O'Donnell TA, Isaacs NJ, Young RL, Li H, Harrington AM et al (2013) Gastric vagal afferent modulation by leptin is influenced by food intake status. J Physiol 591: 1921–1934
21. le Roux CW, Neary NM, Halsey TJ, Small CJ, Martinez-Isla AM, Ghatei MA et al (2005) Ghrelin does not stimulate food intake in patients with surgical procedures involving vagotomy. J Clin Endocrinol Metab 90:4521–4524
22. Schemann M, Grundy D (1992) Electrophysiological identification of vagally innervated enteric neurons in guinea pig stomach. Am J Physiol 263:G709–G718
23. Lawrentjew BJ (1931) Zur Lehre von der Cytoarchitektonik des peripheren autonomen Nervensystems. 1. Die Cytoarchitektonik der Ganglien des Verdauungskanals beim Hunde. Z Mikrosk Anat Forsch 23:527–551
24. Filogamo G, Gabella G (1970) Effects of extrinsic denervation on the synapses of myenteric plexus. J Microscopie 9:281–284
25. Holst MC, Kelly JB, Powley TL (1997) Vagal preganglionic projections to the enteric nervous system characterized with phaseolus vulgaris-leucoagglutinin. J Comp Neurol 381: 81–100
26. Neuhuber WL, Kressel M, Stark A, Berthoud HR (1998) Vagal efferent and afferent innervation of the rat esophagus as demonstrated by anterograde DiI and DiA tracing: focus on myenteric ganglia. J Auton Nerv Syst 70:92–102
27. Furness JB (2006) The enteric nervous system. Blackwell, Oxford
28. Mawe GM (1998) Nerves and hormones interact to control gallbladder function. News Physiol Sci 13:84–90
29. Owyang C, Williams JA (2003) Pancreatic secretion. In: Yamada T (ed) Textbook of gastroenterology. Lippincott, Williams & Wilkins, Philadelphia, pp 340–366
30. Green T, Dockray GJ (1988) Characterization of the peptidergic afferent innervation of the stomach in the rat, mouse and guinea-pig. Neuroscience 25:181–193
31. Tan LL, Bornstein JC, Anderson CR (2008) Distinct chemical classes of medium-sized transient receptor potential channel vanilloid 1-immunoreactive dorsal root ganglion neurons innervate the adult mouse jejunum and colon. Neuroscience 156:334–343
32. Rong W, Hillsley K, Davis JB, Hicks G, Winchester WJ, Grundy D (2004) Jejunal afferent nerve sensitivity in wild-type and TRPV1 knockout mice. J Physiol 560:867–881
33. Gibbins IL, Furness JB, Costa M, MacIntyre I, Hillyard CJ, Girgis S (1985) Co-localization of calcitonin gene related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea-pigs. Neurosci Lett 57:125–130

34. Matthews MR, Cuello AC (1982) Substance P-immunoreactive peripheral branches of sensory neurons innervate guinea pig sympathetic neurons. Proc Natl Acad Sci U S A 79: 1668–1672
35. Ferri A, Blennerhassett P, Wang L, Bercik P, Verdu EF, Marzio L et al (2002) The relationship between chronic colonic inflammation and mechanosensitivity. Gastroenterology 122:A-528
36. Spiller R, Garsed K (2009) Postinfectious irritable bowel syndrome. Gastroenterology 136: 1979–1988
37. Beyak MJ (2010) Visceral afferents – determinants and modulation of excitability. Auton Neurosci 153:69–78
38. Feng B, La JH, Schwartz ES, Gebhart GF (2012) Irritable bowel syndrome: methods, mechanisms, and pathophysiology. Neural and neuro-immune mechanisms of visceral hypersensitivity in irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol 302: G1085–G1098
39. Furness JB, Costa M (1974) The adrenergic innervation of the gastrointestinal tract. Ergeb Physiol 69:1–51
40. Kyloh M, Nicholas S, Zagorodnyuk VP, Brookes SJ, Spencer NJ (2011) Identification of the visceral pain pathway activated by noxious colorectal distension in mice. Front Neurosci 5:1–7
41. Ness TJ, Gebhart GF (1990) Visceral pain: a review of experimental studies. Pain 41: 167–234
42. Larsson M, Arvidsson S, Ekman C, Bayati A (2003) A model for chronic quantitative studies of colorectal sensitivity using balloon distension in conscious mice – effects of opioid receptor agonists. Neurogastroenterol Motil 15:371–381
43. Lynn P, Zagorodnyuk V, Hennig G, Costa M, Brookes S (2005) Mechanical activation of rectal intraganglionic laminar endings in the guinea pig distal gut. J Physiol 564:589–601
44. Zagorodnyuk VP, Kyloh M, Nicholas S, Peiris H, Brookes SJ, Chen BN et al (2011) Loss of visceral pain following colorectal distension in an endothelin-3 deficient mouse model of Hirschsprung's disease. J Physiol 589:1691–1706
45. Brierley SM, Jones RCW III, Gebhart GF, Blackshaw LA (2004) Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice. Gastroenterology 127:166–178
46. Brookes SJ, Dinning PG, Gladman MA (2009) Neuroanatomy and physiology of colorectal function and defaecation: from basic science to human clinical studies. Neurogastroenterol Motil 21:9–19
47. Olsson C, Chen BN, Jones S, Chataway TK, Costa M, Brookes SJH (2006) Comparison of extrinsic efferent innervation of guinea pig distal colon and rectum. J Comp Neurol 496: 787–801
48. Gonella J, Bouvier M, Blanquet F (1987) Extrinsic nervous control of motility of small and large intestines and related sphincters. Physiol Rev 67:902–961
49. Nadelhaft I, Booth AM (1984) The location and morphology of preganglionic neurons and the distribution of visceral afferents from the rat pelvic nerve: a horseradish peroxidase study. J Comp Neurol 226:238–245
50. Gillis RA, Quest JA, Pagani FD, Norman WP (1989) Control centers in the central nervous system for regulating gastrointestinal motility. In: Wood JD (ed) Handbook of physiology: the gastrointestinal system (chapter 17), vol I. American Physiological Society, New York, pp 621–683
51. Vizzard MA, Brisson M, de Groat WC (2000) Transneuronal labeling of neurons in the adult rat central nervous system following inoculation of pseudorabies virus into the colon. Cell Tissue Res 299:9–26
52. de Groat WC, Krier J (1978) The sacral parasympathetic reflex pathway regulating colonic motility and defaecation in the cat. J Physiol 276:481–500

53. Maggi CA, Giuliani S, Santicioli P, Patacchini R, Meli A (1988) Neural pathways and pharmacological modulation of defecation reflex in rats. Gen Pharmacol 19:517–523
54. Shimizu Y, Chang EC, Shafton AD, Ferens DM, Sanger GJ, Witherington J et al (2006) Evidence that stimulation of ghrelin receptors in the spinal cord initiates propulsive activity in the colon of the rat. J Physiol 576:329–338
55. Lynch AC, Antony A, Dobbs BR, Frizelle FA (2001) Bowel dysfunction following spinal cord injury. Spinal Cord 39:193–203
56. Ferens DM, Habgood MD, Saunders NR, Tan YH, Brown DJ, Brock JA et al (2011) Stimulation of defecation in spinal cord-injured rats by a centrally acting ghrelin receptor agonist. Spinal Cord 49:1036–1041
57. Hulten L (1969) Reflex control of colonic motility and blood flow. Acta Physiol Scand 335 (Suppl):77–93
58. Dütsch M, Eichhorn U, Wörl J, Wank M, Berthoud HR, Neuhuber WL (1998) Vagal and spinal afferent innervation of the rat esophagus: a combined retrograde tracing and immunocytochemical study with special emphasis on calcium-binding proteins. J Comp Neurol 398: 289–307
59. Swenson O (2002) Hirschsprung's disease: a review. Pediatrics 109:914–918
60. Furness JB, Poole DP (2012) Involvement of gut neural and endocrine systems in pathological disorders of the digestive tract. J Anim Sci 90:1203–1212
61. Matsuda NM, Miller SM, Evora PRB (2009) The chronic gastrointestinal manifestations of Chagas disease. Clinics 64:1219–1224
62. Di Nardo G, Blandizzi C, Volta U, Colucci R, Stanghellini V, Barbara G et al (2008) Review article: molecular, pathological and therapeutic features of human enteric neuropathies. Aliment Pharmacol Ther 28:25–45
63. Lundgren O (2002) Enteric nerves and diarrhoea. Pharmacol Toxicol 90:109–120
64. Pavlov JP (1902) The work of the digestive glands. Charles Griffin & Co. Ltd., London
65. O'Leary JP, Woodward ER, Hollenbeck HI, Dragstedt LR (1976) Vagotomy and drainage procedure for duodenal ulcer: the results of seventeen years' experience. Ann Surg 183: 613–618
66. Cannon WB, Newton HF, Bright EM, Menkin V, Moore RM (1929) Some aspects of the physiology of animals surviving complete exclusion of sympathetic nerve impulses. Am J Physiol 89:84–107
67. Bingham JR, Ingelfinger FJ, Smithwick RH (1950) The effect of sympathectomy on abdominal pain in man. Gastroenterology 15:18–31
68. Kang CM, Lee HY, Yang HJ, Jang HJ, Gil YC, Kim KS et al (2007) Bilateral thoracoscopic splanchnicectomy with sympathectomy for managing abdominal pain in cancer patients. Am J Surg 194:23–29
69. Denny-Brown D, Robertson EG (1935) An investigation of the nervous control of defecation. Brain 58:256–310
70. Brehmer A (2006) Structure of enteric neurons. Adv Anat Embryol Cell Biol 186:1–94
71. Brehmer A, Rupprecht H, Neuhuber W (2010) Two submucosal nerve plexus in human intestines. Histochem Cell Biol 133:149–161
72. Timmermans JP, Adriaensen D, Cornelissen W, Scheuermann DW (1997) Structural organization and neuropeptide distribution in the mammalian enteric nervous system, with special attention to those components involved in mucosal reflexes. Comp Biochem Physiol 118A: 331–340
73. Brookes SJH, Costa M (2002) Cellular organisation of the mammalian enteric nervous system. In: Brookes SJH, Costa M (eds) Innervation of the gastrointestinal tract. Taylor and Frances, New York, pp 393–467
74. Furness JB, Jones C, Nurgali K, Clerc N (2004) Intrinsic primary afferent neurons and nerve circuits within the intestine. Prog Neurobiol 72:143–164

75. Kirchgessner AL, Tamir H, Gershon MD (1992) Identification and stimulation by serotonin of intrinsic sensory neurons of the submucosal plexus of the guinea pig gut: activity-induced expression of Fos immunoreactivity. J Neurosci 12:235–248
76. Bertrand PP, Kunze WAA, Bornstein JC, Furness JB, Smith ML (1997) Analysis of the responses of myenteric neurons in the small intestine to chemical stimulation of the mucosa. Am J Physiol 273:G422–G435
77. Kunze WAA, Furness JB, Bertrand PP, Bornstein JC (1998) Intracellular recording from myenteric neurons of the guinea-pig ileum that respond to stretch. J Physiol 506:827–842
78. Furness JB, Kunze WAA, Bertrand PP, Clerc N, Bornstein JC (1998) Intrinsic primary afferent neurons of the intestine. Prog Neurobiol 54:1–18
79. Spencer NJ, Smith TK (2004) Mechanosensory S-neurons rather than AH-neurons appear to generate a rhythmic motor pattern in guinea-pig distal colon. J Physiol 558(2):577–596
80. Spencer NJ, Dickson EJ, Hennig GW, Smith TK (2006) Sensory elements within the circular muscle are essential for mechanotransduction of ongoing peristaltic reflex activity in guinea-pig distal colon. J Physiol 576(2):519–531
81. Mazzuoli G, Schemann M (2009) Multifunctional rapidly adapting mechanosensitive enteric neurons (RAMEN) in the myenteric plexus of the guinea pig ileum. J Physiol 587:4681–4693
82. Durnin L, Sanders KM, Mutafova-Yambolieva VN (2013) Differential release of b-NAD^+ and ATP upon activation of enteric motor neurons in primate and murine colons. Neurogastroenterol Motil 25:e194–e204
83. Rivera LR, Poole DP, Thacker M, Furness JB (2011) The involvement of nitric oxide synthase neurons in enteric neuropathies. Neurogastroenterol Motil 23:980–988
84. Sanders KM, Smith TK (1986) Motoneurones of the submucous plexus regulate electrical activity of the circular muscle of the canine proximal colon. J Physiol 380:293–310
85. Furness JB, Lloyd KCK, Sternini C, Walsh JH (1990) Projections of substance P, vasoactive intestinal peptide and tyrosine hydroxylase immunoreactive nerve fibres in the canine intestine, with special reference to the innervation of the circular muscle. Arch Histol Cytol 53:129–140
86. Timmermans J-P, Hens J, Adriaensen D (2001) Outer submucous plexus: an intrinsic nerve network involved in both secretory and motility processes in the intestine of large mammals and humans. Anat Rec 262:71–78
87. Samarasinghe DD (1972) Some observations on the innervation of the striated muscle in the mouse oesophagus-an electron microscope study. J Anat 112:173–184
88. Rodrigo J, Polak JM, Fernandez L, Ghatei MA, Mulderry P, Bloom SR (1985) Calcitonin gene-related peptide immunoreactive sensory and motor nerves of the rat, cat, and monkey esophagus. Gastroenterology 88:444–451
89. Vanner S, MacNaughton WK (2004) Submucosal secretomotor and vasodilator reflexes. Neurogastroenterol Motil 16(Suppl 1):39–43
90. Cooke HJ, Shonnard K, Wood JD (1983) Effects of neuronal stimulation on mucosal transport in guinea pig ileum. Am J Physiol 245:G290–G296
91. Keast JR (1987) Mucosal innervation and control of water and ion transport in the intestine. Rev Physiol Biochem Pharmacol 109:1–59
92. Cooke HJ, Reddix RA (1994) Neural regulation of intestinal electrolyte transport. In: Johnson LR (ed) Physiology of the gastrointestinal tract, 3rd edn. Raven Press, New York, pp 2083–2132
93. Keast JR, Furness JB, Costa M (1985) Investigations of nerve populations influencing ion transport that can be stimulated electrically, by serotonin and by a nicotinic agonist. Naunyn Schmiedebergs Arch Pharmacol 331:260–266
94. Furness JB, Costa M, Gibbins IL, Llewellyn Smith IJ, Oliver JR (1985) Neurochemically similar myenteric and submucous neurons directly traced to the mucosa of the small intestine. Cell Tissue Res 241:155–163

95. Brookes SJH, Steele PA, Costa M (1991) Calretinin immunoreactivity in cholinergic motor neurones, interneurones and vasomotor neurones in the guinea-pig small intestine. Cell Tissue Res 263:471–481
96. Schwartz CJ, Kimberg DV, Sheerin HE, Field M, Said SI (1974) Vasoactive intestinal peptide stimulation of adenylate cyclase and active electrolyte secretion in intestinal mucosa. J Clin Invest 54:536–544
97. Banks MR, Farthing MJG, Robberecht P, Burleigh DE (2005) Antisecretory actions of a novel vasoactive intestinal polypeptide (VIP) antagonist in human and rat small intestine. Br J Pharmacol 144:994–1001
98. Furness JB, Alex G, Clark MJ, Lal VV (2003) Morphologies and projections of defined classes of neurons in the submucosa of the guinea-pig small intestine. Anat Rec 272A: 475–483
99. Modlin IM, Bloom SR, Mitchell SJ (1980) Experimental evidence for vasoactive intestinal peptide as a cause of the watery diarrhea syndrome. Gastroenterology 75:1051–1054
100. Margolskee RF, Dyer J, Kokrashvili Z, Salmon KSH, Ilegems E, Daly K et al (2007) T1R3 and gustducin in gut sense sugars to regulate expression of Na^+-glucose cotransporter 1. Proc Natl Acad Sci U S A 104:15075–15080
101. Gerspach AC, Steinert RE, Schönenberger L, Graber-Maier A, Beglinger C (2011) The role of the gut sweet taste receptor in regulating GLP-1, PYY, and CCK release in humans. Am J Physiol 301:E317–E325
102. Gorboulev V, Schürmann A, Vallon V, Kipp H, Jaschke A, Klessen D et al (2012) Na^+-D-glucose cotransporter SGLT1 is pivotal for intestinal glucose absorption and glucose-dependent incretin secretion. Diabetes 61:187–196
103. Shirazi-Beechey SP, Moran AW, Batchelor DJ, Daly K, Al-Rammahi M (2011) Influences of food constituents on gut health glucose sensing and signalling; regulation of intestinal glucose transport. Proc Nutr Soc 70:185–193
104. Sigalet DL, Wallace L, De Heuval E, Sharkey KA (2010) The effects of glucagon-like peptide 2 on enteric neurons in intestinal inflammation. Neurogastroenterol Motil 22: 1318–e350
105. Stearns AT, Balakrishnan A, Rhoads DB, Tavakkolizadeh A (2010) Rapid upregulation of sodium-glucose transporter SGLT1 in response to intestinal sweet taste stimulation. Ann Surg 251:865–871
106. Schubert ML, Peura DA (2008) Control of gastric acid secretion in health and disease. Gastroenterology 134:1842–1860
107. Pfannkuche H, Reiche D, Sann H, Schemann M (1998) Different subpopulations of cholinergic and nitrergic myenteric neurones project to mucosa and circular muscle of the guinea-pig gastric fundus. Cell Tissue Res 292:463–475
108. Jodal M, Lundgren O (1989) Neurohormonal control of gastrointestinal blood flow. In: Wood JD (ed) Handbook of physiology: the gastrointestinal system. American Physiological Society, Washington, DC, pp 1667–1711
109. Thiefin G, Taché Y, Leung FW, Guth PH (1989) Central nervous system action of thyrotropin-releasing hormone to increase gastric mucosal blood flow in the rat. Gastroenterology 97:405–411
110. Ito S, Ohga A, Ohta T (1988) Gastric relaxation and vasoactive intestinal peptide output in response to reflex vagal stimulation in the dog. J Physiol 404:683–693
111. Makhlouf GM, Grider JR, Schubert ML (1989) Identification of physiological function of gut peptides. In: Makhlouf GM (ed) Handbook of physiology: the gastrointestinal system. American Physiological Society, Washington, DC, pp 123–131
112. Onaga T, Zabielski R, Kato S (2002) Multiple regulation of peptide YY secretion in the digestive tract. Peptides 23:279–290
113. Poitras P, Trudel L, Miller P, Gu CM (1997) Regulation of motilin release: studies with ex vivo perfused canine jejunum. Am J Physiol 272:G4–G9

114. Krammer HJ, Kuhnel W (1993) Topography of the enteric nervous system in Peyer's patches of the porcine small intestine. Cell Tissue Res 272:267–272
115. Kulkarni-Narla A, Beitz AJ, Brown DR (1999) Catecholaminergic, cholinergic and peptidergic innervation of gut-associated lymphoid tissue in porcine jejunum and ileum. Cell Tissue Res 298:275–286
116. Green BT, Lyte M, Kulkarni-Narla A, Brown DR (2003) Neuromodulation of enteropathogen internalization in Peyer's patches from porcine jejunum. J Neuroimmunol 141:74–82
117. Vulchanova L, Casey MA, Crabb GW, Kennedy WR, Brown DR (2007) Anatomical function for enteric neuroimmune interactions in Peyer's patches. J Neuroimmunol 185:64–74
118. Chiocchetti R, Mazzuoli G, Albanese V, Mazzoni M, Clavenzani P, Lalatta-Consterbosa G et al (2008) Anatomical evidence for ileal Peyer's patches innervation by enteric nervous system: a potential route for prion neuroinvasion? Cell Tissue Res 332:185–194
119. Ichikawa S, Eda N, Uchino S (1992) Close association of peptidergic nerves with lymphocytes in canine and monkey ileal villi. Okajimas Folia Anat Jpn 69(5):199–208
120. Stead RH, Dixon MF, Bramwell NH, Riddell RH, Bienenstock J (1989) Mast cells are closely apposed to nerves in the human gastrointestinal mucosa. Gastroenterology 97:575–585
121. Gwynne RM, Bornstein JC (2007) Synaptic transmission at functionally identified synapses in the enteric nervous system: roles for both ionotropic and metabotropic receptors. Curr Neuropharmacol 5:1–17
122. Pompolo S, Furness JB (1993) Origins of synaptic inputs to calretinin immunoreactive neurons in the guinea-pig small intestine. J Neurocytol 22:531–546
123. Portbury AL, Pompolo S, Furness JB, Stebbing MJ, Kunze WAA, Bornstein JC et al (1995) Cholinergic, somatostatin-immunoreactive interneurons in the guinea pig intestine: morphology, ultrastructure, connections and projections. J Anat 187:303–321
124. Young HM, Furness JB (1995) Ultrastructural examination of the targets of serotonin-immunoreactive descending interneurons in the guinea-pig small intestine. J Comp Neurol 356:101–114
125. Mann PT, Southwell BR, Ding YQ, Shigemoto R, Mizuno N, Furness JB (1997) Localisation of neurokinin 3 (NK3) receptor immunoreactivity in the rat gastrointestinal tract. Cell Tissue Res 289:1–9
126. Smith TK, Spencer NJ, Hennig GW, Dickson EJ (2007) Recent advances in enteric neurobiology: mechanosensitive interneurons. Neurogastroenterol Motil 19:869–878
127. Bieger D, Hopkins DA (1987) Viscerotopic representation of the upper alimentary tract in the medulla oblongata in the rat: the nucleus ambiguus. J Comp Neurol 262:546–562
128. Jean A (2001) Brain stem control of swallowing: neuronal network and cellular mechanisms. Physiol Rev 81:929–969
129. Ingelfinger FJ (1958) Esophageal motility. Physiol Rev 38:533–584
130. Neuhuber WL, Wörl J, Berthoud HR, Conte B (1994) NADPH-diaphorase-positive nerve fibers associated with motor endplates in the rat esophagus: new evidence for co-innervation of striated muscle by enteric neurons. Cell Tissue Res 276:23–30
131. Kuramoto H, Kato Y, Sakamoto H, Endo Y (1996) Galanin-containing nerve terminals that are involved in a dual innervation of the striated muscles of the rat esophagus. Brain Res 734: 186–192
132. Wörl J, Mayer B, Neuhuber WL (1997) Spatial relationships of enteric nerve fibers to vagal motor terminals and the sarcolemma in motor endplates of the rat esophagus: a confocal laser scanning and electron-microscopic study. Cell Tissue Res 287:113–118
133. Wu M, Majewski M, Wojtkiewicz J, Vanderwinden J-M, Adriaensen D, Timmermans J-P (2003) Anatomical and neurochemical features of the extrinsic and intrinsic innervation of the striated muscle in the porcine esophagus: evidence for regional and species differences. Cell Tissue Res 311:289–297
134. Izumi N, Matsuyama H, Ko M, Shimizu Y, Takewaki T (2003) Role of intrinsic nitrergic neurones on vagally mediated striated muscle contractions in the hamster oesophagus. J Physiol 551:287–294

135. Breuer C, Neuhuber WL, Wörl J (2004) Development of neuromuscular junctions in the mouse esophagus: morphology suggests a role for enteric coinnervation during maturation of vagal myoneural contacts. J Comp Neurol 475:47–69
136. Reynolds RPE, El-Sharkawy TY, Diamant NE (1984) Lower esophageal sphincter function in the cat: role of central innervation assessed by transient vagal blockade. Am J Physiol 246: G666–G674
137. Diamant NE, Akin A (1972) Effect of gastric contraction of the lower esophageal sphincter. Gastroenterology 63:38–44
138. Franzi SJ, Martin CJ, Cox MR, Dent J (1990) Response of canine lower esophageal sphincter to gastric distension. Am J Physiol 259:G380–G385
139. Kelly KA (1981) Motility of the stomach and gastroduodenal junction. In: Johnson LR (ed) Physiology of the gastrointestinal tract. Raven Press, New York, pp 393–410
140. Kelling G (1903) Untersuchungen über die Spannungszustände der Bauchwand, der Magen- und der Darmwand. Z Biol 44:161–267
141. Cannon WB, Lieb CW (1911) The receptive relaxation of the stomach. Am J Physiol 29: 267–273
142. Abrahamsson H, Jansson G (1969) Elicitation of reflex vagal relaxation of the stomach from pharynx and esophagus in the cat. Acta Physiol Scand 77:172–178
143. Abrahamsson H, Jansson G (1973) Vago-vagal gastro-gastric relaxation in the cat. Acta Physiol Scand 88:289–295
144. Wilbur BG, Kelly KA (1973) Effect of proximal gastric, complete gastric, and truncal vagotomy on canine gastric electric activity, motility and emptying. Ann Surg 178:295–303
145. Abrahamsson H (1973) Vagal relaxation of the stomach induced from the gastric antrum. Acta Physiol Scand 89:406–414
146. Andrews PLR, Davis CJ, Bingham S, Davidson HI, Hawthorn J, Maskell L (1990) The abdominal visceral innervation and the emetic reflex: pathways, pharmacology, and plasticity. Can J Physiol Pharmacol 68:325–345
147. Cannon WB (1911) The mechanical factors of digestion. Edward Arnold, London
148. Cannon WB (1912) Peristalsis, segmentation and the myenteric reflex. Am J Physiol 30: 114–128
149. Andrews PLR, Grundy D, Scratcherd T (1980) Reflex excitation of antral motility induced by gastric distension in the ferret. J Physiol 298:79–84
150. Moore FD, Chapman WP, Schulz MD, Jones CM (1946) Transdiaphragmatic resection of the vagus nerves for peptic ulcer. N Engl J Med 234:241
151. Mroz CT, Kelly KA (1977) The role of the extrinsic antral nerves in the regulation of gastric emptying. Surg Gynecol Obstet 145:369–377
152. Daniel EE, Sarna SK (1976) Distribution of excitatory vagal fibers in canine gastric wall to control motility. Gastroenterology 71:608–613
153. Schemann M, Wood JD (1989) Synaptic behaviour of myenteric neurones in the gastric corpus of the guinea-pig. J Physiol 417:519–535
154. Beani L, Bianchi C, Crema A (1971) Vagal non-adrenergic inhibition of guinea-pig stomach. J Physiol 217:259–279
155. Meulemans AL, Helsen LF, Schuurkes JAJ (1993) Role of NO in vagally-mediated relaxations of guinea-pig stomach. Naunyn Schmiedebergs Arch Pharmacol 347:225–230
156. Hennig GW, Brookes SJH, Costa M (1997) Excitatory and inhibitory motor reflexes in the isolated guinea-pig stomach. J Physiol 501:197–212
157. Sanders KM, Koh SD, Ward SM (2006) Interstitial cells of Cajal as pacemakers in the gastrointestinal tract. Annu Rev Physiol 68:307–343
158. Furness JB (2012) The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol 9:286–294
159. Gwynne RM, Thomas EA, Goh SM, Sjövall H, Bornstein JC (2004) Segmentation induced by intraluminal fatty acid in isolated guinea-pig duodenum and jejunum. J Physiol 556:557–569

160. Ferens D, Baell J, Lessene G, Smith JE, Furness JB (2007) Effects of modulators of Ca^{2+}-activated, intermediate-conductance potassium channels on motility of the rat small intestine, in vivo. Neurogastroenterol Motil 19:383–389
161. Wright EM, Loo DDF (2000) Coupling between Na^{+}, sugar, and water transport across the intestine. Ann N Y Acad Sci 915:54–66
162. Sjövall H, Abrahamsson H, Westlander G, Gillberg R, Redfors S, Jodal M et al (1986) Intestinal fluid and electrolyte transport in man during reduced circulating blood volume. Gut 27:913–918
163. Podewils LJ, Mintz ED, Nataro JP, Parashar UD (2004) Acute, infectious diarrhea among children in developing countries. Semin Pediatr Infect Dis 15:155–168
164. Kuntz A, Saccomanno G (1944) Reflex inhibition of intestinal motility mediated through decentralized prevertebral ganglia. J Neurophysiol 7:163–170
165. Szurszewski JH, Ermilov LG, Miller SM (2002) Prevertebral ganglia and intestinofugal afferent neurones. Gut 51:i6–i10
166. Semba T (1954) Studies on the entero-gastric reflexes. Hiroshima J Med Sci 2:323–327
167. Schapiro H, Woodward ER (1959) Pathway of enterogastric reflex. Proc Soc Exp Biol Med 101:407–409
168. Lin HC, Zhao X-T, Wang L (1997) Intestinal transit is more potently inhibited by fat in the distal (ileal brake) than in the proximal (jejunal brake) gut. Dig Dis Sci 42:19–25
169. Castelucci P, Robbins HL, Furness JB (2003) $P2X_2$ purine receptor immunoreactivity of intraganglionic laminar endings in the mouse gastrointestinal tract. Cell Tissue Res 312: 167–174
170. Berthoud HR, Kressel M, Raybould HE, Neuhuber WL (1995) Vagal sensors in the rat duodenal mucosa: distribution and structure as revealed by in vivo DiI tracing. Anat Embryol 191:203–212
171. Lomax AE, Sharkey KA, Furness JB (2010) The participation of the sympathetic innervation of the gastrointestinal tract in disease states. Neurogastroenterol Motil 22:7–18

Chapter 4
Intestinal Barrier Function and the Brain-Gut Axis

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Abstract The luminal-mucosal interface of the intestinal tract is the first relevant location where microorganism-derived antigens and all other potentially immunogenic particles face the scrutiny of the powerful mammalian immune system. Upon regular functioning conditions, the intestinal barrier is able to effectively prevent most environmental and external antigens to interact openly with the numerous and versatile elements that compose the mucosal-associated immune system. This evolutionary super system is capable of processing an astonishing amount of antigens and non-immunogenic particles, approximately 100 tons in one individual lifetime, only considering food-derived components. Most important, to develop oral tolerance and proper active immune responses needed to prevent disease and inflammation, this giant immunogenic load has to be managed in a way that physiological inflammatory balance is constantly preserved. Adequate functioning of the intestinal barrier involves local and distant regulatory networks integrating the so-called brain-gut axis. Along this complex axis both brain and gut structures participate in the processing and execution of response signals to external and internal changes coming from the digestive tract, using multidirectional pathways to communicate. Dysfunction of brain-gut axis facilitates malfunctioning of the intestinal barrier, and vice versa, increasing the risk of uncontrolled immunological reactions that may trigger mucosal and brain low-grade inflammation, a putative first step to the initiation of more permanent gut disorders. In this chapter, we describe the structure, function and interactions of intestinal barrier, microbiota and brain-gut axis in both healthy and pathological conditions.

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_4,

Abbreviations

ACTH	Corticotropin
CNS	Central nervous system
CRF	Corticotropin-releasing-factor
DSS	Dextran sulphate sodium
ENS	Enteric nervous system
GALT	Gut-associated lymphoid tissue
GCs	Goblet cells
HNPs	Human neutrophil peptides
HPA	Hypothalamic pituitary-adrenal axis
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
JAMs	Junctional adhesion molecules
MAPKs	Mitogen-activated protein kinases
MARVEL	MAL and related proteins for vesicle trafficking and membrane link
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MUC	Mucins
NGF	Nerve growth factor
NLRs	Nod-like receptors
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular patterns
POFUT1	Protein O-fucosyltransferase 1
PRR	Pattern recognition receptors
RELM	Resistin-like molecule
TJs	Tight junctions
TNBS	Trinitrobenzene sulphonic acid
ZO	Zonula occludens

Introduction

The survival of living organisms greatly depends on the ability of species and individuals to constantly provide a series of complex and dynamic repository responses to counteract internal and environmental threats. This functional equilibrium, named homeostasis, relies upon the adequate integration of every generated response to a threat. At the gastrointestinal level, the mucosal surfaces are the first location where immunogenic particles, environmental toxins and microorganism-derived antigens gain access to the immune system [1]. The luminal side of the mucosa of the ileum and jejunum is coated with hundreds of tiny finger-like structures called villi, which in turn are composed by myriads of microvilli, rendering a final physical contact area of about 400 m^2. This enormous epithelial surface area favours nutrient absorption and water and electrolyte transport across. However, it also designed to select which luminal antigens should face the

components of the mucosal-associated immune system. This selection process is aimed at preventing the generation of inadequate pro-inflammatory signals [2]. Mucosal processing of antigens and non-immunogenic molecules will at the end, determine whether tolerogenic or non-tolerogenic immune responses are raised to keep homeostasis [3].

The intestinal mucosal barrier consists of different consecutive layers, including the intestinal flora and external mucus, the columnar epithelium and extracellular matrix below, and the innermost *lamina propria*. Within the *lamina propria* we can find blood and lymph vessels, a plethora of resident immune cells (plasma cells, lymphocytes, macrophages, eosinophils, mast cells, dendritic cells, etc.), and a significant number of intrinsic and extrinsic nerve terminals (Fig. 4.1). All of these components may display effector and modulatory functions relevant to the control of inflammation, absorption and secretion, transport of macromolecules and metabolic processes [4]. Considerable evidence now supports the existence of multidirectional communication between the components of this local regulatory network [5, 6]. Communication is driven by the release of chemical mediators, such as neuropeptides, neurohormones, neurotransmitters, cytokines, chemokines, growth factors, and other regulatory molecules.

The regulation of gut physiology is also achieved through the activity of both the enteric nervous system (ENS) and the central nervous system (CNS). ENS is an extensive neural network, also known as the second brain, containing approximately 100 million neurons embedded in the gastrointestinal lining, similar number to the spinal cord [7]. The ENS contains sensory neurons, inter-neurons, and motor neurons, which primarily control motility, absorption and secretion, but also visceral sensitivity. In addition, the ENS is wired with multiple terminals from ascending and descending CNS pathways that help to control gut function. To understand gut physiology and pathology, it is of particular importance to consider the role of the autonomic nervous system, and the hypothalamic pituitary-adrenal axis (HPA) because both systems also establish a vast and complex array of integrative and bidirectional interactions between the brain and the gut, the brain-gut axis.

The Intestinal Barrier

The intestinal barrier has evolved to guarantee homeostasis through the execution of basic weeping off functions, such as water secretion, to wash off harmful substances that may be present in the intestinal lumen, and by the development of a programme, that includes active immunological surveillance. One of the first steps to fight unwanted or harmful stimuli involves the release of mucus, defensins, secretory-immunoglobulin A, and other chemical mediators to the lumen [8]. In addition, the importance of maintaining epithelial permeability tight to prevent the passage of noxious substances, was emphasized in the early 1990s [9], and reiterated by many authors thereafter.

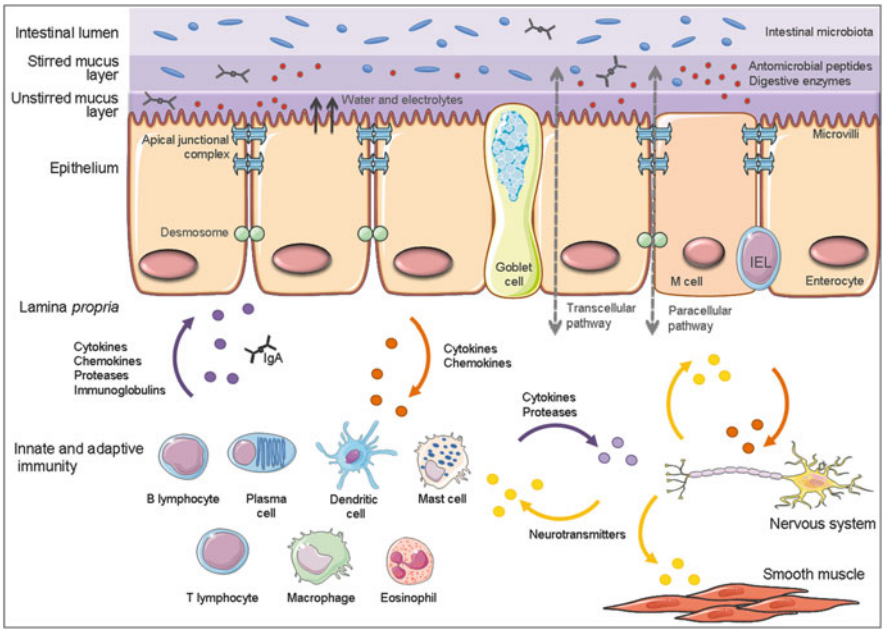

Fig. 4.1 Intestinal barrier function. The intestinal barrier has evolved to guarantee homeostasis through the execution of basic weeping off functions, such as water secretion and intestinal peristaltism, and by the development of immunological surveillance. This barrier is composed by several levels of protection aimed at preventing and selecting toxin and antigen penetration across. The most external laters harbours mucus, enzymes, antimicrobial peptides and the intestinal microbiota. Just below, a single-cell layer of epithelial cells, sealed by intercellular junctions, regulates the transcellular and paracellular passage of substances. Intermingled goblet cells secrete mucins that dissolve in water to form mucus, a major contributor to the retention of secretions containing antibacterial peptides and digestive enzymes, and to keep epithelial hydration. The epithelium also displays microbial recognition receptors and is able to release immune mediators. Lamina propria leukocytes produce proteases and cytokines to modify epithelial secretory activity and permeability range of the epithelium. M cells are found in the follicle-associated epithelium of the Peyer's patches and transport antigens from the luminal side to immune cells across the epithelial barrier. IgA is produced by plasma cells, and transported through, and secreted by, the epithelium to the luminal side. Both, the central and the enteric nervous system, interact with the immune system, the smooth muscle and the epithelium to regulate immune responses, absorption and secretion, motility, and also visceral sensitivity. *Note*: *IEL* intraepithelial lymphocyte

Structure and Function of Intestinal Barrier

Mucus

The entire intestinal mucosal surface is covered by a layer of mucus gel, thicker than 100 μm secreted by goblet cells (GCs). Mucus protects the epithelial lining from luminal sheer forces, adhesion and invasion by microorganisms, dietary, chemical and radiation toxins, and other antigens present in the intestinal lumen

[10]. The mucus layer also contributes to the retention of mucosal secretions containing antibacterial peptides and digestive enzymes [11, 12] and keeps epithelial hydration. Mucus seems to participate in epithelial renewal, differentiation and integrity, and relates to other biological processes [13]. More recently, mucus has also been shown to enhance oral tolerance by imprinting dendritic cells with anti-inflammatory properties through the assembly of a galectin-3-dectin-1-FcγRIIB receptor complex that activated β-catenin, interfering with the expression of inflammatory, but not tolerogenic cytokines by dendritic cells [14].

Components of mucus include water, phospholipids, the negatively charged mucins (MUC), which provide a chemical barrier to protect the underlying epithelium, and a variety of trefoil factors and other antimicrobials such as secretory IgA [15], cathelicidins and defensins that provide the physical and immune protection against luminal agents [16]. Mucus secreted at the apical brush border binds the glycocalyx to form a viscoelastic gel with hydrophobic and surfactant properties, dependent on the presence of phospholipids at the most apical part. Hydrophobicity helps to fight enteric bacteria and to regulate gut permeability [17].

MUC represent the most abundant component of the mucus gel. MUC are huge glycoproteins composed of a central protein backbone rich in serine, threonine and proline. These glycoproteins are highly glycosylated by attached oligosaccharides, which contain blood group structures and are initiated by *N*-acetyl-galactosamine that is O-linked onto serine or threonine at the protein core [18–20]. These O-linked oligosaccharides are responsible for MUC properties. Up to 20 different MUC genes have been identified to date (MUC1 to MUC20) [21], with site and cell-specific expression. Several secreted mucins (MUC2, MUC5AC, MUC5B, and MUC6) function as extracellular viscous secretions whereas others appear as membrane-associated mucins (MUC1, MUC3 and MUC4) in the glycocalyx [22]. MUC1–4 represent the most abundant secreted mucins in the human intestine. The first identified human secretory mucin was MUC2 that is also the principal secreted MUC [23], and is normally restricted to GCs [24]. In mice, it has been shown that colonic mucus consists of two layers with similar protein composition, being MUC2 the major structural component. The inner layer is firmly attached to the epithelium and functions as a barrier to prevent bacterial invasion while the outer layer is a loose matrix usually colonized by bacteria [25]. Thickness of the inner mucus layer varies down along the intestine according to luminal concentration of bacteria, being thicker at the highly colonized colonic segment, and thinner at the less colonized small intestine [26]. Baseline secretion of MUC is a constitutive pathway where small vesicles transport MUC directly to the cell surface where immediate and full exocytosis of their contents takes place. The release and secretion of packaged MUC is a different pathway regulated by specific stimuli including microbes and their products, and neuroendocrine and inflammatory/immune mediators. Mucus production is tightly regulated by different protein families, such as MUC and protein O-fucosyltransferase 1 (POFUT1) family members. Dysfunction of mucus secretion can lead to the development of intestinal inflammation as shown by the susceptibility of MUC2 KO mice to develop spontaneous colitis, and by a more severe intestinal response to the administration of

dextran sulphate sodium (DSS) [27]. These mice also display impaired host resistance to parasitic infection [28], and over-enhanced susceptibility to *Salmonella enterica* serovar *typhimurium* [29]. Decreased production and alteration of the O-glycosylation profile of MUC2 has been associated with increased inflammation in ulcerative colitis [30, 31]. Moreover, increased susceptibility to ulcerative colitis [32] and Crohn's disease [33] has been linked to a rare variable number of tandem repeat alleles of the MUC3 gene. Mice defective in intestinal POFUT1 exhibit chronic intestinal inflammation in association with an alteration of mucus-associated flora, goblet cell hyperplasia and hypertrophy and elevated production of mucus [34].

Resistin-like molecule (RELM)-β is a cysteine-rich protein also present in the mucus layer and specifically produced by intestinal GCs. RELM-β upregulates MUC2 and M1/MUC5AC gene expression in the human colonic HT29 cell line. Pretreatment of murine colon with RELM-β significantly attenuates trinitrobenzene sulphonic acid (TNBS)-induced colitis [35] while RELM-β deficient mice show increased susceptibility to T-cell-dependent TNBS-induced colitis. Therefore, available evidence suggests that RELM-β plays an important role in colonic inflammation [36].

Trefoil factors, a group of small cysteine-rich peptides, are also essential protective components of the mucus layer and contribute to mucosal repair, particularly, the trefoil factor 3 synthesized and secreted by intestinal GCs [37, 38]. Trefoil factor 3 deficient mice are highly susceptible to DSS, chemotherapy and radiation-induced colitis [39, 40], and display prominent hypoxia-elicited increases in intestinal permeability [41].

Epithelial Lining

The intestinal epithelium is a single polarized continuous layer of columnar cells of only 20 μm thick that covers the intestinal surface and separates the intestinal lumen from the internal milieu. Although it functions primarily as a physical barrier, it also regulates the absorption of dietary nutrients, water and electrolytes. The passage of molecules from the intestinal lumen to the *lamina propria* takes place mainly through two different routes: (1) The paracellular pathway, which allows small molecules (<600 Da) diffuse through tight junctions (TJs) located between adjacent intestinal epithelial cells; and, (2) The transcellular pathway, which allows the passage of larger particles through the epithelial cells via endocytosis or exocytosis processes [42].

The intestinal epithelium contains several stem cell-derived cellular types, such as absorptive enterocytes, GCs, Paneth cells, enteroendocrine cells, and M cells, as shown in panel 1 of Fig. 4.2. This epithelial population renews every 3–5 days from pluripotential stem cells located in the intestinal crypts to ensure cellular integrity all along the intestinal epithelium. Pluripotential stem cells migrate to the tip of the villus where final differentiation takes place [43]. Signalling cascades such as the *wnt* and the Notch pathway are involved in epithelial proliferation and differentiation, essential processes to regulate homeostasis in the intestinal epithelium [44].

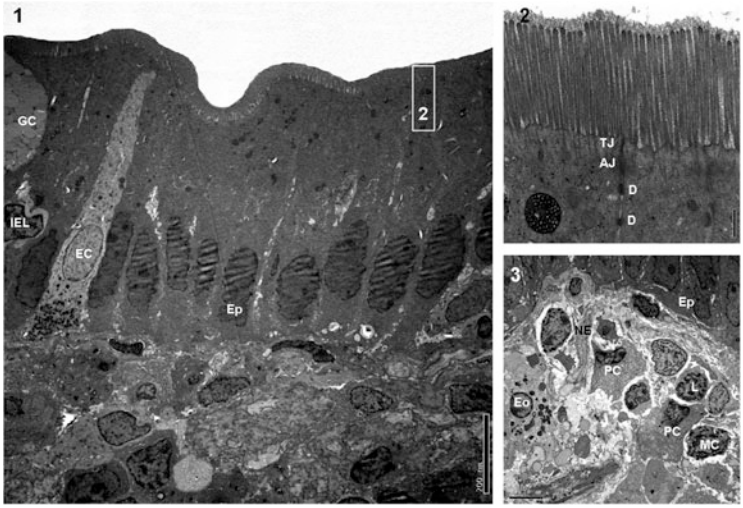

Fig. 4.2 Ultrastructure of the intestinal mucosa. Transmission electron micrographs of the intestinal epithelium and the lamina *propria* of the human jejunum. The intestinal mucosa is responsible for nutrient absorption and water secretion, which require a selectively permeable barrier. **Panel 1—Intestinal epithelium**. The epithelium functions primarily as a physical barrier between the external environment and the internal milieu. It is composed by enterocytes, secretory cells and immune cells, all supported on the basal side by a basement membrane underneath which the lamina *propria* harbors blood and lymph vessels, resident immune cells and nerve terminals. *GC* goblet cell, *IEL* intraepithelial lymphocyte, *EC* enterochromaffin cell, *Ep* epithelial cell. **Panel 2—Intercellular junctions**. The epithelial cells are polarized cells bound together through specific junctions. The apical junctional complex delineates the apical and the basal regions of the epithelial cells. It limits the uptake of microbial and food-derived antigens and prevents the passage of cellular elements across. TJs are located at the most apical site of the epithelium followed by the subjacent adherens junction and the desmosomes. *TJ* tight junction, *AJ* adherens junction, *D Desmosome*. **Panel 3—Lamina *propria***. Most of the immune elements of the intestinal barrier are located in the lamina *propria*, where they develop innate and adaptive responses in coordination with the nervous system and the epithelium. *Eo* eosinophil, *NE* nerve endings, *PC* plasma cell, *MC* mast cell, *L* lymphocyte, *Ep* epithelial cell

Enterocytes

Enterocytes are key elements of the epithelial lining. Although, the most important endeavour of these cells is to maintain the integrity of the intestinal physical barrier, enterocytes reinforce barrier strength by also developing immunologic activity. Enterocytes express innate immune receptors [45], act as non-professional antigen presenting cells, and release several chemokines and cytokines such as fractalkine [46] or thymic stromal lymphopoietin [47] involved in leukocyte recruitment and in dendritic cell regulation.

Enterocytes are tightly bonded to each other through the apical junctional complex that separates the apical membrane from the basolateral membrane. This apical junctional complex is composed TJs, adherens junctions, and desmosomes,

as shown in panel 2 of Fig. 4.2. The junctional complex limits the uptake of microbial and food derived antigens and prevents the passage of cellular elements across. TJs are located at the most apical site of the epithelium and composed of intracellular and surface-membrane proteins. Intracellular proteins are zonula occludens (ZO)-1, ZO-2 and ZO-3, as well as cingulin. Surface-membrane or transmembrane proteins include occludin, claudins, and junctional adhesion molecules (JAMs). TJs seal the intercellular space and regulate intestinal permeability. Adherens junctions are located below TJs and mainly composed by e-cadherin, catenin, and actin filaments. These protein complexes provide the necessary strength to hold the cells together.

Occludin was the first TJ transmembrane protein identified. It belongs to the TJ-associated MAL and related proteins for vesicle trafficking and membrane link (MARVEL) proteins, and contains a MARVEL domain. The function of occludin remains to be elucidated. On one hand, occludin deficient mice do not show alterations in TJ assembly and permeability [48], but, on the other hand, occludin seems to play a role in the regulation of integrity rather than in the de novo assembly of the TJs [21]. Furthermore, in vitro observations suggest that occludin localization to the TJ complex is regulated by phosphorylation [49]. Regulation of occludin phosphorylation implicates several kinases including protein kinases C, mitogen-activated protein kinases (MAPKs), Rho kinases, and the Src-Family kinases [50]. When occludin is highly phosphorylated on serine and threonine residues, it is selectively located at the TJ. In contrast, occludin dephosphorylation at those residues by protein phosphatases, results in redistribution of the protein to the cytoplasm [24].

The claudin family of transmembrane proteins consists of 24 members with a molecular weight ranging from 20 to 27 kDa. Each member shows a specific organ and tissue distribution. This protein family plays a central role in the regulation of barrier function. Some claudins make up pores that allow preferential passage of specific ions, while others reduce the transit of specific ions. The strength, size, and ion selectivity of TJs is determined by claudins, as reflected by massive transepidermal water loss and death of mice within one day of birth affecting claudin-1 deficient mice [51]. Moreover, segmental barrier properties along the crypt-villus axis and throughout the length of the intestine do correlate with the disposition of claudins [52, 53]. In the human intestine, both ileal and colonic mucosa express tightening claudins-1, -3, -4, -5 and -7 [54, 55]. However, the expression of the permeability mediator claudin-2 is restricted to the crypt, in the colon [30, 56], yet detected in the crypt and the villus, in the small bowel [31]. Differences in the expression and distribution of claudins may reflect adaptation to specific physiological functions carried out by the different segments down the intestinal tract.

A third group of transmembrane receptors found at TJs is the family of JAMs. JAMs have been implicated in the construction and assembly of TJs [57], and in the regulation of intestinal permeability and inflammation [58]. JAM-A deficient mice display increased intestinal permeability and inflammatory cytokine production, and marked epithelial apoptosis to DSS-induced colitis [59]. More recently, reduced intestinal JAM-A expression has been described in irritable bowel

syndrome (IBS) patients, possibly contributing to intestinal barrier dysfunction in these patients [60]. JAMs are also present on blood cells, such as leukocytes, thereby contributing to the process of trans-endothelial migration [61].

The TJ transmembrane proteins, claudins, occludin, and JAMs are linked to the actomyosin fibers of the cytoskeleton by members of the ZO family [62]. This association to the peri-junctional actomyosin ring seems crucial for the dynamic regulation of permeability at paracellular spaces. Interestingly, only ZO-1 and ZO-2 are relevant for claudin recruitment, TJ formation and for epithelial barrier function [63].

Far from being static, TJs are quite mobile structures that readily adapt to changing conditions and challenging stimuli. Regulation of intestinal permeability involves different functional pathways. Fast changes in permeability occur usually via myosin light chain kinase (MLCK)-mediated cytoskeleton contraction, and by endocytosis of TJ proteins [64, 65]. In contrast, lasting permeability disturbances involve the transcriptional modulation of TJ proteins, epithelial cell apoptosis and ultrastructural alterations in the epithelium [66].

Phosphorylation of myosin II regulatory light chain (MLC) induces actomyosin cytoskeleton contraction and increased TJ junction permeability. Rho GTPases have been shown to regulate TJs through redistribution of ZO-1, and reorganization of JAM-1 away from the TJ membrane [67]. Up-regulation of zonulin expression increased intestinal permeability to bacterial and gliadin exposure. In fact, this zonulin-mediated intestinal barrier defect has been advocated to play a central role in the origin of celiac disease [68] and type 1 diabetes [69].

Secretory Cells

The intestinal epithelium also houses different types of specialized epithelial called secretory cells that contribute to the reinforcement of the intestinal epithelial barrier, mainly goblet cells, Paneth cells and enteroendocrine cells.

GCs are scattered through the epithelial lining. GCs that mainly secrete mucins, but also trefoil peptides, RELM-β and Fc-γ binding protein. GC distribution varies throughout the gastrointestinal tract, the number increasing from the duodenum to the distal colon. The number of GCs is probably regulated by the intestinal microbiota because germ-free mice have less and smaller GCs than regular mice [70].

Paneth cells are located at the base of the crypts of Lieberkühn. Similar to the other intestinal epithelial cell types, they evolve from stem cells at the bottom of the crypt. Contrary to other cell types, Paneth cells migrate downwards, to the bottom of the crypt, where they synthesize and secrete antimicrobial peptides and other proteins to the intestinal lumen. Among them, lysozyme, α-defensins, TNF-α, and secretory phospholipase A2 type IIA, contribute to maintain host-microbe homeostasis and to protect stem cells from pathogens [71, 72]. Certain defects in Paneth may be linked to the pathogenesis of Crohn's disease [73, 74] and necrotizing enterocolitis [75, 76].

Gut enteroendocrine cells spread all along the intestinal epithelium where they function as highly specialized chemoreceptors sensing changes in luminal osmolarity, pH and nutrient composition. Although they represent less than 1 % of the entire gut epithelial population, enteroendocrine cells constitute the largest endocrine organ of the human body. Products released by enteroendocrine cells include hormones, such as ghrelin, somatostatin, cholecystokinin, gastric inhibitory polypeptide, glucagon-like peptides and peptide YY, and neurotransmitters such as serotonin [77]. Enteroendocrine cells inform the brain-gut axis mostly through the activation of neural pathways [78].

The Intestinal Immune System

Mucosa-associated lymphoid tissue is a diverse and diffuse defence system found at most mucosal surfaces of the body, such as the respiratory system and the eye conjunctiva. The immune response generated by this system provides generalized immunization at all mucosal surfaces [79]. About 70 % of whole body's immune cells reside within the gastrointestinal tract shaping the gut-associated lymphoid tissue (GALT), which is conformed in two different compartments: the organized immune inductive sites, and the diffuse effector sites.

Diffuse GALT is composed of two lymphocyte populations distributed at both sides of the basal lamina. Intraepithelial lymphocytes are found between epithelial cells, above the basal lamina. *Lamina propria* lymphocytes reside in *lamina propria* along with many other types of immune cell, such as eosinophils, dendritic cells, mast cells, macrophages or plasma cells (panel 3 of Figs. 4.2 and 4.3). The majority of intraepithelial lymphocytes are CD8+ T cells that function as surface gatekeepers of the intestinal barrier because the constantly monitor and respond against luminal bacteria and other antigens. *Lamina propria* lymphocytes constitute a much more heterogeneous population, approximately 50 % of which correspond to plasma cells, 30 % to T lymphocytes, and the remaining 20 % to macrophages, dendritic cells, mast cells and eosinophils. Resident B lymphocytes complete their maturation into plasma cells, mostly producing IgA, but IgM and IgG. Activated T and B-lymphocytes express α4β7 integrin and mucosal endothelial cells of Peyer's patches, mesenteric lymph nodes and *lamina propria* of the small and large intestine constitutively express the mucosal addressin cell adhesion molecule-1 that interacts with α4β7 integrin to recirculate lymphocytes between the blood and the gastrointestinal tract [80].

Inductive sites of the GALT include organized lymphoid structures in the small intestine such as Peyer's patches, mesenteric lymph nodes, isolated lymphoid follicles, and lymphocytes and antigen-presenting cells. Peyer's patches are macroscopic lymphoid aggregates found at the submucosal levels in the antimesenteric border of the intestine. The follicle-associated epithelium covering Peyer's patches contains M cells, another special cell type that plays a role in monitoring the gut lumen and maintaining intestinal barrier function. M cells display several unique properties including apical microfolds instead of microvilli, no mucus layer, and a

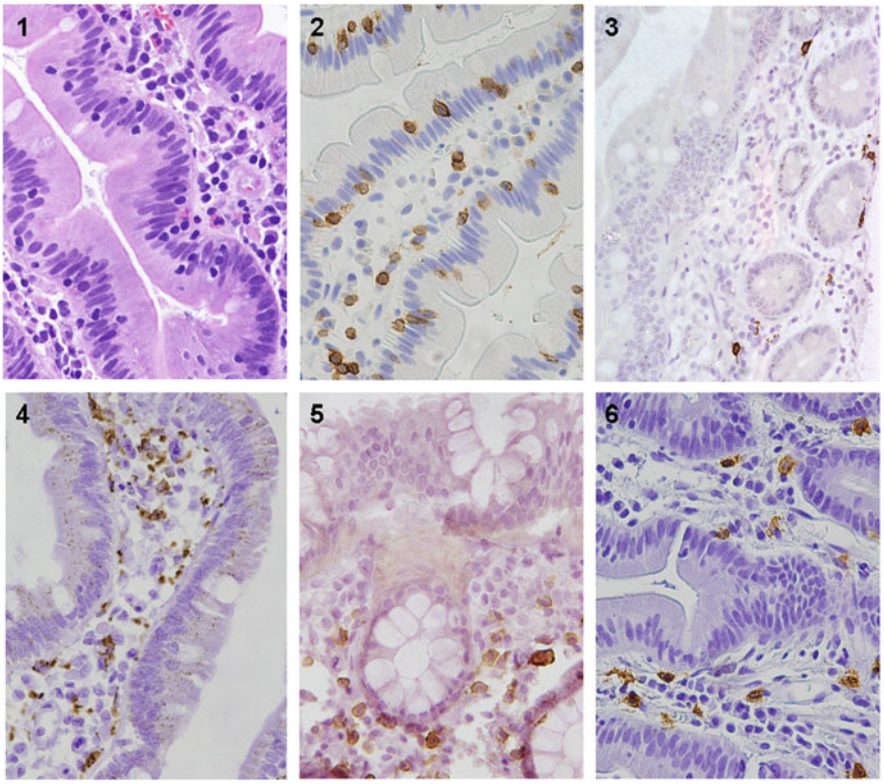

Fig. 4.3 Resident immunocytes in the intestinal mucosa. The majority of the immune cells within the body reside in the gastrointestinal tract (Gut-associated lymphoid tissue, GALT), and are distributed in two different compartments: the organized inductive sites, and the diffuse effector sites. The diffuse GALT is composed of intraepithelial lymphocytes, between the epithelial cells, and the lamina *propria* lymphocytes, which reside below the basal lamina, along with other immune cells. The figure shows intestinal micrographs (×400 magnification) processed for H&E staining to identify mucosal eosinophils (*1*), and immunohistochemistry for T-lymphocytes (*2*, $CD3^+$), B-lymphocytes (*3*, $CD20^+$), macrophages (*4*, $CD68^+$), plasma cells (*5*, $CD138^+$), and mast cells (*6*, $CD117^+$)

reduced glycocalyx, which facilitate the capture of luminal antigens and microorganisms and their transport to contact underlying immune cells [81]. Peyer's patches also contain antigen-presenting cells, mainly dendritic cells, but also macrophages. These antigen-presenting cells capture luminal antigens (taken up by M cells in the Peyer's patch dome), to further process and present them to immunocompetent cells in association with the major histocompatibility complex.

Innate immunity is present in both animals and plants [82]. It serves the host defence via immediate, but non-specific, responses to a wide variety of pathogens. The main components of innate immune response include pattern recognition receptors (PRRs), and antimicrobial peptides.

PPRs are a class protein that responds to small molecular sequences consistently found on pathogens, named pathogen-associated molecular patterns (PAMP). PRRs include Toll-like receptors (TLRs) and Nod-like receptors (NLRs).

The TLR family consists of at least 13 transmembrane receptors containing a large leucine-rich repeats extracellular domain that recognizes different bacterial, viral, parasite or self-derived ligands, such as lipopolysaccharide, peptidoglycan, muramyl dipeptide, lipoteichoic acids, and bacterial DNA. After activation upon PAMP recognition, TLRs initiate downstream signalling cascades, leading to transcriptional responses and to the initiation of both innate immune responses (macrophage activation and induction of antimicrobial peptides for various cell types) and the adaptive immune response (induction of T cell responses and maturation of dendritic cells) [83]. In many tissues, mast cells, dendritic cells, monocytes/macrophages and B cells express TLRs [84]. Healthy intestinal epithelial cells express relatively low levels of TLRs, such as TLR-4, perhaps explaining why lipopolysaccharide does not induce a potent inflammatory response in normal intestine [85]. By contrast, and consistent with the idea that chronic intestinal inflammation may be the result of uncontrolled responses to components of the intestinal bacterial flora, the intestinal epithelium of patients with inflammatory bowel disease (IBD) shows increased expression of TLR-4 [86]. The cellular localization of TLRs is also influenced by the polarized epithelial cell organization. TLR5 is expressed on the basolateral surface of intestinal epithelia only, where if becomes stimulated by luminal flagellin exposure when disruption of the epithelial barrier. Therefore, its localization prevents inappropriate stimulation by flagellin, but allows recognition of invasive pathogens [87]. Similarly, TLR9 activation through apical and basolateral surface domains induces distinct transcriptional responses. Whereas basolateral TLR9 strongly stimulates proinflammatory chemokine secretion, through NF-kappaB activation, apical TLR9 stimulation invokes a unique response in which ubiquitinated IkappaB accumulates in the cytoplasm preventing NF-kappaB activation conferring mucosal tolerance towards microbial exposure [88].

NLR constitutes a large family of 23 intracellular PRRs, being nucleotide-binding oligomerization domain (NOD)1, NOD2 and NALP3 the most extensively described. NOD1 and NOD2 recognize intracellular bacterial cell products, and NALP3 responds to multiple stimuli to form a multi-protein complex termed the NALP3 inflammasome, which promotes the release of the IL-1 family of cytokines. Most NLRs share a similar structure consisting of a centrally located NOD, a C-terminal leucine-rich repeat that detects PAMPs, and a variable N-terminal domain that is critical for downstream signalling through the recruitment of adaptors or effector molecules [89]. NOD1 recognizes γ-D-glutamyl-*meso*-diaminopimelic acid, which is found in the peptidoglycan structures of all gram-negative as well as in several gram-positive bacteria [90]. In contrast, NOD2 recognizes muramyl dipeptide, which is found in nearly all gram-positive and gram-negative organisms [91]. Upon ligand recognition, NOD1 and NOD2 induce the activation of NF-kappaB and MAPKs pathways leading to the activation of both innate and adaptive immune responses. In contrast, other NLRs such as Ipaf and

cryopyrin respond to microbial components through the assembly of multiprotein complexes termed "inflammasomes" that promote caspase-1 activation to generate the proinflammatory cytokines IL-1β and IL-18 [92]. NOD1 is expressed by intestinal epithelial cells [93] while NOD2 expression is predominantly found in monocytes and Paneth cells [73]. Both NOD1 and NOD2 have been shown to modulate inflammation and mediate efficient clearance of bacteria from the mucosal tissue during *Salmonella* colitis [94]. In addition, NOD2-deficient mice display an increased load of commensal resident bacteria, and a diminished ability to prevent intestinal colonization by pathogenic bacteria [95]. NOD-2 mutations have been identified in Crohn's disease patients and could be related to an impaired release of antimicrobial peptides from Paneth cells [96].

Antimicrobial peptides are endogenous antibiotics that are constitutively expressed in intestinal epithelial cells, yet may be also inducible in immune cells and Paneth cells [97]. They include compounds such as lactoferrin, hepcidin, bactericidal/permeability increasing protein, lysozyme and overall, defensins and cathelicidins.

Defensins are a family of small cationic peptides (29–45 amino acids) that exhibit a wide and potent antimicrobial activity spectrum against gram-negative, and gram-positive bacteria, fungal and yeast, parasites, viruses, and even tumor cells [98]. Defensins have been identified in both prokaryotes and eukaryotes. Although structurally different, most defensins display cationic and amphiphilic properties which confer them the capacity to permeabilize the bacterial cell membrane. In mammals, these peptides are expressed in mucosal epithelial cells and phagocytes, but also are released into the intestinal lumen, several grams daily, by Paneth cells [99]. Defensins act as effector and regulatory molecules of the innate immune response. In addition, defensins also enhance adaptive response acting on phagocytic cells and mast cells to induce the release of inflammatory mediators and to regulate the complement system. Defensins also interact with dendritic cells and T cells to increase antigen-specific immune response [100].

These peptides are classified as α and β-defensins according to their disulphide bond pairing pattern. The human α-defensins 1–4, conventionally referred as to neutrophil defensin (human neutrophil peptide, HNP), although defensins HNP1-3 are also expressed in epithelial cells of inflammed mucosa [101]. In contrast, human α-defensin 5 and 6 (HD5 and HD6) are only expressed in Paneth cells of the small intestine [102]. HD5 has been shown to induce IL-8 expression on intestinal epithelial cells [103], and to protect mice from DSS colitis and *Salmonella* infection [104]. More recently, HD6 has been shown to form fibrils and nanonets that surround and entangle bacteria to protect the small intestine against invasion by diverse enteric pathogens [105].

Human β-defensin-1 is constitutively expressed in the small intestine and the colon. In contrast, Human β-defensins-2-4 expression is inducible [106] in inflammatory conditions such as IBD [107, 108] or infection by enteroinvasive bacteria [109].

The other major class of antimicrobial peptides is the cathelicidin group. In mammals, about 35 members have been identified, but only one in humans:

hCAP18/LL37 [110]. Although regarded as neutrophil specific, hCAP18/LL37is also expressed in other leukocytes, keratinocytes and epithelial cells of the respiratory, genitourinary and gastrointestinal tract [111], and in human breast milk [112].

Expression of hCAP18/LL37 in human colonic epithelial cells has been related to cell differentiation [113]. Infection of intestinal epithelial cells by *Shigella* spp. inhibits the expression of hCAP18/LL37 [114], while bacterial components such as sodium butyrate [115] or TLR-ligands such as bacterial DNA [116] induce its expression.

Acquired immunity is restricted to vertebrates and constitutes a second line of defence against pathogens. It is driven by B and T lymphocytes through specific receptors and confers protection against re-exposure to the same antigen. Antigen binding to these receptors results in clonal expansion of these cells and the initiation of a directed immune response. Functionally speaking, within the adaptive immunity, we can distinguish inductive and effector compartments. Antigen presentation and naive T and B-lymphocytes activation occurs in the inductive compartment. In the effector compartment sensitized cells against different antigens extravasate and differentiate to carry out the destruction of pathogens. IgA secretion has been shown to be regulated through TLR-signalling [117] but also by changes in the composition of intestinal Microbiota [118].

Intestinal Barrier Dysfunction

Stress, Hormones and Neurotransmitters

Stress represents a threat to the internal homeostasis. In response to stress, a coordinated response is initiated to maintain stability through the autonomic, endocrine, and immune systems. The main systems activated during the stress response are the sympatho-adrenomedullary, a component of the sympathetic division of the autonomic nervous system, and the HPA axis. The autonomic nervous system provides, through its sympathetic and parasympathetic arms, the fastest response to stressor exposure, leading to rapid alterations in physiological state through neural innervation of end organs. Stress activation of the HPA axis stimulates the *parvocellular* neurons in the *paraventricular nucleus* of the hypothalamus to secrete corticotropin-releasing-factor (CRF), which in turn travels to the anterior pituitary to promote the synthesis of corticotropin (ACTH) [119]. ACTH, when released into the systemic circulation, activates the adrenal cortex to induce cortisol and corticosterone secretion that circulate through the bloodstream to reach every tissue [120]. Adaptation to stress through the activation of the sympatho-adrenomedullary system and the HPA axis to maintain homeostasis is called “allostasis”. However, excessive stress exposure impairs this adaptive response, eventually predisposing these subjects to the development of disease or to

exacerbation of previous existing ones [121], specially in stress-sensitive disorders, like IBS.

At the experimental level, different type of stresses, acute and chronic, physical or psychological, have been shown to influence properties of the intestinal barrier function, including as ion and water secretion, intestinal permeability, mucus secretion, and also intestinal flora. Ion and water secretion allows the intestine to wash away noxious substances present in the intestinal lumen, preventing adhesion to the mucosal surfaces and penetration to the *lamina propria*. The jejunum of rats submitted to restraint stress or cold restraint stress was found to show an increase its baseline short-circuit current, indicative of enhanced anion secretion [122]. Later, it was observed that peripheral CRF and repetitive exposure to water avoidance stress reproduced stress-induced rat jejunal and colonic epithelial barrier dysfunction via cholinergic and adrenergic nerves and mast cells [123, 124]. More recently, it has been shown that chronic psychosocial stress also activates mucosal mast cells and increases baseline short-circuit current in both the jejunum and the colon [125]. In humans, studies using jejunal segmental perfusion techniques reveal that acute physical or psychological stress either reduce net water absorption or increase secretion in healthy subjects and in patients with food allergy [126, 127] through the parasympathetic nervous system and mast cell activation [128]. More recently, we have extended these observations to show that in healthy female volunteers that intestinal water secretion during cold pain stress was significantly reduced in those with moderate background stress compared to those with low stress [129]. This observation could indicate a loss of regulatory mechanisms in subjects suffering from continuous life stress.

Both paracellular and transcellular permeability to small and large molecules increased in response to acute and chronic stress in the rodent jejunum and colon [130–133]. Several mechanisms, including mast cells, CRF [134], MLCK, and cytokines like interferon gamma, and interleukin-4 [135] have been implicated. In humans, it is known that surgery, trauma, and gastrointestinal infections [136] increase intestinal permeability. CRF has been shown to enhance transcellular uptake of macromolecules in human colonic mucosa via CRF-R1 and CRF-R2 receptors, located on subepithelial mast cells [137]. Unpublished observations from our group indicate that intravenous CRF increased intestinal permeability in healthy subjects and in IBS patients [138]. Acute psychological stress also increases small intestinal permeability in humans and peripheral CRF reproduces the effect of stress and mast cell stabilization blocks the effect of both stress and CRF, suggesting the involvement of mast cells [139]. Cold pain stress also increased intestinal permeability in female healthy subjects, although this response was larger in women with moderate background stress. Increased intestinal permeability has been found in diarrhoea prone IBS patients [140]. These findings provide new insight into the complex interplay between the central nervous system and gastrointestinal function in man.

Acute stress causes mucin release in the rat colon, along with enhanced secretion of rat mast cell protease II and prostaglandin 2. These changes were reproduced by intravenous or intracerebral injection of CRF in non-stressed rats, and were

inhibited by the administration of a CRF antagonist or a mast cell stabilizer [141]. In addition, stress-induced release of mucin was abolished in mast-cell deficient mice, highlighting a key role of mast cells in stress-mediated mucin release [142]. In contrast, rats submitted to chronic stress displayed mucus depletion along with increased bacterial adhesion and penetration into enterocytes [143].

Stress can also induce microbiological changes in the intestinal flora. Maternal separation in infant rhesus monkeys decreased faecal bacteria, especially *Lactobacilli*, and increased their susceptibility to opportunistic bacterial infections [144]. Similarly, prenatal stress reduced the overall numbers of *Bifidobacteria* and *Lactobacilli* in the newborn infants [145]. Interestingly probiotic treatment ameliorates stress-induced changes in the gastrointestinal tract [146] and attenuates the observed *Lactobacilli* reduction in maternally-deprived rat pups [147]. In addition to these microbiological changes, dexamethasone administration in rats enhanced bacterial adherence to the mucosa, decreased secretory-immunoglobulin A secretion, and increased intestinal permeability [148]. More recently, Söderholm et al. showed that chronic psychological stress in rats, leads to an increased antigen and bacterial uptake in follicle associated epithelium from Peyer's patches [149] as well as in the villous ileal and colonic epithelium. Emotional stress during take-off in cosmonauts induced changes in faecal *Bifidobacteria* and *Lactobacillus*, as well as an increase in *Escherichia coli*, whereas a substantial increase in *Enterobacteria* and *Clostridia* was found after the flight [150]. These stress-induced changes in the faecal flora have been related to catecholamine release into the intestinal lumen and/or into the systemic circulation, as the addition of various catecholamines to cultures of gram negative bacteria resulted in dramatic increases in growth of *E. coli*, *Yersinia enterocolitica* and *Pseudomonas aeruginosa* [151].

Mast cells are known to modulate stress-mediated responses of the epithelial barrier function, to orchestrate the mucosal immune function and to participate in the defence against bacteria [152, 153]. To exert these functions, enteric mast cells are strategically located within the gastrointestinal tract, developing an optimal sensory and effector interaction within the local regulatory neuroendocrine networks. Upon activation, mast cells act as effector cells, through the selective (piecemeal degranulation) (Fig. 4.4) or massive release (anaphylactic degranulation) of preformed or newly produced biological mediators. More relevant to stress-mediated inflammation is their ability to communicate, bidirectionally, with both the enteric, autonomic and central nervous systems. Anatomical contacts between mast cells and enteric nerve fibres have been demonstrated in the human gastrointestinal mucosa and these contacts increase, when inflammation is present [154]. An increase in the nerve-to-mast cell proximity in the colonic mucosa of IBS patients has been positively correlated with the severity and frequency of abdominal pain [155]. This mast cell-enteric nerve interaction provides a physical substrate for bidirectional communication between the CNS and the gut, by which stress might influence gastrointestinal physiology. This is reflected in vivo by the release of mast cell products into the lumen of the human small intestine after cold stress, which is accompanied by increased epithelial secretion [128]. Mast cell mediators released after degranulation can sensitize mesenteric afferents and

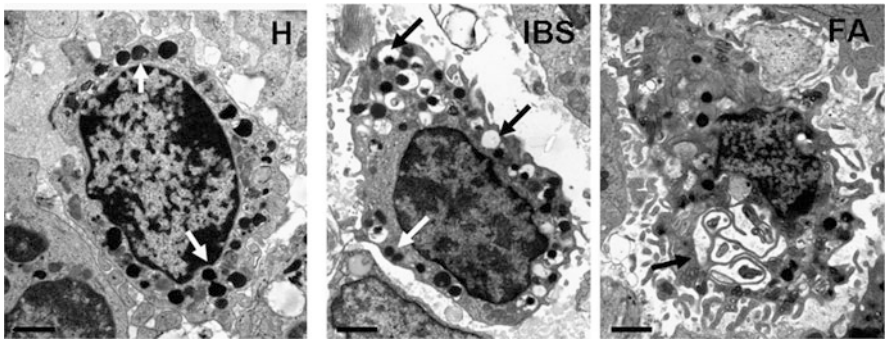

Fig. 4.4 Intestinal mast cells. Enteric mast cells are known to modulate the epithelial barrier function, to orchestrate the mucosal immunity and to participate in the defence against bacteria. They are strategically located within the gastrointestinal tract, developing sensory and effector interactions within the local regulatory neuroendocrine networks. Upon activation, mast cells act as effector cells, through the selective (piecemeal degranulation) or massive release (anaphylactic degranulation) of preformed or newly produced mediators. The figure shows transmission electron micrographs of ultrastructural characteristics of mucosal mast cells: a resting mast cell in health (*H*), with granules filled (*white arrows*) and no signs of degranulation; piecemeal degranulation in a mast cell from a patient with irritable bowel syndrome (*IBS*), identified by partial or total emptiness of granules content (*black arrows*) and intact granules (*white arrow*); and anaphylactic degranulation in a mast cell from a food allergy patient (*FA*), identified by fusion of granule membranes devoid of content (*black arrow*). *Barr* indicates 2 μm

nociceptive receptors [156]. Among the potential mast cell mediators involved, both histamine and serotonin induce intestinal secretion of water, electrolytes and mucus. In addition, mast cells from IBS patients release more histamine and tryptase than intestinal mast cells from normal subjects [157] a fact that has been linked to the generation of visceral hypersensitivity, through the activation of proteinase-activated receptors type 2. These receptors can modulate enteric neurotransmission, secretion, motility, epithelial permeability, and visceral sensitivity, and are also known to regulate intestinal inflammation [158]. However, altered expression of histamine H1 and H2 receptor subtypes has recently been reported in mucosal biopsies from distal gut of IBS patients, suggesting that these receptors could also play a role in these processes [159].

CRF and related peptides are the most important neuroendocrine factors mediating the effects of stress, both at the central and peripheral level. CRF urocortin (Ucn) 1, Ucn 2 and Ucn 3 exert their effects after binding to G protein-coupled receptor subtypes, CRF-R1 and CRF-R2, signalling through cAMP [160]. After physical or psychological stress, neural or immune release of CRF and urocortins mediate autonomic, hormonal, and behavioural responses to stress and stimulate the ENS to modulate gastrointestinal motility and secretion [161–163]. Increased CRF and urocortin expression has been demonstrated in the colonic mucosa of IBD patients [164, 165].

Vasoactive intestinal peptide is also involved in the regulation of chloride secretion, mucin release, paracellular permeability and epithelial cell proliferation

[166, 167]. Psychological stress increases vasoactive intestinal peptide levels in the small intestine of mice [168] and vasoactive intestinal peptide has been implicated in the regulation of the intestinal barrier function, through its direct effect on tight junction-associated protein, ZO-1, in epithelial cells [169].

Substance P participates in gut inflammation by interacting mainly with the neurokinin-1 receptor, expressed on nerves, epithelial, endothelial and smooth muscle cells, and immune cells, such as mast cells, macrophages, and T cells [170]. This neuropeptide has been found to stimulate macrophage and eosinophil secretion of pro-inflammatory cytokines, to increase NK cell activity and migration, and to activate the release of chemokines from leukocytes. It also induces the release of vasoactive mediators from mast cells, contributing to chloride secretion, intestinal permeability, vascular leakiness and oedema at sites of inflammation, modulating diarrhoea, inflammation, and motility [171]. Substance P mediates stress-induced CRF expression in mice eosinophils, and eosinophil-derived CRF is responsible for mast cell activation and consequently, epithelial barrier dysfunction [172].

Nerve growth factor (NGF) has been involved in the development of stress-induced barrier dysfunction [173] and hyperalgesia during inflammation [174, 175]. These effects seem to be mediated by CRF and mast cells [176, 177]. Maternal deprivation has been shown to induce hyperalgesia to rectal distension and to enhance colon permeability in association with elevated NGF expression [173]. A subsequent study from the same group showed that CRF, acting through its receptor CRF-R1, stimulated NGF release from mast cells, which in turn increased gut paracellular permeability [178]. More recently, norepinephrine has been shown to induce visceral sensitivity to colorectal distension by increasing the expression of NGF in the rat colon wall [179]. These findings support the importance of NGF in stress-induced visceral hypersensitivity, but also in stress-induced barrier dysfunction.

Sex steroids also play a role in modulating intestinal barrier, although conflicting results have been described. Estrogen can bind to two different receptors named estrogen receptor-α and β. Estrogen receptor-α mediates estrogen signalling in the development of secondary sex characteristics, and the regulation of the menstrual cycle and sperm maturation [180]. In contrast, estrogen receptor-β is mainly expressed in epithelial cells and is the most abundant estrogen receptor in the colon [181]. Both progesterone and estradiol have been shown to reduce chloride secretion in intestinal epithelial cells [182, 183], whereas estradiol has also been found to reinforce epithelial permeability [184], and to up-regulate JAM-A and occludin expression [185].

Other hormones have been involved in the regulation of intestinal barrier function (Table 4.1).

Table 4.1 Hormones and intestinal barrier

Hormone	Function	References
Glucagon-like peptide 2	Decreases intestinal permeability	[292, 293]
Growth hormone	Decreases intestinal permeability	[294, 295]
Insulin-like growth factor 1	Decreases intestinal permeability	[296, 297]
Ghrelin	Decreases intestinal permeability	[298]
KdPT	Decreases intestinal permeability	[299]

KdPT a tripeptide derivative of the C-terminus of α-melanocyte-stimulating hormone

Infections

Intestinal pathogens have developed specific strategies to gain access to the *lamina propria*. Strategies include direct TJ disruption, the production of toxins that induce fluid and electrolyte secretion, and the activation of the inflammatory cascade [186]. *Vibrio cholerae* can directly alter TJs through its cytotoxin hemagglutinin protease, a metalloproteinase that disrupts occludin-ZO-1 interactions leading to TJ and cytoskeleton anchorage destabilization [187]. In addition, other toxins have been involved in TJ disruption by *V. cholerae* such as the RTX toxin, that crosslinks actin inducing cell rounding and increased permeability [188], or the ZO toxin, that fragments ZO-1 and occludin and disrupts the actin cytoskeleton [189, 190]. *Clostridium difficile* infection produces two distinct exotoxins, Toxin A and B (TcdA and TcdB), that through RhoA GTPases inactivation cause actin filament disaggregation and cell rounding, resulting in increased paracellular permeability [191, 192]. Recent findings suggest that toxin A could even disrupt directly TJ proteins [193]. *Clostridium perfringens* enterotoxin utilizes claudin-3 and 4 as receptors [194] to bind the enterocyte surface where it forms small protein complexes in the plasma membrane that interact with other proteins forming a large complex, that at the end triggers massive permeability changes [195]. Enteropathogenic *E. coli* infection directly disrupts TJ through occludin dephosphorylation and dissociation from TJs to the cytoplasma [196] and MLC phosphorylation [197] enhancing intestinal permeability.

Intestinal Microbiota

Intestinal microbiota has been shown to influence intestinal barrier function and the brain-gut axis [198, 199]. Intestinal microflora displays several important functions to maintain gut homeostasis, such as nutrient digestion, vitamin and hormone production and most importantly, protection from microbial colonization, achieved through competition for intestinal nutrients and for attachment sites [200]. Probiotics are live microorganisms which, when consumed in adequate amounts, confer a health benefit on the host. Increasing evidence suggests that probiotics implement intestinal epithelial homeostasis and enhance barrier tightness and integrity. In contrast with pathogens, probiotics have been shown to increase

occludin expression [201], and to enhance ZO-2 expression in parallel to its redistribution towards the cell boundaries via silencing of PKCζ [202] thereby leading to TJ stabilization and the restoration of the epithelial barrier. Specific *Lactobacilus salivarus* strains prevent hydrogen peroxide-induced reduction in transepithelial resistance when added to *Caco-2* cell monolayers [203]. Similarly, *Lactobacillus rhamnosus* GG improves intestinal barrier function in the immature murine gut through the induction of claudin 3 expression [204], the regulation of apoptosis and the promotion of cytoprotective responses [205]. Interestingly, probiotics have also demonstrated beneficial effects in other tissues such as the skin barrier [206] or the respiratory tract [207, 208].

There is a significant body of evidence indicating that probiotics can also prevent intestinal barrier damage in conditions such as IBD or experimental stress. In rats, DSS-induced colitis was ameliorated by *Lactobacillus reuteri* decreasing the bacterial translocation from the intestine to mesenteric lymph nodes [209]. *E. coli* Nissle 1917 has been shown to confer protection against murine DSS colitis-associated increase in mucosal permeability through up-regulation of ZO-1 expression [210]. Moreover, a probiotic mixture of *Lactobacillus acidophilus*, *Bifidobacterium lactis*, *Lactobacillus plantarum and Bifidobacterium breve* helped to maintain the integrity of colonic mucosal barrier in the DSS model by down-regulating macrophage nitric oxide production and by enhancing mucus production [211]. In this model, the administration of a probiotic mixture prevented not only the decrease in TJ proteins expression, but also the increase of epithelial apoptotic ratio induced by acute colitis [212]. Furthermore, in patients with severe pouchitis, probiotics were able to restore the mucosal barrier, as they decreased *E. coli* K12 passage through the intestinal epithelium in Ussing chambers [213].

Probiotics also play a role in stress-induced intestinal damage and psychiatric comorbidity. *Lactobacillus farciminis* has been shown to suppress stress-induced hyperpermeability and endotoxemia, and to prevent HPA axis response and neuroinflammation in rats submitted to partial restraint stress [214]. Probiotic administration to mice submitted to food and mobility restriction increased IgA producing cells, CD4+ cells in the *lamina propria* of the small intestine, and secretory IgA in the lumen and also reduced the levels of IFN-γ [215]. *Bifidobacterium lactis* CNCM I-2494 has been shown to suppress gut hypersensitivity and colonic barrier disruption induced by partial restraint stress in rats [216, 217]. In the last years, attention has been also pointed to the potential role of microbiota in the pathophysiology of psychiatric disorders such as depression and anxiety [218] and neurodevelopmental disorders such as autism. Interestingly, treatment with the human commensal *Bacteroides fragilis* restores gut permeability, alters microbial composition, and ameliorates defects in communicative, stereotypic, anxiety-like and sensorimotor behaviors in a mouse model of the autism spectrum disorder [219]. Since psychiatric comorbidities are highly common in functional gastrointestinal disorders, the emerging role of microbiota and probiotics in the regulation of intestinal and brain barrier function and its implication in behavioral changes in the host certainly will boost investigations in this field in the years ahead.

Inflammatory Mediators

Several inflammatory mediators have been involved in intestinal barrier regulation. In vitro experiments with epithelial cell monolayers demonstrated that interferon-γ and TNF-α induce epithelial barrier dysfunction through MLCK up-regulation and MLC phosphorylation [220, 221], although they can also disrupt intestinal permeability through down-regulation of occludin transcription [222] and up-regulation of the channel-forming TJ protein claudin-2 expression. In addition, TNF-α but also IL-1β have been shown to inhibit electrogenic sodium absorption in the rat distal colon [223], and mice injected with TNF-α present diarrhoea as a consequence of Na+/H+ exchange inhibition [224].

Similarly, IL-13 and IL-4 increased paracellular permeability in a dose- and time-dependent fashion and IL-4, but not IL-13, stimulated chloride secretion in T84 cells [225] through a PI3K pathway [226]. In contrast, IL-10 has been identified as a protector cytokine in barrier function as the addition of this cytokine to T84 cells prevents interferon-γ-induced disruption of T84 monolayer barrier integrity and limits chloride secretion [227]. Moreover, IL-10 deficient mice display increased intestinal permeability [228] and most importantly, develop spontaneous colitis [229], suggesting that increased permeability predisposes to intestinal inflammation.

Although, beyond the limits of this chapter. It is to know that many other cytokines have been involved in barrier function such as IL-17A, IL-17F, IL-22, and IL-26, interferon-α, interferon-β, transforming growth factor-α, and -β [230, 231].

Nutritional Factors

Some dietary compounds are able to induce intestinal barrier dysfunction in susceptible individuals such as in celiac disease and food allergy. The gliadin fraction of wheat gluten is the environmental triggering of celiac disease. In genetically predisposed subjects gluten exposure may lead to increased intestinal permeability and inflammation. Recent evidence has shown that the increase in intestinal permeability occurs through the activation of the zonulin pathway in a MyD88-dependent fashion [232]. The protein zonulin is the target of the Zot toxin of the *V. cholerae* and has been show to play a pivotal role in TJ regulation in different autoimmune disorders such as type 1 diabetes and celiac disease [233]. Food allergies are adverse reactions against food antigens that are IgE and mast cell mediated. Altered intestinal permeability has also been involved in the pathophysiology of food allergy, as these patients display an enhancement of intestinal permeability even in the absence of food allergens [234]. Moreover, patients under tacrolimus treatment have been shown to develop new-onset food allergies that could be related to tacrolimus-induced increase in intestinal permeability [235].

In contrast with these observations, several diet products such as glutamine or butyrate have been shown to exert a protective effect on the intestinal barrier. Butyrate, a short chain fatty acid produced by intestinal microbial fermentation of dietary fibres, maintains intestinal barrier function through an increase in mucus production [236] and an enhancement in TJ protein expression [237]. Glutamine has also been shown to protect intestinal barrier function through the regulation of TJ proteins such as claudin-1, and occludin [238].

Drugs and Toxins

Ethanol has been shown to promote separation of ZO-1 proteins in Caco-2 monolayers and disassembly and displacement of perijunctional actin and myosin filaments from the perijunctional areas and MLCK activation [239]. Recent findings point to one of its metabolites, acetaldehyde, as the main toxic product for intestinal barrier because it raises tyrosine phosphorylation of ZO-1, e-cadherin, and β-catenin [240]. Further investigations revealed that the deleterious effects of ethanol require the presence of resident microflora, to oxidize ethanol into acetaldehyde in situ, and downstream mast cell activation [241], and that the ethanol-mediated increase in intestinal permeability is modulated through iNOS-mediated activation of RhoA [242] and IL-22 [243].

NSAIDs can increase intestinal permeability. Several factors play a role in NSAIDs-induced intestinal barrier dysfunction. In vitro experiments with gastric epithelial monolayers showed that barrier dysfunction was associated with decreased expression of claudin 7 and involved phosphorylation of p38 MAPK [244]. NSAIDs also affect intestinal barrier through inhibition of intestinal epithelial restitution by decreasing calpain activity and membrane-associated expression of calpain-2 [245], and also through the increase of intestinal NO synthase [246].

Other drugs causing intestinal barrier dysfunction appear in Table 4.2. It is of particular interest the development of new drugs, such as larazotide, that may decrease intestinal permeability in celiac disease by acting on TJs.

Other Disorders Associated with Barrier Dysfunction

Many other conditions such as chronic kidney disease [247], type 1 diabetes [248], primary biliary cirrhosis and primary sclerosing cholangitis [249], liver cirrhosis [250], alcoholic liver disease [251], autoimmune thyroiditis [252] and IgA nephropathy [253] have been associated with TJ dysfunction. In addition, some life threatening conditions have been related to intestinal barrier dysfunction and translocation of bacteria or/and endotoxin from gastrointestinal tract. In this line, hemorrhagic shock has been associated with increased intestinal permeability and bacterial translocation [254] through mucus damage and the generation of free radical species [255]. Estrogens exert a protective role against hemorrhagic shock-induced gut and lung injury by the activation of estrogen receptor-α, β or both [256]

Table 4.2 Drugs and intestinal barrier

Drug	Effect on permeability	References
Ethanol	Increase	[239, 241]
NSAIDs	Increase	[244, 245]
Methotrexate	Increase	[300]
Corticosteroids	Increase	[301]
Omeprazole	Increase	[302]
Cyclophosphamide	Increase	[303]
Tacrolimus	Increase	[304, 305]
Vitamin D	Decrease	[306]
Larazotide/AT1001	Decrease	[307–310]
Anti-TNF monoclonal antibodies	Decrease	[311]
Heparin	Decrease	[312]

receptors. Similarly, gut inflammation and loss of gut barrier function has been related to splachnic ischemia-reperfusion through HIF-1 activation [257]. Multiple injured patients also show an increased intestinal permeability that correlates with IL-6 levels [258]. Severe burn injury also results in the loss of intestinal barrier function involving MLCK-dependent MLC phosphorylation signalling pathway [259] and p38 MAPK activation [260] in a TLR-4-dependent process [261].

Intestinal Barrier and Disorders of the Brain-Gut Axis

The pathophysiology of several gastrointestinal disorders involves intestinal barrier dysfunction and dysregulation of brain-gut interactions, particularly functional gastrointestinal disorders including IBS and functional dyspepsia. In recent years, due to new imaging techniques, such as positron emission tomography, it has been possible to characterize the role of the CNS in modulating gut motility and visceral pain in patients with functional gastrointestinal diseases. There is significant overlap between the brain regions responsible for modulating visceral sensitivity and regions involved in emotion processing in these patients. IBS patients display a higher activation of the anterior cingulate cortex in response to rectal distension [262] that correlates with the presence of psychosocial disorders when compared to healthy subjects [263].

At the peripheral level, mucosal inflammation, increased intestinal permeability and visceral hypersensitivity are findings associated with clinical manifestations of IBS. Mast cells play a key role in IBS pathophysiology because they modulate intestinal permeability, and target visceral afferents involved in abdominal pain [155]. Stress has been associated with the development, exacerbation and perpetuation of IBS through the brain-gut-axis. Early life stress plays a major role in the vulnerability of individuals to develop IBS in adult life [264–266]. Post-traumatic stress syndrome or sexual abuse are also important risk factors in the development of IBS and functional gastrointestinal disorders [267] and both acute psychological

and physical stress have been associated with enhancement of visceral sensitivity [268] and small-intestine motility in IBS [269].

Functional dyspepsia is characterized by postprandial fullness and early satiation or by epigastric pain or burning in the absence of an organic cause Functional dyspepsia has been show to share some of the pathophysiological features of IBS. Particularly, patients with functional dyspepsia display low-grade inflammation in the duodenal mucosa, characterized by an increased infiltration of mucosal mast cells and eosinophils, and increased duodenal permeability [270]. Acute gastroenteritis has been shown to be a risk factor for functional dyspepsia development [271], as well as the presence of psychosocial comorbidities such as anxiety and depression [272], and life stress [273].

Stress, acting through the brain-gut axis, also modulates intestinal inflammatory conditions such as IBD. Social Gibbon monkeys submitted to social upheaval develop spontaneous colon inflammation [274]. Intracolonic infusion of TNBS induced a significantly higher inflammatory reaction in maternally deprived rats than in control animals [275]. Collins et al. [276] found that rats recovering from TNBS-induced colitis and submitted to mild restraint stress displayed a significant increase in myeloperoxidase activity. Moreover, overt inflammation was induced when animals were exposed to stress in combination with a small dose of TNBS, suggesting an additive effect [277]. In keeping with these findings, a significant association between stress and relapse in IBD has been reported, especially in patients with ulcerative colitis [278, 279]. Although the mechanism underlying the association between stress and IBD remains unclear, disturbances of brain-gut axis, peripheral neuroendocrine-immune interactions and altered intestinal barrier function [280–284] have been demonstrated in IBD patients [285].

Finally, a heterogeneous group of conditions associated with chronic manifestations affecting the CNS and the gut may possibly reflect the existence of primary or secondary alterations of brain-gut axis, intestinal microbiota and barrier function. This is the case of diabetes and the metabolic syndrome [286], liver encephalopathy [287], neuropsychiatric disorders [288], autism [289], chronic fatigue [290] or fibromyalgia [291], although the ultimate pathophysiological mechanisms are not well known.

Acknowledgements Supported in part by the Fondo de Investigación Sanitaria and Ciberehd, Instituto Carlos III, Subdirección General de Investigación Sanitaria, Ministerio de Ciencia e Innovación (PI12/00314, Alonso, C.; CM08/00229, Lobo, B; CM10/00155, Pigrau, M; CP10/00502, PI13/00935, Vicario, M; PI11/00716 & CB06/04/0021, Santos, J.).

References

1. Turner JR (2009) Intestinal mucosal barrier function in health and disease. Nat Rev Immunol 9(11):799–809
2. Kayama H, Nishimura J, Takeda K (2013) Regulation of intestinal homeostasis by innate immune cells. Immune Netw 13(6):227–234

3. Knoop KA, Miller MJ, Newberry RD (2013) Transepithelial antigen delivery in the small intestine: different paths, different outcomes. Curr Opin Gastroenterol 29(2):112–118
4. Wood JD (2007) Neuropathophysiology of functional gastrointestinal disorders. World J Gastroenterol 13(9):1313–1332
5. Sansonetti PJ (2004) War and peace at mucosal surfaces. Nat Rev Immunol 4(12):953–964
6. Santos J, Bienenstock J, Perdue MH (2002) Innervation of lymphoid tissue and functional consequences of neurotransmitter and neuropeptide release. In: Brostoff J, Challacombe SJ (eds) Food allergy and intolerance. W.B. Saunders, London, pp 51–67
7. Hall JE (2011) General principles of gastrointestinal function. Guyton and Hal textbook of medical physiology, 12th edn. Saunders Elsevier, p 755. ISBN: 978-1416045748
8. Shao L, Serrano D, Mayer L (2001) The role of epithelial cells in immune regulation in the gut. Semin Immunol 13(3):163–176
9. Yamada T, Sartor RB, Marshall S, Specian RD, Grisham MB (1993) Mucosal injury and inflammation in a model of chronic granulomatous colitis in rats 2. Gastroenterology 104 (3):759–771
10. Gibson P, Rosella O, Nov R, Young G (1995) Colonic epithelium is diffusely abnormal in ulcerative colitis and colorectal cancer. Gut 36:857–863
11. Farhadi A, Banan A, Fields J, Keshavarzian A (2003) Intestinal barrier: an interface between health and disease. J Gastroenterol Hepatol 18(5):479–497
12. Liévin-Le Moal V, Servin AL (2006) The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. Clin Microbiol Rev 19(2):315–337
13. Corfield AP, Carroll D, Myerscough N, Probert CS (2001) Mucins in the gastrointestinal tract in health and disease. Front Biosci 6:D1321–D1357
14. Shan M, Gentile M, Yeiser JR, Walland AC, Bornstein VU, Chen K, He B, Cassis L, Bigas A, Cols M, Comerma L, Huang B, Blander JM, Xiong H, Mayer L, Berin C, Augenlicht LH, Velcich A, Cerutti A (2013) Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. Science 342(6157):447–453
15. Brandtzaeg P (1995) Molecular and cellular aspects of the secretory immunoglobulin system. APMIS 103:1–19
16. Bevins CL, Salzman NH (2011) Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. Nat Rev Microbiol 9(5):356–368
17. Qin X, Caputo FJ, Xu DZ, Deitch EA (2008) Hydrophobicity of mucosal surface and its relationship to gut barrier function. Shock 29(3):372–376
18. Shirazi T, Longman RJ, Corfield AP, Probert CS (2000) Mucins and inflammatory bowel disease. Postgrad Med J 76(898):473–478
19. Allen A, Pearson JP (1993) Mucus glycoproteins of the normal gastrointestinal tract. Eur J Gastroenterol Hepatol 5:193–199
20. Rhodes JM (1997) Mucins and inflammatory bowel disease. Q J Med 90:79–82
21. Kim YS, Ho SB (2010) Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep 12(5):319–330
22. Pigny P, Guyonnet-Duperat V, Hill AS, Pratt WS, Galiegue-Zouitina S, d'Hooge MC, Laine A, Van-Seuningen I, Degand P, Gum JR, Kim YS, Swallow DM, Aubert JP, Porchet N (1996) Human mucin genes assigned to 11p15.5: identification and organization of a cluster of genes. Genomics 38(3):340–352
23. Gum JR Jr, Hicks JW, Toribara NW, Siddiki B, Kim YS (1994) Molecular cloning of human intestinal mucin (MUC2) cDNA. Identification of the amino terminus and overall sequence similarity to prepro-von Willebrand factor. J Biol Chem 269(4):2440–2446
24. Tytgat KM, Büller HA, Opdam FJ, Kim YS, Einerhand AW, Dekker J (1994) Biosynthesis of human colonic mucin: Muc2 is the prominent secretory mucin. Gastroenterology 107 (5):1352–1363

25. Johansson ME, Larsson JM, Hansson GC (2011) The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc Natl Acad Sci U S A 108(Suppl 1):4659–4665
26. Atuma C, Strugala V, Allen A, Holm L (2001) The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. Am J Physiol Gastrointest Liver Physiol 280(5):G922–G929
27. Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, Büller HA, Dekker J, Van Seuningen I, Renes IB, Einerhand AW (2006) Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 131(1):117–129
28. Hasnain SZ, Wang H, Ghia JE, Haq N, Deng Y, Velcich A, Grencis RK, Thornton DJ, Khan WI (2010) Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. Gastroenterology 138(5):1763–1771
29. Zarepour M, Bhullar K, Montero M, Ma C, Huang T, Velcich A, Xia L, Vallance BA (2013) The mucin Muc2 limits pathogen burdens and epithelial barrier dysfunction during *Salmonella enterica* serovar Typhimurium colitis. Infect Immun 81(10):3672–3683
30. Einerhand AW, Renes IB, Makkink MK, van der Sluis M, Buller HA, Dekker J (2002) Role of mucins in inflammatory bowel disease: important lessons from experimental models. Eur J Gastroenterol Hepatol 14:757–765
31. Larsson JM, Karlsson H, Crespo JG, Johansson ME, Eklund L, Sjövall H, Hansson GC (2011) Altered O-glycosylation profile of MUC2 mucin occurs in active ulcerative colitis and is associated with increased inflammation. Inflamm Bowel Dis 17(11):2299–2307
32. Kyo K, Parkes M, Takei Y, Nishimori H, Vyas P, Satsangi J, Simmons J, Nagawa H, Baba S, Jewell D, Muto T, Lathrop GM, Nakamura Y (1999) Association of ulcerative colitis with rare VNTR alleles of the human intestinal mucin gene, MUC3. Hum Mol Genet 8(2):307–311
33. Kyo K, Muto T, Nagawa H, Lathrop GM, Nakamura Y (2001) Associations of distinct variants of the intestinal mucin gene MUC3A with ulcerative colitis and Crohn's disease. J Hum Genet 46:5–20
34. Guilmeau S, Flandez M, Bancroft L, Sellers RS, Tear B, Stanley P, Augenlicht LH (2008) Intestinal deletion of Pofut1 in the mouse inactivates notch signaling and causes enterocolitis. Gastroenterology 135(3):849–860
35. Krimi RB, Kotelevets L, Dubuquoy L, Plaisancié P, Walker F, Lehy T, Desreumaux P, Van Seuningen I, Chastre E, Forgue-Lafitte ME, Marie JC (2008) Resistin-like molecule beta regulates intestinal mucous secretion and curtails TNBS-induced colitis in mice. Inflamm Bowel Dis 14(7):931–941
36. Hogan SP, Seidu L, Blanchard C, Groschwitz K, Mishra A, Karow ML, Ahrens R, Artis D, Murphy AJ, Valenzuela DM, Yancopoulos GD, Rothenberg ME (2006) Resistin-like molecule beta regulates innate colonic function: barrier integrity and inflammation susceptibility. J Allergy Clin Immunol 118(1):257–268
37. Taupin D, Podolsky DK (2003) Trefoil factors: initiators of mucosal healing. Nat Rev Mol Cell Biol 4:721–732
38. Kindon H, Pothoulakis C, Thim L, Lynch-Devaney K, Podolsky DK (1995) Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein. Gastroenterology 109(2):516–523
39. Mashimo H, Wu DC, Podolsky DK, Fishman MC (1996) Impaired defense of intestinal mucosa in mice lacking intestinal trefoil factor. Science 274:262–265
40. Beck PL, Wong JF, Li Y, Swaminathan S, Xavier RJ, Devaney KL, Podolsky DK (2004) Chemotherapy- and radiotherapy-induced intestinal damage is regulated by intestinal trefoil factor. Gastroenterology 126:796–808
41. Furuta GT, Turner JR, Taylor CT, Hershberg RM, Comerford K, Narravula S, Podolsky DK, Colgan SP (2001) Hypoxia-inducible factor 1-dependent induction of intestinal trefoil factor protects barrier function during hypoxia. J Exp Med 193:1027–1034

42. Menard S, Cerf-Bensussan N, Heyman M (2010) Multiple facets of intestinal permeability and epithelial handling of dietary antigens. Mucosal Immunol 3(3):247–259
43. Booth C, Potten CS (2000) Gut instincts: thoughts on intestinal epithelial stem cells 1. J Clin Invest 105(11):1493–1499
44. Jeon MK, Klaus C, Kaemmerer E, Gassler N (2013) Intestinal barrier: molecular pathways and modifiers. World J Gastrointest Pathophysiol 4(4):94–99
45. Pott J, Hornef M (2012) Innate immune signalling at the intestinal epithelium in homeostasis and disease. EMBO Rep 13(8):684–698
46. Muehlhoefer A, Saubermann LJ, Gu X, Luedtke-Heckenkamp K, Xavier R, Blumberg RS, Podolsky DK, MacDermott RP, Reinecker HC (2000) Fractalkine is an epithelial and endothelial cell-derived chemoattractant for intraepithelial lymphocytes in the small intestinal mucosa. J Immunol 164(6):3368–3376
47. Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, Malefyt Rde W, Omori M, Zhou B, Ziegler SF (2007) TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. Annu Rev Immunol 25:193–219
48. Saitou M, Fujimoto K, Doi Y, Itoh M, Fujimoto T, Furuse M, Takano H, Noda T, Tsukita S (1998) Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. J Cell Biol 141(2):397–408
49. Rao R (2009) Occludin phosphorylation in regulation of epithelial tight junctions. Ann N Y Acad Sci 1165:62–68
50. Dörfel MJ, Huber O (2012) Modulation of tight junction structure and function by kinases and phosphatases targeting occludin. J Biomed Biotechnol 2012:807356
51. Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, Noda T, Kubo A, Tsukita S (2002) Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol 156(6):1099–1111
52. Cereijido M, Contreras RG, Shoshani L, Flores-Benitez D, Larre I (2008) Tight junction and polarity interaction in the transporting epithelial phenotype. Biochim Biophys Acta 1778:770–793
53. Fihn BM, Sjoqvist A, Jodal M (2000) Permeability of the rat small intestinal epithelium along the villus-crypt axis: effects of glucose transport. Gastroenterology 119:1029–1036
54. Schumann M, Günzel D, Buergel N, Richter JF, Troeger H, May C, Fromm A, Sorgenfrei D, Daum S, Bojarski C, Heyman M, Zeitz M, Fromm M, Schulzke JD (2012) Cell polarity-determining proteins Par-3 and PP-1 are involved in epithelial tight junction defects in coeliac disease. Gut 61(2):220–228
55. Zeissig S, Bürgel N, Günzel D, Richter J, Mankertz J, Wahnschaffe U, Kroesen AJ, Zeitz M, Fromm M, Schulzke JD (2007) Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. Gut 56:61–72
56. Escaffit F, Boudreau F, Beaulieu JF (2005) Differential expression of claudin-2 along the human intestine: implication of GATA-4 in the maintenance of claudin-2 in differentiating cells. J Cell Physiol 203(1):15–26
57. Liu Y, Nusrat A, Schnell FJ, Reaves TA, Walsh S, Pochet M, Parkos CA (2000) Human junction adhesion molecule regulates tight junction resealing in epithelia. J Cell Sci 113 (Pt 13):2363–2374
58. Laukoetter MG, Nava P, Lee WY, Severson EA, Capaldo CT, Babbin BA, Williams IR, Koval M, Peatman E, Campbell JA, Dermody TS, Nusrat A, Parkos CA (2007) JAM-A regulates permeability and inflammation in the intestine in vivo. J Exp Med 204(13):3067–3076
59. Vetrano S, Rescigno M, Cera MR, Correale C, Rumio C, Doni A, Fantini M, Sturm A, Borroni E, Repici A, Locati M, Malesci A, Dejana E, Danese S (2008) Unique role of junctional adhesion molecule-a in maintaining mucosal homeostasis in inflammatory bowel disease. Gastroenterology 135(1):173–184

60. Wilcz-Villega EM, McClean S, O'Sullivan MA (2013) Mast cell tryptase reduces junctional adhesion olecule-A (JAM-A) expression in intestinal epithelial cells: implications for the mechanisms of barrier dysfunction in irritable bowel syndrome. Am J Gastroenterol 108 (7):1140–1151
61. Ebnet K, Suzuki A, Ohno S, Vestweber D (2004) Junctional adhesion molecules (JAMs): more molecules with dual functions? J Cell Sci 117(Pt 1):19–29
62. Tsukita S, Katsuno T, Yamazaki T, Umeda K, Tamura A, Tsukita S (2009) Roles of ZO-1 and ZO-2 in establishment of the belt-like adherens and tight junctions with paracellular permselective barrier function. Ann N Y Acad Sci 1165:44–52
63. Umeda K, Ikenouchi J, Katahira-Tayama S, Furuse K, Sasaki H, Nakayama M, Matsui T, Tsukita S, Furuse M, Tsukita S (2006) ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. Cell 126:741–754
64. Shen L, Turner JR (2006) Role of epithelial cells in initiation and propagation of intestinal inflammation. Eliminating the static: tight junction dynamics exposed. Am J Physiol Gastrointest Liver Physiol 290(4):G577–G582
65. Utech M, Mennigen R, Bruewer M (2010) Endocytosis and recycling of tight junction proteins in inflammation. J Biomed Biotechnol 2010:484987
66. Schulzke JD, Bojarski C, Zeissig S, Heller F, Gitter AH, Fromm M (2006) Disrupted barrier function through epithelial cell apoptosis. Ann N Y Acad Sci 1072:288–299
67. Hopkins AM, Walsh SV, Verkade P, Boquet P, Nusrat A (2003) Constitutive activation of Rho proteins by CNF-1 influences tight junction structure and epithelial barrier function. J Cell Sci 116(Pt 4):725–742
68. Fasano A, Not T, Wang W, Uzzau S, Berti I, Tommasini A, Goldblum SE (2000) Zonulin, a newly discovered modulator of intestinal permeability, its expression in coeliac disease. Lancet 358:1518–1519
69. Watts T, Berti I, Sapone A, Gerarduzzi T, Not T, Zielke R, Fasano A (2005) Role of the intestinal tight junction modulator zonulin in the pathogenesis of type I diabetes in BB diabetic-prone rats. Proc Natl Acad Sci U S A 102:2916–2921
70. Kandori H, Hirayama K, Takeda M, Doi K (1996) Histochemical, lectin-histochemical and morphometrical characteristics of intestinal goblet cells of germfree and conventional mice. Exp Anim 45:155–160
71. Lin PW, Simon PO Jr, Gewirtz AT, Neish AS, Ouellette AJ, Madara JL, Lencer WI (2004) Paneth cell cryptdins act in vitro as apical paracrine regulators of the innate inflammatory response. J Biol Chem 279(19):19902–19907
72. Vaishnava S, Behrendt CL, Ismail AS, Eckmann L, Hooper LV (2008) Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. Proc Natl Acad Sci U S A 105:20858–20863
73. Lala S, Ogura Y, Osborne C, Hor SY, Bromfield A, Davies S, Ogunbiyi O, Nuñez G, Keshav S (2003) Crohn's disease and the NOD2 gene: a role for paneth cells. Gastroenterology 125 (1):47–57
74. Ogura Y, Lala S, Xin W, Smith E, Dowds TA, Chen FF, Zimmermann E, Tretiakova M, Cho JH, Hart J, Greenson JK, Keshav S, Nuñez G (2003) Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. Gut 52(11):1591–1597
75. McElroy SJ, Underwood MA, Sherman MP (2013) Paneth cells and necrotizing enterocolitis: a novel hypothesis for disease pathogenesis. Neonatology 103(1):10–20
76. Underwood MA (2012) Paneth cells and necrotizing enterocolitis. Gut Microbes 3(6):562–565
77. Sternini C, Anselmi L, Rozengurt E (2008) Enteroendocrine cells: a site of 'taste' in gastrointestinal chemosensing. Curr Opin Endocrinol Diabetes Obes 15(1):73–78
78. Flemström G, Sjöblom M (2005) Epithelial cells and their neighbors. II. New perspectives on efferent signaling between brain, neuroendocrine cells, and gut epithelial cells. Am J Physiol Gastrointest Liver Physiol 289(3):G377–G380

79. Bienenstock J, McDermott M, Befus D, O'Neill M (1978) A common mucosal immunologic system involving the bronchus, breast and bowel 4. Adv Exp Med Biol 107:53–59
80. Campbell DJ, Kim CH, Butcher EC (2003) Chemokines in the systemic organization of immunity. Immunol Rev 195:58–71
81. Gill N, Wlodarska M, Finlay BB (2011) Roadblocks in the gut: barriers to enteric infection. Cell Microbiol 13(5):660–669
82. Nürnberger T, Brunner F, Kemmerling B, Piater L (2004) Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev 198:249–266
83. Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. Annu Rev Immunol 21:335–376
84. Akira S, Takeda K (2004) Toll-like receptor signalling. Nat Rev Immunol 4(7):499–511
85. Abreu MT, Vora P, Faure E, Thomas LS, Arnold ET, Arditi M (2001) Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. J Immunol 167(3):1609–1616
86. Cario E, Podolsky DK (2000) Differential alteration in intestinal epithelial cell expression of Toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun 68 (12):7010–7017
87. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL (2001) Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J Immunol 167:1882–1885
88. Lee J, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, Lee HK, Shen C, Cojocaru G, Shenouda S, Kagnoff M, Eckmann L, Ben-Neriah Y, Raz E (2006) Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. Nat Cell Biol 8 (12):1327–1336
89. Franchi L, McDonald C, Kanneganti TD, Amer A, Núñez G (2006) Nucleotide-binding oligomerization domain-like receptors: intracellular pattern recognition molecules for pathogen detection and host defense. J Immunol 177(6):3507–3513
90. Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jéhanno M, Viala J, Tedin K, Taha MK, Labigne A, Zähringer U, Coyle AJ, DiStefano PS, Bertin J, Sansonetti PJ, Philpott DJ (2003) Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science 300 (5625):1584–1587
91. Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ, Sansonetti PJ (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 278(11):8869–8872
92. Tschopp J, Martinon F, Burns K (2003) NALPs: a novel protein family involved in inflammation. Nat Rev Mol Cell Biol 4:95–104
93. Kim JG, Lee SJ, Kagnoff MF (2004) Nod1 is an essential signal transducer in intestinal epithelial cells infected with bacteria that avoid recognition by Toll-like receptors. Infect Immun 72:1487–1495
94. Geddes K, Rubino S, Streutker C, Cho JH, Magalhaes JG, Le Bourhis L, Selvanantham T, Girardin SE, Philpott DJ (2010) Nod1 and Nod2 regulation of inflammation in the Salmonella colitis model. Infect Immun 78:5107–5115
95. Petnicki-Ocwieja T, Hrncir T, Liu YJ, Biswas A, Hudcovic T, Tlaskalova-Hogenova H, Kobayashi KS (2009) Nod2 is required for the regulation of commensal microbiota in the intestine. Proc Natl Acad Sci U S A 106:15813–15818
96. Wehkamp J, Harder J, Weichenthal M, Schwab M, Schäffeler E, Schlee M, Herrlinger KR, Stallmach A, Noack F, Fritz P, Schröder JM, Bevins CL, Fellermann K, Stange EF (2004) NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. Gut 53(11):1658–1664
97. Ho S, Pothoulakis C, Koon HW (2013) Antimicrobial peptides and colitis. Curr Pharm Des 19(1):40–47
98. Papagianni M (2003) Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. Biotechnol Adv 21(6):465–499

99. Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415(6870):389–395
100. Yang D, Biragyn A, Hoover DM, Lubkowski J, Oppenheim JJ (2004) Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. Annu Rev Immunol 22:181–215
101. Cunliffe RN, Kamal M, Rose FR, James PD, Mahida YR (2002) Expression of antimicrobial neutrophil defensins in epithelial cells of active inflammatory bowel disease mucosa. J Clin Pathol 55(4):298–304
102. Cunliffe RN (2003) Alpha-defensins in the gastrointestinal tract. Mol Immunol 40:463–467
103. Ishikawa C, Tanabe H, Maemoto A, Ito T, Watari J, Kono T, Fujiya M, Ashida T, Ayabe T, Kohgo Y (2010) Precursor processing of human defensin-5 is essential to the multiple functions in vitro and in vivo. J Innate Immun 2(1):66–76
104. Salzman NH, Ghosh D, Huttner KM, Paterson Y, Bevins CL (2003) Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. Nature 422:522–526
105. Chu H, Pazgier M, Jung G, Nuccio SP, Castillo PA, de Jong MF, Winter MG, Winter SE, Wehkamp J, Shen B, Salzman NH, Underwood MA, Tsolis RM, Young GM, Lu W, Lehrer RI, Bäumler AJ, Bevins CL (2012) Human α-defensin 6 promotes mucosal innate immunity through self-assembled peptide nanonets. Science 337(6093):477–481
106. Harder J, Bartels J, Christophers E, Schroder JM (2001) Isolation and characterization of human beta-defensin-3, a novel human inducible peptide antibiotic. J Biol Chem 276 (8):5707–5713
107. Wehkamp J, Fellermann K, Herrlinger KR, Baxmann S, Schmidt K, Schwind B, Duchrow M, Wohlschlager C, Feller AC, Stange EF (2002) Human beta-defensin 2 but not beta-defensin 1 is expressed preferentially in colonic mucosa of inflammatory bowel disease. Eur J Gastroenterol Hepatol 14:745–752
108. Fahlgren A, Hammarstrom S, Danielsson A, Hammarstrom ML (2004) beta-defensin-3 and -4 in intestinal epithelial cells display increased mRNA expression in ulcerative colitis. Clin Exp Immunol 137:379–385
109. O'Neil DA (2003) Regulation of expression of beta-defensins: endogenous enteric peptide antibiotics. Mol Immunol 40(7):445–450
110. Cowland JB, Johnsen AH, Borregaard N (1995) hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules. FEBS Lett 368(1):173–176
111. Frohm NM, Sandstedt B, Sorensen O, Weber G, Borregaard N, Stahle-Backdahl M (1999) The human cationic antimicrobial protein (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6. Infect Immun 67(5):2561–2566
112. Murakami M, Dorschner RA, Stern LJ, Lin KH, Gallo RL (2005) Expression and secretion of cathelicidin antimicrobial peptides in murine mammary glands and human milk. Pediatr Res 57:10–15
113. Hase K, Eckmann L, Leopard JD, Varki N, Kagnoff MF (2002) Cell differentiation is a key determinant of cathelicidin LL-37/human cationic antimicrobial protein 18 expression by human colon epithelium. Infect Immun 70(2):953–963
114. Islam D, Bandholtz L, Nilsson J, Wigzell H, Christensson B, Agerberth B, Gudmundsson G (2001) Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. Nat Med 7(2):180–185
115. Kida Y, Shimizu T, Kuwano K (2006) Sodium butyrate up-regulates cathelicidin gene expression via activator protein-1 and histone acetylation at the promoter region in a human lung epithelial cell line, EBC-1. Mol Immunol 43(12):1972–1981
116. Koon HW, Shih DQ, Chen J, Bakirtzi K, Hing TC, Law I, Ho S, Ichikawa R, Zhao D, Xu H, Gallo R, Dempsey P, Cheng G, Targan SR, Pothoulakis C (2011) Cathelicidin signaling via the Toll-like receptor protects against colitis in mice. Gastroenterology 141(5):1852–1863
117. He B, Xu W, Santini PA, Polydorides AD, Chiu A, Estrella J, Shan M, Chadburn A, Villanacci V, Plebani A, Knowles DM, Rescigno M, Cerutti A (2007) Intestinal bacteria

trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. Immunity 26:812–826
118. Hapfelmeier S, Lawson MA, Slack E, Kirundi JK, Stoel M, Heikenwalder M, Cahenzli J, Velykoredko Y, Balmer ML, Endt K, Geuking MB, Curtiss R 3rd, McCoy KD, Macpherson AJ (2010) Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science 328:1705–1709
119. Cicchetti D, Cohen DJ (2006) Developmental psychopathology: developmental neuroscience. Wiley, New York
120. de Kloet ER, Joels M, Holsboer F (2005) Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6(6):463–475
121. Gunnar M, Quevedo K (2007) The neurobiology of stress and development. Annu Rev Psychol 58:145–173
122. Saunders PR, Kosecka U, McKay DM, Perdue MH (1994) Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. Am J Physiol 267(5 Pt 1): G794–G799
123. Santos J, Saunders PR, Hanssen NP, Yang PC, Yates D, Groot JA, Perdue MH (1999) Corticotropin-releasing hormone mimics stress-induced colonic epithelial pathophysiology in the rat. Am J Physiol 277(2 Pt 1):G391–G399
124. Santos J, Benjamin M, Yang PC, Prior T, Perdue MH (2000) Chronic stress impairs rat growth and jejunal epithelial barrier function: role of mast cells. Am J Physiol Gastrointest Liver Physiol 278(6):G847–G854
125. Vicario M, Guilarte M, Alonso C, Yang P, Martínez C, Ramos L, Lobo B, González A, Guilà M, Pigrau M, Saperas E, Azpiroz F, Santos J (2010) Chronological assessment of mast cell-mediated gut dysfunction and mucosal inflammation in a rat model of chronic psychosocial stress. Brain Behav Immun 24(7):1166–1175
126. Barclay GR, Turnberg LA (1987) Effect of psychological stress on salt and water transport in the human jejunum. Gastroenterology 93(1):91–97
127. Barclay GR, Turnberg LA (1988) Effect of cold-induced pain on salt and water transport in the human jejunum. Gastroenterology 94(4):994–998
128. Santos J, Saperas E, Nogueiras C, Mourelle M, Antolin M, Cadahia A, Malagelada JR (1998) Release of mast cell mediators into the jejunum by cold pain stress in humans. Gastroenterology 114(4):640–648
129. Alonso C, Guilarte M, Vicario M, Ramos L, Ramadan Z, Antolin M, Martinez C, Rezzi S, Saperas E, Kochhar S, Santos J, Malagelada JR (2008) Maladaptive intestinal epithelial responses to life stress may predispose healthy women to gut mucosal inflammation. Gastroenterology 135(1):163–172
130. Meddings JB, Swain MG (2000) Environmental stress-induced gastrointestinal permeability is mediated by endogenous glucocorticoids in the rat. Gastroenterology 119(4):1019–1028
131. Soderholm JD, Perdue MH (2001) Stress and gastrointestinal tract. II. Stress and intestinal barrier function. Am J Physiol Gastrointest Liver Physiol 280(1):G7–G13
132. Kiliaan AJ, Saunders PR, Bijlsma PB, Berin MC, Taminiau JA, Groot JA, Perdue MH (1998) Stress stimulates transepithelial macromolecular uptake in rat jejunum. Am J Physiol 275 (5 Pt 1):G1037–G1044
133. Saunders PR, Santos J, Hanssen NP, Yates D, Groot JA, Perdue MH (2002) Physical and psychological stress in rats enhances colonic epithelial permeability via peripheral CRH. Dig Dis Sci 47(1):208–215
134. Santos J, Perdue MH (2000) Stress and neuroimmune regulation of gut mucosalfunction. Gut 47(Suppl 4):iv49–51; discussion iv52
135. Ferrier L, Mazelin L, Cenac N, Desreumaux P, Janin A, Emilie D, Colombel JF, Garcia-Villar R, Fioramonti J, Bueno L (2003) Stress-induced disruption of colonic epithelial barrier: role of interferon-gamma and myosin light chain kinase in mice. Gastroenterology 125 (3):795–804

136. Dunlop SP, Hebden J, Campbell E, Naesdal J, Olbe L, Perkins AC, Spiller RC (2006) Abnormal intestinal permeability in subgroups of diarrhea-predominant irritable bowel syndromes. Am J Gastroenterol 101(6):1288–1294
137. Wallon C, Yang PC, Keita AV, Ericson AC, McKay DM, Sherman PM, Perdue MH, Soderholm JD (2008) Corticotropin-releasing hormone (CRH) regulates macromolecular permeability via mast cells in normal human colonic biopsies in vitro. Gut 57(1):50–58
138. Guilarte M, Santos J, Alonso C, Vicario M, Antolin M, Saperas E, Malagelada JR (2004) Corticotropin-releasing hormone (CRH) triggers jejunal mast cell and eosinophil activation in IBS patients. Gastroenterology 126(Suppl 2):A38 (ref type: abstract)
139. Vanuytsel T, van Wanrooy S, Vanheel H, Vanormelingen C, Verschueren S, Houben E, Salim Rasoel S, Tóth J, Holvoet L, Farré R, Van Oudenhove L, Boeckxstaens G, Verbeke K, Tack J (2013) Psychological stress and corticotropin-releasing hormone increase intestinal permeability in humans by a mast cell-dependent mechanism. Gut. doi:10.1136/gutjnl-2013-305690 (Epub ahead of print)
140. Zhou Q, Souba WW, Croce CM, Verne GN (2010) MicroRNA-29a regulates intestinal membrane permeability in patients with irritable bowel syndrome. Gut 59(6):775–784
141. Castagliuolo I, LaMont JT, Qiu B, Fleming SM, Bhaskar KR, Nikulasson ST, Kornetsky C, Pothoulakis C (1996) Acute stress causes mucin release from rat colon: role of corticotropin releasing factor and mast cells. Am J Physiol 271(5 Pt 1):G884–G892
142. Castagliuolo I, Wershil BK, Karalis K, Pasha A, Nikulasson ST, Pothoulakis C (1998) Colonic mucin release in response to immobilization stress is mast cell dependent. Am J Physiol 274(6 Pt 1):G1094–G1100
143. Söderholm JD, Yang PC, Ceponis P, Vohra A, Riddell R, Sherman PM, Perdue MH (2002) Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. Gastroenterology 123(4):1099–1108
144. Bailey MT, Coe CL (1999) Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. Dev Psychobiol 35(2):146–155
145. Bailey MT, Lubach GR, Coe CL (2004) Prenatal stress alters bacterial colonization of the gut in infant monkeys. J Pediatr Gastroenterol Nutr 38(4):414–421
146. Eutamene H, Bueno L (2007) Role of probiotics in correcting abnormalities of colonic flora induced by stress. Gut 56(11):1495–1497
147. Gareau MG, Jury J, MacQueen G, Sherman PM, Perdue MH (2007) Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation. Gut 56(11):1522–1528
148. Spitz JC, Ghandi S, Taveras M, Aoys E, Alverdy JC (1996) Characteristics of the intestinal epithelial barrier during dietary manipulation and glucocorticoid stress. Crit Care Med 24 (4):635–641
149. Velin AK, Ericson AC, Braaf Y, Wallon C, Soderholm JD (2004) Increased antigen and bacterial uptake in follicle associated epithelium induced by chronic psychological stress in rats. Gut 53(4):494–500
150. Lizko NN, Silov VM, Syrych GD (1984) Events in he development of dysbacteriosis of the intestines in man under extreme conditions. Nahrung 28(6–7):599–605
151. Lyte M, Ernst S (1992) Catecholamine induced growth of gram negative bacteria. Life Sci 50 (3):203–212
152. Santos J, Guilarte M, Alonso C, Malagelada JR (2005) Pathogenesis of irritable bowel syndrome: the mast cell connection. Scand J Gastroenterol 40(2):129–140
153. Eutamene H, Theodorou V, Fioramonti J, Bueno L (2003) Acute stress modulates the histamine content of mast cells in the gastrointestinal tract through interleukin-1 and corticotropin-releasing factor release in rats. J Physiol 553(Pt 3):959–966
154. Stead RH, Dixon MF, Bramwell NH, Riddell RH, Bienenstock J (1989) Mast cells are closely apposed to nerves in the human gastrointestinal mucosa. Gastroenterology 97(3):575–585
155. Barbara G, Stanghellini V, De GR, Cremon C, Cottrell GS, Santini D, Pasquinelli G, Morselli-Labate AM, Grady EF, Bunnett NW, Collins SM, Corinaldesi R (2004) Activated

mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. Gastroenterology 126(3):693–702
156. Jiang W, Kreis ME, Eastwood C, Kirkup AJ, Humphrey PP, Grundy D (2000) 5-HT(3) and histamine H(1) receptors mediate afferent nerve sensitivity to intestinal anaphylaxis in rats. Gastroenterology 119(5):1267–1275
157. Barbara G, Wang B, Stanghellini V, De GR, Cremon C, Di NG, Trevisani M, Campi B, Geppetti P, Tonini M, Bunnett NW, Grundy D, Corinaldesi R (2007) Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome. Gastroenterology 132(1):26–37
158. Vergnolle N (2005) Clinical relevance of proteinase activated receptors (pars) in the gut10. Gut 54(6):867–874
159. Sander LE, Lorentz A, Sellge G, Coeffier M, Neipp M, Veres T, Frieling T, Meier PN, Manns MP, Bischoff SC (2006) Selective expression of histamine receptors H1R, H2R, and H4R, but not H3R, in the human intestinal tract. Gut 55(4):498–504
160. Hauger RL, Grigoriadis DE, Dallman MF, Plotsky PM, Vale WW, Dautzenberg FM (2003) International Union of Pharmacology. XXXVI. Current status of the nomenclature for receptors for corticotropin-releasing factor and their ligands. Pharmacol Rev 55(1):21–26
161. Kiank C, Tache Y, Larauche M (2010) Stress-related modulation of inflammation in experimental models of bowel disease and post-infectious irritable bowel syndrome: role of corticotropin-releasing factor receptors. Brain Behav Immun 4(1):41–48
162. la Fleur SE, Wick EC, Idumalla PS, Grady EF, Bhargava A (2005) Role of peripheral corticotropin-releasing factor and urocortin II in intestinal inflammation and motility in terminal ileum. Proc Natl Acad Sci U S A 102(21):7647–7652
163. Kokkotou E, Torres D, Moss AC, O'Brien M, Grigoriadis DE, Karalis K, Pothoulakis C (2006) Corticotropin-releasing hormone receptor 2-deficient mice have reduced intestinal inflammatory responses. J Immunol 177(5):3355–3361
164. Moss AC, Anton P, Savidge T, Newman P, Cheifetz AS, Gay J, Paraschos S, Winter MW, Moyer MP, Karalis K, Kokkotou E, Pothoulakis C (2007) Urocortin II mediates pro-inflammatory effects in human colonocytes via corticotropin-releasing hormone receptor 2alpha 1. Gut 56(9):1210–1217
165. Saruta M, Takahashi K, Suzuki T, Torii A, Kawakami M, Sasano H (2004) Urocortin 1 in colonic mucosa in patients with ulcerative colitis. J Clin Endocrinol Metab 89(11):5352–5361
166. Delgado M, Pozo D, Ganea D (2004) The significance of vasoactive intestinal peptide in immunomodulation. Pharmacol Rev 56(2):249–290
167. Toumi F, Neunlist M, Denis MG, Oreshkova T, Laboisse CL, Galmiche JP, Jarry A (2004) Vasoactive intestinal peptide induces IL-8 production in human colonic epithelial cells via MAP kinase-dependent and PKA-independent pathways. Biochem Biophys Res Commun 317(1):187–191
168. Cao SG, Wu WC, Han Z, Wang MY (2005) Effects of psychological stress on small intestinal motility and expression of cholecystokinin and vasoactive intestinal polypeptide in plasma and small intestine in mice. World J Gastroenterol 11(5):737–740
169. Neunlist M, Toumi F, Oreschkova T, Denis M, Leborgne J, Laboisse CL, Galmiche JP, Jarry A (2003) Human ENS regulates the intestinal epithelial barrier permeability and a tight junction-associated protein ZO-1 via VIPergic pathways. Am J Physiol Gastrointest Liver Physiol 285(5):G1028–G1036
170. Koon HW, Pothoulakis C (2006) Immunomodulatory properties of substance P: the gastrointestinal system as a model. Ann N Y Acad Sci 1088:23–40
171. Wang L, Stanisz AM, Wershil BK, Galli SJ, Perdue MH (1995) Substance P induces ion secretion in mouse small intestine through effects on enteric nerves and mast cells. Am J Physiol 269(1 Pt 1):G85–G92

172. Zheng PY, Feng BS, Oluwole C, Struiksma S, Chen X, Li P, Tang SG, Yang PC (2009) Psychological stress induces eosinophils to produce corticotrophin releasing hormone in the intestine. Gut 58(11):1473–1479
173. Barreau F, Cartier C, Ferrier L, Fioramonti J, Bueno L (2004) Nerve growth factor mediates alterations of colonic sensitivity and mucosal barrier induced by neonatal stress in rats. Gastroenterology 127(2):524–534
174. Kobayashi H, Yamataka A, Fujimoto T, Lane GJ, Miyano T (1999) Mast cells and gut nerve development: implications for Hirschsprung's disease and intestinal neuronal dysplasia. J Pediatr Surg 34(4):543–548
175. Theodosiou M, Rush RA, Zhou XF, Hu D, Walker JS, Tracey DJ (1999) Hyperalgesia due to nerve damage: role of nerve growth factor. Pain 81(3):245–255
176. Pearce FL, Thompson HL (1986) Some characteristics of histamine secretion from rat peritoneal mast cells stimulated with nerve growth factor. J Physiol 372:379–393
177. Marshall JS, Stead RH, McSharry C, Nielsen L, Bienenstock J (1990) The role of mast cell degranulation products in mast cell hyperplasia. I. Mechanism of action of nerve growth factor. J Immunol 144(5):1886–1892
178. Barreau F, Cartier C, Leveque M, Ferrier L, Moriez R, Laroute V, Rosztoczy A, Fioramonti J, Bueno L (2007) Pathways involved in gut mucosal barrier dysfunction induced in adult rats by maternal deprivation: corticotrophin-releasing factor and nerve growth factor interplay. J Physiol 580(Pt 1):347–356
179. Winston JH, Xu GY, Sarna SK (2010) Adrenergic stimulation mediates visceral hypersensitivity to colorectal distension following heterotypic chronic stress. Gastroenterology 138 (1):294–304
180. Dahlman-Wright K, Cavailles V, Fuqua SA, Jordan VC, Katzenellenbogen JA, Korach KS, Maggi A, Muramatsu M, Parker MG, Gustafsson JA (2006) International Union of Pharmacology. LXIV. Estrogen receptors. Pharmacol Rev 58:773–781
181. Konstantinopoulos PA, Kominea A, Vandoros G, Sykiotis GP, Andricopoulos P, Varakis I, Sotiropoulou-Bonikou G, Papavassiliou AG (2003) Oestrogen receptor-β (ERβ) is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumour's dedifferentiation. Eur J Cancer 39:1251–1258
182. Condliffe SB, Doolan CM, Harvey BJ (2001) 17beta-oestradiol acutely regulates Cl- secretion in rat distal colonic epithelium. J Physiol 530(Pt 1):47–54
183. Mayol JM, Arbeo-Escolar A, Alarma-Estrany P, Adame-Navarrete Y, Fernandez-Represa JA (2002) Progesterone inhibits chloride transport in human intestinal epithelial cells. World J Surg 26(6):652–656
184. Wada-Hiraike O, Imamov O, Hiraike H, Hultenby K, Schwend T, Omoto Y, Warner M, Gustafsson JA (2006) Role of estrogen receptor beta in colonic epithelium. Proc Natl Acad Sci U S A 103:2959–2964
185. Braniste V, Leveque M, Buisson-Brenac C, Bueno L, Fioramonti J, Houdeau E (2009) Oestradiol decreases colonic permeability through oestrogen receptor beta-mediated up-regulation of occludin and junctional adhesion molecule-A in epithelial cells. J Physiol 587:3317–3328
186. Berkes J, Viswanathan VK, Savkovic SD, Hecht G (2003) Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation. Gut 52(3):439–451
187. Wu Z, Nybom P, Magnusson KE (2000) Distinct effects of Vibrio cholera haemagglutinin/protease on the structure and localization of the tight junction-associated proteins occludin and ZO-1. Cell Microbiol 2(1):11–17
188. Fullner KJ, Lencer WI, Mekalanos JJ (2001) Vibrio cholerae-induced cellular responses of polarized T84 intestinal epithelial cells are dependent on production of cholera toxin and the RTX toxin. Infect Immun 69:6310–6317

189. Fasano A, Baudry B, Pumplin DW, Wasserman SS, Tall BD, Ketley JM, Kaper JB (1991) Vibrio cholerae produces a second enterotoxin, which affects intestinal tight junctions. Proc Natl Acad Sci U S A 88(12):5242–5246
190. Fasano A, Fiorentini C, Donelli G, Uzzau S, Kaper JB, Margaretten K, Ding X, Guandalini S, Comstock L, Goldblum SE (1995) Zonula occludens toxin modulates tight junctions through protein kinase C-dependent actin reorganization, in vitro. J Clin Invest 96(2):710–720
191. Hecht G, Pothoulakis C, LaMont JT, Madara JL (1988) Clostridium difficile toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. J Clin Invest 82(5):1516–1524
192. Hecht G, Koutsouris A, Pothoulakis C, LaMont JT, Madara JL (1992) Clostridium difficile toxin B disrupts the barrier function of T84 monolayers. Gastroenterology 102(2):416–423
193. Chen M, Pothoulakis C, LaMont T (2002) Protein kinase C signaling regulates ZO-1 translocation and increased paracellular flux of T84 colonocytes exposed to Clostridium difficile toxin A. J Biol Chem 277:4247–4254
194. Katahira J, Sugiyama H, Inoue N, Horiguchi Y, Matsuda M, Sugimoto N (1997) Clostridium perfringens enterotoxin utilizes two structurally related membrane proteins as functional receptors in vivo. J Biol Chem 272(42):26652–26658
195. McClane B (1994) Clostridium perfringens enterotoxin acts by producing small molecule permeability alterations in plasma membranes. Toxicology 87:43–67
196. Simonovic I, Rosenberg J, Koutsouris A, Hecht G (2000) Enteropathogenic *Escherichia coli* dephosphorylates and dissociates occludin from intestinal epithelial tight junctions. Cell Microbiol 2(4):305–315
197. Yuhan R, Koutsouris A, Savkovic SD, Hecht G (1997) Enteropathogenic *Escherichia coli*-induced myosin light chain phosphorylation alters intestinal epithelial permeability. Gastroenterology 113(6):1873–1882
198. Lyte M (2013) Microbial endocrinology in the microbiome-gut-brain axis: how bacterial production and utilization of neurochemicals influence behavior. PLoS Pathog 9(11): e1003726
199. Cryan JF, O'Mahony SM (2011) The microbiome-gut-brain axis: from bowel to behavior. Neurogastroenterol Motil 23(3):187–192
200. Sekirov I, Russell SL, Antunes LC, Finlay BB (2010) Gut microbiota in health and disease. Physiol Rev 90(3):859–904
201. Qin HL, Shen TY, Gao ZG, Fan XB, Hang XM, Jiang YQ, Zhang HZ (2005) Effect of Lactobacillus on the gut microflora and barrier function of the rats with abdominal infection. World J Gastroenterol 11(17):2591–2596
202. Zyrek AA, Cichon C, Helms S, Enders C, Sonnenborn U, Schmidt MA (2007) Molecular mechanisms underlying the probiotic effects of *Escherichia coli* Nissle 1917 involve ZO-2 and PKCzeta redistribution resulting in tight junction and epithelial barrier repair. Cell Microbiol 9:804–816
203. Miyauchi E, O'Callaghan J, Buttó LF, Hurley G, Melgar S, Tanabe S, Shanahan F, Nally K, O'Toole PW (2012) Mechanism of protection of transepithelial barrier function by *Lactobacillus salivarius*: strain dependence and attenuation by bacteriocin production. Am J Physiol Gastrointest Liver Physiol 303(9):G1029–G1041
204. Patel RM, Myers LS, Kurundkar AR, Maheshwari A, Nusrat A, Lin PW (2012) Probiotic bacteria induce maturation of intestinal claudin 3 expression and barrier function. Am J Pathol 180(2):626–635
205. Lin PW, Nasr TR, Berardinelli AJ, Kumar A, Neish AS (2008) The probiotic Lactobacillus GG may augment intestinal host defense by regulating apoptosis and promoting cytoprotective responses in the developing murine gut. Pediatr Res 64(5):511–516
206. Ishii Y, Sugimoto S, Izawa N, Sone T, Chiba K, Miyazaki K. Oral administration of Bifidobacterium breve attenuates UV-induced barrier perturbation and oxidative stress in hairless mice skin. Arch Dermatol Res. 2014 Jan 11

207. Fujita R, Iimuro S, Shinozaki T, Sakamaki K, Uemura Y, Takeuchi A, Matsuyama Y, Ohashi Y (2013) Decreased duration of acute upper respiratory tract infections with daily intake of fermented milk: a multicenter, double-blinded, randomized comparative study in users of day care facilities for the elderly population. Am J Infect Control 41(12):1231–1235
208. Luoto R, Ruuskanen O, Waris M, Kalliomäki M, Salminen S, Isolauri E (2013) Prebiotic and probiotic supplementation prevents rhinovirus infections in preterm infants: a randomized, placebo-controlled trial. J Allergy Clin Immunol S0091–6749(13):01307–01309
209. Dicksved J, Schreiber O, Willing B, Petersson J, Rang S, Phillipson M, Holm L, Roos S (2012) Lactobacillus reuteri maintains a functional mucosal barrier during DSS treatment despite mucus layer dysfunction. PLoS One 7(9):e46399
210. Ukena SN, Singh A, Dringenberg U, Engelhardt R, Seidler U, Hansen W, Bleich A, Bruder D, Franzke A, Rogler G, Suerbaum S, Buer J, Gunzer F, Westendorf AM (2007) Probiotic *Escherichia coli* Nissle 1917 inhibits leaky gut by enhancing mucosal integrity. PLoS One 2(12):e1308
211. Toumi R, Abdelouhab K, Rafa H, Soufli I, Raissi-Kerboua D, Djeraba Z, Touil-Boukoffa C (2013) Beneficial role of the probiotic mixture Ultrabiotique on maintaining the integrity of intestinal mucosal barrier in DSS-induced experimental colitis. Immunopharmacol Immunotoxicol 35(3):403–409
212. Mennigen R, Nolte K, Rijcken E, Utech M, Loeffler B, Senninger N, Bruewer M (2009) Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. Am J Physiol Gastrointest Liver Physiol 296(5):G1140–G1149
213. Persborn M, Gerritsen J, Wallon C, Carlsson A, Akkermans LM, Söderholm JD (2013) The effects of probiotics on barrier function and mucosal pouch microbiota during maintenance treatment for severe pouchitis in patients with ulcerative colitis. Aliment Pharmacol Ther 38 (7):772–783
214. Ait-Belgnaoui A, Durand H, Cartier C, Chaumaz G, Eutamene H, Ferrier L, Houdeau E, Fioramonti J, Bueno L, Theodorou V (2012) Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. Psychoneuroendocrinology 37(11):1885–1895
215. Palomar MM, Maldonado Galdeano C, Perdigón G (2014) Influence of a probiotic Lactobacillus strain on the intestinal ecosystem in a stress model mouse. Brain Behav Immun 35:77–85
216. Agostini S, Goubern M, Tondereau V, Salvador-Cartier C, Bezirard V, Lévèque M, Keränen H, Theodorou V, Bourdu-Naturel S, Goupil-Feuillerat N, Legrain-Raspaud S, Eutamene H (2012) A marketed fermented dairy product containing Bifidobacterium lactis CNCM I-2494 suppresses gut hypersensitivity and colonic barrier disruption induced by acute stress in rats. Neurogastroenterol Motil 24(4):376–e172
217. Whelan K, Quigley EM (2013) Probiotics in the management of irritable bowel syndrome and inflammatory bowel disease. Curr Opin Gastroenterol 29(2):184–189
218. Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 13(10):701–712
219. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, Codelli JA, Chow J, Reisman SE, Petrosino JF, Patterson PH, Mazmanian SK (2013) Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell 155(7):1451–1463
220. Zolotarevsky Y, Hecht G, Koutsouris A, Gonzalez DE, Quan C, Tom J, Mrsny RJ, Turner JR (2002) A membrane-permeant peptide that inhibits MLC kinase restores barrier function in in vitro models of intestinal disease. Gastroenterology 123(1):163–172
221. Wang F, Graham WV, Wang Y, Witkowski ED, Schwarz BT, Turner JR (2005) Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. Am J Pathol 166 (2):409–419

222. Mankertz J, Tavalali S, Schmitz H, Mankertz A, Riecken EO, Fromm M, Schulzke JD (2000) Expression from the human occludin promoter is affected by tumor necrosis factor alpha and interferon gamma. J Cell Sci 113(Pt 11):2085–2090
223. Barmeyer C, Amasheh S, Tavalali S, Mankertz J, Zeitz M, Fromm M, Schulzke JD (2004) IL-1beta and TNFalpha regulate sodium absorption in rat distal colon. Biochem Biophys Res Commun 317(2):500–507
224. Clayburgh DR, Musch MW, Leitges M, Fu YX, Turner JR (2006) Coordinated epithelial NHE3 inhibition and barrier dysfunction are required for TNF-mediated diarrhea in vivo. J Clin Invest 116(10):2682–2694
225. Zünd G, Madara JL, Dzus AL, Awtrey CS, Colgan SP (1996) Interleukin-4 and interleukin-13 differentially regulate epithelial chloride secretion. J Biol Chem 271(13):7460–7464
226. Ceponis PJ, Botelho F, Richards CD, McKay DM (2000) Interleukins 4 and 13 increase intestinal epithelial permeability by a phosphatidylinositol 3-kinase pathway. Lack of evidence for STAT 6 involvement. J Biol Chem 275(37):29132–29137
227. Madsen KL, Lewis SA, Tavernini MM, Hibbard J, Fedorak RN (1997) Interleukin 10 prevents cytokine-induced disruption of T84 monolayer barrier integrity and limits chloride secretion. Gastroenterology 113(1):151–159
228. Madsen KL, Malfair D, Gray D, Doyle JS, Jewell LD, Fedorak RN (1999) Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. Inflamm Bowel Dis 5(4):262–270
229. Kühn R, Löhler J, Rennick D, Rajewsky K, Müller W (1993) Interleukin-10-deficient mice develop chronic enterocolitis. Cell 75(2):263–274
230. Blaschitz C, Raffatellu M (2010) Th17 cytokines and the gut mucosal barrier. J Clin Immunol 30(2):196–203
231. Al-Sadi R, Boivin M, Ma T (2009) Mechanism of cytokine modulation of epithelial tight junction barrier. Front Biosci (Landmark Ed) 14:2765–2778
232. Lammers KM, Lu R, Brownley J, Lu B, Gerard C, Thomas K, Rallabhandi P, Shea-Donohue-T, Tamiz A, Alkan S, Netzel-Arnett S, Antalis T, Vogel SN, Fasano A (2008) Gliadin induces an increase in intestinal permeability and zonulin release by binding to the chemokine receptor CXCR3. Gastroenterology 135(1):194–204
233. Wang W, Uzzau S, Goldblum SE, Fasano A (2000) Human zonulin, a potential modulator of intestinal tight junctions. J Cell Sci 113(Pt 24):4435–4440
234. Ventura MT, Polimeno L, Amoruso AC, Gatti F, Annoscia E, Marinaro M, Di Leo E, Matino MG, Buquicchio R, Bonini S, Tursi A, Francavilla A (2006) Intestinal permeability in patients with adverse reactions to food. Dig Liver Dis 38(10):732–736
235. Ozdemir O, Arrey-Mensah A, Sorensen RU (2006) Development of multiple food allergies in children taking tacrolimus after heart and liver transplantation. Pediatr Transplant 10(3):380–383
236. Finnie IA, Dwarakanath AD, Taylor BA, Rhodes JM (1995) Colonic mucin synthesis is increased by sodium butyrate. Gut 36:93–99
237. Ma X, Fan PX, Li LS, Qiao SY, Zhang GL, Li DF (2012) Butyrate promotes the recovering of intestinal wound healing through its positive effect on the tight junctions. J Anim Sci 90 (Suppl 4):266–268
238. Li N, Lewis P, Samuelson D, Liboni K, Neu J (2004) Glutamine regulates Caco-2 cell tight junction proteins. Am J Physiol Gastrointest Liver Physiol 287(3):G726–G733
239. Ma TY, Nguyen D, Bui V, Nguyen H, Hoa N (1999) Ethanol modulation of intestinal epithelial tight junction barrier. Am J Physiol 276:G965–G974
240. Atkinson KJ, Rao RK (2001) Role of protein tyrosine phosphorylation in acetaldehyde-induced disruption of epithelial tight junctions. Am J Physiol 280:G1280–G1288
241. Ferrier L, Bérard F, Debrauwer L, Chabo C, Langella P, Buéno L, Fioramonti J (2006) Impairment of the intestinal barrier by ethanol involves enteric microflora and mast cell activation in rodents. Am J Pathol 168(4):1148–1154

242. Tong J, Wang Y, Chang B, Zhang D, Liu P, Wang B (2013) Activation of RhoA in alcohol-induced intestinal barrier dysfunction. Inflammation 36(3):750–758
243. Rendon JL, Li X, Akhtar S, Choudhry MA (2013) Interleukin-22 modulates gut epithelial and immune barrier functions following acute alcohol exposure and burn injury. Shock 39(1):11–18
244. Oshima T, Miwa H, Joh T (2008) Aspirin induces gastric epithelial barrier dysfunction by activating p38 MAPK via claudin-7. Am J Physiol Cell Physiol 295(3):C800–C806
245. Silver K, Leloup L, Freeman LC, Wells A, Lillich JD (2010) Non-steroidal anti-inflammatory drugs inhibit calpain activity and membrane localization of calpain 2 protease. Int J Biochem Cell Biol 42(12):2030–2036
246. Tanaka A, Kunikata T, Mizoguchi H, Kato S, Takeuchi K (1999) Dual action of nitric oxide in pathogenesis of indomethacin-induced small intestinal ulceration in rats. J Physiol Pharmacol 50(3):405–417
247. Vaziri ND, Yuan J, Norris K (2013) Role of urea in intestinal barrier dysfunction and disruption of epithelial tight junction in chronic kidney disease. Am J Nephrol 37(1):1–6
248. Watts T, Berti I, Sapone A, Gerarduzzi T, Not T, Zielke R, Fasano A (2005) Role of the intestinal tight junction modulator zonulin in the pathogenesis of type I diabetes in BB diabetic-prone rats. Proc Natl Acad Sci U S A 102(8):2916–2921
249. Sakisaka S, Kawaguchi T, Taniguchi E, Hanada S, Sasatomi K, Koga H, Harada M, Kimura R, Sata M, Sawada N, Mori M, Todo S, Kurohiji T (2001) Alterations in tight junctions differ between primary biliary cirrhosis and primary sclerosing cholangitis. Hepatology 33(6):1460–1468
250. Pijls KE, Jonkers DM, Elamin EE, Masclee AA, Koek GH (2013) Intestinal epithelial barrier function in liver cirrhosis: an extensive review of the literature. Liver Int 33(10):1457–1469
251. Rao RK, Seth A, Sheth P (2004) Recent advances in alcoholic liver disease I. Role of intestinal permeability and endotoxemia in alcoholic liver disease. Am J Physiol Gastrointest Liver Physiol 286(6):G881–G884
252. Rebuffat SA, Kammoun-Krichen M, Charfeddine I, Ayadi H, Bougacha-Elleuch N, Peraldi-Roux S (2013) IL-1β and TSH disturb thyroid epithelium integrity in autoimmune thyroid diseases. Immunobiology 218(3):285–291
253. Peng SN, Zeng HH, Fu AX, Chen XW, Zhu QX (2013) Effects of rhein on intestinal epithelial tight junction in IgA nephropathy. World J Gastroenterol 19(26):4137–4145
254. Li Y, Guo M, Shen J, Zheng L, Wang J, Wang P, Li J (2014) Limited Fluid Resuscitation Attenuates lung and intestine injury caused by hemorrhagic shock in rats. J Invest Surg 27:81–87 (Epub 2013 Oct 2)
255. Fishman JE, Levy G, Alli V, Sheth S, Lu Q, Deitch EA (2013) Oxidative modification of the intestinal mucus layer is a critical but unrecognized component of trauma hemorrhagic shock-induced gut barrier failure. Am J Physiol Gastrointest Liver Physiol 304(1):G57–G63
256. Doucet D, Badami C, Palange D, Bonitz RP, Lu Q, Xu DZ, Kannan KB, Colorado I, Feinman R, Deitch EA (2010) Estrogen receptor hormone agonists limit trauma hemorrhage shock-induced gut and lung injury in rats. PLoS One 5(2):e9421
257. Feinman R, Deitch EA, Watkins AC, Abungu B, Colorado I, Kannan KB, Sheth SU, Caputo FJ, Lu Q, Ramanathan M, Attan S, Badami CD, Doucet D, Barlos D, Bosch-Marce M, Semenza GL, Xu DZ (2010) HIF-1 mediates pathogenic inflammatory responses to intestinal ischemia-reperfusion injury. Am J Physiol Gastrointest Liver Physiol 299(4):G833–G843
258. Spindler-Vesel A, Wraber B, Vovk I, Kompan L (2006) Intestinal permeability and cytokine inflammatory response in multiply injured patients. J Interferon Cytokine Res 26(10):771–776
259. Chen C, Wang P, Su Q, Wang S, Wang F (2012) Myosin light chain kinase mediates intestinal barrier disruption following burn injury. PLoS One 7(4):e34946
260. Costantini TW, Peterson CY, Kroll L, Loomis WH, Eliceiri BP, Baird A, Bansal V, Coimbra R (2009) Role of p38 MAPK in burn-induced intestinal barrier breakdown. J Surg Res 156 (1):64–69

261. Peterson CY, Costantini TW, Loomis WH, Putnam JG, Wolf P, Bansal V, Eliceiri BP, Baird A, Coimbra R (2010) Toll-like receptor-4 mediates intestinal barrier breakdown after thermal injury. Surg Infect (Larchmt) 11(2):137–144
262. Mertz H, Morgan V, Tanner G, Pickens D, Price R, Shyr Y, Kessler R (2000) Regional cerebral activation in irritable bowel syndrome and control subjects with painful and nonpainful rectal distention. Gastroenterology 118(5):842–848
263. Drossman DA, Ringel Y, Vogt BA, Leserman J, Lin W, Smith JK, Whitehead W (2003) Alterations of brain activity associated with resolution of emotional distress and pain in a case of severe irritable bowel syndrome. Gastroenterology 124(3):754–761
264. Barreau F, Ferrier L, Fioramonti J, Bueno L (2007) New insights in the etiology and pathophysiology of irritable bowel syndrome: contribution of neonatal stress models. Pediatr Res 62(3):240–245
265. O'Mahony SM, Marchesi JR, Scully P, Codling C, Ceolho AM, Quigley EM, Cryan JF, Dinan TG (2009) Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. Biol Psychiatry 65(3):263–267
266. Klooker TK, Braak B, Painter RC, de Rooij SR, van Elburg RM, van den Wijngaard RM, Roseboom TJ, Boeckxstaens GE (2009) Exposure to severe wartime conditions in early life is associated with an increased risk of irritable bowel syndrome: a population-based cohort study. Am J Gastroenterol 104(9):2250–2256
267. Koloski NA, Talley NJ, Boyce PM (2005) A history of abuse in community subjects with irritable bowel syndrome and functional dyspepsia: the role of other psychosocial variables. Digestion 72(2–3):86–96
268. Murray CD, Flynn J, Ratcliffe L, Jacyna MR, Kamm MA, Emmanuel AV (2004) Effect of acute physical and psychological stress on gut autonomic innervation in irritable bowel syndrome. Gastroenterology 127(6):1695–1703
269. Kellow JE, Langeluddecke PM, Eckersley GM, Jones MP, Tennant CC (1992) Effects of acute psychologic stress on small-intestinal motility in health and the irritable bowel syndrome. Scand J Gastroenterol 27(1):53–58
270. Vanheel H, Vicario M, Vanuytsel T, Van Oudenhove L, Martinez C, Keita AV, Pardon N, Santos J, Söderholm JD, Tack J, Farré R (2014) Impaired duodenal mucosal integrity and low-grade inflammation in functional dyspepsia. Gut 63:262–271 (Epub 2013 Mar 8)
271. Pike BL, Porter CK, Sorrell TJ, Riddle MS (2013) Acute gastroenteritis and the risk of functional dyspepsia: a systematic review and meta-analysis. Am J Gastroenterol 108 (10):1558–1563
272. Van Oudenhove L, Aziz Q (2013) The role of psychosocial factors and psychiatric disorders in functional dyspepsia. Nat Rev Gastroenterol Hepatol 10(3):158–167
273. Van Oudenhove L, Vandenberghe J, Vos R, Fischler B, Demyttenaere K, Tack J (2011) Abuse history, depression, and somatization are associated with gastric sensitivity and gastric emptying in functional dyspepsia. Psychosom Med 73(8):648–655
274. Stout C, Snyder RL (1969) Ulcerative colitis-like lesion in Siamang gibbons. Gastroenterology 57(3):256–261
275. Barreau F, Ferrier L, Fioramonti J, Bueno L (2004) Neonatal maternal deprivation triggers long term alterations in colonic epithelial barrier and mucosal immunity in rats. Gut 53 (4):501–506
276. Collins SM, McHugh K, Jacobson K, Khan I, Riddell R, Murase K, Weingarten HP (1996) Previous inflammation alters the response of the rat colon to stress. Gastroenterology 111 (6):1509–1515
277. Qiu BS, Vallance BA, Blennerhassett PA, Collins SM (1999) The role of CD4+ lymphocytes in the susceptibility of mice to stress-induced reactivation of experimental colitis. Nat Med 5 (10):1178–1182
278. Bitton A, Sewitch MJ, Peppercorn MA, deB Edwardes MD, Shah S, Ransil B, Locke SE (2003) Psychosocial determinants of relapse in ulcerative colitis: a longitudinal study. Am J Gastroenterol 98(10):2203–2208

279. Levenstein S, Prantera C, Varvo V, Scribano ML, Andreoli A, Luzi C, Arca M, Berto E, Milite G, Marcheggiano A (2000) Stress and exacerbation in ulcerative colitis: a prospective study of patients enrolled in remission. Am J Gastroenterol 95(5):1213–1220
280. Buhner S, Buning C, Genschel J, Kling K, Herrmann D, Dignass A, Kuechler I, Krueger S, Schmidt HH, Lochs H (2006) Genetic basis for increased intestinal permeability in families with Crohn's disease: role of CARD15 3020insC mutation? Gut 55(3):342–347
281. D'Inca R, Annese V, Di Leo V, Latiano A, Quaino V, Abazia C, Vettorato MG, Sturniolo GC (2006) Increased intestinal permeability and NOD2 variants in familial and sporadic Crohn's disease. Aliment Pharmacol Ther 23(10):1455–1461
282. Peeters M, Geypens B, Claus D, Nevens H, Ghoos Y, Verbeke G, Baert F, Vermeire S, Vlietinck R, Rutgeerts P (1997) Clustering of increased small intestinal permeability in families with Crohn's disease. Gastroenterology 113(3):802–807
283. D'Inca R, Di Leo V, Corrao G, Martines D, D'Odorico A, Mestriner C, Venturi C, Longo G, Sturniolo GC (1999) Intestinal permeability test as a predictor of clinical course in Crohn's disease. Am J Gastroenterol 94(10):2956–2960
284. Jorgensen J, Ranlov PJ, Bjerrum PJ, Diemer H, Bisgaard K, Elsborg L (2001) Is an increased intestinal permeability a valid predictor of relapse in Crohn disease? Scand J Gastroenterol 36 (5):521–527
285. Langhorst J, Cobelens PM, Kavelaars A, Heijnen CJ, Benson S, Rifaie N, Dobos GJ, Schedlowski M, Elsenbruch S (2007) Stress-related peripheral neuroendocrine-immune interactions in women with ulcerative colitis. Psychoneuroendocrinology 32(8–10):1086–1096
286. Bekkering P, Jafri I, van Overveld FJ, Rijkers GT (2013) The intricate association between gut microbiota and development of Type 1, Type 2 and Type 3 diabetes. Expert Rev Clin Immunol 9(11):1031–1041
287. Summa KC, Voigt RM, Forsyth CB, Shaikh M, Cavanaugh K, Tang Y, Vitaterna MH, Song S, Turek FW, Keshavarzian A (2013) Disruption of the circadian clock in mice increases intestinal permeability and promotes alcohol-induced hepatic pathology and inflammation. PLoS One 8(6):e67102
288. Fetissov SO, Déchelotte P (2011) The new link between gut-brain axis and neuropsychiatric disorders. Curr Opin Clin Nutr Metab Care 14(5):477–482
289. Heberling CA, Dhurjati PS, Sasser M (2013) Hypothesis for a systems connectivity model of autism spectrum disorder pathogenesis: links to gut bacteria, oxidative stress, and intestinal permeability. Med Hypotheses 80(3):264–270
290. Maes M, Coucke F, Leunis JC (2007) Normalization of the increased translocation of endotoxin from gram negative enterobacteria (leaky gut) is accompanied by a remission of chronic fatigue syndrome. Neuro Endocrinol Lett 28(6):739–744
291. Goebel A, Buhner S, Schedel R, Lochs H, Sprotte G (2008) Altered intestinal permeability in patients with primary fibromyalgia and in patients with complex regional pain syndrome. Rheumatology (Oxford) 47(8):1223–1227
292. Hadjiyanni I, Li KK, Drucker DJ (2009) Glucagon-like peptide-2 reduces intestinal permeability but does not modify the onset of type 1 diabetes in the nonobese diabetic mouse. Endocrinology 150(2):592–599
293. Moran GW, O'Neill C, McLaughlin JT (2012) GLP-2 enhances barrier formation and attenuates TNFα-induced changes in a Caco-2 cell model of the intestinal barrier. Regul Pept 178(1–3):95–101
294. Tang ZF, Ling YB, Lin N, Hao Z, Xu RY (2007) Glutamine and recombinant human growth hormone protect intestinal barrier function following portal hypertension surgery. World J Gastroenterol 13(15):2223–2228
295. Yi C, Cao Y, Wang SR, Xu YZ, Huang H, Cui YX, Huang Y (2007) Beneficial effect of recombinant human growth hormone on the intestinal mucosa barrier of septic rats. Braz J Med Biol Res 40(1):41–48

296. Lorenzo-Zúñiga V, Rodríguez-Ortigosa CM, Bartolí R, Martínez-Chantar ML, Martínez-Peralta L, Pardo A, Ojanguren I, Quiroga J, Planas R, Prieto J (2006) Insulin-like growth factor I improves intestinal barrier function in cirrhotic rats. Gut 55(9):1306–1312
297. Dong CX, Zhao W, Solomon C, Rowland KJ, Ackerley C, Robine S, Holzenberger M, Gonska T, Brubaker PL (2014) The intestinal epithelial insulin-like growth factor-1 receptor links glucagon-like peptide-2 action to gut barrier function. Endocrinology 155:370–379 (Epub 2013 Nov 21)
298. Bansal V, Ryu SY, Blow C, Costantini T, Loomis W, Eliceiri B, Baird A, Wolf P, Coimbra R (2010) The hormone ghrelin prevents traumatic brain injury induced intestinal dysfunction. J Neurotrauma 27(12):2255–2260
299. Bettenworth D, Buyse M, Böhm M, Mennigen R, Czorniak I, Kannengiesser K, Brzoska T, Luger TA, Kucharzik T, Domschke W, Maaser C, Lügering A (2011) The tripeptide KdPT protects from intestinal inflammation and maintains intestinal barrier function. Am J Pathol 179(3):1230–1242
300. Hamada K, Shitara Y, Sekine S, Horie T (2010) Zonula Occludens-1 alterations and enhanced intestinal permeability in methotrexate-treated rats. Cancer Chemother Pharmacol 66(6):1031–1038
301. Jung S, Fehr S, Harder-d'Heureuse J, Wiedenmann B, Dignass AU (2001) Corticosteroids impair intestinal epithelial wound repair mechanisms in vitro. Scand J Gastroenterol 36 (9):963–970
302. Hopkins AM, McDonnell C, Breslin NP, O'Morain CA, Baird AW (2002) Omeprazole increases permeability across isolated rat gastric mucosa pre-treated with an acid secretagogue. J Pharm Pharmacol 54(3):341–347
303. Yang J, Liu KX, Qu JM, Wang XD (2013) The changes induced by cyclophosphamide in intestinal barrier and microflora in mice. Eur J Pharmacol 714(1–3):120–124
304. Lee SE, Choi Y, Kim SE, Noh EB, Kim SC (2013) Differential effects of topical corticosteroid and calcineurin inhibitor on the epidermal tight junction. Exp Dermatol 22(1):59–61
305. Gabe SM, Bjarnason I, Tolou-Ghamari Z, Tredger JM, Johnson PG, Barclay GR, Williams R, Silk DB (1998) The effect of tacrolimus (FK506) on intestinal barrier function and cellular energy production in humans. Gastroenterology 115(1):67–74
306. Kong J, Zhang Z, Musch MW, Ning G, Sun J, Hart J et al (2008) Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier. Am J Physiol Gastrointest Liver Physiol 294:G208–G216
307. Paterson BM, Lammers KM, Arrieta MC, Fasano A, Meddings JB (2007) The safety, tolerance, pharmacokinetic and pharmacodynamic effects of single doses of AT-1001 in coeliac disease subjects: a proof of concept study. Aliment Pharmacol Ther 26:757–766
308. Kelly CP, Green PH, Murray JA, Dimarino A, Colatrella A, Leffler DA, Alexander T, Arsenescu R, Leon F, Jiang JG, Arterburn LA, Paterson BM, Fedorak RN, Larazotide Acetate Celiac Disease Study Group (2013) Larazotide acetate in patients with coeliac disease undergoing a gluten challenge: a randomised placebo-controlled study. Aliment Pharmacol Ther 37(2):252–262
309. Gopalakrishnan S, Tripathi A, Tamiz AP, Alkan SS, Pandey NB (2012) Larazotide acetate promotes tight junction assembly in epithelial cells. Peptides 35(1):95–101
310. Gopalakrishnan S, Durai M, Kitchens K, Tamiz AP, Somerville R, Ginski M, Paterson BM, Murray JA, Verdu EF, Alkan SS, Pandey NB (2012) Larazotide acetate regulates epithelial tight junctions in vitro and in vivo. Peptides 35(1):86–94
311. Suenaert P, Bulteel V, Lemmens L, Noman M, Geypens B, Van Assche G et al (2002) Anti-tumor necrosis factor treatment restores the gut barrier in Crohn's disease. Am J Gastroenterol 97:2000–2004
312. Bode L, Salvestrini C, Park PW, Li JP, Esko JD, Yamaguchi Y et al (2008) Heparan sulfate and syndecan-1 are essential in maintaining murine and human intestinal epithelial barrier function. J Clin Invest 118:229–238

Chapter 5
Vagal Pathways for Microbiome-Brain-Gut Axis Communication

Paul Forsythe, John Bienenstock, and Wolfgang A. Kunze

Abstract There is now strong evidence from animal studies that gut microorganism can activate the vagus nerve and that such activation plays a critical role in mediating effects on the brain and behaviour. The vagus appears to differentiate between non-pathogenic and potentially pathogenic bacteria even in the absence of overt inflammation and vagal pathways mediate signals that can induce both anxiogenic and anxiolytic effects, depending on the nature of the stimulus. Certain vagal signals from the gut can instigate an anti-inflammatory reflex with afferent signals to the brain activating an efferent response, releasing mediators including acetylcholine that, through an interaction with immune cells, attenuates inflammation. This immunomodulatory role of the vagus nerve may also have consequences for modulation of brain function and mood.

What is currently lacking are relevant data on the electrophysiology of the system. Certainly, important advances in our understanding of the gut-brain and microbiome- gut-brain axis will come from studies of how distinct microbial and nutritional stimuli activate the vagus and the nature of the signals transmitted to the brain that lead to differential changes in the neurochemistry of the brain and behaviour.

Understanding the induction and transmission of signals in the vagus nerve may have important implications for the development of microbial-or nutrition based therapeutic strategies for mood disorders.

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_5,

Abbreviations

5-HT	5-Hydroxytryptamine
ATP	Adenosine triphosphate
CCK	Cholecystokinin
CNS	Central nervous system
CRF	Corticotropin-releasing factor
DHA	Docosahexaenoic acid
DRG	Dorsal root ganglia
DSS	Dextran sodium sulfate
ENS	Enteric nervous system
EPA	Eicosapentaenoic acid
FDA	Food and Drug Administration
GABA	Gamma-aminobutyric acid
GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1
HPS	Hypothalamic-pituitary-adrenal
IBD	Inflammatory bowel disease
IGLE	Intraganglionic laminar vagal afferent ending
IK_{Ca}	Calcium dependent potassium channel
IPAN	Intrinsic primary afferent neuron
LPS	Lipopolysaccharide
MDD	Major depressive disorder
mRNA	Messenger RNA
NTS	Nucleus of the solitary tract
PSA	Polysaccharide
PUFA	Polyunsaturated fatty acids
PYY	Peptide YY
TNF	Tumor necrosis factor

The Vagus Nerve

The vagus (tenth cranial nerve) innervates the pharynx, larynx and visceral organs. While it contains both motor and sensory fibres, it is the main afferent pathway from the abdominal cavity to the brain. Information from the heart, lungs, pancreas, liver, stomach and intestines are delivered tonically to the brain via sensory fibres in the vagus nerve [1]. Sensory vagal inputs arrive in the nucleus of the solitary tract (NTS) via the nodose ganglion which is chiefly made up of sensory visceral afferent fibres. From there fibres ramify to widespread areas of the CNS, including the cerebral cortex and medulla oblongata.

There are 30,000–80,000 vagal afferent nerves that supply the intestine with a 9:1 ratio of afferent to efferent fibres in peripheral nerve bundles [2–4]. Vagal primary afferents innervate the muscular and mucosal layers of the gut with the

coeliac branch supplying the intestine from proximal duodenum to the distal part of the descending colon [5]. Vagal innervation is densest proximally but is still significant in the colon.

Histological and electrophysiological evidence indicates that visceral afferent endings [3] in the intestine express a diverse array of chemical and mechanosensitive receptors [2]. The chemosensitive receptors are the targets of gut hormones and regulatory peptides such as ghrelin, CCK, GLP-1 and PYY(3–36) that influence the control of food intake and regulation of energy balance [2]. Vagal afferent fibres have been identified in the lamina propria of duodenal and jejunal villi, and crypts of Lieberkühn, but they do not cross the basal membrane to innervate the epithelial layer [5]. Thus, vagal afferents are not in a position to sense luminal nutrients directly unless they arrive intact in the lateral intercellular spaces, but are in close anatomical apposition to the basal membrane of enteroendocrine cells [6].

Intraganglionic laminar vagal afferent endings (IGLEs) are located in the connective tissue capsule of myenteric plexus ganglia, between the longitudinal (outer) and circular (inner) muscle layers. These fibres respond to muscle tension generated by both passive stretch and active contraction of the muscle layers [7]. This type of vagal afferent ending is found in large numbers throughout the oesophagus and gastrointestinal tract and is thought to be important for generating vagal afferent tone which has been associated with balanced interoceptive awareness and emotional well-being. Furthermore experimental data suggesting that changes in visceral sensation can affect the perception and interpretation of external inputs [8, 9] has led to the suggestion that altered sensory vagal inputs can influence our attitude to the outside world. The anterior insular cortex is involved in interpretation of most if not all interoception, and therefore through these vagal and other inputs, represents most of our subjective feelings. It is suggested that pathological changes in sensory vagal inputs may increase the risk of affective behavioural disorders. Chronic sensory vagal inputs might then act as ‘natural’ breaks for augmentation of stress-related behavioural responses via tonic modulation of the neuronal activity in the locus coeruleus and in turn the forebrain [10].

The Vagus and Behaviour

Some of the earliest indication of the role of the vagus in modulating behaviour came from studies of animals exposed to endotoxin. Sickness behaviour is a term used to describe the motivational state responsible for re-organizing perceptions and actions to enable ill individuals to cope better with infection [11]. The associated behaviours include lethargy, depression, anxiety, loss of appetite, sleepiness, hyperalgesia, and reduction in grooming. These behavioural changes are mediated by proinflammatory cytokines particularly IL-1β and TNF [11].

The role of vagal afferents in the induction of sickness behaviour following intraperitoneal administration of the cytokine inducer lipopolysaccharide (LPS) or

IL-1β has been assessed in laboratory animals that have been submitted to subdiaphragmatic vagotomy [12, 13] and these responses have been shown to be entirely dose responsive [14].

A dose of IL-1β or the cytokine inducer lipopolysaccharide (LPS) that induced consistent sickness behaviour in sham-operated animals was no longer able to decrease social exploration in vagotomised rats and mice [15, 16]. In the same manner, vagotomy blocked the depressing effects of LPS on food-motivated behaviour in mice [17].

In contrast to the role of the vagus in mediating sickness and depressive type behaviour it is also emerging that stimulating the vagus can lead to a reduction in anxiety and depression associated behaviours. In one study, rats were exposed to vagus nerve stimulation for 30 min per day for 4 days, and were then subjected to the forced swim test, a well validated assessment of anti-depressant activity. Vagus nerve stimulation significantly reduced immobility time compared to unstimulated controls, reflective of antidepressant effects [18]. Interestingly, the vagal nerve stimulation-induced decreases in immobility were associated with increased swimming behaviour, which has been linked to a predominantly serotonergic mechanism of action [19]. In a subsequent controlled trial, rats received desipramine or vagal nerve stimulation for 2 h at three time points over a 24 h period, prior to undergoing the forced swim test and both treatments resulted in reduced immobility compared to saline control [20]. However, chronic vagal nerve stimulation for 1 month failed to show any behavioural alterations in rats subjected to the forced swim test or the elevated plus maze test, in contrast to treatment with a classical anti-depressant, imipramine [21]. No careful timecourse or analysis of different dose and timing schedules of stimulation appear to have been conducted.

Clinically, vagal stimulation has been used successfully in the treatment of refractory epilepsy [22] and is also an FDA approved alternative treatment for intractable depression. While this treatment for depression is controversial, largely due to a lack of positive sham treatment controlled clinical trials, there have been reports that vagal nerve stimulation is beneficial in at least some patients with depression and may be particularly effective with chronic treatment [23, 24].

The Vagal Anti-Inflammatory Reflex

The vagus innervates tissues known to participate in immune functions and/or contain important immune elements, such as thymus, lung, liver, and gastrointestinal tract. Furthermore, trunks or branches of the vagus are often associated with lymph nodes that drain regions in which immune activation occurs. The functional relevance of vagal innervation of immune tissue has been highlighted by the identification of a neural circuit that controls the inflammatory response in a reflex-like manner. In this system it is suggested that the vagus nerve senses inflammation sending afferent signals to the brain thus activating an efferent

response, releasing mediators including acetylcholine that, through an interaction with immune cells, attenuates inflammation.

This area was first explored by Levine and colleagues [25] who suggested that gut vagal afferents sent signals to the brain and that as a consequence, vagal efferents could inhibit various nociceptive as well as inflammatory peripheral events such as bradykinin induced plasma extravasation in joints. However Tracey and colleagues were the first to highlight and delineate the anti-inflammatory role of the vagus and its mechanism of action. They showed that direct electrical stimulation of the distal end of a subdiaphragmatic sectioned vagus nerve prevented the development of shock in rats through the inhibition of TNF synthesis by macrophages [26]. They considered that inhibition of macrophage function is mediated by Ach released by the vagus acting on specific alpha7nicotinic receptors expressed by the immune cell. Similarly, macrophages have been suggested to be the main target of the anti-inflammatory function of the vagus nerve in a murine model of inflammatory bowel disease (IBD) [27]. However evidence supports the fact that this reflex may not be monospecific for alpha7 and can also be mediated via other nicotinic receptors such as alpha5. In addition to suppressive effects on macrophages the vagus nerve also acts to regulate T cell function. O'Mahony et al. [28] demonstrated that transfer of $CD4^+$ T cells from vagotomized donors into non-vagotomized mice with DSS induced colitis, reduced the number of splenic $Foxp3^+$ regulatory T cells in recipient animals, and was associated with aggravated disease symptoms mimicking the effects of vagotomy on colitis. Sub diaphragmatic vagotomy leads to a dramatic increase in T cell proliferation and production of inflammatory cytokines when compared to cells from sham-operated animals [29]. The effect of vagotomy was not limited to the spleen as lymphocytes isolated from the mesenteric lymph nodes also demonstrated a significant increase in inflammatory cytokine production. Overall these data suggest that $CD4^+$ T cells, in addition to macrophages, are also under tonic inhibitory control from the vagus. Further revisions of the definition of this important function of the efferent vagus have recently been published. The source of the acetylcholine involved in this reflex may not be coming from the vagus but norepinephrine stimulated memory T cells [30], in keeping with the papers listed above [28, 29]. Furthermore B cells in addition to T cells can respond to stimulation by cholecystokinin through release of acetylcholine which controls recruitment of neutrophils but not adaptive immune function [31]. Thus the vagus nerve is intimately involved in many immunoregulatory functions via a number of different cholinergic receptors and through a number of different immune cell types.

These anti-inflammatory efferent responses may be important and play a role in the regulation of mood in healthy conditions as well as in psychiatric disease. They may also mediate the anti-depressive effects of vagal nerve stimulation as outlined before. Immune system dysfunction has been linked to depression [32–34]. Approximately one-third of people with depression, without co-morbid diseases, have higher levels of inflammatory markers such as TNF and C-reactive protein, compared with the normal, non-depressed population [35]. Furthermore, inflammatory illnesses are associated with greater rates of major depression, while patients treated

with cytokines such as interferon for various illnesses, are at increased risk of developing major depressive illness. Conversely, successful treatment with an antidepressant decreases levels of pro-inflammatory cytokines such as IL-6 and TNF [36–38]. While it is as yet unclear whether neurostimulation therapies for depression affect immune function, there is evidence in vagal nerve stimulation treated epilepsy patients that pro-inflammatory cytokine levels were reduced with successful treatment [39, 40].

The huge population of bacteria in the gut, known collectively as the gut microbiome, is largely responsible for the generation and control of the major immunoregulatory pathways that exist to respond to and control external challenges [41]. As we will discuss subsequently in this chapter, there is evidence that commensal bacteria in the gut can directly or indirectly modulate the activity and function of the enteric nervous system and thereby the brain and its functions, including behaviour. Therefore, taken together, the gut microbiome and the vagus nerve may be influencing the brain via various mechanisms. Indeed the inappropriate lack of regulation of inflammation in major depressive disorder (MDD) may be put at the door of a possible imbalance or dysbiosis of the gut microbiome which if rebalanced, might be expected to restore the proper functioning of the central nervous system [42]. This argument is an extension of the "hygiene hypothesis" that many autoimmune, immune and allergic diseases which have recently been shown to be having such epidemic prevalence [43, 44] are doing so as a result of mankind's attention to cleanliness and the eradication of bacteria. In the main, these arguments are based on the evidence that it is likely that evolutionary change in diet, nutrition, environmental factors such as urbanization, concepts of cleanliness and the use of antibiotics may all have conspired to change our previous balanced gut microbiome to one which is not as favourable to immune regulation as it used to be.

Many chronic diseases are associated with mild or moderate inflammation, the evidence for which is present through increase in levels of biomarkers in the blood and also in the tissues themselves (e.g. activated macrophages in obesity). The source(s) of the inflammatory changes noted in association with depression and anxiety are not known, but it has been noted that stress itself is accompanied by evidence of proinflammatory cytokine elevation both experimentally and in clinical conditions and is at least one of the possible causes of raised inflammatory biomarkers [45]. Increased hypothalamic-pituitary-adrenal (HPA) axis activity in chronic stress is known to also be associated with MDD and anxiety syndromes. In this regard it is pertinent that ingestion of beneficial bacteria has been associated with attenuation of HPA axis hyperactivity [46] and also responses to acute stress [47, 48].

Dietary Fatty Acids and the Vagus

Long- and short-chain fatty acids both activate rat jejunal vagal afferent nerve fibers but do so by distinct mechanisms [49]. Short-chain fatty acids such as butyric acid have a direct effect on vagal afferent terminals while the long-chain fatty acids activate vagal afferents via a cholecystokinin (CCK) dependent mechanism. Interaction between long-chain fatty acids and the vagus results in activation of the cholinergic anti-inflammatory pathway [50]. Luyer et al. demonstrated that administration of high fat nutrition reduced circulating levels of TNF and IL-6 in rats subjected to hemorrhagic shock. When these experiments were repeated in vagotomized animals, the administration of the high fat diet no longer prevented the increase in TNF and IL-6 [50]. In addition, nicotine receptor antagonism blocked the ability of dietary fat to suppress the cytokine increase. Similarly, deafferentation abrogates the protective effects of lipid-rich nutrition on systemic inflammation and loss of intestinal integrity following shock [51]. Overall these experiments provide strong evidence of a nutritional anti-inflammatory pathway whereby the intake of dietary fat suppresses cytokine release through activation of peripheral afferent vagus nerves that in turn initiate the cholinergic anti-inflammatory response. In keeping with the findings of Lal et al. [49] the mechanism underlying the protective effects of long chain fatty acids include a role for CCK. Administration of CCK receptor antagonists and specifically antagonists of the peripheral CCK-1 impaired the fat-induced suppression of the shock response [50].

Clinically, studies indicate that dietary n-3 PUFA levels and n-3 PUFA supplementation are related to improved heart rate variability suggesting increased vagal tone [52, 53]. The relationship between the immunomodulatory actions of n-3 PUFA and their effects upon vagal tone has yet to be established. However a number of studies have associated control of inflammation with heart rate variability in humans [54–57] and it is possible that diet-induced activation of the cholinergic anti-inflammatory pathway contributes to the reduced mortality from sepsis and organ damage following early enteral feeding in trauma and surgery patients [58–60].

It has also been suggested [61] that this nutrient activated neural feedback loop could help maintain unresponsiveness of the GI tract to luminal antigens allowing the intestine to perform the dual roles of sensing and absorbing essential nutrients while protecting against invasion from potentially damaging agents.

A number of studies have indicated that long chain fatty acids, and particularly n3-PUFAs such as EPA and DHA, may have potential as therapy or adjunctive treatment in depression and that such effects could also be related to an ability to modulate HPA activity. In animal models, feeding DHA to rats significantly decreased immobility time in the forced swim test, a well validated indication of anti-depressant activity. The DHA induced behavioural change was associated with decreased CRF levels in the hypothalamus and pituitary tissues, an indication of changes in HPA activity [62, 63]. In human studies, Jazayeri et al. [64, 65] reported

that fluoxetine and EPA were equally effective in controlling depressive symptoms and that a fluoxetine and EPA combination was superior to either treatment alone. EPA and fluoxetine, alone or in combination, also decreased serum cortisol after 8 weeks of treatment in depressed patients leading to the suggestion that EPA may exert its therapeutic effects through reduction of HPA hyperactivity [65].

DHA mediated attenuation of HPA may be explained by the demonstration that n-3 PUFAs, can act on $GABA_A$ receptors to potentiate GABAergic inhibitory drive on CRF-secreting hypothalamic neurons [66]. In this regard, it is interesting to note that the decreased anxiety and HPA response to stress of mice fed with *Lactobacillus rhamnosus* is also associated with changes in the central GABAergic system [48].

Bacterial Communication from Gut Lumen to Enteric Nervous System (ENS)

Luminal probiotic bacteria may alter behaviour and brain biochemistry in a variety of ways even in the absence of changes in the inflammatory status of the host [67]. Bacterial products could enter the circulation to pass the blood-brain barrier if they are sufficiently small and lipophilic [68], or they might enter the brain at the circumventricular organs where the barrier is diminished. Since prior vagotomy abolishes behavioural and brain biochemical changes induced by certain probiotic bacteria [48, 67], afferent vagal signalling is a necessary condition for the central effects of these neuroactive microorganisms.

The majority of sensory fibres innervating the intestinal mucosa derive from intrinsic primary afferent neurons (IPANs) of the enteric nervous system (ENS) [69, 70]. Therefore, IPANs are a priori likely targets for the action of neuroactive bacteria leading to alterations in gut motility. This has been substantiated by direct experimentation. Nine day feeding of 10^9 JB-1 cfu caused an increase in the intrinsic excitability of rat colon myenteric IPANs with a decrease in the post action potential slow afterhyperpolarisation (relative refractory period) [71]. These results have been replicated in an acute ex vivo preparation where beneficial bacteria (JB-1 or *Bacteroides fragilis*) or its capsular polysaccharide (PSA) were applied to the epithelium while whole cell recordings were made from nearby IPANs [72]. Application of the bacteria or PSA evoked bursts of action potentials in the IPANs in a sensory (non-synaptic) manner as was demonstrated by blocking all synaptic transmission. The onset latencies of the initial sensory responses were about 8 s which then led to an increase in IPAN intrinsic excitability via entrainment of the reciprocally connected IPAN to IPAN network. The increase in intrinsic excitability depended on IPAN to IPAN slow synaptic transmission via G protein coupled receptors [72]. Because the specific intermediate conductance calcium dependent potassium channel (IK_{Ca}) blocker TRAM-34 mimics the effect of JB-1 on IPAN slow afterhyperpolarisation and intrinsic

excitability we proposed that one of the molecular mechanisms involved may have been the inhibition of IK_{Ca} channels [72, 73]. However, a detailed examination of the effects of JB-1 and other probiotics vs TRAM-34 on neurally mediated propagated motor complexes in the mouse intestine suggests that reduction in the open probability of IK_{Ca} channels is not sufficient to explain the entirety of the neuronal actions of these organisms and that additional ion channel targets or regulatory molecules must be involved [74].

Because the addition of beneficial bacteria reduces IPAN slow afterhyperpolarisation and increases the intrinsic excitability, the question arises whether the absence of gut bacteria altogether might have the opposite effect. In agreement with this notion it has been established that IPANs taken from germ free animals have reduced intrinsic excitability while the slow afterhyperpolarisation is exaggerated beyond that seen in normal healthy animals [75]. This finding suggests that the microbiome itself conditions the normal functioning of IPANs and therefore gastrointestinal motility.

Neuroactive bacteria might alter afferent vagal signalling via two broad sensory modalities. Beneficial luminal bacteria might act on the enteric nervous system to alter the contractile activity of the intestine [73, 74, 76, 77] and this would be sensed by the *intramuscular arrays* and *intraganglionic laminar endings* both of which are vagal mechanoreceptors [78, 79]. Closer to the lumen, the vagus innervates mucosal villi and varied epithelial layer cells [80] with endings that are both chemo-and mechanosensitive [80, 81]. Vagal chemoreceptors could be activated directly by substances such as short chain fatty acids that can be transported across the epithelial barrier to the portal circulation [82] or by paracrine mediators like 5-HT, histamine, CCK, ATP or glucagon-like peptides released by the various mucosal epithelial layer taste cells [83, 84]. That vagal mucosal chemoreceptors fibres might be involved in activation of the "microbiome-gut-brain axis" [83] is substantiated by animal studies where beneficial bacteria were applied to the epithelium at known concentrations and vagal nerve activity recorded.

In a pioneering study, intraduodenal injection of a *Lactobacillus johnsonii* strain increased gastric vagus massed multiunit firing within 15 min of application [85]. However, the authors did not identify single unit chemoreceptors fibres, nor were they able to rule out potentially confounding effects of the probiotic on duodenal motility. Intraluminal JB-1 application appears to reduce single dorsal root fibre discharge [86] as well as blocking nociceptive sensitisation of DRG neurons innervating the rat colon [87]. This is potentially important for behaviour, as the caudal nucleus of the solitary tract receives input from the spinosolitary tract [88, 89]. The caudal nucleus is the part that receives projections from nodose neurons that innervate the intestines [90]. Perez-Burgos et al. [91] recently used an ex vivo mouse jejunum preparation to record from perivascular mesenteric nerves in real time while known concentrations of psychoactive JB-1 bacteria were added to the luminal perfusate. 10^9 cfu/mL JB-1 increased the constitutive firing rate of responsive individual mesenteric nerve single units by about 70 % above baseline rate within 10–15 min of application. This effect persisted even when contractions were abolished by blocking L calcium channels with

nicardipine, demonstrating that the fibres were chemoreceptors. Importantly, the bacteria did not translocate during the period of the experiment. The chemoreceptor fibres were multimodal since they were also strongly activated by raising intraluminal pressure to 30 hPa when the musculature was allowed to contract, and this response was further augmented by addition of JB-1 to the lumen. When vagal fibres were eliminated by subdiaphragmatic vagotomy the excitatory effects of JB-1 on single units was absent demonstrating that vagal but not spinal fibres were activated.

These results have demonstrated for the first time that multimodal chemoreceptor vagal afferents acutely respond to luminal application of a psychoactive probiotic thus delineating the peripheral sensory projection and physical basis for the bacteria's effects on the brain and behaviour.

The Vagus and Gut Bacteria

There is now strong evidence from animal studies that gut microorganisms can activate the vagus nerve and that such activation plays a critical role in mediating effects on the brain and subsequently, behaviour.

Such evidence came early from the study of animals infected with pathogens. Subdiaphragmatic vagotomy attenuated c-fos expression in the PVN of rats inoculated with *Salmonella typhimurium* [92]. Although *S. typhimurium* infection was accompanied by intestinal inflammation subsequent studies have indicated that microorganisms in the gastrointestinal tract can directly activate neural pathways even in the absence of an identified immune response [93]. The anxiogenic effect of orally administered subclinical doses of *Campylobacter jejuni*, in mice was associated with a significant increase in c-Fos expression in neurons bilaterally in the vagal ganglia and activated visceral sensory nuclei in the brainstem. The areas of brainstem activation, the NTS and lateral parabrachial nucleus, participate in neural information processing that ultimately lead to autonomic neuroendocrine and behavioural responses [93].

Non-pathogenic bacteria also activate vagal signalling from gut to brain. Tanida et al. [85] demonstrated that intraduodenal injection of the bacterial strain *L. johnsonii* La1 reduced renal sympathetic nerve activity and blood pressure while enhancing gastric vagal nerve activity. All of these effects could be abolished by pre-treatment with a histaminergic H3-receptor antagonist. Similarly the effects were absent in animals that had bilateral lesions of the hypothalamic suprachiasmatic nucleus, a major regulator of circadian rhythm. These findings suggest that the influence of the bacteria on autonomic neurotransmission and subsequently blood pressure, is mediated centrally, likely through histaminergic nerves and the suprachiasmatic nucleus [85].

Consequently, subdiaphragmatic denervation of vagal nerve fibers surrounding the oesophagus eliminated the ability of *L. johnsonii La1* to reduce renal sympathetic nerve activity and blood pressure indicating that at least some of the effects of

this bacteria on autonomic nerve responses were elicited by interaction with afferent vagal nerve fibers [85].

More recently, it was demonstrated that oral administration of a *L. rhamnosus* strain (JB1) could alter the normal behaviour of adult Balb/c mice [48]. Chronic treatment with the bacteria reduced anxiety-like behaviour as assessed in an elevated plus maze, and decreased the time spent immobile in a forced swim test. In addition, stress-induced plasma corticosterone levels were lower in treated mice, a similar effect to subchronic or chronic treatment with antidepressants that can prevent forced swim stress-induced increases in plasma corticosterone in both mice and rats. Overall, changes induced with *L. rhamnousus* were indicative of reduced anxiety, and decreased depression-like behaviour. Assessment of neural correlates to behavioural changes determined that mice receiving *L. rhamnosus* had alterations in central GABA receptor subunit mRNA expression. *L. rhamnosus* administration decreased expression of GABA type B (GABAB) subunit 1 isoform b (GABAB1b) mRNA in the amygdala and hippocampus, while increasing expression in cortical areas. Expression of GABAAα2 receptor mRNA was reduced in the amygdala and cortical areas, whereas levels were increased in the hippocampus [48]. It is difficult to attribute a causal relationship between behavioural effects observed and neural correlates. However, reduced expression of GABAB1b mRNA, in the amygdala, hippocampus, and locus ceruleus is consistent with the antidepressant-like effect of GABAB receptor antagonists [94] and with studies of GABAB1b-deficient animals, indicating an important role for this subunit in the development of cognitive processes, including those relevant to fear [95, 96]. The experimentally induced changes, especially in GABAA receptors correlates with those seen in benzodiazepine administration, a well characterized tranquillizer [97]. It is also interesting to note that in a recent study of transcriptomes from the mucosa of the proximal small intestines of healthy human subjects following intraduodenal application of different *Lactobacillus* species, there was a strong correspondence between in vivo transcriptional networks altered after consumption of one of the strains, *Lactobacillus casei*, and the response of human cells to the anxiolytic GABA A receptor modulator, Tracazolate [98].

Subdiaphragmatic vagotomy blocked the anxiolytic and antidepressant effects of chronic *L. rhamnosus* ingestion in normal adult Balb/c mice while also preventing the associated alterations in GABAAα2 mRNA expression in the amygdala [48]. Similarly, the ability of *B. longum* to attenuate DSS colitis induced anxiety was abolished by vagotomy [67]. Ingestion of the same bacteria had similar effects on the behaviour of normal healthy mice. However not all beneficial bacteria seem to exert their behavioural effects via the vagus [99]. These data suggest that gut bacteria may affect the brain through both vagal, non-vagal and other possible systemic pathways.

Given what is known of the vagal anti-inflammatory reflex it seems plausible that gut microbiota induced modulation of vagal mediated “periphery to brain” signalling could translate into changes in efferent neural pathways controlling immune responses. As yet, there is no evidence that the vagus nerve contributes to the immunomodulatory effects of gut bacteria and at least one study suggests that

the local protective effect of Lactobacillus and Bifidobacteria strains in models of colitis does not depend on vagal nerves [100].

Conclusions

Overall, studies indicate that vagal pathways mediate signals that can induce both anxiogenic and anxiolytic effects depending on the nature of the stimulus and, interestingly, the vagus appears to differentiate between non-pathogenic and potentially pathogenic bacteria even in the absence of overt inflammation.

It is therefore clear that the involvement of the vagus in microbiota-gut-brain communication is not straightforward or simply dependent on "activation". Even with the dubious assumption that an increase in c-fos expression always reflects an increase in neuronal firing rates, existing anatomical data cannot answer why in some cases vagal activation causes depression and in others, for example, electrical stimulation of the vagus, eases depression. What is currently lacking are relevant data on the electrophysiology of the system. Tanida et al. [85] showed that injecting *L. johnsonii* into rat duodenum increased gastric vagal multiunit firing rate by about 10 % within 15 min, and this slowly grew to a 90 % increase over the baseline 1 h after the injection was delivered. Clearly, much more work of this sort needs to be done and should compare with vagal responses to either anxiogenic or anxiolytic peripheral stimuli.

Electrophysiology may also be utilized to determine the nature of the peripheral signal acting to stimulate the vagus nerve in the gut following exposure to specific bacteria or nutrients. Single chemosensitive vagal afferent units supplying the gut are normally silent or have a low resting discharge of 0–3 Hz [101]. They respond to most luminal molecules by increasing their firing rate. Response latencies vary consistently according to the chemical nature of the stimulus. The short chain fatty acid butyrate had a response onset latency of 2–3 ms [49], the long chain fatty acid sodium oleate had a latency of 15 ms [49], amino acids evoked responses within about 9 ms [3]. The response to casein acid hydrolysate has a latency of 19 ms [102], and glucose takes 20 ms [103]. *S. typhimurium* lipopolysaccharide evoked an increase in the mesenteric nerve discharge within 30 min while LPS from a commensal *E. coli* had no effect [104].

Certainly, important advances in our understanding of the gut-brain and microbiome- gut-brain axis will come from studies of how distinct microbial and nutritional stimuli activate the vagus and the nature of the signals transmitted to the brain that lead to differential changes in the neurochemistry of the brain and behaviour. However, while it appears that the vagus is critical to mediating gut-brain communication by specific bacteria in some model systems, it is by no means the only potential signalling method. Indeed, largely due to technical difficulties, few studies have investigated the role of spinal afferents in mediating bacteria induced changes in behaviour and brain chemistry. It is certainly possible that the observed changes in brain chemistry behaviour induced by gut bacteria

require parallel input from both the vagal and spinal afferents. Furthermore, behavioural changes induced through disruption of the microbiota by antibiotic treatment have been demonstrated to be independent of vagal signalling [99] with some additional evidence that neither sympathetic afferents nor immune modulation is required. This clearly suggests that the bacteria in the gut can communicate to the brain through multiple pathways. Nevertheless understanding the induction and transmission of anxiolytic signals in the vagus nerve may have important implications for the development of microbial-or nutrition based therapeutic strategies for mood disorders.

References

1. Browning KN, Mendelowitz D (2003) Musings on the wanderer: what's new in our understanding of vago-vagal reflexes?: II. Integration of afferent signaling from the viscera by the nodose ganglia. Am J Physiol Gastrointest Liver Physiol 284(1):G8–G14, PubMed PMID: 12488231
2. Blackshaw LA, Brookes SJ, Grundy D, Schemann M (2007) Sensory transmission in the gastrointestinal tract. Neurogastroenterol Motil 19(Suppl 1):1–19, PubMed PMID: 17280582
3. Mei N (1983) Recent studies on intestinal vagal afferent innervation. Functional implications. J Auton Nerv Syst 9(1):199–206, PubMed PMID: 6363498
4. Mei N (1985) Intestinal chemosensitivity. Physiol Rev 65(2):211–237, PubMed PMID: 2858893
5. Wang FB, Powley TL (2007) Vagal innervation of intestines: afferent pathways mapped with new en bloc horseradish peroxidase adaptation. Cell Tissue Res 329(2):221–230, PubMed PMID: 17453246
6. Li Y (2007) Sensory signal transduction in the vagal primary afferent neurons. Curr Med Chem 14(24):2554–2563, PubMed PMID: 17979708
7. Berthoud HR, Lynn PA, Blackshaw LA (2001) Vagal and spinal mechanosensors in the rat stomach and colon have multiple receptive fields. Am J Physiol Regul Integr Comp Physiol 280(5):R1371–R1381, PubMed PMID: 11294756
8. Craig AD (2009) How do you feel now? The anterior insula and human awareness. Nat Rev Neurosci 10(1):59–70, PubMed PMID: 19096369
9. Crucian GP, Hughes JD, Barrett AM, Williamson DJ, Bauer RM, Bowers D et al (2000) Emotional and physiological responses to false feedback. Cortex 36(5):623–647, PubMed PMID: 11195911
10. Zagon A (2001) Does the vagus nerve mediate the sixth sense? Trends Neurosci 24 (11):671–673, PubMed PMID: 11672813
11. Dantzer R, Konsman JP, Bluthe RM, Kelley KW (2000) Neural and humoral pathways of communication from the immune system to the brain: parallel or convergent? Auton Neurosci 85(1–3):60–65, PubMed PMID: 11189027
12. Luheshi GN, Bluthe RM, Rushforth D, Mulcahy N, Konsman JP, Goldbach M et al (2000) Vagotomy attenuates the behavioural but not the pyrogenic effects of interleukin-1 in rats. Auton Neurosci 85(1–3):127–132, PubMed PMID: 11189019
13. Konsman JP, Luheshi GN, Bluthe RM, Dantzer R (2000) The vagus nerve mediates behavioural depression, but not fever, in response to peripheral immune signals; a functional anatomical analysis. Eur J Neurosci 12(12):4434–4446, PubMed PMID: 11122354
14. Hansen MK, O'Connor KA, Goehler LE, Watkins LR, Maier SF (2001) The contribution of the vagus nerve in interleukin-1beta-induced fever is dependent on dose. Am J Physiol Regul Integr Comp Physiol 280(4):R929–R934, PubMed PMID: 11247812

15. Bluthe RM, Walter V, Parnet P, Laye S, Lestage J, Verrier D et al (1994) Lipopolysaccharide induces sickness behaviour in rats by a vagal mediated mechanism. C R Acad Sci III 317 (6):499–503, PubMed PMID: 7987701
16. Laye S, Bluthe RM, Kent S, Combe C, Medina C, Parnet P et al (1995) Subdiaphragmatic vagotomy blocks induction of IL-1 beta mRNA in mice brain in response to peripheral LPS. Am J Physiol 268(5 Pt 2):R1327–R1331, PubMed PMID: 7771597
17. Bret-Dibat JL, Bluthe RM, Kent S, Kelley KW, Dantzer R (1995) Lipopolysaccharide and interleukin-1 depress food-motivated behavior in mice by a vagal-mediated mechanism. Brain Behav Immun 9(3):242–246, PubMed PMID: 8590821
18. Krahl SE, Senanayake SS, Pekary AE, Sattin A (2004) Vagus nerve stimulation (VNS) is effective in a rat model of antidepressant action. J Psychiatr Res 38(3):237–240. PubMed PMID: 15003428
19. Cryan JF, Valentino RJ, Lucki I (2005) Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. Neurosci Biobehav Rev 29 (4–5):547–569, PubMed PMID: 15893822
20. Cunningham JT, Mifflin SW, Gould GG, Frazer A (2008) Induction of c-Fos and DeltaFosB immunoreactivity in rat brain by Vagal nerve stimulation. Neuropsychopharmacology 33 (8):1884–1895, PubMed PMID: 17957222
21. Biggio F, Gorini G, Utzeri C, Olla P, Marrosu F, Mocchetti I et al (2009) Chronic vagus nerve stimulation induces neuronal plasticity in the rat hippocampus. Int J Neuropsychopharmacol 12(9):1209–1221, PubMed PMID: 19309534. Pubmed Central PMCID: 2879889
22. Walsh SP, Kling MA (2004) VNS and depression: current status and future directions. Expert Rev Med Devices 1(1):155–160, PubMed PMID: 16293018
23. Martin JL, Martin-Sanchez E (2012) Systematic review and meta-analysis of vagus nerve stimulation in the treatment of depression: variable results based on study designs. Eur Psychiatry 27(3):147–155, PubMed PMID: 22137776
24. Rizvi SJ, Donovan M, Giacobbe P, Placenza F, Rotzinger S, Kennedy SH (2011) Neurostimulation therapies for treatment resistant depression: a focus on vagus nerve stimulation and deep brain stimulation. Int Rev Psychiatry 23(5):424–436, PubMed PMID: 22200132
25. Miao FJ, Green PG, Levine JD (2004) Mechanosensitive duodenal afferents contribute to vagal modulation of inflammation in the rat. J Physiol 554(Pt 1):227–235, PubMed PMID: 14678504. Pubmed Central PMCID: 1664747
26. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR et al (2000) Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405 (6785):458–462, PubMed PMID: 10839541
27. Ghia JE, Blennerhassett P, Kumar-Ondiveeran H, Verdu EF, Collins SM (2006) The vagus nerve: a tonic inhibitory influence associated with inflammatory bowel disease in a murine model. Gastroenterology 131(4):1122–1130, PubMed PMID: 17030182
28. O'Mahony C, van der Kleij H, Bienenstock J, Shanahan F, O'Mahony L (2009) Loss of vagal anti-inflammatory effect: in vivo visualization and adoptive transfer. Am J Physiol Regul Integr Comp Physiol 297(4):R1118–R1126, PubMed PMID: 19675277. Epub 2009 Aug 12
29. Karimi K, Bienenstock J, Wang L, Forsythe P (2010) The vagus nerve modulates CD4+ T cell activity. Brain Behav Immun 24(2):316–323, PubMed PMID: 19887104, Epub 2009 Nov 1
30. Rosas-Ballina M, Olofsson PS, Ochani M, Valdes-Ferrer SI, Levine YA, Reardon C et al (2011) Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. Science 334(6052):98–101, PubMed PMID: 21921156
31. Reardon C, Duncan GS, Brustle A, Brenner D, Tusche MW, Olofsson PS et al (2013) Lymphocyte-derived ACh regulates local innate but not adaptive immunity. Proc Natl Acad Sci U S A 110(4):1410–1415, PubMed PMID: 23297238. Pubmed Central PMCID: 3557089
32. Connor TJ, Leonard BE (1998) Depression, stress and immunological activation: the role of cytokines in depressive disorders. Life Sci 62(7):583–606, PubMed PMID: 9472719

33. Li M, Soczynska JK, Kennedy SH (2011) Inflammatory biomarkers in depression: an opportunity for novel therapeutic interventions. Curr Psychiatry Rep 13(5):316–320, PubMed PMID: 21671010
34. Miller AH, Maletic V, Raison CL (2009) Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biol Psychiatry 65(9):732–741, PubMed PMID: 19150053. Pubmed Central PMCID: 2680424
35. Dinan TG (2009) Inflammatory markers in depression. Curr Opin Psychiatry 22(1):32–36, PubMed PMID: 19122532
36. Capuron L, Dantzer R (2003) Cytokines and depression: the need for a new paradigm. Brain Behav Immun 17(Suppl 1):S119–S124, PubMed PMID: 12615197
37. Hernandez ME, Mendieta D, Martinez-Fong D, Loria F, Moreno J, Estrada I et al (2008) Variations in circulating cytokine levels during 52 week course of treatment with SSRI for major depressive disorder. Eur Neuropsychopharmacol 18(12):917–924, PubMed PMID: 18805677
38. O'Brien SM, Scully P, Fitzgerald P, Scott LV, Dinan TG (2007) Plasma cytokine profiles in depressed patients who fail to respond to selective serotonin reuptake inhibitor therapy. J Psychiatr Res 41(3–4):326–331. PubMed PMID: 16870211
39. De Herdt V, Bogaert S, Bracke KR, Raedt R, De Vos M, Vonck K et al (2009) Effects of vagus nerve stimulation on pro- and anti-inflammatory cytokine induction in patients with refractory epilepsy. J Neuroimmunol 214(1–2):104–108, PubMed PMID: 19608283
40. Majoie HJ, Rijkers K, Berfelo MW, Hulsman JA, Myint A, Schwarz M et al (2011) Vagus nerve stimulation in refractory epilepsy: effects on pro- and anti-inflammatory cytokines in peripheral blood. Neuroimmunomodulation 18(1):52–56, PubMed PMID: 20639683
41. Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol 9(5):313–323, PubMed PMID: 19343057
42. Raison CL, Lowry CA, Rook GA (2010) Inflammation, sanitation, and consternation: loss of contact with coevolved, tolerogenic microorganisms and the pathophysiology and treatment of major depression. Arch Gen Psychiatry 67(12):1211–1224, PubMed PMID: 21135322. Epub 2010/12/08. eng
43. Okada H, Kuhn C, Feillet H, Bach JF (2010) The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. Clin Exp Immunol 160(1):1–9, PubMed PMID: 20415844. Pubmed Central PMCID: 2841828
44. Strachan DP (2000) Family size, infection and atopy: the first decade of the "hygiene hypothesis". Thorax 55(Suppl 1):S2–S10, PubMed PMID: 10943631, Pubmed Central PMCID: 1765943
45. Godbout JP, Glaser R (2006) Stress-induced immune dysregulation: implications for wound healing, infectious disease and cancer. J Neuroimmune Pharmacol 1(4):421–427, PubMed PMID: 18040814
46. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN et al (2004) Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol 558(Pt 1):263–275, PubMed PMID: 15133062, Pubmed Central PMCID: 1664925, Epub 2004 May 7
47. Gareau MG, Jury J, MacQueen G, Sherman PM, Perdue MH (2007) Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation. Gut 56(11):1522–1528, PubMed PMID: 17339238, Pubmed Central PMCID: 2095679, Epub 2007/03/07. eng
48. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG et al (2011) Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 108(38):16050–16055, PubMed PMID: 21876150, Pubmed Central PMCID: 3179073, Epub 2011/08/31. eng
49. Lal S, Kirkup AJ, Brunsden AM, Thompson DG, Grundy D (2001) Vagal afferent responses to fatty acids of different chain length in the rat. Am J Physiol Gastrointest Liver Physiol 281 (4):G907–G915, PubMed PMID: 11557510

50. Luyer MD, Greve JW, Hadfoune M, Jacobs JA, Dejong CH, Buurman WA (2005) Nutritional stimulation of cholecystokinin receptors inhibits inflammation via the vagus nerve. J Exp Med 202(8):1023–1029, PubMed PMID: 16216887, Pubmed Central PMCID: 2213207
51. Lubbers T, de Haan JJ, Luyer MD, Verbaeys I, Hadfoune M, Dejong CH et al (2010) Cholecystokinin/cholecystokinin-1 receptor-mediated peripheral activation of the afferent vagus by enteral nutrients attenuates inflammation in rats. Ann Surg 252(2):376–382, PubMed PMID: 20585240
52. Billman GE (2012) Effect of dietary omega-3 polyunsaturated Fatty acids on heart rate and heart rate variability in animals susceptible or resistant to ventricular fibrillation. Front Physiol 3:71, PubMed PMID: 22470351, Pubmed Central PMCID: 3313477
53. Christensen JH (2011) Omega-3 polyunsaturated Fatty acids and heart rate variability. Front Physiol 2:84, PubMed PMID: 22110443, Pubmed Central PMCID: 3217222
54. Huston JM, Tracey KJ (2011) The pulse of inflammation: heart rate variability, the cholinergic anti-inflammatory pathway and implications for therapy. J Intern Med 269(1):45–53, PubMed PMID: 21158977
55. Thayer JF, Brosschot JF (2005) Psychosomatics and psychopathology: looking up and down from the brain. Psychoneuroendocrinology 30(10):1050–1058, PubMed PMID: 16005156
56. Weber CS, Thayer JF, Rudat M, Wirtz PH, Zimmermann-Viehoff F, Thomas A et al (2010) Low vagal tone is associated with impaired post stress recovery of cardiovascular, endocrine, and immune markers. Eur J Appl Physiol 109(2):201–211, PubMed PMID: 20052593
57. Sloan RP, McCreath H, Tracey KJ, Sidney S, Liu K, Seeman T (2007) RR interval variability is inversely related to inflammatory markers: the CARDIA study. Mol Med 13 (3–4):178–184. PubMed PMID: 17592552. Pubmed Central PMCID: 1892756
58. Eisner F, Jacob P, Frick JS, Feilitzsch M, Geisel J, Mueller MH et al (2011) Immunonutrition with long-chain fatty acids prevents activation of macrophages in the gut wall. J Gastrointest Surg 15(5):853–859, PubMed PMID: 21384238
59. Long H, Yang H, Lin Y, Situ D, Liu W (2013) Fish oil-supplemented parenteral nutrition in patients following esophageal cancer surgery: effect on inflammation and immune function. Nutr Cancer 65(1):71–75, PubMed PMID: 23368915
60. Lubbers T, Kox M, de Haan JJ, Greve JW, Pompe JC, Ramakers BP et al (2013) Continuous administration of enteral lipid- and protein-rich nutrition limits inflammation in a human endotoxemia model. Crit Care Med 41(5):1258–1265, PubMed PMID: 23388517
61. Tracey KJ (2005) Fat meets the cholinergic antiinflammatory pathway. J Exp Med 202 (8):1017–1021, PubMed PMID: 16230472, Pubmed Central PMCID: 2213208
62. Huang SY, Yang HT, Chiu CC, Pariante CM, Su KP (2008) Omega-3 fatty acids on the forced-swimming test. J Psychiatr Res 42(1):58–63, PubMed PMID: 17070845
63. Jiang LH, Liang QY, Shi Y (2012) Pure docosahexaenoic acid can improve depression behaviors and affect HPA axis in mice. Eur Rev Med Pharmacol Sci 16(13):1765–1773, PubMed PMID: 23208960
64. Jazayeri S, Tehrani-Doost M, Keshavarz SA, Hosseini M, Djazayery A, Amini H et al (2008) Comparison of therapeutic effects of omega-3 fatty acid eicosapentaenoic acid and fluoxetine, separately and in combination, in major depressive disorder. Aust N Z J Psychiatry 42 (3):192–198, PubMed PMID: 18247193
65. Jazayeri S, Keshavarz SA, Tehrani-Doost M, Djalali M, Hosseini M, Amini H et al (2010) Effects of eicosapentaenoic acid and fluoxetine on plasma cortisol, serum interleukin-1beta and interleukin-6 concentrations in patients with major depressive disorder. Psychiatry Res 178(1):112–115, PubMed PMID: 20466437
66. Takeuchi T, Iwanaga M, Harada E (2003) Possible regulatory mechanism of DHA-induced anti-stress reaction in rats. Brain Res 964(1):136–143, PubMed PMID: 12573522
67. Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X et al (2011) The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut-brain communication. Neurogastroenterol Motil 23(12):1132–1139, PubMed PMID: 21988661, Pubmed Central PMCID: 3413724, Epub 2011/10/13. eng

68. Gomes NGM, Campos MG, Órfão JMC, Ribeiro CAF (2009) Plants with neurobiological activity as potential targets for drug discovery. Prog Neuropsychopharmacol Biol Psychiatry 33(8):1372–1389
69. Ekblad E, Winther C, Ekman R, Hakanson R, Sundler F (1987) Projections of peptide-containing neurons in rat small intestine. Neuroscience 20(1):169–188, PubMed PMID: 2436086
70. Keast JR, Furness JB, Costa M (1984) Origins of peptide and norepinephrine nerves in the mucosa of the guinea pig small intestine. Gastroenterology 86(4):637–644, PubMed PMID: 6199254, Epub 1984/04/01. eng
71. Kunze WA, Mao YK, Wang B, Huizinga JD, Ma X, Forsythe P et al (2009) *Lactobacillus reuteri* enhances excitability of colonic AH neurons by inhibiting calcium-dependent potassium channel opening. J Cell Mol Med 13(8B):2261–2270, PubMed PMID: 19210574
72. Mao YK, Kasper DL, Wang B, Forsythe P, Bienenstock J, Kunze WA (2013) *Bacteroides fragilis* polysaccharide A is necessary and sufficient for acute activation of intestinal sensory neurons. Nat Commun 4:1465, PubMed PMID: 23403566
73. Wang B, Mao YK, Diorio C, Wang L, Huizinga JD, Bienenstock J et al (2010) *Lactobacillus reuteri* ingestion and IK(Ca) channel blockade have similar effects on rat colon motility and myenteric neurones. Neurogastroenterol Motil 22(1):98–107, e33. PubMed PMID: 19788711
74. Wu RY, Pasyk M, Wang B, Forsythe P, Bienenstock J, Mao YK et al (2013) Spatiotemporal maps reveal regional differences in the effects on gut motility for *Lactobacillus reuteri* and rhamnosus strains. Neurogastroenterol Motil 25(3):e205–e214, PubMed PMID: 23316914
75. McVey Neufeld KA, Mao YK, Bienenstock J, Foster JA, Kunze WA (2013) The microbiome is essential for normal gut intrinsic primary afferent neuron excitability in the mouse. Neurogastroenterol Motil 25(2):183–e88, PubMed PMID: 23181420
76. Massi M, Ioan P, Budriesi R, Chiarini A, Vitali B, Lammers KM et al (2006) Effects of probiotic bacteria on gastrointestinal motility in guinea-pig isolated tissue. World J Gastroenterol 12(37):5987–5994. PubMed PMID: 17009397. eng
77. Wang B, Mao YK, Diorio C, Pasyk M, Wu RY, Bienenstock J et al (2010) Luminal administration ex vivo of a live *Lactobacillus* species moderates mouse jejunal motility within minutes. FASEB J 24(10):4078–4088, PubMed PMID: 20519636
78. Zagorodnyuk VP, Chen BN, Brookes SJ (2001) Intraganglionic laminar endings are mechano-transduction sites of vagal tension receptors in the guinea-pig stomach. J Physiol 534(Pt 1):255–268, PubMed PMID: 11433006
79. Phillips RJ, Powley TL (2000) Tension and stretch receptors in gastrointestinal smooth muscle: re-evaluating vagal mechanoreceptor electrophysiology. Brain Res Brain Res Rev 34(1–2):1–26, PubMed PMID: 11086184, Epub 2000/11/22. eng
80. Powley TL, Spaulding RA, Haglof SA (2011) Vagal afferent innervation of the proximal gastrointestinal tract mucosa: chemoreceptor and mechanoreceptor architecture. J Comp Neurol 519(4):644–660, PubMed PMID: 21246548
81. Powley TL, Phillips RJ (2002) Musings on the wanderer: what's new in our understanding of vago-vagal reflexes? I. Morphology and topography of vagal afferents innervating the GI tract. Am J Physiol Gastrointest Liver Physiol 283(6):G1217–G1225. PubMed PMID: 12388183. eng
82. Hara H, Haga S, Aoyama Y, Kiriyama S (1999) Short-chain fatty acids suppress cholesterol synthesis in rat liver and intestine. J Nutr 129(5):942–948
83. Raybould HE (2010) Gut chemosensing: interactions between gut endocrine cells and visceral afferents. Auton Neurosci 153(1–2):41–46
84. Bertrand P (2009) The cornucopia of intestinal chemosensory transduction. Front Neurosci 3:48, PubMed PMID: 20582275
85. Tanida M, Yamano T, Maeda K, Okumura N, Fukushima Y, Nagai K (2005) Effects of intraduodenal injection of *Lactobacillus johnsonii* La1 on renal sympathetic nerve activity and blood pressure in urethane-anesthetized rats. Neurosci Lett 389(2):109–114, PubMed PMID: 16118039, Epub 2005/08/25. eng

86. Kamiya T, Wang L, Forsythe P, Goettsche G, Mao Y, Wang Y et al (2006) Inhibitory effects of *Lactobacillus reuteri* on visceral pain induced by colorectal distension in Sprague–Dawley rats. Gut 55(2):191–196, PubMed PMID: 16361309, Pubmed Central PMCID: 1856513, Epub 2005/12/20. eng
87. Kunze WA, Foster JA, Mao Y, Ma XJ, Bienenstock J (eds) (2007) *Lactobacillus* sp (LB) probiotic congestion by rats blocks memory in DRG neurons of repeated colorectal distensions. In: Annual Meeting the Society for Neuroscience, San Diego Convention Center, California, Society for Neuroscience, 12–16 November 2007
88. Menetrey D, Basbaum AI (1987) Spinal and trigeminal projections to the nucleus of the solitary tract: a possible substrate for somatovisceral and viscerovisceral reflex activation. J Comp Neurol 255(3):439–450, PubMed PMID: 3819024, Epub 1987/01/15. eng
89. Menetrey D, De Pommery J (1991) Origins of spinal ascending pathways that reach central areas involved in visceroception and visceronociception in the rat. Eur J Neurosci 3 (3):249–259, PubMed PMID: 12106203, Epub 1991/01/01. Eng
90. Rinaman L (2010) Ascending projections from the caudal visceral nucleus of the solitary tract to brain regions involved in food intake and energy expenditure. Brain Res 1350:18–34, PubMed PMID: 20353764, Pubmed Central PMCID: PMC2909454, Epub 2010/04/01. eng
91. Perez-Burgos A, Wang B, Mao YK, Mistry B, Neufeld KA, Bienenstock J et al (2013) Psychoactive bacteria *Lactobacillus rhamnosus* (JB-1) elicits rapid frequency facilitation in vagal afferents. Am J Physiol Gastrointest Liver Physiol 304(2):G211–G220, PubMed PMID: 23139216
92. Wang X, Wang BR, Zhang XJ, Xu Z, Ding YQ, Ju G (2002) Evidences for vagus nerve in maintenance of immune balance and transmission of immune information from gut to brain in STM-infected rats. World J Gastroenterol 8(3):540–545, PubMed PMID: 12046088
93. Goehler LE, Gaykema RP, Opitz N, Reddaway R, Badr N, Lyte M (2005) Activation in vagal afferents and central autonomic pathways: early responses to intestinal infection with *Campylobacter jejuni*. Brain Behav Immun 19(4):334–344, PubMed PMID: 15944073, Epub 2005/06/10. eng
94. Cryan JF, Slattery DA (2010) GABAB receptors and depression. Current status. Adv Pharmacol 58:427–451, PubMed PMID: 20655491
95. Jacobson LH, Kelly PH, Bettler B, Kaupmann K, Cryan JF (2007) Specific roles of GABA(B (1)) receptor isoforms in cognition. Behav Brain Res 181(1):158–162, PubMed PMID: 17498817, Pubmed Central PMCID: 2980901, Epub 2007/05/15. eng
96. Jacobson LH, Bettler B, Kaupmann K, Cryan JF (2007) Behavioral evaluation of mice deficient in GABA(B(1)) receptor isoforms in tests of unconditioned anxiety. Psychopharmacology (Berl) 190(4):541–553, PubMed PMID: 17171558
97. Jacob TC, Michels G, Silayeva L, Haydon J, Succol F, Moss SJ (2012) Benzodiazepine treatment induces subtype-specific changes in GABA(A) receptor trafficking and decreases synaptic inhibition. Proc Natl Acad Sci U S A 109(45):18595–18600, PubMed PMID: 23091016, Pubmed Central PMCID: 3494928
98. van Baarlen P, Troost F, van der Meer C, Hooiveld G, Boekschoten M, Brummer RJ et al (2011) Human mucosal in vivo transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways. Proc Natl Acad Sci U S A 108(Suppl 1):4562–4569, PubMed PMID: 20823239, Pubmed Central PMCID: 3063594, Epub 2010/09/09. eng
99. Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J et al (2011) The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 141(2):599–609, e1–3. PubMed PMID: 21683077
100. van der Kleij H, O'Mahony C, Shanahan F, O'Mahony L, Bienenstock J (2008) Protective effects of *Lactobacillus rhamnosus* [corrected] and *Bifidobacterium infantis* in murine models for colitis do not involve the vagus nerve. Am J Physiol Regul Integr Comp Physiol 295(4):R1131–R1137, PubMed PMID: 18667710, Epub 2008/08/01. eng

101. Blackshaw LA, Grundy D (1993) Effects of 5-hydroxytryptamine (5-HT) on the discharge of vagal mechanoreceptors and motility in the upper gastrointestinal tract of the ferret. J Auton Nerv Syst 45(1):51–59, PubMed PMID: 8227964
102. Eastwood C, Maubach K, Kirkup AJ, Grundy D (1998) The role of endogenous cholecystokinin in the sensory transduction of luminal nutrient signals in the rat jejunum. Neurosci Lett 254(3):145–148, PubMed PMID: 10214978
103. Hardcastle J, Hardcastle PT, Sanford PA (1978) Effect of actively transported hexoses on afferent nerve discharge from rat small intestine. J Physiol 285:71–84, PubMed PMID: 745132, Pubmed Central PMCID: 1281743
104. Liu CY, Mueller MH, Rogler G, Grundy D, Kreis ME (2009) Differential afferent sensitivity to mucosal lipopolysaccharide from *Salmonella typhimurium* and *Escherichia coli* in the rat jejunum. Neurogastroenterol Motil 21(12):1335–e129, PubMed PMID: 19614870

Chapter 6
The Brain-Gut Axis in Health and Disease

Yasser Al Omran and Qasim Aziz

Abstract The interaction between the brain and the gut has been recognized for many centuries. This bidirectional interaction occurs via neural, immunological and hormonal routes, and is important not only in normal gastrointestinal function but also plays a significant role in shaping higher cognitive function such as our feelings and our subconscious decision-making. Therefore, it remains unsurprising that perturbations in normal signalling have been associated with a multitude of disorders, including inflammatory and functional gastrointestinal disorders, and eating disorders.

Abbreviations

5-HT	5-Hydroxytryptimaine
ACC	Anterior cingulate cortex
Ach	Acetylcholine
ANS	Autonomic nervous system
CCK1R	Cholecystokinin 1 receptor
CNS	Central nervous system
CRH	Corticotropin-releasing factor
DMN	Dorsal motor nucleus of the vagus
EMS	Emotional motor system
FGF19	Fibroblast growth factor 19

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_6,

fMRI	Functional magnetic resonance imaging
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
GLP1	Glucagon-like peptide-1
GPR	G-protein coupled receptor
HPA	Hypothalamic-pituitary-adrenal
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
KLB	Klotho beta
NF- κB	Nuclear factor κB
NPY	Neuropeptide Y
OFC	Orbitofrontal cortex
PAG	Periaqueductal grey
PFC	Prefrontal cortex
TNF-α	Tumor necrosis factor-α
α7nAChR	α7 nicotinic acetylcholine receptor

Introduction

The interaction between the brain and the gut has been recognized for many centuries [1]. However, it was not until the nineteenth and early twentieth centuries that this association was critically evaluated by prominent physiologists, psychiatrists and psychologists [2–6]. From their studies, a bidirectional relationship was assumed, with both a brain to gut modulation of gastrointestinal (GI) function, by stress and emotions, and a gut to brain pathway that transmits physiological information about gut sensory motor function. More recent work demonstrates that these interactions occur via neural, immunological and hormonal routes. Thus the brain-gut axis plays an important role in gut regulating physiological function and its disruption may have pathophysiological consequences. The aim of this chapter is to discuss the basic principles, the latest research and the significance of the brain-gut axis in both health and disease.

Brain to Gut Signalling in Health

The brain signals to the viscera, including the GI tract, through a myriad of neural, hormonal and immunological routes. These include the sympatho-adrenal axis and hypothalamic-pituitary-adrenal (HPA) axis, the two branches of the autonomic nervous system (ANS), and the monoaminergic pathways, which modify dorsal horn excitability and spinal reflexes. The hypothalamus and amygdala are two main subcortical structures that contribute to these routes. They receive inputs from a number of cortical areas, including the anterior cingulate cortex (ACC) and the

medial prefrontal cortex (PFC) [7]. The lateral PFC and the orbitofrontal cortex (OFC) are additionally involved in this signalling process and transmit information relating to homeostatic body states (such as food intake, visceral pain and gut homeostasis) to the medial PFC, which integrates this information. The output from this complex network is integrated into clear motor patterns that are projected to the periaqueductal grey (PAG) [8] and then relayed to the raphe nuclei, the locus coeruleus complex and the dorsal vagal complex in the pons and medulla. This cortico-limbic-pontine network has been labelled the emotional motor system (EMS), with the medial component regulating spinal reflexes related to GI function and the lateral component integrating and modulating motor autonomic, neuroendocrine and pain related patterns [9, 10]. Both components thus modulate gut function. For example, the medial component has been shown to modulate pain-related behaviours in adult rats upon the consumption of food, through activation of the descending serotonergic pain inhibitory pathways [11]. In contrast, the lateral component may influence distinct visceral motor patterns through activation of the regional ANS [12, 13]. This regional ANS can be activated in a plethora of ways; by interoceptive feedback from the gut, descending emotional, cognitive, or during intense periods of emotion such as anger fear and sadness [14, 15].

Role of the Sympathetic Nervous System

The effects of the sympathetic nervous system on GI function have been well established. The gut receives sympathetic innervation from subclasses of post-ganglionic vasoconstrictor, secretion suppressing, and motility suppressing neurons, with the overall effect being to slow GI transit, motility and secretion. These inhibitory effects are mainly fulfilled by modification of cholinergic transmission and by stimulating sphincteric contractions on smooth muscle [16–18]. Additionally, sympathetic innervation may be involved in modification of mucosal immune systems [19], and in mucosa-microflora interactions [20, 21]. The best evidence for this sympathetic-immune interaction comes from the spleen, but this interaction has also been shown in Peyer's patches (lymphoid nodules in the ileum), and in non-follicular mucosa that is in close proximity to other classes of immune cells, and which can influence immune-related activity [21].

Role of the Parasympathetic System

The influence of the parasympathetic nervous system on GI function have also been extensively studied [17, 18]. These wide-ranging influences are initiated from vagal and sacral efferents, which innervate foregut and hindgut structures respectively. For example, in addition to providing input to the stomach, small intestine, and to the proximal portion of the colon, vagal structures may provide input to ganglia

within the ENS to facilitate the cephalic phase of gastric secretion vago-vagal motor reflexes, and to encourage the release of peptides and 5-hydroxytryptimaine (5-HT; a serotonin precursor) containing granules from enteroendocrine and enterochromaffin cells respectively [22]. Also, like the sympathetic nervous system, parasympathetic modulation of immune cells has been reported, with vagal modification of macrophage activation thought to be part of the vago-vagal anti-inflammatory reflex [23]. This reflex is driven by efferent vagal fibres, the majority of which originate from the dorsal motor nucleus of the vagus (DMN) in the medulla, and has been shown to attenuate circulating levels of proinflammatory cytokines.

Overall, the brain to gut interaction (Fig. 6.1), involving sympathetic and parasympathetic neurons, most likely facilitates emotion-related alterations in secretory, motor and immune related activity in the GI tract [24]. These alterations may even be compared to emotion-related alterations in facial expression and posture (facilitated by the medial EMS) to reflect mood and state of mind, perhaps signifying just how strong this interaction normally is.

Gut to Brain Signalling in Health

The ENS has more than 200 million neurons and thus has been colloquially referred to as the "second brain" [25]. This comparison is not surprising when considering that this network covers an area that is 100 times larger than the human surface area of skin and is home to approximately three-quarters of the human body's immune cells [25]. This extensive network has a bidirectional interaction with the brain, and thus may influence it. These are achieved via endocrine, neuronal and immune afferent signalling (Fig. 6.2).

Endocrine Signalling

With over 20 different types found in the body, enteroendocrine cells form the largest endocrine organ [26]. They are involved in the modulation of digestive functions through the ENS network and the modulation of endocrine and paracrine signalling to vagal afferents. Mechanisms that enteroendocrine cells use to achieve this include the activation of Ca^{2+} -dependent fatty acid acyl chains [27], presumable through activation of the G-protein coupled receptor (GPR) 40 [28], depolarization through the opening of mechanosensitive cation channels on microvilli [29], as well as G-protein-coupled taste receptors, and vagal efferents [22, 30].

An exceptionally well-studied type of enteroendocrine cell is the 5-HT releasing enterochromaffin cell, which is partly responsible for why 95 % of an organism's 5-HT is present in the gut [29]. It releases 5-HT on the basolateral side, and possibly the luminal side, in response to shearing forces in the gut [29]. Released 5-HT is

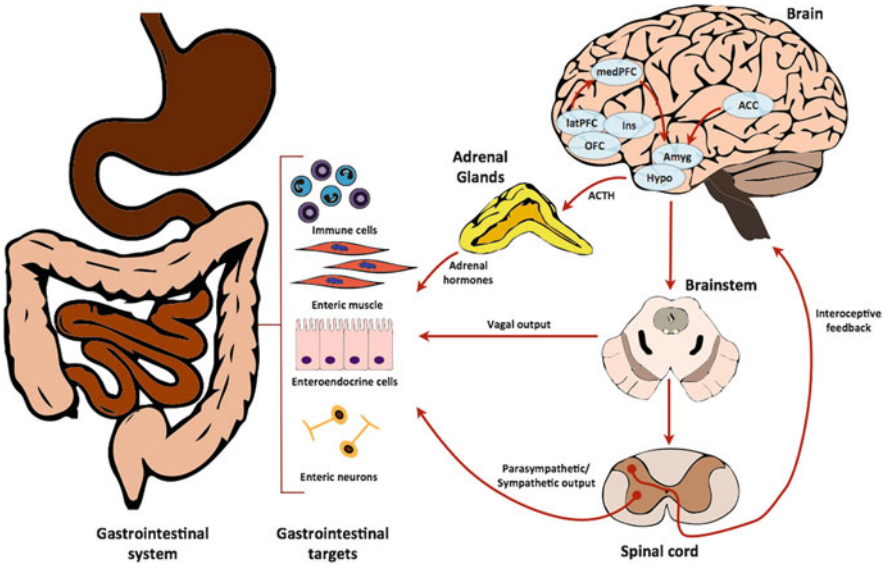

Fig. 6.1 Brain to gut signalling. In response to internal events or environmental factors, specific regions of the brain are activated, which may cause different effects depending on the stimuli. The hypothalamic-pituitary-adrenal axis may be activated to initiate the release of adrenal hormones such as catecholamines and glucocorticoids. Projections from these brain regions to brainstem nuclei, may initiate vagal (parasympathetic) output or these may project to the spinal cord and modulate interoceptive signals such as those relating to gastrointestinal spinal reflexes or pain sensitivity. Also, depending on which spinal cord level is activated, there may be additional parasympathetic or sympathetic outflow. These hormonal and neural outputs have the ability to influence gastrointestinal targets such as immune cells, enteric smooth muscle, enteric neurons and enteroendocrine cells. See text for details. *Abbreviations*: *ACC* anterior cingulate cortex, *ACTH* adrenocorticotrophic hormone, *Amyg* amygdala, *Hypo* hypothalamus, *Ins* insula, *latPFC* lateral prefrontal cortex, *medPFC* medial prefrontal cortex, *OFC* orbitofrontal cortex. Adapted from [104]

needed to initiate peristalsis and secretomotor reflexes, and it is yet to be determined whether it may confer signalling in the central nervous system (CNS).

Neuronal Signalling

The neurons that innervate the GI tract can be divided into primary extrinsic (vagal and spinal afferents) or primary intrinsic afferent neurons, with the intrinsic neurons being much more abundant [31]. Both extrinsic and intrinsic neurons respond to mechanical and chemical noxious stimuli, however it is suggested that luminal signals do not influence afferents nerve terminals directly, they act through multiple reflex loops to optimize gut function [32]. Notably, there are differences in both extrinsic and intrinsic neurons with regards to the importance of these reflex loops,

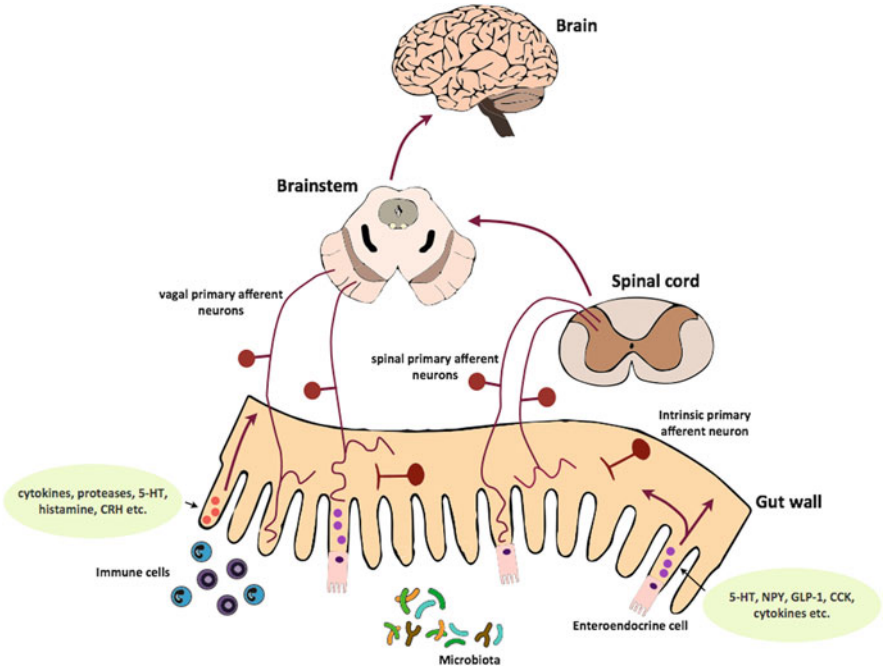

Fig. 6.2 Gut to brain signalling. Gut to brain signalling is achieved through endocrine, neuronal and immune routes. Mechanical and chemical information relating to the luminal environment is signaled through extrinsic (vagal and spinal) primary afferent neurons to the brain. Intrinsic primary afferent neurons are also present and although a direct synaptic connection between extrinsic and intrinsic neurons has yet to be found, they may communicate through intraganglionic laminar endings. Additionally, terminals of extrinsic primary afferent neurons are in close proximity to immune and enteroendocrine cells. Both cell types, in conjunction with enteric microbiota produce a variety of signalling molecules that can activate a number of receptors on extrinsic primary afferent neurons. Therefore, endocrine, neuronal and immune signals are all integrated and are sent to specific brain regions and may alter cognition, mood and emotions. *Abbreviations*: *5-HT* 5-hydroxytryptamine, *CCK* cholecystokinin, *CRH* corticotropin-releasing hormone, *GLP-1* glucagon-like peptide-1, *Neuropeptide Y* NPY. Adapted from [105]

depending on their location. The stomach is predominantly governed by vago-vagal reflexes, thus signals arising from extrinsic and intrinsic neurons are relatively weak. In the intestines, intrinsic primary afferent neurons and enteric motorneurons are important for intestinal function afferents are much stronger in the intestine, which are reliant on these signals [17]. The intrinsic primary afferents feed and regulate signals relating to secretion, propulsion and blood flow indirectly to the CNS, perhaps through specific myenteric ganglia, which receive input from these neurons as well as extrinsic afferents, and relay information to spinal, mesenteric and supraspinal reflex loops that span much larger distances in the bowel [13]. Interestingly, some of these intrinsic afferents are normally unresponsive to mechanical stimuli, and only become responsive during periods of inflammation [33].

Certain subsets of vagal afferents terminate with great proximity to enteroendocrine cells. These terminals contain chemosensitive receptors, which are responsive to the peptides released by these cells [34]. These include receptors for orexigenic (hunger inducing) or anorexigenic (satiety inducing) peptides. Emerging evidence suggests that together with the cholecystokinin 1 receptor (CCK1R), the expression of these receptors can be modulated by diet or nutritional status [35]. Additionally, the receptors for anorexogenic peptides were found to be downregulated by fasting, while those for orexigenic peptides were upregulated, creating a greater impulse to eat. These reports reflect the phenotypic plasticity of vagal afferents depending on the homeostatic state [36].

Immune Signalling

Approximately three-quarters of the body's immune cells are located in the GI tract and is often referred to as the gut-associated lymphoid tissue (GALT), suggesting that the gut may be considered as the body's main defence organ. A single layer of columnar intestinal epithelial cells forms the barrier between 100 trillion microorganisms and the host [37]. These microorganisms are essential for normal physiological functions, as the absence of intestinal bacteria has been shown to compromise the development of the GALT and the subsequent release of antibodies [38, 39]. With the observation that intestinal immune cells remain predominantly hyporeactive to the commensal bacteria that live in symbiosis within us, yet are hyperreactive against pathogens, alludes to the fact that the intestinal immune system can identify commensal bacteria from pathogens and generate appropriate responses in order to maintain normal well-being [40]. As the epithelial layer samples the luminal environment, lymphoid structures (including Peyer's patches) located in the lamina propria in the intestine deal with immune insults [40] through specialised immune cells that sample antigens from or on microorganisms deliver them to antigen processing cells in Peyer's patches, and dendritic cells in the lamina propria, which can extend their dendritic arbours through the epithelial tight junctions to sample the luminal environment. These cells possess a myriad of receptors that can recognise pathogen associated molecular patterns [41]. In addition to being in close proximity and having receptors that are responsive to the products of enteroendocrine cells, vagal afferents are close to, and possess receptors on their terminals that are responsive to immune cells and their products, namely cytokines, proteases, 5-HT, corticotropin-releasing factor (CRH) and histamine [42]. Moreover, immune cells may have functional effects on enteroendocrine cells as has been demonstrated by the increased release of cholecystokinin in an animal model of gut inflammation [43, 44].

Integrated Gut to Brain Signalling

Overall, there is a strong integration of the endocrine, neuronal and immune signals; and these contribute in the transmission of information from the gut to the brain. However, only a portion of these signals are normally consciously perceived. However, a preponderance of evidence suggests that subconscious interoceptive inputs, in conjunction with intestinal microbiota, may effect memory, cognition and emotions [45]. For example, primary extrinsic afferents from the GI tract may project via the vagus nerve to the solitary tract nucleus and through spinal; afferents to laminae I, V, VII and X of the spinal cord. The projections arriving from laminae I, V and the solitary tract are integrated in the parabrachial complex, which is then transmitted to forebrain regions including the hypothalamus and amygdala [46]. The latter of which has been reported to be involved with reward based behavior and emotions, especially fear [47].

Furthermore, collaterals from these ascending extrinsic projections may make further contact to the raphe nuclei and the locus coeruleus complex, and thus may alter the release of 5-HT and noradrenaline respectively, which modify cortical arousal. Projections from Lamina I specifically, have been found to activate the ACC and the insula via the thalamic nuclei [48]. In fact, recent studies have identified the insula as the most likely region for allowing integration of interoceptive information in emotional behavior [49, 50]. This interceptive information is projected to regions of the brain depending on the origin of the signal. For example, both sensory and visceral stimuli are registered in the middle and posterior insula, while gustatory or olfactory inputs are registered in the middle and anterior insula, suggesting that different insular regions may be responsible for the perception of different interoceptive inputs from the GI tract [49, 50]. The activation of the anterior insula, and to a lesser extent the ACC, has additionally been reported in individuals who inhaled pungent odors producing strong feelings of disgust, or in individuals who viewed video clips, showing the altered emotional facial expressions, of others being disgusted by the odors. This suggests that at least at the level of the anterior insula and the ACC, homeostatic reflexes can be activated in the paucity of interoceptive input, and can be activated by the recollection of interoceptive memories [51].

There is emerging evidence that interoceptive memories may develop during infancy, when the gut to brain interactions are beginning to be molded, and positive and negative feeding states are being established. For example, the response to consuming something sweet has been associated with the activation of opioids (associated with a feeling of pleasure) in both in mice and in children [52, 53]. These neurochemical programming of feeding states develop into adulthood and may partially explain why ingestion of food that is high in calories is accompanied by a feeling of pleasure. On the other hand, consumption of potentially harmful food may initiate nausea and vomiting (via 5-HT). In fact, gut-based neurochemicals can signal satiety [via cholecystokinin, glucagon-like peptide-1 (GLP1), and neuropeptide Y (NPY)], hunger (via ghrelin) through enteroendocrine

cells; and can signal pain (via CRH, proteases and cytokines) through intestinal immune cells. Overall, the enteroendocrine, neuronal and immune components of gut-brain signal intermingle with one another and under normal circumstances have great influence in shaping normal homeostatic functions in different aspects of physiology.

Acute Perturbations in Signalling

Substantial evidence suggests that the bidirectional brain-gut interaction can be perturbed leading to acute physiological repercussions. For example, upon activation by possible noxious stimuli such as chemotherapeutic drugs or bacterial toxins, enterochromaffin cells may increase their production of 5-HT, which may activate 5-HT_3 receptors on both extrinsic and intrinsic afferents. This may result not only in hypersecretory and hypermotor reflexes, but also in the activation of brain regions that receive input from ascending afferent pathways. Overactivation of these pathways may be associated with nausea and vomiting in order to expel the harmful contents out of the body. Another example includes vagal-mediated activation of the hypothalamus and limbic brain regions following the release of proinflammatory cytokines in the liver and gut. This results in "sickness responses" that include, fever, depression and withdrawal from usual activity [54]. Additionally, a myriad of inflammatory mediators including cytokines, proteases and neuropeptides may be released by mucosal immune and glial cells, which may result in sensitization of both nociceptive and innocuous ascending spinal pathways, thus amplifying the perception of visceral pain [55, 56]. Finally, the CRH signalling system influences brain-gut signalling. The release of CRH is a normal physiological reaction to stress but may be pathologic if there it is overproduced [57]. Its effects, being endocrine, behavioural, autonomic and visceral, may also be reproduced if administered directly into animal brains [58]. The release of CRH is associated with increased anxiety-like behaviours in animals; and there is also decreased GI secretory and motor responses and acceleration of distal bowel movements, which reduces luminal contents in the GI tract and thus GI metabolic demand. This can result in greater distribution of blood to the skeletomotor and gastrointestinal system for the flight and flight response. It was thought that binding of CRH to the CRH1 receptor might cause these effects [14]. Notably, although these perturbations are usually acute, if severe, they may contribute to chronic diseases.

Chronic Perturbations in Signalling

Perturbations in chronic diseases affect multiple signalling pathways along the brain-gut axis. This makes it difficult to posit specific perturbations to the specific chronic diseases. Additionally, although many disease states may be related to altered signalling along the brain-gut axis, convincing evidence is limited to a few. Taken together, the following section will reflect on well-established perturbations that may render chronic diseases.

Functional Gastrointestinal Disorders (e.g. Irritable Bowel Syndrome)

Functional gastrointestinal disorders form a constellation of disorders characterized by chronic discomfort and pain along various locations in the GI tract, and occur without discernable physical, biological or anatomical abnormalities that would explain their symptoms [59]. Over 40 different disorders have been postulated including functional dyspepsia, chest pain of oesophageal origin and irritable bowel syndrome (IBS); all of which share similar symptoms [59]. However, out of all these disorders, IBS is the most common and most extensively studied [60].

Symptoms specific to irritable bowel syndrome are related to abnormal colonic transit and rectal evacuation such as chronic constipation, diarrhoea and anismus [61, 62]. A number of mechanisms have been found that may cause these symptoms, including luminal and mucosal irritants that alter mucosal permeability, overactivation of immune systems, which subsequently alter motor and sensory conditions in the GI tract.

The increased presence of undigested food in the lumen has been found to be a trigger for the onset of IBS. These include: undigested fats [63], carbohydrates (that lead to the production of short-chain fatty acids) [64]; bile acid malabsorption leading to the production of more bile acid [65]; modifications of bacterial populations in the gut, which may increase colonic secretion and have been associated with mental disease in IBS [66]. These luminal factors and exogenous chemicals trigger the release of several amines and peptides from enteroendocrine cells. However, the abnormal release of 5-HT has been well characterized in IBS [67]; increased 5-HT levels increases motor, sensory and secretory functions and is associated the diarrhoea-predominant IBS, while the decreased 5-HT levels causes the opposite effect and is associated with constipation-predominant IBS [68]. Activation of immune cells may also be involved; as an increased expression of T-lymphocytes in the rectal mucosa has been associated with increased intestinal permeability in a subset of patients with IBS [69]. Genetic predispositions to develop IBS has also been associated with mucosal irritants. For example, a genetic variation of KLB (Arg728Gln) leads to impaired KLB synthesis and prevents fibroblast growth factor 19 (FGF19) bind to the KLB-FGFR4 receptor on

hepatocytes. This reduces the bile acid synthesis negative feedback, causing the release of more bile acid, which is associated with diarrhoea-predominant IBS [70].

There is also evidence that CNS hypervigilance may be involved in IBS. One of the first IBS related symptom reported in children is increased mucosal permeability, which may also be triggered by stress [71]. When CRH was placed on the serosal surface of colonic mucosal biopsy specimens in healthy individuals, there was an increased mast cell-mediated uptake of horseradish peroxidase (used as a marker of permeability) [72]. Increased permeability was also seen when individuals were subjected to a cold stimulus [73]. The connection between increased intestinal permeability and IBS has been supported by observations that increased permeability leads to inflammation and the activation of local reflex pathways, which lead to increased visceral sensations [74]. Additionally, several non-gastrointestinal comorbidities have been associated with IBS, such as fibromyalgia and temporomandibular disorder, which are known to be exacerbated by stress; and the fact that these conditions and IBS may be treated with psychopharmacological agents such as tricyclic antidepressants and serotonin/noradrenaline reuptake inhibitors substantiates the brain's involvement in IBS [75]. Overall, there appears to be bidirectional interaction in the development of IBS, with both interoceptive signals from the gut and peripheral and central sensitization mechanisms associated with modified cognition and altered descending signals to the gut.

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) encompasses a group of inflammatory disorders that affect the GI tract, but the two most common disorders are Crohn's disease and ulcerative colitis. Overwhelming evidence suggests that this disorder is caused by exaggerated responses to enteric microorganisms in a genetically susceptible host [76], but the brain-gut axis may be involved in modulating these responses (Fig. 6.3).

Like in IBS, stress may play a pathological role in IBD through a plethora of mechanisms. The products of mast cells, including numerous cytokines and chemokines, may activate terminals on sympathetic spinal primary afferent neurons [77]. However, during times of stress there is an increase in circulating level of catecholamines that, through α- and β- adrenergic receptors, activate the pro-inflammatory nuclear factor κB (NF- κB) signalling pathway and increase peripheral and central proinflammatory cytokines production, thus potentiating inflammatory insults [78]. Conversely, an anti-inflammatory role of vagal efferent pathways may reduce inflammation in IBD. Acetylcholine (Ach), released at the by vagal efferent terminals have been shown to limit the production of pro-inflammatory cytokines such as IL-1, IL-6 and tumor necrosis factor-α (TNF-α) through activation of the α7 nicotinic acetylcholine receptor (α7nAChR) on the surface of macrophages [79] and thus attenuated intestinal inflammation [80]. In fact chronic vagal nerve stimulation agonists has been shown to improve IBD

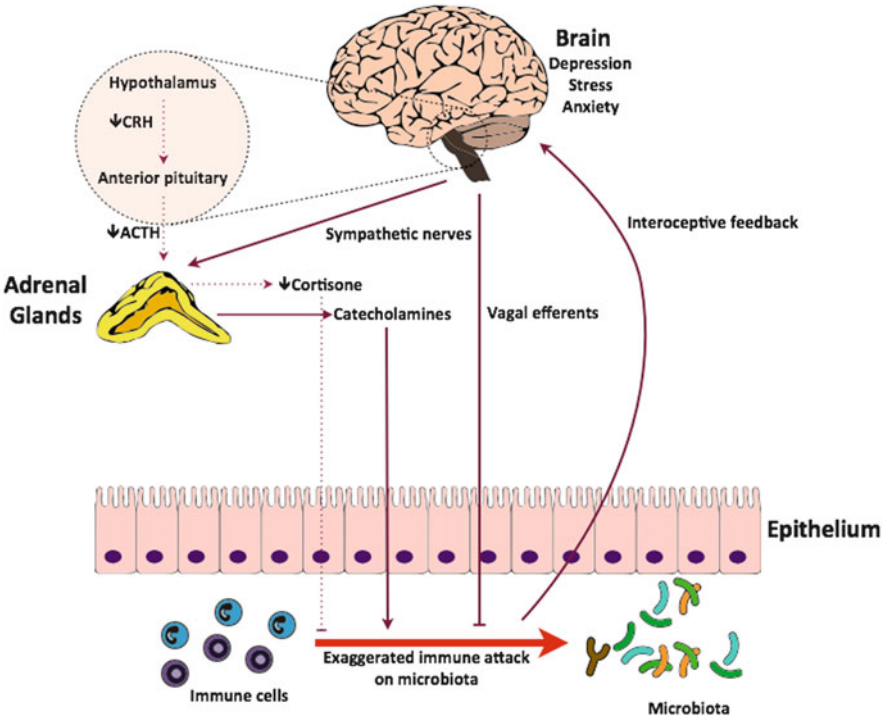

Fig. 6.3 Brain-gut axis in inflammatory bowel disease (IBD). IBD is thought to be a result of inappropriate responses of enteric immune cells against the intestinal microflora. The brain gut axis is involved in modulating these responses. Activation of the sympatho-adrenomedullary axis results in an increase of catecholamines, which activate receptors on immune cells and cause an increased release of inflammatory cytokines. The terminals of vagal efferents are believed to induce an anti-inflammatory effect via the release of acetylcholine on α7 nicotinic acetylcholine receptor (α7nAChR), expressed on the surface of macrophages. Corticotrophin-releasing hormone (CRH) released from the hypothalamus can be attenuated by stress, leading to an attenuated release of plasma corticosterone; its paucity has been associated with IBD. Also depression and anxiety have been shown to increase susceptibility to develop IBD. Alternatively, the gut may signal to the brain via interoceptive feedback that activates descending pain inhibitory systems, which in turn reduces pain in IBD (not shown). *Abbreviations*: *ACTH* adrenocorticotrophic-releasing hormone, *CRH* corticotropin-releasing hormone

symptoms [80], and thus may be a potential alternative to anti-TNF therapy, which is currently the gold standard treatment of moderate to severe IBD [81]. However, stress decreases vagal outflow, and together with the increased levels of catecholamines, there is a greater shift towards intestinal inflammation [82]. This may be achieved by decreased activity of the PFC, which is known to control the parasympathetic tone through modulation of vagal outflow [83], as well as the enhanced activity of the amygdala which is strongly associated with stress [84]. The abnormal

activity of the PFC and the amygdala results in an imbalance between stimulation of the HPA axis and the ANS, which favors greater proinflammatory conditions [85].

The CRH signalling system is additionally affected by stress. Chronic colitis attenuated CRH gene activation in the parvocellular neurosecretory neurons in the hypothalamus, cells that produce CRH and dampened the plasma corticosterone responses to acute environmental stressors [86]. A decreased HPA axis response is associated to a susceptibility to autoimmune and inflammatory disorders, and thus decreased CRH production predisposes to developing IBD [87].

Early life events may influence stress and the development of IBD. Neonatal inflammation has been shown to employ long-term effects in immune regulation and HPA activity [88]. The modulation of stress may harbor a deleterious role of the brain in controlling peripheral immunity. Separation of rat pups from their mothers has been used as a model of early life stress. It produced life long abnormal hyperactivity in the HPA and CRH signalling system, and predisposed rats to visceral hypersensitivity, increased defecation, increased penetration of bacteria into the lamina propria and increased levels of anxiety [89, 90], and these may be due to modulation of immunological responses and microbiota in the intestines [91].

A causal relationship between depression in maternal separation model and the hypersecretion of proinflammatory cytokines and mediators has also been proposed [92]. Mice separated from their mothers at birth exhibit a pattern of behavior reminiscent of depression, and are more vulnerable to inflammation. This relationship is further supported by the fact that treatment with tricyclic antidepressants reversed depressive-like behavior [93]. Additionally, depression has been shown to increase the susceptibility to inflammation under baseline conditions and during periods of stress [94]. However, it may be that the comorbidities of depression and anxiety are auxiliary effects of the main IBD pathology; causality has yet to be determined [95].

Our knowledge of the brain-gut axis in IBD is not limited to preclinical research, brain-imaging studies on patients have also been revealing. Altered sensory experiences are commonly described in patients with IBD; but unlike IBS, persistent visceral hyperalgesia and abdominal pain are variably reported [96]. Using functional magnetic resonance imaging (fMRI), cortical or subcortical areas of activation or deactivation were assessed upon exposure to rectal pain in patients with IBD or in controls. Analysis revealed that the control and IBD groups showed distinctive profiles of response [97]. In particular, activation of both central and peripheral pain inhibitory areas was more prominent in IBD patients which may be a possible mechanism for the lack of visceral hyperalgeisa in IBD patients [98]. Overall, there appears to be a strong interaction between the brain and gut in the modulation of sensations and functions in IBD.

Eating Disorders

Eating disorders are common across society; yet it is still not understood how an assortment of outcomes and situations, including diet, exercise and infections, may result in syndromes such as obesity, anorexia nervosa and bulimia, which harbour many societal problems relating to morbidity, mortality and healthcare costs [99]. Although the causes of these disorders are likely to be multifactorial, the brain-gut axis may have a role to play.

Obese individuals seem to eat beyond their caloric requirements, suggesting that there is an imbalance between homeostatic and hedonic regulation of food intake. This may be due to modulated peripheral signalling processes in the gut, which may encourage greater food take. For example, diet-induced attenuation of gut to brain signals relaying satiety-triggering processes have reported to be affected. These include modulations in cholecystokinin-dependent molecular processes, and the development of insulin and leptin resistance. These cause a switch from an anorexigenic to an orexigenic phenotype [100]. Hedonic causes are also present. Imaging studies suggest that obese subjects may have compromised dopaminergic pathways that regulate neuronal systems related to reward sensitivity, conditioning and control. Provision of food cues (such as viewing or imagining high calorie foods) induced an exaggerated response in the dopaminergic pathways, however actual food intake produced an attenuated response [101]. As many gut produced peptides, including leptin, ghrelin and insulin have the ability to activate the central dopamine pathways, it seems likely that the impairments in satiety responses observed may be also due to modulated interoceptive feedback back to the brain.

On the other hand, individuals with anorexia nervosa or bulimia have an impaired perception of self-image, which drives an obsession with weight loss and a preoccupation with food or food rituals [102]. Although behavioral and brain abnormalities have been reported, potential modifications in the brain-gut axis are not fully understood. However, it has been shown that upon oral sucrose provision, patients with anorexia nervosa had reduced activity in the anterior insula, striatum and ACC. This implies that interoceptive signals from the GI tract that activate dopaminergic pathways might be dysfunctional in these disorders [103].

Conclusion

A substantial amount of progress has been made with regards to our understanding of the brain-gut axis. This includes delineating exact functional neuroanatomical processes, mechanisms and pathways of the ENS, and both how the brain modulates gut function and how the gut modulates activity across different brain regions. These signalling patterns are important in health and their perturbation may contribute to specific disorders that are associated with chronic pain, gut inflammation, psychosocial stressors and eating disorders. There are a multitude of unanswered

questions including what role the enteric microbiota may have in signalling. Through close collaboration with clinical neurophysiologists, neuroradiologists, physicists and even other specialties, gastroenterologists may be able to delve deeper into unknown areas of physiology and pathophysiology and make further advances in our understanding of the gut-brain axis in health and disease.

References

1. Almy TP (1989) Historical perspectives of functional bowel disease. In: Snape WJ (ed) Pathogenesis of functional bowel disease. Plenum, New York, pp 1–11
2. James W (1884) What is an emotion? Mind 9:188–205
3. Cannon WB (1909) The influence of emotional states on the functions of the alimentary canal. Am J Med Sci 137:480–487
4. Pavlov I (1910) The work of digestive glands (English translation from Russian by W. H. Thompson). Griffin, London
5. Wolf S, Wolff HG (1943) Human gastric function: an experimental study of a man and his stomach. Oxford University, New York
6. Beaumont W (1959) Experiments and observations on the gastric juice and the physiology of digestion (Facsimile of the original publication of 1833). Dover, New York
7. Ongur D, Price JL (2000) The organization of networks within the orbital and medial prefrontal cortex of rats, monkeys and humans. Cereb Cortex 10(3):206–219
8. Bandler R, Keay KA (1996) The emotional motor system. In: Holstege G, Bandler R, Saper CB (eds) Progress in brain research. Elsevier, Amsterdam, pp 285–300
9. Holstege G, Bandler R, Saper CB (1996) The emotional motor system. Prog Brain Res 107: 3–6
10. Mayer EA (2000) The neurobiology of stress and gastrointestinal disease. Gut 47(6):861–869
11. Mason P (2011) From descending pain modulation to obesity via the medullary raphe. Pain 152(3 Suppl):S20–S24
12. Valentino RJ, Miselis RR, Pavcovich LA (1999) Pontine regulation of pelvic viscera: pharmacological target for pelvic visceral dysfunctions. Trends Pharmacol Sci 20(6): 253–260
13. Browning KN, Travagli RA (2011) Plasticity of vagal brainstem circuits in the control of gastrointestinal function. Auton Neurosci 161(1–2):6–13
14. Martinez V, Tache Y (2006) CRF1 receptors as a therapeutic target for irritable bowel syndrome. Curr Pharm Des 12(31):4071–4088
15. Welgan P, Meshkinpour H, Ma L (2000) Role of anger in antral motor activity in irritable bowel syndrome. Dig Dis Sci 45(2):248–251
16. Furness JB, Costa M (1974) The adrenergic innervation of the gastrointestinal tract. Ergeb Physiol 69:2–51
17. Furness JB (2006) The enteric nervous system. Blackwell, Oxford
18. Jaenig W (2006) Integrative action of the autonomic nervous system. Cambridge University Press, New York
19. Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES (2000) The sympathetic nerve – an integrative interface between two supersystems: the brain and the immune system. Pharmacol Rev 52(4): 595–638
20. Rhee SH, Pothoulakis C, Mayer EA (2009) Principles and clinical implications of the brain-gut-enteric microbiota axis. Nat Rev Gastroenterol Hepatol 6(5):306–314
21. Lyte M, Vulchanova L, Brown DR (2011) Stress at the intestinal surface: catecholamines and mucosa-bacteria interactions. Cell Tissue Res 343(1):23–32

22. Stephens RL, Tache Y (1989) Intracisternal injection of a TRH analogue stimulates gastric luminal serotonin release in rats. Am J Physiol 256(2 Pt 1):G377–G383
23. Pavlov VA, Tracey KJ (2005) The cholinergic anti-inflammatory pathway. Brain Behav Immun 19(6):493–499
24. Welgan P, Meshkinpour H, Beeler M (1988) Effect of anger on colon motor and myoelectric activity in irritable bowel syndrome. Gastroenterology 94(5 Pt 1):1150–1156
25. Gershon MD (1999) The enteric nervous system: a second brain. Hosp Pract (1995) 34(7): 31-2, 5-8, 41-2 passim
26. Raybould HE (2010) Gut chemosensing: interactions between gut endocrine cells and visceral afferents. Auton Neurosci 153(1–2):41–46
27. McLaughlin JT, Lomax RB, Hall L, Dockray GJ, Thompson DG, Warhurst G (1998) Fatty acids stimulate cholecystokinin secretion via an acyl chain length-specific, Ca2 + -dependent mechanism in the enteroendocrine cell line STC-1. J Physiol 513(Pt 1):11–18
28. Liou AP, Lu X, Sei Y, Zhao X, Pechhold S, Carrero RJ et al (2011) The G-protein-coupled receptor GPR40 directly mediates long-chain fatty acid-induced secretion of cholecystokinin. Gastroenterology 140(3):903–912
29. Gershon MD, Tack J (2007) The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology 132(1):397–414
30. Rozengurt E, Sternini C (2007) Taste receptor signaling in the mammalian gut. Curr Opin Pharmacol 7(6):557–562
31. Clerc N, Furness JB (2004) Intrinsic primary afferent neurones of the digestive tract. Neurogastroenterol Motil 16(Suppl 1):24–27
32. Keita AV, Soderholm JD (2010) The intestinal barrier and its regulation by neuroimmune factors. Neurogastroenterol Motil 22(7):718–733
33. Gold MS, Gebhart GF (2010) Nociceptor sensitization in pain pathogenesis. Nat Med 16(11): 1248–1257
34. Gershon MD (2005) Nerves, reflexes, and the enteric nervous system: pathogenesis of the irritable bowel syndrome. J Clin Gastroenterol 39(5 Suppl 3):S184–S193
35. de Lartigue G, de La Serre CB, Raybould HE (2011) Vagal afferent neurons in high fat diet-induced obesity; intestinal microflora, gut inflammation and cholecystokinin. Physiol Behav 105(1):100–105
36. Brookes SJ, Spencer NJ, Costa M, Zagorodnyuk VP (2013) Extrinsic primary afferent signalling in the gut. Nat Rev Gastroenterol Hepatol 10:286–296
37. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS et al (2006) Metagenomic analysis of the human distal gut microbiome. Science 312(5778):1355–1359
38. Bouskra D, Brezillon C, Berard M, Werts C, Varona R, Boneca IG et al (2008) Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature 456(7221):507–510
39. Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 13(10):701–712
40. Artis D (2008) Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol 8(6):411–420
41. Medzhitov R (2007) Recognition of microorganisms and activation of the immune response. Nature 449(7164):819–826
42. Barbara G, Wang B, Stanghellini V, de Giorgio R, Cremon C, Di Nardo G et al (2007) Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome. Gastroenterology 132(1):26–37
43. McDermott JR, Leslie FC, D'Amato M, Thompson DG, Grencis RK, McLaughlin JT (2006) Immune control of food intake: enteroendocrine cells are regulated by CD4+ T lymphocytes during small intestinal inflammation. Gut 55(4):492–497
44. Mawe GM, Strong DS, Sharkey KA (2009) Plasticity of enteric nerve functions in the inflamed and postinflamed gut. Neurogastroenterol Motil 21(5):481–491

45. Agostini A, Filippini N, Cevolani D, Agati R, Leoni C, Tambasco R et al (2011) Brain functional changes in patients with ulcerative colitis: a functional magnetic resonance imaging study on emotional processing. Inflamm Bowel Dis 17(8):1769–1777
46. Bradley RM, Kim M (2007). In: Bradley RM (ed) The role of the nucleus of the solitary tract in gustatory processing. Taylor & Francis Group, LLC, Boca Raton
47. Li H, Penzo MA, Taniguchi H, Kopec CD, Huang ZJ, Li B (2013) Experience-dependent modification of a central amygdala fear circuit. Nat Neurosci 16(3):332–339
48. Craig AD (1996) An ascending general homeostatic afferent pathway originating in lamina I. Prog Brain Res 107:225–242
49. Mutschler I, Wieckhorst B, Kowalevski S, Derix J, Wentlandt J, Schulze-Bonhage A et al (2009) Functional organization of the human anterior insular cortex. Neurosci Lett 457(2):66–70
50. Kurth F, Zilles K, Fox PT, Laird AR, Eickhoff SB (2010) A link between the systems: functional differentiation and integration within the human insula revealed by meta-analysis. Brain Struct Funct 214(5–6):519–534
51. Wicker B, Keysers C, Plailly J, Royet JP, Gallese V, Rizzolatti G (2003) Both of us disgusted in my insula: the common neural basis of seeing and feeling disgust. Neuron 40(3):655–664
52. Pepino MY, Mennella JA (2005) Sucrose-induced analgesia is related to sweet preferences in children but not adults. Pain 119(1–3):210–218
53. Foo H, Mason P (2009) Analgesia accompanying food consumption requires ingestion of hedonic foods. J Neurosci 29(41):13053–13062
54. Kent S, Bluthe RM, Kelley KW, Dantzer R (1992) Sickness behavior as a new target for drug development. Trends Pharmacol Sci 13(1):24–28
55. Calvo M, Dawes JM, Bennett DL (2012) The role of the immune system in the generation of neuropathic pain. Lancet Neurol 11(7):629–642
56. Sikandar S, Dickenson AH (2012) Visceral pain: the ins and outs, the ups and downs. Curr Opin Support Palliat Care 6(1):17–26
57. Chrousos GP (2009) Stress and disorders of the stress system. Nat Rev Endocrinol 5(7): 374–381
58. Stengel A, Tache Y (2010) Corticotropin-releasing factor signaling and visceral response to stress. Exp Biol Med (Maywood) 235(10):1168–1178
59. Tillisch K, Labus JS (2011) Advances in imaging the brain-gut axis: functional gastrointestinal disorders. Gastroenterology 140(2):407–411 e1
60. Mayer EA, Tillisch K (2011) The brain-gut axis in abdominal pain syndromes. Annu Rev Med 62:381–396
61. Mayer EA (2008) Clinical practice. Irritable bowel syndrome. N Engl J Med 358(16): 1692–1699
62. Tornblom H, Van Oudenhove L, Sadik R, Abrahamsson H, Tack J, Simren M (2012) Colonic transit time and IBS symptoms: what's the link? Am J Gastroenterol 107(5):754–760
63. Ludidi S, Conchillo JM, Keszthelyi D, Koning CJ, Vanhoutvin SA, Lindsey PJ et al (2012) Does meal ingestion enhance sensitivity of visceroperception assessment in irritable bowel syndrome? Neurogastroenterol Motil 24(1):47–53, e3
64. Shepherd SJ, Parker FC, Muir JG, Gibson PR (2008) Dietary triggers of abdominal symptoms in patients with irritable bowel syndrome: randomized placebo-controlled evidence. Clin Gastroenterol Hepatol 6(7):765–771
65. Johnston I, Nolan J, Pattni SS, Walters JR (2011) New insights into bile acid malabsorption. Curr Gastroenterol Rep 13(5):418–425
66. Jeffery IB, O'Toole PW, Ohman L, Claesson MJ, Deane J, Quigley EM et al (2012) An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. Gut 61(7):997–1006
67. Kidd M, Modlin IM, Gustafsson BI, Drozdov I, Hauso O, Pfragner R (2008) Luminal regulation of normal and neoplastic human EC cell serotonin release is mediated by bile

salts, amines, tastants, and olfactants. Am J Physiol Gastrointest Liver Physiol 295(2): G260–G272
68. Hoffman JM, Tyler K, MacEachern SJ, Balemba OB, Johnson AC, Brooks EM et al (2012) Activation of colonic mucosal 5-HT(4) receptors accelerates propulsive motility and inhibits visceral hypersensitivity. Gastroenterology 142(4):844–854 e4
69. Spiller RC, Jenkins D, Thornley JP, Hebden JM, Wright T, Skinner M et al (2000) Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute *Campylobacter enteritis* and in post-dysenteric irritable bowel syndrome. Gut 47 (6):804–811
70. Camilleri M, Katzka DA (2012) Irritable bowel syndrome: methods, mechanisms, and pathophysiology. Genetic epidemiology and pharmacogenetics in irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol 302(10):G1075–G1084
71. Shulman RJ, Eakin MN, Czyzewski DI, Jarrett M, Ou CN (2008) Increased gastrointestinal permeability and gut inflammation in children with functional abdominal pain and irritable bowel syndrome. J Pediatr 153(5):646–650
72. Wallon C, Yang PC, Keita AV, Ericson AC, McKay DM, Sherman PM et al (2008) Corticotropin-releasing hormone (CRH) regulates macromolecular permeability via mast cells in normal human colonic biopsies in vitro. Gut 57(1):50–58
73. Alonso C, Guilarte M, Vicario M, Ramos L, Rezzi S, Martinez C et al (2012) Acute experimental stress evokes a differential gender-determined increase in human intestinal macromolecular permeability. Neurogastroenterol Motil 24(8):740–746, e348–e349
74. Barbara G, Cremon C, De Giorgio R, Dothel G, Zecchi L, Bellacosa L et al (2011) Mechanisms underlying visceral hypersensitivity in irritable bowel syndrome. Curr Gastroenterol Rep 13(4):308–315
75. Dekel R, Drossman DA, Sperber AD (2013) The use of psychotropic drugs in irritable bowel syndrome. Expert Opin Investig Drugs 22(3):329–339
76. Abraham C, Cho JH (2009) Inflammatory bowel disease. N Engl J Med 361(21):2066–2078
77. Bischoff SC (2009) Physiological and pathophysiological functions of intestinal mast cells. Semin Immunopathol 31(2):185–205
78. Johnson JD, Campisi J, Sharkey CM, Kennedy SL, Nickerson M, Greenwood BN et al (2005) Catecholamines mediate stress-induced increases in peripheral and central inflammatory cytokines. Neuroscience 135(4):1295–1307
79. Pavlov VA, Wang H, Czura CJ, Friedman SG, Tracey KJ (2003) The cholinergic anti-inflammatory pathway: a missing link in neuroimmunomodulation. Mol Med 9(5–8): 125–134
80. Meregnani J, Clarencon D, Vivier M, Peinnequin A, Mouret C, Sinniger V et al (2011) Anti-inflammatory effect of vagus nerve stimulation in a rat model of inflammatory bowel disease. Auton Neurosci 160(1–2):82–89
81. D'Haens GR, Panaccione R, Higgins PD, Vermeire S, Gassull M, Chowers Y et al (2011) The London Position Statement of the World Congress of Gastroenterology on Biological Therapy for IBD with the European Crohn's and Colitis Organization: when to start, when to stop, which drug to choose, and how to predict response? Am J Gastroenterol 106(2):199–212
82. Tache Y, Bonaz B (2007) Corticotropin-releasing factor receptors and stress-related alterations of gut motor function. J Clin Invest 117(1):33–40
83. Tracey KJ (2002) The inflammatory reflex. Nature 420(6917):853–859
84. Czeh B, Perez-Cruz C, Fuchs E, Flugge G (2008) Chronic stress-induced cellular changes in the medial prefrontal cortex and their potential clinical implications: does hemisphere location matter? Behav Brain Res 190(1):1–13
85. Straub RH, Herfarth H, Falk W, Andus T, Scholmerich J (2002) Uncoupling of the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis in inflammatory bowel disease? J Neuroimmunol 126(1–2):116–125

86. Kresse AE, Million M, Saperas E, Tache Y (2001) Colitis induces CRF expression in hypothalamic magnocellular neurons and blunts CRF gene response to stress in rats. Am J Physiol Gastrointest Liver Physiol 281(5):G1203–G1213
87. Million M, Tache Y, Anton P (1999) Susceptibility of Lewis and Fischer rats to stress-induced worsening of TNB-colitis: protective role of brain CRF. Am J Physiol 276(4 Pt 1): G1027–G1036
88. Shanks N, Windle RJ, Perks PA, Harbuz MS, Jessop DS, Ingram CD et al (2000) Early-life exposure to endotoxin alters hypothalamic-pituitary-adrenal function and predisposition to inflammation. Proc Natl Acad Sci U S A 97(10):5645–5650
89. Gareau MG, Jury J, Yang PC, MacQueen G, Perdue MH (2006) Neonatal maternal separation causes colonic dysfunction in rat pups including impaired host resistance. Pediatr Res 59(1): 83–88
90. Lippmann M, Bress A, Nemeroff CB, Plotsky PM, Monteggia LM (2007) Long-term behavioural and molecular alterations associated with maternal separation in rats. Eur J Neurosci 25(10):3091–3098
91. O'Mahony SM, Hyland NP, Dinan TG, Cryan JF (2011) Maternal separation as a model of brain-gut axis dysfunction. Psychopharmacology (Berl) 214(1):71–88
92. Irwin MR (2008) Human psychoneuroimmunology: 20 years of discovery. Brain Behav Immun 22(2):129–139
93. Varghese AK, Verdu EF, Bercik P, Khan WI, Blennerhassett PA, Szechtman H et al (2006) Antidepressants attenuate increased susceptibility to colitis in a murine model of depression. Gastroenterology 130(6):1743–1753
94. Danese A, Pariante CM, Caspi A, Taylor A, Poulton R (2007) Childhood maltreatment predicts adult inflammation in a life-course study. Proc Natl Acad Sci U S A 104(4): 1319–1324
95. Tache Y, Bernstein CN (2009) Evidence for the role of the brain-gut axis in inflammatory bowel disease: depression as cause and effect? Gastroenterology 136(7):2058–2061
96. Bernstein CN, Niazi N, Robert M, Mertz H, Kodner A, Munakata J et al (1996) Rectal afferent function in patients with inflammatory and functional intestinal disorders. Pain 66(2–3):151–161
97. Bernstein CN, Frankenstein UN, Rawsthorne P, Pitz M, Summers R, McIntyre MC (2002) Cortical mapping of visceral pain in patients with GI disorders using functional magnetic resonance imaging. Am J Gastroenterol 97(2):319–327
98. Verma-Gandhu M, Verdu EF, Bercik P, Blennerhassett PA, Al-Mutawaly N, Ghia JE et al (2007) Visceral pain perception is determined by the duration of colitis and associated neuropeptide expression in the mouse. Gut 56(3):358–364
99. Finucane MM, Stevens GA, Cowan MJ, Danaei G, Lin JK, Paciorek CJ et al (2011) National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. Lancet 377(9765):557–567
100. Suzuki K, Jayasena CN, Bloom SR (2012) Obesity and appetite control. Exp Diabetes Res 2012:824305
101. Volkow ND, Wang GJ, Baler RD (2011) Reward, dopamine and the control of food intake: implications for obesity. Trends Cogn Sci 15(1):37–46
102. Jauregui-Lobera I (2013) Neuropsychology of eating disorders: 1995–2012. Neuropsychiatr Dis Treat 9:415–430
103. Kaye WH, Fudge JL, Paulus M (2009) New insights into symptoms and neurocircuit function of anorexia nervosa. Nat Rev Neurosci 10(8):573–584
104. Craig AD (2002) How do you feel? Interoception: the sense of the physiological condition of the body. Nat Rev Neurosci 3(8):655–666
105. Mayer EA (2011) Gut feelings: the emerging biology of gut-brain communication. Nat Rev Neurosci 12(8):453–466

Part II
Mechanistic Factors Influencing the Microbiota-Gut-Brain Axis

Chapter 7
Gastrointestinal Hormones and Their Targets

Jens F. Rehfeld

Abstract Gastrointestinal hormones are peptides released from endocrine cells and neurons in the digestive tract. More than 30 hormone genes are currently known to be expressed in the gastrointestinal tract, which makes the gut the largest hormone producing organ in the body. Modern biology makes it feasible to conceive the hormones under five headings: The *structural homology* groups a majority of the hormones into nine families, each of which is assumed to originate from one ancestral gene. The individual hormone gene often has *multiple phenotypes* due to alternative splicing, tandem organization, or differentiated maturation of the prohormone. By a combination of these mechanisms, more than 100 different hormonally active peptides are released from the gut. Gut hormone genes are also *widely expressed* in cells outside the gut, some only in extraintestinal endocrine cells and neurons but others also in other cell types. The extraintestinal cells may synthesize different bioactive fragments of the same prohormone due to *cell-specific processing* pathways. Moreover, endocrine cells, neurons, cancer cells, and, for instance, spermatozoa *release the peptides differentially* (autocrine, endocrine, neurocrine, paracrine, spermiocrine secretion etc.), so the same peptide may act as a blood-borne hormone, a neurotransmitter, a local growth factor, or a fertility factor. The molecular targets of each bioactive peptide are specific G-protein coupled receptors expressed in the cell membranes of different target cells. Also the target cells of gut hormones occur widespread outside the digestive tract.

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_7,

Abbreviations

CCK	Cholecystokinin
CGRP	Calcitonin gene related peptide
EGF	Epidermal growth factor
G-cells	Gastrin-producing cells
GIP	Gastric inhibitory peptide (later renamed glucose-dependent insulinotropic polypeptide)
GLP-1 and -2	Glucagon-like peptide 1 and 2
IGF	Insulin-like growth factor
L-cells	GLP-producing cells
mRNA	Messenger ribonucleic acid
NPY	Neuropeptide Y
PP	Pancreatic polypeptide
PTHrP	Parathyroid Hormone-related Protein
PYY	Peptide YY
TGF-α (alpha) and -β (beta)	Transforming growth factor α (alpha) and -β (beta)
TSH	Thyroidea-stimulating hormone
VIP	Vasoactive intestinal polypeptide

Historical Introduction

The bloodborne regulation by specific messenger molecules was discovered in 1902 in London by Bayliss and Starling [1]. Following up on the observation that acidification in the upper small intestine, the duodenum, stimulated pancreatic secretion, Bayliss and Starling extracted from the duodenal mucosa a substance that released bicarbonate from the denervated pancreas when injected into blood. They gave this substance a very broad name, secretin. In 1905, John Edkins (also from London) suggested that extracts of the antral mucosa [2] contained an acid stimulatory messenger ("gastric secretin"—or simply gastrin). Hence, the first two bloodborne "chemical messengers" to be known in mammalian biology, secretin and gastrin, were both of gastrointestinal origin. Subsequently, also in 1905, Starling proposed the word *hormone* as a general designation for bloodborne messengers [3].

In the following decades, however, other types of hormone came into focus—steroids from the adrenals, ovaries, and testes; protein hormones from the pituitary gland; the thyronins from the thyroid gland; and insulin from the pancreas. The clinical implications and often life-saving effects of these discoveries made the interest for secretin and gastrin fade in the darkness of the bowels. Subsequently, only a small priesthood of physiologists continued to study the hormonal control of digestion. One of them was Andrew Ivy in Chicago, who with his assistant, Eric Oldberg, found a gallbladder emptying hormone [cholecystokinin (CCK)] in

extracts of the small intestine [4]. A stimulator of pancreatic enzyme secretion (pancreozymin) was discovered 15 years later by Harper and Raper in Newcastle [5]. But Jorpes and Mutt showed in the 1960s in Stockholm, however, that CCK and pancreozymin were one and the same peptide hormone [6] for which the acronym CCK is now used.

Secretin, gastrin, and CCK constitute the classical troika of gastrointestinal hormones, but since the early twentieth century, many more have been discovered (Fig. 7.1). In order not to lose overview, this chapter summarizes all the gut hormones, some of their targets, and their major biological activities in Tables 7.1, 7.2 and 7.3, but otherwise presents the general principles governing structure and biogenesis of gastrointestinal hormones and their receptors (see also [7, 8] for longer reviews). Readers interested in details about individual hormones, their targets, receptors, and their effects, should consequently consult multi-author volumes comprising the full range of gastrointestinal endocrinology [9–11]. Also, a shorter review on the history of gastrointestinal endocrinology has recently been published [12].

Comparative Aspects of the Development

Life in multicellular organisms began as a simple tube with only one opening. Take coelenterates, for instance (Fig. 7.2). They live in water that runs into their lumen, and from which nutrients are absorbed into the epithelial cell-lining. Coelenterates have a regulatory system of singular primitive neurons spread out in the wall. Apparently, these neurons release small regulatory peptides. Thus, multicellular life began as an isolated ‘gut’ whose function was controlled by regulatory or hormonal peptides. Consequently, viewing the phylo- and ontogenetical development of life, evolutionists could say that the specific organs and tissues in vertebrate organisms are derivatives of the primordial multicellular structure, the gut. Accordingly, the regulatory or hormonal peptides of the gastrointestinal tract are from the beginning essential caretakers of life; also human life and its disorders.

General Features of Gastrointestinal Hormones

The Structural Homology

Gastrointestinal endocrinology currently encompasses a large number of hormones, neuropeptides and growth factors. Not only have new hormones been found in gut extracts, but also peptides from the central nervous system and hormones first identified in other endocrine organs have been found in endocrine cells and/or neurons in the gastrointestinal tract. Moreover, peptides originally believed to be

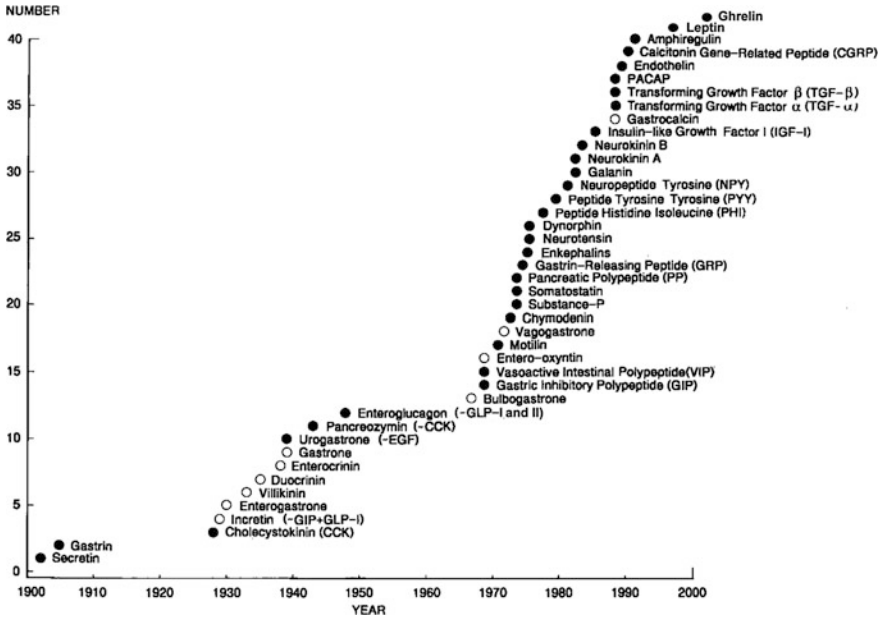

Fig. 7.1 Discovery and identification of regulatory peptides in the gastrointestinal tract 1900–2000. Discovery is indicated by year of first report. *Solid circles* indicated structural identification, and *open circles* indicated hormonal activities that still require structural identification. Some of the unidentified hormonal activities are explained by later identified hormones. For instance, the incretin activity is partly due to gastric inhibitory polypeptide (GIP) and glucagon-like peptide I (GLP-I). Commonly used acronyms are indicated in brackets after full name, except for PACAP, which is an acronym for pituitary adenylate cyclase-activating peptide

classical hormones but later shown to be neurotransmitters have been isolated from gut extracts. Finally, a number of growth factors have now been found in the gut—epidermal growth factor (EGF), originally isolated as the gut hormone, urogastrone, from urine; insulin-like growth factors (IGF) I and II; transforming growth factors (TGF)-α (alpha) and -β (beta); amphiregulin, and others.

The complexity is increased through individual genes for gut regulatory peptides encoding different peptides released in a cell-specific manner. Several principles for gene expression operate to provide such variety. Hence, alternative splicing of the calcitonin gene transcript to express CGRP is not the only example [13]. Also, the secretin gene is expressed in different molecular forms due to alternative splicing [14, 15].

Additional studies indicate that there are still hormonal activities in the gut that are not structurally identified. Perhaps some of the activities can be explained by already identified peptides. Hence, the hormonal stimulation of insulin secretion from the gut, originally called incretin, is today explained by at least two hormones, GIP and truncated GLP-1—probably in combination with other gut hormones, including gastrin and CCK peptides (for review, see [16]), whereas intestinal

Table 7.1 Peptide hormone, neuropeptide and growth factor families in the gastrointestinal tract and the pancreas

Families and members	Major regulatory activity
Secretin family	
Secretin	Stimulates pancreatic bicarbonate secretion
Glucagon	Increases glucose production and amino acid metabolism
Glucagon-like peptide 1 (GLP-1)	Stimulates insulin and inhibits glucagon secretion and gastric emptying
Glucagon-like peptide 2 (GLP-2)	Stimulates mucosal cell growth in intestinal crypts
Gastric inhibitory polypeptide (GIP)	Enhances glucose-stimulated insulin secretion and inhibits gastric secretion
Vasoactive intestinal polypeptide (VIP)	Inhibits gastrointestinal motility and stimulates fluid secretion
Peptide histidine isoleucine (PHI)	VIP-like actions
Growth hormone releasing hormone	Stimulates growth hormone secretion
Pituitary adenylyl cyclase-activating peptide (PACAP)	Contributes to the regulation of gastric acid secretion and gastrointestinal motor function
Gastrin family	
Gastrin	Stimulates gastric acid secretion and gastric mucosal cell growth
Cholecystokinin (CCK)	Stimulates pancreatic enzyme secretion, cell growth, and gallbladder emptying, but inhibits gastric acid secretion
Caerulein Cionin Not expressed in mammals	Cholecystokinin-like activities
Tachykinin family	
Substance P	Stimulates motility
Neurokinin A	Stimulates motility
Neurokinin B	Stimulates motility
Ghrelin family	
Ghrelin	Stimulates appetite and growth hormone secretion
Obestatin	Suppresses food intake (?)
Motilin	Contracts gastrointestinal smooth muscles to stimulate motility
PP-fold family	
Pancreatic polypeptide (PP)	Involved in feeding behavior (?)
Peptide YY (PYY)	Reduces gastric emptying, pancreatic exocrine secretion, and delays intestinal transit
Neuropeptide Y (NPY)	Modulates the contractility in smooth muscle cells
Somatostatin family	
Somatostatin	Inhibits gastric acid, gastrin secretion and other gut functions through endocrine, paracrine, and neurocrine release
Cortistatin	Somatostatin-like activities
Insulin family	
Insulin	Establishes energy resources in fat, liver and muscle cells
Insulin-like growth factor I (IGF-I)	Stimulates growth and differentiation in interaction with other growth factors

(continued)

Table 7.1 (continued)

Families and members	Major regulatory activity
Insulin-like growth factor II (IGF-II)	Stimulates growth and differentiation in interaction with other growth factors
Relaxin	Function in the gastrointestinal tract uncertain
EGF family	
Epidermal growth factor (EGF)	Stimulates growth of epithelial cells and inhibition of gastric acid secretion
Transforming growth factor α (TGFα)	EGF-like activities
Amphiregulin	Growth regulation of epithelial cells
Heparin-binding EGF-like growth factor	EGF-like activities
Opioid peptide family	
Enkephalins	Modulates transmitter activity from nerveplexes
β-endorphins	Modulates transmitter activity from nerveplexes
Dynorphins	Modulates transmitter activity from nerveplexes

Table 7.2 Singular peptide hormones, neuropeptides, and growth hormones in the gastrointestinal tract

Hormones and growth factors	Major regulatory activity
Apelin	Stimulates gastric mucosal growth and cholecystokinin secretion
Bradykinin	Contributes to control alkaline secretion in the duodenal mucosa
Calcitonin gene-related peptide (CGRP)	Modulates blood flow, secretion, and motility
Cocaine and amphetamine regulated transcript (CART)	Increases satiety
Galanin	Stimulates motility and luminal secretion
Gastrin-releasing peptide (GRP)	Stimulates antral gastrin secretion
Neurotensin	Increases the ileal brake
Orexin	Stimulates gut motility (?)
Transforming growth factor β (TGFβ)	Growth, differentiation, and inflammation
Thyrotropin-releasing hormone (TRH)	Releases TSH from epithelial cells in the gut

inhibitory effects on stomach secretion, the gastrone effects, may be explained by combinations of CCK, somatostatin, GIP, and EGF. However, the villikinin, duocrinin, enterocrinin, and the more recently suggested gastrocalcin [17] still await structural identification. At present there is, however, evidence that gastrocalcin may be PTHrP (the parathyroidhomone-related protein) known to be expressed as a paracrine regulator of differentiation and local intercellular signaling [18]. The multiplicity of gut hormones may jeopardize an overview of gut endocrinology. Structural identifications, however, have shown striking homologies between groups of peptides. Consequently, many of the biologically active peptides, hormones, neuropeptides, and growth factors in the gastrointestinal tract can be classified into nine families (Table 7.1). The expression of several hormone

Table 7.3 Receptors and receptor subtypes for some gastrointestinal hormones

Hormones	Receptors and subtypes
Atrial Natriuretic Peptide (ANP)	NP_A, NP_B, NP_C
Brain Natriuretic Peptide (BNP)	NP_A, NP_B, NP_C
C-type Natriuretic Peptide (CNP)	NP_A, NP_B, NP_C
Calcitonin	Calcitonin-R
Calcitonin Gene-Related Peptide (CGRP)	$CGRP_1$, $CGRP_2$
Cholecystokinin (CCK)	CCK_A, CCK_B
Gastric Inhibitory Polypeptide (GIP)	GIP-R
Gastrin	Gastrin/CCK_B
Gastrin-Releasing Peptide (GRP)	CGRP-R
Ghrelin	Ghrelin-R
Glucagon-Like Peptide-1 (GLP-1)	GLP-1-R
Motilin	Motilin-R
Neurotensin	NTR1, NTR2, NTR3
Parathyroid Hormone-related Protein (PTHrP)	PTH-R
Pituitary Adenylate Cyclase Activating Peptide (PACAP)	PAC_1
Peptide Tyrosyl Tyrosyl (PYY)	Y_1, Y_2, Y_3, Y_4, Y_5
Secretin	Secretin-R
Somatostatin	sst_1, sst_{2A}, sst_{2B}, sst_3, sst_4, sst_5
Substance P	NK_1, NK_2, NK_3
Vasoactive Intestinal Polypeptide (VIP)	$VPAC_1$, $VPAC_2$

genes both in the gut and pancreas reflects the intestinal origin of the pancreas. The nature of the homology varies. It may be an overall similarity in the primary structure as, for example, the PP-fold family. The similarity of the tertiary structure in this family is due to homologous residues necessary for stabilization of the three-dimensional structure [19].

Another type of homology is that of the gastrin family which, in addition to mammalian gastrin and CCK, also consists of the protochordean neuropeptide cionin [20] and frogskin peptide cerulein [21]. The decisive homology of this family is concentrated in the primary structure around the active site, the common C-terminal tetrapeptide amide sequence, -Trp-Met-Asp-Phe-NH_2. Comparison between propeptide and gene structures also reveals some similarity, but the family is still defined primarily by the conserved active site sequence and by neighboring O-sulfated tyrosyl residues.

The frequent occurrence of homology among hormones, neuropeptides, and growth factors is not specific for bioactive peptides in the gut. It is a common feature among all kinds of regulatory peptides, enzymes, and other proteins in the organism [22]. Each family is assumed to reflect the phylogenetic evolution by duplication and subsequent mutations of an ancestral gene.

The phylogenetic story shows that gastrointestinal hormones are indeed very old, several hundred million years [23]. So far, the data also support the idea that each hormone family has evolved from a single ancestor. An associated trait is that gastrointestinal hormones have, to a large degree, preserved their tissue-specific

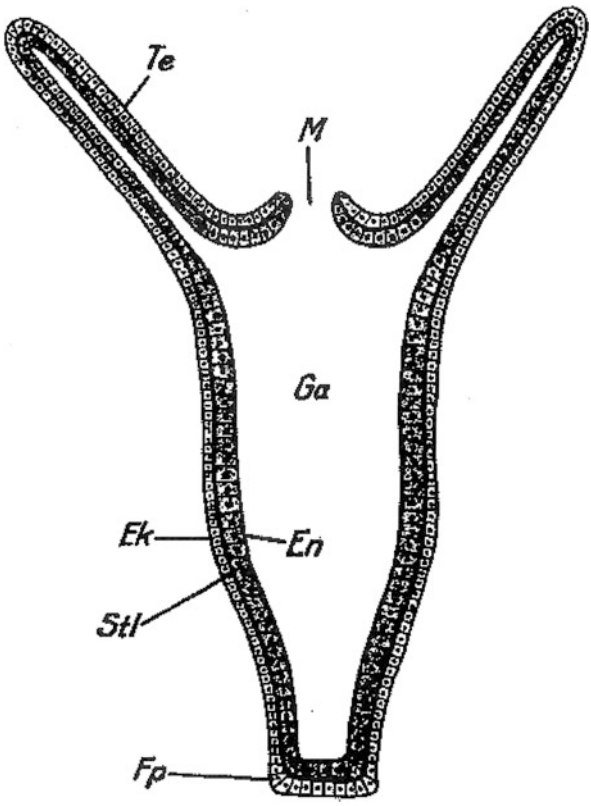

Fig. 7.2 Scheme of the structure of coelenterates with endoderm (*En*), ectoderm (*Ek*), footplate (*Fp*), gaster (stomach *Ga*), mouth (*M*), and tentacles (*Te*)

sites of expression during evolution, both in the primary and secondary sites [24]. Accordingly, the evolution emphasizes the general significance of gut hormones as intercellular messenger molecules.

At present, a few bioactive peptides in the gastrointestinal tract have no relatives or family (Table 7.2). Time will show whether gut peptides still awaiting discovery will show homologies with these peptides.

The Multiple Phenotypes

Three decades ago, one gene was believed to encode one hormonal polypeptide in accordance with what we have learned about the master hormone, insulin. However, more intricate dimensions were added when it became obvious that a single hormone gene often expresses several different bioactive peptides. Today, we know three ways in which a gut hormone gene can express different hormonal peptides.

Alternative Splicing of Transcripts

Alternative splicing was discovered when it was shown that calcitonin gene transcription generates mRNAs encoding either calcitonin peptides or calcitonin-gene related peptides (CGRPs) [13]. CGRPs are now known also to be abundantly expressed in intestinal neurons (see [25] for review). Moreover, a tachykinin gene transcript [26] and the transcript encoded by the secretin gene [14, 15] are also spliced alternatively in the gut. For many years, secretin was believed to exist only as a carboxyamidated peptide of 27 amino acid residues [27]. However, in the mid-1980s, two additional secretins with full bioactivity were identified in porcine gut extracts. One was the immediate precursor of amidated secretin-27, glycine-extended secretin-28, and the other was secretin-30 extended by a Lys-Arg sequence. The existence of glycine- and glycyl-lysyl-arginine-extended forms of secretin and the related VIP is not surprising. They are to be expected from what is known about the biosynthesis of carboxyamidated peptides. The discovery of secretin-71 [14], which contains the sequence of nonamidated secretin-27 N-terminally, followed by a Gly-Lys-Arg extension and a further C-terminal extension of 41 amino acid residues did, however, come unexpectedly (Fig. 7.3). With the exception of an arginine residue, the C-terminal sequence of secretin-71 is identical to the C-terminal 40-amino-acid residue fragment from porcine preprosecretin. Thus, the sequence that corresponds to secretin RNA encoding a 32-amino acid sequence has been spliced out from the primary secretin gene transcript. For reasons mentioned above, secretin-71 has full secretin bioactivity.

Multiple Products of Prohormones with One Active Sequence

The somatostatin and gastrin families represent peptide systems in which the gene encodes only one prohormone that contains only one active site, but where the prohormone is processed in a way to release peptides of different lengths with the same active C-terminus. Although the different bioactive products of the same precursor are bound to the same receptor, their varying clearances from plasma affect their hormonal significance considerably. Hence, it matters whether intestinal proCCK is processed mainly to CCK-58 or to CCK-8 (Fig. 7.3), or whether prosomatostatin is processed to somatostatin-28 or -14. So far, the biosynthesis of gastrin in antral G-cells has been examined particularly thoroughly. It is, therefore, a useful illustration of the second way in which one gastrointestinal hormone gene can encode different bioactive peptides (for reviews, see [7, 28]).

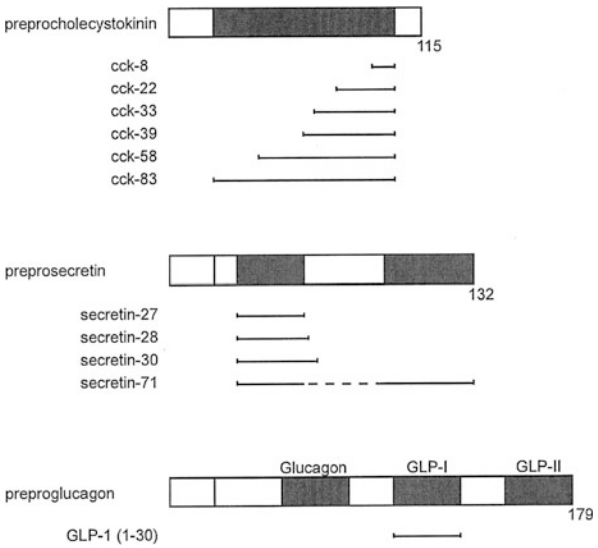

Fig. 7.3 Multiple phenotypes of three gut hormone genes. The cholecystokinin (CCK) gene encodes a prepropeptide which is processed to six CCK peptides varying in length from 83 to 8 amino acid residues through differentiated endoproteolytic cleavage. The six peptides have the same C-terminal bioactive octapeptide sequence. The secretin gene encodes a prepropeptide that through endoproteolytic cleavages and variable C-terminal trimming is processed to three bioactive secretin peptides of almost similar size (secretin-27, -28, and -30). In addition, bioactive secretion-71 is produced by splicing out RNA, encoding the midsequence of preprosecretin (i.e. broken line of secretin-71). The glucagon gene encodes a prepropeptide that through cell-specific endoproteolytic cleavages is processed to either genuine pancreatic glucagon (in pancreatic α-cells) or to glucagon-like peptides I and II (GLP-I, GLP-II) [7]

Differential Processing of Prohormones Containing Two or More Active Sequences

A third way in which one gene can express different bioactive peptides occurs when the gene encodes a propeptide containing different but often homologous peptide hormones or neuropeptides. Gastrointestinal hormones and neuropeptides comprise many examples of such genes of which the opioid-peptide genes, some of the tachykinin genes, the VIP gene, and the glucagon gene amply illustrate the phenomenon. Some of the genes not only encode a peptide precursor containing different bioactive peptides, which is then subjected to tissue-specific posttranslational processing, but the primary transcripts of these gene(s) may also undergo tissue-specific alternative splicing [26].

Proglucagon is an example of a poly-protein precursor that contains three similar but still different peptide sequences in mammals (Fig. 7.3). In pancreatic islet α-cells, proglucagon is processed to release the well-known pancreatic glucagon, whereas the C-terminal part of proglucagon remains silent in that neither GLP-I nor GLP-II is synthesized [29, 30]. The L-cells of the gut also express proglucagon but

process it in a different way to release GLP-I and GLP-II [29, 31]. Although glucagon and, for instance, GLP-I are highly homologous peptides, and both are glucoregulatory, they have separate activities and receptors. Proglucagon also tells another story of interest. Deduction of its structure from cloned cDNA provided the first evidence or suggestion of separate bioactive peptide moieties from the same precursor due to the homologies between the sequences 33–61, 72–107, and 126–158 [32]. Physiological studies, however, showed that the first deduced GLP-I (proglucagon 72–107), which is situated between two dibasic sites in the precursor, is a poorly active peptide. Instead, a truncated form of the original GLP-I, which corresponds to the proglucagon sequence 78–107, turned out to be a highly potent peptide [31, 33]. Thus, bioactive peptide structures cannot be predicted from cDNA and precursor sequences. This also requires exact identification of the released peptides accompanied by physiological studies of their activities.

Widespread Gene Expression

For gastrointestinal hormones, the expression cascade is elaborate and involves multiple processing enzymes with cleavages and derivatizations. Each step may control whether the initial gene transcription results in a bioactive peptide product. Transcription can occur without translation of the transcript, and lack of parallelism between mRNA, propeptide, and the mature bioactive peptide has been described (for review, see [34]). Hence, gene expression in "new" sites in the body requires specification of the sense in which the term expression is meant.

All gut hormones are widely expressed in tissues outside the gastrointestinal tract. For some, the extraintestinal expression is confined mainly to neurons and endocrine cells, especially neurons in the central and peripheral nervous systems. However, several gastrointestinal hormones are also expressed in other cell types and tissues. The literature on extraintestinal expression of gut hormones has become overwhelming. Therefore, the phenomenon will be described for a single hormonal system only (gastrin), which may serve as an example.

The gastrin gene is expressed in several other cell types than the antroduodenal G-cells. Quantitatively, these other cells release only little gastrin to blood in normal organisms since the extra-antral secretion seems to serve local purposes. Besides that, biosynthetic processing is often so different that bioactive gastrins may not even be synthesized. So far, extra-antral expression of progastrin and its products has been encountered in the distal small intestinal and colorectal mucosa [35], endocrine cells in the fetal and neonatal pancreas [36, 37], pituitary corticotrophs and melanotrophs [38, 39], hypothalamopituitary [40] and vagal neurons [41], and human spermatogenic cells [42].

The meaning of extraintestinal synthesis of gastrointestinal hormones is often unknown, but some suggestions can be offered. Local growth regulation is the first possibility. Secondly, it is possible that the low concentration of peptides is without significant function in the adult, but is a relic of a more comprehensive fetal

synthesis. A third possibility is that the low cellular concentration reflects constitutive secretion where the peptides are not stored in secretory granules.

Cell-Specific Prohormone Processing

Gastrointestinal hormone genes and prohormone structures are often so complex and the posttranslational processing so elaborate that the phenotypic result of gene transcription is unpredictable. Hence, the cellular equipment with processing enzymes and their necessary cofactors determine the structure of the particular prohormone product. This cell-specific processing of prohormones applies to all gastrointestinal hormones. But again, gastrin is also one of the most extensively studied gastrointestinal hormones with regard to cell-specific prohormone processing.

Almost every tissue in which progastrin is expressed has its own characteristic processing pattern. Four different patterns are shown in Fig. 7.4. For members of the gastrin family the processing varies with respect to endoproteolytic processing and with respect to amino-acid derivatizations such as tyrosyl sulfations and phenylalanyl amidations. In this context it is worth realizing that the different types of processing my influence each other, presumably by changing the affinity for the various processing intermediates as substrate for the processing enzymes. Thus, tyrosyl sulfation, the earliest posttranslational modification for the gastrin family of prohormones, increases endoproteolytic cleavage efficiency [43], and as endoproteolytic cleavage efficiency increases, so does C-terminal amidation process efficiency.

Cell-Specific Peptide Release

To understand the specific effects of the gastrointestinal peptides, it is necessary to realize that the different types of cells that express the respective genes also release the peptides in different ways. Secretion of gastrointestinal hormones was supposed to be endocrine only, until 30 years ago. But today, three alternative routes of secretion to neighboring cells and one to the secretory cell itself have been discovered (Fig. 7.5). Firstly, the peptides synthesized in neurons are released from synaptosomal vesicles in the nerve terminals to the receptors of adjacent target cells as neurotransmitters. In addition, it is possible that a spill-over of gut hormonal peptides released from peripheral neurons may be transported via blood, analogous to other extraintestinal neuropeptides. It is also possible that some peptidergic neurons expressing gut hormonal peptides, such as hypothalamo-pituitary neurons, release the peptides directly to blood vessels as neurocrine secretion. Secondly, it has been shown that there are specific paracrine cells that release, for instance, somatostatin in the gastrointestinal mucosa [44]. These cells

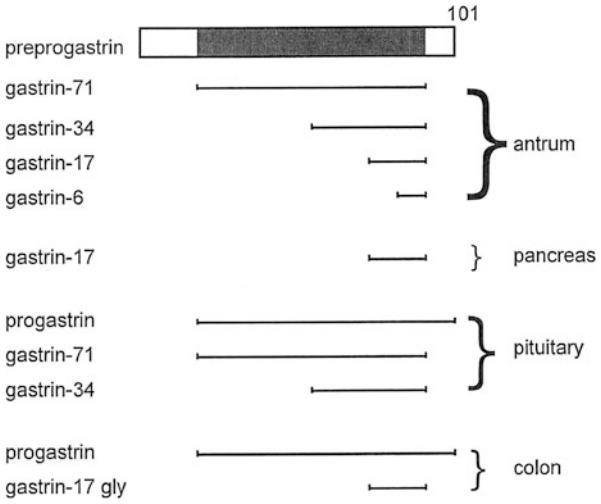

Fig. 7.4 Schematic illustration of cell-specific processing of preprogastrin in antral G-cells, G-cells in fetal and neonatal pancreas, in pituitary corticotrophic cells, and in unidentified cells in the colorectal mucosa [7]

carry peptidergic granules through cytoplasmic extensions to specific target cells in the neighborhood. Paracrine cells can be considered as hybrids of classical endocrine cells and neurons. It is, therefore, possible that a local spillover of peptides from paracrine cells may also reach the circulation.

Cells stimulate their own growth through autocrine secretion. Trophic peptides bind to specific receptors in the membranes of cells in which they are also synthesized (Fig. 7.5). Autocrine secretion is supposed to play a decisive role in tumor and cancer development [45–47]. There is, for instance, evidence to suggest that the growth of certain cultured bronchial carcinoma cells [48], pancreatic tumor cells [49] and gastric and colon cancer cells [50, 51] are stimulated by autocrine secretion of gastrin, and that growth of certain human pancreatic cancer cell lines is stimulated by gastrin and CCK peptides [52].

Cellular release of gastrointestinal peptides also occurs in a fifth way (Fig. 7.5). Spermatogenic cells in mammals express the gastrin, CCK, and PACAP genes [42, 53, 54]. The gastrin and CCK peptides are fully carboxyamidated and, like PACAP [55], concentrated in the acrosome. In accordance with the acrosomal reaction, the peptides are released from the spermatozoon by contact with the jelly-coat of the egg and subsequently bound to receptors in the egg membrane. Defects of the reproductive functions have now been found in PACAP-deficient mice [55].

Acrosomal release may prove an important mechanism of secretion for gut peptides if fertilization of the egg turns out to require such peptides. The release of bioactive peptides from acrosomal granules could be termed *spermiocrine* release (Fig. 7.5).

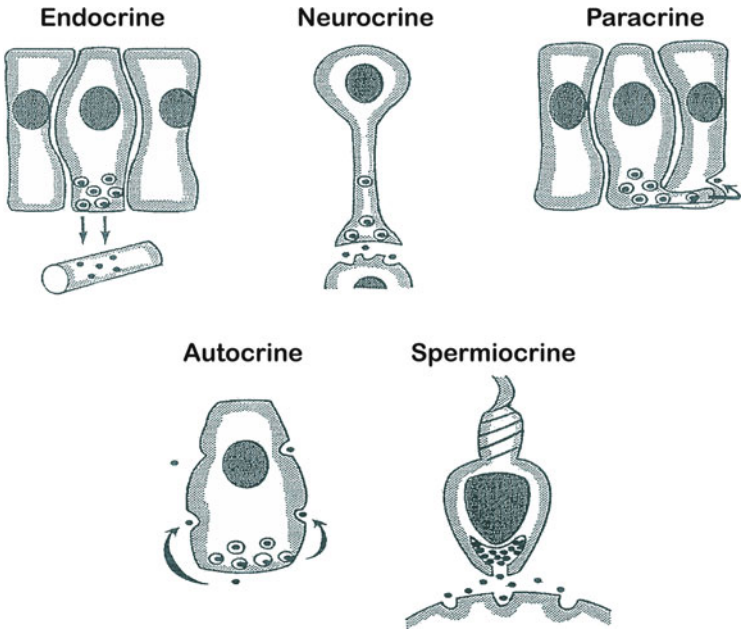

Fig. 7.5 Different types of cell-specific release of regulatory gut peptides: (1) endocrine release to capillaries from classic endocrine cells in the gastrointestinal mucosa; (2) neurotransmitter release from central or peripheral neurons to the synaptic cleft; (3) paracrine release to neighboring cells through short cellular processes; (4) autocrine release to receptors on membrane of same cell that synthesizes and releases the peptides; and (5) spermiocrine release from acrosomal granule of spermatozoa to receptors on egg cell membranes [7]

General Features of Gut Hormone Targets

Target Cells

The molecular targets of gastrointestinal hormones are specific G-protein coupled receptors expressed on a variety of cell membranes in the body. Many of the target cells are located in the gastrointestinal tract: Neurons (including the coordinating myenteric and submucosal nerveplexes); other endocrine gut cells; smooth muscles; secretory cells that release enzymes, amines, acid and bicarbonate etc. The hormonal control of intestinal target cells ensure that digestion, cellular growth turnover and motility of the gut occur in a coordinated manner in order to optimize the utilization of food and the subsequent energy delivery to the body. However, cells of many extraintestinal organs in the body also express receptors for gastrointestinal hormones, which teleologically may provide a reason for the occurrence of hormonal peptides from the gut in general circulation. These extraintestinal organs include for instance endocrine glands (the pituitary; thyroid C-cells; parathyroid glands; islets of Langerhans etc.); the liver; the gallbladder; the pancreas;

the cardiovascular system and the lungs. At low-level most other tissues in the body also express gut hormone receptors, the significance of which is still largely unknown. Finally, many gastrointestinal and extra-intestinal cancers express promiscuously the genes of both gut hormones and their receptors whereby these cancers are often equipped with local autocrine growth promoter mechanisms [56]. The known target functions of each gastrointestinal hormone are outlined in Tables 7.1 and 7.2.

Receptors

The receptors for gut hormones are as mentioned of the G-protein coupled or rhodopsin-like type with seven transmembrane loops. The amino acid chain is often heavily derivatized at for instance phosphorylation and glycosylation sites. Receptors structurally identified so far are listed in Table 7.3 in relation to their specific hormonal ligand. The relationship is often complex because a specific ligand may be bound to several receptors.

The detection of G-protein-coupled receptors in normal tissues is difficult since the number of receptors in normal target organs that is necessary to elicit a functional effect is small, compared, for instance, to the large amount of hormones synthesized in comparable sites. Therefore, the detection methods for receptors are limited and need to be critically evaluated. Several different in vitro techniques have been used to detect G-protein-coupled receptors: measurement of receptor mRNA by PCR techniques is a widely used way to assess receptors in normal and tumor tissue, with the limitations, however, that it is not the receptor protein that is detected and that he morphological correlate is missing (except for in vitro hybridization techniques). The lack of morphology and the high sensitivity of mRNA measurement by PCR imply that small amounts of normal cells expressing the receptors (blood vessel cells, immune cells, endocrine cells, connective tissue, and neurons etc.) may suggest receptor expression of the main target cells present in an organ. Since most tissue samples are highly heterogeneous from a cellular point of view, it is better to use a morphological method for receptor analysis. It is also preferable to detect the receptor protein itself, and if possible, the receptor-binding sites in these proteins, since the binding sites represent the functional molecular basis for peptide hormones [56]. A "gold standard" example is in vitro quantitative somatostatin receptor autoradiography on frozen tissue sections that combines morphology, binding site detection and receptor quantification. Because of limited cellular resolution, receptor autoradiography is optimal for the detection of receptors in larger cell groups. An attractive morphological alternative is immunohistochemical analysis of the receptors on formalin-fixed tissues [57–59] with the limitations that quantification is not possible and that an epitope that may be different from the binding site is identified. The existence of receptor subtypes for G-protein-coupled receptors has made the evaluation of the receptor profiles more complex.

In principle, all the mentioned methods are capable of detecting receptor subtypes. Unfortunately, antibodies raised against the known G-protein-coupled receptors and their subtypes rarely have the necessary reliability for immunohistochemical detection, i.e. the necessary specificity, affinity and titer. Nevertheless, adequate antibodies against the somatostatin receptor, the sst_2 and possibly also sst_5, are now available [59–61], and that is a major progress that eventually may occur also for antibodies to the other hormone receptors [62, 63].

Perspective

Gastrointestinal endocrinology has developed from an appendix of general endocrinology to a biological discipline of its own over the last 40 years. Today it comprises a multitude of more than 100 bioactive peptides expressed in a controlled cell-specific manner all over the body. The peptides participate in intercellular regulation from local control of growth and cell differentiation to acute systemic effects on metabolism all over the body. Thus, in the early 1970s, a revolution changed the fundamental concepts and opened wide perspectives for gastrointestinal hormones in physiology and pathophysiology.

Gastrointestinal peptide hormones must be viewed as evolutionarily conserved intercellular messengers of general significance. There are no obvious boundaries between their role in food intake and digestion and their function in other bodily regulations. Most regulatory peptides (hormones, neuropeptides, growth factors, and cytokines) are probably expressed in the gut, at least at some stage in the phylogenetic or ontogenetic development. Hence, the development of gastrointestinal endocrinology may continue its exponential growth with a broad definition of regulatory peptides. On the other hand, such extension almost deprives the concept of *gastrointestinal* endocrinology of its meaning. And that is exactly what this is all about: Gastrointestinal hormones should be viewed not only as local hormones of specific interest to digestive physiologists and clinical gastroenterologists. They are integrated chemical messengers in the coordination and regulation of many or most bodily functions in mammals. Thus, it is not surprising that today gut hormones are studied not only in physiology and cell biology, but also by microbiologists, psychiatrists, zoologists, cardiologists, diabetologists, and others.

Acknowledgement The skillful and patient secretarial assistance of Connie Bundgaard is gratefully acknowledged. The author also wishes to thank Dr. Jean-Claude Reubi (Berne, Switzerland) for helpful discussion and suggestions for the receptor section.

References

1. Bayliss WM, Starling EH (1902) The mechanism of pancreatic secretion. J Physiol 28 (5):325–353
2. Edkins JS (1906) The chemical mechanism of gastric secretion. J Physiol 34(1–2):133–144
3. Starling EH (1905) The Croonian lecture on the chemical correlation of the function of the body. Lancet II:399–341
4. Ivy AC, Oldberg E (1928) A hormone mechanism for gallbladder contraction and evacuation. Am J Physiol 86:599–613
5. Harper AA, Raper HS (1943) Pancreozymin, a stimulation of the secretion of pancreatic enzymes in extracts of the small intestine. J Physiol 102(1):115–125
6. Jorpes JE, Mutt V (1966) Cholecystokinin and pancreozymin, one single hormone? Acta Physiol Scand 66(1):196–202
7. Rehfeld JF (1998) The new biology of gastrointestinal hormones. Physiol Rev 78 (4):1087–1108
8. Rehfeld JF (2004) A centenary of gastrointestinal endocrinology. Horm Metab Res 36 (11–12):735–741
9. Schultz SG, Mackhlouf GM, Rauner BB (eds) (1989) Handbook of physiology. The gastrointestinal system. Neural and endocrine biology. American Physiology Society, Bethesda
10. Walsh JH, Dockray GJ (eds) (1994) Gut peptides. Raven, New York
11. Taché Y, Goto Y, Ohning G (eds) (2002) Gut-brain peptides in the new millennium. Cure Foundation, Los Angeles
12. Rehfeld JF (2012) Beginnings: a reflection on the history of gastrointestinal endocrinology. Regul Pept 177(Suppl):S1–S5
13. Amara SG, Jonas V, Rosenfeld MG, Onges ES, Evans RM (1982) Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature 298(5871):240–244
14. Gafvelin G, Jörnvall H, Mutt V (1990) Processing of prosecretin: isolation of a secretin precursor from porcine intestine. Proc Natl Acad Sci U S A 87(17):6781–6785
15. Kopin AS, Wheeler MB, Nishtani J, McBride EW, Chang TM, Chey WY et al (1991) The secretin gene: evolutionary history, alternative splicing and developmental regulation. Proc Natl Acad Sci U S A 88(12):5335–5339
16. Rehfeld JF (2011) Incretin physiology beyond glucagon-like peptide 1 and glucose-dependent insulinotropic polypeptide: cholecystokinin and gastrin peptides. Acta Physiol (Oxf) 201 (4):405–411
17. Persson P, Håkanson R, Axelson J, Sundler F (1989) Gastrin releases a blood-calcium lowering peptide from the acid producing part of the stomach. Proc Natl Acad Sci U S A 86 (8):2834–2838
18. Kronenberg HM, Lanske B, Kovacs CS, Chung UI, Lee K, Segre GV, Schipani E, Jüppner H (1998) Functional analysis of the PTH/PTHrP network of ligands and receptors. Recent Prog Horm Res 53:283–301
19. Glover ID, Barlow DJ, Pitts JE, Wood SP, Tickle IJ, Blundell TL et al (1984) Conformational studies on the pancreatic polypeptide hormone family. Eur J Biochem 142(2):379–385
20. Johnsen AH, Rehfeld JF (1990) Cionin: a di-sulfotyrosyl hybrid of cholecystokinin and gastrin from the protochordate Ciona intestinalis. J Biol Chem 265(6):3054–3058
21. Anastasi A, Erspamer V, Endean R (1968) Isolation and amino acid sequence of caerulein, the active decapeptide of the skin of hyla caerulea. Arch Biochem Biophys 125(1):57–68
22. Doolittle RF, Feng DF, Tsang S, Cho G, Little E (1996) Determining divergence times of the major kingdoms of living organisms with a protein clock. Science 271(5248):470–477
23. Johnsen AH (1998) Phylogeny of the cholecystokinin/gastrin family. Front Neuroendocrinol 19(2):73–99

24. Rourke IJ, Rehfeld JF, Møller M, Johnsen AH (1997) Characterization of the cholecystokinin and gastrin genes from the bullfrog, *Rana catesbeiana*: evolutionary conservation of primary and secondary sites of gene expression. Endocrinology 138(4):1719–1727
25. Holzer P (1994) Calcitonin gene-related peptide. In: Walsh JH, Dockray GJ (eds) Gut peptides: biochemistry and physiology. Raven, New York, pp 493–534
26. Nawa H, Kotani H, Nakanishi S (1984) Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing. Nature 312(5996):729–734
27. Mutt V, Jorpes JE, Magnusson S (1970) Structure of porcine secretin. The amino acid sequence. Eur J Biochem 15(3):513–519
28. Dockray GJ, Varro A, Dimaline R, Wang T (2001) The gastrins: their production and biological activities. Annu Rev Physiol 63:119–139
29. Ørskov C, Bersani M, Johnsen AH, Højrup P, Holst JJ (1989) Complete sequences of glucagon-like peptide-1 from human and pig small intestine. J Biol Chem 264 (22):12826–12829
30. Drucker DJ, Erlich P, Asa SL, Brubaker PL (1996) Induction of intestinal epithelial proliferation by glucagon-like peptide 2. Proc Natl Acad Sci U S A 93(15):7911–7916
31. Holst JJ, Ørskov C, Nielsen OV, Schwartz TW (1987) Truncated glucagon-like peptide-I, an insulin-releasing hormone from the distal gut. FEBS Lett 211(2):169–174
32. Bell GI, Santerre RF, Müllenbach GT (1983) Hamster preproglucagon contains the sequence of glucagon and two related peptides. Nature 302(5910):716–718
33. Mojsov S, Weir GC, Habener JF (1987) Insulintropin: glucagon-like peptide I (7-37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas. J Clin Invest 79(2):616–619
34. Rehfeld JF (1990) Posttranslational attenuation of peptide gene expression. FEBS Lett 268 (1):1–4
35. Lüttichau HR, van Solinge WW, Nielsen FC, Rehfeld JF (1993) Developmental expression of the gastrin and cholecystokinin genes in rat colon. Gastroenterology 104(4):1092–1098
36. Larsson LI, Rehfeld JF, Sundler F, Håkanson R (1976) Pancreatic gastrin in foetal and neonatal rats. Nature 262(5569):609–610
37. Bardram L, Hilsted L, Rehfeld JF (1990) Progastrin expression in mammalian pancreas. Proc Natl Acad Sci U S A 87(1):298–302
38. Rehfeld JF (1978) Localisation of gastrins to neuro- and adenohypophysis. Nature 271 (5647):771–773
39. Larsson LI, Rehfeld JF (1981) Pituitary gastrins occur in corticotrophs and melanotrophs. Science 213(4509):768–770
40. Rehfeld JF, Hansen HF, Larsson LI, Stengaard-Pedersen K, Thorn NA (1984) Gastrin and cholecystokinin in pituitary neurons. Proc Natl Acad Sci U S A 81(6):1902–1905
41. Uvnäs-Wallensten K, Rehfeld JF, Larsson LI, Uvnäs B (1977) Heptadecapeptide gastrin in the vagal nerve. Proc Natl Acad Sci U S A 74(12):5707–5710
42. Schalling M, Persson H, Pelto-Huikko M, Odum L, Ekman P, Gottlieb C et al (1990) Expression and localization of gastrin messenger RNA and peptide in human spermatogenetic cells. J Clin Invest 86(2):660–669
43. Bundgaard JR, Vuust J, Rehfeld JF (1995) Tyrosine O-sulfation promotes proteolytic processing of progastrin. EMBO J 14(13):3073–3079
44. Larsson LI, Goltermann N, de Magistris L, Rehfeld JF, Schwartz TW (1979) Somatostatin cell processes as pathways for paracrine secretion. Science 205(4413):1393–1395
45. Sporn MB, Roberts AB (1985) Autocrine growth factors and cancer. Nature 313 (6005):745–747
46. Cuttitta F, Carney DN, Mulshine J, Moody TW, Fedorko J, Fischler A et al (1985) Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. Nature 316(6031):823–826
47. Layton JE, Scanlon DB, Soveny C, Mostyn G (1988) Effects of Bombesin antagonists on the growth of small cell lung cancer cells in vitro. Cancer Res 48(17):4783–4789

48. Sethi T, Rozengurt E (1992) Gastrin stimulates Ca2 + -mobilization and clonal growth in small cell lung cancer cells. Cancer Res 52(21):6031–6035
49. Blackmore M, Hirst BH (1992) Autocrine stimulation of growth of AR4-2J rat pancreatic tumour cells by gastrin. Br J Cancer 66(1):32–38
50. Weinstock J, Baldwin GS (1988) Binding of gastrin(17) to human gastric carcinoma cell lines. Cancer Res 48(4):932–937
51. Hoosein NM, Kiener PA, Curry RC, Brattain MG (1990) Evidence for autocrine growth stimulation of cultured colon tumor cells by gastrin/cholecystokinin-like peptide. Exp Cell Res 186(1):15–21
52. Heald EB, Kramer ST, Smith JP (1992) Trophic effects of unsulfated cholecystokinin on mouse pancreas and human pancreatic cancer. Pancreas 7(5):530–535
53. Persson H, Rehfeld JF, Ericsson A, Schalling M, Pelto-Huikko M, Hökfelt T (1989) Transient expression on the cholecystokinin gene in male germ cells and accumulation of the peptide in the acrosomal granule: possible role of cholecystokinin in fertilization. Proc Natl Acad Sci U S A 86(16):6166–6170
54. Li M, Mbikay M, Nakayama K, Miyata A, Arimura A (2000) Prohormone convertase PC4 processes the precursor of PACAP in the testis. Ann N Y Acad Sci 921:333–339
55. Shintani N, Mori W, Hashimoto H, Imai M, Tanaka K, Tomomoto S et al (2002) Defects in reproductive functions in PACAP-deficient female mice. Regul Pept 109(1–3):45–48
56. Reubi JC (2003) Peptide receptors as molecular targets for cancer diagnosis and therapy. Endocr Rev 24(4):389–427
57. Körner M, Eltschinger V, Waser B, Schönbrunn A, Reubi JC (2005) Value of immunohistochemistry for somatostatin receptor subtype sst2A in cancer tissuer: lessons from the comparison of anti-sst2A antibodies with somatostatin receptor autoradiography. Am J Surg Pathol 29 (12):1642–1651
58. Volante M, Brizzi MP, Faggiano A, La Rosa S, Rapa I, Ferrero A et al (2007) Somatostatin receptor type 2A immunohistochemistry in neuroendocrine tumors: a proposal of scoring system correlated with somatostatin receptor scintigraphy. Mod Pathol 20(11):1172–1182
59. Fischer T, Doll C, Jacobs S, Kolodziej A, Stumm R, Schulz S (2008) Reassessment of sst2 somatostatin receptor expression in human normal and neoplastic tissues using the novel rabbit monoclonal antibody UMB-1. J Clin Endocrinol Metab 93(11):4519–4524
60. Körner M, Waser B, Schönbrunn A, Perren A, Reubi JC (2012) Somatostatin receptor subtype 2A immunohistochemistry using a new monoclonal antibody selects tumors suitable for in vivo somatostatin receptor targeting. Am J Surg Pathol 36(2):242–252
61. Lupp A, Hunder A, Petrich A, Nagel F, Doll C, Schulz S (2011) Reassessment of sst (5) somatostatin receptor expression in normal and neoplastic human tissues using the novel rabbit monoclonal antibody UMB-4. Neuroendocrinology 94(3):255–264
62. Pyke C, Knudsen LB (2013) The glucagon-like peptide-1 receptor – or not? Endocrinology 154(1):4–8
63. Michel MC, Wieland T, Tsujimoto G (2009) How reliable are G-protein-coupled receptor antibodies? Naunyn Schmiedebergs Arch Pharmacol 379(4):385–388

Chapter 8
Microbiome, HPA Axis and Production of Endocrine Hormones in the Gut

Nobuyuki Sudo

Abstract Recent accumulating evidence indicates that the gut microbiome can affect the development and regulation of the hypothalamic-pituitary-adrenal axis and behavior, with central integrative systems being crucial in the successful physiological adaptation of the organism to external stressor. In contrast, host-derived hormones increase the bacterial proliferative capacity and pathogenicity. In the gut lumen, this type of cross-talk between microorganisms and the host is presumed to be performed continually through various kinds of luminal molecules, as numerous types of bacteria and host cells are in close proximity in the gastrointestinal tract of mammals.

We herein focus on bidirectional signaling between the gut microbiome and the host in terms of commensal microbiota affecting the hypothalamic-pituitary-adrenal HPA axis response and behaviors and further discuss the role of gut luminal catecholamines and γ-aminobutyric acid, both of which are presumed to be involved in this signaling.

Abbreviations

ACTH	Adrenocorticotropin hormone
CA	Catecholamines
CRH	Corticotrophin-releasing hormone
DA	Dopamine
E	Epinephrine
EHEC	Enterohemorrhagic *Escherichia coli*
GABA	γ-Aminobutyric acid
GAD	Glutamic acid decarboxylase

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_8,

GC	Glucocorticoids
GUS	β-Glucuronidase
HPA	Hypothalamic-pituitary-adrenal
NE	Norepinephrine
Tir	Translocated intimin receptor

Introduction

Gut microbiota have an estimated mass of 1–2 kg, numbering 100 trillion [1] and together possessing 100 times the number of genes in the human genome [2]. These bacteria not only play a principal role in the postnatal maturation of the mammalian immune system [3], but also aid in the digestion and absorption of macromolecules and act as a barrier to gut pathogens by blocking attachment to gut binding sites [4]. Moreover, it is also rapidly becoming apparent that the gut microbiome plays a major role in the development and regulation of the hypothalamic-pituitary-adrenal (HPA) axis [5] and behavior [6–11].

In contrast, host hormones can signal commensal microbial cells via converging pathways directed to bacterial signaling molecules. Lyte and colleagues first demonstrated in their pioneering studies conducted in the 1990s that some species of pathogens can recognize exogenous catecholamines (CA) in vitro and that such recognition increases the bacterial proliferative capacity [12–15]. Sperandio and colleagues subsequently showed that enterohemorrhagic *Escherichia coli* (EHEC) virulence increases upon exposure to epinephrine (E) and norepinephrine (NE) and that E binds and signals through the QseC receptor [16, 17]. This type of bidirectional communication is called "microbial endocrinology" [15] or "interkingdom signaling" [17, 18], which mediates the symbiotic and pathogenic relationships between the bacteria and mammalian host. Since numerous kinds of bacteria and host cells are in close proximity in the gastrointestinal tract of mammals, interkingdom signaling via various kinds of luminal molecules is presumed to be performed continually in the gut lumen [19] and to participate in the regulation of various pathophysiological functions.

We herein focus on the bidirectional signaling between the gut microbiome and the host in terms of commensal microbiota affecting the HPA axis response and behavior and further discuss the possible involvement of some gut luminal molecules in this signaling.

Gut Microbiota and the Stress Response of the Host

The HPA axis is considered to be a central integrative system, being crucial in the successful physiological adaptation of the organism to stress. During stress, corticotrophin-releasing hormone (CRH) and arginine vasopressin, the principal

hypothalamic regulators of the HPA axis, are released. CRH stimulates the secretion of the adrenocorticotropin hormone (ACTH) from the anterior pituitary into the hypophyseal portal system via collateral fibers in the systemic circulation. ACTH induces the secretion of glucocorticoids (GCs; cortisol in humans and corticosterone in rodents) from the adrenal cortex, the main target of ACTH. GCs regulate multiple bodily functions and prepare the individual to cope with the demands of metabolic, physical and psychological stressors [20].

Critical Role of the Gut Microbiota in Determining the Set Point of the HPA Axis

It is well known that the HPA axis is susceptible to environmental influences, particularly early in life [21, 22]. Since indigenous microbiota constitutes a major environmental force affecting the host physiology, we examined whether these bacteria can alter the development of the HPA response using gnotobiotic mice.

As shown in Fig. 8.1A, the degree of plasma ACTH and corticosterone elevation in response to a 1-h restraint stress was substantially higher in the GF mice than in the SPF mice. When the mice were exposed to ether stimulus, no significant differences in the plasma ACTH or corticosterone response were found in either group of animals (Fig. 8.1B). Monoassociation with *Bifidobacterium infantis*, a representative inhabitant of the neonate gut, lessened the HPA stress response to SPF (Fig. 8.2). The hormonal stress response in the rabbit-derived EPEC-monoassociated mice was substantially higher than that observed in the GF mice, although no such exaggerated response was found in the mice reconstituted with an EPEC mutant strain, ΔTir [23], which is not internalized due to defects in the translocated intimin receptor.

Interestingly, the enhanced HPA stress response of the GF mice was partially corrected at 3 weeks after reconstitution of SPF feces at an early stage of development (Fig. 8.3A), while no such correction was found following reconstitution at a later stage (Fig. 8.3B). Therefore, the microbe-induced reversal of the HPA axis set point extended into adulthood, but only if bacterial colonization occurred before the animals reached 6 weeks of age. Colonization of the adults was ineffective, which suggests a critical window of susceptibility to the effects of bacteria-host interactions.

Recently, animal studies performed by several independent groups have shown the commensal microbiota to be a crucial factor modulating the host behavioral profile [6–9, 11]. In our recent study performed under a strictly contamination-free environment, EX-GF mice, gnotobiotic mice reconstituted with a normal specific pathogen-free microbiota, were less anxious and active than the GF mice based on open field and marble-burying tests [11]. Monoassociation with *Clostridium* (*Brautia*) *coccoides* reduced the anxiety levels; however, it did not affect the

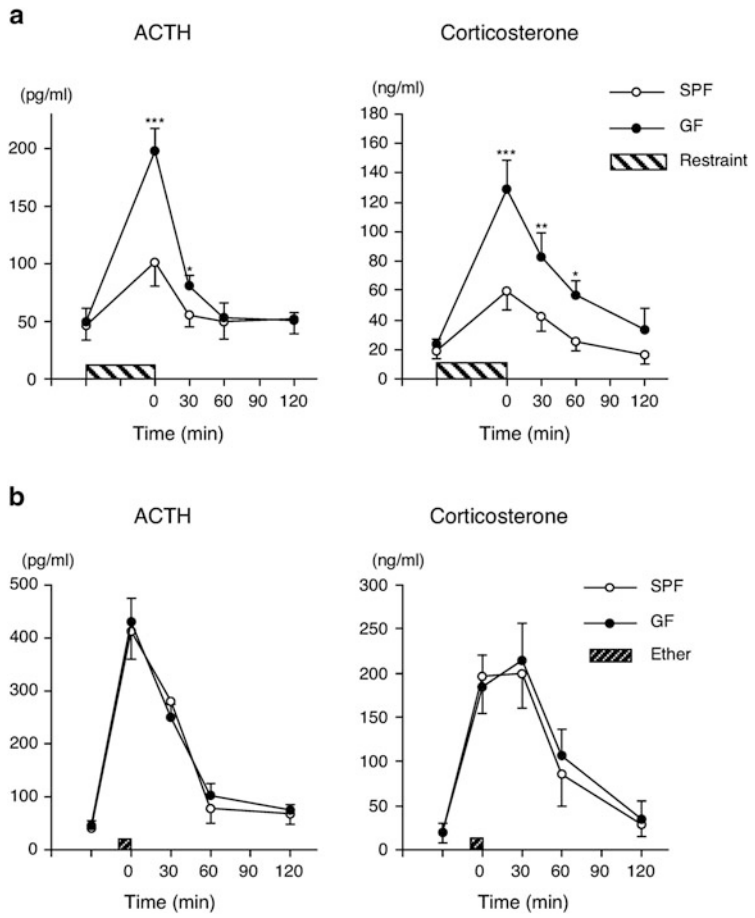

Fig. 8.1 Increased plasma ACTH and corticosterone responses to restraint stress but not to ether exposure in GF mice. **Panel A**: The mice were subjected to a 1-h period of restraint stress (GF, n = 6–11 per each time-point; SPF, n = 6–11 per each time-point). The baseline data were obtained via cardiac puncture in mice killed using cervical dislocation before stress exposure. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ in a post hoc Dunnett's test between GF and SPF. **Panel B**: The GF and SPF mice failed to show any differences in the HPA response to ether exposure (n = 6 per each time point)

locomotor activity. In contrast, colonization with *B. infantis* decreased the locomotor activity while having little effect on the anxiety level.

Therefore, the commensal gut microbiota affects the development and regulation of the biobehavioral stress response of the host.

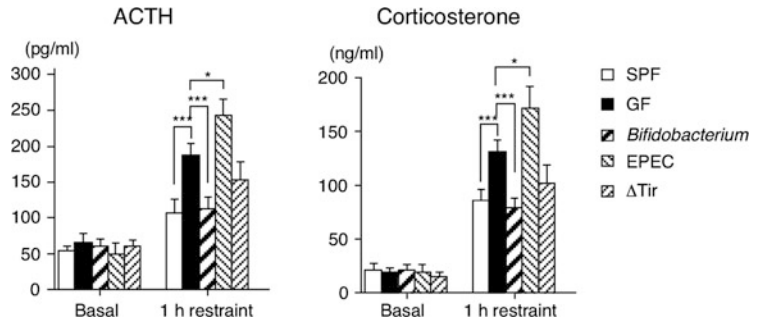

Fig. 8.2 Effects of restraint stress on the plasma ACTH and corticosterone levels in the gnotobiotic mice. The plasma ACTH and corticosterone levels were measured before or immediately after the 1-h restraint test in the GF (n = 20), SPF (n = 18) and monoassociated mice (n = 18–24 per group) at 9 weeks of age. $***P < 0.001$, $*P < 0.05$ according to Dunnett's test. *Bifidobacterium*, EPEC and ΔTir indicate *Bifidobacterium infantis*-, enteropathogenic *E. coli*- and EPEC mutant strain deficient of Tir (translocated intimin receptor)-associated mice, respectively

Microbiota and Stress Resilience

Recently, there has been increasing interest in the individual's response to managing adverse events and stressors [24, 25]. Such an ability to recover from adverse changes, known as "stress resilience," includes psychological and biological processes that allow an individual to avoid or reduce the harmful consequences of extreme stress. Resilient individuals encountering chronic psychosocial stress minimize pathophysiological outcomes, such as extended or exaggerated HPA axis activity [26, 27], that can precipitate stress-related diseases, such as post-traumatic stress disorder, anxiety and major depression [28, 29]. In addition to genetic factors, a broad range of environmental factors contribute to resilience. In fact, a recent elegant study conducted by Lehmann and Herkenham [30] showed that enriched environmental housing (environmental enrichment) confers stress resilience through an infralimbic cortex-dependent neuroanatomical pathway in a mouse model of social defeat stress. Taken together, these findings lead us to the following interesting hypothesis: newborn babies are likely to recognize colonizing bacteria as a stressor when encountering them for the first time because the babies have little capability to discern whether a novel stimulation from the external environment is good or bad. This is supported by the fact that colonization of GF mice by a nonpathogenic bacterium induces a small and transient increase in the plasma corticosterone and IL-6 levels in addition to hypothalamic c-fos activation without eliciting any apparent inflammation of the gut [5]. Such colonizing microbes, however, are not harmful to the host, but rather offer beneficial stimulation for enhancing host resistance to future severe stressors. Selye called this type of stressor "eustress," a positive form of stress usually related to desirable events in a person's life [31]. Therefore, the commensal microbiota may be a "eustress" that

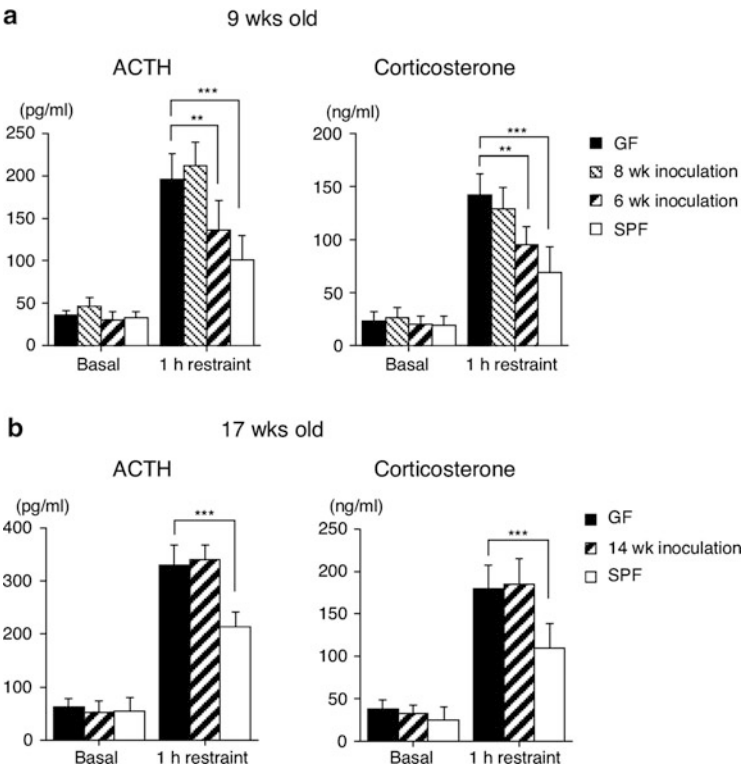

Fig. 8.3 Effects of restraint stress on the plasma ACTH and corticosterone levels in the mice reconstituted with SPF feces. SPF flora-reconstituted mice were established by orally introducing fresh SPF murine feces into the GF mice at either 1 or 3 weeks before being subjected to the stress protocol. Restraint stress was applied to the reconstituted mice at 9 (**panel A**) and 17 (**panel B**) weeks of age (n = 18–24 per group). $***P < 0.001$, $**P < 0.01$ according to Dunnett's test

plays an important role against the development of stress-related disorders, such as anxiety and depression, by providing the host with "stress resilience," similar to environmental enrichment.

Possible Luminal Molecules Mediating Gut Microbe-Host Interactions

The exact mechanisms whereby commensal bacteria interact with the host in the gut and what molecules are involved in this interaction remain to be elucidated. Vagal afferent nerves have been shown to play a role in the signaling from gut microbes to the central nervous system [32]; however, the underlying pathways and molecules are highly complex, and it is unlikely that only one common pathway or

series of molecules is involved. However, we herein pay particular attention to CA and γ-aminobutyric acid (GABA) present in the gut lumen.

CA as an Interkingdom Signal in the Gut Lumen

CA, such as NE and dopamine (DA), are utilized in the central and peripheral nervous systems, which regulate various types of body functions, such as cognitive abilities, mood and gut motility [33]. In addition to the well-established roles of CA, recent accumulating evidence suggests CA to be important interkingdom signal molecules in the gut.

CA Exist in a Biologically Free Form in the Lumen of the Gastrointestinal Tract

Our recent work [34] showed that free NE and DA are present in the lumen of the ileum, cecum and colon. The NE and DA levels in the lumen are the highest in the colon among the three parts of the gut (Fig. 8.4). In contrast, there are no significant differences in the tissue NE or DA levels between the ileum, cecum and colon. Since a large proportion of peripheral CA in the blood and urine exist in a conjugated form that is biologically inactive [35, 36], we investigated the free and glucuronide- and sulfate-conjugated forms of CA in the lumen of the ileum, cecum and colon.

Figure 8.5 shows that almost all of the NE and DA molecules were present in a biologically free form in the lumen of the cecum and colon of the SPF mice, although substantial amounts of glucuronide-conjugated NE and DA were present in the ileum.

Crucial Role of Bacterial β−Glucuronidase in the Generation of a Biologically Active Free Form of CA

The β-glucuronidase (GUS) from *E. coli* is a 290-kDa tetrameric protein that is essentially free of sulfatase activity [37]. Its optimal pH is 6.8, while that of the tissue-type GUS is 4.5 [38]. Since the mean pH in the intestinal lumen ranges from 6.5–7.9 (upper segment of the small intestine) to 6.8–8.0 (colon) in rodents [39–41], we hypothesized that free CA present in the lumen of the cecum and colon are generated via deconjugation by bacterial GUS derived from the gut microbiota. As shown in Fig. 8.6, the luminal free NE and DA levels were lower in the GF mice than in the SPF mice. In addition, more than 90 % of the DA in the GF mice was in the glucuronide-conjugated form in all parts of the digestive tracts examined (Fig. 8.7A), while approximately 40 % of the NE was in the glucuronide-conjugated

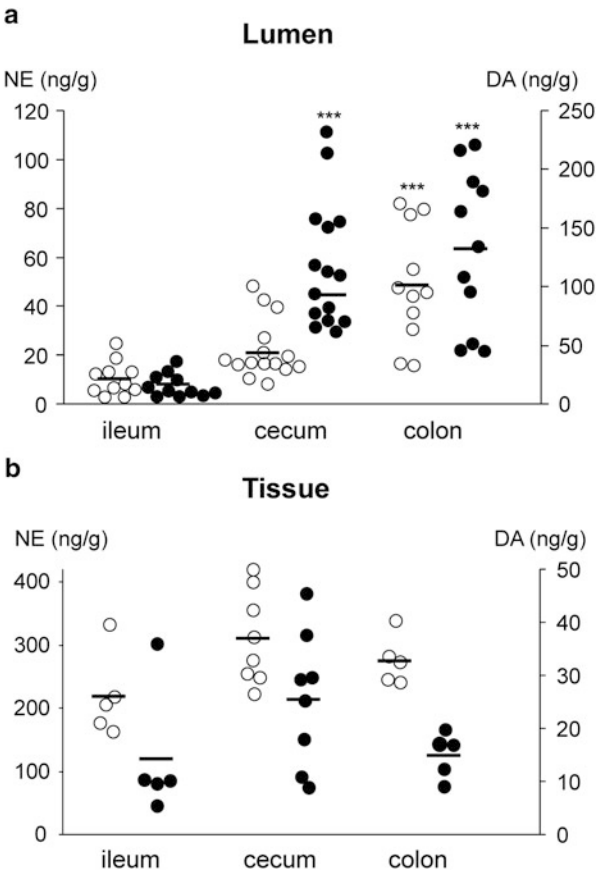

Fig. 8.4 Luminal and tissue CA concentrations in the gastrointestinal tract in the SPF mice. The luminal (n = 11–15, **panel A**) and tissue (n = 5–8, **panel B**) CA levels are shown. The *open* and *closed circles* indicate the NE (*left vertical axis*) and DA (*right vertical axis*) levels, respectively. ***$P < 0.001$ was significantly higher than the corresponding ileum value

form (Fig. 8.7B). The critical role of bacterial GUS in the production of free CA was further verified in additional experiments using gnotobiotic mice.

Association with either a mixture of 46 *Clostridia* species (*Clostridia*) or fecal flora from SPF mice (EX-GF) showed a drastic elevation of the free NE and DA levels (Fig. 8.8). In another set of experiments, the changes in the luminal CA levels were examined in the cecum after GF mice were colonized with either an *E. coli* mutant strain lacking the GUS-encoded gene, *uidA* (JW1609: ΔGUS), or its parent *E. coli* strain (BW25113). Figure 8.9 shows that 70 % of the DA remained in the glucuronide-conjugated form even after the association with ΔGUS, although 25 % of the total DA was in the free form. In contrast, two-thirds of the DA was converted into the free form, while 29 % of the total DA remained conjugated after the association with BW25113. The conjugated form of NE accounted for 29 % of the total following inoculation with ΔGUS, while representing 15 % of the total following inoculation with BW25113. The GUS activity in the cecal lumen of the ΔGUS-gnotobiotic mice was only marginally detectable and almost identical to the GF value (n = 5 per each group, ΔGUS 6.7 ± 0.4 μg PheP/h/mg protein,

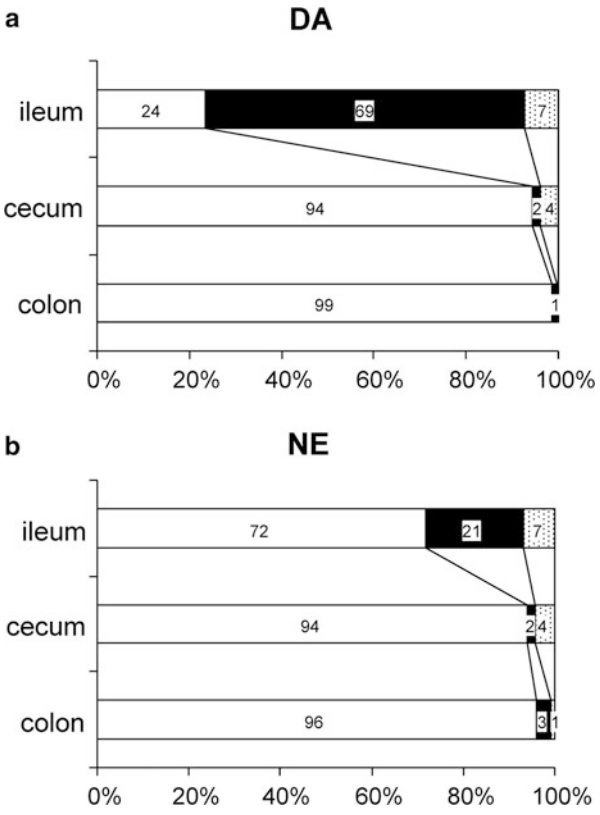

Fig. 8.5 Free and glucuronide- and sulfate-conjugated CA in the gut lumen in the SPF mice. The luminal glucuronide- and sulfate-conjugated DA (n = 6, **panel A**) and NE (n = 6, **panel B**) levels in the ileum, cecum and colon were analyzed with post column HPLC using diphenylethylenediamine as a fluorogenic reagent. The mean value of each form of CA is expressed as the percentage of the total (free CA + conjugated CA). The *open*, *closed* and *dotted bars* indicate the free, glucuronide-conjugated and sulfate-conjugated CA, respectively

GF 6.4 ± 0.3 μg PheP/h/mg protein). On the other hand, the BW25113 *E. coli*-gnotobiotic mice exhibited a small but significant increase in the GUS activity in the cecal lumen in comparison with that observed in the ΔGUS-gnotobiotic mice (n = 4, 11.6 ± 1.3** μg PheP/h/mg protein, $^{**}P < 0.01$ vs. ΔGUS value). Interestingly, the GUS activity in the cecal wall was significantly increased upon exposure to ΔGUS to a comparable level found upon exposure to the BW25113 strain (n = 5 per each group, ΔGUS 3.00 ± 0.25** μg PheP/h/mg protein, BW25113 2.72 ± 0.18* μg PheP/h/mg protein, GF 1.93 ± 0.06 μg PheP/h/mg protein; $^{**}P < 0.01$ and $^{*}P < 0.05$ vs. GF value).

Collectively, these results indicate that the gut microbiota plays a critical role in the generation of luminal free CA via GUS. The bacteria-induced increase in the tissue GUS activity may be involved in this phenomenon.

Can Commensal Bacteria Themselves Produce CA In Vivo?

Russian researchers [42, 43] have reported that some species of microorganisms produce CA in an in vitro culture system. In fact, transcripts that have some

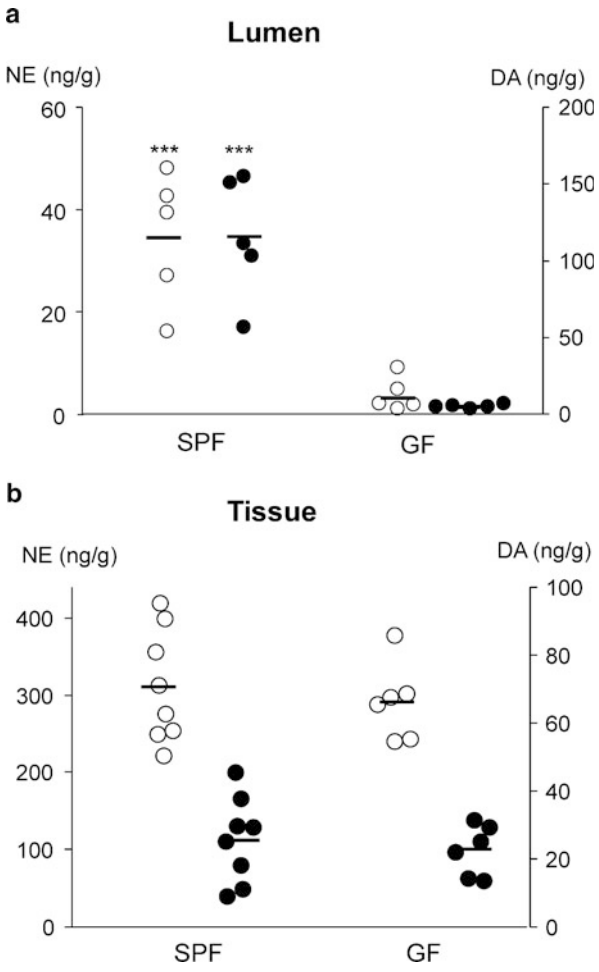

Fig. 8.6 Cecal free CA levels in the lumen and tissue of the SPF and GF mice. The luminal (n = 6–10, **panel A**) and tissue (n = 6–8, **panel B**) free CA levels in the SPF and GF mice are shown. The luminal NE levels (*left vertical axis*) in the SPF and GF mice were 35 ± 5*** ng/g and 3.8 ± 1.3 ng/g, respectively, and the luminal DA levels (*right vertical axis*) in the SPF and GF mice were 115 ± 14*** ng/g and 5.0 ± 0.5 ng/g, respectively. ***$P < 0.001$ was significantly higher than the corresponding GF value

similarity with mammalian tyrosine hydroxylase, a rate-limiting enzyme, are found in some species of bacteria [44, 45]. In our study, no significant differences were observed in the total DA levels (free + conjugated types) of the cecal content between the GF and SPF mice; however, the total NE levels of the cecal and colonic content were substantially higher in the SPF mice than in the GF mice (n = 5 per each group, cecum, GF 7.4 ± 1.7, SPF 36.4 ± 5.6***; colon, GF 6.4 ± 1.3, SPF 62.6 ± 6.7***; ***$P < 0.001$ vs. GF value). These results suggest that gut microbes are a likely source of gut luminal NE. In addition, gut bacteria enriched from murine feces actually contain substantial amounts of NE and a lesser amount of DA (Fig. 8.10). Therefore, it is possible to speculate that gut microbes are an important source of luminal NE. However, some species of bacteria, including *E. coli*, have a functional transporter for CA, such as the bacterial neurotransmitter sodium symporter family member, Leu T [46]. Therefore, there is thus far

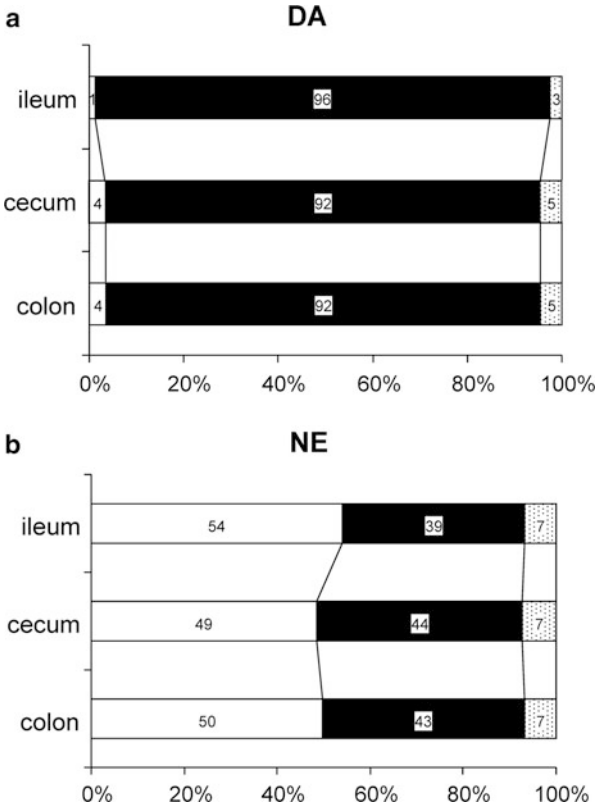

Fig. 8.7 Free and glucuronide- and sulfate-conjugated CA in the gut lumen in the GF mice. The luminal glucuronide- and sulfate-conjugated DA (**panel A**) and NE (**panel B**) levels in the ileum, cecum and colon are shown. The mean value of each form of CA is expressed as the percentage of the total (free CA + conjugated CA). The *open*, *closed* and *dotted bars* indicate free, glucuronide-conjugated and sulfate-conjugated CA, respectively

insufficient evidence to determine whether the NE and DA found in gut microbes originate from bacterial production by tyrosine hydroxylase-like enzyme or if they are obtained from the gut lumen via a Leu T-like transporter.

CA Receptors on Gut Epithelial Cells and Their Functions

DA 1A receptors are identified in the cells at the base of the intestinal crypts of the rat small intestine [47]. Alpha 2-adrenergic receptors are also reported to be present on gut epithelial cells [48]. These findings suggest the physiological importance of luminal CA. In fact, the luminal administration of DA stimulates active ileal ion absorption via α2-adrenergic or dopaminergic receptor activation, demonstrating the role of luminal DA as a proabsorptive modulator of ion and water transport [49, 50]. These results were also confirmed by our recent findings using an in vivo colon loop model in which the injection of ten micromoles of DA into the loop was found to induce a 30 % increase in water absorption out of the gut lumen in comparison to the injection of vehicle without DA (vehicle 55 ± 5 μl/30 min/cm, DA 72 ± 4* μl/30 min/cm; $*P < 0.05$).

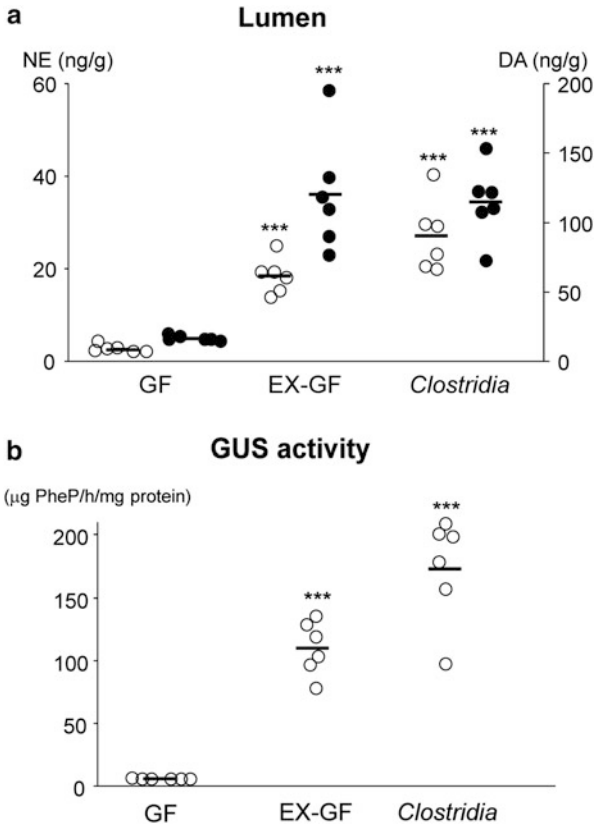

Fig. 8.8 Luminal free CA levels and the GUS activity in the cecum in the gnotobiotic mice. **Panel A**: The cecal luminal contents obtained from EX-GF (n = 6) and *Clostridia* (n = 6)-associated mice were processed for free NE and DA measurement. ***$P < 0.001$ was significantly higher than the corresponding GF value. **Panel B**: The cecal luminal contents of GF, EX-GF and *Clostridia*-associated mice were subjected to measurement of the GUS activity. ***$P < 0.001$ was significantly higher than the corresponding GF value

It is conceivable that luminal CA are involved not only in proabsorptive functions and increases in bacterial pathogenicity, but also a variety of physiological and pathological functions, such as gut motility [51] and modulation of immune reactions [52, 53]. Clarifying such unidentified functions will further support the notion that CA are important molecules mediating between gut microorganisms and the host under pathophysiological conditions.

GABA as an Interkingdom Signal in the Gut

GABA is a four carbon, nonprotein amino acid conserved from bacteria to vertebrates. Organisms have the ability to synthesize GABA from glutamate in a reaction catalyzed by the cytosolic enzyme L-glutamic acid decarboxylase (GAD). Several researchers have found that some bacteria, including *E. coli* and members of the *Lactobacillus* genus, possess a GAD activity, allowing them to

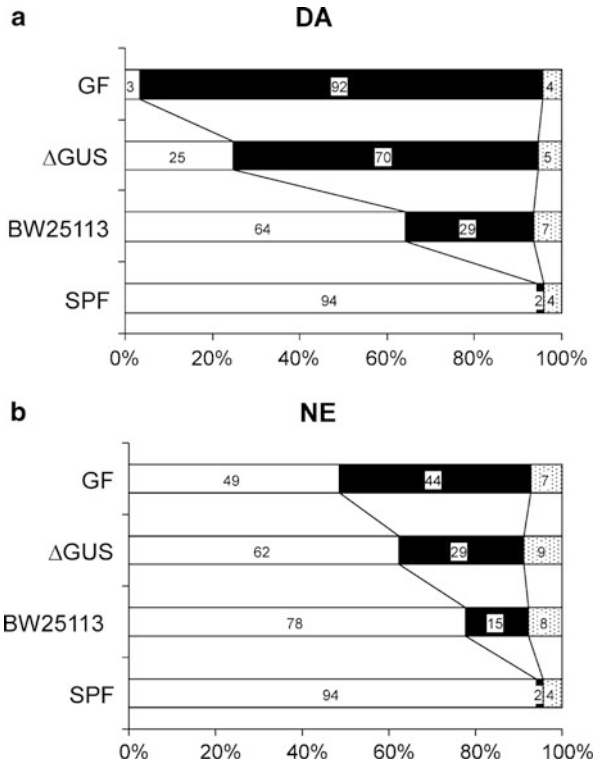

Fig. 8.9 Free and glucuronide- and sulfate-conjugated CA in the cecal lumen in the mice colonized with the *E. coli* mutant strain JW1609 or its parent strain BW25113. The cecal contents were subjected to measurement of the free and glucuronide- and sulfate-conjugated NE (**panel A**) and DA (**panel B**) levels 4 weeks after exposure to *E. coli* mutant strain devoid of GUS (n = 5, JW1609: ΔGUS) or its parent strain (n = 5, BW25113). The mean value of each form of CA is expressed as the percentage of the total (free CA + conjugated CA). The *open*, *closed* and *dotted bars* indicate free, glucuronide-conjugated and sulfate-conjugated CA, respectively

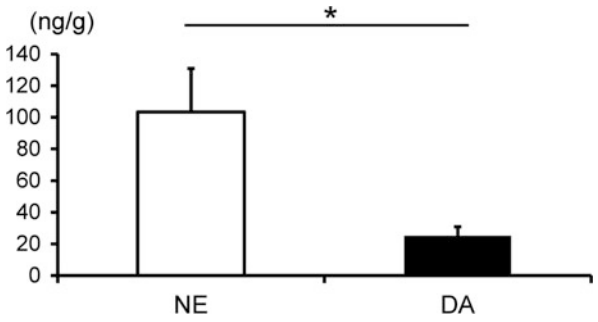

Fig. 8.10 Bacteria-rich fractions enriched from the cecal contents contain NE and DA. Bacteria-rich fractions were enriched from the cecal contents of the SPF mice according to a previously reported method [68]. The enriched fractions were thoroughly disrupted using repeated sonication then processed for CA measurement. A microscopic test revealed that the enriched bacteria had no contaminants, such as epithelia or debris. The NE and DA levels (mean ± standard error) were 103 ± 27 and 25 ± 6 ng/g, respectively (n = 6). $*P < 0.05$ indicates a significant difference between the NE and DA values

convert glutamic acid to GABA [54–56]. The production of GABA by bacteria appears to naturally occur under physiological conditions, as a recent study using metabolomics showed that the gut luminal GABA levels in Ex-GF mice are considerably higher than those observed in GF mice [57]. It is well known that plant-derived GABA mediates communication between organisms belonging to different kingdoms [58]; therefore, the GABA locally produced by the resident microbiota may play an important role as an interkingdom signal in the gut. In fact, a recent publication by Li and colleagues [59] showed that gut epithelial cells express several types of GABA receptors, including β2/3- and π-subunits, on their surface. The authors also demonstrated that endogenous autocrine GABAergic signaling in the mammalian intestinal epithelium upregulates intestinal fluid secretion and becomes intensified in mice with allergic diarrhea. To date, there is no direct evidence demonstrating that gut luminal GABA is actually involved in signaling from the gut to the brain. However, neural and/or humoral interactions between the intestinal GABA system and the brain GABA system comprise a fascinating research theme, as the JB-1 strain of *Lactobacillus rhamnosus* reduces stress-induced anxiety- and depression-related behavior, accompanied by an altered GABAAα2 receptor mRNA expression in the brain [32].

Other Hormones in Microbial Cells

Hormones and hormone-binding proteins with homology to those of vertebrates are reported to be present in fungi, yeast and bacteria [60, 61]. In particular, insulin and insulin-like materials contained in microbes have been the most extensively studied [62–64]. Corticotropin [65] and somatostatin [66] have also been identified in a unicellular organism (*Tetrahymena pyriformis*) and *Bacillus subtilis*, respectively. In this regard, Iyer and colleagues [67] proposed the interesting theory that the evolutionary history of prokaryotic genes encoding many of the enzymes involved in the synthetic and metabolic pathways of CA, histamine, acetylcholine and GABA is best described by scenarios that include late horizontal gene transfer from bacteria. This concept is substantiated by the growing body of evidence showing that bacteria produce small molecules that are formally involved in bacteria–bacteria communication and have now become involved in bacteria-host communication.

While we emphasize the role of CA or GABA in this context, this is but one of many examples of the consequences of the bacterial synthesis of neuroactive molecules that remain to be explored.

Conclusion and Perspectives

We are living in a bacterial world. Bacterial signaling helps us maintain homeostasis, keeping us healthy and happy. Given that the gut microbiome plays a crucial role in the development of the HPA axis and behaviors, gut microbes may play a critical role against the development of stress-related disorders, such as anxiety and depression, by providing the host with the "stress resilience" necessary to adapt to a changing external environment.

Clearly, further studies are called for; however, the recent findings described herein provide strong evidence in this rapidly developing field of research. We foresee a day when a comprehensive view regarding the interactions and pathways involved in the "microbiota-gut-brain axis" will be unraveled.

References

1. Frank DN, Pace NR (2008) Gastrointestinal microbiology enters the metagenomics era. Curr Opin Gastroenterol 24(1):4–10
2. Kurokawa K, Itoh T, Kuwahara T, Oshima K, Toh H, Toyoda A et al (2007) Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. DNA Res 14(4):169–181
3. Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, Koga Y (1997) The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. J Immunol 159(4):1739–1745
4. Finlay BB (1990) Cell adhesion and invasion mechanisms in microbial pathogenesis. Curr Opin Cell Biol 2(5):815–820
5. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN et al (2004) Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol 558(1):263–275
6. Backhed F, Manchester JK, Semenkovich CF, Gordon JI (2007) Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A 104(3):979–984
7. Heijtza RD, Wang S, Anuar F, Qian Y, Bjorkholm B, Samuelsson A et al (2011) Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A 108 (7):3047–3052
8. Neufeld KM, Kang N, Bienenstock J, Foster JA (2011) Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil 23(3):255–264
9. Gareau MG, Wine E, Rodrigues DM, Cho JH, Whary MT, Philpott DJ et al (2011) Bacterial infection causes stress-induced memory dysfunction in mice. Gut 60(3):307–317
10. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F et al (2013) The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. Mol Psychiatry 18(6):666–673
11. Nishino R, Mikami K, Takahashi H, Tomonaga S, Furuse M, Hiramoto T et al (2013) Commensal microbiota modulate murine behaviors in a strictly contamination-free environment confirmed by culture-based methods. Neurogastroenterol Motil 25(6):521–528
12. Lyte M, Ernst S (1992) Catecholamine induced growth of gram-negative bacteria. Life Sci 50 (3):203–212

13. Lyte M, Ernst S (1993) Alpha-adrenergic and beta-adrenergic-receptor involvement in catecholamine-induced growth of gram-negative bacteria. Biochem Biophys Res Commun 190(2):447–452
14. Lyte M, Frank C, Green B (1996) Production of an autoinducer of growth by norepinephrine cultured *Escherichia coli* O157:H7. FEMS Microbiol Lett 139(2–3):155–159
15. Lyte M (2004) Microbial endocrinology and infectious disease in the 21st century. Trends Microbiol 12(1):14–20
16. Clarke MB, Hughes DT, Zhu C, Boedeker EC, Sperandio V (2006) The QseC sensor kinase: a bacterial adrenergic receptor. Proc Natl Acad Sci U S A 103(27):10420–10425
17. Hughes DT, Sperandio V (2008) Inter-kingdom signalling: communication between bacteria and their hosts. Nat Rev Microbiol 6(2):111–120
18. Pacheco AR, Sperandio V (2009) Inter-kingdom signaling: chemical language between bacteria and host. Curr Opin Microbiol 12(2):192–198
19. Rhee SH, Pothoulakis C, Mayer EA (2009) Principles and clinical implications of the brain-gut-enteric microbiota axis. Nat Rev Gastroenterol Hepatol 6(5):306–314
20. Chrousos G, Gold P (1992) The concepts of stress and stress system disorders – overview of physical and behavioral homeostasis. JAMA 267(9):1244–1252
21. Meaney MJ, Aitken DH, van Berkel C, Bhatnagar S, Sapolsky RM (1988) Effect of neonatal handling on age-related impairments associated with the hippocampus. Science 239(4841 Pt 1):766–768
22. Schmidt M, Oitzl MS, Levine S, De Kloet ER (2002) The HPA system during the postnatal development of CD1 mice and the effects of maternal deprivation. Brain Res Dev Brain Res 139(1):39–49
23. Kenny B, DeVinney R, Stein M, Reinscheid DJ, Frey EA, Finlay BB (1997) Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell 91 (4):511–520
24. Bonanno G (2004) Loss, trauma, and human resilience – have we underestimated the human capacity to thrive after extremely aversive events? Am Psychol 59(1):20–28
25. McEwen BS (2007) Physiology and neurobiology of stress and adaptation: central role of the brain. Physiol Rev 87(3):873–904
26. McEwen BS, Stellar E (1993) Stress and the individual. Mechanisms leading to disease. Arch Intern Med 153(18):2093–2101
27. Sapolsky RM (2000) Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Arch Gen Psychiatry 57(10):925–935
28. Southwick SM, Vythilingam M, Charney DS (2005) The psychobiology of depression and resilience to stress: implications for prevention and treatment. Annu Rev Clin Psychol 1:255–291
29. Huhman KL (2006) Social conflict models: can they inform us about human psychopathology? Horm Behav 50(4):640–646
30. Lehmann ML, Herkenham M (2011) Environmental enrichment confers stress resiliency to social defeat through an infralimbic cortex-dependent neuroanatomical pathway. J Neurosci 31(16):6159–6173
31. Selye H (1956) The stress of life. McGraw-Hill, New York
32. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG et al (2011) Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 108(38):16050–16055
33. Eisenhofer G, Kopin IJ, Goldstein DS (2004) Catecholamine metabolism: a contemporary view with implications for physiology and medicine. Pharmacol Rev 56(3):331–349
34. Asano Y, Hiramoto T, Nishino R, Aiba Y, Kimura T, Yoshihara K et al (2012) Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. Am J Physiol Gastrointest Liver Physiol 303(11):G1288–G1295
35. Yoneda S, Alexander N, Vlachakis ND (1983) Enzymatic deconjugation of catecholamines in human and rat plasma and red blood cell lysate. Life Sci 33(10):935–942

36. Gaudin C, Ruget G, Selz F, Cuche JL (1985) Free and conjugated catecholamines in digestive tissues of rats. Life Sci 37(16):1469–1474
37. Kim DH, Jin YH, Jung EA, Han MJ, Kobashi K (1995) Purification and characterization of beta-glucuronidase from *Escherichia coli* HGU-3, a human intestinal bacterium. Biol Pharm Bull 18(9):1184–1188
38. Rod T, Midtvedt T (1977) Origin of intestinal beta-glucuronidase in germfree, mono-contaminated and conventional rats. Acta Pathol Microbiol Immunol Scand B 85(4):271–276
39. Shiga A, Sasaki T, Horii N (1987) Correlations among pH and Mg, Ca, P, Na, K, Cl- and HCO3- contents of digesta in the gastro-intestinal tract of rats. Nihon Juigaku Zasshi 49 (6):973–979
40. Ward FW, Coates ME (1987) Gastrointestinal pH measurement in rats: influence of the microbial flora, diet and fasting. Lab Anim 21(3):216–222
41. Ilett KF, Tee LB, Reeves PT, Minchin RF (1990) Metabolism of drugs and other xenobiotics in the gut lumen and wall. Pharmacol Ther 46(1):67–93
42. Tsavkelova EA, Botvinko IV, Kudrin VS, Oleskin AV (2000) Detection of neurotransmitter amines in microorganisms with the use of high-performance liquid chromatography. Dokl Biochem 372(1–6):115–117
43. Roshchina VV (2010) Evolutionary considerations of neurotransmitters in microbial, plant, and animal cells. In Lyte M, Freestone PP (eds) Microbial Endocrinology. Springer, New York, pp 17–52
44. Solano F, Garcia E, Perez D, Sanchez-Amat A (1997) Isolation and characterization of strain MMB-1 (CECT 4803), a Novel Melanogenic Marine Bacterium. Appl Environ Microbiol 63 (9):3499–3506
45. Hernandez-Romero D, Sanchez-Amat A, Solano F (2006) A tyrosinase with an abnormally high tyrosine hydroxylase/dopa oxidase ratio. FEBS J 273(2):257–270
46. Singh SK, Yamashita A, Gouaux E (2007) Antidepressant binding site in a bacterial homologue of neurotransmitter transporters. Nature 448(7156):952–956
47. Vaughan C, Aherne A, Lane E, Power O, Carey R, O'Connell D (2000) Identification and regional distribution of the dopamine D-1A receptor in the gastrointestinal tract. Am J Physiol Regul Integr Comp Physiol 279(2):R599–R609
48. Lam R, App E, Nahirney D, Szkotak A, Vieira-Coelho M, King M et al (2003) Regulation of Cl- secretion by alpha-adrenergic receptors in mouse colonic epithelium. J Physiol 548 (2):475–484
49. Barry MK, Aloisi JD, Pickering SP, Yeo CJ (1994) Luminal adrenergic agents modulate ileal transport: discrimination between alpha 1 and alpha 2 receptors. Am J Surg 167(1):156–162
50. Barry MK, Maher MM, Gontarek JD, Jimenez RE, Yeo CJ (1995) Luminal dopamine modulates canine ileal water and electrolyte transport. Dig Dis Sci 40(8):1738–1743
51. Strandberg K, Sedvall G, Midtvedt T, Gustafsson B (1966) Effect of some biologically active amines on the cecum wall of germfree rats. Proc Soc Exp Biol Med 121(3):699–702
52. Nakano K, Yamaoka K, Hanami K, Saito K, Sasaguri Y, Yanagihara N et al (2011) Dopamine induces IL-6-dependent IL-17 production via D1-like receptor on CD4 naive T cells and D1-like receptor antagonist SCH-23390 inhibits cartilage destruction in a human rheumatoid arthritis/SCID mouse chimera model. J Immunol 186(6):3745–3752
53. Sarkar C, Basu B, Chakroborty D, Dasgupta PS, Basu S (2010) The immunoregulatory role of dopamine: an update. Brain Behav Immun 24(4):525–528
54. Smith DK, Kassam T, Singh B, Elliott JF (1992) *Escherichia coli* has two homologous glutamate decarboxylase genes that map to distinct loci. J Bacteriol 174(18):5820–5826
55. Nomura M, Kimoto H, Someya Y, Furukawa S, Suzuki I (1998) Production of gamma-aminobutyric acid by cheese starters during cheese ripening. J Dairy Sci 81(6):1486–1491
56. Yokoyama S, Hiramatsu J, Hayakawa K (2002) Production of gamma-aminobutyric acid from alcohol distillery lees by *Lactobacillus brevis* IFO-12005. J Biosci Bioeng 93(1):95–97
57. Matsumoto M, Kibe R, Ooga T, Aiba Y, Kurihara S, Sawaki E et al (2012) Impact of intestinal microbiota on intestinal luminal metabolome. Sci Rep 2:233

58. Shelp BJ, Bown AW, Faure D (2006) Extracellular gamma-aminobutyrate mediates communication between plants and other organisms. Plant Physiol 142(4):1350–1352
59. Li Y, Xiang YY, Lu WY, Liu C, Li J (2012) A novel role of intestine epithelial GABAergic signaling in regulating intestinal fluid secretion. Am J Physiol Gastrointest Liver Physiol 303 (4):G453–G460
60. Lyte M (2010) The microbial organ in the gut as a driver of homeostasis and disease. Med Hypotheses 74(4):634–638
61. Lenard J (1992) Mammalian hormones in microbial cells. Trends Biochem Sci 17(4):147–150
62. LeRoith D, Shiloach J, Roth J, Lesniak MA (1981) Insulin or a closely related molecule is native to *Escherichia coli*. J Biol Chem 256(13):6533–6536
63. LeRoith D, Shiloach J, Heffron R, Rubinovitz C, Tanenbaum R, Roth J (1985) Insulin-related material in microbes: similarities and differences from mammalian insulins. Can J Biochem Cell Biol 63(8):839–849
64. Le Roith D, Shiloach J, Roth J, Lesniak MA (1980) Evolutionary origins of vertebrate hormones: substances similar to mammalian insulins are native to unicellular eukaryotes. Proc Natl Acad Sci U S A 77(10):6184–6188
65. Leroith D, Liotta AS, Roth J, Shiloach J, Lewis ME, Pert CB et al (1982) Corticotropin and beta-endorphin-like materials are native to unicellular organisms. Proc Natl Acad Sci U S A 79 (6):2086–2090
66. LeRoith D, Pickens W, Vinik AI, Shiloach J (1985) *Bacillus subtilis* contains multiple forms of somatostatin-like material. Biochem Biophys Res Commun 127(3):713–719
67. Iyer LM, Aravind L, Coon SL, Klein DC, Koonin EV (2004) Evolution of cell-cell signaling in animals: did late horizontal gene transfer from bacteria have a role? Trends Genet 20(7):292–299
68. Takahashi T, Sakaguchi E (2006) Transport of bacteria across and along the large intestinal lumen of guinea pigs. J Comp Physiol B 176(2):173–178

Chapter 9
Neuropeptides and the Microbiota-Gut-Brain Axis

Peter Holzer and Aitak Farzi

Abstract Neuropeptides are important mediators both within the nervous system and between neurons and other cell types. Neuropeptides such as substance P, calcitonin gene-related peptide and neuropeptide Y (NPY), vasoactive intestinal polypeptide, somatostatin and corticotropin-releasing factor are also likely to play a role in the bidirectional gut-brain communication. In this capacity they may influence the activity of the gastrointestinal microbiota and its interaction with the gut-brain axis. Current efforts in elucidating the implication of neuropeptides in the microbiota-gut-brain axis address four information carriers from the gut to the brain (vagal and spinal afferent neurons; immune mediators such as cytokines; gut hormones; gut microbiota-derived signalling molecules) and four information carriers from the central nervous system to the gut (sympathetic efferent neurons; parasympathetic efferent neurons; neuroendocrine factors involving the adrenal medulla; neuroendocrine factors involving the adrenal cortex). Apart from operating as neurotransmitters, many biologically active peptides also function as gut hormones. Given that neuropeptides and gut hormones target the same cell membrane receptors (typically G protein-coupled receptors), the two messenger roles often converge in the same or similar biological implications. This is exemplified by NPY and peptide YY (PYY), two members of the PP-fold peptide family. While PYY is almost exclusively expressed by enteroendocrine cells, NPY is found at all levels of the gut-brain and brain-gut axis. The function of PYY-releasing enteroendocrine cells is directly influenced by short chain fatty acids generated by the intestinal microbiota from indigestible fibre, while NPY may control the impact of the gut microbiota on inflammatory processes, pain, brain function and behaviour. Although the impact of neuropeptides on the interaction between the gut microbiota and brain awaits to be analysed, biologically active peptides are likely to

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_9,

emerge as neural and endocrine messengers in orchestrating the microbiota-gut-brain axis in health and disease.

Abbreviations

5-HT	5-Hydroxytryptamine
AgRP	Agouti-related protein
APUD	Amine precursor uptake and decarboxylation
BDNF	Brain-derived neurotrophic factor
CRF	Corticotropin-releasing factor
GABA	Gamma-aminobutyric acid
LPS	Lipopolysaccharide
MAMP	Microbe-associated molecular pattern
NPY	Neuropeptide Y
NR2A	NMDA receptor subunit 2A
NTS	Nucleus tractus solitarii
PYY	Peptide YY
TLR4	Toll-like receptor 4

Neuropeptides at the Forefront of the Brain-Gut Axis

Biologically active peptides have been instrumental in the formulation of the concept that brain and gut have much in common. When in the 1960s and 1970s several peptides were discovered to occur both in the brain and gastrointestinal tract, the term "gut-brain axis" was first coined, based on the prevailing concept that the brain would be essential for controlling gut function. The way to this concept was pathed by the so-called APUD (amine precursor uptake and decarboxylation) hypothesis which, owing to common histochemical characteristics, held that amine- and peptide-producing cells of the nervous system, the gut and other organs derive from a common origin in the neural crest [1, 2]. While certain cells of the thyroid, adrenal medulla, carotid bodies and autonomic as well as enteric ganglia originate in fact from the neural crest, the peptide-secreting endocrine cells of the gut do not [2]. Although the APUD hypothesis has not stood the test of time, it was an important contribution to the current understanding of the coordinating function of neuropeptides in many organ systems. We now know that a vast number of neuropeptides is produced by central and peripheral neurons alongside with endocrine cells in the gastrointestinal tract and other endocrinologically active organs [2–4]. Biologically active peptides, particularly neuropeptides, play many diverse roles in the bidirectional data highway between the gut and brain and offer unforeseen opportunities for drug development. At the same time, the multiplicity of messengers (including neuropeptides) also represents a challenge in understanding the complex interactions between gut and brain. Although their precise role in

the microbiota-gut-brain axis has not yet been defined, neuropeptides such as substance P, calcitonin gene-related peptide, neuropeptide Y (NPY), vasoactive intestinal polypeptide, somatostatin and corticotropin-releasing factor (CRF) are candidates to play an important role in this respect.

The Gut-Brain Axis Involves Microbial, Immune, Endocrine and Neural Signalling Pathways: Neuropeptides May Be Involved in Each Pathway

The term "gut-brain axis" refers to the bidirectional communication between the gut and the brain (Fig. 9.1). Apart from the autonomic regulation of digestion by the central, parasympathetic, sympathetic and enteric nervous systems as well as by neuroendocrine factors (derived from the adrenal medulla and cortex), there is ongoing communication from the gut to the brain in health and disease [5, 6]. Thus, visceral information is continuously fed into subcortical regions of the brain including the limbic system and the autonomic and neuroendocrine centres [5]. This information is integrated with other interoceptive information from the body and with contextual information from the environment [5]. Under pathological conditions, the interoceptive input from the periphery may reach the level of consciousness and give rise to the sensation of nausea, discomfort and/or pain [6]. In addition, the brain's output to the gut via autonomic and neuroendocrine pathways may result in gastrointestinal dysfunction. The afferent part of this gut-brain-gut axis has recently been in the focus of investigation in order to understand why gastrointestinal disease such as inflammatory bowel disease and irritable bowel syndrome is associated with pain and a number of psychiatric disturbances including anxiety, neuroticism and depression.

The gut-brain axis uses four major information carriers for the communication between the gut and the brain (Fig. 9.1):

- neural messages carried by vagal and spinal afferent neurons,
- immune messages carried by cytokines,
- endocrine messages carried by gut hormones and
- microbial factors that may directly reach the brain via the blood stream but can also interact with the other three transmission pathways [6–8].

These communication systems are abundantly present in the gastrointestinal tract and, in an evolutionary perspective, are relevant for a number of vital functions:

- The brain with its sensory systems needs to interact with the gut in finding appropriate food and assimilating it for the sake of metabolic survival.
- The gut needs to distinguish between useful and useless as well as dangerous (antigenic, pathogenic, toxic) ingredients of food and sort them accordingly.

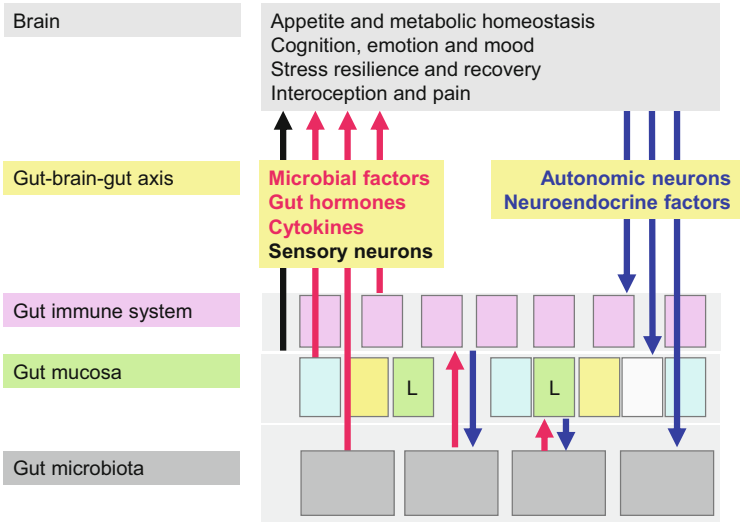

Fig. 9.1 The bidirectional microbiota-gut-brain axis. Four communication pathways (microbial factors, gut hormones, cytokines, sensory neurons) signal from the gut to the brain where they can modify cerebral function and behaviour. Two pathways (autonomic and neuroendocrine outputs) signal from the brain to the gut. *L* denotes endocrine L cells in the intestinal mucosa

- The gut needs to maintain homeostasis with the extensive community of microbes in the intestine, which are important in supporting nutrition, educating the immune system and communicating with other organ systems including the brain.

Each of the communication pathways between the gastrointestinal and central nervous system may involve neuropeptides and structurally related signalling molecules. Ever since their gradual discovery, biologically active peptides have been intimately related to the regulation of digestion and to the communication with the central nervous system. Regulation of food intake (appetite), metabolic homeostasis and pain have been areas that were addressed in particular detail. Neuropeptides comprise a class of evolutionarily well conserved molecules that, by definition, operate as transmitters in the enteric, peripheral and central nervous systems and share transduction mechanisms with other biologically active peptides such as gut hormones. Apart from their origin, it is frequently difficult to distinguish between their function as neuropeptides or gut hormones because they operate often via the same receptors and cellular transduction systems. Thus, neurons as well as endocrine, immune, interstitial, muscle, epithelial and microbial cells can respond to these signalling molecules by expressing the appropriate peptide receptors. The microbiota residing in the mucosa [9] is in immediate vicinity to the endocrine cells of the gastrointestal mucosa which produce more than 20 different gut hormones [10]. Apart from immune mediators, gut hormones may thus play an important role as communicators between the gut microbiota and host functions. Gut hormone

signalling to the brain not only occurs by an endocrine route but may also involve activation of primary afferent neurons, especially in the vagus nerve (e.g., cholecystokinin and ghrelin). Furthermore, it is important to realize that the four communication pathways between the gut and the brain do not operate in isolation but are closely interrelated with each other.

Direct Brain Communication Pathways Used by the Gut Microbiota

With the emerging role of the microbiota a new gut-brain pathway has come to light. Thus, the gut microbiota communicate not only with gastrointestinal epithelial, immune and nerve cells in their immediate neighbourhood but also generate and release molecules that can signal to distant organs. This is true for molecules designated as pathogen-associated molecular patterns or, in a more benevolent vein, microbe-associated molecular patterns (MAMPs) as well as for many other microbial metabolites. There are experimental data to show that a significant part of the metabolites circulating in mammalian blood are derived from the intestinal microbial community [11–15]. Importantly, the presence or absence of the gut microbiota also influences the profile of metabolites (including peptides) present in the brain [16].

While the potential effects of the microbial metabolites on the host are still little understood, it is obvious that they could convey messages around the whole body. Some information in this respect can be derived from the actions of two MAMPs, lipopolysaccharide (LPS) and peptidoglycan components such as mesodiaminopimelic acid. These MAMPs are recognized by pattern recognition receptors of the innate immune system: LPS activates toll-like receptor 4 (TLR4) while the peptidoglycan structures stimulate nucleotide-binding oligomerization domain–containing protein-1 (Nod1) and/or Nod2. Importantly, translocation of peptidoglycan from the gut to the blood impacts on neutrophils in the bone marrow and primes their capacity to defend the body against bacterial infection via stimulating Nod1 [17].

In a similar manner, LPS translocated from the gut through a leaky mucosal barrier carries a microbial message to distant organs including the brain. The behavioural responses to systemic exposure of excess LPS are well characterized in animals and humans and comprise acute sickness [18, 19] and delayed depression-like behaviour [20–24]. LPS originating from the gut microbiota may give rise to alterations in brain function via three different pathways. Following translocation across the intestinal mucosa it may, on the one hand, stimulate the intestinal immune system to produce cytokines which (1) can signal directly to the brain or (2) sensitize/stimulate vagal and spinal afferent neurons [18, 19, 25, 26]. On the other hand, (3) the circulation may carry LPS itself to the central nervous system where it may modify brain function.

The latter possibility need be envisaged because—apart from the innate immune system—there is a widespread expression of TLR4 and other TLRs at several levels of the gut-brain axis. Thus, TLRs are present on gastrointestinal epithelial cells [27, 28], neurons of the enteric nervous system [29, 30], primary afferent neurons [29] and various cell types (neurons, microglial cells and astrocytes) in the brain [31–33]. By stimulating TLR4 and TLRs in the brain, LPS and other bacterial factors can stimulate the generation and release of proinflammatory cytokines and in this way give rise to neuroinflammatory processes. These effects are not only relevant to neurodegeneration and repair [31–33] but may also be involved in the manifestation of psychiatric disorders. Specifically, increased levels of IgA and IgM against LPS of commensal gut bacteria are found in the circulation of patients with depression or chronic fatigue syndrome, and the hypothesis has been put forward that increased translocation of LPS across a leaky gut may be a factor that contributes to these pathologies [34, 35]. Taken all findings together, it would appear, therefore, that the physiological roles of the symbiotic gut microbiota relate not only to the regulation of digestion at the gastrointestinal level but also extend to systemic immunity and brain function.

Neuroactive Factors Released by the Gut Microbiota

There is increasing evidence that the gut microbiota sheds not only ligands for pattern recognition receptors, but also releases factors that target specific neuronal systems involved in the gut-brain axis. Although it remains to be established whether the microbiota can produce neuropeptide-like compounds, they are capable of generating a number of neurotransmitters and neuromodulators [7, 14]. Members of the genera *Candida*, *Streptococcus*, *Escherichia* and *Enterococcus* synthesize 5-hydroxytryptamine (5-HT), members of the genera *Escherichia*, *Bacillus* and *Saccharomyces* generate dopamine and/or noradrenaline, members of the genus *Lactobacillus* produce acetylcholine, and members of the genera *Lactobacillus* and *Bifidobacterium* manufacture gamma-aminobutyric acid (GABA) [7, 14, 36–39]. The release of microbiota-derived dopamine into the lumen of the intestine has been suggested to play a proabsorptive role in the colon [38]. Signalling via opioid and cannabinoid receptors may also be modified by the gut microbiota, a conclusion based on the ability of certain probiotics to alter the expression of opioid and cannabinoid receptors in the gut [7].

Moreover, the microbiota in the intestine is able to produce metabolites with benzodiazepine-like structures and effects [40–42]. Specifically, benzodiazepine receptor ligands originating from the gut microbiota have been proposed to contribute to the encephalopathy associated with fulminant hepatic failure [40]. Under these conditions, benzodiazepine-like molecules are likely to reach the brain at increased concentrations that will enhance neurotransmission via $GABA_A$ receptors and thus contribute to the disease process [40]. The pyrrolobenzodiazepines (e.g., anthramycin) synthesized by a number of gut microbes display not only

benzodiazepine-like but also antibiotic and antineoplastic activities and may thus influence the biology of the microbiota and host alike in many respects. In addition, this circumstance indicates that the gut microbiota is a rich source of yet-to-be-identified compounds with therapeutic potential.

Apart from producing and releasing neuroactive factors, the microbiota modifies the levels of metabolites that are relevant to the synthesis of transmitters in the nervous system. For instance, the concentrations of tryptophan (the precursor of 5-HT), tyrosine (the precursor of dopamine and noradrenaline) and glutamine in the total brain of germ-free mice are lower than in mice that have been re-colonized by the gut microbiota [16]. In the hippocampus of germ-free mice, however, the concentrations of 5-HT and its main metabolite 5-hydroxyindoleacetic acid are higher than in conventionally colonized mice [43]. Colonization of the germ-free animals restores peripheral tryptophan levels to control values but fails to reverse the changes in hippocampal 5-HT levels [43]. The concentrations of tryptophan, 5-HT and tyrosine in the blood plasma are likewise increased in germ-free animals [11, 43], the elevation of tryptophan being likely due to the absence of bacterial tryptophanase [11]. Another explanation could be that the gut microbiota re-directs the metabolism pathways of tryptophan which lead either to the production of 5-HT or kynurenine [7].

Interaction of the Gut Microbiota with Gut Peptides

Due to their spatial vicinity with the gastrointestinal mucosa, the gut microbiota is in a prime position to interact with the epithelial cells and to modify their activity. Among these cells, enteroendocrine cells are poised to govern the activity of cells in and outside the digestive system and in this way also to convey messages from the microbial community in the gut. The enteroendocrine L cells in the distal ileum and colon represent a distinct example of this interactive relationship. These cells are stimulated by particular nutrients and digestive products, which leads to the release of PYY, glucagon-like peptide-1 (GLP-1) and GLP-2 [6, 44, 45]. L cells are also stimulated by short chain fatty acids (e.g., acetate, butyrate, propionate), which particular microbes generate by fermentation of otherwise indigestible carbohydrate fibres. Short chain fatty acids stimulate L cells via activating G protein-coupled receptors such as Gpr41 [6, 44, 45]. The important role of this microbiota-host interaction is underscored by the finding that colonization of the mouse colon with a fermentative human microbial community increases the plasma level of PYY, an effect that is blunted by knockout of Gpr41 [45]. Gpr41 deficiency is associated with a reduced expression of PYY, an increase in intestinal transit rate and an attenuation of energy harvest [45].

Following their release from L cells, PYY and GLP-1 not only inhibit gastric motility and improve glucose homeostasis but also induce satiety and behavioural changes. Thus, butyrate is able to ameliorate aging-related memory decline in rats [46] but has inconsistent effects on anxiety and depression-like behaviour

[47]. Propionate has been shown to evoke autism spectrum disorder-related behaviours in rodents [48, 49].

The interaction between the gut microbiota and intestinal L cells can be modulated by the use of prebiotics (fermentable carbohydrates). Prebiotic supplementation in humans increases the plasma concentrations of GLP-1 and PYY, which is associated with satiety and a decrease of postprandial glucose levels [50]. Experiments in obese mice show that prebiotic treatment causes a change in the composition of the gut microbiota alongside with a decrease of inflammatory tone and an enforcement of mucosal barrier function [51]. The complex interactions between gut microbiota, mucosal function and metabolic homeostasis also involve the endocannabinoid system [52] and GLP-2 which improves intestinal function [51]. These interrelationships suggest that prebiotic supplementation has therapeutic potential as "pharmaco-nutritional" approach to reversing host metabolic alterations linked to intestinal dysbiosis in obesity and diabetes [53].

Given that nutritional status, dietary factors, physical activity and age have an important influence on the composition of the gut microbial community [54, 55] it is not surprising that appetite-regulating hormones other than PYY, GLP-1 and GLP-2 will also interact with the gut microbiota in shaping appetite and metabolic status. Emerging evidence indicates that this applies to ghrelin [55, 56], cholecystokinin [56] as well as leptin [56]. In addition, germ-free mice have a smaller number of enteroendocrine cells than conventionally colonized animals [56].

Interaction of the Gut Microbiota with Brain Function and Behaviour: Emerging Neurochemical Mediators

Accumulating evidence shows that the absence or disturbance of the gut microbiota has a significant impact on brain function and behaviour. There is also some information on the molecular factors that may play an important role in this interrelationship. In a first line of research, germ-free mice have been found to exhibit a number of neurochemical and functional alterations relative to conventionally colonized animals. For instance, the expression of the NMDA receptor subunit 2A (NR2A) in the cortex and hippocampus [57] and of the NR2B unit in the central amygdala [58] is decreased in germ-free mice, as is the expression of the 5-HT receptor 1A (5HT1A) in the dentate granule layer of the hippocampus [58]. In contrast, inconsistent changes in the levels of brain-derived neurotrophic factor (BDNF), a key neurotrophin involved in neuronal growth and survival, have been reported: two studies hold that BDNF in the hippocampus, amygdala and cortex of germ-free mice is decreased [57, 59], while another study purports that the level of BDNF in the hippocampus of germ-free mice is increased [58].

At the behavioural level, germ-free animals exhibit reduced anxiety in three [43, 58, 59] but not one study [60]. This outcome is somewhat surprising, since the hypothalamic-pituitary-adrenal axis in germ-free mice appears to be hyperactive

rather than hypoactive [57]. Germ-free mice also show increased spontaneous motor activity, an observation that may be related to elevated dopamine, noradrenaline and 5-HT turnover in the striatum [59]. With regard to cognition, germ-free mice have deficits in simple non-spatial and working memory tasks [60]. It awaits to be examined whether the cognitive deficits are related to decreased synaptogenesis and a decrease in the expression of synaptic plasticity-related genes [59].

The impact of the gut microbiota on brain function has been confirmed by the impact of antibiotic-induced dysbiosis on the gut-brain axis and by the effects of selective probiotics on behaviour and brain chemistry. Disturbance of the gastrointestinal microbiota with a combination of nonabsorbable antibiotics (neomycin, bacitracin, and pimaricin) increases exploratory behaviour and enhances BDNF expression in the hippocampus [61]. Similar observations have been made with another combination of nonabsorbable antibiotics (neomycin, cefoperazone and ampicillin) which has an anxiolytic-like effect and impairs learning/memory in the object recognition test [62].

Chronic treatment of mice with the probiotic *Lactobacillus rhamnosus* JB-1 has been found to cause region-dependent alterations of $GABA_{A\alpha 2}$ and $GABA_{B1b}$ receptor mRNA in the brain, which are associated with a decrease in the stress-induced corticosterone response and a reduction of anxiety- and depression-related behaviour [63]. Importantly, these neurochemical and behavioural effects of probiotic treatment are prevented by bilateral subdiaphragmatic vagotomy. This role of vagal afferent neurons in communicating between gut bacteria and brain was confirmed by another study in which vagotomy abolished the anxiolytic effect of the probiotic *Bifidobacterium longum* NCC3001 in mice with experimentally induced colitis [64].

Interaction of the Gut Microbiota with Brain Function and Behaviour: The Direct Involvement of Neuropeptides Awaits Exploration

Neuropeptides such as substance P, calcitonin gene-related peptide and NPY are expressed at all levels of the microbiota-gut-brain axis and are likely to play an important role in the bidirectional signalling between the gut and brain. Theoretically, neuropeptide-like molecules may also be produced by certain microbes, and the gut microbiota will respond to neuropeptides and gut hormones if it expresses the relevant receptors. However, direct evidence that these neuropeptides contribute to the communication between the gut microbial community and the central nervous system is sparse. It also remains to be investigated whether alterations in the microbial community within the gut impacts on neuropeptide systems in the central nervous system.

The information available is mostly restricted to peptide level changes associated with manipulation of the intestinal microbiota. For instance, the colonic content of substance P is enhanced following antibiotic-induced dysbiosis of the intestinal microbiota [65]. The expression of neuropeptides in primary afferent neurons (e.g., substance P, calcitonin gene-related peptide) has not yet been addressed in experimental studies, although such studies appear worthwhile in view of two lines of research relating the intestinal microbiota to pain. On the one hand, the establishment of inflammatory hyperalgesia is attenuated in germ-free mice [66]. On the other hand, treatment of rodents with the probiotic *Lactobacillus reuteri* attenuates sensory neuron excitability [67] and alleviates the pain-related response to gastric distension [68]. *Lactobacillus acidophilus* also reduces experimentally evoked visceral pain, an effect that is associated with enhanced expression of opioid and cannabinoid receptors in the intestinal mucosa [69]. *L. paracasei* has been found to attenuate antibiotic-induced visceral hypersensitivity in mice [65], while *L. rhamnosus* GG has a beneficial effect in abdominal pain-related functional gastrointestinal disorders in childhood [70].

Neuropeptide Autoantibodies Under the Control of the Intestinal Microbiota

The gut microbiota is important in educating the immune system to recognize foreign antigens and to tolerate commensal microbes [71]. In this way, the gut microbes can modulate, tune and tame the host immune response [72]. Dysbiosis of the microbial community can lead to the development of autoimmunity [72, 73], and experimental findings indicate that both autoimmune encephalomyelitis [74] and autoimmune demyelination [75] involve the gut microbiota. There is also evidence that the formation of autoantibodies against neuropeptides is governed by intestinal microbes [76–78].

Specifically, IgG and IgA autoantibodies against alpha-melanocyte-stimulating hormone, NPY, PYY, agouti-related protein (AgRP), ghrelin, leptin and some other neuropeptides/peptides involved in appetite control are present in the human blood [76–78]. Numerous intestinal microbes including *Lactobacillus*, *Bacteroides*, *Helicobacter pylori*, *Escherichia coli* and *Candida* species contain proteins that have amino acid sequences identical to these appetite-regulating peptides [78]. The circulating levels of autoantibodies against alpha-melanocyte-stimulating hormone, which are increased in anorexia nervosa and bulimia nervosa, correlate with the psychobehavioural abnormalities of these eating disorders [76]. Vice versa, germ-free rats have decreased levels of circulating IgA autoantibodies against several appetite-regulating peptides, while the levels of antighrelin IgG are increased [78]. A mechanistic analysis in rats has shown that alpha-melanocyte-stimulating hormone autoantibodies are involved in the regulation of feeding and anxiety [78]. It thus appears conceivable that the gut microbiota control appetite and

emotional behaviour indirectly by inciting the formation of autoantibodies against neuropeptides/peptides involved in these processes.

Interaction of Gut Microbiota with Brain Function and Behaviour: A Potential Role for NPY

NPY is a neurotransmitter that in view of its multiple implications in brain function may play a particular role in the microbiota-gut-brain axis. This contention is based on this neuropeptide's involvement in controlling inflammatory processes, pain, emotion, mood, cognition, stress resilience, ingestion and energy homeostasis [6]. Consisting of 36 amino acids, NPY exerts its biological actions via five NPY receptor types, termed Y1, Y2, Y4, Y5 and y6 (a human pseudogene), which are coupled to pertussis toxin-sensitive $G_{i/o}$ protein transduction mechanisms [79]. Y receptors occur at all levels of the gut-brain and brain-gut axis [6, 80–84], the major systems expressing this peptide being enteric neurons, primary afferent neurons, several neuronal pathways throughout the brain and sympathetic neurons (Fig. 9.2).

Within the brain, NPY is one of the most abundant neuropeptides. In the context of the gut-brain axis it is particularly worth noting that NPY occurs in the nucleus of the solitary tract and ventrolateral medulla, periaqueductal grey and locus coeruleus, paraventricular nucleus of the thalamus, hypothalamus (arcuate nucleus, paraventricular nucleus and other regions), septum, hippocampus, amygdala, basal ganglia, nucleus accumbens and cerebral cortex [6, 80–84]. Several important pathways utilizing NPY as a neurotransmitter have been identified. These include noradrenergic neurons originating in the locus coeruleus of the brainstem and issuing both ascending and descending projections in the central nervous system, neurons expressing both NPY and AgRP originating in the arcuate nucleus of the hypothalamus, and distinct pathways operating in the limbic system [80–84]. The major receptor subtypes which NPY acts on are the Y1 and Y2 receptors, which are widely distributed in the central nervous system, while the localization of Y4 and Y5 receptors is restricted to particular regions of the brain [82–85].

The NPY system may impact on the microbiota-gut-brain axis at distinct levels [6, 81–84, 86–91]. It may

- influence the vitality of certain gut bacteria,
- modify gut functions such as motility, secretion and blood flow,
- regulate the activity of the immune system,
- protect against behavioural disturbances caused by peripheral immune challenge,
- inhibit nociceptive transmission in the spinal cord and brainstem,
- protect from the impact of stress on the brain-gut axis,
- regulate food intake and energy homeostasis, and
- play a role in the interoceptive regulation of anxiety and mood.

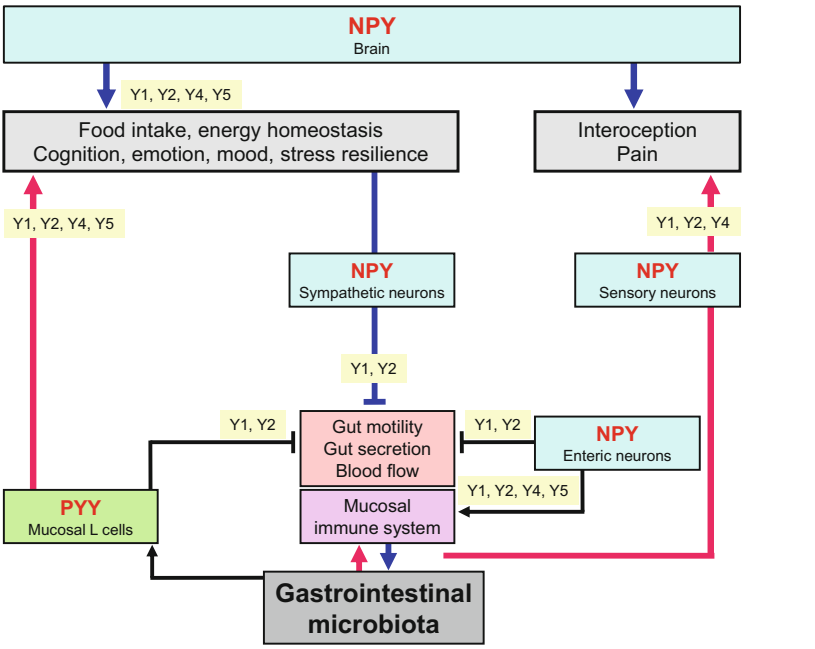

Fig. 9.2 The NPY/Y receptor system in the microbiota-gut-brain axis. The graph shows the major sources of NPY and PYY along the gut-brain axis and the Y receptor subtypes which mediate the effects of these peptides at the different levels of the gut-brain axis. The symbol ⊣ denotes inhibition

Effect of NPY on Gut Bacteria

There is some evidence that the NPY system has an impact on the composition and function of the gut microbiota and its relevance to the gut-brain axis. Similarly to substance P, calcitonin gene-related peptide and vasoactive intestinal polypeptide, NPY has been found to exhibit a direct antimicrobial effect against various gut bacteria including *E. coli*, *Enterococcus faecalis*, and *L. acidophilus* [86].

Effects of NPY on the Immune System

In the context of the microbiota-gut-brain axis it is important to mention that NPY has a distinct impact on immune function, within and outside the gastrointestinal tract [6, 87–89]. NPY released from the sympathetic nerve fibres acts on Y receptors (notably of the Y1, Y2, Y4 and Y5 subtype) expressed by distinct classes of immune cells (e.g., dendritic cells, mononuclear cells, macrophages, granulocytes, T and B lymphocytes) to modify their activity [6, 89–91]. In addition,

NPY acts as a paracrine or autocrine immune mediator, because immune cells (e.g., B and T lymphocytes, macrophages) themselves can produce and release NPY [6, 88, 91]. The effects of NPY include modulation of immune cell trafficking, activation of antigen-presenting cell function, T helper cell differentiation, negative regulation of T cell function, cytokine secretion, phagocytosis and production of reactive oxygen species [6, 87–91].

With this immunological activity profile, NPY regulates inflammatory processes in the gut, given that NPY-containing nerve fibres are in close contact with immune cells in the mouse ileum lamina propria [92]. Specifically, NPY is able to promote colonic inflammation, an effect that is supported by several lines of evidence: (1) NPY knockout mice are largely resistant to the induction of dextran sulfate sodium-induced colitis [93, 94]. (2) The result of NPY deletion is reproduced by treatment with a NPY antisense oligodeoxynucleotide [95] and by knockout or antagonism of Y1 receptors [96]. The antiinflammatory phenotype of Y1 receptor knockout mice results from a defect in antigen-presenting cell function, a reduction of TNF-alpha and IL-12 production by macrophages, and a decrease in the number of effector T cells [90]. Furthermore, experimentally induced colitis is associated with an increase in the colonic synthesis of NPY [93, 95, 97], a reduction of colonic Y1 receptor expression and a loss of the antisecretory action of NPY in the colon [98]. In contrast, the colonic levels of the related gut hormone PYY are decreased in rats with DSS-induced colitis [99]. These experimental data are in line with a decrease of colonic PYY levels in patients with inflammatory bowel disease [100–102], while circulating levels of PYY and NPY are enhanced [103, 104]. The proinflammatory effect of NPY could in part be counterregulated by the vasoconstrictor effect of the peptide [105].

Effect of NPY to Protect from Immune Challenge-Evoked Behavioural Disturbances

Infection and inflammation are increasingly recognized to have an impact on the pathogenesis of mood disorders [18, 19, 106, 107], and clinical evidence suggests that activation of the intestinal immune system by constituents of the intestinal microbiota can give rise to depression [34] and chronic fatigue syndrome [35]. Experimentally, the impact of peripheral immune challenge on brain function and behaviour can be modelled by systemic administration of LPS or Bacille Calmette-Guérin [18, 19, 106, 108]. The signalling pathways whereby peripheral immune challenge alters brain mechanisms involve proinflammatory cytokines such as interleukin-6, tumour necrosis factor-alpha and interferon-gamma, which reach the brain via the circulation but also excite vagal afferent neurons and lead to the expression of cytokines by cerebral microglial cells and astrocytes [18, 19, 106–108].

The effect of peripheral immune challenge on brain function involves several brain areas that express NPY and various Y receptors [6, 81–84]. NPY is involved in the regulation of emotional-affective behaviour [6, 81–84], and there is indirect evidence that NPY-expressing neurons in the arcuate and paraventricular nuclei of the hypothalamus counteract the behavioural responses to immune stress and infection [109–111]. This implication has been confirmed by knockout experiments in which the NPY/Y receptor system has been found to protect against distinct functional disturbances in response to immune challenge. For instance, deletion of NPY as well as NPY plus PYY aggravates the Bacille Calmette-Guérin-induced loss of body weight and markedly delays recovery from this weight loss [112]. This finding attests to an important role of NPY and PYY in maintaining energy homeostasis in the face of immune stimulation [112].

Analogous observations have been made when the behavioural responses to LPS are analysed in Y2 and Y4 knockout mice [6]. Y2 receptor knockout mice are particularly susceptible to the acute action of LPS to attenuate locomotion and suppress social interaction [20]. In contrast, the LPS-induced rise of temperature and circulating corticosterone is suppressed by Y2 receptor knockout [20]. The short-term effect of LPS to enhance anxiety is enhanced in Y2 and Y4 receptor knockout mice [20, 21]. In Y4 receptor knockout mice, the anxiogenic response to LPS persists at least for 4 weeks post-treatment by which time it has waned in WT mice [21]. Depression-related behaviour is enhanced 1 day post-LPS in control and Y2 receptor knockout mice, but not in Y4 receptor knockout mice. Four weeks post-treatment the depressogenic effect of LPS has waned in wildtype mice, but is maintained in Y2 receptor knockout mice and first observed in Y4 receptor knockout mice [21]. Thus, knockout of Y2 and/or Y4 receptors unmasks the ability of immune challenge with LPS to cause a delayed and prolonged increase in anxiety- and/or depression-like behaviour [6]. These findings suggest that NPY acting via Y2 and Y4 receptors prevents the development of long-term anxiety- and depression-like behaviour caused by immune challenge [6, 21]. It awaits examination whether the behavioural disturbances associated with dysbiosis of the gut microbiota are likewise under the control of the NPY/Y receptor system.

Effect of NPY to Inhibit Nociceptive Transmission

Spinal afferent neurons, which contain low amounts of NPY, terminate in the spinal cord where interneurons and descending noradrenergic neurons express appreciable amounts of NPY [6, 113, 114]. An abundant occurrence of Y1 and Y2 receptors in the spinal cord enables NPY to play an important role in the processing of incoming nociceptive information. Germ-line knockout of Y1 receptors or conditional knockdown of NPY is associated with thermal, chemical and mechanical hyperalgesia [6, 113–116]. Peripheral inflammation leads to an upregulation of Y1 receptors in spinal afferent neurons and in the dorsal horn of the spinal cord [117]. While these studies have primarily focused on somatic pain, it remains to be investigated

whether the NPY/Y receptor system also plays a role in the impact of the gut microbiota on visceral pain sensitivity [66–70]. Two major mechanisms whereby NPY controls pain transmission in the spinal cord have been envisaged: inhibition of transmitter release from the terminals of primary afferent neurons, mediated primarily by Y2 receptors, and inhibition of postsynaptic neurons in the dorsal horn, mediated primarily by Y1 receptors [113, 114].

Apart from spinal sensory neurons, vagal afferent neurons terminating in the nucleus tractus solitarii (NTS) have also been established to play a role in visceral nociception, particularly in visceral chemonociception [118]. Y2 and Y4 receptors are the Y receptor subtypes prevailing in the NTS [6, 119], and gene deletion experiments have revealed that endogenous NPY acting via Y2 and Y4 receptors attenuates the chemonociceptive input from the stomach to the brainstem [119].

Effect of NPY to Protect from the Impact of Stress on the Gut-Brain Axis

Inflammation and psychosocial stress have a marked impact on the bidirectional communication between the gut and brain [5]. Since NPY plays a role in stress coping [84], it may also be relevant to the impact of stress on the gut-brain axis. NPY as well as Y1, Y2 and Y5 receptors are widely expressed in cerebral areas critical to the regulation of stress resilience [81–84]. The expression of NPY in the human brain is related to polymorphisms in the NPY gene, and a low NPY expression genotype is associated with negative emotional processing, diminished stress resilience, a risk for major depression, and a reduced antidepressant treatment response [120–122]. If individuals with a low NPY expression genotype are exposed to negative stimuli, there is an exaggerated activation of the amygdala, medial prefrontal cortex and anterior cingulate cortex [120–122]. The concentration of NPY in the cerebrospinal fluid and plasma is reduced in patients with post-traumatic stress disorder, while trauma-exposed individuals who do not develop or have recovered from post-traumatic stress disorder have enhanced plasma levels of NPY [123–125]. It would appear, therefore, that the cerebrospinal and plasma concentration of NPY is a biological correlate of resilience to or recovery from the adverse effects of stress [124]. Animal experiments have confirmed that NPY is involved in the emotional processing of stress [6], and the question arises whether this role also relates to the impact of stress on the microbiota-gut-brain axis.

Since stress can alter the permeability of the gastrointestinal mucosa [126, 127], it is very likely that stress will also alter the interaction between the gut microbiota and the mucosal immune system. CRF is a neuropeptide and gut hormone that is intimately related to stress, and there is considerable evidence that activation of peripheral CRF receptors contributes to stress-related alterations of gut physiology [126]. NPY may likewise be involved because it appears to mediate the effects of stress on many physiological systems including the gastrointestinal and immune

systems [128, 129]. For instance, deletion of NPY alters gastrointestinal, feeding and corticosterone responses to restraint stress, exaggerates stress-induced defaecation and reduces food intake [128]. Trinitrobenzene sulfonic acid-induced colitis increases the NPY concentration in brain and plasma [97], and gastrointestinal inflammation enhances anxiety- and depression-related behaviour, this effect being modified by deletion of NPY and/or PYY [94, 130]. It follows that NPY and PYY participate in the effect of intestinal inflammation on the gut-brain axis. In addition, the depression-like phenotype of PYY knockout animals [94] suggests that alterations in the expression of this gut hormone modify mood and stress coping. This contention is in line with the finding that water avoidance stress lowers the plasma level of PYY, a change that is associated with an increase in gastrointestinal motility [131].

Effects of NPY and PYY to Regulate Food Intake and Energy Homeostasis

The implications of NPY and PYY in gut-brain signalling are particularly well exemplified by their effects on hunger, food intake, satiety and energy balance. These roles have been extensively reviewed elsewhere [10, 132, 133] and may be of particular relevance to the impact of the gut microbiota on metabolic regulation, energy homeostasis and metabolic disorders. PYY is released postprandially from intestinal L cells and acts as a satiety factor, slowing gastrointestinal transit, inhibiting further intake of food and modifying the metabolic status of the organism [10]. In the circulation, PYY is truncated to $PYY_{3\text{-}36}$ which is a relatively selective Y2 receptor agonist. Food intake is inhibited by $PYY_{3\text{-}36}$ both via stimulation of Y2 receptors on vagal afferent neurons and an interaction with Y2 receptors in the hypothalamus [6, 10, 134, 135]. Within the brain, $PYY_{3\text{-}36}$ reduces food intake primarily via activation of Y2 receptors in the arcuate nucleus which is an important centre for integrating peripheral and central signals in the control of appetite and energy homeostasis [136]. NPY, on the other hand, is one of the most potent orexigenic peptides found in the brain [133, 136]. Specifically, it occurs in neurons projecting from the arcuate nucleus to various areas of the hypothalamus in which the orexigenic effect of NPY is primarily mediated by Y1 receptors, although Y5 receptors also contribute [133, 136]. Pathologies associated with a decrease in food intake such as experimental colitis lead to increased release of NPY from the paraventricular nucleus of the hypothalamus [137], again attesting to a role of NPY in gut-brain signalling.

NPY, PYY and Other Gut Peptides in the Interoceptive Regulation of Emotion and Mood

Apart from regulating ingestion and energy homeostasis, gut hormones such as ghrelin, PYY, GLP-1 and GLP-2 have an impact on emotional-affective behaviour. In an evolutionary point of view, co-regulation of appetite and emotional state is an important strategy for survival, given that anxiety would be an adverse condition when there is a need to seek food [6]. Indeed, ghrelin which is released from the upper gastrointestinal tract under conditions of hunger reduces both anxiety-like and depression-related behaviour [138]. Under fed conditions, behaviour is changed to a hedonic state as observed when $PYY_{3\text{-}36}$ is administered to reach postprandial plasma concentrations of the peptide [139]. The ability of PYY to promote hedonic behaviour is supported by the finding that knockout of PYY increases depression-like behaviour but does not alter anxiety [94]. Physiologically, however, emotion and mood under fed conditions will be determined by the presence of a variety of gut hormones such as PYY, GLP-1 and GLP-2 that are released postprandially. Thus, GLP-1 has been found to enhance anxiety-related behaviour [140–142], while GLP-2 attenuates depression-like behaviour [143].

Gut hormones whose release from the enteroendocrine cells is likely to be regulated by the gut microbiota thus provide a constant stream of interoceptive input from the gut to the brain.

Conclusion: The Gut Microbiota Meets Neuropeptides

The gut microbiota has proved as a novel factor relevant to health and disease. How the gut microbiota communicates with distant organs such as the brain is only beginning to emerge. It is very probable that the microbiota will take use of several information carriers from the gut to the brain including microbiota-derived signalling molecules, immune mediators, gut hormones as well as vagal and spinal afferent neurons. Biologically active gut peptides and neuropeptides play a role in several of these communication pathways. This is true for peptides produced by enteroendocrine cells which respond to metabolites generated with the help of the microbiota. PYY, which is one of these peptides, acts via Y receptor types that are also stimulated by the neuropeptide NPY. Neuropeptides are important transmitters in afferent, central and efferent pathways of the bidirectional gut-brain communication network. It remains to be shown whether the gut microbiota itself expresses neuropeptide receptors or releases metabolites that are ligands at neuropeptide receptors. Although a direct link between the gut microbiota and distinct neuropeptide systems has not yet been revealed, NPY, CRF and tachykinins are very likely to emerge as messengers in the microbiota-gut-brain axis.

Acknowledgements This study was supported by the Zukunftsfonds Steiermark (grant 262), the Austrian Science Fund (FWF grants L25-B05, P23097-B18 and P25912-B23), and the Federal Ministry of Science and Research of the Republic of Austria (grant GZ 80.104/2-BrGT/2007).

References

1. Pearse AGE (1969) The cytochemistry and ultrastructure of polypeptide hormone-producing cells of the APUD series and the embryologic, physiologic and pathologic implications of the concept. J Histochem Cytochem 17(5):303–313
2. Strand FL (1999) Neuropeptides: regulators of physiological processes. MIT Press, Cambridge
3. Kastin AJ (2013) Handbook of biologically active peptides, 2nd edn. Academic, San Diego
4. Burbach JP (2010) Neuropeptides from concept to online database www.neuropeptides.nl. Eur J Pharmacol 626(1):27–48
5. Mayer EA, Tillisch K (2011) The brain-gut axis in abdominal pain syndromes. Annu Rev Med 62:381–396
6. Holzer P, Reichmann F, Farzi A (2012) Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut-brain axis. Neuropeptides 46(6):261–274
7. Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 13(10):701–712
8. Collins SM, Surette M, Bercik P (2012) The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol 10(11):735–742
9. Gorkiewicz G, Thallinger GG, Trajanoski S, Lackner S, Stocker G, Hinterleitner T, Gülly C, Högenauer C (2013) Alterations in the colonic microbiota in response to osmotic diarrhea. PLoS One 8(2):e55817
10. Field BC, Chaudhri OB, Bloom SR (2010) Bowels control brain: gut hormones and obesity. Nat Rev Endocrinol 6(8):444–453
11. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, Siuzdak G (2009) Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A 106(10):3698–3703
12. Antunes LC, Han J, Ferreira RB, Lolić P, Borchers CH, Finlay BB (2011) Effect of antibiotic treatment on the intestinal metabolome. Antimicrob Agents Chemother 55(4):1494–1503
13. Tremaroli V, Bäckhed F (2012) Functional interactions between the gut microbiota and host metabolism. Nature 489(7415):242–249
14. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, Pettersson S (2012) Host-gut microbiota metabolic interactions. Science 336(6086):1262–1267
15. Marcobal A, Kashyap PC, Nelson TA, Aronov PA, Donia MS, Spormann A, Fischbach MA, Sonnenburg JL (2013) A metabolomic view of how the human gut microbiota impacts the host metabolome using humanized and gnotobiotic mice. ISME J 7:1933–1943
16. Matsumoto M, Kibe R, Ooga T, Aiba Y, Sawaki E, Koga Y, Benno Y (2013) Cerebral low-molecular metabolites influenced by intestinal microbiota: a pilot study. Front Syst Neurosci 7:9
17. Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN (2010) Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat Med 16 (2):228–231
18. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008) From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 9 (1):46–56
19. McCusker RH, Kelley KW (2013) Immune-neural connections: how the immune system's response to infectious agents influences behavior. J Exp Biol 216(Pt 1):84–98

20. Painsipp E, Herzog H, Holzer P (2008) Implication of neuropeptide-Y Y2 receptors in the effects of immune stress on emotional, locomotor and social behavior of mice. Neuropharmacology 55(1):117–126
21. Painsipp E, Herzog H, Holzer P (2010) Evidence from knockout mice that neuropeptide-Y Y2 and Y4 receptor signalling prevents long-term depression-like behavior caused by immune challenge. J Psychopharmacol 24(10):1551–1560
22. Painsipp E, Köfer MJ, Sinner F, Holzer P (2011) Prolonged depression-like behavior caused by immune challenge: influence of mouse strain and social environment. PLoS One 6(6): e20719
23. Kubera M, Curzytek K, Duda W, Leskiewicz M, Basta-Kaim A, Budziszewska B, Roman A, Zajicova A, Holan V, Szczesny E, Lason W, Maes M (2013) A new animal model of (chronic) depression induced by repeated and intermittent lipopolysaccharide administration for 4 months. Brain Behav Immun 31:96–104
24. Tarr AJ, Chen Q, Wang Y, Sheridan JF, Quan N (2012) Neural and behavioral responses to low-grade inflammation. Behav Brain Res 235(2):334–341
25. Holzer P, Danzer M, Schicho R, Samberger C, Painsipp E, Lippe IT (2004) Vagal afferent input from the acid-challenged rat stomach to the brainstem: enhancement by interleukin-1beta. Neuroscience 129:439–445
26. Hughes PA, Harrington AM, Castro J, Liebregts T, Adam B, Grasby DJ, Isaacs NJ, Maldeniya L, Martin CM, Persson J, Andrews JM, Holtmann G, Blackshaw LA, Brierley SM (2013) Sensory neuro-immune interactions differ between irritable bowel syndrome subtypes. Gut 62:1456–1465
27. Abreu MT (2010) Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol 10(2):131–144
28. Marques R, Boneca IG (2011) Expression and functional importance of innate immune receptors by intestinal epithelial cells. Cell Mol Life Sci 68(22):3661–3673
29. Barajon I, Serrao G, Arnaboldi F, Opizzi E, Ripamonti G, Balsari A, Rumio C (2009) Toll-like receptors 3, 4, and 7 are expressed in the enteric nervous system and dorsal root ganglia. J Histochem Cytochem 57(11):1013–1023
30. Anitha M, Vijay-Kumar M, Sitaraman SV, Gewirtz AT, Srinivasan S (2012) Gut microbial products regulate murine gastrointestinal motility via Toll-like receptor 4 signaling. Gastroenterology 143(4):1006–1016.e4
31. van Noort JM, Bsibsi M (2009) Toll-like receptors in the CNS: implications for neurodegeneration and repair. Prog Brain Res 175:139–148
32. Arroyo DS, Soria JA, Gaviglio EA, Rodriguez-Galan MC, Iribarren P (2011) Toll-like receptors are key players in neurodegeneration. Int Immunopharmacol 11(10):1415–1421
33. Mallard C (2012) Innate immune regulation by toll-like receptors in the brain. ISRN Neurol 2012:701950
34. Maes M, Kubera M, Leunis JC, Berk M (2012) Increased IgA and IgM responses against gut commensals in chronic depression: further evidence for increased bacterial translocation or leaky gut. J Affect Disord 141(1):55–62
35. Maes M, Twisk FN, Kubera M, Ringel K, Leunis JC, Geffard M (2012) Increased IgA responses to the LPS of commensal bacteria is associated with inflammation and activation of cell-mediated immunity in chronic fatigue syndrome. J Affect Disord 136(3):909–917
36. Lyte M (2011) Probiotics function mechanistically as delivery vehicles for neuroactive compounds: microbial endocrinology in the design and use of probiotics. Bioessays 33 (8):574–581
37. Forsythe P, Kunze WA (2013) Voices from within: gut microbes and the CNS. Cell Mol Life Sci 70(1):55–69
38. Asano Y, Hiramoto T, Nishino R, Aiba Y, Kimura T, Yoshihara K, Koga Y, Sudo N (2012) Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. Am J Physiol Gastrointest Liver Physiol 303(11):G1288–G1295

39. Barrett E, Ross RP, O'Toole PW, Fitzgerald GF, Stanton C (2012) Gamma-aminobutyric acid production by culturable bacteria from the human intestine. J Appl Microbiol 113 (2):411–417
40. Yurdaydin C, Walsh TJ, Engler HD, Ha JH, Li Y, Jones EA, Basile AS (1995) Gut bacteria provide precursors of benzodiazepine receptor ligands in a rat model of hepatic encephalopathy. Brain Res 679(1):42–48
41. Hu Y, Phelan V, Ntai I, Farnet CM, Zazopoulos E, Bachmann BO (2007) Benzodiazepine biosynthesis in *Streptomyces refuineus*. Chem Biol 14(6):691–701
42. Gerratana B (2012) Biosynthesis, synthesis, and biological activities of pyrrolobenzodiazepines. Med Res Rev 32(2):254–293
43. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, Dinan TG, Cryan JF (2013) The microbiome-gut-brain axis during early-life regulates the hippocampal serotonergic system in a gender-dependent manner. Mol Psychiatry 18(6):666–673
44. Holst JJ (2007) The physiology of glucagon-like peptide 1. Physiol Rev 87(4):1409–1439
45. Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagisawa M, Gordon JI (2008) Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci U S A 105:16767–16772
46. Reolon GK, Maurmann N, Werenicz A, Garcia VA, Schröder N, Wood MA, Roesler R (2011) Posttraining systemic administration of the histone deacetylase inhibitor sodium butyrate ameliorates aging-related memory decline in rats. Behav Brain Res 221(1):329–332
47. Gundersen BB, Blendy JA (2009) Effects of the histone deacetylase inhibitor sodium butyrate in models of depression and anxiety. Neuropharmacology 57(1):67–74
48. Thomas RH, Meeking MM, Mepham JR, Tichenoff L, Possmayer F, Liu S, MacFabe DF (2012) The enteric bacterial metabolite propionic acid alters brain and plasma phospholipid molecular species: further development of a rodent model of autism spectrum disorders. J Neuroinflammation 9:153
49. MacFabe DF, Cain NE, Boon F, Ossenkopp KP, Cain DP (2011) Effects of the enteric bacterial metabolic product propionic acid on object-directed behavior, social behavior, cognition, and neuroinflammation in adolescent rats: relevance to autism spectrum disorder. Behav Brain Res 217(1):47–54
50. Cani PD, Lecourt E, Dewulf EM, Sohet FM, Pachikian BD, Naslain D, De Backer F, Neyrinck AM, Delzenne NM (2009) Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. Am J Clin Nutr 90(5):1236–1243
51. Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, Geurts L, Naslain D, Neyrinck A, Lambert DM, Muccioli GG, Delzenne NM (2009) Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 58(8):1091–1103
52. Muccioli GG, Naslain D, Bäckhed F, Reigstad CS, Lambert DM, Delzenne NM, Cani PD (2010) The endocannabinoid system links gut microbiota to adipogenesis. Mol Syst Biol 6:392
53. Cani PD, Delzenne NM (2011) The gut microbiome as therapeutic target. Pharmacol Ther 130(2):202–212
54. Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, Harris HM, Coakley M, Lakshminarayanan B, O'Sullivan O, Fitzgerald GF, Deane J, O'Connor M, Harnedy N, O'Connor K, O'Mahony D, van Sinderen D, Wallace M, Brennan L, Stanton C, Marchesi JR, Fitzgerald AP, Shanahan F, Hill C, Ross RP, O'Toole PW (2012) Gut microbiota composition correlates with diet and health in the elderly. Nature 488 (7410):178–184
55. Queipo-Ortuño MI, Seoane LM, Murri M, Pardo M, Gomez-Zumaquero JM, Cardona F, Casanueva F, Tinahones FJ (2013) Gut microbiota composition in male rat models under

different nutritional status and physical activity and its association with serum leptin and ghrelin levels. PLoS One 8(5):e65465
56. Duca FA, Swartz TD, Sakar Y, Covasa M (2012) Increased oral detection, but decreased intestinal signaling for fats in mice lacking gut microbiota. PLoS One 7(6):e39748
57. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, Kubo C, Koga Y (2004) Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. J Physiol 558(Pt 1):263–275
58. Neufeld KM, Kang N, Bienenstock J, Foster JA (2011) Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil 23(3):255–264, e119
59. Heijtz RD, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, Hibberd ML, Forssberg H, Pettersson S (2011) Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A 108(7):3047–3052
60. Gareau MG, Wine E, Rodrigues DM, Cho JH, Whary MT, Philpott DJ, MacQueen G, Sherman PM (2011) Bacterial infection causes stress-induced memory dysfunction in mice. Gut 60(3):307–317
61. Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, Deng Y, Blennerhassett P, Macri J, McCoy KD, Verdu EF, Collins SM (2011) The intestinal microbiota affect central levels of brain-derived neurotrophic factor and behavior in mice. Gastroenterology 141(2):599–609
62. Farzi A, Gorkiewicz G, Holzer P (2012) Non-absorbable oral antibiotic treatment in mice affects multiple levels of the microbiota-gut-brain axis. Neurogastroenterol Motil 24(Suppl 2):78
63. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF (2011) Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 108 (38):16050–16055
64. Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X, Deng Y, Blennerhassett PA, Fahnestock M, Moine D, Berger B, Huizinga JD, Kunze W, McLean PG, Bergonzelli GE, Collins SM, Verdu EF (2011) The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut-brain communication. Neurogastroenterol Motil. 23 (12):1132–1139
65. Verdú EF, Bercik P, Verma-Gandhu M, Huang XX, Blennerhassett P, Jackson W, Mao Y, Wang L, Rochat F, Collins SM (2006) Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 55(2):182–190
66. Amaral FA, Sachs D, Costa VV, Fagundes CT, Cisalpino D, Cunha TM, Ferreira SH, Cunha FQ, Silva TA, Nicoli JR, Vieira LQ, Souza DG, Teixeira MM (2008) Commensal microbiota is fundamental for the development of inflammatory pain. Proc Natl Acad Sci U S A 105 (6):2193–2197
67. Ma X, Mao YK, Wang B, Huizinga JD, Bienenstock J, Kunze W (2009) *Lactobacillus reuteri* ingestion prevents hyperexcitability of colonic DRG neurons induced by noxious stimuli. Am J Physiol Gastrointest Liver Physiol 296(4):G868–G875
68. Duncker SC, Kamiya T, Wang L, Yang P, Bienenstock J (2011) Probiotic *Lactobacillus reuteri* alleviates the response to gastric distension in rats. J Nutr 141(10):1813–1818
69. Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, Dubuquoy C, Merour E, Geboes K, Chamaillard M, Ouwehand A, Leyer G, Carcano D, Colombel JF, Ardid D, Desreumaux P (2007) *Lactobacillus acidophilus* modulates intestinal pain and induces opioid and cannabinoid receptors. Nat Med 13(1):35–37
70. Horvath A, Dziechciarz P, Szajewska H (2011) Meta-analysis: *Lactobacillus rhamnosus* GG for abdominal pain-related functional gastrointestinal disorders in childhood. Aliment Pharmacol Ther 33(12):1302–1310
71. Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, Peterson DA, Stappenbeck TS, Hsieh CS (2011) Peripheral education of the immune system by colonic commensal microbiota. Nature 478(7368):250–254

72. Sathyabama S, Khan N, Agrewala JN (2014) Friendly pathogens: prevent or provoke autoimmunity. Crit Rev Microbiol 40:273–280
73. Chervonsky AV (2013) Microbiota and autoimmunity. Cold Spring Harb Perspect Biol 5(3): a007294
74. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK (2011) Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A 108(Suppl 1):4615–4622
75. Berer K, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, Wekerle H, Krishnamoorthy G (2011) Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. Nature 479(7374):538–541
76. Fetissov SO, Harro J, Jaanisk M, Järv A, Podar I, Allik J, Nilsson I, Sakthivel P, Lefvert AK, Hökfelt T (2005) Autoantibodies against neuropeptides are associated with psychological traits in eating disorders. Proc Natl Acad Sci U S A 102(41):14865–14870
77. Fetissov SO, Hamze Sinno M, Coëffier M, Bole-Feysot C, Ducrotté P, Hökfelt T, Déchelotte P (2008) Autoantibodies against appetite-regulating peptide hormones and neuropeptides: putative modulation by gut microflora. Nutrition 24(4):348–359
78. Fetissov SO, Hamze Sinno M, Coquerel Q, Do Rego JC, Coëffier M, Gilbert D, Hökfelt T, Déchelotte P (2008) Emerging role of autoantibodies against appetite-regulating neuropeptides in eating disorders. Nutrition 24(9):854–859
79. Alexander SPH, Mathie A, Peters JA (2011) Guide to receptors and channels (GRAC), 5th edn. Br J Pharmacol 164(Suppl 1):S1–S324
80. Wettstein JG, Earley B, Junien JL (1995) Central nervous system pharmacology of neuropeptide Y. Pharmacol Ther 65(3):397–414
81. Kask A, Harro J, von Hörsten S, Redrobe JP, Dumont Y, Quirion R (2002) The neurocircuitry and receptor subtypes mediating anxiolytic-like effects of neuropeptide Y. Neurosci Biobehav Rev 26(3):259–283
82. Redrobe JP, Carvajal C, Kask A, Dumont Y, Quirion R (2004) Neuropeptide Y and its receptor subtypes in the central nervous system: emphasis on their role in animal models of psychiatric disorders. Handb Exp Pharmacol 162:101–136
83. Eaton K, Sallee FR, Sah R (2007) Relevance of neuropeptide Y (NPY) in psychiatry. Curr Top Med Chem 7(17):1645–1659
84. Morales-Medina JC, Dumont Y, Quirion R (2010) A possible role of neuropeptide Y in depression and stress. Brain Res 1314:194–205
85. Tasan RO, Lin S, Hetzenauer A, Singewald N, Herzog H, Sperk G (2009) Increased novelty-induced motor activity and reduced depression-like behavior in neuropeptide Y (NPY)-Y4 receptor knockout mice. Neuroscience 158(4):1717–1730
86. El Karim IA, Linden GJ, Orr DF, Lundy FT (2008) Antimicrobial activity of neuropeptides against a range of micro-organisms from skin, oral, respiratory and gastrointestinal tract sites. J Neuroimmunol 200(1–2):11–16
87. Bedoui S, von Hörsten S, Gebhardt T (2007) A role for neuropeptide Y (NPY) in phagocytosis: implications for innate and adaptive immunity. Peptides 28(2):373–376
88. Wheway J, Herzog H, Mackay F (2007) NPY and receptors in immune and inflammatory diseases. Curr Top Med Chem 7(17):1743–1752
89. Dimitrijević M, Stanojević S (2013) The intriguing mission of neuropeptide Y in the immune system. Amino Acids 45(1):41–53
90. Wheway J, Mackay CR, Newton RA, Sainsbury A, Boey D, Herzog H, Mackay F (2005) A fundamental bimodal role for neuropeptide Y1 receptor in the immune system. J Exp Med 202(11):1527–1538
91. Wheway J, Herzog H, Mackay F (2007) The Y1 receptor for NPY: a key modulator of the adaptive immune system. Peptides 28(2):453–458
92. Shibata M, Hisajima T, Nakano M, Goris RC, Funakoshi K (2008) Morphological relationships between peptidergic nerve fibers and immunoglobulin A-producing lymphocytes in the mouse intestine. Brain Behav Immun 22(2):158–166

93. Chandrasekharan B, Bala V, Kolachala VL, Vijay-Kumar M, Jones D, Gewirtz AT, Sitaraman SV, Srinivasan S (2008) Targeted deletion of neuropeptide Y (NPY) modulates experimental colitis. PLoS One 3(10):e3304
94. Painsipp E, Herzog H, Sperk G, Holzer P (2011) Sex-dependent control of murine emotional-affective behaviour in health and colitis by peptide YY and neuropeptide Y. Br J Pharmacol 163(6):1302–1314
95. Pang XH, Li TK, Xie Q, He FQ, Cui DJ, Chen YQ, Huang XL, Gan HT (2010) Amelioration of dextran sulfate sodium-induced colitis by neuropeptide Y antisense oligodeoxynucleotide. Int J Colorectal Dis 25(9):1047–1053
96. Hassani H, Lucas G, Rozell B, Ernfors P (2005) Attenuation of acute experimental colitis by preventing NPY Y1 receptor signaling. Am J Physiol Gastrointest Liver Physiol 288(3): G550–G556
97. Baticic L, Detel D, Kucic N, Buljevic S, Pugel EP, Varljen J (2011) Neuroimmunomodulative properties of dipeptidyl peptidase IV/CD26 in a TNBS-induced model of colitis in mice. J Cell Biochem 112(11):3322–3333
98. Klompus M, Ho W, Sharkey KA, McKay DM (2010) Antisecretory effects of neuropeptide Y in the mouse colon are region-specific and are lost in DSS-induced colitis. Regul Pept 165 (2–3):138–145
99. Hirotani Y, Mikajiri K, Ikeda K, Myotoku M, Kurokawa N (2008) Changes of the peptide YY levels in the intestinal tissue of rats with experimental colitis following oral administration of mesalazine and prednisolone. Yakugaku Zasshi 128(9):347–1353
100. Tari A, Teshima H, Sumii K, Haruma K, Ohgoshi H, Yoshihara M, Kajiyama G, Miyachi Y (1988) Peptide YY abnormalities in patients with ulcerative colitis. Jpn J Med 27(1):49–55
101. El-Salhy M, Danielsson A, Stenling R, Grimelius L (1997) Colonic endocrine cells in inflammatory bowel disease. J Intern Med 242(5):413–419
102. Schmidt PT, Ljung T, Hartmann B, Hare KJ, Holst JJ, Hellström PM (2005) Tissue levels and post-prandial secretion of the intestinal growth factor, glucagon-like peptide-2, in controls and inflammatory bowel disease: comparison with peptide YY. Eur J Gastroenterol Hepatol 17(2):207–212
103. Adrian TE, Savage AP, Bacarese-Hamilton AJ, Wolfe K, Besterman HS, Bloom SR (1986) Peptide YY abnormalities in gastrointestinal diseases. Gastroenterology 90(2):379–384
104. Straub RH, Herfarth H, Falk W, Andus T, Schölmerich J (2002) Uncoupling of the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis in inflammatory bowel disease? J Neuroimmunol 126(1–2):116–125
105. Holzer P (2012) Neural regulation of gastrointestinal blood flow. In: Johnson LR (ed) Physiology of the gastrointestinal tract, 5th edn. Academic, Oxford, pp 817–845
106. Capuron L, Miller AH (2011) Immune system to brain signaling: neuropsychopharmacological implications. Pharmacol Ther 130(2):226–238
107. Maes M, Song C, Yirmiya R (2012) Targeting IL-1 in depression. Expert Opin Ther Targets 16(11):1097–1112
108. Moreau M, André C, O'Connor JC, Dumich SA, Woods JA, Kelley KW, Dantzer R, Lestage J, Castanon N (2008) Inoculation of *Bacillus* Calmette-Guerin to mice induces an acute episode of sickness behavior followed by chronic depressive-like behavior. Brain Behav Immun 22(7):1087–1095
109. McCarthy HD, Dryden S, Williams G (1995) Interleukin-1beta-induced anorexia and pyrexia in rat: relationship to hypothalamic neuropeptide Y. Am J Physiol 269(5 Pt 1):E852–E857
110. Sonti G, Ilyin SE, Plata-Salaman CR (1996) Neuropeptide Y blocks and reverses interleukin-1beta-induced anorexia in rats. Peptides 17(3):517–520
111. McMahon CD, Buxton DF, Elsasser TH, Gunter DR, Sanders LG, Steele BP, Sartin JL (1999) Neuropeptide Y restores appetite and alters concentrations of GH after central administration to endotoxic sheep. J Endocrinol 161(2):333–339

112. Painsipp E, Köfer MJ, Farzi A, Dischinger US, Sinner F, Sperk G, Herzog H, Holzer P (2013) Neuropeptide Y and peptide YY protect from weight loss caused by Bacille Calmette-Guérin in mice. Br J Pharmacol 170:1014–1026
113. Brumovsky P, Shi TS, Landry M, Villar MJ, Hökfelt T (2007) Neuropeptide tyrosine and pain. Trends Pharmacol Sci 28(2):93–102
114. Smith PA, Moran TD, Abdulla F, Tumber KK, Taylor BK (2007) Spinal mechanisms of NPY analgesia. Peptides 28(2):464–474
115. Painsipp E, Sperk G, Herzog H, Holzer P (2010) Delayed stress-induced differences in locomotor and depression-related behaviour in female neuropeptide-Y Y1 receptor knockout mice. J Psychopharmacol 24(10):1541–1549
116. Solway B, Bose SC, Corder G, Donahue RR, Taylor BK (2011) Tonic inhibition of chronic pain by neuropeptide Y. Proc Natl Acad Sci U S A 108(17):7224–7229
117. Ji RR, Zhang X, Wiesenfeld-Hallin Z, Hökfelt T (1994) Expression of neuropeptide Y and neuropeptide Y (Y1) receptor mRNA in rat spinal cord and dorsal root ganglia following peripheral tissue inflammation. J Neurosci 14(11 Pt 1):6423–6434
118. Holzer P (2008) The role of the vagus nerve in afferent signaling and homeostasis during visceral inflammation. In: Jancsó G (ed) Neurogenic inflammation in health and disease. Neuroimmune biology, vol 8. Elsevier, Amsterdam, pp 321–338
119. Wultsch T, Painsipp E, Thoeringer CK, Herzog H, Sperk G, Holzer P (2005) Endogenous neuropeptide Y depresses the afferent signaling of gastric acid challenge to the mouse brainstem via neuropeptide Y type Y2 and Y4 receptors. Neuroscience 136(4):1097–1107
120. Zhou Z, Zhu G, Hariri AR, Enoch M, Scott D, Sinha R, Virkkunen M, Mash DC, Lipsky RH, Hu XZ, Hodgkinson CA, Xu K, Buzas B, Yuan Q, Shen PH, Ferrell RE, Manuck SB, Brown SM, Hauger RL, Stohler CS, Zubieta JK, Goldman D (2008) Genetic variation in human NPY expression affects stress response and emotion. Nature 452(7190):997–1001
121. Domschke K, Dannlowski U, Hohoff C, Ohrmann P, Bauer J, Kugel H, Zwanzger P, Heindel W, Deckert J, Arolt V, Suslow T, Baune BT (2010) Neuropeptide Y (NPY) gene: impact on emotional processing and treatment response in anxious depression. Eur Neuropsychopharmacol 20(5):301–309
122. Mickey BJ, Zhou Z, Heitzeg MM, Heinz E, Hodgkinson CA, Hsu DT, Langenecker SA, Love TM, Peciña M, Shafir T, Stohler CS, Goldman D, Zubieta JK (2011) Emotion processing, major depression, and functional genetic variation of neuropeptide Y. Arch Gen Psychiatry 68(2):158–166
123. Morgan CA, Rasmusson AM, Winters B, Hauger RL, Morgan J, Hazlett G, Southwick S (2003) Trauma exposure rather than posttraumatic stress disorder is associated with reduced baseline plasma neuropeptide-Y levels. Biol Psychiatry 54(10):1087–1091
124. Yehuda R, Brand S, Yang RK (2006) Plasma neuropeptide Y concentrations in combat exposed veterans: relationship to trauma exposure, recovery from PTSD, and coping. Biol Psychiatry 59(7):660–663
125. Sah R, Ekhator NN, Strawn JR, Sallee FR, Baker DG, Horn PS, Geracioti TD (2009) Low cerebrospinal fluid neuropeptide Y concentrations in posttraumatic stress disorder. Biol Psychiatry 66(7):705–707
126. Kiank C, Taché Y, Larauche M (2010) Stress-related modulation of inflammation in experimental models of bowel disease and post-infectious irritable bowel syndrome: role of corticotropin-releasing factor receptors. Brain Behav Immun 24(1):41–48
127. Dinan TG, Cryan JF (2012) Regulation of the stress response by the gut microbiota: implications for psychoneuroendocrinology. Psychoneuroendocrinology 37(9):1369–1378
128. Forbes S, Herzog H, Cox H (2012) A role for neuropeptide Y in the gender-specific gastrointestinal, corticosterone and feeding responses to stress. Br J Pharmacol 166 (8):2307–2316
129. Hirsch D, Zukowska Z (2012) NPY and stress 30 years later: the peripheral view. Cell Mol Neurobiol 32(5):645–659

130. Painsipp E, Wultsch T, Shahbazian A, Edelsbrunner M, Kreissl MC, Schirbel A, Bock E, Pabst MA, Thoeringer CK, Huber HP, Holzer P (2007) Experimental gastritis in mice enhances anxiety in a gender-related manner. Neuroscience 150(3):522–536
131. Liang C, Luo H, Liu Y, Cao J, Xia H (2012) Plasma hormones facilitated the hypermotility of the colon in a chronic stress rat model. PLoS One 7(2):e31774
132. Kirchner H, Tong J, Tschöp MH, Pfluger PT (2010) Ghrelin and PYY in the regulation of energy balance and metabolism: lessons from mouse mutants. Am J Physiol Endocrinol Metab 298(5):E909–E919
133. Yulyaningsih E, Zhang L, Herzog H, Sainsbury A (2011) NPY receptors as potential targets for anti-obesity drug development. Br J Pharmacol 163(6):1170–1202
134. Koda S, Date Y, Murakami N, Shimbara T, Hanada T, Toshinai K, Niijima A, Furuya M, Inomata N, Osuye K, Nakazato M (2005) The role of the vagal nerve in peripheral PYY_{3-36}-induced feeding reduction in rats. Endocrinology 146(5):2369–2375
135. Ueno H, Yamaguchi H, Mizuta M, Nakazato M (2008) The role of PYY in feeding regulation. Regul Pept 145(1–3):12–16
136. Chee MJ, Colmers WF (2008) Y eat? Nutrition 24(9):869–877
137. Ballinger AB, Williams G, Corder R, El-Haj T, Farthing MJ (2001) Role of hypothalamic neuropeptide Y and orexigenic peptides in anorexia associated with experimental colitis in the rat. Clin Sci (Lond) 100(2):221–229
138. Lutter M, Sakata I, Osborne-Lawrence S, Rovinsky SA, Anderson JG, Jung S, Birnbaum S, Yanagisawa M, Elmquist JK, Nestler EJ, Zigman JM (2008) The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. Nat Neurosci 11(7):752–753
139. Batterham RL, ffytche DH, Rosenthal JM, Zelaya FO, Barker GJ, Withers DJ, Williams SC (2007) PYY modulation of cortical and hypothalamic brain areas predicts feeding behaviour in humans. Nature 450(7166):106–109
140. Möller C, Sommer W, Thorsell A, Rimondini R, Heilig M (2002) Anxiogenic-like action of centrally administered glucagon-like peptide-1 in a punished drinking test. Prog Neuropsychopharmacol Biol Psychiatry 26(1):119–122
141. Kinzig KP, D'Alessio DA, Herman JP, Sakai RR, Vahl TP, Figueiredo HF, Murphy EK, Seeley RJ (2003) CNS glucagon-like peptide-1 receptors mediate endocrine and anxiety responses to interoceptive and psychogenic stressors. J Neurosci 23(15):6163–6170
142. Gulec G, Isbil-Buyukcoskun N, Kahveci N (2010) Effects of centrally-injected glucagon-like peptide-1 on pilocarpine-induced seizures, anxiety and locomotor and exploratory activity in rat. Neuropeptides 44(4):285–291
143. Iwai T, Hayashi Y, Narita S, Kasuya Y, Jin K, Tsugane M, Oka J (2009) Antidepressant-like effects of glucagon-like peptide-2 in mice occur via monoamine pathways. Behav Brain Res 204(1):235–240

Chapter 10
Bacterial Neuroactive Compounds Produced by Psychobiotics

Rebecca Wall, John F. Cryan, R. Paul Ross, Gerald F. Fitzgerald, Timothy G. Dinan, and Catherine Stanton

Abstract We recently coined the phrase 'psychobiotics' to describe an emerging class of probiotics of relevance to psychiatry [Dinan et al., Biol Psychiatry 2013;74 (10):720–726]. Such "mind-altering" probiotics may act via their ability to produce various biologically active compounds, such as peptides and mediators normally associated with mammalian neurotransmission. Several molecules with neuroactive functions such as gamma-aminobutyric acid (GABA), serotonin, catecholamines and acetylcholine have been reported to be microbially-derived, many of which have been isolated from bacteria within the human gut. Secreted neurotransmitters from bacteria in the intestinal lumen may induce epithelial cells to release molecules that in turn modulate neural signalling within the enteric nervous system and consequently signal brain function and behaviour of the host. Consequently, neurochemical containing/producing probiotic bacteria may be viewed as delivery vehicles for neuroactive compounds and as such, probiotic bacteria may possibly have the potential as a therapeutic strategy in the prevention and/or treatment of certain neurological and neurophysiological conditions.

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_10,

Abbreviations

5-HT	5-Hydroxytryptamine
AA	Arachidonic acid
ASD	Autism spectrum disorders
CLA	Conjugated linoleic acid
CNS	Central nervous system
DHA	Docosahexaenoic acid
GABA	Gamma-aminobutyric acid
GAD	Glutamate decarboxylase
GF	Germ-free
GIT	Gastrointestinal tract
IPA	Indole-3-propionic acid
LAB	Lactic acid bacteria
LC-PUFA	Long-chain fatty acid
PPAR γ	Peroxisome proliferator-activated receptor gamma
SCFA	Short chain fatty acid
TNF	Tumor necrosis factor

Introduction

We recently coined the phrase 'psychobiotics' to describe an emerging class of probiotics of relevance to psychiatry [1]. Such "mind-altering" probiotics may act via their ability to produce various biologically active compounds, such as peptides and mediators normally associated with mammalian neurotransmission. Several molecules with neuroactive functions such as gamma-aminobutyric acid (GABA), serotonin, catecholamines and acetylcholine have been reported to be microbially-derived, many of which have been isolated from bacteria within the human gut. Secreted neurotransmitters from bacteria in the intestinal lumen may induce epithelial cells to release molecules that in turn modulate neural signalling within the enteric nervous system and consequently signal brain function and behaviour of the host. Consequently, neurochemical containing/producing probiotic bacteria may be viewed as delivery vehicles for neuroactive compounds and as such, probiotic bacteria may possibly have the potential as a therapeutic strategy in the prevention and/or treatment of certain neurological and neurophysiological conditions.

In recent years, interdisciplinary investigation has revealed strong evidence of the existence of a bidirectional signalling between the intestine and the brain, the so called "brain-gut axis". This communication system integrates neural, hormonal and immunological signalling between the gut and the brain and is critical to maintain homeostasis [2]. More recently, however, this axis concept was expanded to the "microbiota-gut-brain axis", when it became clear that not only the intestinal tract itself but also its 100 trillion microbial inhabitants can affect the functioning of the central nervous system (CNS) and consequently mood and behaviour [3, 4]. The

brain communicates with the enteric microbiota directly by releasing signalling molecules into the gut lumen, and indirectly by altering gastric motility, secretion and intestinal permeability [5]. Equally, the enteric microbiota can communicate with the host via epithelial cells, receptor-mediated signalling, and stimulation of cells of the lamina propria [6]. Changes in the composition of the gut microbiota may lead to deterioration in gastrointestinal, neuroendocrine, or immune pathways and relationships, which in turn could lead to alterations in brain-gut interactions and consequently result in disease [7].

Recently, the microbial endocrinology-based theory was introduced which claimed that probiotics (i.e. live microorganisms that, when ingested in adequate amounts, exerts a health benefit on the host [8]) function as pharmacological agents and hence act as drug delivery vehicles due to their ability to synthesize neuroactive compounds [9]. As such, probiotics may affect the brain in a direct manner by producing neurotransmitters and neuromodulators and may therefore have the potential to act as a novel treatment for neuropsychiatric diseases. The delivery of neurochemicals by probiotics may either be in the amount already contained in the bacterium at time of ingestion or what is actively produced by the bacterium once inside the gastrointestinal tract (GIT).

It is well recognized that some bacteria within the human GIT have the capacity to produce many neurotransmitters and neuromodulators. For example, *Lactobacillus* spp. and *Bifidobacterium* spp. have been reported to produce GABA; *Escherichia* spp., and *Bacillus* spp. have been reported to produce norepinephrine; *Streptococcus* spp., *Escherichia* spp. and *Enterococcus* spp. have been reported to produce serotonin; *Bacillus* spp. have been reported to produce dopamine, and *Lactobacillus* spp. have been reported to produce acetylcholine and histamine [10–14]. It is possible that the secreted neurotransmitters from bacteria in the intestinal lumen may induce epithelial cells to release molecules that in turn modulate neural signalling within the enteric nervous system, or act directly on primary afferent axons [15]. Other bacterially-produced metabolites with proven neuroactive functions include short chain fatty acids (SCFAs) and long chain fatty acids such as conjugated linoleic acid (CLA). Table 10.1 provides a list of a range of neuroactive chemicals isolated from bacteria within the human gut (it should be noted that this is representative of neuroactives isolated, but is not a complete and comprehensive list). The production of these metabolites and the aforementioned neuroactives by bacteria naturally inhabiting the human gut will be discussed in this chapter.

Bacterial Metabolites

The microbiome has a capability to produce a spectrum of neuroactive compounds, and although we are still in an early stage of exploring its capacity, there is an expanding volume of evidence supporting the role of our intestinal inhabitants as being factories for neurochemicals. Studies comparing germ-free (GF) animals (lacking gut microbiota) with conventional animals (with a normal gut microbiota)

Table 10.1 Representative list of neurochemicals isolated from bacteria within the human gut

Genus	Neurochemical	References
Lactobacillus, Bifidobacterium	GABA	[10]
Streptococcus, Escherichia, Enterococcus, Lactococcus, Lactobacillus	Serotonin	[17, 49]
Escherichia, Bacillus	Norepinephrine	[14, 49]
Escherichia, Bacillus, Lactococcus, Lactobacillus, Streptococcus	Dopamine	[14, 17, 49]
Lactobacillus, Bacillus	Acetylcholine	[12, 13, 59, 61]
Lactobacillus, Lactococcus, Streptococcus, Enterococcus	Histamine	[11, 66, 67]

have demonstrated that the commensal microbiota influence monoamine levels in specific brain regions of the host brain [3, 16]. Neurochemicals that have been isolated from gut bacteria include GABA, noradrenaline, serotonin, dopamine and acetylcholine [10, 17, 18], which may directly affect the brain. The use of probiotic bacteria that can deliver neurochemicals has further been suggested as a novel treatment for neuropsychiatric diseases [9]. Other bacterial metabolites with neuroactive functions include SCFAs such as propionate and long chain fatty acids such as CLA [19–22].

It is not yet clear as to why certain bacteria harbour the genes responsible for the production of neuroactive molecules. It has been proposed that late horizontal gene transfer can explain the existence of genes encoding many of the enzymes involved in the synthetic and metabolic pathways of catecholamines, acetylcholine, and GABA from bacteria. This concept is concordant with increasing evidence that signalling molecules of quorum-sensing systems, used by bacteria to communicate and coordinate their actions [23] can also bind to mammalian receptors and directly influence the host [24, 25]. Neurotransmitters that are produced by the host can furthermore influence the function of members of the microbiota. As an example, the QseC sensor kinase, present in *Escherichia coli* O157:H7 is a bacterial receptor for host-derived epinephrine/norepinephrine which triggers the transcription of virulence genes in bacteria, a response which can be blocked by adrenergic antagonists [26].

Gamma-Aminobutyric Acid

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the brain regulating many physiological and psychological processes, and dysfunctions in GABA signalling have been linked to anxiety and depression [27]. We have recently demonstrated that human intestinally derived strains of lactobacilli and bifidobacteria produce GABA from monosodium glutamate (MSG) in culture [10] and it has been suggested that microbially produced GABA may have an effect on the brain-gut axis [28]. The production of GABA by commensal bacteria occurs via

the same biosynthetic pathway as in neuronal tissue involving conversion of glutamate by the action of the enzyme glutamate decarboxylase (GAD) and vitamin co-factor pyridoxal phosphate [29]. The GABA-producing capability held by some bacterial strains is thought to protect the organism from the acidic environment encountered in the stomach, since its synthesis involves proton exchange for the uptake of glutamate [30]. Many studies have reported the presence of a *gad* gene in lactic acid bacteria (LAB) [29, 31, 32] and given the heightened interest in the physiological effects associated with GABA, many GABA-enriched fermented food products, using dairy starter cultures with GABA-producing capabilities, have been developed in the past 10 years [31, 33, 34]. The levels of GABA that can be achieved in vitro by probiotic organisms are quite large. For example, in the production of fermented foodstuffs, such as Japanese funa-sushi and Chinese traditional paocai, which uses lactobacilli as starter cultures, GABA levels in the millimolar range have been detected in the final products [35, 36].

The prevalence of GABA-producing lactobacilli and bifidobacteria in the human GIT is not as widespread as it is among food-derived LAB. We screened 91 strains of human-derived lactobacilli and bifidobacteria for their ability to produce GABA from MSG, and found that five strains had the ability [10], with *Lactobacillus brevis* and *Bifidobacterium dentium* being the most efficient GABA producers. *L. brevis* DPC6108 was the most efficient of the strains tested, and it retained the capability to produce GABA in the presence of other gut-derived bacteria (in faecal fermentations). A recent study also demonstrated that GABA production in black soybean milk by *L. brevis* FPA3709 and its administration to rats resulted in an antidepressant effect similar to that of fluoxetine (a common antidepressant drug) but without the side effects of lost appetite and decreased weight [37]. Interestingly, neuronal cells have been shown to respond to nanomolar concentrations of GABA [38]. At the level of gene expression, ingestion of the *Lactobacillus* strain, *Lactobacillus rhamnosus* (JB-1), altered the mRNA expression of both $GABA_A$ and $GABA_B$ receptors. These receptors are implicated in anxiety and depression, and are widely expressed in key brain regions responsible for maintaining normal fear and mood responses [39].

A number of further potential health benefits of GABA have been described, including induction of hypotension, diuretic effects, and tranquilizer effects [40, 41]. Furthermore, GABA has a receptor-mediated role in a number of immunological (i.e. down-regulation of cytokine released by proinflammatory cells release) and intestinal neurophysiological (i.e. secretion of neuropeptides by intrinsic and extrinsic intestinal nerve fibers) processes [38, 42, 43]. Given the broad health benefits associated with GABA, the use of a GABA-secreting bacterium, acting on dietary glutamate, could have potential in both neuropsychiatric diseases and in inflammatory conditions such as inflammatory bowel disease (IBD), however, in vivo studies are required to ascertain whether the host would benefit from microbially- produced GABA.

Serotonin and 5-HT Precursors

Serotonin (5-hydroxytryptamine, 5-HT) is a metabolite of the essential amino acid tryptophan and plays an important role in the regulation of a number of bodily functions, including mood. Today, the vast majority of antidepressant drugs lead to increases in the levels of serotonin in the brain. Serotonin is ubiquitously distributed in nature and has been found in some plants (fruits and nuts) and in both vertebrates and invertebrates animals [44]. Some studies also indicate that bacteria can synthesize serotonin and/or induce its production by the host. Wikoff et al. [45] utilized a metabolomics-based approach to study the metabolic products of the microbiome in mice which may impact health, and unexpectedly found that serotonin plasma levels were nearly threefold higher in conventional mice compared with GF mice, whereas plasma concentrations of tryptophan was 40 % lower in conventional animals than in their GF counterparts. The authors postulated that the increased plasma serotonin levels observed could indirectly result from an as yet undefined host microbe interaction [45]. Furthermore, increased serotonin turnover and altered levels of related metabolites in the striatum [3] and hippocampus [46] of GF mice have been reported. The levels of serotonin in the cortex and hippocampus were also significantly reduced in GF mice [4], suggesting a role for the microbiota in maintaining serotonin levels. Rats that were given *Bifidobacterium infantis* for 14 days had increased concentrations of the serotonin precursor tryptophan in plasma, suggesting that commensal bacteria have the ability to influence tryptophan metabolism [47]. This effect on tryptophan metabolism may be mediated by the impact of the microbiota on the expression of indoleamine-2,3-dioxygenase, a key enzyme in the physiologically dominant kynurenine pathway of tryptophan degradation [48]. Moreover, Özogul [17] investigated the influences of LAB on biogenic amine formation by foodborne pathogens using single and mix cultures. All the LAB species used in their study, including *Lactococcus lactis* subsp. *cremoris* (MG 1363), *L. lactis* subsp. *lactis* (IL1403), *Lactobacillus plantarum* (FI8595) and *Streptococcus thermophilus* (NCFB2392) produced serotonin to some extent [17]. Shishov et al. [49] also demonstrated that *E. coli* K-12 was capable of producing serotonin at nanomolar concentrations in culture.

Catecholamines

Catecholamines such as dopamine and norepinephrine (also known as noradrenaline) are the major neurotransmitters that mediate a variety of the CNS functions, such as motor control, cognition, memory processing, emotion and endocrine regulation [50]. Dysfunctions in catecholamine neurotransmission are implicated in some neurological and neuropsychiatrical disorders, including Parkinson's disease [51], Alzheimer's disease [52] and major depressive disorders [53].

Both norepinephrine and dopamine were identified in bacteria in a study by Tsavkelova et al. [14]. Dopamine, in concentrations from 0.45 to 2.13 mmol/L was found in the biomass of *Bacillus cereus*, *B. mycoides*, *B. subtilis*, *Proteus vulgaris*, *Serratia marcescens*, *S. aureus*, and *E. coli*, and norepinephrine was detected (0.21–1.87 mmol/L) in *B. mycoides*, *B. subtilis*, *P. vulgaris*, and *S. marcescens*. Moreover, it was demonstrated that bacteria, particularly *B. subtilis* may release norepinephrine and dopamine out of the cell and perhaps in this way might participate in intercellular microbe–microbe and microbe–host communications [14]. Shishov et al. [49] used the *E. coli* K-12 strain to investigate the production of catecholamines in vitro and demonstrated that this *E. coli* strain can produce dopamine and norepinephrine and also their precursor, DOPA, in culture. The culture fluid of *E. coli* contained micromolar concentrations of DOPA and nanomolar concentrations of dopamine and norepinephrine [49]. Moreover, Özogul [17] demonstrated the production of dopamine by some LAB species in culture, namely *L. lactis* subsp. *cremoris* (MG 1363), *L. lactis* subsp. *lactis* (IL1403), *L. plantarum* (FI8595) and *S. thermophilus* (NCFB2392). A recent study by Asano et al. [54] demonstrated that bacteria which constitute the normal microbiome in mice are capable of the in vivo production of large quantities of norepinephrine. Furthermore, adoptive transfer of the microbiome of mice that could produce norepinephrine in vivo to GF mice resulted in the in vivo elaboration of norepinephrine within the murine GIT [54]. Interestingly, in many cases, the content of catecholamines found in bacteria is higher than in human blood, for example concentrations of norepinephrine in human blood are found to be 0.04 mmol/L [55].

Acetylcholine

Acetylcholine is a well-known neurotransmitter in the central and peripheral nervous systems that plays a critical role in cognitive function, particularly in memory and learning. It is synthesized by choline acetyltransferase in the CNS and by both choline acetyltransferase and carnitine acetyltransferase in the peripheral system [56, 57]. Acetylcholine has also been identified in non-neuronal tissues, including gastrointestinal, respiratory and urogenital epithelial cells [58]. In addition, acetylcholine has been found to be a component of bacteria and its production was discovered in a strain of *L. plantarum* [13, 59, 60]. Cell free enzyme(s) participating in acetylcholine synthesis were also found in *L. plantarum* [59]. Horiuchi et al. [61] tested three different bacterial strains for acetylcholine content and synthesis, *E. coli* JCM 5491, *Staphylococcus aureus* JCM 2151 and *Bacillus subtilis* PCI 219. Among these, a substantial amount of acetylcholine was detected in *B. subtilis* (55.7 pmol/10^{10} colony forming units), while much smaller amounts were found in *E. coli* (2.22 pmol) and *S. aureus* (0.39 pmol). Although acetylcholine synthesis was detected in all bacterial samples, the levels were low and the authors suggested that an acyltransferase other than choline

acetyltransferase and carnitine acetyltransferase was responsible for acetylcholine synthesis in these bacteria [61].

Histamine

Histamine acts as a modulatory neurotransmitter in the mammalian brain and has an important role in the maintenance of wakefulness, while dysfunction in the histaminergic system has been linked to narcolepsy [62]. Moreover, behavioural studies suggest that the histaminergic system in the brain has important roles in cognitive function [63]. Levels of histamine are decreased in the hippocampus, temporal cortex and hypothalamus of patients with Alzheimer's disease, suggesting that histaminergic neurons undergo degeneration and contribute to cognitive decline in this disorder [64].

Histamine is produced via histidine decarboxylase (HDC) of L-histidine, an essential amino acid for humans that is present in many dietary foods [65]. Some fermentative bacteria, including strains of *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus* and *Enterococcus* have been reported to possess the HDC-gene and to produce histamine at different levels [66, 67]. However, the production of histamine by certain bacterial strains has caused alarm as a health risk in food and as a marker of food spoilage. Ingestion of food containing high concentrations of histamine has been linked with headaches, vomiting and hypertension [68]. Nonetheless, histamine production by the probiotic strain *Lactobacillus reuteri* (ATCC PTA 6475) was recently reported, resulting in a suppression of human proinflammatory tumor necrosis factor (TNF) production [11]. Moreover, histidine supplementation increased the expression of HDC-genes and the production of histamine by *L. reuteri*. In addition to the role of histamine in immunomodulation observed in the study by Thomas et al. [11], luminal production of histamine by *L. reuteri* 6475 may influence signalling in the enteric nervous system. However, in vivo studies are needed to gain a better understanding of the role of bacterially-produced histamine in the gut and that such production does not induce negative side-effects.

Indole-3-Propionic Acid

Indole-3-propionic acid (IPA) is a deamination product of tryptophan and is found in plasma and cerebrospinal fluid [69, 70]. IPA has been shown to be a powerful antioxidant [71] and has been considered as a possible treatment for Alzheimer's disease [72] due to its ability to protect neurons and neuroblastoma cells against oxidative damage and death [73, 74].

Wikoff et al. [45] demonstrated that the production of IPA was completely dependent on the presence of gut microbiota and could also be established by

colonizing germ-free mice with the bacterium *Clostridium sporogenes*. An earlier study by Jellet et al. [75] also demonstrated that IPA was present in the spent bacterial media of *C. sporogenes* and that addition of the precursor tryptophan to the media greatly enhanced the formation of IPA. In addition, IPA was shown to be produced in vitro when human large intestinal contents were incubated with tryptophan and indolelactate [76].

Short-Chain Fatty Acids

Short chain fatty acids (SCFA) are the major products of the bacterial fermentation of carbohydrates and proteins in the GIT [19, 77]. The main compounds are acetic, propionic and n-butyric acids, occurring roughly in molar ratios of 60:20:20 in the colon [78]. Through their absorption and metabolism, the host is able to salvage energy from foodstuffs, particularly resistant starch and fibers that are not digested in the upper part of the GIT. The main site for SCFA production and absorption is the proximal large intestine, where the fermentation of undigested food by colonic bacteria occurs at high rates. Bacteria that produce SCFA include, but are not limited to, *Bacteroides*, *Bifidobacterium*, *Propionibacterium*, *Eubacterium*, *Lactobacillus*, *Clostridium*, Roseburia and Prevotella [19]. SCFA have a multiplicity of effects in the body, and affect epithelial cell transport and metabolism, epithelial cell growth and differentiation, and hepatic control of lipid and carbohydrates, while providing energy sources for muscles and kidneys, as well as the heart and brain [79]. Epithelial cells in the distal colon derive 60–70 % of their energy requirements from bacterial fermentation products [80]. SCFA also act as signalling molecules. Propionate, acetate, and to a lesser extent butyrate are ligands for at least two G protein-coupled receptors (GPCRs), Gpr41 and Gpr43, which are broadly expressed in the distal small intestine, colon and adipocytes [81, 82]. SCFA interaction with Gpr43 can profoundly affect inflammatory responses. For example, mice treated with oral acetate showed a substantial decrease in inflammation. This protection was mediated by acetate binding to Gpr43, because acetate had no effect in Gpr43-deficient mice. Furthermore, it was shown that Gpr43 exhibited enhanced expression in neutrophils and eosinophils, suggesting that SCFA-Gpr43 signalling is one of the molecular pathways whereby commensal bacteria regulate immune and inflammatory responses [83]. Gpr43 is also induced during adipocyte differentiation and exhibits increased levels during high-fat feeding in rodents, suggesting that Gpr43 may also affect adipocyte function [84]. Hong et al. [85] demonstrated that acetate and propionate act on lipid accumulation and inhibition of lipolysis mainly through Gpr43 [85]. Gpr41 has been shown to be implicated in microbiota-dependent regulation of host adiposity and leptin production [86].

SCFA can cross the blood-brain barrier and enter the CNS [87] and are taken up by glia and, to a lesser extent, by neurons, where they are thought to comprise a major energy source in cellular metabolism, particularly during early brain

development [88–90]. They also play a role in cell signalling [91] and neurotransmitter synthesis and release [92]. Moreover, SCFA have been shown to increase the synthesis of dopamine and its related catecholamines through induction of tyrosine hydroxylase, a key enzyme in the synthesis of catecholamines [93]. Propionate, in particular has been shown to alter dopamine, serotonin, and glutamate systems in a manner similar to that observed in autism spectrum disorders (ASD) [94, 95]. Furthermore, intraventricular infusion of propionate in rats was shown to impair social behaviour and cause brain abnormalities, similar to those detected in human autism [96–98] and furthermore to alter brain phospholipid composition [99]. Some clinical studies have also found that a subset of ASD patients have high levels of *Clostridial* and *Bacteriodetes* species in the gut [100, 101], species which are efficient propionate-producers [19]. This highlights that although propionate is beneficial at appropriate levels, such as lowering lipogenesis, serum cholesterol levels and improving insulin sensitivity [102], excessive propionate may have negative effects on health and behaviour.

Butyrate is known to exhibit many important physiological functions in eukaryotic cells [19]. One of the most recognised cellular mechanisms for the action of butyrate is its effects on histone acetylation [103], where the inhibition of histone deacetylase facilitates hyperacetylation of histone proteins to occur, thus facilitating the access of DNA repair enzymes. Interestingly, sodium butyrate has been demonstrated to elicit an antidepressant effect in the murine brain [104]. When injected systemically, sodium butyrate induced a short-lasting, transient acetylation of histones in frontal cortex and hippocampus, in conjunction with dynamic changes in expression of brain-derived neurotrophic factor (BDNF), thereby resulting in an antidepressant-like behavioral response [104].

Long-Chain Fatty Acids

Long-chain fatty acids (LC-PUFAs) play numerous roles in the brain, including structural (forming the physico-chemical properties in the lipid bilayer of cellular membranes) and signalling functions. Moreover, they influence neurogenesis and neurotransmission within the nervous tissue. Arachidonic acid (AA, C20:4n-6) and docosahexaenoic acid (DHA, C22:6n-3) are highly concentrated in the brain and are vital fatty acids for neurological development [105, 106]. Furthermore, there is a growing body of evidence for their role in mental health across the lifespan [107]. Being the major structural components of brain cells [108], AA and DHA influence cell membrane physical properties, enzyme activity, regulation of ion channels and neuroreceptors and their signalling (neurotransmission) [109, 110].

Recently, we have reported that administration of a *Bifidobacterium breve* strain, *B. breve* NCIMB702258 to mice had a significant impact on the fatty acid composition of brain [111]. Mice that received this strain for 8 weeks exhibited significantly higher concentrations of AA and DHA in the brain when compared to unsupplemented mice. Interestingly, this effect was bacterial strain-dependent, as

it was not induced by the *B. breve* strain DPC6330. The mechanism by which the *B. breve* strain alters the fatty acid composition is currently unknown. Possible explanations include modulations of fat-absorption processes in the small intestine and/or desaturase activities involved in the metabolism of fatty acids to the longer-chain unsaturated derivatives caused either directly by the strain administered or by alterations in the gut microbiota. Interestingly, it was previously postulated that different members of the gut microbiota promote fatty acid absorption via distinct mechanisms [112]. Semova et al. [112] used the zebrafish model to investigate how microbiota and diet interact to regulate lipid absorption in the gut epithelium. By comparing GF zebrafish with conventional zebrafish, the authors demonstrated that the microbiota stimulate fatty acid uptake and lipid droplet formation in both the intestinal epithelium and liver [112]. Previous studies have also demonstrated that manipulation of the gut microbiota by probiotics resulted in altered fat composition in the host [113–115]. Although the adult microbiome is not known to be particularly enriched in genes involved in fatty acid metabolism [116], there are indications that interactions between fatty acids and components of the gut microbiota occur which could affect the biological roles of both. However, a deeper knowledge of such interactions and what consequences they have for the host are warranted.

Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) comprises a mixture of positional and geometric isomers of linoleic acid (*cis*-9, *cis*-12 C18:2n-6) characterised by the presence of conjugated double bonds with *cis* or *trans* configurations. CLA is a natural component of ruminant milk and tissue fat as a result of the action of the ruminal microbiota on dietary linoleic acid [117]. A range of health-promoting activities have been attributed to the consumption of CLA, most notably anticarcinogenic, immune-modulatory, anti-obesity and anti-atherosclerotic activities [118–121]. In contrast, in certain conditions, some CLA isomers can also exert potentially negative effects such as liver steatosis and insulin resistance [122, 123]. Regarding the action of CLA on the CNS, it is known that CLA crosses the blood-brain barrier and is incorporated and metabolized in the brain [124]. Dietary CLA has been shown to reduce cerebral prostaglandin E2 in the peripheral and CNS [125], and to exert antiangiogenic actions in the brain [126]. Furthermore, Hunt et al. [127] showed that CLA protects cortical neurons from excitotoxicity at concentrations likely achieved by consumption of CLA as a dietary supplement.

Evidence of the role of the gut microbiota in the endogenous production of CLA was first reported by Chin et al. [128] who observed that increasing the amount of linoleic acid in the diet increased the tissue content of CLA in conventional rats but not in GF rats. Since then, commensal lactobacilli and bifidobacteria from the human GIT, most notably *B. breve* strains and *L. plantarum* strains, have been shown to produce CLA, predominantly the *cis*-9, *trans*-11 (c9, t11) isomer from free linoleic acid [21–23, 129]. These bacteria convert linoleic acid to CLA in a

similar manner to ruminant bacteria via the action of the enzyme linoleic acid isomerase [130], which also has been sequenced in some *Lactobacillus* and *Bifidobacterium* strains [131, 132]. We have reported that the CLA-producing bacterium, *B. breve* NCIMB702258 converts linoleic acid to CLA in the murine gut, resulting in significantly elevated c9, t11 CLA in the liver [133]. Moreover, Bassaganya-Riera et al. [134] demonstrated that administration of the probiotic mixture VSL#3 to mice with colitis resulted in high colonic concentrations of c9, t11 CLA and that this locally produced CLA improved colitis by activating peroxisome proliferator-activated receptor gamma (PPAR γ) in macrophages. Two studies have also reported the in vivo production of the CLA isomer, *trans*-10, *cis*-12 (t10, c12) CLA, by using two strains of human origin, *L. rhamnosus* PL60 and *L. plantarum* PL62. Administration of these two strains to mice resulted in increased t10, c12 CLA concentrations in sera with subsequent reductions in adipose tissue and body weight [135, 136]. Druart et al. [137] further demonstrated that prebiotic supplementation increased CLA content in caecal tissue, by increasing substrate availability and by modulating gut microbiota composition.

Although dietary CLA has been reported to affect the CNS, the consequences of bacterially-produced CLA on nervous system function are yet to be discovered.

Conclusion

Although we are still at the very early stages of understanding the complex communication systems between gut bacteria and the brain, we know that certain bacteria within the human gut have the ability to produce molecules with neuroactive functions which could affect the brain in a direct manner. However, only cultivable bacteria have been tested for their capacity to produce neuroactive compounds in vitro and only a limited number of bacterial strains have been tested up to now. Moreover, in complex microbial ecosystems such as in the human gut, interactions and competition exist between bacteria, which are not studied upon simple culture conditions in vitro. This highlights the need for in vivo studies to elucidate the role of metabolite-producing bacteria and what effect such bacteria, and their components, have on nervous system function and behaviour. Such future studies may also facilitate our understanding of the consequences of neuroactive compound production by the microbiota and how probiotic bacteria can influence the CNS, and could furthermore identify the potential for neurochemical containing/producing probiotic bacteria as a therapeutic strategy in the treatment of certain neurological and neurophysiological conditions. Given that molecular tools have now been developed for many intestinal organisms, the possibility exists now to overproduce neuroactive compounds and/or to regulate their production in response to gut metabolites such as bile.

Acknowledgements This work was supported by the Science Foundation of Ireland—funded Centre for Science, Engineering and Technology, the Alimentary Pharmabiotic Centre.

References

1. Dinan TG, Stanton C, Cryan JF (2013) Psychobiotics: a novel class of psychotropic. Biol Psychiatry 74(10):720–726
2. Collins SM, Surette M, Bercik P (2012) The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol 10(11):735–742
3. Heijtz RD, Wang S, Anuar F, Qian Y, Bjorkholm B, Samuelsson A, Hibberd ML, Forssberg H, Pettersson S (2011) Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A 108:3047–3052
4. Neufeld K, Kang N, Bienenstock J, Foster J (2011) Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil 23:255–264
5. Rhee SH, Pothoulakis C, Mayer EA (2009) Principles and clinical implications of the brain-gut-enteric microbiota axis. Nat Rev Gastroenterol Hepatol 6:306–314
6. O'Mahony SM, Hyland NP, Dinan TG, Cryan JF (2011) Maternal separation as a model of brain-gut axis dysfunction. Psychopharmacology (Berl) 214:71–88
7. Cryan JF, O'Mahony SM (2011) The microbiome-gut-brain axis: from bowel to behaviour. Neurogastroenterol Motil 23:187–192
8. FAO/WHO (2001) Report on Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. ftp://ftp.fao.org/es/esn/food/probio_report_en.pdf
9. Lyte M (2011) Probiotics function mechanistically as delivery vehicles of neuroactive compounds: microbial endocrinology in the design and use of probiotics. Bioessays 33(8): 574–581
10. Barrett E, Ross RP, O'Toole PW, Fitzgerald GF, Stanton C (2012) γ-Amino butyric acid production by culturable bacteria from the human intestine. J Appl Microbiol 113:411–417
11. Thomas CA, Hong T, van Pijkeren JP, Hemarajata P, Trinh DV, Hu W, Britton RA, Kalkum M, Versalovic J (2012) Histamine derived from probiotic *Lactobacillus reuteri* suppress TNF via modulation of PKA and ERK signalling. PLoS One 7(2):e31951
12. Kawashima K, Misawa H, Moriwaki Y, Fujii YX, Fujii T, Horiuchi Y, Yamada T, Imanaka T, Kamekura M (2007) Ubiquitous expression of acetylcholine and its biological functions in life forms without nervous systems. Life Sci 80:2206–2209
13. Marquardt P, Spitznagel G (1959) Bakterielle Acetylcholine Bildung in Kunstlichen Nahrboden. Arzneimittelforschung 9:456–465
14. Tsavkelova EA, Botvinko IV, Kudrin VS, Oleskin AV (2000) Detection of neurotransmitter amines in microorganisms using of high performance liquid chromatography. Dokl Biochem 372:115–117 (in Russian issue 840–842)
15. Forsythe P, Kunze WA (2013) Voices from within: gut microbes and the CNS. Cell Mol Life Sci 70:55–69
16. Nishino R, Mikami K, Takahashi H, Tomonaga S, Furuse M, Hiramoto T, Aiba Y, Koga Y, Sudo N (2013) Commensal microbiota modulate murine behaviors in a strictly contamination-free environment confirmed by culture-based methods. Neurogastroenterol Motil 25:521–e371
17. Özogul F (2011) Effects of specific lactic acid bacteria species on biogenic amine production by foodborne pathogens. Int J Food Sci Technol 46(3):478–484
18. Roshchina VV (2010) Evolutionary considerations of neurotransmitters in microbial, plant, and animal cells. In: Lyte M, Freestone PPE (eds) Microbial endocrinology: interkingdom signaling in infectious disease and health. Springer, New York, pp 17–52
19. Macfarlane GT, Macfarlane S (2012) Bacteria, colonic fermentation, and gastrointestinal health. J AOAC Int 95(1):50–60
20. Coakley M, Ross RP, Nordgren M, Fitzgerald G, Devery R, Stanton C (2003) Conjugated linoleic acid biosynthesis by human-derived *Bifidobacterium* species. J Appl Microbiol 94: 138–145

21. Barrett E, Ross RP, Fitzgerald GF, Stanton C (2007) Rapid screening method for analyzing the conjugated linoleic acid production capabilities of bacterial cultures. Appl Environ Microbiol 73:2333–2337
22. Rosberg-Cody E, Ross RP, Hussey S, Ryan CA, Murphy BP, Fitzgerald GF, Devery R, Stanton C (2004) Mining the microbiota of the neonatal gastrointestinal tract for CLA-producing bifidobacteria. Appl Environ Microbiol 70:4635–4641
23. Hughes DT, Sperandio V (2008) Inter-kingdom signalling: communication between bacteria and their hosts. Nat Rev Microbiol 6:111–120
24. Boontham P, Robins A, Chandran P, Pritchard D, Camara M, Williams P, Chuthapisith S, McKechnie A, Rowlands BJ, Eremin O (2008) Significant immunomodulatory effects of *Pseudomonas aeruginosa* quorum-sensing signal molecules: possible link in human sepsis. Clin Sci (Lond) 115:343–351
25. Telford G, Wheeler D, Williams P, Tomkins PT, Appleby P, Sewell H, Stewart GS, Bycroft BW, Pritchard DI (1998) The *Pseudomonas aeruginosa* quorum-sensing signal molecule *N*-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity. Infect Immun 66: 36–42
26. Clarke MB, Hughes DT, Zhu C, Boedeker EC, Sperandio V (2006) The QseC sensor kinase: a bacterial adrenergic receptor. Proc Natl Acad Sci U S A 103:10420–10425
27. Schousboe A, Waagepetersen HS (2007) GABA: homeostatic and pharmacological aspects. In: Tepper JM, Abercrombie ED, Bolam JP (eds) Gaba and the basal ganglia: from molecules to systems. Elsevier Science, Amsterdam, pp 9–19
28. Bienenstock J, Forsythe P, Karimi K, Kunze W (2010) Neuroimmune aspects of food intake. Int Dairy J 20:253–258
29. Komatsuzaki N, Nakamura T, Kimura T, Shima J (2008) Characterization of glutamate decarboxylase from a high gamma-aminobutyric acid (GABA)-producer, *Lactobacillus paracasei*. Biosci Biotechnol Biochem 72:278–285
30. Higuchi T, Hayashi H, Abe K (1997) Exchange of glutamate and gammaaminobutyrate in a *Lactobacillus* strain. J Bacteriol 179:3362–3364
31. Siragusa S, De Angelis M, Di Cagno R, Rizzello CG, Coda R, Gobbetti M (2007) Synthesis of gammaaminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. Appl Environ Microbiol 73:7283–7290
32. Hiraga K, Ueno YH, Oda KH (2008) Glutamate decarboxylase from *Lactobacillus brevis*: activation by ammonium sulfate. Biosci Biotechnol Biochem 72:1299–1306
33. Park KB, Oh SH (2006) Isolation and characterization of *Lactobacillus buchneri* strains with high gamma-aminobutyric acid producing capacity from naturally aged cheese. Food Sci Biotechnol 15:86–90
34. Rizzello CG, Cassone A, Di Cagno R, Gobbetti M (2008) Synthesis of angiotensin I-converting enzyme (ACE)-inhibitory peptides and gamma-aminobutyric acid (GABA) during sourdough fermentation by selected lactic acid bacteria. J Agric Food Chem 56: 6936–6943
35. Komatsuzaki N, Shima J, Kawamoto S, Momose H (2005) Production of gamma-aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. Food Microbiol 22:497–504
36. Li HX, Gao DD, Cao YS, Xu HY (2008) A high gamma-aminobutyric acid-producing *Lactobacillus brevis* isolated from Chinese traditional paocai. Ann Microbiol 58:649–653
37. Ko CY, Victor Lin HT, Tsai GJ (2013) Gamma-aminobutyric acid production in black soybean milk by *Lactobacillus brevis* FPA 3709 and the antidepressant effect of the fermented product on a forced swimming rat model. Process Biochem 48(4):559–568
38. Krantis A (2000) GABA in the mammalian enteric nervous system. News Physiol Sci 15: 284–290
39. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienestock J, Cryan JF (2011) Ingestion of *Lactobacillus* strain regulates emotional behavior and central

GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 108: 16050–16055
40. Jakobs C, Jaeken J, Gibson KM (1993) Inherited disorders of GABA metabolism. J Inherit Metab Dis 16:704–715
41. Wong CG, Bottiglieri T, Snead OC (2003) GABA, gammahydroxybutyric acid, and neurological disease. Ann Neurol 54:S3–S12
42. Bjurstom H, Wang J, Ericsson I, Bengtsson M, Liu Y, Kumar-Mendu S, Issazadeh-Navikas S, Birnir B (2008) GABA, a natural immunomodulator of T lymphocytes. J Neuroimmunol 205: 44–50
43. Page AJ, O'Donnell TA, Blackshaw LA (2006) Inhibition of mechanosensitivity in visceral primary afferents by GABAB receptors involves calcium and potassium channels. Neuroscience 137:627–636
44. Kema IP, de Vries EG, Muskiet FA (2000) Clinical chemistry of serotonin and metabolites. J Chromatogr B Biomed Sci Appl 747(1–2):33–48
45. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, Siuzdak G (2009) Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A 106:3698–3703
46. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, Dinan TG, Cryan JF (2013) The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. Mol Psychiatry 18:666–673
47. Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TG (2008) The probiotic Bifidobacteria infantis: an assessment of potential antidepressant properties in the rat. J Psychiatr Res 43:164–174
48. Forsythe P, Sudo N, Dinan T, Taylor VH, Bienenstock J (2010) Mood and gut feelings. Brain Behav Immun 24:9–16
49. Shishov VA, Kirovskaia TA, Kudrin VS, Oleskin AV (2009) Amine neuromediators, their precursors, and oxidation products in the culture of *Escherichia coli* K-12. Prikl Biokhim Mikrobiol 45(5):550–554
50. Kobayashi K (2001) Role of catecholamine signalling in brain and nervous system functions: new insights from mouse molecular genetic study. J Investig Dermatol Symp Proc 6:115–121
51. Calabresi P, Castrioto A, Di Filippo M, Picconi B (2013) New experimental and clinical links between the hippocampus and the dopaminergic system in Parkinson's disease. Lancet Neurol 12:811–821
52. Robertson IH (2013) A noradrenergic theory of cognitive reserve: implications for Alzheimers disease. Neurobiol Aging 34(1):298–308
53. Hamon M, Blier P (2013) Monoamine neurocircuitry in depression and strategies for new treatments. Prog Neuropsychopharmacol Biol Psychiatry 45:54–63
54. Asano Y, Hiramoto T, Nishino R, Aiba Y, Kimura T, Yoshihara K (2012) Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. Am J Physiol Gastrointest Liver Physiol 303(11):G1288–G1295
55. Kruk ZL, Pycock CJ (1990) Neurotransmitters and drugs. Chapman and Hall, New York
56. Tucˇek S (1988) Choline acetyltransferase and the synthesis of acetylcholine. In: Whittaker VP (ed) Handbook of experimental pharmacology. The cholinergic synapse, vol 86. Springer, Berlin, pp 125–165
57. Tucˇek S (1882) The synthesis of acetylcholine in skeletal muscles of the rat. J Physiol 322:53–69
58. Wessler I, Kirkpatrick CJ, Racke K (1999) Cholinergic "pitfall": acetylcholine, a universal cell molecule widely distributed in biological systems: expression and function in humans. Clin Exp Pharmacol Physiol 26:198–205
59. Girvin GT, Stevenson JW (1954) Cell free "choline acetylase" from *Lactobacillus plantarum*. Can J Biochem Physiol 32:131–146
60. Rowatt E (1948) The relation of pantothenic acid to acetylcholine formation by a strain of *Lactobacillus plantarum*. J Gen Microbiol 2:25–30

61. Horiuchi Y, Kimura R, Kato N, Fujii T, Seki M, Endo T, Kato T, Kawashima K (2003) Evolutional study on acetylcholine expression. Life Sci 72:1745–1756
62. Panula P, Nuutinen S (2013) The histaminergic network in the brain: basic organization and role in disease. Nat Rev Neurosci 14:472–487
63. Alvarez EO (2009) The role of histamine on cognition. Behav Brain Res 199:183–189
64. Airaksinen MS, Paetau A, Paljarvi L, Reinikainen K, Riekkinen P, Suomalainen R, Panula P (1991) Histamine neurons in human hypothalamus: anatomy in normal and Alzheimer diseased brains. Neuroscience 44:465–481
65. Wu G (2009) Amino acids: metabolism, functions, and nutrition. Amino Acids 37:1–17
66. Landete JM, de las Rivas B, Marcobal A, Munoz R (2008) Updated molecular knowledge about histamine biosynthesis by bacteria. Crit Rev Food Sci Nutr 48:697–714
67. Coton E, Rollan G, Bertrand A, Lonvaud-Funel A (1998) Histamine producing lactic acid bacteria: early detection, frequency and distribution. Am J Enol Vitic 49:199–204
68. Shalaby AR (1996) Significance of biogenic amines in food safety and human health. Food Res Int 29:675–690
69. Morita I, Kawamoto M, Yoshida H (1992) Difference in the concentration of tryptophan metabolites between maternal and umbilical foetal blood. J Chromatogr 576:334–339
70. Young S, Anderson GM, Gauthier S, Purdy WC (1980) The origin of indoleacetic and indolepropionic acid in rat and human cerebrospinal fluid. J Neurochem 34:1087–1092
71. Karbownik M, Reiter RJ, Garcia JJ, Cabrera J, Burkhardt S, Osuna C, Lewinski A (2001) Indole-3-propionic acid, a melatonin-related molecule, protects hepatic microsomal membranes from iron-induced oxidative damage: relevance to cancer reduction. J Cell Biochem 81:507–513
72. Bendheim PE, Poeggeler B, Neria E, Ziv V, Pappolla MA, Chain DG (2002) Development of indole-3-propionic acid (OXIGON) for Alzheimer's disease. J Mol Neurosci 19:213–217
73. Hwang IK, Yoo KY, Li H, Park OK, Lee CH, Choi JH, Jeong YG, Lee YL, Kim YM, Kwon YG, Won MH (2009) Indole-3-propionic acid attenuates neuronal damage and oxidative stress in the ischemic hippocampus. J Neurosci Res 87:2126–2137
74. Chyan YJ, Poeggeler B, Omar RA, Chain DG, Frangione B, Ghiso J, Pappolla MA (1999) Potent neuroprotective properties against the Alzheimer β-amyloid by an exogenous melatonin-related indole structure, indole-3-propionic acid. J Biol Chem 274(31): 21937–21942
75. Jellet JJ, Forrest TP, Macdonald IA, Marrie TJ, Holdeman LV (1980) Production of indole-3-propionic acid and 3-(p-hydroxyphenyl) propionic acid by *Clostridium sporogenes*: a convenient thin-layer chromatography detection system. Can J Microbiol 26(4):448–453
76. Smith EA, Macfarlane GT (1997) Formation of phenolic and indolic compounds by anaerobic bacteria in the human large intestine. Microb Ecol 33:180–188
77. Kovatcheva-Datchary P, Zoetendal EG, Venema K, de Vos WM, Smidt H (2009) Review: tools for the tract: understanding the functionality of the gastrointestinal tract. Therap Adv Gastroenterol 2:s9–s22
78. Cummings JH (1981) Short chain fatty-acids in the human-colon. Gut 22:763–779
79. Cummings JH (1995) In: Gibson GR, Macfarlane GT (eds) Human colonic bacteria: role in nutrition, physiology and health. CRC, Boca Raton, pp 101–130
80. Topping DL, Clifton PM (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev 81(3):1031–1064
81. Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, Fraser NJ, Pike NB, Strum JC, Steplewski KM, Murdock PR, Holder JC, Marshall FH, Szekeres PG, Wilson S, Ignar DM, Foord SM, Wise A, Dowell SJ (2003) The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem 278:11312–11319
82. Xiong Y, Miyamoto N, Shibata K, Valasek MA, Motoike T, Kedzierski RM, Yanagisawa M (2004) Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc Natl Acad Sci U S A 101:1045–1050

83. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, Artis D, Xavier RJ, Teixeira MM, Mackay CR (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 461:1282–1286
84. Ge H, Li X, Weiszmann J, Wang P, Baribault H, Chen JL, Tian H, Li Y (2008) Activation of G protein-coupled receptor 43 in adipocytes leads to inhibition of lipolysis and suppression of plasma free fatty acids. Endocrinology 149(9):4519–4526
85. Hong Y, Nishimura HY, Hishikawa D, Tsuzuki H, Miyahara H, Gotoh C, Choi KC, Feng DD, Chen C, Lee HG, Katoh K, Roh SG, Sasaki S (2005) Acetate and propionate short chain fatty acids stimulate adipogenesis via GPCR43. Endocrinology 146(12):5092–5099
86. Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagizawa M, Gordon JI (2008) Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci U S A 105(43):16767–16772
87. Karuri AR, Dobrowsky E, Tannock IF (1993) Selective cellular acidification and toxicity of weak organic acids in an acidic microenvironment. Br J Cancer 68:1080–1087
88. Maurer MH, Canis M, Kuschinsky W, Duelli R (2004) Correlation between local monocarboxylate transporter 1 (MCT1) and glucose transporter 1 (GLUT1) densities in the adult rat brain. Neurosci Lett 355:105–108
89. Rafiki A, Boulland JL, Halestrap AP, Ottersen OP, Bergersen L (2003) Highly differential expression of the monocarboxylate transporters MCT2 and MCT4 in the developing rat brain. Neuroscience 122:677–688
90. Peinado A, Yuste R, Katz LC (1993) Extensive dye coupling between rat neocortical neurons during the period of circuit formation. Neuron 10:103–114
91. Nakao S, Moriya Y, Furuyama S, Niederman R, Sugiya H (1998) Propionic acid stimulates superoxide generation in human neutrophils. Cell Biol Int 22:331–337
92. DeCastro M, Nankova BB, Shah P, Patel P, Mally PV, Mishra R, La Gamma EF (2005) Short chain fatty acids regulate tyrosine hydroxylase gene expression through a cAMP-dependent signaling pathway. Brain Res Mol Brain Res 142:28–38
93. Shah P, Nankova BB, Parab S, La Gamma EF (2006) Short chain fatty acids induce TH gene expression via ERK-dependent phosphorylation of CREB protein. Brain Res 1107:13–23
94. El-Ansary AK, Ben BA, Kotb M (2012) Etiology of autistic features: the persisting neurotoxic effects of propionic acid. J Neuroinflammation 9:74
95. Mitsui R, Ono S, Karaki S, Kuwahara A (2005) Neural and nonneural mediation of propionate-induced contractile responses in the rat distal colon. Neurogastroenterol Motil 17:585–594
96. Schultz SR, MacFabe DF, Martin S, Jackson J, Taylor R, Boon F, Ossenkopp KP, Caiin DP (2009) Intracerebroventricular injections of the enteric bacterial metabolic product propionic acid impair cognition and sensorimotor ability in the Long-Evans rat: further development of a rodent model of autism. Behav Brain Res 200:33–41
97. Schultz SR, MacFabe DF, Ossenkopp KP, Scratch S, Whelan J, Taylor R, Cain DP (2008) Intracerebroventricular injection of propionic acid, an enteric bacterial metabolic end-product, impairs social behavior in the rat: implications for an animal model of autism. Neuropharmacology 54:901–911
98. MacFabe DF, Cain DP, Rodriguez-Capote K, Franklin AE, Hoffmann JE, Boon F, Taylor AR, Kavaliers M, Ossenkopp KP (2007) Neurobiological effects of intraventricular propionic acid in rats: possible role of short chain fatty acids on the pathogenesis and characteristics of autism spectrum disorders. Behav Brain Res 176:149–169
99. Thomas RH, Meeking MM, Mepham JR, Tichenoff L, Possmayer F, Liu S, MacFabe DF (2012) The enteric bacterial metabolite propionic acid alters brain and plasma phospholipid molecular species: further development of a rodent model of autism spectrum disorder. J Neuroinflammation 9:153

100. Finegold SM, Dowd SE, Gontcharova V, Liu CX, Henley KE, Wolcott RD (2010) Pyrosequencing study of fecal microflora of autistic and control children. Anaerobe 16: 444–453
101. Song Y, Liu C, Finegold SM (2004) Real-time PCR quantification of clostridia in feces of autistic children. Appl Environ Microbiol 70:6459–6465
102. Hosseini E, Grootaert C, Verstraete W, Van de Wiele T (2011) Propionate as a health-promoting microbial metabolite in the human gut. Nutr Rev 69(5):245–258
103. Kruh J, Defer N, Tichonicky L (1995) In: Cummings JH, Rombeau JL, Sakata T (eds) Physiological and clinical aspects of short chain fatty acids. Cambridge University Press, Cambridge, pp 275–288
104. Schroeder FA, Lin CL, Crusio WE, Akbarian S (2007) Antidepressant- like effects of the histone deacetylase inhibitor, sodium butyrate, in the mouse. Biol Psychiatry 62:55–64
105. Haag M (2003) Essential fatty acids and the brain. Can J Psychiatry 48(3):195–203
106. Lauritzen L, Hansen HS, Jørgensen MH, Michaelsen KF (2001) The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. Prog Lipid Res 40:1–94
107. Sinn N, Milte C, Howe PRC (2010) Oiling the brain: a review of randomised controlled trials of omega-3 fatty acids in psychopathology across the lifespan. Nutrients 2(2):128–170
108. O'Brien JS, Sampson EL (1965) Lipid composition of the normal human brain: gray matter, white matter, and myelin. J Lipid Res 6:537–544
109. Heinrichs SC (2010) Dietary ω-3 fatty acid supplementation for optimizing neuronal structure and function. Mol Nutr Food Res 54:447–456
110. Chalon S (2006) Omega-3 fatty acids and monoamine neurotransmission. Prostaglandins Leukot Essent Fatty Acids 75:259–269
111. Wall R, Marques TM, O'Sullivan O, Ross RP, Shanahan F, Quigley EM, Dinan TG, Kiely B, Fitzgerald GF, Cotter PD, Fouhy F, Stanton C (2012) Contrasting effects of *Bifidobacterium breve* NCIMB 702258 and *Bifidobacterium breve* DPC 6330 on the composition of murine brain fatty acids and gut microbiota. Am J Clin Nutr 95(5):1278–1287
112. Semova I, Carten JD, Stombaugh J, Mackey LC, Knight R, Farber SA, Rawls JF (2012) Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. Cell Host Microbe 12:277–288
113. Hoppu U, Isolauri E, Laakso P, Matomäki J, Laitinen K (2012) Probiotics and dietary counselling targeting maternal dietary fat intake modifies breast milk fatty acids and cytokines. Eur J Nutr 51(2):211–219
114. Kaplas N, Isolauri E, Lampi AM, Ojala T, Laitinen K (2007) Dietary counselling and probiotic supplementation during pregnancy modify placental phospholipid fatty acids. Lipids 45:865–870
115. Kankaanpaa PE, Yang B, Kallio HP, Isolauri E, Salminen SJ (2002) Influence of probiotic supplemented infant formula on composition of plasma lipids in atopic infants. J Nutr Biochem 13:364–369
116. Kurokawa K, Itoh T, Kuwahara T, Oshima K, Toh H, Toyoda A, Takami H, Morita H, Sharma VK, Srivastava TP, Taylor TD, Noguchi H, Mori H, Ogura Y, Ehrlich DS, Itoh K, Takagi T, Sakaki Y, Hayashi T, Hattori M (2007) Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. DNA Res 14:169–181
117. Kepler CR, Hirons KP, McNeill JJ, Tove SB (1966) Intermediates and products of the biohydrogenation of linoleic acid by *Butyrinvibrio fibrisolvens*. J Biol Chem 241:1350–1354
118. Gaullier JM, Halse J, Hoye K, Kristiansen K, Fagertun H, Vik H, Gudmundsen O (2004) Conjugated linoleic acid supplementation for 1 y reduces body fat mass in healthy overweight humans. Am J Clin Nutr 79:1118–1125
119. Ip MM, Masso-Welch PA, Ip C (2003) Prevention of mammary cancer with conjugated linoleic acid: role of the stroma and the epithelium. J Mammary Gland Biol Neoplasia 8: 103–118

120. Bassaganya-Riera J, Hontecillas R, Beitz DC (2002) Colonic anti-inflammatory mechanisms of conjugated linoleic acid. Clin Nutr 21:451–459
121. Kritchevsky D, Tepper SA, Wright S, Tso P, Czarnecki SK (2000) Influence of conjugated linoleic acid (CLA) on establishment and progression of atherosclerosis in rabbits. J Am Coll Nutr 19:472S–477S
122. Taylor CG, Zahradka P (2004) Dietary conjugated linoleic acid and insulin sensitivity and resistance in rodent models. Am J Clin Nutr 79:1164S–1168S
123. Clement L, Poirier H, Niot I, Bocher V, Guerro-Millo M, Krief S, Staels B, Besnard P (2002) Dietary *trans*-10, *cis*-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse. J Lipid Res 43:1400–1409
124. Fa M, Diana A, Carta G, Cordeddu L, Melis MP, Murru E, Sogos V, Banni S (2005) Incorporation and metabolism of c9, t11 and t10, c12 conjugated linoleic acid (CLA) isomers in rat brain. Biochim Biophys Acta 1736:61–66
125. Nakanishi T, Koutoku T, Kawahara S, Murai A, Furuse M (2003) Dietary conjugated linoleic acid reduces cerebral prostaglandin E2 in mice. Neurosci Lett 341:135–138
126. Sikorski AM, Hebert N, Swain RA (2008) Conjugated linoleic acid (CLA) inhibits new vessel growth in the mammalian brain. Brain Res 1213:35–40
127. Hunt WT, Kamboj A, Anderson HD, Anderson CM (2010) Protection of cortical neurons from excitotoxicity by conjugated linoleic acid. J Neurochem 115:123–130
128. Chin SF, Storkson JM, Liu W, Albright KJ, Pariza MW (1994) Conjugated linoleic acid (9,11- and 10,12-octadecadienoic acid) is produced in conventional but not germ free rats fed linoleic acid. J Nutr 124:694–701
129. Kishino S, Ogawa J, Omura Y, Matsumura K, Shimizu S (2002) Conjugated linoleic acid production from linoleic acid by lactic acid bacteria. JAOCS 79:159–163
130. Lin TY, Lin CW, Wang YJ (2002) Linoleic acid isomerase activity in enzyme extracts from *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* ssp. shermanii. J Food Sci 67:1502–1505
131. Macouzet M, Robert N, Lee BH (2010) Genetic and functional aspects of linoleate isomerase in *Lactobacillus acidophilus*. Appl Microbiol Biotechnol 87:1737–1742
132. Macouzet M, Lee BH, Robert N (2010) Genetic and structural comparison of linoleate isomerases from selected food-grade bacteria. J Appl Microbiol 109:2128–2134
133. Wall R, Ross RP, Shanahan F, O'Mahony L, O'Mahony C, Coakley M, Hart O, Lawlor P, Quigley EM, Kiely B, Fitzgerald GF, Stanton C (2009) Metabolic activity of the enteric microbiota influences the fatty acid composition of murine and porcine liver and adipose tissues. Am J Clin Nutr 89(5):1393–1401
134. Bassaganya-Riera J, Viladomiu M, Pedragosa M, De Simone C, Carbo A, Shaykhutdinov R, Jobin C, Arthur JC, Corl BA, Vogel H, Storr M, Hontecillas R (2012) Probiotic bacteria produce conjugated linoleic acid locally in the gut that targets macrophage PPAR γ to suppress colitis. PLoS One 7(2):e31238
135. Lee K, Paek K, Lee HY, Park JH, Lee Y (2007) Antiobestity effect of *trans*-10, *cis*-12-conjugated linoleic acid-producing *Lactobacillus plantarum* PL62 on diet-induced obese mice. J Appl Microbiol 103:1140–1146
136. Lee HY, Park JH, Seok SH, Baek MW, Kim DJ, Lee KE, Paek KS, Lee Y, Park JH (2006) Human originated bacteria, *Lactobacillus rhamnosus* PL60, produce conjugated linoleic acid and show anti-obesity effects in diet-induced obese mice. Biochim Biophys Acta 1761: 736–744
137. Druart C, Neyrinck AM, Dewulf EM, De Backer FC, Possemiers S, Van De Wiele T, Moens F, De Vuyst L, Cani PD, Larondelle Y, Delzenne NM (2013) Implication of fermentable carbohydrates targeting the gut microbiota on conjugated linoleic acid production in high-fat-fed mice. Br J Nutr 110:998–1011

Chapter 11
Multidirectional Chemical Signalling Between Mammalian Hosts, Resident Microbiota, and Invasive Pathogens: Neuroendocrine Hormone-Induced Changes in Bacterial Gene Expression

Michail H. Karavolos and C.M. Anjam Khan

Abstract Host-pathogen communication appears to be crucial in establishing the outcome of bacterial infections. There is increasing evidence to suggest that this communication can take place by bacterial pathogens sensing and subsequently responding to host neuroendocrine (NE) stress hormones. Bacterial pathogens have developed mechanisms allowing them to eavesdrop on these communication pathways within their hosts. These pathogens can use intercepted communication signals to adjust their fitness to persist and cause disease in their hosts. Recently, there have been numerous studies highlighting the ability of NE hormones to act as an environmental cue for pathogens, helping to steer their responses during host infection. Host NE hormone sensing can take place indirectly or directly via bacterial adrenergic receptors (BARs). The resulting changes in bacterial gene expression can be of strategic benefit to the pathogen. Furthermore, it is intriguing that not only can bacteria sense NE stress hormones but they are also able to produce key signalling molecules known as autoinducers. The rapid advances in our knowledge of the human microbiome, and its impact on health and disease highlights the potential importance of communication between the microbiota, pathogens and the host. It is indeed likely that the microbiota input significantly in the neuroendocrinological homeostasis of the host by catabolic, anabolic, and signalling processes. The arrival of unwanted guests, such as bacterial pathogens, clearly has a major impact on these delicately balanced interactions. Unravelling the pathways involved in interkingdom communication between invading bacterial pathogens, the resident microbiota, and hosts, may provide novel targets in our continuous search for new antimicrobials to control disease.

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_11, © Springer New York 2014

Abbreviations

AHLs	*N*-acylhomoserine lactones
AI-2	Autoinducer-2
AI-3	Autoinducer-3
BAR	Bacterial adrenergic receptor
DPD	4,5-Dihydroxy-2,3-pentanedione
GI	Gastrointestinal
LPS	Lipopolysaccharide
NE	Neuroendocrine
QS	Quorum sensing

Introduction

During host infection bacterial pathogens use a wide variety of molecular sensors to monitor and adapt to changes in their environment. It is now known that specialised mechanisms allow bacterial pathogens to listen-in on mammalian endocrine signalling molecules such as neuroendocrine (NE) stress hormones [1]. NE hormones such as dopamine, norepinephrine, and epinephrine are synthesised from the amino acid L-tyrosine and are assigned to the chemical family name of catecholamines. The endocrine system represents a complex network of chemical signals or hormones produced by endocrine glands, which relay instructions to target cells located throughout the body. The endocrine system operates intimately with the nervous system to coordinate activities. The responses can work in seconds such as the "flight or fight" reflex in response to fear, to more long-term responses such as growth and developmental processes. The ability to sense the NE landscape of the host may aid pathogens towards their successful adaptation and survival during infection [1–3]. This research area has been termed "microbial endocrinology" and has largely been pioneered by Lyte and colleagues [2, 3]. On infecting the host, bacterial pathogens encounter a variety of chemical signals including the NE stress hormone norepinephrine found abundantly in the gut and epinephrine located predominantly in the bloodstream [4–6]. Lipopolysaccharide (LPS) is present in the outer membrane of Gram-negative bacteria and is potently proinflammatory. Remarkably it has been observed that LPS may act as a signal to stimulate the formation of epinephrine and norepinephrine by macrophages in the bloodstream [7, 8]. This has led to the suggestion that the phagocytic system in some aspects resembles an adrenergic organ composed of scattered cells [7, 8].

Confronted with such an extensive range of chemical signals derived from the host, bacteria engage cooperative decision-making, coordinating their responses to avoid host defences and prolong their survival. Successful pathogens have therefore, developed sophisticated communication tools involving the production and sensing of the critical concentrations of bacterial autoinducer molecules in a process termed quorum sensing (QS) [1, 9]. Following the establishment of a

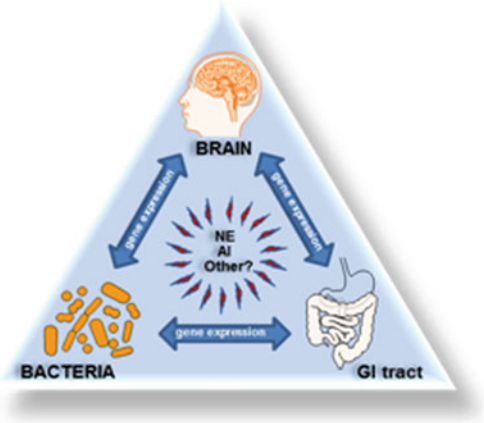

Fig. 11.1 Schematic representation of the role of NE hormones and bacterial autoinducers on interactions between the host, resident microbiota, and bacterial pathogens. NE stress hormones can crosstalk with bacterial autoinducers (*AI*) and other bacterially produced signalling molecules to mediate changes in bacterial or host gene expression. The gastrointestinal (*GI*) tract microbiota may modulate the host NE hormone environment as well as chemically interacting with invading bacterial pathogens. The changing landscape in the production of biologically active host hormones and bacterial signalling molecules, may be sensed by distal organs within the host to ultimately modulate their physiology e.g. brain function. The signalling processes are likely to be complex and multidirectional

critical autoinducer concentration, bacteria detect the autoinducer and synchronise their gene expression and subsequent physiological responses to reflect a "multicellular" response. Interestingly, the more recently described bacterial autoinducer, AI-3, appears to have the ability to "cross-talk" with the host NE stress hormones epinephrine and norepinephrine [10].

It is clear that the interactions of pathogens with their hosts lead to significant changes in gene expression and are hence crucial in shaping the overall result of an infection. Increasing evidence suggests bacteria can directly or indirectly sense host NE stress hormones such as epinephrine and norepinephrine to modulate their gene expression [1, 11–13]. The information in this chapter summarises the major findings on interkingdom signalling and changes in gene regulation, revealing a variety of interesting features and mechanisms for bacterial-host communication (Fig. 11.1).

Bacterial Autoinducers: Chemical Signals in Quorum Sensing and Interkingdom Communication

Quorum sensing (QS) is a key facilitator of bacterial gene regulation and a major component of crosstalk between bacterial and host produced communication signals. The majority of QS mediated communication in Gram-negative bacteria are usually facilitated by *N*-acylhomoserine lactones (AHLs), 2-alkyl-4-quinolones,

and furanones such as autoinducer-2 (AI-2) [14, 15]. The ability to coordinate gene responses via QS creates a major tactical advantage for bacteria in terms of allowing synchronised expression of bacterial survival systems whilst simultaneously modulating the virulence and fitness [16–20].

Many Gram-negative bacteria, but curiously not *Salmonella* or *Escherichia coli*, synthesise AHLs which are freely diffusible autoinducers. AHLs bind to and activate LuxR transcriptional regulators via the LuxNUO signal transduction system as part of QS [21, 22]. On the other hand, Gram-positive bacteria produce post-translationally modified autoinducer peptides derived from the cleavage of larger precursors. These autoinducer peptides are then actively secreted from the bacterial cell, and subsequently bind to membrane receptors. These membrane receptors usually form part of the classical two-component signal transduction system [23–26].

AI-2 is produced by many Gram-negative and also Gram-positive bacteria and may be a universal signal molecule. AI-2 is generated from the molecular rearrangement of 4,5-dihydroxy-2,3-pentanedione (DPD) a by-product of the activity of the enzyme LuxS [27]. DPD can be rearranged in different ways and bacteria may exploit different versions of these products as the AI-2 signalling molecule [28–30]. AI-2 activity has been detected in the culture supernatants of a wide range of bacteria [28, 31]. LuxS has been shown to modulate the regulation of genes encoding virulence factors, antibiotic production, biofilm formation, motility, cell division, and also carbohydrate metabolism [22, 28, 32, 33].

In *E. coli* [10, 34], the LuxS enzyme has a pleiotropic impact on a broad range of metabolic pathways and has also been indirectly implicated in the production of autoinducer-3 (AI-3) [10, 34]. AI-3 regulates the motility and virulence via the two-component signal transduction systems QseBC and QseEF [1, 35]. Remarkably the effects of AI-3 can be mimicked by epinephrine and norepinephrine. AI-3 was first described almost 10 years ago, yet its structure is still unknown, and its importance in quorum sensing remains to be fully determined. Using the existing published methods we and others have not been able to detect AI-3 in culture supernatants and perhaps this can be attributed to a number of reasons including subtle differences in growth conditions [11, 36]. Thus AI-3 appears to be quite an elusive and possibly unstable molecule, so it is now crucial the AI-3 synthase is identified to support these concepts. This should be readily achievable by classic bacterial genetics and AI-3 reporter screens.

NE Hormones Modulate the Fitness of Bacterial Pathogens to Cause Disease by a Variety of Mechanisms: Sensing and Signalling by Bacterial Adrenergic Receptors and Growth Stimulation

A broad range of internal or external signals including stress responses can have a major impact on the functions of the gastrointestinal tract [37]. Consequently these signals can impact on the resident intestinal microbiota. The gastrointestinal tract represents a highly interactive environment where bacteria-host communications can potentially flourish [2, 3].

In mammalian tissues the responses generated by NE hormones depends on the presence of two major types of G-protein coupled adrenergic receptors present on the surface of cells. These are known as α(alpha)-adrenergic and β (beta)-adrenergic receptors and have their distinct set of molecular agonists and antagonists. In EHEC the effects of epinephrine and norepinephrine can be mimicked by the bacterial autoinducer AI-3, providing opportunities for cross-talk between the two signalling systems during infection [10]. This observation raised the possibility of the existence of putative bacterial adrenergic receptors (BARs) [10].

Indeed, the sensor kinase QseC is autophosphorylated on binding either epinephrine or norepinephrine, providing evidence for the existence of adrenergic receptors in bacteria [38]. Furthermore, these adrenergic responses can be inhibited by mammalian α (alpha)- and β (beta)-adrenergic antagonists like phentolamine and propranolol. Remarkably, there is strong specificity in the antagonistic effect with QseC only being blocked by phentolamine [39]. In *E. coli* O157:H7 and *Salmonella* the QseBC system has been proposed as the adrenergic receptor [40–42]. However, new emerging evidence supports the existence of alternative adrenergic receptors. For example, in *S.* Typhimurium it has been demonstrated that QseBC is not required for norepinephrine-enhanced enteritis or intestinal colonisation in calves [43].

In another example of adrenergic receptor antagonist inhibition, increased expression of *virK* and *mig14* in *S.* Typhimurium was blocked by the addition of the β (beta)-adrenergic antagonist propranolol [12]. Some BAR-independent adrenergic phenotypes in bacteria (discussed below) are associated with altered iron uptake by the siderophore enterobactin [44, 45]. A *tonB* mutant, defective in siderophore uptake, showed the same differential gene regulation upon exposure to NE stress hormones as the parent strain, implying that adrenergic regulation operates through a TonB independent mechanism. The *virK* and *mig14* genes are involved in survival and persistence within the host. Genetic deletion of either gene reduces the virulence of *S.* Typhimurium in a mouse infection model, and also reduces survival in macrophages, signifying a possible role in the late stages of infection [46, 47]. The down-regulation of *mig14* by NE stress hormones may hence reduce bacterial persistence and promote clearance of bacteria [12]. These observations highlight the dual role of NE stress hormones in mediating host-bacterial interactions via regulation of gene expression.

Additionally in *Salmonella*, the epinephrine-induced reduction in resistance to polymyxin B was fully reversible by the addition of the β (beta)-adrenergic blocker propranolol. This effect was dependent on the BasSR two component signal transduction system which is the putative epinephrine sensor mediating the antimicrobial peptide response [13]. Epinephrine may, therefore, exert its effect on the *pmr* locus of *S*. Typhimurium through the reversible interaction of the β (beta)-adrenergic blocker with the BasS membrane sensor in a manner similar to the interaction of epinephrine with QseC in *E. coli*. The low (31 %) amino acid sequence identity between BasS and QseC may provide the reason why we observe β-blockage in *Salmonella* as opposed to α (alpha)-blockage in *E. coli*.

The physiological phenotypes of NE stress hormones described above are not linked with QseBC or QseEF signalling [11–13]. This may reflect differences in pathogenesis between *S*. Typhimurium, an invasive pathogen infecting macrophages and epithelial cells and *E. coli*, a mainly non-invasive pathogen which remains in the host intestine. The significant divergence in niches occupied by these two pathogens requires different gene expression patterns for maximum infection efficiency; hence NE stress hormones may modulate different genetic pathways to the advantage or disadvantage of the pathogen.

The inhibition of NE stress hormone-mediated hemolysis by the adrenergic β (beta)-blocker propranolol in the exclusively human pathogen *S*. Typhi is another example of the existence of an additional putative novel bacterial adrenergic receptor. In *S*. Typhi, NE stress hormone-mediated hemolysis is clearly independent of the known *E. coli* O157:H7 adrenergic receptor QseBC and is mediated via the CpxAR two component system [11, 36].

Based on the above observations, it is evident that natural selection ensured there is no monopoly in bacterial adrenergic signalling. Millions of years of co-existence have culminated in a fine tuned bacterial sensing system composed of different BARs, which, through their fastidious specificities, orchestrate the regulatory pathways to modulate the strategic responses of pathogens within their host milieu.

NE stress hormones can modulate the growth of bacteria in iron-limited media, which reflects the in vivo condition within the GI tract [3, 48]. NE stress hormones can efficiently extract iron from host transferrin and lactoferrin and hence these hormones can provide iron for bacteria to use [49, 50]. Although NE stress hormones improve the growth of coagulase-negative *Staphylococci*, the hormones do not have a significant impact on the growth of the important pathogen *Staphylococcus aureus* [51, 52].

Independent of the growth stimulation effects, NE stress hormones modulate the expression of the virulence-associated K99 pilus adhesin enterotoxigenic in *E. coli* [53] to upregulating type 3 secretion in *Vibrio parahaemolyticus* [54]. In *Campylobacter jejuni*, NE stress hormones increase invasion of epithelial cells and disruption of tight junctions [55]. Exposure to NE stress hormones modulates the virulence of *Borrelia burgdorferi* [56], elevates expression of the protease arg-gingipainB virulence factor of *Porphyromonas gingivalis* [57], and in the opportunistic pathogen *Pseudomonas aeruginosa* increases the production of its virulence factors pyocyanin and elastase as well as the secreted *Pseudomonas*

quinolone signal, PQS [70]. Collectively, these examples clearly add support to the role for NE stress hormones in regulating the virulence of bacterial pathogens.

The *S*. Typhimurium epinephrine response is primarily characterised by the upregulation of operons involved in metal homeostasis and oxidative stress [13]. An induction of key *S*. Typhimurium metal transport systems and oxidative stress responses employing manganese internalisation is typical after a 30 min treatment with NE stress hormones [13]. The fact that the oxidative stress regulator OxyR is important for survival in the presence of epinephrine implies that, through affecting iron transport, epinephrine may induce oxidative stress. The sensing of epinephrine may hence provide an environmental cue to initiate the *Salmonella* oxidative stress response in anticipation of forthcoming host-based oxidative stress.

Treatment of *S*. Typhimurium with NE stress hormones reduces its resistance to the human antimicrobial peptide cathelicidin LL-37 [12]. LL-37 is produced in the gastrointestinal tract, bone marrow and macrophages. The peptide possesses antimicrobial activity against a range of Gram-negative and Gram- positive bacteria [58]. It could be speculated that through an increase in sensitivity to LL-37, NE stress hormones may also act as a host defence system to combat infection. This observation may suggest that although bacteria can sense and exploit these host signalling molecules for their benefit, in some instances the host can use the same signals to manipulate the bacteria [12].

Chemical Dialogues Between the Host, Microbiota, and Bacterial Pathogens: A Multidirectional Regulatory Liaison

Most of the studies on BAR dependent or independent signalling discussed above have focused mainly on two enteroinvasive bacterial pathogens. Intriguingly, the microbes that colonise humans hosts, outnumber human cells by tenfold [59]. The collective genomes of these microbial symbionts, referred to as the microbiome, provides a genetic repertoire with the potential to significantly affect host-pathogen-symbiont communication [60]. Data mining of the human microbiome suggests the presence of enzymes and pathways capable of supporting microbiota-based production of known or novel autoinducers [59–61]. Interkingdom signalling in the gut lumen may be crucial for maintaining the physiological balance in the gut microbiome, thus facilitating microbiome homeostasis and preventing dysbiosis. Even in healthy individuals, the diversity of the human microbiome is extraordinary, implying the potential complexity of the interactions in these populations [59, 60].

It is clear that some species of bacteria are able to modulate the production of NE hormones in vitro and possibly in the gut [above; 62–64]. Genes encoding components with high similarity to the molecular machinery needed to produce NE hormones are indeed present in some species of bacteria [65–68]. There is

increasing evidence that gut bacteria may contribute significantly to the levels of gut NE hormones such as norepinephrine [62]. Indeed bacteria may modulate gut motility and/or immune functions via the production of NE hormones to steer gut homeostasis. The complete role of bacterially-induced/produced NE hormones in host gene regulation and microbial survival is yet to be evaluated.

The wide majority of the gut microbiome species are not culturable using traditional laboratory techniques. The size and variation of the gut microbiome only hints at the richness of molecules produced and potentially sensed by these commensal populations. Microbial pathogen infection and insults like antibiotic use, perturb such fine balance leading to dysbiosis in the gut [69]. A metabolomics study in a murine infection model using *S*. Typhimurium revealed a significant alteration in the metabolic profile of multiple pathways involved in host eicosanoid hormone metabolism [69]. It is therefore clear that bacteria can influence their host in a variety of ways including a possibly significant involvement into modulating host NE homeostasis.

Microbiota Are Key Players in Modulating Levels of NE Hormones in the Gastrointestinal Tract

The gastrointestinal microbiota represent a diverse range of balanced bacterial communities with fundamental roles in maintaining good health and preventing disease in humans. A recent study has revealed the involvement of gut microbiota not only in the regulation but also the production of NE hormones in the mouse gastrointestinal (GI) tract [62]. Asano and colleagues investigated the levels of norepinephrine and dopamine through the GI tract by developing a robust HPLC assay [62]. In order to examine the impact of the GI tract microbiota on catecholamine concentration, they determined the levels of norepinephrine and dopamine in specific pathogen-free mice, germ-free mice, and gnotobiotic mice [62]. The lumen of specific pathogen-free mice had much greater levels of neuroendocrine hormones than those found in germ-free mice that harboured no bacteria.

Amazingly the introduction of either, specific-pathogen free mice fecal flora, *Clostridum* species or *E. coli* into germ free mice resulted in a striking elevation in levels of free biologically active norepinephrine and dopamine. An *E. coli* mutant unable to produce β (beta)-glucuronidase was no longer able to elevate levels of free biologically active catecholamines implying a role for this bacterial enzyme in the process [62]. Through this comparative analysis, the investigators identified that the resident gut microbiota and the bacterial enzyme β (beta)-glucuronidase, have major roles in the production of biologically active norepinephrine and dopamine in the GI tract of the host. The study provides important insights into the interactions between the microbiota and host cells, and raises important questions. Can the presence of the gut microbiota: be sensed by the host cells, induce host cells to produce these catecholamine hormones, as well as play a key role in the maturation

of biologically active hormones? This study by Asano and colleagues will undoubtedly have a major impact in the field and stimulating further research.

Conclusions

NE hormones play an essential role in regulating the physiology of mammals producing immediate responses within seconds such as the "fight or flight" response to more long term developmental changes.

It is now widely acknowledged that bacteria can detect, and respond to changes in the NE hormone landscape of their host. These hormones may provide important environmental cues on their in vivo location and the physiological status of the host. This sensing may take place indirectly or directly through BARs. Research from a number of independent laboratories, including ours, vividly demonstrated that *Salmonella* have evolved a number of specialised systems for sensing NE stress hormones. Sensing of the NE hormones subsequently leads to global alterations in bacterial gene regulation patterns. Even transient exposure to NE hormones can have significant impact on the physiology of *Salmonella* modulating its ability to cause disease. There appears to be a delicate balance, which can be shifted to favour the pathogen or the host. The complex interactions between bacteria and their host involving host or bacterially derived NE stress hormones is briefly depicted in Fig. 11.1. In summary, there is increasing evidence to suggest the existence of a complex chemical dialogue between host cells, the microbiota, and invading pathogens. Further studies on understanding the fascinating nature of the bacterial adrenergic receptors and signalling pathways will not only provide colourful biological insights on pathogen-host interactions, but may also identify potential novel targets in the treatment of disease.

References

1. Pacheco AR, Sperandio V (2009) Inter-kingdom signaling: chemical language between bacteria and host. Curr Opin Microbiol 12(2):192–198
2. Lyte M, Vulchanova L, Brown D (2011) Stress at the intestinal surface: catecholamines and mucosa-bacteria interactions. Cell Tissue Res 343(1):23–32
3. Freestone PPE, Sandrini SM, Haigh RD, Lyte M (2008) Microbial endocrinology: how stress influences susceptibility to infection. Trends Microbiol 16(2):55–64
4. Furness JB (2000) Types of neurons in the enteric nervous system. J Auton Nerv Syst 81(1–3): 87–96
5. Eisenhofer G, Aneman A, Hooper D, Rundqvist B, Friberg P (1996) Mesenteric organ production, hepatic metabolism, and renal elimination of norepinephrine and its metabolites in humans. J Neurochem 66(4):1565–1573
6. Aneman A, Eisenhofer G, Olbe L, Dalenbäck J, Nitescu P, Fändriks L et al (1996) Sympathetic discharge to mesenteric organs and the liver. Evidence for substantial mesenteric organ norepinephrine spillover. J Clin Invest 97(7):1640–1646

7. Flierl MA, Rittirsch D, Nadeau BA, Chen AJ, Sarma JV, Zetoune FS et al (2007) Phagocyte-derived catecholamines enhance acute inflammatory injury. Nature 449(7163):721–725
8. Flierl MA, Rittirsch D, Nadeau BA, Sarma JV, Day DE, Lentsch AB et al (2009) Upregulation of phagocyte-derived catecholamines augments the acute inflammatory response. PLoS One 4 (2):e4414
9. Williams P (2007) Quorum sensing, communication and cross-kingdom signalling in the bacterial world. Microbiology 153(12):3923–3938
10. Sperandio V, Torres AG, Jarvis B, Nataro JP, Kaper JB (2003) Bacteria-host communication: the language of hormones. Proc Natl Acad Sci U S A 100(15):8951–8956
11. Karavolos MH, Bulmer DM, Spencer H, Rampioni G, Schmalen I, Baker S et al (2011) Salmonella Typhi sense host neuroendocrine stress hormones and release the toxin haemolysin E. EMBO Rep 12(3):252–258
12. Spencer H, Karavolos MH, Bulmer DM, Aldridge P, Chhabra SR, Winzer K et al (2010) Genome-wide transposon mutagenesis identifies a role for host neuroendocrine stress hormones in regulating the expression of virulence genes in Salmonella. J Bacteriol 192(3): 714–724
13. Karavolos MH, Spencer H, Bulmer DM, Thompson A, Winzer K, Williams P et al (2008) Adrenaline modulates the global transcriptional profile of Salmonella revealing a role in the antimicrobial peptide and oxidative stress resistance responses. BMC Genomics 9(1):458
14. Winson MK, Camara M, Latifi A, Foglino M, Chhabra SR, Daykin M et al (1995) Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 92(20): 9427–9431
15. Bassler BL, Wright M, Silverman MR (1994) Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. Mol Microbiol 13(2):273–286
16. Miller MB, Bassler BL (2001) Quorum sensing in bacteria. Annu Rev Microbiol 55:165–199
17. Fuqua C, Greenberg EP (1998) Cell-to-cell communication in *Escherichia coli* and *Salmonella typhimurium*: they may be talking, but who's listening? Proc Natl Acad Sci U S A 95(12): 6571–6572
18. Winzer K, Hardie KR, Williams P (2002) Bacterial cell-to-cell communication: sorry, can't talk now – gone to lunch! Curr Opin Microbiol 5(2):216–222
19. Winzer K, Hardie KR, Williams P (2003) LuxS and autoinducer-2: their contribution to quorum sensing and metabolism in bacteria. Adv Appl Microbiol 53:291–396
20. Winzer K, Williams P (2001) Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria. Int J Med Microbiol 291(2):131–143
21. Taga ME, Bassler BL (2003) Chemical communication among bacteria. Proc Natl Acad Sci U S A 100:14549–14554
22. Xavier KB, Bassler BL (2003) LuxS quorum sensing: more than just a numbers game. Curr Opin Microbiol 6(2):191–197
23. Lazazzera BA (2000) Quorum sensing and starvation: signals for entry into stationary phase. Curr Opin Microbiol 3(2):177–182
24. Lyon GJ, Novick RP (2004) Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria. Peptides 25(9):1389–1403
25. Dunny GM, Leonard BAB (1997) Cell-cell communication in gram-positive bacteria. Annu Rev Microbiol 51:527–564
26. Schauder S, Bassler BL (2001) The languages of bacteria. Genes Dev 15(12):1468–1480
27. Surette MG, Miller MB, Bassler BL (1999) Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. Proc Natl Acad Sci U S A 96(4):1639–1644
28. Xavier KB, Bassler BL (2005) Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli*. J Bacteriol 187(1):238–248

29. Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL et al (2002) Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415(6871): 545–549
30. Miller ST, Xavier KB, Campagna SR, Taga ME, Semmelhack MF, Bassler BL et al (2004) *Salmonella typhimurium* recognizes a chemically distinct form of the bacterial quorum-sensing signal Al-2. Mol Cell 15(5):677–687
31. Miller MB, Bassler BL (2001) Quorum sensing in bacteria. Annu Rev Microbiol 55:165–199
32. Sircili MP, Walters M, Trabulsi LR, Sperandio V (2004) Modulation of enteropathogenic *Escherichia coli* virulence by quorum sensing. Infect Immun 72(4):2329–2337
33. Vendeville A, Winzer K, Heurlier K, Tang CM, Hardie KR (2005) Making 'sense' of metabolism: autoinducer-2, LuxS and pathogenic bacteria. Nat Rev Microbiol 3(5):383–396
34. Walters M, Sircili MP, Sperandio V (2006) AI-3 synthesis is not dependent on luxS in *Escherichia coli*. J Bacteriol 188(16):5668–5681
35. Rasko DA, Moreira CG, Li DR, Reading NC, Ritchie JM, Waldor MK et al (2008) Targeting QseC signaling and virulence for antibiotic development. Science 321(5892):1078–1080
36. Karavolos MH, Williams P, Khan CMA (2011) Interkingdom crosstalk: host neuroendocrine stress hormones drive the hemolytic behavior of Salmonella typhi. Virulence 2(4):371–374
37. Elenkov IJ, Chrousos GP (2006) Stress system – organization, physiology and immunoregulation. Neuroimmunomodulation 13(5–6):257–267
38. Clarke MB, Hughes DT, Zhu C, Boedeker EC, Sperandio V (2006) The QseC sensor kinase: a bacterial adrenergic receptor. Proc Natl Acad Sci U S A 103(27):10420–10425
39. Clarke MB, Sperandio V (2005) Events at the host-microbial interface of the gastrointestinal tract III. Cell-to-cell signaling among microbial flora, host, and pathogens: there is a whole lot of talking going on. Am J Physiol Gastrointest Liver Physiol 288(6):G1105–G1109
40. Lyte M, Arulanandam BP, Frank CD (1996) Production of Shiga-like toxins by *Escherichia coli* O157:H7 can be influenced by the neuroendocrine hormone norepinephrine. J Lab Clin Med 128(4):392–398
41. Vlisidou I, Lyte M, van Diemen PM, Hawes P, Monaghan P, Wallis TS et al (2004) The neuroendocrine stress hormone norepinephrine augments *Escherichia coli* O157:H7-induced enteritis and adherence in a bovine ligated ileal loop model of infection. Infect Immun 72(9): 5446–5451
42. Kendall MM, Rasko DA, Sperandio V (2007) Global effects of the cell-to-cell signaling molecules autoinducer-2, autoinducer-3, and epinephrine in a luxS mutant of enterohemorrhagic *Escherichia coli*. Infect Immun 75(10):4875–4884
43. Pullinger GD, Carnell SC, Sharaff FF, van Diemen PM, Dziva F, Morgan E et al (2010) Norepinephrine augments *Salmonella enterica*-induced enteritis in a manner associated with increased net replication but independent of the putative adrenergic sensor kinases QseC and QseE. Infect Immun 78(1):372–380
44. Burton CL, Chhabra SR, Swift S, Baldwin TJ, Withers H, Hill SJ et al (2002) The growth response of *Escherichia coli* to neurotransmitters and related catecholamine drugs requires a functional enterobactin biosynthesis and uptake system. Infect Immun 70(11):5913–5923
45. Freestone PPE, Haigh RD, Williams PH, Lyte M (2003) Involvement of enterobactin in norepinephrine-mediated iron supply from transferrin to enterohaemorrhagic *Escherichia coli*. FEMS Microbiol Lett 222(1):39–43
46. Brodsky IE, Ghori N, Falkow S, Monack D (2005) Mig-14 is an inner membrane-associated protein that promotes *Salmonella typhimurium* resistance to CRAMP, survival within activated macrophages and persistent infection. Mol Microbiol 55(3):954–972
47. Detweiler CS, Monack DM, Brodsky IE, Mathew H, Falkow S (2003) virK, somA and rcsC are important for systemic *Salmonella enterica* serovar Typhimurium infection and cationic peptide resistance. Mol Microbiol 48(2):385–400
48. Freestone PPE, Walton NJ, Haigh RD, Lyte M (2007) Influence of dietary catechols on the growth of enteropathogenic bacteria. Int J Food Microbiol 119(3):159–169

49. Freestone PPE, Lyte M, Neal CP, Maggs AF, Haigh RD, Williams PH (2000) The mammalian neuroendocrine hormone norepinephrine supplies iron for bacterial growth in the presence of transferrin or lactoferrin. J Bacteriol 182(21):6091–6098
50. Sandrini SM, Shergill R, Woodward J, Muralikuttan R, Haigh RD, Lyte M et al (2010) Elucidation of the mechanism by which catecholamine stress hormones liberate iron from the innate immune defense proteins transferrin and lactoferrin. J Bacteriol 192(2):587–594
51. Beasley FC, Marolda CL, Cheung J, Buac S, Heinrichs DE (2011) *Staphylococcus aureus* transporters Hts, Sir, and Sst capture iron liberated from human transferrin by Staphyloferrin A, Staphyloferrin B, and catecholamine stress hormones, respectively, and contribute to virulence. Infect Immun 79(6):2345–2355
52. Neal CP, Freestone PPE, Maggs AF, Haigh RD, Williams PH, Lyte M (2001) Catecholamine inotropes as growth factors for *Staphylococcus epidermidis* and other coagulase-negative staphylococci. FEMS Microbiol Lett 194(2):163–169
53. Lyte M, Erickson AK, Arulanandam BP, Frank CD, Crawford MA, Francis DH (1997) Norepinephrine-induced expression of the K99 pilus adhesin of enterotoxigenic *Escherichia coli*. Biochem Biophys Res Commun 232(3):682–686
54. Nakano M, Takahashi A, Sakai Y, Nakaya Y (2007) Modulation of pathogenicity with norepinephrine related to the type III secretion system of vibrio parahaemolyticus. J Infect Dis 195(9):1353–1360
55. Cogan TA, Thomas AO, Rees LEN, Taylor AH, Jepson MA, Williams PH et al (2007) Norepinephrine increases the pathogenic potential of *Campylobacter jejuni*. Gut 56(8): 1060–1065
56. Scheckelhoff MR, Telford SR, Wesley M, Hu LT (2007) *Borrelia burgdorferi* intercepts host hormonal signals to regulate expression of outer surface protein A. Proc Natl Acad Sci U S A 104(17):7247–7252
57. Saito T, Inagaki S, Sakurai K, Okuda K, Ishihara K (2011) Exposure of *P. gingivalis* to noradrenaline reduces bacterial growth and elevates ArgX protease activity. Arch Oral Biol 56 (3):244–250
58. Bals R, Wang X, Zasloff M, Wilson JM (1998) The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. Proc Natl Acad Sci U S A 95(16):9541–9546
59. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI (2007) The Human Microbiome Project. Nature 449(7164):804–810
60. Cho I, Blaser MJ (2012) The human microbiome: at the interface of health and disease. Nat Rev Genet 13(4):260–270
61. Lukáš F, Gorenc G, Kopecný J (2008) Detection of possible AI-2-mediated quorum sensing system in commensal intestinal bacteria. Folia Microbiol 53(3):221–224
62. Asano Y, Hiramoto T, Nishino R, Aiba Y, Kimura T, Yoshihara K et al (2012) Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. Am J Physiol Gastrointest Liver Physiol 303:G1288–G1295. doi:10.1152/ajpgi.00341.2012
63. Tsavkelova EA, Botvinko IV, Kudrin VS, Oleskin AV (2000) Detection of neurotransmitter amines in microorganisms with the use of high-performance liquid chromatography. Dokl Biochem 372(1–6):115–117
64. Roshchina VV (2010) Evolutionary considerations of neurotransmitters in microbial, plant, and animal cells. Microbial endocrinology. Springer, New York, pp 17–52
65. Hernández-Romero D, Sanchez-Amat A, Solano F (2006) A tyrosinase with an abnormally high tyrosine hydroxylase/dopa oxidase ratio. FEBS J 273(2):257–270
66. López-serrano D, Sanchez-Amat A, Solano F (2002) Cloning and molecular characterization of a SDS-activated tyrosinase from *Marinomonas mediterranea*. Pigment Cell Res 15(2): 104–111

67. López-Serrano D, Solano F, Sanchez-Amat A (2007) Involvement of a novel copper chaperone in tyrosinase activity and melanin synthesis in *Marinomonas mediterranea*. Microbiology 153(7):2241–2249
68. Solano F, Garcia E, Perez D, Sanchez-Amat A (1997) Isolation and characterization of strain MMB-1 (CECT 4803), a novel melanogenic marine bacterium. Appl Environ Microbiol 63(9): 3499–3506
69. Antunes LC, Arena ET, Menendez A, Han J, Ferreira RBR, Buckner MMC et al (2011) Impact of Salmonella infection on host hormone metabolism revealed by metabolomics. Infect Immun 79(4):1759–1769
70. Hegde M, Wood T, Jayaraman A (2009) The neuroendocrine hormone norepinephrine increases *Pseudomonas aeruginosa* PA14 virulence through the las quorum-sensing pathway. Appl Microbiol Biotechnol 84(4):763–776

Chapter 12
Influence of Stressor-Induced Nervous System Activation on the Intestinal Microbiota and the Importance for Immunomodulation

Michael T. Bailey

Abstract The body is colonized by a vast population of genetically diverse microbes, the majority of which reside within the intestines to comprise the intestinal microbiota. During periods of homeostasis, these microbes reside within stable climax communities, but exposure to physical, physiological, as well as psychological stressors can significantly impact the structure of the intestinal microbiota. This has been demonstrated in humans and laboratory animals, with the most consistent finding being a reduction in the abundance of bacteria in the genus *Lactobacillus*. Whether stressor exposure also changes the function of the microbiota, has not been as highly studied. The studies presented in this review suggest that stressor-induced disruption of the intestinal microbiota leads to increased susceptibility to enteric infection and overproduction of inflammatory mediators that can induce behavioral abnormalities, such as anxiety-like behavior. Studies involving germfree mice also demonstrate that the microbiota are necessary for stressor-induced increases in innate immunity to occur. Exposing mice to a social stressor enhances splenic macrophage microbicidal activity, but this effect fails to occur in germfree mice. These studies suggest a paradigm in which stressor exposure alters homeostatic interactions between the intestinal microbiota and mucosal immune system and leads to the translocation of pathogenic, and/or commensal, microbes from the lumen of the intestines to the interior of the body where they trigger systemic inflammatory responses and anxiety-like behavior. Restoring homeostasis in the intestines, either by removing the microbiota or by administering probiotic microorganisms, can ameliorate the stressor effects.

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_12, © Springer New York 2014

Abbreviations

ACTH	Adrenocorticotrophic hormone
CFU	Colony forming units
CRH	Corticotrophin release hormone
DGGE	Denaturing gradient gel electrophoresis
GABA	γ-Amino butyric acid
GI	Gastrointestinal
HPA	Hypothalamic-pituitary-adrenal
iNOS	Inducible nitric oxide synthase
mRNA	Messenger ribonucleic acid
NE	Norepinephrine
SDR	Social disruption
SNS	Sympathetic nervous system
TNF-α	Tumor necrosis factor alpha

Introduction

The body is heavily colonized by microorganisms collectively referred to as the microbiota, and it is now realized that all surfaces of the body naturally harbor unique microbial communities. While archea, protists, and viruses are known to reside within these communities, the majority of the microbiota are bacteria that reside within the gastrointestinal tract. Proximal sections of the gastrointestinal (GI) tract, including the stomach and the duodenum, harbor low levels of microorganisms (typically between 100 and 1,000 colony forming units (CFU) per ml of contents), whereas distal sections of the GI tract, including the ileum and the colon, harbor high levels of microorganisms (typically between 10^6 and 10^{12} CFU/ml of contents). In the colon, the microbiota reside as a stable climax community due to the selection of microbes that are best adapted for their given niche [1]. Although this climax community is relatively resistant to change [2], it is well known that factors such as diet and antibiotics can cause transient alterations in microbial community structure [3–5]. This review will discuss the evidence that exposure to different types of stressors can also cause transient alterations in microbial community structure, and will discuss the evidence that even transient alterations in the microbiota may be associated with variations in host immune and behavioral responses.

The Modern Stress Concept

Stress is an intrinsic part of life, and successfully coping with aversive stimuli is essential for organism survival in an ever changing environment. While the concept of stress is intuitive, there is not a single, widely accepted definition of stress. In its simplest form, stress can be broken down into the stimulus that threatens organism homeostasis (called the stressor) and the behavioral and physiological response to this challenge (called the stress response). Thus, a stressor is any stimulus that disrupts internal homeostasis, and can involve psychological, physical, or physiological stimuli. This disruption to homeostasis elicits physiological responses that are aimed at reducing the threat and re-establishing internal homeostasis. Initiation of the stress response to physical or physiological stressors is typically subconscious, but additional cognitive processing occurs in response to psychological stressors. Psychological stressors that are perceived as exceeding available coping strategies set into motion coordinated behavioral and physiological responses that ultimately serve to help the organism adapt to the stressor. Interestingly, the physiological stress responses to physical, physiological, and psychological stressors have many similarities that can be generalized across host species.

Physiological Stress Response

There are two neuroendocrine pathways that are major contributors to the stress response, namely the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS). Activation of the HPA axis occurs through the release of corticotrophin release hormone (CRH) from neurosecretory cells found in the paraventricular nucleus of the hypothalamus. CRH travels a short distance from the hypothalamus to the anterior pituitary gland where it stimulates the release of adrenocorticotrophic hormone (ACTH). The ACTH then travels through the blood and stimulates the release of glucorticoid hormones, namely corticosterone in rodents and cortisol in humans, from the cortex of the adrenal glands. As the name suggests, glucorticoids are important for increasing the bioavailability of glucose via gluconeogenesis in the liver. The glucose is then used by the body to cope with and adapt to the stressful stimulus.

In addition to the HPA axis, the sympathetic branch of the autonomic nervous system becomes activated during stressor exposure. The sympathetic nervous system (SNS) originates in the brain stem from different brain nuclei, such as the locus coeruleus, pons, and medulla, which send projections along the spinal column. After exiting the spinal column, preganglionic SNS neurons synapse in prevertebral ganglia using acetylcholine as the neurotransmitter. The acetylcholine excites postganglionic neurons that innervate virtually every organ in the body using norepinephrine (NE) as the terminal neurotransmitter.

Activation of the SNS occurs very rapidly and is largely responsible for the well-known "fight-or-flight" stress response, that is dependent upon the effects the SNS has on the heart and lungs (i.e., increased heart rate and respiration), blood vessels (i.e., increased vasodilation in skeletal muscle), and internal organs (e.g., increased glycogenolysis in the liver and reduced digestive functions in the gut). Upon stressor termination, the parasympathetic branch of the autonomic nervous system becomes activated and releases the neurotransmitter acetylcholine to restore homeostasis and induce the "rest-and-digest" response.

Stress Exposure and the Gut

Stressor-induced activation of the SNS and the HPA axis are well known to affect the functioning of the gastrointestinal tract. This was first recognized when it was observed that a patient with a gastric fistula produced significantly less gastric acid during fearful periods [6]. Other stressors, including a cold pressure task and mental arithmetic [7, 8] can also reduce gastric acid secretion. Studies in laboratory animals indicate that these stressor effects are due to activation of the autonomic nervous system. Activation of the SNS tends to suppress whereas the PNS enhances gastric acid secretion [9].

Other components of gastrointestinal physiology such as gastrointestinal motility and mucous production are also known to be significantly changed by stressor exposure. For example, GI motility, can be either slowed or enhanced during stressor exposure depending upon the type of stressor and the section of the intestine that is investigated [10, 11]. Stressor exposure has also been shown to affect mucous secretion, depending upon the strength and duration of the stressor. Early life or short-lasting stressors tend to increase mucous secretion throughout the length of the gastrointestinal tract, whereas long-lasting stressors tend to deplete mucous stores and thus decrease mucous levels in the gut [12, 13].

Gastric acid secretion, gastrointestinal motility, and mucous levels can influence the ability of microbes to colonize within the gastrointestinal tract. For example, it is well known that microbes must be able to survive the low acidity in the stomach in order to colonize lower sections of the gut. Reducing acid secretion can in turn alter gut microbiota populations [14]. Similarly, motility has long been recognized as a primary factor controlling microbe levels the length of the GI tract [15]. Pharmacological manipulation of gastrointestinal motility is associated with altered microbial populations [16]. The mucous layer in the gut is also an important factor for the development of microbial community structure, because the mucins that comprise the mucous layer are glycosylated with O-glycans that are an important food source for mucoadherent microbes [17]. Moreover, some microbes, such as members in the genus *Lactobacillus*, contain mucous binding proteins that help them to bind to the intestinal mucous layer [18]. Thus, changing mucous secretion has the potential to change microbial populations.

Impact of the Stress Response on Gut Microbiota and Colonic Inflammation

Studies in this laboratory have been influenced by the findings that gastrointestinal physiological functions that are affected by stressor exposure can also impact gut microbes, because they reflect a possible biological/mechanistic link between stressor-exposure and alterations in the microbiota. However, alteration of gut physiological functioning is not the only potential mechanism by which stress could impact the gastrointestinal microbiota. Direct neurotransmitter/hormone-bacterial interactions might also mediate stressor effects on the gut microbiota.

Neuroendocrine-Bacterial Interactions

The growth of many types of bacteria, including both infectious and commensal organisms, have been shown to be significantly enhanced upon culture with catecholamines, such a norepinephrine (NE), as shown in multiple chapters in this book.

While the effects of neuroendocrine hormones on microbial growth have been amply demonstrated in both in vitro and ex vivo model systems (reviewed in [19]), demonstrating these interactions occur in vivo has been challenging. However, studies involving the use of a neurotoxin to lyse peripheral sympathetic neurons, and thus causing an increase in norepinephrine levels in vivo, indicate that elevated norepinephrine levels leads to bacterial overgrowth in the intestines [20]. The growth of commensal Gram-negative microbes, primarily *Escherichia coli*, was increased by nearly 10,000-fold after elevating NE levels through the use of the neurotoxin [20]. The effects of norepinephrine on bacterial growth was also evident in an ileal loop model, where growth of *Salmonella enterica* in the presence of NE prior to inoculation into an ileal loop was associated with significantly elevated pathogen levels in the ileal loop, and a more severe pathogen-induced disease progression [21].

As these studies demonstrate, there are multiple mechanisms by which host physiology can impact microbial populations in the intestines. And, exposure to stressors that are physical, physiological, or psychological in nature has the capacity to significantly change all of these host physiological processes. These findings have led to testing the general hypothesis that stressor exposure can significantly change microbial populations naturally residing within the gastrointestinal tract.

Culture-Based Findings of Stressor Effects on the Structure of the Microbiota

It has been recognized for over 30 years that changing an animal's environment can lead to gut microbial dysbiosis. In 1974, Tannock and Savage [22] demonstrated that moving mice into a cage lacking bedding, food, and water reduced the number of lactobacilli that could be cultured from the small and large intestines, with the greatest reduction being found in the stomach. Although it was not possible to determine whether the reduction was due to the change in environment, rather than the lack of food and water, this was one of the earliest studies to demonstrate that external factors could impact the microbiota. Subsequent studies confirmed and extended the observation that environmental stimuli can impact the microbiota. For example, chronic sleep deprivation was found to cause a significant overgrowth of microbiota in the distal ileum and cecum [23]. This microbial overgrowth was associated with a translocation of the microbes to the spleen, liver, and regional lymph nodes in sleep-deprived animals [23].

It is becoming increasingly evident that physical and physiological stressor can impact gut microbial populations, but only a few studies have focused on the impact that psychological stressors can have on the microbiota. Data from early studies on the composition of the microbiota in Russian cosmonauts were among the first to suggest that psychological stimuli could impact the composition of the microbiota. The data demonstrated that the intestinal microbiota were significantly different during space flight as compared to training periods on Earth [24]. There are many environmental changes associated with space flight, and it was not clear whether the differences in the microbiota could be due to the stress associated with space flight. However, other studies tracking microbial populations during space training found that periods of emotional stress, such as the stress of confinement, was associated with periods of altered microbial profiles [25], thus suggesting that emotional stress could impact the stability of the intestinal microbiota.

The strongest evidence that stressor exposure can impact microbial populations has come from studies involving laboratory animals. For example, studies demonstrate that separating rhesus monkeys (*Macaca mulatta*) from their mothers was sufficient to significantly change the number of bacteria that could be cultured from the stool. Levels of total cultured bacteria tended to be significantly decreased by 3 days after separation [26], but the most consistent findings occurred when a single type of microbe was cultured. Levels of bacteria in the genus *Lactobacillus* were significantly reduced 3 days after maternal separation [26]. Of importance, this reduction in lactobacilli was significantly correlated with the expression of stress indicative behaviors. Those animals that displayed a larger number of stress-indicative behaviors (such as repetitive lip smacking and cooing) tended to have lower levels of lactobacilli. This effect lasted through 3 days post-separation. Interestingly, as the infant monkeys formed stable social groups by 1 week post-separation, the levels of lactobacilli returned to pre-separation values [26].

Members of the genus *Lactobacillus* are known to have protective effects in the intestines, with one protective effect being the production of proteins and other compounds that have the capacity to kill enteric pathogens. Two enteric pathogens, namely *Shigella flexneri* and *Campylobacter jejuni*, are endemic in monkey colonies. Exposure to maternal separation increased opportunistic infection with *S. flexneri* and *C. jejuni*, and pathogen levels tended to correlate with lactobacilli levels. In general, maternally separated infant monkeys that had high pathogen loads also had low levels of lactobacilli [26]. This study demonstrated that a naturally occurring stressor changed the levels of bacteria that could be cultured from the stool and also reduced the ability of the microbiota to exclude pathogen colonization.

The effects of stressor exposure on the microbiota also extend into the prenatal period. Exposing monkeys to an acoustical startle stressor during gestation significantly changes the development of the intestinal microbiota in the offspring [27]. This was manifest as a reduction in the levels of bifidobacteria and lactobacilli that could be cultured from the stool for the first 6 weeks of life. As with previous studies, this stressor-associated reduction in lactobacilli was associated with an increased incidence of opportunistic infection [27].

Culture-based studies in rodents have also demonstrated that stressor exposure reduces the number of lactobacilli cultured from the stool. Mice that were housed in cages lacking bedding, as well as mice that were exposed to horizontal shaking, for 3 consecutive days were found to have lower lactobacilli levels shed in the feces than did control mice [28]. This reduction in lactobacilli was consistent between the different stressors, and led the authors to suggest that reduction in the lactobacilli could be used as a marker for environmental stressor exposure [28]. A note of caution is needed, however, because one study has found that inbred female mice have low levels of *Enterococcus* and *Lactobacillus* spp., as determined using fluorescent in situ hybridization (FISH), but exposure to water avoidance stress during antibiotic administration causes an increase in this bacterial group, rather than a decrease [29].

The effects of stressor exposure on lactobacilli have primarily been studied in laboratory animals, but one study found that stressor exposure reduced the levels of lactobacilli cultured from humans. Fecal lactobacilli levels were assessed in college students during a low stress period (i.e., the first week of the semester) and a high stress period (i.e., final exam week) to determine whether the stressful period was associated with lower levels of lactobacilli. The final exam period was associated with higher levels of perceived stress, and consistent with results from animal studies, higher perceived stress resulted in lower levels of lactobacilli shed in the stool [30]. It should be noted that the exam period was also associated with significant differences in diet. Because diet can significantly impact microbial populations [31], it is possible that the reductions in lactobacilli were dependent upon changes in diet. However, given results demonstrating stressor-induced reductions in fecal lactobacilli in laboratory animals consuming a standardized laboratory diet [26, 27], it is likely that alterations in the human microbiome during

the stress of the exam week were due to combined effects of the stressor on host physiology and changes in dietary habits.

Culture-Independent Studies of Stressor Effects on Gut Microbial Community Structure and Function

Most studies assessing the effects of stressor exposure on the gut microbiota have relied on culture-based enumeration of only a few types of microbes within a given sample. However, the vast majority of microbes in the gut cannot be cultured due to undefined culture conditions [32]. As a result, there are an increasing number of studies that have utilized culture-independent methods to demonstrate that stressor exposure can affect more than just a few gut microbes; community-wide alterations of the gut microbiota have been demonstrated to occur in response to multiple types of stressors. This was first realized in rats that were separated from their mothers for 3 h per day early in life (i.e., postnatal days 3–12). This maternal separation stressor resulted in significant community-wide alterations in the gut microbiota as assessed using denaturing gradient gel electrophoresis (DGGE) to assess microbial populations in the stool when the rats were 7–8 weeks of age [33]. Studies in this laboratory have also used culture-independent methods to assess the effects of stressor exposure on the intestinal microbiota [34, 35]. Next generation, high throughput 454 FLX pyrosequencing was first used to characterize the microbiota in mice exposed to a prolonged restraint stressor.

Studies Involving Prolonged Restraint

Prolonged restraint is a widely used murine stressor that has been extensively characterized in the literature and is the most commonly used murine stressor in biomedical and biobehavioral research [36]. This stressor involves both a physical component (i.e., physical confinement) and a psychological component that is thought to reflect the animal's perception of burrow collapse and inescapability [36]. Exposure to the prolonged restraint stressor induces a physiological stress response that results in the elevation of endogenous corticosterone, epinephrine, and norepinephrine [36–39]. Thus, mice were exposed to the prolonged restraint stressor to determine the effects of the stress response on the stability of the intestinal microbiota.

In this initial experiment, approximately 100,000 sequences from the cecal contents of 32 mice (approximately 3,000 sequences per mouse) were analyzed to characterize microbial diversity within the cecum. Microbial diversity encompasses both the richness (i.e., the number of different types of bacteria in a community) and the evenness (i.e., the distribution of the individual bacteria). In microbial ecology, there are two primary measures of diversity, with α-diversity assessing diversity of

species within samples and β-diversity assessing diversity between samples. Prolonged restraint affected both α- and β-diversity. Hierarchical clustering analyses indicated that the profile of the top ten most abundant bacterial types was significantly different in the mice exposed to 3, 5, or 7 days of restraint compared to profiles found in control animals [34]. Mice will not eat or drink while in restraining tubes, even if food and water is provided. Because changes in diet can have a profound impact on the microbiota [5, 40], a food and water deprived control group was included in the study. Mice that were restrained for one night had microbial profiles that were similar to food and water deprived control mice. However, as mice were exposed to repeated cycles of the restraint stressor (i.e., 3, 5, or 7 repeated nights of prolonged restraint) microbial profiles were distinct from those found in food and water deprived mice [34]. This indicates that at least some of the effects of the stressor on the microbiota are due to food and water deprivation, but that repeated cycles of the stressor had additional effects on the microbiota that were not accounted for by food and water deprivation.

In addition to changes in microbial community β-diversity, exposure to prolonged restraint also results in changes to α-diversity. Rarefaction analysis indicated that species diversity decreased with repeated cycles of restraint. This is important, because it is generally believed that loss of α-diversity leads to increased susceptibility to enteric infection [41]. Thus, it was hypothesized that mice exposed to the prolonged restraint stressor would have an increased susceptibility to enteric infection [34]. To test this hypothesis, mice were orally challenged with *Citrobacter rodentium*, which is a natural murine colonic pathogen, with pathogenesis and resulting colonic pathology that are nearly indistinguishable from that produced in humans infected with enteropathogenic *E. coli*, and some components of enterohemorrhagic *E. coli* [42–44]. As the infection progresses, the colonic inflammatory response resembles many aspects of the colitis found in patients with inflammatory bowel disease [44, 45].

Challenging mice with *C. rodentium* prior to, or during, exposing to the prolonged restraint stressor significantly increased *C. rodentium* colonization in the colon and increased pathogen-induced colitis marked by increases in inflammatory cytokine (e.g., TNF-α) mRNA levels and increased colonic histopathology [34, 46]. Interestingly, exposing mice to six consecutive nights of prolonged restraint prior to oral challenge with *C. rodentium* increased colonic pathogen levels and mildly increased pathogen-induced colitis [34]. However, exposing mice to the prolonged restraint stressor for 1 night prior to oral challenge with *C. rodentium* and then exposing mice to 6 more nights of prolonged restraint (i.e., through day 5 post-*C. rodentium* challenge) resulted in significantly greater colonic pathology [46]. Simultaneously administering the prolonged restraint stressor and the *C. rodentium* challenge caused outbred CD-1 mice, which are generally considered resistant to *C. rodentium* infection, to develop severe colitis with lesions containing inflammation, epithelial defects, hyperplasia, and dysplasia. In some cases, neutrophilic inflammation extended from the mucosa to the submucosa and was frequently associated with epithelial erosion and ulceration [46].

C. rodentium lack pathogenic mechanisms to cross the intestinal epithelial barrier, and thus are not considered an invasive pathogen. However, simply exposing mice to the prolonged restraint stressor during oral challenge with *C. rodentium* was sufficient to significantly increase the occurrence of *C. rodentium* in the spleen, and also increased circulating levels of IL-6 and anxiety-like behavior. This suggests that stressor exposure during *C. rodentium* challenge disrupted the tight junctions between intestinal epithelial cells that in healthy tissue prevent the passive transfer of non-invasive microbes, fluid, and nutrients form the lumen of the intestines to the interior of the body. Stressor exposure is well known to affect tight junctional protein expression and the permeability of intestinal tissue [47–49]. Our study involving a colonic pathogen suggests that pairing stressor exposure and colonic infection can further degrade colonic epithelial barrier integrity [46].

It is not yet known whether stressor-induced alterations in the intestinal microbiota contribute to the enhancive effects of stressor exposure on *C. rodentium* challenge. However, administering probiotic *Lactobacillus reuteri* (ATCC23272) has beneficial effects on stressor-exposed mice orally challenged with *C. rodentium* [46]. *L. reuteri* is widely recognized to limit inflammation, and in a study involving gnotobiotic mice orally challenged with enterohemorrhagic *E. coli* (which is closely related to *C. rodentium*), *L. reuteri* significantly reduced colonic inflammation [50]. In our studies, *L. reuteri* administration during prolonged restraint significantly enhanced gut barrier integrity. Providing *L. reuteri* to the stressor-exposed mice prevented the ability of *C. rodentium* to translocate from the lumen of the intestines to the spleen. Administering the *L. reuteri* also prevented the increase in circulating IL-6 and the development of anxiety-like behavior in mice exposed to the stressor during *C. rodentium* challenge [46].

Exposure to the prolonged restraint stressor reduces both relative and absolute levels of commensal *L. reuteri* that are associated with colonic tissue (Galley et al., under review). This was observed in a study involving 16s rRNA gene sequencing using the 454 FLX-Titanium pyrosequencing platform followed by real-time PCR to characterize colonic tissue-associated microbiota in mice exposed to the prolonged restraint stressor. Considering the finding that administering probiotic *L. reuteri* to stressor-exposed mice prevents some, but not all, effects of the stressor has led to the hypothesis that stressor-induced alterations in commensal tissue-associated microbiota result in an internal environment that is more conducive to pathogen-induced colonic inflammation. It is further hypothesized that this internal environment leads to increased epithelial permeability and the translocation of pathogenic (as well as commensal) microbes from the lumen of the intestines to the interior of the body where they stimulate increases in inflammatory cytokines that alter the behavior of the host (Fig. 12.1). Further studies are needed to confirm this hypothesis, and to determine whether commensal and probiotic microbes in addition to *L. reuteri* are involved with, or can prevent, stressor-induced increases in colonic inflammation.

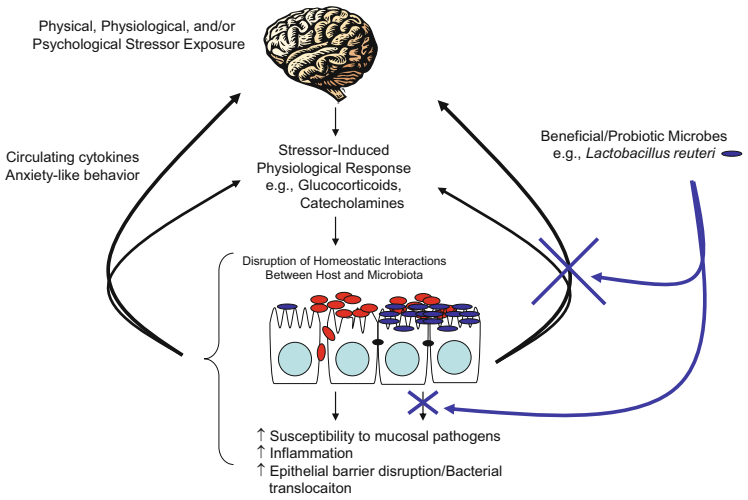

Fig. 12.1 Exposure to physical, physiological, or psychological stressors sets into motion a series of physiological responses that have the capacity to disrupt homeostatic interactions between the host and the gut microbiota. These disrupted homeostatic interactions lead to increases in susceptibility to intestinal infection and inflammation, and enhances epithelial barrier permeability and subsequent translocation from the lumen of the intestines to the interior of the body. The disruptions in epithelial barrier integrity lead to increases in circulating cytokines that have the capacity to change animal behavior and further stimulate the endocrine response. The hypothesis that alterations in the intestinal microbiota are responsible for these disrupted homeostatic interactions comes from data indicating that stressor exposure reduces beneficial microbes, such as bacteria in the genus *Lactobacillus*. Feeding mice lactobacilli to prevent the stressor-induced reduction in *Lactobacillus* spp. prevents the stressor-induced increase in susceptibility to colonic infection and inflammation and prevents disruptions to epithelial barrier integrity

Studies Involving Repeated Social Defeat

The effects of stress on colonic microbiota and inflammatory responses are also evident using a social stressor called social disruption (SDR). Social stressors often involve aggressive interactions between dominant and subordinate animals and are widely used to study the effects of stress on animal behavior and physiological functioning [51–54]. Social disruption involves aggressive interactions between a dominant intruder mouse (i.e., the aggressor) and resident subordinate mice (i.e., the experimental subjects). The aggressive interactions occur over a 2 h period at the beginning of the active cycle, when the aggressor is placed into the cage of the resident subordinate mice. The aggressor physically interacts with the residents for short periods of time until the residents display an upright defeat posture. Because the mice are housed together, the subordinate mice cannot escape and the aggressive intruder mouse will repeatedly attack and defeat the residents. Thus, the residents are exposed to repeated social defeat during the 2 h period.

The SDR stressor involves both physical and psychological components, and the defeated mice develop anxiety-like behaviors [55, 56] and a physiological stress

response marked by elevated corticosterone [57, 58], epinephrine, and norepinephrine [59]. Importantly, exposure to the SDR stressor has well defined effects on systemic immune responses. For example, exposure to the SDR stressor is known to increase circulating levels of cytokines, such as IL-1 and IL-6, even in the absence of infectious challenge [60–62], which is also commonly evident in humans exposed to different types of stressors [63–65]. In addition, exposure to the SDR stressor reduces the sensitivity of splenic monocytes/macrophages to the suppressive effects of glucocorticoid hormones and increases the ability of these splenic monocytes/macrophages to kill target microbes [57, 58, 66–69].

Microbial populations in the cecums of mice exposed to the well-defined SDR stressor were assessed using 454 FLX pyrosequencing. Consistent with results obtained with prolonged restraint, exposure to the SDR stressor resulted in significant changes in both the α and β diversity of the cecal microbiota [35]. Alpha diversity indices, including OTU, ACE, and Chao all demonstrated statistically significant reductions in microbial diversity by 15 h after the last cycle of the SDR stressor. In addition, the relative abundance of 8 out of the top 25 most abundant microbes was significantly affected by exposure to the SDR stressor. These differences were evident immediately after the last cycle of stressor exposure, as well as the morning following the last cycle of the stressor [35] indicating that the effects of the stressor occur rapidly in response to stressor exposure and can persist for at least 15 h after termination of stressor exposure.

The conclusion that stressor exposure can enhance infectious colitis based on studies involving outbred CD-1 mice exposed to prolonged restraint during oral challenge with *C. rodentium* has been confirmed and extended in inbred mice exposed to a social stressor during oral challenge with *C. rodentium*. Inbred C57BL/6 mice were orally challenged with a low dose of *C. rodentium*, and in non-stressed control mice, little pathogen colonization and pathogen-induced colitis occurred with this low infectious dose. However, simply exposing the mice to the SDR stressor again increased both pathogen colonization and associated pathology (Galley et al., under review). Mice exposed to the social stressor during *C. rodentium* challenge had higher levels of mRNA for colonic inflammatory cytokines (e.g., TNF-α), chemokines (e.g., CCL2), and inflammatory mediators (e.g., inducible nitric oxide synthase (iNOS)). In addition, pathogen-induced colonic histopathology, which was mild in mice left undisturbed during oral challenge with *C. rodentium*, was significantly increased in mice exposed to the SDR stressor during challenge with the pathogen. Stressor exposed mice had increases in colonic epithelial cell hyperplasia and dysplasia, as well as epithelial defects, generalized edema and leukocyte infiltration. These effects were not evident in the mice that were not exposed to the stressor during pathogen challenge (Galley et al., under review).

Inflammatory monocytes are recruited to sites of inflammation in response to the chemokine CCL2 and are prolific producers of tissue-damaging TNF-α and iNOS in the colon [70]. Unpublished observations from our laboratory indicate that *L. reuteri* ATCC23272 can inhibit the ability of murine colonic epithelial cells (i.e., CMT-93 cells) to produce CCL2 (Mackos et al., unpublished observations),

while others demonstrate that *L. reuteri* (strain 6475) can inhibit the ability of monocytes to produce TNF-α [71–73]. Thus, mice were administered *L. reuteri* to determine whether the commensal probiotic would attenuate stressor-induced colitis. Daily administration with 1×10^8 CFU of *L. reuteri* after exposure to the SDR stressor significantly reduced the effects of the stressor on *C. rodentium* induced colitis (Galley et al., under review); stressor-induced increases in TNF-α, iNOS, or CCL2 through the peak of *C. rodentium* infection, which occurs on day 12 post-challenge did not occur in probiotic-treated mice. In addition, colonic histopathology was not evident in any of the mice fed the *L. reuteri*, regardless of whether they were exposed to the stressor or not.

Much has been learned about the effects of probiotic microbes on host immune responses over the past 10 years, and it is tempting to speculate on the mechanisms by which *L. reuteri* attenuates stressor-induced colitis. *L. reuteri* has the capacity to limit pathogen growth and replication, particularly in vitro [74]. However, in all of the studies conducted in our laboratory utilizing *C. rodentium* [46], as well as other laboratories using a closely related pathogen (EHEC) [50], *L. reuteri* did not affect pathogen levels in vivo. Mice exposed to either the prolonged restraint stressor or the social stressor during oral challenge with *C. rodentium* had similar pathogen levels with or without being fed *L. reuteri*. These data demonstrate that *L. reuteri* does not attenuate pathogen-induced colitis by reducing pathogen load, and suggests that *L. reuteri* directly suppresses host inflammatory responses.

There are now multiple studies demonstrating that *L. reuteri* produces an immunomodulatory factor(s) when grown to stationary phase in vitro that reduces monocyte inflammatory cytokine production upon stimulation. Some of the effects of *L. reuteri* on stimulated monocytes are thought to be dependent upon bacterial production of histamine that when bound to H2 receptors reduces monocyte activity [73]. However, some strains of *L. reuteri*, such strain as ATCC23272 used in our studies, do not strongly reduce monocyte/macrophage activity, but rather have strong effects on colonic epithelial cells. Administering supernatants from overnight cultures of strain ATCC23272 reduced CCL2, TNF-α, and iNOS production by CMT-93 colonic epithelial cells, but not RAW264.7 macrophages or CD11b + splenic monocytes/macrophages stimulated with *C. rodentium* (Mackos and Bailey, Unpublished Observations). Thus, it is possible that some strains of *L. reuteri* reduce colonic inflammation through effects directly on inflammatory monocytes, whereas other strains might reduce colonic inflammation by reducing the ability of colonic epithelial cells to recruit and activate inflammatory cells, such as inflammatory monocytes and neutrophils.

It is also possible that *L. reuteri* has a more indirect effect on colonic inflammation in stressor-exposed mice. Studies demonstrate that intestinal microbes can impact the activation of the HPA axis and increase glucorticoid levels [75]. This could be of particular importance, because glucocorticoids produced by activation of the HPA axis potently suppress inflammatory responses [76], and reduced glucocorticoid production during stressor exposure as a result of adrenal insufficiency leads to intestinal inflammation during stressor exposure [77]. It is also possible that the production of immunomodulatory neuroendocrine mediators by

L. reuteri, or by commensal microbes affected by *L. reuteri*, are responsible for effects on colonic inflammation in stressor-exposed mice. It has been shown that probiotic microbes can produce immunomodulatory neuroendocrine hormones, such as γ-amino butyric acid (GABA), norepinephrine, dopamine, and serotonin (reviewed in [78]), and it has been hypothesized that this hormone production can be responsible for influencing mucosal immune responses [78]. Thus, it is conceivable that *L. reuteri* does not directly impact host colonic inflammation, but rather stimulates host physiological responses that are known to have suppressive effects on the inflammatory response. Potential pathways by which stress, the microbiota, and probiotics impact colonic inflammation are illustrated in Fig. 12.1.

Microbiota and Stressor-Induced Immunomodulation in Systemic Compartments

Stressor exposure often results in increases in nonspecific inflammatory responses. For example, humans under the chronic stress of caring for a spouse with Alzheimer's disease were found to have increases in circulating IL-6 [79], whereas exposure to acute laboratory stressors, such as different mental tasks, causes increases in IL-1 [80]. The mechanisms by which these stressor-induced increases in inflammatory cytokines occur in otherwise healthy individuals are not completely understood. But data from our laboratory, as well as others, suggest that the intestinal microbiota are involved [35, 81–83]. Mice exposed to the SDR stressor also show evidence of circulating cytokines, and cytokine levels directly correlate with microbiota levels [35]. For example, the relative abundance of three members of the microbiota (i.e., *Coprococcus* spp., *Pseudobutyrivibrio* spp., and *Dorea* spp.) were inversely correlated with the stressor-induced increases in circulating IL-6 [35]. This suggested that the microbiota were somehow involved in stressor-induced increases in circulating cytokines, but it was not until mice were given an oral cocktail of nonabsorbable antibiotics to reduce the microbiota that the link between the microbiota and circulating cytokines began to be clarified. Exposing antibiotic-treated mice to the stressor failed to increase circulating cytokines demonstrating a direct link between the microbiota and circulating cytokines [35]. This initial discovery led to additional studies to determine whether the microbiota are involved in stressor-induced modulation of macrophage microbicidal activity.

Phagocytes from mice lacking microbiota are deficient in their ability to kill target pathogens, including *Streptococcus pneumoniae* and *Staphylococcus aureus* [84]. Colonizing germ free mice by transplanting fecal bacteria from conventional mice in to the germ free mice led to effective bacterial killing by the phagocytes. Because reconstituted germfree mice had detectable levels of bacterial peptidoglycan in circulation, and because mice lacking the peptidoglycan receptor Nod1 were deficient in killing target microbes [84], it is likely that peptidoglycan from the

microbiota is necessary to prime phagocytes for efficient microbicidal activity. This led us to question whether the microbiota are also necessary for the ability of the stress response to prime splenic macrophages for enhanced microbicidal activity.

Exposing conventional mice to the SDR stressor increases the ability of splenic macrophages to kill target microbes, such as *E. coli*, through an increased production of macrophage peroxynitrite [67, 85, 86]. This effect is dependent upon signaling through TLR4 [67] and the IL-1 receptor type 1 [86], and fails to occur in germfree mice that lack any microbiota [85]. Exposing germfree mice to the SDR stressor did not increase macrophage microbicidal activity or peroxynitrite production. However, reconstituting the germfree mice with microbiota allowed the effects of the stressor on splenic macrophage activity to again be manifest [85]. This demonstrates that the microbiota are necessary for stressor-induced increases in microbicidal activity to occur. Ongoing studies are determining the mechanisms by which the microbiota can impact splenic macrophage activity, but as shown in Fig. 12.2, data suggests that the microbiota exert their effects through IL-1R1 and TLR4 signaling.

Conclusions

The dense populations of microbes that naturally colonize the body are well recognized to have beneficial effects on the host, and as microbiota research flourishes, we are becoming increasingly aware of the function of the microbiota in maintaining the health of the host. These functions are in part dependent upon the structure of the microbial communities, and it is thought that structure-function relationships have developed through the co-evolution of the host and its microbiota, such that alterations in one are associated with alterations in the other. This is well-illustrated in animals exposed to different types of stressors. During periods of quiescence, homeostatic interactions occur between the host and its microbiota to maintain beneficial microbial populations in the intestines and limit the induction of host inflammatory responses. As outlined in this chapter, exposing animals to experimental stressors significantly disrupts these homeostatic interactions; stressor-induced alterations in microbiota community structure are associated with increased host inflammatory responses.

There is now accumulating evidence that stressor-induced alterations in microbiota community structure are not just correlated with alterations in host inflammatory responses, but might actually be causally involved in stressor-induced immunomodulation. Exposure to psychological and physical stressors results in the reduction in commensal lactobacilli, with data in mice suggesting that the abundance of colonic tissue-associated *Lactobacillus* spp. are strongly reduced upon stressor exposure. The lactobacilli are known to have several beneficial effects on the health of the host, thus it is somewhat counterintuitive that the stress response, which has evolved to benefit the host in the face of environmental

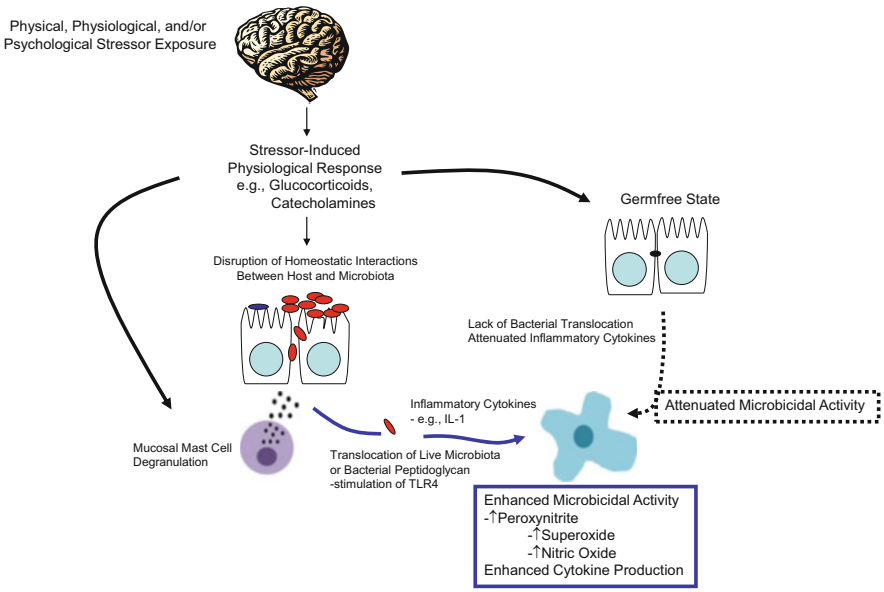

Fig. 12.2 Exposing conventional mice to a social stressor leads to the translocation of gut microbes and their products from the lumen of the intestines to the interior of the body, through a mast cell-dependent mechanism. It is hypothesized that this translocation is responsible for the stressor-induced increase in circulating cytokines, and subsequent enhancement of splenic macrophage microbicidal activity. The gut microbiota are hypothesized to be involved, because germfree mice exposed to the stressor have lower levels of circulating cytokines and macrophage microbicidal activity is not enhanced by stressor exposure

demands, would negatively impact commensal microbes. However, stressor-induced reductions in the lactobacilli might actually be reflective of gastrointestinal physiological responses that are meant to be protective against enteric pathogens. Stressor exposure increases colonic secretions, including the secretion of mucous, and colonic motility. Colonic mucous is a protective buffer that separates potential pathogens from adhering to colonic tissue. Thus, increased colonic mucous secretion, coupled with enhanced colonic motility, would help to flush potential pathogens from the colon. If, however, mucoadherent commensal microbes, such as members of the genus *Lactobacillus*, that naturally suppress colonic inflammation are also flushed from the intestines, it would result in a colonic environment that is conducive to overproduction of inflammatory mediators (Fig. 12.1).

Support for this notion comes from studies demonstrating that exposure to either prolonged restraint or the SDR stressor reduces the abundance of *Lactobacillus* spp., particularly of *L. reuteri*, and leads to increased colonic cytokine and chemokine production upon pathogen challenge. *L. reuteri* reduces colonic cytokine and chemokine production, and feeding stressor exposed mice *L. reuteri* to prevent stressor-induced reductions in *L. reuteri* prevents the exacerbating effects of the

stressor on colonic cytokine and chemokine production. These findings support a causal role for stressor-induced alterations in lactobacilli in the stressor-induced exacerbation of infectious colitis (Fig. 12.1).

In addition to attenuating colonic inflammation, *L. reuteri* helped to reinforce the epithelial barrier. Mice exposed to either prolonged restraint or the SDR stressor during oral challenge with *C. rodentium* are more likely to have *C. rodentium* in the spleen than non-stressed control mice challenged with *C. rodentium*. This is important, because unlike invasive enteric pathogens, such as Salmonella spp., *C. rodentium* does not have mechanisms to invade its host [44]. *C. rodentium* and closely related EPEC stay within the digestive tract, attached to the apical surface of the colonic epithelium during infection of immunocompetent hosts [44]. However, *C. rodentium* can disrupt tight junctions found between colonic epithelial cells via the injection of effector proteins through a type III secretion system. *L. reuteri* are known to prevent inflammation-induced increases in colonic epithelial permeability [87], and can prevent the translocation of *C. rodentium* from the colon to the spleen in stressor-exposed mice, an effect that is associated with altered expression of genes encoding tight junction proteins [46]. These data indicate that an important function of the commensal microbiota is the regulation of tight junctional proteins, and stressor-induced alterations in the microbiota can allow for the loosening of tight junctions and the exacerbation of systemic manifestations of infectious colitis (Fig. 12.1).

Increased epithelial barrier permeability is also evident in uninfected stressor-exposed mice [48, 49, 85]. Several different types of stressors have been shown to increase the permeability of the intestinal barrier through mast cell-dependent mechanisms [47–49]. A defining characteristic of commensal microbes is their inability to invade through an epithelial barrier. Thus, commensal microbes can be maintained within their niche in the body by just a single layer of epithelial cells. However, it is known that stressor exposure can increase the ability of commensal microbes, and their products like lipopolysaccharide and peptidoglycan, to translocate from the lumen of the intestines to the interior of the body [85, 88, 89]. Because stressor-exposed germfree mice do not have increases in circulating cytokines, it is hypothesized that microbes that have translocated into circulation during stressor exposure cause an increase in circulating cytokines, such as IL-1. It is further hypothesized that this microbiota-dependent increase in IL-1 primes phagocytes for enhanced microbicidal activity through TLR4 signaling, because stressor-induced increases in microbicidal activity does not occur in TLR4-/- mice, IL-1R1-/- mice or in germfree mice (Fig. 12.2).

These studies demonstrate that the microbiota are interactively involved in stressor-induced immunomodulation at mucosal surfaces, as well as at systemic sites. Intestinal epithelial cells are important in mediating interactions between the microbiota and host immune responses. As interest in the microbiota continues to grow, it will be of importance to understand the molecular underpinnings through which microbiota, intestinal epithelial cells, and immune system activity are affected by stressor exposure.

References

1. Huffnagle GB (2010) The microbiota and allergies/asthma. PLoS Pathog 6(5):e1000549
2. Allison SD, Martiny JB (2008) Colloquium paper: resistance, resilience, and redundancy in microbial communities. Proc Natl Acad Sci U S A 105(Suppl 1):11512–11519
3. Antonopoulos DA, Huse SM, Morrison HG, Schmidt TM, Sogin ML, Young VB (2009) Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. Infect Immun 77(6):2367–2375
4. Dethlefsen L, Huse S, Sogin ML, Relman DA (2008) The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 6(11):e280
5. Turnbaugh PJ, Backhed F, Fulton L, Gordon JI (2008) Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe 3 (4):213–223
6. Beaumont W (1838) Experiments and observations on the gastric juice and the physiology of digestion. Edinburgh, London
7. Badgley LE, Spiro HM, Senay EC (1969) Effect of mental arithmetic on gastric secretion. Psychophysiology 5(6):633–637
8. Holtmann G, Kriebel R, Singer MV (1990) Mental stress and gastric acid secretion. Do personality traits influence the response? Dig Dis Sci 35(8):998–1007
9. Yang H, Yuan PQ, Wang L, Tache Y (2000) Activation of the parapyramidal region in the ventral medulla stimulates gastric acid secretion through vagal pathways in rats. Neuroscience 95(3):773–779
10. Tache Y, Martinez V, Million M, Wang L (2001) Stress and the gastrointestinal tract III. Stress-related alterations of gut motor function: role of brain corticotropin-releasing factor receptors. Am J Physiol Gastrointest Liver Physiol 280(2):G173–G177
11. Tache Y, Perdue MH (2004) Role of peripheral CRF signalling pathways in stress-related alterations of gut motility and mucosal function. Neurogastroenterol Motil 16(Suppl 1):137–142
12. O'Malley D, Julio-Pieper M, Gibney SM, Dinan TG, Cryan JF (2010) Distinct alterations in colonic morphology and physiology in two rat models of enhanced stress-induced anxiety and depression-like behaviour. Stress 13(2):114–122
13. Shigeshiro M, Tanabe S, Suzuki T (2012) Repeated exposure to water immersion stress reduces the Muc2 gene level in the rat colon via two distinct mechanisms. Brain Behav Immun 26(7):1061–1065
14. Drasar BS, Shiner M, McLeod GM (1969) Studies on the intestinal flora. I. The bacterial flora of the gastrointestinal tract in healthy and achlorhydric persons. Gastroenterology 56(1):71–79
15. Berg RD (1996) The indigenous gastrointestinal microflora. Trends Microbiol 4(11):430–435
16. Stephen AM, Wiggins HS, Cummings JH (1987) Effect of changing transit time on colonic microbial metabolism in man. Gut 28(5):601–609
17. Sonnenburg JL, Xu J, Leip DD et al (2005) Glycan foraging in vivo by an intestine-adapted bacterial symbiont. Science 307(5717):1955–1959
18. Mackenzie DA, Jeffers F, Parker ML et al (2010) Strain-specific diversity of mucus-binding proteins in the adhesion and aggregation properties of *Lactobacillus reuteri*. Microbiology 156 (Pt 11):3368–3378
19. Lyte M (2004) Microbial endocrinology and infectious disease in the 21st century. Trends Microbiol 12(1):14–20
20. Lyte M, Bailey MT (1997) Neuroendocrine-bacterial interactions in a neurotoxin-induced model of trauma. J Surg Res 70(2):195–201
21. Pullinger GD, Carnell SC, Sharaff FF et al (2010) Norepinephrine augments *Salmonella enterica*-induced enteritis in a manner associated with increased net replication but independent of the putative adrenergic sensor kinases QseC and QseE. Infect Immun 78(1):372–380
22. Tannock GW, Savage DC (1974) Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. Infect Immun 9(3):591–598

23. Everson CA, Toth LA (2000) Systemic bacterial invasion induced by sleep deprivation. Am J Physiol Regul Integr Comp Physiol 278(4):R905–R916
24. Lizko NN (1987) Stress and intestinal microflora. Nahrung 31(5–6):443–447
25. Holdeman LV, Good IJ, Moore WE (1976) Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. Appl Environ Microbiol 31 (3):359–375
26. Bailey MT, Coe CL (1999) Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. Dev Psychobiol 35(2):146–155
27. Bailey MT, Lubach GR, Coe CL (2004) Prenatal stress alters bacterial colonization of the gut in infant monkeys. J Pediatr Gastroenterol Nutr 38(4):414–421
28. Sakuma K, Funabashi H, Matsuoka H, Saito M (2013) Potential use of *Lactobacillus* cell density in feces as a non-invasive bio-indicator for evaluating environmental stress during mouse breeding. Biocontrol Sci 18(2):101–104
29. Aguilera M, Vergara P, Martinez V (2013) Environment-related adaptive changes of gut commensal microbiota do not alter colonic toll-like receptors but modulate the local expression of sensory-related systems in rats. Microb Ecol 66(1):232–243
30. Knowles SR, Nelson EA, Palombo EA (2008) Investigating the role of perceived stress on bacterial flora activity and salivary cortisol secretion: a possible mechanism underlying susceptibility to illness. Biol Psychol 77(2):132–137
31. Sartor RB (2006) Microbial and dietary factors in the pathogenesis of chronic, immune-mediated intestinal inflammation. Adv Exp Med Biol 579:35–54
32. Nocker A, Burr M, Camper AK (2007) Genotypic microbial community profiling: a critical technical review. Microb Ecol 54(2):276–289
33. O'Mahony SM, Marchesi JR, Scully P et al (2009) Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. Biol Psychiatry 65(3):263–267
34. Bailey MT, Dowd SE, Parry NM, Galley JD, Schauer DB, Lyte M (2010) Stressor exposure disrupts commensal microbial populations in the intestines and leads to increased colonization by *Citrobacter rodentium*. Infect Immun 78(4):1509–1519
35. Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M (2011) Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain Behav Immun 25(3):397–407
36. Buynitsky T, Mostofsky DI (2009) Restraint stress in biobehavioral research: recent developments. Neurosci Biobehav Rev 33(7):1089–1098
37. Dobbs CM, Vasquez M, Glaser R, Sheridan JF (1993) Mechanisms of stress-induced modulation of viral pathogenesis and immunity. J Neuroimmunol 48(2):151–160
38. Dobbs CM, Feng N, Beck FM, Sheridan JF (1996) Neuroendocrine regulation of cytokine production during experimental influenza viral infection: effects of restraint stress-induced elevation in endogenous corticosterone. J Immunol 157(5):1870–1877
39. Padgett DA, MacCallum RC, Sheridan JF (1998) Stress exacerbates age-related decrements in the immune response to an experimental influenza viral infection. J Gerontol A Biol Sci Med Sci 53(5):B347–B353
40. Sartor RB (2012) Gut microbiota: diet promotes dysbiosis and colitis in susceptible hosts. Nat Rev Gastroenterol Hepatol 9(10):561–562
41. Chang JY, Antonopoulos DA, Kalra A et al (2008) Decreased diversity of the fecal microbiome in recurrent Clostridium difficile-associated diarrhea. J Infect Dis 197(3):435–438
42. Borenshtein D, McBee ME, Schauer DB (2008) Utility of the *Citrobacter rodentium* infection model in laboratory mice. Curr Opin Gastroenterol 24(1):32–37
43. Luperchio SA, Schauer DB (2001) Molecular pathogenesis of *Citrobacter rodentium* and transmissible murine colonic hyperplasia. Microbes Infect 3(4):333–340
44. Mundy R, MacDonald TT, Dougan G, Frankel G, Wiles S (2005) *Citrobacter rodentium* of mice and man. Cell Microbiol 7(12):1697–1706

45. Eckmann L (2006) Animal models of inflammatory bowel disease: lessons from enteric infections. Ann N Y Acad Sci 1072:28–38
46. Mackos AR, Eubank TD, Parry NM, Bailey MT (2013) Probiotic *Lactobacillus reuteri* attenuates the stressor-enhanced severity of *Citrobacter rodentium* infection. Infect Immun 81:3253–3263
47. Cameron HL, Perdue MH (2005) Stress impairs murine intestinal barrier function: improvement by glucagon-like peptide-2. J Pharmacol Exp Ther 314(1):214–220
48. Santos J, Yang PC, Soderholm JD, Benjamin M, Perdue MH (2001) Role of mast cells in chronic stress induced colonic epithelial barrier dysfunction in the rat. Gut 48(5):630–636
49. Soderholm JD, Yates DA, Gareau MG, Yang PC, MacQueen G, Perdue MH (2002) Neonatal maternal separation predisposes adult rats to colonic barrier dysfunction in response to mild stress. Am J Physiol Gastrointest Liver Physiol 283(6):G1257–G1263
50. Eaton KA, Honkala A, Auchtung TA, Britton RA (2011) Probiotic *Lactobacillus reuteri* ameliorates disease due to enterohemorrhagic *Escherichia coli* in germfree mice. Infect Immun 79(1):185–191
51. Bohus B, Koolhaas JM, Heijnen CJ, de Boer O (1993) Immunological responses to social stress: dependence on social environment and coping abilities. Neuropsychobiology 28(1–2):95–99
52. de Groot J, van Milligen FJ, Moonen-Leusen BW, Thomas G, Koolhaas JM (1999) A single social defeat transiently suppresses the anti-viral immune response in mice. J Neuroimmunol 95(1–2):143–151
53. Korte SM, Smit J, Bouws GAH, Koolhaas JM, Bohus B (1990) Behavioral and neuroendocrine response to psychosocial stress in male rats: the effects of the 5-HT1A agonistic ipsapirone. Horm Behav 24:554–567
54. Sgoifo A, Stilli D, de Boer SF, Koolhaas JM, Musso E (1998) Acute social stress and cardiac electrical activity in rats. Aggress Behav 24:287–296
55. Kinsey SG, Bailey MT, Sheridan JF, Padgett DA, Avitsur R (2007) Repeated social defeat causes increased anxiety-like behavior and alters splenocyte function in C57BL/6 and CD-1 mice. Brain Behav Immun 21(4):458–466
56. Wohleb ES, Hanke ML, Corona AW et al (2011) beta-Adrenergic receptor antagonism prevents anxiety-like behavior and microglial reactivity induced by repeated social defeat. J Neurosci 31(17):6277–6288
57. Bailey MT, Avitsur R, Engler H, Padgett DA, Sheridan JF (2004) Physical defeat reduces the sensitivity of murine splenocytes to the suppressive effects of corticosterone. Brain Behav Immun 18(5):416–424
58. Engler H, Engler A, Bailey MT, Sheridan JF (2005) Tissue-specific alterations in the glucocorticoid sensitivity of immune cells following repeated social defeat in mice. J Neuroimmunol 163(1–2):110–119
59. Hanke ML, Powell ND, Stiner LM, Bailey MT, Sheridan JF (2012) Beta adrenergic blockade decreases the immunomodulatory effects of social disruption stress. Brain Behav Immun 26 (7):1150–1159
60. Avitsur R, Kavelaars A, Heijnen C, Sheridan JF (2005) Social stress and the regulation of tumor necrosis factor-alpha secretion. Brain Behav Immun 19(4):311–317
61. Engler H, Bailey MT, Engler A, Stiner-Jones LM, Quan N, Sheridan JF (2008) Interleukin-1 receptor type 1-deficient mice fail to develop social stress-associated glucocorticoid resistance in the spleen. Psychoneuroendocrinology 33(1):108–117
62. Stark JL, Avitsur R, Hunzeker J, Padgett DA, Sheridan JF (2002) Interleukin-6 and the development of social disruption-induced glucocorticoid resistance. J Neuroimmunol 124(1–2):9–15
63. Brydon L, Edwards S, Mohamed-Ali V, Steptoe A (2004) Socioeconomic status and stress-induced increases in interleukin-6. Brain Behav Immun 18(3):281–290
64. Brydon L, Steptoe A (2005) Stress-induced increases in interleukin-6 and fibrinogen predict ambulatory blood pressure at 3-year follow-up. J Hypertens 23(5):1001–1007

65. Steptoe A, Hamer M, Chida Y (2007) The effects of acute psychological stress on circulating inflammatory factors in humans: a review and meta-analysis. Brain Behav Immun 21(7):901–912
66. Avitsur R, Stark JL, Dhabhar FS, Padgett DA, Sheridan JF (2002) Social disruption-induced glucocorticoid resistance: kinetics and site specificity. J Neuroimmunol 124(1–2):54–61
67. Bailey MT, Engler H, Powell ND, Padgett DA, Sheridan JF (2007) Repeated social defeat increases the bactericidal activity of splenic macrophages through a Toll-like receptor-dependent pathway. Am J Physiol Regul Integr Comp Physiol 293(3):R1180–R1190
68. Bailey MT, Kinsey SG, Padgett DA, Sheridan JF, Leblebicioglu B (2009) Social stress enhances IL-1beta and TNF-alpha production by *Porphyromonas gingivalis* lipopolysaccharide-stimulated CD11b + cells. Physiol Behav 98(3):351–358
69. Stark JL, Avitsur R, Padgett DA, Campbell KA, Beck FM, Sheridan JF (2001) Social stress induces glucocorticoid resistance in macrophages. Am J Physiol Regul Integr Comp Physiol 280(6):R1799–R1805
70. Platt AM, Bain CC, Bordon Y, Sester DP, Mowat AM (2010) An independent subset of TLR expressing CCR2-dependent macrophages promotes colonic inflammation. J Immunol 184 (12):6843–6854
71. Jones SE, Versalovic J (2009) Probiotic *Lactobacillus reuteri* biofilms produce antimicrobial and anti-inflammatory factors. BMC Microbiol 9:35
72. Lin YP, Thibodeaux CH, Pena JA, Ferry GD, Versalovic J (2008) Probiotic *Lactobacillus reuteri* suppress proinflammatory cytokines via c-Jun. Inflamm Bowel Dis 14(8):1068–1083
73. Thomas CM, Hong T, van Pijkeren JP et al (2012) Histamine derived from probiotic *Lactobacillus reuteri* suppresses TNF via modulation of PKA and ERK signaling. PLoS One 7(2): e31951
74. Spinler JK, Taweechotipatr M, Rognerud CL, Ou CN, Tumwasorn S, Versalovic J (2008) Human-derived probiotic *Lactobacillus reuteri* demonstrate antimicrobial activities targeting diverse enteric bacterial pathogens. Anaerobe 14(3):166–171
75. Sudo N, Chida Y, Aiba Y et al (2004) Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol 558(Pt 1):263–275
76. Padgett DA, Glaser R (2003) How stress influences the immune response. Trends Immunol 24 (8):444–448
77. Reber SO, Peters S, Slattery DA et al (2011) Mucosal immunosuppression and epithelial barrier defects are key events in murine psychosocial stress-induced colitis. Brain Behav Immun 25(6):1153–1161
78. Lyte M (2011) Probiotics function mechanistically as delivery vehicles for neuroactive compounds: microbial endocrinology in the design and use of probiotics. Bioessays 33 (8):574–581
79. Kiecolt-Glaser JK, Preacher KJ, MacCallum RC, Atkinson C, Malarkey WB, Glaser R (2003) Chronic stress and age-related increases in the proinflammatory cytokine IL-6. Proc Natl Acad Sci U S A 100(15):9090–9095
80. Brydon L, Edwards S, Jia H et al (2005) Psychological stress activates interleukin-1beta gene expression in human mononuclear cells. Brain Behav Immun 19(6):540–546
81. Maslanik T, Tannura K, Mahaffey L et al (2012) Commensal bacteria and MAMPs are necessary for stress-induced increases in IL-1beta and IL-18 but not IL-6, IL-10 or MCP-1. PLoS One 7(12):e50636
82. Maslanik T, Mahaffey L, Tannura K, Beninson L, Greenwood BN, Fleshner M (2013) The inflammasome and danger associated molecular patterns (DAMPs) are implicated in cytokine and chemokine responses following stressor exposure. Brain Behav Immun 28:54–62
83. Fleshner M (2013) Stress-evoked sterile inflammation, danger associated molecular patterns (DAMPs), microbial associated molecular patterns (MAMPs) and the inflammasome. Brain Behav Immun 27(1):1–7

84. Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN (2010) Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat Med 16(2):228–231
85. Allen RG, Lafuse WP, Galley JD, Ali MM, Ahmer BM, Bailey MT (2012) The intestinal microbiota are necessary for stressor-induced enhancement of splenic macrophage microbicidal activity. Brain Behav Immun 26(3):371–382
86. Allen RG, Lafuse WP, Powell ND et al (2012) Stressor-induced increase in microbicidal activity of splenic macrophages is dependent upon peroxynitrite production. Infect Immun 80 (10):3429–3437
87. Ahrne S, Hagslatt ML (2011) Effect of lactobacilli on paracellular permeability in the gut. Nutrients 3(1):104–117
88. Bailey MT, Engler H, Sheridan JF (2006) Stress induces the translocation of cutaneous and gastrointestinal microflora to secondary lymphoid organs of C57BL/6 mice. J Neuroimmunol 171(1–2):29–37
89. Ando T, Brown RF, Berg RD, Dunn AJ (2000) Bacterial translocation can increase plasma corticosterone and brain catecholamine and indoleamine metabolism. Am J Physiol Regul Integr Comp Physiol 279(6):R2164–R2172

Part III
The Microbiota-Gut-Brain Axis in Health and Disease

Chapter 13
The Effects of Inflammation, Infection and Antibiotics on the Microbiota-Gut-Brain Axis

Premysl Bercik and Stephen M. Collins

Abstract Animal studies have demonstrated that the early phase of enteric infection is accompanied by anxiety-like behavior, which is mediated through vagal ascending pathways. Chronic infection alters gut function, including motility and visceral sensitivity, as well as feeding patterns, anxiety and depression-like behavior. These effects are likely immune-mediated, and involve changes in pro-inflammatory cytokines and altered metabolism of kynurenine/tryptophan pathways. Clinical studies have shown that chronic gastrointestinal infections lead to malnutrition and stunting, resulting in impaired cognitive function. Accumulating evidence suggests that in addition to pathogens, the commensal gastrointestinal microbiota also influences gut function and host's behavior. Both animal and clinical studies have demonstrated changes in behavior and brain chemistry after induction of intestinal dysbiosis by administration of antibiotics. This concept of microbiota-gut-brain interactions opens a new field of research aimed at developing microbial-directed therapies to treat a broad spectrum of human conditions, including chronic gastrointestinal and psychiatric disorders.

Abbreviations

BDNF	Brain derived neurotrophic factor
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CRP	C-reactive protein
DGGE	Denaturing gradient gel electrophoresis
ENS	Enteric nervous system
GABA	γ-Aminobutyric acid

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_13,

GBA	Gut brain axis
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IQ	Intelligence quotient
MPO	Myeloperoxidase activity
NMDA	*N*-methyl-D-aspartate
SCFA	Short chain fatty acids
SP	Substance P
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha

Introduction: Gut-Brain Axis

The gut brain axis (GBA) is a bi-directional neuro-humoral communication system that links gut and brain function in health and disease. The axis utilizes neural, endocrine and immunological signaling to integrate the two organs. The clinical importance of this axis is examplified by its role in the Irritable Bowel Syndrome (IBS) most common of all intestinal disorders seen in our society where it generates a huge socio-economic burden [1, 2]. Involvement of the axis is reflected in the high prevalence of psychiatric morbidity in IBS [3]. There is also growing awareness that the GBA plays a role in the natural history of chronic inflammatory bowel diseases (IBD) including Crohn's Disease and Ulcerative Colitis [4, 5]. Patients with depression, anxiety or under psychological stress experience more active disease [5]. It is now evident that the brain monitors and reacts to inflammatory activity in the gut via the vagus nerve in what is known as the inflammatory reflex [6]. Several studies have shown that vagus-mediated central nervous system control of the gut inflammation is tonic and that vagal integrity is critical in regulating chronic inflammation in animal models [7, 8]. The basis of co-existence of psychiatric disorders with IBD is unclear in terms of cause and effect for it is known that chronic intestinal inflammation induces altered behavior in animal models [9].

The intestinal microbiota is a vast consortium of 10^{14} bacteria and represents the most densely packed ecosystem on earth. Emerging data, reviewed below, provides evidence to support the integration of the intestinal microbiota into the GBA [10]. This extended axis involves bidirectional signaling involving neural, hormonal and immunological pathways described in detail below.

Inflammation, Cytokines and the Central Nervous System

There is a growing body of literature linking the immune system with psychiatric disorders. Multiple studies have demonstrated that patients with major depression have elevated levels of pro-inflammatory cytokines and C-reactive protein (CRP) [11, 12]. Depression is increased in patients with chronic illnesses associated with immune activation such as cardiovascular disease [13], rheumatoid arthritis [14], chronic obstructive pulmonary disease [15] and type 1 diabetes [16]. Cognitive function also appears to be decreased in patients with chronic inflammatory disorders [17, 18].

It is well established that acute administration of pro-inflammatory cytokines leads to sickness behavior, which includes depressive-like behavior and fatigue [19]. Therapy with cytokines in cancer patients is associated with changes in CNS function and behavior [20] and psychiatric symptoms are also a frequent side effect of interferon treatment for hepatitis C [21, 22].

The mechanisms by which cytokines can affect central nervous system (CNS) function are not fully elucidated. It has been shown that cytokines can directly activate primary afferents and the vagus nerve, or access the brain via the circumventricular organs, where the blood brain barrier is more permeable [19]. Animal studies have demonstrated that depressive-like behavior is caused by cytokine-induced changes in tryptophan/serotonin metabolism and production of kynurenine, which induces anxiety-like behavior in a dose dependent fashion [23]. Indoleamine 2,3-dioxygenase (IDO) is the extrahepatic enzyme responsible for kynurenine production, which is present in monocytes, macrophages and brain microglia and activated in response to pro-inflammatory cytokines, particularly TNF-α and INF-γ [24]. Patients who receive cytokine immunotherapy and develop major depression exhibit a prolonged decrease in circulating concentrations of tryptophan, which is accompanied by elevated concentrations of kynurenine compared to non-depressed patients [25]. By reducing tryptophan availability, IDO activation may impact brain serotoninergic neurotransmission, as tryptophan is the limiting factor for the synthesis of serotonin that plays a crucial role in the regulation of mood [26]. Furthermore, neuroactive metabolites of IDO activation, such as kynurenine and kynurenic acid, are able to directly affect CNS function [26].

If anxiety and depression are induced by immune mediators, then immunomodulatory treatment should improve mood. Two studies have demonstrated improvement of comorbid depression in patients with psoriasis and rheumatoid arthritis after treatment with etanercept [27, 28]. While infliximab had no overall benefit in a cohort of patients with major depression, it improved depressive symptoms in patients with higher baseline inflammatory biomarkers [29]. Interestingly, in this subset of patients infliximab treatment benefitted a wide range of depressive symptoms, including depressed mood, psychomotor retardation, performance of work and other activities (anhedonia/fatigue), as well as psychic anxiety and suicidal ideation. All together, these data suggest that immune system and its

products affect the brain function and play an important role in, at least in a subset of, patients with psychiatric disorders.

Effects of Infections on Cognitive Function

Accumulating evidence links infectious diseases, mainly of the digestive tract, to the function of the central nervous system. This is evident primarily in children, as from an energy-consumption standpoint, a developing human will have difficulty building a brain and fighting off infectious diseases at the same time, as both are very metabolically costly tasks. A recent study has assessed the worldwide distribution of cognitive ability in relationship to the load of infectious diseases. Using three measures of average national intelligence quotient (IQ), the authors found a robust worldwide correlation between average IQ and parasite stress. Infectious disease remained the most powerful predictor of average national IQ even when controlling for additional factors, such as temperature, distance from Africa, gross domestic product per capita and several measures of education [30]. Treating parasitic infections can positively affect brain function. A double-blind placebo-controlled study examined the effect of treating moderate to high worm burden of non-invasive *Trichuris trichiura* on the cognitive functions of Jamaican school children. Eradication of the infection led to a significant improvement in tests of auditory short-term memory and scanning, and retrieval of long-term memory [31].

Multiple studies have demonstrated that diarrheal diseases affect nutrition and development, including cognitive function in children. Early childhood diarrhea has been associated not only with impaired physical fitness and growth but also with cognitive function 6–9 years later, hindering school performance [32, 33]. A pooled analysis of nine studies, covering five countries and a 20-year period, investigated the effects of the previous history of diarrhea on stunting at age 24 months. The odds of stunting increased multiplicatively with each diarrheal episode, with the adjusted odds of stunting increasing by 1.13 for every five episodes [34]. Stunting in infancy was linked to cognition in a large study in Filipino children that found children aged between 8 and 11 years stunted between birth and age of 2 years, displayed deficits in cognitive ability compared to non-stunted children, especially when stunting was severe [35].

Gastrointestinal Infection Affects Gut-Brain Axis

Animal models have provided insight into mechanisms involved in gut-brain communication during gastrointestinal inflammation. A landmark study by Lyte et al. showed that very early phase of *Campylobacter jejuni* infection causes anxiety-like behavior in mice without any increase in inflammatory mediators,

likely due to activation of vagal ascending pathways [36, 37]. This suggests that the host is able to detect a pathogen before any significant activation of immune system. We have found that chronic infection with *Helicobacter pylori* induces functional and structural changes in the enteric nervous system (ENS), including altered release of acetylcholine upon electrical or chemical stimulation, as well as upregulation of SP and CGRP containing nerves in the stomach and spinal cord, which only partially normalizes 2 months post bacterial eradication [38]. This is accompanied by changes in visceral mechanosensitivity and gastric emptying [39]. Furthermore, the infected mice have altered feeding patterns with increased frequency of eating bouts and decreased amount of food consumed per bout which is associated with decreased proopiomelanocortin expression in the arcuate nucleus, and increased TNFα in the median eminence, which remain up-regulated even 2 months post bacterial eradication. These functional and structural abnormalities in the post-infectious period may be maintained by antigenic mimicry or cross-reactivity, as experiments showed that abnormal peristalsis and visceral hypersensitivity were maintained by feeding crude *Trichinella spiralis* antigen to the previously infected mice [40]. We have also observed that mice with chronic *H. pylori* infection display anxiety-like behavior and possibly impaired learning capacity (Fig. 13.1).

Another series of experiments showed that chronic mild to moderate infection with the non invasive colonic parasite *Trichuris muris* leads to anxiety-like behavior, which is accompanied by decreased brain derived neurotropic factor (BDNF) expression in the hippocampus [41], mildly elevated TNFα and INFγ, and increased levels of kynurenine. The abnormal behavior, but not BDNF levels, was normalized by immunomodulatory treatment with etanercept and budesonide. Interestingly, both behavior and BDNF normalized after administration of the specific probiotic *B. longum*, which likely acts through modulation of ENS and the vagal ascending pathways [9].

Antibiotic Induced Psychosis

Psychosis (highly distorted contact with reality) is a mental disorder, which could arise as a form of an adverse drug reaction [42]. Most classes of antibiotics administered for the treatment of various infections have been recorded to induce transient psychosis in patients. Some of the symptoms presented include, but are not limited to, visual and auditory hallucinations, lost orientation to persons, space and time, delusions, and agitation. Most common antibiotics known to have psychotic inducing effects are penicillins [43, 44], quinolones [42, 45, 46], macrolides [47, 48], sulfonamides [6, 48, 49] and anti-tuberculosis agents [50, 51] The psychotic events generally develop within the first days of treatment and cease after withdrawal of the antibiotic treatment [42]. Several explanations have been put forward to explain this adverse effect, including interaction of the antibiotics with neurotransmitters. Quinolones were proposed to displace GABA from its receptors,

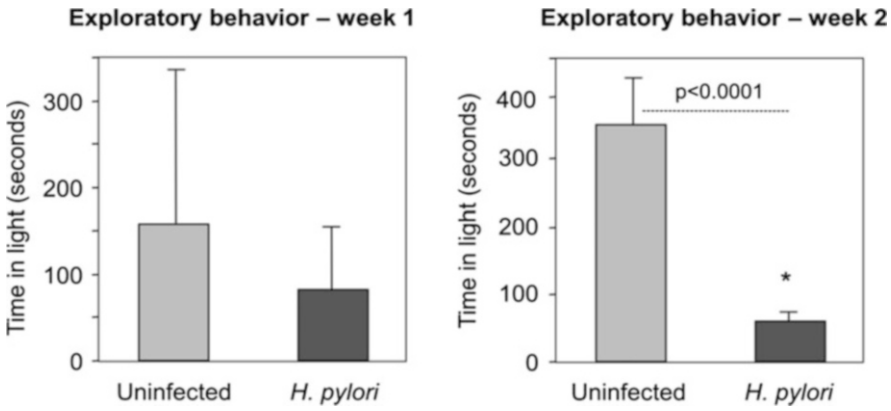

Fig. 13.1 Mice infected with *H. pylori* display anxiety-like behavior. BALB/c mice infected with *H. pylori* for at least 3 months appeared to display anxiety-like behavior when assessed at week 1 using the light preference test. This abnormal behavior became more evident at week 2, as uninfected control mice spent more time in the illuminated compartment, partially due to a learned behavior, while *H. pylori* infected mice did not change their behavior compared to week 1

decreasing GABAergic inhibition and leading to the stimulation of the CNS [42]. - Antibiotic-induced psychosis may be also the result of altered NMDA-receptor function due to the depletion of D-alanine-producing intestinal flora [52]. In our opinion, antibiotic induced intestinal dysbiosis may lead to altered metabolism of multiple neuroactive substances produced by bacteria, including neurotransmitters [53, 54] and short chained fatty acids (SCFA) [55], thus affecting brain function.

Effects of Antibiotics on Gut Function and Behavior: Lesson from Animal Models

Traditionally, insights into host-microbial interactions have used comparison between germ free and colonized young mice. However, many host systems are immature in germ free mice and differences seen may not be congruous with those seen in older mice with a stable microbiota and matured host immune and physiological systems including the brain. A preferred approach is to use interventions that alter the microbiome composition in adult mice. Perturbation of the delicate balance between the microbial composition of the gut and the host results in changes in the gut-brain axis, at the level of the gut and the brain. Exposure of the intestinal microbiota to antimicrobials is a well-established experimental approach to studying the impact of gut bacteria on the host; the impact of antimicrobials on the microbiota is dependent on the class of antibiotics used [56]. The combination of orally administered bacitracin, neomycin and the antifungal agent primaricin for 10 days resulted in increase of the inflammatory state of the gut, as reflected by a small and transient increase in myeloperoxidase (MPO) activity and

in upregulation of the sensory neurotransmitter substance P (SP) in the enteric nervous system. These changes were accompanied by an increase in the pseudoaffective response to colonic distension of the colon [57]. Concomitant treatment with dexamethasone normalized these changes, indicating that the changes in host physiology were mediated by the small increment in intestinal inflammation secondary to the antibiotic-induced change in the microbiota profile. Furthermore, restoration of Lactobacilli following antibiotic administration also ameliorated intestinal function [57]. A similar approach was adopted by Anitha et al. [58] who showed that antibiotic-induced dysbiosis resulted in altered gut transit and that these changes were mediated by Toll-like receptor 4 (TLR4). Antibiotic-induced changes in the intestinal microbiota also induced changes in brain chemistry and behavior. A similar antimicrobial cocktail of bacitracin, neomycin and pimaricin was gavaged to mice over 7 days and behavior was monitored pre-, during and post administration of antibiotics. The microbiota profiles were monitored using denaturing gradient gel electrophoresis (DGGE). While no differences in microbiota profiles were seen between test groups prior to antibiotics, treatment resulted in increased relative abundance of Lactobacilli and Actinobacteria and a decrease in γ-proteobacteria and Bacteroides [59]. This change in microbiota profile was accompanied by a reduction in anxiety, like behavior, as reflected in the step down and the light preference tests when measured 7 days after antibiotic administration. The above-described antibiotic-induced change in microbiota reverted to normal within 2 weeks after cessation of treatment and this was accompanied by a normalization of behavior. The anxiolytic behavior of antibiotic-treated mice was accompanied by an increase in BDNF in the hippocampus, and a decrease in BDNF in the amygdala [59] and these changes normalized 2 weeks post cessation of the antibiotics. Since intra-peritoneal administration of the same antibiotic mixture failed to impact on behavior in mice, we interpreted our findings to mean that the altered microbial profile in the gut was responsible for the changes in behavior and that the latter was mediated by changes in hippocampal and amygdala BDNF. We investigated underlying mechanisms and found that neither surgical bilateral sub-diaphragmatic vagotomy, nor chemical sympathectomy altered the anxiolytic behavioral profile induced by antibiotics [59]. We did not see inflammatory changes in the antibiotic mice nor significant elevations in cytokines, leading us to conclude that the changes observed in behavior most likely reflected intestinal bacterial products either acting directly on the brain, or indirectly via host metabolism. Confirmation of the role of the microbiota in altering behavior was confirmed in subsequent experiments in which we were able to transfer components of behavior phenotype to germ free mice of a different species and behavioral profile via microbial colonization. As there are marked differences in microbiota composition and exploratory behavior between NIH Swiss mice, which are daring and courageous, and BALB/c mice, which are shy and timid, we derived these two mouse strains into germ-free conditions and colonized them with the two sets of microbiota. Colonizing NIH Swiss mice with BALB/c microbiota made them less daring while the exploratory drive increased in BALB/c mice colonized with NIH Swiss bacteria [59]. In agreement with previous

experiments with antibiotic-induced dysbiosis, we observed changes in hippocampal BDNF but did not find any signs of immune activation.

Interestingly, a recent study in a single human using a beta-lactam antibiotic intervention to alter the microbial composition resulted in significant changes in host metabolomic profiles of the gut with changes in anabolic sugar metabolism, the production of acetyl donors and the synthesis and degradation of intestinal/colonic epithelium components being among the most prominent changes [60]. This finding turns attention towards the strong possibility that the shared host-microbiota metabolome is the likely sources of molecules that act directly or as precursors to modify brain function as reflected in the above-described experiments. Taken together, experimental evidence support the integration of the intestinal bacteria into the microbiota-gut-brain axis and demonstrates the utility of using a defined antimicrobial combination to investigate how bacteria access this vitally important axis. Furthermore, we should consider the possible clinical implications of the accumulated data in view of fecal transplant therapy, which is being increasingly used not only for patients with recurrent *C. difficile* infection but also for patients with IBD and IBS. In our opinion, healthy stool donors should be assessed beyond the current recommendations of bacterial and viral pathogen screening, and this should include psychiatric evaluation.

Summary/Conclusions

Changes in the microbial composition of the gut influence the gut-brain axis. This is true in the presence or absence of pathogenic bacteria. It is evident from previous work that the early invasion of the gut by enteric pathogens is monitored by the brain and generates anxiety-like behavior that may modify host behavior to minimize further exposure to the pathogen. More chronic and insidious infections also induce not only anxiety-like behavior and changes in cognition, but may also distort feeding patterns, perhaps in an effort to reduce the chances of further infection. These interpretations are based on a role of the microbiota-gut-brain axis in maintaining homeostasis and the health of the host. In the context of promoting host dysfunction and disease, it is evident that the induction of dysbiosis, either by an enteric infection, exposure to antibiotics, or dietary change can lead to host dysfunction that becomes evident not only in the gut, in conditions such as IBS, but perhaps also in behavioral disorders such as anxiety and depression. This notion opens a new field of research aimed at developing microbial-directed therapies to treat this broad spectrum of human conditions.

References

1. Boivin M (2001) Socioeconomic impact of irritable bowel syndrome in Canada. Can J Gastroenterol 15(Suppl B):8B–11B
2. Sandler RS, Everhart JE, Donowitz M, Adams E, Cronin K, Goodman C et al (2002) The burden of selected digestive diseases in the United States. Gastroenterology 122(5):1500–1511
3. Whitehead WE, Palsson O, Jones KR (2002) Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications? Gastroenterology 122(4):1140–1156
4. Bonaz BL, Bernstein CN (2013) Brain-gut interactions in inflammatory bowel disease. Gastroenterology 144(1):36–49
5. Graff LA, Walker JR, Bernstein CN (2009) Depression and anxiety in inflammatory bowel disease: a review of comorbidity and management. Inflamm Bowel Dis 15(7):1105–1118
6. Tracey KJ (2002) The inflammatory reflex. Nature 420(6917):853–859
7. Ghia JE, Blennerhassett P, El-Sharkawy RT, Collins SM (2007) The protective effect of the vagus nerve in a murine model of chronic relapsing colitis. Am J Physiol Gastrointest Liver Physiol 293(4):G711–G718
8. Ghia JE, Blennerhassett P, Kumar-Ondiveeran H, Verdu EF, Collins SM (2006) The vagus nerve: a tonic inhibitory influence associated with inflammatory bowel disease in a murine model. Gastroenterology 131(4):1122–1130
9. Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X et al (2011) The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut-brain communication. Neurogastroenterol Motil 23(12):1132–1139
10. Collins SM, Surette M, Bercik P (2012) The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol 10(11):735–742
11. Irwin MR, Miller AH (2007) Depressive disorders and immunity: 20 years of progress and discovery. Brain Behav Immun 21:374–383
12. Raison CL, Capuron L, Miller AH (2006) Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends Immunol 27(1):24–31
13. Frasure-Smith N, Lespérance F (2006) Depression and coronary artery disease. Herz 31(Suppl 3):64–68
14. Bruce TO (2008) Comorbid depression in rheumatoid arthritis: pathophysiology and clinical implications. Curr Psychiatry Rep 10(3):258–264
15. Mikkelsen RL, Middelboe T, Pisinger C, Stage KB (2004) Anxiety and depression in patients with chronic obstructive pulmonary disease (COPD). A review. Nord J Psychiatry 58(1):65–70
16. Lustman PJ, Clouse RE (2005) Depression in diabetic patients: the relationship between mood and glycemic control. J Diabetes Complications 2:113–122
17. Elfferich MD, Nelemans PJ, Ponds RW, De Vries J, Wijnen PA, Drent M (2010) Everyday cognitive failure in sarcoidosis: the prevalence and the effect of anti-TNF-alpha treatment. Respiration 80(3):212–219
18. Raftery G, He J, Pearce R, Birchall D, Newton JL, Blamire AM, Isaacs JD (2012) Disease activity and cognition in rheumatoid arthritis: an open label pilot study. Arthritis Res Ther 14 (6):R263 (Epub ahead of print)
19. Kohman RA, Rhodes JS (2013) Neurogenesis, inflammation and behavior. Brain Behav Immun 27(1):22–32
20. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008) From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 9:46–56
21. Renault PF, Hoofnagle JH, Park Y, Mullen KD, Peters M, Jones DB, Rustgi V, Jones EA (1987) Psychiatric complications of long-term interferon alfa therapy. Arch Intern Med 147:1577–1580
22. Keefe B (2007) Interferon-induced depression in hepatitis C: an update. Curr Psychiatry Rep 9:255–261

23. O'Connor JC, André C, Wang Y, Lawson MA, Szegedi SS, Lestage J, Castanon N, Kelley KW, Dantzer R (2009) Interferon-gamma and tumor necrosis factor-alpha mediate the upregulation of indoleamine 2,3-dioxygenase and the induction of depressive-like behavior in mice in response to bacillus Calmette-Guerin. J Neurosci 29(13):4200–4209
24. Wirleitner B, Neurauter G, Schrocksnadel K, Frick B, Fuchs D (2003) Interferon-gamma-induced conversion of tryptophan: immunologic and neuropsychiatric aspects. Curr Med Chem 10:1581–1591
25. Capuron L, Neurauter G, Musselman DL, Lawson DH, Nemeroff CB, Fuchs D, Miller AH (2003) Interferon-alpha-induced changes in tryptophan metabolism: relationship to depression and paroxetine treatment. Biol Psychiatry 54(9):906–914
26. O'Connor JC, Lawson MA, André C, Briley EM, Szegedi SS, Lestage J, Castanon N, Herkenham M, Dantzer R, Kelley KW (2009) Induction of IDO by bacille Calmette-Guérin is responsible for development of murine depressive-like behavior. J Immunol 182 (5):3202–3212
27. Tyring S, Gottlieb A, Papp K, Gordon K, Leonardi C, Wang A, Lalla D, Woolley M, Jahreis A, Zitnik R, Cella D, Krishnan R (2006) Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial. Lancet 367 (9504):29–35
28. Kekow J, Moots R, Khandker R, Melin J, Freundlich B, Singh A (2011) Improvements in patient-reported outcomes, symptoms of depression and anxiety, and their association with clinical remission among patients with moderate-to-severe active early rheumatoid arthritis. Rheumatology (Oxford) 50(2):401–409
29. Raison CL, Rutherford RE, Woolwine BJ, Shuo C, Schettler P, Drake DF, Haroon E, Miller AH (2013) A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: the role of baseline inflammatory biomarkers. Arch Gen Psychiatry 70(1):31–41
30. Eppig C, Fincher CL, Thornhill R (2010) Parasite prevalence and the worldwide distribution of cognitive ability. Proc Biol Sci 277(1701):3801–3808
31. Nokes C, Grantham-McGregor SM, Sawyer AW, Cooper ES, Bundy DA (1992) Moderate to heavy infections of *Trichuris trichiura* affect cognitive function in Jamaican school children. Parasitology 104(Pt 3):539–547
32. Lorntz B, Soares AM, Moore SR, Pinkerton R, Gansneder B, Bovbjerg VE, Guyatt H, Lima AM, Guerrant RL (2006) Early childhood diarrhea predicts impaired school performance. Pediatr Infect Dis J 25(6):513–520
33. Niehaus MD, Moore SR, Patrick PD, Derr LL, Lorntz B, Lima AA, Guerrant RL (2002) Early childhood diarrhea is associated with diminished cognitive function 4 to 7 years later in children in a northeast Brazilian shantytown. Am J Trop Med Hyg 66(5):590–593
34. Checkley W, Buckley G, Gilman RH, Assis AM, Guerrant RL, Morris SS, Mølbak K, Valentiner-Branth P, Lanata CF, Black RE, Childhood Malnutrition and Infection Network (2008) Multi-country analysis of the effects of diarrhoea on childhood stunting. Int J Epidemiol 37(4):816–830
35. Mendez MA, Adair LS (1999) Severity and timing of stunting in the first two years of life affect performance on cognitive tests in late childhood. J Nutr 129(8):1555–1562
36. Lyte M, Varcoe JJ, Bailey MT (1998) Anxiogenic effect of subclinical bacterial infection in mice in the absence of overt immune activation. Physiol Behav 65(1):63–68
37. Goehler LE, Gaykema RP, Opitz N, Reddaway R, Badr N, Lyte M (2005) Activation in vagal afferents and central autonomic pathways: early responses to intestinal infection with *Campylobacter jejuni*. Brain Behav Immun 19(4):334
38. Bercík P, De Giorgio R, Blennerhassett P, Verdú EF, Barbara G, Collins SM (2002) Immune-mediated neural dysfunction in a murine model of chronic *Helicobacter pylori* infection. Gastroenterology 123(4):1205–1215
39. Bercik P, Verdú EF, Foster JA, Lu J, Scharringa A, Kean I, Wang L, Blennerhassett P, Collins SM (2009) Role of gut-brain axis in persistent abnormal feeding behavior in mice following

eradication of *Helicobacter pylori* infection. Am J Physiol Regul Integr Comp Physiol 296(3): R587–R594
40. Bercík P, Wang L, Verdú EF, Mao YK, Blennerhassett P, Khan WI, Kean I, Tougas G, Collins SM (2004) Visceral hyperalgesia and intestinal dysmotility in a mouse model of postinfective gut dysfunction. Gastroenterology 127(1):179–187
41. Bercik P, Verdu EF, Foster JA, Macri J, Potter M, Huang X, Malinowski P, Jackson W, Blennerhassett P, Neufeld KA, Lu J, Khan WI, Corthesy-Theulaz I, Cherbut C, Bergonzelli GE, Collins SM (2010) Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. Gastroenterology 139(6):2102–2112
42. Tomé AM, Filipe A (2011) Quinolones: review of psychiatric and neurological adverse reactions. Drug Saf 34(6):465–488
43. Silber TJ, D'Angelo L (1985) Psychosis and seizures following the injection of penicillin G procaine. Hoigne's syndrome. Am J Dis Child 139(4):335–337
44. Cummings JL, Barritt CF, Horan M (1986) Delusions induced by procaine penicillin: case report and review of the syndrome. Int J Psychiatry Med 16(2):163–168
45. Reeves RR (1992) Ciprofloxacin-induced psychosis. Ann Pharmacother 26:930–931
46. McCue JD, Zandt JR (1991) Acute psychoses associated with the use of ciprofloxacin and trimethoprim-sulfamethoxazole. Am J Med 90(4):528–529
47. Gomez-Gil E, Garcia F, Pintor L et al (1999) Clarithromycin-induced acute psychoses in peptic ulcer disease. Eur J Clin Microbiol Infect Dis 18:70–71
48. Wamer A (2000) Clarithromycin: a precipitant for acute psychotic stress [letter]. Psychosomatics 41:539
49. Weis S, Karagulle D, Kornhuber J et al (2006) Cotrimoxazole-induced psychosis: a case report and review of the literature. Pharmacopsychiatry 39:236–238
50. Kass JS, Shandera WX (2010) Nervous system effects of antituberculosis therapy. CNS Drugs 24(8):655–667
51. Alao AO, Yolles JC (1998) Isoniazid-induced psychosis. Ann Pharmacother 32(9):889–891
52. Shujaath M (2010) Antibiotic-induced psychosis: a link to D-alanine? Med Hypotheses 75 (6):676–677
53. Barrett E, Ross RP, O'Toole PW, Fitzgerald GF, Stanton C (2012) γ-Aminobutyric acid production by culturable bacteria from the human intestine. J Appl Microbiol 113(2):411–417
54. Asano Y, Hiramoto T, Nishino R, Aiba Y, Kimura T, Yoshihara K, Koga Y, Sudo N (2012) Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. Am J Physiol Gastrointest Liver Physiol 303(11):G1288–G1295
55. Van Oudenhove L, McKie S, Lassman D et al (2011) Fatty acid-induced gut-brain signaling attenuates neural and behavioral effects of sad emotion in humans. J Clin Invest 121:3094–3099
56. Perez-Cobas AE, Artacho A, Knecht H, Ferrus ML, Friedrichs A, Ott SJ et al (2013) Differential effects of antibiotic therapy on the structure and function of human gut microbiota. PLoS One 8(11):e80201
57. Verdu EF, Bercik P, Verma-Gandhu M, Huang XX, Blennerhassett P, Jackson W et al (2006) Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 55(2):182–190
58. Anitha M, Vijay-Kumar M, Sitaraman SV, Gewirtz AT, Srinivasan S (2012) Gut microbial products regulate murine gastrointestinal motility via Toll-like receptor 4 signaling. Gastroenterology 143(4):1006–1016 e1004
59. Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J et al (2011) The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 141(2):599–609, 609 e591–593
60. Ferrer M, Martins Dos Santos VA, Ott SJ, Moya A (2013) Gut microbiota disturbance during antibiotic therapy: a multi-omic approach. Gut Microbes 5(1)

Chapter 14
Microbiota, Inflammation and Obesity

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Abstract Interactions between metabolism and immunity play a pivotal role in the development of obesity-associated chronic co-morbidities. Obesity involves impairment of immune function affecting both the innate and adaptive immune system. This leads to increased risk of infections as well as chronic low-grade inflammation, which in turn causes metabolic dysfunction (e.g. insulin resistance) and chronic disease (e.g. type-2 diabetes). Gut microbiota has emerged as one of the key factors regulating early events triggering inflammation associated with obesity and metabolic dysfunction. This effect seems to be related to diet- and obesity-associated changes in gut microbiota composition and to increased translocation of immunogenic bacterial products, which activate innate and adaptive immunity in the gut and beyond, contributing to an increase in inflammatory tone. Innate immune receptors, like Toll-like receptors (TLRs), are known to be up-regulated in the tissue affected by most inflammatory disorders and activated by both specific microbial components and dietary lipids. This triggers several signaling transduction pathways (e.g. JNK and IKKβ/NF-κB), leading to inflammatory cytokine and chemokine (TNF-α, IL-1, MCP1) production and to inflammatory cell recruitment, causing insulin resistance. T-cell differentiation into effector inflammatory or regulatory T cells also depends on the type of TLR activated and on cytokine production, which in turn depends upon gut microbiota-diet interactions. Here, we update and discuss our current understanding of how gut microbiota could contribute to defining whole-body metabolism by influencing diverse components of the innate and adaptive immune system, both locally and systemically.

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_14, © Springer New York 2014

Abbreviations

BMI	Body mass index
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ERS	Endoplasmic reticulum stress
FetA	Fetuin-A
HFD	High-fat diet
IKK	Inhibitory κB kinase
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
IR	Insulin receptor
IRF	Interferon regulatory transcription factor
IRS	Insulin receptor substrate
IRS-1	Insulin receptor substrate 1
LPS	Lipopolysaccharide
LTA	Lipoteichoic acids
M1	"Classically activated" macrophages
M2	"Alternative activated" macrophages
MAPKs	Mitogen-activated protein kinases
M-cells	Microfold cells
MCP	Monocyte chemotactic protein
MDP	Muramyl dipeptide
Meso-DAP	D-Glutamyl-*meso*-diaminopimelic acid
MHC	Major histocompatibility complex
NF- κB	Nuclear factor-κB
NKT	Natural killer T
NLRs	Nod-like receptor family
NOD	Nucleotide oligomerization domain
NOS2	Nitric-oxide synthase 2
PGN	Peptidoglycan
PI3K	Phosphatidylinositol 3-kinase
PI3-K	Phosphatidylinositol 3-kinase
RHM	Recruited hepatic macrophage
ROS	Reactive oxygen species
SAA3	Serum amyloid A3 protein
SFA	Saturated fatty acid
SOC	Suppressor of cytokine signaling
STAT3	Signal transducer and activator of transcription 3
TH1	T helper 1
TLRs	Toll-like receptor family
TNF	Tumor necrosis factor
Tregs	Regulatory T
ZO	Zonula occludens

Introduction

Obesity is associated with immune function impairment, affecting both the innate and the adaptive immune system. These alterations lead to an increased risk of infections and to a state of chronic low-grade inflammation, which is a major cause of metabolic dysfunction (e.g., insulin resistance, metabolic syndrome) and chronic disease (e.g., type 2 diabetes, fatty liver disease, cardiovascular disease, etc.).

Obesity is characterized by infiltration of macrophages and lymphocytes in the adipose tissue and other peripheral organs. This is accompanied by an imbalance in the cytokine network with increased production of pro-inflammatory cytokines, adipokines, acute-phase proteins and other immune mediators [1, 2]. These inflammatory mediators, as well as several transcription factors and kinases, are involved in inflammation-induced metabolic dysfunction such as insulin resistance [3].

Gut microbiota is likely to be one of the factors influencing our predisposition to develop obesity and associated comorbidities. Alterations in the gut microbiota structure have been related to obesity and metabolic dysfunction in murine models [e.g., 4–6] as well as in human observational studies [7, 8]. Differences in microbiota composition in obese animal models could be a consequence of diet and other environmental factors [6, 9–11] and of genotype (e.g., deletion of the leptin gene or its receptor [4, 5]). Notwithstanding, animals with the same genotype and under the same dietary influence (high-fat diet [HFD]) can also develop different metabolic phenotypes (either diabetic or non-diabetic) as a function of their specific gut microbiota profile. This finding suggests that gut microbiota per se determines the risk of developing metabolic dysfunction [12]. This relationship is also supported by studies showing that germ-free mice are protected against diet-induced obesity and by fecal transplantation experiments showing that when microbiota from twins discordant for obesity is transplanted in germ-free mice, these mice develop the corresponding phenotype whether they are fed a low-fat diet or high-saturated fat diet [11], although opposite results have also been published [10].

In humans, many studies associate alterations in gut microbiota structure and function with obesity and markers of metabolic risk, which may also be partly a consequence of diet [7, 13, 14], while effects of the genotype predisposing to obesity on the microbiota are largely unknown. Nonetheless, diet-induced gut microbiota alterations (e.g., an increase in *Firmicutes* and decrease in *Bacteroidetes*) seem to play a role in obesity by, for example, increasing energy harvest and lipid absorption [15, 16].

Gut microbiota is likely to be involved in body weight regulation by influencing the host's metabolic and endocrine network, and the immune system [7]. Colonization of the newborn intestine has an enormous impact on the development of mucosal and systemic immunity, contributing to its ability to discriminate between harmful and innocuous antigens with important effects during early postnatal life through adulthood [17]. The innate immune system is one of the key regulators of the crosstalk between the host and its commensal and pathogenic intestinal bacteria

[18]. Innate immune recognition of specific microbial components is mediated by families of pattern-recognition receptors (e.g. Toll-like receptor family [TLRs] and Nod-like receptor family [NLRs]) which, upon ligand binding, activate different signaling pathways. These can trigger inflammatory responses leading to pathogen clearance or attenuate intestinal inflammation, depending on the stimulus which may also vary depending on gut microbiota composition [19]. These receptors are also activated by dietary lipids and up-regulated in most tissues affected by inflammatory disorders (e.g. adipose tissue, liver, brain) contributing to the inflammatory process leading to insulin resistance [20, 21]. Lymphocyte differentiation into effector or regulatory T (Tregs) cells also depends on the type of TLR activation and cytokine production [19]. Therefore, obesity-associated alterations in lymphocyte distribution and their phenotype may also depend on gut microbiota-diet interactions [22]. Furthermore, recent animal studies report that the intestine, which is the tissue most exposed to "noxious" nutrients (saturated fatty acids) and to a high load of bacterial antigens, is where the inflammatory process associated with diet-induced obesity originates [23, 24].

Therefore, a growing body of scientific evidence supports the notion that the crosstalk between the gut microbiota, diet and immune system activates mediators and signaling pathways, which influence whole body metabolism and disease. Here we update and discuss our current understanding of the specific role that the gut microbiota may play in obesity and metabolic dysfunction (insulin resistance) by influencing host innate and adaptive immunity.

Inflammation Associated with Obesity and Metabolic Dysfunction

Adipose Tissue Inflammation

Adipose tissue inflammation is likely the main contributor to inflammatory signals that lead to metabolic dysfunction (e.g. insulin resistance) in obesity. In fact, expression of pro-inflammatory cytokines in adipose tissue seems to be 100–1,000 times higher than in the liver of subjects with severe obesity and fatty liver disease [25]. Inflammation of this tissue is mainly attributed to macrophage infiltration, which may represent up to 40 % of all cells. This is accompanied by inflammatory cytokine and adipokine production (e.g. tumor necrosis factor (TNF) α, interleukin [IL]-6 and IL-1β and leptin) [1, 2]. Macrophage migration is promoted by adipose tissue-produced chemokines, particularly monocyte chemotactic protein (MCP)-1. Adipose tissue inflammation is also characterized by an increased ratio of "classically activated" macrophages (M1) to "alternative activated" macrophages (M2). M1 are highly inflammatory macrophages via induction of pro-inflammatory cytokines and other factors (e.g. primarily TNF-α IL-1β, IL-6 and resistin [in humans] and inducible nitric oxide synthase [iNOS]). However, M2,

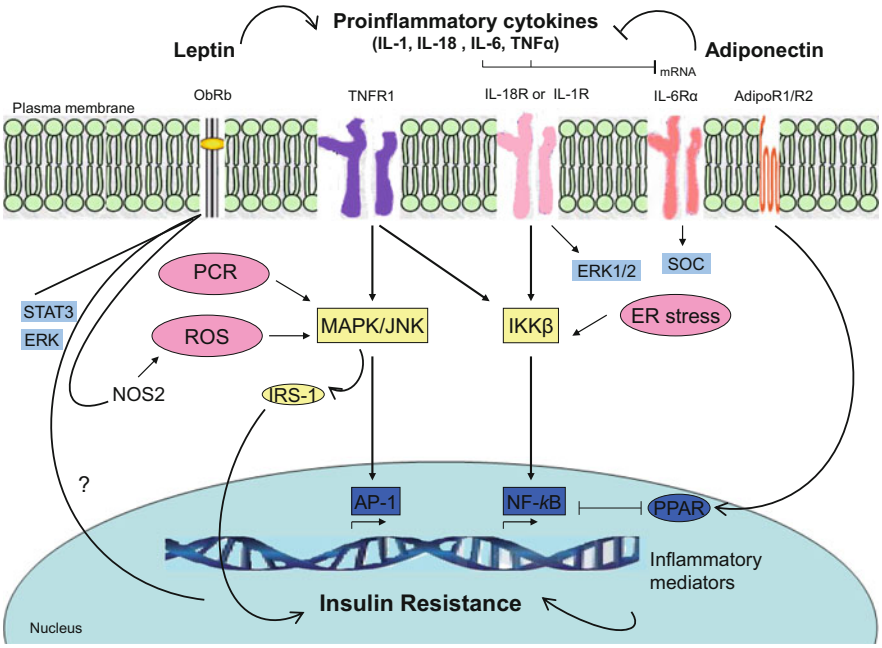

Fig. 14.1 Mode of action of the main cytokines and adipokines and transcriptional factors produced by macrophages and adipose tissue in obesity-associated insulin resistance. TNF-α induces IKKβ/NF-κB and JNK activation promoting the phosphorylation of IRS-1 at serine sites that negatively regulate normal signaling through the insulin receptor/IRS-1 axis and suppress the transcription of adiponectin. IL-1β and IL18 induce insulin resistance by reducing IRS1 expression at a transcriptional level through an extracellular signal-regulated kinase (ERK) dependent mechanism and activating IKKβ/NF-κB. IL-6 may contribute to insulin resistance via induction of SOC proteins and inhibition of adiponectin transcription. Leptin contributes to inflammation by inducing activation of the MAPKs p38 and ERK and of STAT3, leading to pro-inflammatory cytokine production (TNF-α, IL-6 etc). Leptin also activates NOS2 production, leading to increased ROS. Adiponectin, improves insulin sensitivity by suppressing the NF-κB-dependent synthesis of TNF-α and IFNγ, and inducing the anti-inflammatory mediators IL-10 and IL-1RA, as well as by activating PPARγ, which inhibits the NF-κB pathway

which are predominant macrophages in lean adipose tissue, exert anti-inflammatory effects via induction of IL-10 and IL-4 cytokine production [26, 27]. M2 also produce catecholamines to sustain adaptive thermogenesis, increasing thermogenic gene expression and contributing to fatty acid mobilization and energy expenditure in adipose tissue in a macrophage-dependent manner [28].

Figure 14.1 summarizes the mode of action of the main cytokines and adipokines, and transcriptional factors produced by macrophages and adipose tissue in obesity-associated insulin resistance. Insulin regulates glucose homeostasis by activating the insulin receptor (IR). This occurs by IR autophosphorylation, leading to tyrosine phosphorylation of several substrates, such as the insulin receptor substrate (IRS)-1 and -2. Subsequent to tyrosine phosphorylation, IRS-1 and

IRS-2 bind and activate phosphatidylinositol 3-kinase (PI3-K), which increases serine phosphorylation of Akt, leading to glucose transport in muscle and adipose tissue, glycogen synthesis in muscle and liver and lipogenesis in adipose tissue [29]. The proper signaling of this pathway may be disrupted by several mechanisms. These include serine phosphorylation of IRS proteins by protein kinases such as c-Jun N-terminal kinase (JNK) and inhibitory κB kinase (IKK)-β of the nuclear factor-κB (NF-κB) pathway, and decreased tyrosine phosphorylation of IRS-1 [29]. The JNK pathway can be activated by endoplasmic reticulum stress (ERS) activation, which occurs in both adipose tissue and liver [30]. Insulin signaling can also be impaired by increased secretion of pro-inflammatory cytokines of innate immunity, including TNF-α and IL-6, IL-1 and IL-18 as well as IL-17 and IFN-γ produced by T cells [30, 31]. Impaired production or action of adipokines such as leptin and adiponectin may also be involved [30].

TNF-α is overproduced exclusively by activated macrophages in the adipose tissue, and directly causes insulin resistance by acting locally on insulin target cells through paracrine mechanisms. Signaling via TNF-α activates intracellular kinases, such as cJNK and IKK, which inhibit insulin receptor signaling by serine phosphorylation of insulin receptor substrate 1 (IRS-1). Activation of transcription factors AP-1 and NF-κB also exacerbates pro-inflammatory cytokine production by a feedback loop mechanism, whereby proinflammatory cytokine production is increased [31]. TNF-α also suppresses the transcription of adiponectin in adipocyte cell cultures, which explains the reduction in serum adiponectin levels in obese individuals. TNF-α production in adipose tissue can also contribute to increasing circulating levels, which can reach other peripheral tissues (e.g., muscle and liver) and contribute to systemic insulin resistance [31].

Several IL-1 family cytokine members, including pro-inflammatory (e.g., IL-1α, IL-1β, IL-18) and anti-inflammatory components (IL-1 receptor antagonist [IL-1Ra]), are produced by both immunocompetent cells and obese adipose tissue, and play an important role in metabolic inflammation [32]. Neutralization of IL-1 by IL-1Ra can improve hyperglycemia and glycemic control in humans, supporting a role for IL-1 in diabetes and related insulin resistance. Like other inflammatory cytokines, IL-1β and IL18 induce insulin resistance by reducing IRS1 expression at a transcriptional level through an extracellular signal-regulated kinase (ERK) dependent mechanism activating IKKβ/NF-κB [30]. IL-6 is a pro-inflammatory cytokine involved in regulating the acute phase response and in insulin resistance via induction of suppressor of cytokine signaling (SOC) proteins and inhibition of adiponectin transcription [30]. Although preclinical data are not fully conclusive as to whether IL-6 is beneficial or detrimental, in the context of hyperglycemia small clinical trials suggest a beneficial effect of anti-IL-6 therapy. The fact that anti-IL-1 therapies strongly decrease IL-6 levels, also suggests that neutralizing IL-6 is effective and plays a role in metabolic disease [32]. IL-6 also suppresses adiponectin transcription in adipocyte cell cultures such as TNF-α.

Adipocytokines also play different immune roles in the monocyte–macrophage components of the innate immune system, and in T cells of the adaptive immune system [3]. While adiponectin is generally considered anti-inflammatory, leptin and

resistin are considered pro-inflammatory adipocytokines. Through interaction with its receptor (AdipoR1/R2), adiponectin suppresses the NF-κB-dependent synthesis of TNF-α and interferon-γ (IFNγ), and induces IL-10 and IL-1RA production. Adiponectin also induces apoptosis of monocytes and inhibits phagocytosis by macrophages. Adiponectin also decreases T-cell proliferation, reducing the potential allogeneic T-cell response. Nevertheless, adiponectin also has a pro-inflammatory effect in specific circumstances that could be explained by different roles played by the different full-length and globular forms of adiponectin in inflammation and immunity, which are not fully understood yet. In monocytes/macrophages, the mitogen-activated protein kinases (MAPKs) p38 and extracellular-signal-regulated kinase (ERK), signal transducer and activator of transcription 3 (STAT3), are activated by leptin via its OBRb receptor. This activation leads to pro-inflammatory cytokine production, including TNF-α, IL-6 and IL-12. In addition, leptin activates nitric-oxide synthase 2 (NOS2) production, leading to increased reactive oxygen species (ROS), and enhances macrophage phagocytosis, and activation, proliferation and migration of monocytes. Leptin also influences the adaptive immune system, for example by increasing production of the T helper 1 (Th1) cytokines IL-2 and IFNγ, and suppressing Th2 cytokine IL-4 production in T-cell proliferation assays with mouse cells; however, its role in humans is less clear. In monocytes/macrophages, resistin also activates p38, ERK and phosphatidylinositol 3-kinase (PI3K). This adipocytokine also increases the production of TNF, IL-1β, IL-6 and IL-12, contributing to inflammation.

PPARγ is an additional transcriptional factor and a genetic sensor of fatty acids, which is required for fat development and exerts insulin-sensitizing effects. In adipose tissue, PPARγ is also required for the maturation of M2 macrophages and induces adiponectin synthesis. PPARγ expressed by macrophages also inhibits TLR- and IFN-γ-mediated inflammatory responses and is essential for normal skeletal muscle and liver insulin sensitivity [30].

B cells and T lymphocytes also infiltrate the adipose tissue, which can contribute to inflammation and metabolic dysfunction. The sequence of recruitment of each cellular type into adipose tissue is unclear but their functional roles are known to some extent. B cells [33], CD8+ cytotoxic T cells [34], CD4+ Th1 cells [35] and CD4+ Th17 [36] may promote insulin resistance, whereas CD4+ regulatory T (Treg) cells reduce inflammation, likely contributing to improve insulin sensitivity [37, 38]. Treg cells are drastically reduced in obese adipose tissue paralleled to B cell increases [34, 38]. Tregs regulate the macrophage phenotype, inhibiting their polarization into M1-type and preventing macrophage recruitment into tissues [35, 38]. Depletion of Treg cells via administration of diphtheria toxin is also accompanied by substantial decreases in insulin-stimulated insulin-receptor (IR) tyrosine phosphorylation in epididymal fat and liver, supporting a role of this cellular population in glucose metabolism, at least in these tissues [38]. A recent study in mice on a HFD also suggests that CD19+ B lymphocytes are quickly recruited into adipose tissue and activate pro-inflammatory macrophages and T cells, thus adversely influencing glucose metabolism [33]. Adipose tissue-associated B cells can induce major histocompatibility complex (MHC)-dependent pro-inflammatory

cytokine release, including IFNγ, from resident CD4+ and CD8+ T cells, which in turn modulate macrophage polarization. The role of B cells in obesity-associated metabolic dysfunction is also supported by the increased insulin sensitivity found in B-cell-deficient mice on a HFD compared with wild-type mouse controls [33]. CD4 + IFN-γ producing cells can also participate in adipose tissue inflammation and insulin resistance. IFN-γ promotes insulin resistance by (1) reducing insulin-stimulated uptake of glucose in adipocytes parallel to reducing serine/threonine-specific protein kinase Akt phosphorylation and down-regulating the insulin receptor, IRS-1 and the glucose transporter Glut4, which impair insulin signaling; (2) polarizing, activating and stimulating M1 macrophages in adipose tissue and up-regulating T-cell and monocyte chemoattractants (e.g. IP-10 and RANTES and MCP-1 and MCP-2); and (3) inducing STAT1 phosphorylation and SOCS1/3 expression in adipocytes [36]. Increased CD8+ T cell infiltration or increased CD8+/CD4+ ratio have also been described as another critical event driving adipose tissue inflammation since it can contribute to producing critical pro-inflammatory cytokines such as IFN-γ [34, 39]. The CD4+ Th17 cells, producing IL-17, are also detected in visceral adipose tissue, but at low frequencies. IL-17 produced by Th17 cells is a pathogenic mediator of inflammation in numerous autoimmune disorders, for example by triggering NF-κB activation and cytokine release. However, the role of Th17 cells in obesity-related insulin resistance is still unclear and requires further investigation [36].

Liver Inflammation

Two macrophage populations are identified in liver: a resident macrophage population (Kupffer cells) and a recruited hepatic macrophage (RHM) population, which migrated upon weight gain under the influence of the liver-derived MCP-1 and can represent 30–70 % of all hepatic macrophages in obesity [40, 41]. Both types of macrophage populations seem to contribute to chronic hepatic inflammation and insulin resistance. Natural killer T (NKT) cells, which can respond to lipid antigen, may be involved in obesity and glucose tolerance, but evidence from animal models is inconsistent [42, 43]. While mice fed a HFD showed increased expression of NKT cells (defined by CD3+NK1.1+) in adipose tissue, amounts decreased in the liver [44]. Hepatic insulin resistance is a driving force in the pathogenesis of type 2 diabetes Mellitus, coupled with excessive fat storage that ensures liver inflammation. Activation of transcription factor NF-κB and downstream inflammatory signaling pathways systemically and in the liver are considered key events in the etiology of hepatic insulin resistance and also in β-cell dysfunction, although the molecular mechanisms involved are only partly understood [45].

Central Nervous System Inflammation

The central control of energy balance and adjustment of food intake and expenditure mainly occurs in the hypothalamus, where there is a complex interplay between insulin, leptin and other neuro-regulators (e.g. serotonin) partly via IRS/PI3K signaling, which negatively regulates energy balance, reduces food intake and improves insulin signaling [46]. Obesity is associated with hypothalamic inflammation and production of pro-inflammatory cytokines that cause central leptin resistance, leading to reduced central regulation of food intake and energy expenditure. Central nervous system inflammation also contributes to systemic insulin resistance, particularly in the liver, via a brain-liver neuronal signal [47]. In animal models, inhibition of either TLR4 or TNFα reduces hypothalamic inflammation, which is accompanied by reduced hypothalamic resistance to leptin, and improved hepatic insulin signal transduction, reduced steatosis and reduced gluconeogenesis. All these effects are mediated by parasympathetic signals delivered by the vague nerve. Circulating IL-6 is also known to activate the hypothalamic-pituitary-adrenal axis, which is associated with central obesity, hypertension, and insulin resistance [48].

Decreased Immunological Surveillance Associated with Obesity

Obesity is also associated with alterations in the immune defense mechanisms, thus leading to increased risk of infection and decreased response to vaccination. This constitutes an important cause of morbidity and mortality in obese subjects. Epidemiological human studies demonstrate that obese subjects are at a greater risk of nosocomial infections after surgery [49]. Obesity is also an independent risk factor for increased morbidity and mortality related to infection by influenza A (H1N1) virus [49]. Obesity also seems to compromise the efficacy of vaccination against viral infections, as demonstrated in murine models of obesity [50].

The mechanisms underlying obesity-associated risk of infection have been studied in murine models of genetically or diet-induced obesity. In leptin-deficient murine models of obesity (*ob*/*ob* or *db*/*db*) both innate and adaptive immune systems are adversely affected. Leptin activates monocytes/macrophage chemotaxis, phagocytic activity and cytokine production and, consequently, these functions are impaired in leptin-deficient mice [51]. In fact, *ob*/*ob* mice showed impaired immunological protection against different bacterial pathogens due to defective phagocytic activity [52]. Leptin deficiency in mice also leads to an impairment of DC function, characterized by increased production of immunosuppressive cytokines and decreased stimulation of allogenic T cells [53, 54]. T cell reactivity is also impaired in HFD-fed mice that are transgenic for a TRC recognizing a peptide from ovalbumin, indicating that similar defects in immunity occur

in diet-induced obesity. T cells from HFD-fed *naïve* transgenic mice exhibit an inflammatory response against in vitro antigen/mitogen stimulation, which could contribute to chronic obesity-associated low-grade inflammation [55]. In contrast, antigen-experienced T cells from ovalbumin immunized HFD-fed mice produce a Th2 cytokine profile and have reduced proliferation capacity. DCs from HFD-obese mice are also less able to present antigens to T cells, which may influence T cell polarization [55]. All these findings explain this increased susceptibility to infections and hypo-responsiveness to vaccination in obese subjects.

Influence of Gut Microbiota on Inflammation Associated with Obesity and Metabolic Dysfunction via Regulation of Innate Immunity

Gut microbiota is considered one of the factors contributing to chronic-low grade inflammation associated with obesity and metabolic dysfunction (e.g. insulin resistance). The mechanisms by which gut microbiota influences this process are not well understood, but could be related to alterations in gut microbiota composition. Such changes could increase bacterial components that might activate innate immunity locally in the gut and systemically, and increase translocation of immunogenic bacterial products via different routes, thus contributing to inflammation.

The innate immune system is one of the key regulators of the crosstalk between the host and the microbiota (commensal and pathogenic microbes). Innate immune recognition of specific microbial components (e.g. LPS, DNA, etc.) is mediated by families of pattern-recognition receptors, like the TLR family and NOD-like receptor family, which are expressed in epithelial cells and antigen presenting cells (DCs and macrophages). Upon ligand binding, different signaling pathways (e.g. NF-κB, MAPKs/JNK and the interferon regulatory transcription factor [IRF]) are activated, leading to the expression of inflammatory genes encoding cytokines, cytokine receptors, immuno-regulatory proteins, adhesion molecules and stress-associated proteins [18]. These molecules also induce the recruitment of other immune cells (T cells, basophils, neutrophils, DCs and NK cells) that trigger inflammatory responses and can lead to pathogen clearance [18]. These signaling pathways are also responsible for maintaining tolerance to commensal bacteria which, unlike pathogens, attenuate intestinal inflammation via different mechanisms (e.g. inhibiting NF-κB, inducing regulatory T cells, etc.; [19]). TLRs are up-regulated in most tissues affected by inflammatory disorders and activated by dietary lipids, and thus mediate the crosstalk between the gut microbiota, the host innate immune system and whole body metabolism [56]. A schematic representation of the mechanisms by which gut microbiota and dietary lipids could contribute to “metabolic” inflammation by activating innate immunity in the gut and systemically is shown in Fig. 14.2.

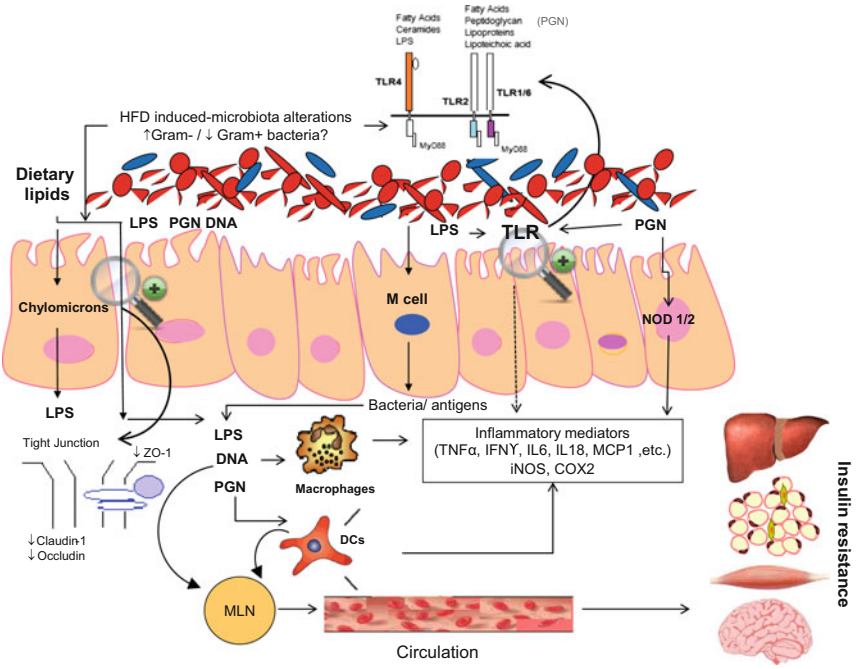

Fig. 14.2 Main mechanisms of action for gut microbiota components and their interactions with dietary lipids in the context of inflammatory processes leading to obesity-associated metabolic dysfunction (insulin resistance). LPS from Gram-negative bacteria activate TLR4/MyD88/NFκB and MAPKs/JNK pathways in epithelial and immunocompetent cells activating inflammatory mediator synthesis, inflammatory cell recruitment and activation of the underlying lymphoid tissue, thus contributing to inflammation. Dietary lipids (saturated fatty acids) increase the expression and activation of innate immune receptors (TLR4, TLR2 and inflammasome) and contribute to translocation of bacterial products (LPS, PGN, etc.) by transcellular and paracellular pathways that activate immunocompetent cells in the gut-associated lymphoid tissue and in peripheral tissues. HFD-induced microbiota imbalances leading to inflammatory cytokine production also alter tight-junctions between enterocytes, increasing paracellular permeability to bacterial antigenic products. Bacteria and bacterial antigenic products can also translocate via M-cells and reach peripheral tissues via DCs. TLR2 recognizes lipoteichoic acids from Gram-positive bacteria and also LPS from Gram-negative bacteria, acting synergically with TLR4 triggering inflammation via NFκB and JNK pathways. NOD1 and NOD2 proteins recognize bacterial peptidoglycan (PGN) and mediate insulin resistance in different tissues via activation of common signaling transduction pathways (MAPKs), expression and production of pro-inflammatory cytokines/chemokines, and impairment of insulin signaling

LPS and TLR4 Signaling

Lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria is currently thought to play a role in both immunity and metabolism through the TLR4/MyD88/NF-κB signaling pathway. Increased LPS plasma levels are associated with an elevated body mass index (BMI) and high-fat feeding, postprandial inflammation

and risk of type-2 diabetes and atherosclerosis in humans [57, 58]. In animal models, increased LPS in plasma (termed "metabolic endotoxemia") has been causally linked to adiposity and obesity-related insulin resistance and inflammatory liver diseases, such as non-alcoholic fatty liver disease and non-alcoholic steatohepatitis [59, 60]. Mice fed a HFD exhibit a significant increase in plasma LPS, associated with changes in the gut microbiota, obesity, inflammation and glucose metabolic dysfunction. The role of circulating LPS per se in metabolic dysfunction is demonstrated by LPS infusion. On reaching the same plasma LPS levels as those measured in HFD-fed mice they reproduce the same phenotype of HFD-fed mice. The role of LPS and TLR4 is proven in mice deficient in CD14, a key molecule in TLR4 signaling activated by LPS, showing that these mice are resistant to inflammation in adipose deposits, liver and muscles, induced by both HFD and chronic LPS administration [60]. The fact the gut microbiota is involved in LPS-induced metabolic dysfunction has been shown by administering antibiotics (norfloxacin and ampicillin) to two different mouse models of insulin resistance (genetically and diet induced), which led to gut microbiota depletion parallel to reduced serum LPS levels, low grade inflammation, obesity and type-2 diabetes [61]. Comparisons between germ-free and conventional mice have also demonstrated the direct role of the gut microbiota in triggering colonic serum amyloid A3 protein (SAA3) expression, which could contribute to inflammation via LPS signaling [62]. This effect is demonstrated to be partially mediated via TLR/MyD88/NF-κB signaling, by comparing wild-type with *Myd88* deficient mice, with the latter gaining less weight on a Western HFD and lower epididymal fat pad and liver masses [62]. A recent study highlights the importance of diet-gut microbiota interactions in this process, reporting that a HFD causes deregulation of the gut microbiota composition (increasing the Firmicutes to Bacteroidetes ratio) leading to increased fecal endotoxin and colonic inflammation, parallel to increased plasma LPS and systemic inflammation. This intestinal inflammation was characterized by increased expression of proinflammatory cytokines, TLR4, iNOS and COX-2, activation of NF-κB and reduced the expression of tight junction-associated proteins claudin-1 and occludin. This study also demonstrates that TLR4 mediates inflammation associated with adiposity and obesity induced by a HFD comparing wild-type with TLR4-deficient mice [24]. Comparing germ-free and conventionally colonized mice has also demonstrated that gut microbiota colonization leads to impaired glucose metabolism and increased macrophage accumulation, and polarization towards a pro-inflammatory M1 phenotype in white adipose tissue in mice fed a standard diet, without HFD feeding [63]. In the same study mice were colonized with an *Escherichia coli* strain, producing immunogenic LPS or not, demonstrating that macrophage recruitment requires LPS, whereas impairment of systemic glucose metabolism is not exclusively LPS-dependent and may involve an additional mechanism [63]. The fact that protection of TLR4-deficient mice from obesity-induced insulin resistance does not require germ-free conditions, also suggests the microbiota is not the only factor activating this signaling pathway triggering metabolic disease [64, 65]. Altogether, these findings demonstrate that LPS-induced TLR4 signaling constitutes one of the links between the gut

microbiota and inflammation that leads to metabolic dysfunction, which is also influenced by diet.

LPS can impair metabolic functions when reaching tissues involved in glucose and lipid metabolism, such as the liver and the adipose tissue, by stimulating TLRs expressed in infiltrated immune cells (e.g., macrophages and dendritic cells) and in obese adipose tissue. In adipose tissue, LPS-stimulated TLR4 activates p65/p50 and p68/p52 NF-κB signal transduction pathway, inducing the expression of inflammatory mediators such as IL-6, TNF-α, and SAA3 protein, possibly impairing insulin sensitivity as explained in a previous section (Fig. 14.1). This signaling pathway can also induce endoplasmic reticulum (ER) stress and JNK activation accompanied by increased IRS-1 serine 307 phosphorylation in the liver, muscles, and adipose tissue, leading to a reduction in insulin sensitivity and signaling [29]. TLR4 signaling also increases expression of iNOS, which reacts with cysteine residues to form S-nitrosothiol adducts, inducing S-nitrosation/S-nitrosylation of the insulin signaling pathway, leading to insulin resistance in the liver, muscles, and adipose tissue. Circulating LPS can also activate monocyte chemo-attractant protein MCP-1, mediating migration of monocytes to peripheral tissues and contributing to the inflammatory process [66].

High intake of saturated fat, which results in increased levels of circulating free-fatty acids and/or lipid accumulation in muscles and liver, is also known to be directly involved in the inflammatory process leading to insulin resistance [21]. Saturated fatty acids (SFAs) trigger both the expression of TLRs and their activation, which may contribute together with the microbiota-derived products to the increased induction of inflammatory cytokines in different tissues, such as adipose tissue and liver [67]. SFAs activate innate immunity components, such as TLR4 and 2 and the inflammasome, thereby triggering kinase activation (JNK and IKK) and inflammatory cytokine production, inhibiting insulin signaling and action [68, 69]. A protein called fetuin-A (FetA), which is a major carrier of free-fatty acids in serum, acts directly as an endogenous ligand of TLR4, thus activating its signaling pathway, promoting insulin resistance in peripheral tissues [70]. In contrast, polyunsaturated free-fatty acids (e.g. Ω-3 fatty acids) can inhibit TLR4 signaling. LPS stimulation also increases cytokine-mediated plasma lipid levels by increasing VLDL lipoprotein synthesis in the liver and inhibiting lipoprotein lipase. In fact, mobilization of lipid stores is considered a mechanism to fuel the host's response against infections; moreover, lipoproteins also seem to help fight against infection by binding and neutralizing LPS [71].

It is still unclear which gut microbiota components constitute a source of LPS in animal models of obesity and in observational human studies. LPS originating from *E. coli* is reportedly sufficient to promote glucose and insulin intolerance and macrophage accumulation in white adipose tissue when mono-colonizing the gut of germ-free mice [63]. In our own studies, compared with standard-diet-fed mice, HFD-fed mice showed increased numbers of *Enterobacteriaceae*, which were reduced by *B. pseudocatenulatum* CECT 7765 administration, parallel to amelioration of metabolic dysfunction; however, LPS translocation was not measured [6]. By contrast, in HFD-fed mice increases in Proteobacteria (which include

enterobacteria) and reductions in Firmicutes and Bacteroidetes by vancomycin administration have been related to reduced body weight gain, TNF-α production and metabolic dysfunction [72]. Other animal studies demonstrated that gut microbiota alterations associated with genetically or HFD-induced obesity do not involved Gram-negative bacteria, which could contribute to LPS increases [24, 73], but reductions in Gram-positive bacteria [74]. A couple of recent human studies support the idea that increased proportions of Proteobacteria are associated with inflammatory and metabolic disease risk markers [8, 13] while other studies do not support such an association [7]. This controversy could partly be due to the influence of confounding factors and differences in methodologies used for microbiota analyses. Furthermore, gut barrier dysfunction associated with diet-induced obesity can lead per se to increased LPS translocation without significant alterations in gut microbial ecology.

Bacterial products may be translocated via different mechanisms, including transcelluar and paracellular pathways. LPS could translocate via a transcellular epithelial pathway together with chylomicrons formed to incorporate dietary long-chain fatty acids in the form of triglycerides, which are finally released into the mesenteric lymph. This LPS translocation mechanism also requires TLR-4 expression by epithelial intestinal cells [75]. In blood, LPS-enriched chylomicrons exchange LPS with other lipoproteins, a process that requires the LPS-binding protein (LBP) and involves the soluble CD14 receptor, facilitating LPS transport to different tissues and blood vessels. LPS can also translocate by a transcellular pathway through intestinal-epithelial microfold cells (M-cells), which are more permeable and responsible for uptake of bacteria and bacterial antigens by the underlying lymphoid tissue, with a preference for Gram-negative bacteria [76]. Murine models of HFD-induced obesity have also demonstrated that live Gram-negative commensal intestinal bacteria (*E. coli*) can translocate to the blood and adipose tissue [77]. This translocation is dependent of innate immunity pattern-recognition receptors (TLR4 and Nod1) demonstrated by the fact it is blocked in mice lacking *CD14* or *Nod1* but increased in *Myd88* knockout and *ob*/*ob* mice. This 'metabolic bacteremia' is thought to be mediated by DCs and reversed by administration of *Bifidobacterium animalis* subsp. *lactis* 420, which also improves the animals' overall inflammatory and metabolic status. This study also suggests that leptin plays a role in intestinal bacterial adherence and translocation in the intestine since leptin treatment reduces translocation in *ob*/*ob* mice [77].

Alcohol ingestion and HFD, common in obese subjects, can also lead to increased intestinal permeability, which is reflected in alterations of tight-junction integrity and related proteins. This might facilitate the translocation of LPS and other bacterial components by a paracellular pathway [74]. Alterations in gut microbiota composition could also contribute to increasing paracellular permeability via alterations in tight-junctions, which could be secondary to excessive activation of inflammatory cytokine production (e.g. TNF-α; [78]). Thus, HFD-fed diabetic mice show alterations in gut bacteria, associated with increased intestinal permeability, characterized by reduced expression of genes coding for two tight

junction proteins ZO-1 and occludin, while antibiotic-treated mice recover normal intestinal epithelial integrity. This reveals the specific role of the microbiota, which seems to be greater than the role of diet in gut permeability [79]. A selective increase in *Bifidobacterium* spp. by feeding *ob/ob* mice with a prebiotic (oligofructose) also reduces the impact of the HFD-induced metabolic endotoxaemia, inflammatory tone and metabolic dysfunction and improves intestinal permeability, demonstrating that these effects are partly mediated by gut microbiota-induced changes [59]. The protective effects of the prebiotic on gut barrier function could also be explained by the reduction in plasma cytokines, known to promote tight-junction disruption, including TNFα, IL1β, IL1α, IL6 and INFγ [59]. These effects could also be attributed to the trophic effect of bacterial fermentation products (short-chain fatty acids [SCFAs] including butyrate) on the gut, leading to increased villus height and crypt depth and thickened mucosal layer [59, 80]. Researchers have also reported that prebiotic-microbiota-induced changes are associated with increased endogenous production of the glucagon-like peptide-2 (GLP-2), whose production may improve mucosal barrier function by increasing the rate of crypt cell proliferation and villus elongation, and reduce apoptosis [59]. In a more recent study, administration of the mucin-degrading bacterium *Akkermansia muciniphila* has also been shown to reverse metabolic endotoxemia and high-fat diet-induced metabolic disorders in mice obesity models, via restoration of gut barrier function and inflammation by increasing the intestinal levels of endocannabinoids (e.g. 2-arachidonoylglycerol and 2-oleoylglycerol) and mucus thickness [81].

TLR-2, Lipoteichoic Acids and LPS

TLR2 recognizes lipoteichoic acids (LTA) from Gram-positive bacteria and also LPS from Gram-negative bacteria, acting synergically with TLR4. Although TLR4-LPS activation is necessary to trigger an innate immune response, TLR2 participates in the up-regulation of genes encoding TNF-α and in the connection between innate and adaptive immunity [82].

In addition, TLR2 can be activated by saturated fatty acids [20]. Thus, TLR2 in conjunction with TLR4 can synergically contribute to insulin resistance in different tissues and constitute one of the links between gut microbiota components and metabolic dysfunction.

The role of TLR2 in metabolic dysfunction was directly evidenced by comparing effects of HFD on *Tlr2*(−/−) mice and *Tlr2*(+/+) mouse controls, showing that knock-outs were protected from the adverse metabolic effects of diet [83]. Glucose tolerance, insulin sensitivity, and insulin secretion were markedly improved, particularly in female *Tlr2*(−/−) mice. This was paralleled by increased fat-burning rates in *Tlr2*(−/−) mice as well as reduced tissue inflammation [83]. The specific role of gut microbiota was shown in studies demonstrating that TLR2-deficient mice, under germ-free conditions, were protected from HFD-induced insulin

resistance, whereas they were not under conventional conditions. TLR2-deficient mice conventionally colonized developed metabolic syndrome parallel to a three-fold increase in Firmicutes and a slight increase in Bacteroidetes, accompanied by decreased Proteobacteria compared to wild-type controls [84]. This phenotype was reproduced when microbiota from conventionally reared TLR2-deficient mice was transplanted to *Bacillus*-monoassociated wild-type lean mice, and was subsequently reversed by antibiotic treatment. These findings prove that gut microbiota can define a specific phenotype regardless of the predisposing genotype for a specific condition. Increased LPS plasma levels may induce insulin resistance by interfering with insulin signaling, as in other models, but insulin resistance in TLR2-deficient mice has particular characteristics. There was activation of TLR4 in liver, muscles, and adipose tissue, associated with endoplasmic reticulum (ER) stress and JNK activation, but no activation of the IKKβ-IκB/NFκB pathway, probably due to lack of TLR4-TLR2 interactions in the knock-outs. While chronic activation of TLR4 by low doses of LPS is sufficient to increase JNK activation, the activation of the IKK/IkB/NF-κB pathway may also depend on the interplay of TLR2 and TLR4 [84].

TLR2 is also involved in regulating intestinal barrier function via modulation of tight-junctions. TLR2 deficiency leads to barrier dysfunction, reflected in decreased expression of the tight-junction protein zonula occludens (ZO)-1 in the ileum, which leads to increased gut permeability and increased LPS translocation and inflammation even in mice fed standard rodent chow. These effects are paralleled to *Bifidobacterium* spp. decreases while their increase leads to reduced gut permeability. Gut microbiota transplantation from TLR2-deficient mice to *Bacillus*-monoassociated wild-type mice also reduces ZO-1 expression in the ileum, proving the role of gut microbe-TLR2 interactions in this phenomenon [84].

TLR5 and Flagellin

TLR5 is expressed in the intestinal mucosa, recognizes flagellin and, upon ligand binding, induces an inflammatory response with TNFα production, contributing to defenses against infection. However, TLR5 may protect against metabolic syndrome as genetically deficient TLR5 mice exhibit hyperphagia and develop the main features of metabolic syndrome, including hyperlipidemia, hypertension, insulin resistance, and increased adiposity [85]. These metabolic dysfunctions correlated with changes in gut microbiota composition (diversity and phylotypes related to murine bacteria). Also gut microbiota transferred from TLR5-deficient mice to wild-type germ-free mice conferred many features of metabolic syndrome to recipients, demonstrating the role of the microbiota in this particular metabolic phenotype via interaction with the innate immune system. Metabolic syndrome in TLR5-deficient mice was exacerbated by a HFD. Food restriction prevented obesity, but not insulin resistance in the TLR5-deficient mice, suggesting that the latter effect is primarily dependent on TLR5-gut microbiota interactions [85].

TLR9 and DNA

TLR9 recognizes special DNA sequences (unmethylated CpG motifs) and activates innate immunity. Translocation of bacterial DNA to the blood stream has been identified in animal models of metabolic dysfunction and also associated with the onset of diabetes and cardiovascular disease risk in humans [86]. Therefore, TLR9 activation constitutes another possible route by which bacterial components may contribute to metabolic diseases.

TLR9 is involved in non-alcoholic fatty liver disease, steatohepatitis and fibrosis, as shown by comparing wild-type and TLR9-deficient mice. In a nonalcoholic steatohepatitis murine model, induced by a choline-deficient amino acid-defined diet, TLR9 signaling induced IL-1β production by Kupffer cells, leading to steatosis, inflammation, and fibrosis [87]. Steatohepatitis and fibrosis were also reduced in mice deficient in *MyD88*, an adaptor molecule for TLR9 and IL-1R signaling [87]. However, the aforementioned studies did not specifically evaluate relationships with the gut microbiota.

NOD1/2 and Peptidoglycan

Nucleotide oligomerization domain (NOD) proteins NOD1 and NOD2 are members of the NOD-like receptor (NLR) family in mammals. These are cytosolic pattern recognition receptors, expressed not only in immune but also in metabolic tissues, which play a role in detecting intracellular microorganisms. These receptors propagate inflammatory signals in response to bacterial peptidoglycan (PGN). NOD1 detects D-glutamyl-*meso*-diaminopimelic acid (*meso*-DAP)-containing PGN found principally in Gram-negative bacteria, whereas NOD2 detects muramyl dipeptide (MDP) present in all bacteria, though more abundant in Gram-positive bacteria [88]. NOD1 is expressed in all cell types and required for NF-κB activation by Gram-negative bacterial infection, once the bacteria have bypassed TLR activation [89]. NOD2 is expressed in monocytes/macrophages and DCs and induced in intestinal epithelial cells by TNF-α. NOD2 mutations have also been associated with defective IL-10 production, and Crohn's disease in humans [90].

PGN levels are lower in the serum of germ-free and antibiotic-treated mice [91]. Germ-free mice are protected from HFD-induced insulin resistance and antibiotic treatment in conventionally colonized mice attenuates the HFD-induced metabolic dysfunctions. Altogether this suggests that PGN is a potential factor linking innate immunity and metabolic dysfunction [91]. The fact mice deficient in NOD1 and NOD2 peptidoglycan receptors are protected from HFD-induced inflammation and insulin intolerance is evidence of causality. Activation of NOD1 causes acute systemic insulin resistance, as demonstrated in mice injected with mimetics of *meso*-diaminopimelic acid-containing PGN or the minimal bioactive PGN motif, which activate NOD1 and NOD2, respectively. Ex vivo, NOD1 ligand

can cause pro-inflammatory cytokine secretion and impaired insulin-stimulated glucose uptake in adipocytes and also cause inflammation and insulin resistance in primary hepatocytes from wild type, but not NOD1(−/−), mice [92]. PGN motifs acting on NOD2, but not those acting on NOD1, induce muscle cell-autonomous insulin resistance [91]. NOD1 mediates insulin resistance by acting on adipocytes/hepatocytes, and NOD2 by acting on myocytes, through mechanisms activating common pathways such as the MAPKs (p38, JNK, ERK1/2) pathway, expression and production of proinflammatory cytokines/chemokines, and impairment of insulin signaling at the level of IRS-1. However, we do not know why these metabolic tissues utilize divergent intracellular innate immune sensors [88]. Overall, it can be concluded that NOD1-activating PGN causes peripheral insulin resistance, involving the complex crosstalk between hepatic and adipose tissues, which is indirectly manifested in skeletal muscles. In contrast, NOD2-activating bacterial PGN motifs cause a milder insulin resistance that affects skeletal muscle.

NLRP6 and NLRP3 Inflammasomes

Inflammasomes are signaling platforms that sense diverse microbial products as well as stress and damage-associated endogenous signals. Inflammasome complexes can be formed by members of the NOD-like receptor family or the PYHIN family AIM2. Upon formation, inflammasomes trigger proteolysis of caspase-1, which cleaves the cytokine precursors of IL-1β and IL-18 to initiate a pro-inflammatory and antimicrobial response. Research has linked inflammasome activation to metabolic disorders, including atherosclerosis, type 2 diabetes, liver disease and obesity [69].

Inflammasome-deficiency is associated with changes in gut microbiota composition, parallel to exacerbated hepatic steatosis and inflammation through influx of TLR4 and TLR9 agonists in the portal circulation, leading to enhanced hepatic TNF-α expression, which drives disease progression [93]. Co-housing of inflammasome-deficient mice with wild-type mice, implying microbiota exchanges by coprophagy, results in exacerbation of hepatic steatosis and obesity in wild-type mice. These findings demonstrate that defective NLRP3 and NLRP6 inflammasome sensing alters interactions between the gut microbiota and the host innate immune system, possibly contributing to metabolic complications [93].

Influence of Gut Microbiota in Macrophage Infiltration in Peripheral Tissues

Different studies show that gut microbiota, and its modulation by dietary intervention, could influence migration and infiltration of macrophages into peripheral tissues, this being a major feature of obesity-induced metabolic dysfunction. For

example, in *ob*/*ob* mice fed a normal diet, prebiotic administration and the consequent increase in intestinal bifidobacterial numbers, reduced several serum inflammatory and anti-inflammatory cytokines (IL-1β, TNF-α, IL-18, and IL-15) as well as the main chemokine (MCP-1) involved in monocyte/macrophage migration and infiltration in the adipose tissue [60].

When mice with HFD-induced obesity and gut microbiota imbalances were administered *B. pseudocatenulatum* CECT 7765, there was a reduction in serum inflammatory cytokines and chemokines of the innate immune system (IL-6 and MCP-1) and a decline in macrophage infiltration in adipose tissue, presumably due to lowered MCP-1 production [6]. These changes in inflammatory markers were accompanied by improvements in glucose tolerance and insulin sensitivity in HFD-fed mice administered *B. pseudocatenulatum* CECT 7765, as well as partial restoration of HFD-induced gut microbiota imbalances [94]. These finding reveal that gut microbiota modulation might help to ameliorate metabolic dysfunction via regulation of macrophage chemoattractants.

Gut microbiota alterations induced by chronic treatment with olanzapine are also suspect to be involved in infiltration of macrophages in adipose tissue and metabolic dysfunction associated with the consumption of this antipsychotic. This hypothesis has been proven by showing that gut microbiota alterations induced by antibiotic administration (neomycin, metronidazole and polymyxin B) to chronically olanzapine treated female rats reduces metabolic alteration caused by olanzapine alone, including body weight gain, uterine fat deposition and plasma free fatty acid levels and macrophage infiltration of adipose tissue [95].

Influence of Gut Microbiota on Adaptive Immunity Alterations Associated with Obesity and Metabolic Dysfunction

Fewer studies report the possible influence of the gut microbiota on the adaptive immune system and its role in the chronic low-grade inflammation associated with metabolic disorders. However, proof of concept of the role played by gut microbiota can be found in studies demonstrating the beneficial effects of intervention with specific bacterial strains on adaptive immune function in animal models of obesity. These beneficial effects seem to be mediated mainly by inducing Tregs, which express the transcription factor Foxp3 and act by limiting proliferation of effector CD4+ T cells, which are often critical in regulating intestinal inflammation [96].

In mice with Western diet-induced obesity (characterized by a CD4(+) Th17-biased immune profile and changes in microbial communities) the administration of *L. reuteri* ATCC 6475 shifted this pro-inflammatory immune cell profile and prevented abdominal fat pathology and age-associated weight gain [22]. The bacterial effects were mediated by induction of Foxp3(+) Tregs and IL-10 in colonic

mesenteric lymph nodes, without significantly influencing gut microbiota composition. Furthermore, these microbe-related beneficial effects were transferable into *naïve* recipients by adoptive transfer of purified *L. reuteri*-induced CD4(+) $Foxp3^+$ T cells [22].

In mice with HFD-induced obesity, *B. pseudocatenulatum* CECT 7765 supplementation increased cytokine production of the adaptive immune system, including the anti-inflammatory cytokine IL-4 [6]. In the context of obesity, this cytokine together with IL-13 contribute to macrophage differentiation into M2 macrophages, which secrete the anti-inflammatory cytokine IL-10, thus helping to control inflammation and promote normal insulin sensitivity [97]. IL-4 also mediates Th2 lymphocyte differentiation and inhibits production of inflammatory cytokines such as IL-1β, TNF-α and IL-6.

A. muciniphila, a newly discovered mucus-degrading bacterium of the human gut, improves glucose tolerance in HFD-fed mice by inducing Foxp3 Tregs in the white adipose tissue. This effect has been related to reduced gene expression of pro-inflammatory cytokines (IL-1β and IL-6) but not to changes in M1/M2 or CD4/CD8 T cell ratios, altered by HFD [39].

A recent study investigating the possible protective effect of *H. pylori* in diet-related disorders reported that it favorably modulates glucose metabolism and suppressed weight gain in *db*/*db* mice (lacking the long isoform of the leptin receptor) and mice with diet-induced obesity, particularly when animals were colonized by a non-pathogenic strain negative for *cag PAI* (cytotoxin-associated gene pathogenicity island) [98]. The effects were mediated by up-regulation of gastric *PPAR γ*-responsive genes (i.e., *CD36* and *FABP4*) parallel to decreased white adipose tissue macrophages and increased adipose tissue Tregs, since the effects were impaired in mice deficient in *PPAR γ* in immune and epithelial cells [98].

Although the precise mechanisms by which microbiota exerts these Treg inductive effects are unknown, short-chain fatty acids (SCFAs) derived from gut microbiota fermentative activity are one of the possible actors [96]. SCFAs contribute to regulating the size and function of the colonic Treg pool, specifically inducing Foxp3+IL-10-producing Tregs but not colonic Foxp3+TGFb+cTregs, colonic Th17 and Th1 or MLN cell and splenic Tregs [96].

TLR2-deficient mice also have lower Tregs in visceral adipose tissue, suggesting this pattern-recognition receptor may also contribute to regulate insulin resistance, an effect that in turn can be influenced by gut microbiota molecules recognized by this receptor [84].

Influence of Gut Microbiota on Decreased Immunological Surveillance Associated with Obesity and Metabolic Dysfunction

There is scarce research into the potential role of gut microbiota in immunological dysfunction, leading to weakened host responses against infections and vaccination. Recent studies have demonstrated that when mice with HFD-induced obesity are

fed *B. pseudocatenulatum* CECT 7765 or *Bacteroides uniformis* CECT 7771, the oxidative burst of macrophages, which reflects their role in phagocytosis, is increased parallel to restoration of HFD-induced microbiota imbalances [6, 94]. Administration of *B. pseudocatenulatum* CECT 7765 or *B. uniformis* CECT 7771 to HFD-fed mice also improved the ability of DCs to activate T-lymphocyte proliferation, a function also adversely affected by HFD-induced obesity in mice [6, 94]. These findings indicate that modifying the gut microbiota may contribute to restoring host defense mechanisms impaired by diet-induced obesity in mice.

Studies of rodents with genetic deficiency in leptin or leptin receptors, reveal obesity-related deficits in macrophage phagocytosis via alterations in phospholipase activation and reduced pro-inflammatory cytokine secretion (e.g. TNF-α and IL-6) in vivo and in vitro. These effects may be due to leptin deficiency as exogenous leptin up-regulated both phagocytosis and proinflammatory cytokine production by macrophages [51]. In leptin-deficient models of obesity (*ob*/*ob* mice), DCs and their role in T cell priming is also adversely affected. DCs from *ob*/*ob* mice are less able to activate allogenic T cells in vitro. The impaired functionality of DCs may be related to increased secretion of the immunosuppressive cytokine TGF-β, rather than to changes in expression of activation markers, which could be due to the absence of leptin in *ob*/*ob* mice [53]. Leptin can improve DCs functions and survival, driving *naïve* T cell polarization toward a Th1 type phenotype by activating NF-κB and exerting an antiapoptotic effect via up-regulation of gene expression (*bcl-2* and *bcl-*x_L). These results demonstrate the ability of leptin to improve DCs and T cells functions and to promote DC survival [54], which could be important in defense against pathogens and in response to vaccination. Decreased leptin plasma concentration in food-deprived animals or malnourished humans impairs immune functions similarly to those detected in leptin-deficient mice.

Similar immune defense mechanism dysfunctions have been demonstrated in a murine model of HFD-induced obesity, the most common form of obesity characterized by hyperleptinemia, presumably due to leptin resistance and, therefore, lack of adequate leptin functionality [6, 94]. Alterations in macrophage, DCs and T-cell function, identified in both models of obesity (genetically or diet-induced), may also be related to obesity-associated alterations in glucose uptake and metabolism, possibly affecting immune cells which are sensitive to insulin because glucose is their major energy source [20].

Conclusions and Future Perspectives

Scientific evidence supports a role of gut microbiota in immunological dysfunctions associated with obesity and metabolic disease, including intestinal and systemic chronic low-grade inflammation, and diminished responses against infections and vaccination. The interdependency of diet and gut microbiota is evident in that diet constitutes a major factor influencing gut microbiota structure and function.

Moreover, both dietary lipids and gut microbes can exacerbate inflammation by activating similar pattern-recognition receptors and signaling pathways of the innate immune system. Furthermore, it has been evidenced that intestinal inflammation is an early event preceding obesity and metabolic disease and the fact that this can be altered by dietary-modulation of the gut microbiota paves the way for novel preventive dietary intervention strategies, designed to combat these disorders. In this context, it is essential to identify the exact immunological processes that are sensitive to gut microbiota interactions within a specific dietary context and to gain a better understanding of the role gut microbiota plays in early responses particularly of the adaptive immune system to high calorie diets.

Acknowledgements This work was supported by grants AGL2011-25169 and Consolider Fun-C-Food CSD2007-00063 from the Spanish Ministry of Economy and Competitiveness (MINECO, Spain). The scholarship of A Moya from MINECO is fully acknowledged.

References

1. Zeyda M, Stulnig TM (2007) Adipose tissue macrophages. Immunol Lett 112(2):61–67
2. Kanneganti TD, Dixit VD (2012) Immunological complications of obesity. Nat Immunol 13 (8):707–712
3. Tilg H, Moschen AR (2008) Inflammatory mechanisms in the regulation of insulin resistance. Mol Med 14(3–4):222–231
4. Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI (2005) Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A 102(31):11070–11075
5. Waldram A, Holmes E, Wang Y, Rantalainen M, Wilson ID, Tuohy KM, McCartney AL, Gibson GR, Nicholson JK (2009) Top-down systems biology modeling of host metabotype-microbiome associations in obese rodents. J Proteome Res 8(5):2361–2375
6. Gauffin Cano PG, Santacruz A, Trejo FM, Sanz Y (2013) *Bifidobacterium* CECT 7765 improves metabolic and immunological alterations associated with obesity in high-fat diet-fed mice. Obesity (Silver Spring). Doi: 10.1002/oby.20330
7. Sanz Y, Rastmanesh R, Agostoni C (2013) Understanding the role of gut microbes and probiotics in obesity: how far are we? Pharmacol Res 69(1):144–155
8. Verdam FJ, Fuentes S, de Jonge C, Zoetendal EG, Erbil R, Greve JW, Buurman WA, de Vos WM, Rensen SS (2013) Human intestinal microbiota composition is associated with local and systemic inflammation in obesity. Obesity (Silver Spring). Doi: 10.1002/oby.20466
9. Burcelin R, Crivelli V, Dacosta A, Roy-Tirelli A, Thorens B (2002) Heterogeneous metabolic adaptation of C57BL/6J mice to high-fat diet. Am J Physiol Endocrinol Metab 282(4):E834–E842
10. Fleissner CK, Huebel N, Abd El-Bary MM, Loh G, Klaus S, Blaut M (2010) Absence of intestinal microbiota does not protect mice from diet-induced obesity. Br J Nutr 104(6):919–929
11. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, Griffin NW, Lombard V, Henrissat B, Bain JR, Muehlbauer MJ, Ilkayeva O, Semenkovich CF, Funai K, Hayashi DK, Lyle BJ, Martini MC, Ursell LK, Clemente JC, Van Treuren W, Walters WA, Knight R, Newgard CB, Heath AC, Gordon JI (2013) Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science 341(6150):1241214
12. Serino M, Luche E, Gres S, Baylac A, Bergé M, Cenac C, Waget A, Klopp P, Iacovoni J, Klopp C, Mariette J, Bouchez O, Lluch J, Ouarné F, Monsan P, Valet P, Roques C, Amar J,

Bouloumié A, Théodorou V, Burcelin R (2012) Metabolic adaptation to a high-fat diet is associated with a change in the gut microbiota. Gut 61(4):543–553
13. Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, Almeida M, Arumugam M, Batto JM, Kennedy S, Leonard P, Li J, Burgdorf K, Grarup N, Jørgensen T, Brandslund I, Nielsen HB, Juncker AS, Bertalan M, Levenez F, Pons N, Rasmussen S, Sunagawa S, Tap J, Tims S, Zoetendal EG, Brunak S, Clément K, Doré J, Kleerebezem M, Kristiansen K, Renault P, Sicheritz-Ponten T, de Vos WM, Zucker JD, Raes J, Hansen T; MetaHIT consortium, Bork P, Wang J, Ehrlich SD, Pedersen O, Guedon E, Delorme C, Layec S, Khaci G, van de Guchte M, Vandemeulebrouck G, Jamet A, Dervyn R, Sanchez N, Maguin E, Haimet F, Winogradski Y, Cultrone A, Leclerc M, Juste C, Blottière H, Pelletier E, LePaslier D, Artiguenave F, Bruls T, Weissenbach J, Turner K, Parkhill J, Antolin M, Manichanh C, Casellas F, Boruel N, Varela E, Torrejon A, Guarner F, Denariaz G, Derrien M, van Hylckama Vlieg JE, Veiga P, Oozeer R, Knol J, Rescigno M, Brechot C, M'Rini C, Mérieux A, Yamada T (2013) Richness of human gut microbiome correlates with metabolic markers. Nature 500 (7464):541–546
14. Karlsson FH, Tremaroli V, Nookaew I, Bergström G, Behre CJ, Fagerberg B, Nielsen J, Bäckhed F (2013) Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature 498(7452):99–103
15. Jumpertz R, Le DS, Turnbaugh PJ, Trinidad C, Bogardus C, Gordon JI, Krakoff J (2011) Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. Am J Clin Nutr 94(1):58–65
16. Semova I, Carten JD, Stombaugh J, Mackey LC, Knight R, Farber SA, Rawls JF (2012) Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. Cell Host Microbe 12(3):277–288
17. Tlaskalová-Hogenová H, Stěpánková R, Kozáková H, Hudcovic T, Vannucci L, Tučková L, Rossmann P, Hrnčíř T, Kverka M, Zákostelská Z, Klimešová K, Přibylová J, Bártová J, Sanchez D, Fundová P, Borovská D, Srůtková D, Zídek Z, Schwarzer M, Drastich P, Funda DP (2011) The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. Cell Mol Immunol 8(2):110–120
18. Lee MS, Kim YJ (2007) Signaling pathways downstream of pattern-recognition receptors and their cross talk. Annu Rev Biochem 76:447–480 (review)
19. Sanz Y, De Palma G (2009) Gut microbiota and probiotics in modulation of epithelium and gut-associated lymphoid tissue function. Int Rev Immunol 28(6):397–413
20. Wolowczuk I, Verwaerde C, Viltart O, Delanoye A, Delacre M, Pot B, Grangette C (2008) Feeding our immune system: impact on metabolism. Clin Dev Immunol 2008:639803
21. Samuel VT, Shulman GI (2012) Mechanisms for insulin resistance: common threads and missing links. Cell 148(5):852–871
22. Poutahidis T, Kleinewietfeld M, Smillie C, Levkovich T, Perrotta A, Bhela S, Varian BJ, Ibrahim YM, Lakritz JR, Kearney SM, Chatzigiagkos A, Hafler DA, Alm EJ, Erdman SE (2013) Microbial reprogramming inhibits Western diet-associated obesity. PLoS One 8(7): e68596
23. Ding S, Chi MM, Scull BP, Rigby R, Schwerbrock NM, Magness S, Jobin C, Lund PK (2010) High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. PLoS One 5(8):e12191
24. Kim KA, Gu W, Lee IA, Joh EH, Kim DH (2012) High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. PLoS One 7 (10):e47713
25. Gerner RR, Wieser V, Moschen AR, Tilg H (2013) Metabolic inflammation: role of cytokines in the crosstalk between adipose tissue and liver. Can J Physiol Pharmacol 91(11):867–872
26. Lumeng CN, Bodzin JL, Saltiel AR (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 117(1):175–184

27. Olefsky JM, Glass CK (2010) Macrophages, inflammation, and insulin resistance. Annu Rev Physiol 72:219–246
28. Nguyen KD, Qiu Y, Cui X, Goh YP, Mwangi J, David T, Mukundan L, Brombacher F, Locksley RM, Chawla A (2011) Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis. Nature 480(7375):104–108
29. Saltiel AR, Kahn CR (2001) Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414(6865):799–806
30. Tilg H, Moschen AR (2006) Adipocytokines: mediators linking adipose tissue, inflammation and immunity. Nat Rev Immunol 6(10):772–783
31. Osborn O, Olefsky JM (2012) The cellular and signaling networks linking the immune system and metabolism in disease. Nat Med 18(3):363–374
32. Wieser V, Moschen AR, Tilg H (2013) Inflammation, cytokines and insulin resistance: a clinical perspective. Arch Immunol Ther Exp (Warsz) 61(2):119–125
33. Winer DA, Winer S, Shen L, Wadia PP, Yantha J, Paltser G, Tsui H, Wu P, Davidson MG, Alonso MN, Leong HX, Glassford A, Caimol M, Kenkel JA, Tedder TF, McLaughlin T, Miklos DB, Dosch HM, Engleman EG (2011) B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies. Nat Med 17(5):610–617
34. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, Otsu M, Hara K, Ueki K, Sugiura S, Yoshimura K, Kadowaki T, Nagai R (2009) CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nat Med 15(8):914–920
35. Winer S, Chan Y, Paltser G, Truong D, Tsui H, Bahrami J, Dorfman R, Wang Y, Zielenski J, Mastronardi F, Maezawa Y, Drucker DJ, Engleman E, Winer D, Dosch HM (2009) Normalization of obesity-associated insulin resistance through immunotherapy. Nat Med 15(8):921–929
36. Winer S, Winer DA (2012) The adaptive immune system as a fundamental regulator of adipose tissue inflammation and insulin resistance. Immunol Cell Biol 90(8):755–762
37. Cipolletta D, Feuerer M, Li A, Kamei N, Lee J, Shoelson SE, Benoist C, Mathis D (2012) PPAR-γ is a major driver of the accumulation and phenotype of adipose tissue Treg cells. Nature 486(7404):549–553
38. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, Lee J, Goldfine AB, Benoist C, Shoelson S, Mathis D (2009) Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. Nat Med 15(8):930–939
39. Shin NR, Lee JC, Lee HY, Kim MS, Whon TW, Lee MS, Bae JW (2014) An increase in the *Akkermansia* spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. Gut 63:727–735 (Epub ahead of print)
40. Obstfeld AE, Sugaru E, Thearle M, Francisco AM, Gayet C, Ginsberg HN, Ables EV, Ferrante AW Jr (2010) C–C chemokine receptor 2 (CCR2) regulates the hepatic recruitment of myeloid cells that promote obesity-induced hepatic steatosis. Diabetes 59(4):916–925
41. Oh DY, Morinaga H, Talukdar S, Bae EJ, Olefsky JM (2012) Increased macrophage migration into adipose tissue in obese mice. Diabetes 61(2):346–354
42. Kotas ME, Lee HY, Gillum MP, Annicelli C, Guigni BA, Shulman GI, Medzhitov R (2011) Impact of CD1d deficiency on metabolism. PLoS One 6(9):e25478
43. Schipper HS, Rakhshandehroo M, van de Graaf SF, Venken K, Koppen A, Stienstra R, Prop S, Meerding J, Hamers N, Besra G, Boon L, Nieuwenhuis EE, Elewaut D, Prakken B, Kersten S, Boes M, Kalkhoven E (2012) Natural killer T cells in adipose tissue prevent insulin resistance. J Clin Invest 122(9):3343–3354
44. Mantell BS, Stefanovic-Racic M, Yang X, Dedousis N, Sipula IJ, O'Doherty RM (2011) Mice lacking NKT cells but with a complete complement of CD8+ T-cells are not protected against the metabolic abnormalities of diet-induced obesity. PLoS One 6(6):e19831
45. Kiechl S, Wittmann J, Giaccari A, Knoflach M, Willeit P, Bozec A, Moschen AR, Muscogiuri G, Sorice GP, Kireva T, Summerer M, Wirtz S, Luther J, Mielenz D, Billmeier U, Egger G, Mayr A, Oberhollenzer F, Kronenberg F, Orthofer M, Penninger JM, Meigs JB, Bonora E, Tilg H, Willeit J, Schett G (2013) Blockade of receptor activator of

nuclear factor-κB (RANKL) signaling improves hepatic insulin resistance and prevents development of diabetes mellitus. Nat Med 19(3):358–363
46. Gerozissis K (2008) Brain insulin, energy and glucose homeostasis; genes, environment and metabolic pathologies. Eur J Pharmacol 585(1):38–49
47. Milanski M, Arruda AP, Coope A, Ignacio-Souza LM, Nunez CE, Roman EA, Romanatto T, Pascoal LB, Caricilli AM, Torsoni MA et al (2012) Inhibition of hypothalamic inflammation reverses diet-induced insulin resistance in the liver. Diabetes 61:1455–1462
48. Berg AH, Scherer PE (2005) Adipose tissue, inflammation, and cardiovascular disease. Circ Res 96(9):939–949 (review)
49. Milner JJ, Beck MA (2012) The impact of obesity on the immune response to infection. Proc Nutr Soc 71(2):298–306
50. Kim YH, Kim JK, Kim DJ, Nam JH, Shim SM, Choi YK, Lee CH, Poo H (2012) Diet-induced obesity dramatically reduces the efficacy of a 2009 pandemic H1N1 vaccine in a mouse model. J Infect Dis 205(2):244–251
51. Matarese G, Moschos S, Mantzoros CS (2005) Leptin in immunology. J Immunol 174 (6):3137–3142
52. Zhou Q, Leeman SE, Amar S (2011) Signaling mechanisms in the restoration of impaired immune function due to diet-induced obesity. Proc Natl Acad Sci U S A 108(7):2867–2872
53. Macia L, Delacre M, Abboud G, Ouk TS, Delanoye A, Verwaerde C, Saule P, Wolowczuk I (2006) Impairment of dendritic cell functionality and steady-state number in obese mice. J Immunol 177(9):5997–6006
54. Mattioli B, Straface E, Quaranta MG, Giordani L, Viora M (2005) Leptin promotes differentiation and survival of human dendritic cells and licenses them for Th1 priming. J Immunol 174(11):6820–6828 [Erratum in: J Immunol. 2005 Sep 1; 175(5):3446]
55. Verwaerde C, Delanoye A, Macia L, Tailleux A, Wolowczuk I (2006) Influence of high-fat feeding on both naive and antigen-experienced T-cell immune response in DO10.11 mice. Scand J Immunol 64(5):457–466
56. Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. Annu Rev Immunol 21:335–376
57. Erridge C, Attina T, Spickett CM, Webb DJ (2007) A high-fat meal induces low-grade endotoxemia: evidence of a novel mechanism of postprandial inflammation. Am J Clin Nutr 86(5):1286–1292
58. Lajunen T, Vikatmaa P, Bloigu A, Ikonen T, Lepäntalo M, Pussinen PJ, Saikku P, Leinonen M (2008) Chlamydial LPS and high-sensitivity CRP levels in serum are associated with an elevated body mass index in patients with cardiovascular disease. Innate Immun 14(6):375–382
59. Cani PD, Delzenne NM (2009) Interplay between obesity and associated metabolic disorders: new insights into the gut microbiota. Curr Opin Pharmacol 9(6):737–743
60. Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, Geurts L, Naslain D, Neyrinck A, Lambert DM, Muccioli GG, Delzenne NM (2009) Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 58(8):1091–1103
61. Membrez M, Blancher F, Jaquet M, Bibiloni R, Cani PD, Burcelin RG, Corthesy I, Macé K, Chou CJ (2008) Gut microbiota modulation with norfloxacin and ampicillin enhances glucose tolerance in mice. FASEB J 22(7):2416–2426
62. Reigstad CS, Lundén GO, Felin J, Bäckhed F (2009) Regulation of serum amyloid A3 (SAA3) in mouse colonic epithelium and adipose tissue by the intestinal microbiota. PLoS One 4(6): e5842
63. Caesar R, Reigstad CS, Bäckhed HK, Reinhardt C, Ketonen M, Lundén GÖ, Cani PD, Bäckhed F (2012) Gut-derived lipopolysaccharide augments adipose macrophage accumulation but is not essential for impaired glucose or insulin tolerance in mice. Gut 61(12):1701–1707
64. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H et al (2006) TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest 116:3015–3025

65. Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM et al (2007) Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. Diabetes 56:1986–1998
66. Shi C, Jia T, Mendez-Ferrer S, Hohl TM, Serbina NV, Lipuma L, Leiner I, Li MO, Frenette PS, Pamer EG (2011) Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands. Immunity 34(4):590–601
67. Sawada K, Ohtake T, Hasebe T, Abe M, Tanaka H, Ikuta K, Suzuki Y, Fujiya M, Hasebe C, Kohgo Y (2013) Augmented hepatic Toll-like receptors by fatty acids trigger the pro-inflammatory state of non-alcoholic fatty liver disease in mice. Hepatol Res. Doi: 10.1111/hepr.12199
68. Glass CK, Olefsky JM (2012) Inflammation and lipid signaling in the etiology of insulin resistance. Cell Metab 15(5):635–645
69. Wen H, Ting JP, O'Neill LA (2012) A role for the NLRP3 inflammasome in metabolic diseases-did Warburg miss inflammation? Nat Immunol 13(4):352–357
70. Pal D, Dasgupta S, Kundu R, Maitra S, Das G, Mukhopadhyay S, Ray S, Majumdar SS, Bhattacharya S (2012) Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. Nat Med 18(8):1279–1285
71. Delzene N, Neyrik M (2008) Prebiotics and lipid metabolism: review of experimental and human data. In: Gibson GR, Roberfroid M (eds) Handbook of prebiotics. CRC, Boca Raton, pp 201–218
72. Murphy EF, Cotter PD, Hogan A, O'Sullivan O, Joyce A, Fouhy F, Clarke SF, Marques TM, O'Toole PW, Stanton C, Quigley EM, Daly C, Ross PR, O'Doherty RM, Shanahan F (2013) Divergent metabolic outcomes arising from targeted manipulation of the gut microbiota in diet-induced obesity. Gut 62(2):220–226
73. Cani PD, Neyrinck AM, Fava F et al (2007) Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia 50:2374–2383
74. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C et al (2007) Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 56:1761–1772
75. Neal MD, Leaphart C, Levy R, Prince J, Billiar TR, Watkins S, Li J, Cetin S, Ford H, Schreiber A, Hackam DJ (2006) Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier. J Immunol 176(5):3070–3079
76. Lapthorne S, Macsharry J, Scully P, Nally K, Shanahan F (2012) Differential intestinal M-cell gene expression response to gut commensals. Immunology 136(3):312–324
77. Amar J, Chabo C, Waget A, Klopp P, Vachoux C, Bermúdez-Humarán LG, Smirnova N, Bergé M, Sulpice T, Lahtinen S, Ouwehand A, Langella P, Rautonen N, Sansonetti PJ, Burcelin R (2011) Intestinal mucosal adherence and translocation of commensal bacteria at the early onset of type 2 diabetes: molecular mechanisms and probiotic treatment. EMBO Mol Med 3(9):559–572
78. Mazzon E, Cuzzocrea S (2008) Role of TNF-alpha in ileum tight junction alteration in mouse model of restraint stress. Am J Physiol Gastrointest Liver Physiol 294:G1268–G1280
79. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM et al (2008) Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 57:1470–1481
80. Cani PD, Knauf C, Iglesias MA, Drucker DJ, Delzenne NM, Burcelin R (2006) Improvement of glucose tolerance and hepatic insulin sensitivity by oligofructose requires a functional glucagon-like peptide 1 receptor. Diabetes 55(5):1484–1490
81. Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, Guiot Y, Derrien M, Muccioli GG, Delzenne NM, de Vos WM, Cani PD (2013) Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci U S A 110(22):9066–9071
82. Laflamme N, Echchannaoui H, Landmann R, Rivest S (2003) Cooperation between toll-like receptor 2 and 4 in the brain of mice challenged with cell wall components derived from gram-negative and gram-positive bacteria. Eur J Immunol 33(4):1127–1138

83. Ehses JA, Meier DT, Wueest S, Rytka J, Boller S, Wielinga PY, Schraenen A, Lemaire K, Debray S, Van Lommel L, Pospisilik JA, Tschopp O, Schultze SM, Malipiero U, Esterbauer H, Ellingsgaard H, Rütti S, Schuit FC, Lutz TA, Böni-Schnetzler M, Konrad D, Donath MY (2010) Toll-like receptor 2-deficient mice are protected from insulin resistance and beta cell dysfunction induced by a high-fat diet. Diabetologia 53(8):1795–1806
84. Caricilli AM, Picardi PK, de Abreu LL, Ueno M, Prada PO, Ropelle ER, Hirabara SM, Castoldi Â, Vieira P, Camara NO, Curi R, Carvalheira JB, Saad MJ (2011) Gut microbiota is a key modulator of insulin resistance in TLR 2 knockout mice. PLoS Biol 9(12):e1001212
85. Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, Sitaraman SV, Knight R, Ley RE, Gewirtz AT (2010) Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science 328(5975):228–231
86. Amar J, Lange C, Payros G, Garret C, Chabo C, Lantieri O, Courtney M, Marre M, Charles MA, Balkau B, Burcelin R (2013) D.E.S.I.R. Study Group. Blood microbiota dysbiosis is associated with the onset of cardiovascular events in a large general population: the D.E.S.I.R. study. PLoS One 8(1):e54461
87. Miura K, Kodama Y, Inokuchi S, Schnabl B, Aoyama T, Ohnishi H, Olefsky JM, Brenner DA, Seki E (2010) Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1beta in mice. Gastroenterology 139(1):323–334.e7
88. Schertzer JD, Klip A (2011) Give a NOD to insulin resistance. Am J Physiol Endocrinol Metab 301(4):E585–E586
89. Fukazawa A, Alonso C, Kurachi K, Gupta S, Lesser CF, McCormick BA, Reinecker HC (2008) GEF-H1 mediated control of NOD1 dependent NF-kappaB activation by Shigella effectors. PLoS Pathog 4(11):e1000228
90. Netea MG, Kullberg BJ, de Jong DJ, Franke B, Sprong T, Naber TH, Drenth JP, Van der Meer JW (2004) NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. Eur J Immunol 34(7):2052–2059
91. Tamrakar AK, Schertzer JD, Chiu TT, Foley KP, Bilan PJ, Philpott DJ, Klip A (2010) NOD2 activation induces muscle cell-autonomous innate immune responses and insulin resistance. Endocrinology 151(12):5624–5637
92. Schertzer JD, Tamrakar AK, Magalhães JG, Pereira S, Bilan PJ, Fullerton MD, Liu Z, Steinberg GR, Giacca A, Philpott DJ, Klip A (2011) NOD1 activators link innate immunity to insulin resistance. Diabetes 60(9):2206–2215
93. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez JP, Shulman GI, Gordon JI, Hoffman HM, Flavell RA (2012) Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 482 (7384):179–185
94. Gauffin Cano P, Santacruz A, Moya Á, Sanz Y (2012) *Bacteroides uniformis* CECT 7771 ameliorates metabolic and immunological dysfunction in mice with high-fat-diet induced obesity. PLoS One 7(7):e41079
95. Davey KJ, Cotter PD, O'Sullivan O, Crispie F, Dinan TG, Cryan JF, O'Mahony SM (2013) Antipsychotics and the gut microbiome: olanzapine-induced metabolic dysfunction is attenuated by antibiotic administration in the rat. Transl Psychiatry 3:e309
96. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS (2013) The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science 341(6145):569–573
97. Wu D, Molofsky AB, Liang HE, Ricardo-Gonzalez RR, Jouihan HA, Bando JK, Chawla A, Locksley RM (2011) Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. Science 332(6026):243–247
98. Bassaganya-Riera J, Dominguez-Bello MG, Kronsteiner B, Carbo A, Lu P, Viladomiu M, Pedragosa M, Zhang X, Sobral BW, Mane SP, Mohapatra SK, Horne WT, Guri AJ, Groeschl M, Lopez-Velasco G, Hontecillas R (2012) *Helicobacter pylori* colonization ameliorates glucose homeostasis in mice through a PPAR γ-dependent mechanism. PLoS One 7 (11):e50069

Chapter 15
Microbiota, Immunoregulatory Old Friends and Psychiatric Disorders

Graham A.W. Rook, Charles L. Raison, and Christopher A. Lowry

Abstract Regulation of the immune system is an important function of the gut microbiota. Increasing evidence suggests that modern living conditions cause the gut microbiota to deviate from the form it took during human evolution. Contributing factors include loss of helminth infections, encountering less microbial biodiversity, and modulation of the microbiota composition by diet and antibiotic use. Thus the gut microbiota is a major mediator of the hygiene hypothesis (or as we prefer, "Old Friends" mechanism), which describes the role of organisms with which we co-evolved, and that needed to be tolerated, as crucial inducers of immunoregulation. At least partly as a consequence of reduced exposure to immunoregulatory Old Friends, many but not all of which resided in the gut, high-income countries are undergoing large increases in a wide range of chronic inflammatory disorders including allergies, autoimmunity and inflammatory bowel diseases. Depression, anxiety and reduced stress resilience are comorbid with these conditions, or can occur in individuals with persistently raised circulating levels of biomarkers of inflammation in the absence of clinically apparent peripheral inflammatory disease. Moreover poorly regulated inflammation during pregnancy might contribute to brain developmental abnormalities that underlie some cases of autism spectrum disorders and schizophrenia. In this chapter we explain how the gut microbiota drives immunoregulation, how faulty immunoregulation and inflammation predispose to psychiatric disease, and how psychological stress drives further

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_15, © Springer New York 2014

inflammation via pathways that involve the gut and microbiota. We also outline how this two-way relationship between the brain and inflammation implicates the microbiota, Old Friends and immunoregulation in the control of stress resilience.

Abbreviations

ASD	Autism spectrum disorders
BH4	Tetrahydrobiopterin
CD	Crohn's disease
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
dACC	Dorsal anterior cingulate cortex
DC	Dendritic cells
DCreg	Regulatory dendritic cells
fMRI	Functional magnetic resonance imaging
GABA	g-Aminobutyric acid
GCR	Glucocorticoid resistance
HPA	Hypothalamic-pituitary adrenal axis
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IDO	Indoleamine-2,3-dioxygenase
IFN-α	Interferon-alpha
IL	Interleukin
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MS	Multiple sclerosis
NO	Nitric oxide
Nod1	Nucleotide-binding oligomerization domain-containing protein-1
PBMCs	Peripheral blood monocyte cells
PET	Positron emission tomography
SCFA	Short chain fatty acids
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphisms
SNS	Sympathetic nervous system
SSRI	Selective serotonin reuptake inhibitors
T1D	Type 1 diabetes
TNF	Tumor necrosis factor
Treg	Regulatory T cells
UC	Ulcerative colitis
XLAAD	X-linked autoimmunity-allergic dysregulation syndrome

Introduction

This chapter concentrates on those gut-brain interactions that operate indirectly via the immune system, rather than via direct neural pathways. At least two sets of findings underlie this aspect of gut-brain interaction. First, we know that persistently raised levels of inflammatory mediators are associated with several psychiatric conditions. This will be discussed with particular reference to depression and to reduced stress resilience. The regulation of background levels of inflammation is dependent upon "learning" inputs to the immune system from appropriate microbial exposures during the prenatal and neonatal periods, and continuing diversity of input in later life. This concept, initially called the "hygiene hypothesis" is becoming renamed the "Old Friends" mechanism, which places it firmly within the field of Darwinian and evolutionary medicine. The various mammalian microbiotas, particularly the gut microbiota, are important components of the Old Friends mechanism, and have a continuing immunoregulatory role in the adult. Secondly, we know that inflammation during pregnancy can lead to abnormal development of the central nervous system (CNS). This will be illustrated by considering autism spectrum disorders (ASD) and schizophrenia, and aspects of epidemiology that suggest the importance of microbe-dependent immunoregulatory effects during pregnancy.

The expression "Hygiene hypothesis" was first published in 1989, following the observation that, when examined at 11 years old, children brought up in families with many older siblings were less likely to have developed allergic disorders. This concept was at first a narrow one, focusing on the notion that childhood infections somehow prevented subsequent allergies. In fact it had been known since the nineteenth century that the environment could modulate the likelihood of developing hay fever, which was increasing amongst wealthy townsfolk, while remaining rare amongst farmers [1]. This protective effect of the farming environment in allergic disorders has subsequently been rigorously confirmed and found to extend to juvenile-onset inflammatory bowel disease (IBD) as well [2]. Moreover to reap protection from these immune-mediated diseases it can be sufficient to expose the pregnant mother to the farming environment, rather than the infant itself [3]. The most recent observations indicate that the farm effect is mostly explained by exposure to increased microbial biodiversity, which was documented by analyzing the bacterial and fungal taxa present in the dust in children's bedrooms [4].

Meanwhile sporadic observations in other branches of medicine have confirmed that allergic disorders are not the only chronic inflammatory conditions that have been increasing in high-income countries, particularly in urban populations. Inflammatory bowel diseases and autoimmune diseases have increased at about the same rate, and in the same places [5, 6] as allergic conditions. Subsequently large epidemiological surveys have shown that childhood infections, originally implicated as the protective mechanism behind the hygiene hypothesis, do not protect against allergic disorders, and may in some cases, such as human rhinoviruses and respiratory syncytial viruses, actually trigger allergic responses. Considered

together, these observations led to a Darwinian reformulation of the hypothesis as the Old Friends mechanism, described in the next section.

Old Friends Mechanism

The Old Friends mechanism states that mammals co-evolved with various microbiotas and commensals (gut, skin, lung etc.), as well as with chronic infectious agents picked up at birth, helminths that persisted for life, and environmental organisms from animals, mud and untreated water with which we were in daily contact. Because all of these categories of organism needed to be tolerated, they took on a role of inducers of immunoregulatory circuits [7, 8]. For example, helminthic parasites need to be tolerated because, although not always harmless, once they are established in the host, efforts by the immune system to eliminate them are typically futile, and merely cause tissue damage [9].

Contact with the "Old Friends" rapidly diminished when industrialization occurred, and mankind started to inhabit a plastic and concrete environment, to consume washed food and chlorine-treated water, and to minimize our contact with mud, animals and feces. This withdrawal of the organisms that drive immunoregulatory circuits results in defective immunoregulation that, depending on the genetic background of any given individual, can manifest itself as a variety of chronic inflammatory disorders, including allergies, IBD and autoimmunity. We know that a failure of immunoregulatory mechanisms really can lead to simultaneous increases in diverse types of pathology. For example, defects in the gene encoding the immunoregulatory transcription factor Foxp3 lead to the X-linked autoimmunity-allergic dysregulation syndrome (XLAAD) that includes aspects of allergy, autoimmunity and enteropathy [10].

The underlying Darwinian principle of the Old Friends mechanism is illustrated in Fig. 15.1. The immune system at birth is analogous to a computer with hardware, some software, but very little data. The minimal data that it does have comes from T lymphocyte selection in the thymus, and probably from transfer of at least some environmental and maternal antigenic material across the placenta. After birth the immune system requires the largest possible exposure to environmental microbial biodiversity in order to build a very broad repertoire of potential effector lymphocytes. Since all life forms are ultimately constructed with similar building blocks, such diversity of "education" can even provide the system with T cells that recognize, for example, some obscure viral pathogen that might be encountered in the future [11].

However in the context of this chapter, still more important than the diverse effector repertoire is the setting up of appropriate immunoregulation. Just as exposure early in life to a wide range of microbial and parasitic organisms trains the immune system regarding what to be on guard against, it also teaches immunity what to profitably ignore because the organisms in question either confer some benefit to the host, or confer no danger or despite posing some risk are not easily

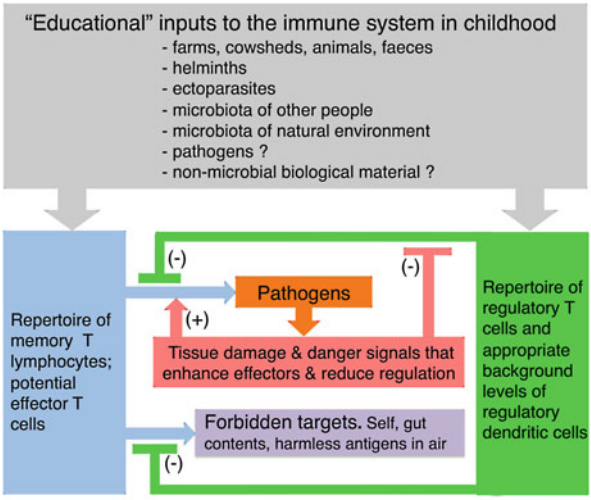

Fig. 15.1 The immune system requires "educational" input. The microbiota of others, tolerated organisms (such as helminths) with which we co-evolved and organisms from the natural environment are required to expand the effector and regulatory branches of the immune system. During subsequent encounters with pathogens, danger signals generated by tissue damage enhance effector mechanisms and attenuate regulatory pathways to permit an appropriate immune response. Adequate background levels of regulatory T cells and dendritic cells and other regulatory mechanisms are required to maintain suppression of responses to "forbidden targets" and to switch off inflammation completely when the danger is eliminated, so that proinflammatory mediators do not continue to circulate

eradicated by immune mechanisms once established. These immunoregulatory inputs benefit the host by teaching the immune system not to waste precious energy engaging in futile battles, by reducing the cost to the host of chronic inflammation and by reducing the risk of destruction of host tissues, either through bystander effects or via the induction of autoimmunity. Because humans in traditional environments were exposed to organisms that dampened, as well as stimulated, immune function, the Old Friends mechanism implies that inflammation should be better regulated in low-income than in high-income countries. At first sight this might seem paradoxical, because the high prevalence of infections in low-income countries might be expected to cause high levels of inflammation [12]. However recent work by McDade et al. [13, discussed in 14] has largely resolved this paradox. The results reveal that in a low-income country where there is still abundant exposure to the immunoregulation-inducing "Old Friends", immunoregulation is efficient, and the inflammatory response is vigorous during an infection, but is terminated when no longer needed, with the result that "resting" C-reactive protein (CRP) is close to zero. These longitudinal results illuminate a previous finding by McDade et al. [15] that *high* levels of microbial exposure in the perinatal period and in infancy correlated with *low* levels of "resting" CRP in adulthood. In contrast, in the USA and other high-income countries there is often constant low-grade inflammation

which tends to be stable across individuals, manifested as chronically raised CRP or interleukin (IL)-6, in the absence of any clinically apparent inflammatory stimulus. Such chronically elevated inflammation greatly increases the risk of subsequent inflammatory disease and cardiovascular problems and has been shown in some studies to predict the future development of depression [16].

Inflammation and Psychiatric Disorders

Inflammation is involved not only in chronic inflammatory disorders such as allergies, autoimmunity and IBD but also in many psychiatric disorders. We have reviewed this topic in detail elsewhere [17, 18]. Briefly, a large subset of depressed individuals has persistently raised levels of proinflammatory cytokines and other downstream inflammatory markers [19, 20], together with a relative deficit in anti-inflammatory mediators and regulatory T cells [fully referenced in 18, 21]. Interestingly, depressed individuals also show exaggerated release of inflammatory mediators in response to psychosocial stressors [22], implying altered immunoregulation (Fig. 15.2), and epidemiological studies in the United Kingdom (UK) showed that raised CRP and IL-6 predicts subsequent risk of depression assessed over a decade later [16].

The possibility that inflammatory mediators might play direct causal roles in depressive pathogenesis has been confirmed for interferon-alpha (IFN-α), interleukin 6 (IL-6), and tumor necrosis factor (TNF). When IFN-α is used therapeutically (to treat viral hepatitis or some cancers) it causes depression-like symptoms in a high percentage of patients. These symptoms have been repeatedly shown to respond to treatment with standard antidepressants, such as selective serotonin reuptake inhibitors (SSRI) [20, 23]. Similarly the cytokine antagonist infliximab, which blocks TNF actions, has been shown to have antidepressant properties, but only in depressed individuals with evidence of increased peripheral inflammation prior to treatment [24].

Finally, when IL-6 is administered to pregnant animals it causes abnormal brain development in the fetus, discussed later in relation to autism [25]. Similarly increased peripheral levels of IL-6 cause increased production of IL-6 in the CNS, and affect neurogenesis in the hippocampus [reviewed in 26]. That IL-6 is directly relevant to the changes seen is supported by the fact that these effects can be blocked by IL-6 receptor antagonists, and knockout mice with non-functional IL-6 genes have enhanced working memory compared to wild type mice [27] and are refractory to peripheral inflammation-induced impairments of spatial memory [28]. In humans raised levels of IL-6 are associated with diminished cognitive performance and reduced hippocampal gray matter [26, 29]. Mechanisms for these effects are discussed in a later section.

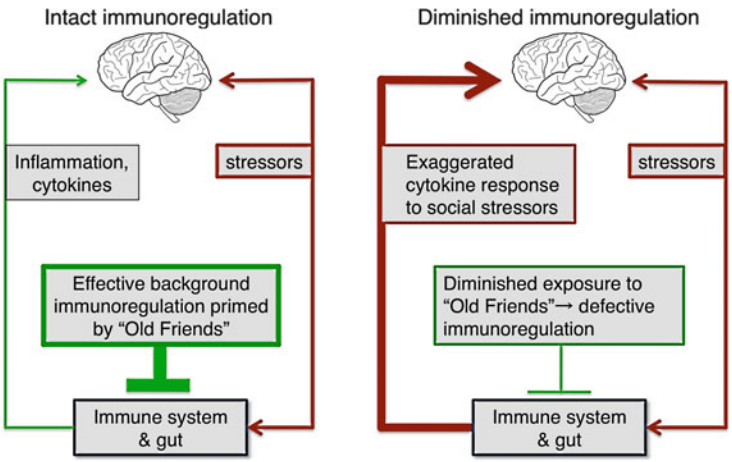

Fig. 15.2 Exaggerated and prolonged cytokine release in response to a psychosocial stressor in individuals with diminished immunoregulation. Populations that have poorly immunoregulatory gut microbiota and reduced exposure to immunoregulation-inducing "Old Friends" such as helminths are susceptible to excessive and prolonged cytokine release in response to psychosocial stressors, which may result in reduced stress resilience and inappropriate triggering of depressive episodes. Reprinted from Rook et al. (2013) *Evolution, Medicine and Pubic Health* (1): 46–64, doi: 10.1093/emph/eot004, by permission of Oxford University Press and the Foundation for Evolution, Medicine, and Public Health

Immunoregulation and Stress Resilience in Developing Countries

The vicious cycle described in Fig. 15.2, considered against the background of the Old Friends mechanism, suggests that in developing countries there will be less release of inflammatory mediators in response to psychosocial stressors, and less psychiatric consequences of such stressors. Recent data support this hypothesis. In experimental animals parental deprivation is a potent inducer of long-term changes to stress responses and immunoregulation [30]. Human studies suggest similar correlations [31]. However in a recent study performed in a developing country, parental absence in childhood was a significant predictor of raised CRP in adulthood, as it would be in a rich country, but *only in a subset* of the cohort raised in hygienic environments [32]. However, adults who had a high level of microbial exposure in infancy were resistant to the long-term proinflammatory effects of this severe childhood stressor [32]. The same was true of perceived stress during the previous month in young adults. CRP correlated with recent perceived stress in subjects with low microbial exposure in infancy, but not in those with high microbial exposure. Again, exposure to immunoregulation-inducing "Old Friends" seemed to provide resistance to the inflammation-inducing effects of psychosocial stressors [32].

This leads to an obvious question. If psychosocial stressors cause depression at least partly by triggering the release of proinflammatory mediators (Fig. 15.2), are inhabitants of developing countries resistant to psychosocial stress-induced depression? If so the prevalence of depression should be increasing in developed countries in parallel with the chronic inflammatory disorders [33], and lower in developing countries than in developed ones. Comparative studies are difficult to do, but this is indeed what data collected by the World Health Organization indicate [34]. Moreover one study failed to find a correlation between depression and raised CRP in a developing country whereas this association is routinely found in rich ones [35].

Urbanization and Immigration to High-Income Countries

If a dysregulated immune system resulting from diminished contact with immunoregulation-inducing "Old Friends" is partly to blame for the increasing prevalence not only of chronic inflammatory disorders such as allergies, autoimmunity and IBD, but also of those psychiatric disorders that can be triggered by inflammatory mediators, then it should be useful to examine urban-rural differences in disease prevalence, and the effect of migration from low-income to rich urban environments. In each case there will be loss of exposure to Old Friends.

Urban Versus Rural

A feature shared by most of the disorders discussed here is a higher prevalence in urban communities compared to rural ones. For example a meta-analysis of high quality studies performed in high-income countries since 1985 found that the prevalence of depression in urban areas was 39 % higher than in rural areas. Similarly, the prevalence of anxiety disorders was 21 % higher in urban than in rural areas [36], though a small minority of studies fails to find this urban-rural difference [37]. Peen et al. [36] also noted an increased urban prevalence of psychiatric disorders in general (38 % more in urban communities). This agrees well with another large meta-analysis that found a significantly raised prevalence of schizophrenia in urban communities [38]. Similarly, a study of all children born in Denmark between 1 January 1984 and 31 December 1998 found that the degree of urbanization of place of birth was very significantly correlated to risk of autism [39].

The urban > rural phenomenon is also well established for chronic inflammatory disorders, where the etiology is known to involve dysregulation of the immune system. Contact with the farming environment, whether early postnatal [40] or prenatal [3, 41] protects against allergic disorders, whereas the prevalence of these conditions increases with increasing urbanization [42]. The same is true for IBD

[43], and for autoimmune diseases such as multiple sclerosis (MS) [44, 45, discussed in 46].

Immigrants

Another striking parallel between chronic inflammatory diseases and psychiatric disorders concerns the effects that immigration has on these conditions. All the diseases discussed here, whether chronic inflammatory [43, 47–49] or psychiatric [50–52], tend to be more common in immigrants than in the birth population from which the immigrant was derived, at least when the migration is from a developing to a high-income country. Other relevant variables include the age of the individual at the time of immigration, and whether the prevalence increases in second generation immigrants, born in the adopted country. A study of these parameters provides some insight into whether the relevant influences, be they psychosocial or immunological, need to occur before birth, or in early childhood, or whether they can still exert their effects on adults.

Immigration and Psychiatric Disorders

Depression is particularly interesting in this respect [53, 54]. Mexicans, Cubans and African/Caribbean peoples were found to have a two to threefold increase in the prevalence of depression if immigration to the USA occurred when the individual was less than 13 years old, or was born in the USA, compared to the prevalence in those who migrated after the age of 13 [53]. But this is not likely due to psychosocial stress related to skin color, because white Eastern European immigrants show the same effect. In sharp contrast, the effect is not seen in immigrants from Western Europe, or from Puerto Rico, which is closely associated with the USA. (These last two populations already have a high prevalence of depression that is not increased by immigrating to, or being born in, the USA) [53]. These findings imply that influences important for depression occur perinatally, or in the early years of life.

The same is true for psychotic disorders [55]. A large Danish study noted that immigration into Denmark when less than 4 years old was associated with a strikingly increased risk for psychotic disorders, whereas the increased risk gradually decreased with older age at migration and disappeared in those immigrating when more than 29 years old [56]. Similarly a large meta-analysis confirmed that schizophrenia was increased amongst first generation immigrants, and further increased amongst second generation immigrants, particularly when the country of origin was a developing one [57]. Again, early events seem crucial.

Age at immigration is irrelevant to an early onset condition such as autism, but autism is strikingly (as much as tenfold) increased in second generation Caribbean or African immigrants born in the UK, compared to children of white UK-born

mothers [52]. *These findings implicate crucial early events in the perinatal period or early childhood as risk factors for depression, schizophrenia and autism.*

Immigration and Chronic Inflammatory Disorders

Migration has clear effects on the prevalence of MS, and the crucial events that confer increased risk for the disease occur very early in life, as is true for the psychiatric disorders [reviewed and referenced in 58, 59]. Iranians who migrate to Sweden have twice the prevalence of MS seen in their birth country [49]. Interestingly, if the second (or later) generation immigrants return to their developing country of origin, they retain their increased susceptibility to MS, which remains higher than in the local population that was not born abroad [60]. A similar phenomenon was seen when people born in the UK (a high MS country) migrated to South Africa (SA: a low MS country). Migration from the UK to SA was protective when the migrant was a child, whereas adult migrants retained their high UK prevalence of MS [61]. Analysis of this and other studies suggests that the environmental factors that protect from or predispose to MS act during the first two decades of life [58, 59]. The same is true for type 1 diabetes (T1D). Here the crucial factor is to have been *born* in the receiving developed country, again suggesting that relevant environmental factors act very early, or even in the prenatal period [48].

The role of migration in conferring risk for allergic disorders has been intensively examined. A study of children adopted into Sweden from developing countries showed that the prevalence of asthma, hay fever and eczema were highest in those adopted when less than 2 years old [62]. Similarly, for Mexican immigrants to the USA, the prevalence of asthma was highest for those born in the USA, while in those not born in the USA, the prevalence of asthma decreased as the age at immigration increased [63]. This effect of age at the time of childhood immigration was also seen in immigrants to Israel from the former Soviet Union or Ethiopia who were assessed when 17 years old [64]. These observations suggest the importance of early environmental influences for allergy/asthma risk, a conclusion that is powerfully supported by evidence that prenatal exposure (i.e. of the pregnant mother) to the farming environment protects the infant against some allergic manifestations [3, 41]. This is discussed later in another context.

Finally, a definitive study of all first- and second-generation immigrants in Sweden between January 1, 1964, and December 31, 2007 showed that some first generation immigrants remain partially protected from both ulcerative colitis (UC) and Crohn's disease (CD), presumably by environmental factors encountered in their countries of origin, but the diseases increased in prevalence in second generation immigrants, relative to first generation immigrants [65]. Similarly, the prevalence of UC in South Asian immigrants to Leicester in the UK was higher in second than in first generation immigrants [66]. This again implicates perinatal factors as potentially causative of this migration effect.

Thus the influence of immigration, acting via factors that occur perinatally or very early in life, is equally consistent and highly apparent for both psychiatric and chronic inflammatory disorders.

Mechanisms of Immunoregulation by Old Friends

Urbanization and immigration from low- to high-income countries cause diminished contact with Old Friends, and correlate with an increased incidence of chronic inflammatory disorders, all of which show evidence of failed immunoregulation [reviewed in 67]. Moreover adverse outcomes in animal models of all of these chronic inflammatory conditions can be prevented or treated with "Old Friends" such as helminths, certain gut commensals or probiotics that induce immunoregulation [68–70]. What are the mechanisms that enable the "Old Friends" to exert immunoregulatory effects? This is a vast topic, and here we outline some of its most studied aspects, particularly those that involve, or occur in, the gut.

Regulation of Innate Immunity

The gut microbiota has been shown to be necessary for priming of innate immunity, measured as the microbicidal activity of splenic macrophages [71]. This microbicidal activity was increased following a social disruption stressor. However, if the mice were germ-free no increase in microbicidal activity was seen [71]. Moreover depletion of microbiota with antibiotics attenuated the stressor-induced macrophage activation, and reduced stressor-induced increases in circulating bacterial cell wall peptidoglycan [71], and eliminated the increases in circulating IL-6 and monocyte chemoattractant protein-1 (MCP-1) usually seen in stressor-exposed mice [72]. These observations were in agreement with an earlier finding that systemic activation of the innate immune system by the gut microbiota involves recognition of *meso*-diaminopimelic acid (*meso*DAP)-containing peptidoglycan found predominantly in Gram-negative bacteria, by the pattern recognition receptor nucleotide-binding, oligomerization domain-containing protein-1 (Nod1) [73]. Similarly abdominal surgery causes systemic release of Nod2-binding bacterial components and consequent rises in several inflammatory biomarkers [74].

Regulatory Macrophages

Regulatory microorganisms can also operate via macrophages. During helminth infections there is expansion of the population of alternatively-activated macrophages, activated by Th2 rather than Th1 cytokines [75]. Such macrophages secrete

IL-10 and TGF-β rather than IL-12, are able to inhibit lymphocyte proliferation in a contact-dependent manner [76, 77], and may be responsible for preventing inflammation in mucosal surfaces such as the lung. However, some helminths drive types of regulatory macrophages that are distinct from alternatively activated macrophages [77]. Several species of filarial nematodes secrete cystatin, a cysteine protease inhibitor that induces macrophages to make IL-10 and IL-12 p40 through activation of intracellular signaling pathways. These can prevent allergic sensitization and airway hyperresponsiveness [78]. Similarly a colon-infiltrating macrophage population induced by Schistosoma infection was shown to prevent colitis in mice [79]. The protection was independent of T cells in general and regulatory T cells (Treg) in particular.

Regulatory B Cells

Helminths also induce regulatory B cells. *S. mansoni* infection prevented anaphylaxis in a mouse model, and this suppression of the effector phase of the allergic response was mediated by IL-10-secreting B cells [80]. These IL-10-secreting $CD1d^{hi}CD5+$ regulatory B cells can also suppress experimental autoimmune encephalomyelitis [81], and have recently been designated B10 cells [82]. They act in part by increasing the number of pulmonary CD4+CD25+Foxp3+ regulatory T cells in the lungs [83]. IL-10+ regulatory B cells are also found in humans [84]. Depletion of human B-cells using rituximab can occasionally exacerbate Th1-mediated conditions, suggesting that the rituximab removed a B-cell-mediated regulatory mechanism [84]. IL-10 production by B cells is increased in multiple sclerosis patients developing intestinal helminth infections [85], but not in patients infected by *Trypanosoma cruzi* [85]. The helminths also increase circulating Treg, as discussed below.

Regulatory Dendritic Cells (DC)

A particularly important immunoregulatory function is the generation of regulatory dendritic cells (DCreg) that tend to drive regulatory rather than inflammatory responses. This is crucial because such DCreg can process gut contents, autoantigens and allergens, and so downregulate responses to the target antigens of the major groups of chronic inflammatory disease. It is likely that health requires the presence of a certain background proportion of DCreg. This could be regarded as a "Treg adjuvant" function. A mixture of several putative probiotic organisms (VSL#3; four lactobacilli, three bifidobacteria, and one streptococcal strains) was found to ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and TGF-β+Treg [86]. In vitro this preparation caused human DC to release more IL-10 and inhibited their ability to drive Th1 cells [87]. Other probiotic strains [88, 89],

and a ubiquitous environmental saprophyte often present in untreated or muddy water [90] also modulate human DC function in vitro so as to induce T cell responses with a more regulatory bias. In the gut some of these DCreg express the integrin alpha chain CD103 and have unique immunoregulatory properties [91]. CD103 is involved in de novo conversion of Foxp3-CD4+ cells to Foxp3+ Treg cells [92]. Conversion of DC to this tolerogenic phenotype is driven locally by TGF-β and retinoic acid (RA). CD103+ DCs express *aldh1a2*, the gene encoding RALDH2. This enzyme is involved in conversion of dietary retinal to RA, which enhances development of FoxP3+ T cells rather than Th17 cells [reviewed in 93]. Some probiotic *Lactobacillus* strains, such as *L. plantarum* WCFS1 induce migration of these CD103+ DCreg as far as the spleen, and bias the response towards Treg [94].

An example of a Treg adjuvant effect that must at some stage involve DC is seen when MS patients become infected with helminths. The disease stops progressing, and circulating Treg appear in the peripheral blood [95, 96]. These Treg recognize the major epitope from myelin basic protein. Thus the immunoregulation caused by the helminths is not merely a bystander effect of IL-10 release, but rather a genuine Treg adjuvant effect that generates regulation specific for the autoantigen. Although the mechanism is not elucidated this is an exciting observation that has led to formal clinical trials [97].

Regulatory T Cells (Treg)

Ultimately many of these immunoregulatory mechanisms result in a relative increase in the numbers of Treg, whether secondary to changes in macrophages, B cells or DC. However some Old Friends release molecules that specifically expand Treg populations. The gut commensal *Bacteroides fragilis* releases a polysaccharide antigen that drives expansion of Treg via TLR-2 [98]. The helminth *Heligmosomoides polygyrus* drives Treg expansion via the TGF-β receptor [99]. Treatment with oral *Lactobacillus reuteri* for 9 days significantly increased the percentage and total number of CD4+CD25+Foxp3+ T cells in the spleens of experimental animals [70]. Colonization of mice by commensal *Clostridium* strains increased TGF−β levels and numbers of Foxp3+ Treg in the colon [100].

Gut Microbiota Diversity and Regulation of Inflammation

Interestingly the diversity of the gut microbiota appears to have consequences for immunoregulation, perhaps for the reasons discussed in relation to Fig. 15.1. From birth our microbiota are constituted by colonization with organisms from our mothers, from other social contacts [101, 102], and from the environment, and then further modified by factors such as diet and antibiotics [103–106]. Thus

lifestyle has major effects on an individual's microbiota and on its diversity. The gut microbiota of children from traditional villages in Burkina Faso is totally different from that of Europeans, and shows greater diversity [104]. There is abundant evidence that diversity of gut microbiota is associated with wellbeing. Mice exhibit at least two enterotypes (bacterial ecosystems in the gut microbiota), one of which has low biodiversity, and correlates with biomarkers of inflammation [107]. In humans reduced biodiversity of the gut microbiota has been associated with a range of inflammatory states, including allergic disorders [108], inflammatory bowel diseases [109–111] and obesity [112]. Loss of diversity in later life is associated with increases in circulating CRP and IL-6 levels [113]. This implies that diversity is associated with effective immunoregulation.

In agreement with this, allergies are less common in children exposed to sources of microbial biodiversity such as farms [40], dogs [114], or the natural environment [115, 116]. Similarly, allergies are reduced where there is evidence of social interactions that promote exchange of microbiota. Indicators of such exchange include infection with orofecally transmitted organisms such as enteroviruses [117], *H. pylori*, *T. gondii*, and hepatitis A virus [118]. There is some evidence that these orofecally transmitted pathogens are themselves immunoregulatory. However it might be much more important that these organisms are markers of transfer of microbiota between individuals. Interestingly mothers who clean their baby's dummy/pacifier by sucking it rather than by sterilizing it have children with less allergic problems [119].

In sharp contrast, lifestyle events that are likely to restrict the diversity of gut microbiota are associated with increased risk of chronic inflammatory disorders. Birth by caesarian section may be a risk factor for allergic disorders [120, 121]. Similarly, excessive antibiotic use during pregnancy [122] or in early childhood is a risk factor for allergic disorders [123, 124] and IBD [125, 126]. And as discussed above, living in a high-income rather than in a low-income country is a risk factor for all of these disorders.

Organisms from the Natural Environment and Microbiota

Clearly microbiota from other people (and animals) can colonize our guts. But do organisms from the natural environment also colonize, or are these organisms "pseudocommensals" that impinge on the skin [116], airways and gut, and have independent immunoregulatory properties? Both mechanisms probably occur, though there are rather limited data on these issues. An interesting animal experiment compared piglets that were housed in a natural outdoor environment, with genetically similar piglets that had been reared in a very clean indoor facility. Firmicutes, in particular *Lactobacillus* strains were dominant in the gut microbiotas of the outdoor piglets, whereas the hygienic indoor piglets had reduced *Lactobacillus* and more potentially pathogenic phylotypes [127]. The indoor piglets also had less diverse gut microbiota, and a more inflammatory pattern of gene

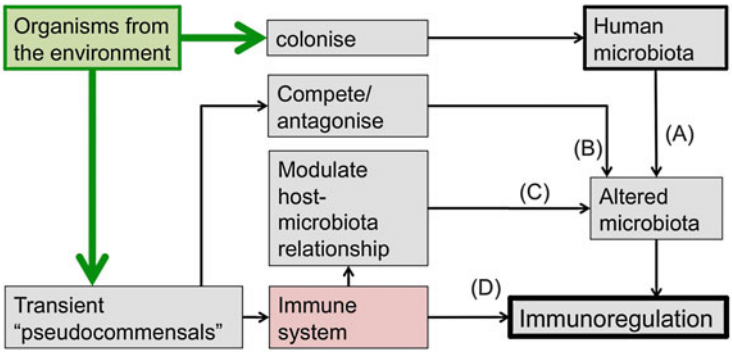

Fig. 15.3 Environmental organisms and immunoregulation. Microbial biodiversity from the environment can modulate immunoregulation by (*D*) directly interacting with the immune system, or (*A*, *B*, *C*) by leading secondarily to altered microbiota. The environmental organism may cause secondary changes to the microbiota by (*A*) colonizing, or (*B*) antagonizing or competing with established microbiota or (*C*) modulating the host immune system-microbiota relationship

expression in ileal biopsies [127]. For example they had increased Type 1 interferon activity, increased MHC Class 1, and upregulation of many chemokines [127], again confirming the correlation between reduced gut microbial biodiversity and poor control of inflammation discussed above.

Were these effects due to direct colonization by immunoregulation-inducing organisms from the outdoor environment [pathway (A) in Fig. 15.3], or did these organisms fail to colonize, but exert indirect effects on the immune system? The answer is unclear, but indirect effects certainly can occur in several ways. Some organisms compete with, or antagonize established organisms [pathway (B)] and so alter the microbiota [128]. Others alter the immune system directly [pathway (D)], or modulate the immune system in ways that lead secondarily to a change in the host-microbiota relationship, which in turn leads to changes in the microbiota [pathway (C) in Fig. 15.3].

The last mechanism is well established in experimental models. Genetic manipulations of the innate immune system that have profound effects on immune function (such as gene knockout) often operate indirectly by altering the gut microbiota. The phenotypic effects can then be transferred to wild-type mice that have not been genetically modified, by transferring the altered microbiota [129, 130]. It is the altered microbiota that is the proximate cause of the altered immunoregulation [129–133].

Is the Gut Still Involved in Immunoregulation by Organisms That Do Not Enter the Gut?

Does this mean that all immunoregulation by "Old Friends" operates indirectly by modulating the immune system, and so secondarily causing changes in the gut microbiota? It is likely that such indirect effects occur, but there could be direct effects too. For example the skin microbiota has at least some immunoregulatory role independent of the gut [134]. Indeed components of the skin microbiota extend into the subepidermal compartments, suggesting subtle and unexplored mechanisms [135]. Moreover psychological stressors cause increased bacterial translocation to lymphoid tissue from both the gut and the skin so the nature of the organisms present on skin is likely to be directly relevant to subsequent effects on immune function [136]. Interestingly, in the British field mouse (*Apodemus*), the burden of the louse *Polyplax serrata* correlated with the state of activation of the innate immune system in the spleen, implying that ectoparasites (fleas, lice, mites, ticks) might also have immunoregulatory roles [137, 138].

The blood nematodes are also of interest because these do not enter the gut at any phase of their life cycles, but they are powerfully immunoregulatory [9]. They cause impaired induction of T-bet and GATA-3 mRNA, Th1/Th2 deficiency and increased Foxp3, TGF-β, CTLA-4, PD-1, ICOS and IDO. Some blood nematodes secrete identifiable immunoregulatory molecules [139, 140].

Nevertheless, in view of the experiments listed in the previous section, it is still possible that in addition to direct effects on the immune system [pathway (D) in Fig. 15.3] these effects also operate indirectly via secondary modification of the gut microbiota [pathway (C) in Fig. 15.3].

Genetics and "Inflammatory Overshoot" in High-Income Countries

In parts of the world where there was a heavy load of organisms that drive potent immunoregulation (such as helminths) there has been selection for single nucleotide polymorphisms (SNP) or other variants to partially compensate for excessive immunoregulation, or to combat new infections such as malaria that spread from gorilla to man about 10,000 years ago [141, 142]. Such proinflammatory SNPs are seen for several proinflammatory cytokines [143], IgE [144] and STAT6, a transcription factor involved in Th2 responses [145]. There is also an increased frequency of the short allele of the serotonin transporter promoter that also has a marked proinflammatory effect [146]. However this results in a dangerous situation. As soon as the immunoregulation-inducing organisms are withdrawn by the modern lifestyle, or after immigration to a high-income country, these genetic variants lead to inflammatory overshoot. The proinflammatory variants become risk factors for chronic inflammatory disorders [143–146].

This is important because work that identifies proximate "causes" for diseases that were rare or nonexistent before the late nineteenth or early twentieth centuries, and that remain rare in low-income countries, may merely be unraveling a gene-environment interaction that would be irrelevant if the microbial status could be returned to that seen in the paleolithic age. For instance, the recent claim to have discovered that the "cause" of Crohn's disease is a genetically determined defect in the homing of neutrophils [147] is difficult to reconcile with the fact that 100 years ago the disease barely existed. But recent environmental changes could conceivably have caused this phenotype to become a risk factor.

How Does Stress Cause Inflammation?

Two major issues were avoided in the discussion of the role of immunoregulation in determining stress resilience (Fig. 15.2). First we did not discuss why stress causes release of inflammatory mediators, and secondly we did not discuss why such mediators trigger depression. Stress leads to activation of the hypothalamic-pituitary adrenal axis (HPA) and the sympathetic nervous system (SNS), and to changes in the microbiota and gut permeability. How do these mechanisms combine to result in raised proinflammatory cytokine levels?

GC Resistance

Depression is commonly associated with hypercortisolaemia and glucocorticoid resistance (GCR) [148]. A recent analysis has revealed that persistently raised levels of inflammatory cytokines cause GCR by impairing the function of glucocorticoid receptors [148], leading to further loss of control of inflammation. This was the suggested mechanism in individuals with recent exposure to severe psychosocial stressors who developed GCR and subsequently released more proinflammatory cytokines in response to an inflammatory stimulus (virus challenge to airways) [149].

Sympathetic Nervous System (SNS)

There is an increase in plasma concentrations of norepinephrine following exposure to a standardized laboratory stressor, the Trier Social Stress Test. This is accompanied by activation of the master regulator of inflammation, nuclear factor-kappa beta (NF-κB), in peripheral blood monocyte cells (PBMCs) [150]. Blocking the effects of activation of the SNS with the β-adrenergic receptor antagonist

propranolol blocked the stressor-induced increases in proinflammatory cytokines, and reduced the development of GCR [151].

Corticotropin-Releasing Hormone (CRH)

Increased expression of corticotropin-releasing hormone (CRH) is found in CSF and in the limbic brain regions in depression [152, 153], but CRH is also involved in the control of gut permeability [154–156]. For example, chronic administration of CRH via minipumps caused colonic barrier dysfunction in rats [155]. Moreover when released in the periphery by T cells, CRH is not only a regulator of intestinal permeability [154, 155], but also a potent pro-inflammatory cytokine [157]. In many cell types CRH activates NF-κB, and stimulates expression of IL-1β, IL-6, and TNF mRNAs [158], so some of the effects of CRH on permeability are secondary to the release of proinflammatory cytokines. Paracellular permeability is controlled by tight junctions, intermediate junctions and desmosomes, which constitute a size- and cation-selective filter for small molecules. However, TNF, IL-17, IFN-γ and nitric oxide (NO) increase permeability. IFN-γ disrupts the tight junctions, and can modify para-epithelial traffic of inflammatory cells. By contrast, the immunoregulatory cytokine TGF-β decreases permeability [154, 155].

The composition of the microbiota, particularly *Lactobacillus* strains and helminth "Old Friends" also modulate permeability. When idiopathic chronic diarrhea in rhesus monkeys was treated with the whipworm *Trichuris trichiura*, clinical improvement was accompanied by striking changes in the microbiota attached to the mucosa [159]. Similarly in a mouse model of IBD, infection with *H. polygyrus* caused an increase in lactobacilli.

Indirect Effects of Psychosocial Stress via the Microbiota

Stress induces changes in the composition of the microbiota of rodents [72], and induces bacterial translocation from gut and skin [136]. The same is true in humans. When sampled within hours of admission to the emergency room fecal bacterial counts were decreased 1,000-fold compared to control subjects, and obligate anaerobes and *Lactobacillus* species were significantly decreased [160]. Similarly, a large change in the microbiota after allogeneic bone marrow transfer was identified as a risk factor for subsequent inflammation and graft-versus host disease [161], implying that stress alters immunoregulation at least partly by altering the microbiota.

The gut microbiota is involved in the activation of the HPA axis by stress, and in the systemic release of cytokines. A social disruption stressor caused increases in circulating IL-6 and MCP-1 that correlated with changes in the composition of the microbiota. But this response was greatly attenuated by pre-treatment with an

antibiotic cocktail to deplete the microbiota [72]. Therefore much of the systemic cytokine response to stress might be secondary to uptake of LPS and other proinflammatory microbial components. Uptake of LPS was measured in another study. Animals subjected to restraint stress show increased portal blood LPS, together with HPA axis activation manifested as increased plasma ACTH and corticosterone, increased hypothalamic CRF and increased IL-1β, IL-6 and TNF. All of these manifestations were blocked by treatment with *Lactobacillus farciminis,* which blocks leakiness due to HPA axis activation [162].

Stress Resilience and the Microbiota

The microbiota might also be involved in the observation that poor stress resilience and an exaggerated cytokine response to environmental [31] or laboratory [22] stressors is characteristic of people who have suffered increased early life stress. We know that early life stress can modulate the microbiota [30, 163]. This interpretation is in agreement with the observation that in a low-income country population the adults who had a high level of microbial exposure in infancy were resistant to the long-term proinflammatory effects of a very severe childhood stressor [32]. In addition to the role in immunoregulation, the microbiota in the first weeks of life also modulates the development of the HPA axis and stress response [164], and the development of the brain [165].

How Does Inflammation Alter Behavior and Cognition?

The mechanisms that cause inflammatory responses to alter behavior and cognition have been extensively reviewed recently [166] and are summarized briefly here. From an evolutionary point of view this link between immunity and psychiatry is explained by the fact that during an acute infection, withdrawal (to conserve resources, fight infection and heal wounds) and hypervigilance (to detect danger) are adaptive responses [167]. Withdrawal and hypervigilance probably result from inflammation-mediated signals to distinct parts of the brain. Positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) have been used to identify brain regions affected by inflammatory cytokines, and the list includes the basal ganglia, the dorsal anterior cingulate cortex (dACC), amygdala, hippocampus, insula, dorsolateral prefrontal cortex, and subgenual ACC [reviewed in 166]. It is suggested that involvement of the basal ganglia is important for withdrawal, while the effects on the dACC are important for hypervigilance. However these states are not sustainable and if prolonged, withdrawal becomes depression and hypervigilance becomes anxiety. This relationship between prolonged inflammatory stimuli and behavioral changes that resemble depression

and anxiety was postulated long ago following observations of "sickness behavior" in mice [168].

Inflammation-associated depression and anxiety are more likely in situations where there is poor control of inflammation [169]. This is seen in high-income countries where persistently high CRP is common [170], as discussed earlier in the context of the Old Friends mechanism. Similarly, depression and anxiety often accompany the chronic inflammatory disorders that are increasing in high-income countries. They are also seen in obesity where fat tissue contains cytokine-secreting activated macrophages, and in people who underwent traumatic childhoods, perhaps because of developmental changes in the HPA axis, brain, and microbiota [31, discussed in 169]. Genetic factors also play a role. For example individuals who have the short allele of the serotonin transporter-linked polymorphic region were more likely to get depression after IFN-α treatment [171].

Cytokines and Cellular Infiltration

Recent studies of patients receiving IFN-α have confirmed in humans many of the mechanisms previously reported in animal studies. For example, recipients of IFN-α develop raised CSF levels of IL-6 and MCP-1, proving that the inflammatory signal is transmitted to the brain [172]. Signals from the inflamed periphery enter the brain via several pathways. First, cytokines can enter the brain in areas such as the circumventricular organs where there is no blood-brain barrier. Perhaps these areas should be regarded as sensory organs of which one role is the detection of inflammation. Signals also pass via activation of endothelial cells within the cerebral vasculature, leading secondarily to release of prostaglandins and NO in the CNS. Furthermore brain endothelium expresses specific cytokine transporters. Cytokines in the periphery can also signal via afferent fibres within the vagus and other sensory nerves. This has been called the "facsimile" mechanism, because the cytokine stimulating the peripheral nerve terminals may subsequently be synthesized de novo and released within the brain.

These various inflammatory signals may secondarily activate local CNS cell populations. The microglia are derived from a subset of CD45+ monocytic cells and enter the CNS during embryogenesis in utero and during early post-natal life. These stable long-lived cells form a network with surveillance functions within the brain parenchyma, and under normal conditions are in a non-activated, non-terminally differentiated state, with low expression of major histocompatibility complex class II [173]. However inflammation propagated to the brain by the pathways listed above can cause these cells to express inflammatory cytokines and to release reactive oxygen and nitrogen species [166]. Activated microglia may also be a source of the MCP-1 mentioned earlier, which recruits monocytes into the brain [174].

Recruited leukocytes can enter the CNS by several routes. In healthy individuals there is background traffic via the choroid plexus or through postcapillary venules

located in the subarachnoid space [175, 176]. However in inflammatory states cells can cross the endothelium of parenchymal post-capillary venules, and so enter the perivascular space. The relevant adhesion molecules are not expressed on these endothelial cells under resting conditions but they are induced by peripheral inflammatory signals such as LPS or TNF, facilitating the MCP-1-driven recruitment [176].

Indoleamine-2,3-Dioxygenase (IDO)

Inflammatory cytokines also activate the enzyme, indoleamine-2,3-dioxygenase (IDO), converting tryptophan to kynurenine. It has been suggested that this can deplete tryptophan sufficiently to cause depression. Recent mouse work showed that inflammation induced by peripheral administration of lipopolysaccharide (LPS) activated IDO and caused mice to display a depression-like behavioral syndrome that could be inhibited by blocking IDO [reviewed in 177]. However, the syndrome could be reproduced by administering kynurenine, which is taken up into the brain by the large amino acid transporter, where it can be further metabolized in microglia, astrocytes and macrophages to quinolinic acid, kynurenic acid and other metabolites [178]. This suggested that depletion of tryptophan was not the primary mechanism of the depression-like syndrome [179] (though this cannot be ruled out as an additional factor because there is evidence that kynurenine can compete with tryptophan for transport into the brain [180]). These mouse findings have been confirmed in patients with hepatitis C who were being treated with IFN-α, in whom there were depressive symptoms, but no reduction in CSF concentrations of tryptophan [181]. The treatment did however cause increased CSF levels of kynurenine, quinolinic acid and kynurenic acid, and also of IFN-α, soluble tumor necrosis factor-α receptor 2 and MCP-1 [181]. This suggested that as in the mouse, the depressogenic effect might involve transport of kynurenine into the brain followed by local generation of further active metabolites, rather than depletion of tryptophan [181]. There is increasing evidence that these neuro-active tryptophan metabolites are important in depression and in schizophrenia, and this topic has been reviewed in detail recently [177].

Transporters and Reuptake

Inflammatory cytokines also increase the expression of the transporters responsible for reuptake of dopamine, norepinephrine and serotonin. For example, in mice, IL-1, TNF and LPS all cause increased expression of the serotonin transporter paralleled by depression-like behavior [182]. In humans administration of IFN-α increases the reuptake and decreases the release of radiolabeled L-DOPA, the precursor of dopamine [183].

Tetrahydrobiopterin (BH4)

Inflammation also disturbs the availability of tetrahydrobiopterin (BH4), which is an essential cofactor for tryptophan hydroxylase and tyrosine hydroxylase. These are the rate-limiting enzymes for the synthesis of serotonin, dopamine and norepinephrine [166]. It is possible that the BH4 gets used up when cytokines drive NO synthase to generate NO, but this pathway is less active in man than in mouse. However BH4 can also be degraded by oxygen radicals and nitrogen radicals. Evidence for depletion of BH4 in the human brain in the presence of inflammatory mediators has recently been obtained in patients receiving IFN-α [184]. In the CSF of treated patients levels of the inactive oxidized form BH2 were increased, while levels of BH4 were inversely correlated with increased IL-6 [184].

Anti-Inflammatory Neurotransmitter Pathways

Inflammation in the CNS may also interfere with neurotransmitter pathways that have anti-inflammatory roles, and so weaken negative feedback on the inflammatory response. For example neural signals transmitted to the periphery via the vagus nerve inhibit cytokine release through a mechanism that requires the α7-subunit-containing nicotinic acetylcholine receptor [185]. This cholinergic anti-inflammatory pathway can be inhibited centrally by mediators such as IL-1 that increase neuronal acetylcholinesterase activity [185]. Similarly IL-1β might reduce signaling by γ-aminobutyric acid (GABA), and so enhance local inflammation [186]. This is due to the fact that GABA-ergic tone is anti-inflammatory because it inhibits the NF-κB and p38 MAPK pathways, and so reduces the response of microglia to LPS or IFN-γ [186].

Abnormal Brain Development

In addition to the postnatal mechanisms described above, inflammation can also act in utero to cause developmental defects in the foetal CNS that lead later to psychiatric problems. The likelihood of this phenomenon occurring will be influenced by the efficiency of immunoregulation in mother and child during pregnancy (Fig. 15.4).

The Old Friends mechanism will be one factor that determines this immunoregulation, though as discussed below, there are also genetic factors. Interestingly autism spectrum disorders (ASD) are increased in towns [39] and in second generation immigrants [52]. These findings parallel the simultaneous increases in chronic inflammatory disorders, and depression in which the Old Friends mechanism likely plays a role [169].

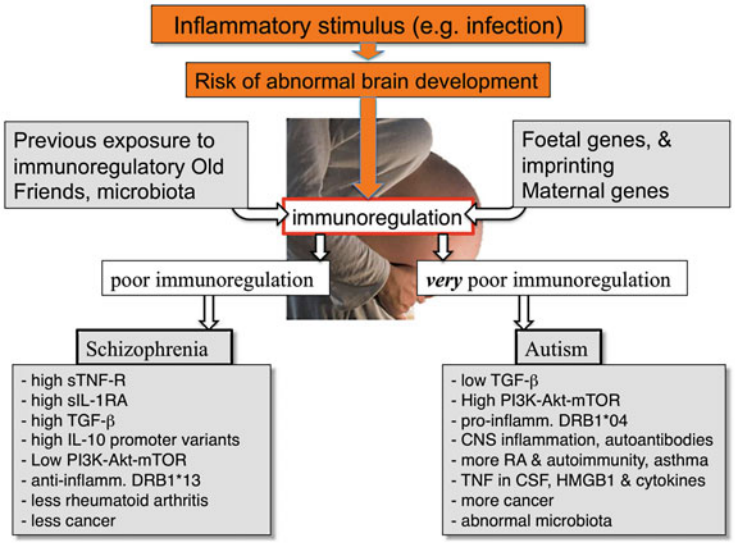

Fig. 15.4 Reduced efficiency of immunoregulation during pregnancy could predispose to inflammatory episodes in utero that lead to neurodevelopmental abnormalities. Such abnormalities are seen in ASD and schizophrenia, and both disorders are accompanied by evidence of failing immunoregulation that is most striking in ASD and in family members. The immunological points listed in the boxes at lower left and right are taken from, and fully explained within, the references in the main text

Maternal Infection, Immunoregulation, Fetal Inflammation and ASD

There has been a significant and genuine increase in the prevalence of autism spectrum disorders (ASD) that cannot be explained only by increased awareness [187]. There is debate about the relative contribution of genetics and environment. A very recent study suggested that "Susceptibility to ASD has moderate genetic heritability and a substantial shared twin environmental component" [188]. Rather than worrying about the relative importance of genes and environment it is important to note that known autism susceptibility genes include a neuronal module and a module enriched for immune genes and glial markers [189]. Another study of interactome networks associated with highly expressed ASD-candidate genes found that immune signaling through NF-κB, TNF, and Jnk were strongly represented and that these interactomes involved glia in addition to neurons [190]. Thus the genetics point to inflammation and the immune system, which could modulate susceptibility to the types of environmental influence discussed in this chapter. A recent study suggests that there might be an underlying immune phenotype (whether genetic or environmental): the immune systems of autistic children and their healthy siblings were found to have similar immune

dysregulation, when compared to the immune systems of matched healthy children [191].

An obvious link between immunoregulation and ASD is provided by evidence that maternal infection during pregnancy increases the risk of ASD in the infant. In one study 13 % of infants developed ASD following exposure to congenital rubella [192]. However it seems that any infection increases the risk, particularly if severe enough to require hospitalization during pregnancy [193]. A study of 1.2 million births in Finland showed that raised maternal CRP early in gestation was associated with increased risk, whatever the cause [194]. Animal work proves that maternal inflammation during pregnancy is transmitted to the foetus. TNF-α and IL-1β expression was upregulated in a dose dependent manner in the fetuses of pregnant rats exposed to LPS [discussed in 195]. Similarly I^{125}-labelled IL-6 administered i.v. to pregnant rats was found in the fetal compartment [196].

Experimental animals exposed to maternal immune activation in utero also display developmental changes measured by MRI, and behavioral changes suggestive of ASD [197]. In pregnant mouse models these effects are dependent upon IL-6, and can be mimicked by administering IL-6 itself rather than an indirect inflammatory stimulus [25]. Interestingly these abnormalities are accompanied by a systemic deficit in $CD4^+$ $TCR\beta^+$ $Foxp3^+$ $CD25^+$ T regulatory cells, and by increased IL-6 and IL-17 production by $CD4^+$ T cells [198]. The behavioural abnormalities can be attenuated by transplanting normal bone-marrow, implicating the immune system both in the development of the syndrome, and in its subsequent maintenance [198].

Inflammation and Faulty Immunoregulation in ASD

The role of inflammation in utero in the development of the CNS abnormalities that accompany at least some cases of human ASD is not in doubt. But there is increasing evidence for an ongoing immunoregulatory deficit in human ASD [199, 200], as in the mouse model mentioned above [198]. ASD patients have increased circulating levels of proinflammatory cytokines, and reduced levels of TGF-β [200], and there is an increased prevalence of asthma and autoimmunity in family members, reinforcing the view that there is an hereditary, or at least familial, immunoregulatory deficit [191, 199, 200]. This tendency to autoimmunity is manifested as brain autoantibodies in plasma from children with ASD, and from their mothers [200].

Studies of autistic brains reveal activation of microglia and astroglia and increased expression of a range of proinflammatory mediators such as TNF, IFN-γ, IL-8 and IL-6 [201–203]. IL-6 is normally expressed at very low levels in the brain, but it is able to cross the placenta [196] and induce an ASD-like state in the offspring when administered to pregnant mice [25]. Overexpression of IL-6 in transgenic mice causes neuroanatomical and neurophysiological alterations

associated with neurological disease [204]. Immunohistochemistry studies confirmed that IL-6 is raised in the cerebellum of autistic brain [205].

Autoantibodies to Brain in ASD

There is an alternative way of interpreting some of the data. There is no doubt that autoantibodies present during fetal life [206], or during inflammatory episodes when blood-brain barrier function is compromised [176], can alter CNS function [207]. Such antibodies have been shown in autism [206] and, as suggested by a murine model, might explain the high frequency of learning disorders in the offspring of mothers suffering from systemic lupus erythematosus (SLE) [208]. The association between autoimmune disease and psychiatric disease has been confirmed in a massive recent study [209]. Patients with MDD have not only a higher frequency of brain-reactive antibodies, but also an increased circulating Th17/Treg ratio [21]. In short, the relationship between immunodysregulation and psychiatric disease might involve additional autoantibody-mediated damage when autoimmunity is one of the forms of chronic inflammatory disorder present in a given individual, even if subclinical. The evidence for this in some cases of ASD is strong [206].

GI Symptoms and Immunoregulation in ASD

Gastrointestinal symptoms are common in ASD. These include diarrhea, constipation, vomiting/reflux, abdominal pain/discomfort, gaseousness, and unusually foul-smelling stools [210] and are similar to the symptoms of irritable bowel syndrome (IBS), which affects 10–20 % of the US population [discussed in 211]. While symptoms are not in doubt, there is controversy about whether these correlate with detectable inflammation in the gut mucosa [discussed in 211]. The observation that low-grade endotoxemia occurs in patients with severe autism tends to suggest that some gut inflammation might be present [212]. Meanwhile the use of modern culture-independent methods has revealed that the gut microbiota of autistics is abnormal [213–215]. Some authors argue that this abnormality might lead to excessive production and absorption of short chain fatty acids (SCFA; for example propionic acid) that in experimental animals induce autism-like states [216].

Brain Development and Schizophrenia

The etiology of schizophrenia is not known but the predominant view is that, like at least some cases of ASD, it can involve abnormalities in brain development occurring during fetal/neonatal life (Fig. 15.4) long before manifestation of the illness in adolescence or early adulthood [173, 217]. There is correlational evidence for this. Raised maternal levels of IL-8 and TNF (but not of IL-6 and IL-1β) in mid gestation were associated with psychosis in the children [173, 218, 219]. As in ASD there is evidence for immunoregulatory problems in adults with schizophrenia, though these are less extreme than in ASD [199]. Interestingly a relationship between ASD and schizophrenia has been postulated that attributes the differing disease manifestations to relative expression of maternal or paternal copies of imprinted genes [220]. It might be possible to reconcile this hypothesis with the differing degrees of immunodysregulation characteristic of the two conditions [199]. It is of particular interest that some genes that predispose to ASD do not need to be expressed in the fetus, involve the immune system and probably act during pregnancy [221].

Conclusions

The study of the gut microbiota by modern molecular methods, and the modulation of the microbiota by modern lifestyle and dietary habits, have led to a vast expansion of our knowledge, and to a tendency for the study of the microbiota to be seen as an independent medical discipline. Meanwhile, the original hygiene hypothesis was seen as a narrow concept dealing mostly with factors affecting allergic disorders in childhood, while an offshoot of the hygiene hypothesis, sometimes known as the "helminth hypothesis" has been applied mostly to the increases in IBD and MS. In this chapter we suggest that these concepts need to be studied together, or even unified under one heading such as the "Old Friends", or "biodiversity" mechanism. They all deal with the issue of the education and regulation of the immune system by microbial contact. The gut microbiota is certainly a major component of this system, but it acts in concert with other environmental inputs that regulate the immune system, including organisms that never enter the gut. By seeing the whole picture we may be able to determine whether immunodysregulation due to divergence of our microbial exposure from that with which we evolved is able to explain the worrying and parallel increases in chronic inflammatory disorders and inflammation-linked psychiatric disorders in high-income countries.

Acknowledgements GAWR is supported by the National Institute for Health Research University College London Hospitals Biomedical Research Centre. CLR receives grant support from the National Center for Complementary and Alternative Medicine (R01AT004698, R01AT004698-01A1S1), the Depressive and Bipolar Disorder Alternative Treatment Foundation and The Brain

and Behavior Research Foundation. He reports the following activities for the previous 2 years: advisory board participation and related travel funds for Pamlab, Lilly and North American Center for Continuing Education; development and presentation of disease state slides for Pamlab, Pfizer and Johnson & Johnson, as well as related travel funds for these activities; development of continuing medical education material for North American Center for Continuing Education and for CME Incite. CAL receives grant support from the National Institute of Mental Health (R01MH065702, R01MH086539, R01DA019921, R01MH075968), the National Science Foundation (NSF- IOS 0921969), the Depressive and Bipolar Disorder Alternative Treatment Foundation, and is the recipient of an NSF CAREER Award (NSF-IOS 0845550) and a NARSAD, Brain & Behavior Research Foundation 2010 Young Investigator Award. He reports the following activities for the previous 2 years: consultant for Enlight Biosciences.

References

1. Blackley CH (1873) Experimental researches on the causes and nature of catarrhus aestivus (Hay-fever and Hay-asthma). Baillière Tindall and Cox, London
2. Radon K, Windstetter D, Poluda AL, Mueller B, von Mutius E, Koletzko S (2007) Contact with farm animals in early life and juvenile inflammatory bowel disease: a case-control study. Pediatrics 120(2):354–361
3. Schaub B, Liu J, Hoppler S, Schleich I, Huehn J, Olek S et al (2009) Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells. J Allergy Clin Immunol 123(4):774–782.e5
4. Ege MJ, Mayer M, Normand AC, Genuneit J, Cookson WO, Braun-Fahrlander C et al (2011) Exposure to environmental microorganisms and childhood asthma. N Engl J Med 364(8): 701–709
5. Stene LC, Nafstad P (2001) Relation between occurrence of type 1 diabetes and asthma. Lancet 357:607
6. Weinstock JV, Elliott DE (2009) Helminths and the IBD hygiene hypothesis. Inflamm Bowel Dis 15(1):128–133
7. Rook GAW (2010) 99th Dahlem conference on infection, inflammation and chronic inflammatory disorders: Darwinian medicine and the 'hygiene' or 'old friends' hypothesis. Clin Exp Immunol 160(1):70–79
8. von Hertzen L, Hanski I, Haahtela T (2011) Natural immunity. Biodiversity loss and inflammatory diseases are two global megatrends that might be related. EMBO Rep 12(11):1089–1093
9. Babu S, Blauvelt CP, Kumaraswami V, Nutman TB (2006) Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. J Immunol 176(5):3248–3256
10. Wildin RS, Smyk-Pearson S, Filipovich AH (2002) Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet 39(8):537–545
11. Su LF, Kidd BA, Han A, Kotzin JJ, Davis MM (2013) Virus-specific CD4(+) memory-phenotype T cells are abundant in unexposed adults. Immunity 38(2):373–383
12. Gurven M, Kaplan H, Winking J, Finch C, Crimmins EM (2008) Aging and inflammation in two epidemiological worlds. J Gerontol A Biol Sci Med Sci 63(2):196–199
13. McDade TW, Tallman PS, Madimenos FC, Liebert MA, Cepon TJ, Sugiyama LS et al (2012) Analysis of variability of high sensitivity C-reactive protein in lowland Ecuador reveals no evidence of chronic low-grade inflammation. Am J Hum Biol 24:675–681

14. Rook G, Raison CL, Lowry CA (2013) Childhood microbial experience, immunoregulation, inflammation and adult susceptibility to psychosocial stressors and depression in rich and poor countries. Evol Med Public Health 2013:14–17
15. McDade TW, Rutherford J, Adair L, Kuzawa CW (2010) Early origins of inflammation: microbial exposures in infancy predict lower levels of C-reactive protein in adulthood. Proc Biol Sci 277(1684):1129–1137
16. Gimeno D, Kivimaki M, Brunner EJ, Elovainio M, De Vogli R, Steptoe A et al (2009) Associations of C-reactive protein and interleukin-6 with cognitive symptoms of depression: 12-year follow-up of the Whitehall II study. Psychol Med 39(3):413–423
17. Rook GAW, Lowry CA (2008) The hygiene hypothesis and psychiatric disorders. Trends Immunol 29:150–158
18. Raison CL, Lowry CA, Rook GAW (2010) Inflammation, sanitation and consternation: loss of contact with co-evolved, tolerogenic micro-organisms and the pathophysiology and treatment of major depression. Arch Gen Psychiatry 67(12):1211–1224
19. Maes M, Scharpe S, Van Grootel L, Uyttenbroeck W, Cooreman W, Cosyns P et al (1992) Higher alpha 1-antitrypsin, haptoglobin, ceruloplasmin and lower retinol binding protein plasma levels during depression: further evidence for the existence of an inflammatory response during that illness. J Affect Disord 24(3):183–192
20. Miller AH, Maletic V, Raison CL (2009) Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biol Psychiatry 65(9):732–741
21. Chen Y, Jiang T, Chen P, Ouyang J, Xu G, Zeng Z et al (2011) Emerging tendency towards autoimmune process in major depressive patients: a novel insight from Th17 cells. Psychiatry Res 188(2):224–230
22. Pace TW, Mletzko TC, Alagbe O, Musselman DL, Nemeroff CB, Miller AH et al (2006) Increased stress-induced inflammatory responses in male patients with major depression and increased early life stress. Am J Psychiatry 163(9):1630–1633
23. Musselman DL, Lawson DH, Gumnick JF, Manatunga AK, Penna S, Goodkin RS et al (2001) Paroxetine for the prevention of depression induced by high-dose interferon alfa. N Engl J Med 344(13):961–966
24. Raison CL, Rutherford RE, Woolwine BJ, Shuo C, Schettler P, Drake DF et al (2013) A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: the role of baseline inflammatory biomarkers. JAMA Psychiatry 70: 31–41
25. Smith SE, Li J, Garbett K, Mirnics K, Patterson PH (2007) Maternal immune activation alters fetal brain development through interleukin-6. J Neurosci 27(40):10695–10702
26. Marsland AL, Gianaros PJ, Abramowitch SM, Manuck SB, Hariri AR (2008) Interleukin-6 covaries inversely with hippocampal grey matter volume in middle-aged adults. Biol Psychiatry 64(6):484–490
27. Braida D, Sacerdote P, Panerai AE, Bianchi M, Aloisi AM, Iosue S et al (2004) Cognitive function in young and adult IL (interleukin)-6 deficient mice. Behav Brain Res 153(2): 423–429
28. Sparkman NL, Buchanan JB, Heyen JR, Chen J, Beverly JL, Johnson RW (2006) Interleukin-6 facilitates lipopolysaccharide-induced disruption in working memory and expression of other proinflammatory cytokines in hippocampal neuronal cell layers. J Neurosci 26(42): 10709–10716
29. McEwen BS, Gianaros PJ (2010) Central role of the brain in stress and adaptation: links to socioeconomic status, health, and disease. Ann N Y Acad Sci 1186:190–222
30. O'Mahony SM, Marchesi JR, Scully P, Codling C, Ceolho AM, Quigley EM et al (2009) Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. Biol Psychiatry 65(3):263–267
31. Danese A, Moffitt TE, Pariante CM, Ambler A, Poulton R, Caspi A (2008) Elevated inflammation levels in depressed adults with a history of childhood maltreatment. Arch Gen Psychiatry 65(4):409–415

32. McDade TW, Hoke M, Borja JB, Adair LS, Kuzawa CW (2013) Do environments in infancy moderate the association between stress and inflammation in adulthood? Preliminary evidence from a birth cohort in the Philippines. Brain Behav Immun 31:23–30
33. Compton WM, Conway KP, Stinson FS, Grant BF (2006) Changes in the prevalence of major depression and comorbid substance use disorders in the United States between 1991–1992 and 2001–2002. Am J Psychiatry 163(12):2141–2147
34. Ustun TB, Ayuso-Mateos JL, Chatterji S, Mathers C, Murray CJ (2004) Global burden of depressive disorders in the year 2000. Br J Psychiatry 184:386–392
35. McDade TW, Borja JB, Adair L, Kuzawa CW (2013) Depressive symptoms are not associated with inflammation in younger and older adults in the Philippines. Evol Med Public Health 2013:18–23
36. Peen J, Schoevers RA, Beekman AT, Dekker J (2010) The current status of urban-rural differences in psychiatric disorders. Acta Psychiatr Scand 121(2):84–93
37. Kovess-Masfety V, Lecoutour X, Delavelle S (2005) Mood disorders and urban/rural settings: comparisons between two French regions. Soc Psychiatry Psychiatr Epidemiol 40(8): 613–618
38. McGrath J, Saha S, Welham J, El Saadi O, MacCauley C, Chant D (2004) A systematic review of the incidence of schizophrenia: the distribution of rates and the influence of sex, urbanicity, migrant status and methodology. BMC Med 2:13
39. Lauritsen MB, Pedersen CB, Mortensen PB (2005) Effects of familial risk factors and place of birth on the risk of autism: a nationwide register-based study. J Child Psychol Psychiatry 46(9):963–971
40. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S et al (2001) Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet 358(9288):1129–1133
41. Ege MJ, Herzum I, Buchele G, Krauss-Etschmann S, Lauener RP, Roponen M et al (2008) Prenatal exposure to a farm environment modifies atopic sensitization at birth. J Allergy Clin Immunol 122(2):407–412, 12 e1–e4
42. Nicolaou N, Siddique N, Custovic A (2005) Allergic disease in urban and rural populations: increasing prevalence with increasing urbanization. Allergy 60(11):1357–1360
43. Hou JK, El-Serag H, Thirumurthi S (2009) Distribution and manifestations of inflammatory bowel disease in Asians, Hispanics, and African Americans: a systematic review. Am J Gastroenterol 104(8):2100–2109
44. Beebe GW, Kurtzke JF, Kurland LT, Auth TL, Nagler B (1967) Studies on the natural history of multiple sclerosis. 3. Epidemiologic analysis of the army experience in World War II. Neurology 17(1):1–17
45. Antonovsky A, Leibowitz U, Smith HA, Medalie JM, Balogh M, Kats R et al (1965) Epidemiologic Study of Multiple Sclerosis in Israel. I. An overall review of methods and findings. Arch Neurol 13:183–193
46. Lowis GW (1990) The social epidemiology of multiple sclerosis. Sci Total Environ 90: 163–190
47. Rottem M, Szyper-Kravitz M, Shoenfeld Y (2005) Atopy and asthma in migrants. Int Arch Allergy Immunol 136(2):198–204
48. Soderstrom U, Aman J, Hjern A (2012) Being born in Sweden increases the risk for type 1 diabetes – a study of migration of children to Sweden as a natural experiment. Acta Paediatr 101(1):73–77
49. Ahlgren C, Oden A, Lycke J (2012) A nationwide survey of the prevalence of multiple sclerosis in immigrant populations of Sweden. Mult Scler 18:1099–1107
50. Breslau J, Borges G, Tancredi D, Saito N, Kravitz R, Hinton L et al (2011) Migration from Mexico to the United States and subsequent risk for depressive and anxiety disorders: a cross-national study. Arch Gen Psychiatry 68(4):428–433
51. Dealberto MJ (2010) Ethnic origin and increased risk for schizophrenia in immigrants to countries of recent and longstanding immigration. Acta Psychiatr Scand 121(5):325–339

52. Keen DV, Reid FD, Arnone D (2010) Autism, ethnicity and maternal immigration. Br J Psychiatry 196(4):274–281
53. Breslau J, Borges G, Hagar Y, Tancredi D, Gilman S (2009) Immigration to the USA and risk for mood and anxiety disorders: variation by origin and age at immigration. Psychol Med 39(7):1117–1127
54. Vega WA, Sribney WM, Aguilar-Gaxiola S, Kolody B (2004) 12-month prevalence of DSM-III-R psychiatric disorders among Mexican Americans: nativity, social assimilation, and age determinants. J Nerv Ment Dis 192(8):532–541
55. Coid JW, Kirkbride JB, Barker D, Cowden F, Stamps R, Yang M et al (2008) Raised incidence rates of all psychoses among migrant groups: findings from the East London first episode psychosis study. Arch Gen Psychiatry 65(11):1250–1258
56. Veling W, Hoek HW, Selten JP, Susser E (2011) Age at migration and future risk of psychotic disorders among immigrants in the Netherlands: a 7-year incidence study. Am J Psychiatry 168(12):1278–1285
57. Cantor-Graae E, Selten JP (2005) Schizophrenia and migration: a meta-analysis and review. Am J Psychiatry 162(1):12–24
58. Milo R, Kahana E (2010) Multiple sclerosis: geoepidemiology, genetics and the environment. Autoimmun Rev 9(5):A387–A394
59. Gale CR, Martyn CN (1995) Migrant studies in multiple sclerosis. Prog Neurobiol 47(4–5): 425–448
60. Cabre P (2009) Environmental changes and epidemiology of multiple sclerosis in the French West Indies. J Neurol Sci 286(1–2):58–61
61. Dean G (1967) Annual incidence, prevalence, and mortality of multiple sclerosis in white South-African-born and in white immigrants to South Africa. Br Med J 2(5554):724–730
62. Hjern A, Rasmussen F, Hedlin G (1999) Age at adoption, ethnicity and atopic disorder: a study of internationally adopted young men in Sweden. Pediatr Allergy Immunol 10(2): 101–106
63. Eldeirawi K, McConnell R, Furner S, Freels S, Stayner L, Hernandez E et al (2009) Associations of doctor-diagnosed asthma with immigration status, age at immigration, and length of residence in the United States in a sample of Mexican American School Children in Chicago. J Asthma 46(8):796–802
64. Pereg D, Tirosh A, Lishner M, Goldberg A, Shochat T, Confino-Cohen R (2008) Prevalence of asthma in a large group of Israeli adolescents: influence of country of birth and age at migration. Allergy 63(8):1040–1045
65. Li X, Sundquist J, Hemminki K, Sundquist K (2011) Risk of inflammatory bowel disease in first- and second-generation immigrants in Sweden: a nationwide follow-up study. Inflamm Bowel Dis 17(8):1784–1791
66. Carr I, Mayberry JF (1999) The effects of migration on ulcerative colitis: a three-year prospective study among Europeans and first- and second-generation South Asians in Leicester (1991–1994). Am J Gastroenterol 94(10):2918–2922
67. Rook GAW (2009) The broader implications of the hygiene hypothesis. Immunology 126: 3–11
68. Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol 9(5):313–323
69. Osada Y, Kanazawa T (2010) Parasitic helminths: new weapons against immunological disorders. J Biomed Biotechnol 2010:743–758
70. Karimi K, Inman MD, Bienenstock J, Forsythe P (2009) *Lactobacillus reuteri*-induced regulatory T cells protect against an allergic airway response in mice. Am J Respir Crit Care Med 179(3):186–193
71. Allen RG, Lafuse WP, Galley JD, Ali MM, Ahmer BM, Bailey MT (2012) The intestinal microbiota are necessary for stressor-induced enhancement of splenic macrophage microbicidal activity. Brain Behav Immun 26(3):371–382

72. Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M (2011) Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain Behav Immun 25(3):397–407
73. Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN (2010) Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat Med 16(2):228–231
74. Kim OY, Monsel A, Bertrand M, Cavaillon JM, Coriat P, Adib-Conquy M (2009) Translocation of bacterial NOD2 agonist and its link with inflammation. Crit Care 13(4):R124
75. Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8(12):958–969
76. Loke P, MacDonald AS, Robb A, Maizels RM, Allen JE (2000) Alternatively activated macrophages induced by nematode infection inhibit proliferation via cell to cell contact. Eur J Immunol 30:2669–2678
77. Elliott DE, Weinstock JV (2012) Helminth-host immunological interactions: prevention and control of immune-mediated diseases. Ann N Y Acad Sci 1247:83–96
78. Schnoeller C, Rausch S, Pillai S, Avagyan A, Wittig BM, Loddenkemper C et al (2008) A helminth immunomodulator reduces allergic and inflammatory responses by induction of IL-10-producing macrophages. J Immunol 180(6):4265–4272
79. Smith P, Mangan NE, Walsh CM, Fallon RE, McKenzie ANJ, van Rooijen N et al (2007) Infection with a helminth parasite prevents experimental colitis via a macrophage-mediated mechanism. J Immunol 178:4557–4566
80. Mangan NE, Fallon RE, Smith P, van Rooijen N, McKenzie AN, Fallon PG (2004) Helminth infection protects mice from anaphylaxis via IL-10-producing B cells. J Immunol 173(10): 6346–6356
81. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF (2008) Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J Clin Invest 118(10):3420–3430
82. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, Tedder TF (2008) A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. Immunity 28(5):639–650
83. Amu S, Saunders SP, Kronenberg M, Mangan NE, Atzberger A, Fallon PG (2010) Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. J Allergy Clin Immunol 125(5):1114–1124 e8
84. Yanaba K, Bouaziz JD, Matsushita T, Magro CM, St Clair EW, Tedder TF (2008) B-lymphocyte contributions to human autoimmune disease. Immunol Rev 223:284–299
85. Correale J, Farez M, Razzitte G (2008) Helminth infections associated with multiple sclerosis induce regulatory B cells. Ann Neurol 64(2):187–199
86. Di Giacinto C, Marinaro M, Sanchez M, Strober W, Boirivant M (2005) Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. J Immunol 174(6):3237–3246
87. Hart AL, Lammers K, Brigidi P, Vitali B, Rizzello F, Gionchetti P et al (2004) Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut 53(11):1602–1609
88. Braat H, van den Brande J, van Tol E, Hommes D, Peppelenbosch M, van Deventer S (2004) *Lactobacillus rhamnosus* induces peripheral hyporesponsiveness in stimulated CD4+ T cells via modulation of dendritic cell function. Am J Clin Nutr 80(6):1618–1625
89. Smits HH, Engering A, van der Kleij D, de Jong EC, Schipper K, van Capel TM et al (2005) Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. J Allergy Clin Immunol 115(6):1260–1267
90. Le Bert N, Chain BM, Rook G, Noursadeghi M (2011) DC priming by *M. vaccae* inhibits Th2 responses in contrast to specific TLR2 priming and is associated with selective activation of the CREB pathway. PLoS One 6(4):e18346

91. Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL et al (2008) Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J Exp Med 205(9):2139–2149
92. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR et al (2007) Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med 204(8):1775–1785
93. Mann ER, Landy JD, Bernardo D, Peake ST, Hart AL, Al-Hassi HO et al (2013) Intestinal dendritic cells: their role in intestinal inflammation, manipulation by the gut microbiota and differences between mice and men. Immunol Lett 150(1–2):30–40
94. Smelt MJ, de Haan BJ, Bron PA, van Swam I, Meijerink M, Wells JM et al (2012) *L. plantarum*, *L. salivarius*, and *L. lactis* attenuate Th2 responses and increase Treg frequencies in healthy mice in a strain dependent manner. PLoS One 7(10):e47244
95. Correale J, Farez M (2007) Association between parasite infection and immune responses in multiple sclerosis. Ann Neurol 61(2):97–108
96. Correale J, Farez MF (2011) The impact of parasite infections on the course of multiple sclerosis. J Neuroimmunol 233:6–11
97. Fleming J, Isaak A, Lee J, Luzzio C, Carrithers M, Cook T et al (2011) Probiotic helminth administration in relapsing-remitting multiple sclerosis: a phase 1 study. Mult Scler 17(6): 743–754
98. Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila TA et al (2011) The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science 332(6032):974–977
99. Grainger JR, Smith KA, Hewitson JP, McSorley HJ, Harcus Y, Filbey KJ et al (2010) Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-beta pathway. J Exp Med 207(11):2331–2341
100. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y et al (2011) Induction of colonic regulatory T cells by indigenous *Clostridium* species. Science 331:337–341
101. Meadow JF, Bateman AC, Herkert KM, O'Connor TK, Green JL (2013) Significant changes in the skin microbiome mediated by the sport of roller derby. Peer J. Doi:10.7717/peerj.53.
102. Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-Lyons D et al (2013) Cohabiting family members share microbiota with one another and with their dogs. Elife 2: e00458
103. Maslowski KM, Mackay CR (2011) Diet, gut microbiota and immune responses. Nat Immunol 12(1):5–9
104. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S et al (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 107(33):14691–14696
105. Dethlefsen L, Huse S, Sogin ML, Relman DA (2008) The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 6(11):e280
106. Cani PD, Delzenne NM (2011) The gut microbiome as therapeutic target. Pharmacol Ther 130(2):202–212
107. Hildebrand F, Nguyen TL, Brinkman B, Yunta RG, Cauwe B, Vandenabeele P et al (2013) Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome Biol 14(1):R4
108. Abrahamsson TR, Jakobsson HE, Andersson AF, Bjorksten B, Engstrand L, Jenmalm MC (2012) Low diversity of the gut microbiota in infants with atopic eczema. J Allergy Clin Immunol 129(2):434–440, 40 e1–e2
109. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L et al (2006) Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut 55(2):205–211
110. Nemoto H, Kataoka K, Ishikawa H, Ikata K, Arimochi H, Iwasaki T et al (2012) Reduced diversity and imbalance of fecal microbiota in patients with ulcerative colitis. Dig Dis Sci 57(11):2955–2964

111. Rehman A, Lepage P, Nolte A, Hellmig S, Schreiber S, Ott SJ (2010) Transcriptional activity of the dominant gut mucosal microbiota in chronic inflammatory bowel disease patients. J Med Microbiol 59(Pt 9):1114–1122
112. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE et al (2009) A core gut microbiome in obese and lean twins. Nature 457(7228):480–484
113. Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S et al (2012) Gut microbiota composition correlates with diet and health in the elderly. Nature 488(7410): 178–184
114. Aichbhaumik N, Zoratti EM, Strickler R, Wegienka G, Ownby DR, Havstad S et al (2008) Prenatal exposure to household pets influences fetal immunoglobulin E production. Clin Exp Allergy 38(11):1787–1794
115. Ege MJ, Mayer M, Schwaiger K, Mattes J, Pershagen G, van Hage M et al (2012) Environmental bacteria and childhood asthma. Allergy 67:1565–1571
116. Hanski I, von Hertzen L, Fyhrquist N, Koskinen K, Torppa K, Laatikainen T et al (2012) Environmental biodiversity, human microbiota, and allergy are interrelated. Proc Natl Acad Sci U S A 109(21):8334–8339
117. Korhonen L, Kondrashova A, Tauriainen S, Haapala AM, Huhtala H, Ilonen J et al (2013) Enterovirus infections in early childhood and the risk of atopic disease – a nested case-control study. Clin Exp Allergy 43(6):625–632
118. Matricardi PM, Rosmini F, Riondino S, Fortini M, Ferrigno L, Rapicetta M et al (2000) Exposure to foodborne and orofecal microbes versus airborne viruses in relation to atopy and allergic asthma; epidemiological study. BMJ 320:412–417
119. Hesselmar B, Sjoberg F, Saalman R, Aberg N, Adlerberth I, Wold AE (2013) Pacifier cleaning practices and risk of allergy development. Pediatrics 131:e1829–e1837
120. Magnus MC, Haberg SE, Stigum H, Nafstad P, London SJ, Vangen S et al (2011) Delivery by Cesarean section and early childhood respiratory symptoms and disorders: the Norwegian mother and child cohort study. Am J Epidemiol 174(11):1275–1285
121. Guibas GV et al (2013) Conception via in vitro fertilization and delivery by cesarean section are associated with pediatric asthma incidence. Clin Exp Allergy 43:1058–1066
122. Stensballe LG, Simonsen J, Jensen SM, Bonnelykke K, Bisgaard H (2013) Use of antibiotics during pregnancy increases the risk of asthma in early childhood. J Pediatr 162(4):832–838 e3
123. Russell G, Helms PJ (1997) Trend in occurrence of asthma among children and young adults. Reporting of common respiratory and atopic symptoms has increased. BMJ 315(7114): 1014–1015
124. Metsala J, Lundqvist A, Virta LJ, Kaila M, Gissler M, Virtanen SM (2013) Mother's and offspring's use of antibiotics and infant allergy to cow's milk. Epidemiology 24(2):303–309
125. Hviid A, Svanstrom H, Frisch M (2011) Antibiotic use and inflammatory bowel diseases in childhood. Gut 60(1):49–54
126. Shaw SY, Blanchard JF, Bernstein CN (2010) Association between the use of antibiotics in the first year of life and pediatric inflammatory bowel disease. Am J Gastroenterol 105(12): 2687–2692
127. Mulder IE, Schmidt B, Stokes CR, Lewis M, Bailey M, Aminov RI et al (2009) Environmentally-acquired bacteria influence microbial diversity and natural innate immune responses at gut surfaces. BMC Biol 7:79
128. Hemarajata P, Versalovic J (2013) Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. Therap Adv Gastroenterol 6(1):39–51
129. Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC et al (2008) Innate immunity and intestinal microbiota in the development of type 1 diabetes. Nature 455(7216):1109–1113
130. Elinav E, Strowig T, Kau AL, Henao-Mejia J, Thaiss CA, Booth CJ et al (2011) NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell 145(5):745–757

131. Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S et al (2010) Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science 328(5975):228–231
132. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T et al (2012) Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 482(7384):179–185
133. Garrett WS, Lord GM, Punit S, Lugo-Villarino G, Mazmanian SK, Ito S et al (2007) Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. Cell 131(1):33–45
134. Naik S, Bouladoux N, Wilhelm C, Molloy MJ, Salcedo R, Kastenmuller W et al (2012) Compartmentalized control of skin immunity by resident commensals. Science 337(6098): 1115–1119
135. Nakatsuji T, Chiang HI, Jiang SB, Nagarajan H, Zengler K, Gallo RL (2013) The microbiome extends to subepidermal compartments of normal skin. Nat Commun 4:1431
136. Bailey MT, Engler H, Sheridan JF (2006) Stress induces the translocation of cutaneous and gastrointestinal microflora to secondary lymphoid organs of C57BL/6 mice. J Neuroimmunol 171(1–2):29–37
137. Friberg IM, Bradley JE, Jackson JA (2010) Macroparasites, innate immunity and immunoregulation: developing natural models. Trends Parasitol 26:540–549
138. Jackson JA, Friberg IM, Bolch L, Lowe A, Ralli C, Harris PD et al (2009) Immunomodulatory parasites and toll-like receptor-mediated tumour necrosis factor alpha responsiveness in wild mammals. BMC Biol 7:16
139. Harnett MM, Melendez AJ, Harnett W (2010) The therapeutic potential of the filarial nematode-derived immunodulator, ES-62 in inflammatory disease. Clin Exp Immunol 159 (3):256–267
140. Kron MA, Metwali A, Vodanovic-Jankovic S, Elliott D (2013) Nematode AsnRS resolves intestinal inflammation in murine T-cell transfer colitis. Clin Vaccine Immunol 20:276–281
141. Liu W, Li Y, Learn GH, Rudicell RS, Robertson JD, Keele BF et al (2010) Origin of the human malaria parasite *Plasmodium falciparum* in gorillas. Nature 467(7314):420–425
142. Sotgiu S, Angius A, Embry A, Rosati G, Musumeci S (2008) Hygiene hypothesis: innate immunity, malaria and multiple sclerosis. Med Hypotheses 70(4):819–825
143. Fumagalli M, Pozzoli U, Cagliani R, Comi GP, Riva S, Clerici M et al (2009) Parasites represent a major selective force for interleukin genes and shape the genetic predisposition to autoimmune conditions. J Exp Med 206(6):1395–1408
144. Barnes KC, Grant AV, Gao P (2005) A review of the genetic epidemiology of resistance to parasitic disease and atopic asthma: common variants for common phenotypes? Curr Opin Allergy Clin Immunol 5(5):379–385
145. Moller M, Gravenor MB, Roberts SE, Sun D, Gao P, Hopkin JM (2007) Genetic haplotypes of Th-2 immune signalling link allergy to enhanced protection to parasitic worms. Hum Mol Genet 16(15):1828–1836
146. Fredericks CA, Drabant EM, Edge MD, Tillie JM, Hallmayer J, Ramel W et al (2010) Healthy young women with serotonin transporter SS polymorphism show a pro-inflammatory bias under resting and stress conditions. Brain Behav Immun 24:350–357
147. Smith AM, Rahman FZ, Hayee B, Graham SJ, Marks DJ, Sewell GW et al (2009) Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. J Exp Med 206(9):1883–1897
148. Pace TW, Hu F, Miller AH (2007) Cytokine-effects on glucocorticoid receptor function: relevance to glucocorticoid resistance and the pathophysiology and treatment of major depression. Brain Behav Immun 21(1):9–19
149. Cohen S, Janicki-Deverts D, Doyle WJ, Miller GE, Frank E, Rabin BS et al (2012) Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk. Proc Natl Acad Sci U S A 109:5995–5999

150. Bierhaus A, Wolf J, Andrassy M, Rohleder N, Humpert PM, Petrov D et al (2003) A mechanism converting psychosocial stress into mononuclear cell activation. Proc Natl Acad Sci U S A 100(4):1920–1925
151. Hanke ML, Powell ND, Stiner LM, Bailey MT, Sheridan JF (2012) Beta adrenergic blockade decreases the immunomodulatory effects of social disruption stress. Brain Behav Immun 26 (7):1150–1159
152. Merali Z, Du L, Hrdina P, Palkovits M, Faludi G, Poulter MO et al (2004) Dysregulation in the suicide brain: mRNA expression of corticotropin-releasing hormone receptors and GABA (A) receptor subunits in frontal cortical brain region. J Neurosci 24(6):1478–1485
153. Lee R, Geracioti TD Jr, Kasckow JW, Coccaro EF (2005) Childhood trauma and personality disorder: positive correlation with adult CSF corticotropin-releasing factor concentrations. Am J Psychiatry 162(5):995–997
154. Gareau MG, Silva MA, Perdue MH (2008) Pathophysiological mechanisms of stress-induced intestinal damage. Curr Mol Med 8(4):274–281
155. Teitelbaum AA, Gareau MG, Jury J, Yang PC, Perdue MH (2008) Chronic peripheral administration of corticotropin-releasing factor causes colonic barrier dysfunction similar to psychological stress. Am J Physiol 295(3):G452–G459
156. Wallon C, Yang PC, Keita AV, Ericson AC, McKay DM, Sherman PM et al (2008) Corticotropin-releasing hormone (CRH) regulates macromolecular permeability via mast cells in normal human colonic biopsies in vitro. Gut 57(1):50–58
157. Calcagni E, Elenkov I (2006) Stress system activity, innate and T helper cytokines, and susceptibility to immune-related diseases. Ann N Y Acad Sci 1069:62–76
158. Zbytek B, Slominski AT (2007) CRH mediates inflammation induced by lipopolysaccharide in human adult epidermal keratinocytes. J Invest Dermatol 127(3):730–732
159. Broadhurst MJ, Ardeshir A, Kanwar B, Mirpuri J, Gundra UM, Leung JM et al (2012) Therapeutic helminth infection of macaques with idiopathic chronic diarrhea alters the inflammatory signature and mucosal microbiota of the colon. PLoS Pathog 8(11):e1003000
160. Hayakawa M, Asahara T, Henzan N, Murakami H, Yamamoto H, Mukai N et al (2011) Dramatic changes of the gut flora immediately after severe and sudden insults. Dig Dis Sci 56(8):2361–2365
161. Jenq RR, Ubeda C, Taur Y, Menezes CC, Khanin R, Dudakov JA et al (2012) Regulation of intestinal inflammation by microbiota following allogeneic bone marrow transplantation. J Exp Med 209:903–911
162. Ait-Belgnaoui A, Durand H, Cartier C, Chaumaz G, Eutamene H, Ferrier L et al (2012) Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. Psychoneuroendocrinology 37(11):1885–1895
163. Bailey MT, Lubach GR, Coe CL (2004) Prenatal stress alters bacterial colonization of the gut in infant monkeys. J Pediatr Gastroenterol Nutr 38(4):414–421
164. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN et al (2004) Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol 558(Pt 1):263–275
165. Heijtz RD, Wang S, Anuar F, Qian Y, Bjorkholm B, Samuelsson A et al (2011) Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A 108(7): 3047–3052
166. Miller AH, Haroon E, Raison CL, Felger JC (2013) Cytokine targets in the brain: impact on neurotransmitters and neurocircuits. Depress Anxiety 30(4):297–306
167. Raison CL, Miller AH (2013) The evolutionary significance of depression in Pathogen Host Defense (PATHOS-D). Mol Psychiatry 18:15–37
168. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008) From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 9(1):46–56
169. Rook GAW, Lowry CA, Raison CL (2013) Microbial old friends, immunoregulation and stress resilience. Evol Med Pubic Health 2013(1):46–64. Doi: 10.1093/emph/eot004

170. McDade TW (2012) Early environments and the ecology of inflammation. Proc Natl Acad Sci U S A 109(Suppl 2):17281–17288
171. Lotrich FE, Ferrell RE, Rabinovitz M, Pollock BG (2009) Risk for depression during interferon-alpha treatment is affected by the serotonin transporter polymorphism. Biol Psychiatry 65(4):344–348
172. Raison CL, Borisov AS, Majer M, Drake DF, Pagnoni G, Woolwine BJ et al (2009) Activation of central nervous system inflammatory pathways by interferon-alpha: relationship to monoamines and depression. Biol Psychiatry 65(4):296–303
173. Hagberg H, Gressens P, Mallard C (2012) Inflammation during fetal and neonatal life: implications for neurologic and neuropsychiatric disease in children and adults. Ann Neurol 71(4):444–457
174. D'Mello C, Le T, Swain MG (2009) Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factor alpha signaling during peripheral organ inflammation. J Neurosci 29(7):2089–2102
175. Kivisakk P, Imitola J, Rasmussen S, Elyaman W, Zhu B, Ransohoff RM et al (2009) Localizing central nervous system immune surveillance: meningeal antigen-presenting cells activate T cells during experimental autoimmune encephalomyelitis. Ann Neurol 65(4):457–469
176. Engelhardt B, Sorokin L (2009) The blood-brain and the blood-cerebrospinal fluid barriers: function and dysfunction. Semin Immunopathol 31(4):497–511
177. Schwarcz R, Bruno JP, Muchowski PJ, Wu HQ (2012) Kynurenines in the mammalian brain: when physiology meets pathology. Nat Rev Neurosci 13(7):465–477
178. Schwarcz R, Pellicciari R (2002) Manipulation of brain kynurenines: glial targets, neuronal effects, and clinical opportunities. J Pharmacol Exp Ther 303(1):1–10
179. O'Connor JC, Lawson MA, Andre C, Moreau M, Lestage J, Castanon N et al (2009) Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. Mol Psychiatry 14(5):511–522
180. Kita T, Morrison PF, Heyes MP, Markey SP (2002) Effects of systemic and central nervous system localized inflammation on the contributions of metabolic precursors to the L-kynurenine and quinolinic acid pools in brain. J Neurochem 82(2):258–268
181. Raison CL, Dantzer R, Kelley KW, Lawson MA, Woolwine BJ, Vogt G et al (2010) CSF concentrations of brain tryptophan and kynurenines during immune stimulation with IFN-alpha: relationship to CNS immune responses and depression. Mol Psychiatry 15(4): 393–403
182. Zhu CB, Lindler KM, Owens AW, Daws LC, Blakely RD, Hewlett WA (2010) Interleukin-1 receptor activation by systemic lipopolysaccharide induces behavioral despair linked to MAPK regulation of CNS serotonin transporters. Neuropsychopharmacology 35(13): 2510–2520
183. Capuron L, Pagnoni G, Drake DF, Woolwine BJ, Spivey JR, Crowe RJ et al (2012) Dopaminergic mechanisms of reduced basal ganglia responses to hedonic reward during interferon alfa administration. Arch Gen Psychiatry 69(10):1044–1053
184. Felger JC, Li L, Marvar PJ, Woolwine BJ, Harrison DG, Raison CL et al (2013) Tyrosine metabolism during interferon-alpha administration: association with fatigue and CSF dopamine concentrations. Brain Behav Immun 31:153–160
185. Pavlov VA, Parrish WR, Rosas-Ballina M, Ochani M, Puerta M, Ochani K et al (2009) Brain acetylcholinesterase activity controls systemic cytokine levels through the cholinergic anti-inflammatory pathway. Brain Behav Immun 23(1):41–45
186. Lee M, Schwab C, McGeer PL (2011) Astrocytes are GABAergic cells that modulate microglial activity. Glia 59(1):152–165
187. Hertz-Picciotto I, Delwiche L (2009) The rise in autism and the role of age at diagnosis. Epidemiology 20(1):84–90

188. Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, Torigoe T et al (2011) Genetic heritability and shared environmental factors among twin pairs with autism. Arch Gen Psychiatry 68(11):1095–1102
189. Voineagu I, Wang X, Johnston P, Lowe JK, Tian Y, Horvath S et al (2011) Transcriptomic analysis of autistic brain reveals convergent molecular pathology. Nature 474(7351):380–384
190. Ziats MN, Rennert OM (2011) Expression profiling of autism candidate genes during human brain development implicates central immune signaling pathways. PLoS One 6(9):e24691
191. Saresella M, Marventano I, Guerini FR, Mancuso R, Ceresa L, Zanzottera M et al (2009) An autistic endophenotype results in complex immune dysfunction in healthy siblings of autistic children. Biol Psychiatry 66(10):978–984
192. Desmond MM, Montgomery JR, Melnick JL, Cochran GG, Verniaud W (1969) Congenital rubella encephalitis. Effects on growth and early development. Am J Dis Child 118(1):30–31
193. Atladottir HO, Thorsen P, Schendel DE, Ostergaard L, Lemcke S, Parner ET (2010) Association of hospitalization for infection in childhood with diagnosis of autism spectrum disorders: a Danish cohort study. Arch Pediatr Adolesc Med 164(5):470–477
194. Brown AS, Sourander A, Hinkka-Yli-Salomaki S, McKeague IW, Sundvall J, Surcel HM (2014) Elevated maternal C-reactive protein and autism in a national birth cohort. Mol Psychiatry 19:259–264
195. Howerton CL, Bale TL (2012) Prenatal programing: at the intersection of maternal stress and immune activation. Horm Behav 62:237–242
196. Dahlgren J, Samuelsson AM, Jansson T, Holmang A (2006) Interleukin-6 in the maternal circulation reaches the rat fetus in mid-gestation. Pediatr Res 60(2):147–151
197. Willette AA, Lubach GR, Knickmeyer RC, Short SJ, Styner M, Gilmore JH et al (2011) Brain enlargement and increased behavioral and cytokine reactivity in infant monkeys following acute prenatal endotoxemia. Behav Brain Res 219(1):108–115
198. Hsiao EY, McBride SW, Chow J, Mazmanian SK, Patterson PH (2012) Modeling an autism risk factor in mice leads to permanent immune dysregulation. Proc Natl Acad Sci U S A 109(31):12776–12781
199. Meyer U, Feldon J, Dammann O (2011) Schizophrenia and autism: both shared and disorder-specific pathogenesis via perinatal inflammation? Pediatr Res 69(5 Pt 2):26R–33R
200. Onore C, Careaga M, Ashwood P (2012) The role of immune dysfunction in the pathophysiology of autism. Brain Behav Immun 26:383–392
201. Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA (2005) Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann Neurol 57(1): 67–81
202. Pardo CA, Vargas DL, Zimmerman AW (2005) Immunity, neuroglia and neuroinflammation in autism. Int Rev Psychiatry 17(6):485–495
203. Li X, Chauhan A, Sheikh AM, Patil S, Chauhan V, Li XM et al (2009) Elevated immune response in the brain of autistic patients. J Neuroimmunol 207(1–2):111–116
204. Campbell IL, Abraham CR, Masliah E, Kemper P, Inglis JD, Oldstone MB et al (1993) Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. Proc Natl Acad Sci U S A 90(21):10061–10065
205. Wei H, Zou H, Sheikh AM, Malik M, Dobkin C, Brown WT et al (2011) IL-6 is increased in the cerebellum of autistic brain and alters neural cell adhesion, migration and synaptic formation. J Neuroinflammation 8:52
206. Nordahl CW, Braunschweig D, Iosif AM, Lee A, Rogers S, Ashwood P et al (2013) Maternal autoantibodies are associated with abnormal brain enlargement in a subgroup of children with autism spectrum disorder. Brain Behav Immun 30:61–65
207. Margutti P, Delunardo F, Ortona E (2006) Autoantibodies associated with psychiatric disorders. Curr Neurovasc Res 3(2):149–157
208. Lee JY, Huerta PT, Zhang J, Kowal C, Bertini E, Volpe BT et al (2009) Neurotoxic autoantibodies mediate congenital cortical impairment of offspring in maternal lupus. Nat Med 15(1):91–96

209. Benros ME, Waltoft BL, Nordentoft M, Ostergaard SD, Eaton WW, Krogh J et al (2013) Autoimmune diseases and severe infections as risk factors for mood disorders: a nationwide study. JAMA Psychiatry 12:1–9
210. Nikolov RN, Bearss KE, Lettinga J, Erickson C, Rodowski M, Aman MG et al (2009) Gastrointestinal symptoms in a sample of children with pervasive developmental disorders. J Autism Dev Disord 39(3):405–413
211. Critchfield JW, van Hemert S, Ash M, Mulder L, Ashwood P (2011) The potential role of probiotics in the management of childhood autism spectrum disorders. Gastroenterol Res Pract 2011:161358
212. Emanuele E, Orsi P, Boso M, Broglia D, Brondino N, Barale F et al (2010) Low-grade endotoxemia in patients with severe autism. Neurosci Lett 471(3):162–165
213. Finegold SM, Dowd SE, Gontcharova V, Liu C, Henley KE, Wolcott RD et al (2010) Pyrosequencing study of fecal microflora of autistic and control children. Anaerobe 16(4): 444–453
214. Adams JB, Johansen LJ, Powell LD, Quig D, Rubin RA (2011) Gastrointestinal flora and gastrointestinal status in children with autism–comparisons to typical children and correlation with autism severity. BMC Gastroenterol 11:22
215. Williams BL, Hornig M, Parekh T, Lipkin WI (2012) Application of novel PCR-based methods for detection, quantitation, and phylogenetic characterization of *Sutterella* species in intestinal biopsy samples from children with autism and gastrointestinal disturbances. MBio 3(1)
216. Thomas RH, Meeking MM, Mepham JR, Tichenoff L, Possmayer F, Liu S et al (2012) The enteric bacterial metabolite propionic acid alters brain and plasma phospholipid molecular species: further development of a rodent model of autism spectrum disorders. J Neuroinflammation 9:153
217. Fatemi SH, Folsom TD (2009) The neurodevelopmental hypothesis of schizophrenia, revisited. Schizophr Bull 35(3):528–548
218. Buka SL, Tsuang MT, Torrey EF, Klebanoff MA, Wagner RL, Yolken RH (2001) Maternal cytokine levels during pregnancy and adult psychosis. Brain Behav Immun 15(4):411–420
219. Brown AS, Hooton J, Schaefer CA, Zhang H, Petkova E, Babulas V et al (2004) Elevated maternal interleukin-8 levels and risk of schizophrenia in adult offspring. Am J Psychiatry 161(5):889–895
220. Crespi B, Badcock C (2008) Psychosis and autism as diametrical disorders of the social brain. Behav Brain Sci 31(3):241–261, discussion 61–320
221. Johnson WG, Buyske S, Mars AE, Sreenath M, Stenroos ES, Williams TA et al (2009) HLA-DR4 as a risk allele for autism acting in mothers of probands possibly during pregnancy. Arch Pediatr Adolesc Med 163(6):542–546

Chapter 16
Microbiota-Gut-Brain Axis and Cognitive Function

Mélanie G. Gareau

Abstract Recent studies have demonstrated a clear association between changes in the microbiota and cognitive behavior. Intestinal dysbiosis, as modeled using GF mice (containing no microbiota), bacterial infection with an enteric pathogen, and administration of probiotics, can modulate cognitive behavior including learning and memory. This chapter will highlight recent findings in both human and animal studies indicating how changes in the composition and diversity of the microbiota can impact behavior and brain physiology in both disease states and in health. Cognitive behavior can not only be affected in cases of intestinal disease, but also manifests changes in extra-intestinal disease conditions.

Abbreviations

5-HT	Serotonin
ANS	Autonomic nervous system
BDNF	Brain derived neurotropic factor
CD	Crohn's disease
CREB	cAMP response element binding protein
CRF	Corticotrophin-releasing factor
DA	Dopamine
DLPFC	Dorsolateral pre-frontal cortex
EPSP	Excitatory postsynaptic potential
GF	Germ-free
GI	gastrointestinal
HE	Hepatic encephalopathy
HPA	Hypothalamus-pituitary-adrenal

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_16,

IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
LPS	Lipopolysaccharide
MS	Maternal separation
NGF	Nerve growth factor
PAMPs	pathogen associated molecular patterns
PGN	Peptidoglycan
SPF	Specific pathogen free
UC	Ulcerative colitis

Introduction

The gut-brain-microbiota axis has recently been demonstrated to play an important role in the establishment and maintenance of cognitive function. In laboratory animals cognition, or learning and memory, is assessed by specific behavioral tests (Table 16.1) targeting spatial (or working) and non-spatial (or recognition) memory. Communication between the gut and the brain can occur via neuronal, endocrine and immunological pathways, highlighting the complexity in deciphering the specific mechanisms involved in mediating normal physiology and homeostasis [1]. Studies involving changes in the composition of the microbiota, either following bacterial infection, administration of antibiotics or probiotics, or in germ-free (GF) mice all demonstrate that modulating the microbiota can impact behavior and cognition. These mouse models involving the presence of an altered microbiota can be used independently or in combination to study the overall impact of pathology, and chronic disease in changing cognitive behavior.

This review will focus on the role of the gut-brain-microbiota axis in mediating alterations in cognition in both human and animal studies and comparing normal and disease states. Specifically, studies utilizing GF mice, enteric infections, and neonatal stress as systems modeling an altered gut-brain-microbiota axis will be described in detail. In addition, studies in healthy human controls as well as those evaluating the effect of intestinal, including inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), or extraintestinal disease states, including diabetes mellitus and hepatic encephalopathy, on cognitive function will be presented.

Microbiota and Cognition

The microbiome has emerged in recent years as a leading factor in establishing normal physiology and function of the host as well as being a causative factor in numerous disease states when inappropriately altered [2]. Changes in the intestinal microbiota, either due to inflammation, infection, or drugs—including administration of antibiotics—can lead to extraintestinal effects, including changes in the brain. Alterations in behavior, including anxiety, depression and cognitive defects,

Table 16.1 Common tests for cognitive behavior

Test	Function tested
Morris water maze	Spatial learning
Fear conditioning	Memory skills; fear levels
Novel object test	Non-spatial memory
T or Y maze	Working (spatial) memory

have recently been demonstrated as additional targets of the microbiome, or more specifically, the presence of dysbiosis [3]. It is well established that the hypothalamus-pituitary-adrenal (HPA) axis is regulated by the microbiota. As evidence of this association, GF mice have increased baseline HPA-axis activation compared to specific pathogen free (SPF) colonized controls, indicated by elevated levels of serum corticosterone [4]. This supports the notion that the microbiota can regulate the development of the central response to stress, at least in rodents.

With respect to cognition, GF mice were recently demonstrated to have a deficit in non-spatial memory and impaired working memory compared to SPF controls at baseline [5]. Exposure to acute psychological stress in the form of one hour of water avoidance, which activates the HPA-axis, did not further affect learning and memory in GF mice [5]. This finding suggests that under GF conditions, the HPA-axis cannot be stimulated by exposure to stress, creating a neuroendocrine system that is vulnerable to external threats. This altered cognition in GF mice is accompanied by reductions in two proteins that play important roles in the regulation of hippocampal-dependent memory, namely brain derived neurotropic factor (BDNF) and c-fos [5]. BDNF is a potent modulator of synaptic plasticity, particularly in the hippocampus during neurogenesis [6] whereas c-fos is an immediate early gene that is a target of CREB, which is required for hippocampus-dependent long-term memory formation [7]. As was observed in protein studies, decreased BDNF mRNA was also demonstrated in the hippocampus of GF mice compared to SPF controls [8, 9]. Taken together, these studies suggest a potential association between the microbiota and BDNF or c-fos levels in regulating brain physiology and memory. Despite these studies, it is not known at this time whether cognitive defects can be normalized by colonization of GF mice either in early life or in adulthood. Numerous subsequent studies have however demonstrated that GF mice display anxiolytic-like behavior, compared to their SPF counterparts [8, 10, 11]. In these studies, conventionalization of mice could normalize behavior, but only in early stages of life [8]. These findings will be discussed in greater detail in other chapters.

Following the discovery that GF mice have altered behavior compared to SPF controls, studies using metabolomics to obtain a complete picture of the influence of the microbiota on brain function were undertaken. Assessment of the cerebral metabolome in GF versus conventionalized controls was recently performed to quantify metabolites that might underlie the gut-brain-microbiota axis, with biosynthetic pathways for dopamine (DA) and serotonin (5-HT) demonstrating significant microbiota-associated changes [12]. These pilot studies revealed a change in brain neurotransmitter levels impacted by the microbiota, which could significantly

impact brain health, development and behavior [12]. Serotonin is important in cognition, with manipulations in the serotonergic system capable of producing changes in cognitive function independent of changes in overall mood [13]. GF mice were demonstrated to have elevated hippocampal and plasma 5-HT levels, suggesting a possible role for the humoral system in the communication of the microbiota with the central nervous system [9]. A sexual dimorphic effect was also observed, with only male GF mice demonstrating these elevations in the serotonergic system in contrast to female mice [9]. Colonization of GF mice post-weaning could reverse the behavioral changes observed, but not the biochemical changes, suggesting that 5-HT is not the only factor that underlies the changes in behavior that are produced by the microbiota [9]. The use of GF mice will continue to provide important knowledge about the role of the microbiota in establishing learning and memory.

In addition to GF status, studies looking at commensal or non-pathogenic organisms have also revealed an important role for the microbiota in mediating cognitive behavior. *Mycobacterium vaccae* is an aerobic bacterium that is considered to be a transient commensal organism, due to its inability to effectively colonize the gastrointestinal tract. In humans, administration of heat killed *M. vaccae* to terminal lung cancer patients was able to improve emotional health and cognitive function, leading to the hypothesis that the immune response to the bacteria involved neurotransmitters, such as 5-HT, resulting in improved mood [14]. In mice, administration of *M. vaccae* decreased maze run time compared to controls in a Hebbs-Williams style complex maze consisting of a close-field test apparatus to study intelligence, suggesting an improvement in learning and memory [15]. Additionally, immunization with *M. vaccae* altered emotional behavior, decreasing the time mice spent immobile during a forced swim test, which was hypothesized to occur as a result of altered serotonergic signaling in the dorsal raphe nucleus of the brain [16]. These studies highlight a role for the immune system, in part via the serotonergic system, in mediating a commensal microbe effect on the brain and cognition.

Stress, Infection and Cognition

Exposure to psychological or physical stress results in activation of the HPA-axis, which subsequently activates the neuroendocrine system and numerous downstream responses. Stress, or the increased perception of stress, has been associated with precipitation of symptoms in patients with IBD, and decreased overall quality of life [17]. Animal models of chronic stress cause changes in the microbiota and intestinal physiology. These include increased macromolecular permeability and an elevated secretory state [18–20]. Furthermore, chronic stress is associated with cognitive deficits, including reduced non-spatial memory [21]. It is therefore not surprising that exposure to stress can also increase susceptibility to a bacterial infection. Chronic physical stress (prolonged restraint stress) was associated with

increased pathogen load following infection with the non-invasive murine pathogen *Citrobacter rodentium* [22]. Exposure to stress can change the composition of the intestinal microbiota [22] and modify microbial-host interactions, resulting in an increase in bacterial attachment and internalization in the epithelium [18]. These altered host-microbe interactions are directly mediated by stress-induced changes in the microbiota, as administration of probiotics was able to normalize these changes [23]. Exposure to stress is also associated with precipitating cognitive impairments. Social defeat stress in mice was sufficient to induce dysfunction in cognitive behavior, including changes in spatial object recognition, using the Y-maze, without affecting anxiety or locomotor activity as assessed by the elevated plus maze [24]. These cognitive effects were mediated in part by the glutaminergic signaling pathway within the hippocampus [24]. Infection with *C. rodentium* alone in wild type mice does not cause changes in memory and cognition, however exposure to a single session of psychological stress (water avoidance stress) in infected mice, but not in uninfected controls led to decreases in both non-spatial recognition memory and working memory [5]. These stress-induced cognitive defects remained in place well after the pathogen had cleared, demonstrating a long lasting effect [5]. Administration of probiotics starting 1 week prior to *C. rodentium* infection was able to prevent these stress-induced changes in behavior [5], again highlighting a role for the microbiota in driving these gut-brain axis effects. The changes in cognition in stressed mice infected with *C. rodentium* were associated with reduced expression of hippocampal BDNF and c-fos [5]. Taken together, this suggests a potential association between stress-induced changes in intestinal physiology and cognition via hippocampal BDNF and c-fos, although this remains speculative at this time. Stress, therefore, plays an important role in the maintenance of the composition of the intestinal microbiota, with profound effects in the context of infection with a bacterial pathogen and changes in the gut-brain axis.

Maternal/offspring interactions are important in shaping the HPA-axis of the offspring and regulating behavior in adulthood. Maternal separation (MS) during the neonatal period in mice increases stress-induced intestinal dysfunction [25, 26]. Exposure to early life stress in rats, using MS, can change the composition of the microbiota both in early life [27, 28] and in adulthood [29]. These changes can increase disease risk and severity in the development of colitis [30] and facilitate infection with a parasite, *Nippostrongylus brasiliensis* [31] in adulthood. MS was also recently demonstrated to cause discordant, biphasic changes in cognition and learning depending on age. Younger rats (2 months) previously subjected to MS demonstrated increased neurogenesis, and decreased repressive histone methylation at the BDNF IV promoter along with increased hippocampal BDNF and improved spatial learning and non-spatial learning [32]. In stark contrast, adult (15 month) rats that had undergone the same stressor regime as neonates demonstrated opposing changes in neurogenesis, epigenetic regulation of BDNF and behavior compared to what was observed in younger rats [32]. This neurological decline in middle-aged rats was prevented by administration of anti-depressants post-exposure to early life stress, suggesting a role for epigenetic changes in

modifying BDNF promoter function and behavior [32]. Similar effects of MS were observed by other groups, who demonstrated an improvement in hippocampal-dependent memory, but not in the learning ability following anti-depressant treatment in rats [33]. A recent study by Aisa et al. [34] demonstrated cognitive defects that were accompanied by decreased nerve growth factor (NGF) expression in the hippocampus. Alternatively, Baudin et al. [35] demonstrated defects in prefrontal cortex-dependent, but not hippocampal-dependent, cognitive function following exposure to MS. Taken together, these studies suggest that the effect of MS on cognition is complex, and may involve different regions of the brain based on the type of cognition being assessed. While the microbiota was not assessed in these studies, MS is known to change the microbiota [28, 29], possibly suggesting its role in mediating the cognitive defects seen in maternally separated rats.

In related studies, epidemiological evidence suggests an association exists between prenatal maternal infection and the increased risk of neurodevelopmental brain disorders in rat and mouse pups [36]. Maternal infection of pregnant rats with *E. coli* is associated with altered cognitive development in the offspring, and is associated with increased hippocampal neuronal apoptosis [37]. Similarly, intracerebellar injection of LPS in neonatal rats results in learning deficits and reduced hippocampal volume in adulthood [38]. Peripheral neonatal bacterial infection also caused impaired memory in adulthood, but only following LPS administration prior to behavioral testing [39]. This phenomenon could be prevented by daily neonatal handling that altered the basal HPA axis. These data further highlight a role for the HPA-axis in regulating cognitive development [39]. Finally, maternal injection of mice with polyI:C during gestation, to mimic viral infection, significantly impaired non-spatial memory and learning in the pups at 3 weeks and 9 weeks of age. Thus, the impact of infection or immune responses to mimetics of infectious agents, including pathogen associated molecular patterns (PAMPs), has long lasting effects in the offspring [40]. At present, the consequences of maternal infection for the microbiota of the offspring are not known, as are any links of these changes with future cognitive abnormalities.

Recent evidence suggests that the cumulative effect of exposure to multiple infectious pathogens, both bacterial and viral, may be associated with changes in behavior. In humans, an elevated infectious burden, defined as a composite serologic measure of exposure to specific common pathogens (e.g. cytomegalovirus, *Helicobacter pylori* and herpes simplex virus), was associated with cognitive impairment as assessed by the mini-mental state examination in a prospective cohort of healthy individuals [41]. These past infections may contribute to cognitive impairments [41]; as a population of home-dwelling elderly individuals who were seropositive for common bacteria and viruses exhibited cognitive impairment [42]. As such these studies suggest that exposure to infectious agents over the course of a lifetime can contribute to determining cognitive function in adults. While it is tempting to speculate that infection alone, and/or the immune response to infectious agents, is the causative factor in cognitive decline, there are no clear data as yet to support precise mechanisms.

IBS/IBD and Memory

Irritable bowel syndrome (IBS) is a prevalent functional gastrointestinal (GI) disorder associated with an altered gut-brain-microbiota axis [43]. Symptoms are often precipitated by exposure to stressors [44], or an enteric pathogen, the latter being termed post-infectious IBS [45]. Patients with IBS, in contrast to healthy controls or patients with organic gastrointestinal disease, display an increased recognition memory to words with a negative emotional connotation [46]. In a separate, more recent study by Gibbs-Gallagher et al., patients with IBS also exhibit altered recall to words and phrases describing GI symptoms versus those associated with respiratory symptoms or neutral phrases [47]. In the Gibbs-Gallagher study, however, patients with organic disease—consisting of patients with asthma—was also skewed towards higher recall of words associated with respiratory illness, suggesting that patients with organic disease may also have a memory bias [47]. This supports the cognitive behavioral theory of IBS, where selective attention to GI sensations may play a role in decreased pain thresholds and consequently increased symptom severity [47]. Similarly, depletion of serotonin in patients with IBS by acute tryptophan depletion induced a significant shift in affective memory bias towards loss of recall of positive words, from a list of emotionally loaded stimulus words versus negative or neutral words, in the absence of overall changes in mood, in contrast to healthy controls [48]. Serotonin plays an important role in the gut-brain axis, mediating behavior and intestinal physiology including motility, secretion and visceral sensitivity. Using imaging techniques, patients with IBS demonstrated latent impairment of cognitive flexibility due to altered activity in the dorsolateral pre-frontal cortex (DLPFC), insula and hippocampus as well as impaired connectivity between the DLPFC and pre-supplementary motor area as determined using the Wisconsin Card Sorting Test [49]. A direct link between serotonin levels and changes in the composition of the microbiota and changes in mood and cognition would be of interest in IBS patients.

Inflammatory bowel disease (IBD) is composed of two distinct diseases, Crohn's disease (CD) and ulcerative colitis (UC). Patients with IBD often have increased intestinal permeability, changes in their microbiota and the presence of inflammation during active disease. A change in the gut-brain-microbiota axis has recently been described in many patients with IBD that present with co-morbid mood disorders, including anxiety and depression, that are found in a subset of patients [50, 51]. These mood disorders not only diminish quality of life directly, but can also increase disease severity [52]. Cognitive function in adult patients with IBD was recently found to be decreased compared to healthy controls, as assessed using a verbal IQ test [53, 54]. Similarly, in an adolescent population, patients with IBD demonstrated decreased mild verbal memory compared to controls with juvenile idiopathic arthritis [55]. This could highlight that inflammation alone may not be a sufficient driver of these functional cognitive impairments. In a pediatric population, administration of steroids to CD patients, but not UC patients, was associated with a negative effect on mood, memory and behavior, compared to non-steroid

treated CD controls, although these differences weren't representative of a marked dysfunction [56]. It would be of significant interest to assess whether changes in mood and memory in patients with IBD is associated with inflammation or the HPA-axis or a combination of the two risk factors.

Diet, Microbiota and Behavior

Dietary habits have been demonstrated to significantly affect the composition of the intestinal microbiota [57]. In humans dietary influence begins after birth, with the choice of feeding modality impacting the composition of the infant microbiota. As evidence of this, changing colonization patterns have been observed in breast milk-fed compared to formula-fed infants [58]. In mice, supplementing the diet with 50 % beef protein for 3 months increased the diversity of the intestinal microbiota, which was accompanied by changes in behavior [59]. These changes included improvements in working and reference memory, along with reduced anxiety in the diet-supplemented group, compared to mice fed standard chow [59]. A potential mechanism of action for the protein enriched diet remains to be determined. Clearly not all dietary alterations are beneficial to host cognition, as feeding mice a Western-style diet high in fat and refined sugar increased anxiety-like behavior and decreased memory function in the setting of low-grade inflammation in an IL-10 deficient mouse model. These deficits could be ameliorated by administration of *Lactobacillus*-containing probiotics [60]. At this point it is uncertain what the complex relationship between the probiotic, inflammation, and diet was on the microbiota and behavior, and whether this was directly due to diet-associated effects of the probiotics or reducing inflammation in this model system.

In a human study involving healthy volunteers, and in particular with no gastrointestinal or psychiatric symptoms, administration of a fermented milk product supplemented with probiotic bacteria resulted in alterations of brain intrinsic connectivity, having effects in the regions that control central processing of emotion and sensation as assessed by neuroimaging using fMRI [61]. In this study, however, this chronic administration of fermented milk products supplemented with probiotic organisms had no effect on the composition of the microbiota compared to the placebo [61]. Whether these changes in brain connectivity are associated with a beneficial role in modulating pain sensitivity, stress responsiveness, mood or anxiety remains to be determined. In contrast, in a group of Vietnamese school children, supplementation with a milk or an inulin fortified milk beverage enhanced weight gain, reduced anemia and increased serum zinc levels compared to the reference control group in a manner that was associated with microbiota changes [62]. Specifically, levels of *Bifidobacteria* and *Bacteroides* spp. increased in both treatment groups, which was coupled with improved short-term memory scores and quality of life compared to children in the control group [62]. However, a third study showed that while administration of a probiotic-containing beverage to a healthy cohort resulted in improved mood, this occurred

only in subjects who were in the bottom third of overall mood scores; and surprisingly, memory was slightly increased in the placebo group compared to the probiotic group [63]. Thus, while these studies suggest that dietary modifications may ultimately be employed as a means of affecting behavior, including cognition, in patients with intestinal diseases, whether this reflects an impact on the microbiota is still controversial. Further, studies in relevant patient groups are still lacking, and it may not be possible to extrapolate from observations in healthy volunteers.

Extraintestinal Impacts of the Gut-Brain-Microbiota Axis

Diabetes mellitus, a metabolic disorder characterized by insulin deficiency or resistance, is accompanied by moderate disturbances in learning and memory, due in part to oxidative stress [64]. Administration of a combination of *Lactobacillus acidophilus*, *Bifidobacterium lactis* and *Lactobacillus fermentum* in the drinking water reversed the behavioral (spatial learning task) and electrophysiological (declined potentiated excitatory postsynaptic potential [EPSP] in the hippocampus) deficits observed in diabetic rats [64]. Performance in the Morris water maze navigation task for spatial learning and memory was restored to control levels, and basic synaptic activity in the hippocampus was normalized [64].

In the setting of liver cirrhosis, hepatic encephalopathy (HE) can develop in a subset of patients, leading to poor cognition and poor survival. HE is thought to occur in the setting of an altered microbiota in the context of increased intestinal permeability [65]. Using a systems biology approach, the presence of *Alcaligeneceae*, *Porphyromonadaceae*, and *Enterobacteriaceae* were found to be strongly correlated with HE, decreased cognition and the presence of inflammation [66]. In a follow up study with patients with milder HE (minimal HE), administration of the antibiotic rifaximin improved cognition in these patients and reduced endotoxemia [67]. This effect of rifaximin is thought to occur via changes in gut bacterial linkages with metabolites, rather than changes in overall microbial abundance [68]. Taken together, these findings may provide supportive evidence for predicting the risk of HE in patients with cirrhosis, as well as a potential role for probiotics or antibiotics in preventing cognitive deficits and endotoxemia in patients.

Impact of the Gut-Brain-Microbiota Axis on Cognitive Function in Health

As demonstrated by studies in GF mice, the microbiota is essential for normal cognitive development [5]. It is therefore not surprising that changing the microbiota, for example by administration of beneficial probiotics, could have an

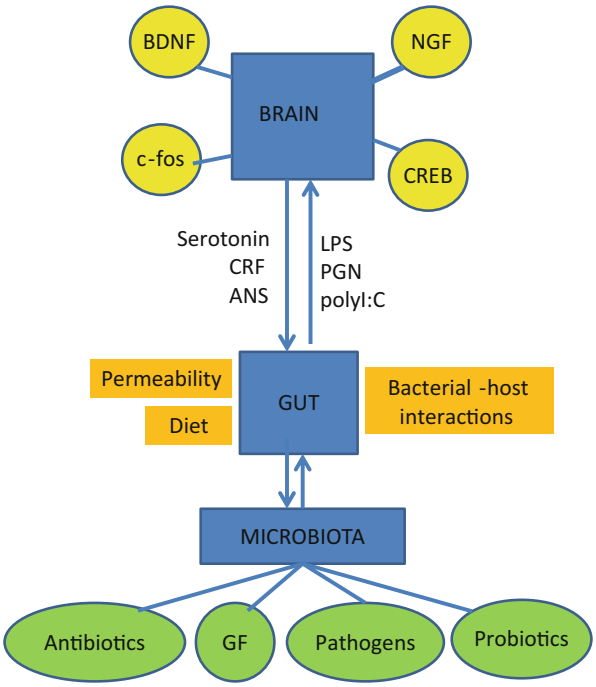

Fig. 16.1 Gut-brain-microbiota and cognition. Each component of the gut-brain-microbiota axis can participate in overall changes in cognitive behavior. Infection with enteric pathogens, administration of antibiotics, treatment with probiotics or mice in a germ-free (GF) state can impact the composition of the microbiota (or lack thereof) and subsequently intestinal physiology. Changes in intestinal physiology can lead to increased intestinal permeability, altered bacterial-host interactions or be modulated by the composition of the diet. Bacterial components, including lipopolysaccharide (LPS), peptidoglycan (PGN) and viral components (mimicked by the use of polyI:C) can all result in changes in the signaling to the brain. Exposure to stress can produce corticotrophin-releasing factor (CRF), neurotransmitters that can signal via the autonomic nervous system (ANS) or activation of the serotonergic system, which can each modulate intestinal function. All these changes can result in alterations in brain, more specifically hippocampus, including expression of nerve-growth factor (NGF), brain-derived neurotropic factor (BDNF), c-fos and subsequently cAMP response element binding protein (CREB), resulting in changes in cognitive function

impact on normal physiology. A recent study by Bravo et al. studied the effect of a potential probiotic (*L. rhamnosus* strain JB-1) on behavior, including fear conditioning to assess the cognitive aspects of anxiety behavior. Enhanced memory consolidation, in the context of decreased hippocampal GABAB1b mRNA, following administration of probiotics suggests a potential mechanism by which *Lactobacillus* species may modulate cognitive behavior [69].

In humans, administration of a probiotic cocktail (composed of *L. helveticus* and *B. longum*) to healthy human volunteers significantly impacted normal behavior. Behavior was studied using the coping checklist, which measures cognitive efforts

to adapt to external stimuli that extend beyond a subject's resources [70]. Subjects receiving probiotics decreased their self-blame score and displayed a higher problem solving score compared to their baseline values, following 30 days of administration of probiotics. This suggests that administration of probiotics may potentially provide benefits on overall mood and cognition in a healthy control population.

Conclusions

Cognitive behavior and function are maintained in part by the gut-brain-microbiota axis. While the mechanistic details still remain to be determined, including specific pathways of microbial communication with various structures in the brain, these recent advances highlight the importance of microbial colonization and communities in mediating appropriate behaviors. Numerous factors can contribute to the brain-gut-microbiota axis at each level, and bi-directional communication can together add significant complexity to the system (Fig. 16.1). Determining the precise mechanisms involved in communication between each step may have important clinical significance in the future. The novel findings that probiotics may have an impact on normal cognitive behaviors is particularly interesting, since it suggests that there exists a certain flexibility within the gut-brain-microbiota axis, well into adulthood and after completion of developmental stages thought to be the critical time for mediating potential changes. Shifting the composition of the microbiota, in part via administration of probiotics, appears to be a viable therapeutic option for modulating both intestinal physiology and behavior, and may improve quality of life in certain patient populations, including IBS and IBD, as well as the general population.

References

1. Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 13(10):701–712
2. Gareau MG, Sherman PM, Walker WA (2010) Probiotics and the gut microbiota in intestinal health and disease. Nat Rev Gastroenterol Hepatol 7(9):503–514
3. Collins SM, Surette M, Bercik P (2012) The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol 10(11):735–742
4. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN et al (2004) Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol 558(Pt 1):263–275
5. Gareau MG, Wine E, Rodrigues DM, Cho JH, Whary MT, Philpott DJ et al (2011) Bacterial infection causes stress-induced memory dysfunction in mice. Gut 60(3):307–317
6. Cowansage KK, LeDoux JE, Monfils MH (2010) Brain-derived neurotrophic factor: a dynamic gatekeeper of neural plasticity. Curr Mol Pharmacol 3(1):12–29

7. Mizuno K, Giese KP (2005) Hippocampus-dependent memory formation: do memory type-specific mechanisms exist? J Pharmacol Sci 98(3):191–197
8. Heijtz RD, Wang S, Anuar F, Qian Y, Bjorkholm B, Samuelsson A et al (2011) Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A 108 (7):3047–3052
9. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F et al (2013) The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. Mol Psychiatry 18(6):666–673
10. Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J et al (2011) The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 141(2):599–609, e1–e3
11. Neufeld KM, Kang N, Bienenstock J, Foster JA (2011) Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil 23(3):255–264, e119
12. Matsumoto M, Kibe R, Ooga T, Aiba Y, Sawaki E, Koga Y et al (2013) Cerebral low-molecular metabolites influenced by intestinal microbiota: a pilot study. Front Syst Neurosci 7:9
13. Schmitt JA, Wingen M, Ramaekers JG, Evers EA, Riedel WJ (2006) Serotonin and human cognitive performance. Curr Pharm Des 12(20):2473–2486
14. O'Brien ME, Anderson H, Kaukel E, O'Byrne K, Pawlicki M, Von Pawel J et al (2004) SRL172 (killed *Mycobacterium vaccae*) in addition to standard chemotherapy improves quality of life without affecting survival, in patients with advanced non-small-cell lung cancer: phase III results. Ann Oncol 15(6):906–914
15. Matthews DM, Jenks SM (2013) Ingestion of *Mycobacterium vaccae* decreases anxiety-related behavior and improves learning in mice. Behav Processes 96:27–35
16. Lowry CA, Hollis JH, de Vries A, Pan B, Brunet LR, Hunt JR et al (2007) Identification of an immune-responsive mesolimbocortical serotonergic system: potential role in regulation of emotional behavior. Neuroscience 146(2):756–772
17. Bonaz BL, Bernstein CN (2013) Brain-gut interactions in inflammatory bowel disease. Gastroenterology 144(1):36–49
18. Soderholm JD, Yang PC, Ceponis P, Vohra A, Riddell R, Sherman PM et al (2002) Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. Gastroenterology 123(4):1099–1108
19. Sun Y, Zhang M, Chen CC, Gillilland M, 3rd, Sun X, El-Zaatari M et al (2013) Stress-induced corticotropin-releasing hormone-mediated NLRP6 inflammasome inhibition and transmissible enteritis in mice. Gastroenterology 144(7):1478–1487 e8
20. Cameron HL, Perdue MH (2005) Stress impairs murine intestinal barrier function: improvement by glucagon-like peptide-2. J Pharmacol Exp Ther 314(1):214–220
21. Sweis BM, Veverka KK, Dhillon ES, Urban JH, Lucas LR (2013) Individual differences in the effects of chronic stress on memory: behavioral and neurochemical correlates of resiliency. Neuroscience 246C:142–159
22. Bailey MT, Dowd SE, Parry NM, Galley JD, Schauer DB, Lyte M (2010) Stressor exposure disrupts commensal microbial populations in the intestines and leads to increased colonization by *Citrobacter rodentium*. Infect Immun 78(4):1509–1519
23. Zareie M, Johnson-Henry K, Jury J, Yang PC, Ngan BY, McKay DM et al (2006) Probiotics prevent bacterial translocation and improve intestinal barrier function in rats following chronic psychological stress. Gut 55(11):1553–1560
24. Wagner KV, Hartmann J, Mangold K, Wang XD, Labermaier C, Liebl C et al (2013) Homer1 mediates acute stress-induced cognitive deficits in the dorsal hippocampus. J Neurosci 33 (9):3857–3864
25. Soderholm JD, Yates DA, Gareau MG, Yang PC, MacQueen G, Perdue MH (2002) Neonatal maternal separation predisposes adult rats to colonic barrier dysfunction in response to mild stress. Am J Physiol Gastrointest Liver Physiol 283(6):G1257–G1263

26. Uchida S, Hara K, Kobayashi A, Funato H, Hobara T, Otsuki K et al (2010) Early life stress enhances behavioral vulnerability to stress through the activation of REST4-mediated gene transcription in the medial prefrontal cortex of rodents. J Neurosci 30(45):15007–15018
27. Gareau MG, Jury J, Yang PC, MacQueen G, Perdue MH (2006) Neonatal maternal separation causes colonic dysfunction in rat pups including impaired host resistance. Pediatr Res 59 (1):83–88
28. Gareau MG, Jury J, MacQueen G, Sherman PM, Perdue MH (2007) Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation. Gut 56(11):1522–1528
29. O'Mahony SM, Marchesi JR, Scully P, Codling C, Ceolho AM, Quigley EM et al (2009) Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. Biol Psychiatry 65(3):263–267
30. Barreau F, Ferrier L, Fioramonti J, Bueno L (2004) Neonatal maternal deprivation triggers long term alterations in colonic epithelial barrier and mucosal immunity in rats. Gut 53 (4):501–506
31. Barreau F, de Lahitte JD, Ferrier L, Frexinos J, Bueno L, Fioramonti J (2006) Neonatal maternal deprivation promotes *Nippostrongylus brasiliensis* infection in adult rats. Brain Behav Immun 20(3):254–260
32. Suri D, Veenit V, Sarkar A, Thiagarajan D, Kumar A, Nestler EJ et al (2013) Early stress evokes age-dependent biphasic changes in hippocampal neurogenesis, BDNF expression, and cognition. Biol Psychiatry 73(7):658–666
33. Couto FS, Batalha VL, Valadas JS, Data-Franca J, Ribeiro JA, Lopes LV (2012) Escitalopram improves memory deficits induced by maternal separation in the rat. Eur J Pharmacol 695 (1–3):71–75
34. Aisa B, Gil-Bea FJ, Marcos B, Tordera R, Lasheras B, Del Rio J et al (2009) Neonatal stress affects vulnerability of cholinergic neurons and cognition in the rat: involvement of the HPA axis. Psychoneuroendocrinology 34(10):1495–1505
35. Baudin A, Blot K, Verney C, Estevez L, Santamaria J, Gressens P et al (2012) Maternal deprivation induces deficits in temporal memory and cognitive flexibility and exaggerates synaptic plasticity in the rat medial prefrontal cortex. Neurobiol Learn Mem 98(3):207–214
36. Meyer U, Feldon J, Fatemi SH (2009) In-vivo rodent models for the experimental investigation of prenatal immune activation effects in neurodevelopmental brain disorders. Neurosci Biobehav Rev 33(7):1061–1079
37. Jiang PF, Zhu T, Gao JD, Gao F, Mao SS, Zhao WT et al (2013) The effect of maternal infection on cognitive development and hippocampus neuronal apoptosis, proliferation and differentiation in the neonatal rats. Neuroscience 246:422–434
38. Wang KC, Fan LW, Kaizaki A, Pang Y, Cai Z, Tien LT (2013) Neonatal lipopolysaccharide exposure induces long-lasting learning impairment, less anxiety-like response and hippocampal injury in adult rats. Neuroscience 234:146–157
39. Bilbo SD, Newsum NJ, Sprunger DB, Watkins LR, Rudy JW, Maier SF (2007) Differential effects of neonatal handling on early life infection-induced alterations in cognition in adulthood. Brain Behav Immun 21(3):332–342
40. Ratnayake U, Quinn TA, Castillo-Melendez M, Dickinson H, Walker DW (2012) Behaviour and hippocampus-specific changes in spiny mouse neonates after treatment of the mother with the viral-mimetic Poly I:C at mid-pregnancy. Brain Behav Immun 26(8):1288–1299
41. Katan M, Moon YP, Paik MC, Sacco RL, Wright CB, Elkind MS (2013) Infectious burden and cognitive function: the Northern Manhattan Study. Neurology 80(13):1209–1215
42. Strandberg TE, Pitkala KH, Linnavuori KH, Tilvis RS (2003) Impact of viral and bacterial burden on cognitive impairment in elderly persons with cardiovascular diseases. Stroke 34 (9):2126–2131
43. Ringel Y, Maharshak N (2013) The intestinal microbiota and immune function in the pathogenesis of irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol 305:G529–G541

44. Stasi C, Rosselli M, Bellini M, Laffi G, Milani S (2012) Altered neuro-endocrine-immune pathways in the irritable bowel syndrome: the top-down and the bottom-up model. J Gastroenterol 47(11):1177–1185
45. Ghoshal UC, Ranjan P (2011) Post-infectious irritable bowel syndrome: the past, the present and the future. J Gastroenterol Hepatol 26(Suppl 3):94–101
46. Gomborone JE, Dewsnap PA, Libby GW, Farthing MJ (1993) Selective affective biasing in recognition memory in the irritable bowel syndrome. Gut 34(9):1230–1233
47. Gibbs-Gallagher N, Palsson OS, Levy RL, Meyer K, Drossman DA, Whitehead WE (2001) Selective recall of gastrointestinal-sensation words: evidence for a cognitive-behavioral contribution to irritable bowel syndrome. Am J Gastroenterol 96(4):1133–1138
48. Kilkens TO, Honig A, van Nieuwenhoven MA, Riedel WJ, Brummer RJ (2004) Acute tryptophan depletion affects brain-gut responses in irritable bowel syndrome patients and controls. Gut 53(12):1794–1800
49. Aizawa E, Sato Y, Kochiyama T, Saito N, Izumiyama M, Morishita J et al (2012) Altered cognitive function of prefrontal cortex during error feedback in patients with irritable bowel syndrome, based on FMRI and dynamic causal modeling. Gastroenterology 143(5):1188–1198
50. Faust AH, Halpern LF, Danoff-Burg S, Cross RK (2012) Psychosocial factors contributing to inflammatory bowel disease activity and health-related quality of life. Gastroenterol Hepatol (N Y) 8(3):173–181
51. Goodhand JR, Wahed M, Mawdsley JE, Farmer AD, Aziz Q, Rampton DS (2012) Mood disorders in inflammatory bowel disease: relation to diagnosis, disease activity, perceived stress, and other factors. Inflamm Bowel Dis 18:2301–2309
52. Goodhand JR, Greig FI, Koodun Y, McDermott A, Wahed M, Langmead L et al (2012) Do antidepressants influence the disease course in inflammatory bowel disease? A retrospective case-matched observational study. Inflamm Bowel Dis 18:1232–1239
53. Dancey CP, Attree EA, Stuart G, Wilson C, Sonnet A (2009) Words fail me: the verbal IQ deficit in inflammatory bowel disease and irritable bowel syndrome. Inflamm Bowel Dis 15 (6):852–857
54. Attree EA, Dancey CP, Keeling D, Wilson C (2003) Cognitive function in people with chronic illness: inflammatory bowel disease and irritable bowel syndrome. Appl Neuropsychol 10 (2):96–104
55. Castaneda AE, Tuulio-Henriksson A, Aronen ET, Marttunen M, Kolho KL (2013) Cognitive functioning and depressive symptoms in adolescents with inflammatory bowel disease. World J Gastroenterol 19(10):1611–1617
56. Mrakotsky C, Forbes PW, Bernstein JH, Grand RJ, Bousvaros A, Szigethy E et al (2013) Acute cognitive and behavioral effects of systemic corticosteroids in children treated for inflammatory bowel disease. J Int Neuropsychol Soc 19(1):96–109
57. Jeffery IB, O'Toole PW (2013) Diet-microbiota interactions and their implications for healthy living. Nutrients 5(1):234–252
58. Hold GL (2014) Western lifestyle: a 'master' manipulator of the intestinal microbiota? Gut 63:5–6
59. Li W, Dowd SE, Scurlock B, Acosta-Martinez V, Lyte M (2009) Memory and learning behavior in mice is temporally associated with diet-induced alterations in gut bacteria. Physiol Behav 96(4–5):557–567
60. Ohland CL, Kish L, Bell H, Thiesen A, Hotte N, Pankiv E et al (2013) Effects of *Lactobacillus helveticus* on murine behavior are dependent on diet and genotype and correlate with alterations in the gut microbiome. Psychoneuroendocrinology 38:1738–1747
61. Tillisch K, Labus J, Kilpatrick L, Jiang Z, Stains J, Ebrat B et al (2013) Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology 144 (7):1394–1401 e4
62. Lien do TK, Nhung BT, Khan NC, Hop le T, Nga NT, Hung NT et al (2009) Impact of milk consumption on performance and health of primary school children in rural Vietnam. Asia Pac J Clin Nutr 18(3):326–334

63. Benton D, Williams C, Brown A (2007) Impact of consuming a milk drink containing a probiotic on mood and cognition. Eur J Clin Nutr 61(3):355–361
64. Davari S, Talaei SA, Alaei H, Salami M (2013) Probiotics treatment improves diabetes-induced impairment of synaptic activity and cognitive function: behavioral and electrophysiological proofs for microbiome-gut-brain axis. Neuroscience 240:287–296
65. Benjamin J, Singla V, Arora I, Sood S, Joshi YK (2013) Intestinal permeability and complications in liver cirrhosis: a prospective cohort study. Hepatol Res 43(2):200–207
66. Bajaj JS, Ridlon JM, Hylemon PB, Thacker LR, Heuman DM, Smith S et al (2012) Linkage of gut microbiome with cognition in hepatic encephalopathy. Am J Physiol Gastrointest Liver Physiol 302(1):G168–G175
67. Patidar KR, Bajaj JS (2013) Antibiotics for the treatment of hepatic encephalopathy. Metab Brain Dis 28(2):307–312
68. Bajaj JS, Heuman DM, Sanyal AJ, Hylemon PB, Sterling RK, Stravitz RT et al (2013) Modulation of the metabiome by rifaximin in patients with cirrhosis and minimal hepatic encephalopathy. PLoS One 8(4):e60042
69. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG et al (2011) Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 108(38):16050–16055
70. Messaoudi M, Lalonde R, Violle N, Javelot H, Desor D, Nejdi A et al (2011) Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. Br J Nutr 105(5):755–764

Chapter 17
The Impact of Microbiota on Brain and Behavior: Mechanisms & Therapeutic Potential

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Abstract There is increasing evidence that host-microbe interactions play a key role in maintaining homeostasis. Alterations in gut microbial composition is associated with marked changes in behaviors relevant to mood, pain and cognition, establishing the critical importance of the bi-directional pathway of communication between the microbiota and the brain in health and disease. Dysfunction of the microbiome-brain-gut axis has been implicated in stress-related disorders such as depression, anxiety and irritable bowel syndrome and neurodevelopmental disorders such as autism. Bacterial colonization of the gut is central to postnatal development and maturation of key systems that have the capacity to influence central nervous system (CNS) programming and signaling, including the immune and endocrine systems. Moreover, there is now expanding evidence for the view that enteric microbiota plays a role in early programming and later response to acute and chronic stress. This view is supported by studies in germ-free mice and in animals exposed to pathogenic bacterial infections, probiotic agents or antibiotics. Although communication between gut microbiota and the CNS are not fully

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_17,

elucidated, neural, hormonal, immune and metabolic pathways have been suggested. Thus, the concept of a microbiome-brain-gut axis is emerging, suggesting microbiota-modulating strategies may be a tractable therapeutic approach for developing novel treatments for CNS disorders.

Abbreviations

5-HT	5-Hydroxytryptamine
APC	Antigen presenting cell
ASD	Autism spectrum disorder
BDNF	Brain derived neurotrophic factor
CNS	Central nervous system
DC	Dendritic cell
DHA	Docosahexaenoic acid
DSS	Dextran sodium sulphate
EC	Enteroendocrine cells
ECC	Enterochromaffin cells
ENS	Enteric nervous system
EPA	Eicosapentaenoic acid
GABA	Gamma-aminobutyric acid
GALT	Gut-associated lymphoid tissues
GI	Gastrointestinal
HDAC	Histone deacetylase
HPA	Hypothalamic-pituitary-adrenal axis
IBS	Irritable bowel syndrome
IDO	Indoleamine-2,3-dioxygenase
NMDAR	*N*-methyl-D-aspartate receptor
NPY	Neuropeptide Y
SCFA	Short chain fatty acid
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TDO	Tryptophan 2,3-dioxygenase
Treg	The regulatory T cells

Introduction

Though still in its early days, the twenty-first century may be seen as the era of the microbiota in scientific discovery. This is due to the significant increase in research investigating the role of the microbiota and in particular the gut microbiota in a wide spectrum of scientific and medical fields. The human microbiota, a collection of various microorganisms, comprises about 1–3 % of total body mass, hosting an impressive 100 trillion bacteria, most of which find their niche in the intestine

[1]. The majority of the microbial cells are comprised of bacteria from 500 to 1,000 different species varying in diversity and stability, adding over eight million genes to the human genome [2–4]. An individual's intestinal microbiota outnumbers somatic cells of the human body by approximately a factor of 10 [3], suggesting that an individual organism can no longer be identified as a single entity but rather as a complex ecosystem. Microbes colonize the digestive tract, reaching high numbers following birth and immediately thereafter [5], evolving and dynamically changing throughout one's lifespan [6]. The importance of microbiota for human health has been known since the early twentieth century, when Metchnikoff, the father of the modern probiotics, hypothesized that rebalancing bacteria in the gut with lactic acid could normalize bowel health and prolong life [7].

We now know that the microbiota plays a crucial role in maintaining physiological homeostasis including digestion, metabolism, growth, development and function of the immune system and resistance to pathogens [8–11]. More recently, an increasing volume of evidence has supported the relationship between the enteric microbiota and brain function [11] both in pre-clinical [12–14] and clinical settings [15–17]. The concept of the gut microbiota contributing to the range of central nervous system (CNS) disorders opens new avenues not only for developing novel therapeutic strategies in treating brain-gut axis dysfunctions, but also managing everyday stress responses.

Microbiota Throughout Lifespan

Although a stable core microbiome is shared among individuals, certain gut microbial populations fluctuate over time, depending on several factors such as mode of delivery, feeding regimen, maternal diet/weight, probiotic and prebiotic use and antibiotic exposure pre-, peri- and post-natally [18]. Bacterial colonization follows a relatively consistent pattern, under the influence of a variety of exogenous and endogenous factors. Exogenous factors include exposure to microorganisms from maternal origin such as gut, vaginal canal, or skin but also the environment in general. Endogenous factors encompass the birth delivery mode (vaginally or via cesarean section), gestational age, the type of feeding (breastfeeding or formula), and antibiotic or drug use [19].

The human host-microbe symbiosis is initiated in early life and its establishment is an intriguing and dynamic biological process. The developing microbiome undergoes its own evolution throughout the host's lifetime, in particular the first 3 years, during which a stable microbiome is established [20–22]. Despite the general dogma that a developing fetus is sterile up until birth [20, 23], increasing evidence suggests that an infant's initial microbiome might in fact be seeded by its mother prior to birth [24, 25] and is then supported by the presence of maternal microbes during birth [26] and breastfeeding [27, 28]. During and shortly after birth, infants are exposed to microbes mainly originating from the mother [29, 30]. Growing evidence suggests that it is this initial inoculation and subsequent

development of the intestinal microbiota in early life that is crucial for healthy development, especially neurodevelopment.

The mode of delivery at birth has recently attracted attention from the scientific community since infants delivered by C-section are more likely to suffer from allergies, asthma and diabetes later in life [21, 31, 32]. Although reasons for these correlations are difficult to tease apart, it has been linked to the crucial role of the early life environment in the development of a healthy microbiome. While the microbial composition of vaginally delivered infants initially resembles that of their mother's vaginal canal, the microbiota of infants delivered via C-section is more similar to the microbiota of their mother's skin [26]. Although infants delivered by C-section exhibit a delayed acquisition of the members (*Firmicutes* and *Bacteroidetes*) which dominate the adult microbiome, their microbiota composition does eventually match that of their vaginally delivered counterparts in later life [33]. It is currently unclear if birth mode can influence brain development and behavior.

In addition to the birth delivery mode, gestational age is thought to contribute to the microbial composition of the host. For example, the microbiota of the pre-term infants lacks two of the main bacterial genera, *Bifidobacterium* and *Lactobacillus*, usually present in full-term infants, and instead display a dominance of the *Proteobacteria* [34]. However, breastfeeding enriched the microbiota of the pre-term infants with the absent microbial species, enhancing the ability of the infant microbiome to utilize human milk oligosaccharides [20]. In addition to the maternal role in the developing infant's microbiome [35], genetic and environmental factors play a role in defining the adult core microbiome. For example, twin studies revealed higher similarities in the microbiota composition between monozygotic and dizygotic twins in comparison to other family members, suggesting a significance of the environmental factors over genetics [36, 37] and that microbial ecologies tend to cluster in family members [33]. The contribution of the genetic background and environmental factors to the microbiota of the host and the subsequent functional outcomes remains to be fully elucidated.

Knowing that the microbiota can significantly interfere with the human metabolic, cognitive, and immune systems, the initiation of the symbiosis especially during prenatal, early postnatal, and adolescence phases appears to be a crucial step for preparing optimal brain development overall and mental health later in life [38–41]. Consequently, understanding the early interaction between the intestinal microbiota and the host opens new avenues for therapeutic interventions, particularly for infants and young children. Unlike our genetic background, our gut microbiota may be modified in the first 2 years of life and possibly throughout pregnancy via the prenatal diet.

The gut microbiome evolves throughout the lifespan and the microbiota diversity declines with ageing, shifting in the dominant species but keeping a stable total number of anaerobic bacteria [42, 43]. It has recently been shown that microbial composition of aged individuals correlated with and was influenced by their residential community, dietary regimen and the health status of the individual [44]. Crucially, the loss of community-associated microbiota correlated with

increased fragility. Because of the geographical and ethnic homogeneity of the studied population, future investigations in heterogeneous cohorts are needed to support the importance of the interactions between diet, the microbiota, health and ageing [45].

The complex ecosystem of the host's microbiota is established at birth and its dynamic nature evolves throughout life span, suggesting its role in maintaining physiological processes potentially via the microbiota-brain-gut axis network. Bi-directional communication between the gut microbiota and CNS embodies several key components which converge to form a complex reflex network with afferents projecting to the CNS structures and efferents innervating the intestinal wall [11].

Interdisciplinary Conceptualization of the Microbiota-Brain-Gut-Axis

The concept of the microbiota-brain-gut axis is becoming increasingly recognized in scientific research, creating multidisciplinary collaborations in the fields of neuroscience, psychiatry, immunology, gastroenterology and microbiology. The initial concept of the brain-gut axis, which describes the complex bi-directional communication system linking the CNS and the gastrointestinal tract, stems largely from studies of the regulation of the digestive tract in the nineteenth century and preceded any notion that microorganisms residing in the gut played a modulatory role in brain function and development. The brain-gut axis plays an important role in maintaining homeostasis and its dysfunction has been implicated in various psychiatric and non-psychiatric disorders [46–51]. In addition, modulation of the brain-gut axis is linked to the stress response and altered behavior with the microbiome being an important factor in the brain-gut axis communication network [9, 46, 49, 52–54].

The microbiota-brain-gut axis is a complex network of communication between the gut, microbiota, and the brain which modulates gastrointestinal and CNS function [49, 52]. It encompasses the CNS, the sympathetic and parasympathetic branches of autonomic nervous system as well as the enteric nervous system (ENS) and the neuroendocrine and neuroimmune systems [46]. Afferent fibers which project from the gut to cortical centers of the brain such as cerebral, anterior and posterior cingulate, insular, and amygdala cortices and as well as effector fibers projecting to the smooth muscle of the gut are the major routes for bi-directional communication along this axis [55]. Most of our knowledge about the microbiota-brain-gut axis is primarily confined to neuronal communication between the ENS and the CNS, however, the exact role of the microbiota has yet to be elucidated.

Pathways of Microbiota-Brain-Gut Communication

Neural Pathways

Within the gut, the microbiota-to-neuron signaling has been shown to depend on signaling within the ENS. The ENS innervation of the gut is a complex network of neurons comprised of sensory, motor, and interneurons that are capable of independently regulating basic GI functions (motility, mucous secretion, and blood flow). Due to the similarity of ENS to CNS and its autonomous nature, the ENS is often referred to as the 'second brain'. The autonomic nervous system interacts with the ENS via its main neurotransmitters, adrenaline, noradrenaline and acetylcholine, and via efferent and afferent neurons, which innervate the gut [56].

Moreover, specific subsets of enteric neurons in the colonic myenteric plexus of rats have recently been shown to be sensitive to microbial manipulation, specifically, a *Lactobacillus reuteri* strain. It was shown to activate calcium-dependent potassium channels in this subset of ENS neurons, which may lead us to postulate that these effects on gut motility and pain perception are mediated by a direct link between microbiota and the ENS [57]. A more recent study has shown electrophysiological properties of myenteric neurons are altered in germ-free mice specifically; decreased excitability in myenteric sensory neurons was found in the absence of intestinal microbiota. Upon colonization of germ-free mice with normal gut microbiota, excitability of after-hyperpolarization sensory neurons in germ-free mice was increased [58].

Another neuronal pathway by which the microbiota can communicate with CNS is via the vagus nerve. The vagus nerve is the major nerve of the parasympathetic division of the autonomic nervous system, which regulates several vital body functions, including heart rate, gut motility, and bronchial constriction [59, 60]. Microbiota can elicit signals via the vagal nerve to the brain and vice versa [61–63] (Fig. 17.1). For example, the behavioral effects mediated by two separate probiotic strains in rodents were dependent on intact vagal nerve activation [64]. Specifically, chronic treatment with *Lactobacillis rhamnosus* induced region-dependent alterations in GABA receptor expression in the brain and reduced stress-induced corticosterone and anxiety- and depression-like symptoms via vagus nerve signaling [13]. Similar effects were observed in an animal model of colitis, where anxiolytic effect of *Bifidobacterium* was absent in vagotomized mice [65]. In contrast to probiotic-mediated effects, antibiotic treatment-induced microbiota alterations in mice did not show a similar dependence on vagal nerve activity [66] suggesting that enteric microbiota communicates with the brain by diverse mechanisms (Fig. 17.1). In addition to neuroanatomical complexity, neurochemistry may play a vital role in modulating microbiota-brain-gut communication.

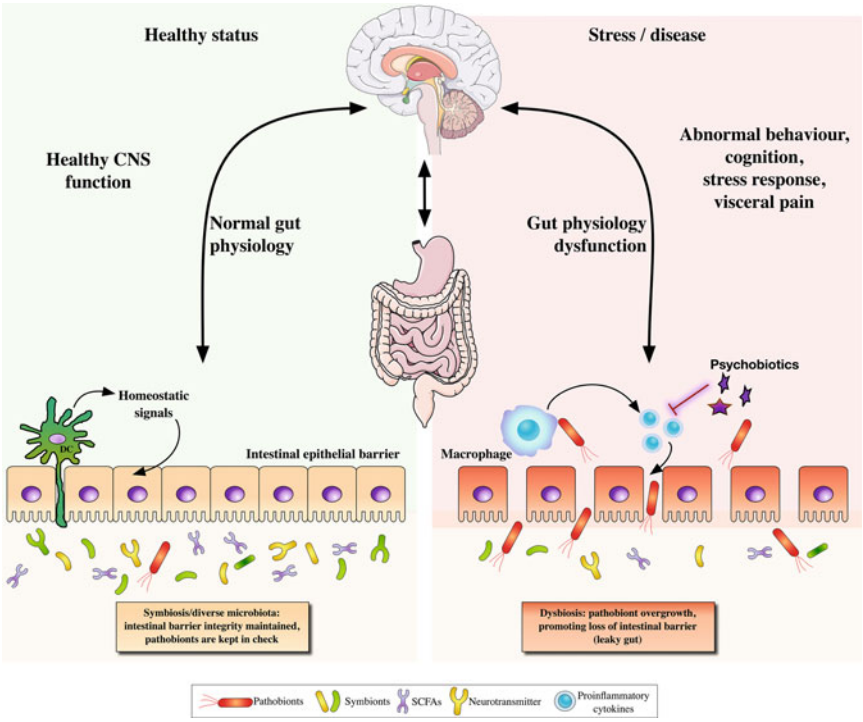

Fig. 17.1 Microbiota-brain-gut axis communication in health and disease (*left hand side*) Under healthy conditions, the predominance of symbiotic bacteria, an intact intestinal barrier, a healthy innate immunity controlling pathobiont overgrowth inside the intestinal tract and healthy gut function support the symbiotic relationship between the CNS function and gut microbiota. (*Right hand side*) Under pathological stress and/or disease conditions, intestinal dysbiosis can adversely influence gut physiology leading to inappropriate brain–gut axis signaling and associated consequences for CNS functions and disease states. Stress at the level of the CNS can also impact on gut function and lead to perturbations of the microbiota. A change the balance of symbionts and pathobionts favoring pathobiont overgrowth, results in dysbiosis. Pathobiont overgrowth leading to perturbations in intestinal microbiota induces inflammation and loss of barrier function (leaky gut), promoting increased translocation of pathogenic bacterial components from the intestinal mucosa to the systemic circulation, where they activate innate immunity characterized by production of pro-inflammatory cytokines, resulting in systemic inflammation and abnormal gut function. These mechanisms potentially lead to impaired CNS function such as altered neurochemistry, cognition, behavior, stress response and visceral pain. *DC* dendritic cells

Serotonin and Tryptophan Metabolism Pathway

Serotonin [5-hydroxytryptamine (5-HT)] is a biogenic amine that functions as a neurotransmitter in the body, both in the CNS and the gut. Peripheral 5-HT is involved in the regulation of GI secretion, gut motility, and pain perception [67, 68], in the brain it plays an important role maintaining mood and cognition [69]. Alterations in serotonin transmission may underlie the pathological symptoms

of both GI and some psychiatric disorders, and, may explain their high co-morbidity [70]. Pharmacotherapy modulating serotonergic neurotransmission, such as tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs), have shown therapeutic efficacy in the treatment of both affective and GI disorders [71–73].

Serotonin synthesis in the brain depends on the availability of its precursor, tryptophan. Within the CNS, tryptophan concentrations are dependent on peripheral supply derived from the diet. Acute tryptophan depletion using tryptophan-restricted diets results in decreased tryptophan plasma levels and consequently decreased CNS serotonin levels in animals [74] and healthy human volunteers [75]. The enteric microbiota appears to play a role in tryptophan availability and metabolism, having an indirect effect on serotonin concentrations in the brain [76]. Further support for the relationship between the gut microbiota and tryptophan metabolism comes from germ-free mouse studies, where the absence of the microbiota in early life resulted in increased plasma tryptophan concentrations and increases in hippocampal serotonin levels in adulthood [41]. Importantly, the former measures are restored following the introduction of bacteria in germ-free mice post weaning [77].

Tryptophan metabolism along the kynurenine pathway, the dominant physiological fate for this essential amino acid and one that is increasingly scrutinized in many disorders of both the brain and gastrointestinal tract [78–80], also warrants consideration. The initial rate-limiting step in this metabolic route is catalyzed by either the ubiquitous indoleamine-2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO) which is of hepatic origin [81]. The activity of these enzymes can be induced by either mediators of inflammation (IDO) or by corticosteroids (TDO) and there is evidence of altered enzyme activity in irritable bowel syndrome (IBS), a disorder associated with altered microbiota profiles [82–84]. The probiotic *Bifidobacterium infantis* affects tryptophan metabolism along this pathway [76] although this does not appear to generalize to all *Bifidobacterium* strains as administration of *Bifidobacterium longum* does not affect kynurenine concentrations [85]. The absence of the microbiota in early life, which increases plasma tryptophan concentrations, also reduces the kynurenine:tryptophan ratio which is used as an index of either IDO or TDO enzyme activity [41]. Importantly, normal enzyme activity is restored following the introduction of bacteria in germ-free mice post weaning.

In summary, the gut microbiota may play a crucial role in tryptophan availability and metabolism and consequently impact on central serotonin concentrations as well as kynurenine and downstream neuroactive metabolites. These effects may be facilitated by indirect immune-mediated or endocrine mechanisms or by a more direct route by modulating tryptophan metabolism at the level of the gut. For example, in certain bacteria, indole is produced from tryptophan by the tryptophanase enzyme [86]. More studies are warranted to elucidate the underlying processes involved in modulation of this microbiota-brain-gut axis communication pathway.

Gut Hormonal Response Pathway

In addition to immune and neural pathways, the gut communicates to the brain via hormonal signaling pathways that involve the release of gut peptides from enteroendocrine cells, which can act directly on the brain. Gut peptides such as orexin, galanin, ghrelin, gastrin, and leptin, modulate feeding behavior, energy homeostasis, circadian rhythm, sexual behavior, arousal, and anxiety [87, 88]. For example, galanin is suggested to modulate the hypothalamic-pituitary-adrenal axis (HPA) response to stress and may act as a link between stress, anxiety, and memory given the established adverse effects of galanin on cognitive function [89, 90]. Similarly, ghrelin may be involved in the modulation of the HPA response to stress or changes in metabolic status [91, 92]. Leptin receptors can be found in limbic structures, and chronic leptin treatment reverses stress-induced behavioral deficits [93], suggesting a potential role for this hormone in emotional processes [94]. Moreover, antidepressant effects of leptin have been shown in diabetic mice [95]. The idea that changes in enteric microbiota composition can alter gut hormone release is supported by probiotic studies [96, 97]. Furthermore, germ-free studies suggest that the gut microbiota mediates and regulates the release of gut peptides [98], yet little is known about the underlying mechanism of the hormonal aspect of the microbiota-brain-gut communication. Neuropeptide Y (NPY) is another target thought to be involved in microbiome brain interactions as it is sensitive to microbiota manipulations and functions both as a neural and endocrine messenger [99]. NPY is present at numerous locations throughout the microbiota-brain-gut axis and have a broad array of functions such as regulation of mood, stress resilience and gastrointestinal motility. The role of the gut hormonal response in the microbiota-brain-gut crosstalk is clearly an area of research that demands more attention and may offer novel therapeutic targets for the brain-gut axis disorders.

Immune System Pathway

The immune system plays an important role in maintaining the delicate balance between the brain and the gut [8, 100]. The intestinal microbiota imprints the mucosal immune system and modulates the immune activation outside the gastrointestinal tract of the host [101]. The gut and the gut-associated lymphoid tissues (GALT) are the largest immune organ of the human body, providing a defensive barrier between externally-derived pathogens and the internal environment [102]. The gut is patrolled by a variety of immune cells such as T-cells (T_{reg}) and antigen presenting cells (APCs), which can traffic from GALT to other peripheral lymphoid sites including the CNS. Immune cell produced within the gut could cross the blood-brain barrier and be reactivated within the CNS by the resident APCs [10]. During homeostasis, a fine balance is maintained between the microbiota and the innate mucosal immune system of the host; but perturbations of the intestinal

microbial composition disturb this equilibrium, resulting in activation of toll-like receptors and consequent alteration of cytokine profiles, which may lead to impaired behavior and cognition. For example, peripheral administration of pro-inflammatory cytokines in rodents induces sickness behaviors such as depressive-like symptoms, disrupted circadian rhythm and reduced appetite [103, 104]. Moreover, the state of the adaptive immune system has been implicated in a range of psychiatric [105] and neurodevelopmental disorders [106].

Despite a growing appreciation of the role played by the intestinal microbiota in immune responses, the precise immunomodulatory mechanisms remain to be elucidated. It has been proposed that immunomodulating effects of probiotic microorganisms may occur through the generation of T_{reg} cell populations and the synthesis of the anti-inflammatory cytokines [107, 108]. Furthermore, colonization of germ-free mice with commensal bacteria promotes T_{reg} and IL-10 production [109], suggesting that alterations in the enteric microbiota may impact behavior by modulating inflammatory responses of the host in the periphery and the brain (Fig. 17.2).

Bacterial Metabolite Pathways

In addition to the above-mentioned potential pathways, bacterial products or metabolites from gut commensals, such as short chain fatty acids (SCFAs), may translocate from the intestinal mucosa to the systemic circulation, where they could interfere with immune regulation and CNS function. SCFAs such as acetate, butyrate or propionate are produced via the fermentation of dietary carbohydrates and have immunomodulatory properties [110]. SCFAs can interact with nerve cells by stimulating the sympathetic nervous system [111] and butyrate in particular has been suggested to modulate brain function via histone deacetylase (HDAC) inhibition [112]. Specifically, systemic injection of butyrate exerted antidepressant-like effects by inducing histone hyperacetylation in mice [113]. Moreover, administration of propionate resulted in autistic-like symptoms in rats [114, 115]. Increasing emphasis is thus being placed on SCFAs as key microbial-induced epigenetic modifiers of brain-gut function [116].

The Microbiome and Behavior

One of the most exciting areas in examining the role of microbiome in health and disease is the effects of the microbiome on behavior. Several approaches including the use of germ-free animals, dietary changes, exposure to adverse stressful events, animals with pathogenic bacterial infections, animals exposed to microbiota-modulating agents such as pro-, pre- and anti-biotics (Table 17.1) have been used to tease apart the role of microbiota on brain and behavior. These data have yielded

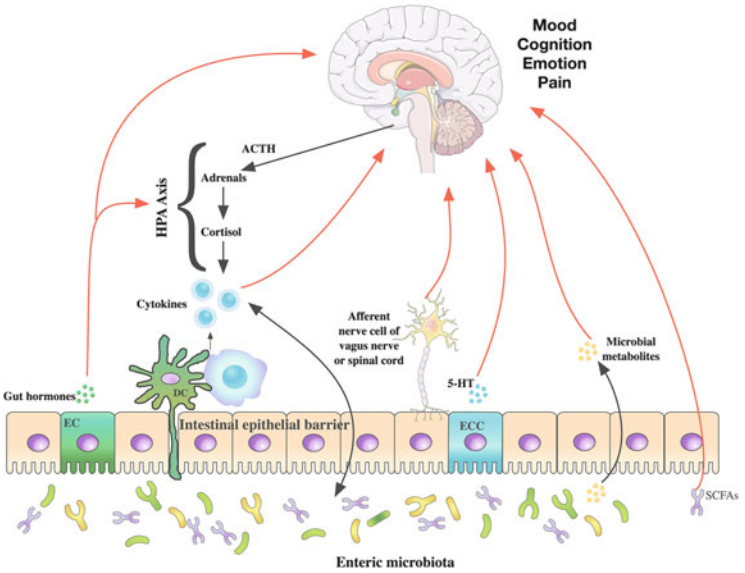

Fig. 17.2 Potential pathways underlying the communication along the microbiota-brain-gut axis. Several pathways have been proposed to understand the communication between the intestinal microbiota and brain function, some of which have been summarized in this figure. These include neuroendocrine (hypothalamus-pituitary-adrenal axis), immune system (neuromodulating cytokines), enteric nervous systems and autonomic nervous systems (vagus nerve). Gut microbes produce tryptophan-related metabolites, gut hormones, 5-hydroxytryptamine (5-HT) from enteroendocrine cells, short chain fatty acids, and neurometabolites GABA, noradrenalin, and dopamine potentially modulating CNS function. Stress and emotions can influence the microbial composition of the gut through the release of stress hormones or sympathetic neurotransmitters that influence gut physiology and alter the microflora balance. *DC* dendritic cell, *EC* enteroendocrine cells, *ECC* enterochromaffin cells

fascinating results highlighting the importance of the microbiome in both health and disease. However, the diversity and multifunctionality of the microorganisms residing in the enteric microbiota add an extra complexity to the equation.

Germ Free Studies

Germ-free animals are powerful tools for examining the relationship of the gut microbiota and brain function. Germ-free animals are maintained in a sterile environment in gnotobiotic units, eliminating the chance of the post-natal colonization of their GI tract, thus, being a 'microbiota-free' control group for the conventionally colonized gut of their counterparts. Despite exaggerated neuroendocrine responses to stress as demonstrated by increased basal levels of plasma corticosterone [117], several independent laboratories have demonstrated

consistent decreases in anxiety-like behavior in germ-free mice when exposed to novel and aversive environments (elevated plus maze, light/dark box, open field) [41, 117, 118] (Table 17.1). Decreased anxiety in germ-free mice is normalized following post-weaning bacterial colonization of germ-free mice. However, this effect is dependent on the time of colonization, be it in adolescence or adulthood [10, 41, 118], proposing neurodevelopmental sensitive time periods [119–121]. In addition to decreased anxiety, germ-free mice show social impairments and increased stereotypical behaviors [122], suggesting that the gut microbiota plays a role in socially-driven behaviors which may be of relevance to certain psychiatric and/or neurodevelopmental disorders (see section "Autism"). At the cognitive level, germ-free mice demonstrate impairments in non-spatial and working memory tasks (novel object recognition and spontaneous alternation assessed in the T-maze) [123]. Further assessment of the cognitive and behavioral phenotype of germ-free animals would allow us to fully appreciate the role of microbiota in the modulation of behavioral and cognitive function of the host.

At the molecular level, germ-free mice have reduced levels of *N*-methyl-D-aspartate receptors (NMDARs), specifically the NR1 and NR2A subunits, in the hippocampus [124], or NR2B subunits in the amygdala [117]. These molecular targets have been shown to play a key role in neuropsychiatric disorders [125]. Moreover, germ-free animals have decreased levels of brain derived neurotrophic factor (BDNF), a key neurotrophin involved in neuronal growth and survival [124]. Interestingly, germ-free effects seem to be sex dependent. While a decrease in hippocampal BDNF mRNA expression was observed in male germ-free animals, a qualitative increase in BDNF mRNA expression was present in female germ-free mice [41], suggesting that BDNF expression differences are related to sex. Moreover, genetic background appears to play a major role in modulating the microbiome-brain-gut axis. In summary, germ-free studies demonstrate utility in teasing apart the mechanisms underlying the microbiota-brain-gut axis communication relevant to brain function.

Antibiotics

Another way to artificially modulate microbiota and induce intestinal dysbiosis is via the administration of the antimicrobial drugs. Several studies demonstrated that antibiotic treatment leads to a microbiota perturbation as demonstrated by in vitro [126] and in vivo experiments [66, 127]. Administration of an antibiotic cocktail in rodents leads not only to microbiota depletion but also an increased visceral pain sensitivity [128], hyperlocomotion and altered BDNF levels in the brain [65]. Interestingly, similar antibiotic treatment in germ-free mice showed no change in behavior, but colonization of the germ-free mice with the microbiota of BALB/C mice resulted in significant increases in anxiety-like symptoms [66], emphasizing the role of the host microbiota on behavior. Further evidence comes from clinical setting, where short-term antibiotic treatment affected evolution of the infant gut

Table 17.1 Studies of microbiota-gut brain axis

Model	Basal behaviour	Behavioural test	Microbiota manipulation	Treatment effect	References
GF (mice)	Reduced anxiety, hyperlocomotion and increased rearing	OF, LDB, EPM	na	na	Diaz Heijtz et al. [118]
GF (mice)	Reduced anxiety	EPM	na	na	Neufeld et al. [117]
GF (mice)	Reduced anxiety	LDB		na	Clarke et al. [182]
C. rodentium infection (mice)	Increased anxiety	OHB	na	na	Lyte et al. [63]
Naive mice		OF, EPM, FST	*L. rhamnosus*	Reduced anxiety	Bravo et al. [13]
		Cued FC		Enhanced fear memory	Bravo et al. [13]
Trichuris muris (nematode parasite) (mice)	Increased anxiety	LDB	na	na	Bercik et al. [85]
DSS induced colitis (mice)	Reduced anxiety	SD	*B. longum*	Normalized	Bercik et al. [65]
C. jejuni (food pathogen) (mice)	Increased anxiety	OHB, EPM			Goehler et al. [204]; Lyte et al. [205]
Naive rat		CDB	*L. helveticus* +*B. longum*	Decreased anxiety	Messaoudi et al. [15]
Maternal separation (rat)	Depressive-like symptoms	FST	*B. infantis*	Normalized	Desbonnet et al. [12]
Myocardial infarction (mice)	Depressive-like symptoms & impaired social interaction	FST, SD	*L. helveticus* +*B. longum*	Normalized	Arseneault-Bréard et al. [206]; Gilbert et al. [207]
Diabetes (rats)	Impaired spatial memory	MWM	*B. lactus* +*L. fermentum*	Improved	Davari et al. [51]
GF (mice)	Impaired (short-term) recognition memory and working memory regardless of stress	NOR, TM	na	na	Gareau et al. [123]

(continued)

Table 17.1 (continued)

Model	Basal behaviour	Behavioural test	Microbiota manipulation	Treatment effect	References
C. rodentium infection (mice)	Impaired (short-term) recognition memory and working memory after stress induction	NOR, TM	*L. rhamnosus* + *L helveticus*	Normalized	Gareau et al. [123]
GF (mice)	Avoidance of conspecifics, increased stereotypical behavior	TCST, grooming	na	na	Desbonnet et al. [122]
GF (mice)	Decreased social investigation	STFP			Desbonnet et al. [122]
GF (mice)	No preference for novel conspecific	TCST			Desbonnet et al. [122]
Maternal immune activation (mice)	Increased anxiety	MB/OF	*B. fragillis*	Normalized	Hsiao et al. [132]
	Impaired social interaction	TCST		No change	
	Increased stereotypical behaviour	Grooming		Normalized	
Antibiotic treatment & GF (mice)	Increased anxiety, hyperlocomotion	LDB, SD, OF	na	na	Bercik et al. [53]
Naive mice		HLB	50 % lean ground beef diet	Increased working and reference memory decreased anxiety	Li et al. [208]

Several behavioral parameters were shown to be altered in germ-free mice or rodents treated with either probiotics or infectious bacteria or parasites. Probiotic treatment effects on the behavioral and cognitive function are shown

na not applicable, *NOR* novel object recognition, *MB* marble burrowing, *TM* T-maze, *TCST* three-chambered sociability test, *STFP* social transmission of food preference, *LDB* light-dark box, *FST* forced swim test, *FC* fear conditioning, *OHB* open hole board, *SD* step-down, *MWM* Morris water maze, *CDB* conditioned defensive burying, *HLB* hole-board

microbiota, disturbing the colonization pattern of *Bifidobacterium* in the first months of life [129]. Antibiotic cocktail treatment provides an attractive alternative to germ-free mice as means to investigate the role of microbiota-brain-gut function. Although antibiotic-induced dysbiosis and consequent behavioral abnormalities provide further support for the role of microbiota in gut-brain communication, the pathways of antimicrobial-based strategies remain unknown and future studies are warranted to extricate the underlying mechanisms.

Probiotics and Prebiotics

Maintaining homeostasis of the gut ecosystem is essential for health, and dietary manipulations such as pre-and-probiotics may present a therapeutic strategy to impact gastrointestinal and CNS-driven disorders. Probiotics are defined as 'live organisms which when administered in adequate amounts confer a health benefit on the host'. *Lactobacillus* and *Bifidobacterium* are the main genera of microorganisms used as probiotics and several studies have demonstrated their beneficial effects on behavior of the host. For example, *L. rhamnosus* treatment decreased anxiety and depressive-like symptoms in mice [13]. Similarly, early-life stress-induced depressive-related behaviors were reduced after treatment with the probiotic *B. infantis*, an effect similar to that of the antidepressant citalopram [12]. Interestingly, the status of the host is critical to the efficacy of probiotics with certain strains exhibiting beneficial effects during pathological states such as IBS without an effect in healthy controls.

L. helveticus reduced anxiety-like behavior and attenuated memory deficit in both naïve and western diet fed mice [130]. Treatment with live *Mycobacterium vaccae* resulted in reduced anxiety and improved cognitive performance [131]. *Bifidobacterium* normalized anxiety-like behavior in the dextran sodium sulphate (DSS)-induced colitis model [65]. Furthermore *Bifidobacterium*, but not *L. rhamnosus*, normalizes anxiety-like behavior in *Trichuris muris* infection [85]. Combining probiotic strains have shown therapeutic efficacy as well. For example, *L. rhamnosus* and *L. helveticus* treatment rescued stress-induced memory impairment in mice [123]. Most recently, *Bacteroides fragilis* treatment in addition to improving GI function and restoring serum metabolites, alleviated some of the autism-related behavioral traits such as anxiety, sensorimotor gating, and stereotypical behaviors in a mouse model of autism [132], providing further support for the potential of probiotic therapy in combating neurodevelopmental disorders. In addition to the ameliorating effects of probiotics on behavior and cognition, probiotics have also proved efficacious in alleviating visceral pain responses [128, 133–135].

Although the use of probiotics in animal studies has demonstrated therapeutic efficacy on anxiety- and depressive-like behaviors, data on the effects of probiotics on depression or anxiety symptoms in humans is rather sparse to date. In a double-blind, placebo-controlled, randomized parallel group clinical trial, combinational

treatment of a mixture of probiotics containing *L. helveticus* and *Bifidobacterium* in a healthy subjects for 30 days resulted in a significantly less psychological distress compared to the placebo group [15]. In a similar study, treatment with a probiotic-containing milk drink resulted in improved mood and cognition in health subjects when compared to the placebo group [136]. In a pilot study, patients suffering from the chronic fatigue syndrome receiving *Lactobacillus casei* daily for 2 months showed significant reduction in anxiety compared to the placebo group [16]. Specifically, treatment with a fermented milk product with probiotic reduced the response of healthy volunteers and altered activity of the brain regions, controlling central regions of emotion and sensation to an emotional faces attention task [137]. Although these beneficial effects have yet to be demonstrated in pathological anxiety, combination of *L. helveticus* and *Bifidobacterium* alleviated psychological distress in healthy subjects [138].

Unlike probiotics, prebiotics have not received as much attention, but have demonstrated promising results [139]. Prebiotics are non-digestible food ingredients that selectively stimulate the growth of *Lactobacilli* and *Bifidobacteria* in the gut [140]. Increasing the proportion of these bacteria with prebiotics has many beneficial effects on the gut, the immune system [141–143], and most recently on the brain function, specifically, increased BDNF expression and NMDAR signaling [14], providing initial support for further investigations of the utility of prebiotics in mental health and potential treatment of psychiatric disorders.

Taken together, studies examining the impact of pro-and-prebiotics on cognition and behavior (see Table 17.1) are in the early stages, yet these preliminary results offer novel therapeutic potential for treating mood and anxiety disorders with psychobiotic-based approaches [107]. Despite promising evidence that certain pre- and/or pro-biotic strains are able to modulate brain function and behavior, caution is warranted when translating and generalizing the pre-clinical data into the clinical domain. Clinical validation and identifying underlying mechanisms of the microbe-based therapeutic effects are essential prior to assessing the actual value of these microbiota-modulating agents in treating disorders of the microbiota-brain-gut axis.

Disorders of the Microbiome-Brain-Gut Axis

In healthy individuals, the normal dominant microbiota is relatively stable and is in a symbiotic rapport with the host. Perturbations in the delicate symbiotic host-microbiota relationship may have serious consequences resulting in various psychiatric and non-psychiatric disorders, collectively known as the disorders of the brain-gut axis.

Stress

Despite the well-established association between stress and psychiatric disorders, the struggle to understand the complex processes by which stress mediates pathological changes that increase vulnerability to disease is on-going [144]. Although stress is a natural occurrence, chronic, severe, and uncontrollable stressors can trigger abnormal changes in brain structure and function with long-term physical and mental stress [145–147]. Chronic stress has been a common denominator in several GI disorders and a key player in microbiota-brain-gut axis dysregulation of the stress-related CNS diseases [9, 11, 38, 148–151]. Animal studies have shown that emotional stressors, such as maternal separation, restraint conditions, crowding, heat stress and acoustic stress negatively impact on the composition of microbiota [152–159]. The association between microbiota and stress-response is further supported by experiments with germ-free mice and rodents treated with probiotics and/or antibiotics. Sudo and colleagues [124] demonstrated an enhanced HPA axis activity in germ-free mice following an acute psychological stress, providing first convincing evidence of the essential role played by microbiota in programming of the stress response. Moreover, increased hippocampal 5-HT and 5-HIAA as well as plasma tryptophan was found in germ-free males [41]. Several independent germ-free studies are summarized in Table 17.1. Furthermore, monoamine neurotransmission has been shown to be increased in the colonized germ-free mice compared with germ-free mice [119], resulting in an altered behavioral phenotype compared to germ-free mice.

Another line of evidence comes from the studies in the maternal separation model, one of the best-characterized animal models in relation to the long-term effects of stress in early life on microbiota [157]. In light of the mutual relationship that exists between the stress response and microbiota, it is not surprising that the period most critical to HPA axis development and programming of the neuroendocrine stress response, early postnatal life, is also an important time-point for the initial establishment of the core gut microbiota. This model and others have been employed to investigate numerous aspects of brain gut interactions more recently the effects of probiotic treatment on fatty acid metabolism of the host [160–162]. This data is particularly relevant in the context of depression with an increasing body of evidence highlighting the role of fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in depressive disorders [163].

One of the principal mechanisms proposed to underlie stress-induced alterations is the “leaky gut” phenomenon, which has also been described in major depression [164] (Fig. 17.1). It is hypothesized that the epithelial barrier of the gastrointestinal tract is compromised as a result of either a stressful event or microbiota dysbiosis, leading to increased intestinal permeability and the consequent translocation of pathobionts across the mucosal lining to sites where direct interaction with immune cells and the ENS can occur [165]. This leads to activation of an immune response characterized by increased production of pro-inflammatory mediators in circulation and eventually the CNS. In support of this hypothesis, pre-treatment with the

probiotic *Lactobacillus farciminis* attenuated the effects of acute restraint stress on intestinal permeability and HPA axis response [166] (Fig. 17.2).

Depression and Anxiety

Depression and general anxiety are disorders with well-established etiological links to the traumatic life events, particularly when experienced in early life and during periods of chronic stress [167, 168]. Although current clinical literature contains few reports that describe the state of the gut microbiota in depressed individuals, data from associated illnesses, such as IBS, which are often accompanied by depressive symptoms, have revealed reduced *Bacteroidetes* and increased *Firmicutes* content in the fecal samples of patients with these disorders [169–171]. These findings emphasize that the microbiota is an attractive target for potential therapeutic strategies whereby altering the host's gut microbiota may infer health benefits to the host.

Indeed, animal studies focusing on the effects of chronic probiotic treatment, both in naïve and stress-related animal models of CNS disorders, have also served as invaluable and informative tools in ascertaining the specific contributions made by gut bacteria to anxious and depressive disorders. Probiotic treatment during the postnatal stress period in maternally separated rat offspring has been shown to normalize basal corticosterone levels [172]. When administered to naïve rodents, *L. rhamnosus* reduced stress-induced corticosterone, which was paralleled by region-dependent alterations in GABA receptor gene expression levels in the brain [13]. Moreover, the neurochemical effects were not found in vagotomized mice, identifying the vagus nerve as a major modulatory communication pathway between the bacteria exposed to the gut and the brain [13]. *B. infantis* altered peripheral cytokine levels and concentrations of the serotonin precursor, tryptophan, which may allude to the development of possible protective mechanisms prior to stress exposure [76]. The therapeutic potential of probiotics in psychiatric conditions has been the topic of intense discussion and additional investigations are required to fully elucidate the role of probiotics in brain function [173, 174].

Despite the lack of clinical data to support the idea to utilize probiotics in treatment of mood disorders, there are sufficient pre-clinical data to support this view. Potential psychobiotics are delivery vehicles for neuroactive compounds, and have a capacity to reduce inflammatory response and reduce HPA activity, a much broader profile than of existing antidepressant treatment options [107]. As not all probiotics are equal in their effects and may not have psychobiotic potential, a careful examination of their efficacy is warranted. There is no doubt that many patients, particularly those with milder symptom profiles, would value the rise of nonconventional antidepressants in the form of psychobiotics.

Taken together, the enteric microbiota has a significant impact on the behavioral, neurochemical and immunological measures relevant to the brain-gut axis disorders with psychobiotics as a promising emerging treatment [107] (Fig. 17.2).

Irritable Bowel Syndrome (IBS)

IBS is a disorder of the brain-gut axis and in addition to gastrointestinal symptoms is associated with frequent comorbidities of depression and anxiety [82]. Although the precise mechanisms of the underlying pathology remains unclear, the role of brain-gut communication in the etiology of IBS has become widely recognized with altered CNS control of visceral pain and inflammatory responses being integral pathophysiological features [175]. Recent neuroimaging studies demonstrated thinning of the anterior cingulate and insular cortex of the IBS patients [176, 177] increased activation of the thalamus, anterior cingulated and prefrontal cortex and microstructural reorganization in the IBS patients which was correlated with symptoms of visceral hypersensitivity, providing further evidence of the abnormal brain function in disease pathology.

Chronic low-grade inflammation is a common feature in many IBS patients and studies have identified several susceptibility genes for IBS involved in innate immunity and recognition of bacteria [178]. Subgroups of IBS patients may have an altered microbiota composition relative to healthy individuals based on the analysis of fecal microbiota [170, 179]. Altered microbiota diversity [180, 181] has been reported in IBS patients, indicating a loss of homeostasis of the intestinal bacterial ecosystem [44]. Although the specific mechanisms by which changes in the gut microbiota lead to IBS symptoms remain unclear, it is hypothesized that an influx of certain microbes such as *Lactobacilli* and *Veillonella* in IBS patients result in a high level of organic acids such as acetic and propionic acid, which in turn may contribute to intestinal discomfort and anxiety [170]. In light of these promising preliminary findings it is not surprising that a positive effect of treatment with microbial-based therapeutics (both non-absorbable antibiotics such as rifaximin and probiotics) has been demonstrated in IBS [140, 182–185].

Autism

Neurodevelopmental disorders are characterized by impaired brain development and behavioral, cognitive, and/or physical abnormalities. Several share behavioral abnormalities in sociability, communication, and/or compulsive activity. Autism spectrum disorders (ASDs) are neurodevelopmental disorders characterized by presence of stereotypical behavior and social interaction deficits [186]. Although the ASD etiology remains unknown, genetic and environmental factors are thought to play a role in the development of ASD [187]. Among several comorbidities in ASDs, gastrointestinal (GI) distress is of particular interest, given its high prevalence and correlation with symptom severity [188, 189]. GI abnormalities in ASDs have been linked to alterations in microbiota composition and function [6, 132, 190–192]. Despite these correlational studies, clinical data interpretation is compromised by high rates of antibiotic use and dietary variations in ASD patients,

which makes it difficult to draw definite conclusions about ASD-related microbiota changes [11]. Studies in germ-free mice demonstrated robust and reproducible social deficits and increases in repetitive behaviors similar to that observed in ASD [122], suggesting that the microbiota is a critical factor in the development of social behavior and the etiology of ASDs. Most recently, studies demonstrated that autism-like behavioral and GI phenotypes are associated with altered microbiota in two separate mouse models of ASDs [132, 190]. Both clinical and pre-clinical studies provide promising evidence indicating an important role for the gut microbiota in the pathogenesis of ASDs, creating opportunities for developing novel therapeutic strategies in managing neurodevelopmental disorders via microbiome-based treatment. Indeed, *B. fragilis* given in early adolescence has been shown to ameliorate some but not all of the behavioral dysfunction in a mouse model of autism [132]. Moreover, whether other neurodevelopmental disorders such as schizophrenia or attention deficit hyperactivity disorder are associated with microbiota changes have yet to be investigated either in animal models or human populations.

Cognition

Cognition, which is a general term used to describe thought processes that contribute to decision making, problem solving, and executive function, is affected in a number of CNS disorders including depression, schizophrenia, autism and Alzheimer's disease. Despite the advances in our understanding of the cognitive processes in the brain [193, 194], cognitive deficits remain a difficult symptom to address mainly due to the lack of efficacious treatments. It is recognized that chronic or uncontrollable stress in early life has a profound effect on the development of cognitive neuronal pathways, which also has a negative impact on cognitive function in later life [195]. Although our knowledge in regards to the role of microbiota in cognition is rather limited, recent studies demonstrated impaired non-spatial memory and social cognition (the ability to distinguish between a novel and previously-encountered mouse) in germ-free mice [122, 123], suggesting a potential link between cognitive processes which may depend on the presence of gut microbiota. Further support of this hypothesis comes from studies demonstrating that treatment with probiotics in naive rodents enhances fear memory [13] and reverses memory deficits observed in infected *Citrobacter rodentium*-infected mice after acute stress exposure [123]. The role of microbiota in cognitive function has also become a topic of interest in IBS, a disorder associated with varying degrees of cognitive impairment [196, 197]. Further investigations into the effects of microbiota, antibiotic and probiotic-based therapies on cognitive performance in both the clinical and preclinical domain are warranted.

Sex Differences

Another important feature of psychiatric conditions is the different prevalence rates reported in males and females. For instance, whereas autism occurs more frequently in males (4:1 male to female ratio [198]) depression and anxiety are more prevalent in females [199]. To date, a limited number of studies have focused on the impact sex of the host may have on the microbiota-brain communication in the context of brain development [41]. Interestingly, the most robust changes in neurochemistry (serotonin), hippocampal neurobiology (BDNF) and behavior (social deficits) have been observed in males [41, 122], which is in line with the neuropathology and symptoms observed in autism [200]. Further studies are needed to explore sex-related factors underlying the conflicting outcomes in brain neurochemistry and function as a result of microbiota-host interactions in males and females. Although establishing the underlying mechanisms of sex differences in microbiota-brain-gut communication may provide insights into the pathogenesis of these disorders, these findings should be addressed with caution when assessing their translational value, requiring more investigations.

Implications and Future Perspectives: Towards Novel Therapies for Brain Disorders

Despite the field of the microbiome-brain-gut research being in its infancy, both pre-clinical and clinical evidence suggest that the gut microbiota plays a key role in the development of various aspects of brain function including anxiety, mood, cognition, and more recently in social behavior. Early pre-weaning and adolescence periods appear to be critical periods for modifying enteric microbiota and potential modulation of abnormal behaviors. Environment in early postnatal life has a crucial impact in the neurodevelopment, thus establishing and targeting vulnerable periods for the microbiota-brain-gut axis will allow identification of critical windows of opportunity, in which restoration of the "normal" core microbiota may have therapeutic value in psychiatric and neurodevelopmental disorders.

Existence of a core healthy microbiota profile remains debatable. Advances in high-throughput screening techniques allow characterization of the microbial community at a genome-level in health and disease, shedding more light on the composition, diversity and functions of the human microbiome. Identifying specific combinations and/or subsets of microorganisms, which are essential for optimal health of the host, will create opportunities for preventive, diagnostic and therapeutic approaches for disorders of the microbiota-brain-gut axis. In line with this idea, advances in fecal transplant strategies have already been attempted to treat metabolic and GI disorders, providing convincing evidence for the benefits of the microbiota-based therapies [201–203]. Despite this progress, fecal microbiota therapy in CNS disorders, especially neurodevelopmental and mood disorders,

have yet to be explored. More pre-clinical research is needed to determine bacterial populations in the gut microbiota that are altered and could be of benefit in CNS disorders prior to introducing this innovative microbial-based therapy into the clinic. Currently, pro- and-prebiotic-driven research offers a potentially safer and more explored alternative to fecal transplantation.

As the century continues and technologies to investigate microbiome-gut-brain interactions improve and are applied to the study of CNS disorders, we will see the advent of psychobiotic-based therapies for a variety of neuropsychiatric disorders. Although the vast majority of studies examining the role of microbiota in brain development and function have yielded promising results, they require validation in the clinical domain. Further studies are warranted to examine the physiological impact of the microbiota on behavior, and consequently the contribution of the gut microbiome in the pathogenesis of psychiatric disorders. With rapid advancements in metagenomic techniques, non-invasive techniques to monitor brain structure, function and signaling, and the development of multidisciplinary collaborations the fast-evolving and exciting field of host-microbiota research area will make significant progress, creating new avenues for microbial-based therapeutics that beneficially influence healthy and pathological brain function.

References

1. Gill SR et al (2006) Metagenomic analysis of the human distal gut microbiome. Science 312 (5778):1355–1359
2. De Filippo C et al (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 107(33): 14691–14696
3. Arumugam M et al (2011) Enterotypes of the human gut microbiome. Nature 473(7346): 174–180
4. Eckburg PB et al (2005) Diversity of the human intestinal microbial flora. Science 308(5728): 1635–1638
5. Benno Y, Sawada K, Mitsuoka T (1984) The intestinal microflora of infants: composition of fecal flora in breast-fed and bottle-fed infants. Microbiol Immunol 28(9):975–986
6. Douglas-Escobar M, Elliott E, Neu J (2013) Effect of intestinal microbial ecology on the developing brain. JAMA Pediatr 167(4):374–379
7. Metchnikoff E. Essais optimistes. 1907, Paris,: A. Maloine. 3 p. l., iii, 438 p
8. Bengmark S (2013) Gut microbiota, immune development and function. Pharmacol Res 69 (1):87–113
9. Collins SM, Surette M, Bercik P (2012) The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol 10(11):735–742
10. Diamond B et al (2011) It takes guts to grow a brain: increasing evidence of the important role of the intestinal microflora in neuro- and immune-modulatory functions during development and adulthood. Bioessays 33(8):588–591
11. Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behavior. Nat Rev Neurosci 13(10):701–712
12. Desbonnet L et al (2010) Effects of the probiotic *Bifidobacterium infantis* in the maternal separation model of depression. Neuroscience 170(4):1179–1188

13. Bravo JA et al (2011) Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 108(38):16050–16055
14. Savignac HM et al (2013) Prebiotic feeding elevates central, *N*-methyl-D-aspartate receptor subunits and D-serine. Neurochem Int 63(8):756–764
15. Messaoudi M et al (2011) Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. Br J Nutr 105(5):755–764
16. Rao AV et al (2009) A randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome. Gut Pathog 1(1):6
17. Tillisch K et al (2013) Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology 144(7):1394–1401, 1401 e1–e4
18. Fouhy F et al (2012) Composition of the early intestinal microbiota: knowledge, knowledge gaps and the use of high-throughput sequencing to address these gaps. Gut Microbes 3(3): 203–220
19. Fanca-Berthon P et al (2010) Intrauterine growth restriction not only modifies the cecocolonic microbiota in neonatal rats but also affects its activity in young adult rats. J Pediatr Gastroenterol Nutr 51(4):402–413
20. Costello EK et al (2012) The application of ecological theory toward an understanding of the human microbiome. Science 336(6086):1255–1262
21. Relman DA (2012) The human microbiome: ecosystem resilience and health. Nutr Rev 70 (Suppl 1):S2–S9
22. Gregory KE (2011) Microbiome aspects of perinatal and neonatal health. J Perinat Neonatal Nurs 25(2):158–162, quiz 163–164
23. Adlerberth I, Wold AE (2009) Establishment of the gut microbiota in Western infants. Acta Paediatr 98(2):229–238
24. Funkhouser LJ, Bordenstein SR (2013) Mom knows best: the universality of maternal microbial transmission. PLoS Biol 11(8):e1001631
25. DiGiulio DB et al (2010) Prevalence and diversity of microbes in the amniotic fluid, the fetal inflammatory response, and pregnancy outcome in women with preterm pre-labor rupture of membranes. Am J Reprod Immunol 64(1):38–57
26. Dominguez-Bello MG et al (2010) Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A 107(26):11971–11975
27. Gronlund MM et al (2007) Maternal breast-milk and intestinal bifidobacteria guide the compositional development of the *Bifidobacterium* microbiota in infants at risk of allergic disease. Clin Exp Allergy 37(12):1764–1772
28. Guaraldi F, Salvatori G (2012) Effect of breast and formula feeding on gut microbiota shaping in newborns. Front Cell Infect Microbiol 2:94
29. Hegde S, Munshi AK (1998) Influence of the maternal vaginal microbiota on the oral microbiota of the newborn. J Clin Pediatr Dent 22(4):317–321
30. Fanaro S et al (2003) Fecal flora measurements of breastfed infants using an integrated transport and culturing system. Acta Paediatr 92(5):634–635
31. Cho CE, Norman M (2013) Cesarean section and development of the immune system in the offspring. Am J Obstet Gynecol 208(4):249–254
32. Romero R, Korzeniewski SJ (2013) Are infants born by elective cesarean delivery without labor at risk for developing immune disorders later in life? Am J Obstet Gynecol 208(4): 243–246
33. Maynard CL et al (2012) Reciprocal interactions of the intestinal microbiota and immune system. Nature 489(7415):231–241
34. Barrett E et al (2013) The individual-specific and diverse nature of the preterm infant microbiota. Arch Dis Child Fetal Neonatal Ed 98:F334–F340

35. Parfrey LW, Knight R (2012) Spatial and temporal variability of the human microbiota. Clin Microbiol Infect 18(Suppl 4):8–11
36. Turnbaugh PJ et al (2009) A core gut microbiome in obese and lean twins. Nature 457(7228): 480–484
37. Lozupone CA et al (2012) Diversity, stability and resilience of the human gut microbiota. Nature 489(7415):220–230
38. Foster JA, McVey Neufeld KA (2013) Gut-brain axis: how the microbiome influences anxiety and depression. Trends Neurosci 36(5):305–312
39. Marques TM et al (2010) Programming infant gut microbiota: influence of dietary and environmental factors. Curr Opin Biotechnol 21(2):149–156
40. Johnson CL, Versalovic J (2012) The human microbiome and its potential importance to pediatrics. Pediatrics 129(5):950–960
41. Clarke G et al (2013) The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. Mol Psychiatry 18(6):666–673
42. Claesson MJ et al (2011) Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A 108(Suppl 1):4586–4591
43. Biagi E et al (2010) Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. PLoS One 5(5):e10667
44. Claesson MJ et al (2012) Gut microbiota composition correlates with diet and health in the elderly. Nature 488(7410):178–184
45. Kinross J, Nicholson JK (2012) Gut microbiota: dietary and social modulation of gut microbiota in the elderly. Nat Rev Gastroenterol Hepatol 9(10):563–564
46. Grenham S et al (2011) Brain-gut-microbe communication in health and disease. Front Physiol 2:94
47. Cryan JF, O'Mahony SM (2011) The microbiome-gut-brain axis: from bowel to behavior. Neurogastroenterol Motil 23(3):187–192
48. Aziz Q, Thompson DG (1998) Brain-gut axis in health and disease. Gastroenterology 114(3): 559–578
49. Mayer EA (2011) Gut feelings: the emerging biology of gut-brain communication. Nat Rev Neurosci 12(8):453–466
50. Bonaz BL, Bernstein CN (2013) Brain-gut interactions in inflammatory bowel disease. Gastroenterology 144(1):36–49
51. Davari S et al (2013) Probiotics treatment improves diabetes-induced impairment of synaptic activity and cognitive function: behavioral and electrophysiological proofs for microbiome-gut-brain axis. Neuroscience 240:287–296
52. Rhee SH, Pothoulakis C, Mayer EA (2009) Principles and clinical implications of the brain-gut-enteric microbiota axis. Nat Rev Gastroenterol Hepatol 6(5):306–314
53. Bercik P (2011) The microbiota-gut-brain axis: learning from intestinal bacteria? Gut 60(3): 288–289
54. Dinan TG, Cryan JF (2012) Regulation of the stress response by the gut microbiota: implications for psychoneuroendocrinology. Psychoneuroendocrinology 37(9):1369–1378
55. O'Mahony SM et al (2011) Maternal separation as a model of brain-gut axis dysfunction. Psychopharmacology (Berl) 214(1):71–88
56. Mertz H (2002) Role of the brain and sensory pathways in gastrointestinal sensory disorders in humans. Gut 51(Suppl 1):i29–i33
57. Kunze WA et al (2009) *Lactobacillus reuteri* enhances excitability of colonic AH neurons by inhibiting calcium-dependent potassium channel opening. J Cell Mol Med 13(8B): 2261–2270
58. McVey Neufeld KA et al (2013) The microbiome is essential for normal gut intrinsic primary afferent neuron excitability in the mouse. Neurogastroenterol Motil 25(2):183–e88
59. Browning KN, Mendelowitz D (2003) Musings on the wanderer: what's new in our understanding of vago-vagal reflexes?: II. Integration of afferent signaling from the viscera by the nodose ganglia. Am J Physiol Gastrointest Liver Physiol 284(1):G8–G14

60. Forsythe P et al (2010) Mood and gut feelings. Brain Behav Immun 24(1):9–16
61. Wang X et al (2002) Evidences for vagus nerve in maintenance of immune balance and transmission of immune information from gut to brain in STM-infected rats. World J Gastroenterol 8(3):540–545
62. Borovikova LV et al (2000) Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405(6785):458–462
63. Lyte M et al (2006) Induction of anxiety-like behavior in mice during the initial stages of infection with the agent of murine colonic hyperplasia *Citrobacter rodentium*. Physiol Behav 89(3):350–357
64. Perez-Burgos A et al (2013) Psychoactive bacteria *Lactobacillus rhamnosus* (JB-1) elicits rapid frequency facilitation in vagal afferents. Am J Physiol Gastrointest Liver Physiol 304 (2):G211–G220
65. Bercik P et al (2011) The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut-brain communication. Neurogastroenterol Motil 23(12):1132–1139
66. Bercik P et al (2011) The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 141(2):599–609, 609. e1–e3
67. Costedio MM, Hyman N, Mawe GM (2007) Serotonin and its role in colonic function and in gastrointestinal disorders. Dis Colon Rectum 50(3):376–388
68. McLean PG, Borman RA, Lee K (2007) 5-HT in the enteric nervous system: gut function and neuropharmacology. Trends Neurosci 30(1):9–13
69. Cryan JF, Leonard BE (2000) 5-HT1A and beyond: the role of serotonin and its receptors in depression and the antidepressant response. Hum Psychopharmacol 15(2):113–135
70. Folks DG (2004) The interface of psychiatry and irritable bowel syndrome. Curr Psychiatry Rep 6(3):210–215
71. Tack J et al (2006) A controlled crossover study of the selective serotonin reuptake inhibitor citalopram in irritable bowel syndrome. Gut 55(8):1095–1103
72. Weilburg JB (2004) An overview of SSRI and SNRI therapies for depression. Manag Care 13 (6 Suppl Depression):25–33
73. Creed F et al (2003) The cost-effectiveness of psychotherapy and paroxetine for severe irritable bowel syndrome. Gastroenterology 124(2):303–317
74. Browne CA et al (2012) An effective dietary method for chronic tryptophan depletion in two mouse strains illuminates a role for 5-HT in nesting behavior. Neuropharmacology 62(5–6): 1903–1915
75. Moore P et al (2000) Clinical and physiological consequences of rapid tryptophan depletion. Neuropsychopharmacology 23(6):601–622
76. Desbonnet L et al (2008) The probiotic *Bifidobacteria* infantis: an assessment of potential antidepressant properties in the rat. J Psychiatr Res 43(2):164–174
77. Wikoff WR et al (2009) Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A 106(10):3698–3703
78. Schwarcz R et al (2012) Kynurenines in the mammalian brain: when physiology meets pathology. Nat Rev Neurosci 13(7):465–477
79. Myint AM (2012) Kynurenines: from the perspective of major psychiatric disorders. FEBS J 279(8):1375–1385
80. Stone TW, Stoy N, Darlington LG (2013) An expanding range of targets for kynurenine metabolites of tryptophan. Trends Pharmacol Sci 34(2):136–143
81. Ruddick JP et al (2006) Tryptophan metabolism in the central nervous system: medical implications. Expert Rev Mol Med 8(20):1–27
82. Clarke G et al (2009) Tryptophan degradation in irritable bowel syndrome: evidence of indoleamine 2,3-dioxygenase activation in a male cohort. BMC Gastroenterol 9:6
83. Clarke G et al (2009) Irritable bowel syndrome: towards biomarker identification. Trends Mol Med 15(10):478–489

84. Fitzgerald P et al (2008) Tryptophan catabolism in females with irritable bowel syndrome: relationship to interferon-gamma, severity of symptoms and psychiatric co-morbidity. Neurogastroenterol Motil 20(12):1291–1297
85. Bercik P et al (2010) Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. Gastroenterology 139(6):2102–2112 e1
86. Li G, Young KD (2013) Indole production by the tryptophanase TnaA in *Escherichia coli* is determined by the amount of exogenous tryptophan. Microbiology 159(Pt 2):402–410
87. Cameron J, Doucet E (2007) Getting to the bottom of feeding behavior: who's on top? Appl Physiol Nutr Metab 32(2):177–189
88. Wren AM, Bloom SR (2007) Gut hormones and appetite control. Gastroenterology 132(6): 2116–2130
89. Rustay NR et al (2005) Galanin impairs performance on learning and memory tasks: findings from galanin transgenic and GAL-R1 knockout mice. Neuropeptides 39(3):239–243
90. Wrenn CC et al (2004) Learning and memory performance in mice lacking the GAL-R1 subtype of galanin receptor. Eur J Neurosci 19(5):1384–1396
91. Giordano R et al (2006) Neuroregulation of the hypothalamus-pituitary-adrenal (HPA) axis in humans: effects of GABA-, mineralocorticoid-, and GH-Secretagogue-receptor modulation. ScientificWorldJournal 6:1–11
92. Jaszberenyi M et al (2006) Mediation of the behavioral, endocrine and thermoregulatory actions of ghrelin. Horm Behav 50(2):266–273
93. Lu XY et al (2006) Leptin: a potential novel antidepressant. Proc Natl Acad Sci U S A 103(5): 1593–1598
94. Finger BC, Dinan TG, Cryan JF (2010) Leptin-deficient mice retain normal appetitive spatial learning yet exhibit marked increases in anxiety-related behaviors. Psychopharmacology (Berl) 210(4):559–568
95. Hirano S, Miyata S, Kamei J (2007) Antidepressant-like effect of leptin in streptozotocin-induced diabetic mice. Pharmacol Biochem Behav 86(1):27–31
96. Lesniewska V et al (2006) Effect on components of the intestinal microflora and plasma neuropeptide levels of feeding *Lactobacillus delbrueckii*, *Bifidobacterium lactis*, and inulin to adult and elderly rats. Appl Environ Microbiol 72(10):6533–6538
97. Di Giancamillo A et al (2008) Effects of orally administered probiotic *Pediococcus acidilactici* on the small and large intestine of weaning piglets. A qualitative and quantitative micro-anatomical study. Histol Histopathol 23(6):651–664
98. Schele E et al (2013) The gut microbiota reduces leptin sensitivity and the expression of the obesity-suppressing neuropeptides proglucagon (Gcg) and brain-derived neurotrophic factor (BDNF) in the central nervous system. Endocrinology 154(10):3643–3651
99. Holzer P, Reichmann F, Farzi A (2012) Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut-brain axis. Neuropeptides 46(6):261–274
100. O'Malley D et al (2014) Differential visceral pain sensitivity and colonic morphology in four common laboratory rat strains. Exp Physiol 99:359–367
101. Macpherson AJ, Uhr T (2004) Compartmentalization of the mucosal immune responses to commensal intestinal bacteria. Ann N Y Acad Sci 1029:36–43
102. Vighi G et al (2008) Allergy and the gastrointestinal system. Clin Exp Immunol 153(Suppl 1):3–6
103. Bilbo SD, Schwarz JM (2012) The immune system and developmental programming of brain and behavior. Front Neuroendocrinol 33(3):267–286
104. Dantzer R (2009) Cytokine, sickness behavior, and depression. Immunol Allergy Clin North Am 29(2):247–264
105. Lotrich FE et al (2011) The role of inflammation in the pathophysiology of depression: different treatments and their effects. J Rheumatol Suppl 88:48–54
106. Ratnayake U et al (2013) Cytokines and the neurodevelopmental basis of mental illness. Front Neurosci 7:180
107. Dinan TG, Stanton C, Cryan JF (2013) Psychobiotics: a novel class of psychotropic. Biol Psychiatry 74:720–726

108. Konieczna P et al (2012) Portrait of an immunoregulatory *Bifidobacterium*. Gut Microbes 3 (3):261–266
109. Macpherson AJ, Uhr T (2002) Gut flora–mechanisms of regulation. Eur J Surg Suppl 587: 53–57
110. Macfarlane S, Macfarlane GT (2003) Regulation of short-chain fatty acid production. Proc Nutr Soc 62(1):67–72
111. Kimura I et al (2013) The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. Nat Commun 4:1829
112. Steliou K et al (2012) Butyrate histone deacetylase inhibitors. Biores Open Access 1(4): 192–198
113. Schroeder FA et al (2007) Antidepressant-like effects of the histone deacetylase inhibitor, sodium butyrate, in the mouse. Biol Psychiatry 62(1):55–64
114. MacFabe DF et al (2007) Neurobiological effects of intraventricular propionic acid in rats: possible role of short chain fatty acids on the pathogenesis and characteristics of autism spectrum disorders. Behav Brain Res 176(1):149–169
115. MacFabe DF et al (2011) Effects of the enteric bacterial metabolic product propionic acid on object-directed behavior, social behavior, cognition, and neuroinflammation in adolescent rats: relevance to autism spectrum disorder. Behav Brain Res 217(1):47–54
116. Stilling RM, Dinan TG, Cryan JF (2014) Microbial genes, brain & behavior – epigenetic regulation of the gut-brain axis. Genes Brain Behav 13(1):69–86
117. Neufeld KM et al (2011) Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil 23(3):255–264, e119
118. Diaz Heijtz R et al (2011) Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A 108(7):3047–3052
119. Nishino R et al (2013) Commensal microbiota modulate murine behaviors in a strictly contamination-free environment confirmed by culture-based methods. Neurogastroenterol Motil 25:521–528
120. Gulati AS et al (2012) Mouse background strain profoundly influences Paneth cell function and intestinal microbial composition. PLoS One 7(2):e32403
121. Olivares M, Laparra JM, Sanz Y (2013) Host genotype, intestinal microbiota and inflammatory disorders. Br J Nutr 109(Suppl 2):S76–S80
122. Desbonnet L et al (2014) Microbiota is essential for social development in the mouse. Mol Psychiatry 19:146–148
123. Gareau MG et al (2011) Bacterial infection causes stress-induced memory dysfunction in mice. Gut 60(3):307–317
124. Sudo N et al (2004) Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol 558(Pt 1):263–275
125. Lakhan SE, Caro M, Hadzimichalis N (2013) NMDA receptor activity in neuropsychiatric disorders. Front Psychiatry 4:52
126. Rea MC et al (2011) Effect of broad- and narrow-spectrum antimicrobials on *Clostridium difficile* and microbial diversity in a model of the distal colon. Proc Natl Acad Sci U S A 108 (Suppl 1):4639–4644
127. Murphy R, Stewart AW, Braithwaite I, Beasley R, Hancox RJ, Mitchell EA; the ISAAC Phase Three Study Group. Antibiotic treatment during infancy and increased body mass index in boys: an international cross-sectional study. Int J Obes (Lond). 2013 Nov 21
128. Verdu EF et al (2006) Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 55(2):182–190
129. Fouhy F et al (2012) High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. Antimicrob Agents Chemother 56(11):5811–5820
130. Ohland CL et al (2013) Effects of *Lactobacillus helveticus* on murine behavior are dependent on diet and genotype and correlate with alterations in the gut microbiome. Psychoneuroendocrinology 38:1738–1747

131. Matthews DM, Jenks SM (2013) Ingestion of *Mycobacterium vaccae* decreases anxiety-related behavior and improves learning in mice. Behav Processes 96:27–35
132. Hsiao EY et al (2013) Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell 155:1451–1463
133. Rousseaux C et al (2007) *Lactobacillus acidophilus* modulates intestinal pain and induces opioid and cannabinoid receptors. Nat Med 13(1):35–37
134. McKernan DP et al (2010) The probiotic *Bifidobacterium infantis* 35624 displays visceral antinociceptive effects in the rat. Neurogastroenterol Motil 22(9):1029–1035, e268
135. Johnson AC, Greenwood-Van Meerveld B, McRorie J (2011) Effects of *Bifidobacterium infantis* 35624 on post-inflammatory visceral hypersensitivity in the rat. Dig Dis Sci 56(11): 3179–3186
136. Benton D, Williams C, Brown A (2007) Impact of consuming a milk drink containing a probiotic on mood and cognition. Eur J Clin Nutr 61(3):355–361
137. Tillisch K et al (2013) Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology 144:1394–1401
138. Yang J et al (2007) Epigenetic marks in cloned rhesus monkey embryos: comparison with counterparts produced in vitro. Biol Reprod 76(1):36–42
139. Petschow B et al (2013) Probiotics, prebiotics, and the host microbiome: the science of translation. Ann N Y Acad Sci 1306(1):1–17
140. Saulnier DM et al (2013) The intestinal microbiome, probiotics and prebiotics in neurogastroenterology. Gut Microbes 4(1):17–27
141. Drakoularakou A et al (2010) A double-blind, placebo-controlled, randomized human study assessing the capacity of a novel galacto-oligosaccharide mixture in reducing travellers' diarrhoea. Eur J Clin Nutr 64(2):146–152
142. van Vlies N et al (2012) Effects of short-chain galacto- and long-chain fructo-oligosaccharides on systemic and local immune status during pregnancy. J Reprod Immunol 94(2): 161–168
143. Vulevic J et al (2008) Modulation of the fecal microflora profile and immune function by a novel trans-galactooligosaccharide mixture (B-GOS) in healthy elderly volunteers. Am J Clin Nutr 88(5):1438–1446
144. Hornig M (2013) The role of microbes and autoimmunity in the pathogenesis of neuropsychiatric illness. Curr Opin Rheumatol 25:488–795
145. McEwen BS (2012) Brain on stress: how the social environment gets under the skin. Proc Natl Acad Sci U S A 109(Suppl 2):17180–17185
146. Nutt DJ, Malizia AL (2004) Structural and functional brain changes in posttraumatic stress disorder. J Clin Psychiatry 65(Suppl 1):11–17
147. Lupien SJ et al (2009) Effects of stress throughout the lifespan on the brain, behavior and cognition. Nat Rev Neurosci 10(6):434–445
148. Bravo JA et al (2012) Communication between gastrointestinal bacteria and the nervous system. Curr Opin Pharmacol 12(6):667–672
149. Bested AC, Logan AC, Selhub EM (2013) Intestinal microbiota, probiotics and mental health: from Metchnikoff to modern advances: part I – autointoxication revisited. Gut Pathog 5(1):5
150. Bested AC, Logan AC, Selhub EM (2013) Intestinal microbiota, probiotics and mental health: from Metchnikoff to modern advances: part III – convergence toward clinical trials. Gut Pathog 5(1):4
151. Scott LV, Clarke G, Dinan TG (2013) The brain-gut axis: a target for treating stress-related disorders. In: Halaris A, Leonard BE (eds) Inflammation in Psychiatry, vol 28. Karger, Basel
152. Tannock GW, Savage DC (1974) Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. Infect Immun 9(3):591–598
153. Timoveyev L et al (2002) Stability to sound stress and changeability in intestinal microflora. Eur Psychiatry 17(Suppl 1):200

154. Suzuki K et al (1983) Effects of crowding and heat stress on intestinal flora, body weight gain, and feed efficiency of growing rats and chicks. Nihon Juigaku Zasshi 45(3):331–338
155. Bailey MT, Coe CL (1999) Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. Dev Psychobiol 35(2):146–155
156. Garcia-Rodenas CL et al (2006) Nutritional approach to restore impaired intestinal barrier function and growth after neonatal stress in rats. J Pediatr Gastroenterol Nutr 43(1):16–24
157. O'Mahony SM et al (2009) Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. Biol Psychiatry 65(3): 263–267
158. Bailey MT et al (2011) Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain Behav Immun 25 (3):397–407
159. Collins SM, Bercik P (2013) Gut microbiota: intestinal bacteria influence brain activity in healthy humans. Nat Rev Gastroenterol Hepatol 10:326–327
160. Barrett E et al (2012) *Bifidobacterium breve* with alpha-linolenic acid and linoleic acid alters fatty acid metabolism in the maternal separation model of irritable bowel syndrome. PLoS One 7(11):e48159
161. Wall R et al (2010) Impact of administered *Bifidobacterium* on murine host fatty acid composition. Lipids 45(5):429–436
162. Wall R et al (2012) Contrasting effects of *Bifidobacterium breve* NCIMB 702258 and *Bifidobacterium breve* DPC 6330 on the composition of murine brain fatty acids and gut microbiota. Am J Clin Nutr 95(5):1278–1287
163. Mocking RJ et al (2013) Relationship between the hypothalamic-pituitary-adrenal-axis and fatty acid metabolism in recurrent depression. Psychoneuroendocrinology 38:1607–1617
164. Maes M (2008) The cytokine hypothesis of depression: inflammation, oxidative & nitrosative stress (IO&NS) and leaky gut as new targets for adjunctive treatments in depression. Neuro Endocrinol Lett 29(3):287–291
165. Gareau MG, Silva MA, Perdue MH (2008) Pathophysiological mechanisms of stress-induced intestinal damage. Curr Mol Med 8(4):274–281
166. Ait-Belgnaoui A et al (2012) Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. Psychoneuroendocrinology 37(11):1885–1895
167. Kendler KS, Thornton LM, Gardner CO (2000) Stressful life events and previous episodes in the etiology of major depression in women: an evaluation of the "kindling" hypothesis. Am J Psychiatry 157(8):1243–1251
168. Caspi A et al (2003) Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science 301(5631):386–389
169. Krogius-Kurikka L et al (2009) Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. BMC Gastroenterol 9:95
170. Tana C et al (2010) Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome. Neurogastroenterol Motil 22(5):512–519, e114-5
171. Jeffery IB et al (2012) An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. Gut 61(7):997–1006
172. Gareau MG et al (2007) Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation. Gut 56(11):1522–1528
173. Craft N, Li H (2013) Response to the commentaries on the paper: *Propionibacterium acnes* strain populations in the human skin microbiome associated with acne. J Invest Dermatol 133:2295–2297
174. Myint AM et al (2013) Tryptophan metabolism and immunogenetics in major depression: a role for interferon-gamma gene. Brain Behav Immun 31:128–133

175. Tillisch K, Mayer EA (2005) Pain perception in irritable bowel syndrome. CNS Spectr 10 (11):877–882
176. Davis KD et al (2008) Cortical thinning in IBS: implications for homeostatic, attention, and pain processing. Neurology 70(2):153–154
177. Ellingson BM et al (2013) Diffusion tensor imaging detects microstructural reorganization in the brain associated with chronic irritable bowel syndrome. Pain 154(9):1528–1541
178. Zucchelli M et al (2011) Association of TNFSF15 polymorphism with irritable bowel syndrome. Gut 60(12):1671–1677
179. Salonen A, de Vos WM, Palva A (2010) Gastrointestinal microbiota in irritable bowel syndrome: present state and perspectives. Microbiology 156(Pt 11):3205–3215
180. Rajilic-Stojanovic M, Smidt H, de Vos WM (2007) Diversity of the human gastrointestinal tract microbiota revisited. Environ Microbiol 9(9):2125–2136
181. Codling C et al (2010) A molecular analysis of fecal and mucosal bacterial communities in irritable bowel syndrome. Dig Dis Sci 55(2):392–397
182. Clarke G et al (2012) Review article: probiotics for the treatment of irritable bowel syndrome–focus on lactic acid bacteria. Aliment Pharmacol Ther 35(4):403–413
183. Moayyedi P et al (2010) The efficacy of probiotics in the treatment of irritable bowel syndrome: a systematic review. Gut 59(3):325–332
184. Parkes GC, Sanderson JD, Whelan K (2010) Treating irritable bowel syndrome with probiotics: the evidence. Proc Nutr Soc 69(2):187–194
185. Pimentel M, Lezcano S (2007) Irritable bowel syndrome: bacterial overgrowth—what's known and what to do. Curr Treat Options Gastroenterol 10(4):328–337
186. Happe F et al (2006) Executive function deficits in autism spectrum disorders and attention-deficit/hyperactivity disorder: examining profiles across domains and ages. Brain Cogn 61(1): 25–39
187. Grabrucker AM (2012) Environmental factors in autism. Front Psychiatry 3:118
188. Coury DL et al (2012) Use of psychotropic medication in children and adolescents with autism spectrum disorders. Pediatrics 130(Suppl 2):S69–S76
189. Buie T et al (2010) Recommendations for evaluation and treatment of common gastrointestinal problems in children with ASDs. Pediatrics 125(Suppl 1):S19–S29
190. de Theije CG et al (2014) Altered gut microbiota and activity in a murine model of autism spectrum disorders. Brain Behav Immun 37:197–206
191. Mulle JG, Sharp WG, Cubells JF (2013) The gut microbiome: a new frontier in autism research. Curr Psychiatry Rep 15(2):337
192. Macfabe DF (2012) Short-chain fatty acid fermentation products of the gut microbiome: implications in autism spectrum disorders. Microb Ecol Health Dis 23
193. Kandel E (2012) The biological mind and art. A conversation with Eric Kandel, MD. Interview by Sue Pondrom. Ann Neurol 72(5):A7–A8
194. Squire LR, Wixted JT (2011) The cognitive neuroscience of human memory since H.M. Annu Rev Neurosci 34:259–288
195. Hedges DW, Woon FL (2011) Early-life stress and cognitive outcome. Psychopharmacology (Berl) 214(1):121–130
196. Kennedy PJ et al (2012) Gut memories: towards a cognitive neurobiology of irritable bowel syndrome. Neurosci Biobehav Rev 36(1):310–340
197. Kennedy PJ et al (2013) Cognitive performance in irritable bowel syndrome: evidence of a stress-related impairment in visuospatial memory. Psychol Med 1–14
198. Fombonne E (2005) Epidemiology of autistic disorder and other pervasive developmental disorders. J Clin Psychiatry 66(Suppl 10):3–8
199. Nolen-Hoeksema S, Larson J, Grayson C (1999) Explaining the gender difference in depressive symptoms. J Pers Soc Psychol 77(5):1061–1072
200. Bethea TC, Sikich L (2007) Early pharmacological treatment of autism: a rationale for developmental treatment. Biol Psychiatry 61(4):521–537

201. Borody TJ, Khoruts A (2012) Fecal microbiota transplantation and emerging applications. Nat Rev Gastroenterol Hepatol 9(2):88–96
202. Damman CJ et al (2012) The microbiome and inflammatory bowel disease: is there a therapeutic role for fecal microbiota transplantation? Am J Gastroenterol 107(10):1452–1459
203. van Nood E et al (2013) Duodenal infusion of donor feces for recurrent *Clostridium difficile*. N Engl J Med 368(5):407–415
204. Goehler LE, Park SM, Opitz N, Lyte M, Gaykema RP (2008) *Campylobacter jejuni* infection increases anxiety-like behavior in the holeboard: possible anatomical substrates for viscerosensory modulation of exploratory behavior. Brain Behav Immun 22(3):354–366
205. Lyte M, Varcoe JJ, Bailey MT (1998) Anxiogenic effect of subclinical bacterial infection in mice in the absence of overt immune activation. Physiol Behav 65(1):63–68
206. Arseneault-Bréard J, Rondeau I, Gilbert K, Girard SA, Tompkins TA, Godbout R, Rousseau G (2012) Combination of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 reduces post-myocardial infarction depression symptoms and restores intestinal permeability in a rat model. Br J Nutr 107(12):1793–1799
207. Gilbert K, Arseneault-Bréard J, Flores Monaco F, Beaudoin A, Bah TM, Tompkins TA, Godbout R, Rousseau G (2013) Attenuation of post-myocardial infarction depression in rats by n-3 fatty acids or probiotics starting after the onset of reperfusion. Br J Nutr 109(1):50–56
208. Li W, Dowd SE, Scurlock B, Acosta-Martinez V, Lyte M (2009) Memory and learning behavior in mice is temporally associated with diet-induced alterations in gut bacteria. Physiol Behav 96(4–5):557–567

Chapter 18
Neuroimaging the Microbiome-Gut–Brain Axis

Kirsten Tillisch and Jennifer S. Labus

Abstract The brain is the most complex organ in the human body, interacting with every other major organ system to continuously maintain homeostasis. Thus it is not surprising that the brain also interacts with our microbiota, the trillions of bacteria and other organisms inhabiting the ecosystem of the human being. As we gather knowledge about the way that our microbiota interact with their local environments, there is also increasing interest in their communication with the brain.

Abbreviations

BOLD	Blood oxygen level dependent
DTI	Diffusion tensor imaging
FA	Fractional anisotropy
fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric acid
HPA	Hypothalamic-pituitary-adrenal
MGBA	Microbe-gut-brain axis
MRI	Magnetic resonance imaging
PET	Positron emission tomography

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_18,

Introduction

The brain is the most complex organ in the human body, interacting with every other major organ system to continuously maintain homeostasis. Thus it is not surprising that the brain also interacts with our microbiota, the trillions of bacteria and other organisms inhabiting the ecosystem of the human being. As we gather knowledge about the way that our microbiota interact with their local environments, there is also increasing interest in their communication with the brain.

Brain-Gut Communication

Bidirectional communication between the brain and gut has been well described (Fig. 18.1) [1–4]. The brain communicates with the gut via the autonomic nervous system (particularly the vagus nerve) and the hypothalamic-pituitary adrenal axis. Descending monoaminergic pathways also act on the dorsal horn and can regulate gut-related sensations. Gastrointestinal motility, secretion, local blood flow, and immune regulation are modulated by the brain, generating stereotypic patterns of gut response which are context specific, such as the classic gastrointestinal stress response of nausea and/or fecal urgency. Thus the local environment of gastrointestinal microbes is continuously adjusted by central influences. These interactions provide a partial explanation for the differences in gut bacterial populations between healthy persons and those with gastrointestinal illness [5–7] or prolonged psychological stress [8]. Similarly, preclinical studies have identified altered fecal bacteria after experimental pre and post-natal stress [9–12].

Completing the bidirectional loop, the brain receives afferent input from the gut, likely from a variety of pathways, as described below. With a surface area far exceeding that of the skin, the gut is the largest interface between the body and the external environment, and contains the body's most numerous population of microbes. The gut also has a vast immune system and complex nervous system through which the microbiota can communicate with the brain. Biologically active compounds such as serotonin, histamine [13], catecholamines [14], gamma-aminobutyric acid (GABA) [15], and others can be produced in various amounts by specific bacteria. Additionally, organisms can stimulate the release of these compounds by gut enterochromaffin cells, leading to central signaling and clinically apparent symptoms [16]. An example of this is the central nausea induced at the nucleus tractus solitarius after rotavirus-stimulated gastrointestinal serotonin release [17]. An alternate pathway by which information may reach the brain from the gut is via neurochemicals secreted into the portal venous system, as is seen in hepatic encephalopathy [18, 19].

The vagus nerve has been shown to be essential in some but not all preclinical studies of microbe-brain interactions and likely plays a key role in the microbe-gut-brain axis (MGBA) in humans [20, 21]. Interoceptive (internal) signals of body

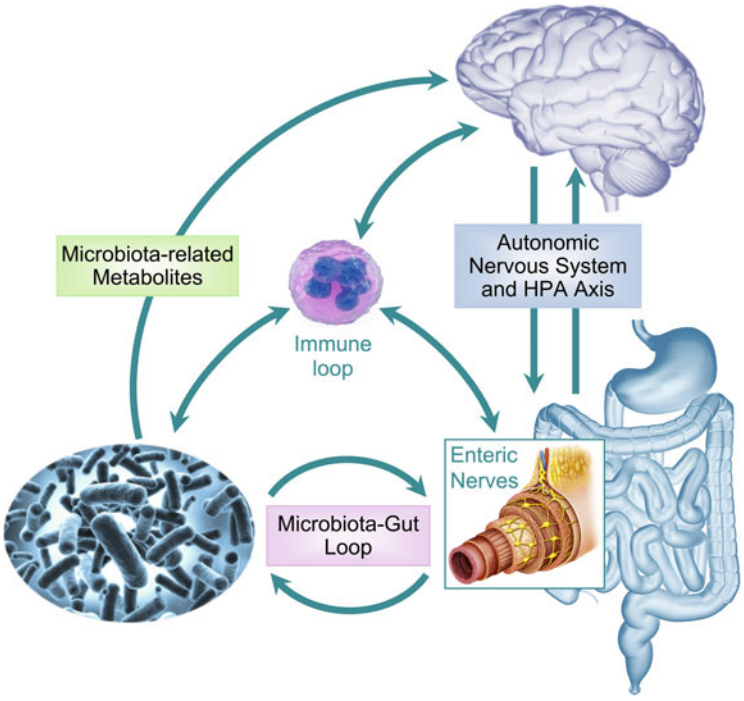

Fig. 18.1 The microbiota-gut-brain axis (MGBA). The traditional gut-brain axis consists of the brain with bidirectional connections to the enteric nervous system of gastrointestinal tract via the autonomic nervous system (sympathetic and parasympathetic branches) and hypothalamic-pituitary-adrenal (HPA) axis. Here the expanded MGBA network is shown. The gastrointestinal microbiota communicate with the brain via enteric nervous system and via metabolic products. The immune system interacts with each member of the MBGA bidirectionally

state are relayed from vagal and spinal afferent nerves to the brain stem and then for further processing in higher cortical centers [22, 23]. It has been proposed that interoceptive input has relevance beyond merely reporting the homeostatic "status" of the body. In the model proposed by Craig and others, interoceptive signals appear to be integrated with emotional and cognitive input primarily in the anterior insula. This combined input is used continuously to create a sense of momentary "self" which can be consciously interpreted as happy, sad, healthy, ill, etc. [24, 25]. Since visceral feedback from the gut and other body sites contributes to our conscious state of wellbeing, it then follows that the gut's luminal organisms also have the opportunity to influence mood states like anxiety or depression [26, 27]. Given the difficulty of gaining access to the cellular workings of the brain in humans, neuroimaging has emerged as a tool to increase our understanding of the MGBA. In the section below, several of the key imaging modalities will be reviewed and their integration into analyses of the MGBA will be discussed.

Neuroimaging in Humans

Functional Neuroimaging Techniques

One of the most common research techniques used to image changes in brain function between groups or after a treatment intervention is functional magnetic resonance imaging (fMRI). This technique is non-invasive, safe, and easy to perform. Functional MRI measures changes in the percentage of oxygenated versus deoxygenated hemoglobin, taking advantage of the differing magnetic properties of the molecules. During an experimental task, when a brain region is more active compared to a baseline or control task, blood flow increases and thus a higher proportion of oxygenated hemoglobin is observed in that area. This change in the regional magnetic properties is measured as the blood oxygen level dependent (BOLD) signal by the scanner and provides an indirect measurement of a change in brain activity. Functional MRI has fairly good spatial resolution of 2–4 mm but does not have the precision of post-mortem studies in animals. Functional MRI has been used successfully to identify differences in brain function in gastrointestinal disease states, such as irritable bowel syndrome and inflammatory bowel disease, as well as in healthy people before and after chronic ingestion of probiotics [28–30].

The other common mode of functional neuroimaging is Positron Emission Tomography (PET). Radiolabeled chemicals are injected into the blood stream and PET measures the emissions regionally throughout the brain. PET has the advantage of measuring physiologic processes more directly via the use of radiolabeled ligands; however it has the drawback of being more invasive and requires radiation exposure. Radioligand PET can be used to explore baseline interactions between regional brain distribution of a variety of signalling systems (including dopamine [31, 32], serotonin [33], substance P/neurokinin-1 [34, 35]) with gut microbiome and metabolomic profiles, as well as assess pre- to post-intervention changes in the MGBA after intervention with specific probiotics. While PET imaging is more invasive and difficult to perform, it has the advantage over fMRI of isolating specific biological processes or pathways for measurement.

The Functional Imaging of the Gut-Brain Axis

The brain-gut axis has been examined using fMRI and PET in humans, particularly in the setting of evoked pain, or anticipation to pain in the esophagus and distal colon. Alterations in resting brain function have also been described in patients with functional gastrointestinal disorders, which are believed to involve brain-gut axis dysfunction [36–38]. Whether these resting brain signal changes represent ongoing gastrointestinal input to the brain or persistent changes in the function of neural circuitry due to chronic disease is not yet known.

Functional MRI has been extensively used to observe changes in brain response after a treatment intervention, most commonly using pharmaceuticals or behavioral interventions, but little has been done to image the effects of antibiotics, probiotics, or dietary interventions in humans [39–41]. Only one study to date has described functional brain changes in response to a probiotic intervention [29]. In this study healthy, normal weight women without any gastrointestinal symptoms, pain or psychiatric disorder, were randomized to treatment with a probiotic, a placebo dairy product or no treatment. The response to an emotional attention task was measured with fMRI before and after the treatment period and the probiotic group showed reductions in response to the emotional task, suggestive of reduced vigilance to negative emotional stimuli. This difference in brain activity was not correlated to any subject reports of mood or gastrointestinal symptoms. Evaluation of the microbiota in that study confirmed that the experimental probiotic could be identified in the stool of the probiotic ingesting subjects but did not show group specific changes in the overall architecture of the microbiota. This is consistent with other studies and suggests that microbial metabolites rather than overall microbial configuration may be the salient result of probiotic ingestion [42]. This initial study suggests that subtle changes in the gut contents can lead to measureable changes in brain function, even in the absence of a conscious awareness of the change. Future studies, which may be able to use microbiome composition, along with metabolomic and metagenomic measurements from stool to correlate with brain function at baseline or after a probiotic intervention, will lead to a better understanding of how the MGBA can be modulated in health and disease.

Structural Neuroimaging

In addition to functional neuroimaging, advances in MR imaging of gray and white matter structure have proven valuable in describing group differences in psychiatric illness and chronic pain syndromes compared to healthy populations. Differences in both white matter and gray matter have been identified in irritable bowel syndrome and functional dyspepsia, both of which are considered to be disorders of the brain-gut axis and which likely are accompanied by alterations in the gut microbiota [43–51]. High resolution structural brain images can be used to produce global (whole-brain), regional, and voxel-level indices of gray matter density and volume as well as cortical thickness, surface area and mean curvature (Fig. 18.2). Network analysis from graph theory has recently been applied to gray matter morphometry to demonstrate alterations in regional topology, providing strong evidence for extensive structural reorganization of cortical and subcortical regions previously implicated in altered brain responses to visceral pain stimuli and their expectation [43]. The biological substrate underlying grey matter changes may involve increased or decreased glial cells, changes in dendritic spines or synapses or less likely, neural degeneration. Gray matter has been shown to remain quite plastic even during adulthood [53–55]. The effects of peripheral factors such as the

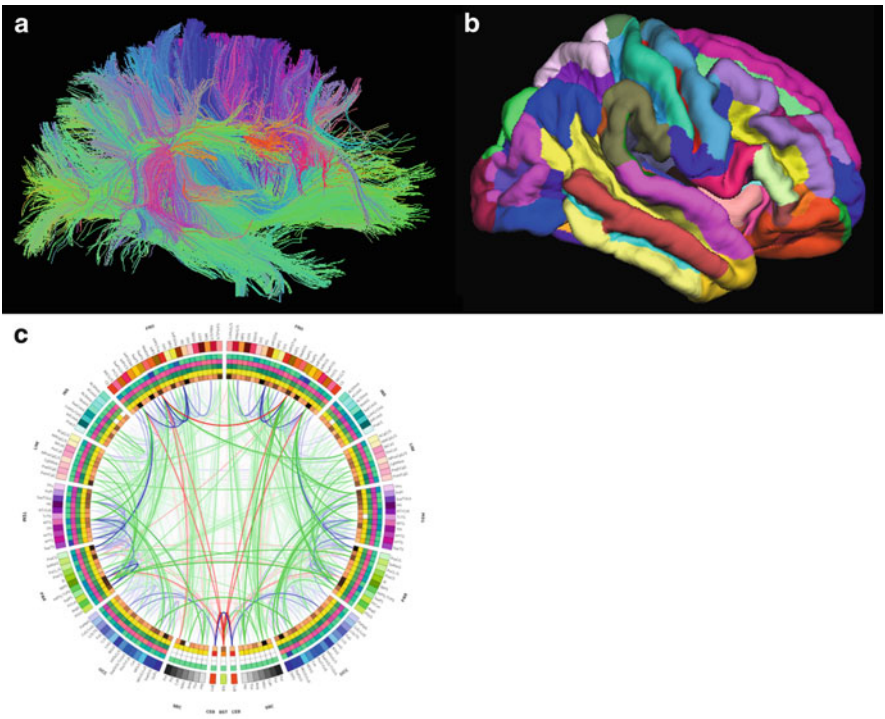

Fig. 18.2 Multimodal neuroimaging. (**a**) White matter tracts in the brain can be visualized with diffusion tensor imaging (DTI). (**b**) The gray matter structure can be viewed with magnetic resonance imaging (MRI) and parcellated into structural or functional regions, measuring characteristic features including volume, cortical thickness and regional curvature. (**c**) Visualization subcortical and cortical brain architecture is depicted using a 'connectogram' [52]. The outer ring shows the brain regions represented by location. The next inner four rings depict the gray matter volume, surface area, cortical thickness, and degree of connectivity. Connectivity between regions was determined using DTI and probabilistic tractography. The color of the links represents the distribution of fractional anisotropy. The number of fiber tracks between regions is represented by the transparency of the line

microbiota on gray matter structure is likely most profound during development, and has been shown in rodent models [56]. However, given that alterations in brain function and behavioral symptom changes occur in response to probiotic interventions in adults, it is likely that structural changes will follow.

Another MRI-based modality of assessing brain structure is diffusion tensor imaging (DTI), which allows the evaluation of white matter integrity and anatomy. DTI can assess the connectivity between gray matter regions via white matter tracts, measuring the fiber pathways that support functional networks. Two main types of DTI analyses are frequently performed [57]. In the first, white matter tract integrity is measured, most commonly expressed as fractional anisotropy (FA), although additional measurements, such as radial or mean diffusivity are also used. This technique assesses the diffusivity of water in the brain tissue. Water molecules

unconstrained by cellular architecture, such as in the CSF, freely move in all directions (isotropic) and thus have a FA value of 0. However, water molecules in dense, parallel white matter tracts containing axons are constrained and have high FA values. Decreases in the FA of white matter tracts can indicate decreased axonal number, myelin integrity, or axonal cytoskeleton integrity. The other DTI analysis method, tractography, allows quantification of fiber density between brain regions, and is commonly used to describe limited or whole brain networks.

It has yet to be clearly defined whether the differences in brain structure in disorders of the brain-gut axis are a result of the chronic condition or a predisposing factor, though there is a great likelihood that both pathways occur. Associations between brain structure and microbiota profiles have not yet been described but provide an opportunity to better understand the interactions between the luminal contents and the brain.

Neuroimaging in Animals

Imaging the brain in animals is also achieved with MRI and PET, as well as more direct radiotracer studies. Rodent fMRI and PET provide fair spatial and temporal resolution but require restraint and/or sedation of the animal to avoid movement, which may confound the interpretation of the functional results. Autoradiography allows neuroimaging in non-sedated, nonrestrained animals. A radiotracer is injected and after the experiment the animal is sacrificed and the brain is cryosectioned to identify regional tracer uptake, allowing a very detailed view of the involved neural circuitry [58]. Using animal imaging in parallel with modulation of the microbiota is likely to inform human studies as animal studies allow for the control of more variables and ability to perform post-mortem studies of the brain.

Incorporation of Behavioral and Gastrointestinal Measurements to Neuroimaging Studies

Preclinical studies have been useful in identifying potential behavioral and peripheral measures that are of particular relevance in examining the MGBA. Modulation of gastrointestinal flora in rodents by using specific bacterial strains, antibiotics, or by using germ-free animals has shown associations with anxiety-like behavior across multiple paradigms [20, 21, 56, 59, 60]. Rodent models of anxiety-like behavior are well developed and show responses to pharmacological agents, such as selective serotonin reuptake inhibitors, indicating the presence of relevant shared core neural circuitry with humans. In humans, measures of anxiety and depression including clinical diagnosis, trait measures and psychological symptoms correlate

with brain structure and function [61–63]. Similar to the findings in rodent models, the ingestion of a *Bifidobacterium* and *Lactobacillius* containing probiotic in healthy humans showed diminished psychological symptoms, including anxiety symptoms in a placebo controlled randomized clinical trial [64]. The central mechanisms through which these symptoms change can be probed with neuroimaging, using symptom measures as covariates. In addition to looking at the interactions between psychological symptoms and brain function when modulating the microbiota in clinical trials, additional gastrointestinal measures such as intestinal permeability, immune activation, motility and visceral sensitivity will be useful in better elucidating gut to brain communication.

Evaluating the MGBA in the Era of Big Data

The ability to analyze the large datasets produced by neuroimaging studies and microbiota profiling has been advancing rapidly [65]. While studies evaluating effects of single organisms or probiotic consortia on the brain will continue to be of great interest; the emerging use of systems biology approaches to the understanding of the relationship between complex structural and functional neural networks and the microbiome is likely to advance our understanding of the MGBA tremendously [66]. Both the microbiome and the brain act within integrated networks for which classical hypothesis driven analytic approaches are not ideal. Agnostically applied multivariate analysis techniques are being used to identify neural networks to develop biomarkers of complex diseases, such as chronic pain, anxiety and depression. These approaches can be utilized to combine complex imaging datasets with genomic, metagenomic and metabolomic data to study the interaction between neural and microbial networks [67]. Since current evidence suggests that the gastrointestinal microflora are likely to play a role in the development and persistence of these disorders, it will be important to look at the interactions between brain phenotypes and the gut microbiome.

Limitations in Neuroimaging of the MGBA

In both the imaging of animal and human MGBA there are a number of limitations. In animals, we have the ability to meticulously manage the presence or absence of specific microorganisms, we are able to image the brain in both direct and indirect ways, and we can observe the effects of various environmental pressures on the developing animal. However, we are faced with the difficulty of translating the relevance of behavior from rodent models to humans, and must deal with the clear differences in the brain between species. As stated by Craig, “A rat is not a monkey is not a human” [68]. He and others [69] have described the difficulties of the bench to clinical translation with a particular focus on interoception and pain processing,

but similar arguments can be made for the study of the stress response, emotion and cognition. If an animal model, as Craig describes in the case of the rodent, lacks the anterior insular cortex, the site in which our subjective sense of physical wellbeing may arise, and if the basic pathways through which the visceral afferents communicate with emotional and cognitive centers vary, then our animal models of complex phenomena must be interpreted with caution.

In humans on the other hand, we have great limitations in our ability to study all three branches of the MGBA precisely. Our access to the gut is limited and most data samples are collected non-invasively, via the stool. This allows us to examine the gut microbiome in broad strokes, but does not differentiate between the luminal and mucosal environment, much less local microenvironments or regional differences throughout the gut [70, 71]. In humans the effects of diet, medications, and external stressors on microbiota content, gastrointestinal motility and immune function are difficult to account for even in the most carefully controlled experiments. Additionally, it is likely that many of the MGBA pathways affected by the microbiota are established early in life, while the brain has its most rapid and dramatic remodeling [72]. Despite these concerns, the combination of human and animal imaging, using a translational or reverse-translational model [73–75] may prove to be the most effective and flexible strategy in evaluating the role of the gut microbiome in brain function, mood and cognition.

Conclusion

Neuroimaging of the MGBA is in its infancy but will clearly be an important modality on the road to understanding the role of microbes in many aspects of health and disease. The current focus on disorders of gastrointestinal disease, such as inflammatory or function bowel diseases, is already shifting to the study of anxiety and depression, metabolic diseases and neurologic disease. With this shift, incorporation of neuroimaging techniques will allow us to measure the rich connectivity between three complex systems: the microbiota, gut and brain.

References

1. Mayer EA (2011) Gut feelings: the emerging biology of gut-brain communication. Nat Rev Neurosci 12(8):453–466
2. Mayer EA, Naliboff BD, Craig AD (2006) Neuroimaging of the brain-gut axis: from basic understanding to treatment of functional GI disorders. Gastroenterology 131(6):1925–1942
3. Hornby PJ (2001) Receptors and transmission in the brain-gut axis. II. Excitatory amino acid receptors in the brain-gut axis. Am J Physiol Gastrointest Liver Physiol 280(6):G1055–G1060
4. Aziz Q, Thompson DG (1998) Brain-gut axis in health and disease. Gastroenterology 114(3): 559–578

5. Nistal E et al (2012) Differences in faecal bacteria populations and faecal bacteria metabolism in healthy adults and celiac disease patients. Biochimie 94(8):1724–1729
6. Carroll IM et al (2012) Alterations in composition and diversity of the intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. Neurogastroenterol Motil 24(6): 521–530, e248
7. Andoh A et al (2012) Multicenter analysis of fecal microbiota profiles in Japanese patients with Crohn's disease. J Gastroenterol 47(12):1298–1307
8. Knowles SR, Nelson EA, Palombo EA (2008) Investigating the role of perceived stress on bacterial flora activity and salivary cortisol secretion: a possible mechanism underlying susceptibility to illness. Biol Psychol 77(2):132–137
9. Bailey MT, Coe CL (1999) Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. Dev Psychobiol 35(2):146–155
10. Bailey MT, Lubach GR, Coe CL (2004) Prenatal stress alters bacterial colonization of the gut in infant monkeys. J Pediatr Gastroenterol Nutr 38(4):414–421
11. O'Mahony SM et al (2009) Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. Biol Psychiatry 65(3): 263–267
12. Garcia-Rodenas CL et al (2006) Nutritional approach to restore impaired intestinal barrier function and growth after neonatal stress in rats. J Pediatr Gastroenterol Nutr 43(1):16–24
13. Thomas CM et al (2012) Histamine derived from probiotic *Lactobacillus reuteri* suppresses TNF via modulation of PKA and ERK signaling. PLoS One 7(2):e31951
14. Asano Y et al (2012) Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. Am J Physiol Gastrointest Liver Physiol 303 (11):G1288–G1295
15. Barrett E et al (2012) Gamma-Aminobutyric acid production by culturable bacteria from the human intestine. J Appl Microbiol 113(2):411–417
16. Uribe A et al (1994) Microflora modulates endocrine cells in the gastrointestinal mucosa of the rat. Gastroenterology 107(5):1259–1269
17. Hagbom M et al (2011) Rotavirus stimulates release of serotonin (5-HT) from human enterochromaffin cells and activates brain structures involved in nausea and vomiting. PLoS Pathog 7 (7):e1002115
18. Butterworth RF (2013) The liver-brain axis in liver failure: neuroinflammation and encephalopathy. Nat Rev Gastroenterol Hepatol 10(9):522–528
19. Lesniewska V et al (2006) Effect on components of the intestinal microflora and plasma neuropeptide levels of feeding *Lactobacillus delbrueckii*, *Bifidobacterium lactis*, and inulin to adult and elderly rats. Appl Environ Microbiol 72(10):6533–6538
20. Bravo JA et al (2011) Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 108(38):16050–16055
21. Bercik P et al (2011) The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 141(2):599–609, 609 e1–e3
22. Craig AD (2009) How do you feel–now? The anterior insula and human awareness. Nat Rev Neurosci 10(1):59–70
23. Craig AD (2002) How do you feel? Interoception: the sense of the physiological condition of the body. Nat Rev Neurosci 3(8):655–666
24. Craig AD (2010) The sentient self. Brain Struct Funct 214(5–6):563–577
25. Craig AD (2011) Significance of the insula for the evolution of human awareness of feelings from the body. Ann N Y Acad Sci 1225:72–82
26. Bajaj JS et al (2013) Modulation of the metabiome by rifaximin in patients with cirrhosis and minimal hepatic encephalopathy. PLoS One 8(4):e60042
27. Dinan TG, Stanton C, Cryan JF (2013) Psychobiotics: a novel class of psychotropic. Biol Psychiatry 74:720–726

28. Tillisch K, Mayer EA, Labus JS (2011) Quantitative meta-analysis identifies brain regions activated during rectal distension in irritable bowel syndrome. Gastroenterology 140(1): 91–100
29. Tillisch K et al (2013) Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology 144(7):1394–1401, 1401 e1–e4
30. Agostini A et al (2011) Brain functional changes in patients with ulcerative colitis: a functional magnetic resonance imaging study on emotional processing. Inflamm Bowel Dis 17(8): 1769–1777
31. Mukherjee J et al (2002) Brain imaging of 18F-fallypride in normal volunteers: blood analysis, distribution, test-retest studies, and preliminary assessment of sensitivity to aging effects on dopamine D-2/D-3 receptors. Synapse 46(3):170–188
32. Olsson H, Halldin C, Farde L (2004) Differentiation of extrastriatal dopamine D2 receptor density and affinity in the human brain using PET. Neuroimage 22(2):794–803
33. Paterson LM et al (2013) 5-HT radioligands for human brain imaging with PET and SPECT. Med Res Rev 33(1):54–111
34. Sprague DR et al (2007) Human biodistribution and radiation dosimetry of the tachykinin NK1 antagonist radioligand [18F]SPA-RQ: comparison of thin-slice, bisected, and 2-dimensional planar image analysis. J Nucl Med 48(1):100–107
35. Jarcho JM et al (2013) Diminished neurokinin-1 receptor availability in patients with two forms of chronic visceral pain. Pain 154(7):987–996
36. Hong JY et al (2013) Patients with chronic visceral pain show sex-related alterations in intrinsic oscillations of the resting brain. J Neurosci 33(29):11994–12002
37. Zeng F et al (2011) Abnormal resting brain activity in patients with functional dyspepsia is related to symptom severity. Gastroenterology 141(2):499–506
38. Van Oudenhove L et al (2010) Abnormal regional brain activity during rest and (anticipated) gastric distension in functional dyspepsia and the role of anxiety: a H(2)(15)O-PET study. Am J Gastroenterol 105(4):913–924
39. Tillisch K et al (2008) Studying the brain-gut axis with pharmacological imaging. Ann N Y Acad Sci 1144:256–264
40. Wise RG, Tracey I (2006) The role of fMRI in drug discovery. J Magn Reson Imaging 23(6): 862–876
41. Mayer EA et al (2002) The effect of the 5-HT3 receptor antagonist, alosetron, on brain responses to visceral stimulation in irritable bowel syndrome patients. Aliment Pharmacol Ther 16(7):1357–1366
42. McNulty NP et al (2011) The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. Sci Transl Med 3(106):106ra106
43. Labus J et al (2014) Irritable bowel syndrome in female patients is associated with alterations in structural brain networks. Pain 155:137–149
44. Holzschneider K, Mulert C (2011) Neuroimaging in anxiety disorders. Dialogues Clin Neurosci 13(4):453–461
45. Ayling E et al (2012) Diffusion tensor imaging in anxiety disorders. Curr Psychiatry Rep 14 (3):197–202
46. May A (2011) Structural brain imaging: a window into chronic pain. Neuroscientist 17(2): 209–220
47. Ellingson BM et al (2013) Diffusion tensor imaging detects microstructural reorganization in the brain associated with chronic irritable bowel syndrome. Pain 154:1528–1541
48. Zeng F et al (2013) Regional brain structural abnormality in meal-related functional dyspepsia patients: a voxel-based morphometry study. PLoS One 8(7):e68383
49. Seminowicz DA et al (2010) Regional gray matter density changes in brains of patients with irritable bowel syndrome. Gastroenterology 139(1):48–57 e2
50. Moayedi M et al (2011) Contribution of chronic pain and neuroticism to abnormal forebrain gray matter in patients with temporomandibular disorder. Neuroimage 55(1):277–286

51. Jiang Z et al (2013) Sex-related differences of cortical thickness in patients with chronic abdominal pain. PLoS One 8(9):e73932
52. Irimia A, Van Horn JD (2012) The structural, connectomic and network covariance of the human brain. Neuroimage 66C:489–499
53. Anderson BJ (2011) Plasticity of gray matter volume: the cellular and synaptic plasticity that underlies volumetric change. Dev Psychobiol 53(5):456–465
54. May A (2011) Experience-dependent structural plasticity in the adult human brain. Trends Cogn Sci 15(10):475–482
55. Gustin SM et al (2012) Pain and plasticity: is chronic pain always associated with somatosensory cortex activity and reorganization? J Neurosci 32(43):14874–14884
56. Sudo N et al (2004) Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol 558(Pt 1):263–275
57. Jellison BJ et al (2004) Diffusion tensor imaging of cerebral white matter: a pictorial review of physics, fiber tract anatomy, and tumor imaging patterns. AJNR Am J Neuroradiol 25(3): 356–369
58. Wang Z et al (2013) Alterations in prefrontal-limbic functional activation and connectivity in chronic stress-induced visceral hyperalgesia. PLoS One 8(3):e59138
59. Matthews DM, Jenks SM (2013) Ingestion of *Mycobacterium vaccae* decreases anxiety-related behavior and improves learning in mice. Behav Processes 96:27–35
60. Neufeld KA et al (2011) Effects of intestinal microbiota on anxiety-like behavior. Commun Integr Biol 4(4):492–494
61. Drevets WC (2003) Neuroimaging abnormalities in the amygdala in mood disorders. Ann N Y Acad Sci 985:420–444
62. Montag C et al (2013) Imaging the structure of the human anxious brain: a review of findings from neuroscientific personality psychology. Rev Neurosci 24(2):167–190
63. Keedwell PA, Linden DE (2013) Integrative neuroimaging in mood disorders. Curr Opin Psychiatry 26(1):27–32
64. Messaoudi M et al (2011) Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. Br J Nutr 105(5):755–764
65. Bullmore E, Sporns O (2009) Complex brain networks: graph theoretical analysis of structural and functional systems. Nat Rev Neurosci 10(3):186–198
66. Hulshoff Pol H, Bullmore E (2013) Neural networks in psychiatry. Eur Neuropsychopharmacol 23(1):1–6
67. Gonzalez I et al (2012) Visualising associations between paired 'omics' data sets. BioData Min 5(1):19
68. Craig AD (2009) A rat is not a monkey is not a human: comment on Mogil (Nature Rev. Neurosci. 10, 283–294 (2009)). Nat Rev Neurosci 10(6):466
69. Mogil JS, Davis KD, Derbyshire SW (2010) The necessity of animal models in pain research. Pain 151(1):12–17
70. Li X et al (2011) A metaproteomic approach to study human-microbial ecosystems at the mucosal luminal interface. PLoS One 6(11):e26542
71. Belkaid Y, Naik S (2013) Compartmentalized and systemic control of tissue immunity by commensals. Nat Immunol 14(7):646–653
72. Stiles J, Jernigan TL (2010) The basics of brain development. Neuropsychol Rev 20(4): 327–348
73. Sinha R, Shaham Y, Heilig M (2011) Translational and reverse translational research on the role of stress in drug craving and relapse. Psychopharmacology (Berl) 218(1):69–82
74. Holschneider DP, Bradesi S, Mayer EA (2011) The role of experimental models in developing new treatments for irritable bowel syndrome. Expert Rev Gastroenterol Hepatol 5(1):43–57
75. Becerra L et al (2013) Parallel buprenorphine phMRI responses in conscious rodents and healthy human subjects. J Pharmacol Exp Ther 345(1):41–51

Chapter 19
The Future of Probiotics for Disorders of the Brain-Gut Axis

Eamonn M.M. Quigley and Fergus Shanahan

Abstract Probiotics, or at the very least products that might have probiotic properties, have been with us for decades, if not centuries, but it has only been in recent years that they have been subjected to serious scientific study. This surge in interest in probiotics has coincided with the era of the microbiome; as more and more is understood about the gut microbiota in health and disease, the therapeutic option of modulating the microbiota through the administration of probiotics has gained a more secure foundation. Regrettably, while a vast literature attests to the beneficial impact of probiotics in a variety of animal models and the mechanisms underlying such positive effects have been dissected in great detail, the data base on probiotics in man remains pretty slender.

To make progress, a number of basic issues need to be addressed: strain characterization and other aspects of quality control need to be rigorously applied and additional steps such as dose optimization, definition of desired site of effect and tailoring of formulation accordingly accomplished before large scale trials, based on appropriately selected study endpoints and employing a clinically meaningful study duration, are embarked upon. Meantime, it is to be hoped that the regulatory climate will have been clarified and appropriate guidelines for the evaluation of probiotics, whether as food or drug, developed. Ultimately, the current terminology may have to be abandoned as evidence for biological and clinical activity for dead bacteria, bacterial components and bacterial products accumulates.

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4_19,

Abbreviations

EFSA	European Food Safety Authority
FGID	Functional gastrointestinal disorder
IBS	Irritable bowel syndrome
IL	Interleukin
SIBO	Small intestinal bacterial overgrowth

Introduction

The reader may be excused for hesitating when confronted by a piece that purports to address the future of an issue or a concept that has been around for almost 100 years; surely the future of such an entity is not in question and certainly, you will say, its course should by now be clearly set? Yet so capricious has been the story of probiotics over this century that it has been only in very recent years that the true potential of this field has come to be appreciated and a few glimpses of the future fleetingly snatched. Before we embark on that most risky and, some would say, doomed, of tasks, namely, predicting the future, let us take stock, firstly, of where we are and, secondly, of the issues that still confront us. Perhaps, if we can crystallize the latter; the future may take care of itself.

Disorders of the Gut-Brain Axis

The list of disorders wherein that bidirectional channel of communication, the brain-gut axis, may play some role is a long one and extends from situations where the brain, the gut and their linkage, through the autonomic nervous system, are affected by the same pathologic process, as in Parkinson's disease [1], to those instances where neurologic symptoms are a consequence of a primarily gastrointestinal pathology, as in the malabsorption syndromes [2], and, finally, to those common gastrointestinal symptoms that afflict us all when stressed and reflect the impact of signals of central origin on a number of gastrointestinal functions [3]. This paradigm is also invoked to explain symptoms in a broad spectrum of common gastrointestinal disorders of uncertain etiology, the functional gastrointestinal disorders (FGIDs) [4]. Several entities have been included under this umbrella ranging from functional heartburn and non-cardiac chest pain, at one end of the gastrointestinal tract, to functional constipation, at the other. Of these disorders, irritable bowel syndrome (IBS) is the best characterized and most thoroughly investigated. While the microbiota has been implicated in the pathophysiology of other FGIDs; the role of *Helicobacter pylori* in functional dyspepsia [5] and descriptions of alterations in the fecal microbiota in chronic constipation [6]

being two examples, investigations of microbiome-host and microbiome-gut-brain interactions have been far more extensive in IBS.

Clinical Evidence for a Role of the Microbiota in Disorders of the Gut-Brain Axis; Lessons from IBS

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal ailments worldwide affecting anywhere from 5 to 15 % of adults in the general population [7]. Despite considerable effort, a biomarker(s) specific for IBS has not been identified [8] and its definition remains entirely clinical, based on the presence of abdominal pain/or discomfort associated with altered bowel habit, often accompanied by symptoms of bloating and distension [9]. The spectrum of symptom severity in IBS is broad with the majority of those affected never seeking medical advice but self-medicating or instituting dietary or life-style measures to control symptoms. At the other end, are a smaller number of individuals whose symptoms are so debilitating that they impose a very significant impact on quality of life. Interestingly, IBS is commonly associated with other FGIDs and has also been linked with extra-intestinal disorders ranging from fibromyalgia and chronic fatigue syndrome to depression; leading to the speculation that there may some common etiological factors for all of these disorders.

Over the years, altered gut motility, visceral hypersensitivity, aberrant brain perception of visceral events, dysregulated stress responses and altered autonomic function have all been invoked to explain the genesis of symptoms in IBS; phenomena that can be collectively incorporated into the concept of the gut-brain axis [10]. Thus, within the spectrum of clinical presentation, symptom severity and natural history that is IBS once can begin to visualize individuals in whom symptoms are primarily of gut origin and, at the other end, where abdominal pain and altered bowel habit are primarily driven by central factors, such as stress or psychopathology.

That the microbiota might be a factor in IBS was first suggested by the observation, in several large series, that IBS could develop de novo in the aftermath of acute enteric bacterial, viral and parasitic infections [11]. More recently, modern sequencing technology has been applied to the study of the microbiota in IBS, in general, and relationships between a variety of clinical and demographic parameters and the microbiota investigated. To date, studies have been largely based on the assessment of fecal samples, despite evidence that fecal and colonic mucosal populations may be quite different in IBS subjects [12–15]. While data remains limited, it is evident that IBS patients have an altered microbiota relative to healthy individuals. Bacterial diversity is reduced [12] and more detailed analyses have identified differences at species and strain level [13–26] among both children [19, 20] and adults [13–18, 21–26] with IBS. Not surprisingly, given the heterogeneity of the IBS phenotype, these results have not been consistent and the sizes of the

study populations involved have not been large enough to encompass the entire symptom and demographic spectrum that is IBS.

More controversial has been the suggestion that small intestinal bacterial overgrowth (SIBO) plays a role in IBS [27, 28]. There are several problems with this proposal; firstly, SIBO, per se, is difficult to define [29]; secondly, the methodology primarily employed in studies supporting a role for SIBO, the lactulose breath hydrogen test, is subject to considerable problems in relation to sensitivity and specificity [29, 30]; and, finally, studies of the prevalence of SIBO in IBS have been highly variable [31]. Nonetheless, if SIBO is indeed a factor in IBS, the potential for SIBO to modulate gut-brain axis communication has already been amply documented in the context of hepatic encephalopathy [32].

Probiotics in IBS

Other clinical evidence apart from the aforementioned supports the potential role of interventions that could modulate the microbiota in IBS. Firstly, modulation of the microbiota could impact on two important functions of colonic bacteria, bile acid metabolism and fermentation and, thereby, alter stool consistency and flatus production, respectively. Secondly, and though its precise mechanism of action remains unclear, the broad spectrum, poorly absorbed, antibiotic rifaximin has been shown, in large clinical trials, to ameliorate the cardinal symptoms of IBS [33]. Thirdly, and most germane to this volume, is evidence for efficacy of certain probiotics in IBS [34].

Probiotics have, indeed, been used on an empirical basis by IBS sufferers for decades and remain popular among those who self-medicate. During the latter half of the last century a number of clinical trials evaluated the efficacy of probiotics in IBS on a more formal basis. While there are many shortcomings with these studies (including but not limited to the clinical definition of the study population, non-randomization, absence of placebo control and small sample sizes) not to mind variations in strain, dose, and method of delivery, when taken together they do suggest a trend towards benefit for probiotics in IBS [35]. Indeed, recent meta-analyses have concluded that probiotics, in general, do benefit patients with IBS [34, 36–40]. What are more difficult to define are the relative benefits of different species or strains. In one of these meta-analyses, for example, it was concluded that *Bifidobacterium* spp. were effective in IBS while *Lactobacilli* were not [34]. A major problem facing any analyst of the literature in this field continues to be the poor quality of many studies: small study populations, variable end-points, and the use of various organisms bedevil their interpretation. Indeed, Brenner and colleagues went so far as to state that only one organism, *Bifidobacterium infantis* 35624, had support for efficacy in IBS based on clinical studies of adequate quality [38]. Since that publication, another strain, *Bifidobacterium lactis* DN-173-010A, has shown promise among IBS subjects with constipation-predominant IBS and prominent bloating [41]. Indeed, the clinical effects of this strain on constipation

Table 19.1 Proposed mechanisms of action of probiotics in irritable bowel syndrome (IBS)

1. Prevention of post-infectious IBS through anti-bacterial or anti-viral actions
2. Normalizing an abnormal microbiota which, in turn, could alleviate symptoms through:
a. Direct effects of bacteria on mucosal functions
b. The elimination of inflammatory/immunogenic stimuli
c. Alterations in microbial metabolism
i. Fermentation of carbohydrates
ii. Deconjugation of bile acids
iii. Production of short-chain fatty acids
d. An impact on motility
e. Modulation of visceral sensation
f. Effects mediated through the microbiome-gut-brain axis
3. Reducing/eliminating small intestinal bacterial overgrowth

and bloating have been supported by evidence that this bacterium accelerates colon transit and reduces abdominal distension [41]. Other strains have shown benefits for specific symptoms, such as bloating [42, 43] or flatulence [44]. While most studies of probiotics in IBS either did not examine relative effects according to IBS sub-type of failed to power adequately for such a sub-group analysis, some have shown benefit exclusively in diarrhea-predominant IBS [45]. How probiotics exert these beneficial actions in IBS is unclear. A number of proposals have been made and these are summarized on Table 19.1. While the proposal that probiotics could influence the central nervous system is based primarily on animal studies [46], a recent brain-imaging study in human volunteers suggests that orally administered probiotics can modulate brain responses [47].

Probiotics 2013; Where Are We?

The concept of probiotics has been with us since the observations of Metchnikoff among Bulgarian peasants in the first decade of the last century. For much of the intervening time, however, the concept has languished in the realm of "alternative" or "natural" medicine and scarcely attracted the interest of either science or conventional medicine. Several factors have, of late, conspired to dramatically change the profile of probiotics and the probiotic concept. These include, firstly, rapid progress, now aided by constantly evolving molecular techniques, in our appreciation of the vital role of the gut microbiota and its interactions with the host in health and disease and, secondly, the application of modern science to the study of probiotics per se. This has resulted in the accurate classification of individual probiotic organisms, as well as detailed descriptions of their genetic, microbiological and immunological properties, and has led to extensive in vivo and in vitro studies of the impact of various probiotics on a variety of biological systems

and, most recently, to well conducted clinical trials of probiotics in specific clinical scenarios in man and domestic animals.

Despite all of this progress several problems persist in relation to these areas that continue to sully the image of probiotics and muddy the field. It is important at this stage to reflect on the most widely accepted (FAO/WHO) definition of a probiotic:

> Live microorganisms which when administered in adequate amounts confer a health benefit on the host [48].

Two issues deserve special emphasis: the focus on "live" organisms and the insistence on conferring "a health benefit on the host". Firstly, while it is readily acknowledged that studies in a number of animal models have demonstrated efficacy for killed bacteria, or even bacterial products or components [49–51], in generating a number of anti-inflammatory and anti-infective effects, this strategy has not, as yet, been explored or validated in man. It seems improbable that effects of probiotics in man will be confined to live organisms so this aspect of the definition will ultimately have to be refined or the term abandoned completely. Secondly, it is obvious from the latter part of this definition that clinical claims in man be they in the augmentation of health or in the treatment of disease, must be supported by credible clinical trial data. Up until the last few years, probiotics were not regulated as drugs and had been able to come on to the market as food supplements or under other designations that have allowed them, to a greater or lesser extent, to make a variety of "health" claims in the absence of supporting data. Recent deliberations from and decisions by the European Food Safety Authority (EFSA) demonstrate a seismic shift in attitude to the extent that the very use of the term probiotic has been deemed to represent a tacit health claim and its use restricted to those "probiotics" that can support such a claim. To date no "probiotic" product, despite their safety and considerable clinical trial data, has received the imprimatur of EFSA. It seems that those who developed and widely promulgated the current definition of a probiotic have now been hoist on their own petard; truly a case of man bites dog. A similar level of scrutiny is now being leveled on these products in North America and elsewhere.

This impasse should have been foreseen. At present the consumer is not being served, not only by the aforementioned issues relating to "health" claims, but also by major problems with quality control. Firstly, it is not unusual for the benefits of a given species or organism to be touted based on evidence derived from studies involving other organisms and species, despite the fact that detailed studies have demonstrated that, in terms of a probiotic property, be it immune modulation [52–55] or anti-bacterial activity [50, 53, 56], there are tremendous differences between different lactobacilli and bifidobacteria, not to mind between lactobacilli and bifidobacteria, for example. No two probiotics are the same and extrapolations from one to another should be resisted at all times. Secondly, an individual who is about to consume a given probiotic preparation should know exactly what he or she is about to take: is it live (if that is necessary for its benefit), what is it's concentration, will the organism survive as it makes contact with acid, bile and digestive enzymes as it transits the gut and what will be the actual concentration of the

organism at its desired site of action? Few probiotic preparations have been characterized and formulated with sufficient rigor to allow the manufacturer to provide answers to these critical questions. Of further concern, critical examinations of the actual constituents of commercially-available probiotic preparations have, in the past, revealed worrying deviations from those included in the product label [57–61].

Nevertheless, evidence for efficacy for specific probiotics in certain clinical conditions continues to accumulate. Most notable have been studies in diarrheal illnesses. Several studies have reported that probiotics may be effective in shortening of the duration of acute diarrheal illnesses in children, such as that related to rotavirus infection [62]. Probiotics also appear to be effective in antibiotic-associated diarrhea [63–65], pouchitis [66, 67], some instances of inflammatory bowel disease [68, 69], and, as already described above, irritable bowel syndrome [55, 70, 71].

The Future of Probiotics

Rather than make wild speculations regarding the future, or even risking modest predictions, we will now attempt to identify those areas where, we believe, the greatest challenges persist and the most important questions remain unanswered.

Quality Control and Regulation

If the field of probiotics is to progress further and gain acceptance within the hallowed halls of science, quality control and appropriate regulation must occur. Inevitably, this will take place on a nation-by-nation basis but, however accomplished, must ensure that the consumer or the prescriber is sufficiently informed of the nature of any given product and assured of the accuracy of its label, including its shelf life, and the validity of health claims. It is incumbent on the medical and scientific communities to actively engage in these processes and to thereby ensure that new requirements and regulations in relation to quality control have scientific credibility and validity. This is a matter of great urgency; failure may result in a gradual ebbing away of confidence in the entire area and the loss of valuable products because the public simply cannot differentiate them from impostors.

Probiotic Characterization

As individual probiotic organisms are subjected to genomic analysis [72] the stage is set for both the accurate definition of each individual organism and the

identification, on the genome, of areas of interest in relation to a particular property or action. This must be the way forward for both the definition of individual organisms and the comparison of their individual characteristics. Parallel developments such as the various collaborative projects defining the human microbiome in health and disease will ultimately lead to a complete description of the microbiome and its metabolic properties and in so doing will facilitate a complete delineation of the interactions (good and bad) between bugs and the host. In so doing, considerable progress should be made in defining the basis for the beneficial actions of probiotic bacteria.

Mechanism of Action

While genomics and metabolomics may suggest certain roles for certain probiotics, these must, ultimately, be further elucidated in appropriate biological systems, including man. Indeed, a further component of the characterization of a probiotic must be the definition of it effects, if any, in a variety of contexts. Does the organism exert anti-bacterial or anti-viral properties, what are its effects on immune responses or metabolic processes? Again a standardized and validated approach to the interrogation of a given organism in relation to a particular use must be developed, where possible. Currently, the methodologies and test systems to be employed to assess the efficacy of an organism against, say *Clostridium difficile*, are well characterized but how does one evaluate the potential impact of an organism in IBS, a disorder whose pathogenesis remains unknown? With regard to the latter, one can only do what the pharmaceutical industry has done for decades, test the organism in relation to putative pathophysiological mechanisms such as, in the case of IBS, dysmotility [73] or visceral hypersensitivity [51, 74, 75]. Proposals to use a probiotic in man must have a plausible scientific rationale; hype and appeals to "being natural" should no longer be sufficient.

Waking the Dead

As emphasized at the outset of this chapter, the current definition of probiotics insists on the inclusion of live organisms. This will undoubtedly change; bacteria are metabolically active organisms that produce a variety of molecules with biological activity [51, 75]. As already mentioned, bacterial DNA has been demonstrated to exert anti-inflammatory activity on certain systems [49, 50]; it seems reasonable to assume that other bacterial components, such as the cell wall or its outer coat, may prove effective in certain contexts. The whole area of bacterial components and bacterial products will be an exceptionally active one in the coming years. In clinical terms, this approach has already shown dividends through

the isolation of probiotic products with specific anti-bacterial activities [76, 77]. There is much more to come.

More Trials!

Performing clinical trials with probiotics is not easy. Quite apart from the aforementioned issues in relation to strain selection for a given indication, the clinical investigator is faced with significant obstacles in choosing formulation, dose and duration of study. Dose is, for the most part, a "black box" in this field, very few dose ranging studies have been attempted and extrapolations from animal studies must always remain mindful of the fact that, weight for weight, probiotic doses used in the mouse or the rat exceed by several orders of magnitude those used in man. We must attempt to get our doses right! Here, however, we encounter the issue of formulation; what may be most acceptable to the patient (e.g. a once a day capsule) may not permit the inclusion of an optimal dose of the organism. These challenges must and will be met; our obligation then is to ensure the performance of clinical trials whose design is optimal for the given indication. Only then can we recommend probiotics to our patients.

Probiotics could, in the future act as vehicles for the targeted delivery of therapeutic molecules to the gut. It has already been shown that probiotics can be genetically engineered to deliver Interleukin (IL)-10 to the intestinal mucosa using an ingenious system which ensures that the organism will not survive outside of the host [78–80].

One great advantage that probiotics currently enjoy in the clinical arena, and in comparison to conventional pharmaceuticals, is that of safety. We must remain vigilant in this area and perform the same rigorous and extensive phase IV, post-marketing, surveys that have become the norm elsewhere. Here again genomic analysis will provide an important supportive role by identifying pathogenicity islands or features that suggest the potential for transference of antibiotic resistance [72].

The changing regulatory climate may alter the approach to clinical trials and pose challenges for potential investigators; specifically who will fund the trials which will be required to satisfy the demands of authorities such as EFSA [81, 82]? If it is decided that a given probiotic product is to be regarded as a food, profit margins will be slim and the target population will, by definition, be the healthy population. Such trials will by virtue of their endpoints require very large numbers of participants and be very expensive. For the food industry, ideal endpoints should be validated biomarkers of risk, of which there are few (e.g. cholesterol for heart disease), not biomarkers of early disease (which will not be applicable in this category). Both issues, size of study population and need for validated biomarkers of risk, pose huge problems for the food industry which is unlikely to be in a position to fund such trials. In other words, it may be cheaper to study probiotics as

drugs within the pharmaceutical sector (paradoxically lower costs and higher margins on licensable product) unless new microbial biomarkers of risk emerge.

New Horizons; Moving Beyond the Gut!

For obvious reasons, including their source and the well-documented interactions between the microbiota and the gut, studies of probiotics have, to date, concentrated in large part on intestinal disorders. Hints to suggest efficacy for probiotics in disorders beyond the gut accumulate with studies illustrating the ability of orally administered probiotics to modulate systemic cytokine patterns in man towards an anti-inflammatory phenotype being of particularly interesting [83, 84]. Experimental and limited clinical data suggest the potential for probiotics to impact on such extra-intestinal disorders as non-alcoholic fatty liver disease [85, 86], arthritis [87], allergy [88], obesity [89] and, as has been amply illustrated in this volume, symptoms arising from and disorders of the central nervous system. The latter is in keeping with very recent and exciting data on the ability of orally administered probiotics and other modifications of the microbiota to influence behavior, mood, cerebral function and morphology in experimental animals [90–92]. Indeed, some preliminary data suggests that probiotics may be able to modulate brain activity in man [47]. Given the pro-inflammatory phenotype associated with such psychiatric disorders as depression and schizophrenia [93, 94] the aforementioned anti-inflammatory actions of some probiotics may be relevant here also. Here, as elsewhere, the development of clinical trial endpoints will be a challenge; psychosocial and behavioral endpoints are symptoms driven and thus prone to considerable placebo responses; objective endpoints are relatively few and those that have been studied, such as brain imaging, are not widely available and expensive. Ongoing studies of the microbiome-gut-brain axis may reveal, not only more objective targets for intervention, but also identify those bacterial components or products that may have optimal biological activity. As our understanding of microbiota-host interactions increases, new applications for probiotics will arise. As the true importance of the microbiota in human homeostasis comes to be recognized the therapeutic potential of probiotics and the broader category of pharmabiotics [95] can begin to be realized.

Conclusion

Having languished for years in the nether world of the “alternative”, probiotics have enjoyed a very recent and very rapid acceleration in scientific investigation and clinical application [96]. In some instances the latter has, regrettably, preceded the former, an approach that, coupled with continuing issues with quality control and regulation, continues to dog the credibility of this area. These hurdles can and will

be overcome and will allow scientifically-based and rigorously tested probiotic products to assume their rightful place in the therapeutic armamentarium.

References

1. Quigley EMM (2008) Gastrointestinal features. In: Factor SA, Weiner WJ (eds) Parkinson's disease – diagnosis and clinical management, 2nd edn. Demos Medical Publishing, New York, pp 99–105
2. Hadjivassiliou M, Sanders DS, Grünewald RA, Woodroofe N, Boscolo S, Aeschlimann D (2010) Gluten sensitivity: from gut to brain. Lancet Neurol 9:318–330
3. Mayer EA (2011) Gut feelings: the emerging biology of gut-brain communication. Nat Rev Neurosci 12:453–466
4. Drossman DA (2006) The functional gastrointestinal disorders and the Rome III process. Gastroenterology 130:1377–1390
5. Suzuki H, Moayyedi P (2013) *Helicobacter pylori* infection in functional dyspepsia. Nat Rev Gastroenterol Hepatol 10:168–174
6. Khalif IL, Konovitch EA, Maximova ID, Quigley EMM (2005) Alterations in the colonic flora and intestinal permeability and evidence of immune activation in chronic constipation. Dig Liver Dis 37:838–849
7. Choung RS, Locke GR 3rd (2011) Epidemiology of IBS. Gastroenterol Clin North Am 40:1–10
8. Clarke G, Quigley EM, Cryan JF, Dinan TG (2009) Irritable bowel syndrome: towards biomarker identification. Trends Mol Med 15:478–489
9. Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC (2006) Functional bowel disorders. Gastroenterology 130:1480–1491
10. Quigley EM (2013) Bugs on the brain; brain in the gut—seeking explanations for common gastrointestinal symptoms. Ir J Med Sci 182:1–6
11. Thabane M, Kottachchi DT, Marshall JK (2007) Systematic review and meta-analysis: the incidence and prognosis of post-infectious irritable bowel syndrome. Aliment Pharmacol Ther 26:535–544
12. Codling C, O'Mahony L, Shanahan F, Quigley EM, Marchesi JR (2010) A molecular analysis of fecal and mucosal bacterial communities in irritable bowel syndrome. Dig Dis Sci 55:392–397
13. Carroll IM, Ringel-Kulka T, Keku TO, Chang YH, Packey CD, Sartor RB, Ringel Y (2011) Molecular analysis of the luminal- and mucosal-associated intestinal microbiota in diarrhea-predominant irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol 301:G799–G807
14. Durbán A, Abellán JJ, Jiménez-Hernández N, Salgado P, Ponce M, Ponce J, Garrigues V, Latorre A, Moya A (2012) Structural alterations of faecal and mucosa-associated bacterial communities in irritable bowel syndrome. Environ Microbiol Rep 4:242–247
15. Parkes GC, Rayment NB, Hudspith BN, Petrovska L, Lomer MC, Brostoff J, Whelan K, Sanderson JD (2012) Distinct microbial populations exist in the mucosa-associated microbiota of sub-groups of irritable bowel syndrome. Neurogastroenterol Motil 24:31–39
16. Kassinen A, Krogius-Kurikka L, Mäkivuokko H, Rinttilä T, Paulin L, Corander J, Malinen E, Apajalahti J, Palva A (2007) The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. Gastroenterology 133:24–33
17. Krogius-Kurikka L, Lyra A, Malinen E, Aarnikunnas J, Tuimala J, Paulin L, Mäkivuokko H, Kajander K, Palva A (2009) Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. BMC Gastroenterol 9:95

18. Rajilić-Stojanović M, Biagi E, Heilig HG, Kajander K, Kekkonen RA, Tims S, de Vos WM (2011) Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. Gastroenterology 141:1792–1801
19. Saulnier DM, Riehle K, Mistretta TA, Diaz MA, Mandal D, Raza S, Weidler EM, Qin X, Coarfa C, Milosavljevic A, Petrosino JF, Highlander S, Gibbs R, Lynch SV, Shulman RJ, Versalovic J (2011) Gastrointestinal microbiome signatures of pediatric patients with irritable bowel syndrome. Gastroenterology 141:1782–1791
20. Rigsbee L, Agans R, Shankar V, Kenche H, Khamis HJ, Michail S, Paliy O (2012) Quantitative profiling of gut microbiota of children with diarrhea-predominant irritable bowel syndrome. Am J Gastroenterol 107:1740–1751
21. Carroll IM, Ringel-Kulka T, Siddle JP, Ringel Y (2012) Alterations in composition and diversity of the intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. Neurogastroenterol Motil 24:521–530
22. Duboc H, Rainteau D, Rajca S, Humbert L, Farabos D, Maubert M, Grondin V, Jouet P, Bouhassira D, Seksik P, Sokol H, Coffin B, Sabaté JM (2012) Increase in fecal primary bile acids and dysbiosis in patients with diarrhea-predominant irritable bowel syndrome. Neurogastroenterol Motil 24:513–520
23. Chassard C, Dapoigny M, Scott KP, Crouzet L, Del'homme C, Marquet P, Martin JC, Pickering G, Ardid D, Eschalier A, Dubray C, Flint HJ, Bernalier-Donadille A (2012) Functional dysbiosis within the gut microbiota of patients with constipated-irritable bowel syndrome. Aliment Pharmacol Ther 35:828–838
24. Jeffery IB, O'Toole PW, Öhman L, Claesson MJ, Deane J, Quigley EM, Simrén M (2012) An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. Gut 61:997–1006
25. Jalanka-Tuovinen J, Salojärvi J, Salonen A, Immonen O, Garsed K, Kelly FM, Zaitoun A, Palva A, Spiller RC, de Vos WM (2013) Faecal microbiota composition and host-microbe cross-talk following gastroenteritis and in postinfectious irritable bowel syndrome. Gut (Epub ahead of print)
26. Durbán A, Abellán JJ, Jiménez-Hernández N, Artacho A, Garrigues V, Ortiz V, Ponce J, Latorre A, Moya A (2013) Instability of the faecal microbiota in diarrhoea-predominant irritable bowel syndrome. FEMS Microbiol Ecol 86:581–589 (Epub 2013 Aug 23)
27. Pimentel M, Chow EJ, Lin HC (2000) Eradication of small intestinal bacterial overgrowth reduces symptoms of irritable bowel syndrome. Am J Gastroenterol 95:3503–3506
28. Pimentel M, Chow EJ, Lin HC (2003) Normalization of lactulose breath testing correlates with symptom improvement in irritable bowel syndrome: a double-blind, randomized, placebo-controlled study. Am J Gastroenterol 98:412–419
29. Quigley EMM (2007) A 51-year old with IBS. Test or treat for bacterial overgrowth? Clin Gastroenterol Hepatol 5:114–1143
30. Quigley EMM, Abu-Shanab A (2009) The diagnosis of small intestinal bacterial overgrowth: the challenges persist! Expert Rev Gastroenterol Hepatol 3:77–87
31. Ford AC, Spiegel BM, Talley NJ, Moayyedi P (2009) Small intestinal bacterial overgrowth in irritable bowel syndrome: systematic review and meta-analysis. Clin Gastroenterol Hepatol 7:1279–1286
32. Quigley EM, Stanton C, Murphy EF (2013) The gut microbiota and the liver pathophysiological and clinical implications. J Hepatol 58:1020–1027
33. Pimentel M, Lembo A, Chey WD, Zakko S et al (2011) TARGET Study Group Rifaximin therapy for patients with irritable bowel syndrome without constipation. N Engl J Med 364:22–32
34. Moayyedi P, Ford AC, Talley NJ, Cremonini F, Foxx-Orenstein A, Brandt L, Quigley E (2010) The efficacy of probiotics in the therapy of irritable bowel syndrome: a systematic review. Gut 59:325–332
35. Quigley EM, Flourie B (2007) Probiotics and irritable bowel syndrome: a rationale for their use and an assessment of the evidence to date. Neurogastroenterol Motil 19:166–172

36. McFarland LV, Dublin S (2008) Meta-analysis of probiotics for the treatment of irritable bowel syndrome. World J Gastroenterol 14:2650–2661
37. Nikfar S, Rahimi R, Rahimi F, Derakhshani S, Abdollahi M (2008) Efficacy of probiotics in irritable bowel syndrome: a meta-analysis of randomized, controlled trials. Dis Colon Rectum 51:1775–1780
38. Brenner DM, Moeller MJ, Chey WD, Schoenfeld PS (2009) The utility of probiotics in the treatment of irritable bowel syndrome: a systematic review. Am J Gastroenterol 104:1033–1049
39. Hoveyda N, Heneghan C, Mahtani KR, Perera R, Roberts N, Glasziou P (2009) A systematic review and meta-analysis: probiotics in the treatment of irritable bowel syndrome. BMC Gastroenterol 9:15
40. Hungin AP, Mulligan C, Pot B, Whorwell P, Agréus L, Fracasso P, Lionis C, Mendive J, Philippart de Foy JM, Rubin G, Winchester C, de Wit N; European Society for Primary Care Gastroenterology (2013) Systematic review: probiotics in the management of lower gastrointestinal symptoms in clinical practice – an evidence-based international guide. Aliment Pharmacol Ther 38:864–886
41. Agrawal A, Houghton LA, Morris J, Reilly B, Guyonnet D, Goupil Feuillerat N, Schlumberger A, Jakob S, Whorwell PJ (2009) Clinical trial: the effects of a fermented milk product containing *Bifidobacterium lactis* DN-173-010 on abdominal distension and gastrointestinal transit in irritable bowel syndrome with constipation. Aliment Pharmacol Ther 29:104–114
42. Kim HJ, Camilleri M, McKinzie S, Lempke MB, Burton DD, Thomforde GM, Zinsmeister AR (2003) A randomized controlled trial of a probiotic, VSL#3, on gut transit and symptoms in diarrhoea-predominant irritable bowel syndrome. Aliment Pharmacol Ther 17:895–904
43. Ringel Y, Ringel-Kulka T, Maier D, Carroll I, Galanko JA, Leyer G, Palsson OS (2011) Clinical trial: probiotic bacteria *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* Bi-07 versus placebo for the symptoms of bloating in patients with functional bowel disorders – a double-blind study. J Clin Gastroenterol 45:518–525
44. Kim HJ, Vazquez Roque MI, Camilleri M, Stephens D, Burton DD, Baxter K, Thomforde G, Zinsmeister AR (2005) A randomized controlled trial of a probiotic combination VSL# 3 and placebo in irritable bowel syndrome with bloating. Neurogastroenterol Motil 17:687–696
45. Andriulli A, Neri M, Loguercio C, Terreni N, Merla A, Cardarella MP, Federico A, Chilovi F, Milandri GL, De Bona M, Cavenati S, Gullini S, Abbiati R, Garbagna N, Cerutti R, Grossi E (2008) Clinical trial on the efficacy of a new symbiotic formulation, Flortec, in patients with irritable bowel syndrome: a multicenter, randomized study. J Clin Gastroenterol 42(Suppl 3 Pt 2):S218–S223
46. Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 13:701–712
47. Tillisch K, Labus J, Kilpatrick L, Jiang Z, Stains J, Ebrat B, Guyonnet D, Legrain-Raspaud S, Trotin B, Naliboff B, Mayer EA (2013) Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology 144:1394–1401
48. Joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. October 2001. www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf?ua=1. Accessed 12 May 2014
49. Rachmilewitz D, Kayatura K, Karmeli F et al (2004) Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. Gastroenterology 126:520–528
50. Jijon H, Backer J, Diaz H, Yeung H, Thiel D, McKagney C, De Simone C, Madsen K (2004) DNA from probiotic bacteria modulates murine and human epithelial and immune function. Gastroenterology 126:1358–1373

51. Kamiya T, Wang L, Forsythe P, Goettsche G, Mao Y, Wang Y, Tougas G, Bienenstock J (2006) Inhibitory effects of *Lactobacillus reuteri* on visceral pain induced by colorectal distension in Sprague-Dawley rats. Gut 55:191–196
52. McCarthy J, O'Mahony L, O'Callaghan L, Sheil B, Vaughan EE, Fitzsimons N, Fitzgibbon J, O'Sullivan GC, Kiely B, Collins JK, Shanahan F (2003) Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. Gut 52:975–980
53. Madsen K, Cornish A, Soper P, McKaigney C, Jijon H, Yachimel C, Doyle J, Jewell L, De Simone C (2001) Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology 121:580–591
54. Hart AL, Lammers K, Brigidi P, Vitali B, Rizzello F, Gionchetti P, Campieri M, Kamm MA, Knight SC, Stagg AJ (2004) Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut 53:1602–1609
55. O'Mahony L, McCarthy J, Kelly P, Shanahan F, Quigley EM (2005) *Lactobacillus* and *Bifidobacterium* in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. Gastroenterology 128:541–551
56. O'Mahony L, O'Callaghan L, McCarthy J, Shilling D, Scully P, Sibartie S, Kavanagh E, Kirwan WO, Redmond RP, Collins JK, Shanahan F (2006) Differential cytokine response from dendritic cells to commensal and pathogenic bacteria in different lymphoid compartments in humans. Am J Physiol 290:G839–G845
57. Hamilton-Miller JM, Shah S, Winkler JT (1999) Public health issues arising from microbiological and labelling quality of foods and supplements containing probiotic microorganisms. Public Health Nutr 2:223–229
58. Coeuret V, Gueguen M, Vernoux JP (2004) Numbers and strains of lactobacilli in some probiotic products. Int J Food Microbiol 97:147–156
59. Szajewska H, Fordymacka A, Bardowski J, Gorecki RK, Mrukowicz JZ, Banaszkiewicz A (2004) Microbiological and genetic analysis of probiotic products licensed for medicinal purposes. Med Sci Monit 10:BR346–BR350
60. Drisko J, Bischoff B, Giles C, Adelson M, Rao RV, McCallum R (2005) Evaluation of five probiotic products for label claims by DNA extraction and polymerase chain reaction analysis. Dig Dis Sci 50:1113–1117
61. Theunissen J, Britz TJ, Torriani S, Witthuhn RC (2005) Identification of probiotic microorganisms in South African products using PCR-based DGGE analysis. Int J Food Microbiol 98:11–21
62. Allen SJ, Okoko B, Martinez E, Gregorio G, Dans LF (2004) Probiotics for treating infectious diarrhoea. Cochrane Database Syst Rev 2, CD003048
63. D'Souza AL, Rajkumar CH, Cooke J et al (2002) Probiotics in prevention of antibiotic associated diarrhea: meta-analysis. BMJ 324:1361–1366
64. Szajewska H, Skorka A, Dylag M (2007) Meta-analysis: *Saccharomyces boulardii* for treating acute diarrhoea in children. Aliment Pharmacol Ther 25:257–264
65. de Vrese M, Marteau PR (2007) Probiotics and prebiotics: effects on diarrhea. J Nutr 137 (3 Suppl 2):803S–811S
66. Gionchetti P, Rizzello F, Helwig U, Venturi A, Lammers KM, Brigidi P, Vitali B, Poggioli G, Miglioli M, Campieri M (2003) Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. Gastroenterology 124:1202–1209
67. Mimura T, Rizzello F, Helwig U, Poggioli G, Schreiber S, Talbot IC, Nicholls RJ, Gionchetti P, Campieri M, Kamm MA (2004) Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. Gut 53:108–114
68. Sheil B, Shanahan F, O'Mahony L (2007) Probiotic effects on inflammatory bowel disease. J Nutr 137(3 Suppl 2):819S–824S
69. Quigley EMM (2007) Probiotics in the management of colonic disorders. Curr Gastroenterol Rep 9:434–440

70. Quigley EMM (2012) Probiotics in the management of functional bowel disorders promise fulfilled? Gastroenterol Clin North Am 41:805–819
71. Whorwell PJ, Altinger L, Morel J, Bond Y, Charbonneau D, O'Mahony L, Kiely B, Shanahan B, Quigley EM (2006) Efficacy of an encapsulated probiotic *Bifidobacterium infantis* 35624 in women with irritable bowel syndrome. Am J Gastroenterol 101:326–333
72. Claesson MJ, Li Y, Leahy S, Canchaya C, van Pijkeren JP, Cerdeno-Tarraga AM, Parkhill J, Flynn S, O'Sullivan GC, Collins JK, Higgins D, Shanahan F, Fitzgerald GF, van Sinderen D, O'Toole PW (2006) Multireplicon genome architecture of *Lactobacillus salivarius*. Proc Natl Acad Sci U S A 103:6718–6723
73. Verdu EF, Bercik P, Bergonzelli GE, Huang XX, Blenerhasset P, Rochat F, Fiaux M, Mansourian R, Corthesy-Theulaz I, Collins SM (2004) *Lactobacillus paracasei* normalizes muscle hypercontractility in a murine model of postinfective gut dysfunction. Gastroenterology 127:826–837
74. Verdu EF, Bercik P, Verma-Gandhu M, Huang X-X, Blenerhasset P, Jackson W, Mao Y, Wang L, Rochat F, Collins SM (2006) Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 55:182–190
75. Ait-Belgnaoui A, Han W, Lamine F, Eutamene H, Fioramonti J, Bueno L, Theodorou V (2006) *Lactobacillus farciminis* treatment suppresses stress-induced visceral hypersensitivity: a possible action through interaction with epithelial cells cytoskeleton contraction. Gut 55:1090–1094
76. Rea MC, Clayton E, O'Connor PM, Shanahan F, Kiely B, Ross RP, Hill C (2007) Antimicrobial activity of lacticin 3,147 against clinical *Clostridium* difficile strains. J Med Microbiol 56 (Pt 7):940–946
77. Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG (2007) Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. Proc Natl Acad Sci U S A 104:7617–7621
78. Vandenbroucke K, Hans W, Van Huysse J, Neirynck S, Demetter P, Remaut E, Rottiers P, Steidler L (2004) Active delivery of trefoil factors by genetically modified *Lactococcus lactis* prevents and heals acute colitis in mice. Gastroenterology 127:502–513
79. Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP, van Deventer SJ, Neirynck S, Peppelenbosch MP, Steidler L (2006) A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. Clin Gastroenterol Hepatol 4:754–759
80. Frossard CP, Steidler L, Eigenmann PA (2007) Oral administration of an IL-10-secreting *Lactococcus lactis* strain prevents food-induced IgE sensitization. J Allergy Clin Immunol 119:952–959
81. Gibson GR, Brummer RJ, Isolauri E, Lochs H, Morelli L, Ockhuizen T, Rowland IR, Schrezenmeir J, Stanton C, Verbeke K (2011) The design of probiotic studies to substantiate health claims. Gut Microbes 2:299–305
82. Binnendijk KH, Rijkers GT (2013) What is a health benefit? An evaluation of EFSA opinions on health benefits with reference to probiotics. Benef Microbes 4:223–230
83. Konieczna P, Groeger D, Ziegler M, Frei R, Ferstl R, Shanahan F, Quigley EMM, Kiely B, Akdis CA, O'Mahony L (2012) *Bifidobacterium infantis* 35624 administration induces Foxp3 + 1T regulatory cells in human peripheral blood – potential role for myeloid and plasmacytoid dendritic cells. Gut 61:354–366
84. Groeger D, O'Mahony L, Murphy EF, Bourke JF, Dinan TG, Kiely B, Shanahan F, Quigley EM (2013) *Bifidobacterium infantis* 35624 modulates host inflammatory processes beyond the gut. Gut Microbes 4:325–339
85. Li Z, Yang S, Lin H et al (2003) Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. Hepatology 37:343–350
86. Abu-Shanab A, Quigley EM (2010) The role of the gut microbiota in nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol 7:691–701
87. Sheil B, McCarthy J, O'Mahony L, Bennett MW, Ryan P, Fitzgibbon JJ, Kiely B, Collins JK, Shanahan F (2004) Is the mucosal route of administration essential for probiotic function?

Subcutaneous administration is associated with attenuation of murine colitis and arthritis. Gut 53:694–700
88. Osborn D, Sinn J (2007) Probiotics in infants for prevention of allergic disease and food hypersensitivity. Cochrane Database Syst Rev 4, CD006475
89. Lee HY, Park JH, Seok SH, Baek MW, Kim DJ, Lee KE, Paek KS, Lee Y, Park JH (2006) Human originated bacteria, *Lactobacillus rhamnosus* PL60, produce conjugated linoleic acid and show anti-obesity effects in diet-induced obese mice. Biochim Biophys Acta 1761:736–744
90. Cryan JF, O'Mahony SM (2011) The microbiome-gut-brain axis: from bowel to behavior. Neurogastroenterol Motil 23:187–192
91. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF (2011) Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 108:16050–16055
92. Desbonnet L, Clarke G, Shanahan F, Dinan TG, Cryan JF (2014) Microbiota is essential for social development in the mouse. Mol Psychiatry 19:146–148 (Epub 2013 May 21)
93. Dinan TG (2009) Inflammatory markers in depression. Curr Opin Psychiatry 22:32–36
94. Dennison U, McKernan D, Cryan J, Dinan T (2012) Schizophrenia patients with a history of childhood trauma have a pro-inflammatory phenotype. Psychol Med 42:1865–1871
95. Shanahan F, Dinan TG, Ross P, Hill C (2012) Probiotics in transition. Clin Gastroenterol Hepatol 10:1220–1224
96. Sanders ME, Guarner F, Guerrant R, Holt PR, Quigley EMM, Sartor RB, Sherman PM, Mayer EA (2013) An update on the use and investigation of probiotics in health and disease. Gut 62:787–796

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M. Lyte and J.F. Cryan (eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, Advances in Experimental Medicine and Biology 817,
DOI 10.1007/978-1-4939-0897-4, © Springer New York 2014