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The role of sex steroid hormones, cytokines and the endocannabinoid system in female fertility

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TABLE OF CONTENTS

BACKGROUND: Marijuana, the most used recreational drug, has been shown to have adverse effects on human reproduction. Endogenous cannabinoids (also called endocannabinoids) bind to the same receptors as those of Δ^9-tetrahydrocannabinol (THC), the psychoactive component of *Cannabis sativa*. The most extensively studied endocannabinoids are anandamide (*N*-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol. The endocannabinoids, their congeners and the cannabinoid receptors, together with the metabolic enzymes and putative transporters form the endocannabinoid system (ECS). In this review, we summarize current knowledge about the relationships of ECS, sex steroid hormones and cytokines in female fertility, and underline the importance of this endocannabinoid–hormone–cytokine network.

METHODS: Pubmed and the Web of Science databases were searched for studies published since 1985, looking into the ECS, sex hormones, type-1/2 T-helper (Th1/Th2) cytokines, leukaemia inhibitory factor, leptin and reproduction.

RESULTS: The ECS plays a pivotal role in human reproduction. The enzymes involved in the synthesis and degradation of endocannabinoids normalize levels of AEA for successful implantation. The AEA degrading enzyme (fatty acid amide hydrolase) activity as well as AEA content in blood may potentially be used for the monitoring of early pregnancies. Progesterone and oestrogen are involved in the maintenance of endocannabinoid levels. The ECS plays an important role in the immune regulation of human fertility.

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CONCLUSIONS: The available studies suggest that tight control of the endocannabinoid–hormone–cytokine network is required for successful implantation and early pregnancy maintenance. This hormone–cytokine network is a key element at the maternal–foetal interface, and any defect in such a network may result in foetal loss.

Key words: endocannabinoids / sex hormones / leukaemia inhibitory factor / leptin / female fertility

Introduction

Endocannabinoids are a group of fatty-acid derivatives that bind to, and activate, the cannabinoid receptors (Di Marzo, 1998) and have several roles in both the central nervous system (CNS) and the periphery (Fride, 2002). Anandamide (*N*-arachidonoylethanolamine: AEA) (Devane *et al*., 1992)—the first endocannabinoid to be identified—was isolated from porcine brain and was closely followed by 2-arachidonoylglycerol (2-AG) (Mechoulam *et al*., 1995; Sugiura *et al*., 1995). Most studies conducted to date involve either AEA or 2-AG, which are prototype members of fatty-acid amides and monoacylglycerols, respectively. However, several novel endocannabinoids have been identified, including *O*-arachidonoylethanolamine (virodhamine) (Porter *et al*., 2002), *N*-arachidonoyldopamine (Bisogno *et al*., 2000) and *N*-arachidonoyltaurine (Saghatelian *et al*., 2004). In addition, *N*-oleoylethanolamine (OEA), *N*-palmitoylethanolamine (PEA) and *N*-stearoylethanolamine are 'endocannabinoid-like' congeners that are thought to exhibit an 'entourage' effect by inhibiting AEA and 2-AG degradation (Ben-Shabat *et al*., 1998; De Petrocellis *et al*., 2004). AEA is released along with OEA and PEA when neurons, somatic cells and reproductive cells are stimulated, and are rapidly removed by re-uptake and hydrolysis to modulate signalling processes (Freund *et al*., 2003). AEA, OEA and PEA are present in human seminal plasma, mid-cycle oviductal fluid, follicular fluid, amniotic fluid and milk (Schuel *et al*., 2002).

Endocannabinoids mimic several actions of the major pharmacologically active component Δ^9-tetrahydrocannabinol (THC) of *Cannabis sativa* (Piomelli, 2004).

The use of *Cannabis* is associated with implantation failure, spontaneous miscarriage, foetal growth restriction and premature birth in humans (Fergusson *et al*., 2002).

Increasing evidence confirms the significance of endocannabinoids in reproductive events such as folliculogenesis, spermatogenesis (Wang *et al*., 2006a, b, c; Taylor *et al*., 2007; Battista *et al*., 2008b), fertilization, oviductal transport, implantation and embryo development (Wang *et al*., 2006a, b, c; Battista *et al*., 2007, 2008a; Taylor *et al*., 2007) it is known that these events are under the control of steroid hormones and cytokines. Several studies have now shown direct effects of these steroids on elements of the ECS (Maccarrone *et al*., 2000a, b, 2003a, b). In this review, we examine the role of sex steroids, cytokines and the ECS in the regulation of female fertility.

Methods

A literature research of Pubmed and the Web of Science databases was performed using the terms 'endocannabinoid system', 'anandamide', 'sex steroid hormones', 'LIF', 'Th1/Th2 cytokines', 'Leptin' and 'reproduction' for studies published between 1985 and the present. We only included articles published in the English language about studies in human and mammals. Studies in non-mammalian species were not included.

The endocannabinoid system

Endocannabinoids, including AEA and 2-AG, bind to G-protein-coupled cannabinoid receptors (CB1 and CB2) (Pertwee and Ross, 2002; Sugiura *et al*., 2002). The biological effects of AEA and 2-AG are terminated by cellular uptake via a putative endocannabinoid membrane transporter (EMT), followed by enzymatic degradation (see below-mentioned text). The endocannabinoids, their congeners and the cannabinoid receptors, together with the metabolic enzymes and purported transporters, form the ECS. This system is summarized in Fig. 1.

Metabolism: biosynthesis, transport and degradation of AEA and 2AG

Biosynthesis

The biosynthesis of AEA occurs on demand. Its precursor is *N*-arachidonoylphosphatidylethanolamine (NAPE), which is formed by the transfer of arachidonic acid (AA) from the *sn*-1 position of 1,2-*sn*-di-arachidonoylphosphatidylcholine to phosphaditylethanolamine. This process is catalyzed by a calcium-dependent *N*-acyltransacylase (Sugiura *et al*., 2002). NAPE is then cleaved into AEA and phosphatidic acid (PA) by NAPE-hydrolyzing phosopholipase D (NAPE-PLD), which is the member of the metallo-β-lactamase family with calcium-sensitive enzyme activity (Okamoto *et al*., 2004; Wang *et al*., 2006a, b, c).

Recently, additional pathways for the synthesis of AEA have been proposed: the double deacylation of NAPE by an α/β hydrolase 4 to generate glycerophospho-AEA, which is then cleaved by a phosphodiesterase to AEA (Simon and Cravatt, 2008); another pathway involves the cleavage of NAPE by a phospholipase C (PLC) to phosphoanandamide, which is followed by dephosphorylation to release AEA (Liu *et al*., 2006). Alternatively, secretory phospholipase A_2 can hydrolyze NAPE to *lyso*-NAPE, which is further hydrolyzed to AEA by a *lyso*-phospholipase D (Sun *et al*., 2004). Figure 2 summarizes the synthetic pathway of AEA. The synthesized AEA is released into the extracellular space, where it may act in an autocrine or paracrine way through activation of cannabinoid receptors (see below-mentioned text) (Piomelli *et al*., 2000).

2-AG is also released from the membranes on demand after the conversion of diacylglycerol (DAG) to 2-AG by *sn*-1-DAG lipase (DAGL). The key intermediate DAG can either be produced from phosphatidylinositol (PI) by PLC activity or alternatively from PA by a PA hydrolase (Bisogno *et al*., 1999). Another pathway for 2-AG synthesis involves the actions of a PI-preferring PLA_1, producing *lyso*-PI, which is then converted to 2-AG by *lyso*-PI-selective PLC (*lyso*-PLC) (Fig. 3).

Transport and degradation

The activity of AEA is terminated first by its removal from the extracellular space via a putative EMT (Ben-Shabat *et al*., 1998) and then by intracellular degradation by either fatty acid amide hydrolases, FAAH-1

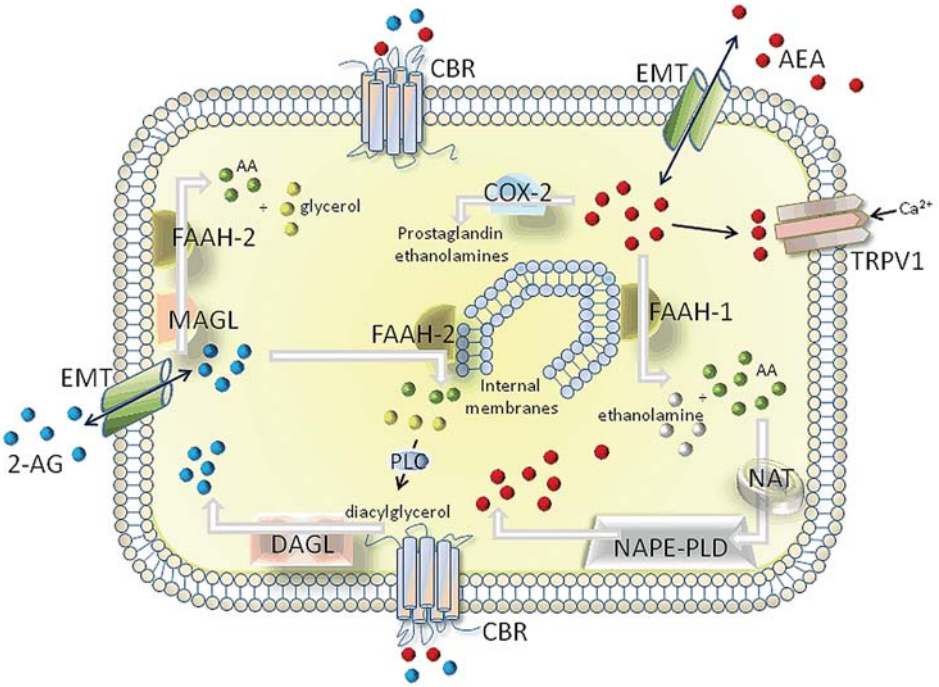

Figure 1 The ECS: synthesis and degradation of AEA and 2-AG (Taylor *et al*., 2010). AEA and 2-AG bind to the putative EMT. AEA can also bind to the transient vanilloid receptor type 1 (TRPV1). AEA is synthesized by the enzymes *N*-acyltransacylase (NAT) and NAPE-PLD and degraded by FAAH1/2 to AA and ethanolamine. COX-2 converts AEA to prostaglandin-ethanolamines. 2-AG is synthesized by *sn*-1-DAGL and degraded by MAGL/FAAH-2 to AA and glycerol. Abbreviations: PLC, phospholipase C; CBR, cannabinoid receptor; EMT, endocannabinoid membrane transporter; AEA, N-arachidonoylethanolamine; 2AG, 2-arachidonoylglycerol; AA, arachidonic acid; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; NAPE, N-arachidonoylphosphatidylethanolamine; NAT, N-acyltransacylase; COX, cyclo-oxygenase; TRPV1, transient vanilloid receptor type 1; PLD, phospholipase D.

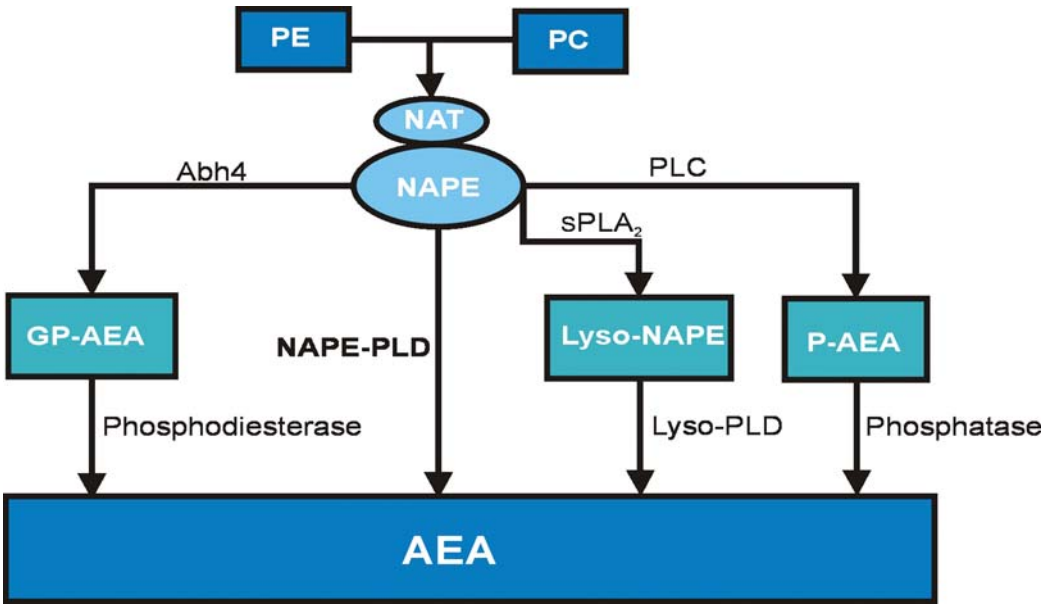

Figure 2 Biosynthetic pathways of AEA. NAPE, produced from membrane phospholipids by NAT, is the key intermediate for the synthetic pathways as described in the text. Abbreviations: PE, phosphatidylethanolamine; PC, 1,2-*sn*-di-arachidonyolphosphatidylcholine; NAT, *N*-acyltransferase; NAPE, *N*-arachidonoylphosphatidylethanolamine; Abh4, α/β hydrolase 4; GP-AEA, glycerophospho-*N*-arachidonoylethanolamine; $sPLA_2$, secretory phospholipase A_2; *lyso*-PLD, *lyso*-phospholipase D; PLC, phospholipase C; pAEA, phosphoanandamide.

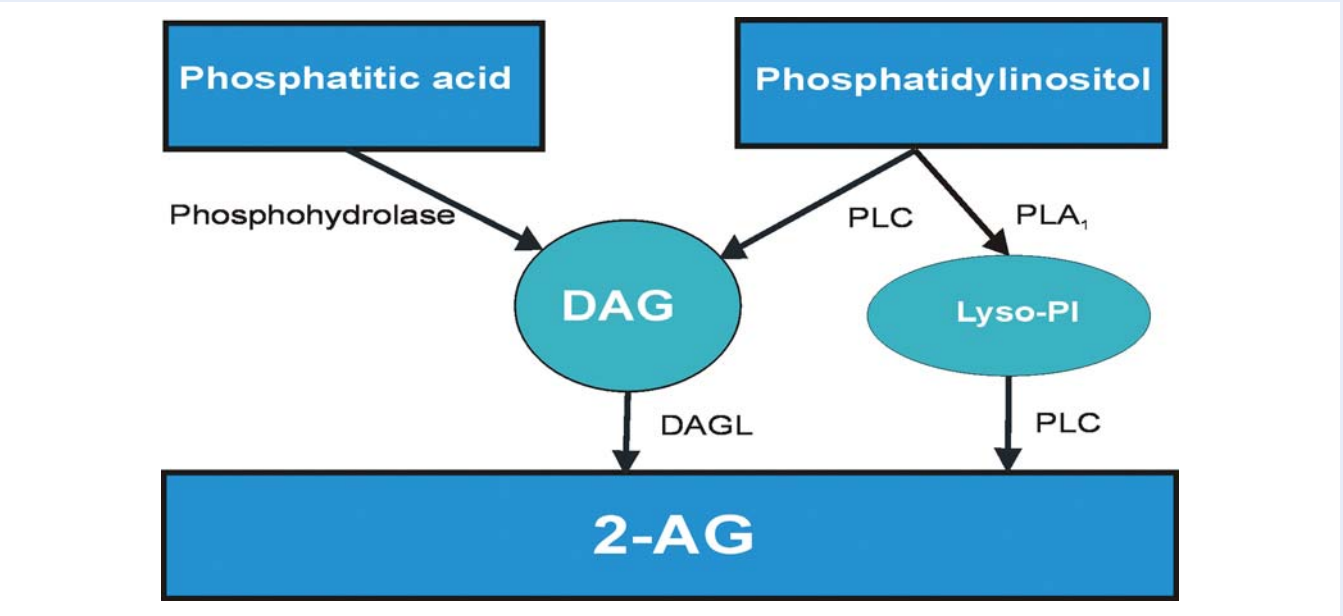

Figure 3 Biosynthetic pathways of 2-AG. 2-AG is produced via a DAG or *lyso*-PI intermediate. DAG can also be produced via PA. Abbreviations: DAG, diacylglycerol; DAGL, *sn*-1-DAG lipase; PLC, phospholipase C; PLA_1, phospholipase A_1; *lyso*-PI, *lyso*-phosphatidylinositol.

(McKinney and Cravatt, 2005) or FAAH-2 (Wei *et al*., 2006) or the *lyso*somal *N*-acylethanolamine-hydrolyzing acid amidase (Tsuboi *et al*., 2005) to AA and ethanolamine (De Petrocellis *et al*., 2004). There are alternative pathways for AEA degradation, such as transformation to 12-hydroxy-AEA by 12-lipoxygenase (12-LOX) (Van der Stelt *et al*., 2002) or inactivation by cyclo-oxygenase (COX)-2 oxidation into prostaglandin-ethanolamide (Rouzer and Marnett, 2008) (Fig. 4a). There is still-controversy about the transmembrane movement of AEA. Cellular models support the hypothesis of a carrier protein for AEA transport in a process of facilitated diffusion (Giuffrida *et al*., 2000; Hillard and Jarrahian, 2000), but this protein has not yet been identified (Glaser *et al*., 2003). Other proposed transport mechanisms include simple diffusion (Kathuria *et al*., 2003) or intracellular sequestration of AEA (McFarland *et al*., 2004). So far, several research groups have shown that AEA cellular uptake is dependent on its concentration gradient and does not require ATP (Hillard *et al*., 1997).

2-AG, on the other hand, is degraded by either FAAH or monoacylglycerol lipase (MAGL) to AA and glycerol (Fergusson *et al*., 2002). MAGL is primarily found in the cytosol and FAAH in membranes of the microsomal and mitochondrial sub-cellular fractions. In addition, COX-2 and LOXs can degrade 2-AG to prostaglandin-glycerol esters (Kozak *et al*., 2002) and hydroxyeicosatetraenoyl-glycerols (Van der Stelt *et al*., 2002), respectively (Fig. 4b).

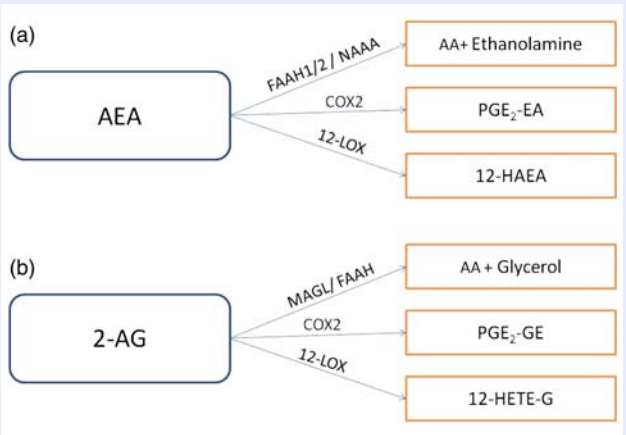

Figure 4 Degradation of AEA and 2-AG. (**a**) AEA is inactivated by FAAH-1, FAAH-2 or NAAA-mediated hydrolysis into AA and ethanolamine, or by COX-2 oxidation into prostaglandin-ethanolamide (PGE_2-EA) or via 12-LOX into 12-hydroxy-AEA (12-HAEA). (**b**) 2-AG signalling is mainly terminated by MAGL or FAAH or by COX-2 oxidation into (PGE_2-GE). 2-AG can also be oxidized to 12-HETE-G via 12-LOX catalysis. Abbreviations: NAAA, *N*-acylethanolamine-hydrolyzing acid amidase.

Endocannabinoid receptors

Classical cannabinoid receptors—CB1 and CB2

Endocannabinoids are ligands for the cannabinoid receptors type 1 (CB1) and type 2 (CB2) (Howlett *et al*., 2002). These are G-protein-coupled seven transmembrane spanning receptors which show 44% overall identity (Devane *et al*., 1988; Howlett *et al*., 2002). CB1 was first described in rat brain (Devane *et al*., 1988) and thought to be present mainly in the CNS, but it is now also known to be present in peripheral tissues, such as the ovary, uterine endometrium, testis, liver, heart, small intestine, urinary bladder and peripheral cells, such as lymphocytes (Bouaboula *et al*., 1993; Pertwee, 1997; Pertwee and Ross, 2002). The CB2 receptor was first isolated from rat spleen and human myeloid cells (Munro *et al*., 1993), and was also thought to be mainly expressed in immune cells (Pertwee, 1997; Pertwee and Ross, 2002) but it has now been localized in other tissues, such as central neurons

(Viscomi *et al*., 2009), embryonic stem cells (ESCs) (Sharov *et al*., 2003), human placenta (Helliwell *et al*., 2004), myometrium (Dennedy *et al*., 2004), ovary (El-Talatini *et al*., 2009a, b), gastrointestinum (Fioramonti and Bueno, 2008), liver (Mallat and Lotersztajn, 2008) and heart (Pacher and Steffens, 2009).

Activation of the CB1 receptor stimulates mitogen-activated protein (MAP) kinases (Bouaboula *et al*., 1995) and inhibits adenylyl cyclase (Paria *et al*., 1995), leading to reduced levels of cyclic adenosine monophosphate (cAMP). Activation of the CB1 receptor results in decreased opening of voltage-gated calcium channels and stimulates potassium channels (Howlett *et al*., 2004). CB2 activation, on the other hand, stimulates MAP kinases and cytosolic PLA_2, but it does not regulate ionic currents. Furthermore, CB2 activation inhibits nitric oxide synthase, whereas CB1 activates it (Howlett *et al*., 2004; Demuth and Molleman, 2006). Nitric oxide has been shown to play an important role in several critical processes in female reproduction, including ovulation, implantation, pregnancy maintenance, labour and delivery (Maul *et al*., 2003).

AEA has a high affinity for the CB1 receptor, whereas 2-AG has a low affinity for the receptor but high efficacy (Sugiura *et al*., 1999). While AEA is only a partial agonist of CB2, 2-AG has a high affinity for the CB2 receptor and is a full agonist for both CB1 and CB2 subtypes (Howlett *et al*., 2004; Demuth and Molleman, 2006). In this context, it should be noted that growing evidence suggests that CB1 is localized within membrane microdomains called 'lipid rafts' (Bari *et al*., 2005), whereas CB2 is not (Bari *et al*., 2006). Additionally, AEA is present in both raft and non-raft domains, whereas 2-AG is present in lipid rafts only (Rimmerman *et al*., 2008). Against this background, it remains to be established whether 2-AG can really bind to CB2 receptors *in vivo*. There is, therefore, a need to classify which CB receptor subtype is activated by which endocannabinoid.

Non-CB1/CB2 G-protein-coupled receptors

In addition to the established cannabinoid receptors CB1 and CB2, two putative CB receptors (GPR55 and GPR119) have been identified. These are G-protein-coupled orphan receptors (McPartland *et al*., 2006) and their associations with the ECS have been discussed in detail in recent reviews (Godlewski *et al*., 2009; Ross, 2009; Moriconi *et al*., 2010).

GPR55 mRNA has been located in various brain regions, testis, ileum, spleen, tonsils and adipose tissue (Brown, 2007). Studies have shown that AEA and 2-AG have no consistent effect on GPR55 (Ryberg *et al*., 2007; Henstridge *et al*., 2009; Yin *et al*., 2009). However, *lyso*-PI appears to be a ligand for GPR55 (Henstridge *et al*., 2009; Yin *et al*., 2009) and triggers extracellular signal-regulated kinase (ERK) phosphorylation and a rise in calcium levels (Oka *et al*., 2007). GPR55 seems to be involved in pain control (Staton *et al*., 2008).

GPR119 mRNA has been found mainly in pancreatic and gastrointestinal tissues (Chu *et al*., 2007; Lauffer *et al*., 2009) and seems to play a role in obesity and diabetes. It has been shown that OEA binds to GPR119 and thereby increases intracellular cAMP (Overton *et al*., 2006). Other effects of GPR119 activation include the stimulation of adenylyl cyclase and protein kinase A activity (Chu *et al*., 2007; Lauffer *et al*., 2009).

Vanilloid receptors

The type-1 vanilloid receptor (TRPV1) (Szallasi and Blumberg, 1999) is a ligand-gated non-selective cationic channel that belongs to the TRP family of proteins. TRPV1 is activated by capsaicin and stimuli, such as heat and protons (Szallasi and Blumberg, 1999). TRPV1 is synthesized in cells outside the peripheral nervous system—for example, keratinocytes, epithelial and endothelial cells (Caterina, 2003), and has also been found in various brain areas (Mezey *et al*., 2000).

Endovanilloids are the endogenous ligands that bind to, and activate, TRPV1 (Di Marzo *et al*., 2001a; Van Der Stelt and Di Marzo, 2004). The first identified endovanilloid was AEA (Zygmunt *et al*., 1999) which, unlike 2-AG, binds to and activates TRPV1 at a cytosolic binding site, triggering non-selective ion-channel activation of protein kinases, calcium influx and release of cytochrome c (Szallasi and Blumberg, 1999; Maccarrone and Finazzi-Agrò, 2003). Cannabinoid and TRPV1 receptors are often found in the same organs, tissues and cells, where they can have opposing or similar functions (Ahluwalia *et al*., 2003; Cristino *et al*., 2006). It is noteworthy that in striatal neurons, AEA inhibits the metabolism and physiological actions of 2-AG at CB1 receptors, through a TRPV1-dependent mechanism (Maccarrone *et al*., 2008).

Based on the different signal transduction pathways activated by AEA and 2-AG, it is understandable that endocannabinoids have different biological roles within the CNS and peripheral tissues (Fride, 2002; Sugiura *et al*., 2006; Smita *et al*., 2007), especially when the receptors are differentially located. One such emerging role is the regulation of reproduction (Battista *et al*., 2007, 2008a; Taylor *et al*., 2007).

ECS and female reproduction

In animal studies, it has been shown that the ECS plays a pivotal role in reproduction. Endocannabinoid signalling pathways are involved in fertilization, oviductal transport, implantation, embryo development and maintenance of early pregnancy (Battista *et al*., 2007, 2008a; Taylor *et al*., 2007). AEA is now thought to be the key link between the developing embryo and the endometrium, ensuring synchronous development of the preimplantation embryo and the endometrium, thereby facilitating to permit embryo implantation during the 'implantation window'.

The metabolically stable AEA-analogue (R-methanandamide) stimulates hyperactive motility of human sperm during *in vitro* capacitation at 0.25 nM, and inhibits hyperactivated motility at 2.5 nM (Schuel *et al*., 2002). These findings suggest that localized differences in AEA concentration may modulate sperm capacitation within the human oviduct.

Studies on cultured bovine oviductal epithelial cells indicate that AEA modulates attachment of sperm to epithelial cells by activating CB1 receptors (Gervasi *et al*., 2009), which suggests an important role of endocannabinoid-signalling in regulating the migration of sperm to the site of fertilization within the oviduct. After fertilization in the oviduct, the fertilized egg undergoes mitotic divisions to form a morula. The morula develops to a blastocyst, which consists of an inner cell mass (ICM) and the trophectoderm. The ICM forms the embryo and the trophectoderm develops to become the placenta and extra-embryonic membranes. A reciprocal interaction between

the blastocyst and a receptive uterus is essential for successful implantation.

Previous studies on mice have localized the expression of CB1 and CB2 receptors in preimplantation embryos, whereas only CB1 receptors are found in the oviduct and uterus (Paria *et al*., 1995, 2001; Wang *et al*., 2004).

In addition, both CB1 and CB2 mRNAs have also been found in the preimplantation mouse embryo; CB1 mRNA is detected from the 4-cell stage to the blastocyst stage and CB2 mRNA is detected from the 1-cell stage onwards (Battista *et al*., 2007) (Fig. 5). CB2 is expressed in the ESCs but not in the trophectoderm and CB1 is found in the trophectoderm (Paria *et al*., 1995). More recently, a systematic study of the presence of elements of the ECS in mouse ESCs has revealed, in addition to classical CB1 and CB2 receptors, also TRPV1 at mRNA, protein and binding levels (Bari *et al*., 2010). Remarkably, ESCs were found to possess the mRNA, protein and activity of the enzymes required to synthesize and degrade AEA (i.e. NAPE-PLD and FAAH) and 2-AG (i.e. DAGL and MAGL), and both endocannabinoids were detected in these cells (Bari *et al*., 2010).

CB1 seems to play an important role in the control of oviductal transport and embryo development. Studies with CB1 knockout and wild-type mice showed pregnancy loss in the knockout group (Paria *et al*., 2001; Wang *et al*., 2004), suggesting that the expression of CB1 in the blastocyst is required for implantation. CB1 deficiency causes embryo retention in the oviduct and resultant ectopic pregnancy. When wild-type females were exposed to the stable AEA analogue methanandamide or to THC the embryos were retained in the oviduct (Wang *et al*., 2004). A more recent study has confirmed low CB1-mRNA expression in the Fallopian tubes and endometrium of women with tubal pregnancies (Horne *et al*., 2008). It seems therefore that both silenced and enhanced cannabinoid signalling can impair embryo development. Furthermore, *in vitro* studies have demonstrated the involvement of the endocannabinoids, via CB1, in the storage and capacitation of boar spermatozoa in the oviduct (Talevi *et al*., 2010). AEA has also been shown to depress motility and capacitation of human spermatozoa (Rossato *et al*., 2005), thereby prolonging the fertile sperm period until the periovulatory signals release the sperm from the oviductal epithelium (Hunter, 2008).

Normal gestation is based on early immunological adaptation involving peripheral T-lymphocytes (Maccarrone and Finazzi-Agrò, 2004). Studies have shown that CB2 is involved in the release of cytokines related to fertility (Correa *et al*., 2005; Borner *et al*., 2006). CB2 receptors have been found in the first trimester human placenta (Helliwell *et al*., 2004) suggesting a role for these receptors in

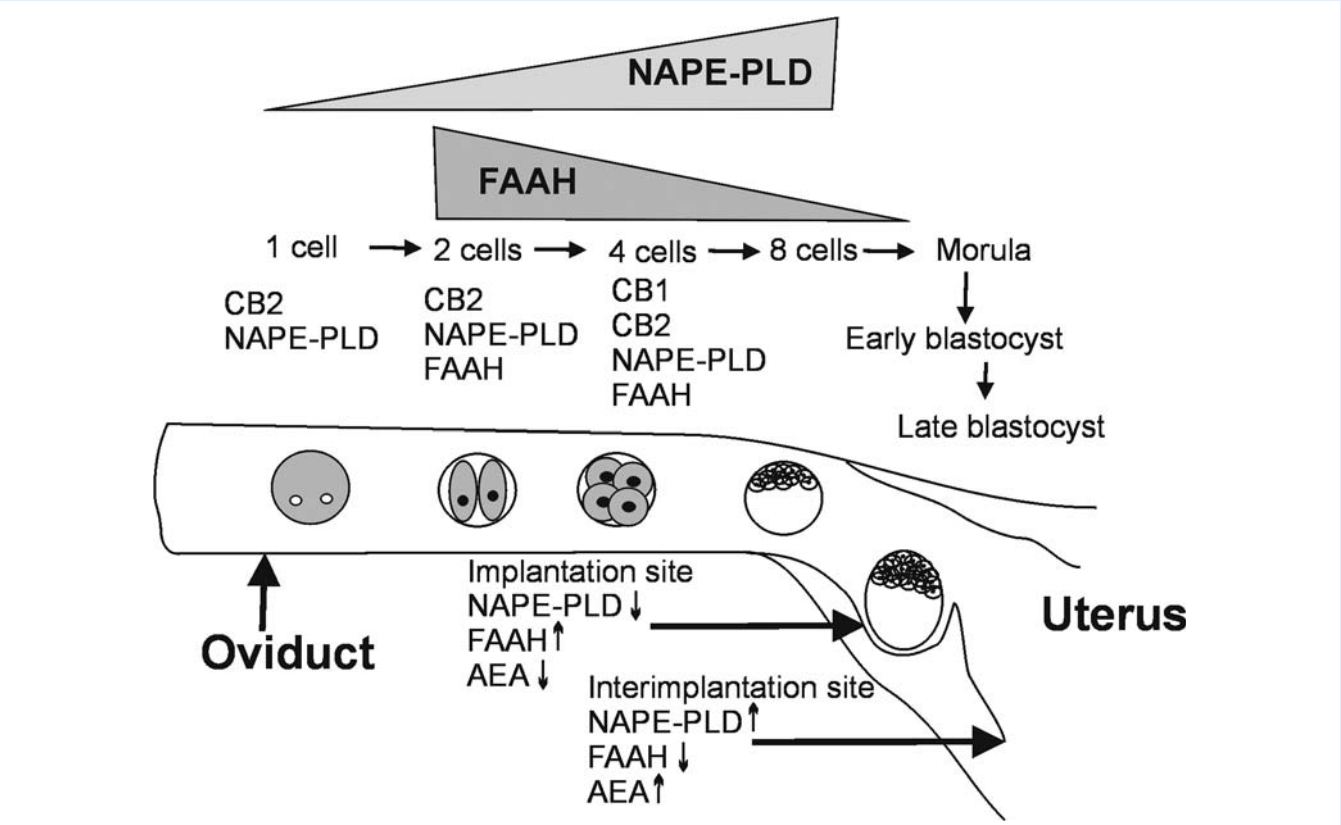

Figure 5 Preimplantation embryo and endocannabinoid signalling in blastocyst implantation. The enzymes NAPE-PLD and FAAH are expressed in the oviduct: NAPE-PLD is more highly expressed at the isthmus than the ampulla, whereas FAAH expression is higher at the ampulla. CB2 mRNA and NAPE-PLD are detected from the 1-cell stage onwards, whereas FAAH is expressed from the 2-cell stage and CB1 mRNA from the 4-cell stage onwards. There are low NAPE-PLD levels and high FAAH levels at the implantation site, resulting in low AEA concentration, which is favourable for implantation. At the inter-implantation site, high NAPE-PLD and low FAAH activity result in high AEA concentrations, which are not conducive to implantation.

placentation and perhaps also in maternal–foetal signalling (Maccarrone, 2008).

The enzymes involved in the synthesis and degradation of endocannabinoids normalize the levels of AEA for successful implantation. NAPE-PLD is present in the cytoplasm of cells in the preimplantation mouse embryos from the 1-cell stage to the blastocyst stage, while FAAH is expressed from the 2-cell stage in the outer cell layers of morulae and trophectoderm. NAPE-PLD is also found in the oviduct, with higher levels at the isthmus and lower levels in the ampullary region, whereas the expression of FAAH is higher in the ampulla (Wang *et al.*, 2006a, b, c) (Fig. 5). The AEA gradient is important for normal embryo development, oviductal transport, implantation and successful pregnancy (Wang *et al.*, 2006a, b, c).

AEA plays an important role in the local regulation of implantation in the uterus (Paria *et al.*, 2001). High levels of NAPE-PLD and low levels of FAAH are present in the inter-implantation sites of the mouse uterus on Day 5–7, whereas high levels of FAAH and low levels of NAPE-PLD, and consequently low AEA levels, are found in the implantation sites (Habayeb *et al.*, 2002; Wang *et al.*, 2007) (Fig. 5). Implantation is associated with a 4-fold reduction in AEA levels at the implantation site (Schmid *et al.*, 1997) and an increase in FAAH activity (Paria *et al.*, 1996).

The implanting blastocyst can also regulate uterine AEA levels by an inhibitory effect of uterine NAPE-PLD (Guo *et al.*, 2005), as well as through the release of a putative lipid 'FAAH activator' (Maccarrone *et al.*, 2004). The 'FAAH activator', produced by both the ICM and trophectoderm, up-regulates FAAH in the uterine cavity, which then reduces AEA levels. Incidentally, a 'FAAH activator' has recently been documented also in mouse ESCs, suggesting that such an entity may be instrumental in regulating FAAH beyond the reproductive events (Bari *et al.*, 2010).

The exposure of 2-cell embryos to high levels of endocannabinoids *in vitro* results in developmental arrest (Paria *et al.*, 1995, 1998). This arrest can be prevented by selective CB1 antagonists (SR141716A, AM251), but not by a specific CB2 antagonist (SR144528), suggesting that the effect of endocannabinoids on the preimplantation embryo is likely mediated via CB1. Indeed, Maccarrone *et al.* (2000a, b) demonstrated that high AEA levels have a pro-apoptotic effect on mouse blastocysts.

Endocannabinoid signalling mediated by CB1 in the embryo is concentration-dependent. Low concentrations of AEA (7 nM) activate the ERK signalling pathway via CB1 and make the blastocyst competent for implantation; conversely, higher levels (28 nM) of AEA cannot activate ERK but inhibit calcium mobilization (Wang *et al.*, 2003). This is clinically relevant as reduced peripheral levels of AEA-hydrolase in women have been shown to be associated with spontaneous miscarriage (Maccarrone *et al.*, 2000a, b) (Table I). A pilot study of women with threatened miscarriage showed that all women who subsequently miscarried had high peripheral AEA levels (greater than 2.0 nM) (Habayeb *et al.*, 2008). Maccarrone *et al.* (2000a, b) also demonstrated in IVF pregnancies that high plasma levels of AEA were associated with failure to achieve an ongoing pregnancy after embryo transfer. Furthermore, it has been shown that women undergoing IVF/ICSI required low AEA levels at the time of implantation for a successful pregnancy (El-Talatini *et al.*, 2009a, b) (Table I). Taken together, the results suggest that FAAH activity as well as AEA content in blood could perhaps be used for the monitoring of early pregnancies. Of note is a recent study in rat, where no correlation was found between plasma levels of endocannabinoids and uterine tissue levels during pregnancy. The absence of a correlation suggests that maternal tissue levels are regulated by *in situ* production and degradation of endocannabinoids (Fonseca *et al.*, 2010). Therefore it would be interesting to investigate this further in humans.

Table I Main effects of the ECS on female fertility.

Effects of low levels	Target	Effects of high levels
Embryo implantation	AEA	Miscarriage
Embryo development		Pro-apoptotic mouse blastocyst
Miscarriage	FAAH	Embryo implantation
		Embryo development
Ectopic pregnancy	CB1	Oviductal transport
	CB2	Embryo development

AEA, arachidonoylethanolamine; FAAH, fatty acid amide hydrolase; CB, cannabinoid receptor.

Although the ECS has not been studied extensively during pregnancy, cross-sectional studies of the levels of AEA in plasma show very distinct patterns. The levels of AEA are highest in the first trimester, fall thereafter and then rise significantly in labour (Habayeb *et al.*, 2004; Lam *et al.*, 2008). Low AEA levels are thus required to maintain the pregnancy, whereas high levels are associated with labour onset (Habayeb *et al.*, 2004). The effects of ECS are summarized in Table I.

Although the precise mechanisms by which endocannabinoids influence reproduction are uncertain, the involvement of COX-2 may be one of them. Maintenance of appropriate AEA levels conducive to implantation and maintenance of pregnancy may be partly dependent upon oxidation by COX-2 (Yu *et al.*, 1997; Kozak *et al.*, 2002), which catalyzes the conversion of AEA to prostanoids (prostaglandin, prostacyclin and thromboxane) and prostamides (prostaglandin-ethanolamides formed from endocannabinoids). COX-2 is an enzyme that is produced during inflammation, carcinogenesis and pyrexia. It is essential in female reproduction as it is involved in several critical processes, including ovulation, fertilization, implantation and decidualization (Lim *et al.*, 1997). Experiments in mice have shown that COX-2 is expressed at the implantation site but is hardly detected at the inter-implantation sites (Wang *et al.*, 2007), and may therefore contribute to the differential concentrations of AEA at these sites.

In addition to the direct effects proposed for endocannabinoids on reproduction, the ECS also interacts with sex steroid hormones and cytokines to regulate reproduction indirectly. In the following sections, we will review the evidence for these interactions.

The endocannabinoids and sex steroid hormones

The role of progesterone

Progesterone is a C-21 steroid hormone that is produced predominantly after ovulation by the corpus luteum and the placenta during

pregnancy and exerts its primary action through the intracellular progesterone receptor.

Progesterone has a number of physiological effects that are amplified in the presence of oestrogen. This amplification by oestrogen may be mediated through the oestrogen receptors, which have been shown to up-regulate the expression of progesterone receptors.

It is well known that reproduction is dependent upon a tight immunoregulation, whereby type-2 T-helper (Th2) cytokines promote fertility and type-1 T-helper (Th1) cytokines inhibit it. Progesterone creates a suitable endometrial environment for implantation and maintains pregnancy by contributing to a protective immune milieu. Progesterone induces the production of the pro-fertility Th2 cytokines and inhibits the anti-fertility Th1 cytokines (Piccinni and Romagnani, 1996).

Progesterone stimulates the release of leukaemia inhibitory factor (LIF) through interleukin (IL)-4, which has also been demonstrated to promote implantation and pregnancy continuation (Maccarrone *et al.*, 2001).

Furthermore, both progesterone and oestrogen are involved in the maintenance of endocannabinoid levels. It has been shown that progesterone up-regulates lymphocyte FAAH activity through the transcription factor Ikaros (Maccarrone *et al.*, 2001, 2003a, b) and thereby decreases AEA levels (Table II) (Fig. 6). However, progesterone has been shown to have a minimal effect on EMT, NAPE-PLD and CB1 expression in lymphocytes (Maccarrone *et al.*, 2001, 2003a, b).

Progesterone and oestrogen have been shown to down-regulate uterine NAPE-PLD expression in mice, possibly leading to a decrease in AEA levels (Guo *et al.*, 2005). However, the activity of uterine FAAH, localized in murine glandular and luminal epithelium, is decreased below basal levels by both progesterone and oestrogen, contrary to the expectation that these should lead to an increase in AEA (Maccarrone *et al.*, 2000a, b) (Table II).

Table II Effects of progesterone and oestrogen on ECS in female fertility.

Hormone/ cytokine	Reproductive process	Effect on ECS
Progesterone	Implantation	Increases FAAH through transcription factor Ikaros and reduces AEA
	Pregnancy maintenance	Increases LIF via IL4
		Promotes pro-fertility Th2 cytokines
Oestrogen	Folliculogenesis	Stimulates NAPE-PLD and increases AEA from endothelial cells
	Implantation	Inhibits FAAH activity and increases AEA content in endothelial cells
		Down-regulates NAPE-PLD and inhibits FAAH in uterine epithelium

LIF, leukaemia inhibitory factor; IL4, interleukin 4; NAPE-PLD, *N*-arachidonoylphosphatidylethanolamine-hydrolyzing phosopholipase D.

Changes in progesterone levels and FAAH expression are well correlated during the menstrual cycle (Lazzarin *et al.*, 2004) in agreement with the finding that progesterone up-regulates the FAAH gene (Maccarrone *et al.*, 2003a, b). However, there seems to be no correlation between plasma levels of AEA and progesterone in normal cycling women (El-Talatini *et al.*, 2010) and in early pregnancy (El-Talatini *et al.*, 2009a, b).

The role of oestrogen

Oestrogens are steroid hormones that diffuse across the cell membrane. Once inside the cell, they bind, to and activate, oestrogen receptors, which up-regulate the expression of many genes.

Oestrogens are produced primarily by developing follicles and the corpus luteum in ovaries, and by the trophoblast cells of the placenta. FSH and LH stimulate the production of oestrogen in the ovaries. Other non-ovarian sources of oestrogens include the liver, adrenal glands and the breasts. 17β-estradiol (E_2) modifies many responses and is known to increase prolactin secretion.

E_2 is thought to be involved in the regulation of the ECS but the evidence for this is not yet robust.

Maccarrone *et al.*, for example, demonstrated that E_2 stimulates NAPE-PLD and inhibits FAAH, stimulating the release of AEA from endothelial cells, which then modulates the cardiovascular and immune systems (Maccarrone *et al.*, 2002a,b,c). In contrast, uterine NAPE-PLD is down-regulated by E_2, suggesting that it induces a decrease in AEA levels (Guo *et al.*, 2005). However, results from a separate study demonstrated decreased activity of murine uterine FAAH by E_2 (Maccarrone *et al.*, 2000a, b) (Table II). The opposite effect of E_2 on NAPE-PLD in different tissues, despite a consistent inhibition of FAAH, suggests that it is hard to predict the effects of E_2 on AEA levels based upon the expression of its metabolic enzymes; to this end, studies that directly measure AEA content may be more appropriate.

We investigated changes in plasma AEA levels during the menstrual cycle of healthy women and found a positive correlation between E_2 and AEA, suggesting that indeed E_2 may be involved in the regulation of AEA (El-Talatini *et al.*, 2010). A positive correlation between E_2 and AEA levels was also demonstrated in non-pregnant women after IVF and embryo transfer (El-Talatini *et al.*, 2009a, b).

The endocannabinoids and cytokines

The role of LIF

LIF, a member of the IL-6 family, plays important roles in the immune and haematopoietic systems. It is, however, also essential for reproduction (Smith *et al.*, 1998). Among its biological roles are cell proliferation, differentiation and survival (Hilton, 1992). Signalling is triggered after binding of the LIF receptor-β (LIF-Rβ) to glycoprotein gp130 (Heinrich *et al.*, 2003). Signal transduction involves several different pathways, but the main ones are Janus kinase/Signal transducer and activator of transcription (JAK/STAT) signalling, Src homology 2-domain-containing tyrosine phosphatase/Ras/ERK signalling and PI-3-kinase/Akt signalling (Auernhammer and Melmed, 2000; Kimber, 2005). Signal transduction can be inhibited by suppressor of

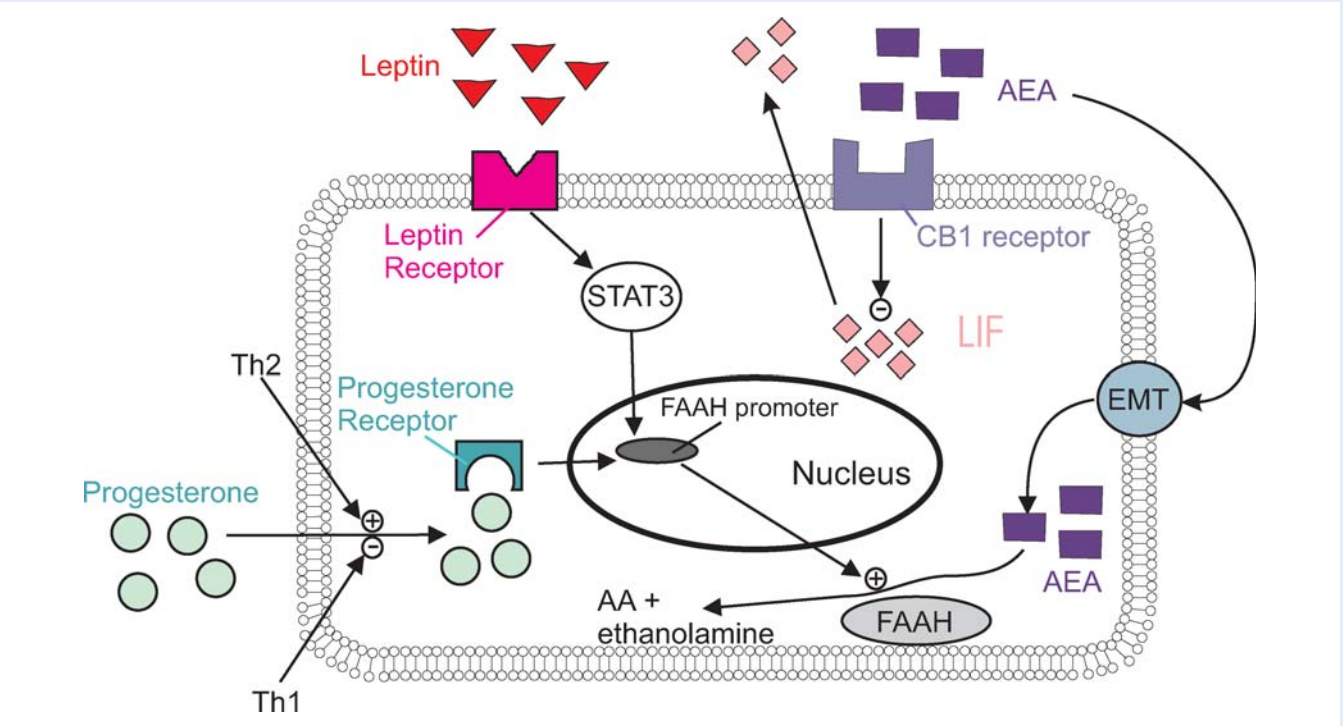

Figure 6 AEA-hormone–cytokine network. AEA is removed from the extracellular space via the putative, not yet identified EMT, and then it is degraded intracellularly by FAAH to AA and ethanolamine. Leptin promotes the up-regulation of the FAAH promoter via STAT3, and thereby decreases AEA levels. Furthermore, progesterone up-regulates the FAAH promoter via the transcription factor Ikaros. AEA reduces LIF release via the CB1 receptor. Progesterone induces pro-fertility Th2 cytokines and inhibits the release of anti-infertility Th1 cytokines.

cytokine-signalling proteins and protein inhibitors of activated STAT (Auernhammer and Melmed, 2000).

Studies with LIF knockout mice showed that they are infertile as a result of failed implantation; however, $LIF^{-/-}$ embryos can implant in wild-type female mice or $LIF^{-/-}$ females after injection of LIF (Stewart *et al.*, 1992; Chen *et al.*, 2000). These results suggest that LIF is critically important for implantation but less so for embryo development. Interestingly, gp130 knockout mice also present with failed embryo implantation (Ernst *et al.*, 2001). *In vitro* studies on mice have demonstrated a role for LIF in blastocyst hatching, trophoblast outgrowth and implantation of cultured mouse embryos (Lavranos *et al.*, 1995; Cai *et al.*, 2000).

LIF expression has been detected in the human endometrium with a peak LIF mRNA concentration in the luteal phase at the time of implantation (Charnock-Jones *et al.*, 1994; Kojima *et al.*, 1994).

Furthermore, LIF is expressed in Fallopian tubes in humans and therefore may be involved in blastocyst development (Keltz *et al.*, 1996). LIF-Rβ mRNA, but not LIF itself, has been demonstrated in human blastocysts (Charnock-Jones *et al.*, 1994).

IL1β and leptin have been found to up-regulate LIF-Rβ in human endometrium (Gonzalez *et al.*, 2004). Studies of cultured endometrial cells have demonstrated that tumour necrosis factor (TNF)-α, and IL6 also stimulate LIF production (Laird *et al.*, 1997). Seminal fluid has also been shown to increase LIF in human endometrial cells (Gutsche *et al.*, 2003). Furthermore, it has also been suggested that the human blastocyst is involved in the regulation of endometrial LIF expression (Perrier d'Hauterive *et al.*, 2004), whereby hCG has a stimulating effect on LIF expression. Conversely, it is known that LIF can stimulate hCG production by the trophoblast (Nachtigall *et al.*, 1996). LIF also enhances blastocyst development and differentiation *in vitro* (Dunglison *et al.*, 1996; Cai *et al.*, 2000).

LIF seems to be involved in decidualization, as high levels of LIF expression are detected in trophoblast and placenta (Hilton, 1992; Nachtigall *et al.*, 1996; Laird *et al.*, 1997; Sharkey *et al.*, 1999). Furthermore, LIF production in cultured endometrial tissue from women affected by idiopathic infertility is lower than that of fertile women (Delage *et al.*, 1995), and lower levels of LIF have also been found in some women with recurrent miscarriage (Piccinni *et al.*, 1998). Additionally, it has been shown that gp130 secretion is reduced in infertile women (Sherwin *et al.*, 2002). LIF levels in uterine flushings have been investigated as a predictor of successful embryo implantation: LIF levels decrease in the late luteal phase of the menstrual cycle (Laird *et al.*, 1997; Sharkey *et al.*, 1999), and increased LIF levels are measured in women who fail to conceive (Ledee-Bataille *et al.*, 2002). A suggested explanation for failed conception is a delayed LIF expression following a delayed development of the endometrium.

The Th1/Th2 balance

T-lymphocytes play a significant role in implantation and successful pregnancy (Piccinni *et al.*, 1998). Th2 cytokines inhibit Th1 cytokine responses, and therefore they allow the survival of the foetus (Piccinni and Romagnani, 1996). Th2 cytokines such as IL-3, IL-4 and IL-10

stimulate trophoblast growth through inhibition of natural killer cells. Th1 cytokines, such as IL-2, IL-12 and interferon (INF)-γ, damage the trophoblast through stimulation of natural killer cells and secretion of TNF-α. In the preceding sections, a case was made for the ECS's role in the immune regulation of human fertility. In this context, it has been found that FAAH expression is regulated by the Th1 and Th2 cytokines: IL-4 and IL-10 enhance FAAH activity, whereas IL-2 and INF-γ reduce FAAH expression (Maccarrone *et al*., 2001). In addition, IL-2 inhibits the release of LIF, and IL-4 stimulates it (Maccarrone *et al*., 2001, 2002a, b, c). AEA reduces the release of LIF from T cells via a CB1 receptor-dependent mechanism (Lim *et al*., 1997; Maccarrone *et al*., 2000a, b, 2001), and thereby carries out its anti-fertility action (Fig. 6).

As stated before, progesterone induces pro-fertility Th2 cytokines and stimulates LIF release through IL-4 (Piccinni *et al*., 1998; Maccarrone *et al*., 2001). Treatment of women with the antiprogesterone RU486 after ovulation resulted in a reduction in LIF expression of the glandular epithelium, but not in the luminal epithelium or stromal cells (Danielsson *et al*., 1997). Furthermore, RU486 had no effect on LIF expression in the Fallopian tube (Li *et al*., 2004), suggesting that there are different regulatory mechanisms in different cells.

The role of leptin

Leptin, a 16 kDa helical cytokine, is a product of the obese (*ob*) gene (Zhang *et al*., 1994) and is produced by adipose tissue, the ovary and the placenta (Henson and Castracane, 2000; Reitman *et al*., 2001; Margetic *et al*., 2002). Leptin was first described in relation to food uptake and energy homeostasis (Friedman and Halaas, 1998). Mutations in *ob* are responsible for the absence of leptin production and for obesity and infertility in homozygous (*ob/ob*) mice (Clement *et al*., 1998). Exogenous leptin can restore fertility in *ob/ob* mice (Chehab *et al*., 1996), and there is now a general consensus that leptin is critical for reproduction (Clarke and Henry, 1999).

Leptin has been shown to regulate the growth and development of the conceptus (Kiess *et al*., 1998) and may be involved in the regulation of angiogenesis, an important process during early pregnancy (Bouloumie *et al*., 1998; Park *et al*., 2001). The human leptin receptor exists in long and short isoforms, which couple to different signal transduction pathways. The long isoform (OB−Rl) couples to the JAK2/STAT3 signalling system (Tartaglia, 1997), whereas the short isoform (OB−Rs) signals through the MAP kinase pathway (Bjorbaek *et al*., 1997). Leptin and its receptors have been located in placental syncytiotrophoblast (Ashworth *et al*., 2000) and the endometrium (Gonzalez *et al*., 2000). Low levels of leptin have been found in women with spontaneous miscarriage in the first trimester (Lage *et al*., 1999). Enhanced leptin secretion from the endometrium occurs in the presence of a blastocyst; therefore, it seems that leptin is also important for implantation (Gonzalez *et al*., 2000). Furthermore, Kawamaru *et al*. (2002) demonstrated that leptin stimulates the development of mouse embryos *in vitro*. However, high levels of leptin interfere with mouse embryo development and hatching and also cause apoptosis in blastocysts (Fedorcsak and Storeng, 2003).

In humans, leptin levels vary in relation to gender and body composition. For example, women of reproductive age have higher serum levels than men (Hickey *et al*., 1996) and post-menopausal women (Shimizu *et al*., 1997). Serum leptin levels also change during the menstrual cycle with lower levels during the follicular phase compared with the secretory phase (Hardie *et al*., 1997). Maternal serum leptin concentrations are greater than those of non-pregnant women, indicating that leptin may play a role in pregnancy maintenance (Hardie *et al*., 1997). During early pregnancy, leptin concentrations rise in conjunction with E_2 levels (Hardie *et al*., 1997). E_2 regulates leptin levels through the leptin promoter (Machinal *et al*., 1999). Leptin concentrations have been demonstrated to correlate well with progesterone levels during the luteal phase of the menstrual cycle and with hCG concentrations during human pregnancy (Hardie *et al*., 1997). Consequently, available evidence suggests a relationship between obesity, leptin levels and reproduction (Linne, 2004; Henson and Castracane, 2006; Metwally *et al*., 2008). In fact, leptin concentrations in plasma are related to the amount of body fat (Considine *et al*., 1996; Hardie *et al*., 1997), and obese women have been shown to have lower conception rates after IVF treatment (Wang *et al*., 2000; Fedorcsak *et al*., 2004); these women are also at increased risk of early pregnancy loss (Fedorcsak *et al*., 2000; Wang *et al*., 2002). Weight reduction before IVF treatment increases the chances of a successful pregnancy (Fedorcsak *et al*., 2004). Successful appetite control and therefore reduction of obesity has been demonstrated from interventions with the CB1 antagonist rimonabant (Leite *et al*., 2009) but there are no data on the effects of rimonabant on leptin levels in women of the reproductive age group.

Leptin is also integrated into the regulation of the endocannabinoid-hormone−cytokine network. Results from studies on *ob/ob*$^{-/-}$ mice demonstrated that leptin reduces the levels of AEA and 2-AG in the hypothalamus (Di Marzo *et al*., 2001b; Kirkham *et al*., 2002). Maccarrone *et al*. (2005) determined that uterine AEA and 2AG are up-regulated in the *ob/ob*$^{-/-}$ mice owing to reduced activity of EMT, FAAH and MAGL as well as increased activity of DAGL, and normal endocannabinoid levels were obtained by treatment with leptin. These results suggest that leptin down-regulates the endocannabinoid signalling pathway.

In human studies, it has been shown that leptin up-regulates the promoter region of the FAAH gene through STAT3 signalling (Maccarrone *et al*., 2003a, b) and concomitantly reduces AEA levels in T cells (Fig. 6). Consequently, inhibition of LIF release by AEA is reduced (Maccarrone *et al*., 2002a, b) and embryo implantation is impaired (Piccinni *et al*., 1998).

Overall, LIF, Th1/Th2 cytokines and leptin are all essential for implantation. It seems, therefore, that a fundamental interaction exists between these substances and the ECS, which ultimately impacts on implantation. Figure 6 summarizes these relationships, which imply that changes in the immunological response are essential for successful implantation and maintenance of pregnancy.

Conclusions

In this review, we have summarized the current knowledge of the cross talk that occurs between the ECS, steroid hormones and cytokines in female fertility. The available data suggest that a tight control of this network is required for successful implantation and maintenance of early pregnancy. This hormone−cytokine network is a key element at the maternal−foetal interface, and any defect in such a network may result in foetal loss (Piccinni *et al*., 1998).

Studies have shown that low plasma AEA levels are required for successful implantation and maintenance of pregnancy (Maccarrone *et al*., 2000a, b; Habayeb *et al*., 2004). FAAH is the key regulator of AEA levels, which directs various preimplantation events. AEA levels in humans inversely correlate with FAAH activity in peripheral lymphocytes (Maccarrone *et al*., 2002a, b, c), and FAAH is also under the control of Th1/Th2 cytokines, Progesterone and leptin (Maccarrone *et al*., 2001, 2003a, b). Taken together, FAAH and AEA assays might be useful in predicting the outcome of assisted reproduction and natural pregnancy in women with threatened miscarriage. On a final note, it should be stressed that a clear correlation between peripheral (blood) alterations of elements in the ECS and dysregulation in the actual reproductive tissues of miscarrying versus healthy women has yet to be established. However, the adverse effects of marijuana smoke and THC on reproductive functions point to processes that are modulated by ECS. THC, unlike endogenous ligands, is slowly metabolized and accumulates in fat deposits within the body and may mimic situations where an excess of endocannabinoids is produced or when re-uptake or removal of endogenous ligands is impaired (Schuel and Burkman, 2006). Future research efforts should be directed to fill this gap, in order to develop ECS-oriented drugs for the treatment of human infertility problems.

Authors' roles

T.K. conducted literature searches and prepared the manuscript. T.H.M., M.M. and J.C.K. were involved in the critical review of the manuscript.

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