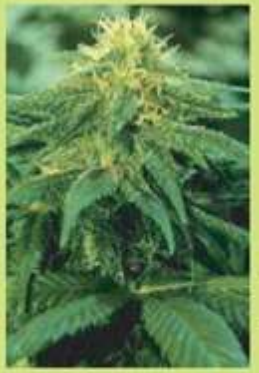
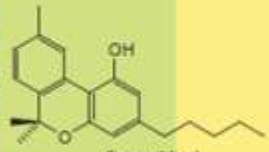
Drug Design for Membrane Proteins
The Endocannabinoid System

The in vivo biological activity of eCBs is under metabolic control, whereby catabolic enzymes play a prominent role. Among them, fatty acid amide hydrolase (FAAH), an integral membrane enzyme that hydrolyses the amide bond of AEA, and also the ester bond of 2-AG, is a key regulator of eCB signalling. The accessibility of human and rat recombinant FAAH enzymes, and the availability of X-ray structures, has recently boosted drug discovery efforts on FAAH. Hence, a better understanding of the key factors able to modulate the eCB concentration by regulating FAAH catalytic activity will allow not only to decipher basic molecular details of a variety of physiological processes, but also to develop more effective therapeutics against different human diseases, such as pain, anxiety, and epilepsy.

The history of the (endo)cannabinoids





Cannabinol

200
The therapeutic properties of cannabis are described in Chinese pharmacopoeia

1838–1840
Sir W.B. O'Shaughnessy assesses methodically the medicinal properties of cannabis

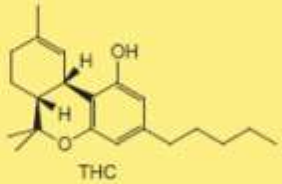
1932
Cahn elucidates part of the structure of cannabinol

Wood *et al.* isolate cannabinol from cannabis resin
1899

Todd *et al.* (and Adams *et al.* in the USA) fully elucidate and synthesize cannabinol
1940

1964
Gaoni and Mechoulam elucidate the structure of THC

Cannabinoid pharmacology is thoroughly investigated
1970–1990



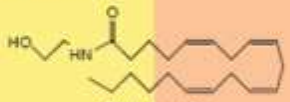
THC

1988
Howlett's group identifies specific THC binding sites in the brain

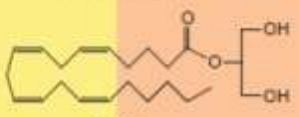
Matsuda *et al.* clone the CB_1 receptor
1990

1992
Mechoulam's group in collaboration with Pertwee's group identify the first endocannabinoid, anandamide

Munro *et al.* clone the CB_2 receptor
1993



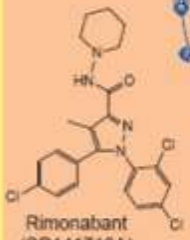
Anandamide



2-AG

1995
Mechoulam's group and Waku's group identify the second endocannabinoid, 2-AG

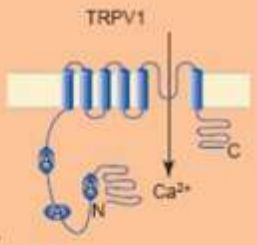
Rinaldi-Carmona *et al.* at Sanofi develop the first CB_1 receptor antagonist
1994



Rimonabant (SR141716A)

1998
House of Lord's report on medical cannabis;
Di Marzo *et al.* propose the existence of interactions between endocannabinoids and vanilloid receptors

Cravatt *et al.* clone the first endocannabinoid-degrading enzyme, FAAH
1996



2003
Bisogno *et al.* clone the first endocannabinoid-biosynthesizing enzymes

Zygmunt *et al.* and Smart *et al.* show that anandamide activates vanilloid receptors
1999–2000

????
Cloning of new cannabinoid receptors; identification of other endocannabinoid enzymes; cloning of the endocannabinoid transporter; more endocannabinoid-based therapies

New drugs

Sativex ® approved for sale in Canada; regulatory approval is filed to sell rimonabant in the USA; the Aberdeen group discovers an allosteric site on CB_1 receptors
2005

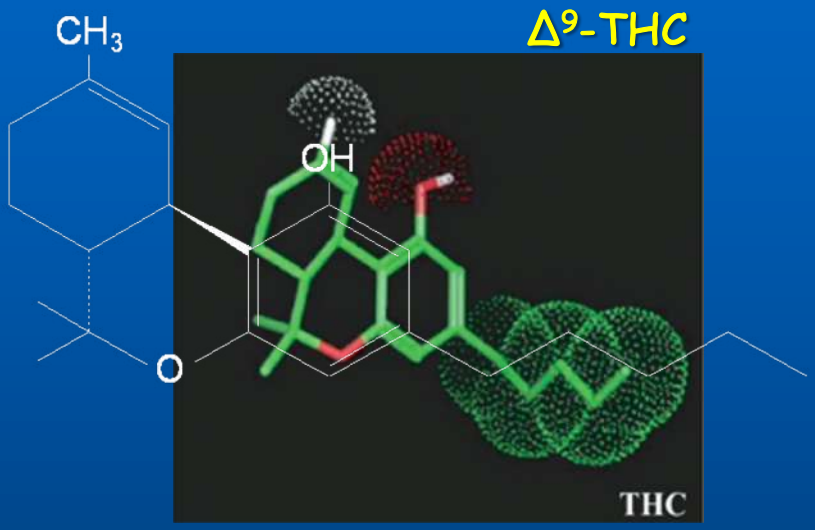
Cannabis research

Cannabinoid research

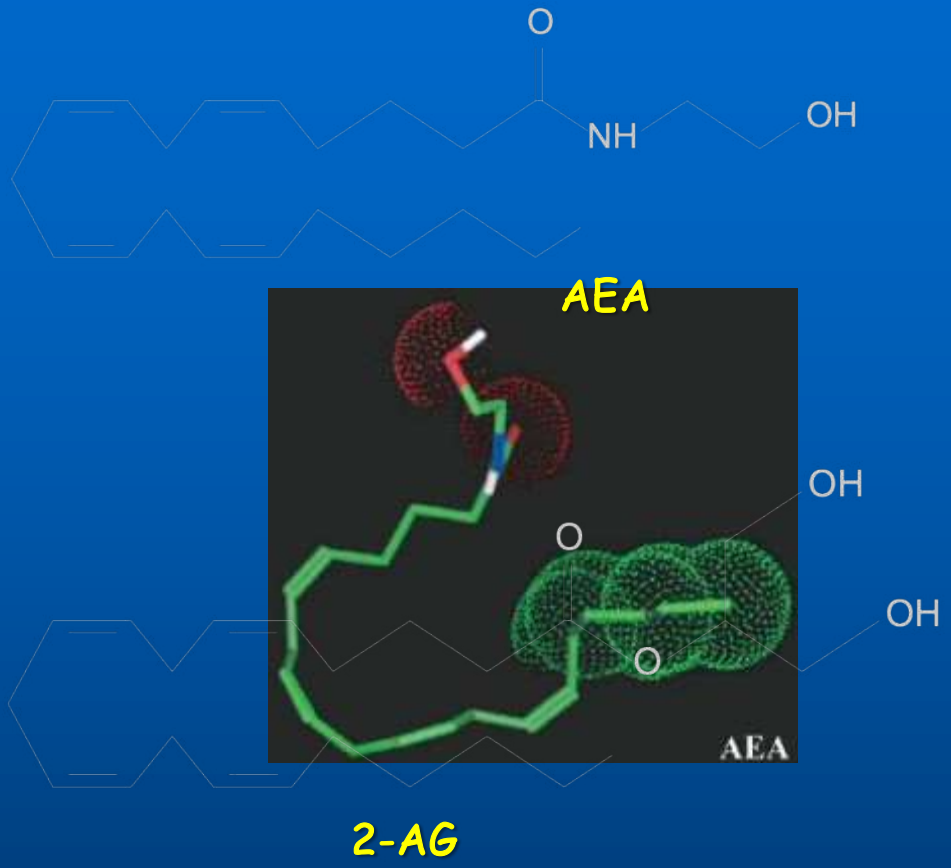
Endocannabinoid research

Di Marzo, 2006

The endocannabinoids



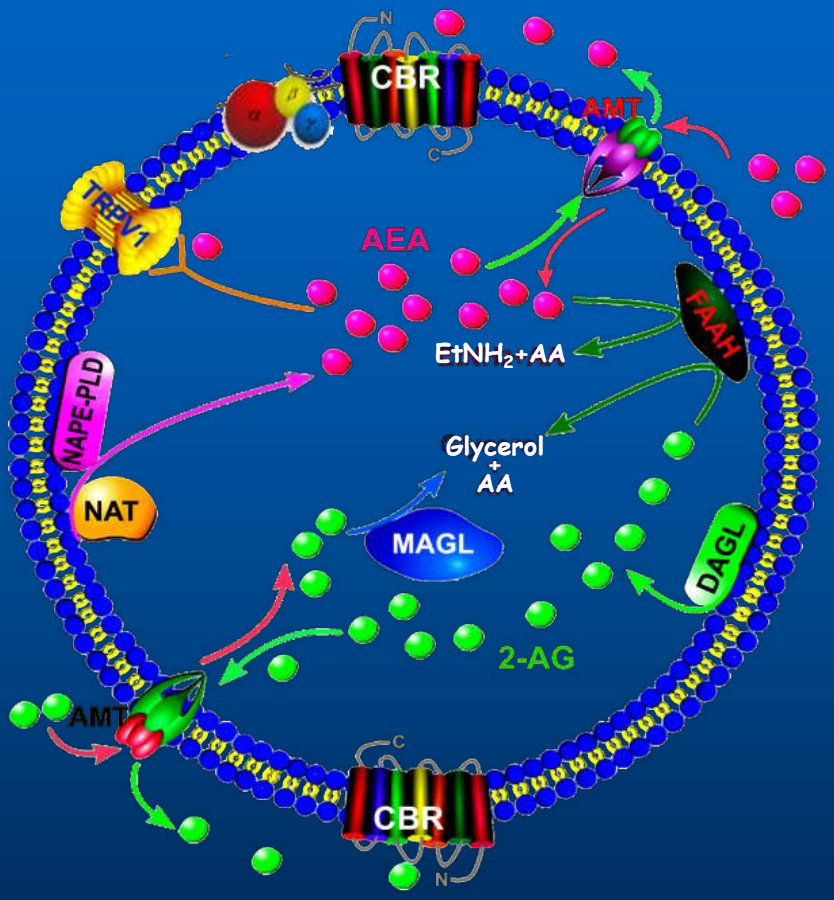
Δ9-THC



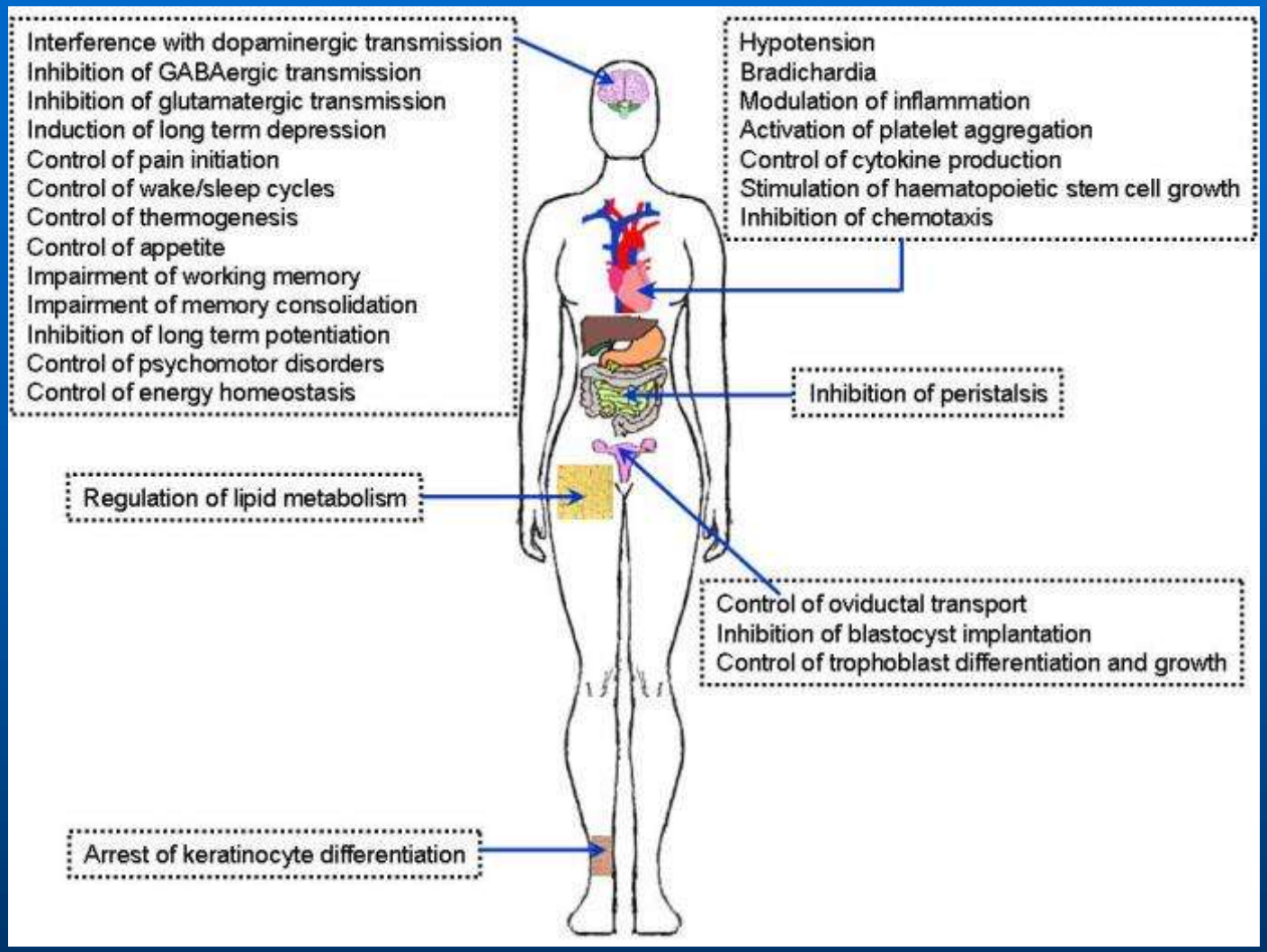
AEA

2-AG

The endocannabinoid system



Biological functions of endocannabinoids



From Maccarrone & Dainese, et al., Annual Review of Nutrition, (2010).

The biochemistry of lipids in Drug Design

saturated fatty acids

Laurate 12:0
Dodecanoic acid

Myristate 14:0
Tetradecanoic acid

Palmitate 16:0
Hexadecanoic acid

Stearate 18:0
Octadecanoic acid

Arachidate 20:0
Eicosanoic acid

Behenate 22:0
Docosanoic acid

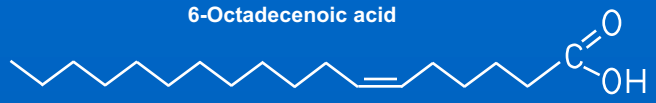
Lignocerate 24:0
Tetracosanoic acid

The biochemistry of lipids in Drug Design

monounsaturated fatty acids

Palmitoleate 16:1 Δ9
9-Hexadecenoic acid

Petroselinate 18:1 Δ6
6-Octadecenoic acid



Vaccenate 18:1 Δ11
11-Octadecenoic acid

Oleate 18:1 Δ9
9-Octadecenoic acid

Gadoleate 20:1 Δ9
9-Eicosenoic acid

Cetoleate 22:1 Δ11
11-Docosenoic acid

Erucate 22:1 Δ113
13-Docosenoic acid

Elaidate 24:1 Δ9 trans
9-trans-Octadecenoic acid

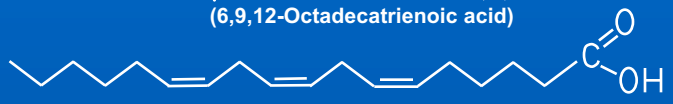
The biochemistry of lipids in Drug Design

polyunsaturated fatty acids

Linoleate 18:2 Δ 9,12
(9,12-Octadecadienoic acid)

α–Linolenate 18:3 Δ 9,12,15
(9,12,15-Octadecatrienoic acid)

γ–Linolenate 18:2 Δ 6,9,12
(6,9,12-Octadecatrienoic acid)



Meadate 20:3 Δ 5,8,11
(5,8,11-Eicosatrienoic acid)

Parinarate 18:4 Δ 9,11,13,15
(9,11,13,15-Octadecatetraenoic acid)

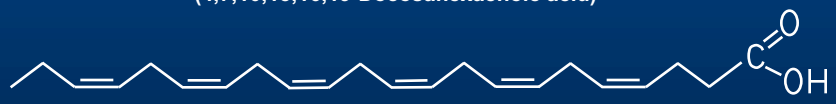
Arachidonate 20:4 Δ 5,8,11,14
(5,8,11,14-Eicosatetraenoic acid)

Timnodonate 20:5 Δ 5,8,11,14,17
(5,8,11,14,17-Eicosapentaenoic acid)

Adrenate 22:3 Δ 7,10,13,16
(7,10,13,16-Docosatetraenoic acid)

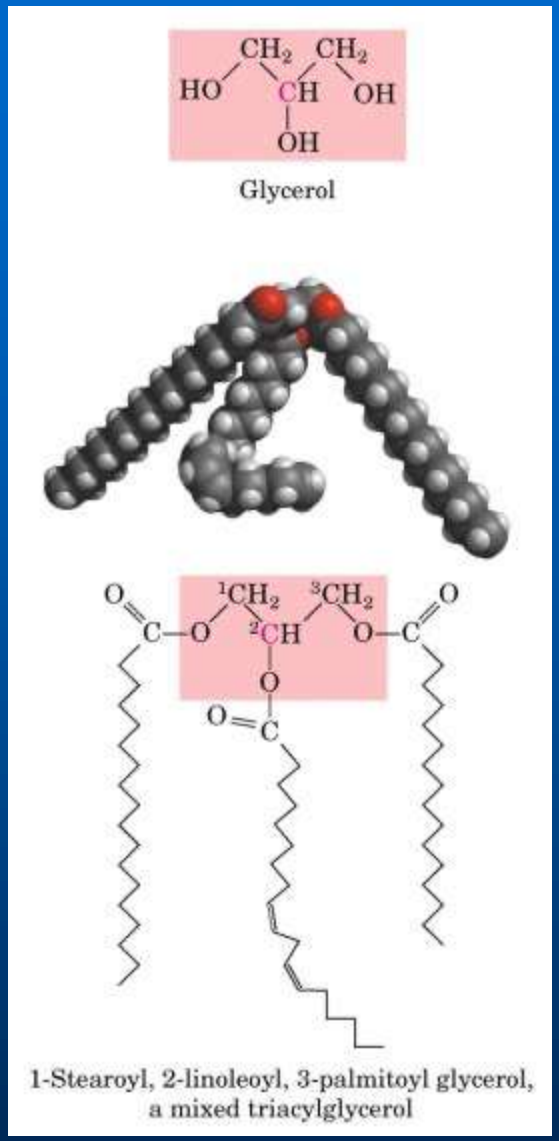
Clupanodonate 22:5 Δ 7,10,13,16,19
(7,10,13,16,19-Docosapentaenoic acid)

Cervonate 22:6 Δ 4,7,10,13,16,19
(4,7,10,13,16,19-Docosahexaenoic acid)

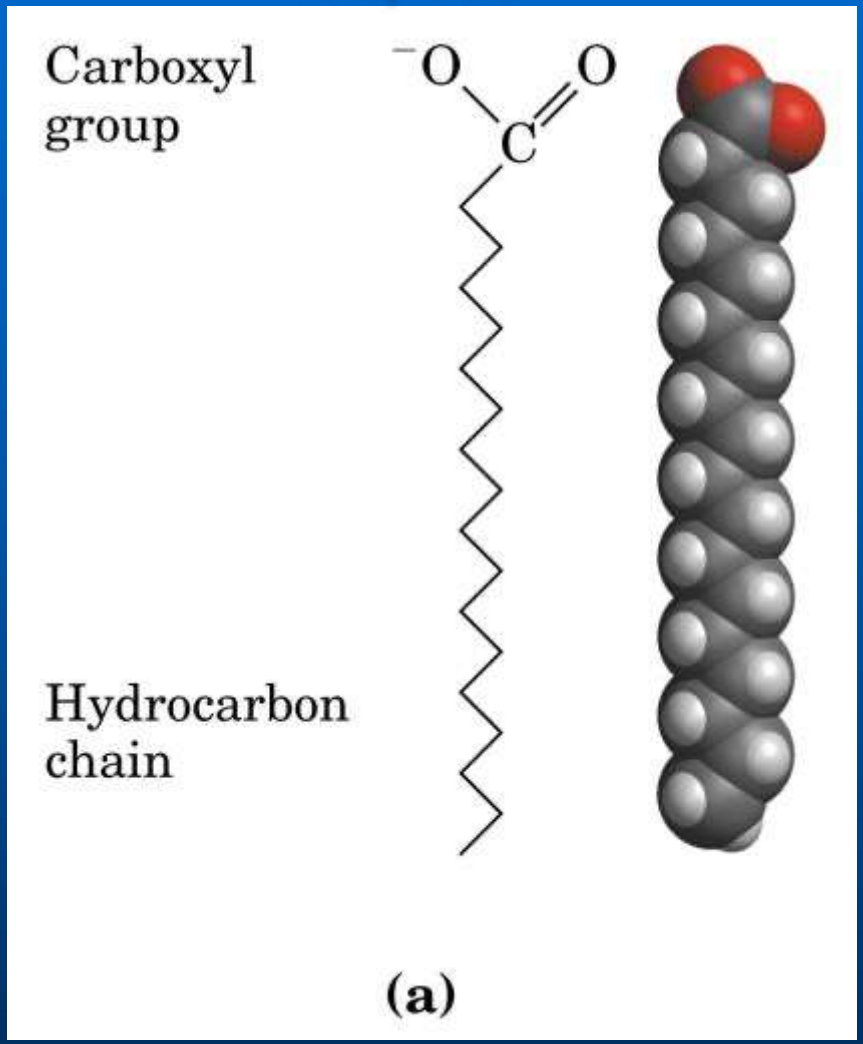


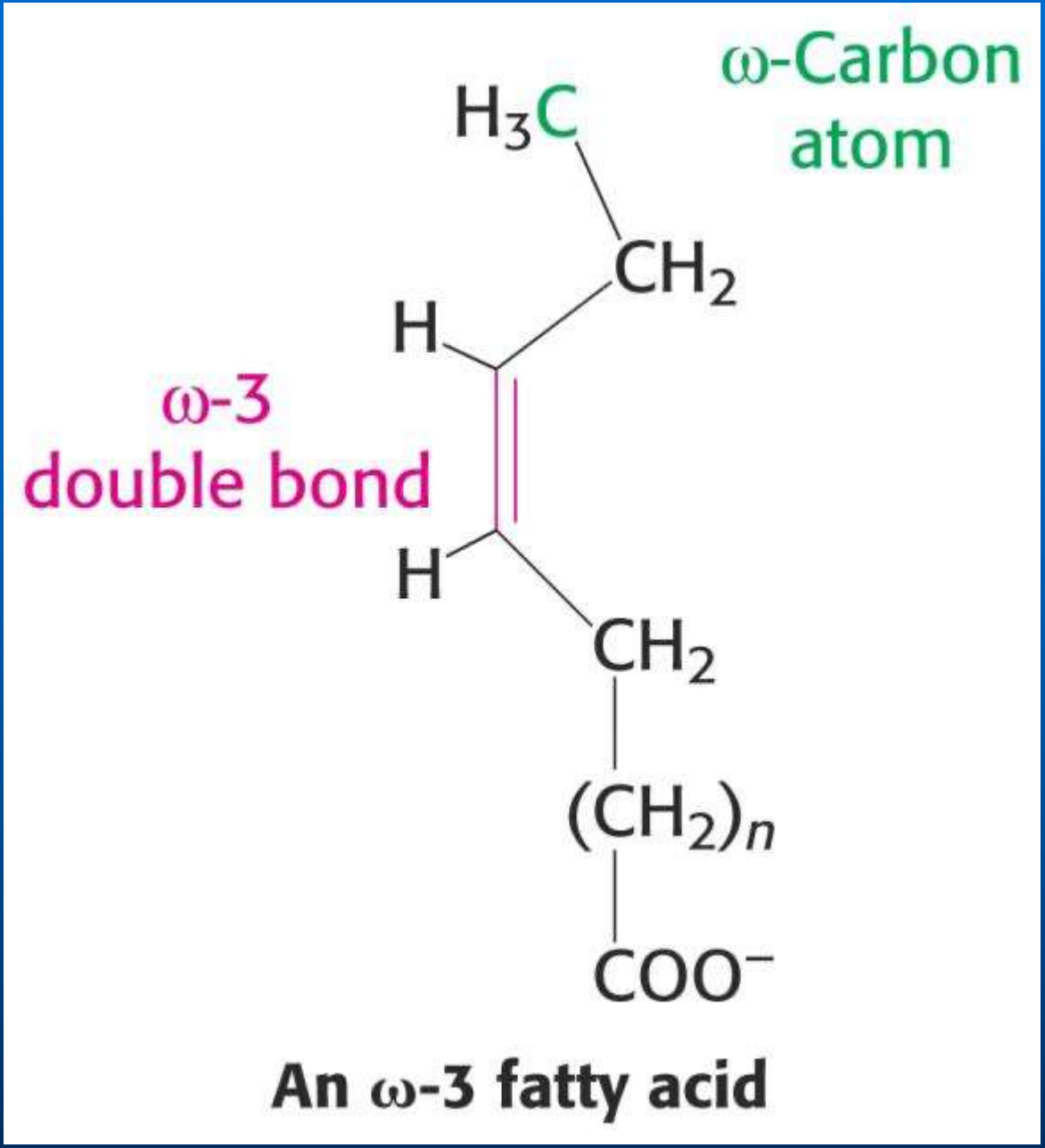
The biochemistry of lipids in Drug Design

Triglyceride

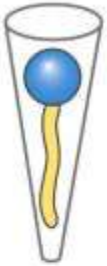


Fatty acid

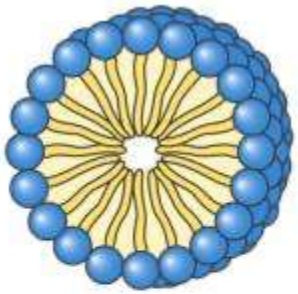




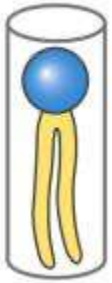
An ω-3 fatty acid



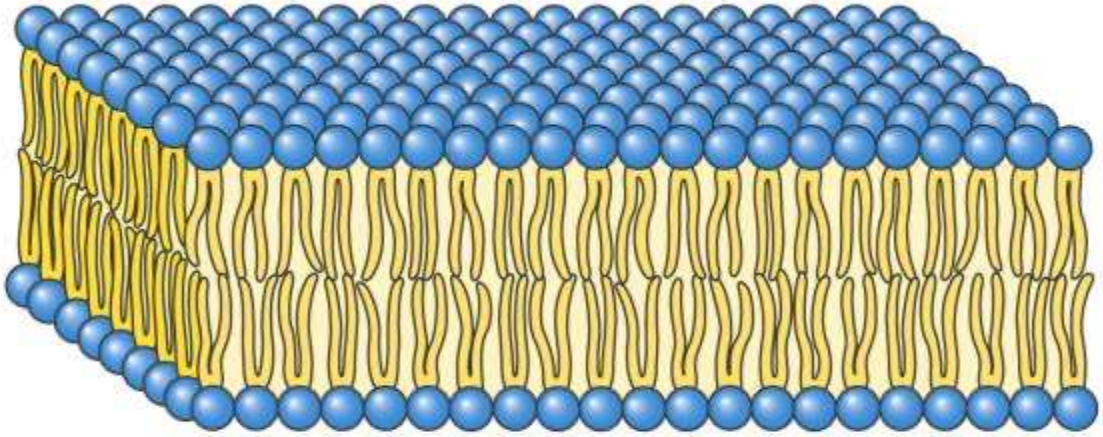
Individual units are wedge-shaped (cross-section of head greater than that of side chain)



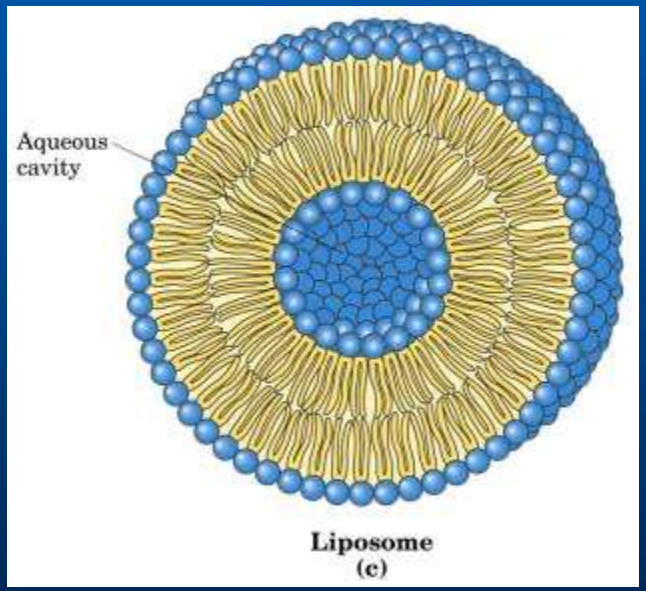
Micelle
(a)



Individual units are cylindrical (cross-section of head equals that of side chain)



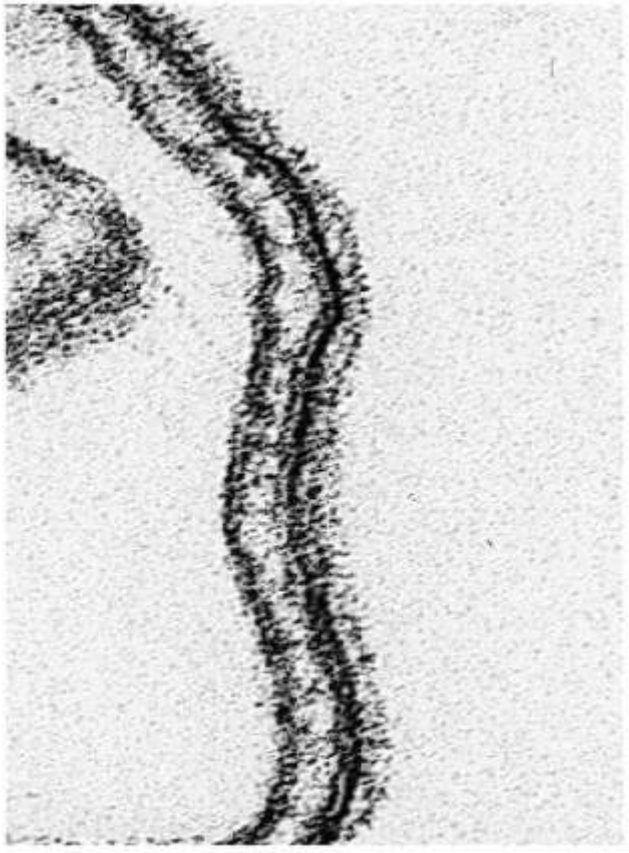
Bilayer
(b)



Liposome
(c)

The cell membranes

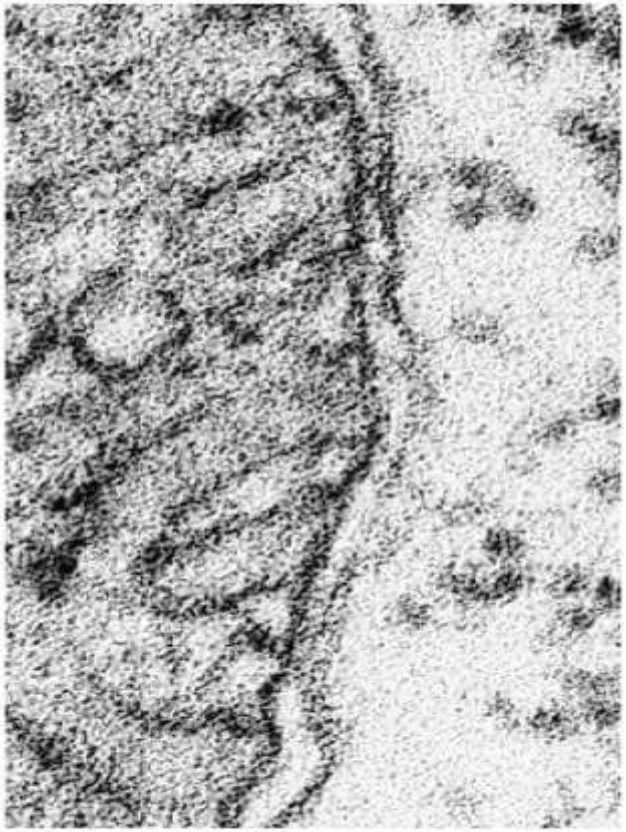
They define the outer boundaries of cells and regulate the traffic of molecules across these borders. In eukaryotic cells divide the interior space into discrete compartments, segregating in their specific internal components and processes.



(a)

Plasma Membrane

- resistant
- flexible
- self-sealing
- selectively permeable
- Support for cellular processes



(c)

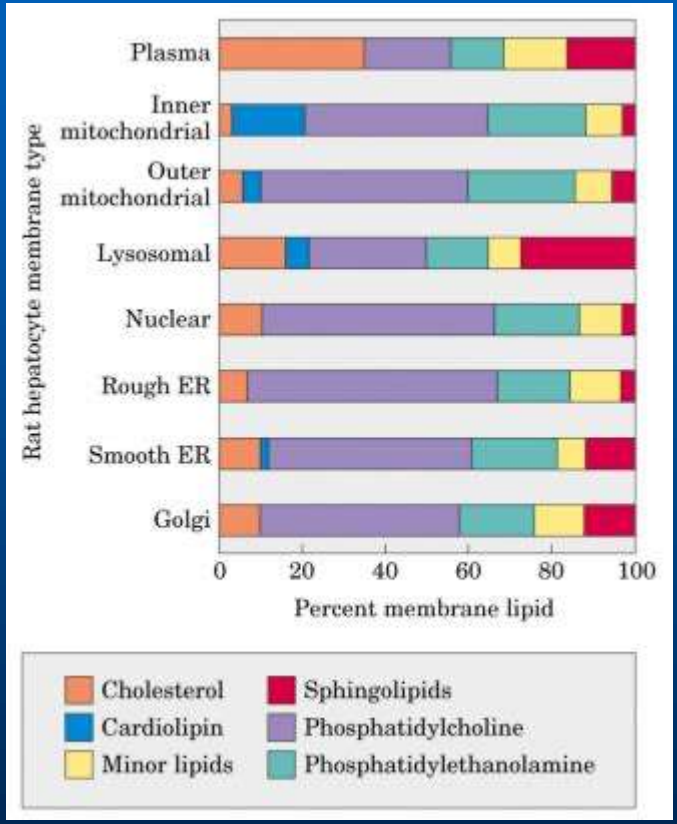
Mitochondrial membrane

table 12–1

Major Components of Plasma Membranes in Various Organisms

	Components (% by weight)				
	Protein	Phospholipid	Sterol	Sterol type	Other lipids
Human myelin sheath	30	30	19	Cholesterol	Galactolipids, plasmalogens
Mouse liver	45	27	25	Cholesterol	—
Maize leaf	47	26	7	Sitosterol	Galactolipids
Yeast	52	7	4	Ergosterol	Triacylglycerols, steryl esters
Paramecium (ciliated protist)	56	40	4	Stigmasterol	—
E. coli	75	25	0	—	—

The relative amounts of lipids and proteins vary depending on the membrane type and reflect the differences of their biological functions



Dietary lipids affect cell membrane composition

- Dietary lipids are used as an energy source, as a structural component in the membranes of cells (cholesterol and phospholipids), as structural components of a small fraction of the proteins in the cell.
- In the case of cholesterol, for the synthesis of detergents that facilitate digestion and absorption of dietary lipids, and for the synthesis of steroid hormones.

Dietary lipids affect cell membrane composition

TABLE 12.1 Some naturally occurring fatty acids in animals

Number of carbons	Number of double bonds	Common name	Systematic name	Formula
12	0	Laurate	*n*-Dodecanoate	$CH_3(CH_2)_{10}COO^-$
14	0	Myristate	*n*-Tetradecanoate	$CH_3(CH_2)_{12}COO^-$
16	0	Palmitate	*n*-Hexadecanoate	$CH_3(CH_2)_{14}COO^-$
18	0	Stearate	*n*-Octadecanoate	$CH_3(CH_2)_{16}COO^-$
20	0	Arachidate	*n*-Eicosanoate	$CH_3(CH_2)_{18}COO^-$
22	0	Behenate	*n*-Docosanoate	$CH_3(CH_2)_{20}COO^-$
24	0	Lignocerate	*n*-Tetracosanoate	$CH_3(CH_2)_{22}COO^-$
16	1	Palmitoleate	*cis*-Δ^9-Hexadecenoate	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COO^-$
18	1	Oleate	*cis*-Δ^9-Octadecenoate	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COO^-$
18	2	Linoleate	*cis,cis*-Δ^9,Δ^{12}-Octadecadienoate	$CH_3(CH_2)_4(CH{=}CHCH_2)_2(CH_2)_6COO^-$
18	3	Linolenate	all-*cis*-$\Delta^9,\Delta^{12},\Delta^{15}$-Octadecatrienoate	$CH_3CH_2(CH{=}CHCH_2)_3(CH_2)_6COO^-$
20	4	Arachidonate	all-*cis*-$\Delta^5,\Delta^8,\Delta^{11},\Delta^{14}$-Eicosatetraenoate	$CH_3(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2COO^-$

The essential fatty acids: eicosanoid precursors

Linoleate 18:2 Δ 9,12
(9,12-Octadecadienoic acid)

α–Linolenate 18:3 Δ 9,12,15
(9,12,15-Octadecatrienoic acid)

Linoleic acid 18:2 ω-6	**Linolenic acid 18:3 ω-3**
↓ Unsaturation, elongation ↓	
Arachidonic acid 20:4 ω-6	**Eicosapentaenoic acid 20:5 ω-3 (EPA)**
↓ Elongation, Unsaturation ↓	
Docosapentaenoic acid 22:5 ω-6	**Docosahexaenoic acid (DHA) 22:6 ω-3**

The biochemistry of lipids in Drug Design

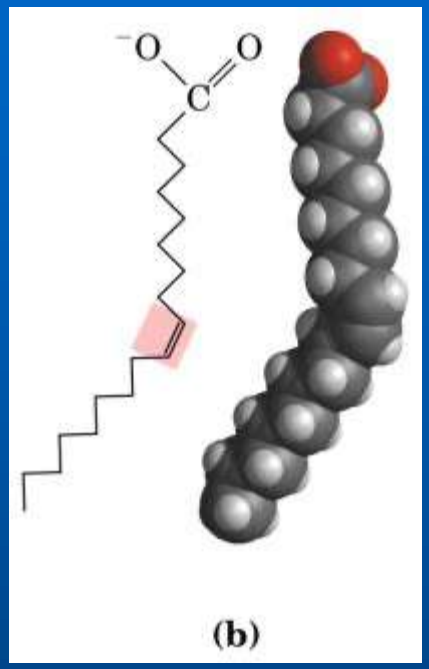
Medium content in fatty acids and vitamin E in different oils and fats

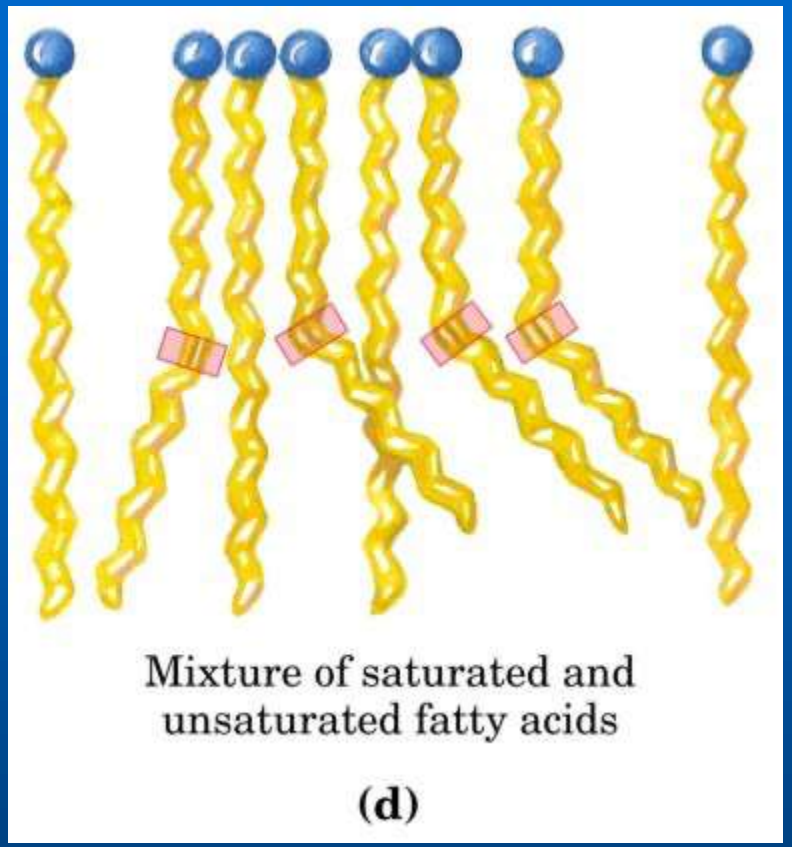
Oil/ fat	Fatty acids (%) saturated	mono-unsaturated	poly-unsaturated	Tocopherols mg/100 g
Olive oil	16	72	9	18
Corn oil	15	31	50	35
Soybean oil	14	23	59	18
Butter	49	24	3	2
Lard	43	43	12	N.D.

The main mono-unsatured fatty acid in olive oil is oleic acid.

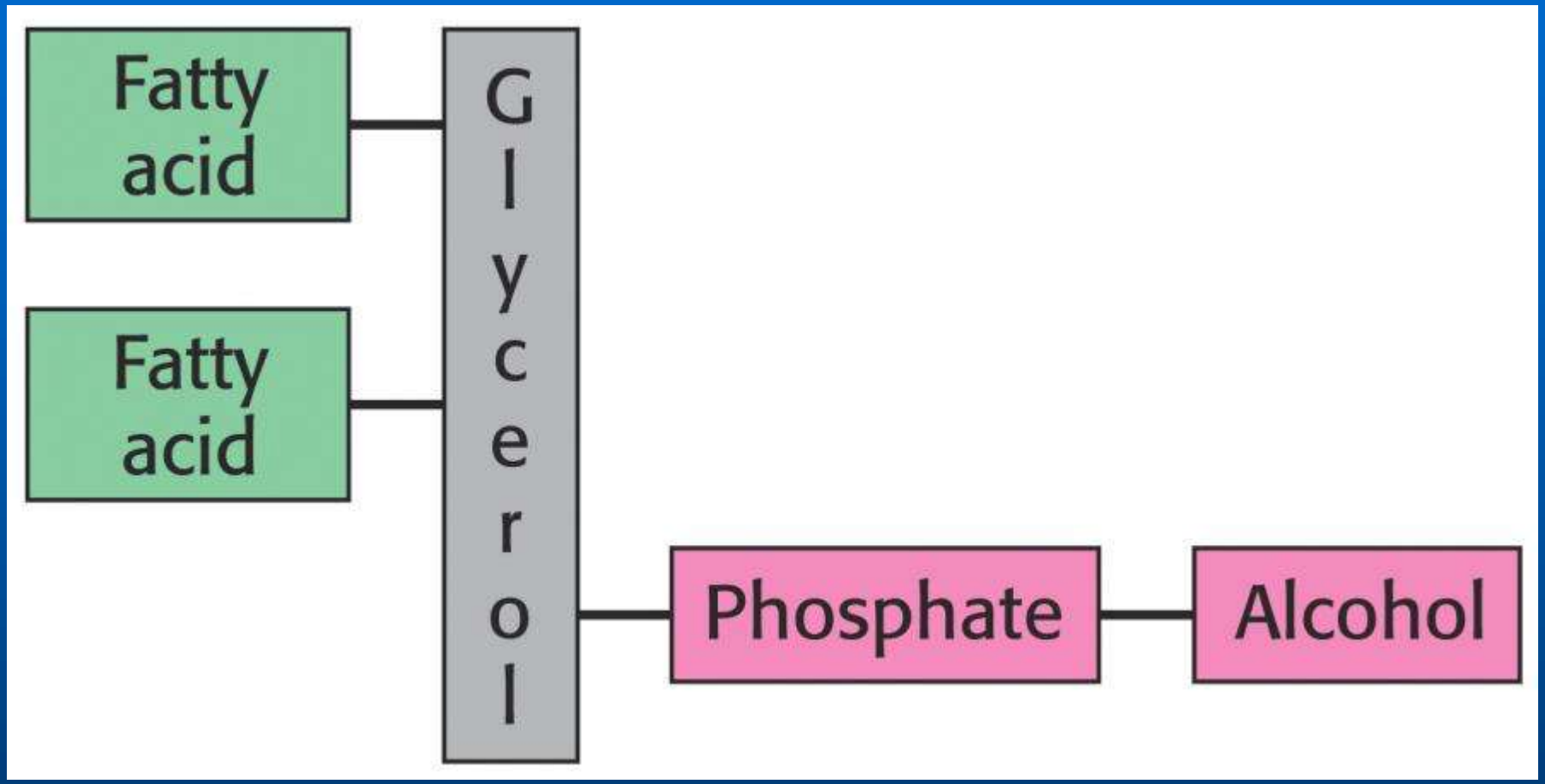
Oleate 18:1 Δ9
9-Octadecenoic acid

The biochemistry of lipids in Drug Design



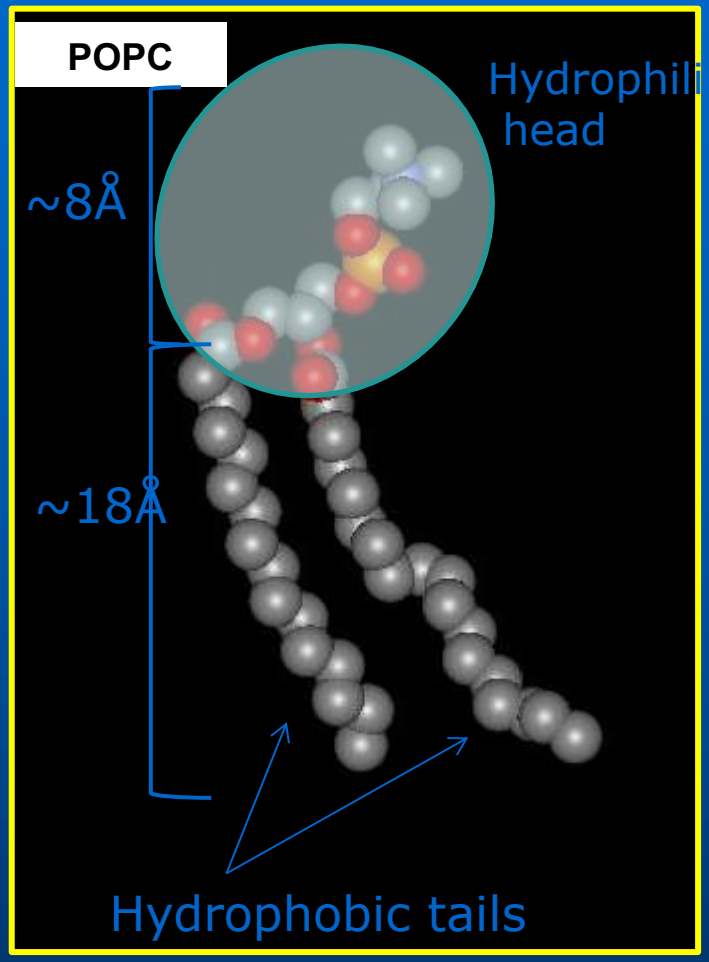


The biochemistry of lipids in Drug Design

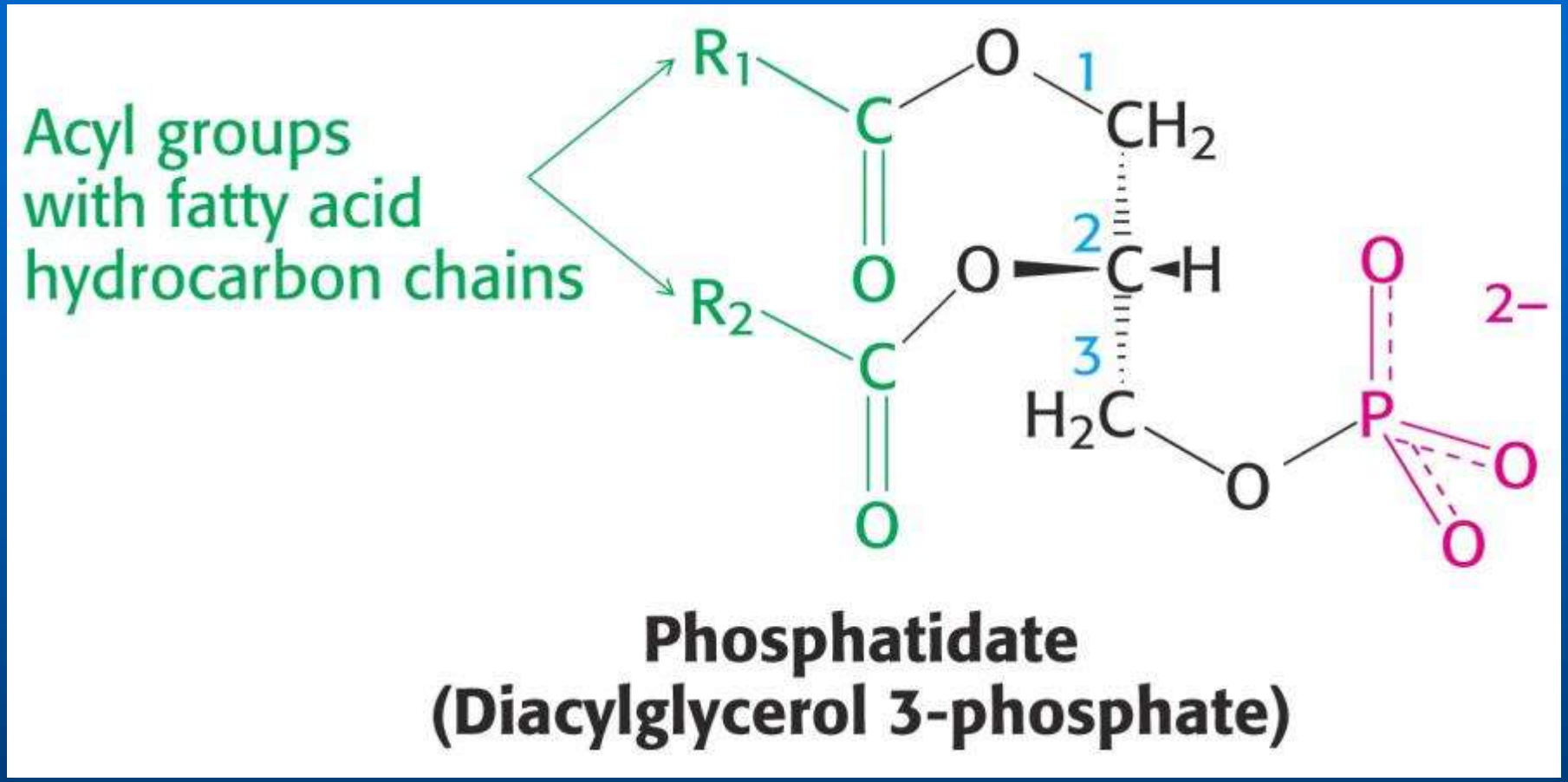


PHOSPHOLIPIDS

Most of the lipids in the bilayer can be more precisely described as **phospholipids**, i.e. lipids featuring a phosphate group at one end of each molecule.
Phospholipids are typically **hydrophilic** at their phosphate ends and **hydrophobic** along their lipid tail regions. In each layer of a plasma membrane, the hydrophobic lipid tails are oriented inwards and the hydrophilic phosphate groups are aligned so that they face outwards, either toward the aqueous cytosol of the cell or toward the external environment. Thus, phospholipids tend to spontaneously aggregate, whenever they are exposed to water.

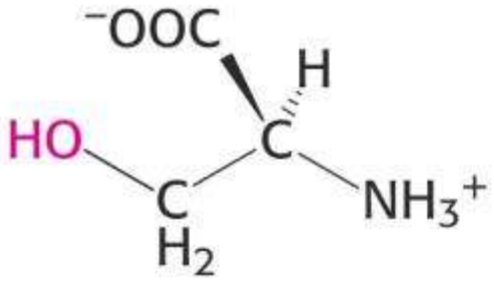


The biochemistry of lipids in Drug Design

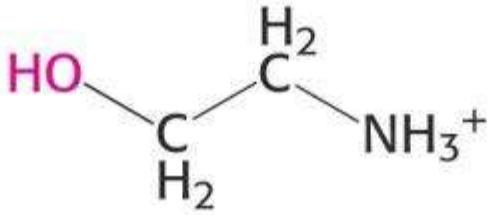


Phosphatidate
(Diacylglycerol 3-phosphate)

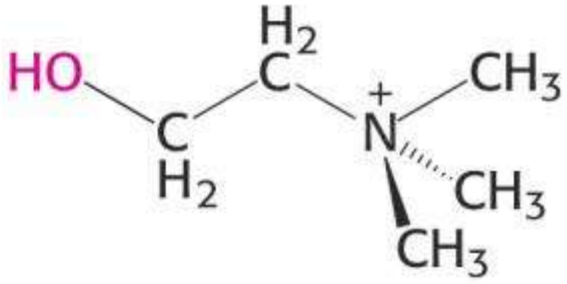
The biochemistry of lipids in Drug Design



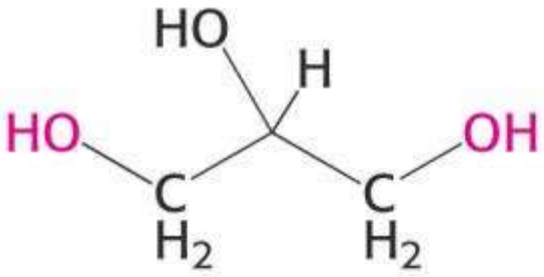
Serine



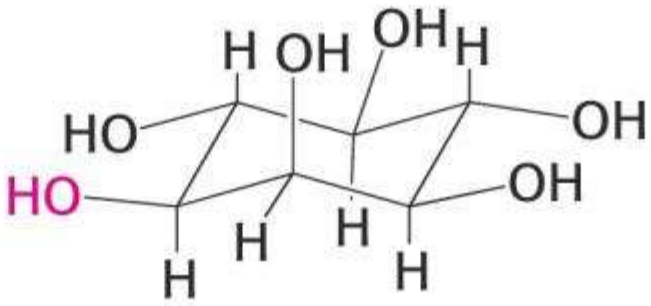
Ethanolamine



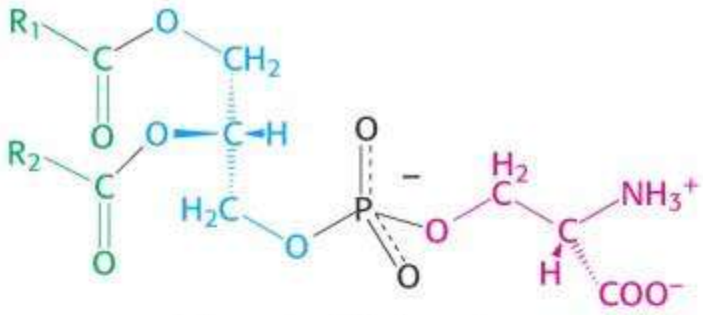
Choline



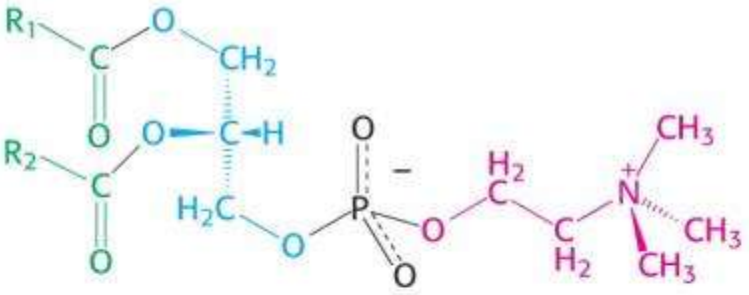
Glycerol



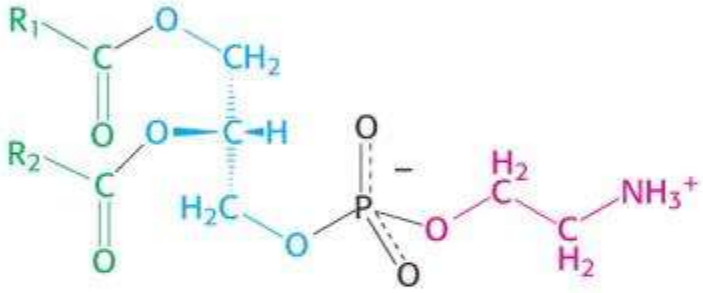
Inositol



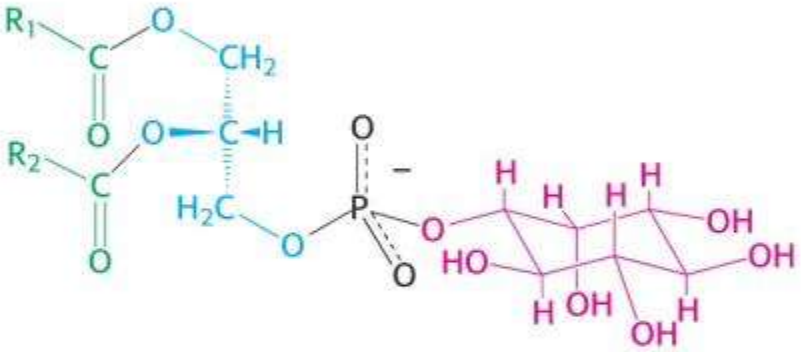
Phosphatidyl serine



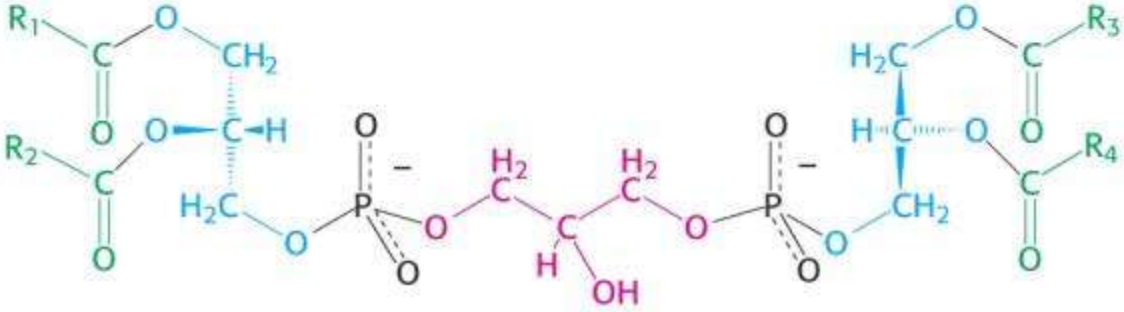
Phosphatidyl choline



Phosphatidyl ethanolamine

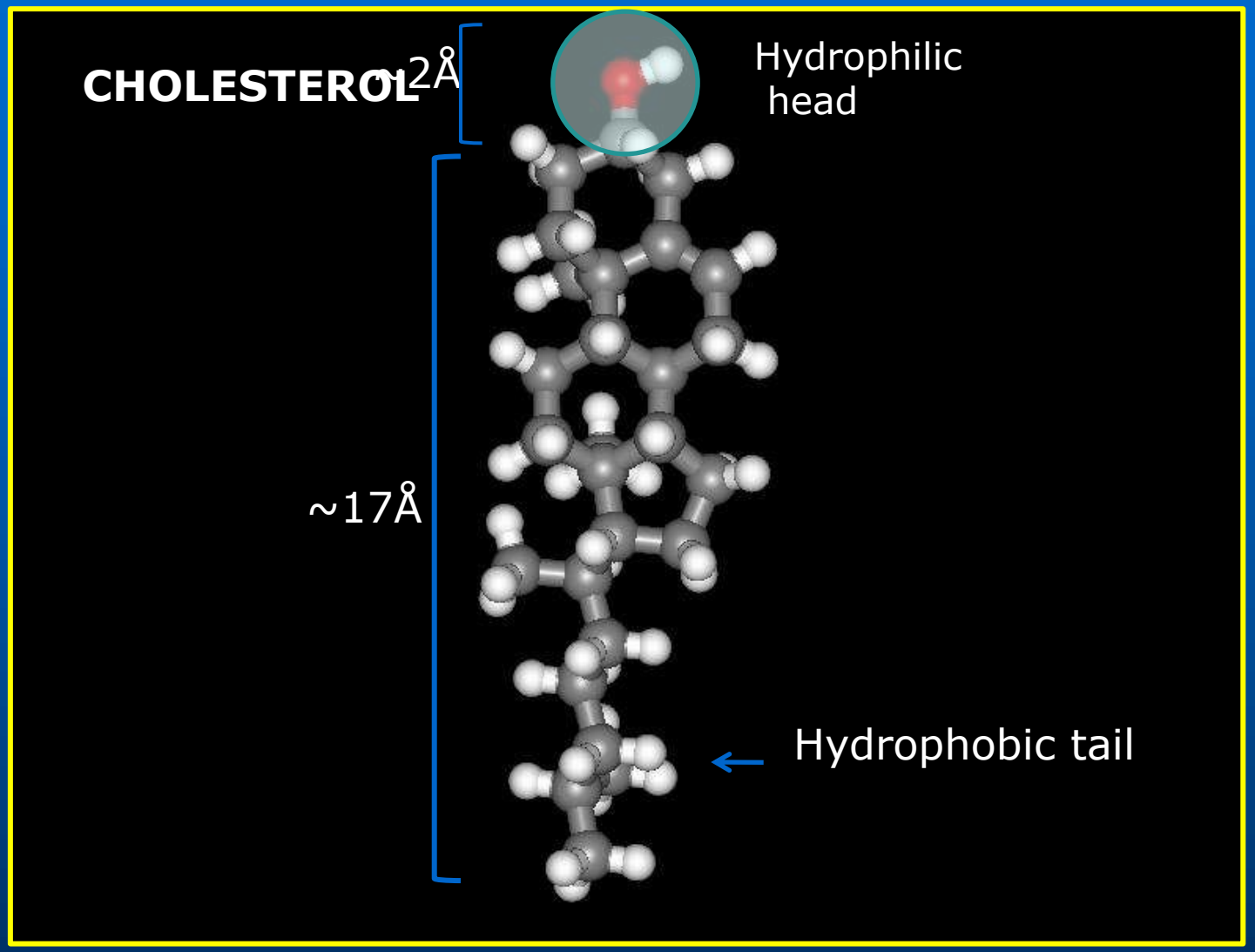


Phosphatidyl inositol

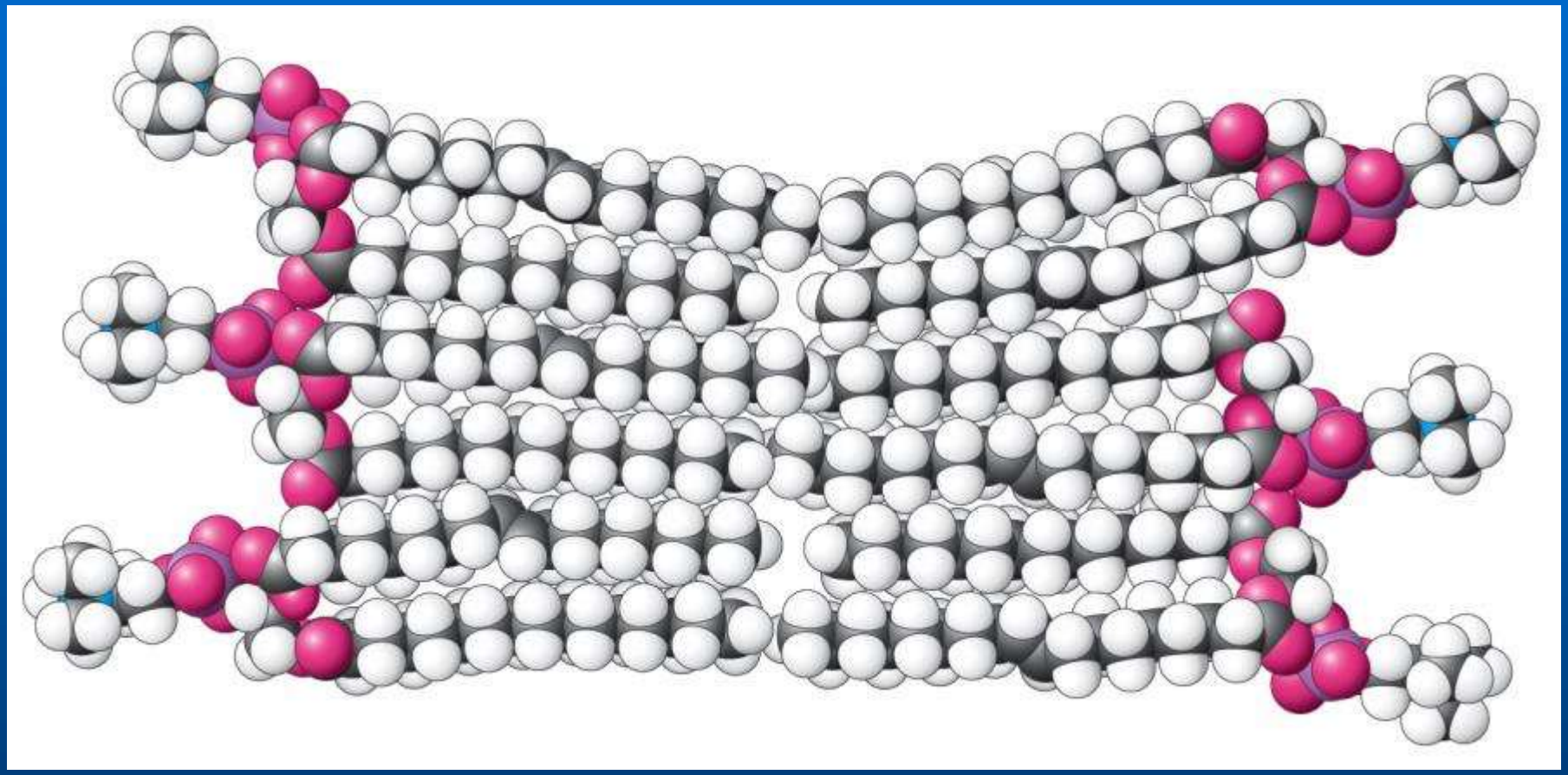


Diphosphatidyl glycerol (cardiolipin)

The biochemistry of lipids in Drug Design



The biochemistry of lipids in Drug Design



Dynamics of the membrane lipids in biological membranes

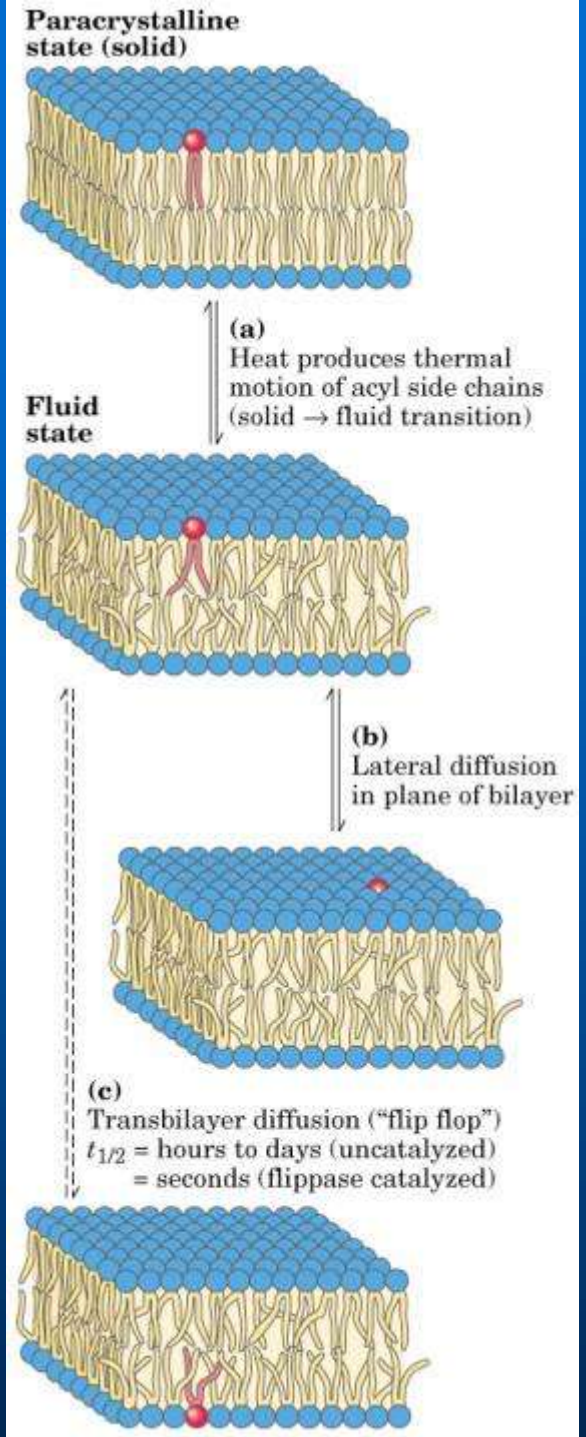


table 12–2

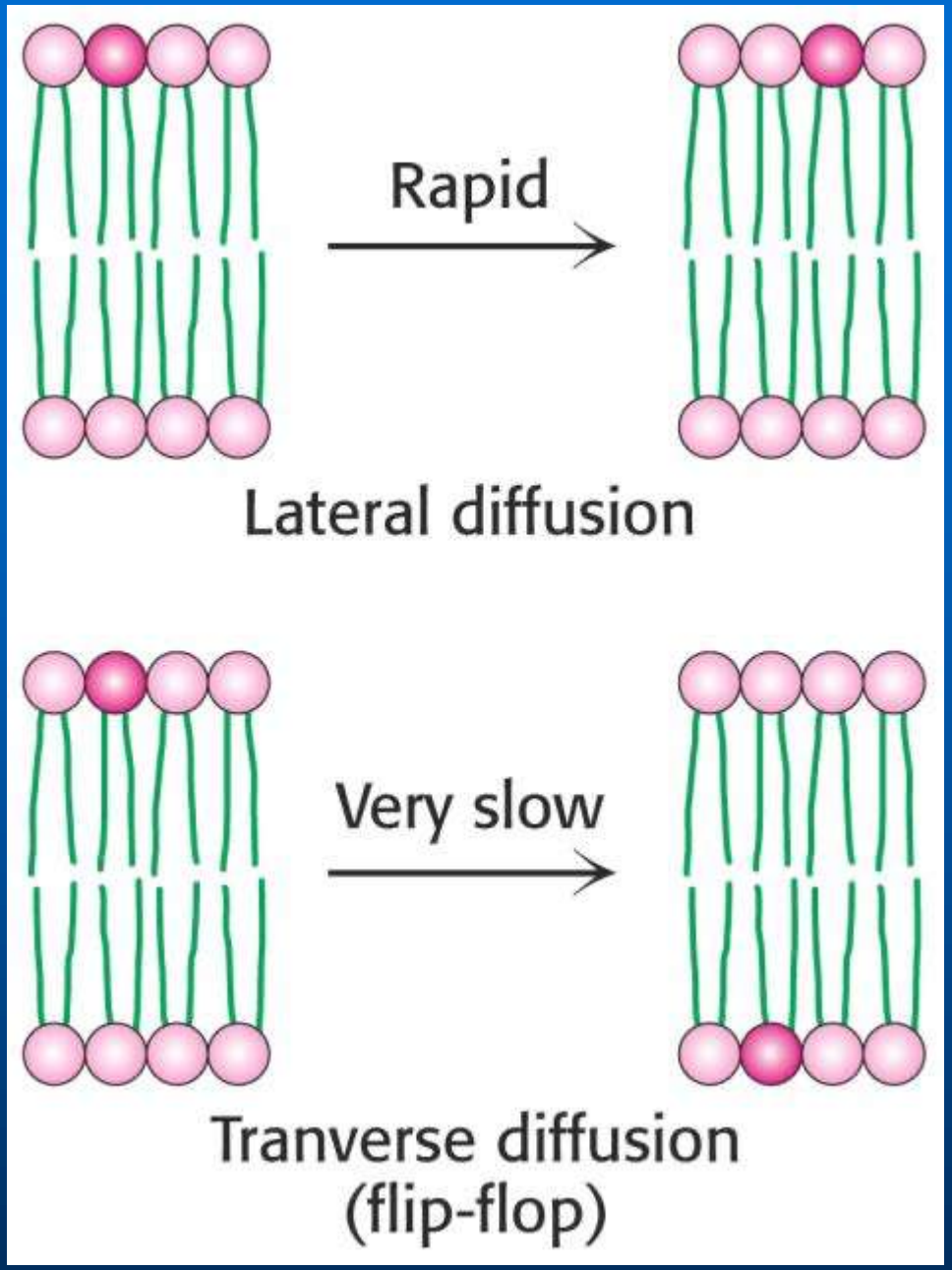
Fatty Acid Composition of *E. coli* Cells Cultured at Different Temperatures

	Percentage of total fatty acids*			
	10 °C	20 °C	30 °C	40 °C
Myristic acid (14:0)	4	4	4	8
Palmitic acid (16:0)	18	25	29	48
Palmitoleic acid (16:1)	26	24	23	9
Oleic acid (18:1)	38	34	30	12
Hydroxymyristic acid	13	10	10	8
Ratio of unsaturated to saturated†	2.9	2.0	1.6	0.38

Source: Data from Marr, A.G. & Ingraham, J.L. (1962) Effect of temperature on the composition of fatty acids in *Escherichia coli*. *J. Bacteriol.* **84,** 1260.

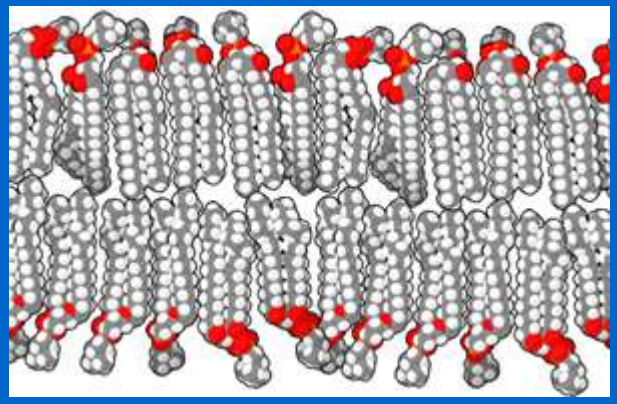
*The exact fatty acid composition depends not only on growth temperature but on growth stage and growth medium composition.

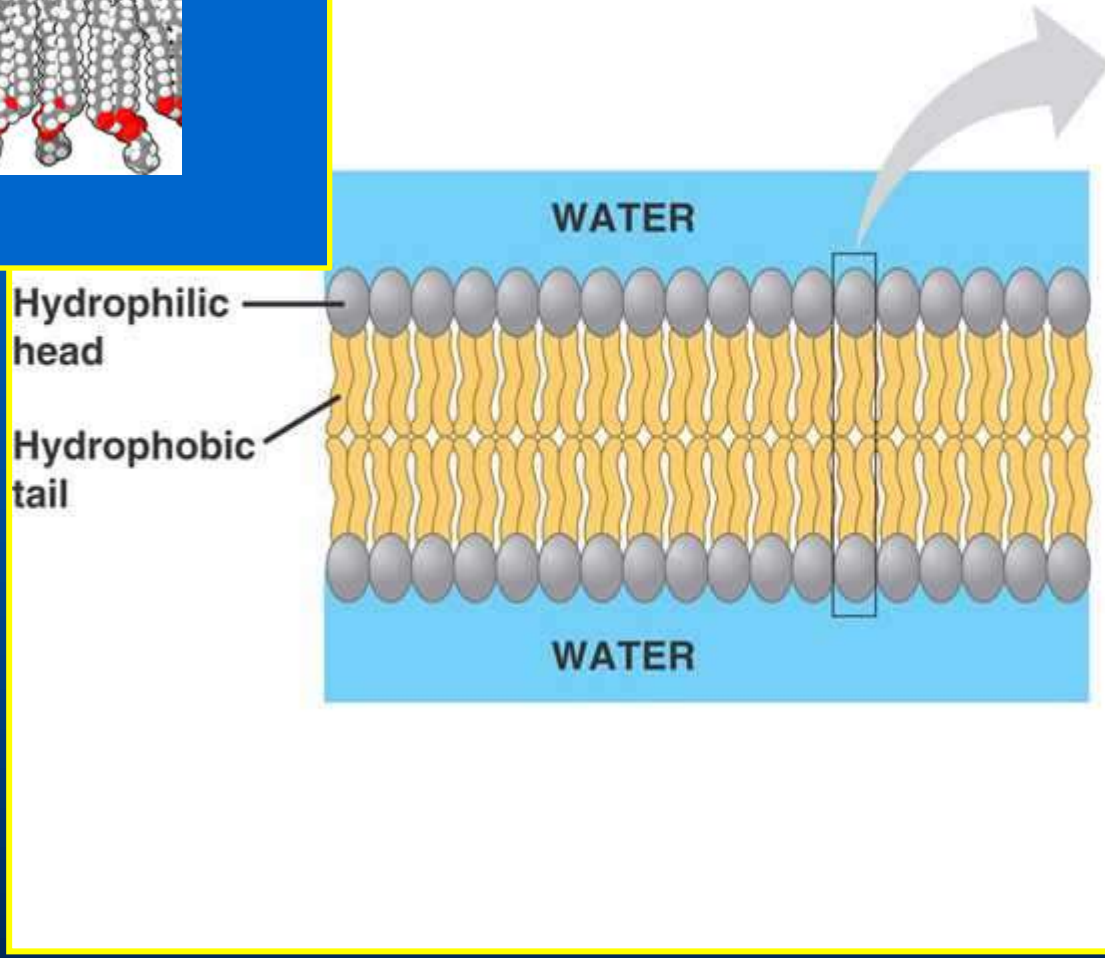
†Calculated as the total percentage of 16:1 plus 18:1 divided by the total percentage of 14:0 plus 16:0. Hydroxymyristic acid was omitted from this calculation.


Rapid
Lateral diffusion
Very slow
Tranverse diffusion
(flip-flop)

THE PHOSPHOLIPID BILAYER

DYNAMIC REPRESENTATION

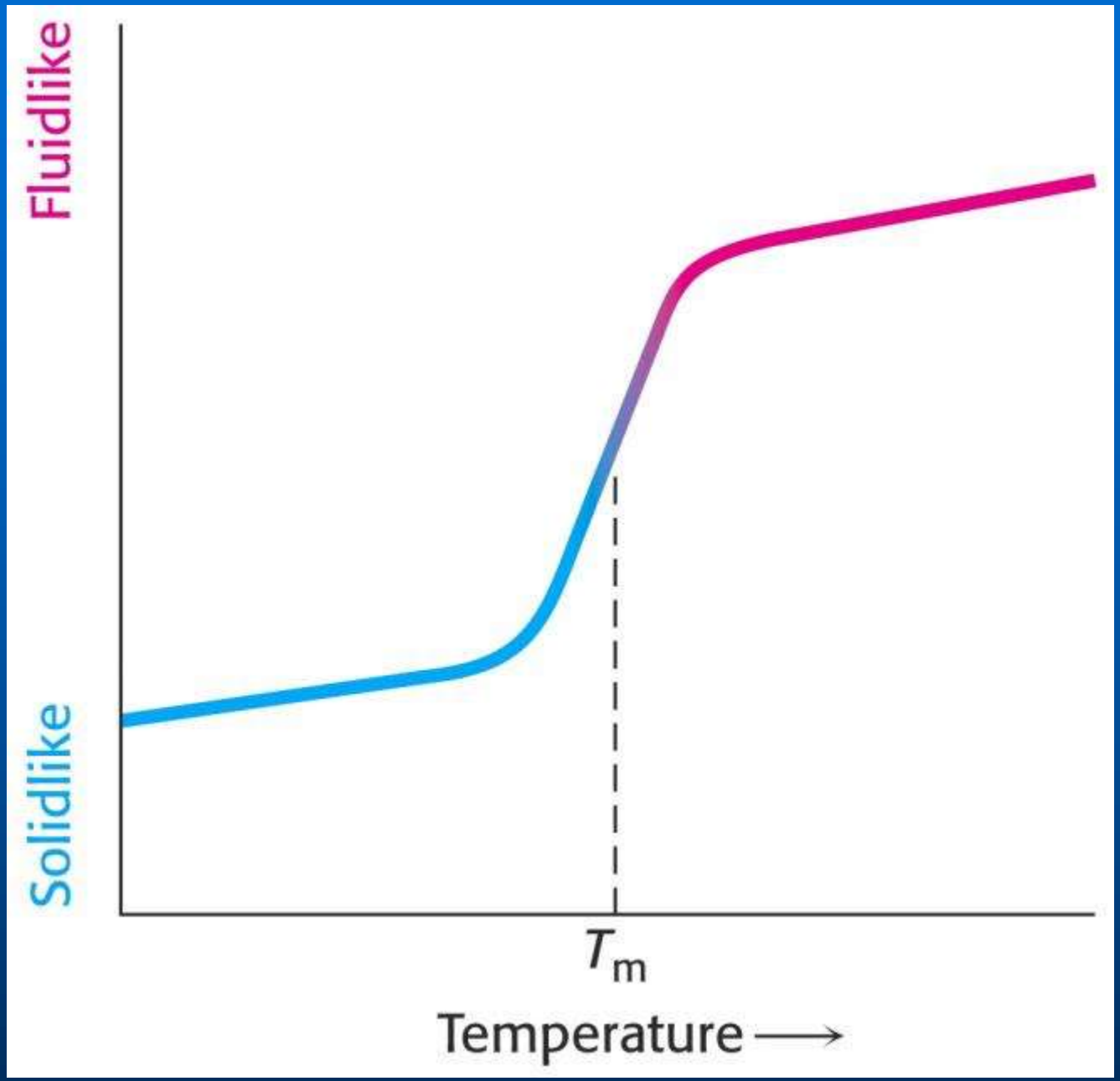




Melting temperature varies as a function of length and unsaturation of fatty acids

TABLE 12.3 **The melting temperature of phosphatidyl choline containing different pairs of identical fatty acid chains**

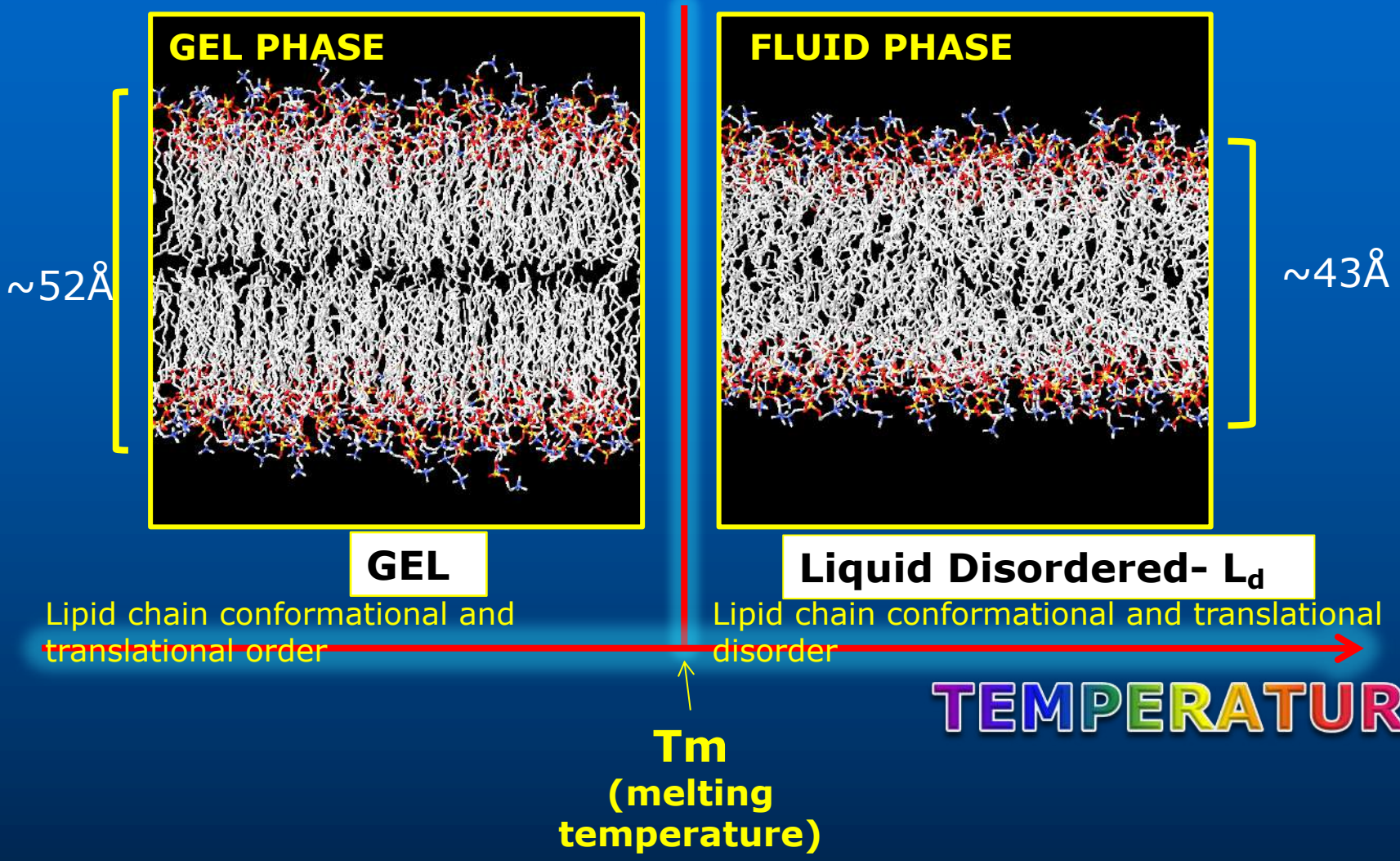
Number of carbons	Number of double bonds	Fatty acid		T_m (°C)
		Common name	Systematic name	
22	0	Behenate	*n*-Docosanote	75
18	0	Stearate	*n*-Octadecanoate	58
16	0	Palmitate	*n*-Hexadecanoate	41
14	0	Myristate	*n*-Tetradecanoate	24
18	1	Oleate	*cis*-Δ^9-Octadecenoate	− 22


Fluidlike
Solidlike
T_m
Temperature ⟶

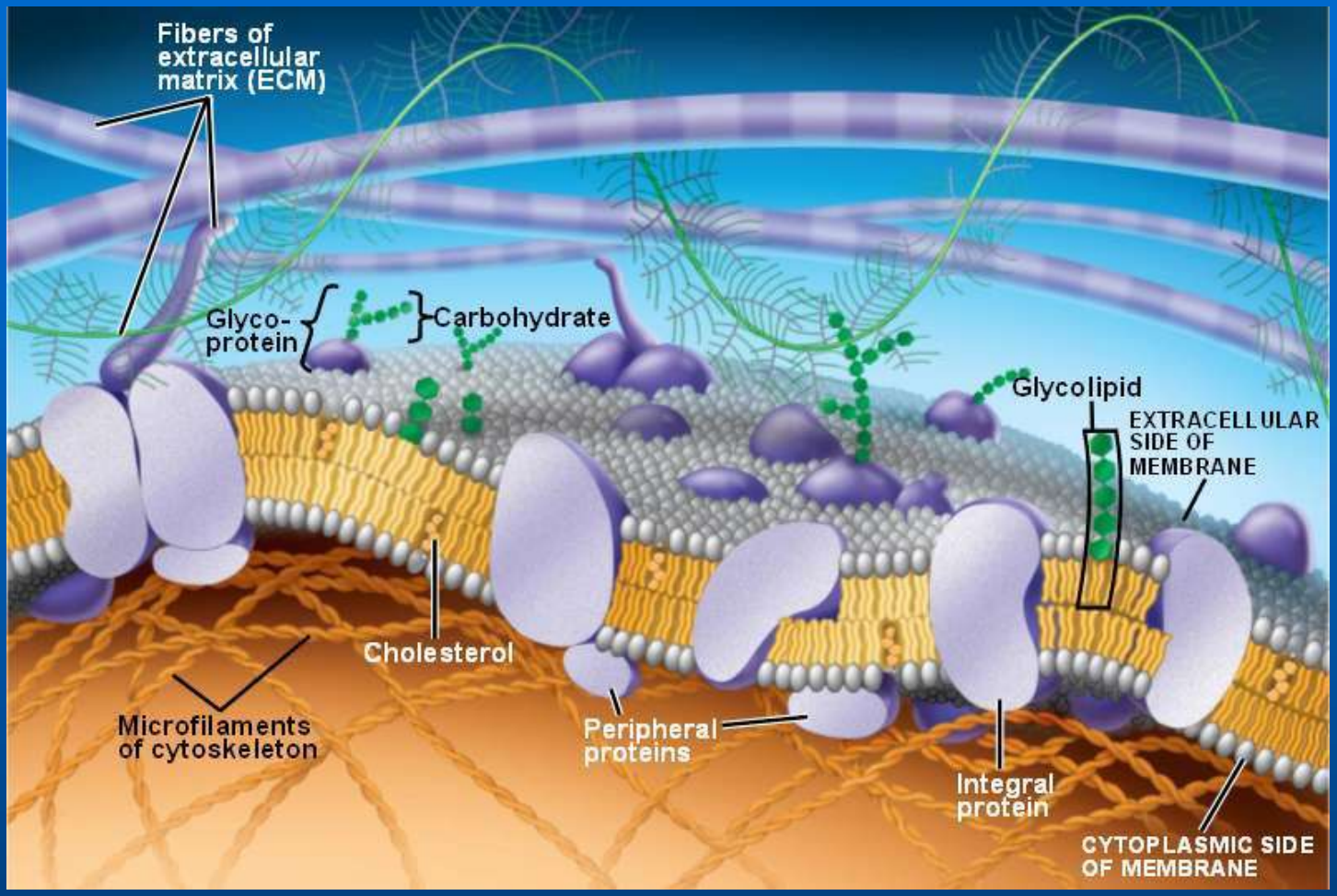
LIPID BILAYER PHASE STATES

The bilayer can adopt a solid gel phase state at lower temperatures …

… but it undergoes phase transition to a fluid state at higher temperatures

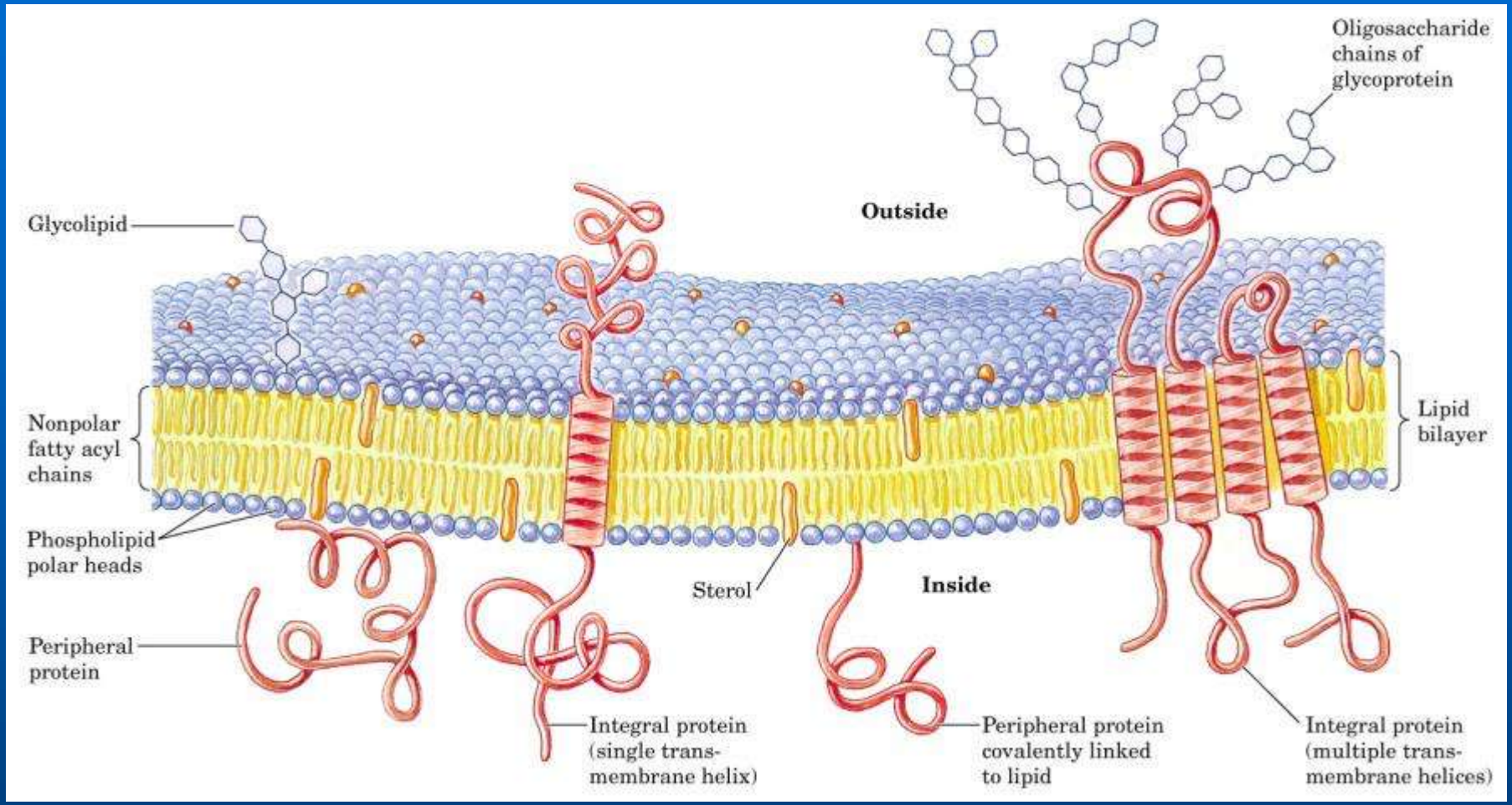


BIOLOGICAL MEMBRANES: The fluid mosaic model



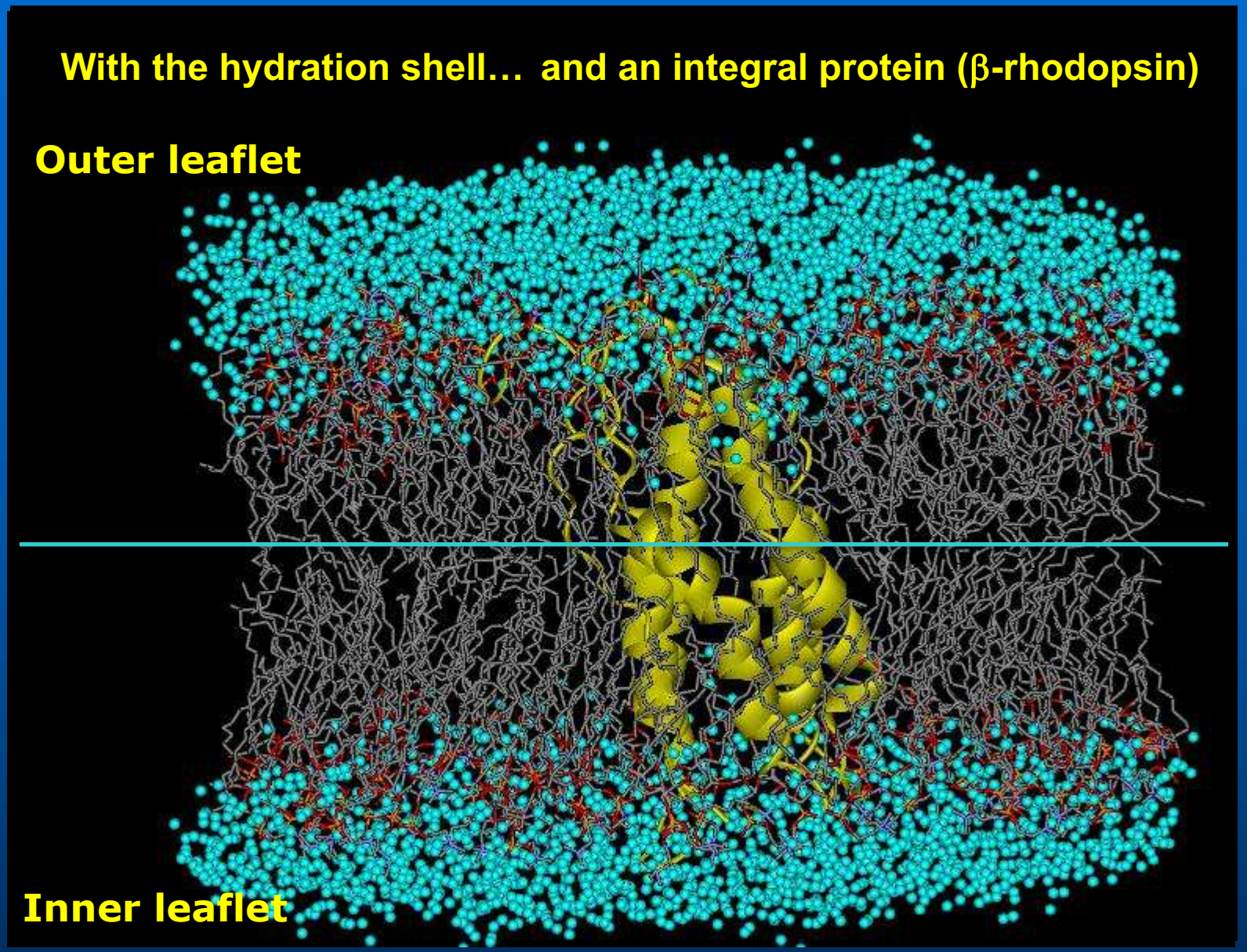
According to the fluid mosaic model of S. J. Singer and G. Nicolson (1972) the **plasma membrane** is a **fluid** structure with a "**mosaic**"
of proteins embedded in or attached to a bilayer of **lipids**.

Fluid mosaic model of membrane structure

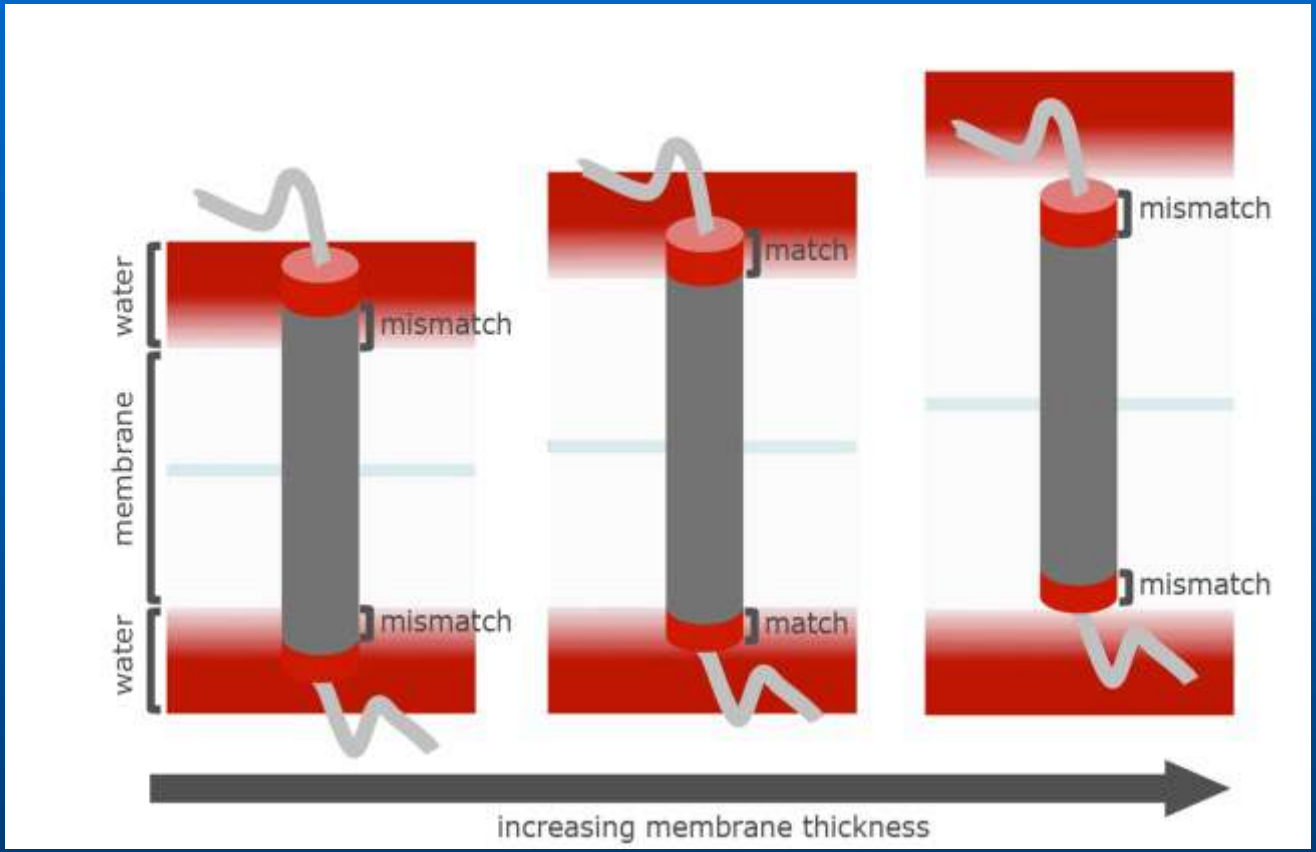


functional asymmetry

Protein/lipid interactions in biological membranes

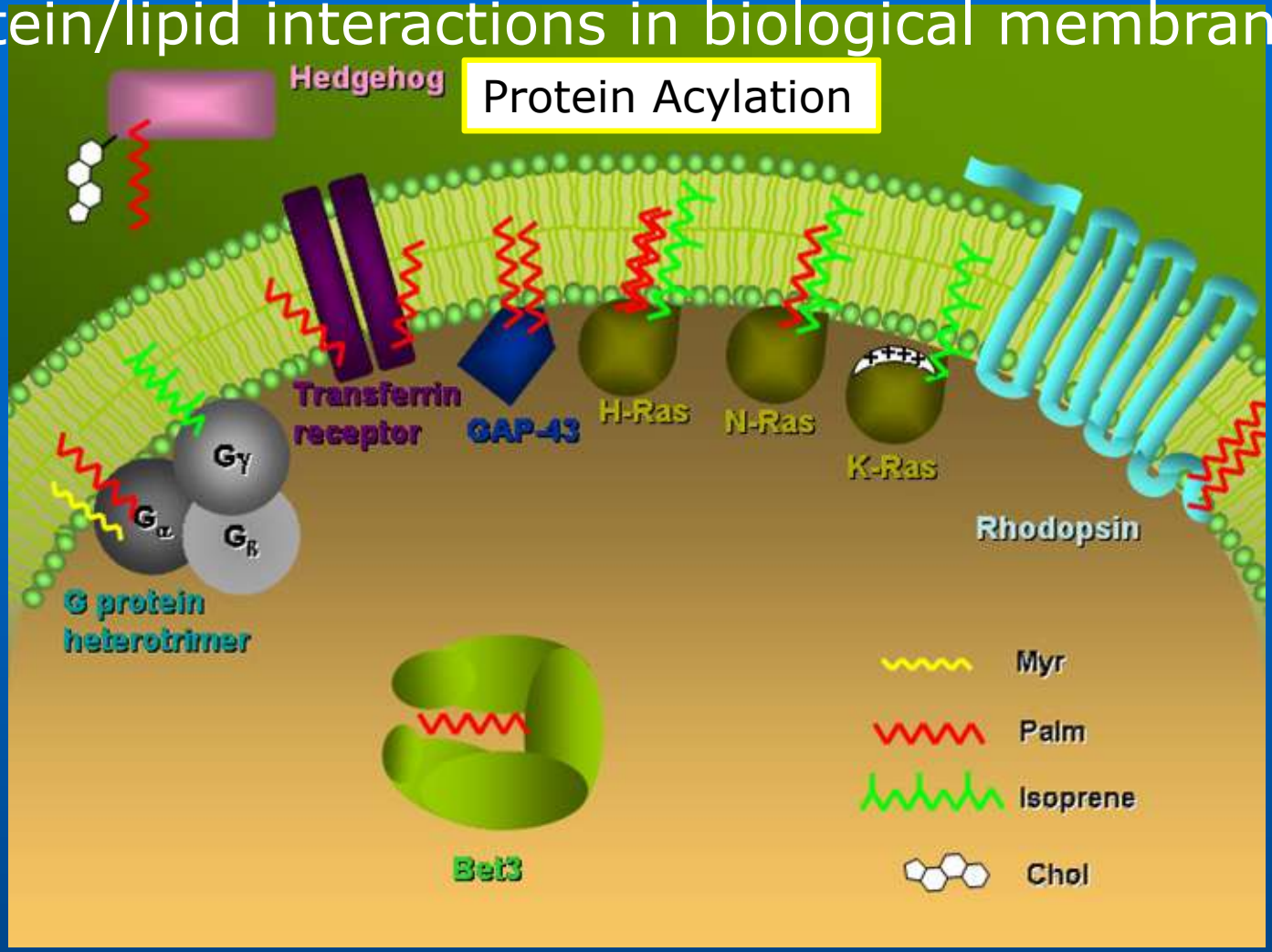


Unspecific effects on GPCRs due to overall properties of the membrane: the hydrophobic mismatch



Sadik & Dainese et al., 2013

Protein/lipid interactions in biological membranes

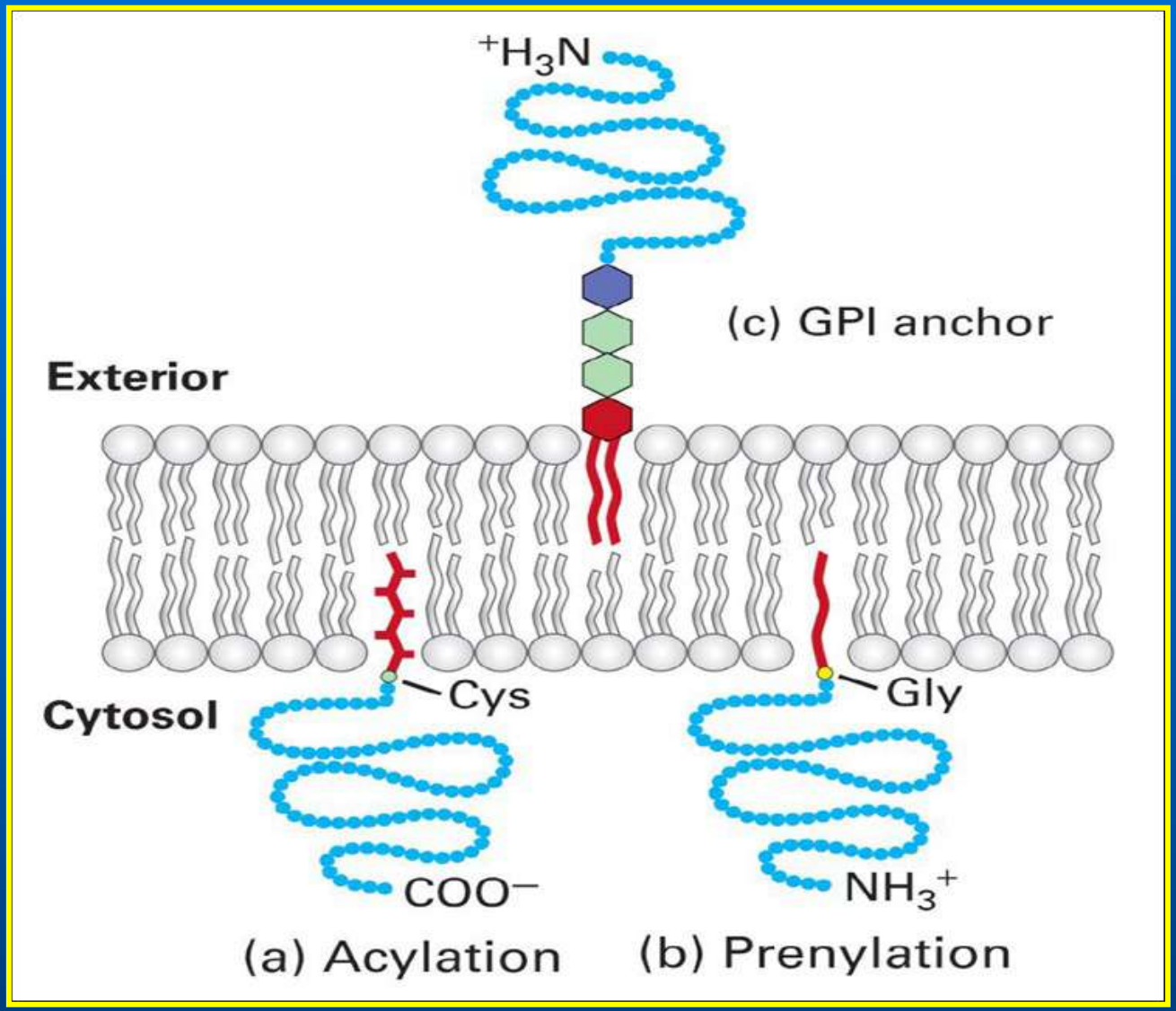


Myristoylation increases the tendency of a protein to become associated with membranes although it usually appears accompanied by other post-translational modification such as palmitoylation or by a poly-basic signal.

Palmitoylation is generally accepted to be a reversible and modulable modification. Very frequently, palmitoylated proteins are targeted to caveolae/rafts.

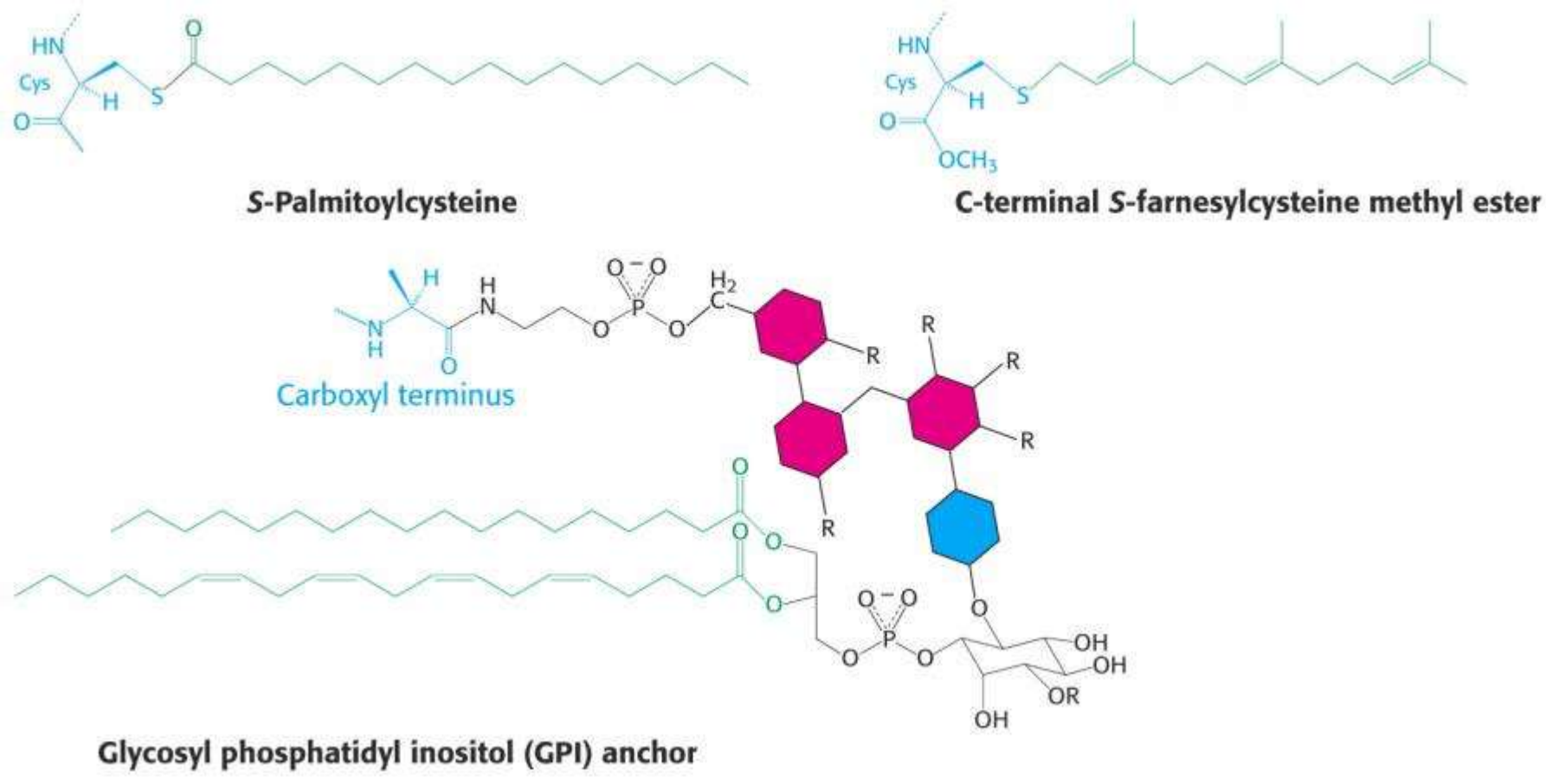
Protein prenylation occurs in proteins of great biological significance, such as the Ras and Rho small GTPases and is frequently a prerequisite for the subsequent protein palmitoylation. Both farnesylated (15 carbons added) or geranygeranylated (20 carbons added) proteins are associated with intracellular membranes. It is essentially an irreversible modification.

Some cellular proteins, such as the morphogen hedgehog are known to become modified through the addition of a cholesterol moiety, a process completely indispensable for biological activity.

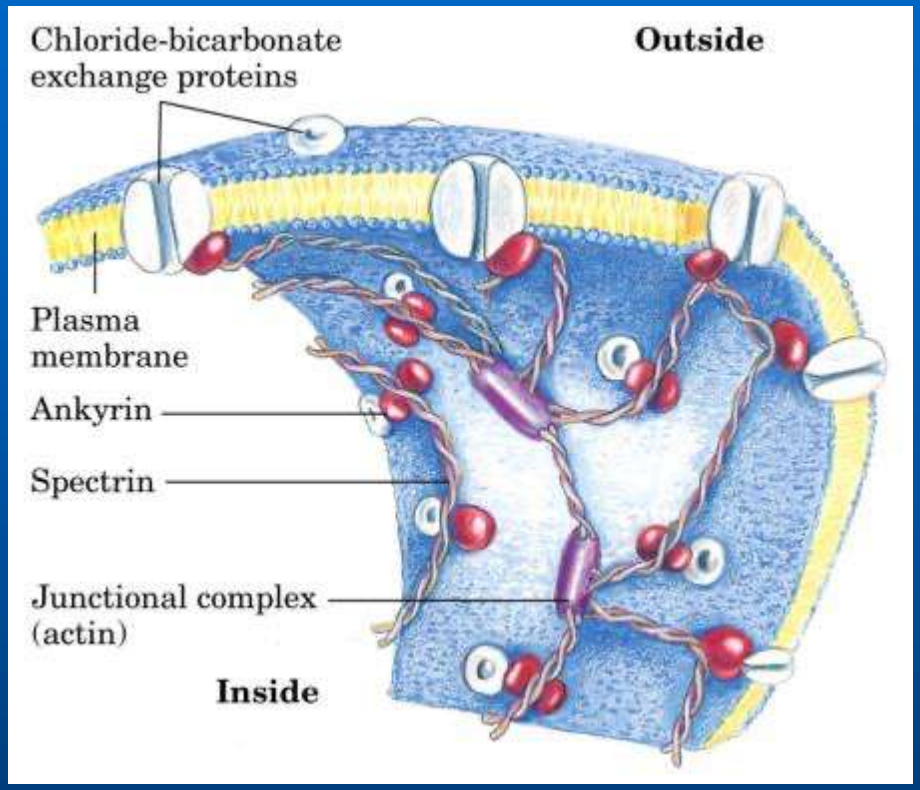


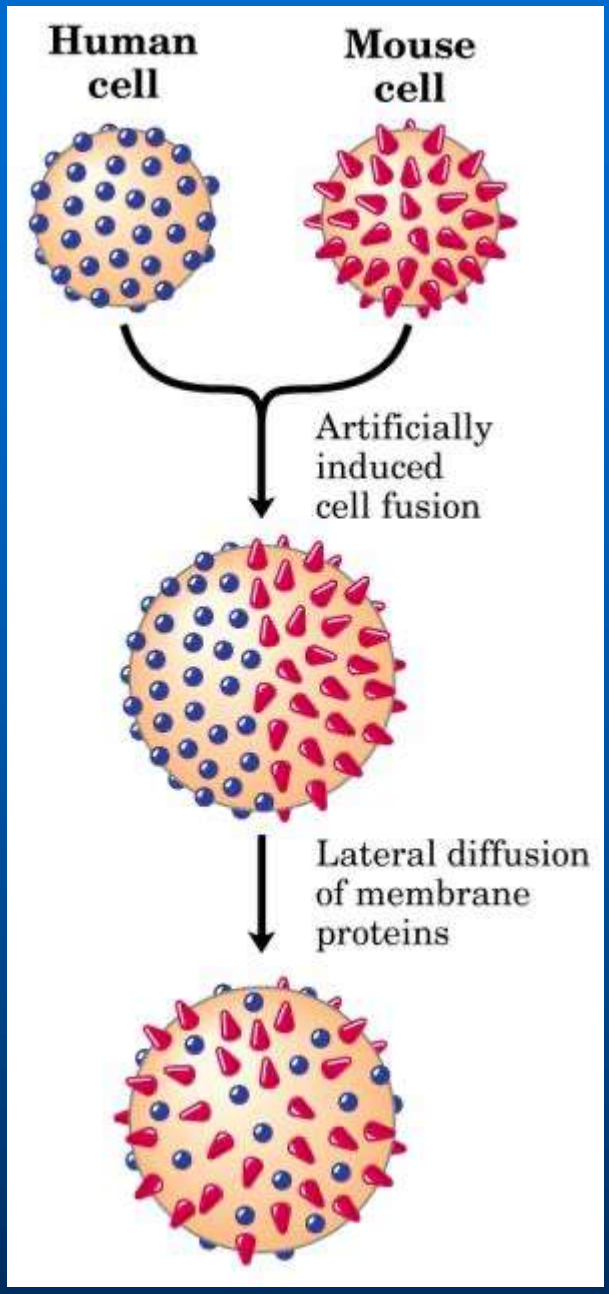
- Glycosylphosphatidylinositol anchors (GPI) link proteins to the outer leaflet
- **Fatty acylation** or **prenylation** links protein to inner leaflet

Protein/lipid interactions in biological membranes



Compartmentalization of proteins

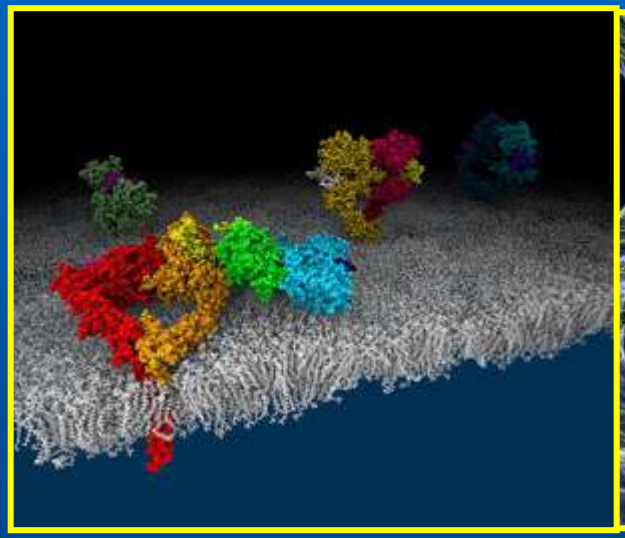




Some proteins are anchored to the internal structures that prevent the lateral diffusion

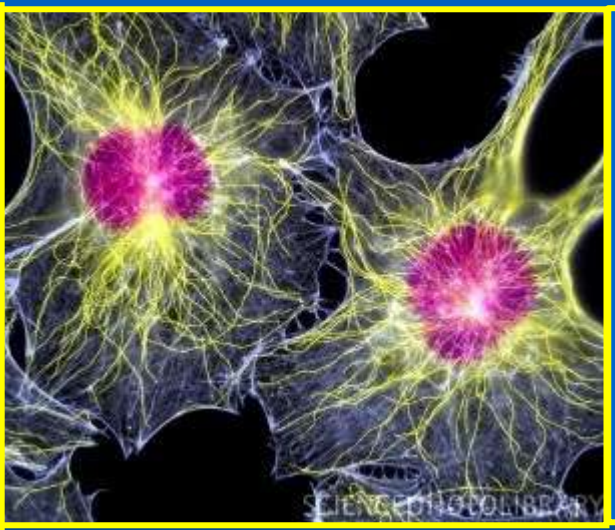
Protein/lipid interactions in biological membranes

However, the plasma membranes contain different lateral compartmentalization that can be classified as:
(a) protein-protein complexes;
(b) pickets and fences, formed by the actin-based cytoskeleton;
(c) lipid rafts.

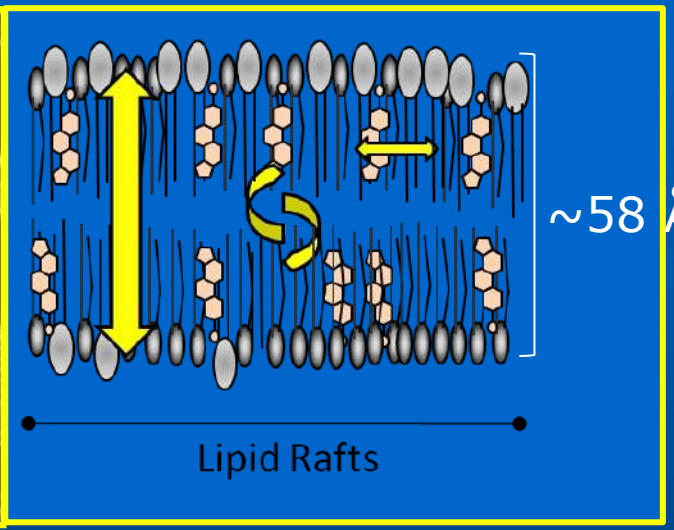


Schematic drawing of epidermal growth factor receptor (EGFR, ErbB1, Her1) molecules on a lipid bilayer. A tetramer (front), two dimers (right) and a monomer in its tethered form (left background) are shown.

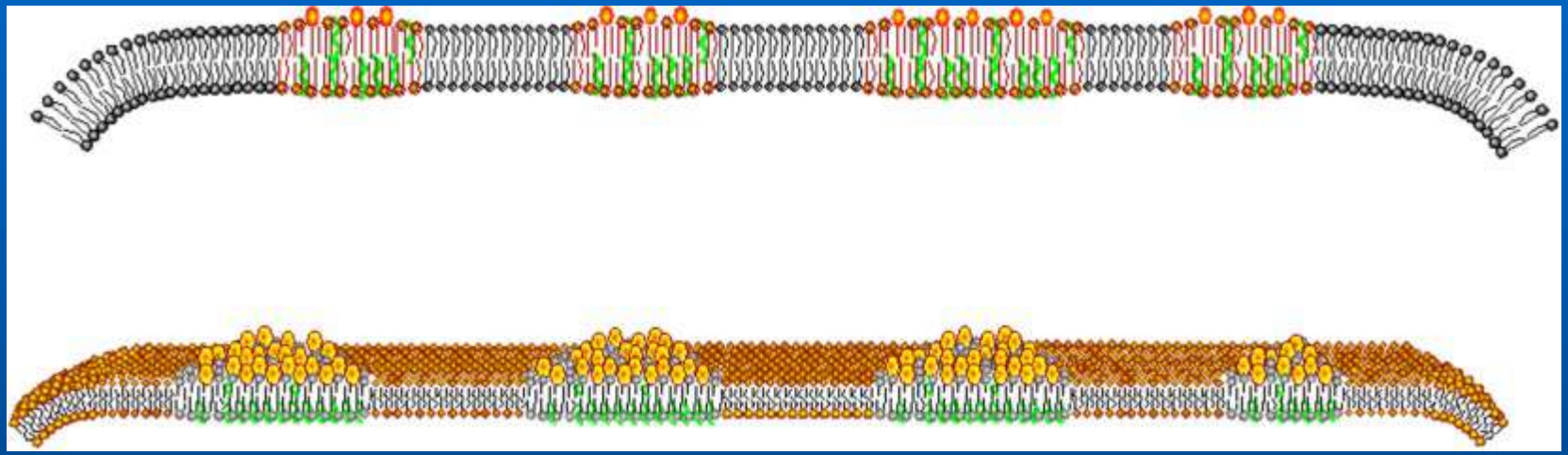
[More at: http://www.theochem.unistuttgart.de/kaestner/gallery.html]



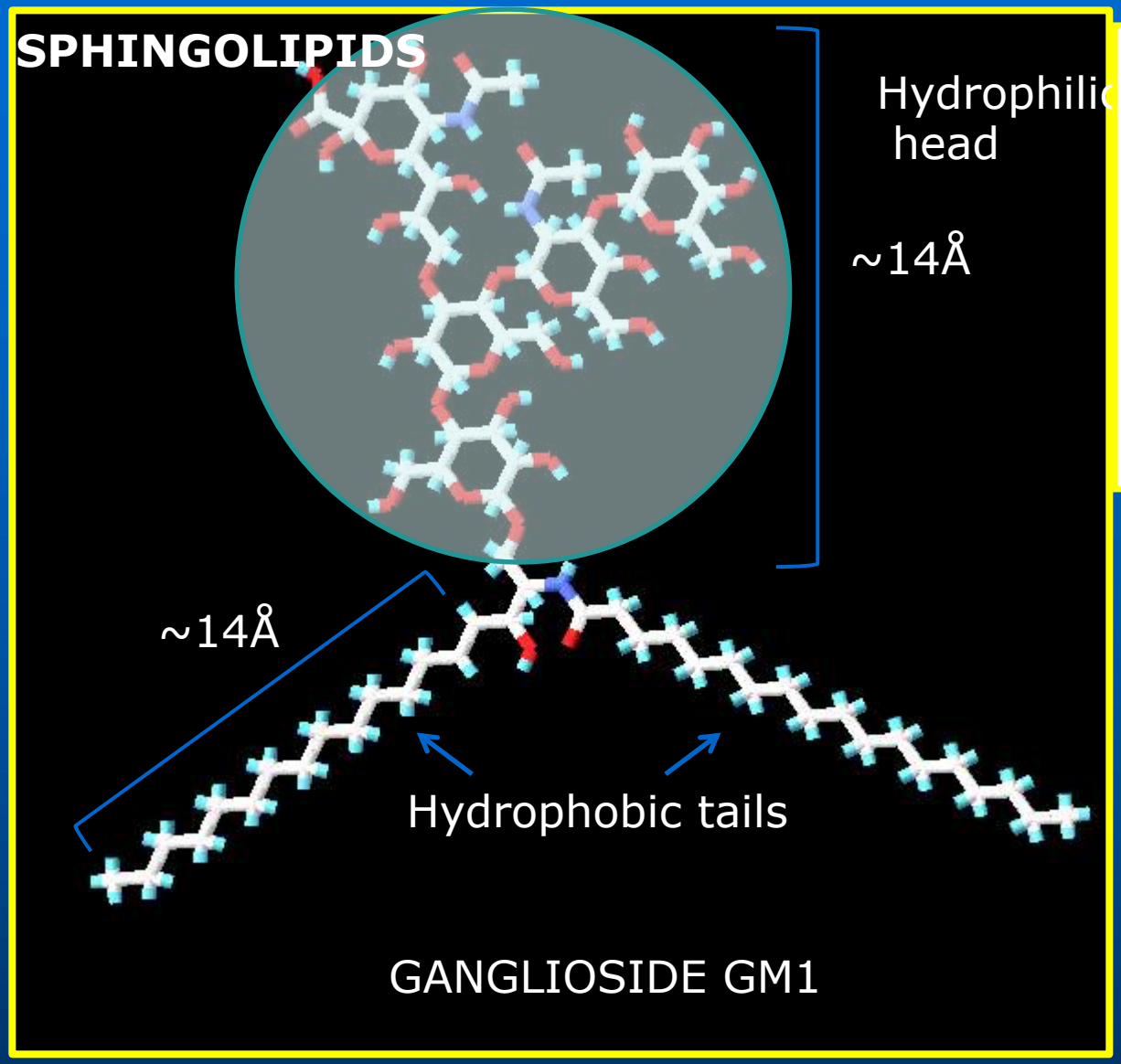
Fibroblast cells showing cytoskeleton

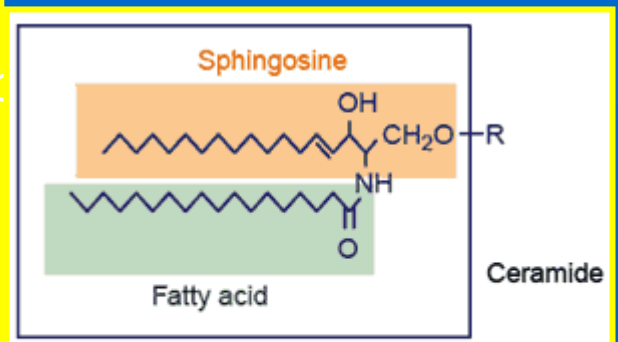


The “lipid rafts”

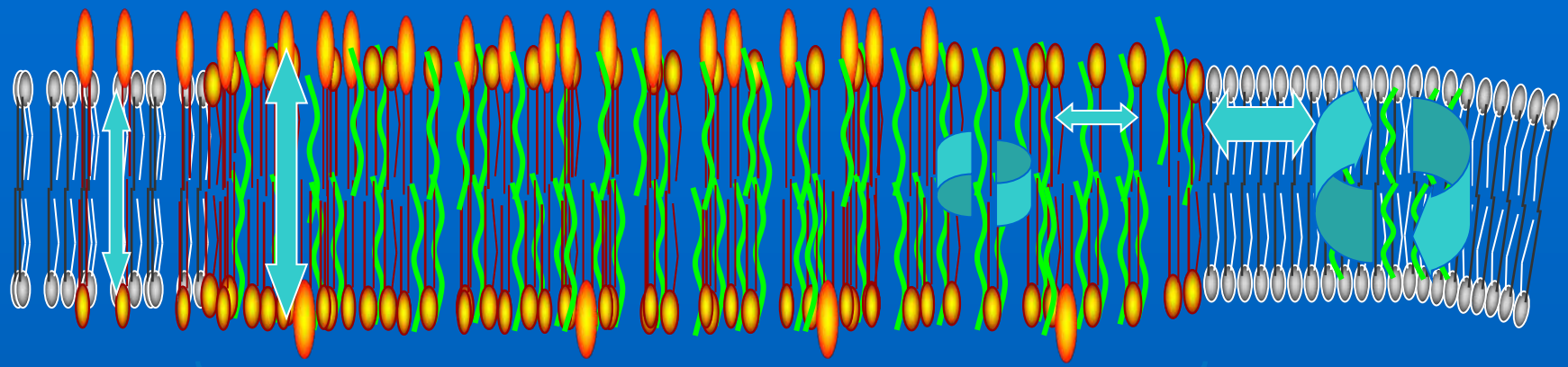


SPHINGOLIPIDS





Substituent (R)	Sphingolipid
H	Ceramide
Phosphocholine	Sphingomyelin
Sugar(s)	Glycosphingolipid



Lipid rafts (LRs)
Liquid ordered phase (Lo)

= Sphingolipids

= Phospholipids

= Cholesterol

= Lateral diffusion

= Rate of flip-flop

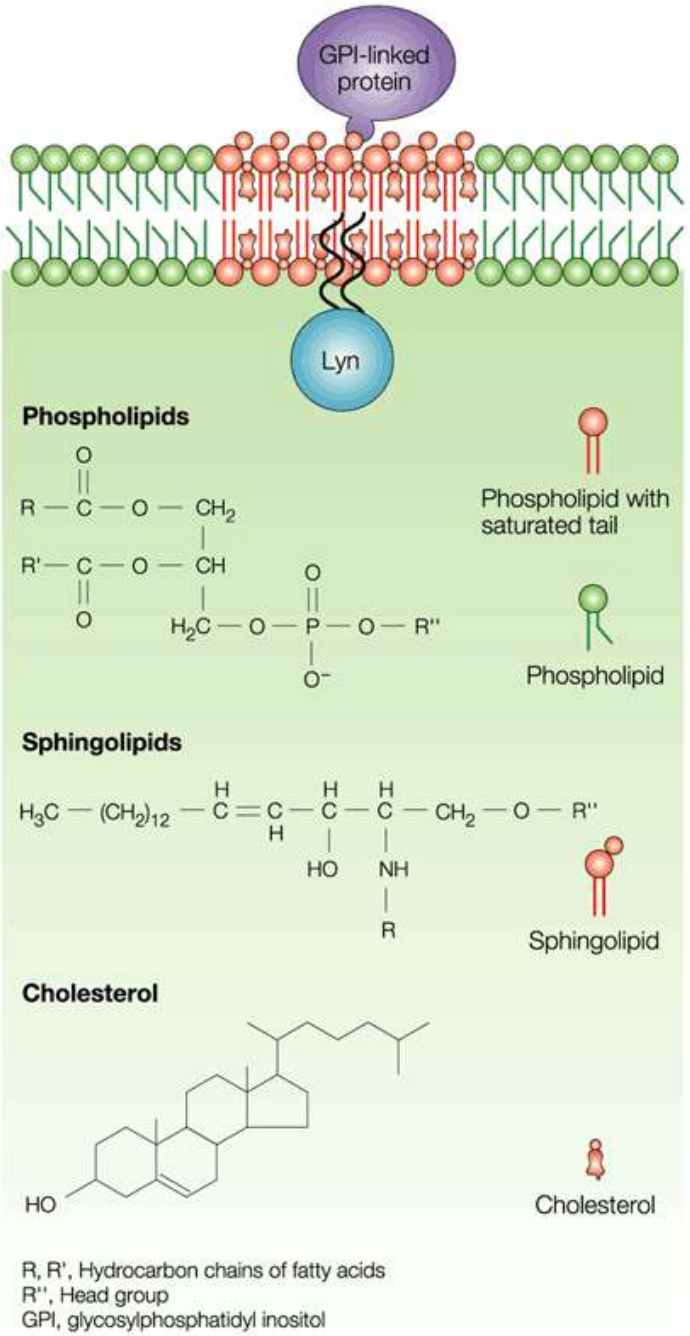
= Membrane thickness

Lipid rafts and B-cell activation

Susan K. Pierce

Nature Reviews Immunology 2, 96-105 (February 2002)

Lipid rafts are sphingolipid- and cholesterol-rich membrane microdomains in the outer leaflet of the plasma membrane. The plasma membrane is composed primarily of sphingolipids, (glycerol)phospholipids and cholesterol. Sphingolipids differ from most phospholipids in that they have long, largely saturated acyl chains that allow them to pack tightly in a bilayer, forming a gel phase in which there is very little lateral movement or diffusion. The gel phase of the sphingolipids is altered by the association with cholesterol, which condenses the packing of the sphingolipids by occupying the spaces between the acyl chains. As a consequence, cholesterol-containing sphingolipid microdomains exist in a **liquid-ordered phase** that is significantly more fluid than the gel phase.



Science. 2010 Jan 1;327(5961):46-50.

Lipid rafts as a membrane-organizing principle

Lingwood D, Simons K

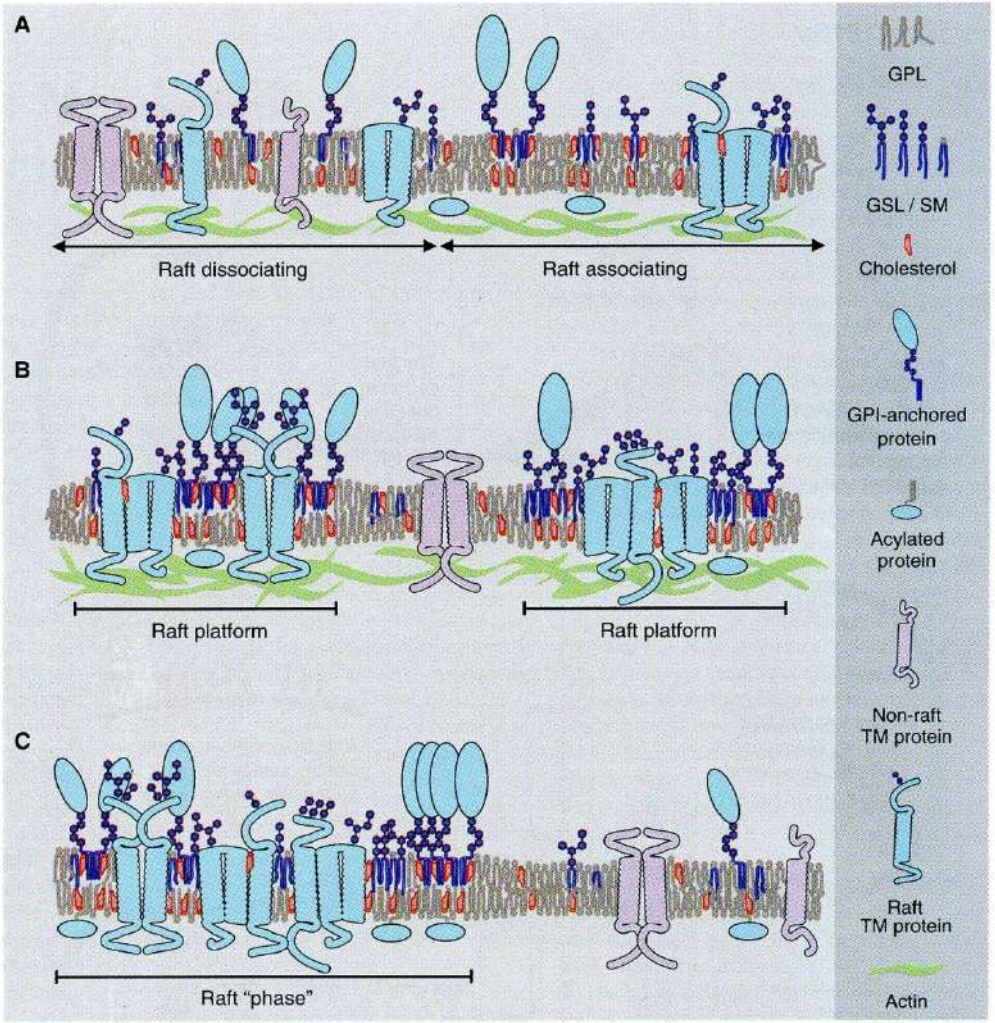


Fig. 2. Hierarchy of raft-based heterogeneity in cell membranes. **(A)** Fluctuating nanoscale assemblies of sterol- and sphingolipid-related biases in lateral composition. This sphingolipid/sterol assemblage potential can be accessed and/or modulated by GPI-anchored proteins, certain TM proteins, acylated cytosolic effectors, and cortical actin. Gray proteins do not possess the chemical or physical specificity to associate with this membrane connectivity and are considered non-raft. GPL, glycerophospholipid; SM, sphingomyelin. **(B)** Nanoscale heterogeneity is functionalized to larger levels by lipid- and/or protein-mediated activation events (e.g., multivalent ligand binding, synapse formation, protein oligomerization) that trigger the coalescence of membrane order–forming lipids with their accompanying selective chemical and physical specificities for protein. This level of lateral sorting can also be buttressed by cortical actin. **(C)** The membrane basis for heterogeneity as revealed by the activation of raft phase coalescence at equilibrium in plasma-membrane spheres. Separated from the influence of cortical actin and in the absence of membrane traffic, multivalent clustering of raft lipids can amplify the functional level to a microscopic membrane phase. Membrane constituents are laterally sorted according to preferences for membrane order and chemical interactions.

Science. 2010 Jan 1;327(5961):46-50.
Lipid rafts as a membrane-organizing principle
Lingwood D, Simons K

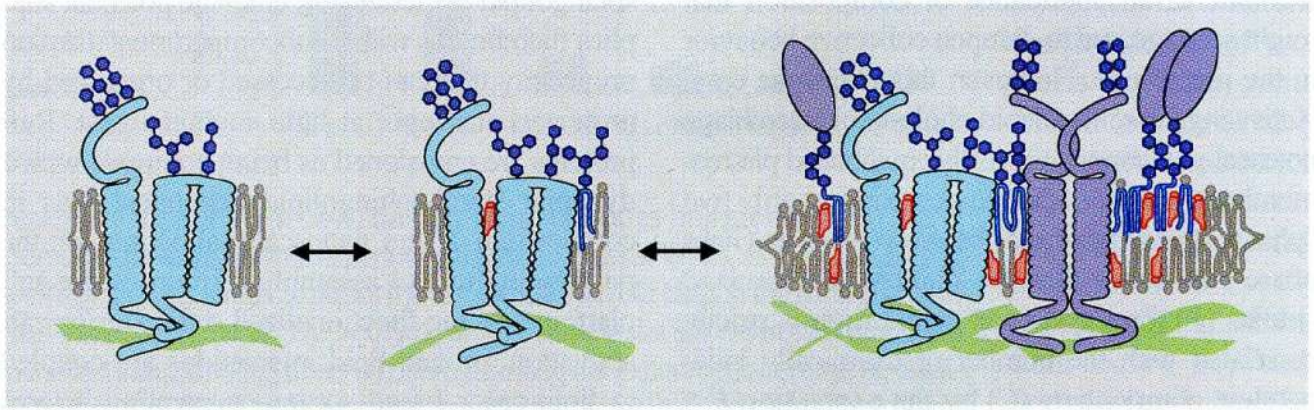


Fig. 3. The lubrication of a raft TM protein by lipid. Membrane proteins bind and/or enrich certain lipids through chemical and physical specificities. These lipids may themselves exhibit sphingolipid/sterol assemblage potential. In this scheme, a TM raft protein (light blue) specifically interacts with sterol and GSL, an interaction that lubricates its inclusion to and the assembly of functionalized (coalesced) raft membrane.

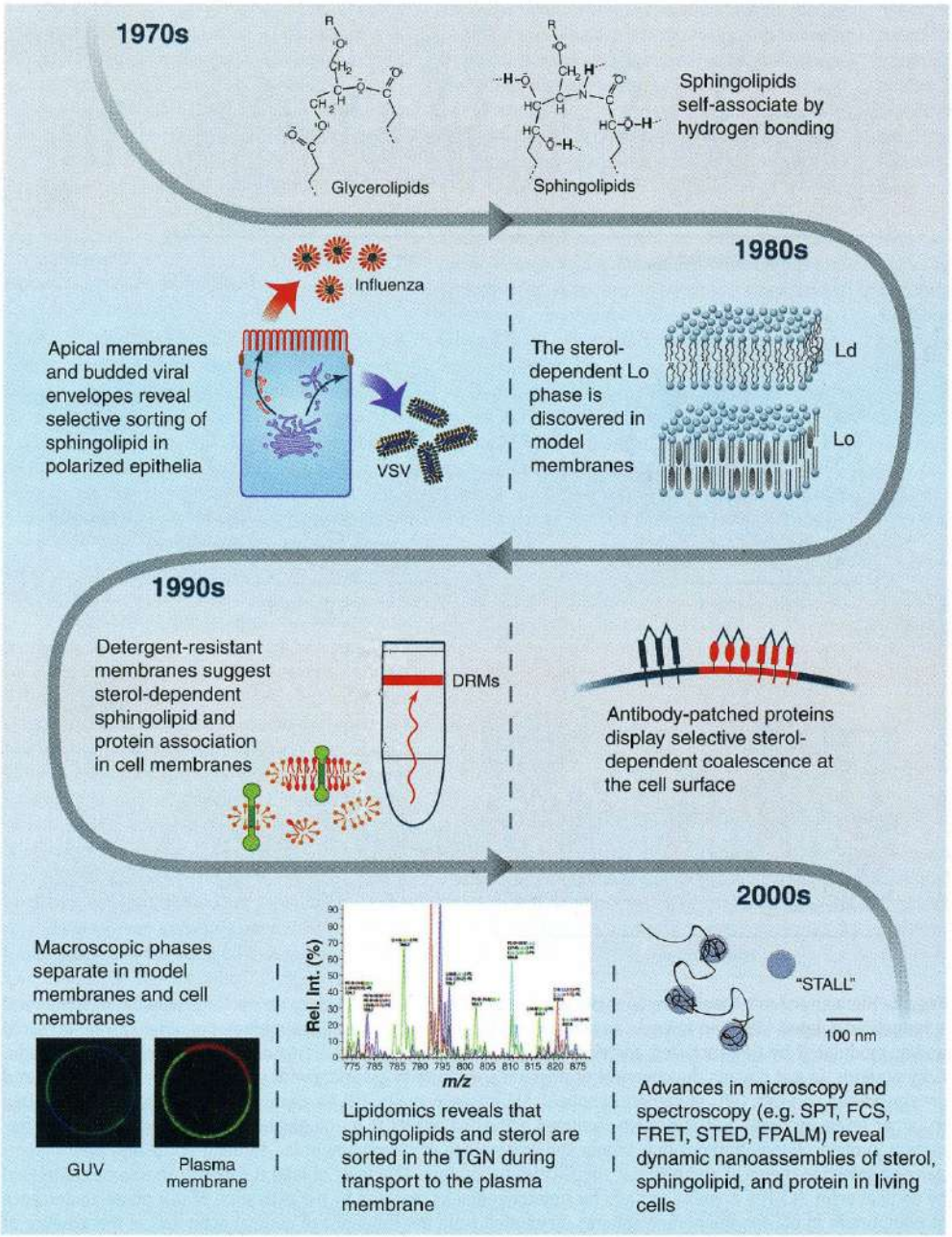
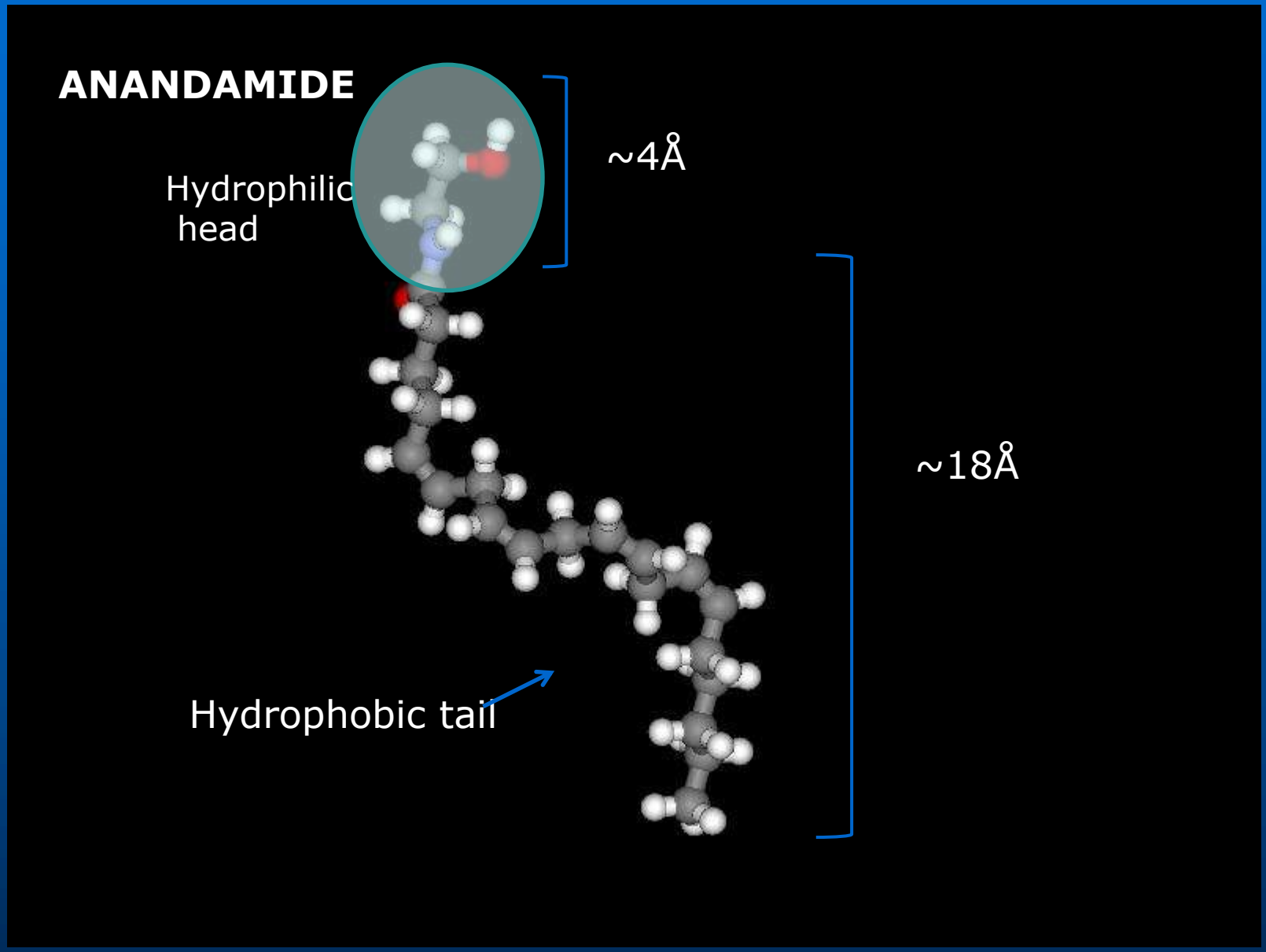
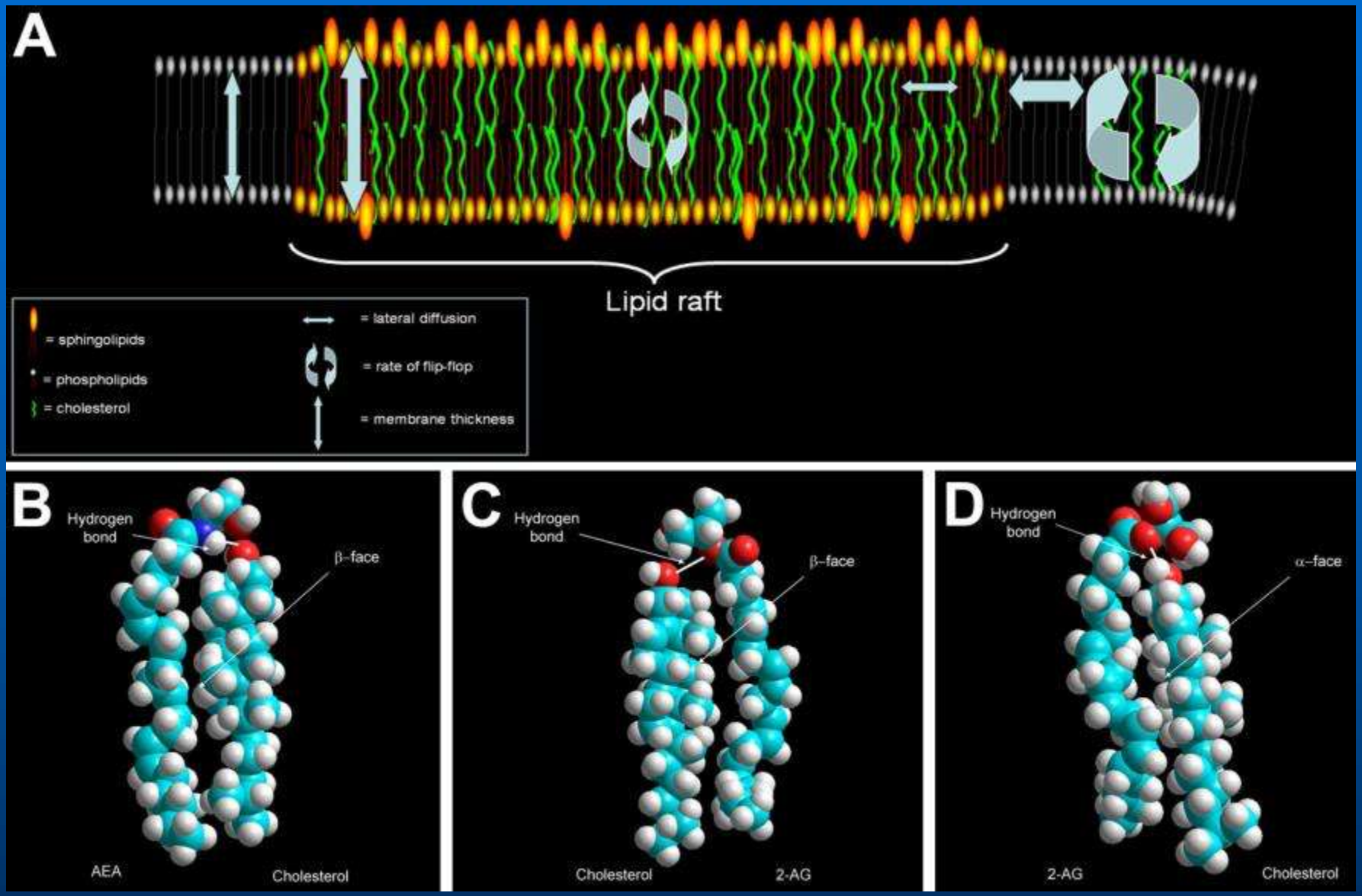


Fig. 1. Evolution of the raft concept for subcompartmentalization in cell membranes. A bold H indicates hydrogen bonding. VSV, vesicular stomatitus virus; DRMs, detergent-resistant membranes; GUV, giant unilamellar vesicle; *m/z*, mass/charge ratio; SPT, single-particle tracking; FCS, fluorescence correlation spectroscopy; FRET, fluorescence resonance energy transfer; STED, stimulated emission depletion; FPALM, fluorescence photoactivation localization microscopy.

Science. 2010 Jan 1;327(5961):46-50.
Lipid rafts as a membrane-organizing principle
Lingwood D, Simons K

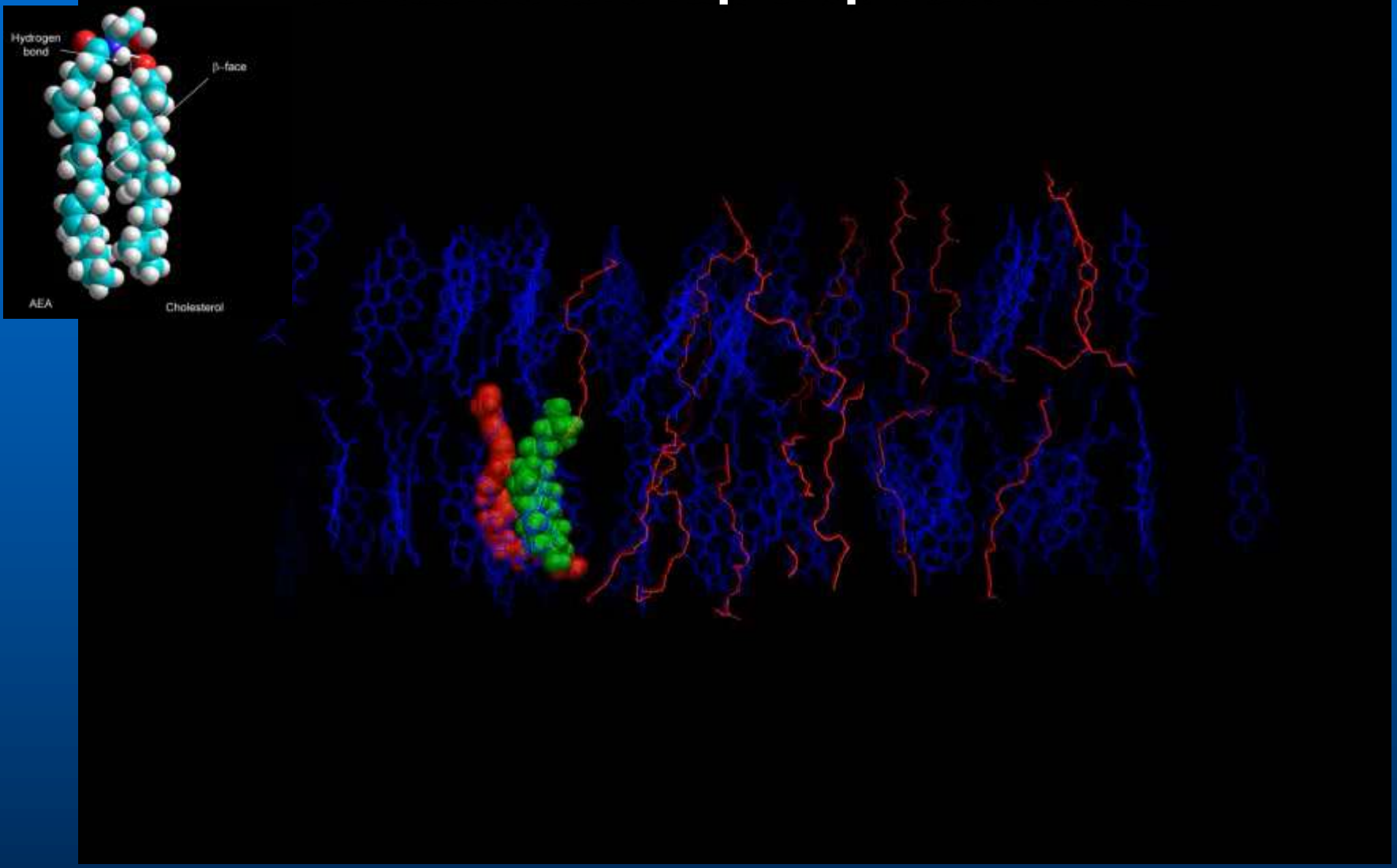

ANANDAMIDE
Hydrophilic head
~4Å
~18Å
Hydrophobic tail

Specific lipid-lipid interaction modulating the diffusion and transport of endocannabinoids



Dainese E, Oddi S, Bari M, Maccarrone M. Modulation of the endocannabinoid system by lipid rafts. Curr Med Chem. 2007;14(25):2702-15.

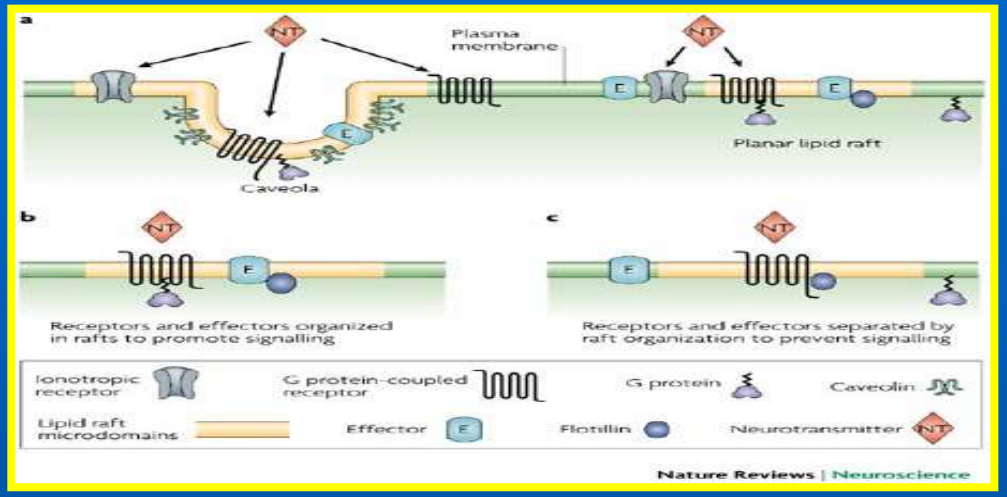
MD shows that the presence of cholesterol enhance the flip-flop rate of AEA



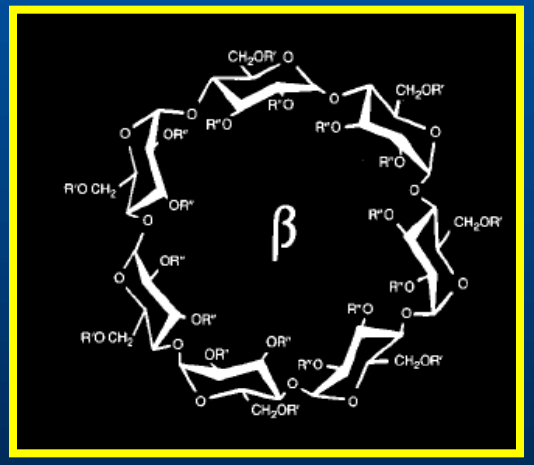
LRs are an ideal platform for GPCRs signaling

Table 1 | Examples of G-protein-coupled receptors that localize in lipid raft/caveolae before ('pre-agonist') and/or after ('post-agonist') treatment with agonists

	Pre-agonist	Post-agonist
Endothelin (ETA and ETB)	+	+
Cholecystokinin (CCK)		+
Muscarinic cholinergic	+	+
Bradykinin (B_1 and B_2)	+	+
Lysophosphatidic acid (LPA-1)	+	
Angiotensin II (AT-1)		+
β_1- and β_2-adrenergic	+	
P_2Y (P_2Y_1)	+	
Adenosine A1	+	+
Sphingosine 1-phosphate (EDG-1)	+	+
Smoothened/patched	+	
Serotonin ($5HT_{2A}$)	+	
Calcium-sensitive	+	
α_1-Adrenergic (α_{1B})	+	
Chemokine CCR_2		+
Metabotropic glutamate (mGlu1)	+	
Gonadotrophin-releasing hormone (GnRH)		+
Oxytocin	+	
Growth-hormone releasing hormone		+
Dopamine [D_1; D(1A)]	+	+
Neurokinin 1	+	
μ-Opioid receptor	+	



(Allen *et al.*, 2007)



(MCD)

Modulation of ECS by membranes
Effect of plasma membrane cholesterol on CB receptors function

Receptor	Treatment			
	Cholesterol depletion		Cholesterol enrichment	
	Binding	*Signaling*	*Binding*	*Signaling*
CB1R	↑	↑	↓	↓
CB2R	↔	↔	↔	↔
β_2-AR	↑	↑	↓	↓
Serotonin$_{1A}$R	↑	↑	↓ or ↔	↓ or ↔

Current Medicinal Chemistry, 2010, 17, 1487-1499 1487
Interaction of Endocannabinoid Receptors with Biological Membranes
E. Dainese, S. Oddi and M. Maccarrone

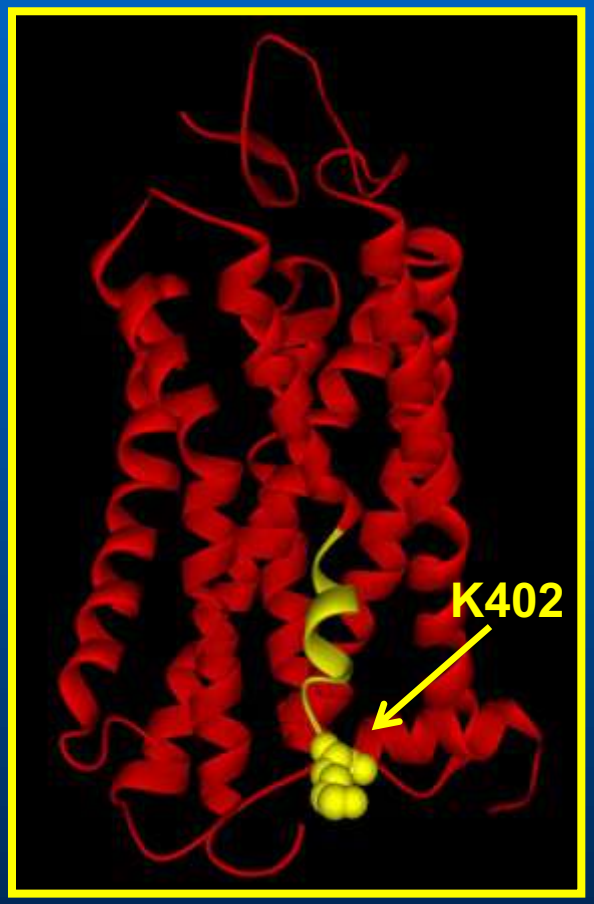
Putative cholesterol binding sites in CBRs: Cholesterol Recognition Aminoacid Consensus (CRAC)

CB2R	NSMVNPVIYALRSGEIRSSAHHCLAHWKKCVR	322
CB1R	NSTVNPIIYALRSKDLRHAFRSMFPSCEGTAQ	420
β_2AR	NSGFNPLIYC-RSPD----FRIAFQELLCLRR	346
SerR	NSLLNPVIYAYFNKD----FQNAFKKIIKCKF	417

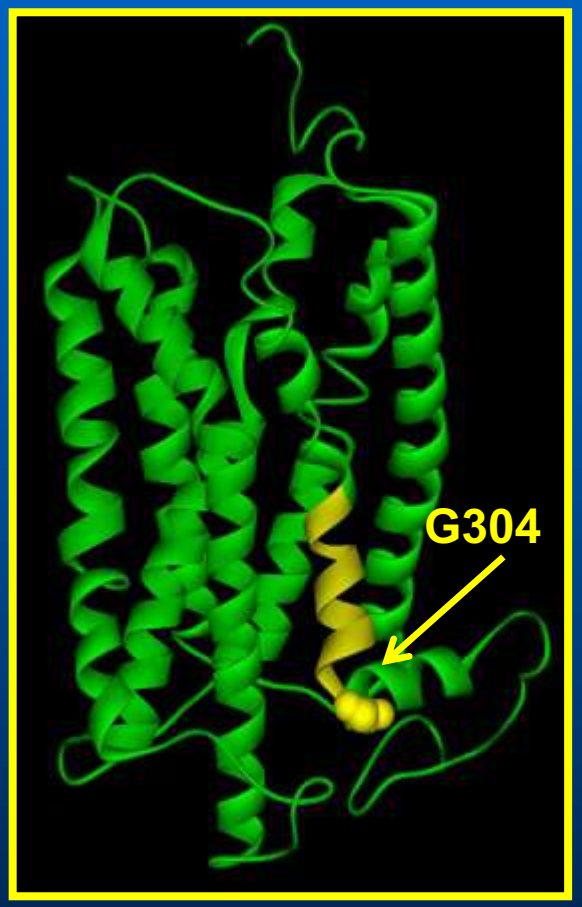
CRAC seq: V/L-X_{1-4}-Y-X_{1-4}-K/R

Transmembrane helix 7

CB1R

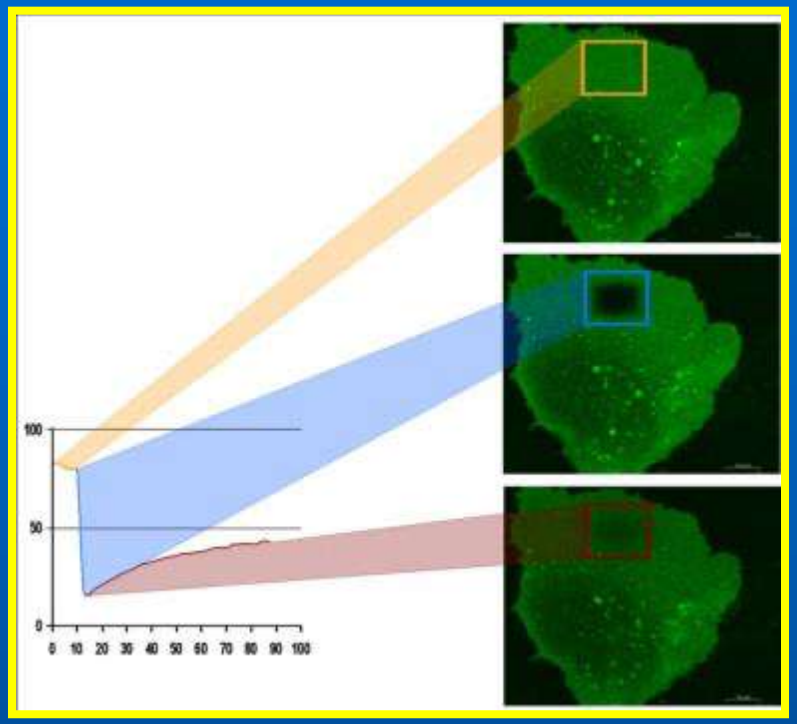


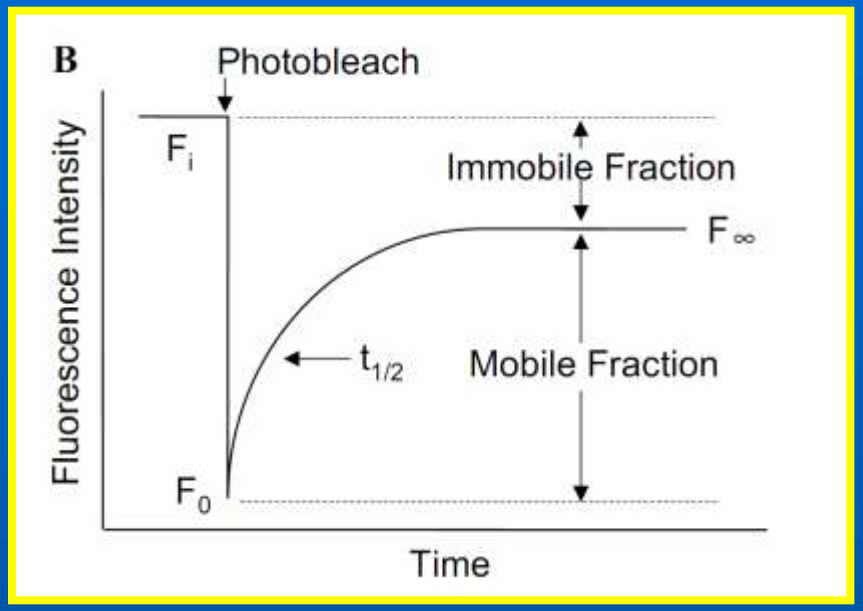
CB2R



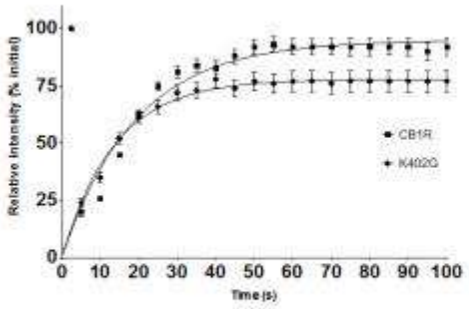
Oddi & Dainese et al., 2011

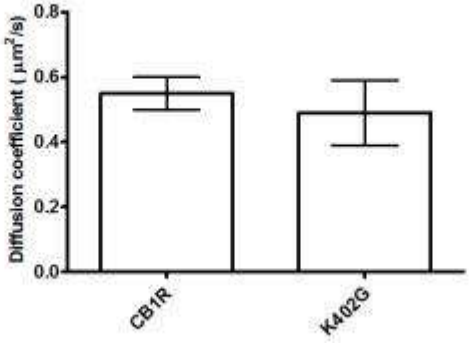
FRAP analysis of CB1-GFPR

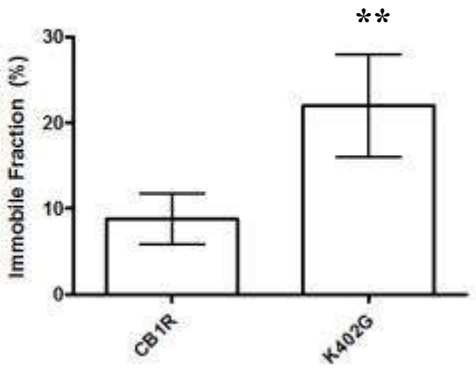




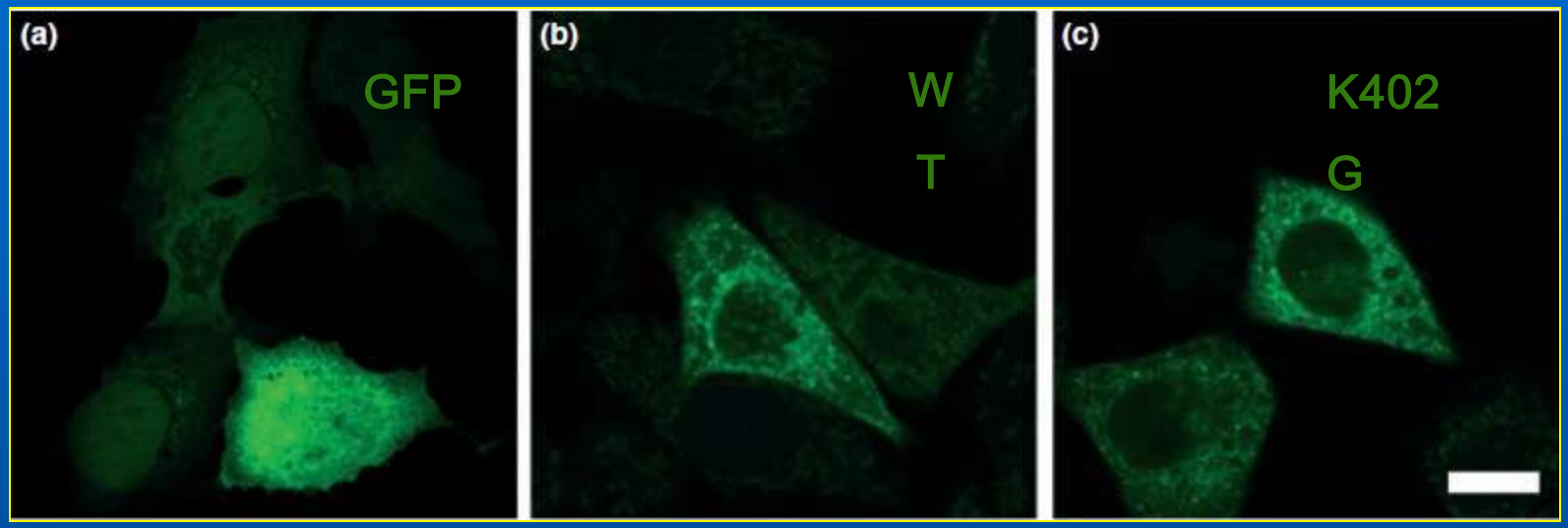
CB1R: WT *versus* K402G (**p < 0.05 *versus* WT)

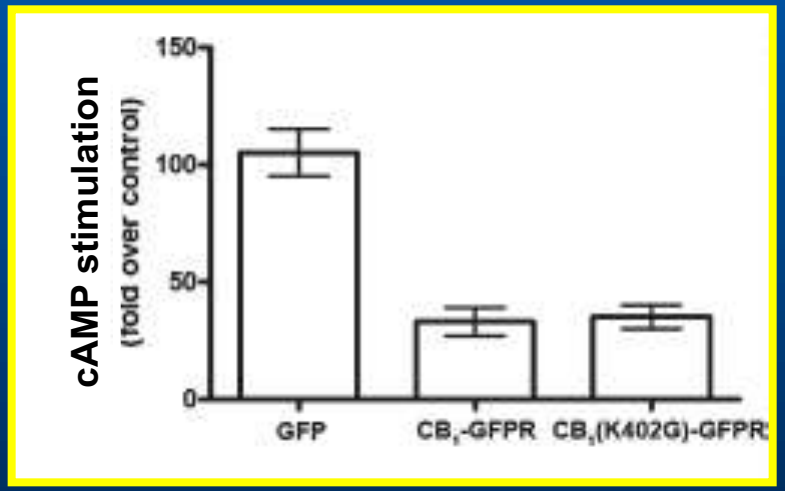






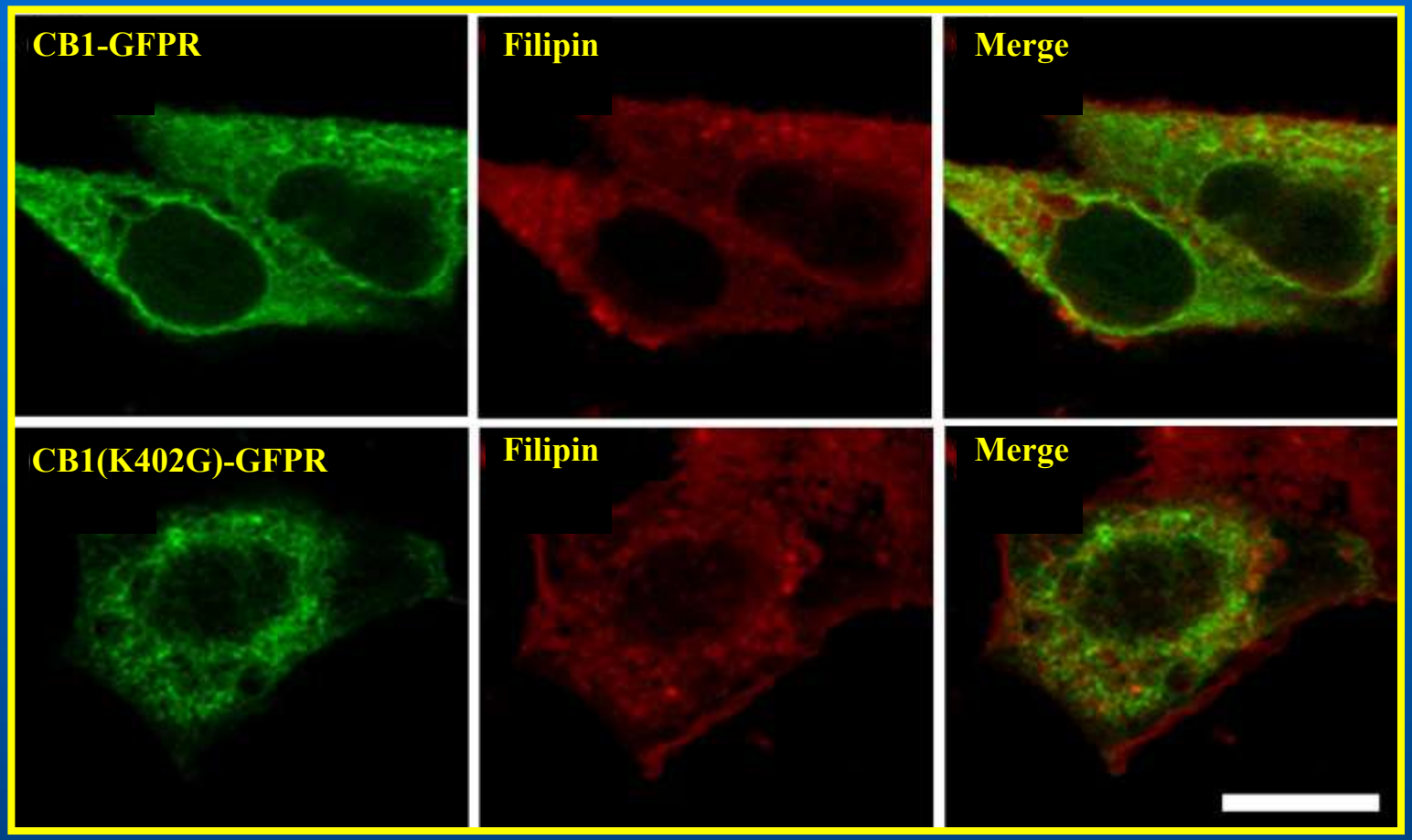
Functionality and intracellular distribution of WT and K402G CB1-GFPR





	WT	K402G
Ratio M/T	0.12 ± 0.02	0.09 ± 0.02
DRM remnant (%	20 ± 5	25 ± 5

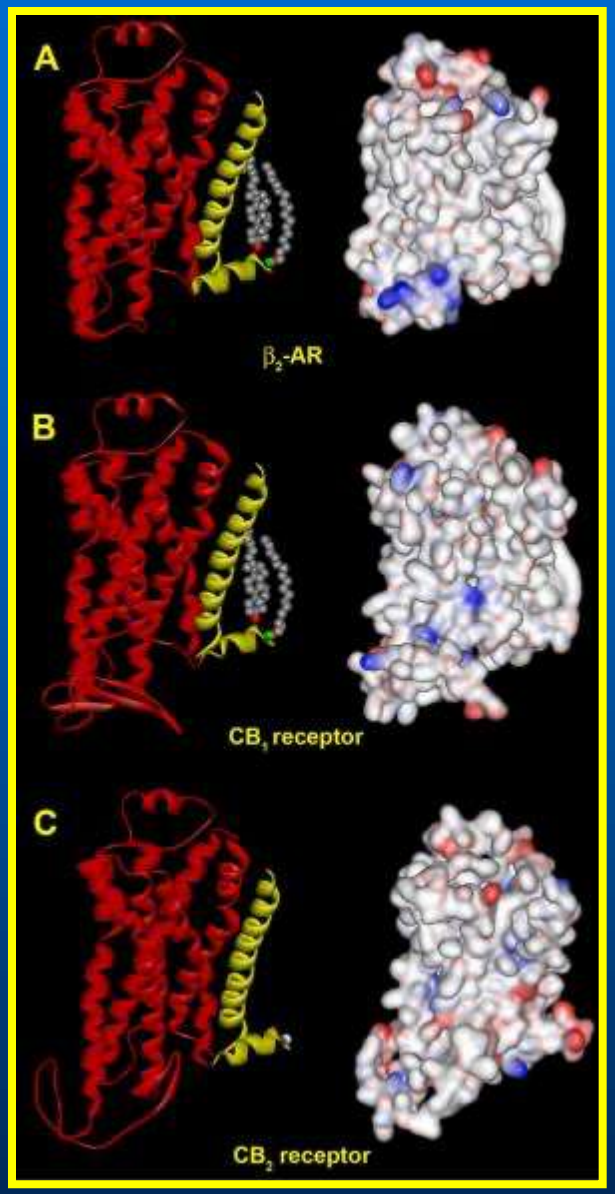
Quantitative colocalization of CB1-GFPR and filipin (cholesterol binder) on the plasma membrane

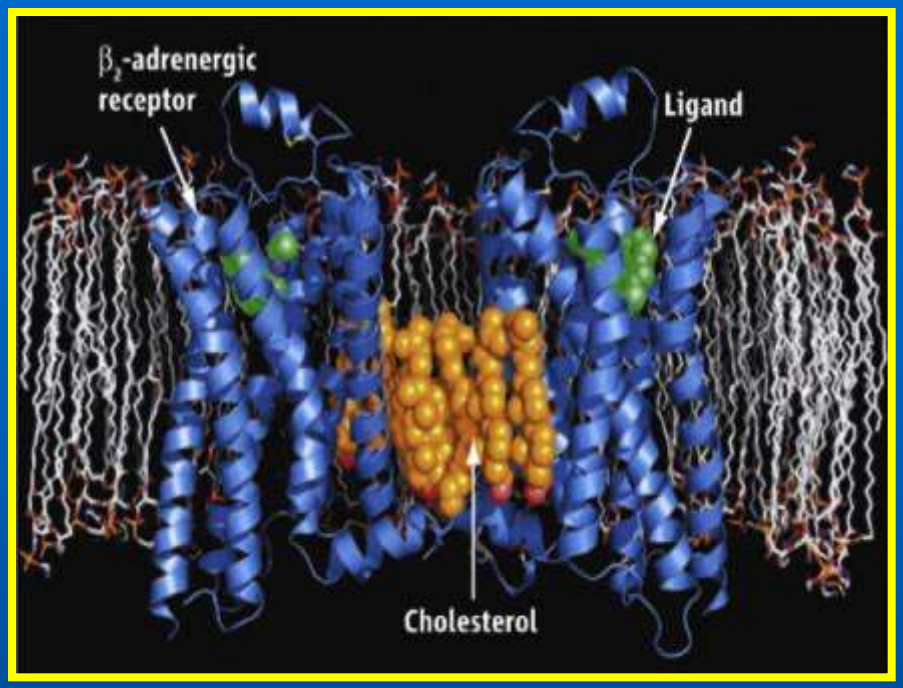


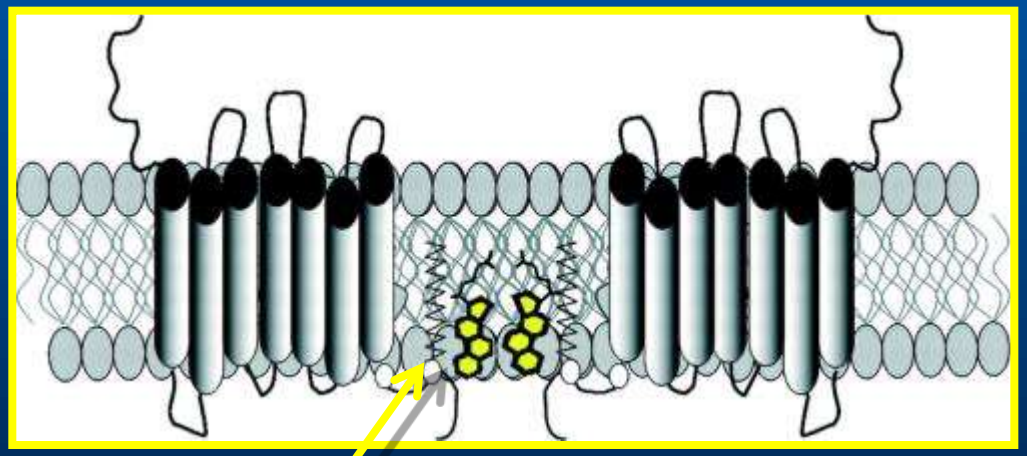
Receptor	M/T ratio	DRM remnant (%)	Pearson's correlation coefficient	Intensity correlation quotient
CB_1-GFPR	0.12 ± 0.02	20 ± 5	0.733 ± 0.039	0.390 ± 0.016
CB_1(K402G)-GFPR	0.09 ± 0.02	25 ± 5	0.560 ± 0.040*	0.274 ± 0.032*

*$p < 0.05$ vs. CB_1-GFPR.

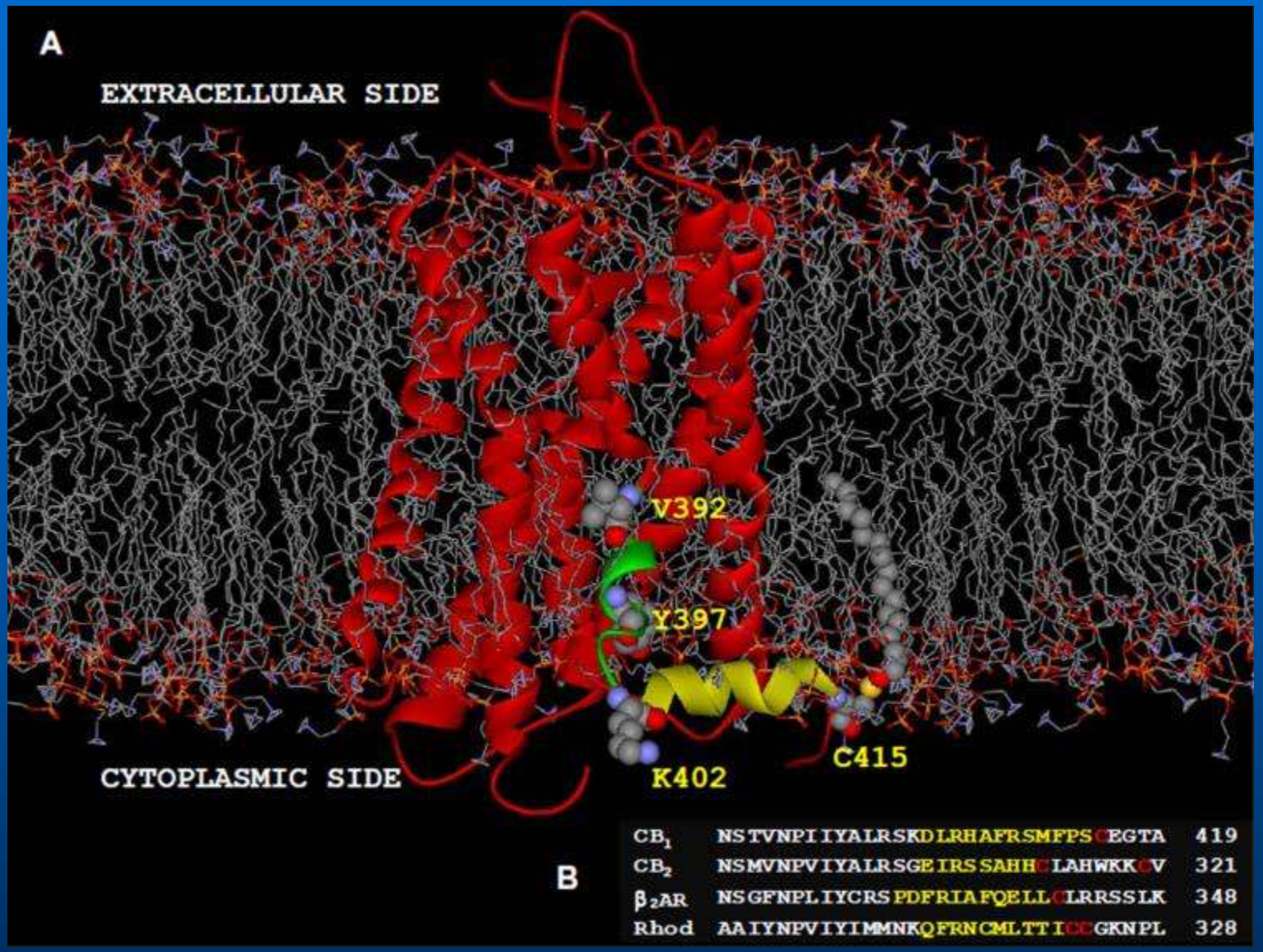
β_2-AR and CB1 receptors share putative palmitoylation sites



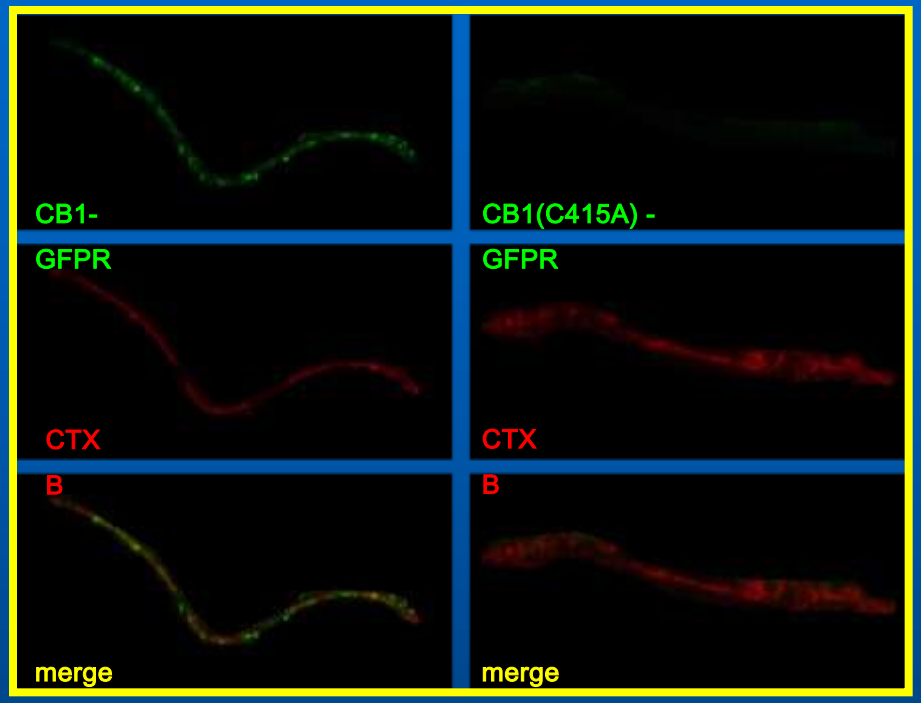




Putative palmitoylation site in CB1R



Cys415 palmitoylation is involved in targeting CB1 receptor to the plasma membrane

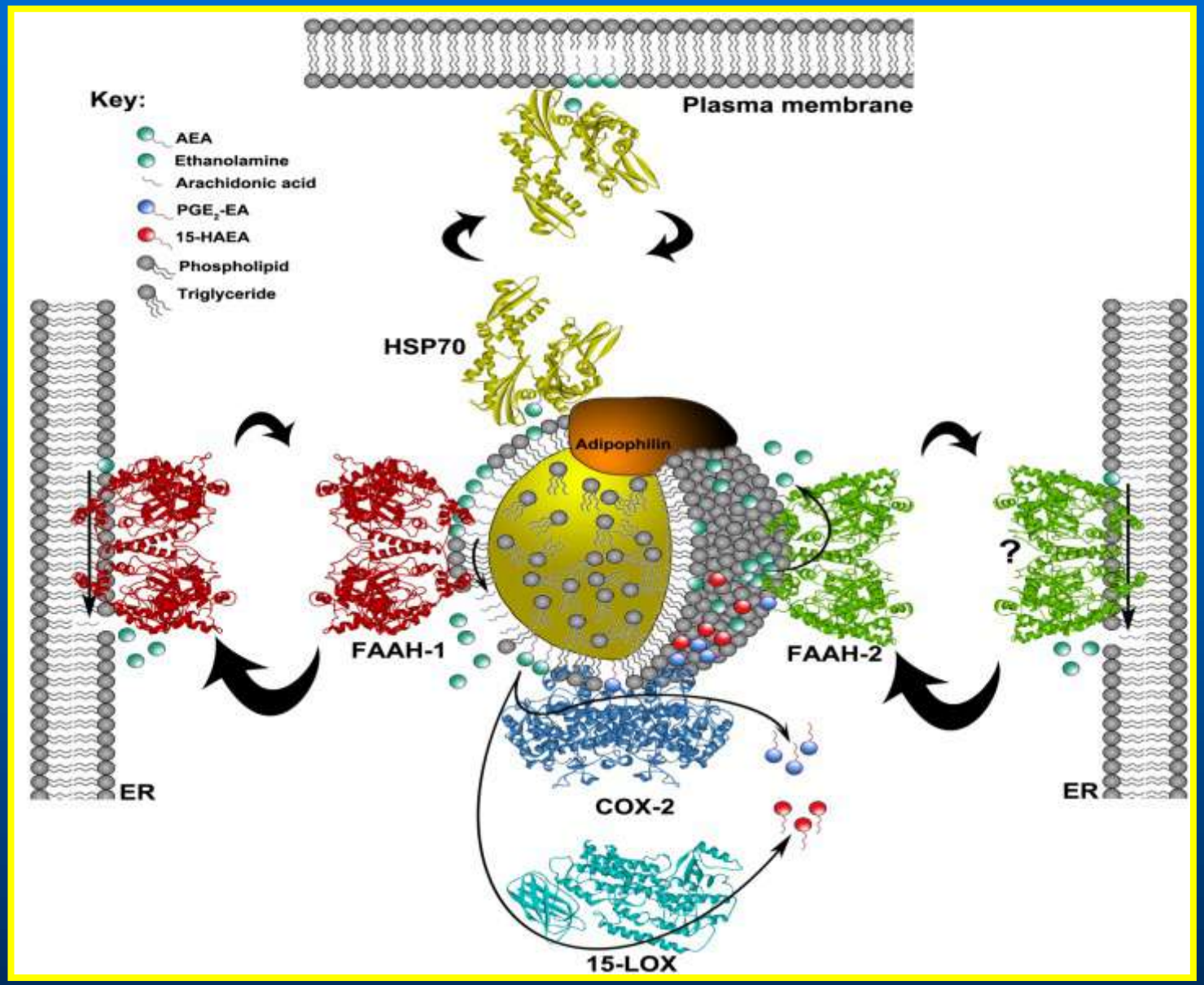


Protein	Pearson's Correlation Coefficie	Overlap Coefficient	Intensity Correlation Quotient
CB1-GFPR	0.74 ± 0.08	0.74 ± 0.07	0.214 ± 0.035
CB1(C415A)-GFPR	0.27 ± 0.07*	0.27 ± 0.04*	0.046 ± 0.009*

*p < 0.01 *versus* CB1R-GFP

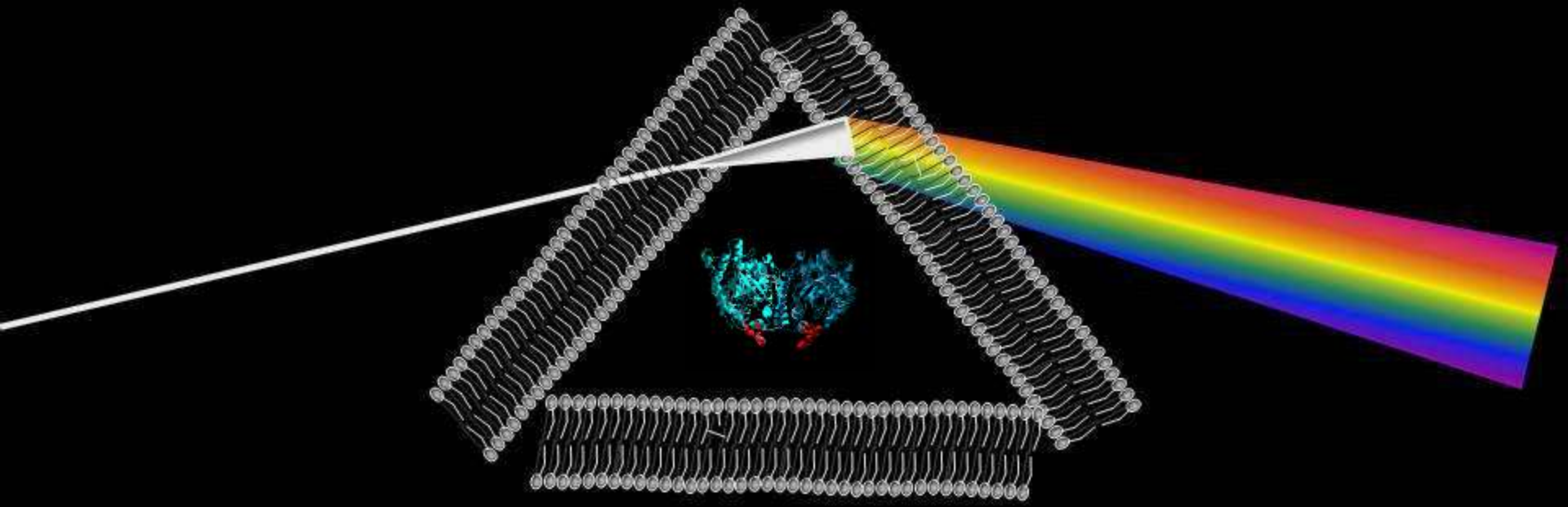
Oddi & Dainese et al., 2012

Lipid-lipid and lipid-protein interactions: trafficking of AEA as a control point of its signalling

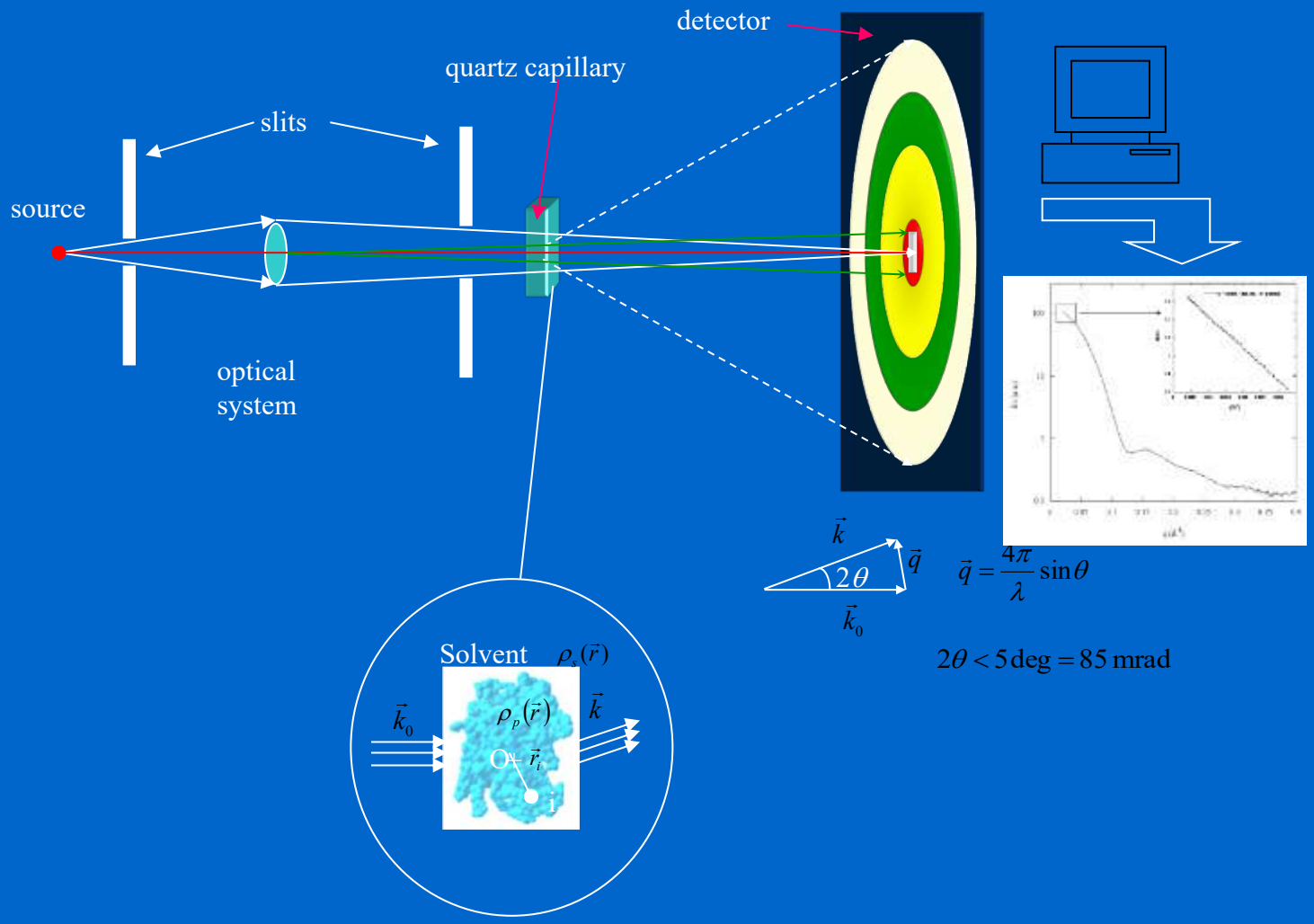


Maccarrone, Dainese & Oddi, 2011

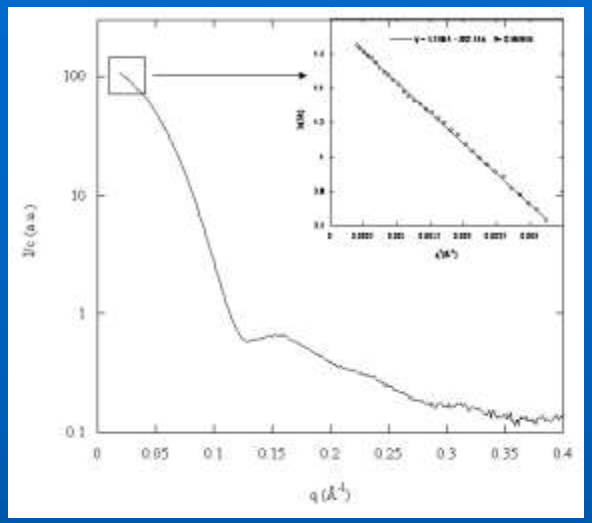
STUDIES OF FAAH-LIPIDS INTERACTIONS BY COMBINING Fluorescence Resonance Energy Transfer (FRET), Small Angle X-ray Scattering (SAXS) and *in silico* APPROACHES

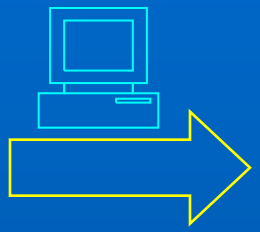


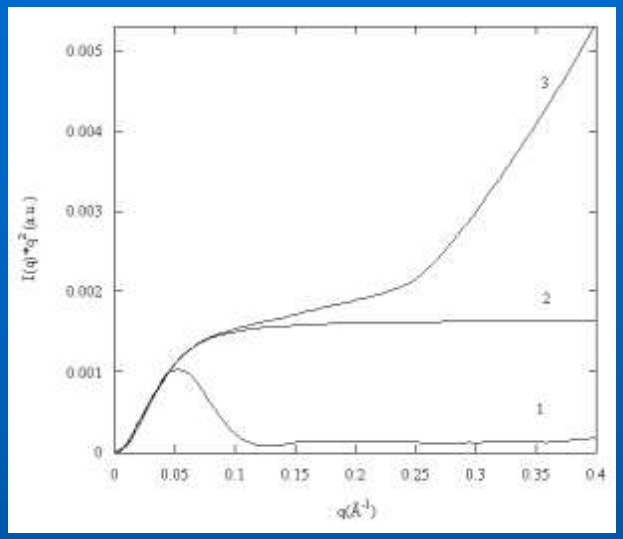
SMALL ANGLE X-RAY SCATTERING

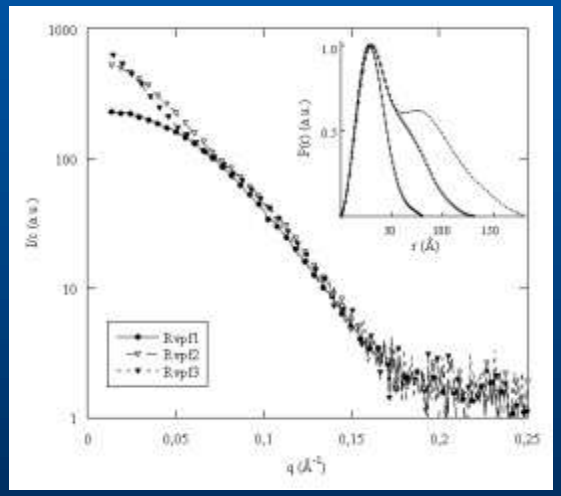


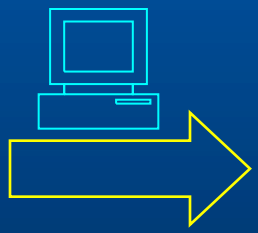
Direct structural parameters and 3D modeling

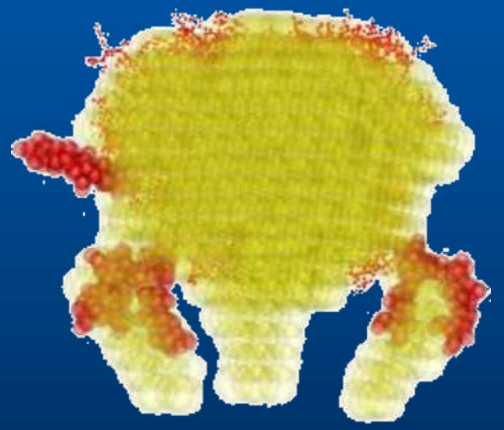








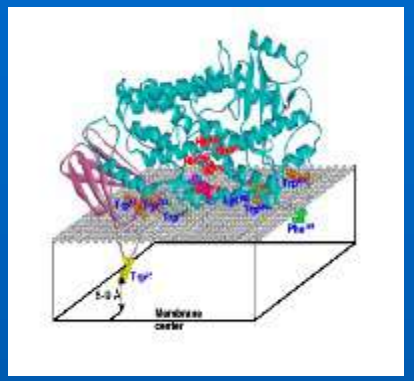




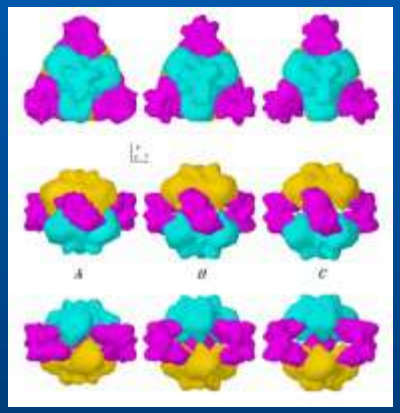
Studies by SAXS: conformational changes of biological macromolecules

Applications to proteins

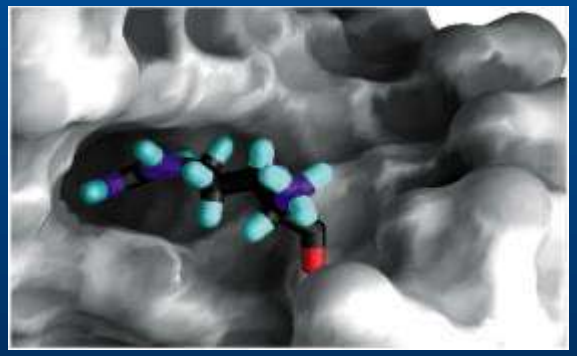
folding-unfolding



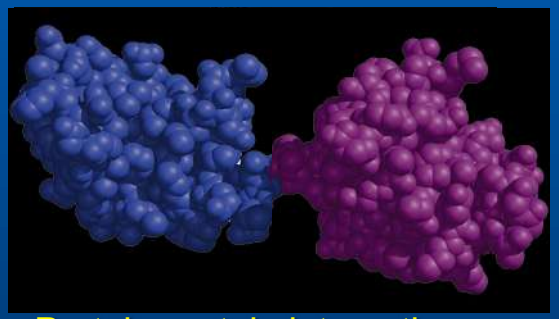
Protein-lipids interactions



Oligomeric organization



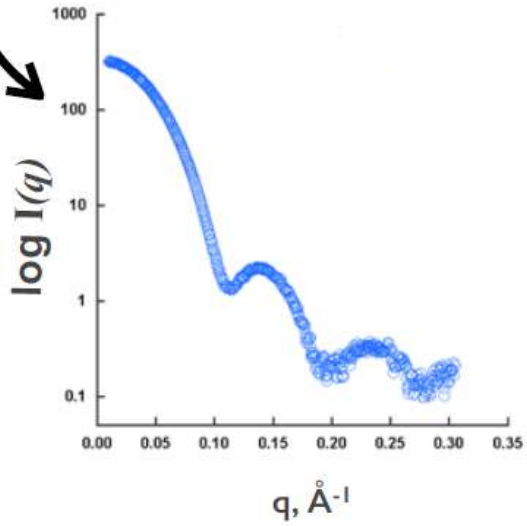
Protein-ligand interactions

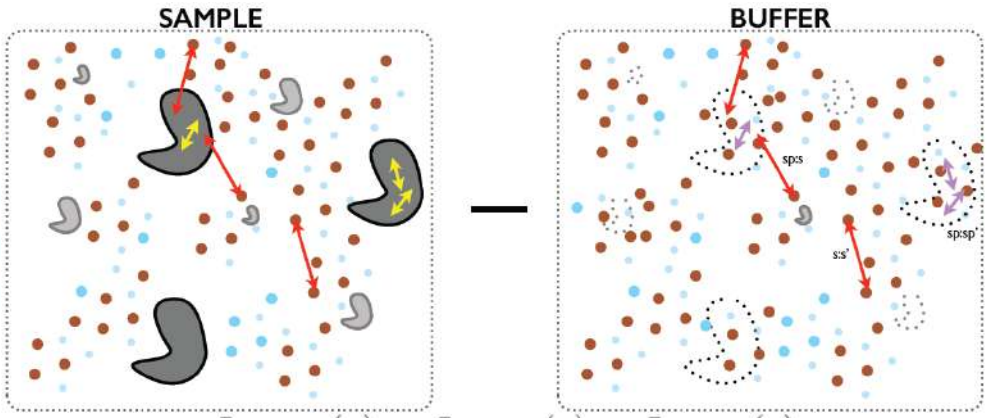


Protein-protein interactions

Basics of a SAXS curve

• I(q) measured intensities



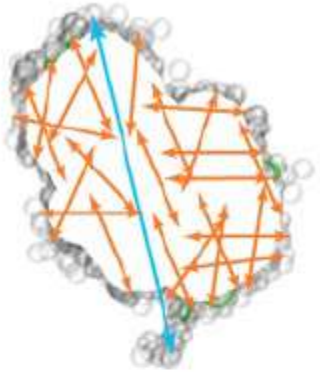


$$I_{particle}(q) = I_{sample}(q) - I_{buffer}(q)$$

low resolution ⟷ high resolution

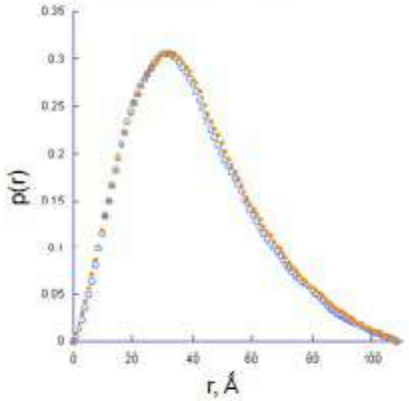
- Features throughout the curve relate to shape
- At low resolution, can approximate particle as a homogenous body of electron density
- larger the object, the faster the I(q) decay

Interatomic Vectors

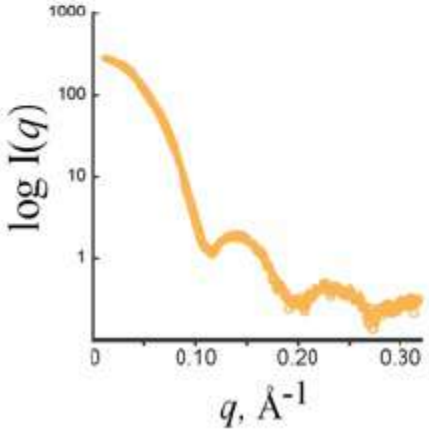


P(r) Function

Defined on $0 < r < d_{max}$

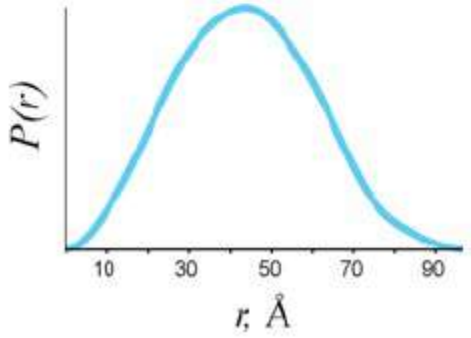


I(q) vs q



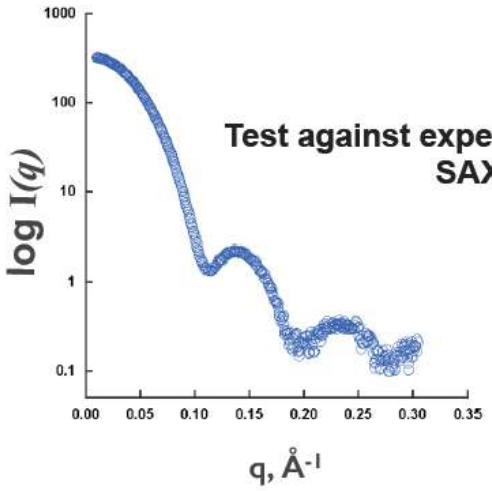
$$I(q) = \int_0^{d_{max}} P(r) \frac{\sin\ q \cdot r}{q \cdot r} dr$$

P(r)-distribution

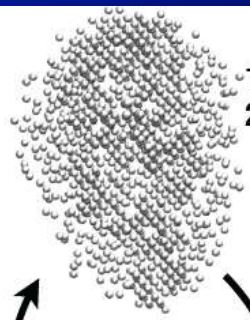


DAMMY ATOMS MODELING

ab initio modeling



Test against experimental SAXS curve

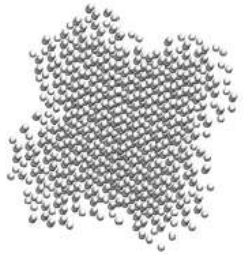


1. Define Search Volume
2. Fill with beads (dummy atoms)
   - proportional to mass

Sample different bead arrangements

- Simulated Annealing
- Genetic Algorithm

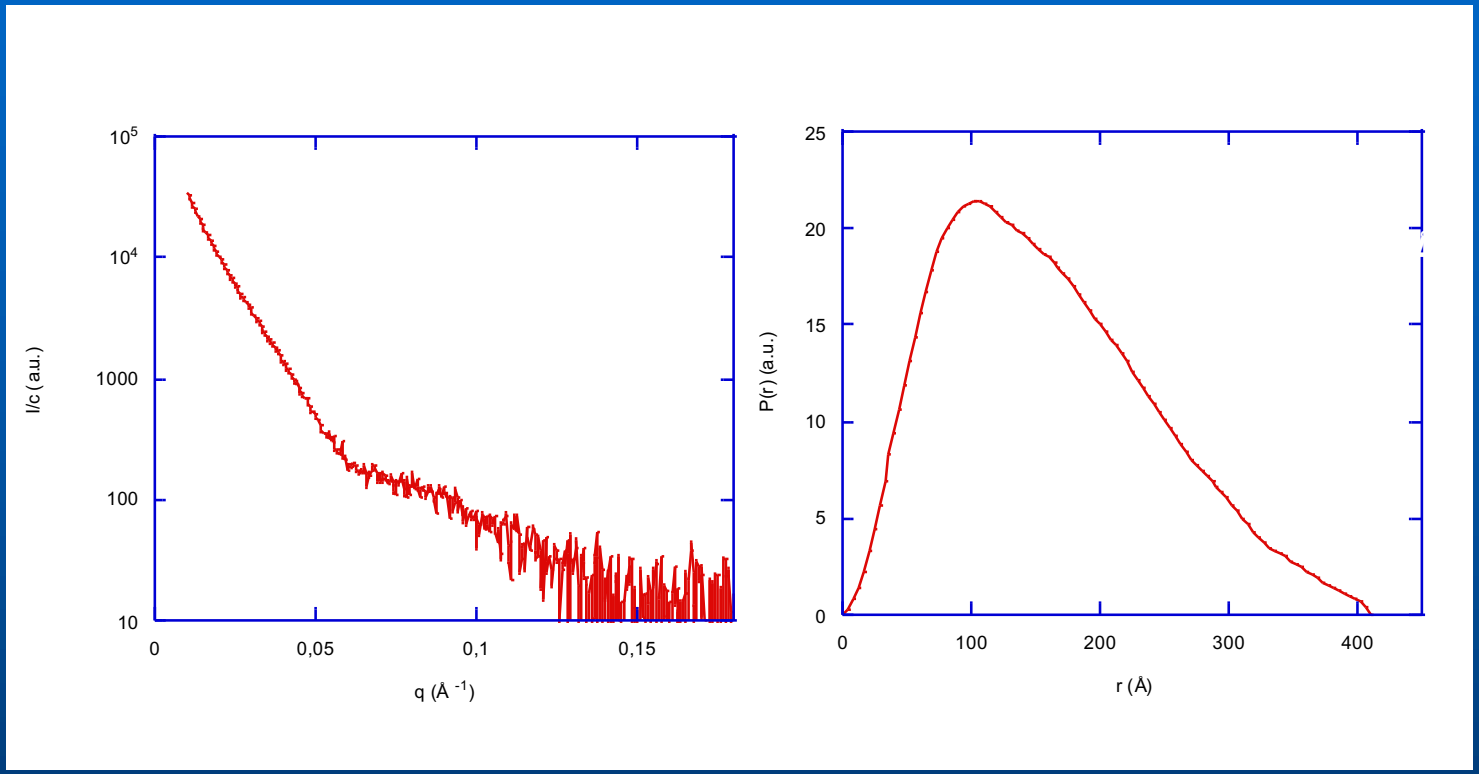
Repeat



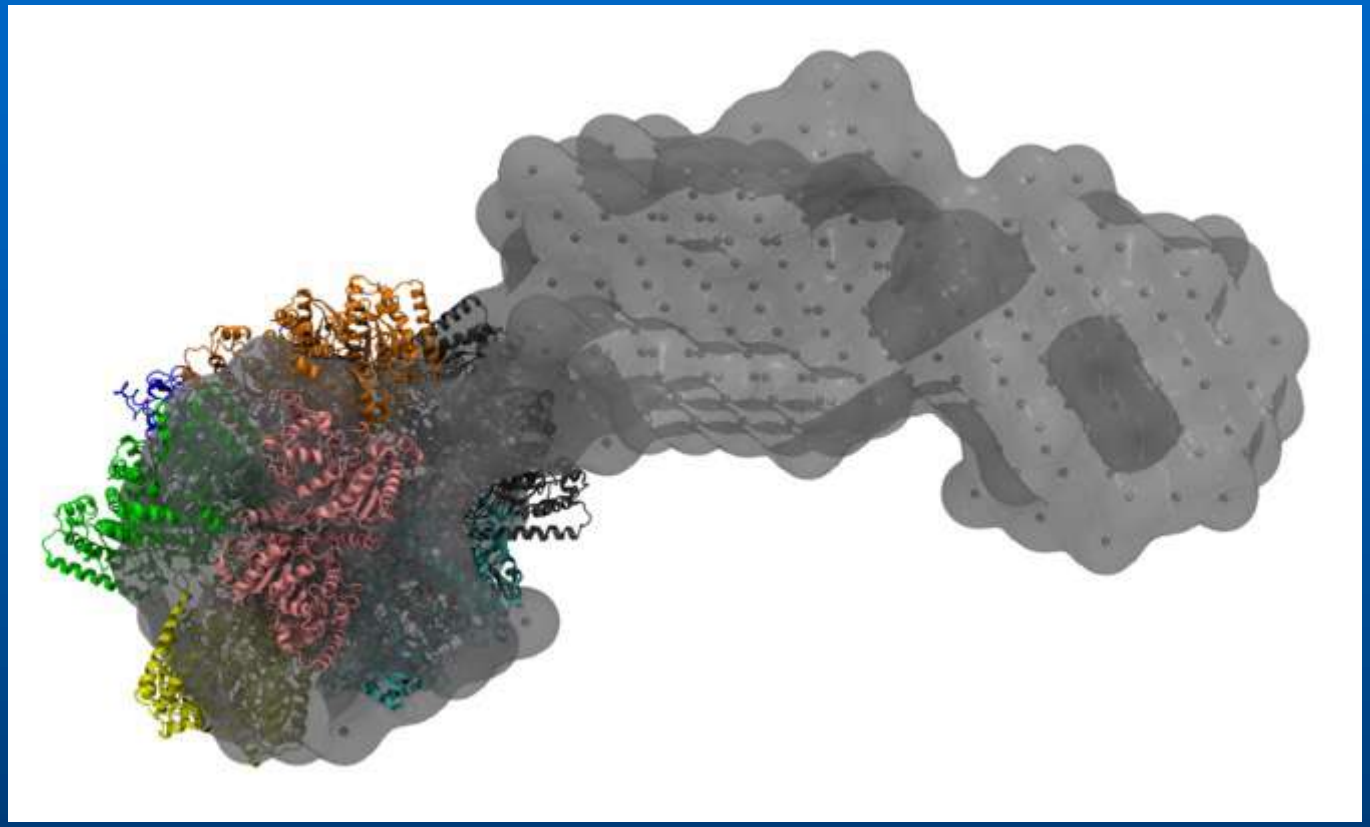
Modeling depends greatly on

1. high quality SAXS data
2. accurate mass constraints

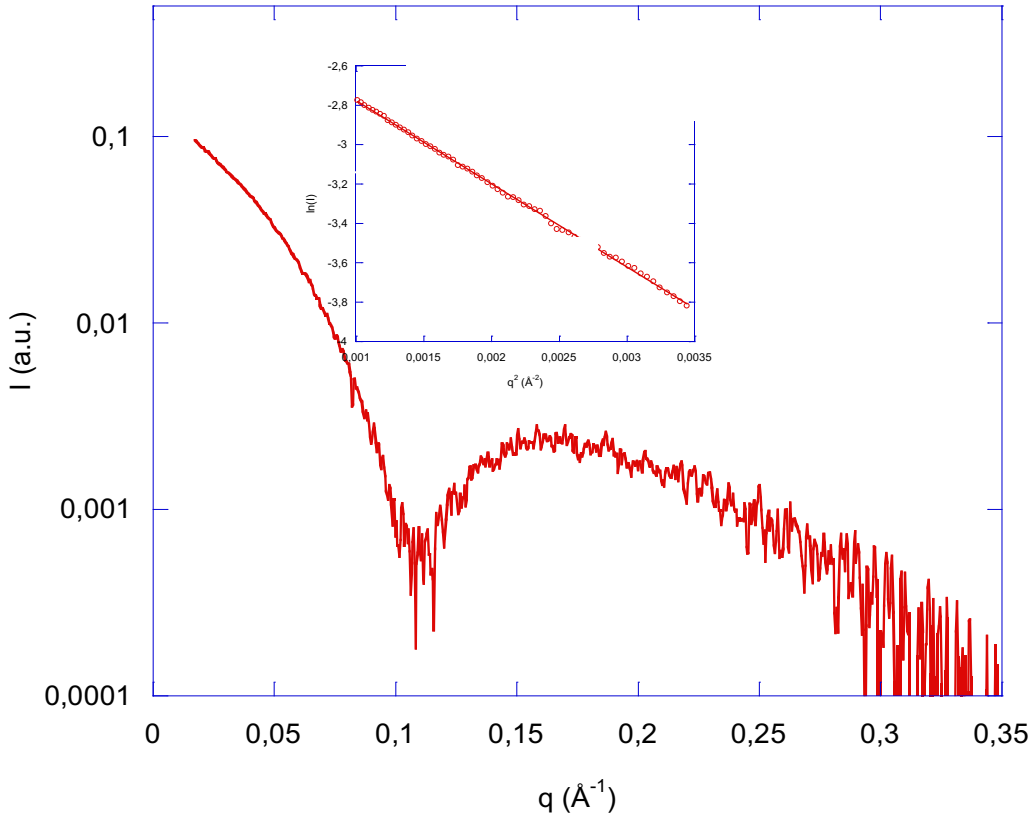
Determination of the oligomerization state of FAAH in solution by small angle X-ray scattering (SAXS)

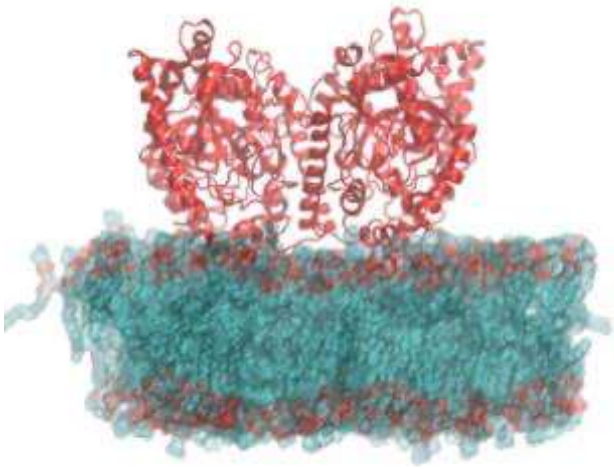


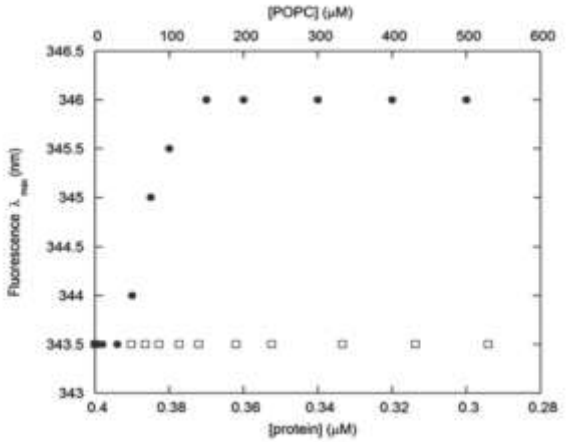
Determination of the oligomerization state of FAAH in solution by small angle X-ray scattering (SAXS)



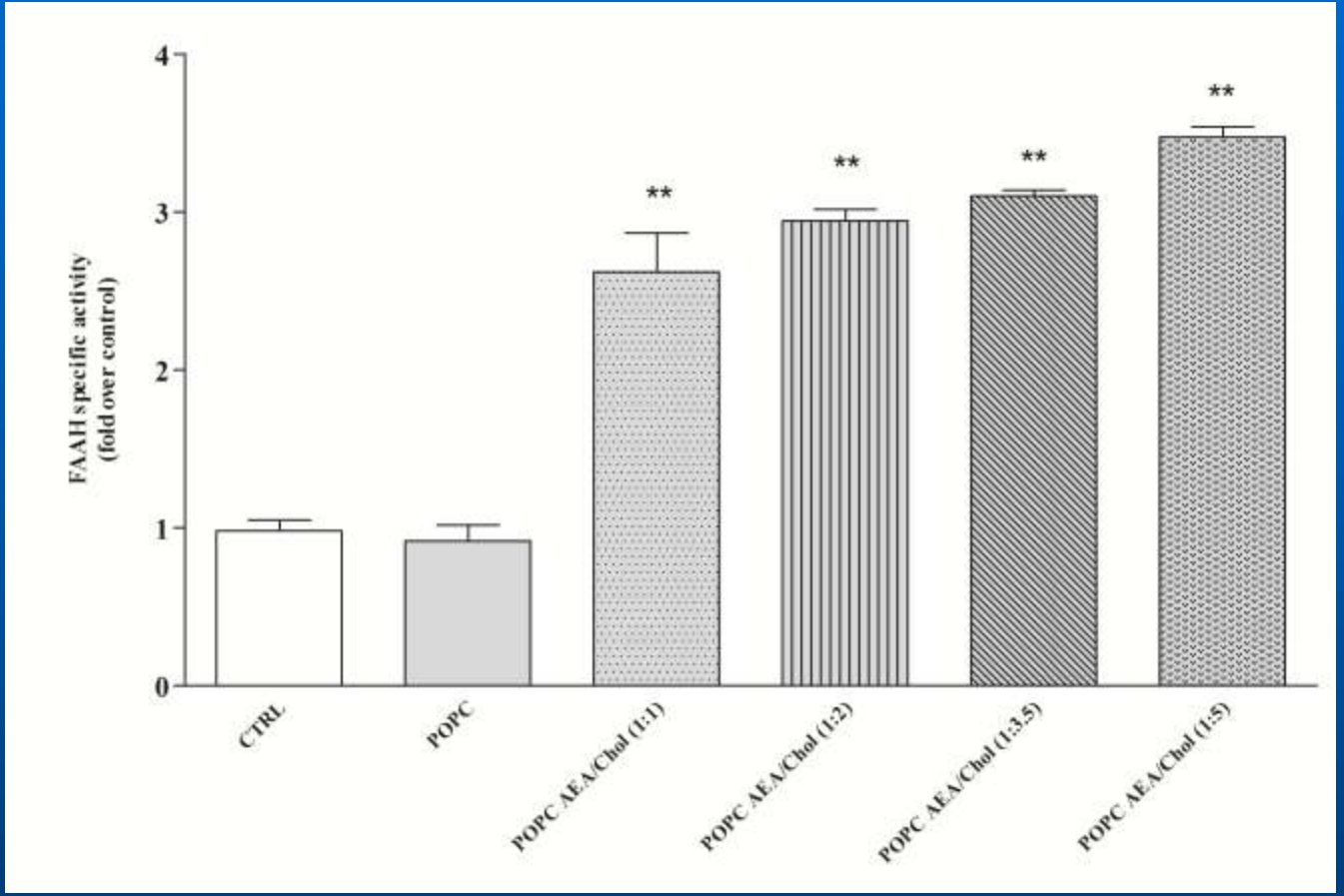
Membrane lipids dissociate these oligomers and stabilize FAAH dimer







FAAH activity is strongly increased by membranes containing AEA and cholesterol



**p < 0.01 *versus* CTRL

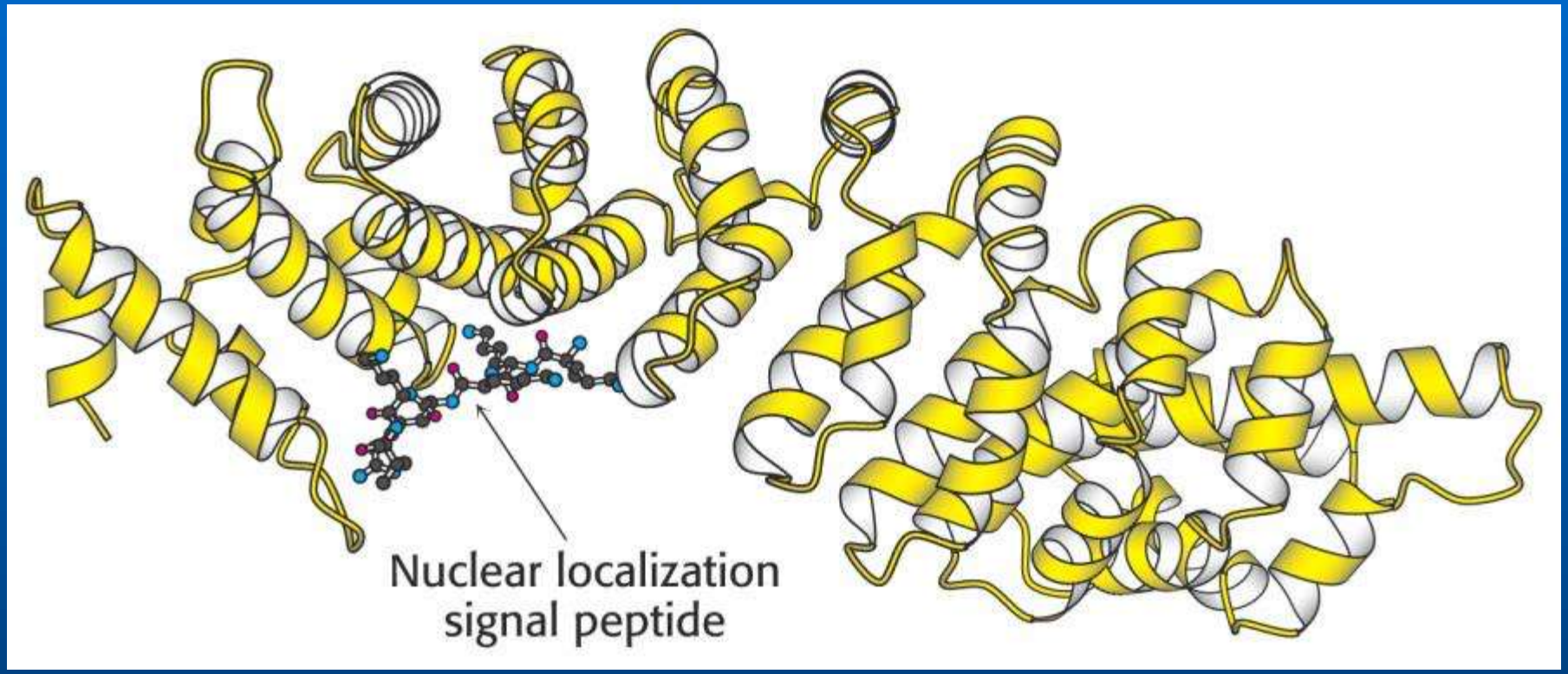
Subcellular distribution of proteins: Protein trafficking

TABLE 12.4 Targeting sequences

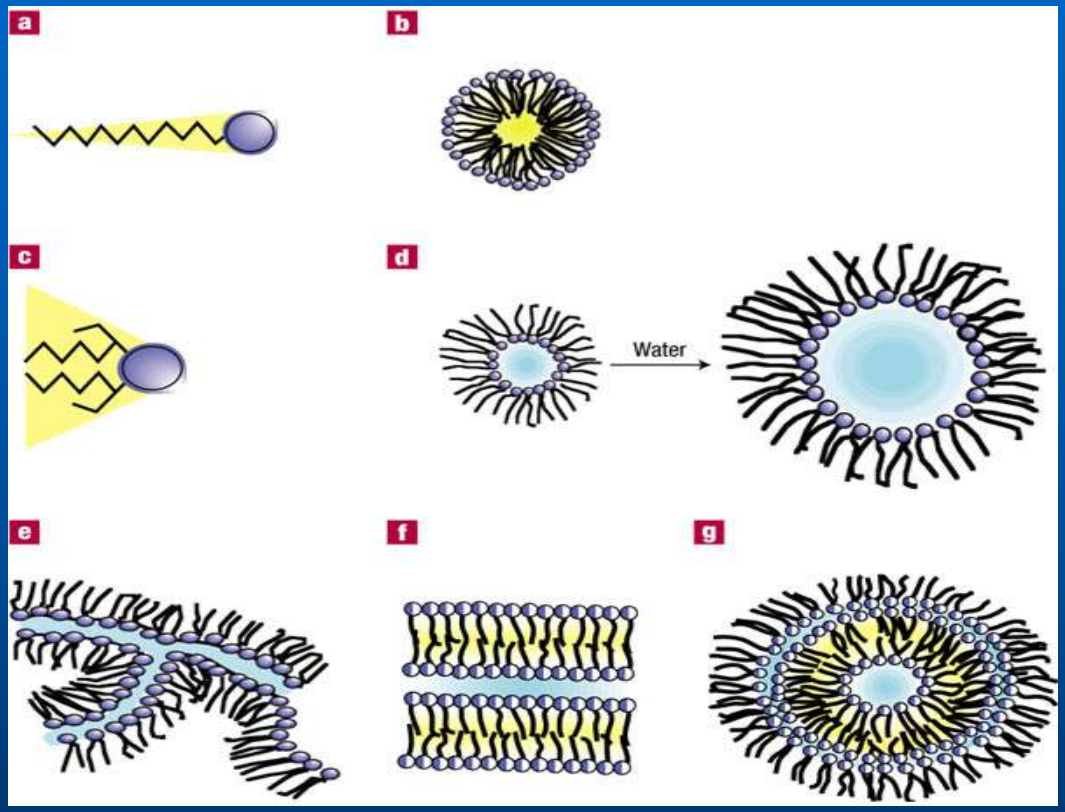
Target	Signal
Nucleus	–KKXK or –$(K/R)_2$–$X_{10–12}$–$(K/R)^*$
Peroxisome	–SKL–COO^-
Mitochondrion	*N*-terminal amphipathic helix
Endoplasmic reticulum	–KDEL–COO^-(ER retention)

*The "/" means that either K or R is required.

Subcellular distribution of proteins: Protein trafficking

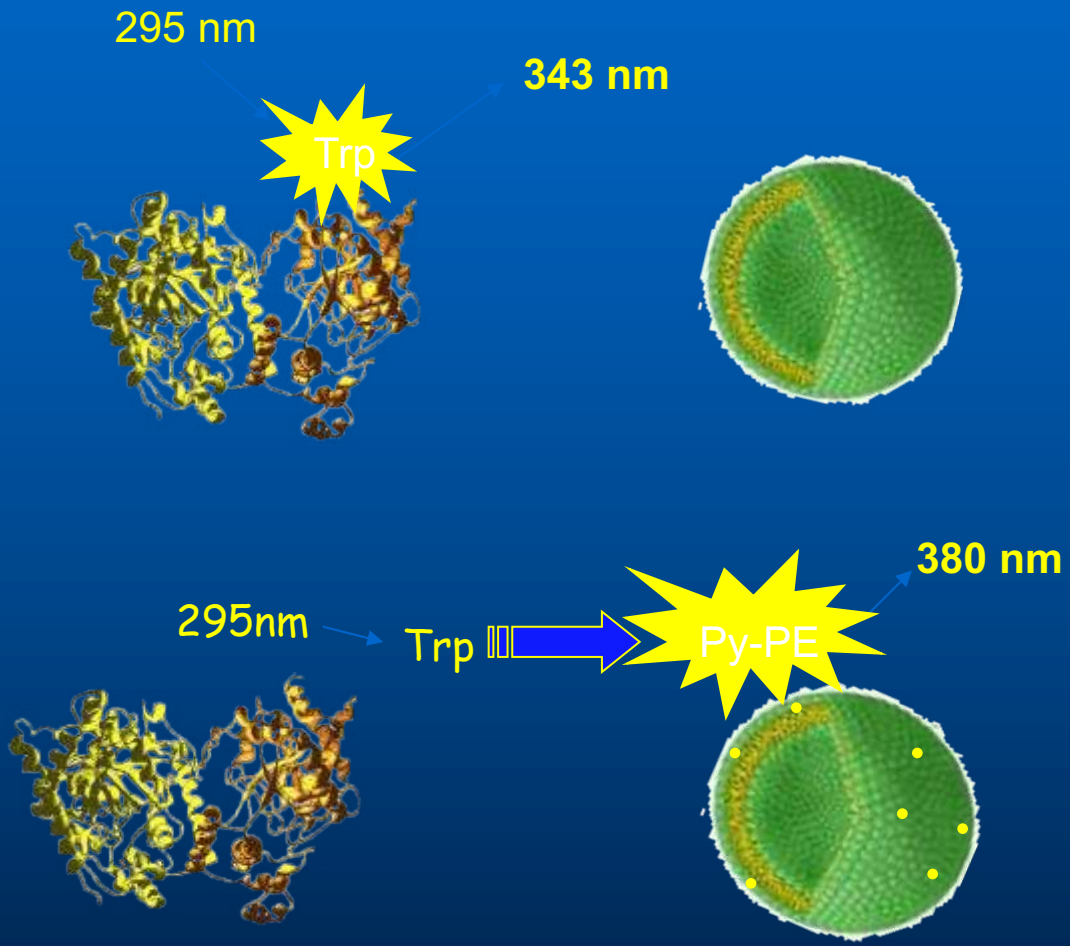


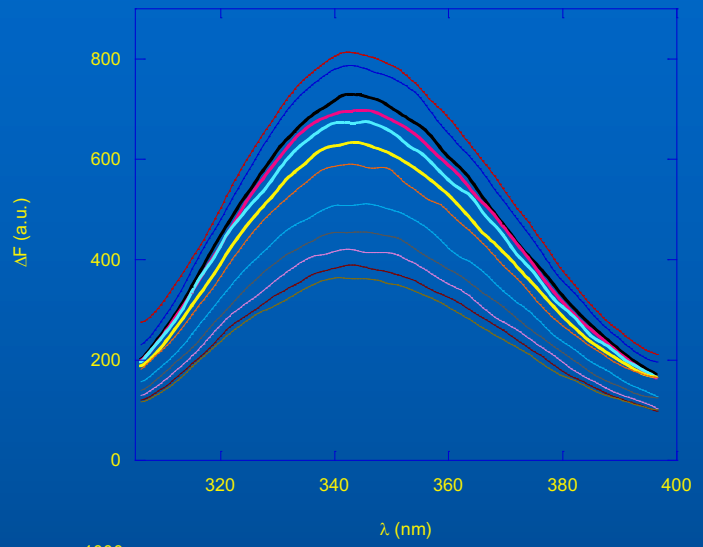
Subcellular distribution of proteins: How lipids affect Protein trafficking?

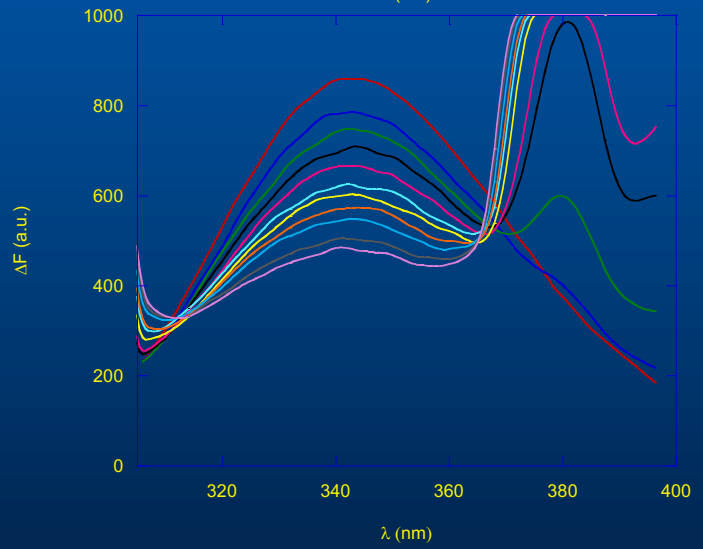


a, Cone-shaped surfactant resulting in **b**, normal micelles.
c, Champagne cork shaped surfactant resulting in **d**, reverse micelles with control of their size by the water content.
e, Interconnected cylinders. **f**, Planar lamellar phase. **g**, Onion-like lamellar phase.

Study of FAAH/membrane interaction by FRET

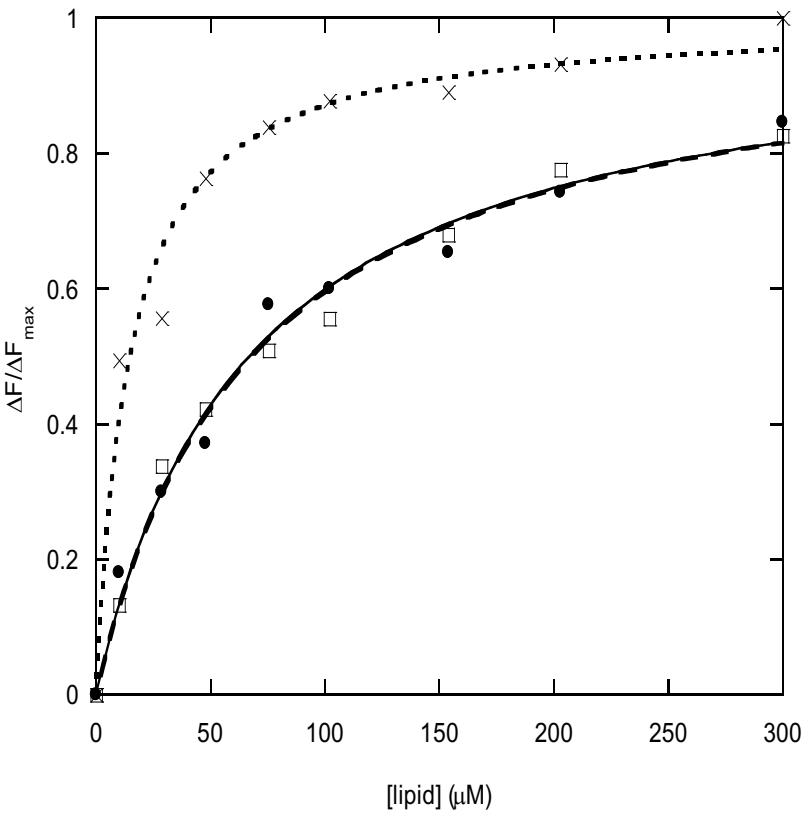


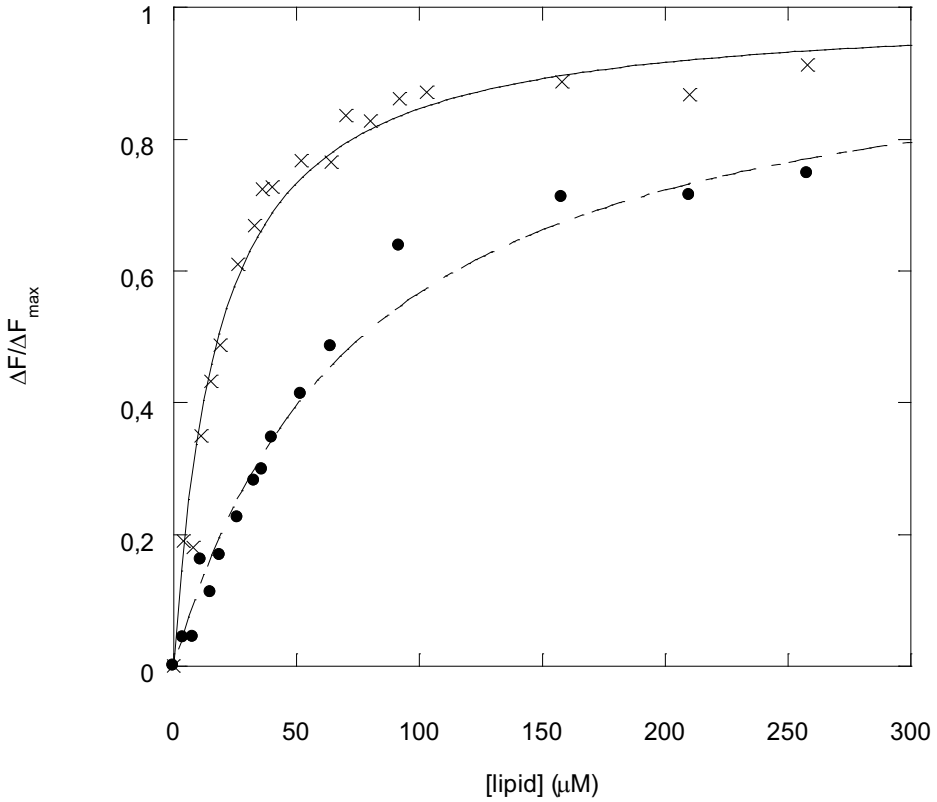




Angelucci CB, Sabatucci A, Dainese E. *Methods Mol Biol*. 2016;1412:267-76.

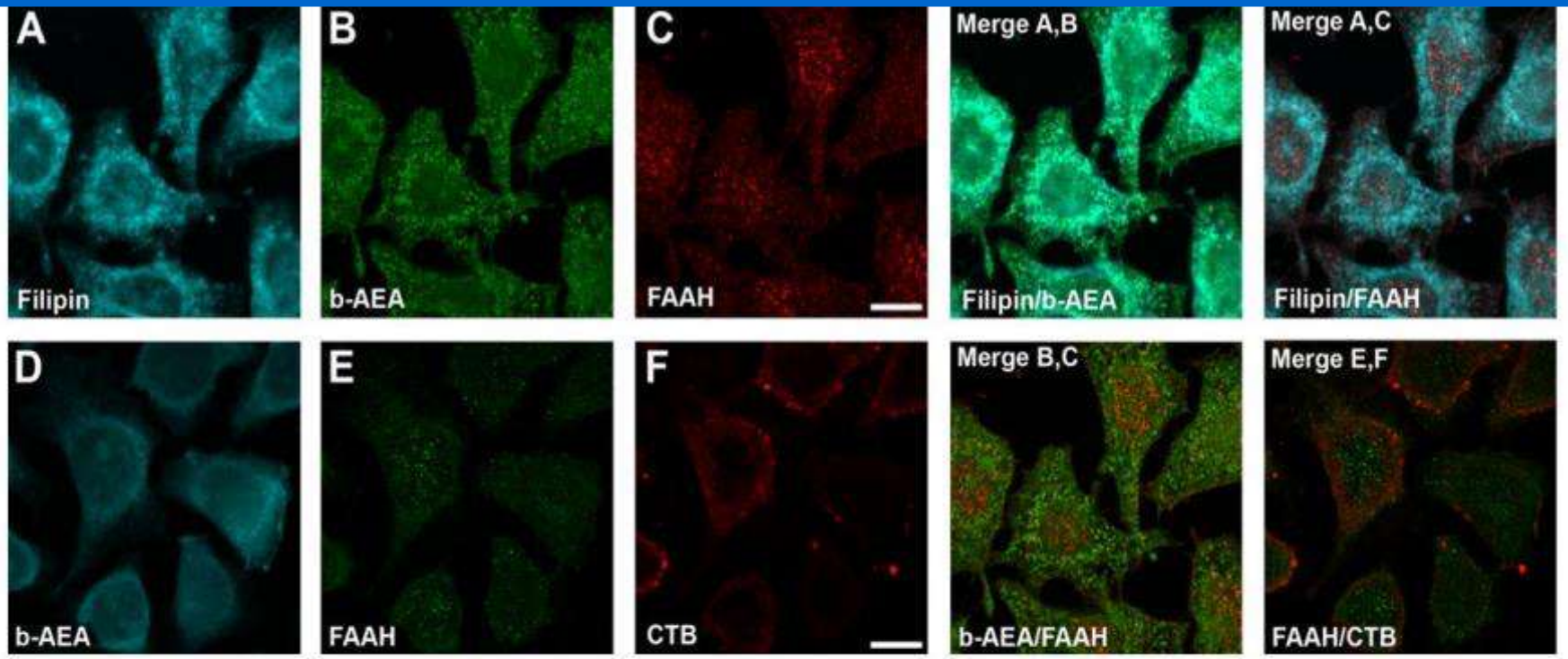
Higher membrane affinity of FAAH to ER membranes containing AEA and cholesterol





Dainese E et al., (2014) Membrane lipids are key modulators of the endocannabinoid-hydrolase FAAH. *Biochem J.* ***457*****(3)**:463-472.

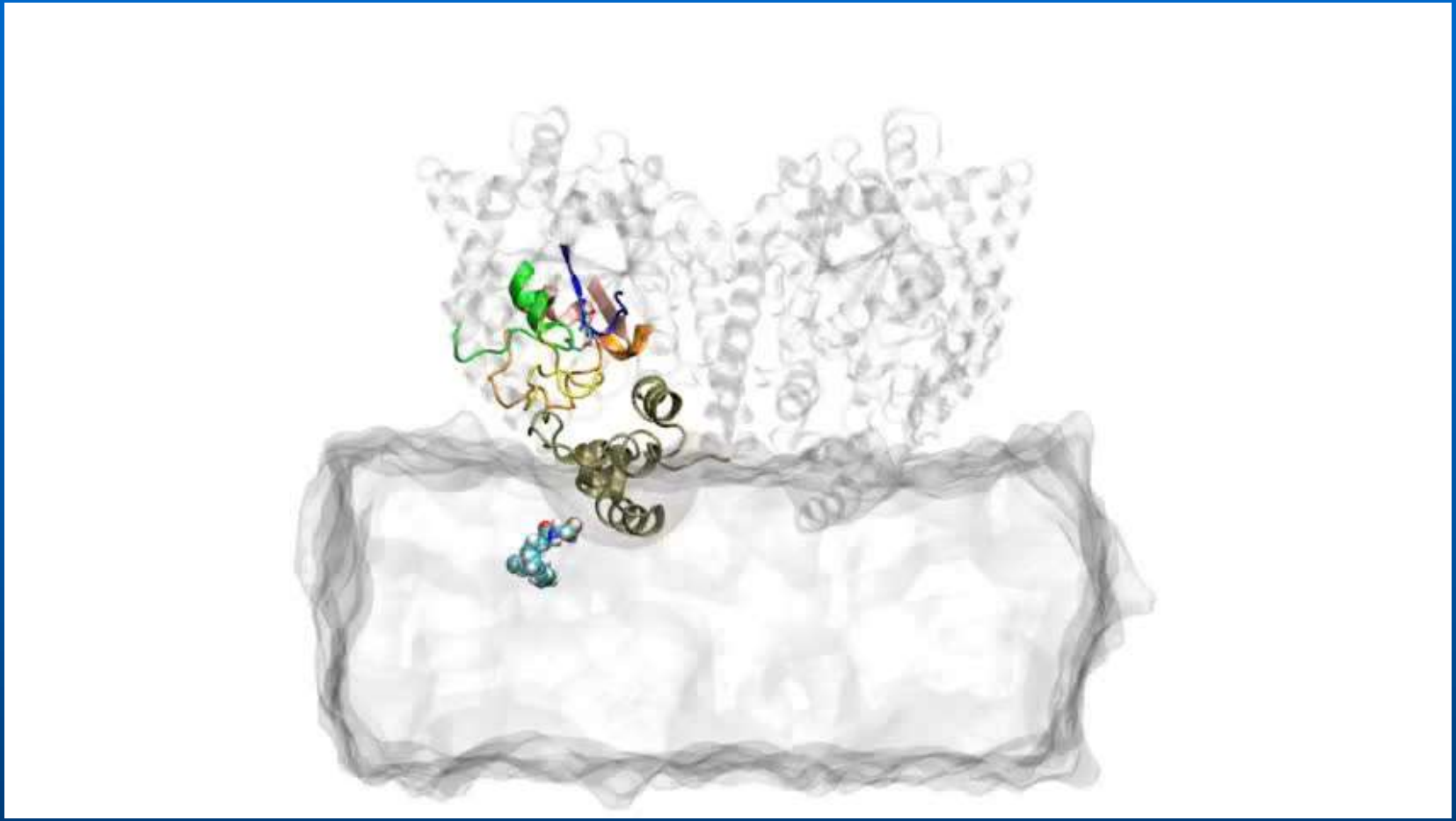
Confocal analysis of the cellular localization of FAAH



Parameter	Filipin/b-AEA	Filipin/rFAAH	b-AEA/rFAAH	FAAH/CTB
Pearson's correlation coefficient (R_r)	0.69 ± 0.03	0.43 ±0.02	0.51 ± 0.05	0.10 ± 0.02

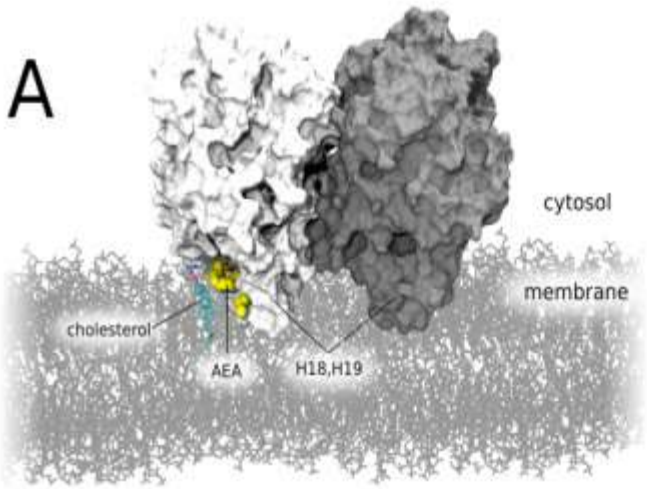
Dainese E et al., (2014) Membrane lipids are key modulators of the endocannabinoid-hydrolase FAAH. *Biochem J.* ***457*****(3)**:463-472.

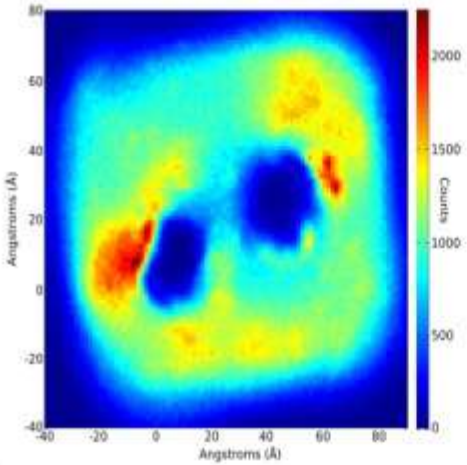
Molecular Dynamics (MD) of the full binding trajectory of AEA into the FAAH active site

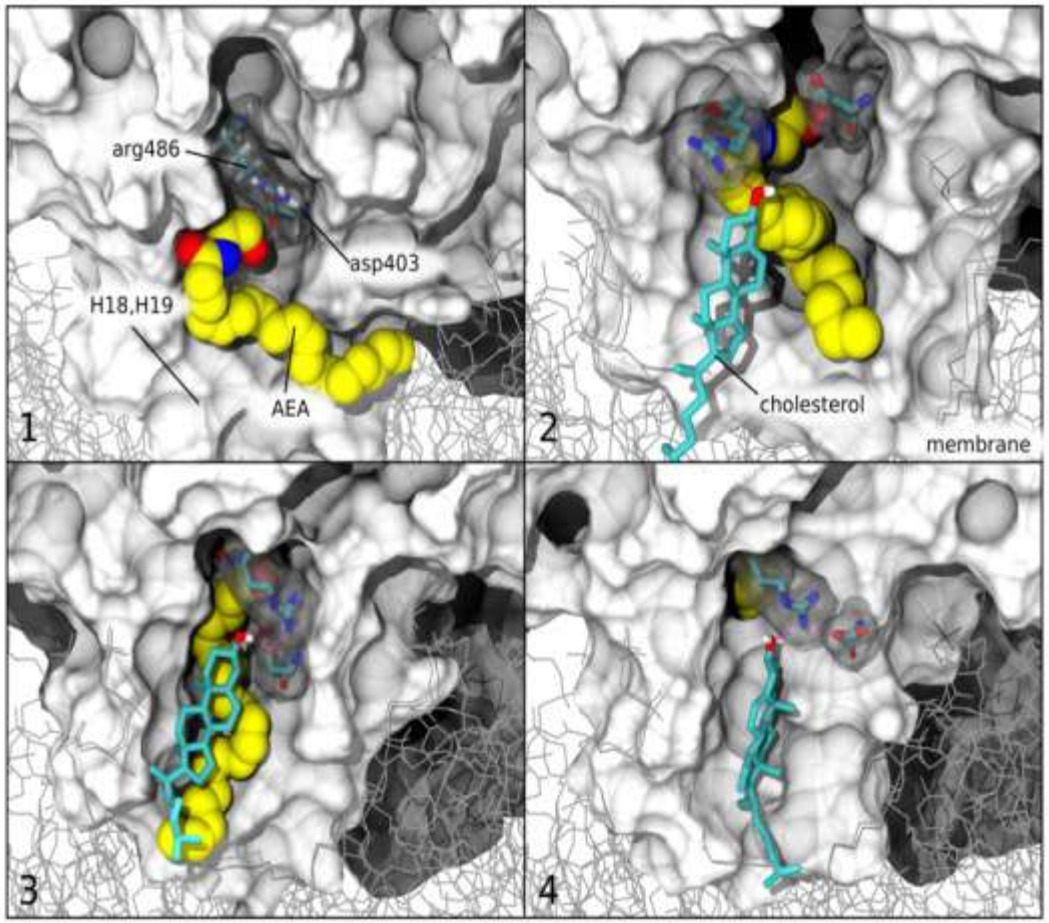


Dainese E et al., (2014) Membrane lipids are key modulators of the endocannabinoid-hydrolase FAAH. *Biochem J.* ***457*****(3)**:463-472.

MD simulations show that cholesterol facilitates the binding of AEA to FAAH by opening the membrane port

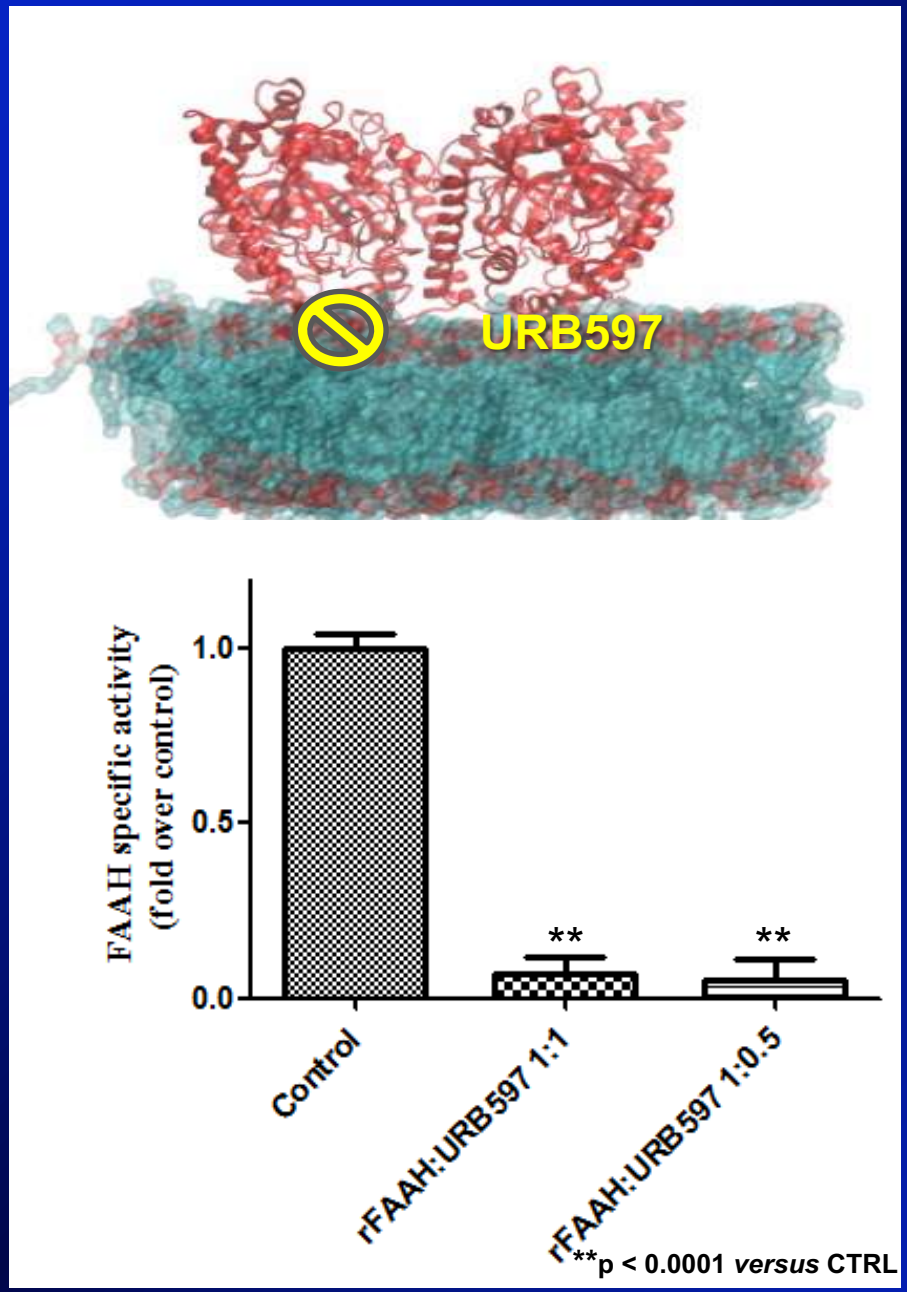






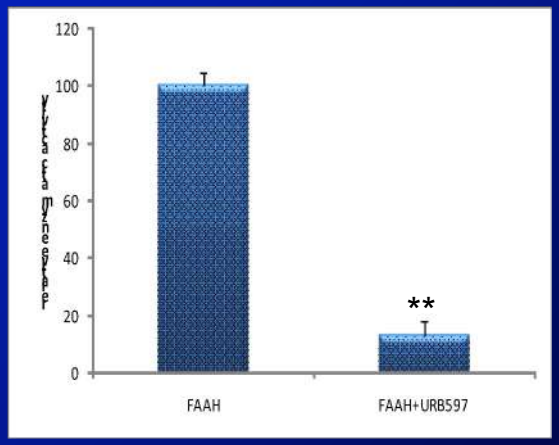
Dainese E et al., (2014) Membrane lipids are key modulators of the endocannabinoid-hydrolase FAAH. *Biochem J.* ***457*****(3)**:463-472.

FAAH enzymatic activity: a complex enzyme

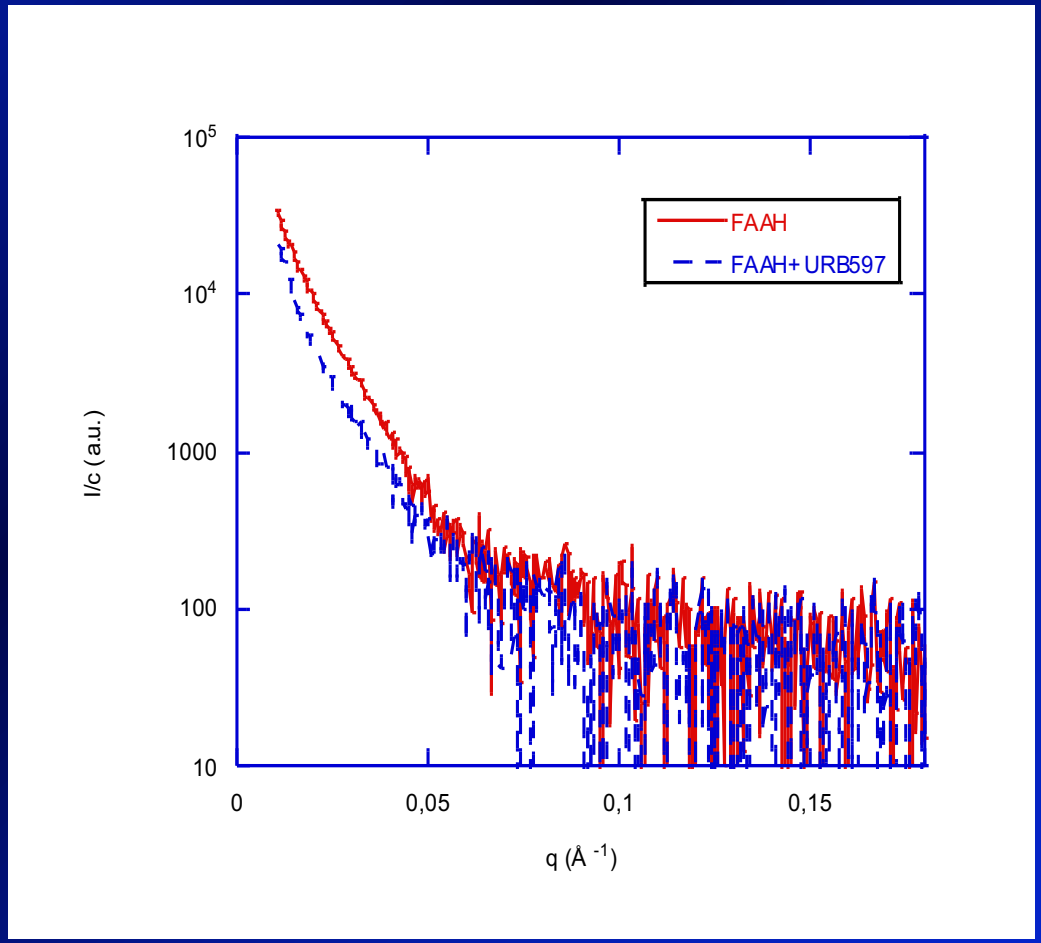


Using also a FAAH(dimer):inhibitor 1:0.5 stoichiometry we obtained a full inhibition of FAAH activity

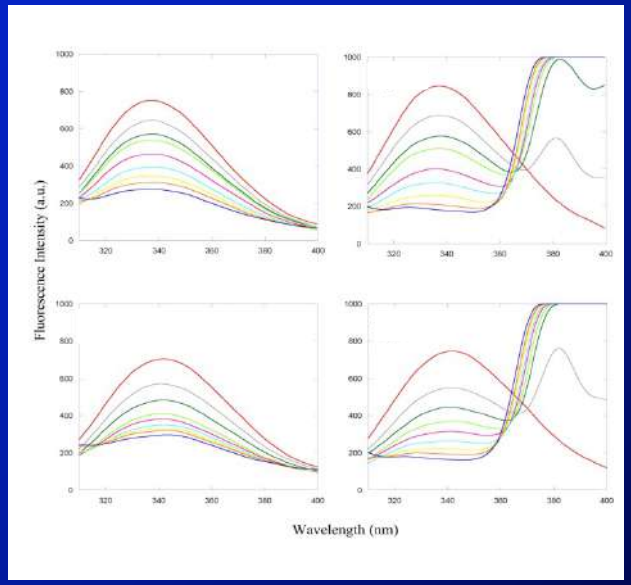
SAXS revealed that at a FAAH:URB597 1:0.5 stoichiometry the inhibitor induces a conformational change leading to a more compact form of FAAH



**p < 0.001 *versus* FAAH

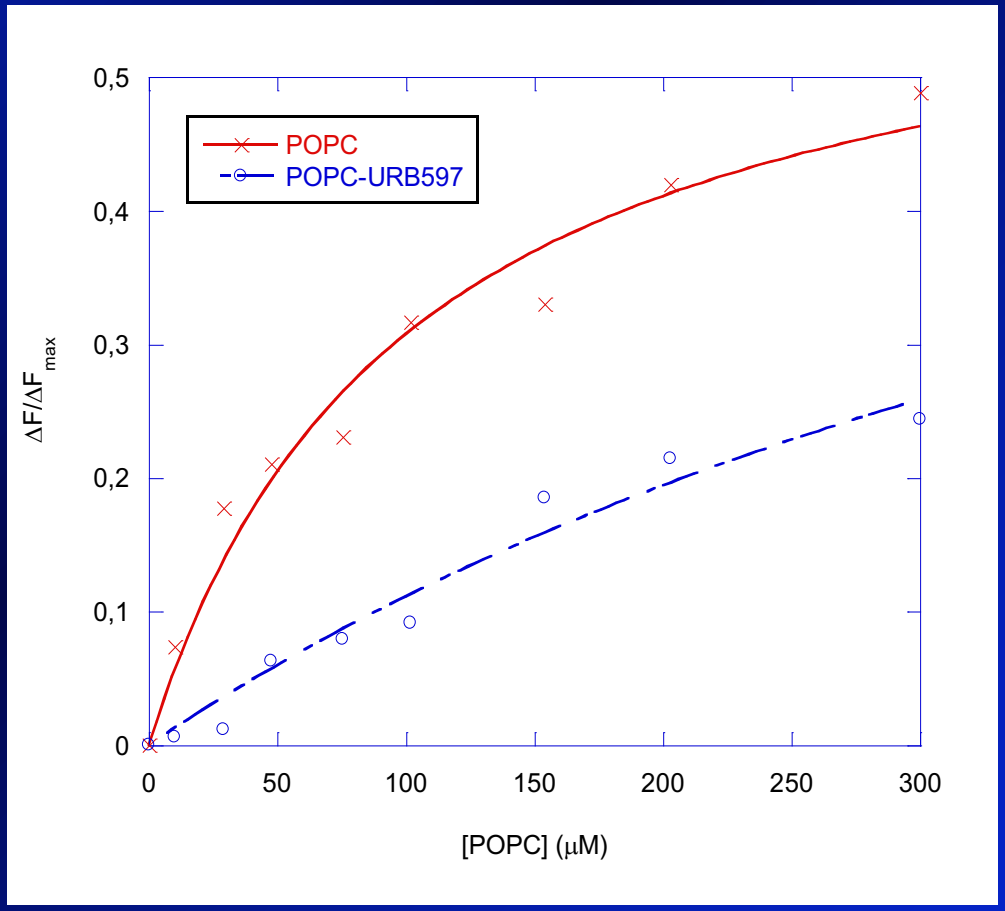


The latter form of FAAH has a reduced membrane binding affinity

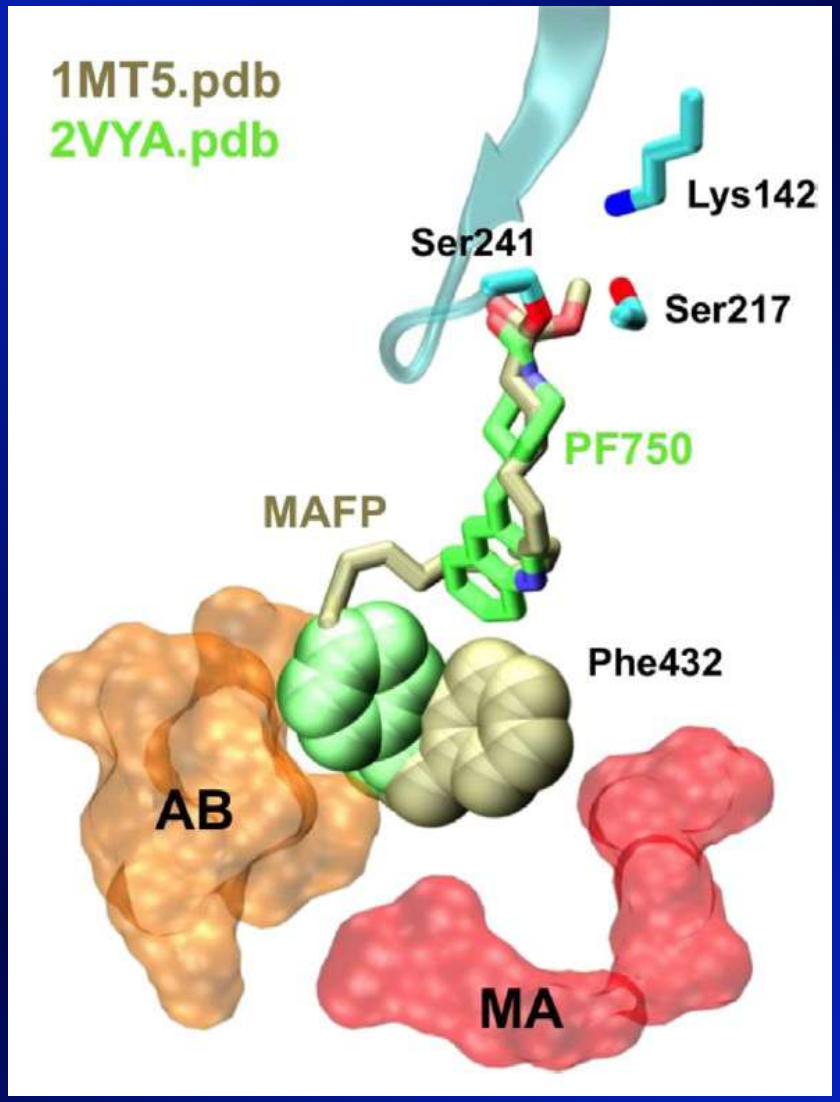


sample	Kd (μM)
FAAH	67 ±10
FAAH + URB597	272 ± 27*

*p < 0.0001 *versus* FAAH+URB597

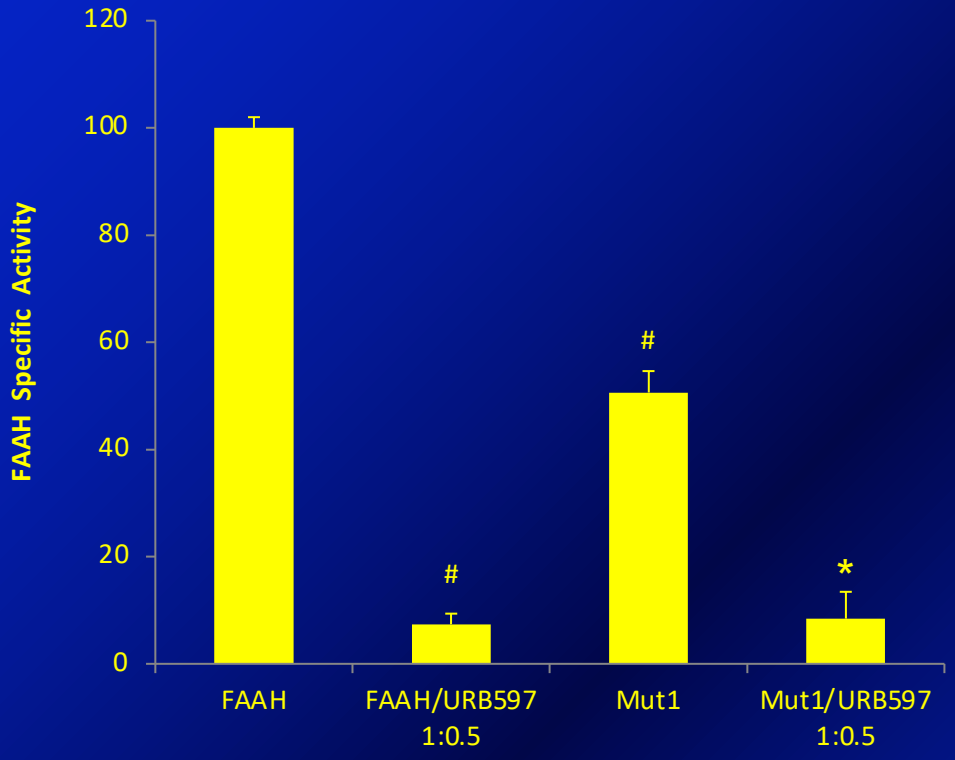


Phe432 was proposed to be involved in the activation of AEA necessary for the hydrolysis



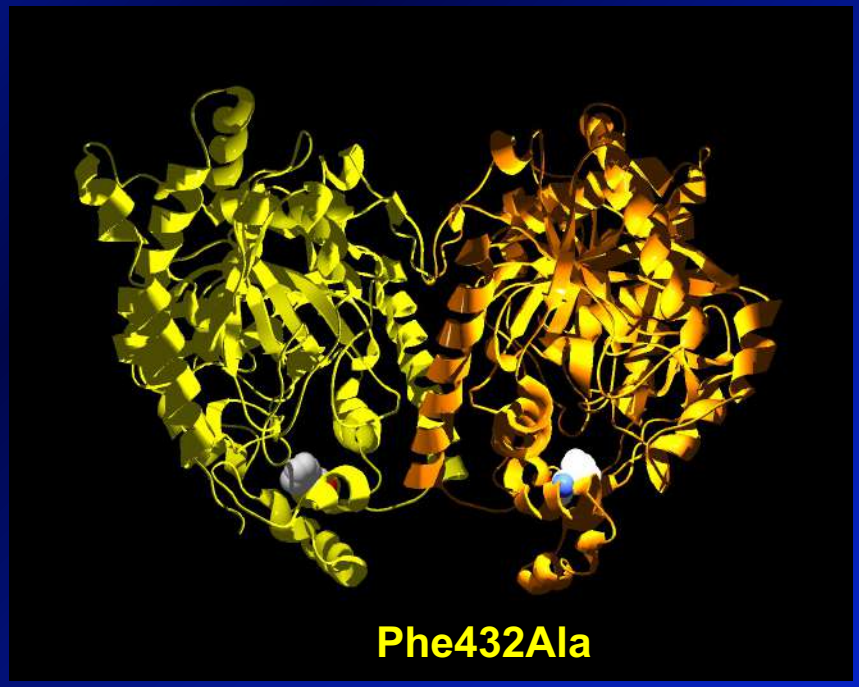
Palermo et al., *European Journal of Medicinal Chemistry* 91 (2015) 15-26

Phe432Ala mutation reduces the FAAH specific activity but not the effect of URB597

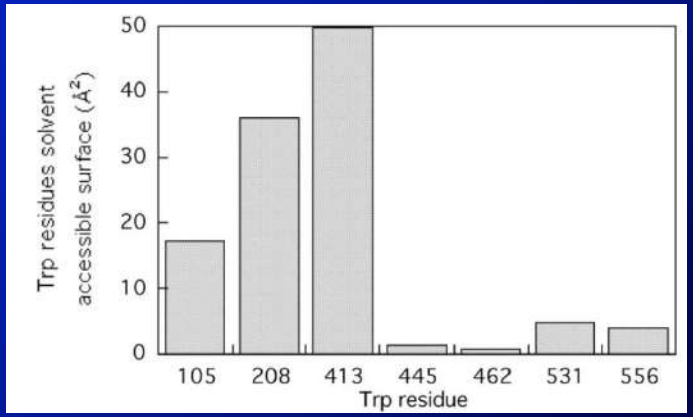


#p < 0.0001 *versus* FAAH

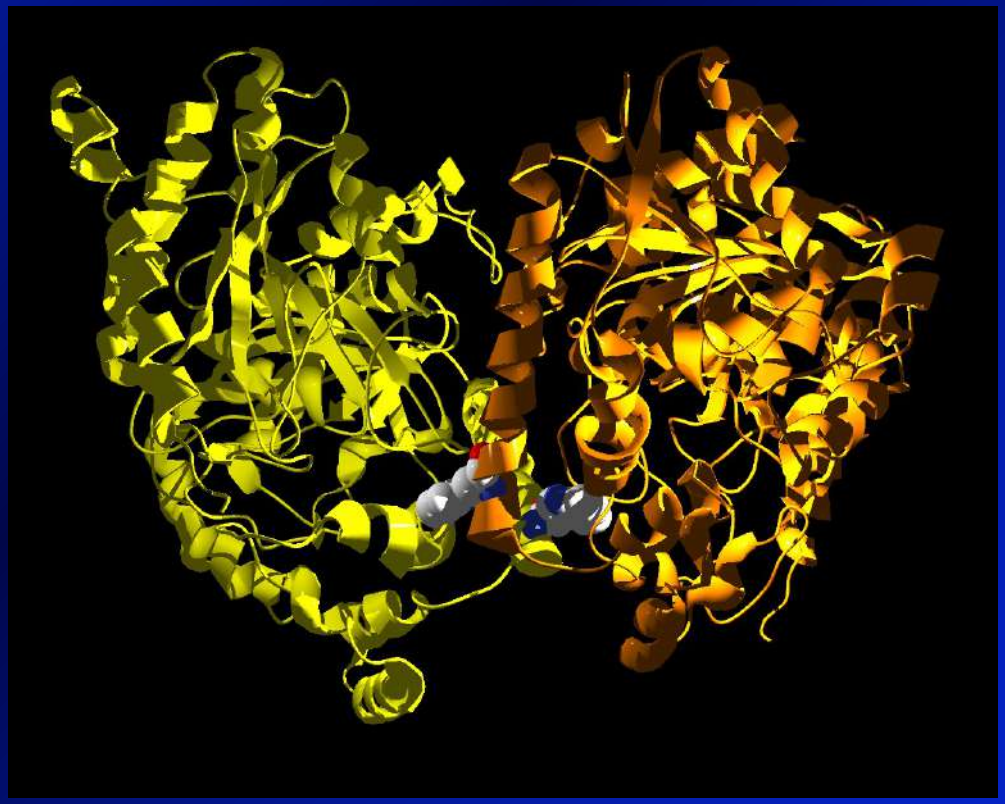
*p < 0.0001 *versus* Mut1



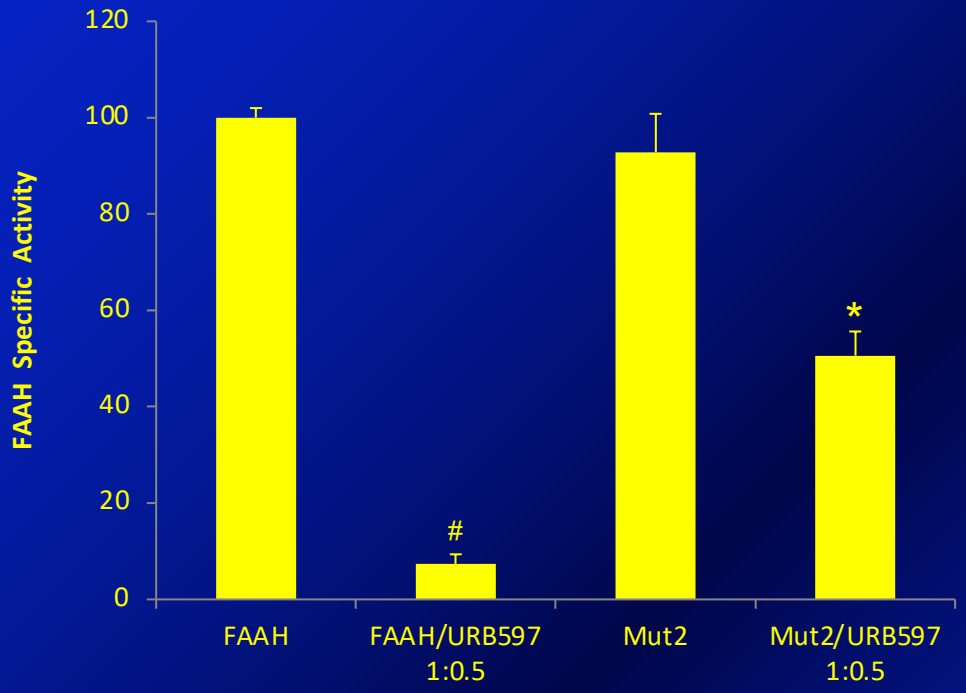
Trp 445 is a fully buried residue proposed to be involved in the inter-subunit interaction

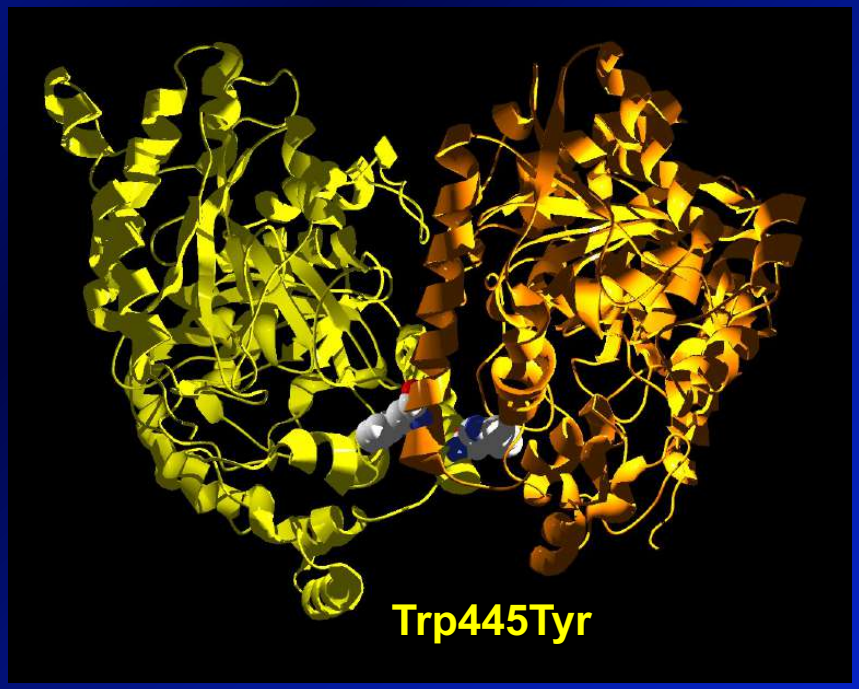


Di Venere & Dainese et al., 2011
Dainese et al., 2014



Trp445Tyr mutation do not alter FAAH specific activity but impairs the effect of inhibitor

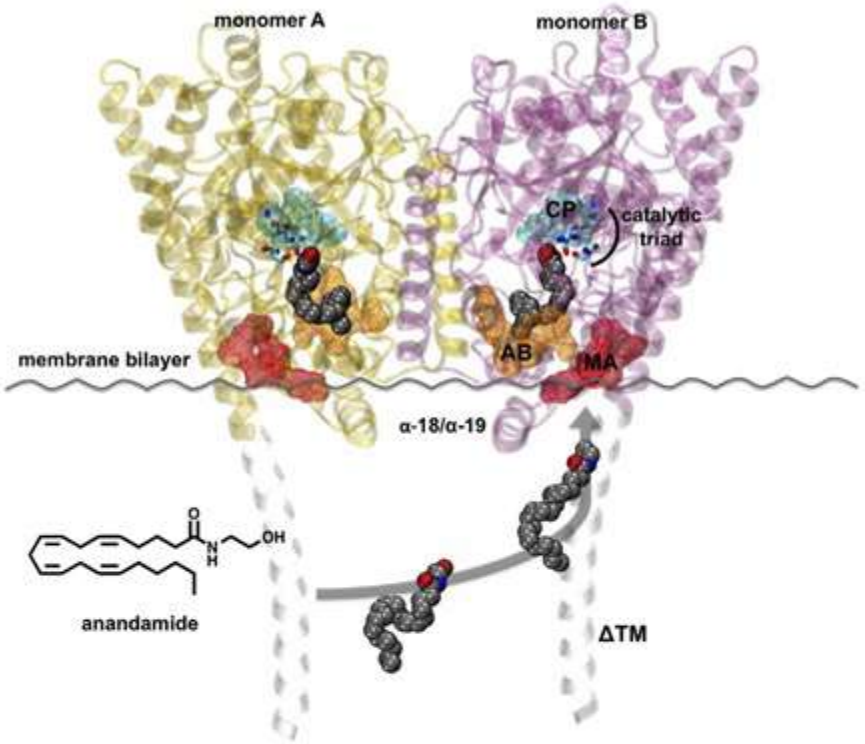




#p < 0.01 *versus* FAAH

*p < 0.01 *versus* FAAH and Mut2

FAAH shows a relative monomer orientation allowing the entrance of AEA from the membrane ports of each subunit



$$FAAH_0 + nAEA \overset{K}{\Leftrightarrow} FAAHn$$

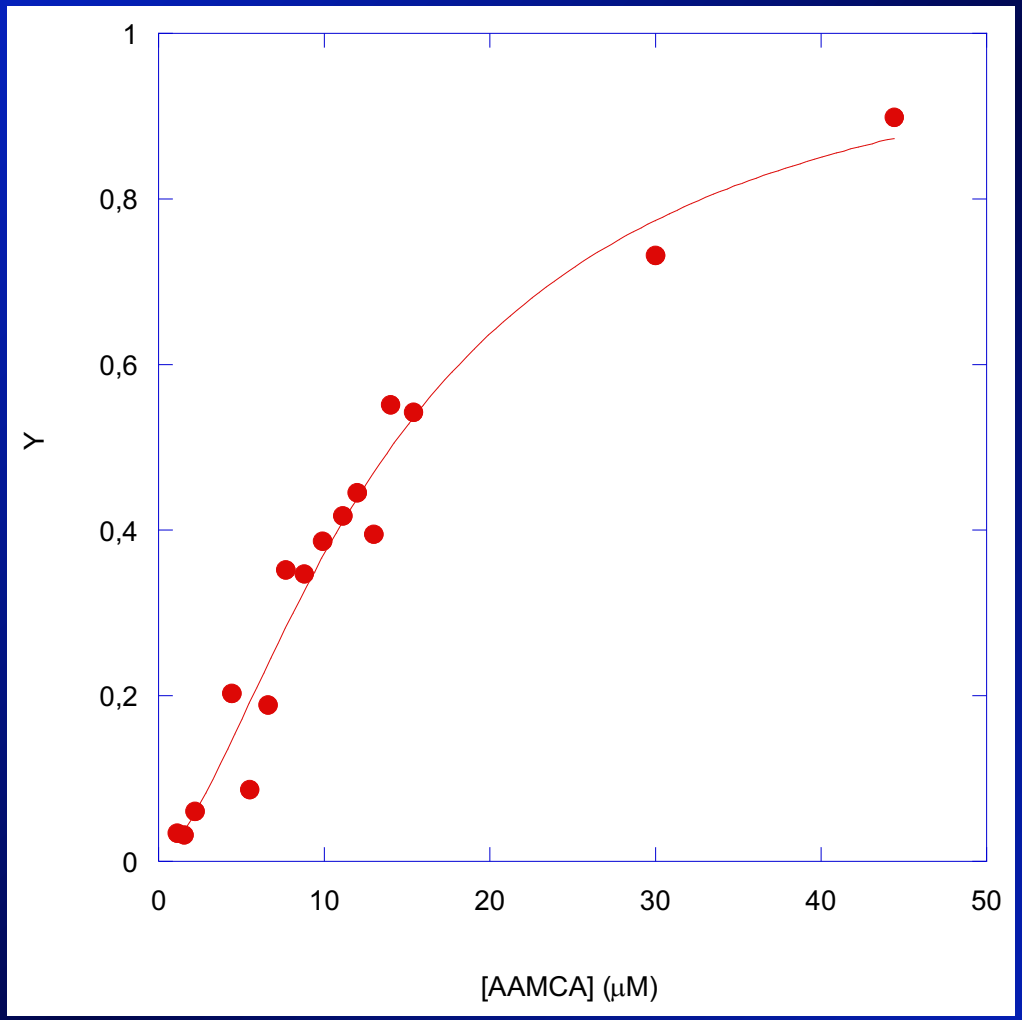
$$Y = \frac{[AEA]^n}{K + [AEA]^n}$$

Y= fraction of the ligand-binding sites on the enzyme which are occupied by the substrate

$$log\left(\frac{Y}{1-Y}\right) = -\log K + n_{Hill}\, log\,[AEA]$$ The Hill equation

n_{Hill} > 1: positively cooperative binding
n_{Hill} < 1: negatively cooperative binding
n_{Hill} = 1: non-cooperative binding

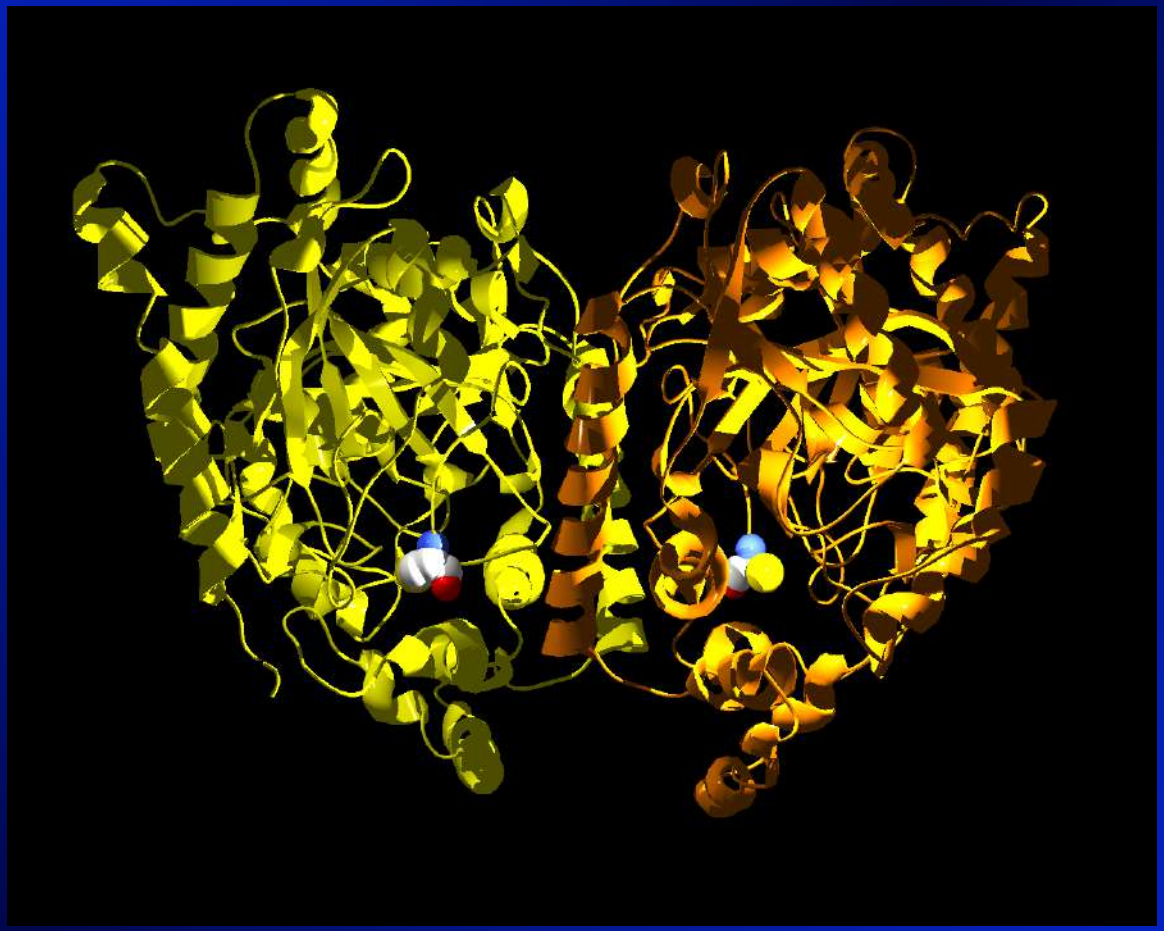
FAAH enzymatic activity: an allosteric enzyme?



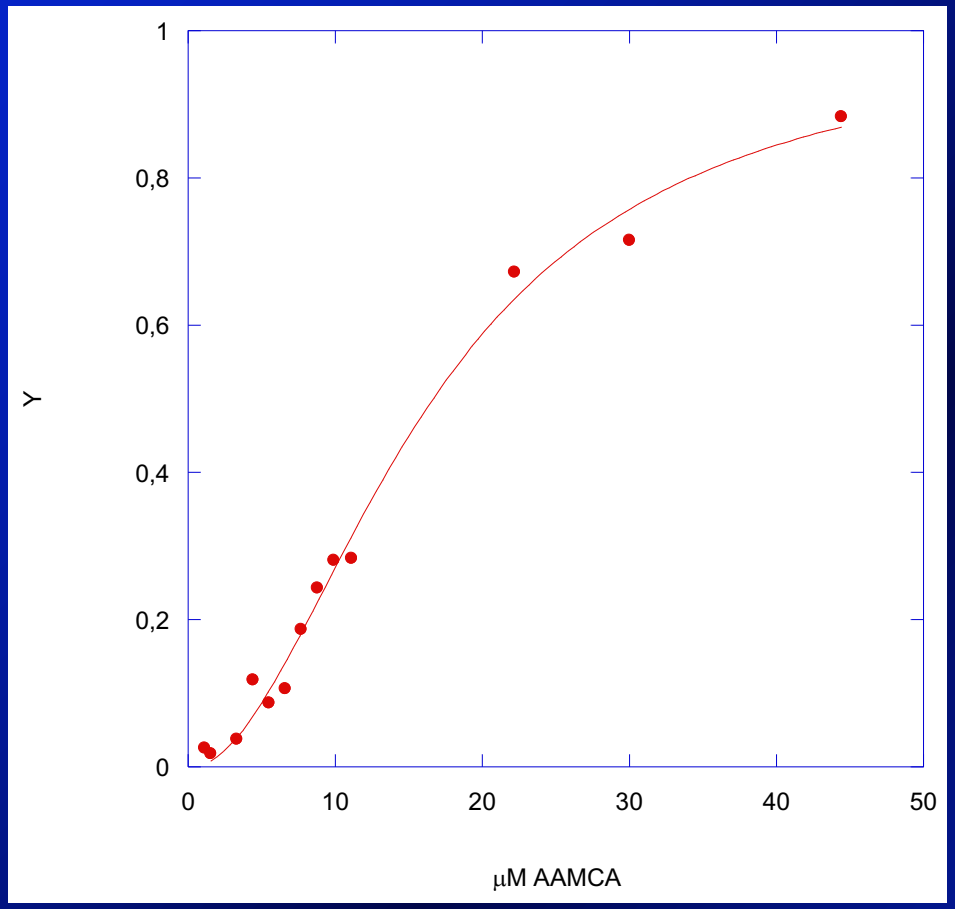
FAAH K = 14.8 ± 3 µM

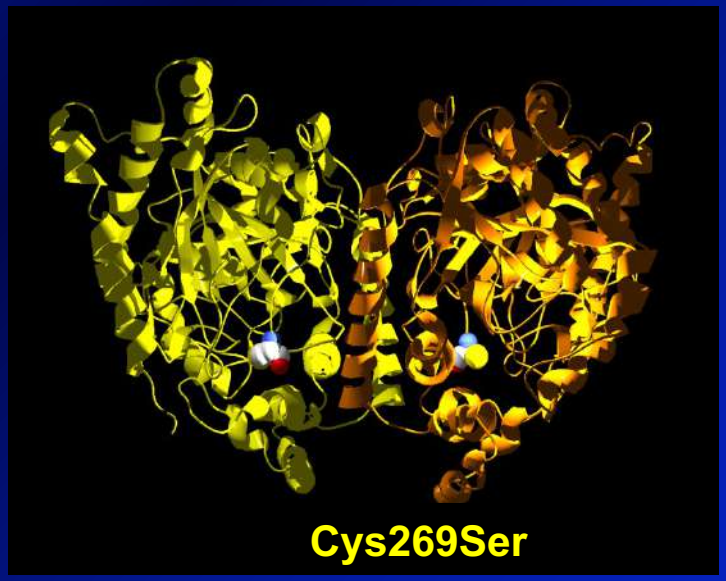
n_{Hill} = 1.5 ± 0.2

The Cys269Ser mutation was introduced in a region of FAAH where structural changes occur upon substrate/inhibitor binding



The FAAH Cys269Ser mutation behaves as a built-in inhibitor reducing the catalytic activity but not the n_{Hill} value

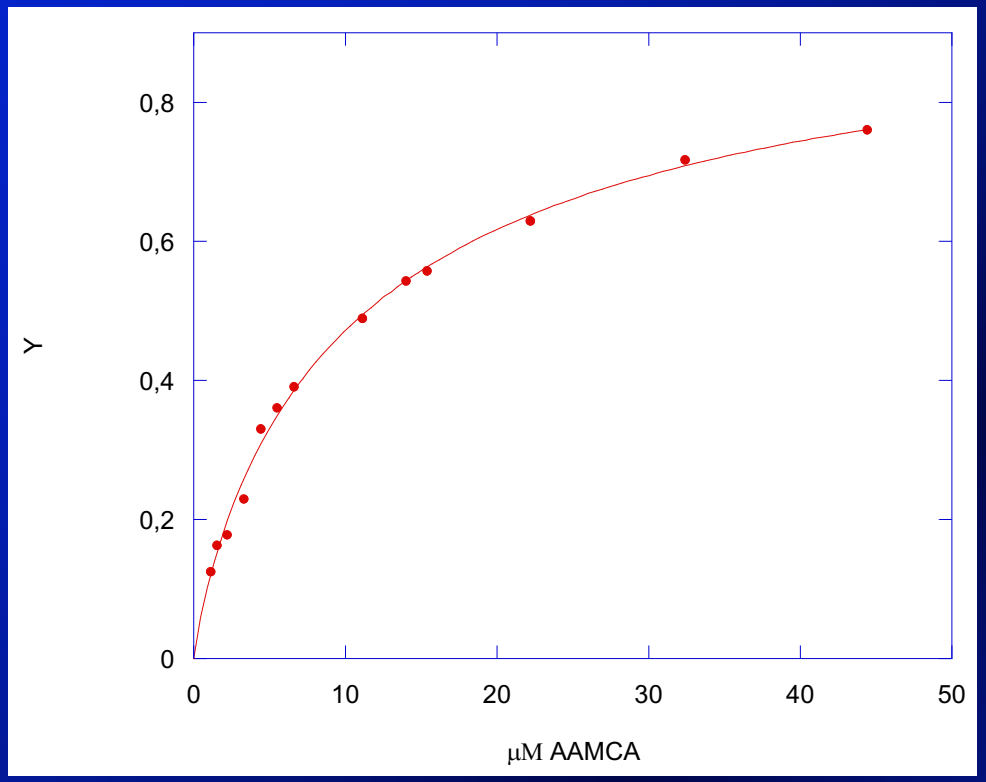


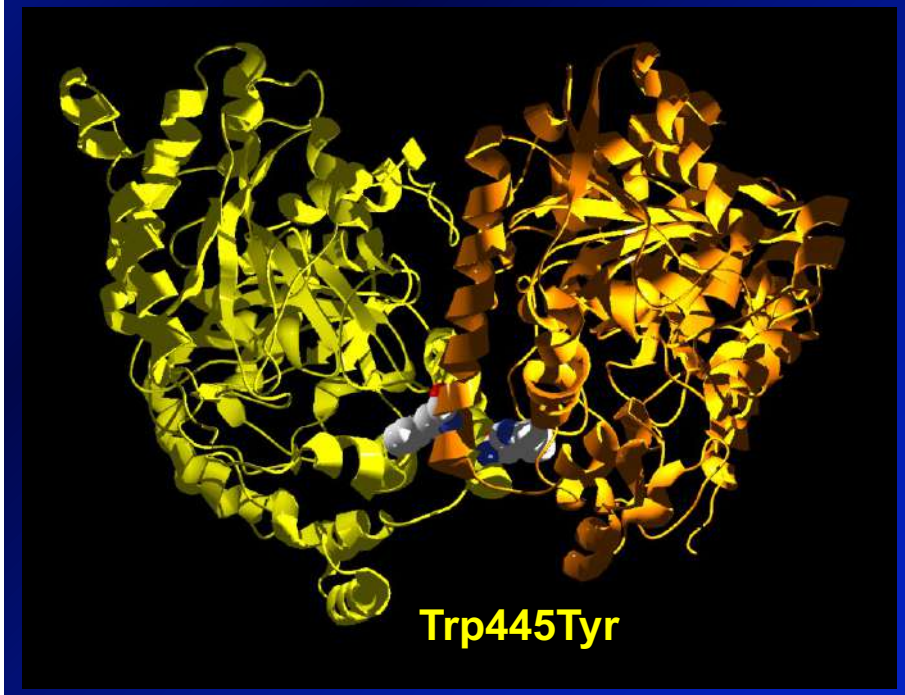


FAAH-C269S K = 16.5 ± 3 µM

n_{Hill}= 1.6 ± 0.2

The Trp445Tyr mutation completely impairs FAAH cooperativity



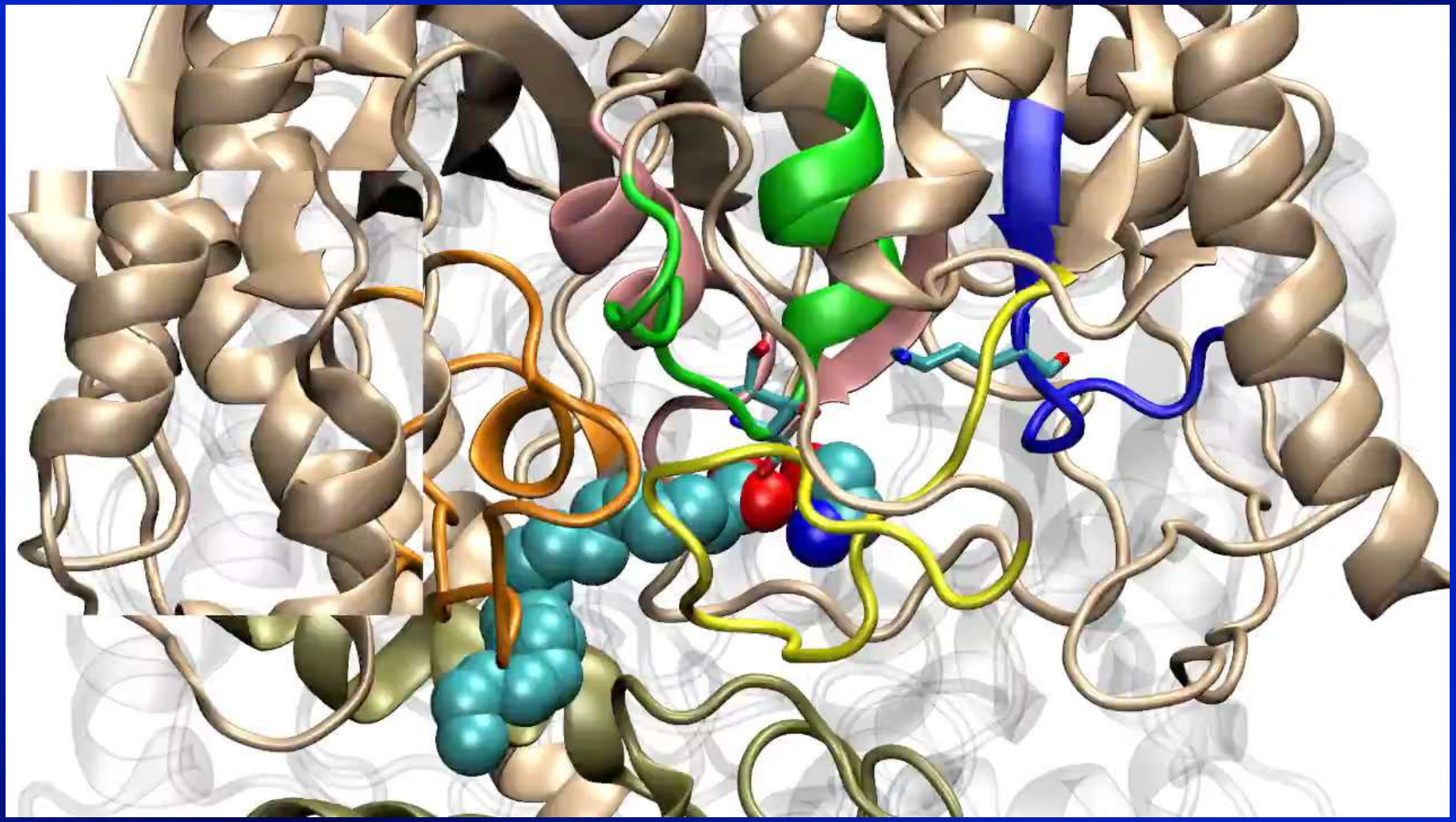


FAAH-W445Y K = 14.0 ± 3 µM

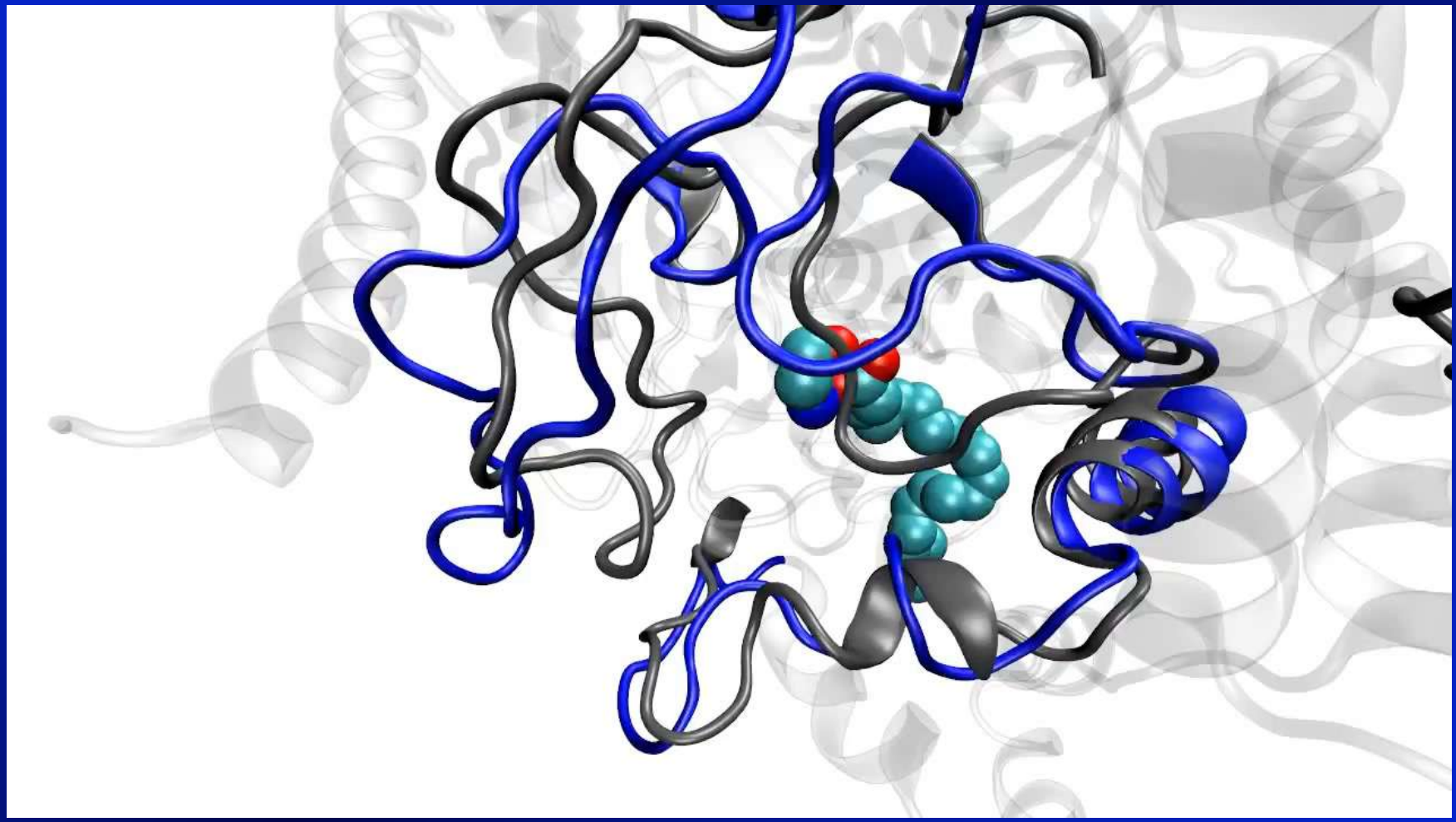
n_{Hill}= 0.9 ± 0.2*

***p < 0.01 *versus* FAAH**

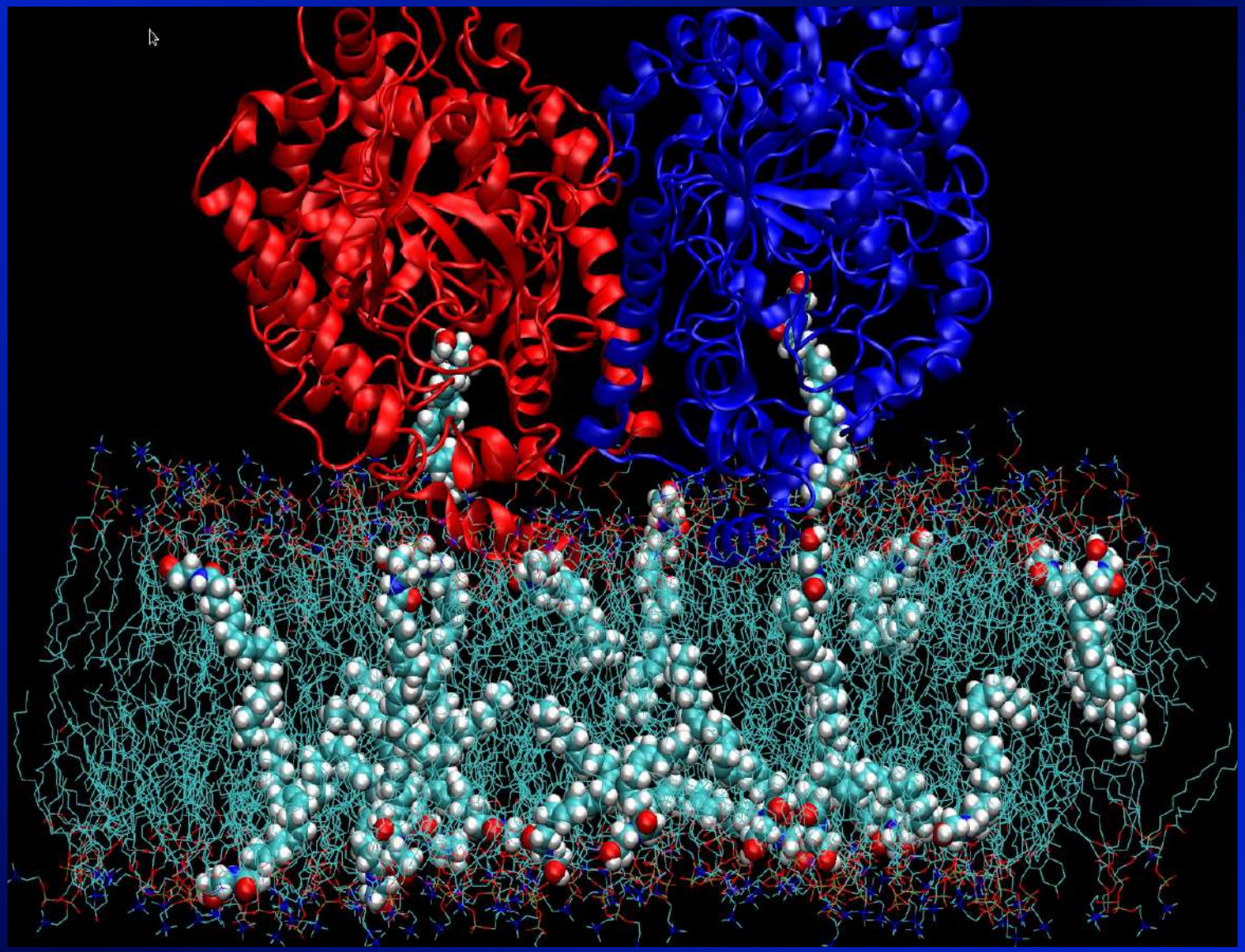
MD simulation of the AEA hydrolysis by FAAH



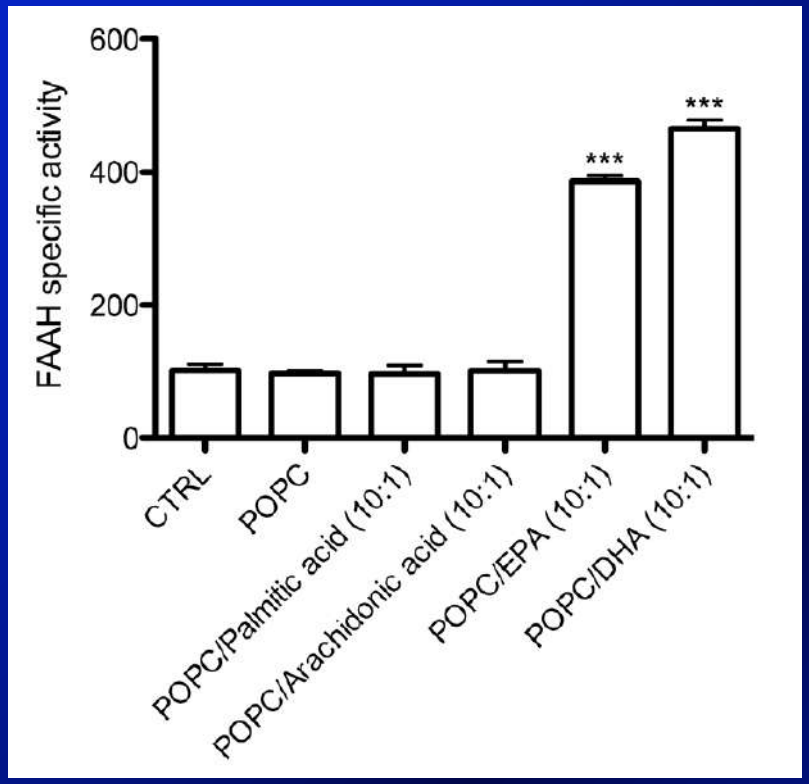
MD simulation suggests the involvement of a conformational change of a specific loop allowing the exit of ethanolamine

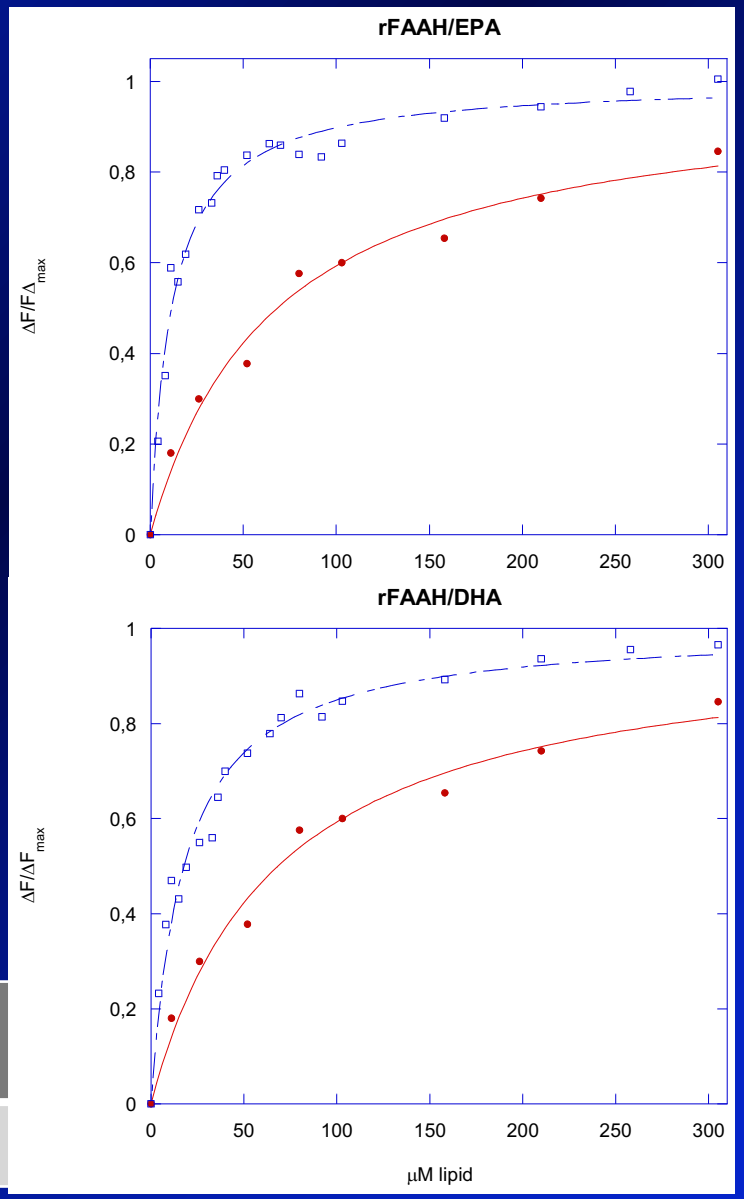


MD simulations confirm the cooperative behaviour of FAAH: the AEA binding to one subunit facilitates the access of another molecule of substrate into the other subunit



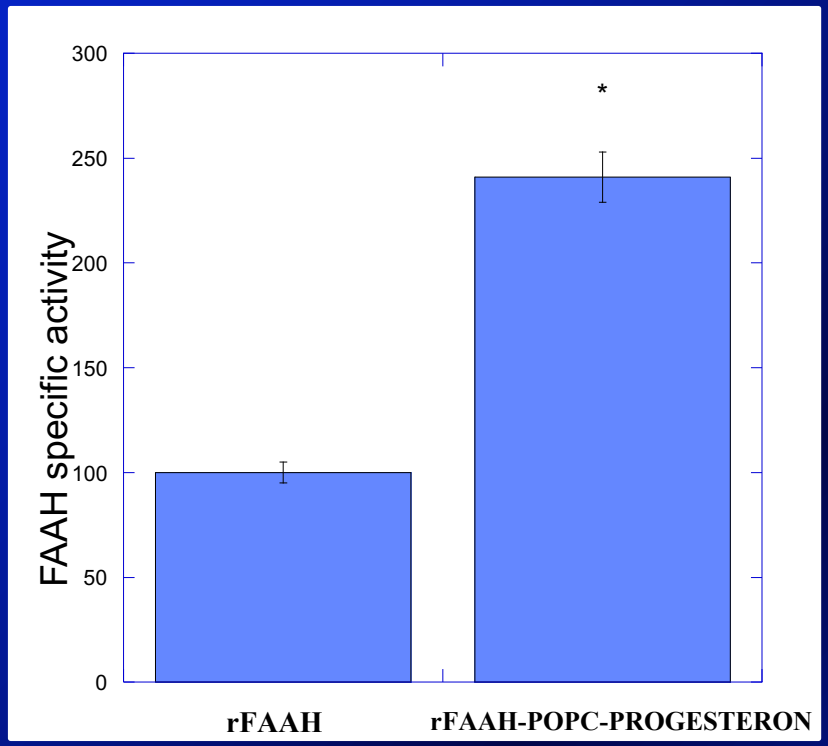
n-3 fatty acids behave as heterotropic effectors of FAAH increasing both the catalytic activity and membrane binding affinity

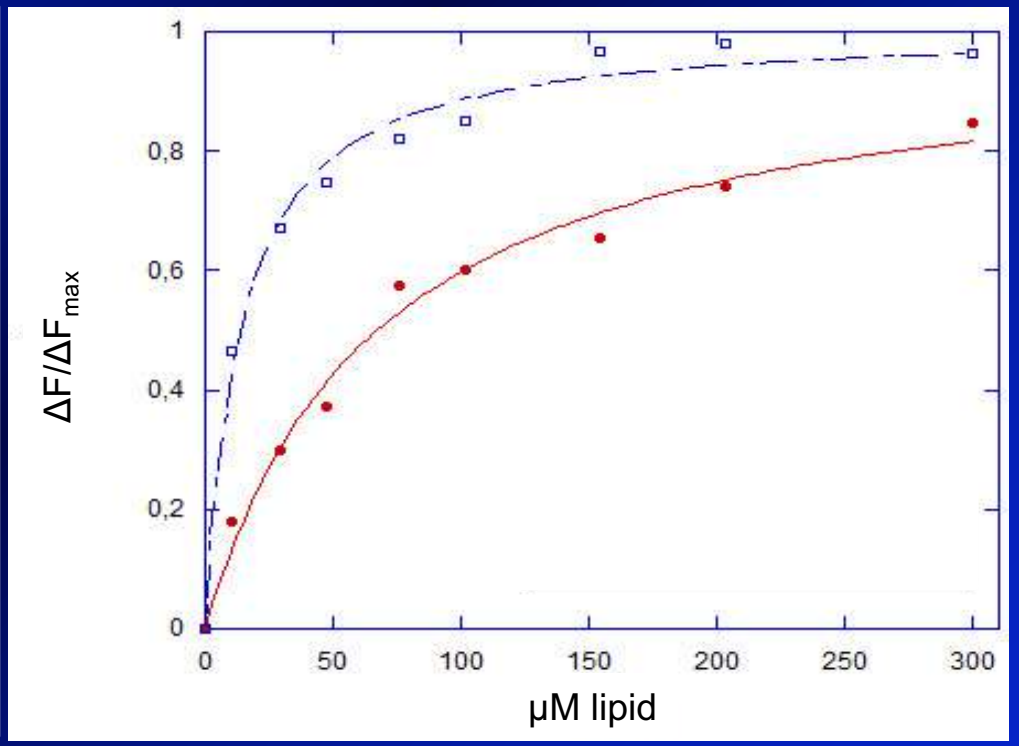




	rFAAH-POPC	rFAAH-EPA	rFAAH-DHA
L½	67 ± 10	11 ± 2	18 ± 2

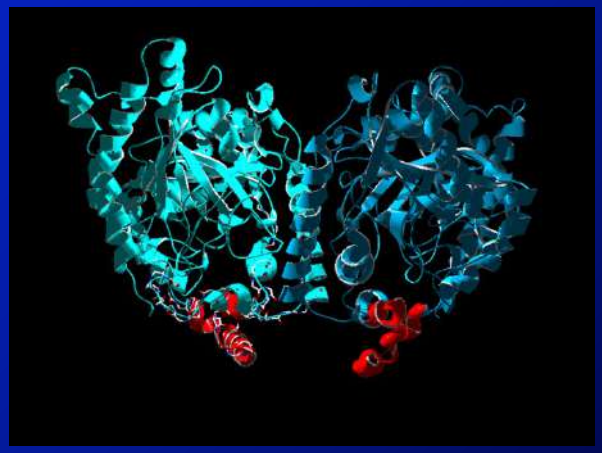
Progesteron is also able to activate FAAH and to increase its membrane binding affinity to membrane



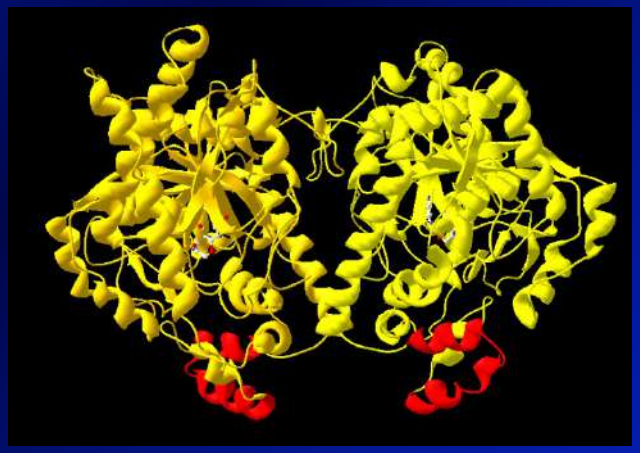


FAAH behaves as an allosteric enzyme

T-STATE



R-STATE



- compact form
- lower catalytic activity
- lower membrane binding affinity
- induced by inhibitors

- relaxed form
- higher catalytic activity
- higher membrane binding affinity
- induced by heterotropic effectors

Trp445Tyr mutation impairs the inter-subunit functional interaction leading to a non allosteric FAAH

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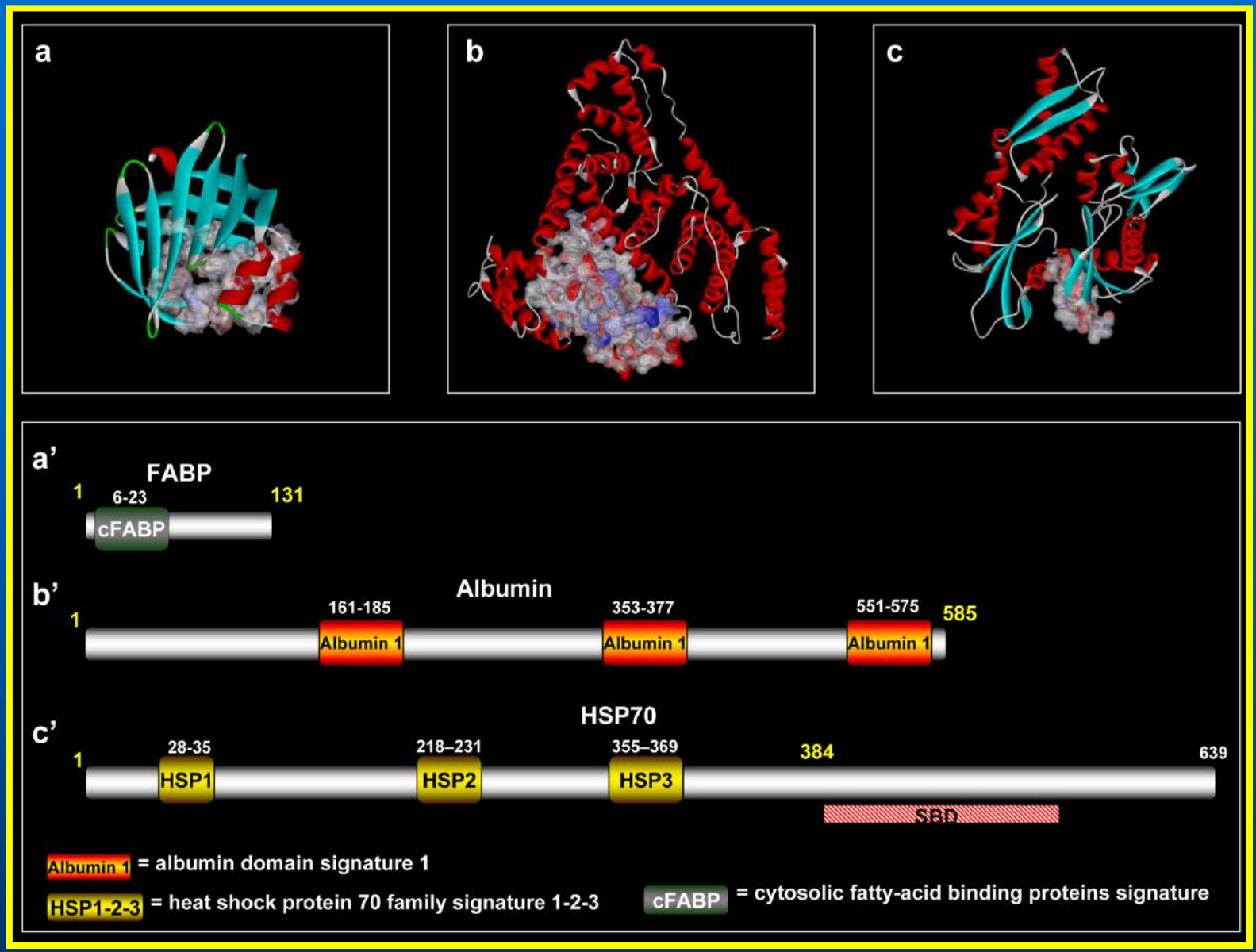
Corrected: Author Correction

OPEN

The endocannabinoid hydrolase FAAH is an allosteric enzyme

Enrico Dainese[1,7*], Sergio Oddi[2,3,7], Monica Simonetti[1], Annalaura Sabatucci[1], Clotilde B. Angelucci[3], Alice Ballone[4], Beatrice Dufrusine[1], Filomena Fezza[5], Gianni De Fabritiis[4] & Mauro Maccarrone[2,6*]

Fatty acid binding proteins studied as drug targets



Maccarrone, Dainese & Oddi, 2011

CONCLUSIONS 1

CRAC seems to be one of the structural determinants responsible for the interaction of CB1 receptor with membrane cholesterol, and for the effect of this lipid on receptor signalling;

Palmitoylation of cysteine 415 may be used by cells to direct CB1 targeting to cholesterol-rich subdomains of the plasma membrane, thus influencing, directly or indirectly, its interaction with some G proteins;

In the presence of AEA and cholesterol embedded within the membrane FAAH shows an increased enzymatic activity probably due to a specific effect of cholesterol, that is able to open the access to the active site of the enzyme.

Future directions

These data imply the development of new high throughput screening assays of FAAH taking into account the role of specific membrane lipids in testing new drugs.

Open avenue to develop new classes of inhibitors by adding to their molecules specific lipids able to increase their diffusion within the membrane and to increase their accessibility to the FAAH enzyme.

The proposed allosteric control of FAAH implies the possibility to discover and design new nonsubstrate molecules able to modulate FAAH activity and to potentiate or attenuate the efficacy of FAAH inhibitors;

Understanding how lipids can modulate or even lead signal transduction to different targets and outcomes will possibly give rise to a brand new area of scientific inquiry with huge impact on human health and disease, well-beyond endocannabinoids themselves.

Take home messages

Membrane lipids composition of cell membranes affect the subcellular localization of membrane proteins;

CRAC seems to be one of the structural determinants responsible for the interaction of GPCRs with membrane cholesterol, and for the effect of this lipid on receptor signalling;

Palmitoylation of GPCRs, and in particular cysteine 415 in CB1 may be used by cells to direct CB1 targeting to cholesterol-rich subdomains of the plasma membrane, thus influencing, directly or indirectly, its interaction with some G proteins;

In the presence of AEA and cholesterol embedded within the membrane FAAH shows an increased enzymatic activity probably due to a specific effect of cholesterol, that is able to open the access to the active site of the enzyme.

Understanding how lipids can modulate ECS system is giving rise to innovative lead compound with therapeutic potential and huge impact on human health and disease.