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The role of the lipid environment in the activity of G protein coupled receptors

Rim Baccouch[1], Estelle Rascol[1], Kaja Stoklosa[1], Isabel D. Alves[1*]

[1]CBMN UMR 5248 CNRS, U. of Bordeaux, B14 allée Geoffroy St. Hilaire, 33600 Pessac, France

G protein coupled receptors (GPCRs) are a class of membrane proteins that sense extracellular signals ranging from light to odorants and small molecules and activate intracellular signaling pathways that control important physiological responses. Being composed of 7 transmembrane helices linked by extracellular and intracellular loops, the great majority of the sequence of these receptors is embedded in the lipid membrane. Therefore, it is expected GPCR structure and function to be impacted by the surrounding lipid environment and the lipid membrane physico-chemical and mechanical properties. A large number of examples from the literature is provided to highlight the role of the lipid nature (lipid headgroup, membrane polyunsaturation and cholesterol) and membrane physical and mechanical properties (curvature elastic stress, membrane thickness and hydrophobic mismatch, fluidity) in the activity of different GPCRs. In addition, lipids are important co-factors being identified in very specific locations in several GPCR structures. GPCRs and G proteins can also be lipid post-translationally modified and such events can significantly impact membrane binding, trafficking and signaling. These aspects are all treated in this review. Understanding how the lipid can modulate GPCR activity is important not only from a fundamental point of view but also due to the fact that certain pathologies, where GPCRs are central targets, have been associated with important lipid imbalance. Establishing a link between the lipid pathological imbalance and the receptor functioning in such environment is thus essential as it can open avenues to potentially innovative therapeutic strategies.

Keywords: G protein coupled receptors; lipids; receptor activation and signaling; lipid membrane physico-chemical properties;

1. Introduction

G protein coupled receptors (GPCRs) are the largest and most diverse family of membrane receptors in the human genome, counting with approximately 800 individual members (1). GPCRs play key roles at many levels of intercellular communication, regulating biological processes from vision, cell growth to synaptic signal transmission. Due to their physiological importance and their good accessibility as cell surface receptors, GPCRs are one of the most important drug targets today and still have a great pharmacological potential (2). Over the last decades, our understanding on how GPCRs function has greatly advanced, mostly triggered by the raise in determination of over 600 GPCR structures by x-ray crystallography and cryo-EM (see, e.g., GPCRdb.org). Structural atomistic details in combination with important biophysical studies have greatly contributed to the understanding of their activation and signaling mechanisms.

GPCRs are integral membrane proteins with seven transmembrane α-helices connected by intracellular and extracellular loops. They associate with heterotrimeric G proteins on the cytosolic side of membranes. Upon receptor activation by light or ligands, GPCRs catalyze GDP/GTP exchange on the G protein alpha subunit, eliciting an intracellular signaling cascade resulting in events such as apoptosis or cell proliferation (3). On the other hand, upon receptor phosphorylation on their intracellular side, GPCRs also bind to β-arrestins leading to its desensitization and internalization but also triggering additional signaling cascades (4) (Fig. 1).

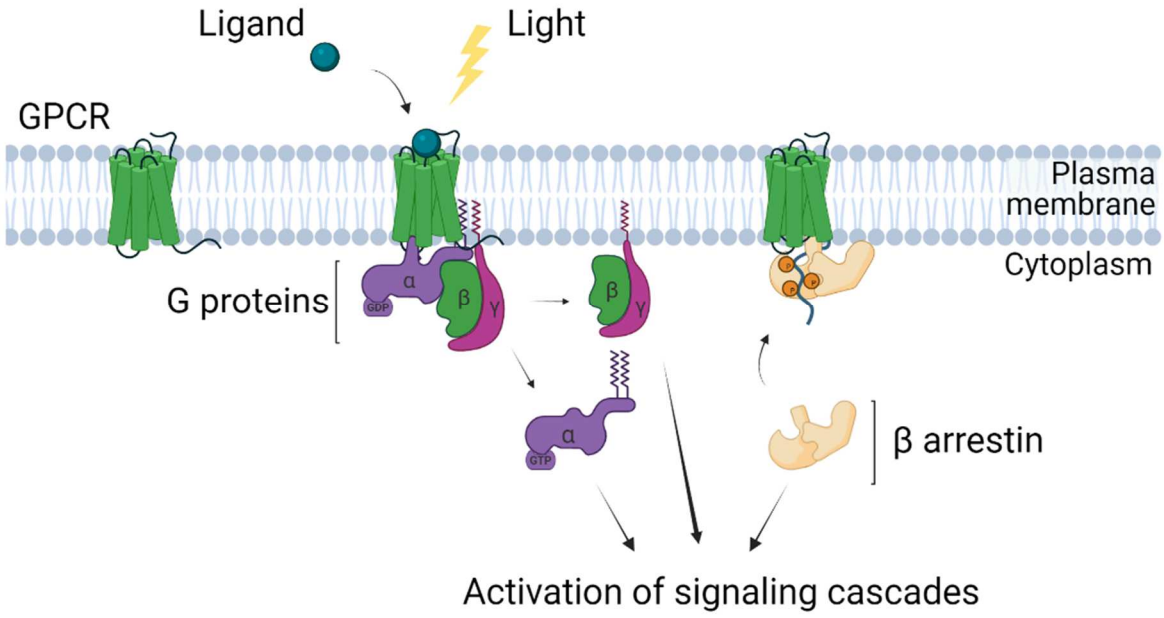


Figure 1. General representation of GPCR ligand or light-activation and main signaling cascades activated by G proteins and β-arrestin. Ligand or light present on the extracellular side interacts with the receptor, triggers its conformational change and the dissociation of the G protein (composed of the αβγ trimer) following GDP/GTP exchange at the α-subunit. Both α and βγ subunits will activate downstream signaling cascades. In parallel, the receptor gets phosphorylated on its intracellular side leading to β-arrestin recruitment that results in both receptor desensitization and the activation of diverse signaling cascades.

The concept of biased signaling that is related to the specificity in the signaling pathway taken upon activation by a specific ligand is extremely important in drug design (5). While originating from the ligand side via its different properties and from the receptor due to potential mutations or isoforms, lipids can provide an additional source by modulating receptor and effector properties and their potential encounters. The current model proposes that receptors explore complex conformational landscapes populated with multiple states of distinct functional properties, and that the relative distribution of these states is modulated by ligands, signaling proteins, and the environment, which all together dictate the signaling output (4). In terms of the environment, the cell membrane does not only provide the physiological environment necessary for the stability of the native fold of membrane proteins but it also modulates their function through an impact on their conformational dynamics and signaling events. GPCR dependence on lipid composition is important because the composition of the plasma membrane varies with age, diet, and certain pathologies, making the modulation of GPCR function through lipids a possible mode of disease etiology (6). Variation in plasma membrane composition across tissues may lead widely distributed GPCRs to behave differently in different tissue types. Therefore, to draw a complete picture of GPCR activation and signaling events it is crucial to include the lipid component.
In this review we tackle the question concerning the role of the cell membrane lipid composition for the activity of GPCRs by addressing both the lipid chemical nature (headgroup type, level of poly-unsaturation, cholesterol) as well as the lipid impact on the membrane physical and mechanical properties (membrane curvature elastic stress, membrane thickness and hydrophobic mismatch, membrane fluidity). On the other hand, we provide an overview about lipids as cofactors in GPCR functioning that establish specific and direct interactions with receptors and at the interface between receptors and G proteins or β-arrestins. We have made efforts in this review to separately present direct lipid impact versus the impact of global/bulk membrane properties on receptor activation and signaling. The two concepts however are very much interconnected and thus are at certain points merged. Finally, the subject of receptor and effector lipidation, more commonly known as post-translational modifications, is presented. The different lipid molecules mentioned in the different reported studies are presented in Figure 2, 3 and Table 1.

2. Lipid impact in GPCR activity – the role of the lipid nature

Regarding the role of the lipid nature, herein we focus on the headgroup type of phospholipids, the level of polyunsaturation as well as on cholesterol. Concerning the headgroup type this factor can directly impact the type of molecular interactions that can be established directly between the receptor or effectors with lipids such as electrostatic interactions and H-bonding. In addition, as a consequence of the size of the headgroup and the relation between its size and shape of the headgroup and the fatty acid chain region, the headgroup type is now known to modulate the physical properties of the membrane (thickness, curvature, membrane packing), which will later be treated in section 3. When considering the hydrophobic core of the lipid membrane, two important aspects are the level of lipid polyunsaturation and the presence of cholesterol, these can highly impact the ordering, packing, fluidity and lateral organization of membranes (e.g. formation of domains). The property of membrane fluidity will be further discussed in detail in section 3.

2.1. The importance of the lipid headgroup

The nature of the phospholipid headgroup constitutes an important parameter regarding lipid modulation of GPCR activation and signaling because of their impact in certain membrane properties: the overall membrane charge; the formation capacity of H-bonds formation, electrostatic bonds or other types of specific interactions between the protein and the lipids; membrane mechanical and physical properties including membrane intrinsic curvature, surface tension and others.

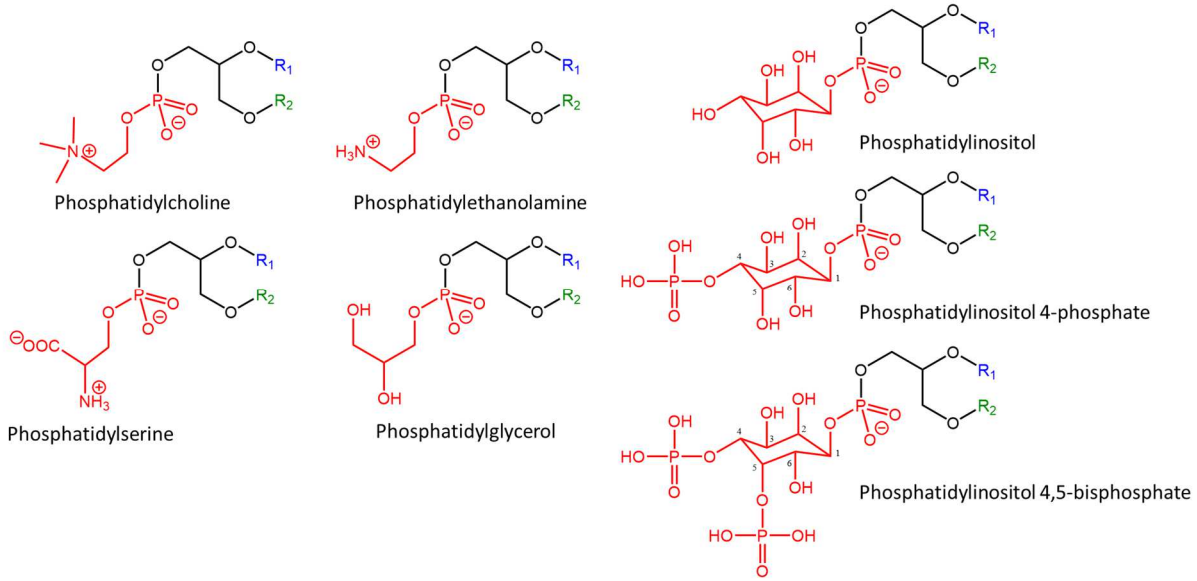


Figure 2. Structures of main lipid headgroups. R1 and R2 represent variable fatty acyl chains. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are two zwitterionic headgroups, with a positive charge on a tertiary amine for PC and a primary amine for PE. The other headgroups are anionic.

Govaerts and collaborators have characterized the effect of biological relevant phospholipids on the β2-adrenergic receptor (β2AR), namely they investigated how the nature of the lipid headgroup impacts the structure and activity of β2AR using biochemical and biophysical approaches (7). They have shown that the phospholipids modulate ligand binding to the receptor and agonist-induced conformational states with the anionic lipids Dioleoyl Phosphatidylglycerol (DOPG), Dioleoyl Phosphatidylserine (DOPS) and Dioleoyl Phosphatidylinositol (DOPI) promoting receptor activation, whereas Dioleoyl Phosphatidyethanolamine (DOPE) favoring the inactive state (Table 1). Interestingly, the effect of lipids on the receptor structure was observed in the absence of ligand demonstrating that it is not due to interactions between the ligands and the lipids. Moreover, negatively charged lipids favor receptor activation in the absence of a bilayer (since the researchers have performed experiments in a detergent-solubilized system enriched with lipids but in absence of liposomes, high-density lipoparticles were employed) and in a dose-dependent manner thus acting as true positive allosteric modulators. According to the authors, it is unlikely that the differences observed between the lipid species are due to differences in the affinity for the receptor as they are observed in nanodiscs, where the very high local concentration of lipids ensures permanent interaction. Their studies indicate that lipids investigated differentially affect the conformational kinetics of the receptor. Indeed, they have observed that the presence of DOPG maintains the cytoplasmic side of the β2AR in an open

conformation for a longer (average) time than the binding of other lipids, leading to a larger apparent stimulation by the nanobody Nb80, with almost complete receptor activation after 10 min. Thus the binding of negatively charged lipids favors transmembrane (TM) 6, opening through specific binding of the headgroup to the protein, most likely on the intracellular side of the receptor. The authors suggest that the strong effect of negatively charged phospholipids indicate ionic interactions between receptor side chains and the lipid headgroup. The stronger effect observed with DOPG as compared to DOPS or DOPI indicates a clear level of specificity in such interactions.

Few years later, the Giraldo team has investigated by long-timescale (total of 24 μs) molecular dynamics simulations (MDS), the allosteric modulation and conformational changes of the β2AR that occur as a result of interactions with anionic (DOPG) and zwitterionic phospholipids dioleoyl phosphatidylcoline (DOPC) and DOPE. Their studies show that net negatively charged lipids stabilize an active-like state of the receptor that is able to bind to G proteins, while net-neutral zwitterionic lipids inactivate the receptor, generating a fully inactive or intermediate states with kinetics depending on the lipid headgroup charge distribution (8). Their studies revealed that such lipid modulation of the receptor activity is governed by the chemistry of protein-interacting lipid headgroups, as modeling studies are performed in absence of ligand and G proteins. The study shows that the intracellular loop 3 (ICL3) establish important electrostatic interactions with the lipid headgroups and influences the conformation of TM6. Moreover, their studies suggest that the nature of the phospholipid headgroup may contribute to controling the receptor's basal activity via receptor deactivation. Indeed, receptor interactions with anionic lipids may provide the energy necessary for the receptor to go from the inactive to the active state when in absence of agonist. Such modeling studies together with experimental ones by Govaerts as presented above, provide important insight into the role of anionic lipids in β2AR activation and signaling.

Besides having an impact on the equilibrium between the inactive and active states of the receptor and its conformational dynamics by the nature of the lipid headgroup, this lipid property can also modulate the recruitment and interactions with effectors as G proteins or β-arrestins. A study by the Grisshammer group on the impact of the anionic lipid POPG on both the ligand activation and G protein recruitment of the Neurotensin 1 receptor (NTS1R) reported that an increased negative charge density dramatically affects the NTS1R-catalyzed GDP/GTPγS nucleotide exchange rates for Gαq, whereas surrounding lipid type shows little influence on agonist binding (9). In a cryo-EM structural study of the serotonin receptor (5-HT_{1A}) bound to the G protein either in absence or presence of ligand, the presence of phosphatidylinositol (PI) at the receptor/G protein interface was revealed (10). The phosphatidylinositol 4-phosphate (PI4P) headgroup is inserted into a cavity formed between the TM3, TM6 and TM7 of the receptor and the helix 5 of the G protein Gαi1. Analysis of GTP hydrolysis activity of the G protein in absence and presence of PI4P, indicated that this lipid enhanced GTP hydrolysis. Such impact in hydrolysis was markedly less in presence of PI or phosphatidylinositol bisphosphate (PIP_2) and almost absent when other anionic lipids as PG and PS were used, demonstrating a certain level of lipid specificity in such impact. One of the two acyl chains of PI4P was found to be sandwiched between two cholesterol molecules, suggesting a role of cholesterol in stabilizing PI4P binding. This study joins a previous study by mass spectrometry that identified key residues on cognate Gα subunits that mediate simultaneous PIP_2 bridging interactions between basic residues on class A receptors (11).

In a recent study combining experimental (FRET switch sensor assays) and computational methods (coarse-grained molecular dynamic simulations; CG-MDS) Baneres and coworkers,

have reported that ghrelin receptor conformational equilibrium was shown to be highly impacted by the presence of PIP_2 in the lipid membrane, that shifted the equilibrium away from the inactive sate, favoring basal and agonist-induced G protein activation (12). By an experimental setup combining CG-MDS and site-directed mutagenesis, their study identified a preferential binding of PIP_2 to specific intracellular sites in the receptor active state.
Following the determination of the structure of certain GPCRs in complex with β-arrestin, it came to light that, as in the case of G-proteins, PIP_2 is a key lipid for mediating β-arrestin binding to GPCRs. Indeed, the cryoEM structure of the NTS1R and β-arrestin complex revealed a bridge between the membrane side of NTS1R in TM1 and TM4 and the C-lobe of arrestin (13). In a recent report, Kobilka and collaborators have shown that GPCRs which only transiently engage β-arrestin require phosphoinositide binding for β-arrestin recruitment. By using NTSR1 as a model system they found that specific phosphorylation sites are linked to this phosphoinositide binding-dependence for arrestin recruitment. Using *in vitro* biochemical, and biophysical assays, they demonstrated that phosphoinositide binding contributes to the stability of a GPCR-β-arrestin complex. This study also shows that phosphoinositides alone are able to promote a partially activated state of arrestin, thereby offering an explanation for how arrestin is able to persist at the plasma membrane once dissociated from a GPCR. Together, these results offer an explanation for how receptors that transiently associate with β-arrestin are able to recruit (and dissociate) β-arrestin in a spatiotemporally resolved manner. Moreover, the results show that arrestin-strongly coupled receptors are able to maintain a stable association with arrestin in subcellular compartments yet regain the ability for further G protein engagement from subcellular structures C. A recent report, suggests phospoinositides to be key players in GPCR endocytosis where β-arrestin-dependent PIP_2 synthesis boosts receptor endocytosis (15).

2.2. The role of the lipid polyunsaturation

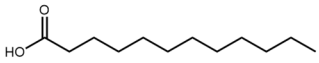
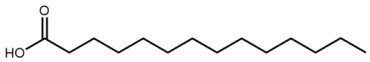
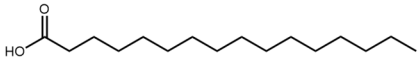
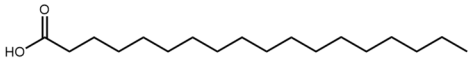
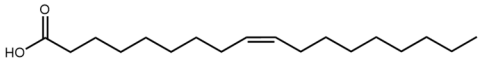
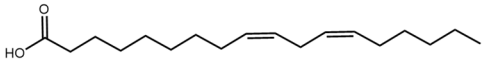
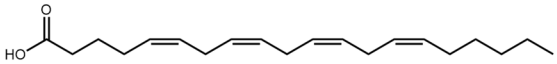
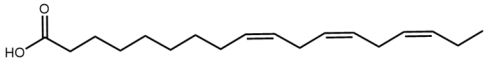
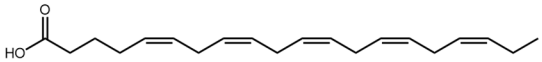
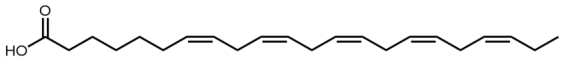
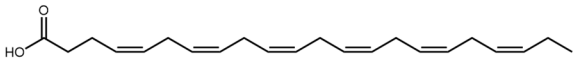
Shorthands	Structure	Common name
C12:0		Lauric acid
C14:0		Myristic acid
C16:0		Palmitic acid
C18:0		Stearic acid
C18:1, n-9		Oleic acid
C18:2, n-6		Linoleic acid
C20:4, n-6		Arachidonic acid
C18:3, n-3		α-Linoleic acid
C20:5, n-3		Eicosapentaenoic acid
C22:5, n-3		Docosapentaenoic acid
C22:6, n-3		Docosahexaenoic acid

Table 1. List of the main fatty acyl chains that can be esterified in membrane phospholipids.

Poly-unsaturated fatty acids (PUFAs) are important components of cell membrane phospholipids that are particularly enriched in certain organs as the brain where many GPCRs are expressed. PUFA level alterations have been associated with several pathologies in which GPCRs are main targets of psychiatric disorders.
Indeed, PUFA levels are substantially decreased in patients with psychiatric disorders as schizophrenia relative to healthy patients (16). PUFAs are classified into two major classes, the n-6 PUFAs and the n-3 PUFAs depending on the position of the first double bond from the methyl terminal end. In fact, n-6 PUFAs have the first double bond at the sixth carbon whereas n-3 PUFAs at the third (17, 18). These classes are derived biosynthetically from two precursors, linoleate (18:2:*n*-6) and α-linolenate (18:3*n*-3) fatty acids, that are not synthesized *de novo* by mammals and have to be provided by the diet (19). Among the different PUFAs, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are the most abundant n-3 fatty acid in human brains (20, 21).

Until recently, unsaturated acyl chains are widely described as important modulators of the spatial regulation of membrane physical properties including fluidity, flexibility and curvature (22–25). A range of evidence from computational and experimental studies indicates that the type of lipid acyl chain directly influences the activity of GPCRs. Pioneer work has been performed on class A GPCR prototype, rhodopsin. While the observations made in rhodopsin

cannot be fully extrapolated to other GPCRs, as rhodopsin is a special and atypical GPCR – the protein/lipid ratio is extremely high, the lipid environment is particularly rich in specific lipids as polyunsaturated ones – this work was crucial and has set up an important basis for future research in GPCR/lipid interactions. Absorption spectroscopy (UV–visible) studies have shown that DHA acyl chains facilitate the transition from the light-activated meta-rhodopsin I form to the G protein-binding intermediate meta-rhodopsin II leading to an efficient and rapid propagation of G protein–coupled signaling (26–29). This finding is consistent with previous studies demonstrating that highest levels of meta-rhodopsin II form occurred in DHA-containing bilayers (30, 31).

Moreover, MDS have revealed that tight DHA-Rhodopsin packing occurs in a small number of regions in well characterized ways, whereas saturated chains packing is relatively nonspecific, with a much larger number of weakly populated associations (32). Accordingly, nuclear magnetic resonance (NMR) experiments on rhodopsin reconstituted into membranes with variable lipid content showed that DHA chains compete with other lipids for contact with a small number of weakly specific sites on rhodopsin (33). Supporting the crucial role of DHA-containing phospholipids for optimal rhodopsin activity, similar studies have showed that DHA deficiency resulted in slower kinetics accompanied by reduced levels of receptor activation and rhodopsin/G protein coupling (34, 35).

Pioneer work on rhodopsin and PUFAs was followed by reports on other GPCRs. *In vivo* studies on the cannabinoid receptors have found that presynaptic cannabinoid CB1 receptors (CB_1Rs), normally responding to endocannabinoids, were uncoupled from $G_{i/o}$ proteins in n-3 PUFAs deficient mice (36). This uncoupling, in a PUFA altered environment, directly impairs CB_1Rs functionality and confirm that long chain n-3 PUFA, especially the DHA, are indispensable to a normal activity of the endocannabinoid system (37).

Receptor dimerization and oligomerization are also thought to be tuned by direct lipid binding or by lipid composition-dependent bilayer properties. Recently, Guixà-González *et al* (38) have combined Martini coarse-grained molecular dynamics (CGMD) simulations and bioluminescence resonance energy transfer (BRET) experiments in order to investigate the effect of membrane DHA on adenosine A2A (A2AR) and dopamine D2 (D2R) receptors oligomeric state stability, highly relevant for brain functioning. By using low- and high-DHA- content membranes models, they demonstrated that A2AR and D2R oligomerization had a clear increase in high (~20%) compared to low DHA content membranes. This work led the author to propose that DHA effect on GPCRs assembly is purely kinetic resulting from a combination of an increase of the receptor lateral diffusion and the rate of spontaneous receptor–receptor interactions due to their partition into DHA-enriched domains. This was supported by similar studies, combining all atom and CGMD simulations with free energy calculations, indicating that DHA promotes A2AR partitioning into the ordered (Lo) phase while in the absence of DHA the receptor partitioning into the disordered (Ld) phase is favored. According to the authors, the reduction of A2AR partitioning to its native ordered environment upon PUFA depletion might impact the activity of this GPCR in various neurodegenerative diseases, where the membrane PUFAs content in the brain are reduced compared to healthy individuals (39). In this context, a recent NMR study of A2A receptor reconstituted in nanodiscs, showed that DHA chains enhanced the activation of the G protein by A2AR. Interestingly, these studies revealed that DHA chains accelerate A2AR conformational exchange and shift the equilibrium towards a conformation displaying a large clockwise rotation of TM6, preferable for G protein binding (40).

All together these studies point to the importance of PUFA, especially DHA acyl chains, in promoting optimal function in G protein-coupled signaling. According to these different studies, bilayers rich in DHA may impact protein function both by a change of general membrane properties as well as by specific interactions with particular regions of the GPCR.

2.3. The role of cholesterol and lipid domains

Cholesterol is a small molecule containing four rings and a short aliphatic chain, with a total of 27 carbons. The second ring presents an unsaturation between carbons 5 and 6, while the first ring exhibits a hydroxyl group in position 3. This molecule is amphiphilic, the rigid planar four-ring backbone is composed of a hydrophobic part, which promotes its insertion in the lipid bilayer forming cell membranes. The hydroxyl group orients cholesterol molecules in the membrane, presenting this hydrophilic group close to the hydrophilic head groups of the phospholipids. Plasma membranes concentrate 80-90% of the total cell cholesterol content, that represents 20-25% of total lipids in the plasma membrane (41), and 25% of the total body content is found in the central nervous system (42). In the membrane, cholesterol interacts preferentially with the saturated acyl chain of phospholipids by its methyl groups corresponding to C18, C19 and the isooctyl chain linked to C17 (43).

The high concentration of cholesterol in plasma membrane and its unique chemical properties lead to a major impact on membrane proteins, including GPCRs (44). The influence of cholesterol on GPCR properties, in particular, on ligand binding, signalling and recycling, may be the result of specific and/or unspecific effects (see review (45)). The unspecific effects correspond to the influence of cholesterol on the physico-chemical properties of the membrane, also called the indirect allosteric modulation. The specific effects on the other hand relate to specific interactions between cholesterol molecules and some residues of the GPCR, also called direct allosteric modulation (review (46)). The distinction of these two different effects is not straightforward, as evidenced from the literature, thus while we have tried to present the two effects in a separate manner in this section and in the rest of this review, it was not always fully accomplished. Another proposed mechanism is the possible location of cholesterol at nonannular sites (45, 47), corresponding to sites that are not at the immediate annulus surrounding the cross-sectional area of the GPCR. Finally, an orthosteric modulation mechanism has also been suggested (48). Specific binding sites can be nonannular ones, with high cholesterol affinity for the receptor at specific positions, that may constitute a favourable environment for lower-affinity and annular cholesterol molecules (49).

The non-superimposable mirror image of cholesterol, named the non-natural enantiomer ent-cholesterol, allows the same physico-chemical properties than cholesterol to the membrane (50). On the other hand, the diastereoisomer, 3-epicholesterol, which differs from natural cholesterol by the orientation of hydroxyl group in position 3, induces larger differences in the physico-chemical properties of the membrane (51–53) (Figure 3).Replacement of natural cholesterol by one of these stereoisomers in the membrane allow to distinguish specific and unspecific effects of cholesterol on GPCRs. Serotonin 1A receptor ($5HT_{1A}$) ligand binding affinity is affected by replacement of natural cholesterol by épi-cholesterol but not by ent-cholesterol, indicating that changes in membrane properties affect ligand binding to this GPCR(53). Molecular dynamic simulations with serotonin 2A and 2B receptor subtypes ($5HT_{2A}$, $5HT_{2B}$) indicated similar sterol density for cholesterol and the two stereoisomers ent- and epi- cholesterol, close to helix 4. One sterol molecule is also able to enter deep between the helices 2, 3 and 4 in the extracellular leaflet. However, the simulations have shown similar helices motion than for ent-cholesterol than natural cholesterol, but while a greater difference was seen for epi-cholesterol (51).

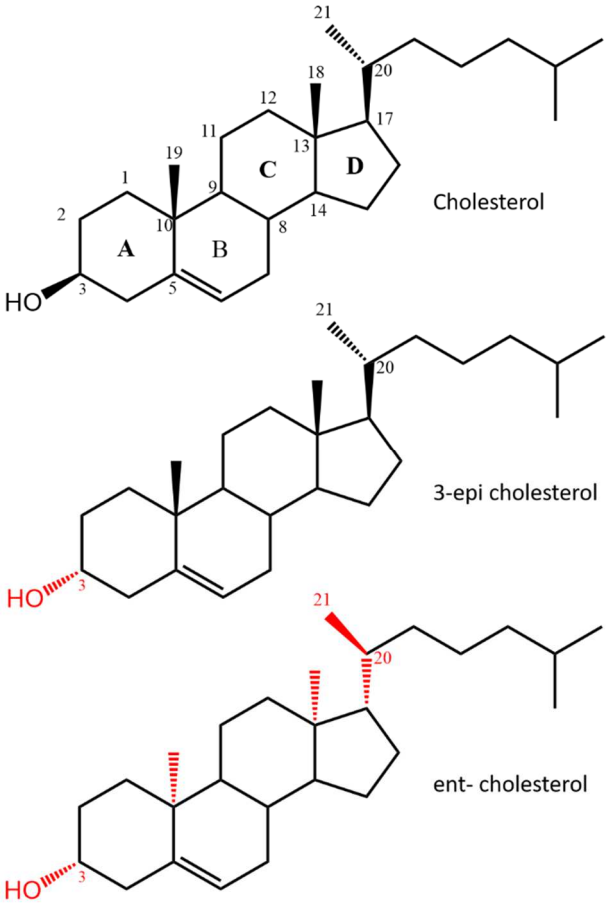


Figure 3. Structures of cholesterol and synthetic cholesterol stereoisomers 3-epi cholesterol and ent-cholesterol. Cholesterol is composed of 4 rings A, B, C, D, substituted by one hydroxyl group in position 3, 2 methyl groups in position 10 (C18) and 13 (C19), and a carbon chain, resulting in 8 asymmetric carbons. Only 5 are represented here, the three other stereo centers being in position 8, 9 and 14. In 3-epicholesterol, only the hydroxyl group in position 3 presents a different configuration. Ent-cholesterol corresponds to the non-superimposable mirror image of the cholesterol, with inversion in the configuration of all the 8 stereocenters.

Ligand binding affinity is one of the easiest parameters to experimentally investigate the allosteric or orthosteric modulation of GPCRs by lipids. Cholesterol impacts ligand binding affinity for several GPCRs (54), but the direction of modulation (increasing or decreasing) is controversial. In a C6 cell line, cholesterol depletion induced by methyl-β-cyclodextrin (MβCD) incubation enhanced the specific binding of A2AR selective antagonist (48)). To avoid any controversial role of cholesterol depletion in mammalian cell line, as it can be the case using MβCD or statin incubations, ligand binding affinity has also been investigated in model membranes. In a report by our team , ligand binding affinity has been investigated after solubilisation, purification and reconstitution of the chemokine receptor CCR5 expressed in *Pichia pastoris* membranes or after reconstitution following cell-free expression (55). For both of these models, CCR5 affinity for maraviroc, a selective antagonist, was drastically reduced in presence of cholesterol. In contrast, binding affinity measured by fluorescence polarization assays, of the chemokine receptor CCR3 for its endogenous and agonist ligand CCL1 increased in a dose-dependent manner with cholesterol concentration in both

styrene/maleic acid lipid particles and proteoliposomes (56). In bovine hippocampal membranes, cholesterol depletion obtained using various MβCD concentrations, reduced the affinity of a selective agonist to the $5HT_{1A}R$(42). Several studies on oxytocin receptor (OTR) have also shown a positive correlation, in cell or reconstituted systems, of cholesterol and agonist binding affinity(54, 57, 58). General influence of cholesterol on ligand binding affinity of GPCRs cannot be drawn since positive, negative and no modulation have been reported. For example, CB1R, cholecystokinin 2 (CCK2), $serotonin_7$ ($5\text{-}HT_7$), or $tachykinin_1$ (NK_1) ligand binding affinity is not influenced by cholesterol levels (summarized in (54)). On the other hand, galanin (GAL2), 5-HT1A, metabotropic glutamate mGlu1, δ-opioid or oxytocin receptors show a positive correlation between cholesterol levels and high ligand affinity.

In the plasma membrane, cholesterol is spontaneously associated to sphingolipids, forming lipid rafts and caveolae. These two types of cholesterol-rich domains have been largely investigated (59). Caveolae are invaginations at the cell surface, stabilized by structural proteins, called caveolins (60). Lipid rafts contain less cholesterol (relative to sphingolipids) than caveolae, and have a shorter lifespan. The composition of these 2 distinct microdomains is unique and influences the biological functions of these membrane structures. Cholesterol-rich domains allow particular physic-chemical properties: they rigidify the membrane in the liquid phase (61), and enhance the ordering of lipids, causing a tighter packing of lipids, associated to an increased thickness of the membrane (62), reducing the dipole potential of the membranes (63) (Figure 4c). The protein composition of cholesterol rich domains, lipid rafts or caveolae, may be driven by acylation of the protein, that contribute to the partitioning in cholesterol-rich liquid order domains while a caveolin-binding domain in the protein sequence may promote association with caveolae (64). Other structural components may contribute to cholesterol-rich domain partitioning of proteins, such as the presence of particular hydrophobic residues in the amino acid sequence of the proteins.

These mechanisms apply for GPCR partitioning in these domains as well as in membrane signalling effectors, which may participate in cholesterol's impact on GPCR signalling (64, 65). For example, of the nine isoforms of adenylyl cyclase, studies suggest that some of them preferentially localized to caveolae (AC5, AC6) while others, depending on the cell type, were not detected (AC2, AC4) in these lipid domains (66). Another important component of GPCR signalling are G proteins. Gαs and Gαi segregates in lipid rafts while Gαq prefers caveolae if present (67). This difference may be due to caveolin binding domain in the Gαq sequence (68). The role of cholesterol in GPCR signalling is tightly linked to the composition of signalling complexes that compose the membrane lipid domains, and affinity of GPCRs and effector proteins to the different domains, all of which can be modulated by the presence of ligands. MβCD-induced cholesterol depletion disrupts CCL4 mediated CCR5 signalling (69). Cholesterol depletion reduced intracellular calcium flux, and Gαi mediated inhibition of cAMP accumulation, after CCR5 stimulation by CCL4. The μ-opioid receptor (MOR) translocates to lipid rafts through its interaction with Gαi2 subunit while its location in non-raft domains is associated to β-arrestin 2 signalling (65). This receptor has been found in lipid raft domains in the absence of ligands. Morphine, the endogenous agonist, induces signalling through Gαi2 which inhibits adenylyl-cyclase, leading to MOR staying in lipid rafts domains. On the contrary, in presence of etorphine, a synthetic agonist, the receptor translocated to non-raft domains disrupting G protein signalling. In contrast, ERK-MAPK activation by β2AR agonist requires receptor translocation to non-raft lipid domains (70). The class 1

metabotropic glutamate receptor association to lipid rafts is promoted by agonists, and this effect is reduced in presence of a non-competitive antagonist (71). The signalling of A2AR, coupled to Gαs is reduced following MβCD-induced cholesterol depletion (72). Cholesterol depletion is associated with a decrease in basal cAMP accumulation, as well as agonist-mediated cAMP accumulation. Forskolin induced cAMP accumulation was not influenced by MβCD. Importantly, the authors didn't observe a difference in ligand binding affinity in their conditions, contrary to Guixa-Gonzalez 2017 (48). This indicates that cholesterol plays an important role in Gαs mediated cAMP accumulation, independently of ligand binding stimulation. As some GPCRs are enriched in cholesterol-rich domains, these signalling complexes may also promote interactions between GPCRs. Homo- or heteromerization, that also play a role in the signalling of some GPCRs, may be influenced by membrane cholesterol content (73).

GPCR's trafficking is also under the modulation of cholesterol membrane concentration. In the case of $5HT_{1A}R$, statin-induced cholesterol depletion switched the internalization mechanism following serotonin stimulation from clathrin- to caveolin-mediated endocytosis (74). On the other hand, the same group observed a different effect when cholesterol depletion was obtained by incubation with MβCD (75). In this case, cholesterol depletion was associated to a concentration dependent inhibition of serotonin-induced endocytosis of the receptor. For a same cholesterol depletion level induced by lovastatin or MβCD (~23%), there is a switch in endocytic pathway with lovastatin but not with MβCD. Such results are quite puzzling and raise questions about the outcome of the two procedures in terms of cholesterol depletion and potential additional cellular effects. While the use of MβCD has been reported by numerous studies, care about the concentrations used is advised as high doses have been associated with unwanted effects in cells (76). Indeed, it should be recalled that MβCD action for cholesterol capture is mechanic and aggressive to the cell membrane due to its action of punching holes in the membrane (77). Statins on the other hand, as they act directly in the cholesterol metabolism have a milder action mode. Both of them however have showed a re-routing of the receptor toward lysosomal degradation instead of endosomal recycling.

3. Lipid impact in GPCR activity – the role of the membrane physico-chemical and mechanical properties

Due to the fact that lipid molecules possess different chemical properties and structures, different morphologies leading to different packing and organization when inserted in lipid membranes, they therefore impact membrane physic-chemical and mechanical properties. Herein, we have focused on: 1) membrane curvature elastic stress, a property that is closely related to the shape and relative volumes of lipid headgroups and fatty acid chain region; 2) membrane thickness that is directly related to fatty acid chain length and other lipid properties that can result in hydrophobic mismatch between the lipid and the receptor; 3) membrane fluidity, it is a property related to the presence of lipid unsaturation in the fatty acid region and the presence of cholesterol in the membrane. Certain authors have investigated this membrane property by the use of external membrane fluidizing agents as described below.

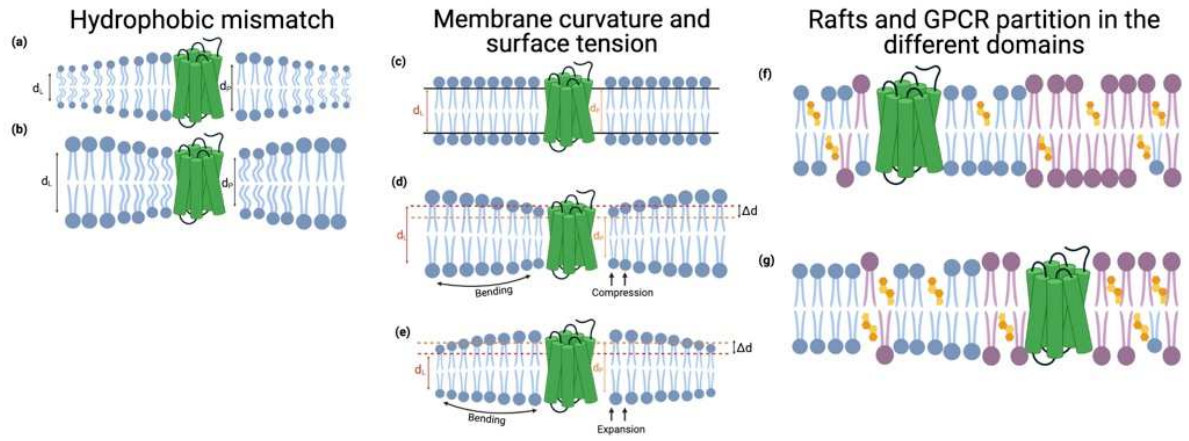


Figure 4. Illustration of some physical and mechanical membrane properties that are modulated by the lipid membrane composition: hydrophobic mismatch, membrane curvature and surface tension, lateral membrane homogeneity – domain (raft) formation. ***Hydrophobic mismatch:*** the lipid bilayer can distort around the GPCR to adapt the lipid membrane (d_L) thickness to that of the protein hydrophobic thickness (d_P) that can be larger (a) or smaller (b) than that of the bilayer. ***Membrane curvature and surface tension*** are involved in the mechanisms that GPCRs and the surrounding lipids adopt to reduce energetically unfavorable scenarios. Changes in GPCR conformation can result in the decrease (d) or increase (e) of their hydrophobic thickness (d_P) which becomes different than that of the lipid membrane (d_L) by the factor Δd. By taking advantage from the fact that different lipids possess different shapes, packing and ordering properties, lipids can reorganize around the receptor resulting in optimal hydrophobic coupling. Local compression or expansion forces of the bilayer adjacent to the protein as well as bending take place in such process. ***Lipid membrane domains or rafts*** occur as a result of the differences in the lipid properties of the membrane, namely the presence of cholesterol, those domains are transitory and kinetically unstable. Partition of GPCRs and effectors in these domains can add in an additional source of signaling complexity, as these domains can function as signaling platforms that control the encounters of the receptors and effectors implicated in the different signaling cascades (f, g). More details are found in the text.

3.1. Lipid membrane curvature elastic stress

Curvature is a ubiquitous feature of biological membranes. It ranges in its extent from slight bending as observed in the plasma membrane to the strongly curved membranes of small cellular vesicles. In cellular membranes the curvature is often determined by specific proteins that are associated with the membrane as scaffold proteins such as clathrin, membrane-binding modules such as BAR domains (highly conserved protein dimerization domains that occur in many proteins involved in the cell's membrane dynamics) and other cytoskeletal components, which can actively bend regions of lipid membranes (78). Integral membrane proteins, in contrast, can influence membranes from their location within the bilayer as every membrane protein energetically favors a certain membrane curvature for its complementarity to its overall molecular shape. Membrane curvature and surface tension are involved in the mechanisms that GPCRs and the surrounding lipids adopt to reduce energetically unfavorable

scenarios. Changes in GPCR conformation can result in a decrease (Fig. 4b) or an increase (Fig. 4a) in their hydrophobic thickness (d_P) which becomes different than that of the lipid membrane (d_L) by the factor Δd (Figure 4c, d, e). By taking advantage from the fact that different lipids possess different shapes, packing and ordering properties, lipids can reorganize around the receptor resulting in optimal hydrophobic coupling. Local compression or expansion forces of the bilayer adjacent to the protein as well as bending take place in such process. Membrane curvature is strongly dependent on the lipid composition of the respective bilayer which intrinsically can possess cylindrical, conical and inverted conical shapes depending on the dimensions of their hydrophobic acyl chains relative to the size of their respective head group. Lipids with small polar headgroups such as PE and polyunsaturated hydrocarbon chains including the six-fold unsaturated DHA, have high tendency to adopt a negative membrane curvature (H_{II} phase) (79–81). Phospholipids such as PC (if not possessing PUFAs chains) on the other hand adopt a more conical shape and contribute to a zero-curvature membrane. For a review on the forces governing the membrane spontaneous curvature please refer to Lee (82).

Most biological membranes contain lipids that, in isolation, prefer to adopt a curved, hexagonal H_{II} phase rather than the normal, planar, bilayer phase (83, 84). The curvature elastic stress in lipid bilayers is related to the intrinsic propensity of the lipid monolayer to curl in a stress-free environment state. PE is mostly found in the internal leaflet of the plasma membrane (the face where G protein binds). Membrane curvature has been shown to impact receptor activation and signaling events as described below. While pioneer and the great majority of studies have implicated rhodopsin, studies on other GPCR systems have been reported and are presented below.

Elastic curvature stress resulting from nonlamellar lipids in bilayer membranes has been shown to affect the activation of rhodopsin, namely the MI-MII equilibrium, shifting it toward the MII state (85). Brown and coworkers have suggested a mechanism by which stored curvature elastic energy could be linked to a mismatch between the hydrophobic thicknesses of rhodopsin and the surrounding lipid bilayer (86, 87). When the hydrophobic thickness of a protein is greater than that of the surrounding lipid bilayer, the system might respond by stretching the fatty acyl chains of the lipids around the protein to provide hydrophobic matching (further discussed in section 3.2). The lipids around the protein will show negative curvature (Fig. 4a, d, e). This process would be highly unfavorable for lipids having a tendency to form zero membrane curvature such as non-unsaturated PC, on the other hand formation of a membrane with negative curvature will be favorable for phospholipids such as PE. Thus, if a membrane protein can adopt two conformational states: 1) in which its hydrophobic thickness matches that of the planar bilayer; 2) in which its hydrophobic thickness is greater than that of the planar bilayer, then the presence of PE will favor the thicker form and thus would promote scenario 2. Such effect could explain why PE favors the MII conformation of rhodopsin, if the hydrophobic thickness of MII is greater than that of MI (86, 87). It has been reported by several authors that upon activation rhodopsin increases its average thickness, plasmon resonance experiments have estimated an average thickness increase of the rhodopsin membrane to about 4 Å upon photoactivation (88). Plasmon-waveguide resonance (PWR) studies of rhodopsin embedded in membranes containing or not PE at different levels, also revealed PE to highly enhance the MI-MII transition with the pka of the transition being shifted by about one unit Moreover, measurements of the interaction of this rhodopsin and its corresponding G protein transducin (αt) in lipid bilayers demonstrated that PE markedly increased receptor affinity for αt upon light activation, while the affinity of

αt for dark-adapted rhodopsin remained unchanged. In contrast, in pure lamellar PC bilayers the affinity of αt for light-activated rhodopsin was substantially lower (89), suggesting membrane curvature impact to go beyond the core of the lipid membrane that surrounds the receptor.

Elastic curvature stress was also shown to affect 5-HT_{1A}R activity in a study conducted by Gutierrez and collaborators where the receptor was reconstituted in GUPs (giant unilamellar protein-vesicles) made from palmitoyl-oleoyl phosphatidylcholine (POPC) and DOPE and its activity was followed by the receptor dependent G protein oligonucleotide exchange (90). Their results indicate that the presence of PE lead to increased receptor activity. The authors suggested as observed and reported for rhodopsin, the presence of DOPE in their GUPs with a lipid/protein ratio of 100:1, and a peak rate of 5-HT_{1A}R activity at 7.5–10 mol % DOPE could be explained in terms of the release of elastic curvature stress due to favorable lipid and protein interactions. This theory requires further investigation.

In contrast to G protein heterotrimers, the activated monomeric αi-subunit has been reported to possess a marked preference for pure lamellar structures (91). This would provoke the rapid exit of activated αi-monomers from the receptor environment where the hexagonal-phase propensity is increased. Both αi and αs have been found in lipid rafts (67). On the other hand, dissociated Gβγ dimers still maintain a high affinity for membranes with a hexagonal propensity(91), which may also influence their distribution in native membranes. The general observation that prenylated proteins are normally not localized in lipid rafts (92) was also corroborated for Gβγ subunits, which were found to be excluded from synthetic lipid rafts (93). This indicates that the Gβγ dimer most probably determines the lipid–protein interaction characteristics of the heterotrimeric G proteins. As such, the Gβγ dimer defines the preference of complete Gαβγ heterotrimers for the hexagonal-phase, thereby masking the lamellar membrane affinity of the Gα-subunit. As a result, one of the functions of the Gβγ dimer could be to shuttle the lamellar membrane phase preferring Gα subunit to the vicinity of the receptor, making it available for instant activation.

An interesting study on how the membrane curvature regulates ligand-specific sorting of GPCRs in living cells was reported by Rosholm and coworkers (94). In this report the authors used fluorescence imaging to establish a quantitative correlation between membrane curvature and sorting of three prototypic class A GPCRs (the neuropeptide Y receptor Y2, the β1 adrenergic receptor, β1AR, and the β2AR) in living cells. It has long been hypothesized that transmembrane proteins such as GPCRs can partition into their sites of action by recognizing the curvature of the membrane (95–97). However, to date there have been only a few examples of live-cell measurements that investigate membrane curvature-dependent protein sorting (98–100). They combined fluorescence microscopy approaches with data fitting by a thermodynamic model to quantify how sorting is mediated by an energetic drive to match receptor shape and membrane curvature. The three receptors displayed distinct curvature-dependent responses to ligand activation, revealing multiple layers of regulation and specificity encoded in the sorting process.

3.2. Membrane thickness and hydrophobic mismatch

The hydrophobic thickness of a lipid membrane is a fundamental property that has a strong effect on transmembrane protein structure and function. Lipid membrane properties such as fatty acid chain length, presence of lipid unsaturation and cholesterol, with consequent lateral lipid heterogeneity, among others, dictate the hydrophobic membrane thickness. Hydrophobic

mismatch is a phenomenon that occurs when the hydrophobic thickness of the transmembrane region of a membrane protein does not match the natural hydrophobic thickness of the membrane in which it is located (Fig. 4a, b) (101). Such mismatch has obvious energetic consequences due to the juxtaposition of energetically unfavorable regions of the membrane lipids and the protein. To deal with that, both lipids and proteins can adapt. There are two different scenarios: 1) the TM domain length of the protein is higher than the hydrophobic membrane thickness, it is called a positive mismatch; 2) the TM domain length is shorter than the hydrophobic membrane thickness, this is called negative mismatch (Fig. 4a-e). To minimize such mismatch, the protein can adapt via structural conformational adaptation, by potentially changing tilting of the helices or by self-aggregation to prevent energetically unfavorable exposure of specific residues. The protein can also laterally diffuse to regions of the membrane where hydrophobic match can be satisfied (Fig. 4f, g). Alternatively, the membrane thickness could deform and adjust to the length of the hydrophobic transmembrane helices in order to avoid an energetic penalty (102, 103). Hydrophobic mismatch could lead to changes in membrane protein folding, conformation, and activity as further discussed below.

Early FRET studies on rhodopsin by Brown's laboratory, revealed that the reduction of membrane thickness or the increase of the protein/lipid molar ratio promote rhodopsin association (104). This observation was assumed to be based on the hydrophobic mismatch induced curvature of the membrane at the protein-lipid interface. Indeed, association of proteins into clusters relieves the curvature free energy. Association of GPCRs to improve the hydrophobic match was since then being extensively analyzed using computational methods (105–109). Periole *et al* developed and employed CGMD models to investigate the molecular basis of how the physicochemical properties of the phospholipid bilayer membrane affect self-assembly of visual rhodopsin. Using systems containing several copies of rhodopsin (up to 16 rhodopsin molecules at a protein-to-lipid ratio of 1:100) embedded in membranes of different thickness, their simulations (for time scales of up to 8 μs) revealed that rhodopsin alters the membrane thickness at the membrane protein interface (108). The shorter the lipid chain length, the more pronounced was the hydrophobic mismatch that induced deformation of the bilayer. The results obtained for four different phospholipid environments showed that localized adaptation of the membrane bilayer to the presence of receptors is reproducibly most pronounced near transmembrane helices 2, 4, and 7. This local membrane deformation appears to be a key factor defining the rate, extent, and orientation preference of protein−protein association. CG-MDS studies by Mondal (long tens of μs simulations) of the spontaneous diffusion-interaction of the β2AR in a POPC lipid bilayer, described the energy penalty associated with the "residual hydrophobic mismatch" (RHM) to be composed of specific local contributions that can be attributed to particular residues (109). Such RHM can be reduced if proteins oligomerize. They have demonstrated the role of the RHM as a mechanistically important component of GPCR oligomerization in a case study of the β2AR oligomerization. They show RHM at TMs 1,4, and 5 of the monomeric GPCR to be substantially alleviated in the oligomeric form.

The modulation by cholesterol, along with its capacity in modulating membrane thickness, of GPCR oligomerization has been extensively investigated by Böckmann and collaborators, supporting that GPCR (mostly chemokine receptors) association is promoted in the presence of cholesterol (110). Interestingly, the impact of cholesterol content in hydrophobic mismatch of GPCRs is not straightforward, as in the case of the β2AR, hydrophobic mismatch was determined to be higher in presence of cholesterol (111).

Hydrophobic mismatch should impact GPCR activity as this property can depend on the GPCR occupied state, the membrane perpendicular length of the GPCR is shorter in an inactive conformation than in an active one. Few studies have addressed the relation between receptor activity and hydrophobic mismatch. Indeed, we should refer to earlier studies on

rhodopsin by the Brown laboratory, who revealed that a membrane composed by a larger variety of lipids has the possibility to harbor regions of distinct thickness and fluidity and was shown to facilitate rhodopsin activation (formation of the light-activated meta-rhodopsin II state)(86).

Years later, NMR measurements have also shown that increasing bilayer thickness favors formation of meta-rhodopsin II, while oligomerization favors meta-rhodopsin I (which corresponds to the dark-adapted, inactive conformation) (112). Light-activation of rhodopsin leads to TM6 motion outside of the protomer which overall expands the receptor surface (113) and consequently increases the hydrophobic mismatch. To compensate this effect, the receptor has three options: association with another receptor, translation to a different membrane domain of overall increased thickness, or both. In a more disperse membrane environment, active MII state rhodopsin receptors may likely diffuse to membrane areas with more suitable properties. Receptor movement/partitioning to membrane regions of increased membrane thickness (raft-like) have been observed by Alves and coworkers by PWR in the case of the human delta-opioid receptor (114). The study revealed that receptor partitioning was dictated by its bound-state, being preferentially incorporated in thinner membrane regions (POPC-rich domains) in the absence of any ligand, while agonist-bound proteins tended to move into increased thickness membrane regions (sphingomyelin-rich domains) (114).

A very recent study on the ghrelin receptor has specifically investigated the impact of membrane thickness on the conformation of the receptor and receptor mediated G protein activation. This was done by the use of nanodiscs composed of different phospholipids that resulted in membrane thickness differences of 5-6 Å without any changes in membrane fluidity (12). The study suggests that changing the membrane thickness of the bilayer had an impact on the conformational features of the active/active-like conformation of the isolated receptor in the absence of the G protein. Specifically, the thinner the membrane the shorter the distance between the cytoplasmic ends of TM1 and TM6, with a difference in the 5 Å range. The same trend was observed in the presence of the ligand ghrelin. Further, to assess whether these differences in GHSR conformation had an impact on G protein activation, the authors measured receptor-catalyzed GTP turnover. Together with energy transfer assays, their studies revealed that while it did not impact the assembling of the receptor/G protein complex, the membrane thickness did affect the receptor catalyzed G protein activation.

One could ask whether hydrophobic mismatch could be relevant in cellular membranes. Chattopadhyay and colleagues have nicely addressed this question and reminded that in eukaryotic cells there is a gradient of increasing bilayer thickness from the endoplasmic reticulum to Golgi to the plasma membrane (115). All membrane proteins traverse this path and hydrophobic mismatch has been proposed to play a crucial role in such sorting (116).

Another interesting type of mismatch can occur between posttranslational lipid modifications of the GPCR and membrane lipids in the bilayer center. This type of mismatch is caused by a misalignment between acyl chains of membrane lipids and the posttranslational receptor modification. Such phenomenon has been computationally observed by Olausson *et al* for the palmitoylated rhodopsin receptor by a detailed analysis of the behavior of the highly flexible post-translational lipid modifications of rhodopsin from multiple-microsecond all-atom molecular dynamics simulations. In this study they found that lower order parameters of the posttranslational linked palmitoyl chains, combined with the smaller number of methylene groups of the membrane resulted in an energetically disfavored mismatch of approximately 3 Å to the center of the membrane (117) in comparison to the lipid chains (stearic acid (STEA) and DHA) of the membrane. The authors suggest that such membrane-centered mismatch could have an effect on the curvature stress and the hydrophobic mismatch within the bilayer which in turn could drive the equilibrium between different substrates of the membrane-embedded rhodopsin.

3.3. Membrane fluidity

Membrane fluidity, or the reciprocal microviscosity is a property that depends on the lipid composition, especially the type of acid moieties in the phospholipids (with varied length and unsaturation levels) and the amount of cholesterol know to order or rigidify fluid membranes. A general rule is that increasing fatty acid chain length decreases membrane fluidity while increasing the degree of unsaturation increases fluidity (118). Alterations in membrane lipid composition, namely affecting membrane fluidity, have been shown to occur upon aging, have been associated with both metabolic and pathological conditions as well as with nutritional interventions (to cite a few:(119–124)). Membrane fluidity was among the first properties shown to impact the activity of proteins (125) and GPCRs are not an exception. Gurdal and coworkers found a correlation between low membrane fluidity and impaired coupling of Gαs with the β2AR, which mediates vasorelaxation and as a result reduction of blood pressure, in aortas of rats (126). In contrast, another study carried out in crude membrane preparations from rat lung showed the opposite relationship, concluding that the age related alteration in coupling between the receptor and the G protein is difficult to explain by alterations in membrane fluidity (127).

Besides numerous studies implicating cholesterol having an impact on membrane fluidity, cholesterol has also been shown to modulate GPCR activation and signaling (as described in section 2.3), with some reports directly addressing this question. The serotonin receptors have been investigated regarding the impact of membrane fluidity on the receptor activity and conformational equilibrium. Gutierrez *et al* have performed a systematic analysis on the impact of membrane ordering and thus fluidity on the functional activity of the human serotonin 5-HT1A receptor. This was done with the use of synthetic bilayers (GUPs) of controlled lipid content together with a fluorescent reporting system that detects GPCR-catalyzed activation of G protein by measuring receptor-catalyzed oligonucleotide exchange (90). They show that increasing the concentrations of the ordering components (cholesterol and brain sphingomyelin) in POPC membranes results in increased receptor activity as measured by the receptor-catalyzed oligonucleotide exchange. Moreover, they have used a series of cholesterol analogues possessing different membrane ordering profiles in membranes and show that the magnitude of the response they observe in terms of GTPase activity is directly correlated with increased membrane ordering. Their results show that membrane order, induced by sterols and sphingomyelin, is the key determinant of 5-HT1A receptor activity as revealed by the highest rates of receptor-catalyzed oligonucleotide exchange. Nonetheless, the mechanisms by which such effects take place are not determined.

A more recent study by Yoshida *et al*, reported on the impact of membrane fluidity in another human serotonin receptor subtype, the 5-HT2B (128). They have used reconstituted systems (nanodiscs) to control the membrane composition fluidity by incorporating phospholipids with varied fatty acid chain composition: POPC (C16 with no unsaturation and C18 with one unsaturation), DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine; C12 with no unsaturation), DOPC (two C18 with one unsaturation) and DMPC (1,2-dimyristoyl-snglycero-3-phosphocholine; C16 with no unsaturation). They characterized the membrane fluidity of the different membranes by all-atom MDS (50 nsec) that suggest, as previous reports, that the membrane of DLPC possessed the highest fluidity of the four phospholipids, and also suggested that the DMPC membrane was more rigid than those composed of the other three phospholipids. In support of this conclusion, the gel-liquid crystal transition temperature of

DMPC is higher than that of the others as reported (129). They have used microscale thermophoresis and SPR to measured ligand binding affinity (of agonist serotonin, 5-HT) to the receptor and have found that DMPC totally abolished ligand binding. Surface plasmon resonance (SPR) studies revealed that binding affinities depend mainly on the dissociation rate (k_{off}), suggesting that increased membrane fluidity may stabilize the activated 5-hydroxytryptamine 2B receptor (5-HT2BR)-ligand complex. Based on these biophysical analyses, they hypothesize that membrane fluidity influences the equilibrium between active and inactive forms of 5-HT2BR and have used thermal shift assays to get further insight into this. The results implied that the increased membrane fluidity shifted the equilibrium to favor the active form of the receptor. In contrast, the decreased membrane fluidity, observed in the DMPC would prevent the shift of conformational equilibrium toward the active form of the receptor. Simulation studies suggest that the fluidity of the phospholipid membrane would lower the energy barrier between conformational states of this GPCR by forming the intramolecular interaction of TM1-TM7. This interaction in the receptor would in turn make 5-HT binding more favorable, and make the receptor-ligand complex more stable, leading to the slower k_{off} in the SPR. Conformational changes of TM1-TM7 have been shown to be responsible for the activation of A2AR and β2AR receptors in previous reports (130), thus the present findings nicely reveal the close relationship between GPCR conformations and membrane fluidity at atomic resolution.

Cell membrane fluidity can also be directly altered by treating cells with external agents such as alcohols. Care must be taken in the use of this procedure to certify that such treatment does not impact cell viability or other cell mechanisms. Chachisvilis and coworkers have used benzyl alcohol, a known enhancer of this membrane property to investigate the impact of membrane fluidity in the activity and dynamics of the bradykinin B2 receptor in endothelial cells (131). By using time-resolved fluorescence microscopy along with FRET to follow the receptor conformation, the authors found that membrane fluidization leads to significant increase in the activity of the receptor, a process that is blocked by an antagonist of the receptor, and that overall membrane fluidity along with cell membrane tension affects the conformational dynamics of this receptor.

4. Lipids as cofactors

While the bulk of the lipid molecules in contact with a GPCR act as a solvent for the receptor, interacting with it relatively non-specifically and impacting membrane physical and mechanical properties, some receptors may also interact with much greater specificity with a small number of specific lipid molecules often essential for activity and acting as cofactors. Cholesterol is certainly one of the most commonly found lipid cofactors in GPCRs, often revealed following structural determination. Nonetheless, with the increasing number of structures determined over the last year, many other lipids besides cholesterol have emerged as cofactors as presented below.

An interesting study by Watts and collaborators, directly measured binding constants by electron spin resonance (ESR) between the rat NTS1R in its oligomeric form and the lipids: PC, PE, PS and cholesterol (132). The measurements of the relative lipid affinities indicated that both PC and PE, showed no relative selectivity for the receptor. As the authors have previously reported that for NTS1R in reconstituted systems, PE is important for effective binding of neurotensin (133), they suggest that this lipid exerts a bulk collective effect on the receptor rather than specific binding. PS showed approximately double the affinity for the

receptor compared to zwitterionic PC and PE, while the negatively charged fatty acid showed about five times the affinity compared to that of the zwitterionic lipids. The differences in affinity could, in principle, be due to exposed positively charged residues at the lipid headgroup region of the bilayer as the authors describe in detail herein (132). As per cholesterol, no relative selectivity was found for this receptor, despite the fact that it influences receptor oligomerization and signaling.

The Govaerts team has also reported on the strong effect of negatively charged lipids as PG on agonist binding and activation of the β2AR (7). While their data cannot rule out the possibility that such receptor modulation arises from changes in membrane bulk properties (as discussed in section 2.1), the strong effect observed with negatively charged lipids suggest ionic interactions between receptor side chains and lipid headgroups.

The importance of anionic lipid interactions was also revealed by a coarse-grained molecular dynamics simulation study, where the polyanionic lipid PIP_2 was shown to enhance the interaction between the A2AR and Gs protein (134). The study revealed 4 PIP_2 binding sites in the receptor all located at the intracellular rim of the receptor at interfaces between the different helices that occurred via arginine residue. Besides PIP_2 sites, other lipid binding sites were revealed by this study: GM3 that exhibited five receptor interactions placed in different locations from the N-terminus to the interfaces between TM helices and intra and extracellular loops; cholesterol covering nearly all the hydrophobic grooves between the transmembrane helices of the receptor. The most interesting finding of this study, regards the dual role of PIP_2 on A2AR activation that involves both stabilization of the characteristic outward tilt of TM6 and enhancement of Gs protein association, thus demonstrating that these bound lipids allosterically regulate the functional properties of this receptor. A recent study on the ghrelin receptor that employed FRET measurements between a fluorescent modified PIand fluorescently labelled amino-acids in different receptor locations, allowed to locate PIP_2 binding sites to the extracellular part of the receptor (135). Then MDS and site-directed mutagenesis (details found in section 2.1) have further refined 3 hotspots for PIP_2 binding to the receptor located at: the intracellular parts of TM1 and TM4 that bound two PIP_2 molecules; the interface between TM5 and TM6 and bound two PIP_2; the interface between TM7 and H8 and bound 1 PIP_2. Interestingly this third site was also found in the NTS1R (11) and A2AR (134).

As mentioned above, high resolution structure determination of several GPCRs allowed direct visualization of lipid molecules in very specific receptor locations and at interfaces between the receptor and effector proteins. In a recent report that determined the structures of the serotonin receptor ($5\text{-}HT_{1A}$) in different bound states in interaction with G protein complexes, PI was revealed to be placed at the receptor/G protein interface (10). More precisely, the PI was found inserted into a cavity formed between TM3, TM6 and TM7 of the receptor and the α5 helix of Gαi1. The study revealed phosphatidylinositol to be important for the enzymatic activity of the G protein, with a 2,4-fold enhancement in GTP hydrolysis activity in presence of this lipid. Furthermore, mutation of the residues implicated in such lipid/receptor interaction resulted in reduced G protein activation and abolished the regulatory function of phosphatidylinositol. The cryo-electron microscopy structure of full-length human NTSR1 in complex with truncated human β-arrestin 1 was also determined recently by the Kobilka laboratory (13). Besides the fact that this study revealed that phosphorylation of the receptor was essential to ensure receptor/β-arrestin interactions, the study highlight the importance of a PIP_2 molecule forming a bridge between the membrane side of NTSR1 transmembrane segments 1 and 4 and the C-lobe of arrestin. The presence of this lipid was also confirmed by

mass spectrometry analysis and fluorescence studies and determined a binding affinity of PIP_2 to both the receptor and β-arrestin in the low μM region. A recent study from the same team, further reported on the importance of PIP_2 in stabilizing the receptor-arrestin complex and offering control of the complex assembly and dynamics (14). Namely, using the PIP-binding deficient mutant of arrestin, the study revealed that the three components (receptor, β-arrestin and PIP) act in a concerted manner to provide a mechanism for release of arrestin from GPCRs with insufficient phosphorylation, allowing for the rapid recycling.

Specific cholesterol binding sites, also called nonannular cholesterol, have also been identified which may play a physiological role and influence GPCR activity (136). A cholesterol recognition amino acid consensus (CRAC) motif has been first described in a mitochondrial membrane protein (137). This motif corresponds to a sequence comprising -L/V-$(X)_{1-5}$-Y-$(X)_{1-5}$-R/K-, where $(X)_{1-5}$ represents one to five residues of the same amino acid. It is not specific to GPCRs, and can be located in different TM domains of the GPCRs. For example, a CRAC motif has been recently observed in TM7, co-localized with the highly conserved NPxxY motif found in class A GPCRs (138). This motif has been found in 38% of about 285 class A GPCRs analysed structures. However, the presence of the CRAC motif in the sequence is not predictive for cholesterol binding sites as illustrated by this recent work on CCK1R and CCK2R (138). For the first one, ligand binding and downstream signalling are modulated by cholesterol membrane levels. For the second one, even if highly homologous, the activity is not affected by cholesterol levels. Both of them share several cholesterol recognition motif sites, in particular CRAC sequences on TM3, TM5 or TM7, but cholesterol has not been observed to bind on these positions by CGMD simulations. In CCK1R, cholesterol has been found to interact importantly with TM3 but with residues upper to the CRAC motif. On the other hand, no cholesterol interaction with CCK2R has been found. A mirror image of CRAC, also called CARC, has also been described, where R/K is found on the N-terminus and L/V on the C-terminus (139). Another cholesterol binding site has been identified first for the β2AR (140), called cholesterol consensus motif (CCM), where a charged residue arginine or lysine in the lower part of TM4 interacts with hydrogen bonds with the hydroxyl group of the ring A of cholesterol. This residue is followed by hydrophobic residues such as isoleucine, valine or leucine upper on TM4. An aromatic residue, tyrosine or tryptophan upper on TM4 is highly conserved among class A GPCRs (94%), and seems to highly contribute in the interaction with the sterol ring and the edge of ring D. Another residue, considered of least importance for cholesterol binding is a phenylalanine, a tyrosine or a tryptophan localized on the bottom of TM2. Concerning the β2AR, CGMD simulations have shown a good correlation between cholesterol localization found in the crystal structure and the residency time measured using a CGMD approach (138). Even if CCM motif is conserved in 44% of human class A GPCRs, it doesn't always correlate with cholesterol binding sites observed in high resolution crystal structures or molecular simulations. Indeed, these general binding sites, determined based on the sequence or on the crystal structure, are not predictive for cholesterol specific interaction and other cholesterol preferential localizations has also been evidenced, also called "cholesterol hot-spots" that can be specific for each receptor families (111). Complementary experimental data are now necessary to confirm physiologically relevant cholesterol specific binding sites and its role for GPCR structure and dynamics.

5. The importance of GPCR and G protein post-translational lipid modifications

When considering protein/lipid interactions, one must not forget co- and post-translational modifications of the signaling proteins as the receptor, G proteins, G protein coupled effector enzymes and receptor kinases (141–144). Lipid modifications consist in the covalent binding of a hydrophobic lipid molecule to the signaling protein and comprise: myristoylation, palmitoylation and isoprenylation.

In case of GPCRs, and on the contrariry to G proteins, protein lipidation is not needed for tight membrane attachment. GPCRs can be palmitoylated through the attachment of a palmitate to one or more cysteine residues via a thioester bond. As this thioester bond is cleavable, the palmitoylation state of a receptor can be used to regulate its activity. Depalmitoylation seems to be regulated by the bound state of the receptor, being accelerated upon agonist binding as demonstrated for certain receptors (dopamine D1, serotonin 4A, delta opioid and adrenergic receptors but not for all serotonin 1A, for example) (41). Palmitoylation usually occurs in the cytoplasmic (C-terminal tail) of the receptor positioned 10 to 14 amino acids downstream of the last transmembrane domain. At this position, palmitoylation has a profound effect on the local conformation of this domain by the creation of an additional intracellular loop. Although being the common location, GPCR palmitoylation can also occur in other locations, for instance in the intracellular loops as evidenced in case of the vasopressin receptor (145). Indeed, up to three palmitate groups can be found on GPCRs and different palmitoylation profiles can result in various conformations of the carboxy-terminal tail, which may select for certain G protein interactions (41). Palmitoylation influences many aspects of GPCR signaling. The palmitoylation state of certain receptors can preferentially direct signaling through particular G proteins upon binding of the same ligand, thus being a source of biased signaling. Palmitoylation can influence the phosphorylation state of the receptor, modulating desensitization and internalization, and can also control internalization independently to phosphorylation. It has been suggested that palmitate binding in the endoplasmic reticulum ensures correct processing and trafficking of receptors, and, once at the cell membrane, may target GPCRs to lipid rafts. However, not all palmitoylated receptors associate with rafts and not all raft-associated GPCRs are palmitoylated (146).

The other type of lipid modification in GPCRs is isoprenylation, a lipid modification that occurs upstream of S-palmitoylation on many protein substrates, facilitating membrane localization and activity of key intracellular signaling proteins. Such lipidation has been shown to exist in the prostacyclin receptor but also in other proteins of the signaling machinery such as Ras-type proteins and small GTPases where its deregulation has been connected to pathologies (147) (148). A second lipid modification can further increase the hydrophobicity of the protein, its membrane affinity and membrane residence time. As palmitoylation occurs after isoprenylation, this first signal may also be required to permit an initial interaction with membranes thereby facilitating palmitoylation (149).

G proteins ($\alpha\beta\gamma$) bind tightly and often reversibly to the cell membrane, thus covalent lipid modifications are essential to mediate such process. G protein α subunits ($G\alpha$) are modified attheir N-terminus by the fatty acids myristate and/or palmitate, while γ subunits of $\beta\gamma$ dimers ($G\beta\gamma$) are modified by farnesyl or geranylgeranyl isoprenoids at their C-terminus. Myristoylation occurs exclusively on $G\alpha$ of the αi family (150, 151). The N-myristoyltransferase catalyzes the amide bond attachment of the 14-carbon saturated fatty acid myristate to the extreme N-terminus of a protein. This modification occurs co-translationally and requires a glycine at the extreme N-terminus of a substrate protein; thus, a

prerequisite is the removal of the initiating methionine by a methionyl amino peptidase. Not all extreme N-terminal glycines, however, are myristoylated and this is an irreversible modification.

Most mammalian Gα proteins undergo palmitoylation via cysteine residues in the N-terminal amino acids of Gα, although rarer palmitoylation can also occur via N-terminal glycine residues, as observed for the αs subunit (152). Thus, it is possible that αs subunits possess dual palmitoylation, the functional significance for that is yet not understood. As with GPCR palmitoylation the process is reversible. Changes in palmitoylation can clearly affect the subcellular localization and trafficking of αs, or other Gα, but understanding the functional significance of such regulated changes in localization is quite challenging.

All mammalian G protein γ subunits are isoprenylated (153) via thioether attachment of either the 20 carbon geranylgeranyl or 15 carbon farnesyl moiety to a C-terminal cysteine in a CaaX motif, in which the cysteine is followed by two aliphatic amino acids and the X amino acid that specifies recognition by either well-characterized geranylgeranyl or farnesyl transferases. After irreversible isoprenylation, most isoprenylated proteins undergo the additional obligate modifications of proteolysis of the C-terminal three -aaX residues and then carboxy methylation of the new isoprenylcysteine C-terminus (154). The role of such subsequent modifications is surprisingly still not clear. Carboxy methylation does appear to increase membrane binding of farnesylated proteins, most likely by a general increase in hydrophobicity. On the other hand, the greater hydrophobicity of the geranylgeranyl lipid compared to farnesyl makes it a strong membrane anchor that does not seem to require methylation to increase strength of membrane binding. Prenylation of γ subunits is necessary for the membrane localization of heterotrimeric G proteins and for functional heterotrimeric GPCRs. The deficiency in G protein gamma subunit prenylation has also been shown to disrupt GPCR signaling in the zebrafish signaling (155).

Overall, besides the fact that post-translational modifications are essential to ensure G protein anchoring to the membrane and the lack of such modifications results in important signaling defects, the additional roles of such modifications in both GPCRs and G proteinG proteins is far from being totally understood.

5. Conclusions

The modulation of GPCR activity by lipids has set up the interest of researchers from as early as the 80s, with pioneering studies being mostly done in bovine rhodopsin. This GPCR was chosen, certainly as a result of the ease of obtaining high amounts of this receptor from natural sources (bovine retinas) at low cost, their intrinsic higher stability relative to other receptors (due to the fact that the ligand retinal is naturally present in the ligand pocket) and the facility in following protein activation by classical fluorescence spectroscopy and flash photolysis assays commonly available in most laboratories. Rhodopsin was also largely investigated by MDS as presented in this review. While studies on rhodopsin have greatly contributed to understand GPCR mechanisms including lipid/receptor interplay, one cannot forget that results obtained with rhodopsin cannot be blindly transposed to other GPCRs. Indeed, rhodopsin's special natural properties such as very high protein/lipid ratios in native samples and peculiar composition of their lipid environment (e.g. high levels of polyunsaturated fatty acids as DHA) and the presence of the antagonist in the binding pocket, make this GPCR quite atypical.

Lipid impact in GPCR functioning regained some interest by the end of the nineties where lipid rafts have become popular and particularly investigated. Several laboratories have determined receptor localization in or out of such domains as well as how ligands could affect such receptor partitioning.

With the exploding number of high-resolution structures of GPCRs over the last few years by cryo-EM and X-ray studies, it became evident that specific lipids occupy very particular locations in such structures. The combination of diverse structural information obtained from both static and solution-based fluorescence and NMR techniques applied to cellular systems and to receptors in reconstituted model membranes (liposomes, nanodiscs, etc) have all highly contributed to gain insight into the implication of such lipids in protein activity and signaling events. Moreover, computational approaches are well placed and have highly contributed to link this information together into a cohesive picture.

It would be interesting in the next years to make a bridge between lipid imbalance observed in different pathologies, some of which may result from aging, as well as the impact of such lipid alterations on the functioning of the key GPCR targets in such pathologies. With the high potential of lipids such as allosteric modulators of GPCRs and associated effector molecules, it remains to be seen whether the development of drugs aiming to alter lipid-protein interaction will become a valuable therapeutic strategy in the future.

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