Molecular Modeling and Simulation of Membrane Lipid-Mediated Effects on GPCRs

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Abstract: Functioning of G protein-coupled receptors (GPCRs) is tightly linked to the membrane environment, but a molecular level understanding of the modulation of GPCR by membrane lipids is not available. However, specific receptor-lipid interactions as well as unspecific effects mediated by the bulk properties of the membrane (thickness, curvature, etc.) have been proposed to be key regulators of GPCR modulation. In this review, we examine computational efforts made towards modeling and simulation of (i) the complex behavior of membrane lipids, (ii) membrane lipid-GPCR interactions as well as membrane lipid-mediated effects on GPCRs and (iii) GPCR oligomerization in a native-like membrane environment. We propose that, from the perspective of computational modeling, all three of these components need to be addressed in order to achieve a deeper understanding of GPCR functioning. Presently, we are able to simulate numerous lipid properties applying advanced computational techniques, although some barriers, such as the time-length of these simulations, need to be overcome. Implementing three-dimensional structures of GPCRs in such validated membrane systems can give novel insights in membrane-dependent receptor modulation and formation of higher order receptor complexes. Finally, more realistic GPCR-membrane models would provide a very useful tool in studying receptor behavior and its modulation by small drug-like ligands, a relevant issue for drug discovery.

Keywords: G protein-coupled receptor, lipid bilayer, molecular dynamics, hydrophobic mismatch, GPCR regulation, oligomerization.

INTRODUCTION

G protein-coupled receptors (GPCRs) are the largest protein superfamily in the human genome [1]. As transmembrane proteins, they sit at the interface between the extra- and intracellular environment mediating signal transduction into the cells and are validated targets for between 40-60% of approved drugs [2].

Recent research has clearly demonstrated that membrane lipids significantly modulate GPCR functioning. It is known that the degree of unsaturation of the phospholipid acyl chains, the specificity of their polar head groups, and the cholesterol content play a crucial role in the modulation of this class of transmembrane proteins. Membrane can induce both specific effects due to the interaction of GPCRs with specific embedded lipids and unspecific effects modulated by the overall properties of the membrane (thickness, curvature, etc.). Moreover, some lateral organization of the membrane - named microdomains - could both provide more specificity and diversity in the signaling cascade by influencing the compartmentalization of the different components involved or by directly modulating the intrinsic activities of the GPCRs located therein. Specific microdomains with high content of cholesterol and sphingolipids, such as lipid rafts, are well-known modulators of the activity of a number of GPCRs. For these membrane proteins a raft domain provides a more organized platform for the proper assembly of signaling complexes, and prevents crosstalk between different pathways [3]. When not activated by ligands, some GPCRs are almost exclusively associated to microdomains whereas others are present in a small amount. For instance, under basal conditions rhodopsin, β_1- and β_2-adrenergic and serotonin $5HT_{1A}$ receptors were shown to be localized at level of lipid rafts [4-6], and in some

cases, such as for the β_2-adrenergic receptor [7], the treatment with agonist causes the translocation of the receptor out of lipid rafts region of the membrane. By contrast, in other cases the raft localization of GPCRs is positively modulated by ligand binding, this is the case of muscarinic M_2 receptors [8], angiotensin AT_2 receptors [9], bradykinin B_1 and B_2 receptors [10], cholecystokinin receptors [11] and endothelin ET_A receptors [12] that upon stimulation with agonist were shown to translocate to specific microdomains of the membrane (i.e. flask shaped invaginations named caveolae). Concerning the cannabinoid receptors, it has been reported that the cannabinoid CB_1 receptor is localized into the lipid raft region of the membrane [13], and attraction to these microdomains is dynamically modified by agonist/antagonist binding [13-15]. Cannabinoid CB_1 receptor localization at level of cholesterol-rich regions of the membrane has shown also to be mediated both by the presence of a cholesterol interaction/recognition amino acid consensus sequence [16] and, as reported for other GPCRs [7], by a constitutive palmitoylation of a cysteine residue [17]. On the other hand, the cannabinoid CB_2 receptor is not localized at level of lipid rafts [18, 19], and its signaling is not modulated by membrane cholesterol [18].

Other raft-associated GPCRs, such as the endothelin receptor, are apparently unaffected by agonist binding [20], and in the case of the human oxytocin receptor, it has been shown that high-affinity agonist (but not antagonist) binding is highly dependent on the cholesterol content of the plasma membrane [21], but only a small fraction of oxytocin receptors is raft-associated [22, 23]. However, it has to be taken into account that in some cases the activation of specific G proteins could lead to their migration from non- raft to lipid raft regions of the membrane, thus indirectly influencing the localization of GPCRs. Thus, overall these data indicate that cholesterol can be involved in modulating very selective receptor-induced signaling events also when it is not embedded within lipid rafts.

The aim of this review is to illustrate the importance of the membrane environment in GPCR functioning in three different aspects: (i) the complex behavior of membrane lipids, (ii) membrane lipid-GPCR interactions and membrane lipid-mediated effects on GPCRs as well as (iii) GPCR oligomerization in a native-like membrane environment. We propose that, from the perspective

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of computational modeling, all three of these components need to be addressed concurrently in order to achieve a deeper understanding of GPCRs' functioning. Therefore, this paper is organized as follows. In the first section, we examine the computational efforts made towards modeling the complexity of the lipid membrane. In the following section, we report the progresses made in modeling receptor-membrane interactions as well as membrane-mediated effects on GPCRs. Finally, in the last two sections, we overview the most recent understanding of the phenomena of GPCR oligomerization and discuss the effects of oligomerization on GPCR functioning as well as the modulating effects of lipid properties on oligomerization and thus on GPCR activity. We also consider the computational challenge posed by the prospect of understanding membrane-dependent GPCR oligomerization at atomic level accuracy and propose general methodologies by which such challenges may be achieved. We conclude with an outlook on the forthcoming developments that are likely to contribute to the field.

MODELING THE BIOPHYSICAL PROPERTIES OF MEMBRANE LIPIDS

Lipids are one of the leading players in the cell and molecular research arena. The emerging understanding of this role has led to the creation of concepts such as the *lipidome* and has even given rise to a new 'omic' science, the so-called lipidomics [24]. Part of this new science studies membrane lipids in much greater depth in an attempt to establish their real contribution to membrane functioning. We know that lipids are involved in widespread disorders such as obesity, neurodegenerative diseases and even cancer [25]. Although we have traditionally considered lipid membranes as passive solvents we now know that lipids do have biological functions and take part in key biological events. They are not just a matrix where proteins reside but are actually involved in important cell events such as domains formation, membrane trafficking and signal transduction [26].

The specific content of both proteins and lipids regulates the global biophysical properties of cell membranes. These properties greatly rely on the membrane content as the lipid to protein ratio in native plasma membranes is around 50:1 [27, 28], a fact sometimes overlooked. Besides, optimal activity of membrane proteins is significantly determined by particular lipid requirements [29]. Consequently, to understand membrane proteins dynamics one unavoidably needs to make room for understanding membrane lipids. The major lipid components of biomembranes are phospholipids and cholesterol Fig. (**1**), the most abundant of which are the phospholipids. The amphipathic nature of phospholipids is conferred by the presence of a polar head and hydrophobic tails Fig. (**1**). In eukaryotic cell membranes, around half the number of membrane phospholipids consist of phosphatidylcholine [30]. The other half consists of phospholipids that confer essential structural properties to the membrane, for example, the curvature stress partially imposed by phosphatidylethanolamines [31]. Yet, not all phospholipids are phosphoglycerides and so key lipids such as sphingomyelin can make up a quarter of the total plasma membrane lipids in mammal cells [31]. Along with phospholipids, cholesterol is a key lipid component of biological membranes that has an essential condensing effect on biological membranes [32].

Thus, simulations of protein-membrane systems need an adequate characterization of membrane lipid composition. However, current simulations of protein-membrane systems have certain limitations in representing the complex nature of lipid bilayers. Most of these simulations use phosphatidylcholine-lipids as the only representative of phospholipids and, furthermore, in many of them the cholesterol is not even present. Another example of such misrepresentation when simulating protein-membrane systems is the use of symmetric membrane leaflets despite the real asymmetry present in cell membranes. Biological membranes are asymmetric, that is, the

lipid composition on each leaflet is not even and significantly varies across different cellular compartments and different cell types [33, 34]. In fact, molecular simulations of asymmetric membrane leaflets describe the important role of such compartmentalization and highlight its possible impact on protein function [35]. Furthermore, membrane heterogeneity is not only present transversally, but also laterally since membranes segregate certain lipids and proteins to form dynamic assemblies called lipid microdomains [36]. The biological importance of lipid rafts [26, 27], a type of microdomain [37], perfectly shows the impact of membrane heterogeneity on the biophysical properties of cell membranes.
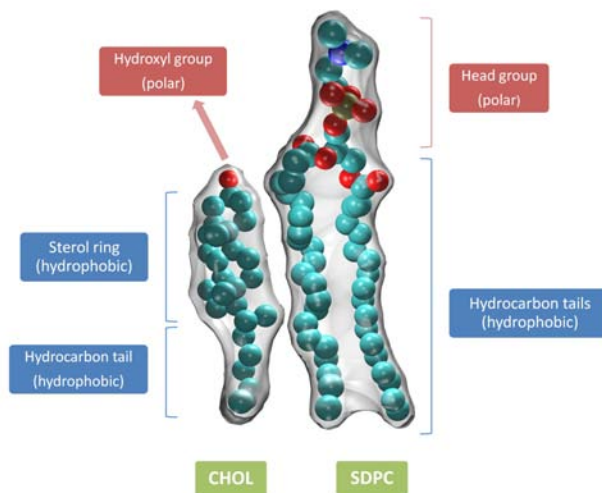

Fig. (1). Amphipathic structure of cholesterol and phospholipids. The figure shows a snapshot of one cholesterol molecule (left) near one SDPC (1-stearoyl-2-docosahexaenoyl-sn-glyerco-3-phosphatidylcholine) molecule (right), used here as an example phospholipid. There are different head groups in biomembrane phospholipids, namely, phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphoinositides (PI). Phospholipids classification is based on these groups, which determine many lipid properties providing cell membranes with idiosyncrasy. Both cholesterol and SDPC are amphipathic molecules, that is, they have both polar and hydrophobic regions. In addition, the length and degree of saturation of hydrocarbon tails provide the cell with a tool to regulate the physicochemical nature of membranes. SDPC contains both saturated and unsaturated lipid tails. This figure shows how its right tail has regular length and is fully saturated (stearic acid, C18:0) whereas the left tail is longer and have 6 double bonds (docosahexaenoic acid, C22:6). This snapshot is taken during a molecular dynamics (MD) trajectory of a complex lipid mixture made of 4 different phospholipid plus cholesterol. Carbon atoms are depicted in cyan, phosphorous in ochre, nitrogen in blue and oxygen atoms in red. Hydrogen atoms are not depicted for clarity.

In practice, biological membranes are difficult to study both experimentally and computationally due to their complexity. For this reason, experimental [38] and computational [39] model membranes normally consist of either pure component or two-three component mixtures. Monte Carlo [40, 41] and Molecular dynamics (MD) simulations of these mixtures are effective computational tools to describe, at the molecular level, the biophysical properties of lipid bilayers (reviewed in [42]). As for any other computer simulation, lipids can be studied at various levels of detail depending on both the goal of the study and the computational resources available. While simple coarse-grained models [43] can run over longer time periods, atomistic models [44] provide a more detailed description of membrane and protein properties. In both cases, the use of an adequate force field is essential for modeling the bio-

physical properties of membrane lipids, which must be consistent with experimental data. The analysis of certain parameters of the simulated system can provide valuable data on properties such as membrane and lipid structure, density profiles, rotational and lateral motions or inter/intra molecular interactions.

The overall membrane structure is inherently determined by the lipid structure. One of the key structural parameters when studying lipid models is the average area per lipid frequently calculated from the area of the simulation box. The area per lipid can be used to monitor system equilibration and provides hints on the condensation degree of the membrane. Calculating this parameter is rather trivial for pure bilayer systems where values should be in agreement with experiments. However, in binary [45] or more complex mixtures, although feasible, this calculation becomes more complicated due to the presence of different molecular species [46]. Changes in the average area per lipid of a lipid bilayer normally correlate with changes in the overall membrane thickness. The electron density profiles are commonly used to assess this thickness by measuring the so-called 'phosphate-to-phosphate' distance Fig. (**2**). This parameter allows studying membrane condensation effects driven by, among other reasons, the influence of cholesterol [47] and the nature of phospholipid tails [48] Fig. (**2**). Moreover, electron density profiles provide interesting data on the extent of the interaction between opposite monolayers or 'interdigitation'. Thus, the interdigitation phenomenon is visible in lipid bilayers rich in long and saturated lipid tails Fig. (**2**). Membrane thickness is normally averaged over the simulation and then compared with experimental X-ray diffraction data.
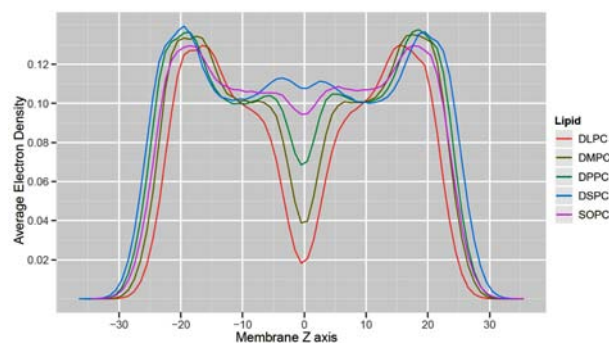

Fig. (2). Electron density profiles of lipid tails in ternary mixtures of phospholipids with different carbon lengths. All 5 systems depicted contain cholesterol, POPC and one extra phospholipid at a ratio of 1:1:1. This extra phospholipid varies in the number of carbons or the number of double bonds for each system. Hence, DLPC (C12:0), DMPC (C14:0), DPPC (C16:0) and DSCP (C18:0) provide an increase in tail length while SOPC (C18:1) is the only unsaturated lipid with one double bond. This figure shows how the *phosphate-to-phosphate* distance is lower for short-tail lipids, that is, membranes with longer lipids are thicker (up to 4 Å per leaflet). Besides, the degree of interdigitation clearly varies upon increasing the length of the hydrocarbon chains. Interestingly, the insertion of a double bond (from SOPC) reduces the amount of interdigitation (unpublished data). DLPC - 1,2-dilauroyl phosphatidylcholine; DMPC - 1,2-dimyristoyl phosphatidylcholine ; DPPC - 1,2-dipalmitoyl phosphatidylcholine; DSPC -1,2-distearoyl phosphatidylcholine; POPC - 1-palmitoyl-2-oleoyl phosphatidylcholine; SOPC - 1-stearoyl-2-oleoyl phosphatidylcholine;

The analysis of the chain structure of individual lipid species provides valuable data on the disorder and fine-structure of the membrane. NMR experiments use the deuterium order parameter, S_{CD}, for measuring the disorder of phospholipid hydrocarbon tails by studying the orientation of their C-H bonds. The MD simulations approach to approximate such disorder relies on calculating the average of the angle formed between each C-H bond in the lipid tail

and the bilayer normal [49]. One can calculate this angle, θ, for each phospholipid tail, average it over a set of MD trajectories and then compute S_{CD}, defined [49] as an interesting example is the study of the average cholesterol tilt angle with respect to the membrane normal. This angle should be lower in more ordered membranes due to the effect of packing. Both S_{CD} and angle distributions are frequently used in phase behavior modeling of membrane domains, such as lipid rafts.
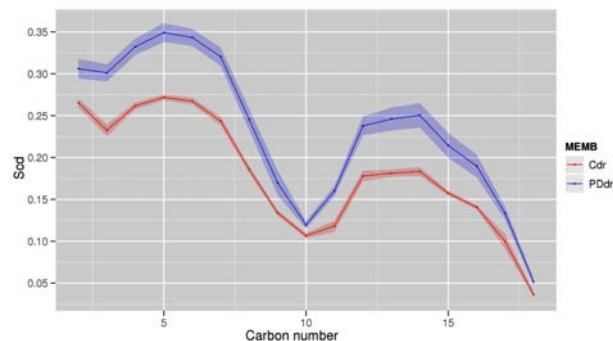

Fig. (3). Comparison of deuterium order parameters, S_{CD}, between two membranes. The figure shows S_{CD} values of the *sn*-2 chain for DOPC (1,2 - dioleoyl phosphatidylcholine). These values correspond to the average S_{CD} over the simulation of two multicomponent lipid mixtures (4-5 different phospholipid plus cholesterol). PDdr, in blue, corresponds to a less ordered mixture containing more saturated phospholipids and more cholesterol while CDdr, in red, contains more unsaturated phospholipids and less cholesterol. The PDdr membrane shows significantly higher S_{CD} values when compared to CDdr. The local minimum at carbon 10 in both membranes is a typical fingerprint of this lipid [131] and corresponds to the only double bond present at this hydrocarbon tail (unpublished data).

On the other hand, the study of the inner distribution of lipids within the membrane provides key information on the forces driving such distribution. In molecular simulations, the so-called radial distribution function (RDF) is used to describe the atomic distribution of the system around a reference atom. The RDF gives the probability of finding a pair of atoms as a function of their radial distance. Hence, this correlation function describes the profile of the coordination shells formed by the components of the system during the simulation [52]. This analysis defines the radial symmetry of the molecules but yields, however, no information on their angular symmetry which is also important [50, 53]. Angular symmetry information is very valuable when studying asymmetric structures such as the steroid ring of cholesterol. This planar ring defines two asymmetric faces, the rough one with off-plane methyl groups is the β-face while the smooth one is the α-face. Thus, cholesterol interacts differently with their counterparts and the rest of membrane components through its α- and β-faces [54]. Bivariate correlation functions involving both orientation and position, the so-called two-dimensional RDFs, can be useful to study such subtle structural data [50, 53, 54]. The angular information allows an accurate description of the coordination shells established between membrane components see Fig. (**4**). Therefore, RDFs and, especially, two-dimensional RDFs can help to reveal the molecular interaction network behind a certain membrane structure.

In addition to the former structural parameters, the lateral motion of membrane components can provide useful information on membrane organization. Lipid lateral diffusion is an essential process of membrane dynamics, implicated in the formation of membrane domains. Motion parameters such as root mean square displacements and diffusion coefficients are frequently used to describe lipid lateral diffusion [55, 56]. Yet, the insufficient timescale of current atomistic simulations does not allow a straightforward
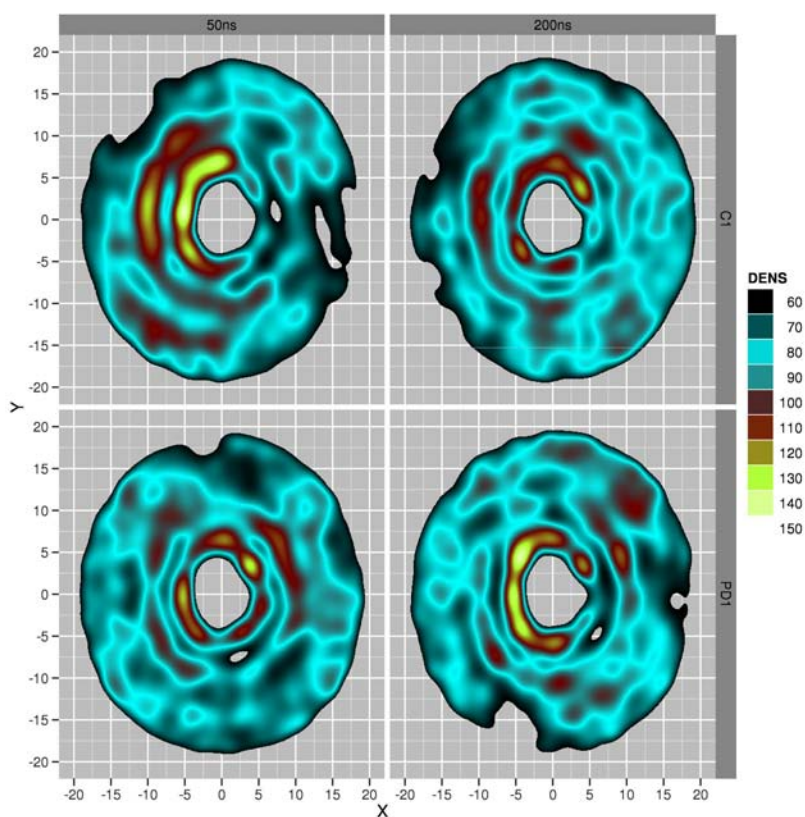
Fig. (4). Two-dimensional distribution function plots of sphingomyelin around cholesterol. These plots show, for two different hydrated bilayers (C1 and PD1), the average position and orientation of the sphingomyelin tails around cholesterol molecules at two stages of the simulation, 50 and 200 ns. PD1 corresponds to a highly saturated and cholesterol-enriched bilayer (40%) whereas C1 is a bilayer rich in unsaturated and polyunsaturated phospholipids with a lower concentration of cholesterol (30%). The color-scale gradient represents the density of sphingomyelin tail carbons, densities below 60 are not plotted for clarity. Cholesterol molecules are aligned to the plot center and while the rough β-face extends to x > 0 the smooth α-face does so for x < 0. Sphingomyelin coordination shells are visible at high densities (brown to white). Interestingly, PD1 membrane displays a clear tendency of sphingomyelin tails to interact mainly with the α-face of cholesterol after 200 ns (unpublished data).

interpretation of diffusion coefficients [57]. Furthermore, the apparent concerted diffusion that exists in pure bilayers and lipid mixtures makes this interpretation even more controversial [57]. What's more, recent theoretical studies point to a concerted diffusion of both proteins and lipids within biological membranes and highlight the importance of protein-lipid interactions on membrane dynamics [58].

All in all, MD analysis can be a useful tool for modeling the biophysical properties of membrane lipids. Membrane structure can be thoroughly modeled, from general structural parameters such as area per lipid and electron density profiles to more fine-structure analysis such as order parameters, angle distributions and RDFs. The study of lipid lateral diffusion in current membrane simulations can also help understand membrane dynamics although its interpretation still needs a careful approach. In this sense, it is worth to bearing in mind the importance of considering protein-lipid interactions on the overall biophysical properties of biological membranes.

MODELING MEMBRANE LIPID-GPCR INTERACTIONS AND MEMBRANE LIPID-MEDIATED EFFECTS ON GPCRs

There is plenty of experimental data suggesting that GPCR functioning is tightly linked to its lipid environment. Nevertheless, the aforementioned structural aspects of membrane lipids highlight the complexity of simulating biophysically realistic membrane bilayers. There is clear evidence that the length and saturation degree

of lipids, the specificity of their polar head groups as well as the cholesterol content play a crucial role in the modulation of transmembrane proteins. The mechanism of such membrane-mediated GPCR modulation remains for some aspects elusive. It is unclear if this effect is primarily mediated by specific receptor-membrane interaction or rather by an unspecific effect based on the alteration of membrane properties (thickness, curvature, etc.). First advances towards all-atomistic simulations of GPCR in a more native environment involved embedding the receptor in an explicit homogenous one-component membrane bilayer. This setup has been used to obtain relevant insights into receptor dynamics (e.g. ionic lock), ligand or ion binding [59]. Very recent studies focus also on the contribution of membrane lipid-dependent effects on GPCRs' functioning (Table **1**) [60-65]. In this context, some studies reach beyond the simplistic one-component membrane-receptor approach by including different lipid components to observe more specific membrane lipids effects on GPCRs (Table **1**) [66-72]. To what extent we can simulate membrane lipid-dependent GPCR properties to an atomic level is addressed in the next two sections highlighting studies that focus on specific and unspecific membrane effects and followed by a third section about major challenges.

Specific Membrane Effects on GPCRs

One of the best-studied GPCR in the context of its membrane environment is bovine rhodopsin. Experimentally, it has been demonstrated that rhodopsin reacts very sensitively to its lipid environment [73-76]. A high content of polyunsaturated lipid chains desta-

Table 1. **Systems Parameters of the Discussed Work**

GPCR	Membrane Composition	Force Field	Software	Total Simulation time	Reference
Rhodopsin	DOPC	CHARMM [197]	CHARMM [197]	40 ns	Crozier *et al.* [60]
Rhodopsin	SDPC	CHARMM [197]	CHARMM [197]	12.4 ns	Feller *et al.* [61]
Rhodopsin	System 1: DMPC System 2: PLPC System 3: DPPC System 4: POPC	OPLS [198]	GROMACS 3.2 [199]	80 ns	Cordomí *et al.* [65]
Cannabinoid CB$_1$ receptor	POPC	CHARMM22 [200]	NAMD [201]	105 ns	Shim *et al.* [62]
Rhodopsin	SDPC: SDPE: CHO (2:2:1)	CHARMM27 [202]	BlueMatter [203]	118 ns	Pitman *et al.* [66]
Gonadotropin-releasing hormone receptor	DPPC	GROMOS96 [204]	GROMACS 3.2 [199]	245 ns	Jardón-Valadez *et al.* [63]
Rhodopsin	SDPC: SDPE: CHO (2:2:1)	CHARMM27 [202]	CHARMM [197], BlueMatter [203]	2.6 μs	Grossfield *et al.* [67, 68]
Rhodopsin	SDPC: SDPE: CHO (2:2:1)	CHARMM27 [202]	BlueMatter [203]	1.6 μs	Khelashvili *et al.* [69]
Rhodopsin	SDPC: SDPE: CHO (2:2:1)	CHARMM27 [202]	CHARMM [197], BlueMatter [203]	4.3 μs	Olausson *et al.* [70]
Metabotropic glutamate receptor 2	System1: SDPC: CHO (3:1) System 2: SDPC: CHO (4:0)	CHARMM27 [202]	ACEMD [205]	>3.5 μs	Bruno *et al.* [118]
Rhodopsin, Serotonin 5HT$_{2A}$ receptor bound to the full agonist serotonin, partial agonist LSD, inverse agonist ketan-serin	System 1: diC$_{14:1}$PC System 2: diC$_{16:1}$PC System 3: diC$_{18:1}$PC System 4: diC$_{20:1}$PC System 5: SDPC:POPC:CHO (7:7:6)	CHARMM27 [202]	NAMD [201]	> 2 μs	Mondal *et al.* [71] Shan *et al.* [72]
Adenosine A$_{2A}$ receptor with and without the antagonist ZM241385	POPC	AMBER99 [206]	Desmond v20109 [207]	≈ 3 μs	Lyman *et al.* [64]

CHO - Cholesterol; diC$_{14:1}$PC - 1,2-ditetradecenoyl phosphatidylcholine; diC$_{16:1}$PC - 1,2-dihexadecenoyl phosphatidylcholine; diC$_{18:1}$PC - 1,2-dioctadecenoyl phosphatidylcholine; diC$_{20:1}$PC - 1,2-diicosaenoyl phosphatidylcholine; POPC - 1-palmitoyl-2-oleoyl phosphatidylcholine; SDPC - 1-stearoyl-2-docosahexaenoyl phosphatidylcholine; SDPE - 1-stearoyl-2-docosahexaenoyl phosphatidylethanolamine.

bilizes the native state of rhodopsin enhancing the kinetics of the photocycle [77, 78]. In contrast, cholesterol seems to stabilize the native state of rhodopsin slowing down its kinetics [77-79]. Rhodopsin was the first GPCR structure to be known at atomic resolution [80] laying the groundwork for all-atomistic computational studies of a GPCR in monounsaturated [81-83] and polyunsaturated membranes [61, 66].

Action of Different Membrane Components on GPCRs

A first computational insight into rhodopsin-lipid interaction was given by Crozier *et al.* using a mono-component membrane of 1,2-dioleoyl phosphatidylcholine (DOPC) [60]. They observed in a 40 ns simulation different lipid accessibility for transmembrane helices of rhodopsin. This ranged from relatively weak lipid coupling to transmembrane helix (TMH) 3 to strong and more broadly coupling to TMH 6. As rigid body movement of in particular TMH 6 is implicated in transition between different receptor states, interaction of this helix with chemically diverse lipid types could impact receptor transition [60].

Similar lipid accessibility to TMHs has been observed in a model for the gonadotropin-releasing hormone receptor (GnRHR) simulated in a one-component 1,2-dipalmitoyl phosphatidylcholine (DPPC) environment (TMH 2,3 < TMH 1,4,5,6,7) [63]. In addition, those authors find that a particular lipid tail can act as a stable intermediary connecting TMHs 2, 3 and 4 through interaction with hydrophobic TMH residues on the protein surface. They suggest that this lipid molecule may constitute part of the GnRHR structure determining receptor stability and dynamic [63].

A peculiar case of lipid interaction has been described for the cannabinoid CB$_1$ receptor embedded in a one-component 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) membrane system

and monitored during 105 ns [62]. This study shows that TMH5 and TMH6 are rather loosely packed in the middle of the helical bundle due to the kink of TMH6 induced by P6.50 of the CWxP motif. At this location, hydrophobic residues form a hydrophobic pocket, which is quickly occupied by a lipid tail and establishes a firm interaction throughout the entire simulation. Interestingly, the lipid tail penetrates into the receptor as deep as approximately 10 Å reaching the core of the binding pocket. Based on this finding, it has been proposed that lipids may have a potential role in ligand binding [62]. In fact, that a lipid molecule can intercalate inside the ligand-binding pocket has been recently captured in the X-ray structure of the A$_{2A}$ adenosine receptor (PDB 4EIY) [84].

A first attempt in studying interaction of saturated/unsaturated lipid species with rhodopsin has been undertaken by Feller *et al.* [61] using a mono-component membrane of 1-stearoyl-2-docosahexaenoyl phosphatidylcholine (SDPC, see Fig. (**1**)), a lipid molecule containing a completely saturated (stearoyl-) and partially unsaturated (docosahexaenoyl-) tail. They observed in a relatively short simulation (12.5 ns) that there is a preference for unsaturated lipid chains of SDPC, namely ω-3 docosahexaenoyl chains, to interact with the protein thereby excluding contacts with saturated stearoyl chains of SDPC [61]. This work supported the view that at least some of the observed lipid-dependent effects on rhodopsin known at that time [85] arise from direct molecular interactions.

In addition, the effect of the lipid saturation degree and chain length on structural features of rhodopsin has been addressed in a systematic study by Cordomí *et al.* [65]. The receptor was studied in pure one-component lipid bilayers of saturated lipids (1,2-dimyristoyl phosphatidylcholine (DMPC), and DPPC) and unsaturated lipids (POPC, and 1-palmitoyl-2-linoleoyl phosphatidylcho-

line (PLPC)). The evolution of the Lenard Jones (LJ) contribution to the lipid-protein interaction energy along individual 20 ns MD trajectories for each lipid type was monitored. The magnitude of the LJ contribution can be used to evaluate contact surface and hydrophobic matching between the protein and the bilayer. Comparison of the LJ contribution in different lipid systems shows that LJ contribution correlates with the chain length and with the presence of double bounds, which the authors associate to a better matching of the acyl chains to the protein. The energetically preferred binding of unsaturated lipids to rhodopsin is consistent with the work of Feller *et al.* [61].Those observations were reproduced by Pitman *et al.* [66] studying rhodopsin in a ternary membrane mixture of SDPC, 1-stearoyl-2-docosahexaenoyl phosphatidylethanolamine (SDPE) and cholesterol (2:2:1). In addition, they found that the unsaturated docosahexaenoic acid (DHA) forms contacts deep inside the protein, suggesting that this kind of protein-lipid interaction could contribute to the observed destabilization of rhodopsin when placed into a DHA-rich environment. In a continuation of this study, the same group accumulated 2.6 µs of rhodopsin evolving in an identical three-component membrane system, which supported frequent DHA-rhodopsin interactions on the 100 ns time scale [67]. Fig. (**5a**) highlights some receptor regions, in particular TMH3-4, TMH1-7 and TMH6, which interact preferentially with DHA chains. Also saturated chains (stearic acid, STEA, Fig. (**5b**)) and cholesterol Fig. (**5c**) are reported to bind to the rhodopsin. Unsaturated STEA preferably interacts with regions in TMH3-4, TMH6-7 and TMH5 whereas cholesterol forms frequent interactions with receptor regions TMH1 and helix 8 (H8), TMH6-7 and TM3-4. Another important outcome of this study is that the DHA-receptor interaction is found to be specific whereas interactions of STEA and cholesterol with the receptor seem to be relatively non-specific. The preferred binding of unsaturated lipid chains such as DHA to rhodopsin is, in fact, consistent with recent NMR experiments [86] and possibly entropically driven [68]. All types of chains (saturated/unsaturated) experience an entropic penalty when associating with the protein. However, this penalty is far lower for unsaturated ω-3 chains than saturated chains [68]. The final implication of the authors is that that such specific DHA-rhodopsin interaction, which involves grooves between receptor´s helices, can weaken the interhelical packing which in turn affects rhodopsin stability, kinetics and function [67].

Also, the type of lipid head groups (see lipid structure in Fig. (**1**)) affects the conformational states of rhodopsin as demonstrated by Alves *et al.* using plasmon-waveguide resonance spectroscopy [87]. Accordingly, photolysis of rhodopsin in the presence of a phosphatidylethanolamine (PE) head group favors the activated MII state of rhodopsin compared to a phosphatidylcholine (PC) head group. However, to our knowledge, no computational study has addressed such head group effect on functionally linked rhodopsin conformations. Grossfield *et al.* state that it could be difficult to entirely capture such head group effect in a ternary mixture of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE) and cholesterol by a computationally approach since simulation times of 100 ns might not be sufficient to allow large-scale lateral reorganization of the membrane, i.e. allowing the preferentially segregation of one head group at the protein surface [67]. Nevertheless, in this scenario, some insight at the molecular level of the head group effect could be obtained by studying and comparing a simplified system of rhodopsin in a pure POPE membrane system with a pure POPC for which no lateral re-organization needs to be taken into account.

Several experimental studies have demonstrated that cholesterol plays also an important role on rhodopsin function [77-79, 88, 89]. In this context, cholesterol has been shown to stabilize the inactive receptor states in retinal rod cells, thus reducing signaling efficiency. At a molecular level, the aforementioned computational work showed only a non-specific binding of cholesterol when com-

pared to DHA tails of the SDPC [66, 67]. Khelashvili *et al.* eventually tackled the open question about the potential role of cholesterol in modulating rhodopsin function in a simulation of 1.6 µs using a ternary membrane mixture of SDPC, SDPE and cholesterol (2:2:1) [69]. Calculation of the spatial distribution of cholesterol helped to identify three regions of the receptor which maintained close contact to cholesterol during microsecond dynamics: (i) the extracellular end of TMH7 [69] which is in agreement with high-density sterol area from electron microscopy data [90, 91], (ii) intracellular parts of TMH1-2 and 4 [69], a region suggested in a X-ray crystallography structure of the β₂-adrenergic receptor [92, 93], and (iii) intracellular ends of TMH2 and 3. Through correlation analysis, the authors connected local effects of cholesterol to structural rearrangements relevant for GPCR function. The authors' final conclusion is that direct cholesterol-receptor interactions with residues in the extracellular end of TMH2-TMH3 bundle and the intracellular side of the TMH1-2-4 network modulates a GPCR microdomain consisting of TMH1-TMH2-TMH7-H8, which is known to be critical for GPCR activation [94-97].
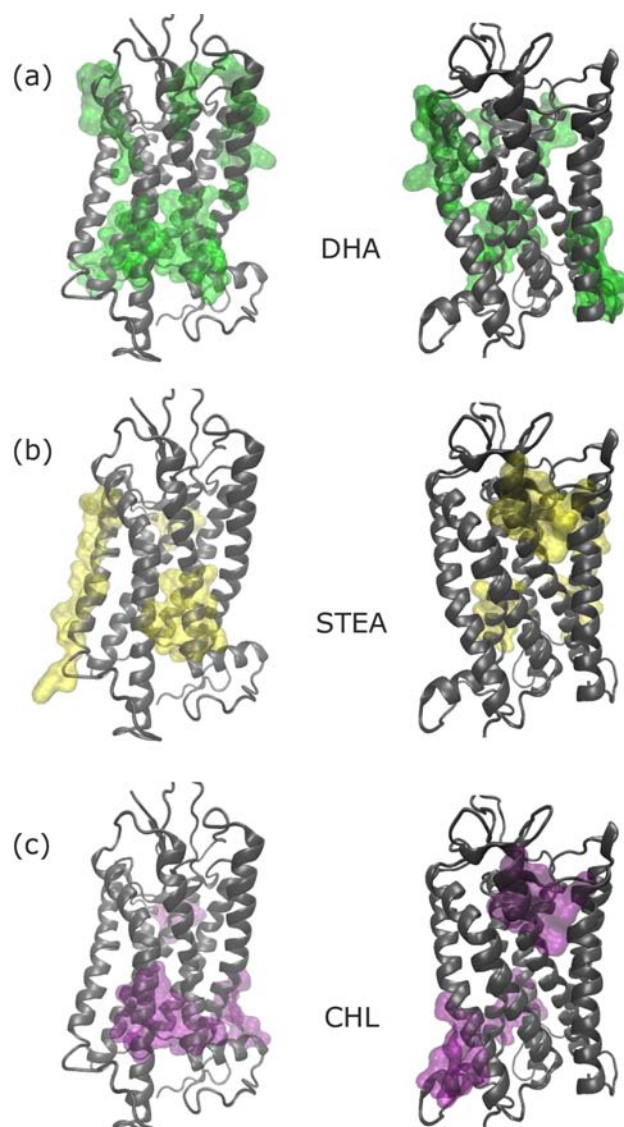

Fig. (5). Direct lipid contacts depicting residue groups (transparent surface) which interact with (**a**) docosahexaenoic acid (DHA), in green, (**b**) stearic acid (STEA), in yellow, and (**c**) cholesterol (CHL), in purple, identified in microsecond time scale simulations [67].

Finally, Lyman *et al.* studied the adenosine A_{2A} receptor with and without the antagonist ZM241385 in a cholesterol-free POPC membrane collecting a total simulation time of approximately 3 μs [64]. According to their simulation outcome, the A_{2A} receptor showed to be stable in the presence of the antagonist but instable when absent. Briefly, the authors find that receptor instability in the absence of the ligand is characterized by a rapid and dramatic opening of the binding pocket in which in particular TMH2 undergoes a significant conformational change. As a result, a gap created between TMH1 and TMH2 allows the entrance of a lipid head into the empty and open binding site remaining there for the rest of the simulation. The location of this lipid entrance is part of a region which has been earlier predicted [67] and later observed [93] to be a cholesterol binding site between TMH1, 2, 3 and 4, the cholesterol consensus motif which requires three residues to interact with the cholesterol molecule. Interestingly, placing two cholesterol molecules into the cholesterol binding site resulted in a stabilization of the receptor, in particular of TMH2, preventing lipids to enter the binding pocket. The authors have suggested that the observed cholesterol-dependent A_{2A} receptor stabilization could explain the experimental finding that the adenosine A_{2A} receptor activates only in the presence of cholesterol and agonist [98].

Posttranslational Lipid Modification

Numerous GPCRs including rhodopsin [99], CB_1 receptor [17], β_2-adrenergic receptor [100], human prostacyclin receptor [101], thyrotropin-releasing hormone receptor [102], the human prostanoid thromboxane A_2 receptor [103] and the chemokine CCR5 receptor [104] are found to be post-translationally modified by palmitic acid. This palmitoylation is mainly found in H8, a domain that follows the 7TM helical bundle on the C-terminal of GPCRs see Fig. (**6**).

Different functions have been ascribed to a palmitoylation in H8: (i) to act as lipid anchor for GPCR driving the subcellular location and diffusion properties of GPCRs [104], (ii) to be required for receptor internalization [103] and (iii) to modulate G protein signaling [17, 100, 103, 105, 106]. Computationally, Olausson *et al.* addressed this question for rhodopsin by simulating rhodopsin in a

2:2:1 mixture of SDPE, SDPC and cholesterol analyzing a total simulation time of 4.3 μs for 26 individually generated membrane systems [70]. They studied two different palmitoylation sites in close proximity (Cys322 and Cys323) of H8 Fig. (**6**). Extensive sampling in a realistic membrane environment revealed that polyunsaturated fatty acids, such as the DHA surround the palmitoylation more frequently than saturated fatty acids such as stearoyl acid (STEA) [70] which is in agreement with earlier findings that rhodopsin is favorably solvated by unsaturated acids [61, 67]. However, this also suggests that the palmitoylation tail, a completely saturated acid chain, does not establish the more preferred interaction with saturated phospholipids (STEA). Moreover, the authors find, by calculating lipid order parameters, that the Cys322-palmitoylation (closer to the protein) is less ordered due to adopting specific kinked conformations by frequently interacting with TMH1 of rhodopsin. In contrast, the Cys323-palmitoylation is higher ordered adopting less kinked conformations as a result of a reduced stabilizing interaction with rhodopsin. Based on this findings the authors question the common notion that palmitoylation acts mainly as a non-specific anchor. In particular, the frequently observed C322-palmitoylation contacts with transmembrane helices could potentially stabilize or destabilize functionally linked rhodopsin states. In contrast, the authors suggest for the C323-palmitoylation, which exerts less protein contacts but more membrane lipid contacts, a potential function as lipid anchor and determinant for diffusion properties of the rhodopsin receptor as well as for subcellular localization [70]. A role of S-palmitoylation of H8 in the modulation of protein diffusional mobility and in the receptor localization was also demonstrated in cannabinoid CB_1 receptor by fluorescence recovery after photobleaching (FRAP) [17].

Unspecific Membrane Effects on GPCRs

Unspecific effects on functionally linked conformational GPCR states can be driven by bulk properties of membranes such as membrane thickness among others (see section on modeling the biophysical properties of membrane lipids). One particular phenomena of interest is the hydrophobic mismatch, defined as the difference
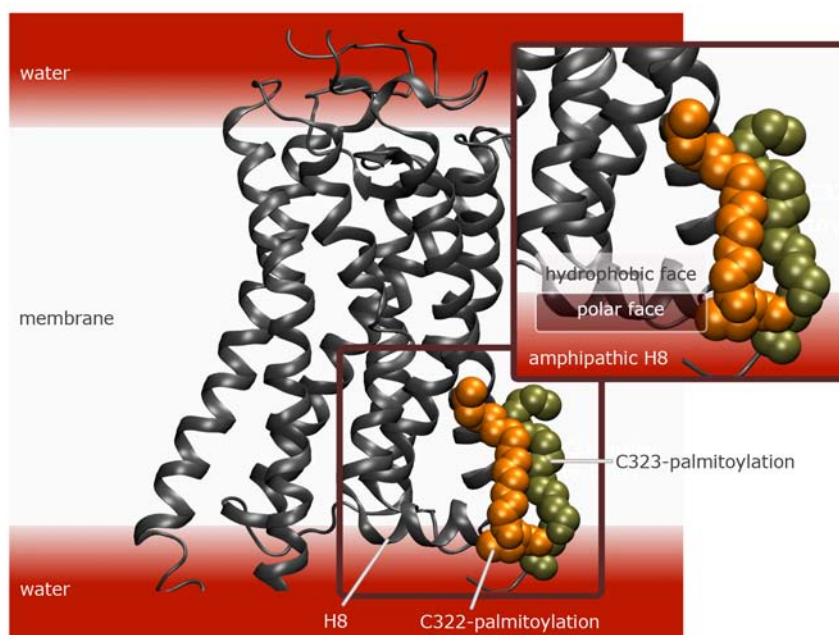

Fig. (6). Posttranslational palmitoylation at helix 8 (H8) of rhodopsin showing a C322- (orange spheres) and C323-palmitoylation (tan spheres). The inset depicts the amphipathic properties of helix 8 (H8), highlighting its hydrophobic and polar face enabling a parallel location to the membrane surface in a polar/hydrophobic environment.

between the length of the hydrophobic part of a transmembrane protein and the equilibrium hydrophobic bilayer thickness see Fig. (**7**) [107]. An optimal membrane thickness enables the GPCR to match the hydrophobic outer surface of the 7TM helical bundle to the hydrophobic lipid chains of the membrane bilayer Fig. (**7b**). An alteration of the membrane towards a smaller thickness would provoke an energetically unfavorable exposure of hydrophobic side chains to water Fig. (**7a**). An inverse situation in which polar GPCR residues are exposed to hydrophobic lipid side chains would occur as the membrane thickness increases Fig. (**7c**).

In this respect, it was suggested that membrane thickness could deform and adjust to the length of the hydrophobic transmembrane helices in order to avoid an energetic penalty (theory 1) [108, 109]. This theory 1 is based on the fact that acyl chains of fluid lipid phases are considered disordered and flexible, which may allow them to expand to match the hydrophobic length of the protein or squeeze if needed [108]. Another theory (theory 2) is that an optimal match between membrane thickness and the hydrophobic GPCR outer surface can be reached by an adjustment of the membrane-incorporated protein [109, 110] mediated by e.g. flexing and tilting of transmembrane helices as suggest for the lactose permease [111]. Therefore, when hydrophobic mismatch occurs, local reorganization of the lipid takes place in order to match the hydrophobic region of the transmembrane protein, thus inducing curvature in the membrane [107, 112]. Applied to GPCRs, the mismatch-driven membrane adjustment to 7TM helical GPCRs has been addressed by simulating rhodopsin in four different membrane systems varying chain length and saturation degree (DMPC, DPPC, POPC, and PLPC) for 20 ns each [65]. On one hand, the authors observed that, in dependence on the hydrophobic mismatch between lipid and protein, the lipids are able to accommodate to the protein surface (hydrophobic matching) [65], which is consistent with theory 1. On the other hand, the study shows that kinks of individual helices and the secondary elements of the TMH remain relatively unaffected by the lipid environment, whereas rather the tilt of the whole protein is affected [65], thus giving also importance to theory 2. An even more detailed insight into the dynamics and energetics of a mismatch-driven membrane adjustment to 7TM helical GPCRs has been obtained in an elegant study [71] combining the elastic theory of membrane deformations [113, 114] with atomistic MD simula-

tion (accumulated simulation time > 2 μs). Basically, this approach takes into account two key contributions to the energy penalty of a hydrophobic mismatch: (i) membrane-deformation energy penalty and (ii) residual hydrophobic exposure energy penalty which corresponds to the surface area of residues exposed to unfavorable hydrophobic-polar interaction. Four different membranes systems (S1-4) of pure monounsaturated PC membranes of different thickness (carbon C14 to C20) as well as a ternary mixture (S5) of SDPC, POPC, and cholesterol (7:7:6) were studied for two different GPCRs: rhodopsin in system S1-5, and the serotonin $5HT_{2A}$ receptor just in system S5. This study computationally shows that, around the transmembrane protein, thicker bilayers (monounsaturated PC membranes with carbon $\geq$ C20 and 7:7:6 mixture of SDPC/POPC/Cholesterol) locally become thinner whereas thinner bilayers (monounsaturated PC membranes with carbons < C20) become thicker. This indicates that membrane deformation/curvature is a mechanism to reduce hydrophobic mismatch between the hydrophobic length of the protein and the bilayers hydrophobic thickness [71] which is in agreement with theory 2. However, the deformation is limited as it leaves hydrophobic exposure of transmembrane domains to a certain extent in all systems. Remarkably, those authors suggest that the pattern of such residual hydrophobic exposure around the receptor is linked to the type of the bound ligand [71, 72]. Simulated complexes of the $5HT_{2A}$ receptor with the full agonist serotonin show thinner membranes around TMH4 and TMH6 then a $5HT_{2A}$ complex with the inverse agonist ketanserin. On the other hand, a thicker bilayer is found at TM1 for partial agonist LSD when compared to serotonin and ketanserin; thus yielding differential ligand-dependent hydrophobic exposure between receptor and membrane. Interestingly, the authors propose that one possible mechanism to reduce this energy penalty for residual exposure is the formation of receptor-receptor complexes in which such unfavorably hydrophobic exposed regions interact with each other. Provided that hydrophobic mismatch is a driving force for receptor oligomerization, this finding suggests that ligands can regulate the extent of GPCR oligomerization as well as influence which TMH participates in the dimerization interface [71, 72]. In fact, the authors' conclusion of ligand-sensitive GPCR oligomerization is supported by studies for the dopamine D_2R [115], the $5HT_{2C}$ receptor [116] and the β2 adrenergic receptor [117].
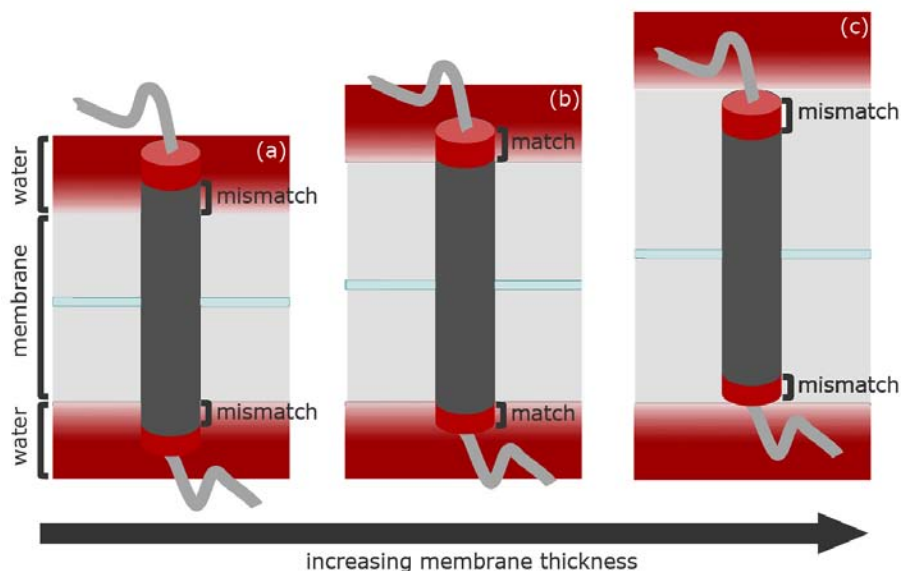

Fig. (7). Hydrophobic mismatch of a single transmembrane domain in dependence of the membrane thickness. (**a**) The hydrophobic transmembrane length (grey) contacts unfavorably the polar region (red) of its environment which consists of polar heads of the membrane and water molecules. (**b**) Polar (red) / hydrophobic parts (grey) of the transmembrane domain matches the polar environment (red) and the lipid environment (grey). (**c**) Polar protein regions (red) contact unfavorably hydrophobic membrane regions (grey).

Through such unspecific effects, lipids can also affect the structure of specific GPCR domains. A very recent computational study observed a hydrophobic mismatch effect on the metabotropic glutamate receptor 2, a class C GPCR [118], inducing different conformational states of the H8 domain. H8 is a very peculiar structural domain in GPCRs with a polar and a hydrophobic face that allows this domain to adopt an approximately planar position in between the hydrophobic membrane and the polar intracellular region Fig. (**6**, inset). The important functional role of H8 in G protein-coupling [119] and β-arrestin binding [120] has been experimentally demonstrated for class A GPCRs. Bruno *et al.* [118] have very recently studied the unspecific effect of lipids on the H8 domain of the metabotropic glutamate receptor 2 by extended MD simulations. In agreement with experimental data, they were able to simulate a realistic membrane environment in which the presence of cholesterol has a condensing effect on the membrane thickness when compared to a cholesterol-depleted system. Based on significant differences in membrane thickness, the authors show that in a cholesterol-rich environment the H8 domain nicely match the amphipathic surrounding Fig. (**6**, inset). In contrast, they show how in the thinner and cholesterol-depleted system the H8 domain is exposed to a purely polar environment. Thus, the authors highlight the increased structural flexibility of GPCR H8 domain by adopting different conformational states driven by a hydrophobic mismatch. This study illustrates that such membrane-thickness-related phenomena can be computationally captured applying modern molecular dynamics simulations.

Another interesting type of mismatch can occur between post-translational 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 post-translational receptor modification as it has been computationally observed for the aforementioned palmitoylated rhodopsin receptor [70]. Olausson *et al.* found that lower order parameters S_{CD} (for details on computing S_{CD} see previous section on modeling the biophysical properties of membrane lipids) of the posttranslational-linked palmitoyl chains, combined with the smaller number of methylene groups compared to the lipid chains (STEA and DHA) of the membrane, results in an energetically disfavored mismatch of approximately 3 Å to the center 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.

Challenges in Simulating Realistic Lipid Effects on GPCR Function

To what extent can we simulate specific and unspecific membrane lipid-dependent GPCR properties? According to the herein reviewed studies (listed in Table **1**), simulations of GPCR embedded in a membrane can give a general insight into specific membrane interactions such as lipid accessibility to TMH domains [60] which is basically driven by the GPCR architecture. Thus, more buried TMHs (e.g. TMH3) are less accessible than exposed TMHs (e.g. TMH6). More importantly, such experiments can also point out the molecular details of specific lipid binding as observed for unsaturated lipids at rhodopsin [61, 65, 66] and experimentally supported by NMR experiments [86]. Even the ability of lipids to intercalate into the binding pocket (experimentally seen in [84]), which may have consequences for ligand binding and receptor stability, has been observed for the cannabinoid CB_1 receptor [62], the rhodopsin receptor [66] and the adenosine A_{2A} receptor [64]. All in all, specific lipid-GPCR interactions are observable to a certain extent using all-atom systems of GPCRs embedded an explicit membrane environment. In this line, a very recent and interesting study [121] provides both computational and experimental proof of

an unprecedented binding specificity between a transmembrane domain and sphingolipids, a key component of cellular membranes.

On the other hand, the molecular details of unspecific membrane effects which are driven by bulk properties of membranes such as membrane thickness are also accessible to a certain extent using molecular dynamics simulations. In this respect, it has been shown that (i) membrane deformation [65, 71, 72] and (ii) the tilt of the entire protein [65] could be valid mechanisms to reduce an unfavorable mismatch between membrane thickness and the hydrophobic GPCR outer surface. In addition, an unspecific cholesterol effect on GPCR conformational stability, ascribed to a cholesterol-condensing membrane effect, has been computationally observed and may play a role in experimentally observed cholesterol-dependent GPCR functioning [118].

However, there are general key aspects limiting the modeling of realistic membrane effects on GPCRs. The first key aspect refers to the availability of structural information on the GPCR of interest being crucial to obtain reliable information on lipid-GPCR interaction. The past 4 years have seen remarkable progress in GPCR crystallography making high-resolution structures now available for rhodopsin, adrenergic, adenosine, chemokine, dopaminergic, histaminergic, muscarinic ([122] and included references) and very recently the sphingosine 1-phosphate receptor [123] and the opioid receptors [124-126]. In this scenario, also homology modeling of closely-related GPCRs becomes easier and the resulting structures are more reliable. A recent assessment has demonstrated that a sequence similarity of just 37% allows accurate predictions [127, 128] whereas one has to take care when modeling more distantly-related GPCRs [128].

A second important aspect in modeling realistic membrane effects is the quality of the force field. Besides the membrane receptor which is standardly well described by common force fields, different types of lipids with relevance for the scientific problem have to be accurately described too. In this respect, the CHARMM-GUI Membrane Builder [129] presently provides a list of 32 lipid molecules differing in length, saturation degree and polar head groups to build heterogeneous lipid bilayers [130]. Using those force field parameters, realistic biophysical parameters can be computationally reproduced as demonstrated in simulations of pure membrane systems [35, 50, 51, 53, 54, 131-138], as well as in GPCR-membrane systems [118] capturing membrane-thickness-mediated alterations of receptor conformation. Yet, common force fields do not cover some key lipid components present in biological membranes and membrane microdomains such as sphingolipids. Thus, when simulating biologically relevant structures, such as membrane microdomains [50, 139, 140], one needs to parameterize membrane lipids such as sphingomyelin. Some key structural differences between phospholipids and sphingomyelin are well represented by these customized force fields [132, 137, 141-144].

A third important aspect is the length of the simulation time in terms of sufficient sampling for drawing meaningful conclusions. Usually, membrane construction protocols assume that lipid types are randomly distributed in the heterogeneous membrane. Therefore, prior to the analysis of lipid-receptor interactions of a GPCR in a heterogeneous membrane, the researcher needs to consider whether lateral reorganization of lipids is present. When this is the case, one needs also to check whether such lipid reorganization has converged, i.e. if certain lipids segregate from the rest of the bilayer and/or their repositioning around the receptor. For pure phospholipid membranes or binary systems (i.e. one phospholipid plus cholesterol) typical convergence of most biophysical properties occurs within 5-20 ns [39, 50, 139]. However, more complex systems such as ternary membrane mixtures or membrane-protein systems can show a lack of convergence even during the first 100 ns [145]. Since it is extremely difficult to evaluate convergence from a single long trajectory, an improved approach is to average the computa-

tional outcome over numerous trajectories of individually generated GPCR-membrane systems [67]; i.e. in each system the receptor is exposed to a different randomly generated and dynamically evolving membrane environment. Still, one should always consider these results as a tendency and try to increase the statistical power of the simulated data by running more than one replicate each starting from individual starting coordinates.

In summary, significant challenges can be expected when simulating GPCRs in more native (multi-component) lipid bilayer systems, to understand the fine details of lipid-mediated effects on GPCR functionality. The reason, as above mentioned, is the intricacy of reaching a fully converged lipid organization through sufficient sampling time due to current limitations in computational power. Nevertheless, these approaches are becoming a key complement of experiments, where molecular dynamics simulations can help confirming the specificity of certain lipid-protein interactions.

MEMBRANE-DEPENDENT OLIGOMERIZATION OF GPCRS

The classical paradigm for understanding GPCRs has been that they exist as monomers, the corresponding ligand binding and signal transduction processes based on this assumption. However, this paradigm has been challenged over the last decade, due to compelling evidence that GPCRs can form both functional hetero- and homo-oligomers [146-152]. A wealth of biophysical and biochemical techniques have established this fact (reviewed in reference [153]) and include earlier studies of radioligand binding, cross-linking and radiation activation, more recently complemented by co-immunoprecipitation, Fluorescence Resonance Energy Transfer (FRET) and Fluorescence Correlation Spectroscopy (FCS), Bioluminescence Resonance Energy Transfer (BRET) and Bimolecular Fluorescence Complementation (BiFC). A notable breakthrough was the discovery of rhodopsin dimers [154-156] using Atomic Force Microscopy [150], from which the first three-dimensional molecular model of rhodopsin oligomerization was proposed [157]. The latest milestone has been the determination of the first X-ray crystal structure of two non-rhodopsin GPCR dimers, that of the chemokine CXCR4 receptor [158] and the μ-opioid receptor [124] leading to a wider acceptance of the GPCR oligomerization concept by the scientific community.

Apart from the fact that crosstalk between GPCR monomers is involved in some diseases such as schizophrenia and Parkinson's disease [159], the exact physiological relevance of GPCR oligomerization is not well understood. Although GPCR monomers are capable of activation, dimerization has been shown to increase efficiency [156]. Furthermore, conformational changes at the TMH4 dimer interface has been related to the GPCR activation mechanism [115], whilst selective agonist activation for opioid heterodimers over homodimers has also been reported [160]. These studies attempt to answer a number of questions that stem from the observation of oligomerization. Specifically, are both monomers in a dimer activated for signal transduction or is there asymmetric activation [161]? If the latter mechanism is possible, can a ligand, by binding to one monomer, induce a conformational change and activation of the other monomer (trans-activation)? Could control of such receptor properties be achieved by selective agonists and/or antagonists that favorably bind one subunit?

Furthermore, as activation efficiency may be related to oligomerization, understanding the transient stability of oligomers under various environmental conditions becomes of paramount importance. It is here that the role of the membrane in determining GPCR function is highlighted. As mentioned earlier, cell membranes contain microdomains as lipid rafts and caveolae which are characterized by a peculiar lipid composition. Such domains seem to spatially organize specific G-proteins at the membrane, leading to a compartmentalization that could facilitate or prevent interac-

tions with their receptors and/or effectors [162]. The membrane composition affects the diffusivity of proteins within it, thus impacting the diffusional properties of GPCR monomers [163]. The slower diffusion properties of GPCRs in lipid rafts may act to enrich or deplete the number of GPCRs within lipid rafts [164]; which may in turn enhance or reduce GPCR dimerization. Moreover, dimerizing proteins can spontaneously self-organize into clusters of higher-order structures via diffusion-limited partner switching [165].

The bulk physical properties of the membrane itself may affect oligomerization of GPCRs. An example stems from the consequences of hydrophobic mismatch between hydrophobic membrane and transmembrane region thickness Fig. (**7**), which causes local reorganization of the lipid around the protein in order to match the hydrophobic region of the transmembrane protein, thus inducing curvature in the membrane [107, 112]. It is this curvature that in turn creates a strain field in the lipid bilayer and which gives rise to a membrane-mediated attractive potential between transmembrane proteins, explained by a simple elasticity theory [166], leading to oligomerization [107, 112]. An alternative contribution may arise from the fact that membrane deformation/curvature is limited, leaving residual hydrophobic exposure [71] between receptor and membrane. Those unfavorably exposed hydrophobic regions of one monomer may interact with unfavorably exposed regions of a second monomer thus driving GPCR dimerization [71].

Varying hydrophobic bilayer thickness also changes the mean separation between nearest monomer neighbors as the lipid adjusts to match the transmembrane hydrophobic region [167]. Experimental insertion of α-helices into membranes indicates that lipid packing around helices is different to that of larger proteins [168]. However, the tilt angle of α-helices, which has been linked to activation of GPCRs [169], has been shown to affect the hydrophobic mismatch [170] which in turn leads to helix self-association [171]. Thus, in addition to the specific compartmentalization conferred through microdomains, varying bilayer hydrophobic thickness, protein-lipid interactions, the curvature and hydrophobic properties of the membrane itself all modulate oligomerization [107, 112]. Finally, one can hypothesize that GPCRs amplify or attenuate their own activation signal by manipulating the biophysical properties of the membrane through helix-tilting, adjustment of hydrophobic mismatch, subsequent attraction of other GPCRs and the formation of oligomers that result in enhanced response.

Computational Progress and Challenges in Predicting Physiologically Relevant Dimers/Oligomers

Although crystal structures of GPCR monomers have existed for a number of years [80, 172], those of dimers are not numerous and only emerged relatively recently, including the CXCR4 chemokine [158], μ-opioid [124], dark rhodopsin [99], opsin [173] and κ-opioid [125] receptors. Without existing crystal structures of dimers at a variety of interfaces, investigations of oligomerization properties of GPCRs have thus far relied on a number of structure-based computational tools such as protein-protein docking, coarse-grained and all-atom molecular dynamics simulations, normal mode analysis as well as a number of sequence-based tools (reviewed in reference [174]). Here we focus specifically on the progress made by coarse-grained and all-atom simulations in the membrane environment.

One of the greatest challenges has been choosing which dimeric or higher order structure to use. A number of model structures have been built [115, 118, 161, 175, 176] (reviewed in [59, 174]) for exploring various dimeric interfaces, the principal one being the symmetrical TM4-TM5 interface but also the TM5-TM6 and TM1-TM2 interfaces [177].

All-atom molecular dynamics simulations are then useful to explore the flexibility of the oligomeric system and its relation to

function. For example, several studies have shown that a significantly different conformational space is sampled in one monomer as compared to another [161, 178]. This has led to the suggestion that asymmetry in conformational change is related to function in the oligomeric state.

The transience and affinity of GPCR dimerization is of great interest and relates directly to the differential favorability of possible interfaces. Coarse-grained simulations have explored the free energy of association of the δ-opioid homodimer using umbrella sampling methods which calculate a potential of mean force (PMF) as a function of monomer centre-centre distance [179]. This provides an estimation of the dissociation rate constant (K_D); the association rate constant (k_{on}) can be estimated using diffusion-limited reaction rate theory [180] and the corresponding dissociation rate constant (k_{off}) can be calculated from: $k_{off} = k_{on}/K_D$. Such a method has provided insight into the favorable interfaces involving the TMH4 helix. Importantly, while reviewing this manuscript three different papers studying GPCR oligomerization process by molecular simulations appeared in the literature. On the one hand, Johnston *et al.*, using again both coarse-grained and all-atom simulations, show that TMH1/8 interfaces of β1- and β2-adrenergic homodimers are more stable than TMH3/4 interfaces [181]. On the other hand, Periole *et al.*, through coarse-grained simulations and umbrella sampling calculations, conclude that upon oligomerization of rhodopsin a preferred dimer interface is established between TMH1/2 and TMH8 [182]. Lastly, another coarse-grained study shows the importance of different lipid organization on the hydrophobic mismatch between GPCRs and lipid membrane, thus, potentially impacting oligomerization [183].

Both coarse-grained and all-atom molecular dynamics approaches have advantages and drawbacks. All-atom molecular dynamics provide the necessary atomic level detail required to understand specific interactions at the dimer interface; however an analysis of the convergence of long GPCR simulations shows that the microsecond timescale is probably not sufficient to accurately characterize properties of interest [145, 184]. Coarse-grained simulations can access the required timescale to see reversible GPCR associations and self-assembly; they have also explored the relation of the lipid bilayer thickness to dimerization. However, they lack the detail required to accurately discriminate between near-diffusional and atomic-level contacts. Furthermore, the role of lipids in modulating the energetics and kinetics of dimerization, and thus the subsequent function of GPCRs, is still largely unexplored.

Towards An Atomistic Understanding of Membrane-Dependent GPCR Oligomerization

The deposition of a handful of crystal structures of GPCR dimers in the Protein Data Bank marks a noticeable change in our capacity to investigate the process of GPCR dimerization using MD simulation methods. One recently deposited structure is that of the CXCR4 receptor dimer see Fig. (**8a**). Such structures provide atomic level references for developing and validating computational methods to discriminate between possible alternate GPCR interfaces. We envisage the forthcoming understanding of the energetics and kinetics of dimerization in the context of both translational and rotational diffusion as well as the role of lipid constitution and thickness in modulating such properties. Quantitative and qualitative methods that achieve this will need to be able to recapture the atomic level detail of the specific interactions in such existing crystal structures.

An ideal ab-initio approach to understand dimerization would be to implement free rotational and diffusional exploration for two GPCR monomers within an MD simulation allowing multiple reversible binding/unbinding events. This would permit the energetic and kinetic description of dimerization in terms of the rotational and translation degrees of freedom of the corresponding monomers

and reveal the thermodynamic order of the dimeric interfaces. The role of the lipid could then be explored in terms of the effect on the energetics and kinetics of association and rotation. Filizola and co-workers [179] have investigated the free energy of the dimer in terms of the centre-centre distance (r) of the two monomers and the local angular reaction coordinates (θA, θB) of the TMH4 helix see Fig. (**8b**). Extending this concept to allow complete rotational exploration of each monomer, one can envisage an energetic landscape in the three-dimensional reaction coordinate space (r, θA, θB), allowing direct understanding of the rotation and translation dependent energetics. Such a landscape would also allow the thermodynamic effects of changes in the lipid constitution to be quantified. For example, it may emerge that different lipid constitutions favor different dimeric interfaces.

However, significant technological and possibly methodological challenges may need to be met in order to achieve such a quantitative treatment at the atomic-level. The spatial and temporal scale at which an event of interest happens determines the amount of computation that is required. Smaller systems are faster to simulate, processes that occur on a faster timescale require less simulation to obtain statistically relevant sampling. Given that the experimentally determined diffusion coefficient for GPCRs is 0.08 μm²/s [185], the timescale at which an encounter complex will form in a 15 nm² simulation box is of the order of 100 μs. Statistically relevant sampling leading to an energetic and kinetic description across rotational and translation degrees of freedom may thus require well into milliseconds of simulation time.

We compare this requirement to the current state of the art in MD. Typically, the speed of various MD codes and/or architectures on which they are run are provided in terms of the number of ns/day on a benchmark system such as the dihydrofolate reductase (DHFR) one, consisting of 23,000 atoms [186].

There are many MD codes as well as types of computational architecture on which they can run, ranging from scalable codes designed to run on large supercomputing facilities to codes optimized for use on smaller clusters. Various codes also make use of programmable graphical processing units (GPUs) [187] which greatly accelerate computation over central processing units (CPUs), whilst projects exist that harness distributed computational power (both CPU and GPU) from volunteer communities [186, 188]. Dedicated machine architectures for computing MD simulations have also emerged [189]. Such supercomputational facilities are now capable of the order of 10 μs/day of either continuous or aggregate computational sampling. However, construction of the chemokine CXCR4 receptor dimer system embedded in membrane and solvated with a reasonable number of water molecules results in a model of greater than 100,000 atoms see Fig. (**8c**) and is thus neither ideal for small- nor large-system optimized codes. Studies of diffusion-based monomer encounter within the membrane would require a significant initial separation and a significantly larger system size. This diminishes the speed of the simulation, we estimate to between 1-10 μs/day on state-of-the-art computational resources. Therefore, such an investigation is beyond the limits of current molecular simulation, although likely not for long.

On the other hand, a host of methodologies have been developed over the last two-decades that aim to determine binding free energies whilst overcoming the limitations imposed by lack of sampling (see review by [190]). Here, we suggest ways in which such methods may find future use in answering the challenging problem of GPCR dimerization. As mentioned before, umbrella sampling techniques have already been applied to coarse-grained GPCR dimer models [179]. To determine protein-ligand binding free energies, an umbrella potential can be applied at various intervals along a set of reaction coordinates and then the system sampled at each interval [191, 192]. Provided that sufficient overlap in sampling exists between proximate intervals, the PMF can be calculated
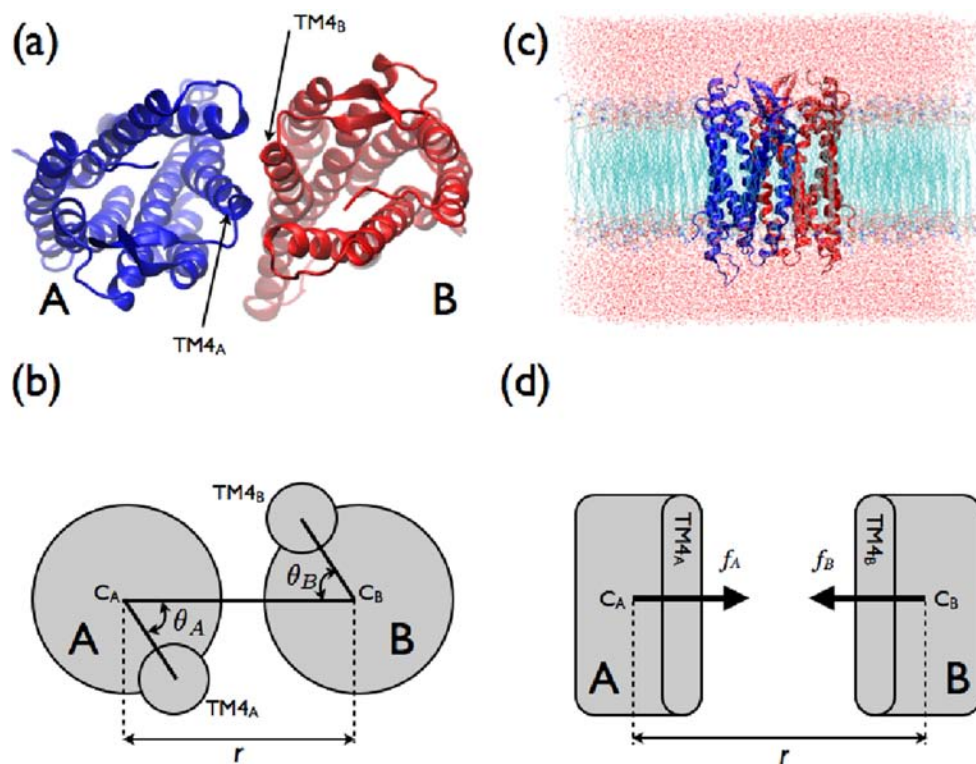
Fig. (8). Oligomerization of GPCRs. (**a**) Ribbon representation of the crystal structure of the chemokine CXCR4 homodimer [158]. Monomer A is in red, monomer B in blue. The dimerization interface involves interactions between the TM4 helix of each monomer. (**b**) Schematic (adapted from Provasi *et al.* [179]) showing possible reaction coordinates along which to investigate the possible dimerization interfaces of GPCR. These are the centre-centre distance of each monomer (r) and the angles, θA and θB that lie between the centre line and the corresponding centre of the TM4 helix of each monomer, A and B respectively. (**c**) Typical set up of an all atom molecular dynamics simulation required to model a GPCR dimer (in this case showing the chemokine CXCR4 dimer) containing a 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) membrane surrounding the dimer, water molecules and ions. The system size is over 123,000 atoms. (**d**) A schematic representation of a guiding-potential experimental set-up to facilitate GPCR interface exploration. The centers of each monomer feel a guiding potential that exerts a centrally directed force. This accelerates association beyond diffusion-limitations. Optimization of such a procedure in conjunction with other methods for accelerating conformational sampling and binding may prove useful in recapturing the energetics and/or kinetics of dimerization across different interfaces.

along the reaction coordinates, using methods such as the weighted histogram analysis method [193]. Metadynamics is another methodology that has gained ground [194, 195]. The method iteratively applies biasing potentials along designated reaction coordinates/collective variables that allow a system to explore conformational space more rapidly than in a free system. Once sufficient sampling is reached along the reaction coordinates, inverting the sum of the applied potentials allows reconstruction of the energetics along those reaction coordinates and thus quantification of the various energy basins. An interesting feature of such models is that predefined guiding potentials can be used in conjunction with the umbrella potentials in order to limit the degree of non-relevant sampling by either the protein and/or ligand [196] and then handled a posteriori in the ultimate calculation to obtain the overall free energy of binding.

Consider a system with two monomers that are initially separated by a substantial distance see Fig. (**8d**). A harmonic potential can be set up within molecular dynamics simulations such that a centrally directed force is exerted on each monomer resulting in faster association than just diffusion. If the applied potential is too soft, the acceleration in association is negligible compared with diffusion. In contrast, an overly hard potential will cause rapid association at the cost of inaccurate conformational sampling of the correct association interface. An optimization process is therefore likely in order to find the right balance between accuracy and acceleration. We propose that methodologies that make use of such a combination of features may prove useful in the near future.

CONCLUSION

GPCRs and membrane lipids are strongly interrelated, a fact that should be considered when modeling and interpreting the dynamics of GPCRs. Despite the scarcity of molecular dynamics studies where the heterogeneity of membrane lipids is well represented, today we can adequately model their biophysical properties. While basic structural parameters of model membranes such as the area per lipid, electronic density profile, chain order parameters, tilt angles or radial distribution functions correlate well with experiments, diffusional properties of membrane components cannot be approached in the same manner. The potential concerted diffusion between lipids and proteins points towards the importance of studying lipid-protein interactions when modeling transmembrane proteins. As we discussed in this review, MD studies of GPCRs embedded in more realistic membrane environment have revealed both the specific and unspecific nature of protein-lipid interactions. Thus, membrane lipid composition has a clear impact on GPCR structure through specific lipid interactions such as palmitoylation or cholesterol binding. On the other hand, lipids can also influence protein dynamics through unspecific effects such as the increased membrane thickness caused by cholesterol that ultimately leads to a hydrophobic mismatch.

Lipids not only affect GPCR individually but may enhance or diminish GPCR oligomerization. Coarse-grained and MD simulations have tried to elucidate both the exact interface of GPCR dimers and the effect of the membrane environment in such an impor-

tant event. Since GPCR dimers have only been crystallized very recently, except for rhodopsin, the computational understanding of GPCR oligomerization is still in its infancy. In the last part of this review we have discussed the use of particular methodologies where the combination of classic and new approaches could help studying lipid dependent GPCR dimerization.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

ABBREVIATIONS

BiFC	=	Bimolecular Fluorescence Complementation
BRET	=	Bioluminescence Resonance Energy Transfer
CPUs	=	Central Processing Units
diC$_{14:1}$PC	=	1,2-ditetradecenoyl Phosphatidylcholine
diC$_{16:1}$PC	=	1,2-dihexadecenoyl Phosphatidylcholine
diC$_{18:1}$PC	=	1,2-dioctadecenoyl Phosphatidylcholine
diC$_{20:1}$PC	=	1,2-diicosaenoyl Phosphatidylcholine
DHA	=	Docosahexaenoic Acid
DHFR	=	Dihydrofolate Reductase
DLPC	=	1,2-dilauroyl Phosphatidylcholine
DMPC	=	1,2-dimyristoyl Phosphatidylcholine
DOPC	=	1,2-dioleoyl Phosphatidylcholine
DPPC	=	1,2-dipalmitoyl Phosphatidylcholine
DSPC	=	1,2-distearoyl Phosphatidylcholine
FRET	=	Fluorescence Resonance Energy Transfer
FCS	=	Fluorescence Correlation Spectroscopy
GPCRs	=	G protein-Coupled Receptors
GPUs	=	Graphical Processing Units
H8	=	Helix 8
K$_D$	=	Dissociation Rate Constant
k$_{on}$	=	Association Rate Constant
k$_{off}$	=	Dissociation Rate Constant
MD	=	Molecular Dynamics
PA	=	Phosphatidic Acid
PC	=	Phosphatidylcholine
PE	=	Phosphatidylethanolamine
PI	=	Phosphoinositides
PLPC	=	1-palmitoyl-2- linoleoyl Phosphatidylcholine
PMF	=	Potential of Mean Force
POPC	=	1-palmitoyl-2-oleoyl Phosphatidylcholine
POPE	=	1-palmitoyl-2-oleoyl Phosphatidylethanolamine
PS	=	Phosphatidylserine
RDF	=	Radial Distribution Function
SCD	=	Deuterium Order Parameters
SDPC	=	1-stearoyl-2-docosahexaenoyl Phosphatidylcholine
SDPE	=	1-stearoyl-2-docosahexaenoyl Phosphatidylethanolamine
SOPC	=	1-stearoyl-2-oleoyl Phosphatidylcholine
STEA	=	Stearic Acid
TMH	=	Transmembrane Helix
7TM	=	Seven Transmembrane

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