Chapter 16
Interaction of Membrane Cholesterol with GPCRs: Implications in Receptor Oligomerization

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Abstract G protein-coupled receptors (GPCRs) are the largest family of proteins involved in signal transduction across cell membranes, and represent major drug targets in all clinical areas. Oligomerization of GPCRs and its implications in drug discovery constitute an exciting area in contemporary biology. In this review, we have highlighted the role of membrane cholesterol and the actin cytoskeleton in GPCR oligomerization, using a combined approach of homo-FRET and coarse-grain molecular dynamics simulations. In the process, we have highlighted experimental and computational methods that have been successful in analyzing different facets of GPCR association. Analysis of photobleaching homo-FRET data provided novel information about the presence of receptor oligomers under varying conditions. Molecular dynamics simulations have helped to pinpoint transmembrane helices that are involved in forming the receptor dimer interface, and this appears to be dependent on membrane cholesterol content. This gives rise to the exciting and challenging possibility of age and tissue dependence of drug efficacy. We envision that GPCR oligomerization could be a game changer in future drug discovery.

Keywords GPCR • GPCR oligomerization • Membrane cholesterol • Actin cytoskeleton • Homo-FRET • Coarse-grain simulation

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16.1 G protein-Coupled Receptors (GPCRs)

G protein-coupled receptors (GPCRs) are cellular nanomachines that comprise the largest and most diverse group of proteins in mammals, and are involved in transfer of information from outside the cell to the cellular interior [7, 40, 49]. GPCRs are typically seven transmembrane domain proteins and include >800 members which are encoded by ~5% of human genes [63]. Cellular signaling by GPCRs involves their activation by ligands present in the extracellular milieu, and the subsequent transduction of signals to the interior of the cell through concerted changes in their transmembrane domain structure [12]. GPCRs regulate physiological responses to a variety of stimuli that include endogenous ligands such as biogenic amines, peptides, glycoproteins, lipids, nucleotides, Ca^{2+} ions and various exogenous ligands for sensory perception such as odorants, pheromones, and even photons. As a consequence, GPCRs mediate multiple physiological processes such as neurotransmission, cellular metabolism, secretion, cellular differentiation, growth, inflammatory and immune responses. Since GPCRs play a central role in cellular signaling and are implicated in pathophysiology of several disorders [17, 20], they have emerged as major drug targets in all clinical areas [9, 16, 20, 21]. It is estimated that ~50% of clinically prescribed drugs and 25 of the 100 top-selling drugs target GPCRs [50, 57].

16.2 Role of Membrane Cholesterol in GPCR Function

Since GPCRs are integral membrane proteins, interaction of membrane lipids with them constitutes an important area of research in contemporary biology. In particular, membrane cholesterol has been reported to have a modulatory role in the function of a number of GPCRs. Extensive work has been carried out in case of GPCRs such as the serotonin$_{1A}$ receptor, the β_2-adrenergic receptor, and opioid and cannabinoid receptors. In all these cases, membrane cholesterol has been shown to regulate receptor function, dynamics and oligomerization. Details of these effects have been described in previous reviews [13, 22, 31, 34, 46, 52]. We therefore prefer to direct interested readers to these reviews.

Current understanding of the mechanistic basis of GPCR-cholesterol interaction appears to indicate that specific effects in terms of cholesterol binding to certain regions (sequences) of the receptor play a role in these changes [33, 36], although global membrane effects cannot be ruled out [37]. Some of these regions (such as the cholesterol recognition/interaction amino acid consensus (CRAC) motif) have been identified in GPCRs [23]. The involvement of these regions in regulation of GPCRs by membrane cholesterol is being investigated for various types of GPCRs by experimental [32, 48] and simulation approaches [51]. Detailed molecular dynamics simulations have revealed that cholesterol binding on GPCRs is weak and dynamic, with an occupancy time ranging between ns and μs. The emerging model regarding the energy landscape of cholesterol association with GPCRs corresponds

to a series of shallow minima interconnected by low energy barriers [52]. A consequence of such interactions is the conformational plasticity exhibited by GPCRs induced by membrane cholesterol [44].

16.3 GPCR Oligomerization: Pitfalls of Commonly Used Hetero-FRET Approach to Monitor Receptor Oligomerization

GPCR oligomerization is an interesting and exciting aspect of contemporary receptor biology since it is believed to be an important determinant for GPCR function and cellular signaling [1, 18, 26, 29, 38, 53]. Such oligomerization is implicated in proper folding of receptors, thereby providing the framework for efficient and controlled signal transduction. The potential implications of oligomerization are far reaching, specially keeping in mind the role of GPCRs as major drug targets [14]. Evidence of GPCR dimers or higher-order oligomers has been reported in the last few years [1, 11, 24, 35, 60] and implicated in receptor trafficking, signaling and pharmacology.

Oligomerization of GPCRs in live cell membranes has been studied extensively utilizing fluorescence resonance energy transfer (FRET) approaches such as hetero-FRET (FRET between two different fluorophores) and bioluminescence resonance energy transfer [26]. The major intrinsic complication of hetero-FRET measurements arise from the use of receptors conjugated to two different probes, and a lack of control in their relative expression levels, the so-called 'Bystander FRET' [6, 8, 28]. A source of possible error is the often misunderstood inverse sixth power distance dependence of FRET [6, 41]. Another complication arises from the 'bleed-through' problem [6, 41]. This is usually manifested by the emission of one fluorophore being detected in the photomultiplier channel for the second fluorophore, due to broad bandwidths and asymmetrical spectral profiles.

In contrast, homo-FRET (FRET between two identical fluorophores) is a simpler variant of energy transfer because it takes place between like fluorophores and therefore requires only a single type of fluorophore. Fluorophores with a relatively small Stokes' shift will have a greater probability of undergoing homo-FRET. In addition, homo-FRET measurements can provide an estimate of higher-order oligomerization [62], which is a serious limitation with hetero-FRET measurements. This is important, specially in the microheterogeneous membrane environment, where multiple types of oligomeric clusters can coexist. Importantly, homo-FRET is manifested by a reduction in fluorescence anisotropy, a parameter that is largely independent of the concentration of fluorophores [58]. Homo-FRET leads to depolarization of the emission because of the lack of correlation between the orientation of the initially photoselected donor and the secondarily excited molecules [25].

16.4 Role of Membrane Cholesterol and the Actin Cytoskeleton in GPCR Oligomerization: Homo-FRET Approach

In view of the advantages of homo-FRET, we previously utilized this approach to explore the oligomerization state of the serotonin$_{1A}$ receptor [11]. Homo-FRET was assayed by the increase in fluorescence anisotropy upon progressive photobleaching of the receptor, in which fluorescence depolarization due to energy transfer was prevented by photobleaching of FRET acceptors [59]. Our results showed that the initial anisotropy of serotonin$_{1A}$ receptors tagged to enhanced yellow fluorescent protein (EYFP) in control cells was significantly low (~0.22) compared to the fundamental anisotropy (r_o) of EYFP (0.38; from [4]) (see Fig. 16.1a). The observed depolarization of emission was attributed to energy transfer (homo-FRET) between receptor molecules of the oligomers. Fig. 16.1a shows that there is a steady increase in fluorescence anisotropy of serotonin$_{1A}$-EYFP receptors with progressive photobleaching, which is expected for a system undergoing homo-FRET.

We utilized a previously developed theoretical formalism for deducing the type of oligomers from such anisotropy enhancement upon photobleaching data [62]. This formalism relies on the difference between the extrapolated and predicted (0.38) anisotropy values at 100% photobleaching limit for predicting oligomeric state, such that, larger the difference greater the fraction of higher-order oligomers (see Fig. 16.1b). In other words, with increasing oligomerization, the extrapolated anisotropy shows higher deviation from the predicted (fundamental) anisotropy. The predicted variation of fluorescence anisotropy with increased photobleaching for a homogeneous distribution of monomers, dimers, trimers, and tetramers (assuming an anisotropy of 0.38 for monomers) is shown in the inset of Fig. 16.1b. Due to experimental limitation of achieving very high degree of photobleaching (low signal-to-noise ratio), we compared the linearly extrapolated anisotropy with the predicted anisotropy to infer the presence of higher-order oligomers (see Fig. 16.1b). On the basis of the observed increase in fluorescence anisotropy upon progressive photobleaching (Fig. 16.1a), and the analysis of data based on the difference between the extrapolated anisotropy and the predicted anisotropy (Fig. 16.1b), we proposed the presence of constitutive oligomers of the serotonin$_{1A}$ receptor [11].

Utilizing this approach, we explored the role of membrane cholesterol and the underlying actin cytoskeleton on the oligomerization status of the serotonin$_{1A}$ receptor. Our results showed that actin cytoskeletal destabilization led to a reduction in the initial anisotropy and increase in the difference between the extrapolated anisotropy and the predicted anisotropy compared to control conditions (Fig. 16.1a, b). This suggested increased contribution from higher-order oligomers under such condition. In contrast, cholesterol depletion led to an increase in initial anisotropy, and reduction in the difference between the extrapolated anisotropy and the predicted anisotropy relative to control (Fig. 16.1c). These results show that cholesterol depletion effectively reduced the population of higher-order oligomers. Taken together, these results showed the presence of constitutive oligomers of the serotonin$_{1A}$ recep-

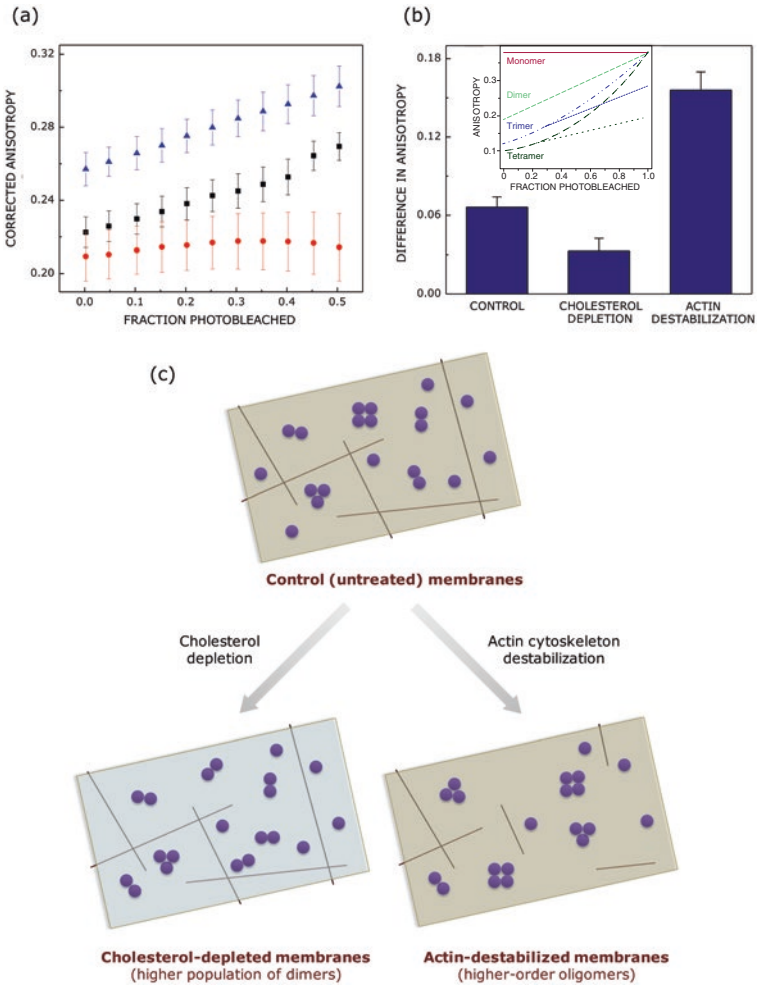

Fig. 16.1 GPCR oligomerization: the role of cholesterol and the actin cytoskeleton. (**a**) Fluorescence anisotropy enhancement profiles of EYFP-tagged serotonin$_{1A}$ receptors upon photobleaching. Anisotropies corrected for microscopic aperture-induced depolarization upon photobleaching as a function of the photobleached fraction of fluorescently tagged receptors are plotted for control (untreated) cells (■, *black*), cells treated with MβCD (▲, *blue*) and upon cytochalasin D treatment (●, *red*). (**b**) Difference between extrapolated (to complete photobleaching) and simulated (see *inset*) anisotropies in various conditions. The extent of receptor oligomerization can be determined using the difference between the anisotropy upon linear extrapolation of the photobleaching data (from (**a**)) to complete photobleaching, and the simulated anisotropy (from the *inset* which shows the simulation of the enhancement in anisotropy upon increasing photobleaching for a homogeneous population of monomers, dimers, trimers and tetramers using the formalism developed by Yeow and Clayton [62]). The magnitude of deviation of extrapolated anisotropy from simulated anisotropy is indicative of the extent of oligomerization. Adapted and modified from [11]. (**c**) A schematic representation of the effect of cholesterol depletion and destabilization of the actin cytoskeleton (shown as *rods* underlying the membrane) on oligomerization of the serotonin$_{1A}$ receptor (shown as *circles* on the membrane in *top view*). Receptors are present in heterogeneous oligomeric states in untreated (control) cell membranes. Depletion of cholesterol leads to an increase in the proportion of receptor dimers. Higher order oligomers of the serotonin$_{1A}$ receptor are observed upon destabilization of the actin cytoskeleton

tor and reorganization of higher-order oligomers in response to membrane cholesterol depletion, and actin cytoskeleton destabilization. These results are further supported by careful analysis of the organization of the EYFP-tagged serotonin$_{1A}$ receptor using time-resolved fluorescence anisotropy decay [35].

16.5 Computational Approaches to Explore GPCR Oligomerization

Computational methods, especially coarse-grain molecular dynamics simulations, have been effectively used to study the association of several GPCRs in molecular details. These simulations are limited to the sub-ms time regime, and have been able to capture the association, but not the dissociation of GPCRs. It should be noted that diffusion of GPCRs in cell membranes is slower than in model membranes, and association and dissociation of GPCRs could be diffusion-limited. Importantly, the factors that affect GPCR diffusion, such as membrane lipid composition, could be intricately linked to GPCR association. For instance, cholesterol depletion has been reported to induce dynamic confinement of the serotonin$_{1A}$ receptor in living cells [47].

16.5.1 Molecular Determinants of GPCR Dimers: Dependence on Protein-Lipid Interactions

Computational approaches represent powerful tools to analyze the molecular determinants of GPCR association. Coarse-grain simulations have been used to analyze the association of several GPCRs, such as the β_2-adrenergic receptor [42], rhodopsin [39] and the serotonin$_{1A}$ receptor [44]. Several protein-protein contact interfaces have been predicted in these receptors. Although the dimer interfaces are dependent on the receptor, the main sites at which association occurs appear to be common and are schematically shown in Fig. 16.2 for the β_2-adrenergic receptor. Two specific sites have been identified, involving predominantly transmembrane helices I/II and IV/V. Homo-interfaces, *i.e.*, a symmetric interface with the same site of both receptors, have also been reported. In addition, a role for transmembrane helix VI has been reported. Further, hetero-interfaces comprising of different transmembrane helices, but from the same sites on the receptor, have been reported. For instance, a I/II-IV/V interface has been reported in case of the β_2-adrenergic receptor [42]. Interestingly, the dimer interfaces in the opioid receptor family (μ, δ and κ subtypes) suggested a similar dimer interface across the different subtypes [45]. For the hetero-dimers, similar sites of dimer interface have been reported in the A_{2A} adenosine-D_2 dopamine receptor complex [15]. Since several interfaces have been reported, the next question that arises is related to the relative energetics of these interfaces. Coarse-grain simulations have been used to analyze the energetics of these

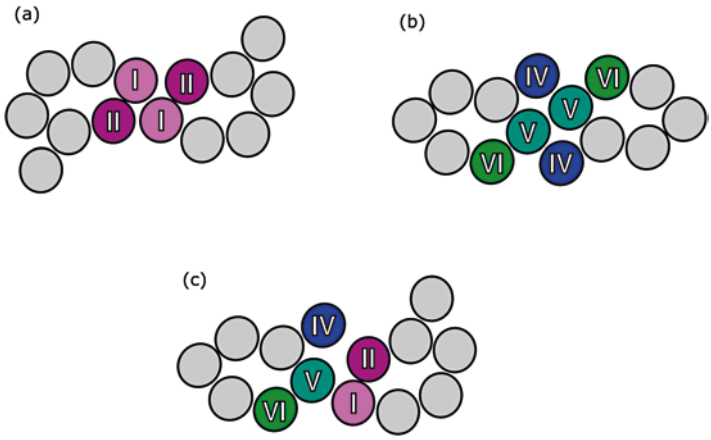

Fig. 16.2 A schematic representation of the predominant GPCR dimer interfaces observed in simulations of the β_2-adrenergic receptor. It appears that the predominant sites at which receptor association takes place are common to many receptors. The interacting helices at the receptor dimer interfaces are shown in panels (**a**–**c**)

interfaces. Importantly, the energetics of these dimer interfaces is of the order of kT. The values should be treated as qualitative due to the nature of the coarse-grain force-field and the free-energy calculations. Nonetheless, these results point toward several alternate dimer structures that could interconvert at room temperature.

From the studies on single component lipid membranes, we were able to identify a few specific interfaces. The dimerization of the β_2-adrenergic receptor was addressed by systematically varying the cholesterol concentration in the membrane [42]. The main interfaces described above, namely helices I/II and IV/V were observed (Fig. 16.3a–c). However, an intrinsic cholesterol dependence was observed in the simulations. In the absence of cholesterol, the dimer interface comprised only of transmembrane helices IV and V. At high cholesterol concentrations, transmembrane helices I and II were observed at the dimer interface. At intermediate concentrations, hetero-interfaces comprising of these two sites were observed. Interestingly, the two homo-interfaces have been observed in the crystal structure of a related receptor, the β_1-adrenergic receptor (Fig. 16.3d, e), although the lipid dependence is not clear in the crystal structure [19]. We believe that these interfaces are of similar energetics, and the membrane composition tunes the interactions of the receptors to modulate dimerization. In addition, a mixed bilayer of POPC/cholesterol has been used to analyze oligomerization in the β_2-adrenergic receptor [30]. However, these authors were not able to discern differences in the oligomerization pattern, possibly due to a lack of sampling.

A more comprehensive study was performed for the serotonin$_{1A}$ receptor, in which more subtle changes were observed relative to the β_2-adrenergic receptor [44]. Similar to the β_2-adrenergic receptor, cholesterol was found to modulate the dimer interface, although the structural and dynamic determinants of the dimer interfaces were found to vary. The time-course of dimer formation of two seroto-

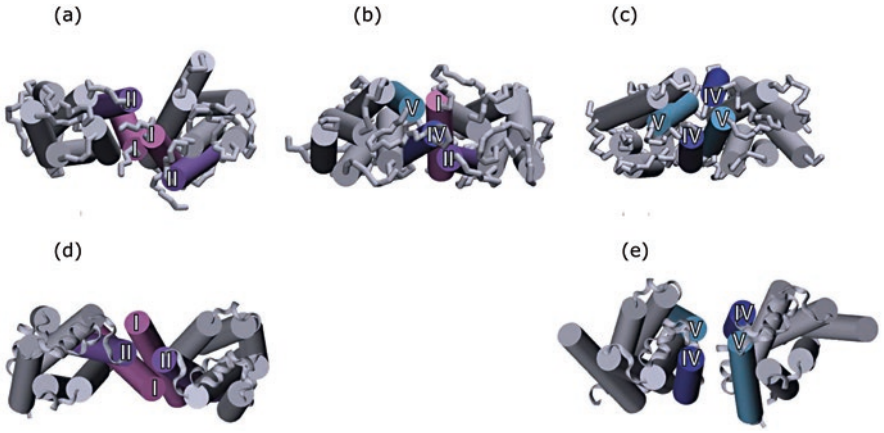

Fig. 16.3 Representative dimer interfaces of the β_2-adrenergic receptor in membrane bilayers of varying cholesterol concentration: (**a**) high (50 mol%) (b) intermediate (30 mol%) and (**c**) in the absence of cholesterol. Panels (**d**) and (**e**) represent dimer interfaces of the β_1-adrenergic receptor obtained from crystallography (PDB: 4GPO) (Adapted and modified from [42])

nin_{1A} receptor monomers at varying cholesterol concentration is shown in Fig. 16.4. The most striking feature was that the number of dimers formed at low cholesterol concentrations was higher than that at high cholesterol concentrations. Additionally, it was observed that a 'tight' dimer interface comprising only of transmembrane helix I was formed in the absence of cholesterol. In the presence of cholesterol, the flexibility of this dimer interface was higher and it formed a transmembrane helix I/II-I/II type of an interface. Additionally, several dimer interfaces comprising of transmembrane helices IV,V and VI were observed.

For the hetero-dimers, a unique modulation of the dimer interface has been reported in adenosine A_{2A}-dopamine D_2 receptors [15]. Polyunsaturated lipids, such as omega 3 fatty acids have been observed to modulate the dimer interfaces. A clear distinction in the kinetics of dimer formation was observed at high and low concentrations of omega 3 fatty acids. A comparison of the dimer interfaces of the opioid receptors (μ, δ and κ subtypes) suggested a similar dimer interface across the different subtypes, although an effect of the interfacial lipids was observed [45]. Preliminary results suggest a similar cholesterol dependence for the adenosine-dopamine receptor pairs (Prasanna et al. unpublished observations). The modulation of dimer interface upon varying cholesterol and other lipids that was hinted at with experimental studies, appears to be better resolved with simulations.

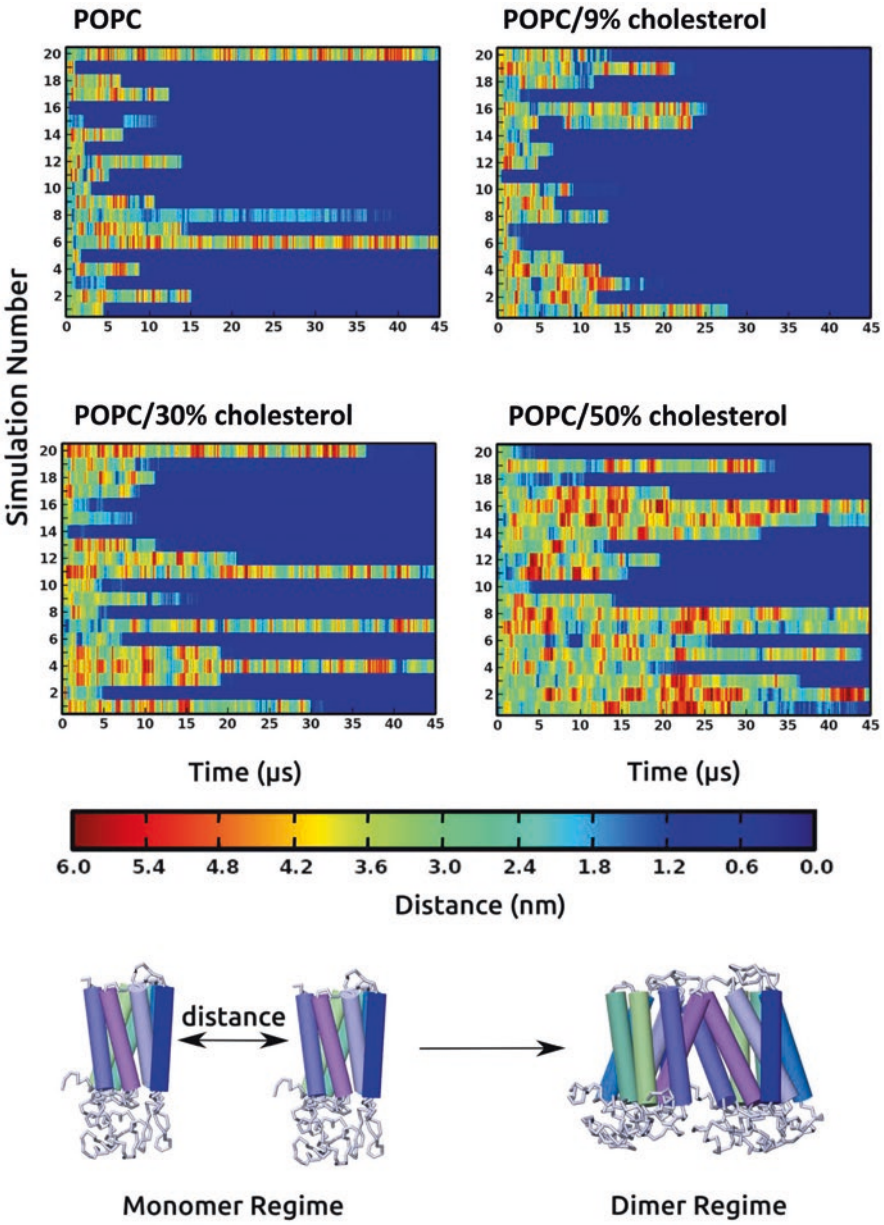

Fig. 16.4 Dimerization of the serotonin$_{1A}$ receptor is dependent on membrane cholesterol content. A schematic representation showing the minimum distance between two receptors in POPC membranes with increasing cholesterol content. Coarse-grain molecular dynamics simulations show that receptor dimerization is dependent on membrane cholesterol concentration. As apparent from the figure, lower number of dimers are observed at higher cholesterol concentrations. The entire range of distances between the receptor monomers is color coded and shown as a scale bar. The *bottom panel* shows the two receptors in monomer and dimer regimes (Adapted and modified from [44])

16.5.2 How Does Cholesterol Influence GPCR Dimerization?

The exact molecular mechanism of membrane cholesterol modulation of GPCR dimers is not clear [33, 36]. A recurrent theme pertains to the presence of lipid microdomains due to the presence of cholesterol. A more likely alternative view is that cholesterol modulates the energetics of the dimer interfaces, by destabilizing or stabilizing certain dimer conformations. For example, it has been proposed that membrane cholesterol interacts with the receptor and helps in forming a cholesterol-mediated dimer ([61]; see Fig. 16.5a for a schematic representation). On the other hand, unfavorable membrane perturbations due to hydrophobic mismatch have been proposed as non-specific mechanisms of GPCR dimer formations ([31]; see Fig. 16.5b).

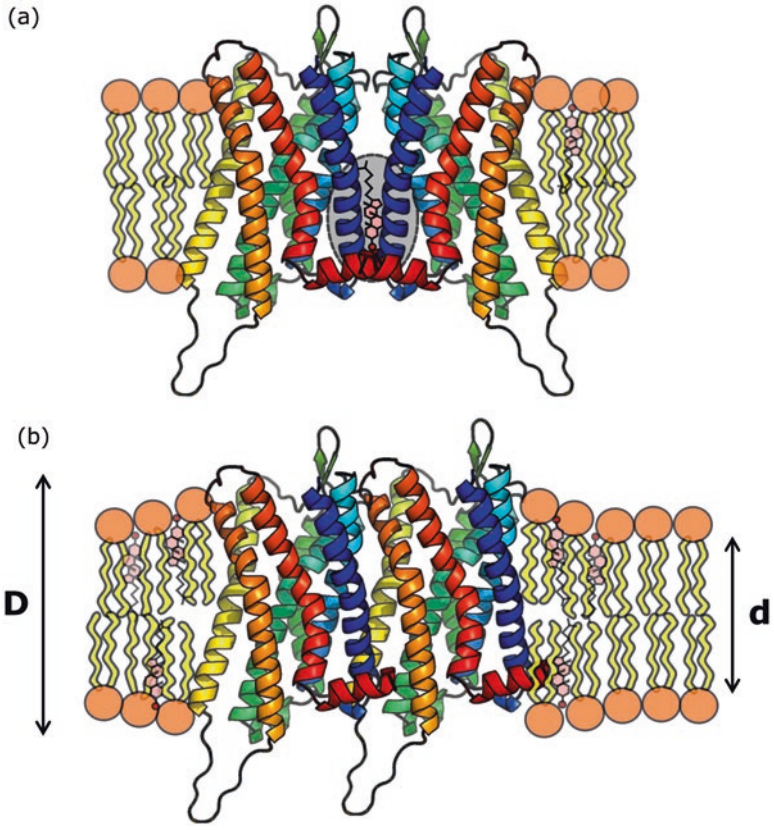

Fig. 16.5 Cholesterol-mediated mechanisms of modulation of GPCR dimerization. (**a**) Cholesterol may directly associate with GPCRs at inter-receptor (or inter-helical sites). (**b**) Hydrophobic mismatch between the length of the hydrophobic stretch in the receptor transmembrane region 'D' and membrane hydrophobic thickness 'd' can be induced by local concentration of membrane cholesterol

To test which of these mechanisms help to explain the cholesterol-mediated GPCR dimer interfaces in β_2-adrenergic receptor, we monitored both cholesterol binding sites and changes in the local membrane thickness. A direct correlation was observed between the occupancy of cholesterol at transmembrane helix IV and the dimer interface comprising of transmembrane helix IV [42]. We would like to suggest that cholesterol interaction sites are better represented as 'high-occupancy sites' or 'hot-spots', rather than binding sites [51, 52]. Along with the cholesterol interactions, the transmembrane helices that occur at the dimer interface (such as transmembrane helices I and IV) display large perturbations in their vicinity [43]. Taken together, these results suggest that non-specific effects direct the formation of dimers, while direct interaction of cholesterol with the receptor dictate the relative populations of the possible dimer species.

A similar effect is observed in case of the serotonin$_{1A}$ receptor dimer interface that could be related to both specific cholesterol interactions, and the non-specific effects [44]. An important difference observed with the β_2-adrenergic receptor was that the transmembrane helix I-II/I-II interface appears to be stabilized with increasing cholesterol concentration. This could be directly correlated to the cholesterol interaction sites that differ between these receptors. The most favorable cholesterol interaction site in the β_2-adrenergic receptor was at transmembrane helix IV, as opposed to several cholesterol interaction sites of comparable occupancies, including transmembrane helices I, V and VI in the serotonin$_{1A}$ receptor. This leads to the destabilization of the transmembrane helix IV/V interface in the β_2-adrenergic receptor in the presence of cholesterol, and an opposing stabilization of the flexible helix I-II/I-II interface in the dimer regime in the serotonin$_{1A}$ receptor. The take-home message is that cholesterol-mediated effects in GPCR dimers appear to be receptor-specific. A comprehensive approach involving both experimental and simulation inputs would help better understand this.

16.6 Future Perspectives: What Lies Ahead

Membrane cholesterol dependence of GPCR oligomerization opens up the important and interesting possibility of age and tissue dependence of drug efficacy. Cellular cholesterol is known to be developmentally regulated, also in a cell type/cell cycle dependent manner [27, 54, 55]. This could imply that the organization of dimers is age and cell type dependent. In addition, the tissue-dependent organization of GPCRs [5] could be important in the context of drug efficacy and specificity.

GPCRs act as crucial signaling hubs in higher eukaryotes. Although GPCRs represent the most predominant therapeutic targets, a large fraction of the GPCR receptorome remains unexplored from the drug discovery perspective [2]. It is estimated that ~150 GPCRs represent orphan receptors whose endogenous ligands and functions are yet to be established. These orphan receptors would be very useful in future drug discovery efforts. GPCR oligomerization and crosstalk incorporates

another dimension to this complex process of drug discovery. The exciting possibility of homo- and hetero-oligomerization of GPCRs provides tremendous diversity and potential to future drug discovery. Drugs that may prefer either GPCR dimers or monomers are already under consideration for novel drug development [21]. One such attempt involves development of drugs that block the activity of heterodimers of angiotensin II receptor type 1 (AT_1R) and chemokine type 2 receptor (CCR2) for the treatment of chronic kidney disease [3]. Similarly, post-synaptic heterodimers of adenosine A_{2A} receptor and dopamine D_2 receptor are believed to be crucial in the context of use of adenosine A_{2A} antagonists in the treatment of Parkinson's disease [10]. Knowledge of receptor oligomerization state under various pathophysiological conditions is of greater significance in the pharmacology of GPCRs since oligomerization gives rise to pharmacological diversity [56], opening new avenues for therapeutics.

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