Chapter 6

Molecular determinants of GPCR oligomerization

Madhura Mohole[1], G. Aditya Kumar[2], Durba Sengupta[1] and Amitabha Chattopadhyay[2]
[1]CSIR-National Chemical Laboratory, Pune, India; [2]CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India

6.1 Introduction

GPCRs are transmembrane receptors that transfer signals from the outside of the cell to the inside of the cell (Pierce et al., 2002; Rosenbaum et al., 2009). Due to their central role in cell signaling, they constitute ~50% of the current therapeutic drugs in the market (Cooke et al., 2015; Jacobson, 2015). These receptors consist of seven α-helical transmembrane helices alternately connected by extracellular and intracellular loops (Pierce et al., 2002). Ligands such as photons, peptides, and neurotransmitters bind to the lumen or the extracellular domain and promote conformational changes leading to G-protein coupling and signal propagation on the intracellular side (Venkatakrishnan et al., 2013). GPCRs have been reported to function as monomeric signaling units, supported in part by crystallography (Whorton et al., 2007). However, complex ligand binding kinetics of these receptors had originally suggested the presence of GPCR dimers and oligomers (Limbird et al., 1975). Biochemical (Maggio et al., 1993) and biophysical (Angers et al., 2000; McVey et al., 2001) studies were able to comprehensively detect GPCR association (Bouvier, 2001; Devi, 2001). Receptor pairs such as dopamine D_2 and D_3 receptor (Lee et al., 2000a; Nimchinsky et al., 1997), serotonin and dopamine receptors (Lee et al., 2000b) and opioid receptors (George et al., 2000) have been shown to associate in homo or heteromeric forms. A dynamic equilibrium exists in these associated forms, and the receptors may exhibit a dynamic interconversion between the oligomer states. For instance, the N-formyl peptide receptor (FPR) and the M1 muscarinic receptor were shown to interconvert between monomers and dimers with a submillisecond dimer lifetime (Hern et al., 2010; Kasai et al., 2011). For the β_1- and β_2-adrenergic receptor homodimers, this association time was reported to be of a timescale of seconds (Calebiro et al., 2013).

The association of GPCRs could lead to interreceptor functional crosstalk that was first demonstrated using receptor coexpression studies in α_{2c}-adrenergic receptor (α_{2C}AR) and M3 muscarinic receptor (Maggio et al., 1993). Constitutive heterodimerization of the GABA receptors B(R1) and B(R2) receptors was shown to be required for receptor trafficking from the endoplasmic reticulum to the plasma membrane (Bowery and Enna, 2000; Marshall et al., 1999; White et al., 1998). In the somatostatin receptors 1 and 5, it was shown that the function of the monomer and dimer differ and could lead to altered ligand binding in the presence of receptor dimers (Pfeiffer et al., 2001; Rocheville et al., 2000). In the serotonin receptor subtypes, a ligand binding deficient $serotonin_{2C}$ receptor mutant was shown to abolish ligand binding to the wild type receptor, suggesting a role of the dimer in signal initiation (Herrick-Davis et al., 2005). Similarly, heterodimerization of $serotonin_{1A}$ receptors with $serotonin_7$ receptors was shown to differentially regulate receptor signaling and trafficking (Renner et al., 2012). The associations between GPCRs and their functional consequences in terms of ligand binding, G-protein coupling, co-desensitization of receptors and altered pharmacology are now well established (Breitwieser, 2004; Ferre et al., 2014; George et al., 2002; Gurevich and Gurevich, 2008; Milligan, 2004, 2007; Terrillon and Bouvier, 2004).

Although the presence of GPCR dimers and oligomers has been extensively reported, the molecular determinants that control and modulate receptor oligomerization are less well understood. Several experimental and computational studies have implicated several factors such as membrane cholesterol and lipids, as well as inherent sequence and structural characteristics (Chakraborty and Chattopadhyay, 2015; Periole, 2016). In this article, we will discuss the factors and molecular mechanisms underlying GPCR oligomerization. We have focused on the $serotonin_{1A}$ receptor to highlight experimental and computational approaches to probe receptor association.

GPCRs. https://doi.org/10.1016/B978-0-12-816228-6.00006-4

6.2 Structural features of GPCR dimers

The key structural features for GPCR association appear to be receptor-specific. For instance, the GABA receptors that belong to class C GPCRs, primarily dimerize through the large extracellular loop domain characteristic of this class of GPCRs (Marshall et al., 1999). In contrast, the metabotropic glutamate receptors belonging to the same subclass were reported to associate with both the extracellular domain and the transmembrane domains (Wu et al., 2014). For class A GPCRs, early studies were able to show the importance of the transmembrane domains and helix VIII at the dimer/oligomer interfaces (Devi, 2001). Similarly, transmembrane helix VI was implicated at the dimer interface in some GPCRs.

The molecular details of receptor-receptor interfaces have been elucidated from crystallography studies. Most GPCRs that have been crystallographically resolved are monomers, although a few dimer structures have been reported. The crystal structures of the β_1-adrenergic receptor (β_1AR) (Huang et al., 2013), adenosine A_1 receptor (A_1AR) (Glukhova et al., 2017), the CXC Chemokine Receptor 4 (CXCR4) (Wu et al., 2010) are shown in Fig. 6.1A–C. In addition, the structure of the β_2-adrenergic receptor (β_2AR) that forms a dimer via crystal contacts (Cherezov et al., 2007) is shown in Fig. 6.1E. Several different transmembrane helices constitute the dimer interface; transmembrane helices I/II in β_1AR, II/III in A_1AR and IV/V in CXCR4. Interestingly in β_2AR, transmembrane helix I and helix VII are involved in crystal contacts together with cholesterol molecules (Fig. 6.1E). In the metabotropic glutamate receptor 1 (mGluR1) dimer, several cholesterol molecules were observed, but only a few direct contacts between the transmembrane helices (Fig. 6.1D).

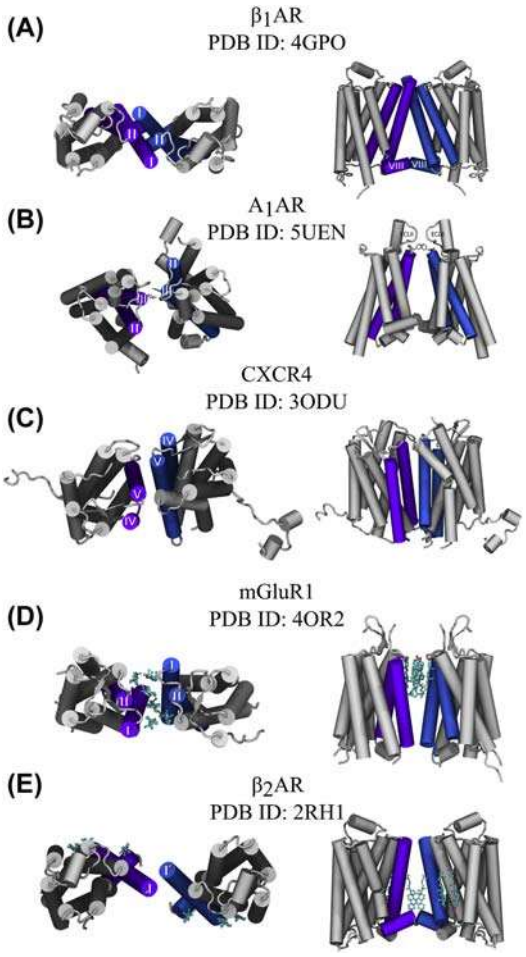

FIGURE 6.1 Experimentally determined structures of GPCR dimers. The crystal structures of the (A) β_1-adrenergic receptor (β_1AR), (B) adenosine A_1 receptor (A_1AR), (C) the CXC Chemokine Receptor 4 (CXCR4), (D) the metabotropic glutamate receptor 1 (mGluR1), and (E) β_2-adrenergic receptor (β_2AR). The left and right columns depict a top view and side view of the receptors, respectively. The transmembrane helices participating in receptor-receptor interactions are shown in violet and blue color for each monomer. The remaining receptor domains are shown in gray. The intracellular and extracellular domain fusion proteins (used in crystallography) are not shown for clarity. In panels (D and E) the cholesterol molecules that mediate the GPCR dimer states are shown in cyan with the headgroup hydroxyl moiety in red.

In addition, several dimer interfaces have been elucidated by molecular dynamics simulations (Mondal et al., 2013; Periole et al., 2012; Prasanna et al., 2014, 2016; Pluhackova et al., 2016). The dimer interfaces probed by both crystallography and simulations suggest a receptor-specific dimerization with varying transmembrane domains. However, the structures of GPCR dimers only appear to reflect a given state and several factors, such as membrane effects, have been observed to modulate GPCR association.

6.3 Role of cholesterol in GPCR oligomerization: monitoring serotonin$_{1A}$ receptor oligomerization utilizing time-resolved fluorescence anisotropy

One of the earliest and most comprehensive membrane effects have been shown with cholesterol, a predominant component of eukaryotic membranes with a cell-type dependent abundance (typically ~25−40 mol%). Several GPCRs are now known to be functionally modulated by membrane cholesterol (Jafurulla et al., 2019; Paila and Chattopadhyay, 2009; Sengupta et al., 2017). In particular, ligand binding, G-protein coupling and receptor stability of the serotonin$_{1A}$ receptor have been shown to be dependent on membrane cholesterol (Patra et al., 2015; Pucadyil and Chattopadhyay, 2004, 2007; Saxena and Chattopadhyay, 2012). An emerging role of cholesterol in receptor association is now becoming evident. For example, as described in Fig. 6.1, a few crystal structures of GPCRs (β_2AR and mGluR1) have shown cholesterol associated with receptor dimers (Hanson et al., 2008; Jafurulla et al., 2019). These structures indicate a possible role of cholesterol in the dimer conformations.

Several fluorescence based-approaches have been developed and tested to analyze the effects of cholesterol in GPCR organization. Chattopadhyay and coworkers have used photobleaching homo-FRET (Ganguly et al., 2011), analysis of time-resolved fluorescence anisotropy (Paila et al., 2011) and photobleaching image correlation spectroscopy (pbICS) (Chakraborti et al., 2018) to comprehensively analyze cholesterol-dependent serotonin$_{1A}$ receptor oligomerization at varying time scales. These results, taken together, have provided evidence for cholesterol-dependent GPCR organization at a wider spatiotemporal range. We will describe below the analysis of time-resolved fluorescence anisotropy approach.

Fluorescence resonance energy transfer (FRET) is a popular and powerful spectroscopic approach to monitor the oligomerization of membrane proteins in live cells. FRET involves the nonradiative transfer of energy from a donor to an acceptor fluorophore via a dipolar mechanism, which exhibits a sixth power distance dependence between the two molecules. FRET between two different fluorophores (hetero-FRET) is commonly utilized to monitor oligomerization in heterologously expressed systems. However, this method is associated with caveats arising from intensity-based hetero-FRET measurements due to the use of receptors conjugated to two different fluorophores. Moreover, the lack of stringent control over relative expression levels of the two fluorophores and bystander FRET in an overcrowded system are major limitations associated with hetero-FRET measurements (for a comprehensive discussion, see Chakraborty and Chattopadhyay, 2015; Clayton and Chattopadhyay, 2014). Homo-FRET offers a suitable alternative to overcome the disadvantages of hetero-FRET since it involves energy transfer between identical fluorophores. The excitation and emission spectra of fluorophores exhibiting homo-FRET should have considerable overlap. It is for this reason that fluorophores with relatively small Stokes' shift offer a greater probability of homo-FRET. Importantly, homo-FRET manifests itself by the appearance of a new decay process in the time-resolved anisotropy decay. The fact that time-resolved anisotropy is independent of fluorophore concentration to a large extent becomes useful in homo-FRET measurements to explore the oligomeric status of GPCRs. Further, homo-FRET is usually accompanied by no change in fluorescence lifetime or intensity.

Homo-FRET arising due to energy transfer between the constituents of an oligomer could be analyzed in terms of time-resolved anisotropy decay (see Fig. 6.2), a phenomenon that avoids the limitations of intensity-based measurements. The unresolved fast component of the observed anisotropy decay is interpreted as homo-FRET between the two molecules undergoing oligomerization. Utilizing this approach, we previously applied time-resolved fluorescence anisotropy decay to monitor oligomerization of the serotonin$_{1A}$ receptor (tagged to enhanced yellow fluorescence protein (EYFP)) and its modulation with membrane cholesterol (Paila et al., 2011). In general, the time-resolved decay in fluorescence anisotropy is a combination of (1) depolarization due to rotational motion of the fluorophore giving rise to the longer late component, and (2) homo-FRET arising due to energy transfer between fluorophores within Förster distance (the shorter early component). The time-resolved fluorescence anisotropy decay can, therefore, be expressed as:

$$r(t) = r_0\{\beta_1 \exp(-t/\phi_1) + \beta_2 \exp(-t/\phi_2)\} \quad (6.1)$$

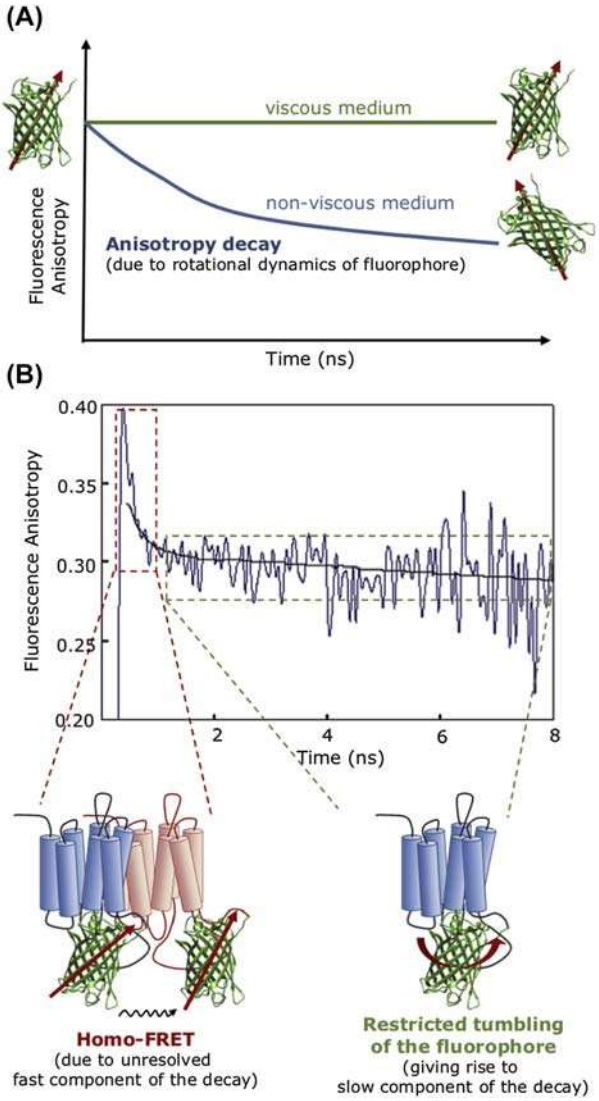

FIGURE 6.2 The photophysical principle underlying the analysis of GPCR oligomerization utilizing time-resolved fluorescence anisotropy. (A) A schematic representation of the time-resolved fluorescence anisotropy decay of a fluorophore (EYFP) in (1) a viscous medium that inhibits rotational tumbling of the fluorophore (shown as a *green line*), and (2) a nonviscous medium that allows free rotational dynamics (shown as a *blue line*). The anisotropy of the fluorophore observed in a completely viscous medium is known as *fundamental anisotropy* (r_0), which represents the maximum value of anisotropy achieved by the fluorophore in the absence of rotational diffusion or energy transfer. The fundamental anisotropy is an intrinsic property of a fluorophore which depends *only* on the angle between the absorption and the emission dipoles (transition moments). This angle changes if excitation or emission wavelength is changed. On the other hand, rotational motion of the fluorophore in a nonviscous medium results in depolarization of fluorescence which gets manifested as a time-dependent reduction in fluorescence anisotropy. (B) A representative time-resolved fluorescence anisotropy decay profile of EYFP-tagged serotonin$_{1A}$ receptor heterologously expressed in CHO cells. The fluorescence anisotropy decay could be resolved into two components. The initial fast component of the decay reflects decay in fluorescence anisotropy due to homo-FRET, and the relatively slow component of the decay is indicative of the restricted tumbling of the fluorophore. Analysis of the observed fluorescence anisotropy decay provides information on receptor oligomerization. *(A) Adapted and modified from Sharma et al., (2004) with permission; (B) Adapted and modified with permission from Paila, Y.D., Kombrabail, M., Krishnamoorthy, G., Chattopadhyay, A., 2011. Oligomerization of the serotonin1A receptor in live cells: a time-resolved fluorescence anisotropy approach. J. Phys. Chem. B 115 11439–11447. (copyright 2018 American Chemical Society). See text for more details.*

where r_0 is the fundamental anisotropy of EYFP, and β_1 and β_2 are amplitudes of the first and second rotational correlation times ϕ_1 and ϕ_2, respectively, such that $\beta_1 + \beta_2 = 1$. Fig. 6.2B shows a representative time-resolved fluorescence anisotropy decay profile of serotonin$_{1A}$ receptors tagged to EYFP. The initial fast component (ϕ_1) of the decay is indicative of homo-FRET between EYFP molecules, while the relatively slow component (ϕ_2) represents restricted tumbling of the fluorophore (Fig. 6.2B). Since the correlation time ϕ_1 (ascribed to homo-FRET) is too fast to be resolved, we can assume that

$\phi_1 << t$ (the first observable time point). Under this condition, the first term in the right-hand side of Eq. (6.1) becomes negligible, so that

$$r(t) = r_0\beta_2 \exp(-t/\phi_2)$$

Therefore, $r_0\beta_2$ corresponds to apparent initial anisotropy (r_{in}^{app}) estimated from the first few data points of anisotropy versus time curves in the actual experiment. At $t = 0$, $r(t) = r(0) = r_0$, and Eq. (6.1) can be written as

$$r_0 = r_0\{\beta_1 \exp(-0/\phi_1) + \beta_2 \exp(-0/\phi_2)\} \tag{6.2}$$

Since $\exp(-0/\phi_1) = 1$ and $\exp(-0/\phi_2) = 1$, Eq. (6.2) can be written as

$$r_0 = r_0\{\beta_1 + \beta_2\}$$

$$r_0 = r_0\beta_1 + r_0\beta_2 \tag{6.3}$$

Since $r_0\beta_2$ corresponds to r_{in}^{app}, Eq. (6.3) becomes

$r_0 = r_0\beta_1 + r_{in}^{app}$, which can be rearranged as

$$\beta_1 = (r_0 - r_{in}^{app})/r_0 \tag{6.4}$$

The values of β_1, the amplitude of the fast component of the decay, provide an estimate of the extent of receptor oligomerization. Analysis of results obtained showed that the serotonin$_{1A}$ receptor exists as constitutive oligomers in live cells. Interestingly, results showed that acute depletion of membrane cholesterol utilizing methyl-β-cyclodextrin (but not with chronic depletion using statin, the inhibitor of the rate-limiting step in biosynthesis of cholesterol) resulted in enhanced oligomerization of the receptor. The reasons behind this differential effect is not known, although a recent study has highlighted the difference in membrane environments in these two cases (Sarkar et al., 2017).

6.4 GPCR dimerization and cholesterol dependency: computational approaches

Computational studies, especially coarse-grain molecular dynamics simulations, have complemented experimental approaches. In particular, the molecular details underlying the association have been probed (Sengupta and Chattopadhyay, 2015; Sengupta et al., 2018). In general, protein-protein docking studies have been used to analyze the dimer conformers, but are unable to probe receptor dynamics. Molecular dynamics simulations have been able to probe receptor association, but a complete sampling of receptor and membrane dynamics remains challenging.

To probe the molecular effects of cholesterol on receptor dimerization, we studied association of the serotonin$_{1A}$ receptor in POPC and POPC/cholesterol membranes (Prasanna et al., 2016). In the simulations, the receptors dimerize within the first few microseconds and reveal several stable as well as transient dimer interfaces. To achieve a comprehensive sampling of several dimer conformers, coarse-grain simulations were performed at the microsecond timescale using the Martini coarse-grain force field. It was observed that the conformational plasticity of the dimers directly correlated with membrane cholesterol concentration (see Fig. 6.3). Further, the diversity and number of dissociation events are dependent on cholesterol concentration. The multiple conformations sampled in the simulations were classified into three main conformers depending on the transmembrane helices at the dimer interface. Each simulation showed a diversity of dimer conformers that were sampled. For example, the dimer interface comprised of transmembrane helices I–I (conformer A) in POPC membranes. With increasing cholesterol concentration, conformers B (transmembrane helices IV, V and VI), C (transmembrane helices I, II and transmembrane helices IV and V) and conformer A′ (transmembrane helices I and II) were more populated. The population of conformer A (transmembrane helix I–I interface) was the maximum in POPC bilayers and completely absent in POPC membranes with 30% and 50% cholesterol. Instead, a more flexible and dynamic dimer interface was observed (conformer A′) with transmembrane helices I/II-I/II. The absence of the conformer A in the presence of higher cholesterol concentration could be correlated with increased cholesterol occupancy in the monomeric regime at this site (transmembrane helix I). The occurrence of multiple interfaces was attributed to the enhanced receptor "plasticity" due to the presence of cholesterol at the nonannular sites (interhelical sites). In addition, membrane perturbations were observed in the presence and absence of cholesterol that could be related to the different dimer conformers. Cholesterol, therefore, appears to modulate serotonin$_{1A}$ receptor dimerization by a combination of effects.

Similarly, β_2AR receptor dimerization was reported to be dependent on cholesterol concentration (Prasanna et al., 2014). In the absence of cholesterol, transmembrane helices IV and V were observed at the dimer interface, although at

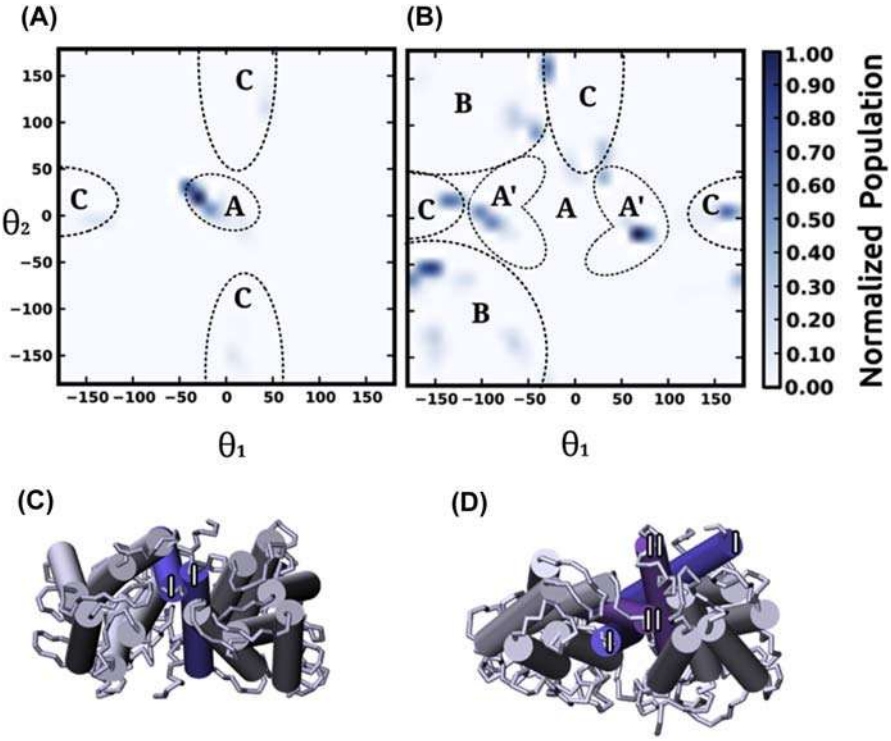

FIGURE 6.3 Cholesterol-dependent serotonin$_{1A}$ receptor dimer interfaces. The normalized population of the relative orientations of the serotonin$_{1A}$ receptor dimer in (A) POPC bilayers and (B) POPC/cholesterol bilayers with 50% cholesterol concentration. The relative dimer orientations are defined by the angles θ_1 and θ_2 and have been broadly mapped to four conformations (A, B, C, and A′). The top view of two of the representative conformers, A and A′, are shown in panels (C) and (D), respectively. *Adapted and modified from Prasanna, X., Sengupta, D., Chattopadhyay, A., 2016. Cholesterol-dependent conformational plasticity in GPCR dimers. Sci. Rep. 6 31858.*

higher cholesterol concentrations the nonsymmetric dimer interface was formed with transmembrane helices I/II and IV/V. At a cholesterol concentration of about 50%, a symmetric interface with transmembrane helices I and II was observed. In this case, a high cholesterol occupancy site at transmembrane helix IV was shown to result in the absence of this transmembrane helix at the dimer interface, together with membrane perturbations. In the case of CXCR4 receptors, a study combining Martini coarse-grain and atomistic simulations reported a novel symmetric transmembrane helices III/IV dimer interface in the presence of cholesterol (Pluhackova et al., 2016). The dimer interface at these helices supported a previous bioluminescence resonance energy transfer (BRET) study that reported the decrease in BRET efficiency upon addition of a peptide corresponding to the sequence of transmembrane helix IV, which competes for this site (Wang et al., 2006). Interestingly, two cholesterol molecules were observed to be temporarily stacked at the interface at the site of dimerization. The predominant interface formed by transmembrane helices for the cholesterol-depleted system was observed to be absent in the simulations with cholesterol. The authors suggested that the different conformers observed were related to the higher occupancy of cholesterol at an inter-helical site between transmembrane helices I and VII (Pluhackova et al., 2016).

A schematic representation of these direct and indirect cholesterol effects in GPCR association is depicted in Fig. 6.4. The direct effects arise from specific GPCR cholesterol interaction sites in the monomer and dimer that modulate the relative populations of interfaces comprising these sites. Additionally, membrane thickness due to cholesterol can modulate GPCR oligomerization by local perturbation at the receptor. These indirect effects are related to the hydrophobic mismatch (the difference between membrane thickness and length of the transmembrane helix) that arises due to differences in membrane thickness in the presence and absence of cholesterol (Fig. 6.4). Although cholesterol appears to modulate association of several GPCRs, the effects appear to be receptor-specific.

6.5 Energy landscapes of GPCR oligomerization

To understand the molecular basis of the effects of membrane cholesterol discussed above, we need to consider the relative energetics of the different oligomer states. Fig. 6.5 depicts a proposed energy landscape comprising of three distinct wells corresponding to the monomer, dimer and oligomer states. Although GPCR oligomers exhibit diverse spatial and structural

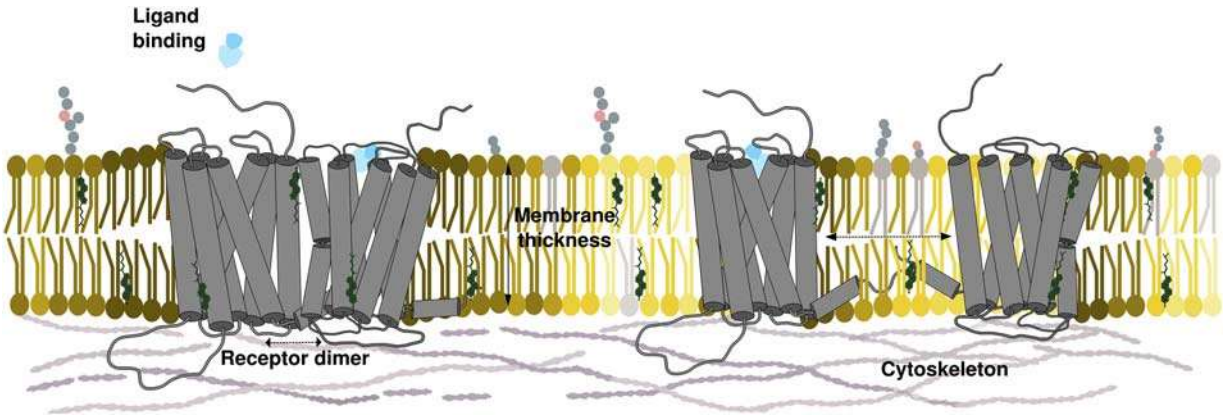

FIGURE 6.4 Membrane modulation of GPCR monomers and dimers. A schematic representation of GPCR monomers and dimers embedded in a membrane is shown. Hot-spots of cholesterol interactions and varying membrane thickness that directly or indirectly modulate GPCR oligomerization are represented. The lipid molecules in the membrane bilayer are shown in shades of yellow and cholesterol molecules are shown in green. The receptor monomers are shown in gray color. A representative ligand is shown in blue. The underlying cytoskeleton that has been reported to modulate GPCR association is shown in tan.

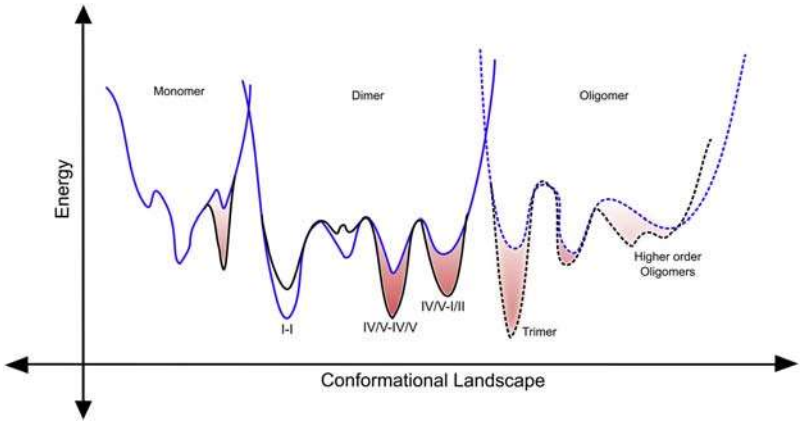

FIGURE 6.5 The conformational landscape of GPCR oligomerization. A schematic representation of the conformational landscape of a representative GPCR, the serotonin$_{1A}$ receptor, is shown in the presence (black) and absence (blue) of membrane cholesterol. The serotonin$_{1A}$ monomer is energetically stable with one deep minima (blue), corresponding to an inactive receptor. A different conformer (active state, R*) is stabilized by cholesterol interactions (black) at specific interaction sites. As the number of receptors increase, multiple minima represent possible conformers in the dimer and oligomer states. The possible conformers in the dimer state are marked (I−I, IV/V-IV/V, IV/V−I/II) depending on the transmembrane helices at the dimer interface. The oligomer state is depicted in *dotted lines* due to the lack of molecular-level details of the conformational landscape.

assemblies (Jonas et al., 2015), we have simplified it to include only the main conformers discussed here. This conformational landscape is dynamic and is modulated by a large number of factors, such as membrane cholesterol. For instance, the pbICS data suggests that in the absence of cholesterol (blue line), the relative energies of the three states gives rise to a favorable dimer state (Chakraborty et al., 2018). In the presence of cholesterol (black line), the relative populations of these oligomeric states could vary, stabilizing a different oligomeric state, for instance, the trimer.

Cholesterol effects appear to modulate the local energy minima in each of the oligomeric states as well. In the monomeric form, the cholesterol-receptor interactions could stabilize the receptor in a certain minima, such as the active state R*, in line with the experimentally observed modulation of ligand binding and G-protein coupling. Computational studies have shown that receptor-receptor associations in the membrane sample multiple intermediate dimer conformers. For serotonin$_{1A}$ receptor this interface was mainly observed to consist of transmembrane helix I from both receptors (I−I interface) in the absence of cholesterol. Cholesterol alters this association by selectively interacting with certain transmembrane regions on the receptor surface and also by increasing the membrane thickness to amplify the hydrophobic mismatch. The relative population (and energetics) of the I−I conformer is destabilized in the presence of cholesterol,

leading to different conformers being energetically more favorable. Such multiple intermediate dimer configurations that are explored represent local/shallow minima on the energy landscape (Fig. 6.5). The relative energetics of these states are comparable, although a quantitative estimate is difficult (Johnston et al., 2012; Periole et al., 2012). Since cholesterol can exert its effect in multiple ways, it is not *a priori* clear which conformer could be most favorable. In addition, there could be a crosstalk between cholesterol and other membrane components, or even the nature of the ligand, making the underlying energy landscape much more complicated than depicted. A systematic exploration of the various energy states and molecular modulators is required to comprehensively understand receptor organization.

6.6 Membrane physical properties: indirect effects

The indirect effects on GPCR oligomerization are induced through various membrane physical properties like membrane thickness leading to hydrophobic mismatch, fluidity, and lipid diffusion (Fig. 6.4). For instance, in β_2AR, membrane perturbations were observed at the transmembrane helices IV and V in POPC membranes and were subsequently observed to constitute the dimer interface (Prasanna et al., 2014, 2015). In the presence of cholesterol, increased membrane thickness alleviates perturbations at transmembrane helices IV and V, but increases the mismatch at transmembrane helices I and II. Interestingly, the receptors now appear to associate at this face of the receptor (Prasanna et al., 2015). Similarly, in the serotonin$_{1A}$ receptor, the dimer interface appears to be correlated with the transmembrane helices with the maximum membrane perturbations around them, together with direct cholesterol interactions (Prasanna et al., 2016). Interestingly, it was experimentally shown that varying cholesterol concentrations leads to varying membrane viscosity as well (Pal et al., 2016). As a result, it is difficult to decompose the direct and indirect effects in these complex membrane environments.

Periole and coworkers analyzed the association of rhodopsin in a series of single component bilayers to systematically analyze the effect of membrane thickness (Periole et al., 2007). The authors observed an increase in bilayer deformation led to an increased protein-protein association, as indicated by increased buried surface area. A similar effect was observed in β_2AR by Weinstein and coworkers (Mondal et al., 2013). A cell membrane with its diverse lipids would naturally exhibit varying lipid effects, and further work is needed to understand these indirect membrane effects in GPCR organization.

6.7 Emerging roles of membrane diversity on GPCR organization

Phospholipids, sphingolipids, and other membrane components have been shown to directly or indirectly tune GPCR organization. The rate of association of A_{2A} and D_2 receptor heterodimers was shown to be enhanced in membranes containing docosahexanoic acid in a study combining BRET and coarse-grain simulations (Guixà-González et al., 2016). Sphingolipids have been shown to modulate the organization of higher-order oligomers of the serotonin$_{1A}$ receptor by homo-FRET measurements (Ganguly et al., 2011). In fact, a more general role of interfacial lipids in stabilizing membrane protein oligomers has been suggested (Gupta et al., 2017). A large-scale mass spectroscopic study was able to identify several membrane proteins with interfacial lipids (Gupta et al., 2017). The authors suggest that these interfacial lipids contribute to oligomer stability and could be able to drive transient and stable interactions. For instance, in the μ-opioid receptor (MOR) they predict two dimer interfaces (transmembrane helices I-II/I-II and V-VI/V-VI) of which the former could be stabilized by interfacial lipids. In natural membranes, there would be both synergy and competition between these various membrane components that would determine the dynamic GPCR organization.

6.8 Ligand binding could drive GPCR association

A role of external stimuli and ligand binding has been reported in GPCR oligomerization, although in a receptor-dependent manner. For instance, increased oligomerization of the serotonin$_{1A}$ receptor was observed in the presence of serotonin, its natural agonist (Ganguly et al., 2011). Interestingly, the treatment with an antagonist (p-MPPI) led to reduced oligomerization of the receptor. In β_2AR, inverse agonists ICI-118,551, carazolol, and carvedilol were found to promote receptor oligomerization with a typical dimer interface of transmembrane helices I, VII and helix VIII (Fung et al., 2009). In contrast, no effect was observed in FPR in the presence of the ligand (Kasai et al., 2011; Kasai and Kusumi, 2014). The millisecond dynamic monomer-dimer equilibrium appeared to be unperturbed by addition of the ligand. In this context, it is interesting to note that the binding of a bivalent ligand was also shown to enhance the dimerization in type-2 cholecystokinin receptor (CCK2) and MOR (Zheng et al., 2009). It remains unclear whether the bivalent ligand stabilizes the preformed dimers or initiates dimerization of the receptors.

6.9 Cytoskeleton modulates GPCR association

An understanding of the spatiotemporal control of transmembrane proteins by the actin cytoskeleton is now emerging (Clayton and Chattopadhyay, 2016). To investigate the interplay between receptor and actin cytoskeleton, Chattopadhyay and co-workers explored the role of the actin cytoskeleton in the dynamics of the serotonin$_{1A}$ receptor (Ganguly et al., 2008). They analyzed the diffusion of the serotonin$_{1A}$ receptor by fluorescence recovery after photobleaching (FRAP) and showed that with increasing destabilization of the actin cytoskeleton, there was a gradual increase in the mobile fraction of the receptor. The increase in the mobile fraction with increasing cytoskeletal destabilization could be due to the gradual release of receptors that were previously confined (or corralled) by the actin network. Subsequently, it was observed that the actin cytoskeleton could directly modulate the organization of the receptor (Ganguly et al., 2011). Another interesting evidence on the crosstalk between GPCR organization and actin cytoskeleton comes from mathematical modeling to compare to single particle tracking measurements (Deshpande et al., 2017). The model predicts a continuous change in the percentage of receptor dimers, with increasing corral size as well as the probability of hopping. The monomer-dimer equilibrium of the GPCRs predicted are in line with previous experimental findings (Kasai et al., 2011). The model reveals a complex interplay between cytoskeletal components and their influence on receptor association kinetics within the features of the membrane landscape. The results suggest a functional coupling between receptor and actin and modulation of the dynamics of receptor organization.

6.10 Implications and future perspectives

The detection of the GPCR oligomers has been rapidly increasing with *state-of-the-art* experimental and computational approaches. Although the association of several GPCRs has been shown to have a role in receptor pharmacology, such as altered ligand binding and G protein coupling, the molecular determinants of the association are still being unraveled. For instance, the role of cholesterol and membrane-mediated effects in GPCR association has been extensively reported for many receptor types. The varying cholesterol abundance with age could imply a relation to disease progression (Prasanna et al., 2016; Chakraborty et al., 2018). The role of membrane composition in general, and cholesterol in particular needs to be considered for several GPCRs homo- and hetero-dimer pairs that are implicated in diseases such as schizophrenia (serotonin$_{1A}$ receptor) and Parkinson's disease (adenosine$_{2A}$ receptor - dopamine D_2 receptor). Understanding the role of the actin cytoskeleton in GPCR association is a new frontier in GPCR biology. Reports that cholesterol depletion and actin stability are intricately related suggest a dynamic interplay in GPCR organization. These varied effects essentially point toward the complex biology of this family of receptors, and a thorough investigation of the intrinsic and extrinsic determinants of GPCR association is necessary. Several factors have been reported to drive and modulate GPCR association that could act either alone or in concert to regulate the receptor organization and, thereby, downstream signaling cascades. Understanding the complex interplay of these determinants would thus ensure a comprehensive understanding of GPCR mediated signaling and its pathophysiology.

Acknowledgements

D.S. and A.C. gratefully acknowledge the project (EMR/2016/002294) from the Science and Engineering Research Board (Govt. of India). A.C. gratefully acknowledges support from SERB Distinguished Fellowship (Department of Science and Technology (Govt. of India). M.M. and G.A.K. thank the Department of Biotechnology (Govt. of India) and the Council of Scientific and Industrial Research (Govt. of India) for the award of Senior Research Fellowships, respectively. A.C. is a Distinguished Visiting Professor at Indian Institute of Technology, Bombay (Mumbai), and Adjunct Professor at RMIT University (Melbourne, Australia), Tata Institute of Fundamental Research (Mumbai) and Indian Institute of Science Education and Research (Kolkata), and an Honorary Professor at the Jawaharlal Nehru Center for Advanced Scientific Research (Bangalore).

References

Angers, S., Salahpour, A., Joly, E., Hilairet, S., Chelsky, D., Dennis, M., Bouvier, M., 2000. Detection of β_2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc. Nat. Acad. Sci. U.S.A. 97, 3684–3689.

Bouvier, M., 2001. Oligomerization of G-protein-coupled transmitter receptors. Nat. Rev. Neurosci. 2, 274–286.

Bowery, N.G., Enna, S.J., 2000. γ-aminobutyric acid(B) receptors: first of the functional metabotropic heterodimers. J. Pharmacol. Exp. Ther. 292, 2–7.

Breitwieser, G.E., 2004. G protein-coupled receptor oligomerization: implications for G protein activation and cell signaling. Circ. Res. 94, 17–27.

Calebiro, D., Rieken, F., Wagner, J., Sungkaworn, T., Zabel, U., Borzi, A., Cocucci, E., Zürn, A., Lohse, M.J., 2013. Single-molecule analysis of fluorescently labeled G-protein-coupled receptors reveals complexes with distinct dynamics and organization. Proc. Nat. Acad. Sci. U.S.A. 110, 743–748.

Chakraborty, H., Chattopadhyay, A., 2015. Excitements and challenges in GPCR oligomerization: molecular insight from FRET. ACS Chem. Neurosci. 6, 199–206.

Chakraborty, H., Jafurulla, M., Clayton, A.H.A., Chattopadhyay, A., 2018. Exploring oligomeric state of the serotonin$_{1A}$ receptor utilizing photobleaching image correlation spectroscopy: implications for receptor function. Faraday Discuss 207, 409–421.

Cherezov, V., Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G.F., Thian, F.S., Kobilka, T.S., Choi, H.-J., Kuhn, P., Weis, W.I., Kobilka, B.K., Stevens, R.C., 2007. High-resolution crystal structure of an engineered human β_2-adrenergic G protein-coupled receptor. Science 318, 1258–1265.

Clayton, A.H.A., Chattopadhyay, A., 2014. Taking care of bystander FRET in a crowded cell membrane environment. Biophys. J. 106, 1227–1228.

Clayton, A.H.A., Chattopadhyay, A., 2016. Spatiotemporal control of transmembrane proteins through the cytoskeleton: an evolving story. Biophys. J. 110, 1036–1037.

Cooke, R.M., Brown, A.J.H., Marshall, F.H., Mason, J.S., 2015. Structures of G protein-coupled receptors reveal new opportunities for drug discovery. Drug Discov. Today 20, 1355–1364.

Deshpande, S.A., Pawar, A.B., Dighe, A., Athale, C.A., Sengupta, D., 2017. Role of spatial inhomogenity in GPCR dimerisation predicted by receptor association-diffusion models. Phys. Biol. 14, 036002.

Devi, L.A., 2001. Heterodimerization of G-protein-coupled receptors: pharmacology, signaling and trafficking. Trends Pharmacol. Sci. 22, 532–537.

Ferre, S., Casadó, V., Devi, L.A., Filizola, M., Jockers, R., Lohse, M.J., Milligan, G., Pin, J.-P., Guitart, X., 2014. G protein-coupled receptor oligomerization revisited: functional and pharmacological perspectives. Pharmacol. Rev. 66, 413–434.

Fung, J.J., Deupi, X., Pardo, L., Yao, X.J., Velez-Ruiz, G.A., DeVree, B.T., Sunahara, R.K., Kobilka, B.K., 2009. Ligand-regulated oligomerization of β_2-adrenoceptors in a model lipid bilayer. EMBO J. 28, 3315–3328.

Ganguly, S., Clayton, A.H.A., Chattopadhyay, A., 2011. Organization of higher-order oligomers of the serotonin$_{1A}$ receptor explored utilizing homo-FRET in live cells. Biophys. J. 100, 361–368.

Ganguly, S., Pucadyil, T.J., Chattopadhyay, A., 2008. Actin cytoskeleton-dependent dynamics of the human serotonin$_{1A}$ receptor correlates with receptor signaling. Biophys. J. 95, 451–463.

George, S.R., Fan, T., Xie, Z., Tse, R., Tam, V., Varghese, G., O'Dowd, B.F., 2000. Oligomerization of μ- and δ-opioid receptors. Generation of novel functional properties. J. Biol. Chem. 275, 26128–26135.

George, S.R., O'Dowd, B.F., Lee, S.P., 2002. G-protein-coupled receptor oligomerization and its potential for drug discovery. Nat. Rev. Drug Discov. 1, 808–820.

Glukhova, A., Thal, D.M., Nguyen, A.T., Vecchio, E.A., Jőrg, M., Scammells, P.J., May, L.T., Sexton, P.M., Christopoulos, A., 2017. Structure of the adenosine A_1 receptor reveals the basis for subtype selectivity. Cell 168, 867–877.

Guixà-González, R., Javanainen, M., Gómez-Soler, M., Cordobilla, B., Domingo, J.C., Sanz, F., Pastor, M., Ciruela, F., Martinez-Seara, H., Selent, J., 2016. Membrane omega-3 fatty acids modulate the oligomerisation kinetics of adenosine A_{2A} and dopamine D_2 receptors. Sci. Rep. 6, 19839.

Gupta, K., Donlan, J.A.C., Hopper, J.T.S., Uzdavinys, P., Landreh, M., Struwe, W.B., Drew, D., Baldwin, A.J., Stansfeld, P.J., Robinson, C.V., 2017. The role of interfacial lipids in stabilizing membrane protein oligomers. Nature 541, 421–424.

Gurevich, V.V., Gurevich, E.V., 2008. How and why do GPCRs dimerize? Trends Pharmacol. Sci. 29, 234–240.

Hanson, M.A., Cherezov, V., Roth, C.B., Griffith, M.T., Jaakola, V.-P., Chein, E.Y.T., Velasquez, J., Kuhn, P., Stevens, R.C., 2008. A specific cholesterol binding site is established by the 2.8 Å structure of the human β_2-adrenergic receptor. Structure 16, 897–905.

Hern, J.A., Baig, A.H., Mashanov, G.I., Birdsall, B., Corrie, J.E.T., Lazareno, S., Molloy, J.E., Birdsall, N.J.M., 2010. Formation and dissociation of M_1 muscarinic receptor dimers seen by total internal reflection fluorescence imaging of single molecules. Proc. Nat. Acad. Sci. U.S.A. 107, 2693–2698.

Herrick-Davis, K., Grinde, E., Harrigan, T.J., Mazurkiewicz, J.E., 2005. Inhibition of serotonin 5-hydroxytryptamine2C receptor function through heterodimerization. Receptor dimers bind two molecules of ligand and on G-protein. J. Biol. Chem. 280, 40144–40151.

Huang, J., Chen, S., Zhang, J.J., Huang, X.-Y., 2013. Crystal structure of oligomeric β_1-adrenergic G protein-coupled receptors in ligand-free basal state. Nat. Struct. Mol. Biol. 20, 419–425.

Jacobson, K.A., 2015. New paradigms in GPCR drug discovery. Biochem. Pharmacol. 98, 541–555.

Jafurulla, M., Kumar, G.A., Rao, B.D., Chattopadhyay, A., 2019. A critical analysis of molecular mechanisms underlying membrane cholesterol sensitivity of GPCRs. Adv. Exp. Med. Biol. 1115, 21–52.

Johnston, J.M., Wang, H., Provasi, D., Filizola, M., 2012. Assessing the relative stability of dimer interfaces in G protein-coupled receptors. PLoS Comput. Biol. 8, e1002649.

Jonas, K.C., Fanelli, F., Huhtaniemi, I.T., Hanyaloglu, A.C., 2015. Single molecule analysis of functionally asymmetric G protein-coupled receptor (GPCR) oligomers reveals diverse spatial and structural assemblies. J. Biol. Chem. 290, 3875–3892.

Kasai, R.S., Kusumi, A., 2014. Single-molecule imaging revealed dynamic GPCR dimerization. Curr. Opin. Cell Biol. 27, 78–86.

Kasai, R.S., Suzuki, K.G.N., Prossnitz, E.R., Koyama-Honda, I., Nakada, C., Fujiwara, T.K., Kusumi, A., 2011. Full characterization of GPCR monomer-dimer dynamic equilibrium by single molecule imaging. J. Cell Biol. 192, 463–480.

Lee, S.P., O'Dowd, B.F., Ng, G.Y.K., Varghese, G., Akil, H., Mansour, A., Nguyen, T., George, S.R., 2000a. Inhibition of cell surface expression by mutant receptors demonstrates that D2 dopamine receptors exist as oligomers in the cell. Mol. Pharmacol. 58, 120–128.

Lee, S.P., Xie, Z., Varghese, G., Nguyen, T., O'Dowd, B.F., George, S.R., 2000b. Oligomerization of dopamine and serotonin receptors. Neuropsychopharmacology 23, S32–S40.

Limbird, L.E., De Meyts, P., Lefkowitz, R.J., 1975. β-Adrenergic receptors: evidence for negative cooperativity. Biochem. Biophys. Res. Commun. 64, 1160–1168.

Maggio, R., Vogel, Z., Wess, J., 1993. Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular "cross-talk" between G-protein-linked receptors. Proc. Natl. Acad. Sci. U.S.A. 90, 3103–3107.

Marshall, F.H., Jones, K.A., Kaupmann, K., Bettler, B., 1999. $GABA_B$ receptors – the first 7TM heterodimers. Trends Pharmacol. Sci. 20, 396–399.

McVey, M., Ramsay, D., Kellett, E., Rees, S., Wilson, S., Pope, A.J., Milligan, G., 2001. Monitoring receptor oligomerization using time-resolved fluorescence resonance energy transfer and bioluminescence resonance energy transfer. The human δ-opioid receptor displays constitutive oligomerization at the cell surface, which is not regulated by receptor occupancy. J. Biol. Chem. 276, 14092–14099.

Milligan, G., 2004. G protein-coupled receptor dimerization: function and ligand pharmacology. Mol. Pharmacol. 66, 1–7.

Milligan, G., 2007. G protein-coupled receptor dimerisation: molecular basis and relevance to function. Biochim. Biophys. Acta 1768, 825–835.

Mondal, S., Johnston, J.M., Wang, H., Khelashvili, G., Filizola, M., Weinstein, H., 2013. Membrane driven spatial organization of GPCRs. Sci. Rep. 3, 2909.

Nimchinsky, E.A., Hof, P.R., Janssen, W.G.M., Morrison, J.H., Schmauss, C., 1997. Expression of dopamine D_3 receptor dimers and tetramers in brain and in transfected cells. J. Biol. Chem. 272, 29229–29237.

Paila, Y.D., Chattopadhyay, A., 2009. The function of G-protein coupled receptors and membrane cholesterol: specific or general interaction? Glycoconj. J. 26, 711–720.

Paila, Y.D., Kombrabail, M., Krishnamoorthy, G., Chattopadhyay, A., 2011. Oligomerization of the $serotonin_{1A}$ receptor in live cells: a time-resolved fluorescence anisotropy approach. J. Phys. Chem. B 115, 11439–11447.

Pal, S., Chakraborty, H., Bandari, S., Yahioglu, G., Suhling, K., Chattopadhyay, A., 2016. Molecular rheology of neuronal membranes explored using a molecular rotor: implications for receptor function. Chem. Phys. Lipids 196, 69–75.

Patra, S.M., Chakraborty, S., Shahane, G., Prasanna, X., Sengupta, D., Maiti, P.K., Chattopadhyay, A., 2015. Differential dynamics of the $serotonin_{1A}$ receptor in membrane bilayers of varying cholesterol content revealed by all atom molecular dynamics simulation. Mol. Membr. Biol. 32, 127–137.

Periole, X., 2016. Interplay of G protein-coupled receptors with the membrane: insights from supra-atomic coarse grain molecular dynamics simulations. Chem. Rev. 117, 156–185.

Periole, X., Huber, T., Marrink, S.-J., Sakmar, T.P., 2007. G protein-coupled receptors self-assemble in dynamics simulations of model bilayers. J. Am. Chem. Soc. 129, 10126–10132.

Periole, X., Knepp, A.M., Sakmar, T.P., Marrink, S.J., Huber, T., 2012. Structural determinants of the supramolecular organization of G protein-coupled receptors in bilayers. J. Am. Chem. Soc. 134, 10959–10965.

Pfeiffer, M., Koch, T., Schröder, H., Klutzny, M., Kirscht, S., Kreienkamp, H.-J., Höllt, V., Schulz, S., 2001. Homo- and heterodimerization of somatostatin receptor subtypes. Inactivation of sst_3 receptor function by heterodimerization with sst_{2A}. J. Biol. Chem. 276, 14027–14036.

Pierce, K.L., Premont, R.T., Lefkowitz, R.J., 2002. Seven-transmembrane receptors. Nat. Rev. Mol. Cell Biol. 3, 639–650.

Pluhackova, K., Gahbauer, S., Kranz, F., Wassenaar, T.A., Böckmann, R.A., 2016. Dynamic cholesterol-conditioned dimerization of the G protein coupled chemokine receptor type 4. PLoS Comput. Biol. 12, e1005169.

Prasanna, X., Chattopadhyay, A., Sengupta, D., 2015. Role of lipid-mediated effects in β_2-adrenergic receptor dimerization. Adv. Exp. Med. Biol. 842, 247–261.

Prasanna, X., Chattopadhyay, A., Sengupta, D., 2014. Cholesterol modulates the dimer interface of the β_2-adrenergic receptor via cholesterol occupancy sites. Biophys. J. 106, 1290–1300.

Prasanna, X., Sengupta, D., Chattopadhyay, A., 2016. Cholesterol-dependent conformational plasticity in GPCR dimers. Sci. Rep. 6, 31858.

Pucadyil, T.J., Chattopadhyay, A., 2004. Cholesterol modulates ligand binding and G-protein coupling to $serotonin_{1A}$ receptors from bovine hippocampus. Biochim. Biophys. Acta 1663, 188–200.

Pucadyil, T.J., Chattopadhyay, A., 2007. Cholesterol depletion induces dynamic confinement of the G-protein coupled $serotonin_{1A}$ receptor in the plasma membrane of living cells. Biochim. Biophys. Acta 1768, 655–668.

Renner, U., Zeug, A., Woehler, A., Niebert, M., Dityatev, A., Dityateva, G., Gorinski, N., Guseva, D., Abdel-Galil, D., Frőhlich, M., Dőring, F., Wischmeyer, E., Richter, D.W., Neher, E., Ponimaskin, E.G., 2012. Heterodimerization of serotonin receptors $5\text{-}HT_{1A}$ and $5\text{-}HT_7$ differentially regulates receptor signalling and trafficking. J. Cell Sci. 25, 2486–2499.

Rocheville, M., Lange, D.C., Kumar, U., Sasi, R., Patel, R.C., Patel, Y.C., 2000. Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. J. Biol. Chem. 275, 7862–7869.

Rosenbaum, D.M., Rasmussen, S.G.F., Kobilka, B.K., 2009. The structure and function of G-protein-coupled receptors. Nature 459, 356–363.

Sarkar, P., Chakraborty, H., Chattopadhyay, A., 2017. Differential membrane dipolar orientation induced by acute and chronic cholesterol depletion. Sci. Rep. 7, 4484.

Saxena, R., Chattopadhyay, A., 2012. Membrane cholesterol stabilizes the human $serotonin_{1A}$ receptor. Biochim. Biophys. Acta 1818, 2936–2942.

Sengupta, D., Chattopadhyay, A., 2015. Molecular dynamics simulations of GPCR-cholesterol interaction: an emerging paradigm. Biochim. Biophys. Acta 1848, 1775–1782.

Sengupta, D., Kumar, G.A., Chattopadhyay, A., 2017. Interaction of membrane cholesterol with GPOCRs implications in receptor oligomerization. In: Giovanni, G., Herrick-Davis, K., Milligan, G. (Eds.), G Protein-Coupled Receptor Dimers. Springer, Heidelberg, pp. 415–429.

Sengupta, D., Prasanna, X., Mohole, M., Chattopadhyay, A., 2018. Exploring GPCR-lipid interactions by molecular dynamics simulations: excitements, challenges and the way forward. J. Phys. Chem. B 122, 5727–5737.

Sharma, P., Varma, R., Sarasij, R.C., Gousset, K., Krishnamoorthy, G., Rao, M., Mayor, S., 2004. Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. Cell 116, 577–589.

Terrillon, S., Bouvier, M., 2004. Roles of G-protein-coupled receptor dimerization. From ontogeny to signalling regulation. EMBO Rep. 5, 30–34.

Venkatakrishnan, A.J., Deupi, X., Lebon, G., Tate, C.G., Schertler, G.F., Babu, M.M., 2013. Molecular signatures of G-protein-coupled receptors. Nature 494, 185–194.

Wang, J., He, L., Combs, C.A., Roderiquez, G., Norcross, M.A., 2006. Dimerization of CXCR4 in living malignant cells: control of cell migration by a synthetic peptide that reduces homologous CXCR4 interactions. Mol. Cancer Ther. 5, 2474–2483.

White, J.H., Wise, A., Main, M.J., Green, A., Fraser, N.J., Disney, G.H., Barnes, A.A., Emson, P., Foord, S.M., Marshall, F.H., 1998. Heterodimerization is required for the formation of a functional $GABA_B$ receptor. Nature 396, 679–682.

Whorton, M.R., Bokoch, M.P., Rasmussen, S.G.F., Huang, B., Zare, R.N., Kobilka, B., Sunahara, R.K., 2007. A monomeric G protein-coupled receptor isolated in a high-density lipoprotein particle efficiently activates its G protein. Proc. Natl. Acad. Sci. U.S.A. 104, 7682–7687.

Wu, B., Chien, E.Y.T., Mol, C.D., Fenalti, G., Liu, W., Katritch, V., Abagyan, R., Brooun, A., Wells, P., Bi, F.C., Hamel, D.J., Kuhn, P., Handel, T.M., Cherezov, V., Stevens, R.C., 2010. Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. Science 330, 1066–1071.

Wu, H., Wang, C., Gregory, K.J., Han, G.W., Cho, H.P., Xia, Y., Niswender, C.M., Katritch, V., Meiler, J., Cherezov, V., Conn, P.J., Stevens, R.C., 2014. Structure of a class C GPCR metabotropic glutamate receptor 1 bound to an allosteric modulator. Science 344, 58–64.

Zheng, Y., Akgün, E., Harikumar, K.G., Hopson, J., Powers, M.D., Lunzer, M.M., Miller, L.J., Portoghese, P.S., 2009. Induced association of μ opioid (MOP) and type 2 cholecystokinin (CCK_2) receptors by novel bivalent ligands. J. Med. Chem. 52, 247–258.