A Critical Analysis of Molecular Mechanisms Underlying Membrane Cholesterol Sensitivity of GPCRs

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Abstract G protein-coupled receptors (GPCRs) are the largest and a diverse family of proteins involved in signal transduction across biological membranes. GPCRs mediate a wide range of physiological processes and have emerged as major targets for the development of novel drug candidates in all clinical areas. Since GPCRs are integral membrane proteins, regulation of their organization, dynamics, and function by membrane lipids, in particular membrane cholesterol, has emerged as an exciting area of research. Cholesterol sensitivity of GPCRs could be due to direct interaction of cholesterol with the receptor (specific effect). Alternately, GPCR function could be influenced by the effect of cholesterol on membrane physical properties (general effect). In this review, we critically analyze the specific and general mechanisms of the modulation of GPCR function by membrane cholesterol, taking examples from representative GPCRs. While evidence for both the proposed mechanisms exists, there appears to be no clear-cut distinction between these two mechanisms, and a combination of these mechanisms cannot be ruled out in many cases. We conclude that classifying the mechanism underlying cholesterol sensitivity of GPCR function merely into these two mutually exclusive classes could be somewhat arbitrary. A more holistic approach could be suitable for analyzing GPCR–cholesterol interaction.

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Abbreviations

7-DHC	7-Dehydrocholesterol
7-DHCR	3β-Hydroxy-steroid-Δ^7-reductase
24-DHCR	3β-Hydroxy-steroid-Δ^{24}-reductase
AY 9944	*trans*-1,4-*bis*(2-chlorobenzylaminoethyl)cyclohexane dihydrochloride
CB	Cannabinoid receptor
CCK	Cholecystokinin receptor
CCM	Cholesterol consensus motif
CCR5	CC chemokine receptor 5
CRAC	Cholesterol recognition/interaction amino acid consensus
CXCR4	CXC chemokine receptor 4
GalR2	Galanin receptor 2
GPCR	G protein-coupled receptor
MβCD	Methyl-β-cyclodextrin
MI	Metarhodopsin I
MII	Metarhodopsin II
mGluR	Metabotropic glutamate receptor
SLOS	Smith–Lemli–Opitz syndrome
Smo	Smoothened
T2R4	Bitter taste receptor 4

1 G Protein-Coupled Receptors as Signaling Hubs and Drug Targets

The G protein-coupled receptor (GPCR) superfamily is the largest and an extremely diverse family of proteins implicated in information transfer across biological membranes [1–3]. They are characterized by seven transmembrane domain topology and include >800 members which are encoded by ∼5% of genes in humans [4]. Signaling by GPCRs involves their activation by a wide variety of extracellular ligands that trigger the transduction of signals into the cellular interior through concerted structural rearrangements in their transmembrane and extramembranous domains [5, 6].

GPCRs are involved in the modulation of cellular responses to stimuli that encompass a variety of endogenous and exogenous ligands which even include photons. As a result, GPCRs mediate several essential physiological processes such as

neurotransmission, cellular metabolism, secretion, cellular differentiation, growth, and inflammatory/immune responses. GPCRs have therefore emerged as popular targets for the development of novel drug candidates in all clinical areas ranging from disorders of the central nervous system to cancer [7–11]. Importantly, ~50% of clinically prescribed drugs and 25 of the 100 top selling drugs target GPCRs [12–14]. However, only a small number of GPCRs are currently targeted by drugs [15, 16]. This presents the exciting possibility that the receptors which are not identified yet could be potential drug targets for diseases that pose a challenge to the available repertoire of drugs.

The role of membrane lipids in GPCR organization, dynamics, structure, and function has emerged as an exciting area in GPCR biology. GPCRs are integral membrane proteins with their transmembrane helices traversing the membrane seven times and as a consequence a major part of these receptors is surrounded by membrane lipids. For example, in case of rhodopsin, molecular dynamics simulations show that the lipid–protein interface corresponds to ~38% of the total surface area of the receptor [17]. In such a scenario, it is only realistic that the membrane lipid environment would modulate GPCR structure and function. Cellular membranes comprise of a wide variety of lipids, each of which uniquely modulates the physicochemical properties of the bilayer [18, 19]. Phospholipids, sphingolipids, and cholesterol constitute major lipid components of cell membranes, among which cholesterol has been extensively studied in the context of the organization, dynamics, structure, and function of GPCRs.

2 Membrane Cholesterol in GPCR Function

Cholesterol is a crucial and representative lipid in higher eukaryotic cell membranes and plays a key role in membrane organization, dynamics, function, and sorting. The unique molecular structure of cholesterol has been intricately fine-tuned over a very long timescale of natural evolution [20, 21]. The chemical structure of cholesterol comprises of the 3β-hydroxyl group, the rigid tetracyclic fused ring, and the flexible isooctyl side chain (Fig. 1a). The 3β-hydroxyl group (sole polar group) helps cholesterol anchor at the membrane interface and is believed to form hydrogen bonds with polar residues of membrane proteins. The tetracyclic fused ring and the isooctyl side chain constitute the apolar component of cholesterol. An inherent asymmetry about the plane of the sterol ring is generated by methyl substitutions on one of its faces (Fig. 1b). The protruding methyl groups (constituting the rough β face) are believed to participate in van der Waals interactions with the side chains of branched amino acids such as valine, leucine, and isoleucine. The other side of the sterol ring (constituting the smooth α face) exhibits favorable van der Waals interaction with the saturated fatty acyl chains of phospholipids (Fig. 1c; [22–24]). Cholesterol is nonrandomly distributed in specific domains (or pools) in biological and model membranes [22, 25–28]. Membrane cholesterol is essential for a range

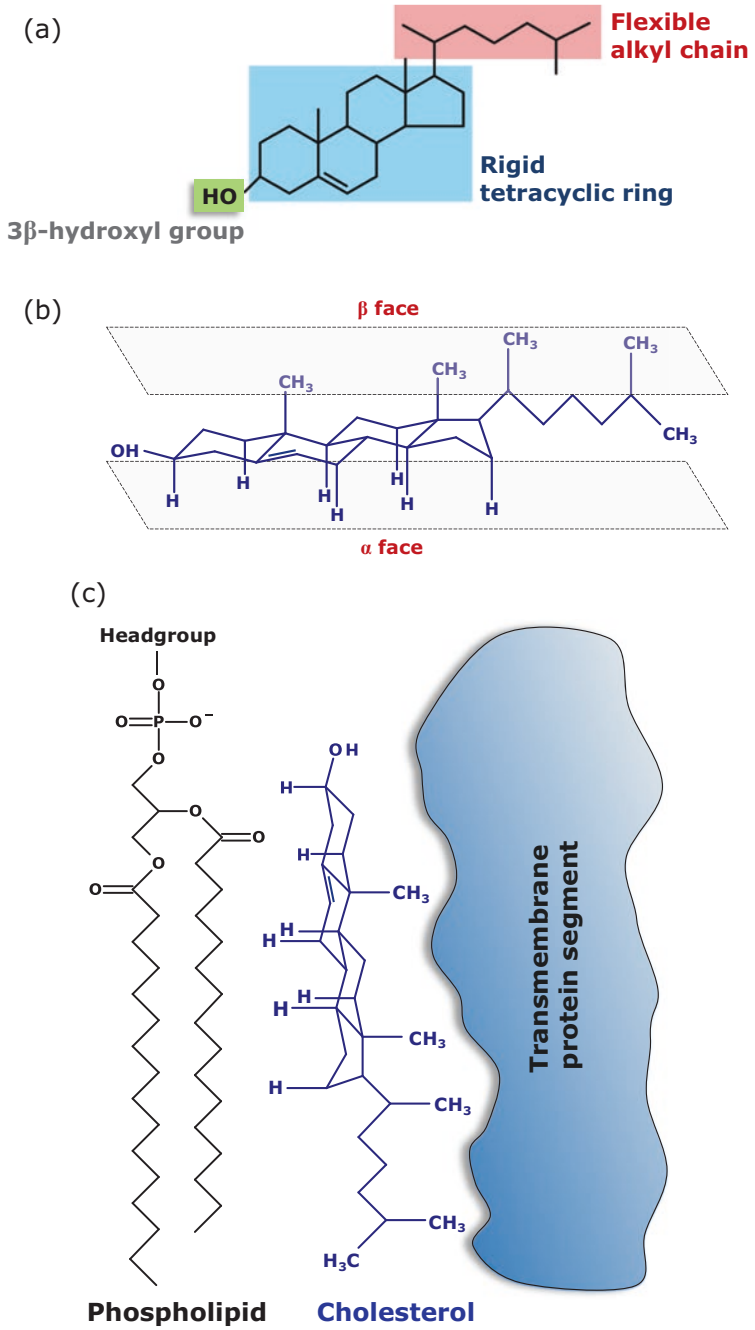

Fig. 1 Structural features of cholesterol and its orientation with respect to membrane components: (**a**) Chemical structure of cholesterol with its three structurally distinct regions (shown as shaded boxes): the 3β-hydroxyl group, the rigid tetracyclic fused ring, and the flexible isooctyl side chain.

of cellular processes such as membrane sorting and trafficking [29], signal transduction [30], and the entry of pathogens [31–35].

Membrane cholesterol has been shown to modulate the organization, dynamics, and function of several GPCRs (reviewed in [3, 36–42]). Understanding such dependence of the function of GPCRs on membrane cholesterol assumes significance since the function of GPCRs has been found to be compromised in pathological conditions with misregulated cholesterol metabolism [43]. In addition, cholesterol exhibits an inherent diversity in terms of its distribution across cell, tissue, and organ types. For example, although the central nervous system constitutes ~2% of the body mass, it accounts for ~25% of the cholesterol content in the body [44, 45]. Moreover, cellular cholesterol content is age-dependent [46] and developmentally regulated [47].

In spite of several studies showing the importance of cholesterol in GPCR function, the exact molecular mechanism underlying this remains elusive [48, 49]. The cholesterol dependence of the function of GPCRs could be attributed to either specific (direct) interaction or general (indirect) effect of membrane cholesterol on physical properties of the membrane in which the receptor is embedded. A combination of specific and general effects is yet another possibility. In this review, we discuss the cholesterol sensitivity of GPCRs with examples highlighting specific and general effects of membrane cholesterol on GPCR function, along with experimental strategies to explore such interactions.

3 Strategies to Explore Cholesterol Sensitivity of GPCRs

The mechanism of action of cholesterol on GPCRs has been explored using a battery of experimental strategies, each of which provides a unique perspective to address the molecular basis of these interactions. The strategies commonly used to study such interactions rely on the modulation of cholesterol content or its availability in membranes in order to probe its role in supporting the function and organization of GPCRs. These techniques, when used judiciously, could be helpful in delineating the specific and general effects of cholesterol on GPCR function. We discuss below a few important strategies that are used to explore the nature of the interaction of membrane cholesterol with GPCRs.

Fig. 1 (continued) (**b**) Two faces of cholesterol: asymmetry is due to the methyl groups on one plane of the sterol ring of cholesterol resulting in a rough (β) face, leaving the other plane with axial hydrogen atoms (smooth (α) face). (**c**) A schematic showing the possible orientation of cholesterol with respect to membrane components (phospholipid and transmembrane protein segment). The smooth α face of cholesterol contributes to favorable van der Waals interaction with the saturated fatty acyl chains of phospholipids and the rough β face interacts with uneven transmembrane domains of integral membrane proteins. Adapted and modified from [22]. See text for more details

3.1 Solubilization and Reconstitution

Solubilization is an important method used to understand the structural and functional aspects of GPCRs. Solubilization involves the isolation of the receptor from its native membrane environment and dispersing it in a relatively purified state using suitable amphiphilic detergents. The process of solubilization leads to dissociation of proteins and lipids which are held together in the native membrane, ultimately resulting in the formation of small clusters of protein, lipid, and detergent in an aqueous solution [50–54]. Solubilization has been utilized as an effective strategy to study GPCR–lipid interactions and probe lipid specificity by reconstitution of the receptor with specific lipids [54, 55]. The process of reconstitution involves removal of detergent, followed by incorporation of the receptor into membrane-mimics such as micelles, bicelles, liposomes, nanodiscs, and planar lipid bilayers [55, 56]. This strategy has been earlier utilized to explore the role of cholesterol in the function of the serotonin$_{1A}$ receptor [54]. Using this strategy, we further explored the structural stringency of cholesterol in the function of the serotonin$_{1A}$ receptor by reconstituting the solubilized receptor with close structural analogs (biosynthetic precursors and stereoisomers) of cholesterol [57–60].

3.2 Inhibition of Cholesterol Biosynthesis

Biosynthesis of cholesterol is carried out in a stringently regulated multi-step enzymatic pathway [61]. A physiologically relevant approach to study the role of cholesterol in GPCR function is metabolic (chronic) depletion by inhibiting specific enzymes in its biosynthetic pathway. A common strategy that has been used to chronically deplete cellular cholesterol is the use of statins [62, 63]. Statins are competitive inhibitors of HMG-CoA reductase, the enzyme that catalyzes the rate-limiting step in the cholesterol biosynthetic pathway (Fig. 2a; [64]). In addition, distal inhibitors such as AY 9944 (*trans*-1,4-*bis*(2-chlorobenzylaminoethyl)cyclohexane dihydrochloride) that inhibits 3β-hydroxy-steroid-Δ^7-reductase (7-DHCR), and triparanol which inhibits 3β-hydroxy-steroid-Δ^{24}-reductase (24-DHCR) have been extensively utilized [65, 66]. Inhibition of 7-DHCR and 24-DHCR that catalyze final steps in the Kandutsch-Russell pathway [67] and Bloch pathway [68] results in the accumulation of 7-dehydrocholesterol (7-DHC) and desmosterol, respectively (Fig. 2a). Importantly, malfunctioning of 7-DHCR and 24-DHCR has been identified as major factors for lethal neuropsychiatric disorders such as Smith–Lemli–Opitz syndrome (SLOS) and desmosterolosis [69, 70]. Therefore, inhibitors of 7-DHCR and 24-DHCR have been successfully utilized to generate cellular and animal model systems to study these disease conditions [65, 66, 71, 72]. We previously utilized this strategy to generate a cellular model for SLOS using AY 9944, and explored the function of the serotonin$_{1A}$ receptor (an important neurotransmitter receptor) in this neuropsychiatric disease condition [43].

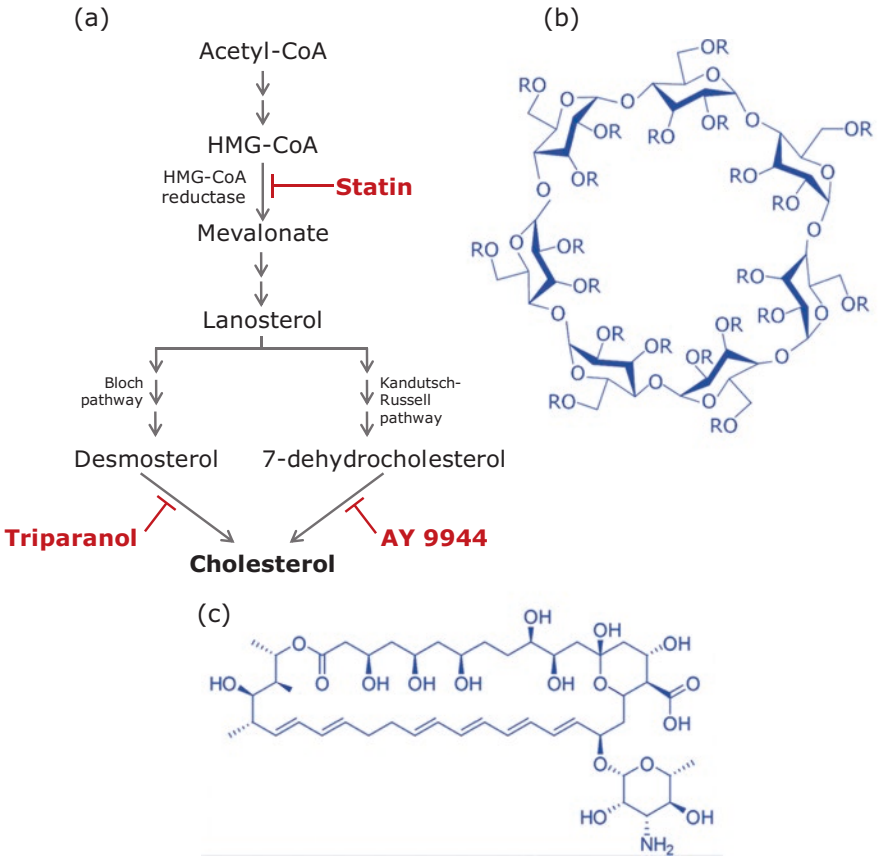

Fig. 2 Strategies to explore cholesterol-dependence of GPCR function. (**a**) A schematic representation of biosynthetic inhibitors of cholesterol. The role of cholesterol in GPCR function can be analyzed utilizing inhibitors of cholesterol biosynthesis that allow chronic depletion of cholesterol in a physiologically relevant manner. Statins inhibit the first rate-limiting step that involves the conversion of HMG-CoA to mevalonate at an early step in the cholesterol biosynthetic pathway. Inhibitors of the final steps in the Kandutsch-Russel and Bloch pathways of cholesterol biosynthesis include AY 9944 and triparanol that inhibit the synthesis of cholesterol from their immediate precursors, 7-dehydrocholesterol and desmosterol, respectively. (**b**) The chemical structure of methyl-β-cyclodextrin (MβCD), a specific carrier of cholesterol that selectively depletes membrane cholesterol. R denotes a methyl group. (**c**) Chemical structure of nystatin, a representative complexing agent

3.3 Specific Carriers

A commonly utilized strategy for acute and specific modulation of membrane cholesterol content is by using specific carriers. Methyl-β-cyclodextrin (MβCD), a member of the cyclodextrin family, is an oligomer of seven methylated-glucose residues that exhibits specificity for cholesterol over other membrane lipids

(see Fig. 2b; [34, 73, 74]). MβCD has been utilized as the carrier of choice to study the effect of cholesterol on GPCR function, organization, and dynamics in a large number of studies [36, 37]. The relatively small size and polar nature of MβCD allows its close interaction with membranes, thereby enabling efficient and selective modulation of cholesterol content. This strategy has been utilized to explore the cholesterol-dependent function of several GPCRs such as rhodopsin [75], oxytocin [76], galanin [77], serotonin$_{1A}$ [78, 79], cannabinoid [80–82], and bitter taste T2R4 receptors [83]. We have successfully utilized MβCD for controlled modulation of membrane cholesterol to study its role in the function of the serotonin$_{1A}$ receptor [78, 79, 84]. We further utilized MβCD to replace cholesterol with its various close structural analogs in order to explore the structural stringency of cholesterol for supporting receptor function [54]. Interestingly, we have recently shown that although both inhibition of cholesterol biosynthesis and specific carriers modulate cholesterol levels in cell membranes, the actual effect could differ a lot (even at same cholesterol concentrations), since the membrane dipolar environment in these cases turn out to be very different [85].

3.4 Enzymatic Oxidation

Specific modulation of membrane cholesterol could also be achieved by its oxidation using the enzyme cholesterol oxidase. Cholesterol oxidase catalyzes the oxidation of cholesterol to 4-cholestenone at the membrane interface [86], thereby modifying the chemical nature of cholesterol without physical depletion from membranes. Oxidation of cholesterol exhibits mild effect on global membrane properties relative to its physical depletion, and minimizes nonspecific effects of cholesterol modulation. This strategy has been earlier utilized to explore the structural specificity of cholesterol (the hydroxyl group in particular) in the function of several GPCRs such as the serotonin$_{1A}$ receptor [87, 88], oxytocin and cholecystokinin (CCK) receptors [76], galanin-GalR2 receptors [77], rhodopsin [89], and chemokine receptors CXCR4 and CCR5 [90].

3.5 Complexing Agents

Modulating availability of cholesterol in the membrane, rather than physical depletion, is yet another method to explore the cholesterol sensitivity of GPCR function. Cholesterol-complexing agents such as digitonin, filipin, nystatin, amphotericin B, and perfringolysin O [91–95] at appropriate concentrations partition into membranes and sequester cholesterol, thereby making it unavailable for interaction with GPCRs. These agents could be used to address the interaction of cholesterol with GPCRs by restricting cholesterol availability. Figure 2c shows the chemical

structure of nystatin, a representative complexing agent. This strategy has been earlier utilized to probe the requirement of membrane cholesterol for the function of the serotonin$_{1A}$ [96, 97], oxytocin [76], and galanin [77] receptors.

4 Mechanisms of Cholesterol Sensitivity of GPCRs

Cholesterol sensitivity of GPCRs is well documented. However, the underlying molecular mechanism remains elusive. The ongoing efforts to understand the structural and functional correlates underlying cholesterol sensitivity of GPCR function have provided evidence in favor of both specific interaction and general (membrane) effects. We discuss below representative studies on cholesterol sensitivity of GPCRs.

4.1 Specific Requirement of Membrane Cholesterol for GPCRs

4.1.1 Serotonin$_{1A}$ Receptor

The serotonin$_{1A}$ receptor is a key neurotransmitter GPCR that is implicated in the generation and modulation of various cognitive, behavioral, and developmental functions [98–102]. The serotonin$_{1A}$ receptor is the most well-studied GPCR in terms of specificity of cholesterol in the organization, dynamics, and function of the receptor. Earlier work from our laboratory has comprehensively demonstrated the specific requirement of membrane cholesterol for the function of the serotonin$_{1A}$ receptor utilizing an array of experimental approaches. By modulating the availability of membrane cholesterol by employing (1) MβCD [57, 78], (2) biosynthetic inhibitors such as statin [63] and AY 9944 [43], and (3) complexing agents such as nystatin [96] and digitonin [97], we have shown the requirement of cholesterol in receptor function. We generated a cellular model for SLOS (a fatal neuropsychiatric disorder) using AY 9944 and showed that the function of the serotonin$_{1A}$ receptor is compromised under this disease-like condition [43]. We have recently generated a rat model of SLOS by oral feeding of AY 9944 to dams for brain metabolic NMR studies. Importantly, enzymatic oxidation of cholesterol [87, 88] led to a change in receptor function, without any appreciable effect on membrane order (as reported by fluorescence anisotropy measurements), *thereby suggesting specific requirement of cholesterol for receptor function*. We further demonstrated the structural stringency of cholesterol in supporting the function of the serotonin$_{1A}$ receptor by replacing cholesterol with its immediate biosynthetic precursors (7-DHC and desmosterol) [58, 59, 103] and stereoisomers of cholesterol ([60]; reviewed in [54]). In addition, we showed that the stability of the serotonin$_{1A}$ receptor is enhanced in the presence of cholesterol using biochemical approaches [104], molecular modeling [105], and all atom molecular dynamics simulations [106]. Taken together, these studies bring out the cholesterol sensitivity of the serotonin$_{1A}$ receptor function, which in some cases (such as treatment with cholesterol oxidase) could have a specific mechanism.

4.1.2 Oxytocin Receptor

The oxytocin receptor plays an important role in several neuronal functions and in reproductive biology [107]. Cholesterol dependence of oxytocin receptor function was explored using multiple approaches [76, 108]. Modulation of membrane cholesterol content using MβCD resulted in a change in the affinity state of the receptor for oxytocin, with the receptor in a high affinity state in the presence of cholesterol [108]. In addition, utilizing cholesterol-complexing agent filipin, mere complexation of cholesterol was shown to be sufficient to modulate receptor function [76]. Importantly, treatment with cholesterol oxidase modulated the function of the receptor without a significant change in membrane order. The structural stringency of cholesterol for the function of the oxytocin receptor was demonstrated by replacing cholesterol with an array of its structural analogs [76]. Further, the oxytocin receptor was shown to be more stable in the presence of cholesterol [109]. These results point out the role of specific mechanism in the cholesterol-dependent function of the oxytocin receptor.

4.1.3 Galanin Receptor

Galanin receptors upon binding to the neuropeptide galanin mediate diverse physiological functions in the peripheral and central nervous systems. The requirement of cholesterol for galanin receptor (GalR2) function was shown by modulating cholesterol content in cellular membranes using MβCD or by culturing cells in lipoprotein-deficient serum [77]. Depletion of membrane cholesterol led to decrease in affinity of ligand binding to the receptor. In addition, complexation of cholesterol with filipin and enzymatic oxidation of cholesterol led to significant reduction in ligand binding activity of the receptor. The mechanistic basis of cholesterol sensitivity was evident from experiments in which cholesterol was replaced with its structural analogs, thereby implying a possible specific mechanism responsible for cholesterol sensitivity of GalR2 [77].

4.1.4 Chemokine Receptors

Chemokine receptors are important GPCRs implicated in immunity and infection. A wide range of chemokines bind to these receptors and mediate specific immune responses. Membrane cholesterol has been shown to be essential for stabilizing the functional conformation and signaling of CCR5 and CXCR4 receptors, members of the chemokine receptor family [90, 110, 111]. The cholesterol sensitivity of the function of CCR5 was shown using conformation-specific antibodies, whose binding to the receptor exhibited cholesterol dependence [110]. Treatment with cholesterol oxidase [90] resulted in reduction in binding of epitope-specific antibodies to CCR5 along with loss in receptor function. In addition, replacement of cholesterol with 4-cholesten-3-one showed reduction in specific ligand binding to the receptor [110]. Similar results were observed for CXCR4 where depletion or oxidation of membrane cholesterol resulted in reduction in binding of conformation-specific

antibodies and signaling of the receptor [90, 111]. These effects were reversed upon replenishment with membrane cholesterol.

4.1.5 Bitter Taste Receptors

The human bitter taste receptors (T2Rs) are chemosensory receptors with significant therapeutic potential [112]. Earlier work from our laboratory has shown that the T2R4 receptor, a representative member of the bitter taste receptor family, exhibits cholesterol sensitivity in its signaling [83]. The molecular basis of such cholesterol dependence of receptor function could be attributed to the putative cholesterol recognition/interaction amino acid consensus (CRAC) motif (see below), since mutation of a lysine residue in the CRAC sequence led to loss of cholesterol sensitivity of the receptor [83].

4.1.6 Cannabinoid and Cholecystokinin Receptors

Cannabinoid receptors are activated by endocannabinoids which mediate a variety of physiological and neuroinflammatory processes, and are implicated in several neurodegenerative and neuroinflammatory disorders. The cholesterol sensitivity of type-1 cannabinoid (CB1) receptors was shown from dependence of specific ligand binding and signaling of the receptor on membrane cholesterol [80, 81, 113]. Importantly, such a sensitivity of CB1 receptor function to membrane cholesterol is lost upon mutation of a lysine residue in the putative CRAC sequence. Interestingly, the type-2 cannabinoid (CB2) receptor has glycine instead of lysine (as in CB1 receptor) in the CRAC sequence [113] and does not show cholesterol dependence for its function [82, 113]. These studies point toward the possible involvement of the CRAC motif in cholesterol sensitivity of CB1 receptors.

Similar observations were reported for subtypes of cholecystokinin CCK1 and CCK2 receptors [114, 115]. CCK1 receptors were shown to be sensitive to membrane cholesterol by analyzing active conformation of the receptor, probed using fluorescence of a specific fluorescent ligand and intracellular calcium response [114]. Interestingly, a closely related subtype CCK2 receptor has been shown to be insensitive to membrane cholesterol [115]. Importantly, mutation in CRAC motif region in CCK1 receptor resulted in the loss of its cholesterol sensitivity.

4.2 Structural Evidence in Support of GPCR–Cholesterol Interaction

The specificity of cholesterol for the function of GPCRs has gained support from recently reported high-resolution crystal structures of GPCRs with bound cholesterol molecules. Crystal structures of several GPCRs have been resolved with bound cholesterol molecules over the last decade (see Table 1). Cholesterol was found to

Table 1 GPCR structures with bound cholesterol[a]

Receptor	PDB ID	# Chol[b]	Reference
β_2-adrenergic receptor	2RH1	3	[116]
	3D4S	2	[117]
	3NYA, 3NY8, 3NY9	2	[118]
	3PDS	1	[119]
	5JQH	1	[120]
	5D5A, 5D5B	3	[121]
	5X7D	2	[122]
	5D6L	3	[123]
Adenosine A_{2A} receptor	4EIY 5K2A, 5K2B, 5K2C, 5K2D	3 3	[124] [125]
	5IU4, 5IU7, 5IU8, 5IUA 5IUB	4 3	[126] [126]
	5UVI	3	[127]
	5NLX, 5NM2, 5NM4	3	[128]
	5MZJ, 5N2R 5MZP	3 4	[129] [129]
	5JTB	3	[130]
	5VRA	3	[131]
	6AQF	3	[132]
	5OLH, 5OLO 5OM4, 5OLV, 5OM1, 5OLG, 5OLZ	3 4	[133] [133]
κ-opioid receptor	6B73	1	[134]
μ-opioid receptor	4DKL	1	[135]
	5C1M	1	[136]
Metabotropic glutamate receptor 1	4OR2	6 (per dimer)	[137]
Smoothened	5L7D	1 (per dimer)	[138]
	6D35	1	[139]
Serotonin$_{2B}$ receptor	4IB4	1	[140]
	4NC3	1	[141]
	5TVN	1	[142]
Cannabinoid receptor 1	5XR8, 5XRA	1	[143]
CC chemokine receptor type 9	5LWE	1 (per dimer)	[144]
Endothelin receptor type-B	5X93	1	[145]
US28 in complex with the chemokine domain of human CX3CL1	4XT1 5WB2	2 2	[146]
$P2Y_1$ receptor	4XNV	1	[147]
$P2Y_{12}$ receptor	4PXZ 4NTJ	1 2	[148] [149]

[a]The list was generated by searching the PDB database for GPCR structures with cholesterol as a small molecule ligand

[b]Number of cholesterol molecules bound per GPCR monomer

be bound between transmembrane helices (interhelical) within the receptor or between monomers of a receptor dimer. Interestingly, cholesterol sensitivity has been demonstrated in few of these GPCRs. We discuss below examples of GPCRs (see Fig. 3) which display cholesterol sensitivity in their function.

4.2.1 β_2-Adrenergic Receptor

One of the first high-resolution crystal structures of a GPCR with bound cholesterol molecules was for the β_2-adrenergic receptor, in which three cholesterol molecules were found per receptor monomer (Fig. 3a; [116]). In addition, in a subsequent structure, two cholesterol molecules were identified in a shallow cleft formed by transmembrane helices I–IV of the receptor (Fig. 3b; [117]). Importantly, this structure was instrumental in defining one of the putative cholesterol interaction sites in GPCRs, the cholesterol consensus motif (CCM) (see below). The cholesterol dependence of the stability and function of the β_2-adrenergic receptor has been previously reported [150–153].

4.2.2 Adenosine A_{2A} Receptor

The high-resolution crystal structure of the adenosine A_{2A} receptor showed three bound molecules of cholesterol, all of them located at the extracellular half of the transmembrane helices of the receptor (Fig. 3c; [124]). The three cholesterol molecules were found between transmembrane helices II/III, V/VI, and VI/VII. Interestingly, transmembrane helix VI which is implicated in ligand binding appears to be stabilized by cholesterol [124], and could provide structural basis for the reported cholesterol sensitivity of adenosine A_{2A} receptor function [154].

4.2.3 Opioid Receptors

In case of κ-, μ-, and δ-opioid receptors, cholesterol has been shown to modulate the affinity of ligand binding and signaling [62, 155, 156]. Recent crystal structures of the κ-opioid receptor ([134]; Fig. 3d) and μ-opioid receptor [135, 136]; Fig. 3e) showed cholesterol bound to transmembrane helices of the receptors. Cholesterol was found to interact with the transmembrane helices VI and VII of the μ-opioid receptor.

4.2.4 Metabotropic Glutamate Receptor

Unlike class A GPCRs discussed above in which transmembrane domains constitute predominant sites for ligand binding, the metabotropic glutamate receptor mGluR belongs to class C and has large extracellular domain(s) responsible for

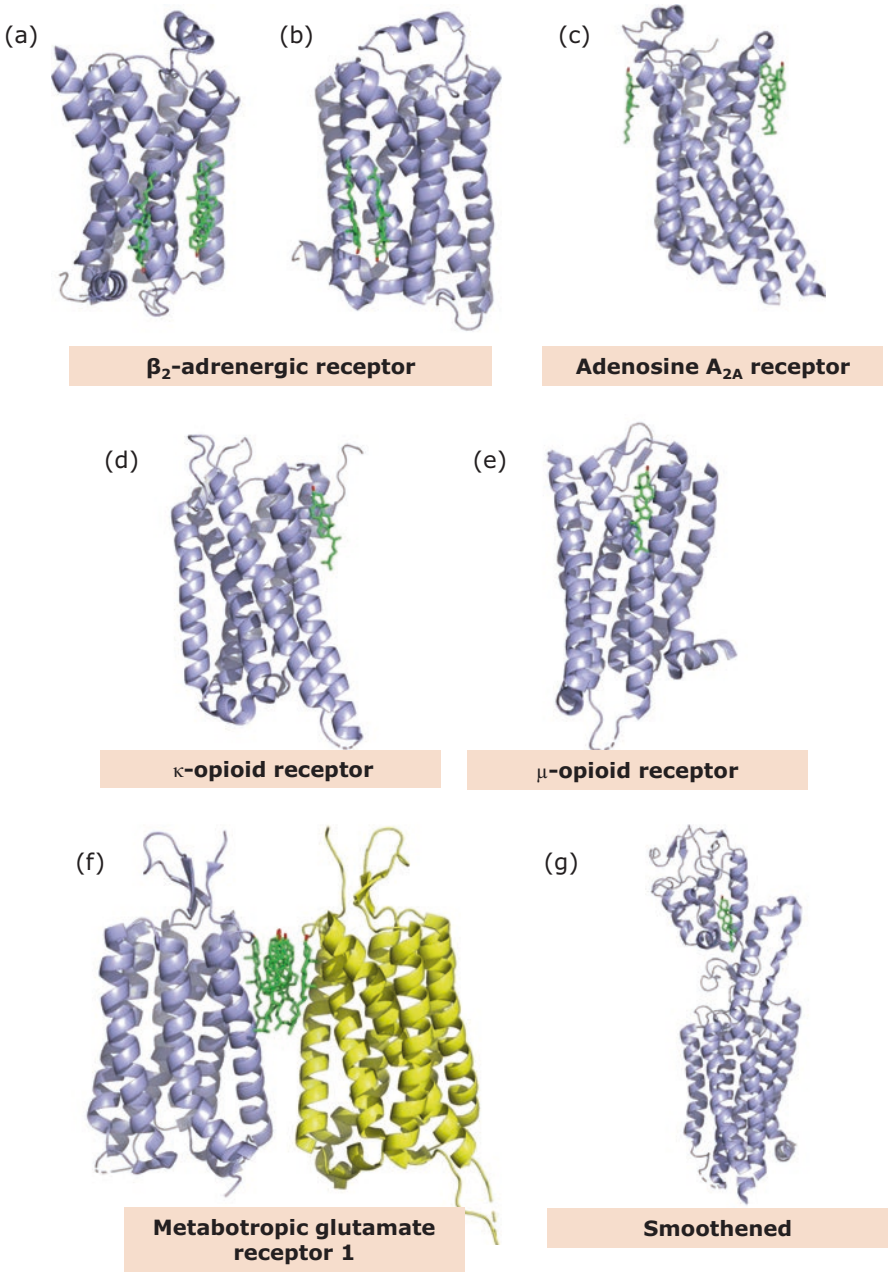

Fig. 3 Crystal structures of representative GPCRs with bound cholesterol molecules. Bound cholesterol molecules have been identified in crystal structures of several GPCRs (the corresponding PDB IDs are indicated in parentheses): (**a**, **b**) β_2-adrenergic receptor (2RH1, 3D4S), (**c**) adenosine A_{2A} receptor (4EIY), (**d**) κ-opioid receptor (6B73), (**e**) μ-opioid receptor (4DKL), (**f**) metabotropic glutamate receptor 1 (4OR2), and (**g**) smoothened (5L7D). Snapshots of cholesterol-bound

ligand binding. It has been earlier shown that membrane cholesterol modulates the ligand binding affinity and signaling of the mGluR [157, 158]. However, it was not clear how membrane cholesterol could modulate ligand binding at the extracellular domain of the receptor. The structural basis of such modulation of receptor function by membrane cholesterol was recently shown in a cholesterol-bound crystal structure of the mGluR [137]. In the receptor structure, six cholesterol molecules were bound symmetrically in the extracellular side of transmembrane helices I and II at the dimer interface (Fig. 3f). These structural evidences could form the basis of the observed role of cholesterol in mGluR function.

4.2.5 Smoothened Receptor

One of the most compelling functional correlates of cholesterol interaction with GPCRs was shown in the recently reported structure of the sterol binding frizzled (class F) GPCR, smoothened (Smo) [138, 139, 159]. Smo is a component of the hedgehog signaling pathway involved in embryonic development and programmed cell death, and the role of cholesterol in this pathway is well documented [160]. Cholesterol acts as the endogenous activator of Smo by inducing conformational changes in the receptor that stimulates the hedgehog pathway. The structure of Smo showed a cholesterol molecule bound to the extracellular cysteine-rich domain of the receptor which is crucial for transduction of hedgehog signals (Fig. 3g). Importantly, the structure helped to predict key residues for this interaction, mutating which impaired hedgehog signaling [159].

We would like to end this section with a cautionary note. Although crystallography is an excellent technique to resolve detailed high-resolution structures of GPCRs, it suffers from some inherent limitations. Despite the fact that the extramembranous regions of GPCRs play crucial roles in receptor function and signaling [161–163], the flexible loops corresponding to these regions are generally stabilized using a monoclonal antibody or replaced with lysozyme [116, 164, 165], since the inherent conformational flexibility of the loops poses a problem for crystallography. In addition, crystallography is often carried out in detergent dispersions or lipidic cubic phases using a heavily engineered (mutated) and antibody-bound receptor. In spite of the popularity of lipidic cubic phase membranes for GPCR crystallization [166], the physiological significance of bound cholesterol molecules in GPCR crystal structures in lipidic cubic phases is not clear [167]. It is possible that the bound cholesterol molecules and the CCM site could be specific to membrane lipid environment (which is different in lipidic cubic phase relative to the lamellar phase). It would therefore be prudent to be careful in extrapolating bound cholesterol in crystal structures of GPCRs to their cholesterol-sensitive function.

Fig. 3 (continued) (cholesterol shown in green with its hydroxyl group in red) structures of GPCRs were generated from their respective PDB structures using PyMOL Molecular Graphics System (version 2.0.6 Schrödinger, LLC). Function of these GPCRs has been shown to be sensitive to membrane cholesterol. See text and Table 1 for more details

4.3 Cholesterol Interaction Motifs

The specific association of cholesterol with GPCRs that could possibly mediate cholesterol-dependent function is proposed to be manifested through conserved sequence motifs on these receptors. We discuss here few putative cholesterol interaction motifs that have been identified in GPCRs.

4.3.1 Cholesterol Recognition/Interaction Amino Acid Consensus (CRAC) Motif

CRAC motif is one of the most well-studied sequence motifs proposed to be implicated in the interaction of proteins with cholesterol. The CRAC motif is characterized by the sequence -L/V-$(X)_{1-5}$-Y-$(X)_{1-5}$-R/K- (from N-terminus to C-terminus of the protein), where $(X)_{1-5}$ represents between one and five residues of any amino acid [24, 168]. Subsequent to the first report on the presence of CRAC motif in the peripheral-type benzodiazepine receptor [169], the motif has been identified in several membrane proteins such as HIV transmembrane protein gp41 [170], caveolin-1 [171], and receptors implicated in pathogen entry [35]. We reported, for the first time, the presence of CRAC motifs in representative GPCRs such as rhodopsin, β_2-adrenergic receptor, and the serotonin$_{1A}$ receptor [172].

We have previously shown that the serotonin$_{1A}$ receptor consists of three CRAC motifs in transmembrane helices II, V, and VII ([172]; see Fig. 4a). Interestingly, coarse-grain molecular dynamics simulations identified high cholesterol occupancy at the CRAC motif in transmembrane helix V of the serotonin$_{1A}$ receptor ([173]; see Fig. 4b). A characteristic feature of these sites is the inherent dynamics exhibited by cholesterol, ranging from ns to μs timescale. The corresponding energy landscape of cholesterol association with GPCRs can be described as a series of shallow minima, interconnected by low energy barriers (see Fig. 4c; [40]). Ongoing work in our laboratory aims to elucidate the role of CRAC motifs in the function of the serotonin$_{1A}$ receptor. In addition, CRAC motifs have been identified and correlated to cholesterol-dependent function of GPCRs such as CB1 [113], CCK1 [115], and bitter taste T2R4 receptors [83]. Importantly, as described earlier (see Sect. 4.1), mutation of key residues in the respective CRAC motifs in these GPCRs led to the modulation of cholesterol sensitivity of their function.

4.3.2 CARC: An Inverted CRAC Motif

The search for cholesterol interaction sites led to the recent identification of CARC, a motif which is similar to CRAC sequence, but with opposite orientation along the polypeptide chain, i.e., -(K/R)-X_{1-5}-(Y/F)-X_{1-5}-(L/V)- [24, 174]. The CARC motif was first identified in the nicotinic acetylcholine receptor and was found to be

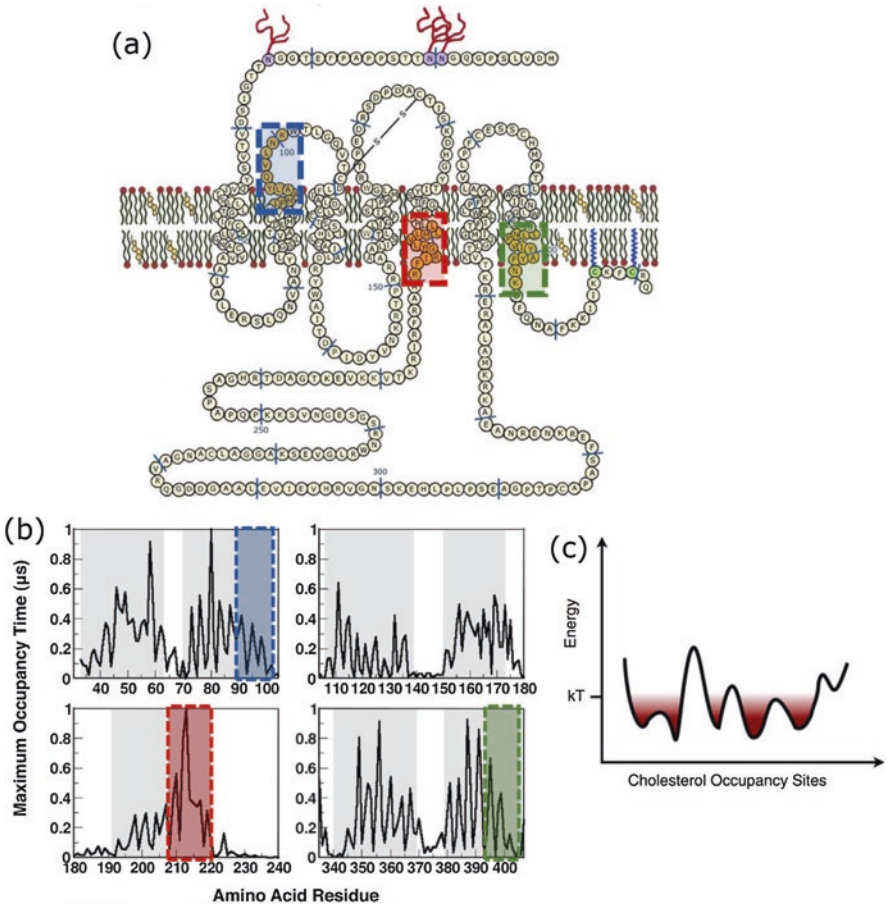

Fig. 4 (**a**) A schematic representation depicting the topological features and amino acid sequence of the human serotonin$_{1A}$ receptor embedded in a membrane bilayer consisting of phospholipids and cholesterol. The serotonin$_{1A}$ receptor consists of three CRAC motifs in transmembrane helices II (boxed in blue), V (boxed in red), and VII (boxed in green). Adapted and modified from [172]. (**b**) Residue-wise maximum occupancy of cholesterol at the serotonin$_{1A}$ receptor, obtained by coarse-grain molecular dynamics simulations. Maximum occupancy time (defined as the longest time a given cholesterol molecule is bound at a particular residue) of cholesterol at each amino acid of the serotonin$_{1A}$ receptor was averaged over simulations carried out at varying concentrations of cholesterol. Transmembrane helices are represented as gray bands, and CRAC motifs are highlighted as in (**a**). The high cholesterol occupancy observed at the CRAC motif on transmembrane helix V is noteworthy. Adapted and modified with permission from [173] (copyright 2018 American Chemical Society). (**c**) Energy landscape corresponding to cholesterol interaction sites in GPCRs. The interaction of cholesterol with GPCRs is weak, yet dynamic with varying occupancy times ranging from ns to μs timescale. This feature of the interaction of cholesterol with GPCRs is reflected in the energy landscape of cholesterol interaction which shows a series of shallow minima interconnected by low energy barriers. Adapted from [40]

conserved over natural evolution among members of this family of receptors [174]. Interestingly, the CARC motif was found in several GPCRs such as rhodopsin, β_2-adrenergic receptor, δ-opioid receptor, galanin receptor type 1, metabotropic glutamate receptor, and chemokine receptor CXCR4 [174]. Some of these receptors display cholesterol sensitivity in their function. The simultaneous presence of the CARC and CRAC motifs in two leaflets of the membrane bilayer in membrane proteins has been proposed as a potential "mirror code" [175].

4.3.3 Cholesterol Consensus Motif (CCM)

CCM was one of the first putative cholesterol interaction sites identified in GPCRs from the crystal structure of the β_2-adrenergic receptor [117]. On the basis of homology, the CCM site has been defined as [4.39-4.43(R,K)]-[4.50(W,Y)]-[4.46(I,V,L)]-[2.41(F,Y)] (according to the Ballesteros–Weinstein nomenclature [176]). We have previously shown high cholesterol occupancy at the CCM site located at the groove of transmembrane helices II and IV of the β_2-adrenergic receptor using coarse-grain molecular dynamics simulations [177]. We have earlier identified a characteristic CCM in the serotonin$_{1A}$ receptor which was found to be evolutionarily conserved [49].

However, it should be noted that mere presence of cholesterol interaction motif(s) does not necessarily translate to cholesterol-dependence of receptor function. For example, the neurotensin receptor 1 does not exhibit cholesterol sensitivity for its function, although the receptor has CCM in its sequence [178].

4.3.4 The Accessibility Issue: Nonannular Binding Sites

In the context of cholesterol binding sites in GPCRs, we previously proposed that these sites could represent "nonannular" binding sites whose possible locations could be inter or intramolecular (interhelical) protein interfaces [49]. Transmembrane proteins are surrounded by a shell (or annulus) of lipid molecules, termed as "annular" lipids [179]. The rate of exchange of lipids between the annular lipid shell and the bulk lipid phase was shown to be approximately an order of magnitude slower than the rate of exchange of bulk lipids [37, 179]. In addition, it was previously proposed that cholesterol binding sites could be "nonannular" in nature [180, 181]. Nonannular sites are characterized by relative lack of accessibility (due to their location in deep clefts or cavities on the protein surface) to the annular lipids [182], and therefore it is proposed that lipids in these sites are difficult to be replaced by competition with annular lipids [181]. Binding to the nonannular sites is considered to be more specific compared to annular sites. Interestingly, a recent study, employing experimental and simulation approaches, has proposed that membrane cholesterol could enter the deep orthosteric ligand binding pocket in the adenosine A_{2A} receptor [183].

5 General Effects of Membrane Cholesterol on GPCRs

The influence of cholesterol on bulk (global) membrane properties has been extensively studied. Cholesterol has been shown to modulate membrane physical properties such as fluidity, curvature, phase, elasticity, dipole potential, and thickness [184–193]. Such effects of cholesterol on general membrane properties have been shown to modulate the organization and function of GPCRs (see Fig. 5; [75, 76, 194–196]).

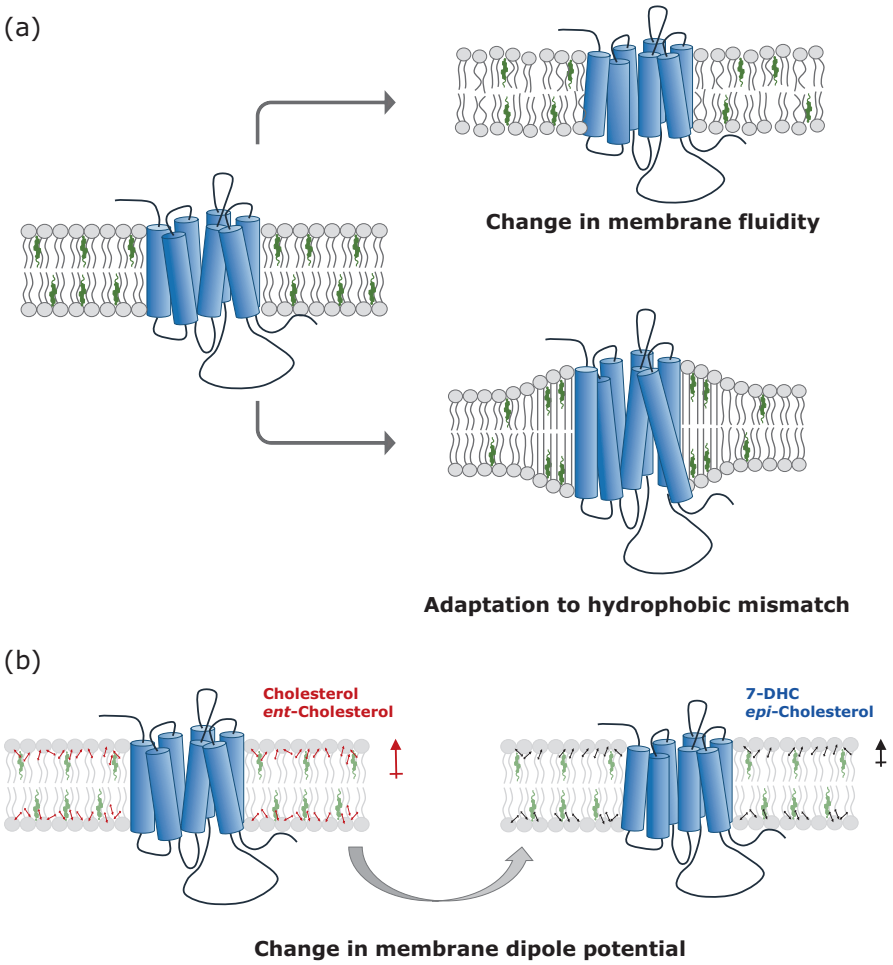

Fig. 5 A schematic representation depicting general effects of cholesterol on membrane physical properties. (**a**) Changes in membrane fluidity and adaptation to hydrophobic mismatch could modulate GPCR function. (**b**) Dipole potential of membranes containing cholesterol and its close structural analogs. Membranes containing cholesterol and *ent*-cholesterol exhibit higher dipole potential (shown as arrows, the length of which represents the magnitude of dipole potential) relative to 7-dehydrocholesterol (7-DHC) and *epi*-cholesterol. Such changes in membrane dipole potential have implications in GPCR function. See text for more details

As discussed above, the specific requirement of cholesterol has been implicated in the function of several GPCRs. The other mechanism by which membrane cholesterol could modulate GPCR function is by affecting general (bulk) membrane properties. What follows below is a brief overview of some of the studies on representative GPCRs where cholesterol-induced modulation of general membrane properties has been implicated in receptor function.

5.1 Rhodopsin

Rhodopsin is a photoreceptor of retinal rod cells and upon exposure to light, undergoes a series of conformational changes. Light-activated rhodopsin exists in equilibrium with a number of intermediates, collectively termed metarhodopsins. Cholesterol is known to regulate the activation of rhodopsin by influencing the equilibrium between the inactive metarhodopsin I (MI) and active metarhodopsin II (MII) states of the receptor [197, 198]. Membrane cholesterol has been shown to stabilize the inactive MI state of the receptor by inducing ordering of membrane lipids, thereby reducing the equilibrium MII (active state) concentration [199]. By increasing the lipid acyl chain ordering, cholesterol reduces the free volume in membrane bilayers [75, 200]. This change in free volume by cholesterol is implied in the observed shift in equilibrium of MI and MII states of rhodopsin [75]. Interestingly, the extent of MII formation displayed a positive correlation with free volume in membranes over a range of cholesterol concentration.

In addition, a variety of mechanisms such as adaptation of rhodopsin to bilayer thickness (in case of hydrophobic mismatch) and membrane curvature have been proposed to regulate MI-MII equilibrium [195]. Interestingly, cholesterol is known to modulate membrane thickness [186] and induce membrane curvature [188]. It is therefore possible that the observed effects of cholesterol on rhodopsin function (MI-MII equilibrium) could be partly due to its effect on membrane thickness (hydrophobic mismatch) and curvature.

5.2 Serotonin$_{1A}$ Receptor

The role of cholesterol in the function of the serotonin$_{1A}$ receptor has been well worked out by our laboratory [3, 37, 40, 42, 201]. Utilizing multiple approaches, we showed that serotonin$_{1A}$ receptors exhibit stringent requirement for cholesterol to support their function, with evidence pointing toward a specific mechanism in many cases (see Sect. 4.1). However, the role of bulk membrane effects of cholesterol on the receptor function cannot be ruled out. With an overall objective of addressing the role of membrane physical properties in receptor function, we monitored the microviscosity of membranes of varying cholesterol content using a fluorescent molecular rotor which allows measurement of membrane viscosity through its

characteristic viscosity-sensitive fluorescence depolarization [196]. A noteworthy feature of our results was that specific agonist binding by the serotonin$_{1A}$ receptor exhibited close correlation with membrane viscosity. This prompted us to speculate that global membrane properties modulated by cholesterol are important in the function of the serotonin$_{1A}$ receptor.

Along similar lines, we measured membrane dipole potential of membranes of varying cholesterol content using an electrochromic fluorescent probe [202]. This provides a convenient method to measure dipole potential, utilizing the probe fluorescence, which is sensitive to the electric field in which the probe is localized [192]. Membrane dipole potential is the potential difference within the membrane bilayer, generated due to the nonrandom arrangement (orientation) of amphiphile dipoles in the membrane interfacial region [203]. Importantly, membrane dipole potential has been shown to play a role in the function of membrane proteins and peptides [204, 205]. In this case too, we noted a correlation between membrane dipole potential and receptor activity [202], reinforcing the above conclusion that global membrane properties could be crucial for the function of the serotonin$_{1A}$ receptor, even if that may not be the whole story.

5.3 Cholecystokinin Receptor

The function of cholecystokinin receptors has been shown to be sensitive to membrane cholesterol content [76]. Interestingly, replacement of membrane cholesterol with sterol analogs that restored membrane fluidity (to levels comparable to membrane fluidity when cholesterol was used) supported the function of the receptor. The specific ligand binding to the receptor exhibited a positive correlation with membrane fluidity, thereby implying the general effect of cholesterol on receptor function.

6 Conclusions

While examples of membrane cholesterol sensitivity of GPCR function have increased over the years, the mechanism underlying such phenomena remains elusive. The notion that cholesterol sensitivity of GPCR function has two underlying mutually exclusive mechanisms appears somewhat arbitrary (although may have provided some early insights). A major reason for this is the fact that it is not always possible to dissect out specific and general effects in a cooperative molecular assembly such as membranes. We would like to illustrate this with recent work done by us [60, 206] and others [207]. In our ongoing work on the role of membrane cholesterol on the function of the serotonin$_{1A}$ receptor, we utilized two stereoisomers of cholesterol, *ent*-cholesterol and *epi*-cholesterol [60]. These are enantiomer and diastereomer of cholesterol, respectively. While *ent*-cholesterol is the non-superimposable

mirror image of natural cholesterol, only the orientation of the hydroxyl group at carbon-3 is inverted relative to natural cholesterol in *epi*-cholesterol. Interestingly, *ent*-cholesterol is often used to distinguish specific interaction of cholesterol from nonspecific effects [208–211]. Typically, enantiomers are characterized by identical physicochemical properties (except for the direction of rotation of plane-polarized light).

We showed that *ent*-cholesterol, but not *epi*-cholesterol, could replace cholesterol in supporting the function of the serotonin$_{1A}$ receptor [60]. In other words, our results demonstrated that the requirement of membrane cholesterol for the serotonin$_{1A}$ receptor function is diastereospecific, but not enantiospecific. A direct implication of these results is that a key structural feature of natural cholesterol in terms of its ability to support the function of the serotonin$_{1A}$ receptor is the *equatorial* configuration of the 3-hydroxyl group. We attributed these results to the fact that *epi*-cholesterol, differing with cholesterol only in the *axial* orientation of the 3-hydroxyl group, was unable to support receptor function. We therefore concluded that the interaction of membrane cholesterol with the serotonin$_{1A}$ receptor is specific in nature [60]. A recent paper reported the detailed physical properties of membranes containing *epi*-cholesterol determined by atomistic molecular dynamics simulations [207]. A closer examination of this paper reveals that physical properties of membranes such as lipid headgroup area, tilt angle, order parameter, and extent of interdigitation are different for membranes containing cholesterol and *epi*-cholesterol. Similar observations were also reported earlier [212]. In addition, we earlier reported that dipole potential of membranes containing cholesterol and *epi*-cholesterol is very different ([206]; see Fig. 5b). Keeping in mind all of the above, whether the difference in receptor function reported by us [60] could be due to these differences in membrane physical properties, or difference in specific interaction due to the orientation of the 3-hydroxyl group, remains a moot question. At this point in time, it is not easy to dissect out a precise answer to this question with available approaches. In addition, specific and general effects of cholesterol may not be mutually exclusive and the observed effect could be a combination of both. Clearly, a judicious combination of experimental and computational approaches would provide more holistic insight into the mechanism of cholesterol sensitivity of GPCR function.

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