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The function of G-protein coupled receptors and membrane cholesterol: specific or general interaction?

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Abstract Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics and function. The G-protein coupled receptors (GPCRs) are the largest class of molecules involved in signal transduction across membranes and constitute ~1–2% of the human genome. GPCRs have emerged as major targets for the development of novel drug candidates in all clinical areas due to their involvement in the generation of multitude of cellular responses. Membrane cholesterol has been reported to have a modulatory role in the function of a number of GPCRs. This effect could either be due to specific molecular interaction between cholesterol and GPCR, or due to alterations in the membrane physical properties induced by cholesterol. Alternatively, membrane cholesterol could modulate receptor function by occupying the 'nonannular' sites around the receptor. In this review, we have highlighted the nature of cholesterol dependence of GPCR function taking a few known examples.

Keywords Membrane cholesterol · G-protein coupled receptor · Serotonin$_{1A}$ receptor · Specific effect · General effect

Abbreviations

5-HT	5-Hydroxytryptamine
7-DHC	7-Dehydrocholesterol
8-OH-DPAT	8-Hydroxy-2(di-*N*-propylamino)tetralin
CCK	Cholecystokinin
DPH	1,6-Diphenyl-1,3,5-hexatriene
GPCR	G-protein coupled receptor
FRET	Fluorescence resonance energy transfer
MβCD	Methyl-β-cyclodextrin
SLOS	Smith–Lemli–Opitz syndrome

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Role of membrane cholesterol in the function of G-protein coupled receptors

Biological membranes are complex non-covalent assemblies of a diverse variety of lipids and proteins that allow cellular compartmentalization, thereby imparting an identity to the cell and its organelles. Since a significant portion of integral membrane proteins remains in contact with the membrane [1], and reaction centers in them are often buried within the membrane, the structure and function of membrane proteins often depend on their interactions with the surrounding lipids [2, 3]. Cholesterol is a major representative lipid in higher eukaryotic cellular membranes and is crucial in organization, dynamics, function, and sorting of membranes [4, 5]. Cholesterol is often found distributed nonrandomly in domains or pools in biological and model membranes [4–8]. Many of these domains (sometimes termed as 'lipid rafts') are believed to be important for the maintenance of membrane structure and function. The idea of such specialized membrane domains assumes significance in cell biology since physiologically important functions such as membrane sorting and trafficking [9] and signal transduction processes [10], in addition to the entry of pathogens [11, 12], have been attributed to these domains. Cholesterol plays a vital role in the function and organization of membrane proteins and receptors [13, 14].

The G-protein coupled receptor (GPCR) superfamily is one of the largest and most diverse protein families in mammals, whose primary function is to transduce signal across membranes [15–17]. Cellular signaling by GPCRs involves their activation upon binding to ligands present in the extracellular environment and the subsequent transduction of signals to the interior of the cell through concerted changes in their transmembrane domain structure [18]. GPCRs are prototypical members of the family of seven transmembrane domain proteins and include >800 members, which together constitute ~1–2% of the human genome [19]. They are involved in the generation of cellular responses to a diverse array of stimuli that include biogenic amines, peptides, glycoproteins, lipids, nucleotides and even photons. As a consequence, these receptors mediate multiple physiological processes such as neurotransmission, cellular metabolism, secretion, cellular differentiation, growth, inflammatory and immune responses. For this reason, GPCRs have emerged as major targets for the development of novel drug candidates in all clinical areas [20–23]. Interestingly, although GPCRs represent 30–50% of current drug targets, only a small fraction of all GPCRs are presently targeted by drugs [24]. This points out the exciting possibility that the receptors, which are not yet recognized, could be potential drug targets for diseases that are difficult to treat by currently available drugs.

Importantly, membrane cholesterol has been shown to modulate the function of a number of GPCRs. From the available data on the role of cholesterol on GPCR function (see Table 1), it appears that there is a lack of consensus in the manner in which cholesterol modulates receptor function. For example, while cholesterol is found to be essential for the proper function of several GPCRs, the function of rhodopsin has been shown to be inhibited by the presence of cholesterol. This brings out the necessity for a detailed mechanistic analysis of the effects of cholesterol on the specific receptor system. In the following section, an effort will be made to critically analyze some of the available literature data on the role of membrane cholesterol in GPCR function, with an overall objective to distinguish specific and general effects.

Table 1 GPCRs whose function is modulated by membrane cholesterol

GPCR	References
Rhodopsin	[25–27, 64]
Cholecystokinin (CCK)	[13, 28, 29]
Galanin (GAL2)	[30]
Serotonin$_{1A}$ (5-HT$_{1A}$)	[14, 31–33]
Serotonin$_7$ (5-HT$_7$)	[34]
Metabotropic glutamate[a]	[35, 36]
δ Opioid	[37]
κ Opioid	[38]
μ Opioid	[39]
Oxytocin	[28, 40–43]
β_2-adrenergic	[44–46]
Chemokine (CXCR4, CCR5)	[47–49]
Neurokinin (NK1)	[50, 51]
Cannabinoid (CB1)	[52, 53]
M_2 muscarinic	[54]

[a] These studies were carried out in the *Drosophila* eye where the major sterol present is ergosterol

Effect of membrane cholesterol on the function of GPCRs: general or specific effect?

The effect of cholesterol on the structure and function of integral membrane proteins and receptors has been a subject of intense investigation [13, 14]. For example, it has been proposed that cholesterol can modulate the function of GPCRs in two ways: (1) through a direct/specific interaction with the GPCR, which could induce a conformational change in the receptor [43, 55], or (2) through an indirect way by altering the membrane physical properties in which the receptor is embedded [3, 56] or due to a combination of both factors. There could be yet another manner in which membrane cholesterol could affect structure and function of membrane proteins. For example, it has been reported that for the nicotinic acetylcholine receptor (which requires cholesterol for its function), cholesterol is proposed to be present at the 'nonannular' sites around the receptor (annular sites are binding sites of lipids in the immediate annulus surrounding the cross sectional area of the membrane protein) [57]. These nonannular sites, initially postulated for Ca^{2+}/Mg^{2+}–ATPase [58, 59], are characterized by occlusion of phospholipids. It has been suggested that the possible locations for the nonannular sites could be either inter- or intramolecular protein interfaces [58]. In the context of GPCRs, it is interesting to note that many GPCRs are believed to function as oligomers [60]. More importantly, cholesterol has been shown to improve stability of GPCRs such as the β_2-adrenergic receptor [61], and appears to be a necessary component for crystallization of the receptor, since it facilitates receptor–receptor interaction and consequent oligomerization [62]. Since a possible location of the nonannular sites is interprotein interfaces [58], it is possible that cholesterol molecules located between individual receptor molecules (see later, Fig. 1) occupy nonannular sites and modulate receptor structure and function. We will discuss below a few known examples of GPCRs for which the mechanism of cholesterol dependence of function has been addressed.

Rhodopsin, the photoreceptor of retinal rod cells, undergoes a series of conformational changes upon exposure to

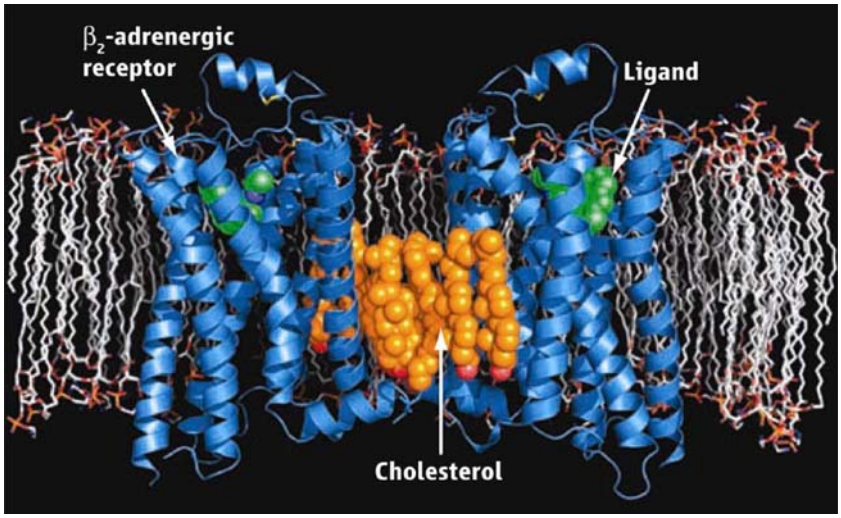

Fig 1 Presence of cholesterol molecules in the recently reported crystal structure of human β_2-adrenergic receptor. The figure depicts the structure of the human β_2-adrenergic receptor (in *blue*) embedded in a lipid bilayer. Cholesterol molecules between two receptor molecules are shown in *orange* (reproduced from [62], with permission from AAAS)

light. The light activated receptor exists in equilibrium with various intermediates collectively called metarhodopsins. The state of equilibrium is sensitive to the presence of cholesterol in the membrane [25, 26]. An increase in the amount of cholesterol in the membrane shifts this equilibrium toward the inactive conformation of the protein. The inhibitory effect of cholesterol on rhodopsin function has been explained by direct as well as indirect modes of action. Direct interaction between rhodopsin and cholesterol has been investigated using Fluorescence Resonance Energy Transfer (FRET) between the tryptophan residues in the receptor and a fluorescent cholesterol analogue, cholestatrienol [63]. Interestingly, in presence of ergosterol, FRET was observed between the tryptophan of rhodopsin and cholestatrienol, indicating a specific interaction between rhodopsin and cholesterol. In addition, this study postulated the presence of one sterol molecule per molecule of receptor present at the lipid–protein interface. On the other hand, the indirect mode of action has been rationalized based on the free-volume theory of membranes, which relates the alteration in membrane physical properties due to the presence of cholesterol to receptor function [26]. The conversion of the photointermediates, metarhodopsin I to metarhodopsin II, upon exposure to light involves an expansion of the protein in the plane of the bilayer [65], which occupies the available partial free volume from the surrounding bilayer. The presence of cholesterol in the membrane has been reported to inhibit the formation of metarhodopsin II, due to its role in reducing the partial free volume in the membrane [66]. Importantly, FRET approaches have indicated an inherent property of rhodopsin to partition out of cholesterol-rich regions of the membrane [67]. These results have recently been reinforced by molecular dynamics simulation with rhodopsin in a membrane containing a mixture of cholesterol and polyunsaturated phospholipids [68].

GPCRs such as oxytocin and cholecystokinin (CCK) receptors have been shown to require membrane cholesterol for their function [28, 29, 40–43]. Interestingly, while the interaction between the oxytocin receptor and cholesterol is believed to be specific, the function of the CCK receptor appears to be dependent on the physical properties of membranes, which are a function of cholesterol content. This is demonstrated by the fact that these receptors displayed different types of correlation, when fluorescence anisotropy of the popular membrane probe DPH was correlated with the ligand binding activity. In case of the CCK receptor, ligand binding showed linear increase with measured anisotropy values [28]. In contrast to this, the ligand binding activity of the oxytocin receptor showed a slight reduction with cholesterol depletion followed by a sharp decline, when the membrane cholesterol content reached a certain critical level (~57% of the original cholesterol content). This shows that membrane cholesterol could affect the ligand binding activity of the oxytocin receptor by a cooperative mechanism. Hill analysis of cholesterol content *vs.* ligand binding revealed that the oxytocin receptor binds several molecules of cholesterol ($n \geq 6$) in a positively cooperative manner [13, 43]. These conclusions are further supported by structure–activity analysis of the oxytocin and cholecystokinin receptor using a variety of cholesterol analogues substituting for membrane cholesterol [28]. In order to assess the specific structural features of cholesterol that are required to maintain the high-affinity state of the oxytocin receptor, cyclodextrins were used to replenish cholesterol-depleted membranes with a broad range of cholesterol analogues that were subtly different from cholesterol either in the head group, the steroid ring, or in the hydrocarbon tail. Interestingly, ligand binding of the oxytocin receptor could be restored only with certain analogues, thereby indicating to a specific structural feature in cholesterol to support

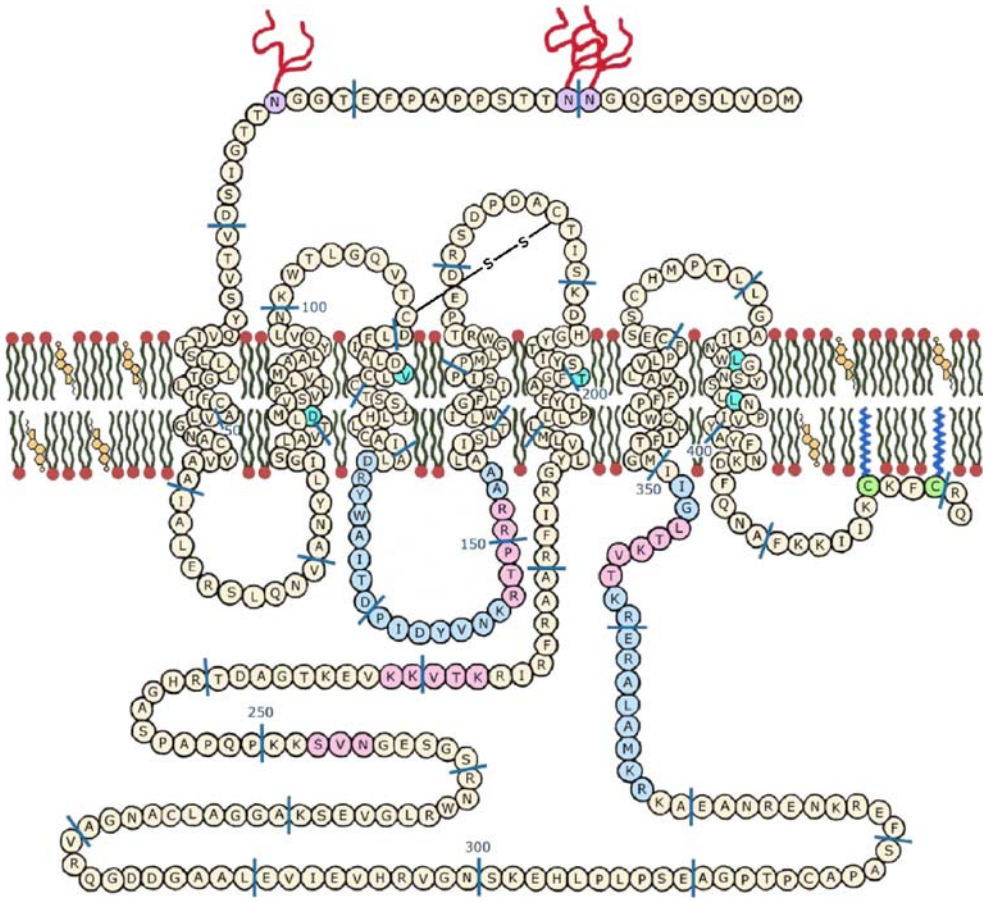

Fig 2 A schematic representation of the membrane embedded human serotonin$_{1A}$ receptor showing its topological and other structural features. The membrane is shown as a bilayer of phospholipids and cholesterol, representing typical eukaryotic membranes. The amino acids in the receptor sequence are shown as circles and are marked for convenience. Seven transmembrane stretches, each composed of 20–26 amino acids, are depicted as α-helices. The potential sites (shown in *lavender*) for N-linked glycosylation (depicted as *branching trees in red*) on the amino terminus are shown. A putative disulphide bond between Cys^{109} and Cys^{187} is shown. Transmembrane domains contain residues (shown in *cyan*) that are important for ligand binding. The receptor is stably palmitoylated (shown in *blue*) at residues Cys^{417} and/or Cys^{420} (shown in *green*). *Light blue circles* represent contact sites for G-proteins. *Light pink circles* represent sites for protein kinase mediated phosphorylation. Further structural details of the receptor are available in [14] and [71]. Adapted and modified from [71]. It is probable, based on comparison with known crystal structures of similar GPCRs such as rhodopsin and β_2-adrenergic receptor that there are motionally restricted water molecules that could be important in inducing conformational transitions in the transmembrane portion of the receptor

receptor function. Although cholesterol depletion reduces ligand binding to the cholecystokinin receptor, this effect could be reversed with most analogues of cholesterol that could restore membrane order. The ligand binding of the CCK receptor therefore was supported by each of the cholesterol analogues and was well correlated with the corresponding fluorescence anisotropy values. However, similar effects on the oxytocin receptor could be demonstrated only with certain analogues that structurally resembled cholesterol in some critical features. Taken together, this data provide support for a specific molecular interaction between the oxytocin receptor and cholesterol. Further, molecular modeling studies have indicated a putative docking site (involving residues on the surface of transmembrane segments 5 and 6) for cholesterol in the oxytocin receptor that is absent in the CCK receptor [69]. In addition, it has been reported that cholesterol stabilizes the oxytocin receptor against thermal inactivation and protects the receptor from proteolytic degradation [70]. It has also recently been shown that cholesterol promotes cooperativity the binding of ligands to the M_2 muscarinic receptor [54].

Pang *et al.* have shown that membrane cholesterol is required for the ligand binding of the subtype 2 galanin receptor (GalR2) and intracellular signaling of the receptor [30]. The role of membrane cholesterol in modulating ligand binding to the galanin receptor was examined by treating membranes with MβCD or by culturing cells expressing the receptor in lipoprotein-deficient serum. These studies revealed a marked reduction in galanin binding to the receptor in cholesterol-deficient membranes. Importantly, replenishment of cholesterol to cholesterol-depleted membranes restored galanin binding to normal levels. This

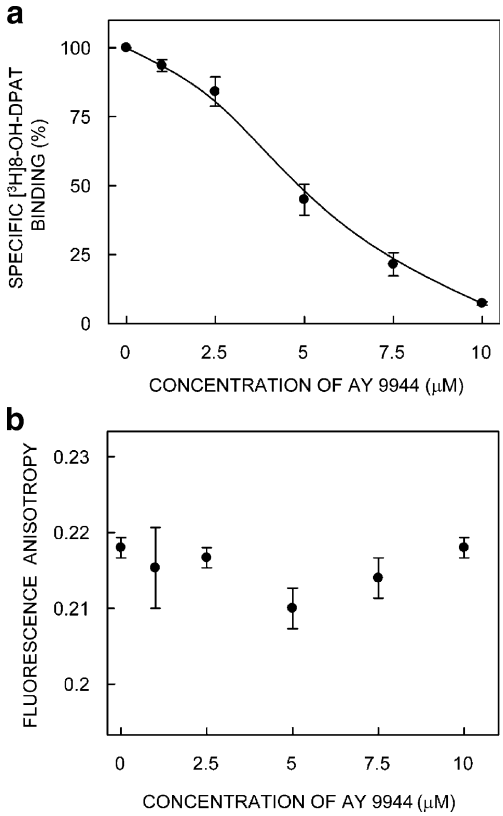

Fig 3 Ligand binding function of the human serotonin$_{1A}$ receptor is impaired in cellular model of the Smith–Lemli–Opitz syndrome. CHO cells stably expressing the human serotonin$_{1A}$ receptor were treated with varying concentrations of the inhibitor AY 9944 and specific [^{3}H]8-OH-DPAT binding to the serotonin$_{1A}$ receptor was measured (shown in *panel a*). Values (means±standard error) are expressed as a percentage of specific binding for control cell membranes without AY 9944 treatment. *Panel b* shows that the overall membrane order is unaltered in SLOS-like condition. The overall membrane order was estimated in control cell membranes and in membranes of cells treated with varying concentrations of AY 9944, using fluorescence anisotropy of the membrane probe DPH. Means±standard error of the measured anisotropy values are shown. Adapted and modified from [79]

interaction appears to be specific to cholesterol as only a limited number of cholesterol analogues were able to rescue galanin binding. In addition, treatment of membranes with filipin, a cholesterol-binding agent, or with cholesterol oxidase markedly reduced galanin binding. Hill analysis suggested that several molecules of cholesterol ($n \geq 3$) could bind in a positively cooperative manner to GalR2 [30].

The serotonin$_{1A}$ receptor: an important member of the GPCR superfamily in the context of membrane cholesterol dependence for receptor function

The serotonin$_{1A}$ receptor is an important neurotransmitter receptor and is the most extensively studied of the serotonin receptors for a number of reasons [71]. Serotonin receptors have been classified into at least 14 subtypes on the basis of their pharmacological responses to specific ligands, sequence similarities at the gene and amino acid levels, gene organization, and second messenger coupling pathways [72]. The serotonin$_{1A}$ receptor is the first among all the types of serotonin receptors to be cloned as an intronless genomic clone (G-21) of the human genome which cross-hybridized with a full length β-adrenergic receptor probe at reduced stringency [71, 73]. Sequence analysis of this genomic clone (later identified as the serotonin$_{1A}$ receptor gene) showed 43% amino acid homology with the β_2-adrenergic receptor in the transmembrane domain. The serotonin$_{1A}$ receptor was therefore initially discovered as an 'orphan' receptor and was identified ('deorphanized') later [74]. The human gene encodes a protein of 422 amino acids (see Fig. 2). Serotonergic signaling plays a key role in the generation and modulation of various cognitive, developmental and behavioral functions. Interestingly, mutant (knockout) mice lacking the serotonin$_{1A}$ receptor exhibit enhanced anxiety-related behavior, and represent an important animal model for the analysis of complex traits such as anxiety disorders and aggression in higher animals [75, 76].

The modulatory role of cholesterol on the ligand binding activity and G-protein coupling of the hippocampal serotonin$_{1A}$ receptor has been shown in our laboratory using various approaches, which include treatment with (i) MβCD, which physically depletes cholesterol from membranes [31, 32] (ii) the sterol-complexing agent digitonin [33], and (iii) the sterol-binding antifungal polyene antibiotic nystatin [77]. While treatment with MβCD physically depletes cholesterol from membranes, treatment with other agents modulates the availability of membrane cholesterol without physical depletion. In addition, metabolic depletion of cholesterol using cholesterol lowering agents such as statins resulted in the reduction of the ligand binding of serotonin$_{1A}$ receptors (Shrivastava, S., Pucadyil, T.J., Chattopadhyay, A., unpublished observations). The underlying tenet brought out by these data implies that it is the *non-availability of cholesterol*, rather than the manner in which its availability is modulated, is crucial for ligand binding of the serotonin$_{1A}$ receptor. Importantly, replenishment of membranes with cholesterol using MβCD–cholesterol complex led to recovery of ligand binding activity to a considerable extent.

In order to test the stringency of the requirement of cholesterol for the function of the serotonin$_{1A}$ receptor, we treated membranes with cholesterol oxidase, which catalyzes the oxidation of cholesterol to cholestenone. The results showed that oxidation of membrane cholesterol led to inhibition of the ligand binding activity of the serotonin$_{1A}$ receptor without altering overall membrane order [78]. To further explore the specificity of cholesterol requirement, we have recently generated a cellular model

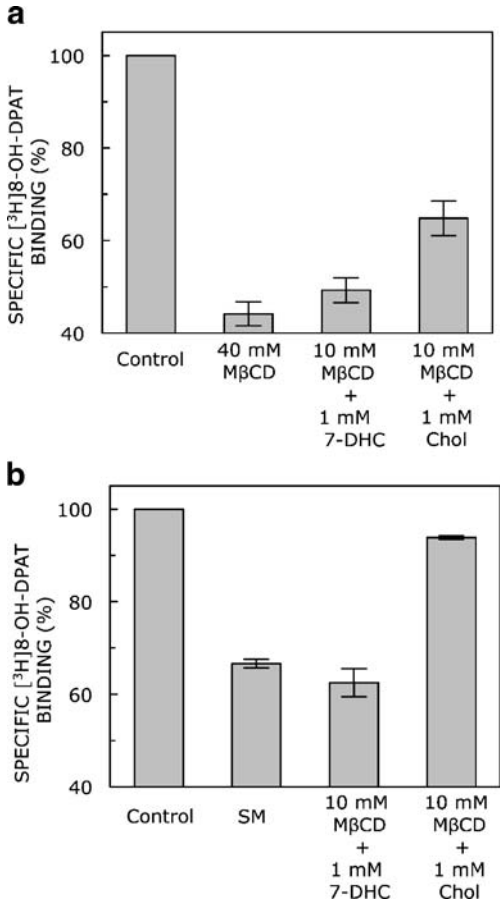

Fig 4 (**a**) Effect of replenishment of 7-DHC and cholesterol into cholesterol-depleted membranes on the specific binding of the [^{3}H]8-OH-DPAT to the hippocampal serotonin$_{1A}$ receptor. Cholesterol depletion in native hippocampal membranes was achieved using 40 mM MβCD followed by replenishment with 7-DHC and cholesterol. Values (means±standard error) are expressed as percentages of the specific binding obtained in native membranes. (**b**) Effect of replenishment of 7-DHC or cholesterol into solubilized membranes (denoted as SM) on specific binding of the [^{3}H]8-OH-DPAT to the hippocampal serotonin$_{1A}$ receptor. Solubilized membranes were replenished with 7-DHC or cholesterol, using the corresponding sterol: MβCD complex. Values (means±standard error) are expressed as percentages of specific ligand binding obtained in native membranes. Adapted and modified from [81] and [82]

of the Smith–Lemli–Opitz Syndrome (SLOS) using cells stably expressing the human serotonin$_{1A}$ receptor [79]. SLOS is a congenital and developmental malformation syndrome associated with defective cholesterol biosynthesis in which the immediate biosynthetic precursor of cholesterol (7-DHC) is accumulated [80]. The cellular model of SLOS was generated by metabolically inhibiting the biosynthesis of cholesterol, utilizing a specific inhibitor (AY 9944) of the enzyme required in the final step of cholesterol biosynthesis. SLOS serves as an appropriate condition to delineate the specific and global effects of cholesterol in the function of the serotonin$_{1A}$ receptor, since the two aberrant sterols that get accumulated in SLOS, *i.e.*, 7- and 8-DHC, differ from cholesterol only in a double bond. Figure 3a shows a progressive and drastic reduction in the specific ligand binding with increasing concentrations of AY 9944 used. In addition, our results show that the G-protein coupling and downstream signaling of serotonin$_{1A}$ receptors are impaired in SLOS-like condition, although the membrane receptor level does not exhibit any reduction. Importantly, metabolic replenishment of cholesterol using serum partially restored the ligand binding activity of the serotonin$_{1A}$ receptor. Figure 3b shows that the overall membrane order, as monitored with anisotropy measurements of the fluorescent probe DPH, does not exhibit a significant change in SLOS-like condition. Interestingly, we have recently shown that 7-DHC does not support the function of the serotonin$_{1A}$ receptor without any change in overall membrane order [81, 82]. This is shown in Fig. 4a, where cholesterol depletion from native hippocampal membranes followed by replenishment with 7-DHC, did not result in restoration of the ligand binding to the serotonin$_{1A}$ receptor, in spite of recovery of the membrane order (Fig. 5) [81]. In addition, solubilization of the hippocampal serotonin$_{1A}$ receptor is accompanied by loss of membrane cholesterol, which results in a reduction in specific ligand binding activity and overall membrane order [82]. Replenishment of cholesterol to solubilized membranes restores the cholesterol content of the membrane and significantly enhances specific ligand binding activity (Fig. 4b) and overall membrane order (Fig. 5). Importantly, replenishment of solubilized hippocampal membranes with 7-DHC does not restore ligand binding activity of the serotonin$_{1A}$ receptor (Fig. 4b), in spite of

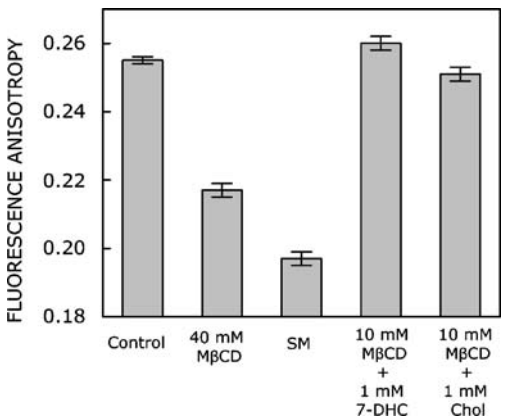

Fig 5 Effect of replenishment of 7-DHC and cholesterol into cholesterol-depleted and solubilized membranes on fluorescence anisotropy (means±standard error) of the membrane probe DPH. Cholesterol depletion was carried out using 40 mM MβCD. Membranes (cholesterol-depleted or solubilized) were replenished with 7-DHC or cholesterol, using the corresponding sterol:MβCD complex. Adapted and modified from [81] and [82]

recovery of the membrane order (Fig. 5). Interestingly, we have recently shown that the effects of 7-DHC and cholesterol on membrane organization and dynamics are considerably different [83]. We therefore conclude that the requirement for maintaining ligand binding activity is more stringent than the requirement for maintaining membrane order. Taken together, these results indicate that the molecular basis for the requirement of membrane cholesterol in maintaining the ligand binding activity of serotonin$_{1A}$ receptors could be specific interaction, although global bilayer effects may not be ruled out.

Conclusion and future perspectives

As mentioned earlier, GPCRs are involved in a multitude of physiological functions and represent important drug targets. Although the pharmacological and signaling features of GPCRs have been studied widely, aspects related to their interaction with membrane lipids have been addressed in relatively few cases. In this context, the realization that lipids such as cholesterol could influence the function of GPCRs has remarkably transformed our idea regarding the function of this important class of membrane proteins. Very recently, it has been possible to resolve closely associated lipid molecules in the crystal structures of GPCRs. For example, tightly bound cholesterol molecules have been reported in the recently reported crystal structure of β_2-adrenergic receptor [62] (see Fig. 1). The presence of such tightly bound cholesterol molecules in GPCR structures indicates local (specific) interaction between GPCR and cholesterol. With progress in deciphering molecular details on the nature of this interaction, our overall understanding of GPCR function in health and disease would improve significantly thereby enhancing our ability to design better therapeutic strategies to combat such diseases.

It has been postulated that glycosphingolipids and cholesterol occur in laterally segregated lipid domains (sometimes termed as 'lipid rafts') [84, 85]. Keeping in mind the crucial role of glycolipids in cellular function [86], the involvement of these lipids in GPCR-cholesterol interaction promises to be an intriguing area of research. In a broader sense, the diversity of lipids found in natural membranes, combined with the ability of cells to modulate their membrane lipid composition under conditions of a variety of stress (or shock), vastly increase the potential by which lipids can exert their influence on receptor function. As in the case of many other membrane proteins, low expression levels of the GPCRs in natural membranes, and inherent difficulties in solubilizing [87] and purifying them have posed considerable challenges in addressing various issues related to membrane biology of GPCRs. Nonetheless, cultured cells heterologously expressing GPCRs have made it possible to address important aspects related to membrane organization and function of GPCRs. The development of newer and more sensitive technologies that determine the interactions of GPCRs with membrane lipids and their influence on receptor function in a more native-like membrane environment [88] would provide a more comprehensive understanding of GPCR function.

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