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Structural Stringency and Optimal Nature of Cholesterol Requirement in the Function of the Serotonin$_{1A}$ Receptor

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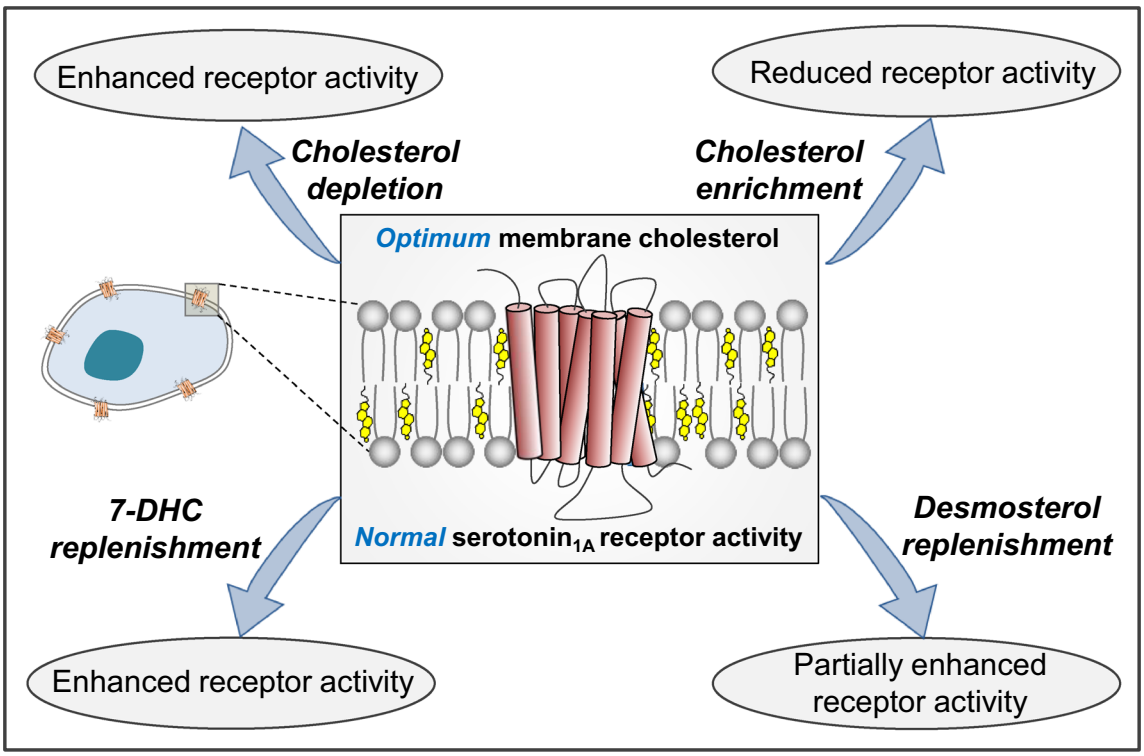
Abstract

The role of membrane cholesterol in modulating G protein-coupled receptor (GPCR) structure and function has emerged as a powerful theme in contemporary biology. In this paper, we report the subtlety and stringency involved in the interaction of sterols with the serotonin$_{1A}$ receptor. For this, we utilized two immediate biosynthetic precursors of cholesterol, 7-dehydrocholesterol (7-DHC) and desmosterol, which differ with cholesterol *merely* in a double bond in their chemical structures in a position-dependent manner. We show that whereas 7-DHC could not support the ligand binding function of the serotonin$_{1A}$ receptor in live cells, desmosterol could partially support it. Importantly, depletion and enrichment of membrane cholesterol over basal level resulted in an increase and reduction of the basal receptor activity, respectively. These results demonstrate the relevance of optimal membrane cholesterol in maintaining the activity of the serotonin$_{1A}$ receptor, thereby elucidating the relevance of cellular cholesterol homeostasis.

Graphic Abstract



Keywords Serotonin$_{1A}$ receptor · 7-Dehydrocholesterol/desmosterol · Optimum membrane cholesterol

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Introduction

G protein-coupled receptors (GPCRs) are the largest class of receptors in the plasma membrane that transduce extracellular signals to the cellular interior. They are characterized by a seven transmembrane domain architecture connected by three extracellular and three intracellular loops (Pierce et al. 2002; Rosenbaum et al. 2009; Venkatakrishnan et al. 2013; Chattopadhyay 2014; Pal and Chattopadhyay 2019). GPCRs are crucial communication hubs on the cell membrane and facilitate a wide array of cellular signaling processes upon binding to a diverse variety of ligands (Ghosh et al. 2014; Wacker et al. 2017). As a consequence of the varied range of physiological processes regulated by GPCRs, these receptors are the most popular therapeutic targets and ~ 40% of approved drugs in the current market act on GPCRs (Insel et al. 2019). Due to the intimate association of GPCRs with the plasma membrane due to their heptahelical architecture, the interaction of GPCRs with lipids in their immediate microenvironment assumes relevance. In this context, the interplay between GPCRs and membrane cholesterol has emerged as a major area of research in GPCR biology. Extensive work using biochemical, biophysical and computational approaches has shown that cholesterol affects various facets of GPCR function that include ligand binding, G-protein coupling, downstream signaling, endocytosis and oligomerization (Pucadyil and Chattopadhyay 2006; Paila and Chattopadhyay 2010; Oates and Watts 2011; Jafurulla and Chattopadhyay 2013; Sengupta and Chattopadhyay 2015; Gimpl 2016; Sengupta et al. 2018; Jafurulla et al. 2019; Kumar and Chattopadhyay 2020).

The serotonin$_{1A}$ receptor (Pucadyil et al. 2005; Müller et al. 2007; Sarkar et al. 2018) is one of the most comprehensively studied neurotransmitter GPCRs of the serotonin receptor family. The serotonin$_{1A}$ receptor mediates a multitude of neurological functions associated with the neurotransmitter serotonin, the endogenous ligand for the receptor. As a consequence of its indispensable role in neurological functions, the agonists and antagonists of the serotonin$_{1A}$ receptor represent major drugs in the development of therapeutics against neuropsychiatric disorders such as anxiety (Akimova et al. 2009), depression (Kaufman et al. 2016), schizophrenia and Parkinson's disease (Ohno 2011; Miyazaki and Asanuma 2016). As a consequence of the broad range of physiological processes regulated by the serotonin$_{1A}$ receptor, the spectrum of therapeutic interventions targeting the receptor has broadened beyond its role in neurological function (Fiorino et al. 2014).

Cholesterol is one of the most abundant lipids in higher eukaryotic cell membranes (Mouritsen 2005; van Meer et al. 2008) that plays a unique functional role in the organization, dynamics and function of membranes (Simons and Ikonen 2000; Mouritsen and Zuckermann 2004; Kumar and Chattopadhyay 2016). In higher eukaryotes, cellular cholesterol biosynthesis takes place in a stringently regulated multi-step pathway concerted by more than twenty enzymes (Kandutsch and Russell 1960; Bloch 1983; Nes 2011). From a structural standpoint, cholesterol is an amphiphilic molecule with a hydrophobic near-planar fused tetracyclic steroid ring and a polar 3β-hydroxyl group (see Fig. 1). The uniquely crafted structural and stereochemical features of cholesterol, contributing to its preferential interaction with membrane proteins, have been fine-tuned over a long time span of natural evolution (Bloch 1983; Kumar and Chattopadhyay 2016). According to the Bloch hypothesis, the structure of cholesterol has been selected over ~ 2.5 billion years of natural evolution for its ability to support complex cellular functions in higher eukaryotes by optimizing membrane physicochemical properties such as fluidity, permeability and microviscosity (Brown and Galea 2010). As a consequence, the biosynthetic precursors of cholesterol should possess properties that gradually support cellular function in higher organisms as they progress along the biosynthetic pathway toward cholesterol.

Cholesterol biosynthesis in cells begins with acetyl-CoA, that feeds into the mevalonate pathway to generate lanosterol (Fig. 1). Subsequently, cholesterol is synthesized from lanosterol either via desmosterol (the Bloch pathway) or 7-dehydrocholesterol (7-DHC) (the Kandutsch–Russel pathway) as immediate precursors (Nes 2011; Mitsche et al. 2015). 7-DHC is reduced to cholesterol by the enzyme 3β-hydroxy-steroid-Δ^7-reductase (7-DHCR) in the final step of the Kandutsch–Russel pathway (Kandutsch and Russell 1960). 7-DHC differs with cholesterol *only* in a double bond at the 7th position in the sterol ring (see Fig. 1). Accumulation of 7-DHC has been associated with the neurological disorder Smith–Lemli–Opitz syndrome (SLOS), a clinical manifestation of defective cholesterol biosynthesis (due to mutations in the gene encoding 7-DHCR) that results in severe congenital and developmental defects (Waterham and Wanders 2000; Porter 2008; Witsch-Baumgartner 2008). On the other hand, in the Bloch pathway, desmosterol is the immediate biosynthetic precursor of cholesterol and it differs only in an extra double bond at the 24th position in the flexible alkyl side chain (Fig. 1). Although desmosterol differs from cholesterol *merely* by a double bond, its accumulation, due to mutations in the gene encoding 3β-hydroxy-steroid-Δ^{24}-reductase (24-DHCR), leads to desmosterolosis, resulting in developmental and neurological disorders (FitzPatrick et al. 1998). For this reason, comparative studies of the effects of cholesterol and its biosynthetic precursors on membrane protein function assume relevance.

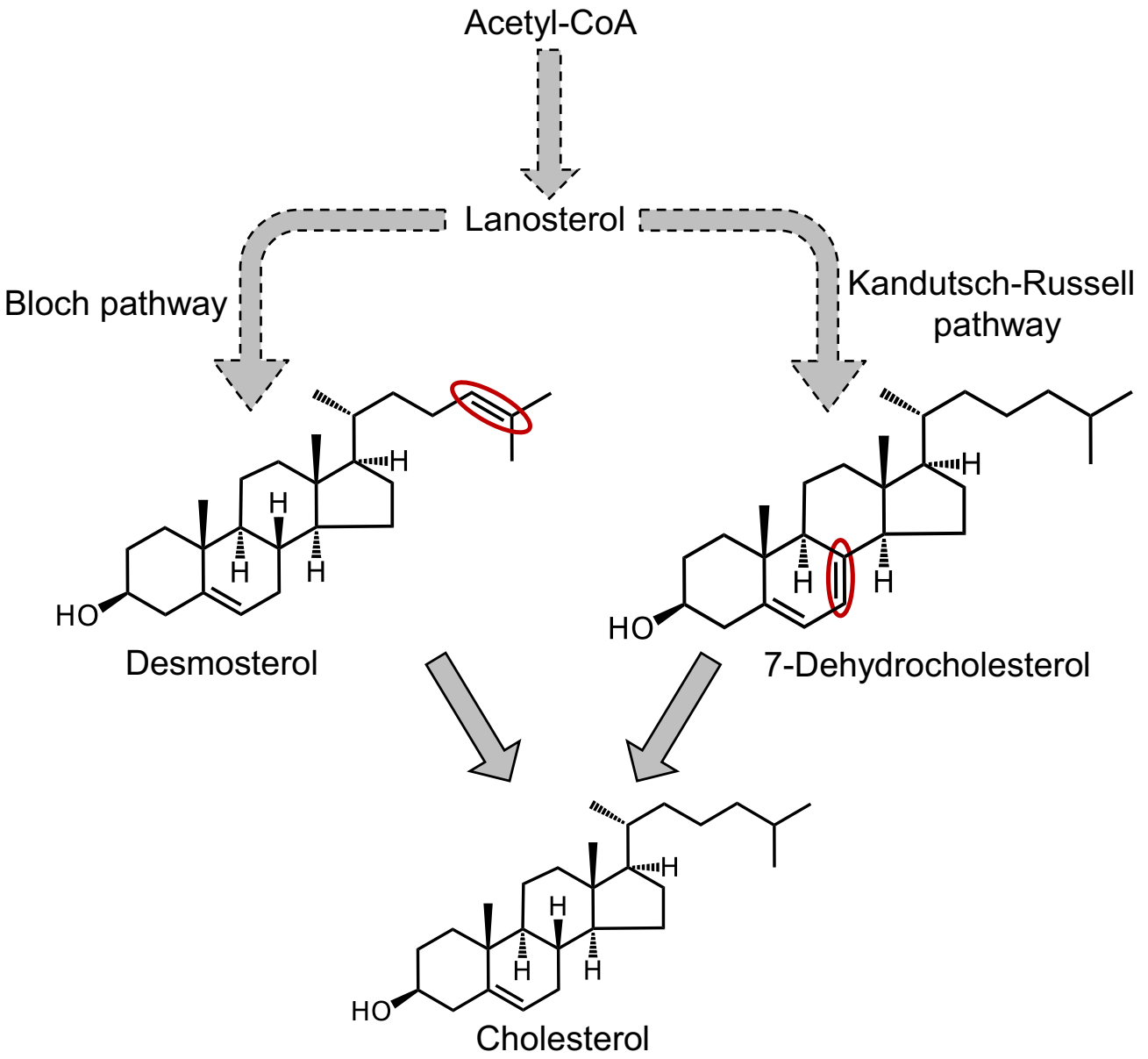

Fig. 1 Chemical structures of cholesterol, 7-dehydrocholesterol (7-DHC) and desmosterol and their relative positions in cholesterol biosynthetic pathway. 7-DHC and desmosterol are the immediate biosynthetic precursors of cholesterol in the Kandutsch–Russell and Bloch pathways of cholesterol biosynthesis, respectively. 7-DHC differs with cholesterol only in a double bond at the 7th position in the sterol ring, whereas desmosterol differs only in a double bond at the 24th position in the flexible alkyl side chain (highlighted in their chemical structures). See text for more details

Cholesterol sensitivity of GPCR function could be ascribed either to direct interaction of cholesterol with the receptor, or to the ability of cholesterol to modulate the receptor microenvironment (such as fluidity, hydrophobic thickness and dipole potential) (Paila and Chattopadhyay 2009; Clarke 2019; Jafurulla et al. 2019), or a combination of both. Previous studies have shown that structural features of cholesterol, such as a free 3β-OH group, a planar $\Delta^{5(6)}$ double bond, a branched 7-carbon alkyl chain at the 17th position and angular methyl groups, are critical for its complex biological function (Kumari et al. 1982; Ranadive and Lala 1987; Róg et al. 2007; Pöyry et al. 2008). As a result, immediate biosynthetic precursors of cholesterol are known to give rise to membrane properties, such as dipole potential (Haldar et al. 2012), lateral pressure profile and packing (Berring et al. 2005; Megha et al. 2006; Ollila et al. 2007), tilt angle (Aittoniemi et al. 2006) and membrane temporal heterogeneity (Shrivastava et al. 2020), that are strikingly different from those observed in membranes containing cholesterol. In this overall context, we have previously

shown that membrane cholesterol is crucial for the function of the serotonin$_{1A}$ receptor both in native (Pucadyil and Chattopadhyay 2004, 2005) and heterologous (Pucadyil and Chattopadhyay 2007) systems.

In the present work, to explore the stringency of the requirement of key structural features associated with membrane cholesterol in modulating serotonin$_{1A}$ receptor function, we monitored whether its immediate biosynthetic precursors (7-DHC and desmosterol) could support the ligand binding function of the serotonin$_{1A}$ receptor in live cells. Our results show that cholesterol depletion from plasma membrane of live cells leads to an increase in the specific agonist binding to the serotonin$_{1A}$ receptor. Importantly, we demonstrate that replenishment with 7-DHC does not restore ligand binding of the serotonin$_{1A}$ receptor, although replenishment with cholesterol (subsequent to cholesterol depletion) results in complete recovery of ligand binding. Interestingly, we observed that desmosterol could partially restore the ligand binding activity of the serotonin$_{1A}$ receptor. To further explore the role of optimum requirement of membrane cholesterol content in the ligand binding function of the serotonin$_{1A}$ receptor, we enriched cellular membranes with additional cholesterol. Our results show that enrichment of membrane cholesterol results in a significant decrease in ligand binding of the receptor, thereby demonstrating that optimum plasma membrane cholesterol is necessary for the function of the serotonin$_{1A}$ receptor. Taken together, we believe that our results have potential implications in understanding the interaction of membrane sterols with neuronal receptors in diseases related to defective cholesterol biosynthesis (Waterham 2006; Porter and Herman 2011) and cellular cholesterol homeostasis.

Materials and Methods

Materials

Cholesterol, 7-dehydrocholesterol (7-DHC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), EDTA, gentamycin sulfate, methyl-β-cyclodextrin (MβCD), $MgCl_2$, $MnCl_2$, Na_2HPO_4, phenylmethylsulfonyl fluoride (PMSF), polyethylenimine, penicillin, serotonin, sodium bicarbonate, streptomycin and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). Desmosterol was obtained from Avanti Polar Lipids (Alabaster, AL). DMEM/F-12 [Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Ham) (1:1)], fetal calf serum and Geneticin (G418) were from Invitrogen/Life Technologies (Grand Island, NY). Bicinchoninic acid (BCA) reagent for protein estimation was from Pierce (Rockford, IL). Amplex Red cholesterol assay kit was purchased from Molecular Probes/Invitrogen (Eugene, OR). [^{3}H]8-hydroxy-2(di-*N*-propylamino)tetralin ([^{3}H]8-OH-DPAT, specific activity of 141.1 Ci/mmol) was purchased from MP Biomedicals (Santa Ana, CA). GF/B glass microfiber filters were from Whatman International (Kent, UK). Precoated silica gel 60 thin layer chromatography plates were from Merck (Darmstadt, Germany). All other chemicals and solvents used were of the highest available purity. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Cells and Cell Culture

Chinese hamster ovary (CHO-K1) cells stably expressing the human serotonin$_{1A}$ receptor (termed as CHO-5HT$_{1A}$R) were maintained in DMEM/F-12 (1:1) medium supplemented with 2.4 g/l of sodium bicarbonate, 10% (v/v) fetal calf serum, 60 µg/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml gentamycin sulfate and 0.2 mg/ml G418 (complete medium) in a humidified atmosphere with 5% CO_2 at 37 °C.

Cholesterol Depletion of Cells in Culture

CHO-5HT$_{1A}$R cells were grown for 3 days followed by incubation in serum-free DMEM/F-12 (1:1) medium for 3 h prior to treatment. Cholesterol depletion was carried out by treating cells with 10 mM MβCD for 30 min in serum-free DMEM/F-12 (1:1) medium in a humidified atmosphere with 5% CO_2 at 37 °C followed by a wash with PBS.

Sterol Replenishment of Cholesterol-Depleted Cells in Culture

Cholesterol-depleted cells were replenished with 7-DHC, desmosterol or cholesterol using respective sterol-MβCD complex. The complex was prepared by dissolving the required amounts of 7-DHC or desmosterol or cholesterol and MβCD in a ratio of 1:10 (mol/mol) in water by constant shaking at room temperature (~23 °C). Stock solution of the complex (containing 2 mM 7-DHC or desmosterol or cholesterol:20 mM MβCD) was freshly prepared before each experiment. Sterol replenishments were carried out by incubating the cholesterol-depleted cells with 1 mM of the respective sterol:10 mM MβCD complex for 10 min in serum-free DMEM/F-12 medium in a humidified atmosphere with 5% CO_2 at 37 °C followed by a wash with PBS.

Cholesterol Enrichment of Cells in Culture

CHO-5HT$_{1A}$R cells were enriched with cholesterol using a water soluble cholesterol-MβCD complex. Stock solution of the cholesterol-MβCD complex (containing 2:20 mM cholesterol:MβCD) was prepared by dissolving the required amounts of cholesterol and MβCD in water by constant shaking at room temperature (~23 °C). The stock solution

was freshly prepared before each experiment. CHO-5HT$_{1A}$R cells were incubated with the cholesterol-MβCD complex (final concentration 1:10 mM cholesterol:MβCD) for 10 min in serum-free DMEM/F-12 medium in a humidified atmosphere with 5% CO_2 at 37 °C followed by a wash with PBS.

Cell Membrane Preparation

Cell membranes of control and sterol-modulated CHO-5HT$_{1A}$R cells were prepared by differential centrifugation as described previously (Kalipatnapu et al. 2004). CHO-5HT$_{1A}$R cells were harvested by treatment with ice-cold hypotonic buffer containing 10 mM Tris, 5 mM EDTA and 0.1 mM PMSF (pH 7.4). Cells were then homogenized for 10 s at 4 °C with a Polytron homogenizer at maximum speed. The cell lysate was centrifuged at 500×*g* for 10 min at 4 °C and the resulting post-nuclear supernatant was centrifuged at 40,000×*g* for 30 min at 4 °C. The final pellet was resuspended in 50 mM Tris buffer (pH 7.4), homogenized using a hand-held Dounce homogenizer and used immediately for radioligand binding assays. The total protein concentration in the isolated membranes was determined using the BCA reagent (Smith et al. 1985). In case of cholesterol-enriched membranes, cholesterol content in cell membranes prepared from CHO-5-HT$_{1A}$R cells was estimated using the Amplex Red cholesterol assay kit (Amundson and Zhou 1999) and normalized to cell membrane protein content.

Estimation of Membrane Phospholipid Contents

Total phospholipid content from cellular lysates was estimated subsequent to lipid extraction by the Bligh and Dyer method with some modifications (Bligh and Dyer 1959). Briefly, cell membranes containing ~ 100 µg protein were treated with methanol/chloroform/water (2:1:0.8, v/v/v) for ~ 6 h with intermittent vortexing and subsequently phase separated using water/chloroform (1:1, v/v). This was followed by centrifugation of samples at low speed (500 × g for 10 min), and the bottom layer containing lipids in chloroform was collected and dried under a stream of nitrogen with gentle warming (~35 °C). Total phospholipid content from the extracted lipids was determined following digestion with perchloric acid as described previously (McClare 1971) using Na_2HPO4 as a standard. DMPC was used as an internal standard to assess the extent of lipid digestion.

Radioligand Binding Assay

Receptor radioligand binding assays with cell membranes isolated from control and sterol-modulated CHO-5HT$_{1A}$R cells were carried out as described previously (Kalipatnapu et al. 2004). Tubes in duplicate containing ~ 100 µg total membrane protein in a volume of 1 ml of buffer (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM $MnCl_2$ (pH 7.4)) were incubated with the radiolabeled specific agonist [^{3}H]8-OH-DPAT for 1 h at room temperature (~ 23 °C). The final concentration of the radioligand in each assay tube was 0.5 nM. Nonspecific binding was determined by performing the assay in presence of 10 µM serotonin. The binding reaction was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B filters (1.0 µm pore size) which were pre-soaked in 0.5% (w/v) polyethylenimine for 1 h (Bruns et al. 1983). Following this, filters were washed three times with 5 ml of cold water (~ 4 °C), dried and the retained radioactivity was measured in a Packard Tri-Carb 2900 liquid scintillation counter (Perkin Elmer, Waltham, MA) using 5 ml of scintillation fluid.

Quantitation of 7-DHC, Desmosterol and Cholesterol by Thin Layer Chromatography

Total lipids were extracted from isolated cell membranes in control, cholesterol-depleted and sterol-replenished conditions according to Bligh and Dyer method (Bligh and Dyer 1959). The lipid extracts were dried under a stream of nitrogen at ~ 37 °C and then dissolved in a mixture of chloroform/methanol (1:1, v/v). The extracted lipids were separated by thin layer chromatography (TLC) on precoated silica gel TLC plates that were impregnated with 3% (w/v) silver nitrate in 97% methanol (v/v), dried briefly and activated at ~ 120 °C for 15 min. In case of 7-DHC-replenished membranes, lipids were separated using *n*-heptane/ethyl acetate (2:1, v/v) as a solvent system (Aufenanger et al. 1986), whereas sterols were resolved in chloroform/diethyl ether/acetic acid (97:2.3:0.5, v/v/v) solvent system for desmosterol-replenished membranes (Copius-Peereboom and Beekes 1965). In order to achieve maximum sterol separation, for desmosterol-replenished membranes, TLC was run three times in the same solvent and the plate was dried after each run. The separated lipids were visualized by charring with a solution containing cupric sulfate (10%, w/v) and phosphoric acid (8%, v/v) at ~ 170 °C (Baron and Coburn 1984). 7-DHC, desmosterol and cholesterol bands were identified with the help of standards. The TLC plates were scanned and sterol band intensities were analyzed using Adobe Photoshop (version 10.0, Adobe Systems, San Jose, CA). Intensities of the sterols from all samples on the TLC plate were normalized to the intensity of the cholesterol band obtained from untreated cells.

Statistical Analysis

Significance levels were calculated using two-tailed unpaired *t*-test with 95% confidence interval using GraphPad Prism software (version 4.0, San Diego, CA). All plots were generated

using Microcal Origin (version 9.7, OriginLab, Northampton, MA).

Results

Membrane Cholesterol Depletion Enhances Ligand Binding

Membrane cholesterol depletion has proved to be a convenient tool to address cholesterol-dependent function of membrane proteins. This is achieved by physical depletion of membrane cholesterol using sterol carriers such as methyl-β-cyclodextrin (MβCD), a water soluble carbohydrate polymer with seven residues of methylated-glucose (Kilsdonk et al. 1995; Christian et al. 1997). MβCD can selectively extract cholesterol from membranes by including it in a central nonpolar cavity under carefully controlled conditions (Zidovetzki and Levitan 2007; Mahammad and Parmryd 2015; Vahedi and Farnoud 2020). We carried out modulation of membrane sterols using MβCD in CHO-K1 cells expressing the human serotonin$_{1A}$ receptor. We have previously showed that the human serotonin$_{1A}$ receptor heterologously expressed in CHO-K1 cells displays pharmacological characteristics similar to native hippocampal receptors and can therefore be used to reliably explore aspects of receptor biology (Kalipatnapu et al. 2004). Figures 2a, b show membrane lipid composition of control, cholesterol-depleted and sterol-replenished (subsequent to cholesterol depletion) cells, separated using thin layer chromatography. The respective sterols were identified by authentic standards and their contents were quantified using the chromatograms (Fig. 2c). Figure 2c shows that treatment with 10 mM MβCD in live cells resulted in ~ 49% reduction in membrane cholesterol content. The reduction of cholesterol was accompanied by ~ 65% increase in specific agonist ([^{3}H]8-OH-DPAT) binding to the serotonin$_{1A}$ receptor (Fig. 4). Importantly, replenishment with 1 mM cholesterol (using a pre-formed cholesterol/MβCD complex (1:10, mol/mol)) resulted in complete recovery of specific agonist binding to ~ 101% relative to control cells (Fig. 4), when cholesterol content was ~ 111% of that in control cells (without MβCD) (see Fig. 2c). These results highlight the regulation of specific ligand binding activity of the serotonin$_{1A}$ receptor by membrane cholesterol. In addition, these results point out the selective nature of MβCD in terms of its ability to modulate membrane cholesterol (the phospholipid content remains unchanged (Fig. 3)).

Differential Effects of 7-DHC and Desmosterol on Ligand Binding Activity

In order to explore whether replenishment with immediate biosynthetic precursors of cholesterol could restore ligand binding function of the serotonin$_{1A}$ receptor, we carried out sterol replenishment of cholesterol-depleted cells using the complex of MβCD and 7-DHC or desmosterol. The total sterol contents (cholesterol + 7-DHC or, cholesterol + desmosterol) were found comparable to total cholesterol content of control cells, when sterol replenishment was carried out using 1 mM 7-DHC or 1 mM desmosterol (using a pre-formed sterol/MβCD complex (1:10, mol/mol)) (Fig. 2c). This suggests that extent of loading of 7-DHC and desmosterol was similar to what was obtained with 1 mM cholesterol (Fig. 2c). Importantly, the phospholipid content in cells remained unaltered under these conditions (Fig. 3). However, the specific agonist binding to the serotonin$_{1A}$ receptor could not be restored when replenishment was carried out using 7-DHC (~ 172% of control cells, see Fig. 4), although the extent of total sterol content was comparable to control cells (Fig. 2c). We therefore conclude that 7-DHC is not capable of supporting the ligand binding function of the human serotonin$_{1A}$ receptor in live cells. We have previously shown this to be true for native serotonin$_{1A}$ receptors in hippocampal membranes (Singh et al. 2007) and for membranes isolated from CHO-5HT$_{1A}$R cells (Paila et al. 2008).

Interestingly, the specific agonist binding to the serotonin$_{1A}$ receptor could be partially restored when replenishment was carried out using 1 mM desmosterol (Fig. 4). The specific agonist binding to the serotonin$_{1A}$ receptor in desmosterol-replenished cell membranes (subsequent to cholesterol depletion) was ~ 139% of the untreated (control) cells (compared to ~ 165% in cholesterol-depleted cells). This indicates a partial (yet significant) reduction of specific agonist binding upon desmosterol-replenishment (subsequent to cholesterol depletion) relative to cholesterol-depleted cells. These results reveal the stringent requirement of the fine-tuned structural features of cholesterol necessary for regulating the ligand binding activity of the human serotonin$_{1A}$ receptor.

Relevance of Optimal Membrane Cholesterol in Maintaining Receptor Activity

To monitor the effect of enhancement of membrane cholesterol over control levels on ligand binding function of the serotonin$_{1A}$ receptor, we enriched plasma membrane cholesterol in cells using the water soluble cholesterol-MβCD complex. Such a complex has previously been shown to efficiently enrich cholesterol in the plasma membrane of macrophages (Viswanathan et al. 2015; Kumar et al. 2016). As shown in Fig. 5a, treatment of control (untreated) cells with cholesterol complexed with MβCD was able to increase cholesterol level to ~ 136% of control cells. Importantly, enrichment of membrane cholesterol had no significant effect on membrane phospholipid content (Fig. 3). Next, we monitored the effect of such enrichment in cholesterol content in the plasma membrane

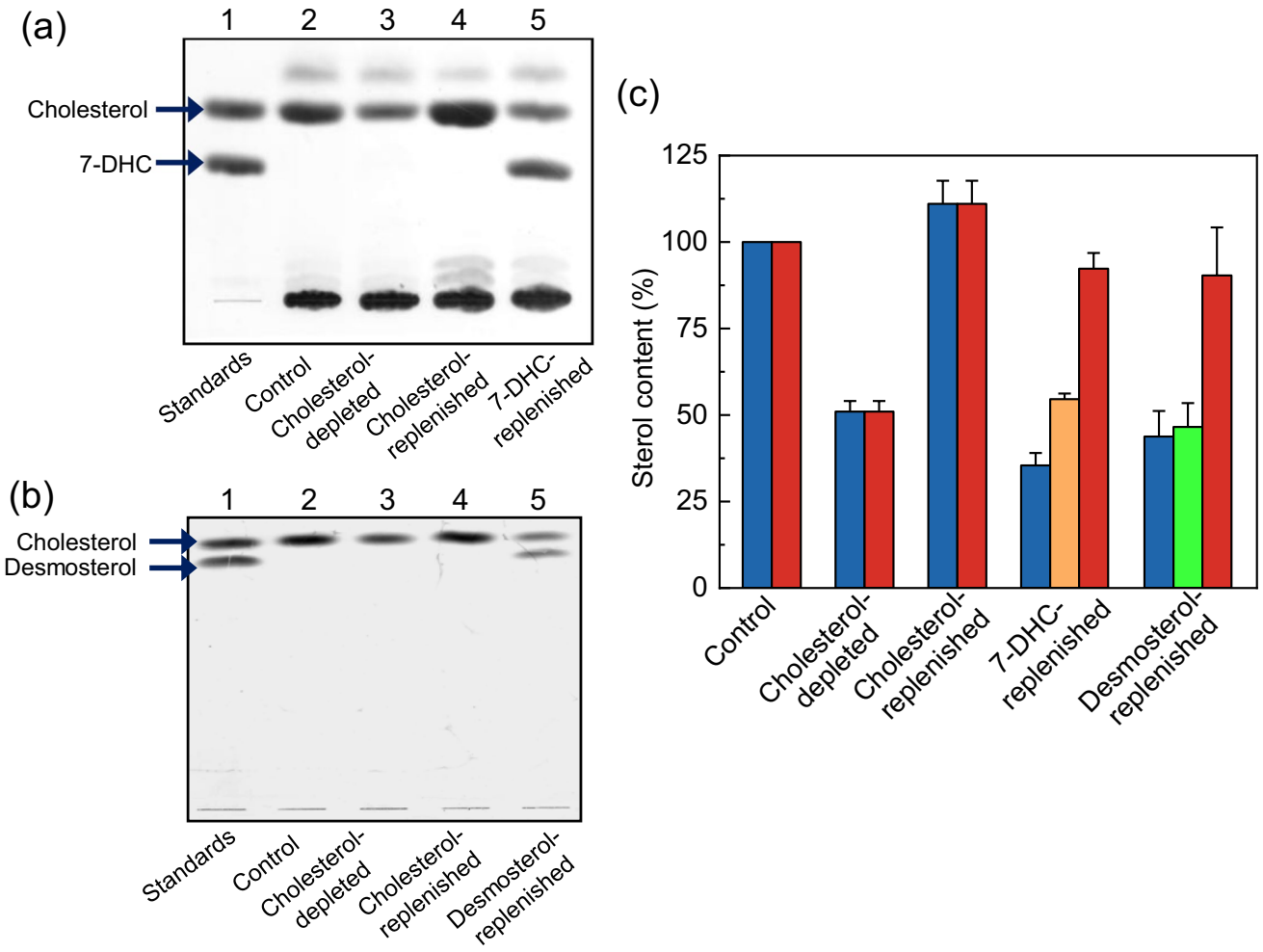

Fig. 2 Representative thin layer chromatograms of total membrane lipids extracted from control and sterol-modulated CHO-5HT$_{1A}$R cells. **a** The chromatogram shows lipids extracted from control membranes (lane 2), membranes treated with 10 mM MβCD (lane 3, cholesterol-depleted), and membranes treated with 10 mM MβCD followed by replenishment with either cholesterol using 1 mM cholesterol:10 mM MβCD complex (lane 4, cholesterol-replenished) or 7-DHC using 1 mM 7-DHC:10 mM MβCD complex (lane 5, 7-DHC-replenished). The arrows represent positions of cholesterol and 7-dehydrocholesterol on the thin layer chromatogram identified using standards in lane 1. **b** The chromatogram shows lipids extracted from control membranes (lane 2), membranes treated with 10 mM MβCD (lane 3, cholesterol-depleted), and membranes treated with 10 mM MβCD followed by replenishment with either cholesterol using 1 mM cholesterol:10 mM MβCD complex (lane 4, cholesterol-replenished) or desmosterol using 1 mM desmosterol:10 mM MβCD complex (lane 5, desmosterol-replenished). The arrows represent positions of cholesterol and desmosterol on the thin layer chromatogram identified using standards in lane 1. **c** Estimation of sterol content of control, cholesterol-depleted and cholesterol-/7-DHC-/ or desmosterol-replenished cell membranes. Cholesterol (blue bar), 7-DHC (orange bar), desmosterol (green bar) and total sterol (red bar) were quantified by densitometric analysis of the chromatogram. Values are expressed as percentages of the cholesterol content of control (untreated) cell membranes. Total sterol content of membranes was obtained by the addition of cholesterol and 7-DHC or desmosterol contents. Data represent means ± SE of three independent experiments. See "Materials and Methods" section for more details

on ligand binding function of the serotonin$_{1A}$ receptor. Figure 5b shows the specific agonist binding to the serotonin$_{1A}$ receptor in cholesterol-enriched cells. As apparent from the figure, enrichment of cholesterol results in significant reduction (~35%) in specific binding of the agonist to the receptor relative to control cells. These results, combined with our observation on the increase in binding of specific agonist to the serotonin$_{1A}$ receptor upon cholesterol depletion (Fig. 5b), demonstrate the requirement of optimal levels of cholesterol in cell membranes for ligand binding. In other words, the ligand binding feature of the receptor is perturbed both above and below the optimum level of membrane cholesterol (see Fig. 6).

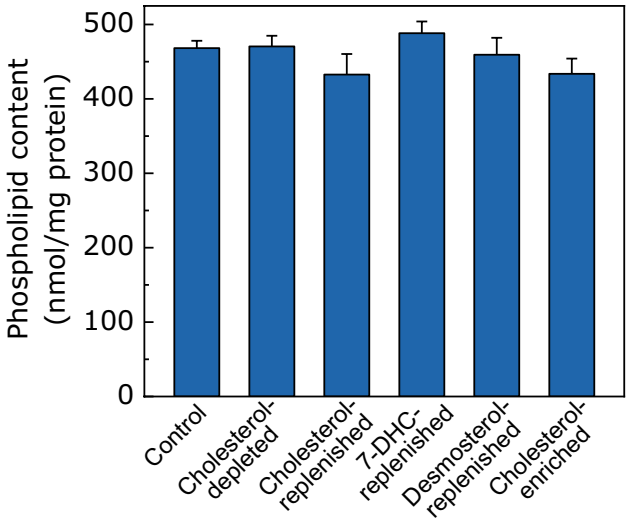

Fig. 3 Effect of sterol modulation on total phospholipid contents in cell membranes of CHO-K1 cells. Values are expressed as percentages of phospholipid in cell membranes of sterol-modulated cells relative to untreated (control) cell membranes. Data represent means ± SE of three independent experiments. See "Materials and Methods" section for other details

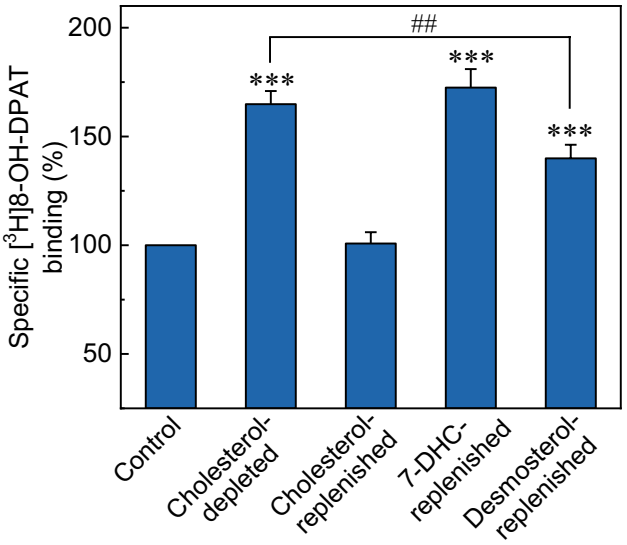

Fig. 4 Effect of replenishment of cholesterol, 7-DHC and desmosterol into cholesterol-depleted live cells on specific binding of [^{3}H]8-OH-DPAT to the human serotonin$_{1A}$ receptor. CHO-K1 cells expressing the human serotonin$_{1A}$ receptor were treated with 10 mM MβCD and were replenished with cholesterol, 7-DHC or desmosterol using 1 mM of respective sterol:10 mM MβCD complex. Values are expressed as percentages of specific binding obtained in membranes isolated from control (untreated) cells. Data shown are means ± SE from at least five independent experiments (***corresponds to significant ($p < 0.001$) differences in the specific binding of [^{3}H]8-OH-DPAT to cholesterol-depleted, 7-DHC-replenished and desmosterol-replenished cells relative to control cells; ## corresponds to significant ($p < 0.01$) difference in the specific binding of [^{3}H]8-OH-DPAT to desmosterol-replenished cells relative to cholesterol-depleted cells). See "Materials and Methods" section for more details

Discussion

The role of membrane cholesterol in GPCR organization, dynamics and function has emerged as an exciting area of research in GPCR biology. The commonly used strategies to probe role of cholesterol in supporting the function of GPCRs rely on the modulation of membrane cholesterol content or its availability in membranes (Jafurulla et al. 2019). In this context, we have previously shown that metabolic (chronic) depletion of cholesterol by inhibiting specific enzymes in its biosynthetic pathway, significantly affects the function of the human serotonin$_{1A}$ receptor heterologously expressed in CHO-K1 cells (Paila et al. 2008; Shrivastava et al. 2010). However, chronic cholesterol depletion using inhibitors are often complicated by the fact that the effects could be pleiotropic in nature due to side effects (Liao and Laufs 2005; Sahu et al. 2019) and could even induce cell cycle arrest (Singh et al. 2013). In addition, we have recently shown, by measurements of membrane dipole potential, that the molecular scenarios associated with acute and chronic cholesterol depletion are strikingly different, even if cholesterol contents are comparable in a given condition (Sarkar et al. 2017). In this context, sterol carriers such as MβCD offer specific modulation of membrane cholesterol content, when used judiciously (Zidovetzki and Levitan 2007). The polar nature of MβCD allows its close interaction with membranes, giving rise to fast kinetics of cholesterol efflux which results in efficient modulation of cholesterol content. Since MβCD is water soluble, it is easy to be separated from the membrane after cholesterol depletion.

In this work, to explore the stringent requirement of membrane cholesterol in maintaining serotonin$_{1A}$ receptor activity, we carried out cholesterol depletion from live cells using MβCD and subsequent replenishment with structurally close immediate biosynthetic precursors of cholesterol. Our results show that cholesterol depletion from plasma membrane of live cells resulted in an increase in the specific agonist binding to the serotonin$_{1A}$ receptor which was reversed upon replenishment with cholesterol. Importantly, replenishment with 7-DHC (subsequent to cholesterol depletion) did not reverse ligand binding of the serotonin$_{1A}$ receptor, although desmosterol could partially restore the activity of the serotonin$_{1A}$ receptor. This brings out the interesting message that even an extra double bond in sterol structure could be unfavorable for receptor activity, that too in a position-dependent manner, thereby implying both subtlety and stringency involved in receptor-sterol interaction. In addition, by enrichment of membrane cholesterol over basal level, we demonstrated the role of optimal membrane cholesterol

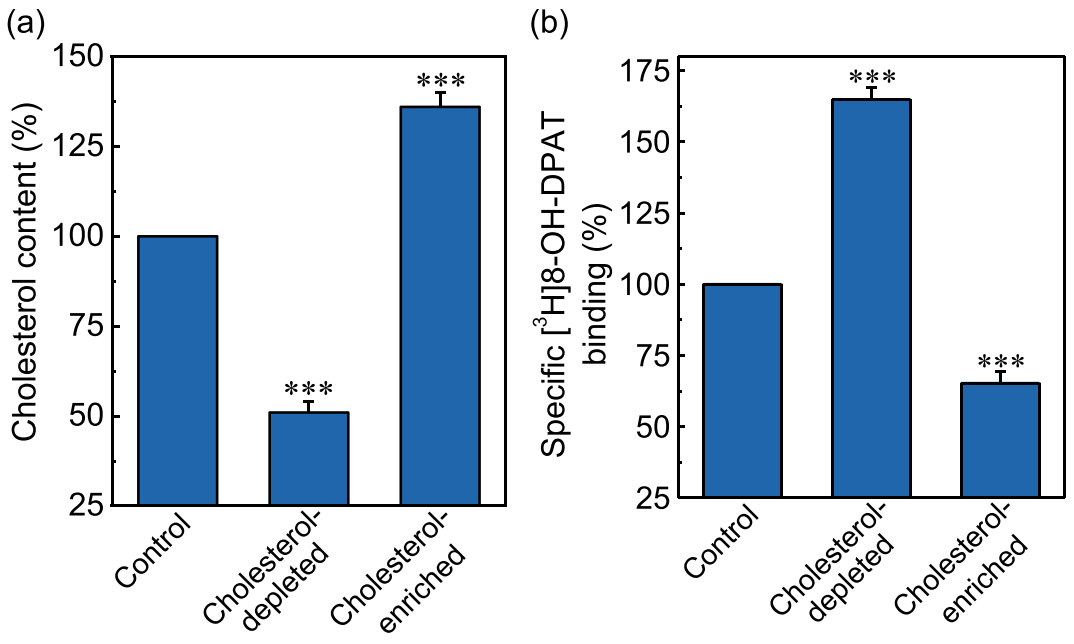

Fig. 5 An optimum level of membrane cholesterol is necessary for the activity of the human serotonin$_{1A}$ receptor. **a** Cholesterol content in cell membranes under control, cholesterol-depleted and cholesterol-enriched conditions. CHO-K1 cells expressing the human serotonin$_{1A}$ receptor were treated with 10 mM MβCD to deplete membrane cholesterol and cholesterol enrichment was carried out using 1 mM cholesterol:10 mM MβCD complex. Values are normalized to cholesterol content in control (untreated) cells. Data represent means ± SE of at least three independent experiments (***corresponds to significant ($p < 0.001$) difference in cholesterol content in cholesterol-depleted and cholesterol-enriched cells relative to control cells). **b** Effect of cholesterol enrichment on the specific binding of the agonist [^{3}H]8-OH-DPAT to the human serotonin$_{1A}$ receptor. Values are expressed as percentages of specific binding obtained in membranes prepared from control (untreated) cells. Data shown are means ± SE from at least three independent experiments (***corresponds to significant ($p < 0.001$) differences in the specific binding of [^{3}H]8-OH-DPAT to cholesterol-depleted and cholesterol-enriched cells relative to control cells). See "Materials and Methods" section for more details

necessary for maintaining the activity of the serotonin$_{1A}$ receptor.

The ability of desmosterol to support the ligand binding function of the serotonin$_{1A}$ receptor merits comment. Previous work by us (Shrivastava et al. 2008) and others (Huster et al. 2005; Mouritsen et al. 2017) have demonstrated that the effects exerted by cholesterol and desmosterol on membrane organization and dynamics are similar. For example, we showed that cholesterol and desmosterol share common characteristics in terms of lipid packing at various regions (depths) and long-range diffusion (pyrene excimer/monomer measurements) (Shrivastava et al. 2008). In addition, by utilizing NMR, ESR, and fluorescence spectroscopic approaches using laurdan, it was shown that properties such as lipid packing are very similar in the presence of cholesterol and desmosterol (Huster et al. 2005). This was further supported by results from monolayer studies showing that the lateral pressure profile of 7-DHC is different from that of cholesterol and desmosterol in DPPC monolayers (Berring et al. 2005). Interestingly, sterol analysis in a mutant L-cell fibroblast (Rothblat et al. 1970) and murine macrophage-like cells (Rodríguez-Acebes et al. 2009) previously showed that desmosterol could support the function of membrane cholesterol. In addition, results from *Dhcr24* gene knockout mice (Dhcr24$^{-/-}$) (Wechsler et al. 2003) and cell lines derived using *Dhcr24* gene knockout (Lu et al. 2006; Heverin et al. 2007) showed that the absence of cholesterol could be suitably balanced by the presence of desmosterol. Interestingly, *Dhcr24* gene knockout mice were viable and showed a mild phenotype although they are sterile and small in size (Wechsler et al. 2003). In this context, we have previously showed that desmosterol could replace cholesterol for ligand binding function of the serotonin$_{1A}$ receptor in solubilized native membranes (Singh et al. 2011). Our present results showing that desmosterol is capable of partially supporting the ligand binding function of the serotonin$_{1A}$ receptor is in overall agreement with the above reports.

Interestingly, the observed increase in agonist binding (Fig. 4) of the serotonin$_{1A}$ receptor upon cholesterol depletion in CHO-K1 cells appears inconsistent with our earlier results where we reported that cholesterol depletion from native hippocampal membranes (endogenously expressing the serotonin$_{1A}$ receptor) led to reduction of agonist binding (Pucadyil and Chattopadhyay 2004; Singh et al. 2007). A possible reason for such difference in ligand binding function of the receptor could be due to cholesterol depletion in

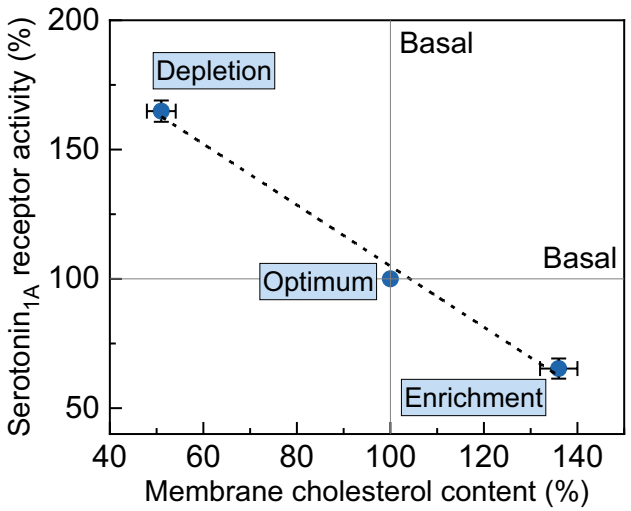

Fig. 6 The role of optimum membrane cholesterol in supporting the activity of the human serotonin$_{1A}$ receptor. Specific [^{3}H]8-OH-DPAT binding to serotonin$_{1A}$ receptors (values taken from Fig. 5b) and corresponding values of membrane cholesterol content (from Fig. 5a) are shown. Linear regression analysis yielded a correlation coefficient (r) ~ −0.99. Reduction or enrichment of membrane cholesterol from its basal level perturbs receptor activity, thereby highlighting the requirement of an *optimum* cholesterol level in membranes for ligand binding

intact live cells in culture and not from isolated membranes. In other words, there could be unique reorganization in the immediate membrane microenvironment around the receptor while it is localized in the plasma membrane, that takes place *only* upon cholesterol depletion of intact cells in culture and not in isolated cell membranes. Such reorganization could be plausible in cell membranes that are spatially connected and coupled to the underlying cytoskeleton, which is absent in isolated membranes (Pucadyil and Chattopadhyay 2007). This is relevant in light of the emerging relationship between membrane cholesterol and the actin cytoskeleton (Sarkar et al. unpublished observations).

The requirement of optimum membrane cholesterol for receptor function is an emerging theme (recently reviewed in Meza et al. 2020). The modulation of membrane protein function, in response to reduction or enrichment of cholesterol from the basal level, has previously been reported for the Na^+,K^+-ATPase (Cornelius 1995), $GABA_A$ receptor (Sooksawate and Simmonds 2001) and cholecystokinin type 1 receptor (Harikumar et al. 2005). Our present results showing increase and reduction in ligand binding function of the serotonin$_{1A}$ receptor upon cholesterol depletion and enrichment further emphasizes these observations. In a cautionary note, whether such cholesterol-induced regulation of receptor activity holds good for other cholesterol-sensitive GPCRs is not clear and requires careful examination of data on a case-by-case basis. Interestingly, a similar trend of membrane protein function in response to varying membrane cholesterol was previously reported for the type 1 cannabinoid receptor (CB1R) (Bari et al. 2005a, b) and inward-rectifier K^+ channel (K_{ir}) (Romanenko et al. 2002). While enrichment of membrane cholesterol decreased the ligand binding efficiency of CB1R and K_{ir} current density, cholesterol depletion enhanced the ligand binding efficiency of CB1R and increased the density of the current through K_{ir} (Romanenko et al. 2002; Bari et al. 2005a, b). On a broader perspective, we recently showed the requirement of optimum cholesterol for pathogen entry into host cells. For example, we showed requirement of optimal host membrane cholesterol for the entry of *Leishmania* (Kumar et al. 2016) and *Mycobacterium* (Viswanathan et al. 2015) into macrophages. We believe that these cases elucidate the relevance of cellular cholesterol homeostasis.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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