

## Research Article

Membrane cholesterol oxidation in live cells enhances the function of serotonin<sub>1A</sub> receptors

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## ABSTRACT

The serotonin<sub>1A</sub> (5-HT<sub>1A</sub>) receptor is an important neurotransmitter receptor that belongs to the G protein-coupled receptor (GPCR) family. It is implicated in a variety of cognitive and behavioral functions and serves as an important drug target for neuropsychiatric disorders such as anxiety and depression. Previous work from our laboratory has demonstrated that membrane cholesterol plays an important role in the function of the serotonin<sub>1A</sub> receptor. Our earlier results highlighted several structural features of cholesterol essential for receptor function. In order to explore the importance of the hydroxyl group of cholesterol in the function of the serotonin<sub>1A</sub> receptor, we utilized cholesterol oxidase to oxidize the hydroxyl group of cholesterol to keto group. Our results show that the oxidation of the hydroxyl group of cholesterol in live cells resulted in enhancement of agonist binding and G-protein coupling to the receptor with no appreciable change in overall membrane order. These results extend our understanding of the structural requirements of cholesterol for receptor function.

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## 1. Introduction

G protein-coupled receptors (GPCRs) constitute a superfamily of seven transmembrane domain proteins that serve as communication interface in transferring information from external environment to the cellular interior (Pierce et al., 2002; Rosenbaum et al., 2009; Chattopadhyay, 2014). As highly versatile membrane sensors, GPCRs are known to mediate cellular responses to a diverse variety of stimuli in several physiological processes. Since GPCRs play a central role in cellular signaling and are implicated in pathophysiology of several disorders (Insel et al., 2007; Heng et al., 2013), it is not surprising that they have emerged as major drug targets in all clinical areas (Insel et al., 2007; Lagerström and Schiöth, 2008; Heilker et al., 2009; Jacobson, 2015; Cooke et al., 2015).

All GPCRs share a common feature of seven transmembrane helices and depending on the family and subgroup they belong to, they transduce signals through multiple G-protein subtypes (Rosenbaum et al., 2009). Recent advances in high resolution structural characterization of GPCRs have provided valuable insights into receptor activation mechanisms (Katritch et al., 2013). Despite such advances, the influence of the membrane environment and surrounding lipids on GPCR structure and function remains relatively less explored (Oates and Watts, 2011; Chattopadhyay, 2014). An important membrane lipid which is most studied in this context is cholesterol. Recent evidence from functional and structural studies has highlighted the possible close interaction of cholesterol with GPCRs which could influence the organization and function of these receptors (Paila and Chattopadhyay, 2010; Oates and Watts, 2011; Joseph et al., 2012; Chattopadhyay, 2014; Sengupta and Chattopadhyay, 2015; Gimpl, 2016).

A GPCR which is extensively studied in terms of cholesterol sensitivity of its organization, dynamics and function is the serotonin<sub>1A</sub> receptor (Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2010; Jafurulla and Chattopadhyay, 2013). The serotonin<sub>1A</sub> (5-HT<sub>1A</sub>) receptor is an important neurotransmitter receptor and is implicated in behavior, learning, development and cognition (Pucadyil et al., 2005a; Kalipatnapu and Chattopadhyay, 2007; Müller et al., 2007). As a result, the serotonin<sub>1A</sub> receptor has

**Abbreviations:** 5-HT<sub>1A</sub> receptor, 5-hydroxytryptamine-1A receptor; BCA, bicinchoninic acid; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; GTP-γ-S, guanosine-5'-O-(3-thiotriphosphate); 8-OH-DPAT, 8-hydroxy-2-(di-*N*-propylamino)tetralin; PMSF, phenylmethylsulfonyl fluoride; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene.

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emerged as a crucial target in developing new drugs to treat a range of diseases such as anxiety, depression, schizophrenia, Parkinson's disease and cancer (Celada et al., 2013; Fiorino et al., 2014; Miyazaki and Asanuma, 2016). Previous work from our laboratory has demonstrated the essential role of membrane cholesterol in the organization, dynamics and function of the serotonin<sub>1A</sub> receptor (reviewed in Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2010; Jafurulla and Chattopadhyay, 2013; Chattopadhyay et al., 2015). Our previous results have shown that the immediate biosynthetic precursors of cholesterol (differing merely in a double bond with cholesterol) could not support the receptor function (Paila et al., 2008; Singh et al., 2009), suggesting that the interaction between cholesterol and the serotonin<sub>1A</sub> receptor is considerably stringent. In order to further explore structural stringency and the importance of the hydroxyl group of cholesterol in the function of the serotonin<sub>1A</sub> receptor, in this work, we oxidized the hydroxyl group of cholesterol (to keto group) utilizing cholesterol oxidase. Our results show that the oxidation of the hydroxyl group of cholesterol in live cells enhances the agonist binding and G-protein coupling to the serotonin<sub>1A</sub> receptor.

## 2. Materials and methods

### 2.1. Materials

Cholesterol, cholesterol oxidase (EC 1.1.3.6 from *Pseudomonas fluorescens*), 4-cholestenone, DMPC, EDTA, gentamycin sulfate, MgCl<sub>2</sub>, MnCl<sub>2</sub>, penicillin, PMSF, polyethylenimine, serotonin, streptomycin, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). DPH and TMA-DPH were from Molecular Probes/Invitrogen (Eugene, OR). The concentration of a stock solution of DPH (or TMA-DPH) prepared in methanol was calculated using molar extinction coefficient ( $\epsilon$ ) of 88,000 M<sup>-1</sup> cm<sup>-1</sup> at 350 nm in methanol. D-MEM/F-12 [Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (Ham) (1:1)], fetal calf serum, and geneticin (G 418) were from Invitrogen (Carlsbad, CA). Bicinchoninic acid (BCA) assay reagent for protein estimation was obtained from Pierce (Rockford, IL). Amplex Red cholesterol assay kit was from Molecular Probes/Invitrogen (Eugene, OR). GTP- $\gamma$ -S was from Roche Applied Science (Mannheim, Germany). [<sup>3</sup>H]8-OH-DPAT (sp. activity 135 Ci/mmol) was obtained from DuPont New England Nuclear (Boston, MA). All other chemicals were of the highest purity available. GF/B glass microfiber filters were from Whatman International (Kent, UK). Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

### 2.2. Methods

#### 2.2.1. Cells and cell culture

Chinese hamster ovary (CHO) cells stably expressing the human serotonin<sub>1A</sub> receptor (termed as CHO-5-HT<sub>1A</sub>R cells) were cultured as described earlier (Kalipatnapu et al., 2004).

#### 2.2.2. Treatment of cells with cholesterol oxidase

Cells at a density of  $5 \times 10^5$  were grown for 3 days in 100 mm petridishes. After pre-incubation in serum-free D-MEM/F-12 (1:1) medium for 3 h at 37 °C, cells were treated with increasing concentrations of cholesterol oxidase in serum-free D-MEM/F-12 medium for 30 min at 37 °C. Cells were then washed with PBS and processed for membrane preparation.

#### 2.2.3. Cell membrane preparation

Cell membranes were prepared as described earlier (Kalipatnapu et al., 2004). Protein concentration of membranes was determined using the BCA assay (Smith et al., 1985).

#### 2.2.4. Estimation of cholesterol content of cell membranes

Membrane cholesterol levels were estimated using the Amplex Red cholesterol assay (Amundson and Zhou, 1999). Cholesterol levels were normalized to total protein content of cell membranes.

#### 2.2.5. Estimation of inorganic phosphate

The concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid (McClure, 1971) using Na<sub>2</sub>HPO<sub>4</sub> as standard. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

#### 2.2.6. Radioligand binding assays

Receptor binding assays with membranes isolated from control and cholesterol oxidase treated CHO-5-HT<sub>1A</sub>R cells were carried out as described earlier (Kalipatnapu et al., 2004) with ~100  $\mu$ g of total protein and 0.29 nM of radiolabeled agonist [<sup>3</sup>H]8-OH-DPAT.

#### 2.2.7. Determination of efficiency of G-protein coupling

The efficiency of G-protein coupling to the serotonin<sub>1A</sub> receptor in control and cholesterol oxidase treated cells was determined by performing agonist ([<sup>3</sup>H]8-OH-DPAT) binding assays in presence of increasing concentrations of GTP- $\gamma$ -S, as described earlier (Chattopadhyay et al., 2005). The half maximal inhibition concentrations (IC<sub>50</sub>) of GTP- $\gamma$ -S were calculated by nonlinear regression fitting of the data to a four parameter logistic function (Higashijima et al., 1987):

$$B = a[1 + (x/I)^s]^{-1} + b \quad (1)$$

where B is the specific binding of the agonist in presence of GTP- $\gamma$ -S normalized to binding observed at lowest concentration of GTP- $\gamma$ -S used, x denotes concentration of GTP- $\gamma$ -S, a is the range ( $y_{\max} - y_{\min}$ ) of the fitted curve on the ordinate (y-axis), I is the IC<sub>50</sub> concentration, b is the background of the fitted curve ( $y_{\min}$ ) and s is the slope factor.

#### 2.2.8. Fluorescence anisotropy measurements

Anisotropy changes in the hydrophobic core and interfacial regions of the membrane were measured by fluorescence anisotropy experiments using DPH and TMA-DPH, as described previously (Pucadyil and Chattopadhyay, 2004). Steady state fluorescence was measured in a Hitachi F-4010 spectrofluorometer (Tokyo, Japan) with excitation and emission wavelengths set at 358 and 430 nm, and excitation and emission slits with bandpass of 1.5 and 20 nm. Fluorescence anisotropy measurements were performed using a Hitachi Glan-Thompson polarization accessory. The excitation slit was kept less to reduce any photoisomerization of DPH. Fluorescence was measured with a 30 s interval between consecutive openings of the excitation shutter to reverse any photoisomerization of DPH (Chattopadhyay and London, 1984). The optical density of the samples measured at 358 nm was always less than 0.15. Anisotropy values were calculated from the equation (Lakowicz, 2006):

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (2)$$

where  $I_{VV}$  and  $I_{VH}$  are fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G (grating correction factor) is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light ( $I_{HV}/I_{HH}$ ).

### 2.2.9. Thin layer chromatography of membrane lipids

Thin layer chromatography of membrane lipids extracted from control and cholesterol oxidase (1 U/ml) treated cells was carried out as described earlier (Pucadyil et al., 2005b). After separation of lipids, the TLC plate was sprayed with 0.01% (w/v) primuline solution (van Echten-Decker, 2000) and lipid bands were visualized under ultraviolet light.

### 2.2.10. Statistical analysis

Significance levels were estimated using Student's two-tailed paired *t*-test using Graphpad Prism version 4.0 (San Diego, CA). Plots were generated using GRAFIT program, version 3.09b (Erithacus Software, Surrey, UK).

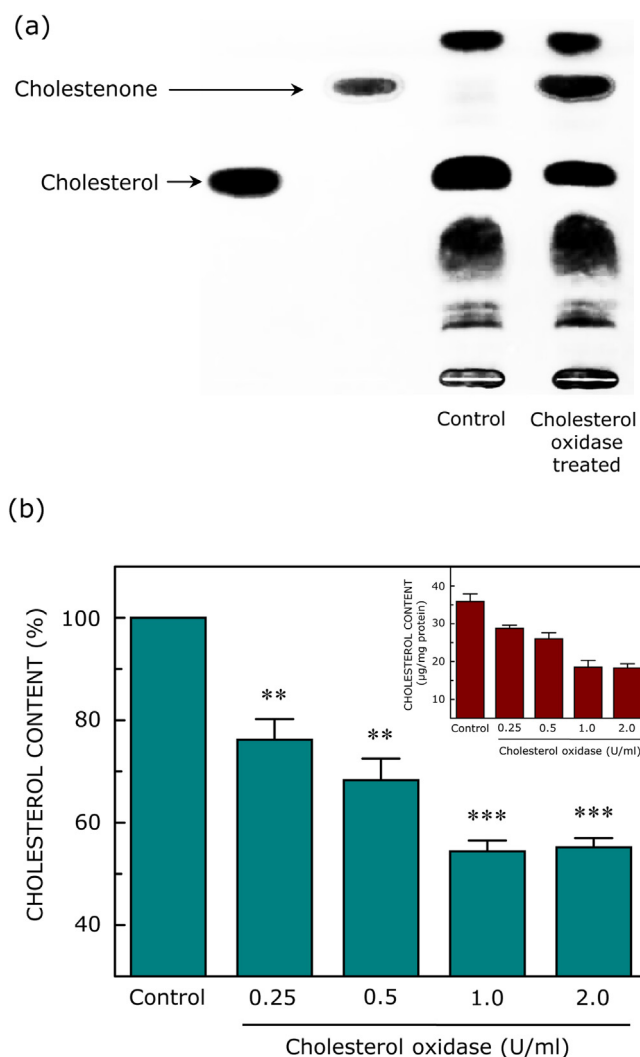
## 3. Results

### 3.1. Modulation of membrane cholesterol content using cholesterol oxidase

In order to modulate the 3 $\beta$ -hydroxyl group of cholesterol, we treated CHO-5-HT<sub>1A</sub>R cells with cholesterol oxidase, which has been shown to act at the membrane interface and catalyze the oxidation of cholesterol to 4-cholestenone (Sampson and Vrielink, 2003; Vrielink and Ghisla, 2009). A thin layer chromatogram of lipids extracted from membranes of control (untreated) and cholesterol oxidase treated cells is shown in Fig. 1a. The figure shows reduction in cholesterol and generation of 4-cholestenone in cell membranes upon treatment with cholesterol oxidase. Since treatment with cholesterol oxidase results in the oxidation of the hydroxyl group of cholesterol to a keto group, we utilized Amplex Red cholesterol assay (a method which could only quantitate unoxidized form of cholesterol) to quantitate membrane cholesterol from cholesterol oxidase treated cells. Fig. 1b shows that the treatment of CHO-5-HT<sub>1A</sub>R cells with cholesterol oxidase resulted in a concentration-dependent reduction of (unoxidized) membrane cholesterol upto an enzyme concentration of 1 U/ml, beyond which a plateau was reached. The figure shows that the reduction in membrane cholesterol content exhibited a plateau at ~45%. These results show that increasing the concentration of enzyme beyond certain concentration does not increase the oxidation of membrane cholesterol, which suggests that the accessibility of membrane cholesterol to the enzyme could be the rate-limiting factor. Similar saturating effect on oxidation of membrane cholesterol with longer exposure to cholesterol oxidase has been reported earlier (El Yandouzi and Le Grimellec, 1992, 1993; Gimpl et al., 1997) and was attributed to existence of different pools of membrane cholesterol (heterogeneous distribution) with varying accessibility to the enzyme.

### 3.2. Oxidation of membrane cholesterol enhances specific ligand binding to the serotonin<sub>1A</sub> receptor

To examine the functional correlate of the enzymatic oxidation of cholesterol on the serotonin<sub>1A</sub> receptor, we monitored specific agonist ([<sup>3</sup>H]8-OH-DPAT) binding to the serotonin<sub>1A</sub> receptor in membranes isolated from cells treated with cholesterol oxidase. Fig. 2 shows that the specific agonist binding to the serotonin<sub>1A</sub> receptor is enhanced upon oxidation of membrane cholesterol. The figure shows that treatment with 1 U/ml and 2 U/ml of cholesterol oxidase results in ~38% and 46% increase (compared to control membranes) in agonist binding to the receptor. These results are reminiscent of our previous results of enhanced ligand binding to serotonin<sub>1A</sub> receptors upon physical depletion of membrane cholesterol from live cells using methyl- $\beta$ -cyclodextrin (M $\beta$ CD) (Pucadyil and Chattopadhyay, 2007). It is interesting to note that these results show that enzymatic modification of functionality of

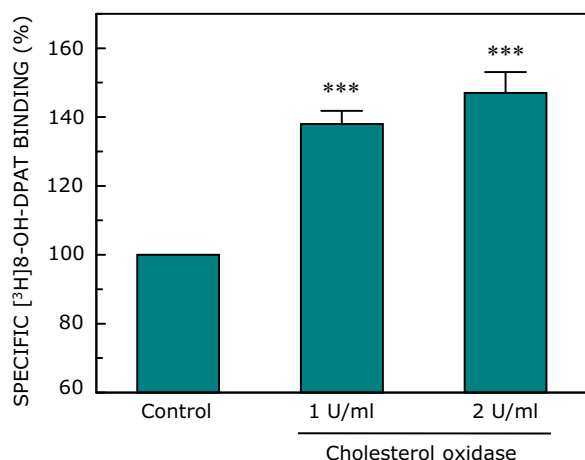


**Fig. 1.** Membrane cholesterol content of cells treated with cholesterol oxidase. (a) A representative thin layer chromatogram of membrane lipids extracted from control and cholesterol oxidase (1 U/ml) treated cells. The chromatogram shows reduction in total cholesterol and generation of 4-cholestenone in cell membranes treated with cholesterol oxidase. Cholesterol and 4-cholestenone standards are shown. (b) Cholesterol content was estimated in membranes isolated from CHO-5-HT<sub>1A</sub>R cells treated with cholesterol oxidase using Amplex Red cholesterol assay. Values are normalized to the membrane cholesterol content of untreated (control) cells. Data represent means  $\pm$  S.E. of at least four independent experiments (\*\* and \*\*\* correspond to significant ( $p < 0.01$  and  $p < 0.001$ ) difference in membrane cholesterol content of cells treated with cholesterol oxidase relative to control cells). The inset shows cholesterol content in  $\mu\text{g}/\text{mg}$  protein. See Section 2 for more details.

cholesterol by its mere conversion to cholestenone is sufficient to mimic the effects of physical depletion of cholesterol, in terms of ligand binding to serotonin<sub>1A</sub> receptors. These results therefore provide further insight into structural details of cholesterol necessary for optimum functioning of the serotonin<sub>1A</sub> receptor.

### 3.3. The efficiency of G-protein coupling is enhanced upon cholesterol oxidation

The classical signaling pathway of GPCRs is the activation of heterotrimeric G-proteins upon binding of specific agonists to the receptor. The affinity of agonist binding is dependent on G-protein coupling status of the receptor, with high affinity toward receptors coupled to G-proteins (Sundaram et al., 1993; Harikumar and Chattopadhyay, 1999). Agonist binding to GPCRs is therefore



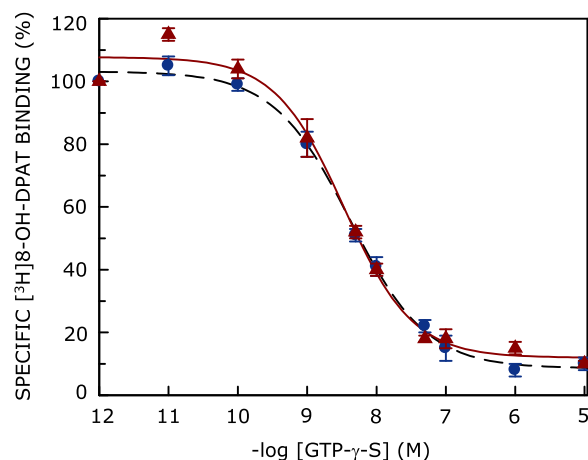
**Fig. 2.** Specific agonist binding to the serotonin<sub>1A</sub> receptor is enhanced upon treatment with cholesterol oxidase. CHO-5-HT<sub>1A</sub>R cells were treated with 1 and 2 U/ml cholesterol oxidase and specific agonist ([<sup>3</sup>H]8-OH-DPAT) binding to the serotonin<sub>1A</sub> receptor in membranes isolated from cells treated with cholesterol oxidase was measured. Values are expressed as percentages of specific ligand binding obtained in untreated (control) membranes. Data represent means  $\pm$  S.E. of at least five independent experiments (\*\*\*) corresponds to significant ( $p < 0.001$ ) difference in specific agonist binding to membranes from cholesterol oxidase treated cells relative to control cells. See Section 2 for more details.

sensitive to guanine nucleotides. The serotonin<sub>1A</sub> receptor has been previously shown to couple with and signal through G<sub>i/o</sub> class of G-proteins (Raymond et al., 1993, 1999). Specific agonist (8-OH-DPAT) binding to serotonin<sub>1A</sub> receptors has earlier been shown to be sensitive to the status of G-protein coupling of the receptor (Sundaram et al., 1993; Harikumar and Chattopadhyay, 1999). Previous work from our laboratory has shown that agonist binding to the serotonin<sub>1A</sub> receptor undergoes a transition from a high-affinity to a low-affinity state upon disruption of G-protein coupling to the receptor utilizing GTP- $\gamma$ -S (a non-hydrolyzable analogue of GTP that uncouples the normal cycle of interaction between the G-proteins and the receptor) (Harikumar and Chattopadhyay, 1999).

Fig. 3 shows a characteristic reduction in the binding of specific agonist [<sup>3</sup>H]8-OH-DPAT to the serotonin<sub>1A</sub> receptor in presence of GTP- $\gamma$ -S in a concentration dependent manner. The efficiency of G-protein coupling to the receptor is inferred from the half-maximal inhibition concentration (IC<sub>50</sub>) of GTP- $\gamma$ -S (i.e., 50% inhibition of specific agonist binding). IC<sub>50</sub> values obtained in control and cholesterol oxidase treated cells are shown in Table 1. The table shows that the treatment with 1 U/ml of cholesterol oxidase results in a significant ( $p < 0.05$ ) reduction in the IC<sub>50</sub> value relative to control. This implies that the efficiency of G-protein coupling to the serotonin<sub>1A</sub> receptor exhibits significant enhancement upon oxidation of membrane cholesterol and could be a contributing factor to the observed increase in the specific agonist binding to the receptor (Fig. 2).

### 3.4. The effect of cholesterol oxidation on the function of serotonin<sub>1A</sub> receptors is independent of the overall membrane order

In order to further explore the possible reason for the observed enhancement of agonist binding and G-protein coupling to the serotonin<sub>1A</sub> receptor, we monitored overall membrane order upon treatment with cholesterol oxidase. Fluorescence anisotropy is sensitive to packing of membrane components and is correlated to the rotational diffusion of membrane embedded probes (Lakowicz, 2006). Since the membrane represents a two-dimensional anisotropic fluid (Haldar et al., 2011), any possible change in membrane order could be non-uniform. It is therefore prudent to



**Fig. 3.** Effect of cholesterol oxidase treatment on the efficiency of G-protein coupling. The efficiency of G-protein coupling of the serotonin<sub>1A</sub> receptor was monitored by the sensitivity of specific agonist ([<sup>3</sup>H]8-OH-DPAT) binding in the presence of GTP- $\gamma$ -S, a non-hydrolyzable analogue of GTP. The figure shows the effect of increasing concentrations of GTP- $\gamma$ -S on the specific binding of the agonist [<sup>3</sup>H]8-OH-DPAT to the receptor in membranes isolated from control (●) and 1 U/ml cholesterol oxidase treated (▲) cells. Values are expressed as percentage of specific agonist binding at the lowest concentration of GTP- $\gamma$ -S ( $10^{-12}$  M). The curves are nonlinear regression fits to the experimental data using Eq. (1). The half maximal inhibition concentration (IC<sub>50</sub>) of GTP- $\gamma$ -S, reflecting the efficiency of G-protein coupling to the receptor in control and cholesterol oxidase treated cells, are shown in Table 1. Data points represent means  $\pm$  S.E. of at least three independent experiments. See Section 2 and Table 1 for more details.

monitor the change in membrane order at more than one location (depth). We monitored the change in membrane order upon cholesterol oxidase treatment at the hydrophobic core and interfacial regions of the membrane utilizing two depth-specific fluorescent membrane probes, DPH and TMA-DPH. DPH is located in the hydrophobic region of the membrane (on an average  $\sim 8$  Å from the center of the membrane), while the amphipathic TMA-DPH is localized in the shallower region of the membrane, with its DPH moiety located at  $\sim 11$  Å from the center of the membrane, at the interfacial region (Kaiser and London, 1998).

The effect of enzymatic oxidation of cholesterol on fluorescence anisotropy of DPH and TMA-DPH is shown in Fig. 4. The figure shows that the treatment with cholesterol oxidase does not alter fluorescence anisotropy of these membrane probes in a major way. Taken together, these results show that membrane order does not appear to be a major factor for the observed enhancement of agonist binding and G-protein coupling to the serotonin<sub>1A</sub> receptor.

## 4. Discussion

Cholesterol oxidases are water-soluble enzymes of bacterial origin that catalyze the first step in the degradation of cholesterol by oxidizing cholesterol to cholestenone (4-cholestenone) (Sam-  
son and Vrielink, 2003; Vrielink and Ghisla, 2009). In case of

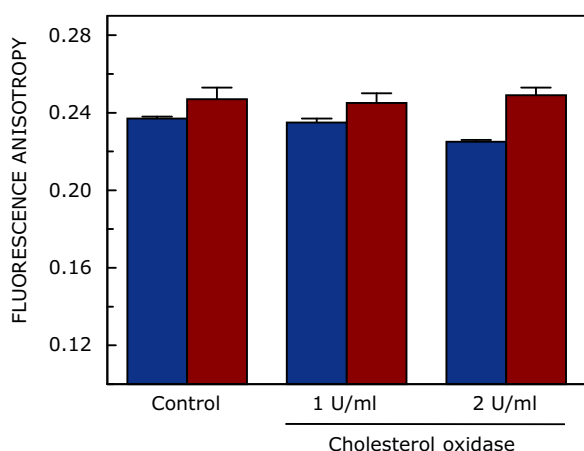
**Table 1**  
Effect of cholesterol oxidase treatment on the efficiency of G-protein coupling<sup>a</sup>.

Treatment	IC <sub>50</sub> (nM)
Control	4.3 $\pm$ 0.1
Cholesterol oxidase 1 U/ml	3.4 $\pm$ 0.3 <sup>b</sup>

<sup>a</sup> The efficiency of G-protein coupling to the receptor is inferred from the half maximal inhibition concentration (IC<sub>50</sub>) of GTP- $\gamma$ -S for specific [<sup>3</sup>H]8-OH-DPAT binding to the receptor. Data represent means  $\pm$  S.E. of at least three independent experiments. See Section 2 and Fig. 3 for more details.

<sup>b</sup> The decrease in IC<sub>50</sub> value, reflecting the increase in efficiency of G-protein coupling to the receptor, was found to be significant ( $p < 0.05$ ).





**Fig. 4.** Effect of cholesterol oxidase treatment on fluorescence anisotropy of differentially localized membrane probes. CHO-5-HT<sub>1A</sub>R cells were treated with 1 and 2 U/ml cholesterol oxidase and fluorescence anisotropy of membrane location-specific probes DPH (blue) and TMA-DPH (maroon) was measured in membranes isolated from cells that were treated with cholesterol oxidase. Fluorescence anisotropy measurements were performed with membranes containing ~50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (~23° C). Values represent means  $\pm$  S.E. of at least four independent experiments. See Section 2 for more details. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bacteria, cholesterol oxidase serves in utilizing host cholesterol as carbon source (in nonpathogenic species) and for infection in host cells (in pathogenic species). Cholesterol oxidase has previously been used as a tool to probe localization and heterogenous distribution of cholesterol in membranes (Patzner et al., 1978; Lange, 1992; El Yandouzi and Le Grimellec, 1992, 1993). In this work, we utilized cholesterol oxidase to explore the importance of the hydroxyl group of cholesterol in the function of the serotonin<sub>1A</sub> receptor. An important question in this context is whether cholesterol oxidase affects any specific pool of membrane cholesterol. It has been reported earlier that cholesterol is less susceptible to oxidation by cholesterol oxidase in the presence of sphingomyelin (Patzner et al., 1978; El Yandouzi and Le Grimellec, 1992; Gimpl et al., 1997). Keeping this in mind, it could be possible that the oxidized cholesterol may initially come from the pool that does not strongly interact with the receptor (relatively free cholesterol) followed by membrane reorganization.

The function of many GPCRs exhibits sensitivity to their membrane lipid environment (Oates and Watts, 2011; Soubias and Gawrisch, 2012; Jafurulla and Chattopadhyay, 2013; Chattopadhyay, 2014). While membrane lipids could influence receptor organization and function by altering membrane physical properties, specific interaction(s) between lipids and GPCRs have been proposed for such membrane lipid sensitivity of GPCR function (Paila and Chattopadhyay, 2009; Gimpl, 2016). As mentioned above, earlier studies from our laboratory have shown that the serotonin<sub>1A</sub> receptor exhibits specific requirement for membrane cholesterol for maintaining its function. Importantly, the serotonin<sub>1A</sub> receptor was found to be more compact (Paila et al., 2011), with lower conformational flexibility (Patra et al., 2015) and displayed increased stability (Saxena and Chattopadhyay, 2012) in the presence of membrane cholesterol. In addition, our earlier studies utilizing close structural analogues of cholesterol such as desmosterol and 7-dehydrocholesterol (which are immediate biosynthetic precursors of cholesterol), and stereoisomers of cholesterol have revealed the structural stringency of cholesterol required for maintaining receptor function (Chattopadhyay et al., 2015). Utilizing coarse-grain molecular dynamics simulation, we have recently shown the preferential occupancy of membrane

cholesterol at specific sites on the receptor (Sengupta and Chattopadhyay, 2012, 2015). A prominent site for cholesterol occupancy is the cholesterol recognition/interaction amino acid consensus (CRAC) motif, previously identified by us in GPCRs (Jafurulla et al., 2011). In this context, our present results, along with our recent report utilizing stereoisomers of cholesterol (Jafurulla et al., 2014), highlight the structural stringency of membrane cholesterol (in particular the presence and orientation of 3 $\beta$ -hydroxyl group of cholesterol) for maintaining optimum function of the serotonin<sub>1A</sub> receptor.

In addition, membrane anisotropy measurements with fluorescent membrane probes, known to be localized at different regions of the membrane, show that the overall membrane order at different locations of the membrane is not appreciably different between control and cholesterol oxidase treated cells. Although there is ~45% reduction in membrane cholesterol content upon treatment with cholesterol oxidase (see Fig. 1b), our results showing no appreciable change in membrane order are not surprising (Fig. 4). This is because treatment with cholesterol oxidase merely results in oxidation of the hydroxyl group of cholesterol to a keto group, with the bulk of the cholesterol molecule (including the steroid nucleus) still present in the membrane.

It is important to mention here that earlier results from our laboratory have shown that oxidation of membrane cholesterol by cholesterol oxidase in isolated hippocampal membranes exhibited reduction in agonist binding to serotonin<sub>1A</sub> receptors (Pucadyil et al., 2005b). The present results appear contradictory to our previous observations. We believe that such apparent discrepancy in these results could be due to differential membrane reorganization in live cells relative to isolated membranes. Long timescale detailed molecular dynamics simulations may be able to address this issue in future. Nonetheless, it is interesting to note that such opposite trend in receptor activity upon modulation of membrane cholesterol (by M $\beta$ CD) from either live cells or isolated membranes has been reported earlier by us (Pucadyil and Chattopadhyay, 2007). We conclude that adequate caution should be exercised while analyzing data from live cells and isolated membranes.

In this context, it is interesting to note that cholesterol oxidase has been earlier utilized to explore the role of cholesterol in the function of other GPCRs such as rhodopsin (Boesze-Battaglia and Albert, 1990), oxytocin and cholecystokinin receptors (Gimpl et al., 1997), galanin-GalR2 receptors (Pang et al., 1999), and chemokine receptors CXCR4 and CCR5 (Nguyen and Taub, 2003). These studies were carried out by treatment of either isolated membranes or live cells with cholesterol oxidase. Treatment of isolated membranes with cholesterol oxidase resulted in reduction in specific agonist binding to oxytocin and galanin receptors, while rhodopsin receptors exhibited enhanced function and there was no effect on cholecystokinin receptors. On the other hand, in the case of chemokine receptors CXCR4 and CCR5, treatment of human T cells with cholesterol oxidase resulted in inhibition of binding of chemokines and chemokine-mediated cell signaling. Although limited, these studies highlight that the effect of oxidation of cholesterol on receptor function cannot be generalized for GPCRs and the results could vary depending on the receptor.

In summary, our results show that in live cells the specific agonist binding to the serotonin<sub>1A</sub> receptor is enhanced upon oxidation of membrane cholesterol utilizing cholesterol oxidase. Importantly, this change in receptor function is accompanied by a significant change in the efficiency of G-protein coupling to the receptor, with no appreciable change in overall membrane order. These results therefore highlight the importance of the hydroxyl group of cholesterol for maintaining the optimum function of the serotonin<sub>1A</sub> receptor. Taken together, these results along with our previous results, indicate that the molecular mechanism involved

in the observed influence of membrane cholesterol on the function of the serotonin<sub>1A</sub> receptor could mainly be *via* specific interaction, although global bilayer effects could not be completely ruled out.

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