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The sterol-binding antibiotic nystatin differentially modulates ligand binding of the bovine hippocampal serotonin$_{1A}$ receptor☆

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Abstract

We have monitored the ligand binding of the bovine hippocampal 5-HT$_{1A}$ receptor following treatment with the sterol-binding antifungal antibiotic nystatin. Nystatin considerably inhibits the specific binding of the antagonist to 5-HT$_{1A}$ receptors in a concentration-dependent manner. However, the specific agonist binding does not show significant changes. Fluorescence polarization measurements of membrane probes incorporated at different locations in the membrane revealed a substantial decrease in the membrane order in the interior of the bilayer. Experiments with cholesterol-depleted membranes indicate that the action of nystatin is mediated through membrane cholesterol. These results represent the first report on the effect of a cholesterol-perturbing agent on the ligand-binding activity of this important neurotransmitter receptor.

Keywords: Nystatin; 5-HT$_{1A}$ receptor; Cholesterol; 8-OH-DPAT; *p*-MPPF; Bovine hippocampus

Serotonin (5-hydroxytryptamine or 5-HT) is an intrinsically fluorescent [1], biogenic amine which acts as a neurotransmitter and is found in a wide variety of sites in the central and peripheral nervous system [2]. Serotonergic signaling appears to play a key role in the generation and modulation of various cognitive and behavioral functions such as sleep, mood, pain, addiction, locomotion, sexual activity, depression, anxiety, alcohol abuse, aggression, and learning [3–5]. Disruptions in serotonergic systems have been implicated in the etiology of mental disorders such as schizophrenia, migraine, depression, suicidal behavior, infantile autism, eating disorders, and obsessive compulsive disorder [4,6,7].

Serotonin exerts its diverse actions by binding to distinct cell surface receptors which have been classified into many groups [8]. Serotonin receptors are members of a superfamily of seven transmembrane domain receptors [9] that couple to GTP-binding regulatory proteins (G-proteins) [10]. Among the 14 subtypes of serotonin receptors, the G-protein-coupled 5-HT$_{1A}$ receptor is the best characterized for a variety of reasons [11]. The hippocampal 5-HT$_{1A}$ receptor is negatively coupled to the adenylate cyclase through G$_i$-proteins [12]. We have earlier solubilized and partially purified the 5-HT$_{1A}$ receptor from bovine hippocampus in a functionally active form [13,14]. In addition, we have shown modulation of ligand binding by metal ions [15,16], agents that perturb G-proteins [17,18], alcohols [19,20], local anesthetics [21], and covalent modifications of the disulfide and sulfhydryl groups [11].

Lipid–protein interactions play a crucial role in maintaining the structure and function of integral membrane proteins and receptors [22]. We have recently shown the requirement of membrane cholesterol in modulating ligand-binding activity of the 5-HT$_{1A}$ receptor from the bovine hippocampus [23]. This was achieved by the use of methyl-β-cyclodextrin (MβCD)

☆ *Abbreviations:* BCA, bicinchoninic acid; 5-HT, 5-hydroxytryptamine; DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; 8-OH-DPAT, 8-hydroxy-2-(di-*N*-propylamino)tetralin; MβCD, methyl-β-cyclodextrin; *p*-MPPF, 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine; *p*-MPPI, 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-iodobenzamido]ethyl-piperazine; PMSF, phenylmethylsulfonyl fluoride; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene; Tris, *tris*-(hydroxymethyl)aminomethane.

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which physically depletes cholesterol from membranes. Treatment of bovine hippocampal membranes with MβCD therefore resulted in specific removal of membrane cholesterol without any change in phospholipid content. Removal of cholesterol from bovine hippocampal membranes in this manner resulted in a reduction in ligand binding to the 5-HT_{1A} receptor [23]. If cholesterol is necessary for ligand binding of the 5-HT_{1A} receptor, modulating cholesterol availability by other means could affect ligand binding. In this report, we have tested this proposal by treating the membranes with the sterol-binding antifungal polyene antibiotic nystatin [24–26]. Nystatin specifically interacts with cholesterol to sequester it in the membrane thereby effectively reducing the ability of cholesterol to interact with and exert its effects on other membrane components such as receptors. In this work, we monitored ligand binding of the 5-HT_{1A} receptor in hippocampal membranes treated with nystatin. Our results show that while ligand binding to this receptor is perturbed by nystatin treatment, there are interesting differences from what was observed with cholesterol depletion by MβCD treatment.

Materials and methods

Materials. BCA, DMPC, DPH, EDTA, EGTA, MβCD, $MgCl_2$, $MnCl_2$, Na_2HPO_4, nystatin, *p*-MPPI, PMSF, TMA-DPH, Tris, iodoacetamide, polyethylenimine, serotonin, sodium azide, and sucrose were obtained from Sigma Chemical (St. Louis, MO, USA). [^{3}H]8-OH-DPAT (specific activity 135.0 Ci/mmol) and [^{3}H]*p*-MPPF (specific activity 70.5 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA, USA). BCA reagent kit for protein estimation was obtained from Pierce (Rockford, IL, USA). All other chemicals used were of the highest available quality. GF/B glass microfiber filters were from Whatman International (Kent, UK). Fresh bovine brains were obtained from a local slaughterhouse within 10 min of death and the hippocampal region was carefully dissected out. The hippocampi were immediately flash-frozen in liquid nitrogen and stored at −70 °C until further use.

Preparation of native hippocampal membranes. Native hippocampal membranes were prepared as described earlier [15]. Bovine hippocampal tissue (~100 g) was homogenized as 10% (w/v) in a polytron homogenizer in buffer A (2.5 mM Tris, 0.32 M sucrose, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 0.24 mM PMSF, and 10 mM iodoacetamide, pH 7.4). The homogenate was centrifuged at 900*g* for 10 min at 4 °C. The supernatant was filtered through four layers of cheesecloth and the pellet was discarded. The supernatant was further centrifuged at 50,000*g* for 20 min at 4 °C. The resulting pellet was suspended in 10 vol of buffer B (50 mM Tris, 1 mM EDTA, 0.24 mM PMSF, and 10 mM iodoacetamide, pH 7.4) using a hand-held Dounce homogenizer and centrifuged at 50,000*g* for 20 min at 4 °C. This procedure was repeated until the supernatant was clear. The final pellet (native membranes) was resuspended in a minimum volume of buffer C (50 mM Tris, pH 7.4), homogenized using a hand-held Dounce homogenizer, flash-frozen in liquid nitrogen, and stored at −70 °C until further use. Protein concentration was determined using the BCA reagent with bovine serum albumin as a standard [27].

Radioligand-binding assays. Receptor-binding assays were carried out as described earlier [21] in the presence of increasing concentrations of nystatin. Stock solutions of nystatin (4 mM) were prepared in water and briefly sonicated in a bath sonicator (Laboratory Supplies, Hicksville, NY, USA) before use. Tubes in duplicate containing 0.5 mg total protein in a volume of 1 ml buffer D (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, and 5 mM $MnCl_2$, pH 7.4) for agonist binding or in 1 ml buffer E (50 mM Tris, 1 mM EDTA, pH 7.4) for antagonist-binding assays were used. Tubes were incubated with the radiolabeled agonist [^{3}H]8-OH-DPAT (final concentration in assay tube being 0.29 nM) or antagonist [^{3}H]*p*-MPPF (final concentration in assay tube being 0.5 nM) for 1 h at room temperature (23 °C) in presence of increasing concentrations of nystatin. Non-specific binding was determined by performing the assay either in the presence of 10 μM serotonin (for agonist-binding assays) or in the presence of 10 μM *p*-MPPI (for antagonist-binding assays). The binding reaction was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B 2.5 cm diameter glass microfiber filters (1.0 μm pore size) which were presoaked in 0.15% (w/v) polyethylenimine for 1 h [28]. The filters were then washed three times with 3 ml cold water (4 °C), dried, and the retained radioactivity was measured in a Packard Tri-Carb 1500 liquid scintillation counter using 5 ml scintillation fluid.

Cholesterol depletion of native membranes. Native hippocampal membranes were depleted of cholesterol using MβCD as described previously [23,34]. Briefly, membranes resuspended at a protein concentration of 2 mg/ml were treated with 40 mM MβCD in buffer C at room temperature (23 °C) with constant shaking for 1 h. Membranes were then spun down at 50,000*g* for 5 min, washed with buffer C, and resuspended in the same buffer. Cholesterol was estimated using the Amplex Red cholesterol assay kit [29]. Concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid [30] using Na_2HPO_4 as a standard. DMPC was used as an internal standard to assess lipid digestion.

Fluorescence polarization measurements. Fluorescence polarization experiments were carried out with membranes containing 50 nmol of total phospholipids suspended in 1.5 ml buffer C as described earlier [23,34] in presence of increasing concentrations of nystatin. Stock solutions of the fluorescent probes (DPH and TMA-DPH) were prepared in methanol. The amount of probe added was such that the final probe concentration was 1 mol% with respect to the total phospholipid content. This ensures optimal fluorescence intensity with negligible membrane perturbation. Membranes were vortexed for 1 min after addition of the probe and kept in the dark for 1 h before measurements. Background samples were prepared the same way except that the probe was omitted. The final probe concentration was 0.33 μM in all cases and the methanol content was low (0.03%, v/v). Control experiments showed that at this concentration of methanol, the ligand-binding properties of the receptor are not altered.

Steady state fluorescence was measured in a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes at room temperature (23 °C). Excitation and emission wavelengths were set at 358 and 430 nm. Excitation and emission slits with nominal bandpasses of 1.5 and 20 nm were used. The excitation slit was kept low to avoid any photoisomerization of DPH. In addition, fluorescence was measured with a 30 s interval between successive openings of the excitation shutter (when the sample was in the dark in the fluorimeter) to reverse any photoisomerization of DPH and TMA-DPH [31]. The optical density of the samples measured at 358 nm was 0.15 ± 0.01 which increased in presence of nystatin. To avoid scattering artifacts in these experiments, fluorescence polarization was measured after membrane samples were diluted with buffer C [32]. The fluorescence polarization values reported in Fig. 2 are of the most diluted samples (three times with buffer C). Fluorescence polarization measurements were performed using a Hitachi polarization accessory. Polarization values were calculated from the equation [33]:

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}, \quad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G is the instrumental correction factor and is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light and is equal to I_{HV}/I_{HH}. All experiments were done with multiple sets of samples and average values of fluorescence polarization are shown in Fig. 2.

Results

We monitored the ability of nystatin to affect the ligand binding of 5-HT_{1A} receptors in native hippocampal membranes. As mentioned earlier, nystatin specifically interacts with cholesterol [26] to sequester it in the membrane thereby effectively reducing the ability of cholesterol to interact with other membrane constituents such as receptors. Fig. 1 shows the effect of increasing concentrations of nystatin on ligand binding to bovine hippocampal 5-HT_{1A} receptors. It is apparent from the figure that nystatin inhibits the specific binding of the radiolabeled antagonist [^{3}H]*p*-MPPF to 5-HT_{1A} receptors to a considerable extent in a concentration-dependent manner. Thus, the specific antagonist binding to 5-HT_{1A} receptors reduces by ~66% when nystatin is used at an 8-fold molar excess over membrane cholesterol. In contrast to this, the specific radiolabeled agonist [^{3}H]8-OH-DPAT binding to 5-HT_{1A} receptors does not exhibit significant alterations in presence of similar concentrations of nystatin. The difference in the effects of nystatin on the agonist and antagonist binding to 5-HT_{1A} receptors points toward a specific mode of action of nystatin on the receptor.

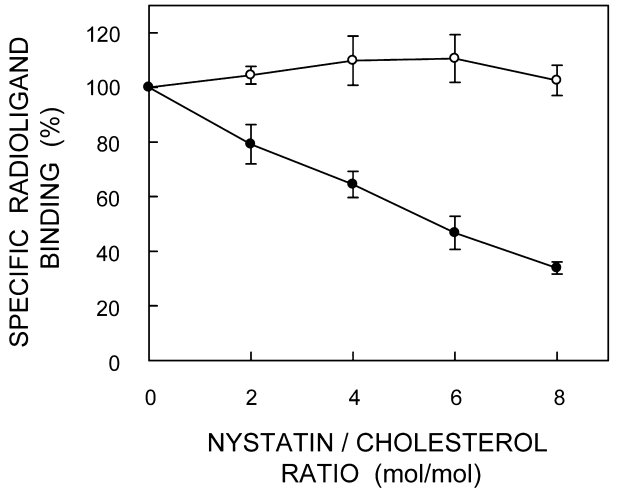

Fig. 1. Effect of increasing concentrations of nystatin on the specific binding of the agonist [^{3}H]8-OH-DPAT (○) and antagonist [^{3}H]*p*-MPPF (●) to the 5-HT_{1A} receptor in bovine hippocampal membranes. Nystatin concentrations are expressed as the ratio of nystatin to membrane cholesterol (mol/mol) and were based on the cholesterol content of bovine hippocampal membranes which was estimated to be ~433 nmol/mg protein, similar to the previously reported value [34]. Values are expressed as a percentage of the specific binding obtained in the absence of nystatin. The data shown are means ± SE of duplicate points from four independent experiments. See Materials and methods for other details.

The agonist 8-OH-DPAT and antagonist *p*-MPPF have earlier been shown to specifically bind to bovine hippocampal 5-HT_{1A} receptors with high affinity [15–17,23]. The greater sensitivity of the antagonist [^{3}H]*p*-MPPF binding of 5-HT_{1A} receptors to the sterol-binding agent nystatin could indicate a stringent requirement of the native-like distribution of cholesterol in membranes to support the antagonist-binding function of these receptors. We have recently shown the requirement of membrane cholesterol for the agonist [^{3}H]8-OH-DPAT [23] and the antagonist [^{3}H]*p*-MPPF (T.J. Pucadyil and A. Chattopadhyay, unpublished observations) binding of 5-HT_{1A} receptors. Thus, physical depletion of cholesterol from membranes using MβCD reduces agonist and antagonist binding of 5-HT_{1A} receptors to similar extents. In the backdrop of these results, it is interesting that the treatment of hippocampal membranes with the sterol-binding agent nystatin affects the antagonist [^{3}H]*p*-MPPF but not the agonist [^{3}H]8-OH-DPAT binding to 5-HT_{1A} receptors. From the present results, it appears that the mere presence of cholesterol (even in complexed form) could be sufficient to support the agonist binding to 5-HT_{1A} receptors whereas the antagonist binding requires the presence of native-like distribution of cholesterol in the membrane. These results thus further refine the role of membrane cholesterol in modulating ligand binding to 5-HT_{1A} receptors.

Nystatin is a membrane-active polyene antibiotic that effectively partitions into membranes [35]. It has been proposed that nystatin forms a 1:1 (mol/mol) complex with membrane cholesterol and forms channels in the membrane [36]. A large portion of any given transmembrane protein, the 5-HT_{1A} receptor in this case, remains in contact with the membrane lipid environment. This raises the obvious possibility that the overall membrane order and dynamics could be an important modulator of receptor structure and function [22,37]. In order to examine any change in membrane order induced by the partitioning of nystatin, we monitored the steady state fluorescence polarization of two membrane probes, DPH and TMA-DPH. DPH and its derivatives represent popular membrane probes for monitoring organization and dynamics in membranes [38]. Fluorescence polarization is correlated to the rotational diffusion [33] of membrane embedded probes which is sensitive to the packing of fatty acyl chains and cholesterol. Since the membrane is considered to be a two-dimensional anisotropic fluid, any possible change in membrane order may not be uniform or restricted to a unique location in the membrane. It is therefore important to monitor the change in membrane order at multiple regions in the membrane to obtain a

comprehensive understanding of any change in membrane (lipid) dynamics. DPH and TMA-DPH differ in their orientation and location in the membrane. While DPH is known to partition into the hydrophobic core of the membrane [39], the amphipathic TMA-DPH is oriented in the membrane bilayer with its positive charge localized at the lipid–water interface [40].

The change in fluorescence polarization of DPH and TMA-DPH incorporated in hippocampal membranes treated with increasing concentrations of nystatin is shown in Fig. 2. The fluorescence polarization of DPH and TMA-DPH in hippocampal membranes (without any nystatin) is found to be 0.332 and 0.359, respectively. The higher polarization of TMA-DPH compared to DPH is indicative of the shallower location and therefore of greater restriction in the rotational mobility of TMA-DPH in the membrane, as has been observed earlier for anthroyloxy-labeled fluorescent membrane probes [41]. The fluorescence polarization of DPH shows a decrease with increasing concentrations of nystatin, with a ~9% decrease when nystatin is used at a 6-fold molar excess over membrane cholesterol. The corresponding change in fluorescence polarization of TMA-DPH is much smaller. Thus, the percentage change in fluorescence polarization is higher for DPH compared to TMA-DPH in presence of nystatin indicating a greater membrane disordering effect in the hydrophobic interior of the membrane than in the shallower region of the membrane. It is worth mentioning here that the presence of nystatin contributed significantly to the optical density of membrane samples used for these studies. To avoid any scattering artifacts in the determination of polarization values, fluorescence polarization was measured after membrane samples were sufficiently diluted, as described earlier [32]. The fluorescence polarization values reported in Fig. 2 are of the most diluted samples (three times with buffer C) which ensured accuracy in the determination of polarization values. Thus, the presence of nystatin substantially decreased the membrane order in the interior of the bilayer and is accompanied by a greater reduction in the antagonist binding compared to the agonist-binding activity of the 5-HT_{1A} receptor.

The nature and specificity of nystatin and sterol (cholesterol in mammalian membranes) interaction has been analyzed quite extensively [26]. Studies on ion permeability induced by the presence of nystatin–sterol pore complexes in model membranes suggest that the action of nystatin is considerably enhanced in the presence of membrane sterols. This is evident from the ~10-fold higher amounts of nystatin required to bring about a similar increase in ion permeability in membranes lacking sterols [26]. To further analyze the specificity of the action of nystatin in reducing the antagonist binding to 5-HT_{1A} receptors, we performed similar experiments as described in Fig. 1 with cholesterol-depleted membranes. These experiments (shown in Fig. 3) indicate that the prior depletion of ~87% cholesterol (estimated using the Amplex Red assay, see Materials and

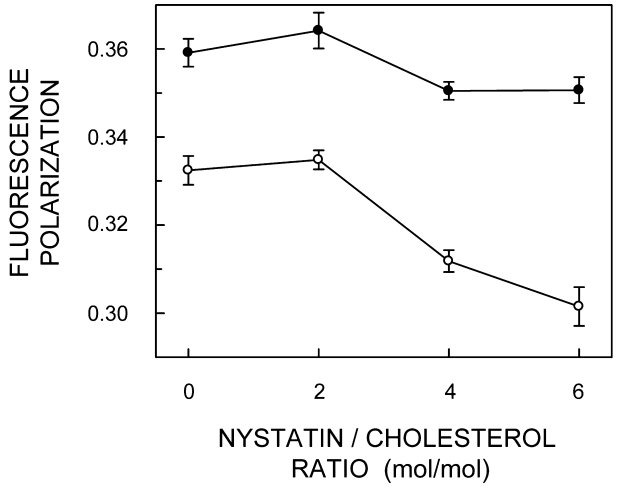

Fig. 2. Effect of increasing concentrations of nystatin on fluorescence polarization of membrane probes DPH (○) and TMA-DPH (●). Fluorescence polarization experiments were carried out with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (23 °C) that were diluted three times to avoid scattering artifacts. Nystatin concentrations are expressed as the ratio of nystatin to membrane cholesterol (mol/mol). The data shown represent means ± SE of at least six independent experiments. See Materials and methods for other details.

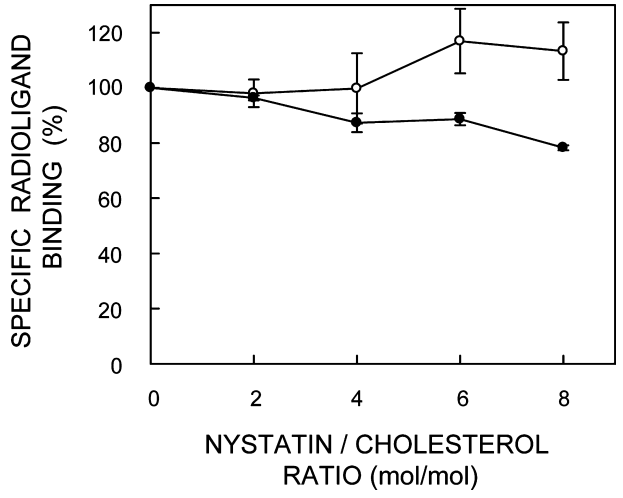

Fig. 3. Effect of increasing concentrations of nystatin on the specific binding of the agonist [^{3}H]8-OH-DPAT (○) and antagonist [^{3}H]*p*-MPPF (●) to the 5-HT_{1A} receptor from bovine hippocampal membranes depleted of cholesterol using 40 mM MβCD. Nystatin concentrations are expressed as the ratio of nystatin to membrane cholesterol (mol/mol) and were based on the cholesterol content of MβCD-treated bovine hippocampal membranes which was estimated to be ~57 nmol/mg protein, similar to the previously reported value [34]. Values are expressed as a percentage of the specific binding obtained in the absence of nystatin. The values are means ± SE of duplicate points from three independent experiments. See Materials and methods for other details.

methods) using 40 mM MβCD greatly attenuates the effect of nystatin on the specific antagonist [^{3}H]*p*-MPPF binding to 5-HT_{1A} receptors. Thus, the specific antagonist binding to 5-HT_{1A} receptors reduces by ~22% when nystatin is used at an 8-fold molar excess over membrane cholesterol in cholesterol-depleted membranes (Fig. 3), as compared to ~66% reduction observed for control membranes (Fig. 1). As observed earlier (Fig. 1), the specific agonist [^{3}H]8-OH-DPAT binding shows no significant variation under these conditions. These results indicate that the action of nystatin to reduce the antagonist binding to 5-HT_{1A} receptors is mediated through membrane cholesterol.

Discussion

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function, and sorting [42,43]. It is often found distributed non-randomly in domains or pools in biological and model membranes [44–46]. Many of these domains are believed to be important for the maintenance of membrane structure and function. The membrane organization of G-protein-coupled receptors, such as 5-HT_{1A} receptors, in relation to these domains assumes significance in light of their role in health and disease [47]. Recent evidence has indicated that a spatiotemporally organized system rather than a freely diffusible system of receptors and G-proteins is responsible for rapid and specific propagation of extracellular stimuli to intracellular signaling molecules [48,49]. It has been proposed that G-protein-coupled receptors are not uniformly present on the plasma membrane but are concentrated in specific membrane microdomains [48–51], some of which are presumably enriched in cholesterol [43,46]. Importantly, the integrity of some of these domains is maintained by the presence of cholesterol [52]. In this regard, the analysis of membrane protein function under conditions that affect membrane cholesterol content, availability, and distribution assumes greater significance.

This report represents one of the first studies on the effect of agents that perturb membrane cholesterol on the ligand-binding activity of this important neurotransmitter receptor. These results demonstrate interesting differences in the manner by which cholesterol modulates ligand-binding activity of hippocampal 5-HT_{1A} receptors depending on the exact approach used to perturb membrane cholesterol (i.e., by depletion with agents such as MβCD or complexation with nystatin). In addition, our results show the important role played by cholesterol in regulation of ligand-binding activity of the 5-HT_{1A} receptor. More importantly, these results are relevant in the general context of the influence of the membrane lipid environment on the activity of G-protein-coupled transmembrane receptors.

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