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Membrane cholesterol depletion enhances ligand binding function of human serotonin$_{1A}$ receptors in neuronal cells

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ABSTRACT

Membrane lipid composition of cells in the nervous system is unique and displays remarkable diversity. Cholesterol metabolism and homeostasis in the central nervous system and their role in neuronal function represent important determinants in neuropathogenesis. The serotonin$_{1A}$ receptor is an important member of the G-protein coupled receptor superfamily, and is involved in a variety of cognitive, behavioral, and developmental functions. We report here, for the first time, that the ligand binding function of human serotonin$_{1A}$ receptors exhibits an increase in membranes isolated from cholesterol-depleted *neuronal* cells. Our results gain pharmacological significance in view of the recently described structural evidence of specific cholesterol binding site(s) in GPCRs, and could be useful in designing better therapeutic strategies for neurodegenerative diseases associated with GPCRs.

Introduction

The G-protein coupled receptor (GPCR) superfamily is the largest and most diverse protein family in mammals, involved in signal transduction across membranes [1]. GPCRs are increasingly recognized as major targets for the development of novel drug candidates in all clinical areas [2,3]. The serotonin$_{1A}$ (5-HT$_{1A}$) receptor is an important neurotransmitter receptor belonging to the GPCR superfamily. It is the most extensively studied of the serotonin receptors for a number of reasons [4,5]. Serotonergic signaling plays a key role in the generation and modulation of various cognitive, behavioral and developmental functions [6]. This is supported by the fact that the agonists and antagonists of the serotonin$_{1A}$ receptor represent major classes of molecules with potential therapeutic effects in anxiety- and stress-related disorders. Interestingly, mutant (knockout) mice lacking the serotonin$_{1A}$ receptor exhibit enhanced anxiety-related behavior, and represent an important animal model for genetic vulnerability to complex traits such as anxiety disorders and aggression in higher animals [7,8]. In the context of increasing pharmacological relevance of the serotonin$_{1A}$ receptor, a transmembrane protein, its interaction with surrounding lipids assumes significance in modulating the function of the receptor in healthy and diseased states [5].

Cholesterol is an essential and representative lipid in higher eukaryotic cellular membranes and is crucial in membrane organization, dynamics, function, and sorting [9–11]. It is often found distributed non-randomly in domains in biological and model membranes. Many of these domains (sometimes termed as 'lipid rafts') are believed to be important for the maintenance of membrane structure and function. Nonetheless, characterizing the spatiotemporal resolution of these domains has proven to be challenging [11–14]. The idea of such specialized membrane domains assumes relevance in the cellular context since physiologically important functions such as membrane sorting and trafficking [15] signal transduction processes [16], and the entry of pathogens [17–19] have been attributed to these domains. We have earlier shown that membrane cholesterol is essential for the function of the serotonin$_{1A}$ receptor (reviewed in [5] and [20]).

Mammalian cells in culture heterologously expressing membrane receptors represent convenient systems to address problems in receptor biology due to higher expression levels of receptors [21]. An important consideration in such expression systems is selecting a cell type which is derived from the tissue of natural occurrence of the receptor. This is particularly true for receptors of neural origin since the membrane lipid composition of cells in the nervous system is unique and displays remarkable diversity [22–24]. Lipids found in neuronal membranes are often necessary for maintaining the structure and function of neuronal receptors. Keeping this in mind, we earlier reported the pharmacological and functional characterization of the human serotonin$_{1A}$ receptor stably expressed in HN2 cells [25], which are a hybrid cell line between hippocampal

Abbreviations: 5-HT$_{1A}$ receptor, 5-hydroxytryptamine-1A receptor; 8-OH-DPAT, 8-hydroxy-2(di-*N*-propylamino)tetralin; BCA, bicinchoninic acid; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; GPCR, G-protein coupled receptor; MβCD, methyl-β-cyclodextrin; PMSF, phenylmethylsulfonyl fluoride.

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cells and mouse neuroblastoma. Our results showed that the human serotonin$_{1A}$ receptor expressed in HN2 cells displays characteristic features found in the native receptor isolated from bovine hippocampus and represents a realistic model system for the receptor. In this paper, we monitored the function of the human serotonin$_{1A}$ receptor stably expressed in HN2 cells upon depletion of membrane cholesterol from live cells. Cholesterol depletion from cell membranes was achieved using methyl-β-cyclodextrin (MβCD). The corresponding changes in membrane dynamics were monitored by fluorescence anisotropy of the membrane probe DPH. Importantly, our results show that the ligand binding function of serotonin$_{1A}$ receptors is enhanced in membranes isolated from cholesterol-depleted neuronal cells. This constitutes the first report describing functional changes in serotonin$_{1A}$ receptors upon membrane cholesterol depletion in neuronal cells.

Materials and methods

Materials. Cholesterol, MβCD, DMPC, DPH, EDTA, $MgCl_2$, $MnCl_2$, Na_2HPO_4, PMSF, serotonin, polyethylenimine, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). DMEM (Dulbecco's modified Eagle's medium), fetal calf serum, and geneticin (G 418) were from Invitrogen Life Technologies (Carlsbad, CA). Amplex Red cholesterol assay kit was from Molecular Probes (Eugene, OR). BCA reagent for protein estimation was from Pierce (Rockford, IL). [^{3}H]8-OH-DPAT (sp. activity 106 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). 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. All other chemicals and solvents used were of the highest available purity.

Cells and cell culture. The intronless human genomic clone G-21 [26] which encodes the human serotonin$_{1A}$ receptor was used to generate stable transfectants in HN2 cells which are a hybrid cell line between hippocampal cells and mouse neuroblastoma [27]. These cells stably expressing the human serotonin$_{1A}$ receptor are referred to as HN2-5-HT$_{1A}$R cells [25]. HN2-5-HT$_{1A}$R cells were maintained as described earlier [25,28].

Cholesterol depletion of neuronal cells in culture. Cells at density of 2×10^6 in 150 cm^2 flasks were grown for 3 days in DMEM supplemented with 10% serum, followed by incubation in serum-free DMEM for 3 h at 37 °C. Cholesterol depletion was carried out by treating cells with increasing concentrations of MβCD in serum-free DMEM for 30 min at 37 °C, followed by wash with 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4 buffer.

Cell membrane preparation. Cell membranes were prepared as described earlier [25]. Total protein concentration in isolated membranes was determined using the BCA assay [29].

Radioligand binding assay. Receptor binding assays in membranes isolated from control and cholesterol-depleted HN2-5-HT$_{1A}$R cells were carried out as described earlier [25,30] with ~200 μg total protein. The concentration of [^{3}H]8-OH-DPAT used was 0.29 nM.

Analysis of cholesterol and phospholipid contents. Cholesterol content in cell membranes was estimated using the Amplex Red cholesterol assay kit [31]. Total phospholipid content of membranes was determined subsequent to digestion with perchloric acid [32] using Na_2HPO_4 as standard. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

Fluorescence anisotropy measurements. Steady state fluorescence anisotropy measurements were carried out in a Hitachi F-4010 spectrofluorometer using the fluorescent probe DPH incorporated in membranes isolated from control and cholesterol-depleted HN2-5-HT$_{1A}$R cells, as mentioned earlier [33]. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy values were calculated from the equation [34]:

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

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 grating correction factor and is equal to I_{HV}/I_{HH}. Experiments were done with multiple sets of samples and average values of fluorescence anisotropy are shown in Fig. 4.

Statistical analysis: Significance levels were estimated using Student's two-tailed unpaired t-test using Graphpad Prism software version 4.0 (San Diego, CA).

Results and discussion

We monitored specific binding of the serotonin$_{1A}$ receptor agonist [^{3}H]8-OH-DPAT to membranes isolated from HN2-5-HT$_{1A}$R cells. Fig. 1 shows that the binding of the [^{3}H]8-OH-DPAT is linear over a broad range (30–200 μg) of total protein. These results suggest that under the conditions of the assay, there is no depletion of the radiolabel during the course of the assay. These conditions are therefore appropriate for analyzing binding parameters of the receptor using [^{3}H]8-OH-DPAT [35].

MβCD is a water-soluble cyclic oligosaccharide, and has earlier been shown to extract cholesterol from membranes in a selective and efficient manner by including it in a central nonpolar cavity [36]. Fig. 2 shows the cholesterol content in membranes isolated from control and cholesterol-depleted HN2-5-HT$_{1A}$R cells. Treatment of cells with increasing concentrations of MβCD results in a progressive depletion of membrane cholesterol. When membranes were treated with 10 mM MβCD, the cholesterol content was reduced to ~35% of that of control (without treatment). The concentration range of MβCD was carefully chosen to minimize any possible change in membrane phospholipid content. The phospholipid content remains invariant under these conditions (see inset of Fig. 2).

Fig. 3 shows the increase in specific [^{3}H]8-OH-DPAT binding in membranes isolated from HN2-5-HT$_{1A}$R cells upon treatment with increasing concentrations of MβCD. For example, specific agonist binding is enhanced by ~47% of the control (in the absence of

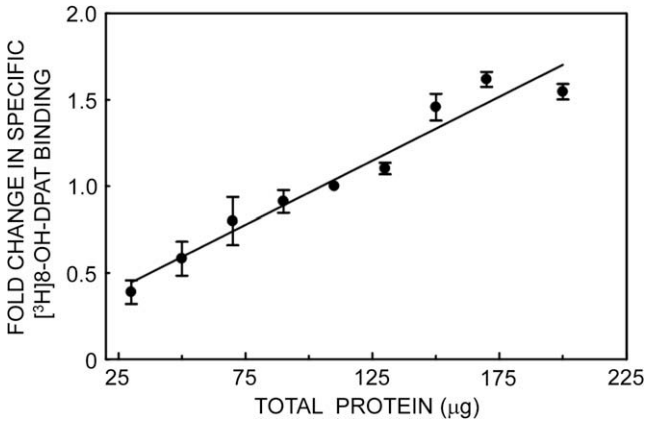

Fig. 1. Fold change in specific binding of the agonist [^{3}H]8-OH-DPAT to serotonin$_{1A}$ receptors from HN2-5-HT$_{1A}$R cell membranes with increasing amounts of total membrane protein. Values have been normalized with respect to specific binding obtained with 110 μg total protein in the assay. Concentration of [^{3}H]8-OH-DPAT used in the assay was 0.29 nM. Data represent means ± SE from three independent measurements. See Materials and methods for other details.

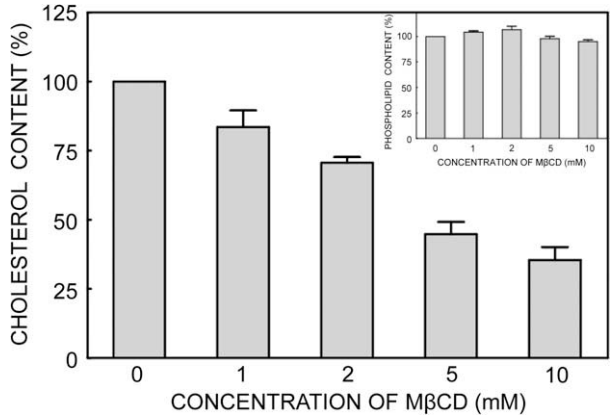

Fig. 2. Lipid contents of membranes isolated from HN2-5-HT$_{1A}$R cells upon cholesterol depletion. The figure depicts cholesterol contents in membranes isolated from control cells and cells treated with increasing concentrations of MβCD. The inset shows the corresponding phospholipid content in membranes isolated from cholesterol-depleted HN2-5-HT$_{1A}$R cells. Values are expressed as percentages of the respective lipid content in control (without MβCD treatment) cell membranes. Data shown are means ± SE from at least four independent measurements. See Materials and methods for other details.

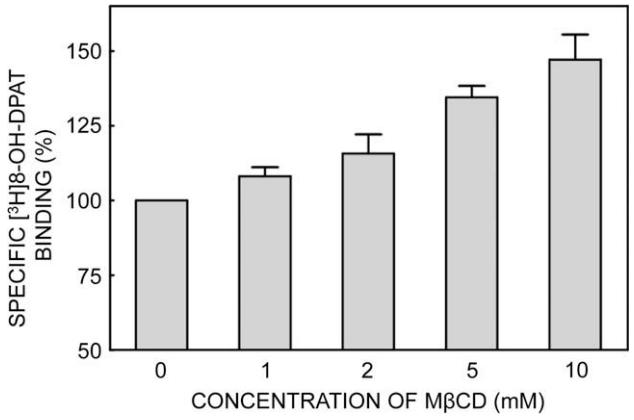

Fig. 3. Effect of cholesterol depletion on specific binding of the agonist to human serotonin$_{1A}$ receptors in membranes isolated from control and MβCD-treated HN2-5-HT$_{1A}$R cells. The figure shows specific [^{3}H]8-OH-DPAT binding to receptors in membranes isolated from cells treated with increasing concentrations of MβCD. Values are expressed as percentages of specific binding obtained in control cells without MβCD treatment. Data represent means ± SE from at least six independent measurements performed in duplicate. See Materials and methods for other details.

MβCD treatment), when 10 mM MβCD was used. This shows that removal of cholesterol from intact HN2-5-HT$_{1A}$R cells leads to enhancement of specific agonist binding to serotonin$_{1A}$ receptors. To the best of our knowledge, these results constitute the first report on change in ligand binding function of serotonin$_{1A}$ receptors upon cholesterol modulation in neuronal cells.

The observed increase in specific ligand binding function upon reduction in membrane cholesterol content could be due to an alteration in overall membrane organization. In order to monitor the change in overall membrane order, we monitored the steady state fluorescence anisotropy of the membrane probe, diphenylhexatriene (DPH). DPH is a rod-like molecule and partitions into the bilayer, irrespective of the phase (ordered/disordered) of the membrane [37]. Fluorescence anisotropy is correlated to the rotational diffusion of membrane embedded probes such as DPH [34], which is sensitive to the packing of lipid acyl chains. This is due to the fact that fluorescence anisotropy depends on the degree to which the probe is able to reorient after excitation, and probe

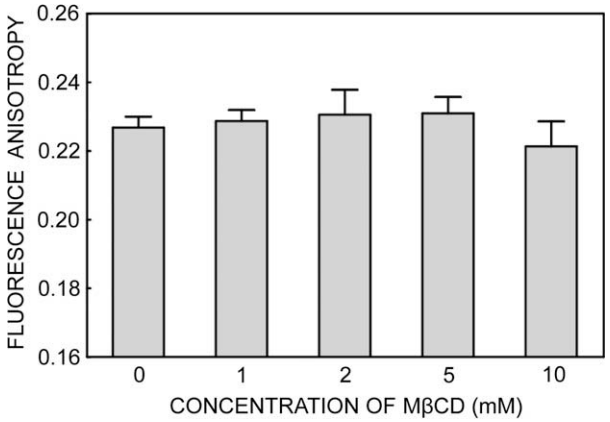

Fig. 4. Steady state fluorescence anisotropy of the membrane probe DPH in membranes isolated from cholesterol-depleted HN2-5-HT$_{1A}$R cells. Fluorescence anisotropy measurements were carried out with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (~23 °C). The excitation wavelength was 358 nm and emission was monitored at 430 nm. Data represent means ± SE from at least four independent measurements. See Materials and methods for other details.

reorientation is a function of local lipid packing. Fig. 4 shows the steady state fluorescence anisotropy of DPH in membranes isolated from control and cholesterol-depleted HN2-5-HT$_{1A}$R cells. Interestingly, the fluorescence anisotropy does not exhibit any appreciable change in membranes isolated from cholesterol-depleted cells. This implies that the overall membrane order is not significantly ($p > 0.05$) altered upon cholesterol depletion.

A useful approach for performing pharmacological studies on GPCRs is to use a functional receptor system that converts receptor–ligand interaction into a cellular signal which allows to monitor the relationship between concentration and response [38]. In the past few years, there have been an increasing number of genetically engineered recombinant receptor systems for monitoring drug–receptor interactions. This has led to a corresponding increase in the testing of new drugs in recombinant receptor systems. However, differences in host membrane lipid composition often complicate interpretation of drug testing results in such systems, and can lead to receptors with characteristics different from native receptors. For example, it has earlier been reported that although the rat cortical serotonin$_{1A}$ receptor exists only in the high affinity state in its native environment, it displays both high and low affinity when expressed in HEK293 cells [39]. It is therefore judicious to work with cellular systems in which the membrane lipid composition closely mimics the native lipid environment. Our choice of HN2 cells for exploring the interaction between membrane cholesterol and serotonin$_{1A}$ receptors is based on the observation that cell lines of neural origin represent realistic models for understanding signal transduction in neuronal cells.

Cholesterol metabolism and homeostasis in the central nervous system and their role in neuronal function is only beginning to be addressed [40]. Previous work from our laboratory has shown that depletion of cholesterol from isolated hippocampal membranes using MβCD leads to the reduction in ligand binding of the serotonin$_{1A}$ receptor [41]. In this context, our present observation of increase in agonist binding of serotonin$_{1A}$ receptors in membranes isolated from cholesterol-depleted HN2-5-HT$_{1A}$R cells appears interesting. Our present results imply that specific ligand binding to serotonin$_{1A}$ receptors upon cholesterol depletion is influenced by whether cholesterol depletion is carried out in isolated membranes or from intact cells. These results assume additional relevance since they represent the first report on change in ligand binding function of serotonin$_{1A}$ receptors upon cholesterol depletion in neuronal cells. This is important in the context of the recent

observation that cholesterol depletion from neuronal and non-neuronal cells differentially modulates the function of the G-protein coupled δ-opioid receptor [42]. Overall, our results are relevant in understanding the interaction of membrane cholesterol with GPCRs, particularly in view of the recently described structural evidence of specific cholesterol binding site(s) in GPCRs [43].

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