

## Differential effects of cholesterol and 7-dehydrocholesterol on the ligand binding activity of the hippocampal serotonin<sub>1A</sub> receptor: Implications in SLOS

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### Abstract

The requirement of membrane cholesterol in maintaining ligand binding activity of the hippocampal serotonin<sub>1A</sub> receptor has previously been demonstrated. In order to test the stringency of the requirement of cholesterol, we depleted cholesterol from native hippocampal membranes followed by replenishment with 7-dehydrocholesterol. The latter sterol is an immediate biosynthetic precursor of cholesterol differing only in a double bond at the 7th position in the sterol ring. Our results show, for the first time, that replenishment with 7-dehydrocholesterol does not restore ligand binding activity of the serotonin<sub>1A</sub> receptor, in spite of recovery of the overall membrane order. The requirement for restoration of ligand binding activity therefore is more stringent than the requirement for the recovery of overall membrane order. These novel results have potential implications in understanding the interaction of membrane lipids with this important neuronal receptor under pathogenic conditions such as the Smith-Lemli-Opitz syndrome.

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**Keywords:** Smith-Lemli-Opitz syndrome; Cholesterol; 7-Dehydrocholesterol; Ligand binding activity; Serotonin<sub>1A</sub> receptor; Fluorescence anisotropy

Seven transmembrane domain G-protein coupled receptors (GPCRs) constitute one of the largest family of proteins in mammals and account for ~2% of the total proteins coded by the human genome [1]. GPCRs represent major targets for the development of novel drug candidates in all clinical areas [2]. The serotonin<sub>1A</sub> (5-HT<sub>1A</sub>) receptor is an important GPCR and is the most extensively studied among the serotonin receptors. The serotonin<sub>1A</sub> receptor is involved in a variety of cognitive, behavioral, and developmental functions (for a recent review, see [3]). The seroto-

nin<sub>1A</sub> receptor agonists and antagonists represent major classes of molecules with potential therapeutic effects in anxiety- or stress-related disorders. Interestingly, mice lacking the serotonin<sub>1A</sub> receptor exhibit enhanced anxiety-related behavior [4] and represent an important animal model for genetic vulnerability to conditions such as anxiety disorders and aggression [5].

Cholesterol is an abundant and essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function, and sorting [6]. Interestingly, the central nervous system which accounts for only 2% of the body mass contains ~25% of free cholesterol present in the whole body [7]. Brain cholesterol is synthesized *in situ* and is developmentally regulated. As a result, a number of neurological diseases share a common etiology of defective cholesterol metabolism in the brain [8]. In the Smith-Lemli-Opitz syndrome (SLOS), for example, the marked abnormalities in brain development and function leading to serious neurological and mental dys-

*Abbreviations:* 5-HT<sub>1A</sub> receptor, 5-hydroxytryptamine-1A receptor; 7-DHC, 7-dehydrocholesterol; 8-OH-DPAT, 8-hydroxy-2(di-*N*-propylamino)tetralin; 7-DHCR, 3 $\beta$ -hydroxy-steroid- $\Delta^7$ -reductase; DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; GPCR, G-protein coupled receptor; M $\beta$ CD, methyl- $\beta$ -cyclodextrin; PMSF, phenylmethylsulfonyl fluoride; SLOS, Smith-Lemli-Opitz syndrome.

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functions have their origin in the fact that the major input of brain cholesterol comes from the *in situ* synthesis and such synthesis is defective in this syndrome [9]. SLOS is caused by mutations in  $3\beta$ -hydroxy-steroid- $\Delta^7$ -reductase (7-DHCR), an enzyme required in the final step of cholesterol biosynthesis. Elevated plasma levels of 7- and 8-dehydrocholesterol and the ratio of their contents to that of cholesterol are representative parameters for diagnosis of SLOS. The effect of alteration in the cholesterol content of neuronal membranes on membrane dynamics and protein/receptor function therefore represents an important determinant in the analysis of neurogenesis and several neuropathologies.

We have previously shown the requirement of membrane cholesterol in maintaining ligand binding activity of the hippocampal serotonin<sub>1A</sub> receptor [10,11]. In order to further examine the stringency of cholesterol requirement, we have tested whether 7-DHC, an immediate biosynthetic precursor of cholesterol differing only in its unsaturation at 7th position in the sterol ring (see Fig. 1), can support the ligand binding activity of the hippocampal serotonin<sub>1A</sub> receptor.

## Materials and methods

**Materials.** Cholesterol, 7-DHC, M $\beta$ CD, DMPC, DPH, EDTA, EGTA, MgCl<sub>2</sub>, MnCl<sub>2</sub>, iodoacetamide, PMSF, serotonin, sucrose, polyethylenimine, sodium azide, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BCA reagent for protein estimation was from Pierce (Rockford, IL, USA). [<sup>3</sup>H]8-OH-DPAT (sp. activity 135 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA, USA). GF/B glass microfiber filters were from Whatman International (Kent, UK). All solvents used were of analytical grade. Pre-coated silica gel 60 thin-layer chromatography plates were from Merck (Darmstadt, Germany). All other chemicals used were of the highest purity available. Water was purified through a Millipore (Bedford, MA, USA) Milli-Q system and used throughout. 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  $-70^{\circ}\text{C}$  till further use.

**Preparation of native hippocampal membranes.** Native hippocampal membranes were prepared as described earlier [10]. Protein concentration was determined using the BCA reagent with bovine serum albumin as a standard [12].

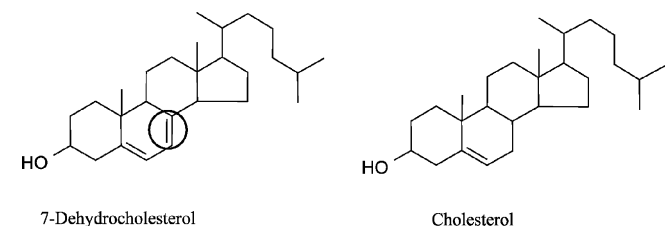


Fig. 1. Chemical structures of cholesterol and 7-dehydrocholesterol. The principal route of cholesterol synthesis in humans is the Kandutsch-Russell pathway [20]. In this pathway, the immediate precursor of cholesterol is 7-dehydrocholesterol which differs only in its unsaturation at 7th position in the sterol ring (highlighted in its chemical structure). Elevated levels of 7-dehydrocholesterol have been characterized as a diagnostic parameter of the Smith-Lemli-Opitz syndrome. See text for more details.

**Radioligand binding assays.** Receptor binding assays were carried out as described earlier [10] using 0.5 mg total protein. Briefly, tubes in duplicate with 0.5 mg protein in a total volume of 1 ml of buffer (50 mM Tris, 1 mM EDTA, 10 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, pH 7.4) were incubated with the radiolabeled agonist [<sup>3</sup>H]8-OH-DPAT (final concentration in assay tube being 0.29 nM) for 1 h at 25 °C. Nonspecific binding was determined by performing the assay in the presence of 10  $\mu\text{M}$  serotonin. 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  $\mu\text{m}$  pore size), which were presoaked in 0.15% polyethylenimine for 1 h. Filters were then washed three times with 3 ml of cold water (4 °C), dried and the retained radioactivity was measured in a Packard Tri-Carb 1500 liquid scintillation counter using 5 ml of scintillation fluid.

**Cholesterol depletion of native membranes.** Native hippocampal membranes were depleted of cholesterol using M $\beta$ CD as described previously [10]. Briefly, membranes resuspended at a protein concentration of 2 mg/ml were treated with 40 mM M $\beta$ CD in 50 mM Tris, pH 7.4, buffer at 25 °C with constant shaking for 1 h. Membranes were then spun down at 50,000g for 5 min at 4 °C, washed with 50 mM Tris, pH 7.4, buffer and resuspended in the same buffer.

**7-Dehydrocholesterol and cholesterol replenishment of cholesterol-depleted membranes.** Cholesterol-depleted hippocampal membranes were replenished with 7-dehydrocholesterol (7-DHC) or cholesterol using either 7-DHC-M $\beta$ CD or cholesterol-M $\beta$ CD complex which are soluble in water. The complex was prepared by dissolving the required amounts of 7-DHC or cholesterol and M $\beta$ CD in a ratio of 1:10 (mol/mol) in 50 mM Tris, pH 7.4, buffer by constant shaking at 25 °C. Stock solutions (typically 2 mM 7-DHC (or cholesterol):20 mM M $\beta$ CD) of this complex were freshly prepared before each experiment. 7-DHC and cholesterol replenishment were carried out at a protein concentration of 2 mg/ml by incubating the cholesterol-depleted membranes with 1 mM 7-DHC (or cholesterol):10 mM M $\beta$ CD complex for 1 h in 50 mM Tris, pH 7.4, buffer at 25 °C under constant shaking. Membranes were then spun down at 50,000g for 5 min at 4 °C, washed with 50 mM Tris, pH 7.4, buffer and resuspended in the same buffer.

**Estimation of 7-DHC and cholesterol by thin-layer chromatography.** Lipid extraction from native, cholesterol-depleted, and membranes replenished with 7-DHC or cholesterol after cholesterol depletion using 40 mM M $\beta$ CD was carried out according to Bligh and Dyer [13]. The lipid extracts were dried under a stream of nitrogen at 45 °C. The dried extracts were resuspended in a mixture of chloroform/methanol (1:1, v/v). 7-DHC and cholesterol were resolved by thin-layer chromatography (TLC). TLC plates were impregnated with a 3% (w/v) silver nitrate solution in 97% methanol, allowed to dry briefly and activated at 120 °C for 15 min. Total lipid extracts were separated using *n*-heptane/ethylacetate (2:1, v/v) as the solvent system [14]. The separated lipids were visualized by charring with a solution containing cupric sulfate (10%, w/v) and phosphoric acid (8%, v/v) at 150 °C. 7-DHC and cholesterol were used as standards to identify 7-DHC and cholesterol bands on the thin-layer chromatogram run with lipid extracts from native, cholesterol-depleted, and 7-DHC or cholesterol replenished hippocampal membranes. The TLC plates were scanned and lipid band intensities were analyzed using the Adobe Photoshop software version 5.0 (Adobe Systems, San Jose, CA, USA). Intensities of the sterols (7-DHC and cholesterol) from all samples on the TLC plate were normalized to the intensity of the cholesterol band obtained from the native membrane.

**Estimation of inorganic phosphate.** Concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid [15] 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.

**Fluorescence anisotropy measurements.** Fluorescence anisotropy measurements were carried out with fluorescent membrane probe DPH with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes at room temperature (23 °C) as described earlier [16]. 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 optical

density of the samples measured at 358 nm was  $0.15 \pm 0.01$ . The anisotropy values remained identical even after dilution of membrane samples, indicating the absence of any scattering artifact [17]. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy values were calculated from the equation [18]:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

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

## Results and discussion

Cholesterol depletion of native hippocampal membranes was carried out using the water-soluble compound M $\beta$ CD which has previously been shown to selectively and efficiently extract cholesterol from membranes [10]. Fig. 2 shows the extents of replenishment of 7-DHC and cholesterol into hippocampal membranes treated with 40 mM M $\beta$ CD. Treatment of membranes with 40 mM M $\beta$ CD results in ~61% reduction in the cholesterol content (Fig. 2B). This is accompanied by a corresponding reduction (~56%) in the specific [ $^3$ H]8-OH-DPAT binding (see Fig. 3). Replenishment with 1 mM cholesterol resulted in recovery of specific agonist binding to ~65% of native membranes (Fig. 3) even when ~87% of the cholesterol could be replenished (Fig. 2B). In order to monitor whether replenishment with 7-DHC could restore the specific [ $^3$ H]8-OH-DPAT binding, cholesterol-depleted membranes were replenished with 0.5 and 1 mM 7-DHC. Importantly, the total sterol content (7-DHC + cholesterol) appears to be comparable to native membranes, especially when loading was carried out with 1 mM 7-DHC (Fig. 2B). Interestingly, even when loading was carried out with 1 mM 7-DHC, the specific agonist binding could not be restored (Fig. 3) in spite of the fact that the extent of loading of 7-DHC was similar to what was obtained with 1 mM cholesterol (Fig. 2B). We interpret this to be due to the inability of 7-DHC in restoring the specific agonist binding activity of the hippocampal serotonin $_{1A}$  receptor.

The observed difference between cholesterol and 7-DHC, in terms of being able to restore the ligand binding activity (Fig. 3), could be due to an alteration in overall membrane organization (order). In order to monitor whether there is a change in overall membrane order when cholesterol-depleted membranes were replenished with cholesterol or 7-DHC, fluorescence anisotropy measurements were carried out with the membrane probe, DPH. Fluorescence anisotropy measured using probes such as DPH is correlated to the rotational diffusion of membrane embedded probes [18], which is sensitive to the packing of lipid chains. Fig. 4 shows that the fluorescence anisotropy of DPH exhibits a significant reduction upon cholesterol depletion from native membranes. Interestingly, when cholesterol-depleted membranes were replenished with either

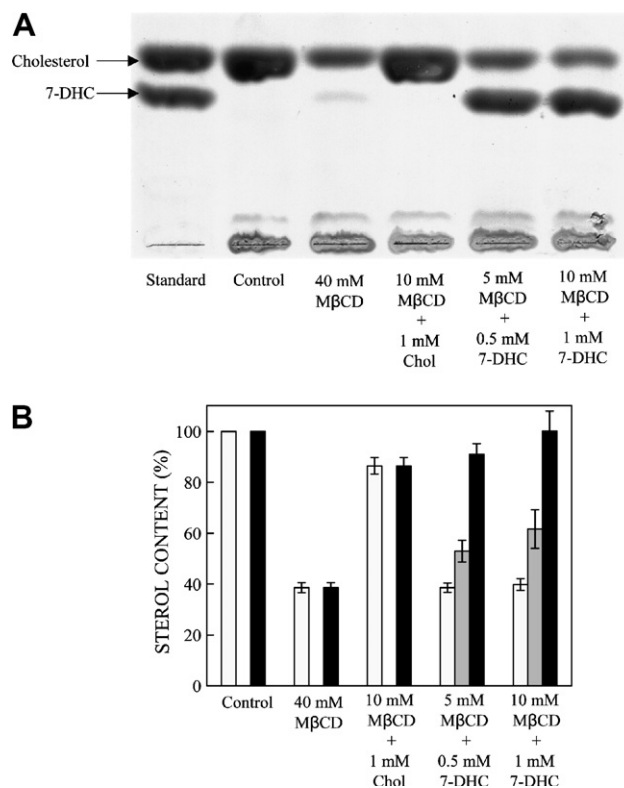


Fig. 2. Estimation of sterol content of native and cholesterol-depleted hippocampal membranes, and cholesterol-depleted membranes replenished with varying concentrations of sterols (7-DHC and cholesterol). Total lipids extracted from native membranes, membranes treated with 40 mM M $\beta$ CD, and cholesterol-depleted membranes replenished with varying concentrations of M $\beta$ CD and sterols were separated by thin layer chromatography as shown in (A). The lanes represent lipids extracted from native membranes (lane 2), membranes treated with 40 mM M $\beta$ CD (lane 3), and membranes treated with 40 mM M $\beta$ CD followed by replenishment with 1 mM cholesterol (lane 4), 0.5 mM of 7-DHC (lane 5) and 1 mM 7-DHC (lane 6). The arrows represent positions of cholesterol and 7-DHC on the thin layer chromatogram identified using standards in lane 1. Cholesterol (open bars), 7-DHC (gray bars) and total sterol (black bars) were quantified by densitometric analysis of the thin layer chromatogram and are shown in (B). Values are expressed as percentages of the cholesterol content of native membranes without any treatment and total sterol content of membranes were obtained by the addition of 7-DHC and cholesterol contents. Data represent means  $\pm$  SE of at least three independent experiments. See Materials and methods for other details.

cholesterol or 7-DHC, the fluorescence anisotropy was found to be similar to that of native (control) membranes. This indicates that the overall membrane order is unaltered upon replenishment. The requirement for restoration of ligand binding activity is therefore more stringent than the requirement for the recovery of overall membrane order.

We have earlier reported that membrane cholesterol is required in maintaining ligand binding activity of the hippocampal serotonin $_{1A}$  receptor [11]. In order to test the stringency of the requirement of cholesterol in maintaining the function of the hippocampal serotonin $_{1A}$  receptor, in this paper we have replaced cholesterol with 7-DHC, the

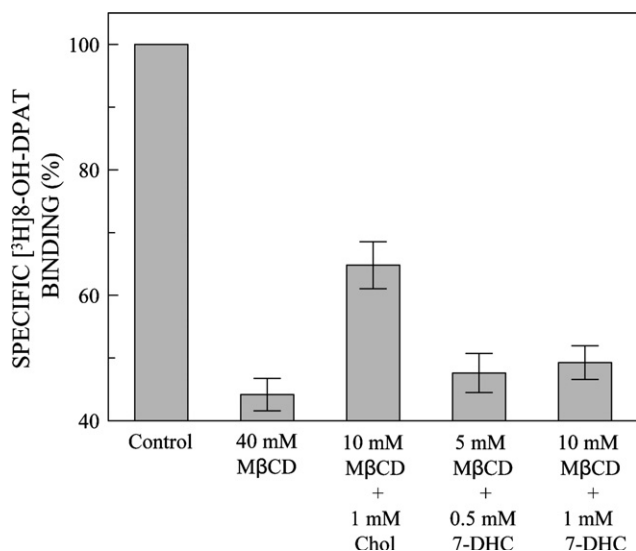


Fig. 3. Effect of replenishment of 7-DHC and cholesterol into cholesterol-depleted membranes on the specific binding of the [ $^3$ H]8-OH-DPAT to the hippocampal serotonin $_{1A}$  receptor. Native membranes were treated with 40 mM MβCD and were replenished with varying concentrations of 7-DHC and cholesterol. Values are expressed as percentages of the specific binding obtained in native membranes. The data shown are means  $\pm$  SE from at least five independent experiments. See Materials and methods for other details.

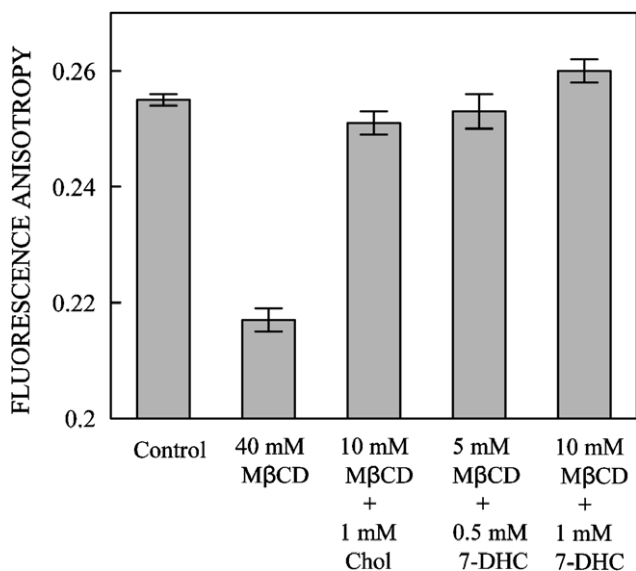


Fig. 4. Effect of replenishment of 7-DHC and cholesterol into cholesterol-depleted membranes on fluorescence anisotropy of the membrane probe DPH. Cholesterol depletion was carried out using 40 mM MβCD. Fluorescence anisotropy experiments were performed with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (23 °C). The values represent the means  $\pm$  SE of duplicate points from at least three independent experiments. See Materials and methods for other details.

only difference between the two molecules being the double bond in 7-DHC at the 7th position. Our results show that 7-DHC does not support the ligand binding activity of the hippocampal serotonin $_{1A}$  receptor. This is in spite of the

fact that replenishment with 7-DHC restores overall membrane order to that of native membranes. We have previously shown that membrane cholesterol oxidation leads to inhibition of the ligand binding activity of the hippocampal serotonin $_{1A}$  receptor without changing membrane order [19]. Taken together, our results indicate that the molecular basis for the requirement of membrane cholesterol in maintaining the ligand binding activity of serotonin $_{1A}$  receptors could be specific interaction, although global bilayer effects may not be ruled out. These results have potential implications in understanding the interaction of membrane lipids with this important neuronal receptor under SLOS-like condition in which 7-DHC accumulates due to mutations in the *DHCR7* gene.

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