Signaling by the human serotonin₁A receptor is impaired in cellular model of Smith–Lemli–Opitz Syndrome

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1. Introduction

The Smith–Lemli–Opitz Syndrome (SLOS) [1] is an autosomal recessive disorder characterized clinically by mental retardation, physical deformities, failure to thrive and multiple congenital anomalies [2,3]. SLOS is caused by mutations in the gene encoding Δ7-reductase (Δ7-DHCR), an enzyme required in the final step of cholesterol biosynthesis [4,5]. To date, close to 100 different mutations in the DHCR7 gene have been identified which lead to the disease [6]. SLOS is ranked as one of the most serious recessive genetic conditions [3,7]. Reduced levels of plasma cholesterol along with elevated levels of 7-dehydrocholesterol (7-DHC) (and its positional isomer 8-dehydrocholesterol, 8-DHC) and the ratio of their concentrations to that of cholesterol are representative parameters for diagnosis of SLOS [8]. Although SLOS has devastating effects on the nervous system, the relationship of SLOS with neuronal receptors and their membrane lipid interactions, which play a crucial role in the function of the nervous system, remains an unexplored area. Cholesterol is an important lipid in this context since it is known to regulate the function of membrane receptors [9], especially neuronal receptors [10], thereby affecting neurotransmission. Importantly, a possible role of cholesterol in a variety of neurological disorders is well documented [11].

Previous work from our laboratory has shown the requirement of membrane cholesterol in the function of an important neurotransmitter G-protein coupled receptor, the serotonin₁A receptor (recently reviewed in [10]). Serotonin receptors represent one of the largest, evolutionarily ancient, and highly conserved families of seven transmembrane G-protein coupled receptors (GPCRs) [12,13]. Among the 14 subtypes of serotonin receptors, the G-protein coupled serotonin₁A (5-HT₁A) receptor is the best characterized for a variety of reasons [14]. Serotonin₁A receptors appear to play a key role in the generation and modulation of various cognitive, behavioral and developmental functions such as sleep, mood, addiction, depression, anxiety, alcohol abuse, aggression and learning [15]. This is
supported by the fact that the agonists and antagonists of the serotonin1A receptor represent major classes of molecules with potential therapeutic effects in anxiety- or stress-related disorders. Interestingly, mutant (knockout) mice lacking the serotonin1A receptor exhibit enhanced anxiety-related behavior, and represent an important animal model for the analysis of complex traits such as anxiety disorders and aggression in higher animals [16,17]. Keeping in mind the pharmacological relevance of the serotonin1A receptor, which is a transmembrane protein, its interaction with the surrounding lipid environment assumes greater significance in modulating its function in healthy and diseased states.

In this work, we have generated a cellular model of SLOS using CHO cells stably expressing the human serotonin1A receptor (CHO-5-HT1AR) by metabolically inhibiting the biosynthesis of cholesterol. To achieve this, we utilized AY 9944, which is a specific metabolic inhibitor of 7-DHCR [18]. AY 9944 treatment results in a reduction of cholesterol with a concomitant accumulation of 7- and 8-DHC, thereby mimicking SLOS (see Fig. 1). We explored the function of the human serotonin1A receptor under these conditions by monitoring ligand binding, G-protein coupling and downstream signaling of the receptor. Our results show that the function of the serotonin1A receptor is impaired under SLOS-like condition. Importantly, metabolic replenishment of cholesterol partially restored the ligand binding activity of the serotonin1A Receptor. These results are significant since intake of dietary cholesterol is the recommended treatment for SLOS patients.

2. Materials and Methods

2.1. Materials

Cholesterol, DMPC, AY 9944, EDTA, 7-DHC, coprostanol, MgCl₂, MnCl₂, 8-OH-DPAT, penicillin, streptomycin, gentamycin sulfate, polyethylenimine, PMSF, serotonin, sodium bicarbonate, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). DMEM/F-12 (1:1) [Dulbecco’s modified Eagle medium:nutrient mixture F-12 (Ham) (1:1)], lipofectamine, fetal calf serum, and geneticin (G-418) were from Invitrogen Life Technologies (Carlsbad, CA). N,N-di(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Supelco (Belleville, PA). Trypt-P-G-S was from Roche Applied Science (Mannheim, Germany). BCA reagent for protein estimation was from Pierce (Rockford, IL). Forskolin and IBMX were obtained from Calbiochem (San Diego, CA). [3H]-OH-DPAT (sp. activity = 1350 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). The cyclic [3H]AMP assay kit was purchased from Amersham Biosciences (Buckinghamshire, U.K.). GF/B glass microfiber filters were from Whatman International (Kent, U.K.). All other chemicals and solvents used were of the highest available purity. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

2.2. Methods

2.2.1. Cell culture and treatment with AY 9944

CHO cells stably expressing the human serotonin1AR receptor (termed as CHO-5-HT1AR) and CHO cells stably expressing the human serotonin1AR receptor tagged to enhanced yellow fluorescent protein (termed as CHO-5-HT1AR-EYFP) were maintained in DMEM/F-12 (1:1) supplemented with 2.4 g/l of sodium bicarbonate, 10% fetal calf serum, 60 µg/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml gentamycin sulfate, and 200 µg/ml geneticin in a humidified atmosphere with 5% CO₂ at 37 °C. Stock solution of AY 9944 was prepared in water and added to cells grown for 24 h (final concentration of AY 9944 was 1–10 µM) and incubated in 5% serum for 63-66 h. Control cells were grown under similar conditions without AY 9944 treatment.

2.2.2. Cell membrane preparation

Cell membranes were prepared as described earlier [19]. Total protein concentration in the isolated membranes was determined using the BCA assay [20].

2.2.3. Estimation of inorganic phosphate

The concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid as described earlier [21] using Na₂HPO₄ as standard.

2.2.4. Gas chromatography and mass spectrometry

GC-MS analysis was carried out on an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a model 5973i mass selective detector and 7683 series injector. For membrane sterol measurements, cellular membranes were prepared and total lipids were extracted in chloroform–methanol [22], after the addition of coprostanol as an internal recovery standard. The chloroform phase obtained was dried.

Fig. 1. Generation of a cellular model of Smith–Lemli–Opitz syndrome. The principal route of cholesterol synthesis in humans is the Kandutsch–Russell pathway [29]. In this pathway, the immediate precursor of cholesterol is 7-DHC. The reduction of C7(8) double bond of 7-DHC to yield cholesterol is catalyzed by 3β-hydroxy-steroid-Δ₇-reductase (7-DHCR) in an NADPH-dependent manner. Mutations in this enzyme cause SLOS, a severe developmental disorder associated with multiple congenital and morphogenetic anomalies. Reduced levels of cholesterol, along with elevated levels of dehydrocholesterol (7-DHC+8-DHC), have been characterized as a diagnostic parameter of the SLOS. 8-DHC, a positional isomer of 7-DHC, is formed due to an enzyme-catalyzed isomerization of 7-DHC. 7-DHC (and 8-DHC) differs with cholesterol only in a double bond at the 7th (or 8th) position in the sterol ring (highlighted in their chemical structures). We have generated a cellular model for SLOS by treating CHO cells stably expressing the human serotonin1AR receptor (CHO-5-HT1AR) with AY 9944, a specific metabolic inhibitor of 7-DHCR, by altering the cholesterol and dehydrocholesterol levels, thereby mimicking the condition of mutated 7-DHCR (see text for more details).
under nitrogen. Sterols were identified and quantitated using GC-MS as described previously [23] with some modifications. Briefly, an aliquot of the extract was hydrolyzed in 1 M NaOH in ethanol for 1 h at 70 °C, extracted with n-hexane, and converted into trimethylsilyl ether derivatives followed by injection into a capillary column. This was a chemically bonded, fused silica, nonpolar CP-Sil BC8 (Varian, Middleburg, The Netherlands) capillary column (30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness). To achieve optimal separation of sterols, the column oven temperature was maintained at 250°C with a solvent delay of 5 min from the time of injection. Helium at the rate of 1.2 ml/min was used as the carrier gas under constant flow mode. The inlet and interface temperatures were kept at 250 and 280 °C, respectively. The ion source and quadrupole temperatures were kept at 230 and 150 °C, respectively. Mass spectra were scanned from 30 to 600 Da under scan mode. The ions at m/z 329, 351 and 370 were used under Selected Ion Monitoring (SIM) mode for the estimation of cholesterol, 7-DHC, and 8-DHC, respectively. Peak areas were obtained individually for each compound under SIM mode in the concentration range of 100–1000 ppm for cholesterol, 10–100 ppm for both 7-DHC and coprostanol, and the calibration curves were constructed. The absolute concentrations of various sterols in the samples were calculated from the calibration curves. The concentration of coprostanol is used to calculate the recovery of the steroids. Membrane sterol contents are expressed as cholesterol to total sterol ratio and cholesterol to dehydrocholesterol (7-DHC+8-DHC) ratio.

2.2.5. Radioligand binding assays

Receptor binding assays were carried out as described earlier [19] with ~50 μg total protein. The concentration of [3H]8-OH-DPAT in each assay tube was 0.29 nM.

2.2.6. Saturation binding assays

Saturation binding assays were carried out with increasing concentrations (0.1–7.5 nM) of the radiolabeled agonist [3H]8-OH-DPAT as described previously [19,24]. The protein content for the saturation binding assays was in the range of 75–95 μg. The dissociation constant (KD) and maximum binding sites (Bmax) were calculated by non-linear regression analysis of binding data using GraphPad Prism software version 4.00 (San Diego, CA). Data obtained after regression analysis were used to plot graphs with the GRAFIT program version 3.07b (Erithacus Software, Surrey, U.K.).

2.2.7. GTP–γ-S sensitivity assay

In order to estimate the efficiency of G-protein coupling, GTP–γ-S sensitivity assays were carried out as described earlier [24]. The concentrations of GTP–γ-S leading to 50% inhibition of specific agonist binding (IC50) were calculated by non-linear regression fitting of the data to a four parameter logistic function [25]:

\[ B = \frac{a}{1 + \left(\frac{x}{b}\right)^c} + d \]  

where B is the specific binding of the agonist normalized to agonist binding at the lowest concentration of GTP–γ-S, x denotes the concentration of GTP–γ-S, a is the range [ymin–y0%] of the fitted curve on the ordinate (y-axis), I is the IC50 concentration, b is the background of the fitted curve [y0%], and c is the slope factor.

2.2.8. Estimation of cyclic AMP content in cells

The ability of agonists such as 8-OH-DPAT to affect the forskolin-stimulated increase in cAMP levels in CHO-5-HT3R cells was assessed as described earlier [24]. The amount of cAMP in an aliquot of the supernatant was estimated using the cyclic [3H] AMP assay system which is based on the protein binding method described previously [26]. Agonist dependent dose–response curves were analyzed according to the four parameter logistic function using Eq. (1).

2.2.9. Fluorescence anisotropy measurements

Fluorescence anisotropy experiments were carried out using the fluorescent probe DPH with membranes prepared from cells that were treated with varying concentrations of AY 9944, containing 50 nmol of total phospholipids suspended in 1.5 ml of 50 mM Tris, pH 7.4 buffer, as described earlier [21]. 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 nm and 20 nm were used. The optical density of the samples measured at 358 nm was 0.15±0.01. The anisotropy values remained identical even after dilution of membrane samples, indicating the absence of any scattering artifact [27]. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy (r) values were calculated from the equation [28]:

\[ r = \frac{l_{xx} - G_{hi}}{l_{xx} + 2G_{hi}} \]

where lxx and lyy 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

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**Fig. 2. Identification of sterols.** Panels A and C show representative selected ion chromatograms of sterols obtained from total lipid extracts from membranes of CHO-5-HT3R cells treated with 5 and 10 μM AY 9944, respectively. An additional peak (besides 7-DHC) was observed for concentrations beyond 2.5 μM of AY 9944. This was identified as 8-DHC, a positional isomer of 7-DHC. Peaks 1, 2 and 3 correspond to cholesterol, 7-DHC and 8-DHC, respectively. Panels B and D show the mass spectra of 7-DHC and 8-DHC from lipid extracts. 8-DHC has a shorter retention time compared to 7-DHC. The distinctive patterns of m/z ratios of 7-DHC and 8-DHC are shown in panels B and D.
the ratio of the efficiencies of the detection system for vertically and horizontally polarized light and is equal to \( \frac{I_{HV}}{I_{HH}} \). All experiments were done with multiple sets of samples and average values of fluorescence anisotropy are shown in Fig. 6.

2.2.10. Metabolic replenishment of cholesterol with serum

After treatment with 5 µM AY 9944, CHO-5-HT\(_{1A}\)-R cells were grown for 3 days in D-MEM/F-12 (1:1) supplemented with 2.4 g/l of sodium bicarbonate, 10% fetal calf serum, 60 µg/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml gentamycin sulfate, and 200 µg/ml geneticin in a humidified atmosphere with 5% CO\(_2\) at 37 °C to achieve metabolic replenishment of cholesterol.

Cell membranes were prepared from CHO-5-HT\(_{1A}\)-R-EYFP (control), AY 9944 treated and cholesterol-replenished cells as previously described[19] with an addition of 1:20 dilution of freshly added protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). 60 µg of total protein from each sample was mixed with 1:4 v/v of 4× electrophoresis sample buffer and boiled for 30 min at 37 °C. Sample mixtures were loaded and separated on 10% SDS-PAGE.

After electrophoresis, gel proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech, Little Chalfont, U.K.) using semi-dry transfer apparatus (Amersham Pharmacia Biotech, Little Chalfont, U.K.). The non specific binding sites were blocked with 10% fat-free dry milk in PBS/Tween 20, pH 7.4 for 1 h at room temperature. To monitor the expression of 5-HT\(_{1A}\)-R-EYFP, blots were probed with antibodies raised against GFP (BD Biosciences, San Jose, CA). 1:10 000 dilution in PBS/Tween 20, pH 7.4 for 1 h at room temperature. To monitor the levels of β-actin, which acts as a loading control, membranes were probed with antibodies raised against β-actin (Chemicon International, Temecula, CA; diluted 1:800 in PBS/Tween 20), incubated overnight at 4 °C. Membranes were washed with PBS/Tween 20 (washing buffer) for 30 min and the wash buffer was changed every 10 min. Membranes were then incubated with 1:4000 dilution of a secondary antibody (horseradish peroxidase conjugated-anti-mouse antibody) in PBS/Tween 20, pH 7.4 for 1 h at room temperature. Membranes were then washed and developed using the enhanced chemiluminescence detection reagents (Amersham Biosciences, Buckinghamshire, U.K.). 5-HT\(_{1A}\)-R-EYFP and β-actin were detected using the chemiluminescence detection system (Chemi-Smart 5000, Vilber Lourmat). 5-HT\(_{1A}\)-R-EYFP and β-actin levels were quantitated using Bio-Profile (Bio-1D+, version 11.9).

2.2.12. Statistical analysis

Significance levels were estimated using student’s two-tailed paired t-test using Microcal Origin software version 5.0 (OriginLab Corp., Northampton, MA).

3. Results

3.1. Quantification of sterols using gas chromatography–mass spectrometry (GC–MS)

With an overall goal of addressing lipid–protein interactions in healthy and diseased states, we developed a cellular model of SLOS using AY 9944, a specific metabolic inhibitor of 7-DHCR, on CHO-5-HT\(_{1A}\)-R cells (see Fig. 1 for more details). Sterols (cholesterol, 7-DHC and 8-DHC) were separated, identified and quantitated using GC–MS (Figs. 2 and 3). 7-DHC content in control cells was below the detection level. We observed an increase in 7-DHC content up to 2.5 µM of AY 9944 (see Fig. 3A), beyond which an additional peak appeared (Fig. 2C). This peak was identified as 8-DHC (Fig. 2C and D), a positional isomer of 7-DHC (see Fig. 1). The identities of the trimethylsilyl ethers of the compounds were established by comparison with authentic standards for cholesterol and 7-DHC. The peak for 8-DHC was identified using its mass spectra reported earlier [30,31]. Importantly, the ratios of sterols generated using AY 9944 in these cases (see Fig. 3) were comparable to the ratios reported in SLOS patients [32–34]. It should be mentioned here that we estimated phospholipid contents under identical conditions by performing lipid

Fig. 3. Quantitation of sterols in membranes from CHO-5-HT\(_{1A}\)-R cells treated with AY 9944. Effect of increasing concentrations of AY 9944 on sterol content of membranes from CHO-5-HT\(_{1A}\)-R cells. Cholesterol and 7-DHC were identified with authentic standards. 8-DHC was identified using its mass spectra reported earlier [31,32]. Sterols were separated and quantitated using GC–MS analysis. Panel A shows the relative contents of membrane cholesterol (gray bars), 7-DHC (open bars) and 8-DHC (hatched bars) from CHO-5-HT\(_{1A}\)-R cells treated with increasing concentrations of AY 9944 used, and normalized to total sterol (cholesterol + 7-DHC + 8-DHC) content. The cell membrane cholesterol to total sterol ratio is shown in (B). The ratio of cholesterol to total sterol was normalized to control cells. The ratio between cholesterol and total dehydrocholesterol (7-DHC + 8-DHC) is shown in (C). Data represent means ±S.E. of at least three independent experiments. See Materials and methods for other details.
phosphate assays. The change in phospholipid content was found to be negligible, even when the highest concentration of AY 9944 was used.

3.2. Ligand binding activity of the human serotonin$_{1A}$ receptor is reduced in SLOS-like condition

Since SLOS is associated with neurological deformities and malfunction, exploring the function of neuronal receptors and their membrane lipid interactions under these conditions assumes significance. We explored the ligand binding function of the human serotonin$_{1A}$ receptor under SLOS-like condition. Fig. 4A shows the effect of AY 9944 treatment on specific $[^3H]$8-OH-DPAT binding to serotonin$_{1A}$ receptors in CHO-5-HT$_{1A}$R cells. As shown in the figure, there is a progressive and drastic reduction in the specific $[^3H]$8-OH-DPAT binding with increasing concentrations of AY 9944 used. The reduction in agonist binding correlates well with the reduction in ratio between cholesterol to total sterol as well as the cholesterol to dehydrocholesterol ratio (shown in Fig. 3B and C). The agonist binding of the human serotonin$_{1A}$ receptor is reduced to ~8% of the original value upon treatment with 10 µM AY 9944. Importantly, the reduction in agonist binding is not due to a decrease in the expression level of the human serotonin$_{1A}$ receptor (see later, Fig. 8). Although the requirement of membrane cholesterol in maintaining the ligand binding activity of the serotonin$_{1A}$ receptor has previously been reported by us [35], this result represents the first observation that defective cholesterol biosynthesis could result in loss of specific agonist binding to the serotonin$_{1A}$ receptor.

The saturation binding analysis of the specific agonist $[^3H]$8-OH-DPAT binding to serotonin$_{1A}$ Receptors is shown in Fig. 4B and Table 1. The results of saturation binding analysis with the agonist $[^3H]$8-OH-DPAT, carried out with membranes prepared from AY 9944 treated and control cells, revealed that the reduction in ligand binding can

![Image](https://example.com/image.png)
the presence of GTP-γ-S, caused by receptor activation. We have previously shown that in the normal cycle of guanine nucleotide exchange at the G-o class of G-proteins in CHO cells, perturbation of receptor-G-protein interaction in SLOS-like condition. The serotonin1A receptor agonists such as GTP-γ-S with an estimated half maximal inhibition concentration (IC50) of 3.75 nM for control cells. Data represent means±S.E. of duplicate points from at least three independent experiments. See Materials and methods for other details.

3.3. Ligand-dependent downstream signaling efficiency of the human serotonin1A receptor is reduced in SLOS-like condition

Most of the seven transmembrane domain receptors are coupled to G-proteins, and therefore guanine nucleotides are known to modulate ligand binding. The serotonin1A receptor agonists such as 8-OH-DPAT are known to specifically activate the G-o class of G-proteins in CHO cells [36]. Agonist binding to such receptors therefore displays sensitivity to agents such as GTP-γ-S, a non-hydrolyzable analogue of GTP, that uncouple the normal cycle of guanine nucleotide exchange at the Go subunit caused by receptor activation. We have previously shown that in the presence of GTP-γ-S, serotonin1A receptors undergo an affinity transition, from a high affinity G-protein coupled to a low-affinity G-protein uncoupled state [37]. In agreement with these results, Fig. 5A shows a characteristic reduction in binding of the agonist [3H]8-OH-DPAT in the presence of a range of concentrations of GTP-γ-S with an estimated half maximal inhibition concentration (IC50) of 3.75 nM for control cells. The inhibition curve in case of cells treated with AY 9944 displays a significant (~2.5-fold, p<0.03) shift toward higher concentrations of GTP-γ-S with an increased IC50 value of 9.92 nM. This implies that the agonist binding to the serotonin1A receptor in SLOS-like condition is less sensitive to GTP-γ-S indicating that the G-protein coupling efficiency is reduced under these conditions. This suggests a possible perturbation of receptor-G-protein interaction in SLOS-like condition.

In addition to ligand binding properties, we monitored the function of serotonin1A receptors in CHO-5-HT1A-R cells by measuring its ability to catalyze downstream signal transduction processes upon stimulation with the specific agonist, 8-OH-DPAT. Serotonin1A receptor agonists such as 8-OH-DPAT are known to specifically activate the G-o class of G-proteins in CHO cells, which subsequently reduce cAMP levels in cells [36]. As shown in Fig. 5B, the forskolin-stimulated increase in cAMP levels is inhibited by 8-OH-DPAT with a half maximal inhibition concentration (IC50) of 11.97 nM in control cells. In cells treated with AY 9944, the IC50 value is increased to a considerable extent (~4-fold, p<0.03) to 50.68 nM. This points out that the downstream signaling efficiency of the human serotonin1A receptor is considerably reduced under this condition.

3.4. Reduction in the function of the human serotonin1A receptor in SLOS-like condition is independent of the overall membrane order

In order to explore any possible change in overall membrane order upon AY 9944 treatment, we measured the fluorescence anisotropy of the fluorescent probe DPH. Fluorescence anisotropy measured using probes such as DPH is correlated to the rotational diffusion of membrane embedded probes [28], which is sensitive to the packing of lipid fatty 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 reorientation is a function of local lipid packing. DPH, which is a rod-like hydrophobic molecule, partitions into the interior (fatty acyl chain region) of the bilayer. Fig. 6 shows the effect of increasing concentrations of AY 9944 on the fluorescence anisotropy of the membrane probe DPH incorporated into CHO-5-HT1A-R cell membranes. The fluorescence anisotropy of DPH appears to be more or less invariant over the concentration range of AY 9944 used. These results therefore suggest that the overall (global) membrane order is not significantly altered in SLOS-like condition and changes in ligand binding activity and downstream signaling could be due to specific (local) effects (see later).
3.5. Metabolic cholesterol replenishment restores the ligand binding activity of the human serotonin<sub>1A</sub> receptor

Treatment of SLOS consists of reduction of 7-DHC levels on one hand, and to supplement deficient cholesterol, on the other hand. This is generally achieved by providing supplemental cholesterol in the diet [7,38,39]. This is due to the fact that the supplemented dietary cholesterol would reduce cholesterol precursors by feedback inhibition of HMG-CoA reductase. In order to check the reversibility of the changes induced by AY 9944 (i.e., reduction of cholesterol and accumulation of dehydrocholesterol) in SLOS-like condition in CHO-5-HT<sub>1A</sub>R cells, we performed metabolic replenishment with serum cholesterol. This was carried out by incubating cells treated with AY 9944 for 3 days in serum (see Materials and methods). Under these conditions, a significant fraction of cholesterol could be restored, along with a concomitant reduction in 8-DHC levels, and an overall decrease in combined dehydrocholesterol levels (Fig. 7A), resulting in significant recovery of the ligand binding activity (see Fig. 7B).

3.6. The expression level of the serotonin<sub>1A</sub> receptor is not reduced in SLOS-like condition

The impaired ligand binding activity and signaling of the human serotonin<sub>1A</sub> receptor observed in SLOS-like condition could be due to reduced expression levels of serotonin<sub>1A</sub> receptors. In order to explore this possibility, we performed Western blot analysis of 5-HT<sub>1A</sub>R-EYFP in cell membranes prepared from control, AY 9944 treated and cholesterol-replenished CHO-5-HT<sub>1A</sub>R-EYFP cells (see Fig. 8). For these experiments, we chose to use the receptor tagged to EYFP (5-HT<sub>1A</sub>R-EYFP) since no monoclonal antibodies for the serotonin<sub>1A</sub> receptor are available yet, and the polyclonal antibodies have been reported to give variable results on Western blots [40]. We have earlier shown that EYFP fusion to the serotonin<sub>1A</sub> receptor does not affect the ligand binding properties, G-protein coupling and signaling functions of the receptor [41]. Fig. 8B shows that the levels of the serotonin<sub>1A</sub> receptor in membranes are not reduced in SLOS-like condition. The receptor level is slightly increased (~1.4-fold compared to control) following AY 9944 treatment. This increase, however, was found to be not significant (p > 0.05). After metabolic replenishment with serum cholesterol, the receptor level increased to ~1.6-fold. It has been previously reported that serotonin<sub>1A</sub> receptor levels in CHO cells could increase upon induction of stress [42].

4. Discussion

SLOS is a congenital and developmental malformation syndrome associated with defective cholesterol biosynthesis. Patients diagnosed with SLOS typically show growth and mental retardation, microcephaly, cleft palate, syndactyly, urogenital abnormalities, and a variety of other anatomical defects. SLOS is clinically characterized by reduced levels of plasma cholesterol coupled with accumulation of 7- and 8-DHC. Learning disabilities and mental retardation are present in at least 95% of SLOS patients [38] which could be attributed to impaired neurotransmission. The relationship between clinical severity and biochemical parameters in SLOS is an ongoing area of research. It has been reported previously that reduced cholesterol levels are associated with increased clinical severity, while the correlation between 7-DHC and clinical severity is weak [43]. These results are supported using SLOS animal model, which shows that cholesterol deficit but not accumulation of precursor sterols, is the major cause of abnormal embryogenesis in SLOS [44]. Importantly, cholesterol is an essential lipid in this context since a number of neurological diseases share a common etiology of defective cholesterol metabolism in the brain [45]. As mentioned earlier, we have previously shown the requirement of membrane cholesterol in the function of the serotonin<sub>1A</sub> receptor [10,35]. Based on this evidence, it appears that reduction in cholesterol levels could contribute to the impairment of function of the serotonin<sub>1A</sub> receptor under SLOS-like condition, although effects of accumulation of dehydrocholesterol may not be ruled out.

Serotonin<sub>1A</sub> receptors are members of a superfamily of seven transmembrane domain receptors [46] that couple to and transduce signals via G-proteins [14]. In this work, we have monitored the effect of metabolic inhibition of cholesterol biosynthesis on the function of the human serotonin<sub>1A</sub> receptor in CHO-5-HT<sub>1A</sub>R cells. These results show that ligand binding activity, G-protein coupling and downstream signaling of serotonin<sub>1A</sub> receptors are impaired in SLOS-like condition. The potential clinical relevance of this observation stems from the fact that defective cholesterol biosynthesis constitutes a common theme for a number of genetically inherited disorders [47]. These results assume greater significance in the light of recent findings that symptoms of anxiety and major depression are apparent in humans upon long-term administration of statins which are inhibitors of the first enzyme (HMG-CoA reductase) in the cholesterol biosynthesis pathway [48]. Interestingly, SLOS carriers have been reported to be at greater risk of suicidal behavior [49] and a recent study has shown that serotonin<sub>1A</sub> receptors play a key role in the biology of suicidal behavior [50].

Importantly, our results show that the receptor level is not reduced under this condition. This implies that the fraction of functional receptors is reduced upon AY 9944 treatment resulting in a higher fraction of non-functional receptors. Interestingly, these results could be related to an earlier report describing abnormal development of serotonergic neurons in SLOS mouse model [51]. In addition, modulation of serotonergic transmission has been implicated in cholesterol-lowering therapy [52]. Our results assume significance in the light of the fact that SLOS patients suffer from impaired neurotransmission. It should be mentioned here that although we have monitored the levels of membrane sterols and clinical reports from patient samples rely on plasma...
sterols, it has been earlier reported that modulation of membrane and plasma sterols are positively correlated [53]. Based on our results, it could be possible that the function of serotonin\textsubscript{1A} receptors is reduced in SLOS patients, since the membrane sterol levels we obtained match well with reported serum sterol levels in SLOS patients [32–34]. Clearly, further work is needed in this area to support this hypothesis.

The effect of cholesterol on the structure and function of integral membrane proteins has been a subject of intense investigation [9,10]. It has been proposed that such effects could occur either due to a specific molecular interaction with membrane proteins [54], or due to alterations in the membrane physical properties induced by the presence of cholesterol [55,56], or due to a combination of both factors. We have earlier shown that oxidation of membrane cholesterol with cholesterol oxidase leads to inhibition of the ligand binding activity of the serotonin\textsubscript{1A} receptor [57]. In the present work, we have utilized a cellular model of SLOS in order to delineate the specific and global effects of cholesterol in the function of the serotonin\textsubscript{1A} receptor. SLOS serves as an appropriate condition to test this since the two aberrant sterols that get accumulated in SLOS, i.e., 7- and 8-DHC, differ with cholesterol only in a double bond. Our results show that the overall membrane order, as monitored with the fluorescent probe DPH, does not exhibit significant change in SLOS-like condition (Fig. 6). Interestingly, we have recently shown that 7-DHC does not support the function of the serotonin\textsubscript{1A} Receptor without any change in overall membrane order [58]. We therefore conclude that the requirement for maintaining ligand binding activity is more stringent than the requirement for maintaining membrane order. Taken together, these results indicate that the molecular basis for the requirement of membrane cholesterol in maintaining the ligand binding activity of serotonin\textsubscript{1A} receptors could be specific interaction, although global bilayer effects may not be ruled out.

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function and sorting. It is often found distributed non-randomly in domains or pools in biological membranes [59,60]. Many of these domains (sometimes termed as ‘lipid rafts’) are believed to be important for the maintenance of membrane structure and function, although characterizing the spatiotemporal resolution of these domains is proving to be challenging [60,61]. The idea of such membrane domains gains significance since physiologically important functions such as cellular membrane sorting and trafficking [62], and signal transduction [63] have been implicated to these putative domains. In addition, membrane cholesterol has been shown to modulate the entry of pathogens to host cells [64]. It would be interesting to compare to what extent 7- and 8-DHC could mimic cholesterol in this regard. It has been previously reported using a number of approaches that membrane domains formed by 7-DHC differ with those formed by cholesterol in protein composition [65], packing [66] and stability [67–69]. In addition, model membranes mimicking SLOS membranes have been reported to exhibit atypical membrane organization [23] and curvature [70]. It is therefore possible that mere replacement of cholesterol with 7-DHC may significantly affect aspects of membrane organization such as near neighbor interactions (7-DHC is more polar than cholesterol) and accessible surface area. Interestingly, it has been reported that the activity of inward-rectifier K\textsuperscript{+} channels is modulated even by optical isomers of cholesterol thereby exemplifying the specificity of interaction [71].

Dietary cholesterol has been proposed as a potential therapy in the treatment of SLOS and currently a lot of research is focused on developing a formula as food supplement for SLOS patients [33]. We have metabolically replenished cholesterol in cells pre-treated with AY 9944 with serum and found that the ligand binding activity of the human serotonin\textsubscript{1A} receptor could be recovered to a significant extent (Fig. 7B). Metabolic replenishment of cholesterol is relevant since the only feasible treatment for SLOS patients is regular intake of dietary cholesterol. It has been reported earlier that administration of dietary cholesterol can partially reverse some of the behavioral abnormalities [39]. It is possible that the impaired function of receptors such as the serotonin\textsubscript{1A} receptor in SLOS patients could be regained to some extent after cholesterol administration. Taken together, our results could be potentially useful in understanding the molecular basis that underlie the pathophysiology of this disorder and could provide novel insight in formulating future treatment for the disease.

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