

RESEARCH ARTICLE

Late endosomal/lysosomal accumulation of a neurotransmitter receptor in a cellular model of Smith-Lemli-Opitz syndrome

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Abstract

Smith-Lemli-Opitz syndrome (SLOS) is a congenital and developmental malformation syndrome associated with defective cholesterol biosynthesis. It is characterized by accumulation of 7-dehydrocholesterol (the immediate biosynthetic precursor of cholesterol in the Kandutsch-Russell pathway) and an altered cholesterol to total sterol ratio. Because SLOS is associated with neurological malfunction, exploring the function and trafficking of neuronal receptors and their interaction with membrane lipids under these conditions assume significance. In this work, we generated a cellular model of SLOS in HEK-293 cells stably expressing the human serotonin_{1A} receptor (an important neurotransmitter G-protein coupled receptor) using AY 9944, an inhibitor for the enzyme β -hydroxy-steroid- Δ^7 -reductase (7-DHCR). Using a quantitative flow cytometry based assay, we show that the plasma membrane population of serotonin_{1A} receptors was considerably reduced under these conditions without any change in total cellular expression of the receptor. Interestingly, the receptors were trafficked to sterol-enriched LysoTracker positive compartments, which accumulated under these conditions. To the best of our knowledge, our results constitute one of the first reports demonstrating intracellular accumulation and misregulated traffic of a neurotransmitter GPCR in SLOS-like conditions. We believe these results assume relevance in our overall understanding of the molecular basis underlying the functional relevance of neurotransmitter receptors in SLOS.

KEYWORDS

7-DHC, altered trafficking, AY 9944, cholesterol, serotonin_{1A} receptor, SLOS

1 | INTRODUCTION

The Smith-Lemli-Opitz syndrome (SLOS)¹ is a congenital error of cholesterol biosynthesis which results in developmental, behavioral and cognitive abnormalities.²⁻⁵ SLOS is caused due to mutation(s) in the gene encoding β -hydroxy-steroid- Δ^7 -reductase (7-DHCR), the enzyme which catalyzes the conversion of 7-dehydrocholesterol (7-DHC) to cholesterol in the last step of the Kandutsch-Russell pathway of cholesterol biosynthesis (Figure 1A).^{6,7}

SLOS is relatively common in the Caucasian population with an estimated incidence between 1:10,000 and 1:70,000 and a carrier frequency of 1:30,^{8,9} and more than 150 mutations in the gene (*DHCR7*)

encoding 7-DHCR have been reported that lead to the disorder.¹⁰ The affected patients exhibit anatomical defects such as syndactyly, polydactyly, cleft palate, microcephaly and abnormal gums. These developmental abnormalities are believed to be due to defective signaling of Sonic Hedgehog, the protein implicated in development and pattern formation.¹¹⁻¹³ SLOS is characterized by reduced levels of plasma cholesterol along with accumulation of 7-DHC and its positional isomer 8-dehydrocholesterol (8-DHC).⁴ Interestingly, the ratio of these sterols to cholesterol has been reported to be linearly related to impairment in cognitive and adaptive function, with the amount of 7-DHC accumulation being the most important determinant.⁵ Although learning and cognitive disabilities are well established for

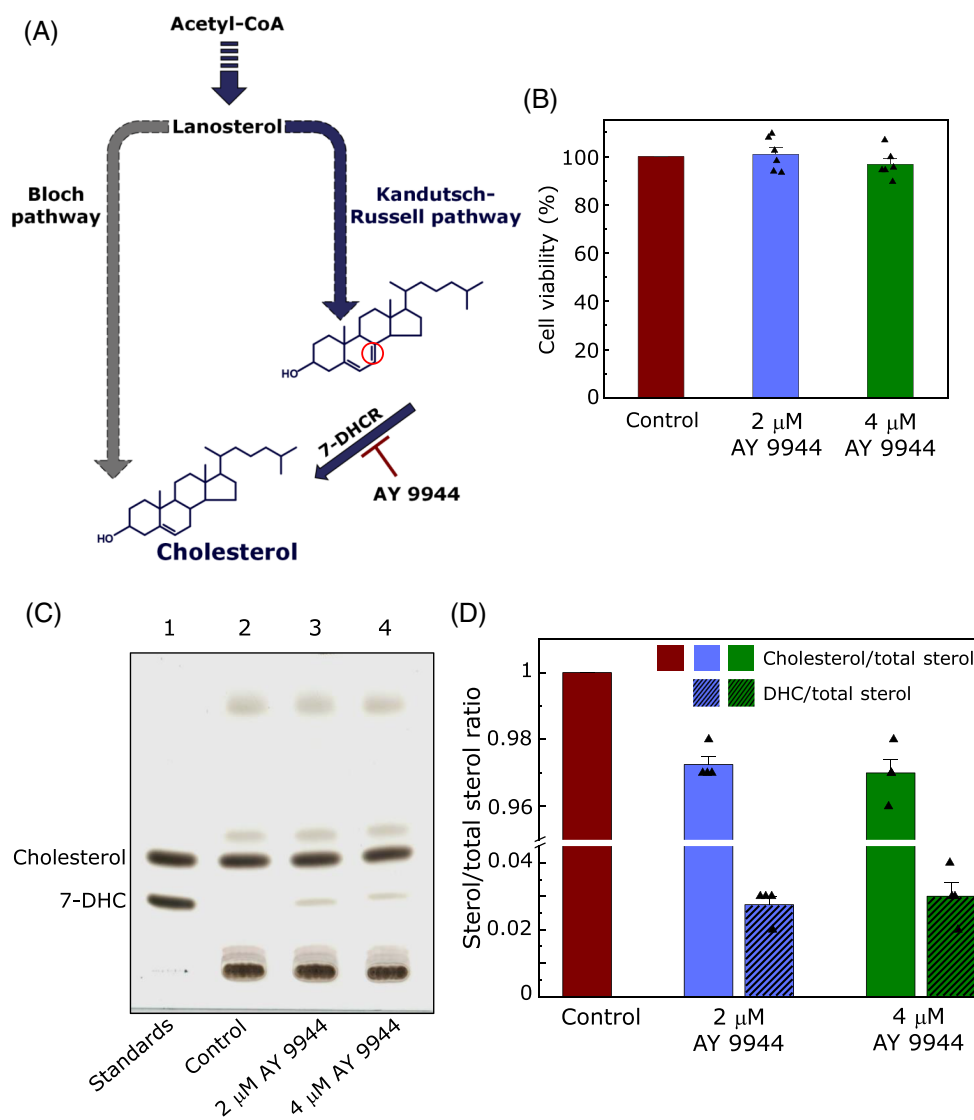


FIGURE 1 Generating a cellular model of Smith-Lemli-Opitz syndrome. A, Two known pathways of cholesterol biosynthesis are the Kandutsch-Russell and the Bloch pathway. 7-DHC, the immediate biosynthetic precursor of cholesterol in the Kandutsch-Russell pathway, differs from cholesterol *merely* by the presence of an additional double bond (highlighted with a red circle). Mutation(s) in the enzyme (7-DHCR) catalyzing the conversion of 7-DHC to cholesterol leads to Smith-Lemli-Opitz syndrome, a metabolic disorder characterized by accumulation of 7-DHC and an altered cholesterol to total sterol ratio. We generated a cellular model of SLOS in HEK-5-HT_{1A}R cells (HEK-293 cells stably expressing N-terminal myc-tagged human serotonin_{1A} receptors) using AY 9944, a metabolic inhibitor of 7-DHCR. B, Viability of HEK-5-HT_{1A}R cells is not affected upon AY 9944 treatment. HEK-5-HT_{1A}R cells were treated with 2 and 4 μ M AY 9944 for 66 hours and cellular viability was measured using MTT assay. Values are expressed as percentage viability of cells treated with AY 9944 normalized to control (in the absence of AY 9944) cells. C, Representative thin layer chromatogram showing lipids extracted from cell lysates prepared from HEK-5-HT_{1A}R control cells and treated with AY 9944. The chromatogram shows cholesterol and 7-DHC standards (lane 1), lipids extracted from cellular lysates under control (untreated, lane 2), and treated with 2 μ M (lane 3) and 4 μ M (lane 4) AY 9944. D, Quantitation of cholesterol/total sterol ratio (maroon, solid blue and solid green bars) and DHC/total sterol ratio (hatched blue and hatched green bars) was performed using densitometric analysis. Values are expressed as relative sterol content normalized to total sterol from corresponding samples in each case. Data represent means \pm SEM from four independent experiments. See “Materials and Methods” for more details

patients suffering from SLOS, the role of neuronal receptors and their interaction with membrane lipids in this context are relatively less explored.

Serotonin receptors are neurotransmitter receptors that belong to the superfamily of G protein-coupled receptors (GPCRs) and are implicated in the modulation of cognitive and behavioral

functions.¹⁴⁻¹⁶ The serotonin_{1A} receptor is an important neurotransmitter receptor in the family of serotonin receptors and is implicated in a variety of cognitive, behavioral and developmental functions such as sleep, mood, aggression, anxiety and depression.¹⁷⁻²⁴ With an overall goal of understanding the mechanistic basis underlying the impaired neuronal function observed in SLOS, we have previously

shown that functional aspects of the serotonin_{1A} receptor, such as ligand binding, G-protein coupling and cellular signaling, are affected in SLOS-like conditions.²⁵⁻²⁸

Extensive work over the past decade has highlighted the importance of spatiotemporal organization of GPCRs in their cellular function.²⁹⁻³¹ Although GPCRs have been classically considered to be plasma membrane resident signaling hubs, their subcellular localization has emerged as an important feature associated with regulation of their function.³²⁻³⁴ This raises an interesting possibility of changes in cellular localization of GPCRs such as the serotonin_{1A} receptor in disorders such as SLOS. In this work, we have monitored the subcellular localization and trafficking of the serotonin_{1A} receptor under SLOS-mimicking conditions. To achieve this, we generated a cellular model of SLOS in HEK-293 cells stably expressing N-terminal myc-tagged human serotonin_{1A} receptors (HEK-5-HT_{1A}R cells) by metabolically inhibiting the last step in the Kandutsch-Russell pathway of cholesterol biosynthesis³⁵⁻³⁷ using AY 9944, which inhibits the enzyme 7-DHCR.^{26,38} Chronic treatment of cells with AY 9944 resulted in accumulation of 7-DHC and an altered cholesterol to total sterol ratio. We monitored plasma membrane population of the serotonin_{1A} receptor under these conditions using a quantitative flow cytometry based assay recently developed by us.³⁹ Our results show a considerable reduction in the plasma membrane population of serotonin_{1A} receptors in cells treated with AY 9944 without any change in the total cellular expression of the receptor. Interestingly, we observed a considerable increase in sterol-enriched LysoTracker positive compartments inside cells, with accumulation of serotonin_{1A} receptors in these compartments in SLOS-mimicking conditions. These results point toward an altered trafficking regime of the serotonin_{1A} receptor in these conditions. To the best of our knowledge, our results constitute one of the first reports demonstrating intracellular accumulation and misregulated traffic of a GPCR in SLOS-like conditions. From a broader perspective, these results contribute to our overall understanding of the molecular basis underlying receptor trafficking under conditions of defective sterol biosynthesis.

2 | RESULTS

2.1 | Generating a cellular model of Smith-Lemli-Opitz syndrome

We previously showed that serotonin_{1A} receptors stably expressed in HEK-5-HT_{1A}R cells exhibit characteristic ligand binding and G-protein coupling, and undergo clathrin-mediated endocytosis upon agonist stimulation.³⁹ In this work, with an overall goal of understanding the role of membrane lipids in trafficking of the serotonin_{1A} receptor and its implications in health and disease, we developed a cellular model of SLOS in HEK-5-HT_{1A}R cells. For this, we used AY 9944,³⁸ a specific metabolic inhibitor which inhibits the enzyme (7-DHCR) that catalyzes the conversion of 7-DHC to cholesterol in the last step of the Kandutsch-Russell pathway of cholesterol biosynthesis (Figure 1A).³⁵⁻³⁷ Notably, AY 9944 has been extensively utilized for mimicking SLOS in cellular and animal

models.⁴⁰⁻⁴² We chose AY 9944 concentrations carefully to ensure that cellular viability was not affected under our experimental conditions, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Figure 1B). SLOS is characterized by accumulation of 7-DHC accompanied by an altered cholesterol to total sterol ratio. In order to validate the cellular model, we quantified cholesterol to total sterol ratio using thin layer chromatography (TLC) upon AY 9944 treatment (Figure 1C). Because 7-DHC could be converted to 8-DHC (the positional isomer of 7-DHC), the observed TLC band corresponding to the 7-DHC standard could be a combination of 7-DHC and 8-DHC and is often termed as DHC to indicate a combination of both dehydrocholesterol species. As shown in Figure 1C, we did not observe any band corresponding to 7-DHC standard under control condition. However, bands corresponding to 7-DHC standard could be visualized upon chronic treatment (66 hours) with 2 and 4 μ M AY 9944. Densitometric analysis of the TLC bands corresponding to 2 and 4 μ M AY 9944 treatment showed cholesterol/total sterol ratio of 0.97 (Figure 1D). Notably, the ratio of sterols obtained upon AY 9944 treatment was comparable to cholesterol/total sterol ratios reported in SLOS patients.⁴³

2.2 | Reduction in plasma membrane serotonin_{1A} receptor population under SLOS-like conditions without any change in its total cellular expression

We have previously shown that in normal condition, the serotonin_{1A} receptor predominantly localizes on the plasma membrane in HEK-5-HT_{1A}R cells.³⁹ Since GPCRs are considered to be canonical plasma membrane resident receptors involved in signal transduction, we monitored the effect of SLOS-like conditions on the plasma membrane population of serotonin_{1A} receptors using a quantitative flow cytometric assay, previously developed by us.³⁹ In this assay, we exclusively label the plasma membrane associated serotonin_{1A} receptor population with anti-myc antibody Alexa Fluor 488 conjugate. Relative shifts in the flow cytometric histogram (quantified as mode count, which represents counts in the modal channel of the histogram) are indicative of changes in the plasma membrane receptor population (Figure 2A). We observed that upon treatment with AY 9944, there was considerable reduction in the plasma membrane population of the serotonin_{1A} receptor. For example, upon treatment with 2 μ M AY 9944 for 66 hours, we observed \sim 42% reduction in the plasma membrane receptor population relative to control. Upon treatment with 4 μ M AY 9944, the reduction in plasma membrane receptor population was \sim 64% (Figure 2B). These results show that the plasma membrane population of the serotonin_{1A} receptor exhibits considerable reduction under SLOS-like conditions.

In order to explore whether the decrease in plasma membrane population of the serotonin_{1A} receptor upon AY 9944 treatment was due to reduction in the overall expression of the receptor, we performed Western blot analysis from whole cell protein extract under control and AY 9944 treated conditions (Figure 2C). Upon quantitation using densitometric analysis of bands corresponding to the

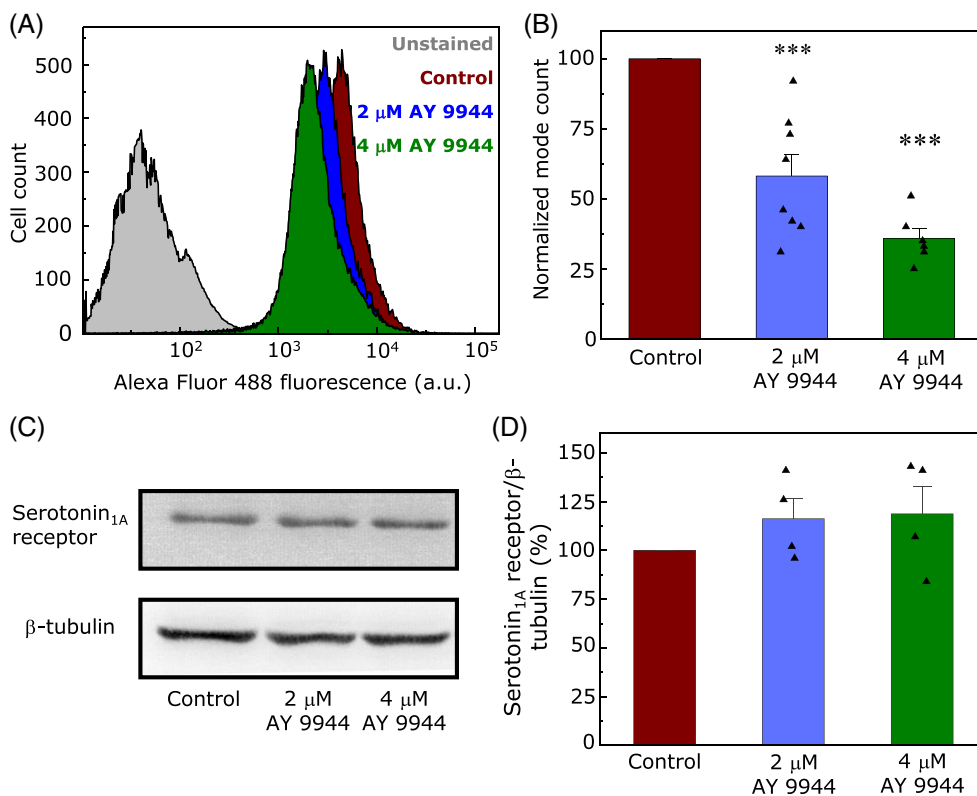


FIGURE 2 Plasma membrane population of the serotonin_{1A} receptor is reduced upon AY 9944 treatment without any change in its total cellular expression. A, HEK-5-HT_{1A}R cells were treated with 2 and 4 μM AY 9944 for 66 hours. Cells were subsequently fixed and labeled with anti-myc antibody Alexa Fluor 488 conjugate. Overlays of representative flow cytometric histograms showing plasma membrane population of the serotonin_{1A} receptor under control condition (maroon), and treated with 2 (blue) and 4 (green) μM AY 9944. The histogram corresponding to unstained cells (gray) is shown as reference. B, Quantitative flow cytometric estimates of plasma membrane receptor population under these conditions. Values are normalized to mode count in control cells. Data represent means ± SEM from at least four independent experiments (***) represents a significant [$P < .001$] difference in mode count associated with AY 9944 treated cells relative to control cells. C, Western blot showing total cellular expression of the serotonin_{1A} receptor upon 2 and 4 μM AY 9944 treatment for 66 hours. Corresponding β-tubulin expression levels are shown below as loading controls. D, Total cellular expression of the serotonin_{1A} receptor was analyzed using densitometric analysis and normalized to β-tubulin. Values are represented as percentage expression of the serotonin_{1A} receptor normalized to control cells in each case. Data represent means ± SEM from four independent experiments. See “Materials and Methods” for more details

serotonin_{1A} receptor, we did not observe any significant change in the total cellular expression of the receptor upon chronic treatment (66 hours) with 2 and 4 μM AY 9944 (Figure 2D). Taken together with our observations on the reduction of the plasma membrane receptor population (Figure 2A,B), these results suggest a possible effect of SLOS-like conditions on trafficking of the serotonin_{1A} receptor to the plasma membrane.

2.3 | Accumulation of LysoTracker positive compartments upon AY 9944 treatment

Since SLOS is associated with cholesterol metabolism and lysosomes play a key role in regulating cellular distribution of cholesterol,^{44,45} we measured cellular distribution of lysosomes using LysoTracker red which stains acidic compartments inside cells (*i.e.*, late endosomes/lysosomes). Notably, we found a considerable increase in LysoTracker red staining in cells treated with AY 9944 relative to control cells

(Figure 3A). We further quantified LysoTracker red staining under these conditions using flow cytometry. We observed a significant increase in mean fluorescence intensity of LysoTracker red in AY 9944 treated cells relative to control condition (Figure 3B,C).

2.4 | Serotonin_{1A} receptor accumulates in LysoTracker positive compartments under SLOS-like conditions

To further explore the effect of increased LysoTracker positive compartments in SLOS-mimicking conditions, we tested whether serotonin_{1A} receptors were trafficked to these compartments upon AY 9944 treatment. As shown in Figure 4A, we observed a significant increase in colocalization of serotonin_{1A} receptors with LysoTracker in these conditions. We further measured the extent of colocalization using Manders' colocalization coefficient⁴⁶ upon chronic treatment (66 hours) with 2 and 4 μM AY 9944, and observed a

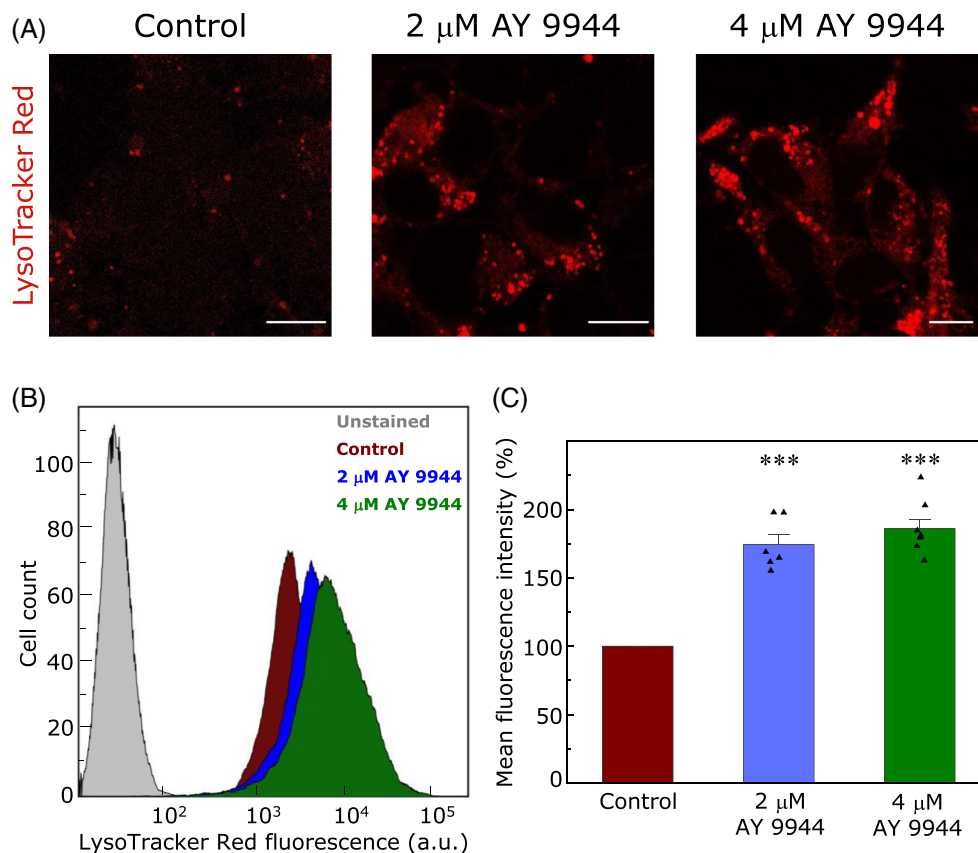


FIGURE 3 Increase in cellular LysoTracker positive compartments upon AY 9944 treatment. HEK-5-HT_{1A}R cells were treated with AY 9944 for 66 hours and subsequently stained with 0.3 μM LysoTracker red at 37°C for 30 minutes. A, Representative confocal microscopic images of the mid-plane section showing LysoTracker red staining under control and 2 and 4 μM AY 9944 treatment conditions. B, Overlays of representative flow cytometric histograms showing LysoTracker red stained population under control condition (maroon), and treated with 2 (blue) and 4 (green) μM AY 9944. The histogram corresponding to unstained cells (gray) is shown as a reference. C, Total LysoTracker red positive population quantified using flow cytometry. Data represent mean fluorescence intensity in control, and 2 and 4 μM AY 9944 treatment conditions. Values are represented as percentages of mean fluorescence intensity normalized to control condition. Data represent means ± SEM from three independent experiments (***) represents a significant [$P < .001$] difference in percentage mean fluorescence intensity associated with AY 9944 treated cells relative to control cells). The scale bars represent 10 μm. See “Materials and Methods” for more details

concentration-dependent increase in colocalization of the serotonin_{1A} receptor with LysoTracker positive compartments (Figure 4B). These results point toward directed trafficking of serotonin_{1A} receptors to late endosomal/lysosomal pools in SLOS-like conditions.

2.5 | LysoTracker positive compartments are sterol enriched

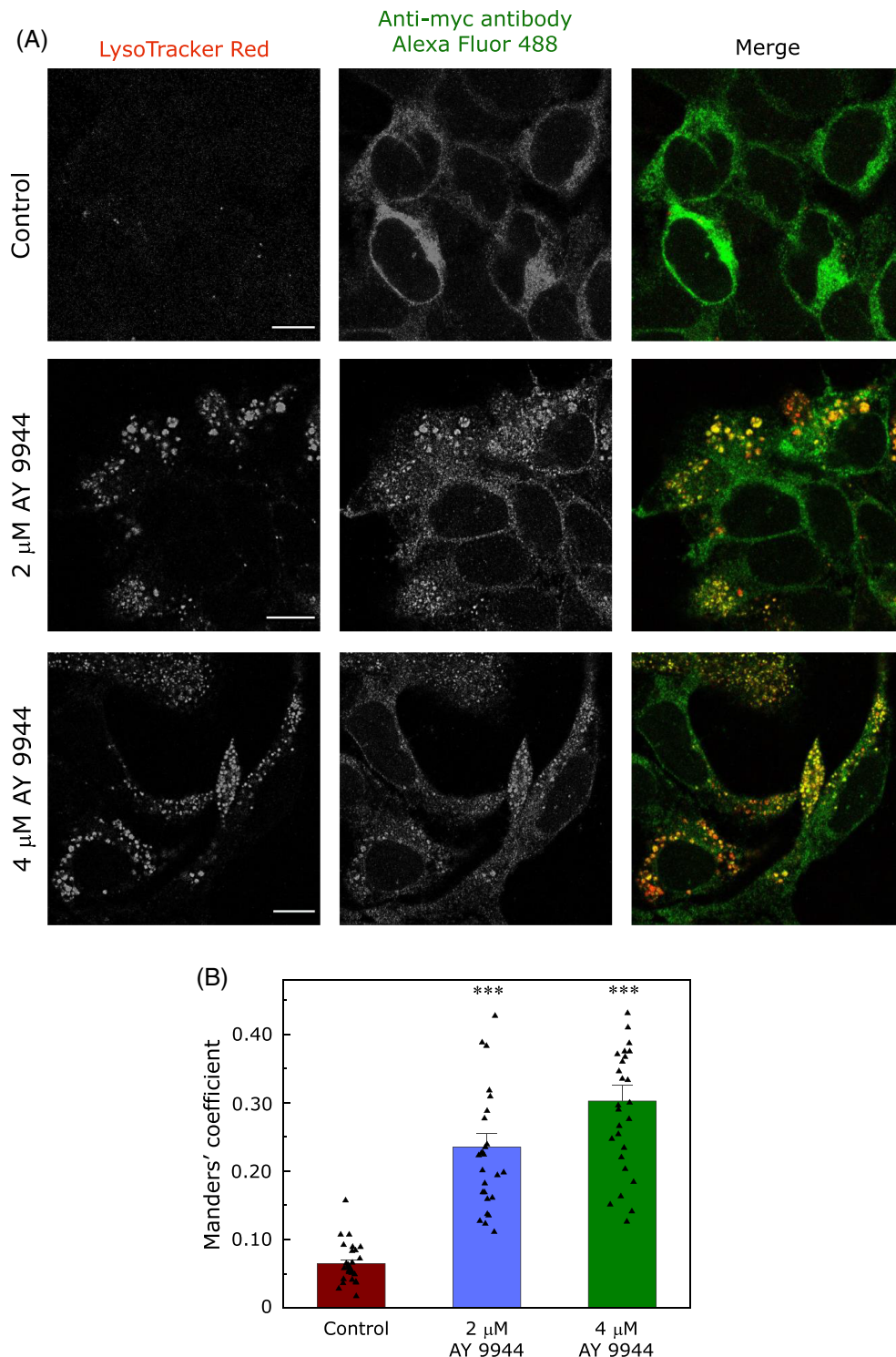
Since SLOS is associated with defects in cellular cholesterol metabolism, we monitored the distribution of free sterol in AY 9944 treated conditions using filipin. Filipin is an intrinsically fluorescent polyene antibiotic which stains unesterified sterols and allows the study of cellular distribution of sterols.^{47,48} We observed a significant increase in cellular filipin staining upon chronic treatment with AY 9944 (see Figure 5A). These results, combined with our observation of increased LysoTracker positive compartments, prompted us to monitor the colocalization of filipin with LysoTracker red. As shown in Figure 5A,

there was a visibly high overlap of filipin with LysoTracker red. Quantification of colocalization using Manders' colocalization coefficient exhibited significant increase in colocalization of filipin with LysoTracker red upon AY 9944 treatment (shown in Figure 5B). Taken together with our results shown in Figures 3 and 4, these observations show that under SLOS-like conditions, the serotonin_{1A} receptor exhibits a novel trafficking pattern directed toward sterol-enriched late endosomal/lysosomal pools.

3 | DISCUSSION

SLOS is an autosomal recessive disorder which is caused due to defective cholesterol biosynthesis.^{1–5} SLOS is characterized by the accumulation of 7-DHC (the immediate biosynthetic precursor of cholesterol in the Kandutsch-Russell pathway) and an altered cholesterol to total sterol ratio.^{6,7} Till date, there is no specific drug available to treat SLOS. Although some therapies are recommended by clinicians,

FIGURE 4 The serotonin_{1A} receptor is trafficked to LysoTracker positive compartments upon AY 9944 treatment. HEK-5-HT_{1A}R cells were treated with AY 9944 for 66 hours and subsequently stained with 0.3 μ M LysoTracker red at 37°C for 30 minutes. Cells were fixed, permeabilized and stained with anti-myc antibody Alexa Fluor 488 conjugate. A, Representative confocal microscopic images of the mid-plane section showing colocalization between LysoTracker positive compartments (red) and serotonin_{1A} receptors (green) under control, and 2 and 4 μ M AY 9944 treatment conditions. The scale bars represent 10 μ m. B, Quantitative estimates of colocalization of the serotonin_{1A} receptor with LysoTracker red, analyzed using Manders' colocalization coefficient (M). Data represent means \pm SEM from 27 measurements from three independent experiments (***) represents a significant [$P < .001$] difference in Manders' colocalization coefficient associated with colocalization of the serotonin_{1A} receptor with LysoTracker red upon AY 9944 treatment relative to control cells. See "Materials and Methods" for more details



none of them have gone through the rigor of a comprehensive clinical trial.⁴⁹ Owing to the developmental, behavioral and cognitive abnormalities associated with SLOS, the role of neuronal receptors in the etiology of the disorder assumes relevance. The serotonin_{1A} receptor represents an important member of the neurotransmitter GPCR family involved in cognitive, behavioral and developmental functions, and therefore serves as an important target for the development of novel therapeutics in neuropathological conditions such as SLOS.^{15,16,20-23}

However, the mechanistic basis underlying the role of the serotonin_{1A} receptor and its interaction with membrane lipids in SLOS is relatively less explored. In this context, previous work from our laboratory has demonstrated impaired ligand binding, G-protein coupling and cellular signaling by the serotonin_{1A} receptor under SLOS-like conditions.²⁵⁻²⁸ In this work, we show that the plasma membrane population of serotonin_{1A} receptors exhibits a considerable reduction in a cellular model of SLOS generated by treating cells with AY 9944. Our results

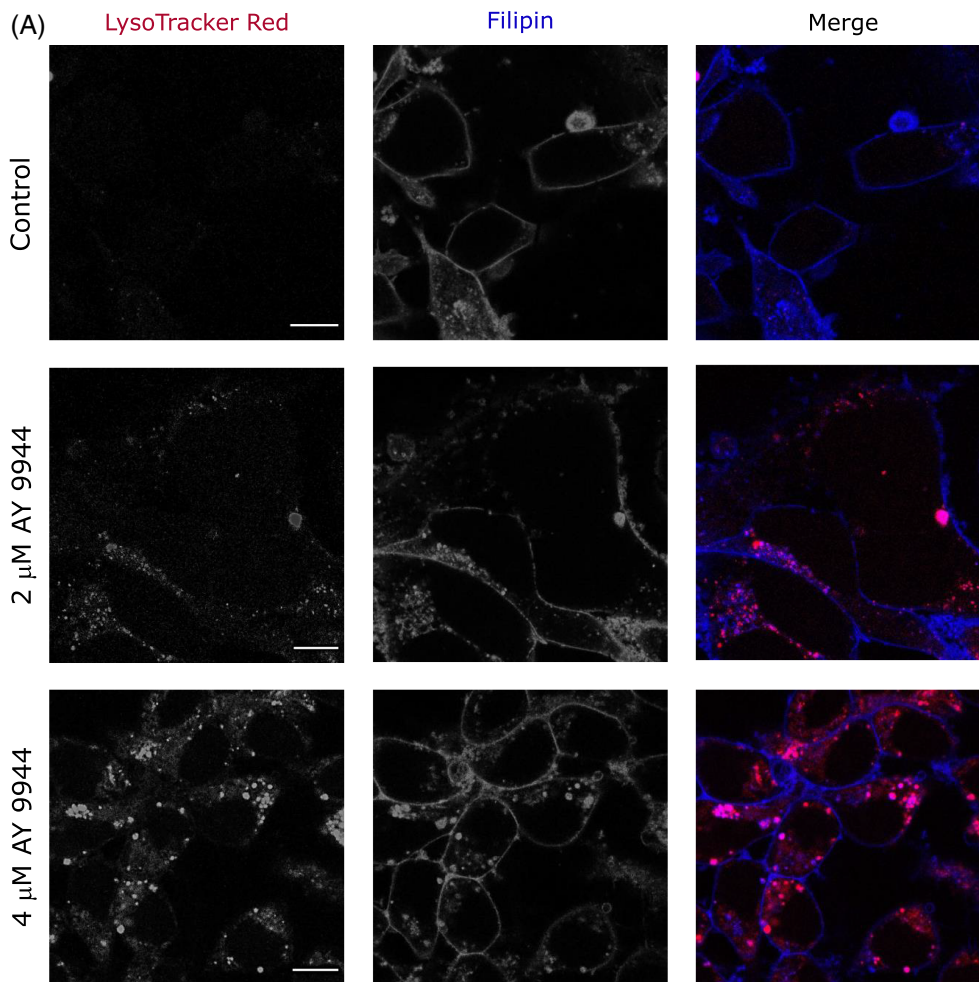
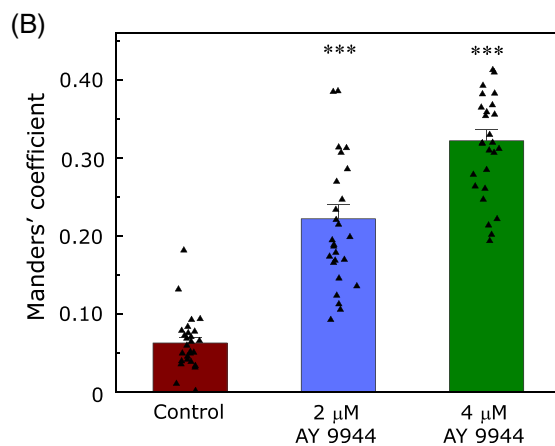


FIGURE 5 LysoTracker positive compartments accumulated upon AY 9944 treatment are sterol-enriched. HEK-5-HT_{1A}R cells were treated with AY 9944 for 66 hours and stained with 0.3 μM LysoTracker red at 37°C for 30 minutes. Cells were subsequently fixed and stained with filipin. A, Representative confocal microscopic images of the mid-plane section showing colocalization between LysoTracker positive compartments (red) and filipin (blue) under control, 2 and 4 μM AY 9944 treatment conditions. The scale bars represent 10 μm. B, Quantitative estimates of colocalization of filipin with LysoTracker red, analyzed using Manders' colocalization coefficient. Data represent means ± SEM from 27 measurements from three independent experiments (***) represents a significant [$P < .001$] difference in Manders' colocalization coefficient associated with colocalization of filipin with LysoTracker red upon AY 9944 treatment relative to control cells). See "Materials and Methods" for more details



demonstrate a considerable increase in sterol-enriched LysoTracker positive compartments in this condition, a hallmark of lysosomal storage disorders such as Niemann-Pick type C (NPC) disease.^{45,50,51} In addition, our results show that serotonin_{1A} receptors are trafficked to these compartments upon AY 9944 treatment, indicating an altered trafficking pathway associated with SLOS-mimicking conditions. Notably, accumulation of lysosomal inclusion bodies has been reported in fibroblasts obtained from SLOS patients.⁵² At this point, we cannot rule out any possible contribution of lysosomal

accumulation of AY9944 in the observed increase in sterol-enriched LysoTracker positive compartments, since AY 9944 belongs to the family of cationic amphiphilic drugs (CAD).⁵³

The accumulation of 7-DHC is an important clinical manifestation of SLOS⁵ and most of the effects are believed to arise either from altered sterol ratios or due to derivatives of 7-DHC formed under these conditions. Although 7-DHC differs from cholesterol *merely* by the presence of an additional double bond at the 7th position in the sterol ring (marked by a red circle in Figure 1A), it exhibits differential

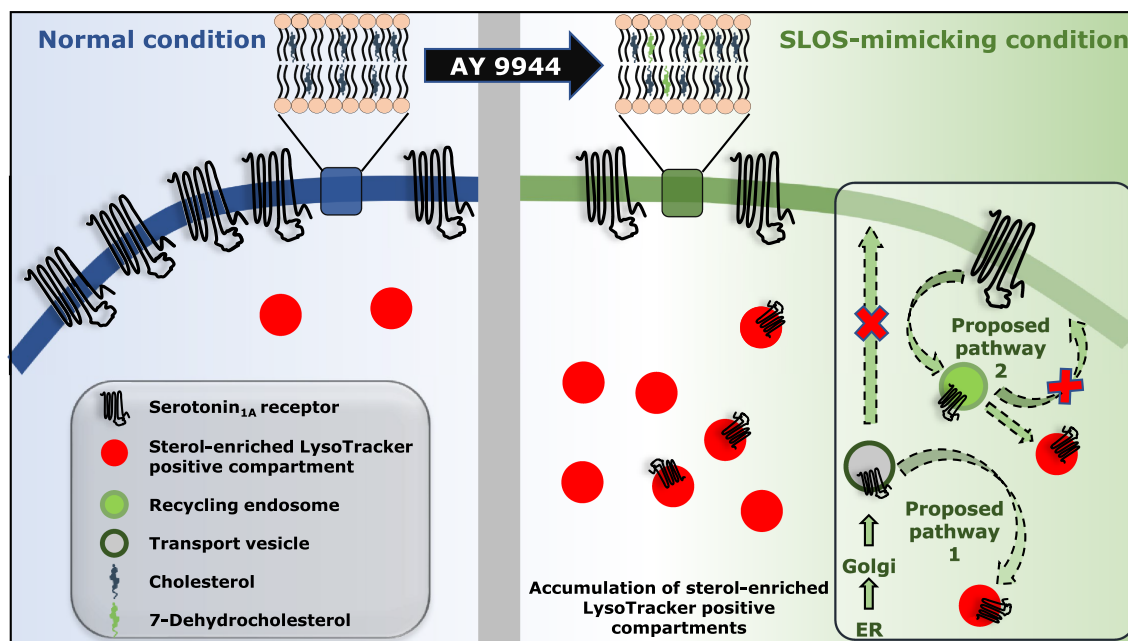


FIGURE 6 SLOS-like conditions alter subcellular localization and trafficking of the serotonin_{1A} receptor. A schematic representation showing localization of serotonin_{1A} receptors under SLOS-mimicking conditions upon chronic treatment of cells stably expressing the receptors with AY 9944, a specific inhibitor of the enzyme 7-DHCR. A significant reduction in plasma membrane population of the serotonin_{1A} receptors associated with enhanced colocalization with sterol-enriched LysoTracker positive compartments was observed under SLOS-mimicking conditions. Two putative mechanisms underlying the altered subcellular localization of the serotonin_{1A} receptor in SLOS-like conditions are shown in a box on the far right: (i) receptors are targeted to these compartments during biosynthetic traffic (proposed pathway 1) instead of being sorted along the ER-Golgi-plasma membrane pathway, or (ii) serotonin_{1A} receptors are routed toward LysoTracker positive compartments in the course of constitutive internalization and recycling to the plasma membrane (proposed pathway 2) (see text for other details)

effects on membrane physical properties. Cholesterol is known to fine-tune lipid-protein interactions by increasing the thickness of the membrane with increasing cholesterol content.⁵⁴ Such increase in membrane thickness along the endoplasmic reticulum-Golgi-plasma membrane pathway has been implicated in efficient sorting of membrane proteins and receptors.⁵⁵ What would be the effect on this process if we replace a fraction of membrane cholesterol with 7-DHC? Is 7-DHC as efficient in this process of organelle-specific lipid-protein interaction as cholesterol? It appears that 7-DHC is not as effective as cholesterol in increasing membrane thickness.^{56,57} There are additional differences in physical properties between membranes containing 7-DHC and cholesterol. For example, it has been reported that membranes prepared from skin fibroblasts of SLOS patients display increased 7-DHC content and abnormal membrane fluidity.⁵⁸ In addition, membranes containing 7-DHC exhibit greater sterol tilt angle,⁵⁶ membrane packing^{59,60} and phase behavior^{61,62} relative to membranes containing cholesterol. In this context, we have previously shown that 7-DHC differs from cholesterol in its ability to increase membrane dipole potential.^{63,64} In a recent work, using fluorescence lifetime distribution analysis of a membrane interfacial probe, we demonstrated that membranes containing 7-DHC exhibit reduced interfacial temporal heterogeneity relative to membranes containing cholesterol.⁶⁵ Such differential effects on membrane physical properties could affect function of membrane proteins.^{25-27,55,66,67} Altered functioning of membrane proteins such as NPC1 owing to such

changes in membrane properties could be a cause for secondary NPC-like storage defects observed in SLOS.⁴⁴

Importantly, accumulation of exogenously derived (from LDL) free cholesterol has been previously reported in cultured fibroblasts from SLOS patients.⁵² Such accumulation of free cholesterol could drive partitioning of serotonin_{1A} receptors to these compartments. As discussed above, this is supported by the cholesterol-induced increase in membrane thickness observed along the membrane protein biosynthetic trafficking and sorting pathway.^{55,68} Accumulation of additional compartments enriched in cholesterol could perturb the intracellular biosynthetic itinerary of membrane proteins such as serotonin_{1A} receptors (Figure 6, proposed pathway 1). Another possibility is that the receptors could be re-routed during the course of constitutive internalization and recycling to the plasma membrane (Figure 6, proposed pathway 2). This is supported by reports in which cholesterol accumulation has been shown to cause a defect in late endosome motility and intra-endosomal trafficking.^{69,70} Interestingly, slow recycling of transferrin receptors has been reported in NPC cells⁷¹ and accumulation of endosomal cholesterol has been shown to affect recycling of transferrin in Niemann-Pick type A and C lipid storage disease fibroblasts.⁷²

Defective trafficking of GPCRs has been implicated in diseases such as nephrogenetic diabetes insipidus, retinitis pigmentosa and cancer.⁷³⁻⁷⁵ Recent work from our group has shown that statin-induced chronic cholesterol depletion affects the mechanism of endocytosis and the intracellular trafficking of the serotonin_{1A} receptor.⁷⁶

In this overall context, our present results showing reduced plasma membrane localization of the serotonin_{1A} receptor and its accumulation in sterol-enriched LysoTracker positive compartments highlight a novel trafficking defect associated with SLOS-like conditions. Looking ahead, the use of cellular systems expressing *DHCR7* harboring naturally occurring mutations observed in SLOS could serve as a genetic model to monitor the effects of the SLOS phenotype and the associated functional aspects of neurotransmitter receptors. Although HEK-293 cells offer a convenient and extensively studied system to explore receptor trafficking, these cells may not represent a native environment for the serotonin_{1A} receptor. It would be interesting to explore the effect of the SLOS phenotype on the trafficking of neurotransmitter receptors in a more native system. We envision that a detailed mechanistic exploration of such altered trafficking and localization of the serotonin_{1A} receptor in SLOS-like conditions could be critical in the development of novel therapeutic interventions against SLOS.

4 | MATERIALS AND METHODS

4.1 | Materials

AY 9944 (Cat #: C5364), cholesterol, 7-DHC, Tris, EDTA, poly-L-lysine, glycine, penicillin, streptomycin, gentamycin sulfate, MTT, filipin and doxycycline were obtained from Sigma Chemical Co. (St. Louis, Missouri). DMEM/F-12 [Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Ham) (1:1)], fetal bovine serum (FBS), LysoTracker red, β -tubulin loading control monoclonal antibody (BT7R) (Cat #: MA5-16308, RRID: AB_2537819), cholesterol esterase (obtained from Amplex red cholesterol assay kit) and hygromycin B were from Invitrogen/Life technologies (Grand Island, New York). Bicinchoninic acid (BCA) reagent for protein estimation was from Pierce (Rockford, Illinois). Anti-myc tag antibody Alexa Fluor 488 conjugate (9E10) (Cat #: 16-308, RRID: AB_568801) and myc-tag (9B11) mouse mAb (Cat #: 2276S, RRID: AB_331783) were obtained from Millipore (Bedford, Massachusetts) and Cell Signaling Technology, Inc. (Danvers, Massachusetts), respectively. Methanol and pre-coated silica gel 60 thin layer chromatography plates were from Merck (Darmstadt, Germany). HRP goat anti-mouse IgG antibody (Cat #: 405306, RRID: AB_315009) was from BioLegend (San Diego, California) and bovine serum albumin (BSA) was from Himedia Laboratories (Mumbai, India). Acetone and chloroform were from Spectrochem (Mumbai, India). *n*-Heptane and ethyl acetate were obtained from Sisco Research Laboratories (Mumbai, India). All other chemicals used were of the highest purity available. Water was purified through a Millipore (Bedford, Massachusetts) Milli-Q system and used throughout.

4.2 | Methods

4.2.1 | Cell culture

HEK-293 cells stably expressing N-terminal myc-tagged serotonin_{1A} receptors (HEK-5-HT_{1A}R cells) were grown as described previously.³⁹

Briefly, cells were grown in DMEM/F-12 medium supplemented with 10% FBS, 60 μ g/mL penicillin, 50 μ g/mL streptomycin, 50 μ g/mL gentamycin sulfate in a humidified atmosphere with 5% CO₂ at 37°C. Hygromycin B (250 μ g/mL) was used to select cells expressing the serotonin_{1A} receptor. Receptor expression was induced with 1 μ g/mL doxycycline 24 hours prior to experiment.

4.2.2 | Treatment with AY 9944

HEK-5-HT_{1A}R cells plated on poly-L-lysine coated dishes were treated with AY 9944 as described previously.²⁶ Stock solution of AY 9944 was prepared in water. Cells were incubated for 24 hours after seeding followed by treatment with 2 and 4 μ M AY 9944 for 66 hours in DMEM/F-12 medium supplemented with 5% FBS, 60 μ g/mL penicillin, 50 μ g/mL streptomycin and 50 μ g/mL gentamycin sulfate. Control cells were grown under identical conditions except treatment with AY 9944.

4.2.3 | MTT viability assay

Cellular viability upon AY 9944 treatment was determined using MTT assay as described previously.⁷⁷ Cells were plated in a 96-well plate and treated with AY 9944. Subsequently, cells were incubated for 1 hour at 37°C with MTT prepared in serum-free DMEM/F-12 media at a final concentration of 0.5 mg/mL. The media was washed off and DMSO was added and kept for 10 minutes at room temperature (~23°C) to dissolve the formazan crystals. Absorbance was measured at 560 nm using an EnSpire 2300 Multimode Plate Reader (Perkin Elmer, Waltham, Massachusetts).

4.2.4 | Thin layer chromatography for estimation of sterols

Lipids were isolated from cellular lysates obtained from control and AY 9944 treated samples. Briefly, cells were scraped, collected in ice-cold PBS and sonicated (5 minutes with 15/30 seconds on/off cycles) using a Sonics Vibra-Cell VCX 500 sonifier (Sonics & Materials Inc, Newton, Connecticut) fitted with a titanium microtip. Cellular lysates were treated with 0.2 U/mL cholesterol esterase at 37°C for 1 hour to hydrolyze esterified cholesterol, if any. Total protein concentration in cell lysates was measured using BCA assay.⁷⁸ Lipids from cellular lysates were extracted according to the Bligh and Dyer method.⁷⁹ Lipids were dried under a stream of nitrogen at ~40°C and kept under a high vacuum till further use. Sterol content in the lipid mixture under various treatment conditions was estimated by thin layer chromatography (TLC). Precoated silica gel TLC plates were impregnated with 3% (w/v) silver nitrate in methanol and activated at ~100°C for 15 minutes. Samples for TLC were prepared by dissolving the dried lipids in chloroform/methanol (1:1, v/v) just before use. Sterols were separated using *n*-heptane/ethyl acetate (2:1, v/v) as a solvent

system.^{25,80} The TLC plate was sprayed with 10% (w/v) cupric sulfate solution containing 8% (v/v) orthophosphoric acid followed by charring at $\sim 150^{\circ}\text{C}$ to visualize separated lipids. Cholesterol and 7-DHC bands were identified with the help of standards. TLC plates were scanned and sterol band intensities were estimated using densitometric analysis of the chromatogram using Adobe Photoshop CS3 (Adobe Systems, San Jose, California) software. To quantify sterol ratios, a calibration curve was plotted from TLC developed using a range of known cholesterol/7-DHC (mol/mol) standards (see Figure S1). Cholesterol/total sterol ratios in AY 9944 treated samples were evaluated using this calibration curve.

4.2.5 | Flow cytometric analysis

Plasma membrane receptor population of serotonin_{1A} receptors was monitored using a quantitative flow cytometric assay.³⁹ Briefly, cells were scraped subsequent to treatment and collected in ice-cold PBS. Cells were fixed using 4% (w/v) formaldehyde solution in PBS for 30 minutes and the plasma membrane serotonin_{1A} receptor population was labeled using anti-myc antibody Alexa Fluor 488 conjugate (1:100 dilution) in PBS containing 2% serum for 1 hour, followed by washing and resuspending in PBS. Data were acquired using a Gallios flow cytometer (Beckman Coulter Inc., Brea, California) and analyzed in Kaluza analysis software version 2.1. Data were collected for 10 000 cells by exciting Alexa Fluor 488 at 488 nm and emission was collected using a 525/40 nm bandpass filter. Change in mode count values upon AY 9944 treatment was analyzed with respect to control condition.

To quantify LysoTracker positive compartments, cells were incubated with 0.3 μM LysoTracker red in serum-free DMEM/F-12 for 30 minutes at 37°C . Media was washed off and cells were scraped in ice-cold PBS, washed and resuspended in ice-cold PBS. Data were acquired using a BD LSRFortessa flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey). Data were collected by exciting LysoTracker red at 561 nm and emission was collected using 585/15 nm bandpass filter. Change in mean fluorescence intensity was analyzed with respect to control condition.

4.2.6 | Western blot analysis of total cellular expression of serotonin_{1A} receptors

Total cellular protein was extracted from control and AY 9944 treated cells using radioimmunoprecipitation assay (RIPA) lysis buffer in presence of protease inhibitor cocktail. Samples were prepared by incubating 20–40 μg protein in electrophoresis sample buffer for 30 minutes at 37°C . Samples were loaded and separated on a 12% SDS-PAGE gel. The separated proteins were then transferred to a nitrocellulose membrane using a semi-dry transfer apparatus (Amersham Pharmacia Biotech, Little Chalfont, UK). The nitrocellulose membrane was blocked for non-specific binding sites using 5% (w/v) BSA prepared in Tris buffer saline containing 0.1% (v/v) Tween 20 (TBST) for 1 hour at room temperature ($\sim 23^{\circ}\text{C}$). The membrane

was probed overnight at 4°C with myc-tag mouse mAb (1:5000 dilution) in 5% (w/v) BSA in TBST to detect serotonin_{1A} receptors. The membrane was washed three times with TBST and probed with HRP goat anti-mouse IgG antibody (1:10 000 dilution) for 1 hour at room temperature ($\sim 23^{\circ}\text{C}$). Bands were detected using enhanced chemiluminescence detection reagent and imaged in Chemi-Smart 5000 chemiluminescence detection system (Vilber Lourmat, Marne-la-Vallée, France). β -tubulin was used as a loading control and the membrane was probed using β -tubulin monoclonal antibody (1:5000 dilution). Band intensities of serotonin_{1A} receptors and β -tubulin were quantified by densitometric analysis using Adobe Photoshop CS3 (Adobe Systems, San Jose, California) software. The intensities of the bands corresponding to serotonin_{1A} receptors were normalized to β -tubulin band intensities.

4.2.7 | Confocal fluorescence imaging

HEK-5-HT_{1A}R cells were plated on poly-L-lysine coated glass coverslips and were treated with AY 9944 as described above. Upon completion of AY 9944 treatment, cells were incubated with 0.3 μM LysoTracker red in serum-free DMEM/F-12 medium for 30 minutes at 37°C and subsequently washed with ice-cold PBS. Cells were fixed using 4% (w/v) formaldehyde solution in PBS for 10 minutes at room temperature ($\sim 23^{\circ}\text{C}$) and washed with ice-cold PBS. For filipin staining, cells fixed with formaldehyde were stained with 50 $\mu\text{g}/\text{mL}$ filipin in PBS for 45 minutes at room temperature ($\sim 23^{\circ}\text{C}$) in dark. Cells were washed with ice-cold PBS. For staining cellular serotonin_{1A} receptor population, fixed cells (or subsequent to filipin staining of cells) were permeabilized with 0.05% (v/v) Triton X-100 (prepared in ice-cold PBS) for 5 minutes on ice. Cells were washed with ice-cold PBS 4–5 times and blocked using 2% (w/v) BSA for 15 minutes at room temperature ($\sim 23^{\circ}\text{C}$). Cells were labeled with anti-myc antibody Alexa Fluor 488 conjugate (1:100 dilution) in PBS containing 2% BSA for 1 hour at room temperature ($\sim 23^{\circ}\text{C}$), followed by washing with ice-cold PBS and mounted in Vectashield antifade mounting medium. Images of z-sections were acquired on a Zeiss LSM 880 confocal microscope (Jena, Germany) using a $\times 63/1.4$ NA oil immersion objective under one airy condition with a fixed step size of 0.5 μm . LysoTracker red was excited at 543 nm and emission was collected between 566 and 690 nm. Serotonin_{1A} receptors were imaged by exciting anti-myc antibody Alexa Fluor 488 conjugate at 488 nm and emission was collected between 490 and 543 nm. Filipin was imaged using excitation at 405 nm and emission was collected between 410 and 460 nm.

4.2.8 | Quantifying colocalization

Two-channel confocal microscopic images were analyzed for colocalization using Manders' colocalization coefficient as described previously with some modifications.³⁹ Briefly, Manders' colocalization coefficient was calculated for z-sections spanning the entire cellular

volume using automatic threshold values using the JACoP plug-in for ImageJ (National Institutes of Health, Bethesda, Maryland).

4.2.9 | Statistical analysis

Significance levels were analyzed using Student's two-tailed unpaired t-test with 95% confidence interval using GraphPad Prism software (version 4.0, San Diego, California). Plots were generated using OriginPro (version 2021b, OriginLab, Northampton, Massachusetts).

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTION

Ashwani Sharma: Performed experiments; analyzed data; wrote the manuscript. **G. Aditya Kumar:** Analyzed data; designed experiments; wrote the manuscript. **Amitabha Chattopadhyay:** Designed experiments; wrote the manuscript; conceptualized the project, edited the manuscript, organized access to research facilities and funding, and provided overall supervision and mentoring.

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