Effect of Hypoxia on the Function of the Human Serotonin\textsubscript{1A} Receptor

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ABSTRACT: Cellular hypoxia causes numerous pathophysiological conditions associated with the disruption of oxygen homeostasis. Under oxygen-deficient conditions, cells adapt by controlling the cellular functions to facilitate the judicious use of available oxygen, such as cessation of cell growth and proliferation. In higher eukaryotes, the process of cholesterol biosynthesis is intimately coupled to the availability of oxygen, where the synthesis of one molecule of cholesterol requires 11 molecules of O\textsubscript{2}. Cholesterol is an essential component of higher eukaryotic membranes and is crucial for the physiological functions of several membrane proteins and receptors. The serotonin\textsubscript{1A} receptor, an important neurotransmitter G protein-coupled receptor associated with cognition and memory, has previously been shown to depend on cholesterol for its signaling and function. In this work, in order to explore the interdependence of oxygen levels, cholesterol biosynthesis, and the function of the serotonin\textsubscript{1A} receptor, we developed a cellular hypoxia model to explore the function of the human serotonin\textsubscript{1A} receptor heterologously expressed in Chinese hamster ovary cells. We observed cell cycle arrest at G1/S phase and the accumulation of lanosterol in cell membranes under hypoxic conditions, thereby validating our cellular model. Interestingly, we observed a significant reduction in ligand binding and disruption of downstream cAMP signaling of the serotonin\textsubscript{1A} receptor under hypoxic conditions. To the best of our knowledge, our results represent the first report linking the function of the serotonin\textsubscript{1A} receptor with hypoxia. From a broader perspective, these results contribute to our overall understanding of the molecular basis underlying neurological conditions often associated with hypoxia-induced brain dysfunction.

KEYWORDS: hypoxia, cholesterol, lanosterol, cell cycle, serotonin\textsubscript{1A} receptor, cAMP

INTRODUCTION

Oxygen (O\textsubscript{2})-producing cyanobacteria changed the course of natural evolution nearly 2.5 billion years ago.\textsuperscript{1} With increasing O\textsubscript{2} levels in the atmosphere (finally reaching \textasciitilde21\%), evolution of more complex aerobic organisms was initiated,\textsuperscript{2} and O\textsubscript{2} became integral to various biochemical pathways.\textsuperscript{3} The sterol biosynthetic pathway became oxygen-dependent, and in due course of evolution, it developed into the current pathway.\textsuperscript{4,5} The concept of hypoxia or oxygen deficiency in cells emerged as oxygen became crucial for cellular survival.\textsuperscript{6} Every tissue has an ideal oxygen pressure ($P_{O_2}$) that maintains tissue metabolism which is regulated by a number of extrinsic and intrinsic factors.\textsuperscript{7,8} A continued exposure to hypoxia results in pathophysiology associated with heart disease, cancer, cerebrovascular disease, and chronic lung disease.\textsuperscript{9} Cerebral hypoxia is caused by impaired blood flow as a result of cerebral or myocardial ischemia\textsuperscript{7,10} and exposure to diminished oxygen pressure in high altitude.\textsuperscript{10} Interestingly, hypoxia induced by Covid-19 (termed “happy hypoxia”) has been recently reported to pose a novel challenge to physicians treating Covid patients.\textsuperscript{11,12} Although human brain comprises only 2% of the body weight, it consumes 20% of oxygen supply from our body in spite of its relatively small size.\textsuperscript{13} As a consequence, prolonged hypoxia cannot be sustained by the brain, leading to seizures, coma, and even death.\textsuperscript{7,14}

There are inbuilt oxygen sensors at the cellular level capable of detecting oxygen deficiency and directing cells toward either an adaptive response or a stress response, while balancing oxygen homeostasis.\textsuperscript{15} These responses could vary, depending on whether the hypoxia is severe (0.01% O\textsubscript{2}), causing the inhibition of nucleotide synthesis, or moderate (\textasciitilde1% O\textsubscript{2}), causing numerous biological reactions to deviate from their normal functions.\textsuperscript{16} Cellular hypoxia\textsuperscript{17} alters cell proliferation in two distinct ways, either by apoptosis or by growth arrest.\textsuperscript{18} An imbalance in cellular O\textsubscript{2} homeostasis has implications in
cellular lipid metabolism in general\textsuperscript{19} and cholesterol metabolism in particular.\textsuperscript{5} The synthesis of one molecule of cholesterol requires 11 molecules of \( \text{O}_2 \) (see Figure 1), and the stepwise progression from squalene, lanosterol demethylation, and finally to cholesterol is believed to be in agreement with the increasing oxygen concentration along the evolutionary timescale.\textsuperscript{23} Lack of cellular oxygen required for removing the methyl groups at 4\( \alpha \), 4\( \beta \), and 14\( \alpha \) in lanosterol to convert to zymostenol. The final oxygen-requiring step in cholesterol synthesis consumes one molecule of oxygen, resulting in the reduction of lanosterol to 7-dehydrocholesterol. Lack of oxygen slows down lanosterol conversion, leading to its accumulation in cells, ultimately disrupting the cholesterol biosynthetic pathway. The solid and dashed arrows represent single and multistep reactions. See text for more details.

![Figure 1. Evolutionary link between oxygen and cholesterol biosynthetic pathway. Biosynthesis of cholesterol begins with acetyl-CoA that feeds into the mevalonate pathway to generate lanosterol. Lanosterol-to-cholesterol conversion includes multiple steps. This oxygen-intensive conversion utilizes nine molecules of oxygen to remove methyl groups at 4\( \alpha \), 4\( \beta \), and 14\( \alpha \) in lanosterol to convert to zymostenol. The final oxygen-requiring step in cholesterol synthesis consumes one molecule of oxygen, resulting in the reduction of lanosterol to 7-dehydrocholesterol. Lack of oxygen slows down lanosterol conversion, leading to its accumulation in cells, ultimately disrupting the cholesterol biosynthetic pathway. The solid and dashed arrows represent single and multistep reactions. See text for more details.](image-url)
Generating a Cellular Hypoxia Model. Cells grown in vitro are usually maintained at an optimum oxygen concentration for their growth in normal atmospheric oxygen pressure in a regular humidified incubator (21% O₂ and 5% CO₂). To mimic hypoxic conditions, we developed a cellular hypoxia model (Figures 2 and S1) and cultured cells in a humidified incubator where the oxygen concentration could be regulated using excess of nitrogen. We cultured cells using 1% O₂ and 5% CO₂ for 6, 12, and 24 h to mimic hypoxia.17,22 We utilized CHO-K1 cells stably expressing the human serotonin₁A receptor with (or without) an enhanced yellow fluorescent protein (EYFP) tag at its C-terminal (denoted as CHO-5-HT₁AR-EYFP and CHO-5-HT₁AR, respectively) for this purpose. We previously showed that the human serotonin₁A receptor stably expressed in CHO-K1 cells retains all the characteristics of the native receptor expressed in hippocampus in terms of ligand binding, G-protein coupling, and signaling, even when labeled with EYFP.57,58 In order to avoid the uptake of exogenous cholesterol from the serum present in a complete culture medium, we cultured cells in a serum-free medium under hypoxic conditions.59 To rule out any possible effect of the serum-free medium itself, control cells were grown in both complete and serum-free culture media under normoxic conditions for 24 h (see Figures 2 and S1).

In the cholesterol biosynthetic pathway, demethylation of lanosterol and subsequent conversion to cholesterol consume 10 molecules of oxygen (Figure 1). The hallmark of cellular hypoxia is slow lanosterol demethylation and subsequent accumulation of lanosterol in the cells due to oxygen deficiency.21,23 In order to validate the cellular model of hypoxia, we checked the accumulation of lanosterol under hypoxic conditions. For this, lipid extracts were prepared from cell membranes, and lipids were separated by thin-layer chromatography. Figure 3a shows a representative thin-layer chromatogram showing the lipid composition of cells under various conditions. As shown in the figure, we did not observe any band corresponding to lanosterol under control conditions. However, bands corresponding to lanosterol could be visualized after 12 h of exposure to hypoxia, and a considerable amount of lanosterol could be detected when cells were grown under hypoxic conditions for 24 h. Densitometric analysis of the TLC bands showed lanosterol/total sterol ratio of 0.01 and 0.05 in cells exposed to hypoxic condition for 12 and 24 h, respectively (Figure 3b). These results validate our cellular model for hypoxia.

Membrane Cholesterol Content Does Not Change in Cells under Hypoxia. As described above, we observed a distinct accumulation of lanosterol under hypoxic conditions. However, no apparent change in cholesterol level was found under hypoxia when analyzed using densitometric analysis of the chromatogram (Figure 3b). We therefore used a more sensitive fluorometry-based Amplex Red assay for the quantification of cholesterol under these conditions.60 Importantly, our control experiments showed that the presence of lanosterol does not interfere with this assay (data not shown). Figure 3c shows the cholesterol content in cells cultured under normoxic and hypoxic conditions. As shown in the figure, we did not observe any significant change in the cholesterol content in all cases.

Cell Viability under Hypoxic Conditions. Low oxygen can affect cellular functions, and cells are known to activate apoptosis under prolonged hypoxic treatment.61 In addition, the accumulation of lanosterol leads to toxicity and is associated with its inability to maintain the normal physiological properties of cell membranes essential to carry out crucial functions.25,61,62 We therefore chose the duration of hypoxic treatment carefully to ensure that cell viability was not compromised in our experimental conditions. Apoptosis was measured utilizing a flow cytometry-based assay using FITC-Annexin V and propidium iodide (PI). Whereas apoptosis is
characterized by phosphatidylserine (PS) externalization on the cell surface\textsuperscript{64} and significant membrane defects in later phases, necrosis is characterized by significant membrane defects that do not involve externalization of PS. Externalized PS binds to Annexin,\textsuperscript{65} and the fluorescence from the tagged FITC is quantitated to determine the extent of apoptosis. On the other hand, PI intercalates with nucleic acids, and its fluorescence allows discrimination between the late apoptotic phase and necrosis. Figure S2 shows the representative flow cytometric dot plots with four distinct quadrants marked as viable cells (Annexin-FITC and PI-negative cells), early apoptosis (only Annexin-FITC-positive cells), late apoptosis (Annexin-FITC and PI double-positive cells), and necrosis (only PI-positive cells). We observed that $\sim$99\% cells across all treatment conditions did not exhibit apoptosis and were comparable to the control cells grown in complete media.

**Cell Cycle Progression under Hypoxic Conditions.** Hypoxia is known to induce cell cycle arrest at the G1/S interphase.\textsuperscript{18} In order to validate the effect of hypoxia on the cell cycle progression in our cellular model, we utilized a flow cytometry-based assay to quantitate the number of cells in various phases of the cell cycle. The phases of cell cycle could be identified on the basis of the changes in the cellular DNA content in a population of cells using flow cytometry acquired upon PI labeling. In order to estimate the effect of hypoxia, the distribution of cells (\textit{i.e.}, number of cells in G1, S, G2/M, and sub-G1 phases) was determined under various conditions and plotted in Figure 4. The figure shows that the distribution of control cells grown in complete medium in G1, S, G2/M, and sub-G1 phases was $\sim$59, $\sim$16, $\sim$23, and $\sim$2\%, respectively. In the case of cells cultured under hypoxic conditions, we observed a significant increase in cell numbers in the G1 phase across all time points. These results indicate the arrest of cell cycle at the G1/S interphase.

**Ligand Binding to the Serotonin\textsubscript{1A} Receptor under Hypoxic Conditions.** Ligand binding to GPCRs is the first step toward initiation of signaling in response to an extracellular stimulus. In order to monitor the changes in ligand binding to the serotonin\textsubscript{1A} receptor upon hypoxia, we carried out whole cell ligand binding of the serotonin\textsubscript{1A} receptor. Importantly, we previously showed that the serotonin\textsubscript{1A} receptor expressed in CHO-K1 cells with the C-
Figure 4. Effect of hypoxia on cell cycle. Cell cycle arrest was monitored in cells cultured under hypoxic conditions for 6, 12, and 24 h. Cell numbers in G1, S, G2/M, and sub-G1 phases are represented by gray, green, maroon, and blue bars, respectively. Data represent means ± SE from at least three independent experiments (*** and **** correspond to significant \( p < 0.01 \) and \( p < 0.001 \) difference in the number of cells at G1 phase under hypoxic conditions and cells grown in serum-free medium under normoxic conditions relative to cells grown in complete medium under normoxic conditions). See Methods for more details.

Figure 5. Effect of hypoxia on agonist binding to the serotonin1A receptor. Specific binding of the radiolabeled agonist \(^{[3]H}\)8-OH-DPAT to serotonin1A receptors in intact CHO-5-HT1AR-EYFP cells exposed to hypoxic conditions for 6, 12, and 24 h (blue bars). The corresponding data for cells maintained under normoxic conditions are also shown (orange bars). Values are expressed as percentages of specific radioligand binding normalized to control cells grown in complete medium under normoxic conditions. Data represent means ± SE from at least three independent experiments (*** and **** correspond to significant \( p < 0.01 \) and \( p < 0.001 \) difference in specific \(^{[3]H}\)8-OH-DPAT binding in cells cultured under hypoxic conditions for 12 and 24 h, respectively, relative to cells grown in complete medium under normoxic conditions; # and ### correspond to significant \( p < 0.05 \) and \( p < 0.001 \) difference in specific \(^{[3]H}\)8-OH-DPAT binding in cells cultured under hypoxic conditions for 12 and 24 h, respectively, relative to cells grown in serum-free medium under normoxic conditions). See Methods for more details.
together, these results show that the signaling efficiency of the serotonin1A receptor is compromised in a hypoxic condition.

**DISCUSSION**

Hypoxia is a physiological stress condition caused due to the lack of oxygen in cells, which leads to organ dysfunction and tissue necrosis. Hypoxia could cause widespread brain damage and atrophy, leading to amnesia, depression, and anxiety. As cognitive disability, depression, and anxiety are commonly associated with the malfunction of the serotonin1A receptor, we explored the association between hypoxia and the function of the serotonin1A receptor. For this, we generated a cellular hypoxia model mimicking the effect of moderate hypoxia, characterized by the accumulation of lanosterol in cells and the arrest of cell cycle at the G1 phase. Interestingly, membrane cholesterol has been reported to regulate the cell cycle progression. We previously showed that the presence of immediate biosynthetic precursors of cholesterol (such as 7-dehydrocholesterol and desmosterol) could induce cell cycle arrest. We observed that a prolonged exposure (12 and 24 h) of cells to hypoxic conditions led to reduction in ligand binding and cAMP signaling by the serotonin1A receptor. Interestingly, the progressive accumulation of lanosterol in cells exposed to prolonged hypoxia (12 and 24 h) correlated with the dysfunction of the serotonin1A receptor.

The serotonin1A receptor is primarily expressed in the central nervous system, as evident from the neurological functions associated with the receptor. Autoradiography using specific radiolabeled agonists and antagonists of the receptor showed that the serotonin1A receptor is highly expressed in the limbic forebrain regions such as the hippocampus, raphe nuclei, amygdala, hypothalamus, and cortex, whereas the lowest density is found in the extrapyramidal areas such as the basal ganglia, substantia nigra, and in the adult cerebellum. Notably, under normoxic conditions, the partial pressure of oxygen ($pO_2$) in brain is ∼34 mmHg (4.6% $pO_2$). A drop in oxygen levels below this value would create a hypoxic condition in the brain.

Defects in cholesterol biosynthesis have been reported to cause impaired synaptic transduction and neurodegenerative disorders. Cholesterol is synthesized in the CNS and accounts for ∼25% of the total cholesterol content in the body. Accumulation of cholesterol biosynthetic precur-

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**Figure 6.** Effect of hypoxia on the expression of the serotonin1A receptor in CHO-5-HT1A-R-EYFP cells. (a) Receptor expression was analyzed using a flow cytometry-based assay using the EYFP-tagged serotonin1A receptor. Representative flow cytometric histograms showing the population of the serotonin1A receptor tagged to EYFP at its C-terminal under hypoxic conditions. CHO-K1 cells expressing an untagged (without EYFP) version of the serotonin1A receptor were used to gate EYFP-negative (gray regions denoted as EYFP-negative) and EYFP (green regions denoted as EYFP-positive) population of cells. Quantification of the serotonin1A receptor expression as measured by (b) percentage of EYFP-positive cells from flow cytometric histograms and (c) mean fluorescence intensity. Values are expressed as percentage of EYFP-positive cells in each condition. Data represent means ± SE from at least three independent experiments (no significant differences observed in the receptor expression in cells cultured under hypoxic conditions relative to control cells in both cases). See Methods for more details.
Figure 7. Effect of hypoxia on cAMP signaling mediated by the serotonin\textsubscript{1A} receptor. The efficiency of the serotonin\textsubscript{1A} receptor to inhibit forskolin-stimulated increase in cAMP levels by activation with 10 \textmu M serotonin in CHO-S-HT\textsubscript{1A}R cells exposed to hypoxic conditions. Data are normalized to cAMP levels in the presence of 50 \textmu M IBMX + 10 \textmu M forskolin (forskolin-stimulated) for each condition. Data represent means \pm SE of at least four independent experiments (*, **, *** correspond to significant (p < 0.05 and p < 0.0001) difference in reduction in cAMP content in cells cultured under hypoxic conditions for 12 and 24 h, respectively, relative to cells grown in complete medium under normoxic conditions; # and ##### correspond to significant (p < 0.05 and p < 0.001) difference in reduction in cAMP content in cells cultured under hypoxic conditions for 12 and 24 h, respectively, relative to cells grown in serum-free medium under normoxic conditions). See Methods for more details.

Cells in the brain, as a consequence of the inhibition of cholesterol pathway, has been associated with various neurological diseases.\textsuperscript{80,85} The serotonin\textsubscript{1A} receptor, a critical neurotransmitter receptor in the GPCR family, displays a stringent requirement for cholesterol, both in terms of its structure\textsuperscript{86,87} and optimal level\textsuperscript{88} for its organization, dynamics, and function. Cholesterol sensitivity to GPCR function can be attributed to the direct interaction of membrane cholesterol with GPCRs, or by cholesterol-induced changes in the global lipid bilayer properties, or a combination of both mechanisms.\textsuperscript{89} In this context, cholesterol interaction motifs represent putative sites on receptors that could be involved in facilitating specific interactions with cholesterol.\textsuperscript{90} Notably, we recently showed that a key lysine residue (K101), that forms a part of the cholesterol recognition/interaction amino acid consensus (CRAC) motif in the transmembrane helix 2 of the serotonin\textsubscript{1A} receptor, is crucial in sensing altered membrane cholesterol levels.\textsuperscript{70} In general, changes in membrane properties due to sterol modulation have earlier been shown to lead to the modulation of membrane phospholipids.\textsuperscript{91} On a cautionary note, we would therefore like to mention that changes in lipidome (other than sterols) upon hypoxia have been previously reported.\textsuperscript{92−95} In this context, we observed the appearance of a few nonidentified bands on the thin-layer chromatogram under hypoxic conditions, apart from the marked accumulation of lanosterol (Figure 3a). The contribution of nonsterol lipids in the impaired function of the serotonin\textsubscript{1A} receptor, therefore, may not be ruled out.

Taken together, our results highlight the impaired function of the serotonin\textsubscript{1A} receptor under hypoxic conditions, which could be relevant in understanding cerebral hypoxia that has been a major point of concern for mountaineers and soldiers stationed at high altitudes.\textsuperscript{96} We believe that our results constitute one of the first reports linking altered serotonin\textsubscript{1A} receptor function with hypoxia and could provide opportunities for novel therapeutics in hypoxia-related disorders. From a broader perspective, our work represents an important step in understanding the link between oxygen, sterol biosynthesis, and function of neuronal receptors, thereby contributing to our overall understanding of the molecular basis underlying neurological conditions often associated with hypoxia-induced brain dysfunction.

METHODS

Materials. Bovine serum albumin, cholesterol, trypsin, EDTA, forskolin, gentamycin sulfate, 3-isobutyl-1-methylyanthine (IBMX), genetin (G418), lanosterol, sodium bicarbonate, penicillin, streptomycin, phenylmethysulfonil fluoride (PMSF), polyethylenimine, serotonin, Tris, PI, RNase A, and staurosporine were purchased from Sigma Chemical Co. (St. Louis, MO). Biochinnonic acid (BCA) reagent for protein estimation was obtained from Pierce (Rockford, IL). \textsuperscript{[3]}H\textsubscript{8}-Hydroxy-2(d-N-propylamino)tetralin (\textsuperscript{[3]}H\textsubscript{8}-OH-DPAT, specific activity: 141.1 Ci/mmol) was obtained from MP Biomedicals (Santa Ana, CA). DMEM/F-12 [Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (Ham) (1:1)] and fetal calf serum were obtained from Gibco/Life Technologies (Grand Island, NY). Amplex Red cholesterol assay kit was purchased from Molecular Probes/Invitrogen (Eugene, OR). GF/B glass microfilter was obtained from Whatman International (Kent, UK). Homogeneous time-resolved fluorescence (HTRF) CAMP-Gi assay kit was purchased from CisBio Bioassays (Codolet, France). Precocoated silica gel 60 thin-layer chromatography plates were obtained from Merck (Darmstadt, Germany). ApoAlert Annexin V apoptosis kit was purchased from Clontech Laboratories (Mountain View, CA). All chemicals used were of the highest available purity. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Cell Culture under Normoxic and Hypoxic Conditions. CHO-K1 cells stably expressing human serotonin\textsubscript{1A} receptors tagged to EYFP at its C-terminal (CHO-S-HT\textsubscript{1A}R-EYFP) were maintained in DMEM/F-12 (1:1) medium, supplemented with 2.4 g/L of sodium bicarbonate, 10% (v/v) fetal calf serum, 60 \mu g/mL penicillin, 50 \mu g/mL streptomycin, 50 \mu g/mL gentamycin sulfate (complete medium), and 0.3 mg/mL G418 in a humidified atmosphere with 5% CO\textsubscript{2} at 37 °C for 72 h. CHO-S-HT\textsubscript{1A}R R cells (receptors without EYFP tag) were maintained in complete medium with 0.2 mg/mL G418 under the same conditions for 72 h (or 84 h or 90 h, depending on the time in the hypoxic chamber so that the total time remains 96 h in all cases). Cells were cultured under hypoxic conditions at 1% O\textsubscript{2} and 5% CO\textsubscript{2} at 37 °C in a humidified HERA CELL 240i CO\textsubscript{2} incubator (where O\textsubscript{2} concentration could be regulated using excess nitrogen) in serum-free medium for 6, 12, or 24 h. Two control groups of cells were grown in complete and serum-free media under normoxic conditions (21% O\textsubscript{2} and 5% CO\textsubscript{2} at 37 °C) for 24 h. We plated \textasciitilde10\textsuperscript{5} cells in 35 mm culture dish for CAMP assay, \textasciitilde5 \times 10\textsuperscript{5} cells in 100 mm culture dish (for quantifying receptor expression, apoptosis assay, radioligand binding assay, and cell cycle analysis), and \textasciitilde15 \times 10\textsuperscript{5} cells in 150 mm culture dish (for thin-layer chromatography and cholesterol estimation).

Cell Membrane Preparation. Subsequent to treatments, cells were washed with phosphate-buffered saline (PBS) and harvested by treatment with ice-cold hypotonic buffer containing 10 mM Tris, 5 mM EDTA, and 0.1 mM PMSF (pH 7.4). Cells were then homogenized for 10 s using a Polytron homogenizer at a maximum speed. The homogenized cells were centrifuged at 500g for 10 min at 4 °C. The resulting supernatant was centrifuged at 40,000g for 30 min at 4 °C, and pellets were suspended in 50 mM Tris buffer, pH 7.4. The total protein concentration in isolated membranes was determined using BCA assay.\textsuperscript{97}

Detection of Lanosterol and Cholesterol by Thin-Layer Chromatography. Total lipids were extracted from isolated cell membranes according to the Bligh and Dyer method.\textsuperscript{98} The extracted lipids were dried using a stream of nitrogen at 70 °C and dissolved to EYFP at its C-terminus (CHO-5-HT\textsubscript{1A}R-EYFP) were maintained in the hypoxic chamber so that the total time remains 96 h in all cases. The resulting supernatant was centrifuged at 40,000g for 30 min at 4 °C, and pellets were suspended in 50 mM Tris buffer, pH 7.4. The total protein concentration in isolated membranes was determined using BCA assay.\textsuperscript{97}
visualized by thin-layer chromatography using precoated silica gel plates. Sterols were resolved in a dual solvent system, using ethyl acetate/benzene (1:5, v/v) as the first solvent system and heptane/benzene (97:3, v/v) as the second solvent system. The separated lipids were visualized by charring with a solution containing cupric sulfate (10%, w/v) and orthophosphoric acid (8%, v/v) at 150 °C. Lanosterol and sterol bands were identified using respective standards. The 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.

**Estimation of Cholesterol Content.** The cholesterol content in cell membranes was estimated using the Amplex Red cholesterol assay kit and normalized to the total cellular protein estimated using the BCA assay.

**Detection of Apoptosis.** The percentage of apoptotic cells was assessed by flow cytometry using an Annexin V-FITC/PI apoptosis detection kit as per manufacturer’s protocol. CHO-5-HT1A-R, CHO-5-HT1AR-EYFP cells were collected after treatment with 0.1% (w/v) trypsin–EDTA and washed with complete DMEM/F-12 medium. The cells were further washed with 1× binding buffer and incubated with 5 μL of Annexin V-FITC (stock concentration, 20 μg/mL) and 10 μL of PI (stock concentration, 50 μg/mL) at a density of ~5 × 10^6 cells/mL in 1× binding buffer for 15 min at room temperature (~23 °C) in the dark. The samples were analyzed using a Gallios flow cytometer (Beckman Coulter, Brea, CA). FITC and PI were excited at 488 nm, and the emission was collected using 525/40 nm and 620/30 nm bandpass filters, respectively. As a positive control, cells were treated with 5 μM staurosporine for 24 h in a humidified atmosphere with 5% CO2 at 37 °C. The acquired data were analyzed using Kaluza analysis software (version 1.5a, Beckman Coulter, Brea, CA).

**Flow Cytometric Analysis of Cell Cycle.** CHO-5-HT1A-R cells were harvested using 0.1% (w/v) trypsin–EDTA, centrifuged for 5 min at 500g, and fixed using ice-cold 70% (v/v) ethanol for 10 min. Subsequently, cells were centrifuged, and DNA was labeled in PBS containing 2% (v/v) FCS with 50 μg/mL PI and 200 μg/mL RNase A. Cells were incubated for 15 min in the staining solution on ice, centrifuged, and resuspended in PBS containing 2% (v/v) FCS. The distribution of cells in the different phases of cell cycle was monitored using a Gallios flow cytometer (Beckman Coulter, Brea, CA), and the acquired data were analyzed using Kaluza analysis software (version 1.5a, Beckman Coulter, Brea, CA). The excitation was set at 488 nm, and emission was collected using a 620/30 nm bandpass filter, while 10,000 cells were analyzed in each condition. To distinguish single cells from multiplets of cells, we used a “pulse-processing” protocol, where fluorescence from multiplets of cells was excluded using a fluorescence pulse width and fluorescence pulse area display.

**Radioligand Binding Assay in Live Cells.** CHO-5-HT1A-R-EYFP cells were harvested using PBS containing 0.25 mM EDTA. Cells were spun at 500g for 5 min and resuspended in serum-free DMEM/F-12 medium for counting using a hemocytometer. Cells (~10^6) in serum-free medium were incubated at 25 °C for 15 min in the presence of 1 nM [3H]8-OH-DPAT. Nonspecific binding was obtained by performing the assay in the presence of 10 μM unlabeled serotonin. Ligand binding was terminated by rapid filtration under vacuum through a Millipore multiprot filtration apparatus using Whatman GF/B glass microfiber filters of 1 μM pore size that were presoaked in 0.3% (v/v) polyethyleneimine for 3 h. This was followed by washing the filters three times with 5 mL of ice-cold water (~4 °C). After subsequent drying of the filters, the remaining radioactivity was measured using ~5 mL scintillation fluid in a Packard Tri-Carb 2900 liquid scintillation counter (PerkinElmer, Waltham, MA).

**Flow Cytometric Analysis of Receptor Expression Level.** CHO-5-HT1A-R-EYFP cells were collected in PBS containing 0.25 mM EDTA, spun at 500g for 5 min, and resuspended in PBS containing 2% (v/v) FCS. The receptor population from 10,000 cells in each condition was quantified using a Gallios flow cytometer (Beckman Coulter, Brea, CA), and data were acquired and analyzed using Kaluza analysis software (version 1.5a, Beckman Coulter, Brea, CA). Excitation was set at 488 nm, and emission was collected using a 525/40 nm bandpass filter. CHO-5-HT1AR (serotonin receptor without EYFP tag) cells were used to gate EYFP-negative population.

**Cellular Signaling Assay.** CHO-5-HT1A-R cells were treated with 50 μM IBMX (basal), 50 μM IBMX + 10 μM forskolin (forskolin-stimulated), or 50 μM IBMX + 10 μM forskolin + 10 μM serotonin (agonist treatment) and incubated for 30 min at 37 °C. The phosphodiesterase inhibitor IBMX was present during all treatments to prevent the breakdown of cAMP. Incubation was performed under hypoxic conditions for cells previously grown under hypoxia, whereas control cells were incubated under normoxic conditions. After discarding the media, cells were washed once with PBS, lifted using a cell scraper, counted using a hemocytometer, and added at 6000 cells/well to a low-volume HTRF 96-well plate (CisBio Bioassays). The ability of the serotonin receptor to inhibit the forskolin-stimulated increase in cAMP levels was assessed using the fluorescence resonance energy transfer (FRET)-based HTRF cAMP-Gi assay kit (CisBio Bioassays). Fluorescence was measured at 620 nm (cAMP-cryptate donor emission) and 655 nm (anti-cAMP-d2 acceptor emission) upon excitation of the donor at 320 nm using the EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA). cAMP levels were calculated as a ratio of the acceptor/donor emission. Values for serotonin-induced cAMP reduction were normalized to cAMP levels in the presence 50 μM IBMX + 10 μM forskolin-treated cells.
Notes
The authors declare no competing financial interest.

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ABBREVIATIONS
FITC, fluorescein isothiocyanate; PI, propidium iodide; ER, endoplasmic reticulum; BCA, bicinchoninic acid; EDTA, ethylenediaminetetraacetic acid; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; cAMP, adenosine 3′,5′-cyclic monophosphate; FRET, fluorescence resonance energy transfer; IBMX, 3-isobutyl-1-methylxanthine; 8-OH-DPAT, 8-hydroxy-2-(di-N-propylamino)tetralin

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