

# Metabolic Depletion of Sphingolipids Reduces Cell Surface Population of the Human Serotonin<sub>1A</sub> Receptor due to Impaired Trafficking

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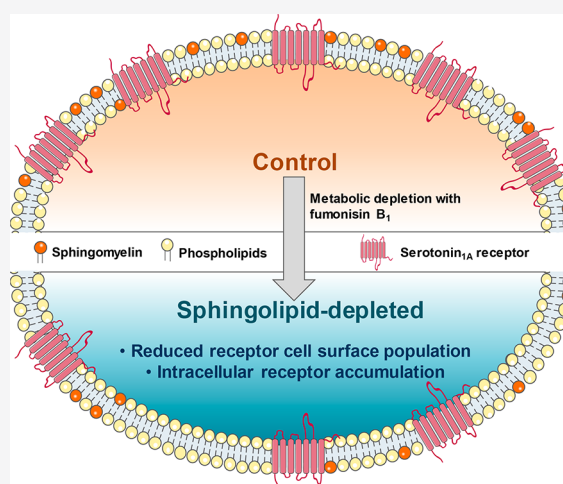
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**ABSTRACT:** Sphingolipids and their metabolites are increasingly implicated in the pathogenesis of many metabolic and neurological diseases. It has been postulated that sphingolipids coalesce with cholesterol to form laterally segregated lipid domains that are involved in protein sorting and trafficking. In this work, we have explored the effect of metabolic depletion of sphingolipids on cell surface expression of the human serotonin<sub>1A</sub> receptor, a neurotransmitter G protein-coupled receptor. We used fumonisins B<sub>1</sub> (FB<sub>1</sub>), a fungal mycotoxin, to inhibit sphingolipid biosynthesis in HEK-293 cells stably expressing the human serotonin<sub>1A</sub> receptor. Our results obtained using flow cytometric analysis and confocal microscopic imaging show that the cell surface population of the serotonin<sub>1A</sub> receptor is reduced under sphingolipid-depleted condition. Western blot analysis confirmed that there was no significant difference in total cellular level of the serotonin<sub>1A</sub> receptor upon depletion of sphingolipids. Interestingly, the effect of FB<sub>1</sub> on serotonin<sub>1A</sub> receptor population was reversed upon replenishment with sphingolipids. These results indicate that sphingolipid depletion does not alter total cellular receptor levels, but impairs serotonin<sub>1A</sub> receptor trafficking to the cellular plasma membrane. These results could provide mechanistic insights into the role of sphingolipids in modulation of neurotransmitter receptor signaling and trafficking in health and disease.

**KEYWORDS:** Sphingolipids, sphingomyelin, fumonisins B<sub>1</sub>, GPCR, serotonin<sub>1A</sub> receptor, trafficking



## INTRODUCTION

Sphingolipids are crucial constituents of eukaryotic cell membranes and account for ~10–20% of total membrane lipids.<sup>1</sup> They play a key role in the regulation of various cellular functions, such as cell signaling, proliferation, senescence, and in several neurological diseases.<sup>2,3</sup> Sphingolipids such as sphingomyelin are reservoirs of ceramide and sphingosine-1-phosphate, which serve as bioactive molecules in various cellular processes.<sup>4</sup> Sphingolipids are more abundant in the plasma membrane relative to intracellular membranes.<sup>5</sup> It has been proposed that sphingolipids coalesce with cholesterol to form laterally segregated domains termed “lipid rafts”,<sup>6</sup> which concentrate signaling molecules for effective signal transduction and play a crucial role in protein sorting and entry of pathogens into cells.<sup>7–11</sup> Due to differential lipid composition of such microdomains, some transmembrane proteins have been proposed to be preferentially localized in these domains. This indicates that sphingolipids are involved in membrane protein sorting and trafficking.<sup>12</sup> Importantly, sphingolipids modulate conformation and function of neurotransmitter

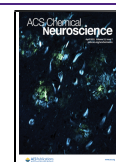
receptors, thereby regulating brain development and function, and are implicated in several neurological disorders.<sup>3,13–15</sup> In this context, monitoring the trafficking of neurotransmitter receptors under sphingolipid-depleted condition assumes relevance.

G protein-coupled receptors (GPCRs) are integral membrane proteins with a seven transmembrane domain architecture and are involved in signal transduction across the cell membrane in response to a diverse variety of exogenous and endogenous ligands.<sup>16–19</sup> Due to their versatile function, GPCRs represent major drug targets across all clinical areas.<sup>19–22</sup> The serotonin<sub>1A</sub> receptor is a representative

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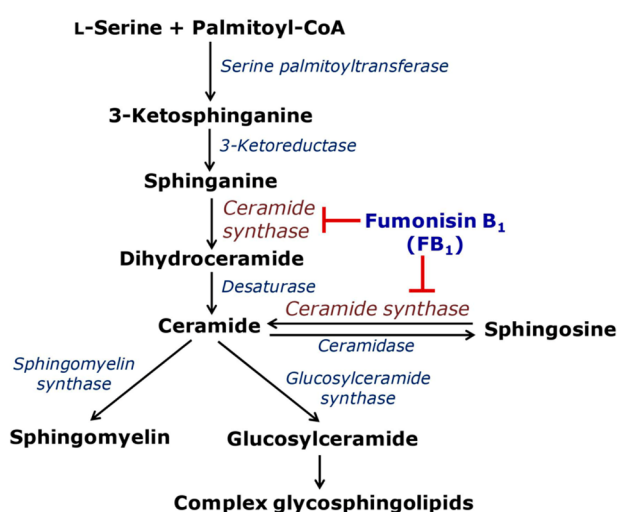
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member of the serotonin receptor family of GPCRs and is one of the most studied neurotransmitter GPCRs due to a number of reasons.<sup>23–28</sup> Serotonin<sub>1A</sub> receptors play a vital role in the generation and modulation of cognitive, behavioral, and developmental functions such as sleep, mood, addiction, depression, anxiety, and learning.<sup>29,30</sup> Previous work from our group has shown that sphingolipids modulate function and dynamics (lateral diffusion) of the serotonin<sub>1A</sub> receptor.<sup>31–35</sup>

Cellular biosynthesis of sphingolipids can be inhibited using fumonisin B<sub>1</sub> (FB<sub>1</sub>). FB<sub>1</sub> is a fungal mycotoxin from *Fusarium* species and is the most abundant among the family of fumonisins.<sup>36</sup> FB<sub>1</sub> is structurally similar to sphingoid bases, which are sphingolipid metabolic intermediates such as sphingosine and sphinganine (sphingosine lacking *trans*-C4–C5 double bond), and thereby acts as a competitive inhibitor of the enzyme sphinganine *N*-acetyltransferase (ceramide synthase) (see Figure 1).<sup>37,38</sup> In this work, we modulated



**Figure 1.** Metabolic pathway of sphingolipid biosynthesis. The *de novo* biosynthesis of sphingolipids is initiated by the enzyme serine palmitoyltransferase in the endoplasmic reticulum. Subsequent reactions lead to synthesis of ceramides, which are incorporated into complex sphingolipids (predominantly in the Golgi), such as sphingomyelin and glycosphingolipids. Fumonisin B<sub>1</sub> (FB<sub>1</sub>) is a competitive inhibitor of the enzyme ceramide synthase, which introduces fatty acid groups to sphinganine in *de novo* biosynthesis of sphingolipids and sphingosine (derived from sphingolipid catabolic pathway), thereby inhibiting the synthesis of ceramide and complex sphingolipids. See text for other details.

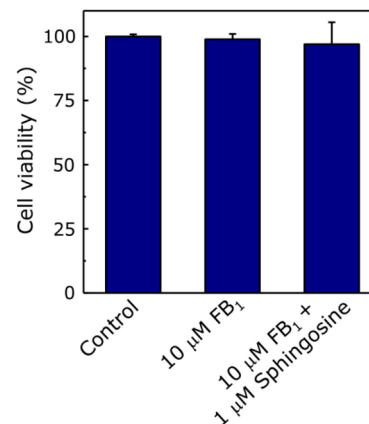
sphingolipid levels in HEK-293 cells stably expressing N-terminal myc-tagged human serotonin<sub>1A</sub> receptor (termed HEK-5-HT<sub>1A</sub>R cells) by metabolically inhibiting sphingolipid biosynthesis using FB<sub>1</sub>. We observed that chronic treatment with FB<sub>1</sub> resulted in reduction in sphingomyelin level in HEK-5-HT<sub>1A</sub>R cells. We subsequently monitored the plasma membrane population of the serotonin<sub>1A</sub> receptor under these conditions using a quantitative flow cytometric assay. Our results show a significant reduction in the plasma membrane population of serotonin<sub>1A</sub> receptors in sphingolipid-depleted cells. Interestingly, the effect of FB<sub>1</sub> on cell surface population of the serotonin<sub>1A</sub> receptor could be reversed when we replenished cellular sphingolipids using sphingosine. In addition, we show that the reduction in the cell surface population of the serotonin<sub>1A</sub> receptor was not due to reduction in total cellular expression of the receptor, thereby

indicating that sphingolipid depletion could impair trafficking of the serotonin<sub>1A</sub> receptor to the plasma membrane. These results assume significance in our overall understanding of the role of sphingolipids underlying neurotransmitter receptor function.

## RESULTS AND DISCUSSION

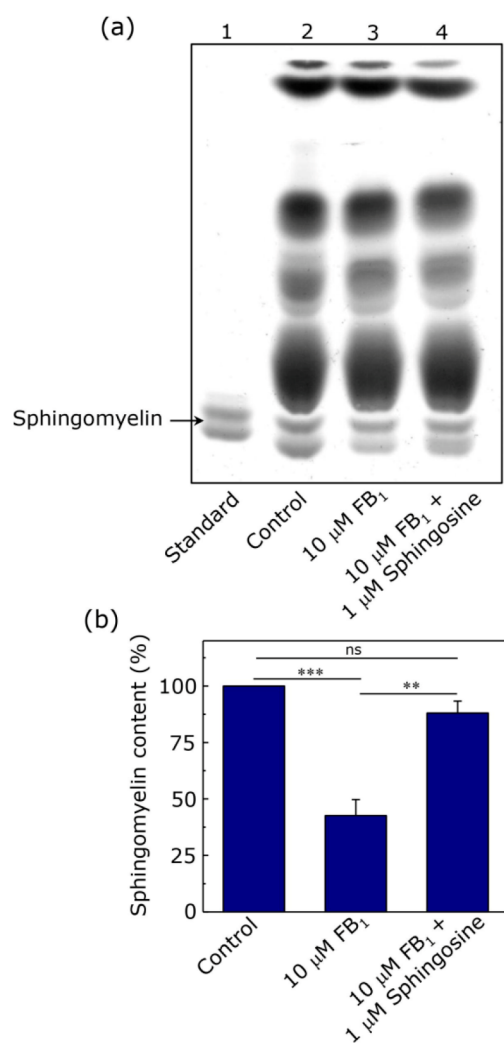
**FB<sub>1</sub> Treatment Reduces Sphingolipid Content of HEK-5-HT<sub>1A</sub>R Cells.** We used HEK-293 cells stably expressing serotonin<sub>1A</sub> receptors (generated by transfection with a construct encoding the receptor with an N-terminal myc-tag)<sup>39</sup> for measuring receptor population on the cell surface. This stable cell line allows us to fluorescently label the serotonin<sub>1A</sub> receptor with anti-myc antibody and monitor receptor expression on the cell surface. We previously showed that the serotonin<sub>1A</sub> receptor in HEK-5-HT<sub>1A</sub>R cells retains all characteristics of the native receptor in terms of ligand binding, G-protein coupling, cellular signaling, and receptor trafficking.<sup>39,40</sup>

With an overall goal of understanding the role of sphingolipids in regulating the plasma membrane population of the human serotonin<sub>1A</sub> receptor, we treated HEK-5-HT<sub>1A</sub>R cells with FB<sub>1</sub> to metabolically deplete cellular sphingolipid content. FB<sub>1</sub> is a competitive inhibitor of the enzyme ceramide synthase that catalyzes acylation of sphinganine in *de novo* biosynthesis of sphingolipids and utilizes sphingosine derived from sphingolipid turnover (see Figure 1). We chose to use a low concentration of FB<sub>1</sub> (10  $\mu$ M) and ensured that cell viability was not compromised as measured by MTT assay (see Figure 2). In order to estimate sphingolipid content of cells,



**Figure 2.** Effect of FB<sub>1</sub> treatment on cell viability. The viability of HEK-5-HT<sub>1A</sub>R cells was assessed by MTT assay under sphingolipid-depleted (10  $\mu$ M FB<sub>1</sub> treatment) and sphingolipid-replenished (with 1  $\mu$ M sphingosine following 10  $\mu$ M FB<sub>1</sub> treatment) conditions. Values are expressed as percentages of viability normalized to control cells (without FB<sub>1</sub> treatment). Data represent means  $\pm$  SE of at least three independent experiments. See the Methods section for other details.

total lipids were extracted from membranes of control and FB<sub>1</sub>-treated cells and separated using thin layer chromatography (TLC). Since sphingomyelin constitutes the major fraction of total cellular sphingolipids,<sup>41,42</sup> we quantitated cellular sphingomyelin content as a representative indicator of total cellular sphingolipids. A representative chromatogram showing sphingomyelin content in control and FB<sub>1</sub>-treated cells is shown in Figure 3a. Sphingomyelin band intensities were analyzed using densitometric analysis of the chromatogram as



**Figure 3.** Estimation of sphingomyelin content in cell membrane following FB<sub>1</sub> treatment. (a) Total lipids extracted from HEK-5-HT<sub>1A</sub>R cell membranes of control, sphingolipid-depleted and sphingolipid-replenished cells separated by TLC. Lane 2 shows lipids extracted from control cells, whereas lanes 3 and 4 show lipids extracted from cells treated with 10  $\mu$ M FB<sub>1</sub> (sphingolipid-depleted) and cells replenished with 1  $\mu$ M sphingosine following FB<sub>1</sub> treatment (sphingolipid-replenished), respectively. The position of the sphingomyelin band was identified by using a standard in lane 1. (b) Sphingomyelin content was quantified using densitometric analysis of the chromatogram. Values are expressed as percentages of sphingomyelin content normalized to control cells (without FB<sub>1</sub> treatment). Data represent means  $\pm$  SE of at least three independent experiments (\*\*\*) corresponds to significant ( $p < 0.001$ ) difference in sphingomyelin content in FB<sub>1</sub>-treated cells relative to control cells, \*\* corresponds to significant ( $p < 0.01$ ) difference in sphingomyelin content in sphingolipid-replenished cells relative to FB<sub>1</sub>-treated cells, and ns corresponds to lack of significant difference in sphingomyelin content in sphingolipid-replenished cells relative to control cells as estimated using one-way ANOVA, followed by Bonferroni's multiple comparisons test). See the [Methods](#) section for other details.

described in the [Methods](#) section and are shown in [Figure 3b](#). The figure shows that the sphingomyelin content of HEK-5-HT<sub>1A</sub>R cells was reduced by  $\sim 58\%$  following treatment with 10  $\mu$ M FB<sub>1</sub>. Importantly, FB<sub>1</sub> treatment did not alter membrane cholesterol content of cells (data not shown).

### Sphingolipid Depletion Reduces Cell Surface Population of the Human Serotonin<sub>1A</sub> Receptor.

We used a quantitative flow cytometric assay, previously developed by us,<sup>39,43</sup> to monitor the effect of sphingolipid depletion on the expression of serotonin<sub>1A</sub> receptors on the plasma membrane of HEK-5-HT<sub>1A</sub>R cells. This assay allows us to fluorescently label the N-terminal myc-tagged serotonin<sub>1A</sub> receptor with anti-myc Alexa Fluor 488 conjugate antibody to exclusively monitor the receptor population on the cell surface. We quantified changes in the plasma membrane receptor population from relative shifts in the flow cytometric histogram using mode counts (representing cell counts in the modal (peak) channel of the histogram). We observed that there was a considerable shift of the flow cytometric histogram (see [Figure 4a](#)) toward lower fluorescence intensity upon FB<sub>1</sub> treatment (red histogram) relative to control condition (blue histogram). This indicates a considerable reduction in cell surface receptor population of the serotonin<sub>1A</sub> receptor upon sphingolipid depletion. To obtain a quantitative estimate of the population of serotonin<sub>1A</sub> receptors on the plasma membrane, we plotted the mode count values (averaged over five independent experiments) of control and sphingolipid-depleted conditions in [Figure 4b](#). The figure shows that the receptor population on the cell surface was significantly reduced by  $\sim 22\%$  in HEK-5-HT<sub>1A</sub>R cells treated with 10  $\mu$ M FB<sub>1</sub>, relative to control cells. This result suggests that the receptor expression on the plasma membrane of HEK-5-HT<sub>1A</sub>R cells is reduced upon metabolic depletion of sphingolipids.

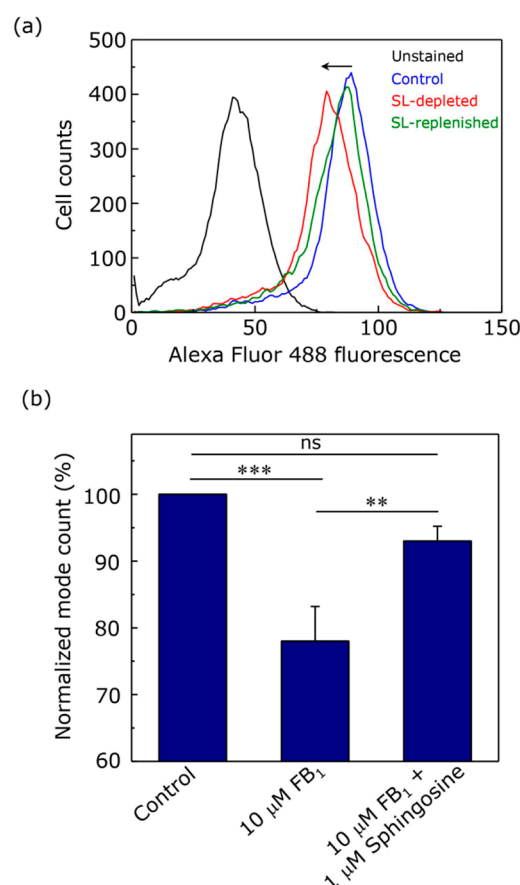
### Replenishment of Sphingolipids Restores Cell Surface Population of the Human Serotonin<sub>1A</sub> Receptor.

In order to explore the specific requirement of sphingolipids in the cell surface expression of the serotonin<sub>1A</sub> receptor, we replenished sphingolipids in cells which were subjected to metabolic sphingolipid depletion. For this, we exogenously added sphingosine in the cell culture medium that serves as a metabolic intermediate to replenish sphingolipids. We extracted total lipids from sphingolipid-replenished cells and quantified the sphingomyelin content using TLC (see [Figure 3a](#)). The figure shows that the sphingomyelin content was restored up to  $\sim 88\%$  upon metabolic replenishment of sphingolipids relative to control cells (see [Figure 3b](#)). Subsequently, the cell surface expression level of the serotonin<sub>1A</sub> receptor was monitored using flow cytometric assay. [Figure 4b](#) shows that, upon sphingolipid replenishment, the cell surface population of the serotonin<sub>1A</sub> receptor was restored to  $\sim 93\%$  relative to control cells (green histogram in [Figure 4a](#)). This suggests that the reduction in the cell surface population of the serotonin<sub>1A</sub> receptor due to metabolic depletion of sphingolipids is reversible. Taken together, these observations clearly demonstrate the specific requirement of sphingolipids in cell surface expression of the serotonin<sub>1A</sub> receptor.

### Sphingolipid Depletion Does Not Alter Total Cellular Expression of the Serotonin<sub>1A</sub> Receptor.

To determine whether the reduction in the cell surface population of the serotonin<sub>1A</sub> receptor upon metabolic depletion of sphingolipids could be due to reduced biosynthesis, we carried out Western blot analysis from whole cell lysates under control and sphingolipid-depleted conditions. [Figure 5a](#) shows protein bands corresponding to the serotonin<sub>1A</sub> receptor and  $\beta$ -tubulin (loading control) under control and sphingolipid-depleted conditions. Upon quantitation using densitometric analysis of

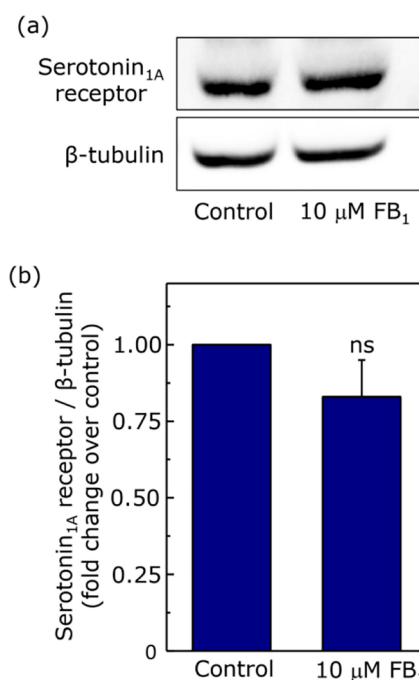




**Figure 4.** Effect of sphingolipid depletion on cell surface population of the serotonin<sub>1A</sub> receptor. (a) An overlay of representative flow cytometric histograms corresponding to unstained (black), control (blue), sphingolipid-depleted (SL-depleted, red), and sphingolipid-replenished (SL-replenished, green) conditions. HEK-5-HT<sub>1A</sub>R cells were fixed and labeled with anti-myc antibody Alexa Fluor 488 conjugate following treatment. A shift in the modal channel (peak) toward lower value on the fluorescence axis (indicated by an arrow) in case of sphingolipid-depleted cells (red) indicates a reduction in serotonin<sub>1A</sub> receptor population on the cell surface. (b) A quantitative estimate of the serotonin<sub>1A</sub> receptor population on the plasma membrane of HEK-5-HT<sub>1A</sub>R cells quantified by flow cytometric assay. Values are expressed as percentages of mode count normalized to control cells (without FB<sub>1</sub> treatment). Data represent means  $\pm$  SE of at least five independent experiments (\*\*\* corresponds to significant ( $p < 0.001$ ) difference in mode count in FB<sub>1</sub>-treated cells relative to control cells, \*\* corresponds to significant ( $p < 0.01$ ) difference in mode count in sphingolipid-replenished cells relative to FB<sub>1</sub>-treated cells, and ns corresponds to lack of significant difference in mode count in sphingolipid-replenished cells relative to control cells as estimated using one-way ANOVA, followed by Bonferroni's multiple comparisons test). See the [Methods](#) section for other details.

bands corresponding to the serotonin<sub>1A</sub> receptor, we did not observe any significant reduction in total cellular expression of serotonin<sub>1A</sub> receptors due to metabolic sphingolipid depletion (see [Figure 5b](#)). These results suggest that the reduction in cell surface population of the serotonin<sub>1A</sub> receptor was not due to reduced protein synthesis but could possibly be due to impaired trafficking of the serotonin<sub>1A</sub> receptor to the cell surface under sphingolipid-depleted condition.

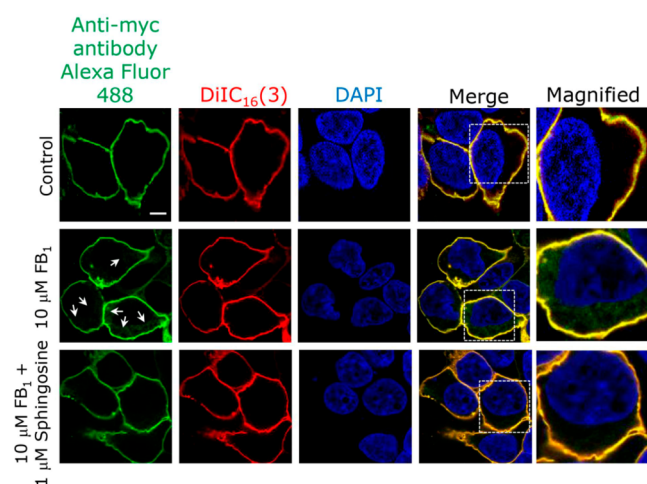
In order to validate our observations, in a complementary approach, we performed confocal microscopic imaging of the serotonin<sub>1A</sub> receptor under sphingolipid-depleted condition.



**Figure 5.** Total cellular expression level of the serotonin<sub>1A</sub> receptor following sphingolipid depletion. Western blot analysis of N-terminal myc-tagged serotonin<sub>1A</sub> receptor expression levels in control and FB<sub>1</sub>-treated HEK-5-HT<sub>1A</sub>R cells. (a) The N-terminal myc-tagged serotonin<sub>1A</sub> receptors with corresponding  $\beta$ -tubulin (loading control) were probed with antibodies directed against myc-tag and  $\beta$ -tubulin, respectively. (b) Expression level of the serotonin<sub>1A</sub> receptor was quantified by using densitometric analysis. Values are expressed as fold change in serotonin<sub>1A</sub> receptor expression level over  $\beta$ -tubulin and normalized to control condition (without FB<sub>1</sub> treatment). Data represent means  $\pm$  SE of at least three independent experiments (ns denotes lack of significant difference between expression level of serotonin<sub>1A</sub> receptors in sphingolipid-depleted cells relative to control cells). See the [Methods](#) section for other details.

[Figure 6](#) shows representative confocal microscopic images of HEK-5-HT<sub>1A</sub>R cells with membranes labeled with DiI<sub>C</sub><sub>16</sub>(3) (red) and serotonin<sub>1A</sub> receptors labeled with anti-myc antibody Alexa Fluor 488 conjugate (green), subsequent to permeabilization of cells. In control condition (in absence of FB<sub>1</sub> treatment), serotonin<sub>1A</sub> receptors were predominantly localized in the plasma membrane (see top row in [Figure 6](#)). Upon metabolic depletion of sphingolipids, we observed a considerable accumulation of the intracellular pool of serotonin<sub>1A</sub> receptors (see middle row of [Figure 6](#), highlighted with arrows), reinforcing our flow cytometric and Western blot data. Interestingly, there was significant clearance of the intracellular pool of the serotonin<sub>1A</sub> receptor upon sphingolipid replenishment (see bottom row in [Figure 6](#)). Taken together with our observations on the reduction of the cell surface receptor population under sphingolipid-depleted condition ([Figure 4](#)), these results suggest a crucial role of sphingolipids in the trafficking of the serotonin<sub>1A</sub> receptor to the plasma membrane.

Sphingolipids are widely implicated in neuronal growth, development, and survival<sup>14,44</sup> and in the progression of several neurological disorders.<sup>3</sup> In particular, recent evidence has shown the involvement of sphingolipids in the pathogenesis of Alzheimer's disease<sup>45–47</sup> and Parkinson's disease.<sup>48–50</sup> Interestingly, imbalance in serotonergic signaling is implicated in



**Figure 6.** Confocal microscopic imaging of the serotonin<sub>1A</sub> receptor upon modulation of sphingolipids. Representative confocal microscopic images of control, sphingolipid-depleted, and sphingolipid-replenished cells. The plasma membrane of HEK-5-HT<sub>1A</sub>R cells was labeled with DiIC<sub>16</sub>(3) (red). Subsequently, cells were fixed and permeabilized, and serotonin<sub>1A</sub> receptors were labeled with anti-myc antibody Alexa Fluor 488 conjugate (green). The nucleus was visualized using DAPI (blue). The regions shown in the box in the merge image are zoomed and shown in the far right panel (magnified) for clarity. The accumulation of the intracellular pool of serotonin<sub>1A</sub> receptors due to metabolic depletion of sphingolipids is highlighted with arrows (see the image on the extreme left in middle row) and showed in the magnified panel on the far right. Upon replenishment of sphingolipids, reduction in intracellular pool of serotonin<sub>1A</sub> receptors was observed (bottom panel). The scale bar represents 10  $\mu$ m. See the [Methods](#) section for other details.

several neuropsychiatric disorders such as Parkinson's disease, schizophrenia, Alzheimer's disease, anxiety, and depression.<sup>51,52</sup> In particular, the serotonin<sub>1A</sub> receptor is shown to play a key role in the pharmacotherapy of Parkinson's disease and schizophrenia.<sup>53,54</sup> However, the mechanistic basis underlying the role of serotonin<sub>1A</sub> receptors and its interaction with membrane lipids in neurological diseases associated with altered sphingolipid metabolism is relatively less explored. Previous work from our laboratory has demonstrated impaired ligand binding, G-protein coupling, cellular signaling, and lateral dynamics of the serotonin<sub>1A</sub> receptor under sphingolipid-depleted condition.<sup>31–35</sup> In this work, we show that the plasma membrane population of the serotonin<sub>1A</sub> receptor exhibits a considerable reduction upon metabolic depletion of sphingolipids upon treatment with FB<sub>1</sub>. The total cellular expression of the receptor remains invariant under these conditions. Importantly, the cell surface population of the serotonin<sub>1A</sub> receptor was restored to control levels upon replenishment of sphingolipids using sphingosine, indicating that the effect of sphingolipid depletion on the cell surface population of the serotonin<sub>1A</sub> receptor is reversible.

The effect of sphingolipids on the sorting and trafficking of GPCRs could be attributed to structural features of GPCRs that could facilitate their preferential association with sphingolipids.<sup>55</sup> Sphingolipids have been reported to interact directly with several membrane proteins, which are involved in diverse physiological processes such as endocytosis, protein sorting, ion conductance, and GPCR function.<sup>15,56,57</sup> We previously showed that specific binding motifs for sphingolipids such as the sphingolipid-binding domain (SBD)<sup>58</sup> and

sphingolipid-binding motif (SBM)<sup>59</sup> are present in the human serotonin<sub>1A</sub> receptor and are evolutionary conserved.<sup>60–63</sup> On the other hand, sphingolipid-dependent membrane protein organization and function could arise due to modulation in membrane physical properties induced by sphingolipids. For example, sphingomyelin is known to affect sorting of membrane proteins by modulating membrane thickness along with cholesterol and has been implicated in efficient sorting of membrane proteins and receptors.<sup>56,64,65</sup> We have previously shown that metabolic depletion of sphingolipids by FB<sub>1</sub> leads to an increase in membrane fluidity which could be due to disruption of ordered sphingomyelin-rich domains.<sup>31</sup> Such effects on membrane physical properties due to altered sphingolipid content could be manifested at a cellular level in terms of altered membrane protein function and trafficking.<sup>66</sup>

It has been previously reported that depletion of sphingolipids (such as sphingomyelin or glycosphingolipids) leads to impaired trafficking and cell surface targeting of the nicotinic acetylcholine receptor.<sup>67</sup> Based on their results, the authors proposed a “chaperone-like” role of sphingolipids at early stages of the nicotinic acetylcholine receptor biosynthetic pathway, affecting receptor trafficking to the cell surface. In case of GPCRs, it was shown that cholesterol and sphingolipids have differential effects on the conformation, function, and trafficking of the cholecystokinin receptor.<sup>68</sup> In this backdrop, our present results showing reduced plasma membrane population of the serotonin<sub>1A</sub> receptor, without affecting total cellular receptor content, suggest the possibility of trafficking defects of GPCRs associated with altered sphingolipid condition. We conclude that sphingolipid depletion could impair trafficking of membrane receptors to the plasma membrane and could provide mechanistic insight into sphingolipid-mediated regulation of GPCR function and trafficking in healthy and diseased conditions.

## METHODS

**Materials.** D-erythro-sphingosine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-terazolium bromide (MTT), doxycycline, fumonisins B<sub>1</sub>(FB<sub>1</sub>), gentamycin, penicillin, poly-L-lysine, streptomycin, sodium bicarbonate, Triton X-100, Tris base, and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO).  $\beta$ -Tubulin monoclonal antibody (BT7R), DMEM/F-12 [Dulbecco's modified Eagle's medium/nutrient mixture F-12(Ham) (1:1)], fetal calf serum (FCS), and hygromycin B were obtained from Invitrogen/Life technologies (Grand Island, NY). Formaldehyde solution (~37–41%, w/v) was purchased from Merck (Darmstadt, Germany). Anti-myc tag antibody Alexa Fluor 488 conjugate and precoated silica gel 60 thin layer chromatography plates were from Millipore (Bedford, MA). Protease inhibitor cocktail was purchased from Roche Applied Science (Mannheim, Germany). Bicinchoninic acid (BCA) reagent for protein estimation was from Pierce (Rockford, IL). Myc-tag mouse mAb (9B11) was from Cell Signaling Technology, Inc. (Danvers, MA). HRP goat anti-mouse IgG antibody was purchased from BioLegend (San Diego, CA). Porcine brain sphingomyelin was purchased from Avanti Polar Lipids (Alabaster, AL). DiIC<sub>16</sub>(3) was purchased from Molecular Probes/Invitrogen (Eugene, OR). Vectashield mounting medium containing DAPI was purchased from Vector Laboratories Inc. (Burlingame, CA). All solvents used were of analytical grade. All other chemicals used were of highest purity available. Water was purified through a Millipore Milli-Q system (Bedford, MA) and used throughout.

**Cell Culture.** HEK-5-HT<sub>1A</sub>R cells were maintained in DMEM/F-12 complete medium [DMEM/F-12 (1:1) media supplemented with 2.4 g/L of sodium bicarbonate, 10% (v/v) FCS, 60  $\mu$ g/mL penicillin, 50  $\mu$ g/mL streptomycin, 50  $\mu$ g/mL gentamycin sulfate, and 250  $\mu$ g/mL hygromycin B] in a humidified atmosphere with 5% CO<sub>2</sub> at 37

°C. Receptor expression was induced with 1  $\mu$ g/mL doxycycline for 24 h prior to experiments.

**Treatment with FB<sub>1</sub>.** Stock solution (1 mM) of FB<sub>1</sub> was prepared in water, and cells grown in poly-L-lysine coated plates for 24 h were treated with 10  $\mu$ M FB<sub>1</sub> in DMEM/F-12 medium containing 5% (v/v) FCS and incubated for 60 h.<sup>31</sup> Control cells were grown under similar conditions without FB<sub>1</sub>.

**Metabolic Replenishment of Sphingolipids Using Sphingosine.** Following treatment with 10  $\mu$ M FB<sub>1</sub> for 60 h, HEK-5-HT<sub>1A</sub>R cells were grown for 24 h in DMEM/F-12 complete medium supplemented with 1  $\mu$ M sphingosine in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C to achieve metabolic replenishment of sphingolipids.<sup>31</sup>

**MTT Assay for Cell Viability.** MTT assay was performed to assess the viability of HEK-5-HT<sub>1A</sub>R cells under treatment conditions as described previously.<sup>69</sup> HEK-5-HT<sub>1A</sub>R cells were plated in a 96-well plate, and treatments were carried out as described above. Subsequently, MTT was dissolved in serum-free DMEM/F-12 medium and added to the cells at a final concentration of 0.5 mg/mL, followed by incubation at 37 °C for 1 h. Formazan crystals formed upon reduction of MTT salt by mitochondrial enzymes in live cells were then dissolved in DMSO after removing the medium. The color obtained was measured by absorbance at 540 nm using an EnSpire 2300 Multimode Plate Reader (PerkinElmer, Waltham, MA).

**Cell Membrane Preparation.** Cell membranes from control, sphingolipid-depleted, and sphingolipid-replenished cells were prepared as described previously.<sup>70</sup> Briefly, confluent HEK-5-HT<sub>1A</sub>R cells were harvested in ice-cold hypotonic buffer containing 10 mM Tris and 5 mM EDTA, pH 7.4. Cells were then homogenized for 10 s at 4 °C at maximum speed using a Polytron homogenizer. The cell lysate was centrifuged at 500  $\times$  g for 10 min at 4 °C, and the resulting postnuclear supernatant was centrifuged at 40 000  $\times$  g for 30 min at 4 °C. The pellet obtained was finally resuspended in 50 mM Tris buffer, pH 7.4, and kept at -70 °C until further use. Total protein concentration in isolated membranes was determined using BCA reagent.<sup>71</sup>

**Estimation of Sphingomyelin Content by Thin Layer Chromatography.** Total lipid extraction from membranes isolated from control, sphingolipid-depleted, and sphingolipid-replenished cells were performed according to the Bligh and Dyer method.<sup>72</sup> Lipid extracts from ~1 mg protein were dried under a stream of nitrogen at ~45 °C, and the dried lipids were then dissolved in chloroform/methanol (1:1 v/v). Extracted lipids were separated by TLC on precoated silica TLC plate using chloroform/methanol/0.22% (w/v) aqueous CaCl<sub>2</sub> (60:35:8, v/v/v) as a solvent system.<sup>73</sup> A sphingomyelin standard was run to identify the sphingomyelin band in lipid extracts. Sphingomyelin bands were quantified based on densitometric analysis using ImageJ (NIH, Bethesda, MD), and the values were normalized to control cells (without FB<sub>1</sub> treatment).

**Flow Cytometric Analysis of Cell Surface Population of the Serotonin<sub>1A</sub> Receptor.** The receptor population on the plasma membrane of cells was monitored using a quantitative flow cytometric method as described previously.<sup>39,43</sup> In brief, cells were scraped after treatment and collected in ice-cold PBS. Cells were subsequently fixed with 4% (w/v) formaldehyde in PBS for 30 min on ice and stained with anti-myc antibody Alexa Fluor 488 conjugate (1:100 dilution) in PBS containing 2% (v/v) FCS for 60 min on ice. Cells were then washed and resuspended in PBS. The plasma membrane receptor population from ~10 000 cells was quantified using MoFlo XDP flow cytometer (Brea, CA), and Summit analysis software (version 5.4.0) was used to acquire and analyze data. Alexa Fluor 488 was excited at 488 nm, and emission was collected using a 529/28 nm band-pass filter. Changes in mode count (cell count value in the peak channel) values in each case were analyzed with respect to control condition.

**Western Blot Analysis of Serotonin<sub>1A</sub> Receptor Expression Level Following FB<sub>1</sub> Treatment.** Total cellular protein was extracted from control and FB<sub>1</sub>-treated HEK-5-HT<sub>1A</sub>R cells by using radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor cocktail (1:20 dilution). Samples were prepared by incubating 25  $\mu$ g of protein in electrophoresis sample buffer for 30

min at 37 °C. The samples were loaded and separated on a 12% SDS-PAGE gel. The separated proteins were transferred to a nitrocellulose membrane using a semidry transfer apparatus (Amersham Pharmacia Biotech, Little Chalfont, UK). The nitrocellulose membrane was incubated in 5% (w/v) BSA (prepared in tris buffer saline containing 0.1% (v/v) Tween 20 (TBST)) for 1 h at room temperature (~23 °C). To detect the N-terminal myc-tagged serotonin<sub>1A</sub> receptor, the membrane was probed with myc-tag mouse mAb (1:5 000 dilution) in 5% (w/v) BSA (in TBST) overnight at 4 °C. Subsequently, the membrane was washed 3 times with TBST and incubated with HRP goat anti-mouse IgG antibody (1:10 000 dilution) for 1 h at room temperature (~23 °C).  $\beta$ -Tubulin monoclonal antibody (1:5000 dilution) was used to detect  $\beta$ -tubulin (loading control). Protein bands were detected using enhanced chemiluminescence detection reagents (BioRad, Hercules, CA), and images were acquired in a Chemi-Smart 5000 chemiluminescence detection system (Vilber Lourmat, Marne-la-Vallée, France). Band intensities were quantified by densitometric analysis using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA) software. Intensities of the bands corresponding to serotonin<sub>1A</sub> receptors were normalized to  $\beta$ -tubulin band intensities.

**Confocal Microscopic Imaging of Serotonin<sub>1A</sub> Receptor Cellular Localization.** Cells were plated on poly-L-lysine coated glass coverslips, and sphingolipid modulation was carried out as described above. To obtain representative confocal microscopic images for receptor distribution, cells were washed with ice-cold PBS and placed on ice. Cell membranes were labeled with DiI<sub>16</sub>(3) as described previously<sup>39</sup> with some modifications. Briefly, cells were incubated with 8  $\mu$ M DiI<sub>16</sub>(3) in PBS for 10 min on ice. Subsequently, cells were washed with ice-cold PBS, fixed with 4% (w/v) formaldehyde, and permeabilized with 0.5% (v/v) Triton X-100 on ice. Cells were subsequently stained with the anti-myc antibody Alexa Fluor 488 conjugate (1:100 dilution) for 60 min on ice, washed, and mounted using Vectashield mounting medium containing DAPI. Images were acquired on a Zeiss (Jena, Germany) LSM 880 confocal microscope. N-terminal myc-tagged serotonin<sub>1A</sub> receptors were imaged by exciting anti-myc antibody Alexa Fluor 488 conjugate at 488 nm and collecting the emission between 495 and 535 nm. To image plasma membranes, DiI<sub>16</sub>(3) was excited at 543 nm, and the emission was collected between 550 and 620 nm. Images of z-sections were acquired with a 63 $\times$ /1.4 NA oil immersion objective under 1 airy condition with a fixed step size of 0.5  $\mu$ m. Images shown are from a midplane section of the acquired z-stack.

**Statistical Analysis.** Significance levels were estimated using one-way ANOVA, followed by Bonferroni's multiple comparisons test or Student's two tailed unpaired *t*-test using GraphPad Prism, version 4.0 (San Diego, CA). Plots were generated using OriginPro, version 8.0 (OriginLab, Northampton, MA).

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## Author Contributions

A.K. performed experiments. A.K. and P.S. analyzed data and prepared figures. A.K., P.S., and A.C. designed experiments. A.K., P.S., and A.C. wrote the manuscript. A.C. edited the manuscript, organized access to research facilities and funding, and provided overall supervision and mentoring.

## Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS:

BCA, bichinchoninic acid; FB<sub>1</sub>, fumonisins B<sub>1</sub>; FCS, fetal calf serum; GPCR, G protein-coupled receptor; HEK-5-HT<sub>1A</sub>R, HEK-293 cells stably expressing N-terminal myc-tagged human serotonin<sub>1A</sub> receptors; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PBS, phosphate buffered saline

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