Metabolic Depletion of Sphingolipids Impairs Ligand Binding and Signaling of Human Serotonin$_{1A}$ Receptors[†]

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ABSTRACT: Sphingolipids are essential components of eukaryotic cell membranes and are thought to be involved in a variety of cellular functions. Mycotoxins such as fumonisins can disrupt sphingolipid metabolism, and treatment with fumonisins represents an efficient approach to modulate cellular sphingolipid levels. In this work, we modulated sphingolipid levels in CHO cells stably expressing the human serotonin$_{1A}$ receptor by metabolically inhibiting the biosynthesis of sphingolipids using fumonisin B$_1$. Serotonin$_{1A}$ receptors belong to the family of seven-transmembrane domain receptors that couple to G-proteins and are implicated in the generation and modulation of various cognitive, behavioral, and developmental functions. We explored the function of serotonin$_{1A}$ receptors under sphingolipid-depleted conditions by monitoring ligand binding, G-protein coupling, and downstream signaling of the receptor. Importantly, our results show that the function of the serotonin$_{1A}$ receptor is impaired upon metabolic depletion of sphingolipids, although the membrane receptor level does not exhibit any reduction. Interestingly, we find that the replenishment of sphingolipids using sphingosine results in restoration of ligand binding of serotonin$_{1A}$ receptors, demonstrating that the loss of ligand binding due to metabolic depletion of sphingolipids is reversible. These novel results demonstrate that sphingolipids are necessary for ligand binding and downstream signaling of the human serotonin$_{1A}$ receptor. We discuss possible mechanisms of specific interaction of sphingolipids with the serotonin$_{1A}$ receptor that could involve the proposed "sphingolipid-binding domain" (SBD).

Sphingolipids are essential and indispensable components of eukaryotic cell membranes and constitute 10−20% of the total membrane lipids (*1*). They are implicated in the regulation of cell growth, differentiation, and neoplastic transformation through participation in cell−cell communication, and possible interaction with receptors and signaling systems. Sphingolipids such as sphingomyelin are regarded as reservoirs for bioactive second messengers such as ceramide and sphingosine 1-phosphate (*2*). Sphingolipids are abundant in the plasma membrane compared to intracellular membranes. Their distribution in the bilayer appears to be heterogeneous, and it has been postulated that sphingolipids and cholesterol exist in laterally segregated lipid domains (sometimes termed "lipid rafts") (*3, 4*). Many of these domains are believed to be important for the maintenance of membrane structure and function, although analyzing the spatiotemporal resolution of these domains is proving to be challenging (*5*). The idea of such membrane domains gains significance since physiologically important functions such as cellular

membrane sorting, trafficking (*6*), signal transduction (*7*), and the entry of pathogens into cells (*8*) have been attributed to these domains.

Sphingolipid levels in cells can be modulated using fumonisins. Fumonisins have been extensively used to explore functions of sphingomyelin and complex sphingolipids (*9*). Fumonisins make up a group of naturally occurring mycotoxins, which are ubiquitous contaminants of corn and other grain products, produced by *Fusarium verticelloides* and several other *Fusarium* species (*10, 11*). There are at least 14 known fumonisins; fumonisin B$_1$ (FB$_1$)[1] is the most abundant (*12*). FB$_1$ is structurally similar to sphingoid bases such as sphinganine and sphingosine (see Figure 1), which are intermediates in sphingolipid metabolism. FB$_1$ inhibits the reaction catalyzed by sphinganine *N*-acetyltransferase (ceramide synthase) (*11, 13*). Consumption of FB$_1$ through contaminated corn has been reported to induce neurotoxicity (*9, 14*) and esophageal and liver cancer in humans (*15*). Although little is known about the molecular mechanism of action by which these mycotoxins induce carcinogenic effects, disruption of the sphingolipid metabolism appears

[1]Abbreviations: 5-HT$_{1A}$, 5-hydroxytryptamine-1A; 5-HT$_{1A}$R-EYFP, 5-hydroxytryptamine-1A receptor tagged with EYFP; 8-OH-DPAT, 8-hydroxy-2-(di-*N*-propylamino)tetralin; BCA, bicinchoninic acid; DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; EYFP, enhanced yellow fluorescent protein; FB$_1$, fumonisin B$_1$; GPCR, G-protein-coupled receptor; GTP-γ-S, guanosine 5′-*O*-(3-thiotriphosphate); *p*-MPPF, 4-(2′-methoxy)phenyl-1-[2′-(*N*-2′′-pyridinyl)-*p*-fluorobenzamido]ethylpiperazine; PMSF, phenylmethanesulfonyl fluoride; SBD, sphingolipid-binding domain; TLC, thin layer chromatography.
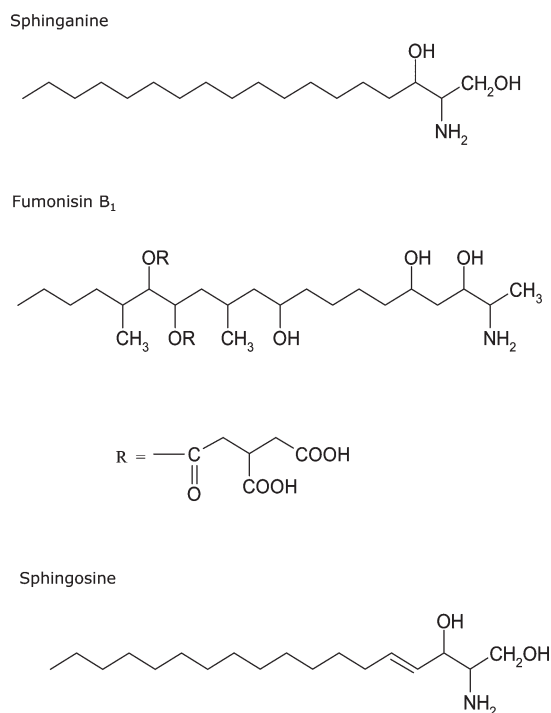
Sphinganine

Fumonisin B$_1$

Sphingosine

FIGURE 1: Chemical structures of sphinganine, fumonisin B$_1$, and sphingosine. Fumonisin B$_1$ is a potent and competitive inhibitor of ceramide synthase (*N*-acetyltransferase), the enzyme that catalyzes the acylation of sphinganine in *de novo* biosynthesis of sphingolipids and the reutilization of sphingosine derived from sphingolipid turnover. See the text for details.

to be a major factor. It has been previously demonstrated that inhibition of sphingolipid biosynthesis using FB$_1$ results in depletion of cellular (glyco)sphingolipids and significantly affects axonal growth, suggesting that sphingolipids may play a vital role in regulating neuronal development (*16*, *17*). Sphingolipids have been demonstrated to regulate apoptosis, survival, and regeneration of cells in the nervous system. In addition, the role of sphingolipids in the development and progression of several neurological diseases such as Alzheimer's disease is well-documented (*18*), which could be due to impaired neurotransmission. Modulating sphingolipid levels and monitoring the function of an important neurotransmitter receptor therefore assume relevance.

The serotonin$_{1A}$ (5-HT$_{1A}$) receptor is an important neurotransmitter receptor belonging to the G-protein-coupled receptor (GPCR) superfamily and is the most extensively studied among the serotonin receptors for a number of reasons (*19*). Serotonin$_{1A}$ receptors play a key role in the generation and modulation of various cognitive, behavioral, and developmental functions such as sleep, mood, addiction, depression, anxiety, aggression, and learning (*20*). This is supported by the fact that agonists and antagonists of the serotonin$_{1A}$ receptor represent major classes of molecules with potential therapeutic effects in anxiety- or stress-related disorders. Interestingly, mutant (knockout) mice lacking the serotonin$_{1A}$ receptor exhibit enhanced anxiety-related behavior and represent an important animal model for the analysis of complex traits such as anxiety disorders and aggression in higher animals (*21*). In the context of increasing pharmacological relevance of the serotonin$_{1A}$ receptor, a transmembrane protein, its interaction with the surrounding lipids such as sphingolipids assumes significance in modulating the function of the receptor in healthy and diseased states (*22*).

In this work, we have modulated sphingolipid levels in CHO cells stably expressing the human serotonin$_{1A}$ receptor (CHO-5-HT$_{1A}$R) by metabolically inhibiting the biosynthesis of sphingolipids. To achieve this, we utilized FB$_1$, which is a specific inhibitor of ceramide synthase. FB$_1$ treatment results in reduction of sphingomyelin levels in CHO-5-HT$_{1A}$R cells. We explored the function of the human serotonin$_{1A}$ receptor under these conditions by monitoring ligand binding, G-protein coupling, and downstream signaling of the receptor. Our results show that the function of the serotonin$_{1A}$ receptor is impaired upon metabolic depletion of sphingolipids, although the membrane receptor level does not exhibit any reduction. Interestingly, we show here that the effect of sphingolipids on the ligand binding of serotonin$_{1A}$ receptors caused by metabolic depletion of sphingolipids is reversible.

EXPERIMENTAL PROCEDURES

Materials. DMPC, fumonisin B$_1$, EDTA, MgCl$_2$, MnCl$_2$, 8-OH-DPAT, penicillin, streptomycin, gentamycin sulfate, polyethylenimine, PMSF, *p*-MPPI, primuline, serotonin, sodium bicarbonate, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). D-MEM/F-12 [Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Ham) (1:1)], fetal calf serum, and Geneticin (G 418) were from Invitrogen Life Technologies (Carlsbad, CA). GTP-γ-S was from Roche Applied Science (Mannheim, Germany). Porcine brain sphingomyelin was purchased from Avanti Polar Lipids (Alabaster, AL). BCA reagent for protein estimation was from Pierce (Rockford, IL). Forskolin and IBMX were obtained from Calbiochem (San Diego, CA). [^{3}H]-8-OH-DPAT (specific activity of 135.0 Ci/mmol) and [^{3}H]-*p*-MPPF (specific activity of 70.5 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA). The cyclic [^{3}H]AMP assay kit was purchased from Amersham Biosciences (Buckinghamshire, U.K.). GF/B glass microfiber filters were from Whatman International (Kent, U.K.). Precoated silica gel 60 thin layer chromatography plates were from Merck (Darmstadt, Germany). All other chemicals and solvents used were of the highest available purity. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Cell Culture and FB$_1$ Treatment. CHO cells stably expressing the human serotonin$_{1A}$ receptor (termed CHO-5-HT$_{1A}$R) and CHO cells stably expressing the human serotonin$_{1A}$ receptor tagged with enhanced yellow fluorescent protein (termed CHO-5-HT$_{1A}$R-EYFP) were maintained in D-MEM/F-12 (1:1) supplemented with 2.4 g of sodium bicarbonate per liter, 10% fetal calf serum, 60 μg/mL penicillin, 50 μg/mL streptomycin, 50 μg/mL gentamycin sulfate, and 200 μg/mL Geneticin in a humidified atmosphere with 5% CO$_2$ at 37 °C. Stock solutions (1 mM) of FB$_1$ were prepared in water and added to cells grown for 24 h (final concentration of FB$_1$ of 2$-$6 μM) and incubated in 5% serum for 63$-$66 h. Control cells were grown under similar conditions without FB$_1$.

Cell Membrane Preparation. Cell membranes were prepared as described previously (*23*). The total protein concentration was determined using the BCA assay (*24*).

Estimation of Sphingomyelin by Thin Layer Chromatography. Total lipid extraction from membranes of control and FB$_1$-treated cells was conducted according to the method of Bligh and Dyer (*25*). Lipid extracts were dried under a stream of nitrogen at 45 °C. The dried extracts were dissolved in a chloroform/methanol mixture (1:1, v/v). Total lipid extracts were

resolved by thin layer chromatography (TLC) using a chloroform/methanol/acetic acid/water mixture (25:15:4:2, v/v/v/v) as the solvent system (*26*). We visualized the separated lipids under ultraviolet light by spraying a fluorescent solution of 0.01% (w/v) primuline prepared in acetone (*27*). The sphingomyelin standard was used to identify sphingomyelin bands on TLC plates run with total lipid extracts obtained from control, FB$_1$-treated, and sphingomyelin-replenished cells. The sphingomyelin bands were scraped from TLC plates; lipids were re-extracted with a chloroform/methanol mixture (1:1, v/v) from samples, and the phosphate content was estimated and normalized to the phosphate content obtained from control samples.

Estimation of Inorganic Phosphate. The concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid (*28*) using Na$_2$HPO$_4$ as the standard. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

Radioligand Binding Assays. Receptor binding assays were conducted as described previously (*23*) with ∼50 μg of total protein. The concentrations of [^{3}H]-8-OH-DPAT and [^{3}H]-*p*-MPPF in each assay tube were 0.29 and 0.5 nM, respectively.

GTP-γ-S Sensitivity Assay. To estimate the efficiency of G-protein coupling, GTP-γ-S sensitivity assays were conducted as described previously (*29*). The concentrations of GTP-γ-S leading to 50% inhibition of specific agonist binding (IC$_{50}$) were calculated by nonlinear regression fitting of the data to a four-parameter logistic function (*30*):

$$B = a/[1 + (x/I)^s] + b \qquad (1)$$

where *B* is specific binding of the agonist normalized to agonist binding at the lowest concentration of GTP-γ-S, *x* denotes the concentration of GTP-γ-S, *a* is the range ($y_{max} - y_{min}$) of the fitted curve on the ordinate (*y*-axis), *I* is the IC$_{50}$ concentration, *b* is the background of the fitted curve (y_{min}), and *s* is the slope factor.

Estimation of Cyclic AMP Content in Cells. The ability of ligands to affect the forskolin-stimulated increase in cAMP levels in CHO-5-HT$_{1A}$R cells was assessed as described previously (*29*). The amount of cAMP in an aliquot of the supernatant was estimated using the cyclic [^{3}H]AMP assay system which is based on the protein binding method described previously (*31*). Agonist-dependent dose−response curves were analyzed according to a four-parameter logistic function:

$$C = a/[1 + (x/I)^s] + b \qquad (1')$$

where *C* is the cAMP level normalized to the level of cAMP in cells stimulated with 10 mM forskolin, *x* denotes the concentration of 8-OH-DPAT, *a* is the range ($y_{max} - y_{min}$) of the fitted curve on the ordinate (*y*-axis), *I* is the IC$_{50}$ concentration of 8-OH-DPAT for half-maximal reduction of cAMP, *b* is the background of the fitted curve (y_{min}), and *s* is the slope factor.

Fluorescence Anisotropy Measurements. Fluorescence anisotropy experiments were conducted using the fluorescent probe DPH with membranes prepared from control cells and cells that have been treated with varying concentrations of FB$_1$, containing 50 nmol of total phospholipids suspended in 1.5 mL of 50 mM Tris buffer (pH 7.4), as described previously (*22, 32*). Steady state fluorescence was measured in a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes at room temperature (∼23 °C). Excitation and emission

wavelengths were set at 358 and 430 nm, respectively. Excitation and emission slits with nominal bandpasses of 1.5 and 20 nm were used. The optical density of the samples measured at 358 nm was ∼0.15. The anisotropy values remained identical even after dilution of membrane samples, indicating the absence of any scattering artifact (*32*). Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy (*r*) values were calculated from the equation (*33*)

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

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. *G* is the grating correction factor, the ratio of the efficiencies of the detection system for vertically and horizontally polarized light, and equal to I_{HV}/I_{HH}. All experiments were conducted with multiple sets of samples, and average values of fluorescence anisotropy are shown in Figure 5.

Metabolic Replenishment of Sphingolipids Using Sphingosine. Following treatment with 6 μM FB$_1$, CHO-5-HT$_{1A}$R cells were grown for 24 h in D-MEM/F-12 (1:1) supplemented with 1 μM sphingosine, 2.4 g/L sodium bicarbonate, 10% fetal calf serum, 60 μg/mL penicillin, 50 μg/mL streptomycin, and 50 μg/mL gentamycin sulfate in a humidified atmosphere with 5% CO$_2$ at 37 °C to achieve metabolic replenishment of sphingolipids.

Confocal Microscopy and Live Cell Imaging. To visualize the serotonin$_{1A}$ receptor, CHO cells stably expressing the serotonin$_{1A}$ receptor tagged with enhanced yellow fluorescent protein (termed CHO-5-HT$_{1A}$R-EYFP) were used (*34*). CHO-5-HT$_{1A}$-R-EYFP cells were plated at a density of 5×10^4 cells on a 40 mm glass coverslip and were grown in D-MEM/F-12 medium with or without FB$_1$. Coverslips were washed twice with 3 mL of HEPES-Hanks buffer (pH 7.4) and mounted on an FCS2 closed temperature-controlled Bioptechs (Butler, PA) chamber. The chamber was gently perfused with 10 mL of the same buffer and was allowed to reach 37 °C, which took ∼10 min. Images were acquired on an inverted Zeiss (Jena, Germany) LSM 510 Meta confocal microscope, with a 63×, 1.2 NA water immersion objective using the 514 nm line of an argon laser. EYFP fluorescence emission was collected using the 535−590 nm bandpass filter.

Western Blot Analysis. Cell membranes were prepared from CHO-5-HT$_{1A}$R-EYFP cells (control), FB$_1$-treated cells, and cells subjected to FB$_1$ treatment followed by sphingosine treatment as described previously (*29*). Freshly prepared protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) was added to membranes in a 1:20 dilution. Western blot analysis was performed as described previously (*22*).

Statistical Analysis. Significance levels were estimated using Student's two-tailed paired *t*-test using Origin version 5.0 (OriginLab Corp., Northampton, MA).

RESULTS

Quantitation of Sphingomyelin upon Metabolic Depletion Using FB$_1$. CHO cells stably expressing the human serotonin$_{1A}$ receptor (CHO-5-HT$_{1A}$R) were treated with FB$_1$, to achieve metabolic depletion of sphingolipids. FB$_1$ is a potent and competitive inhibitor of ceramide synthase, the enzyme that
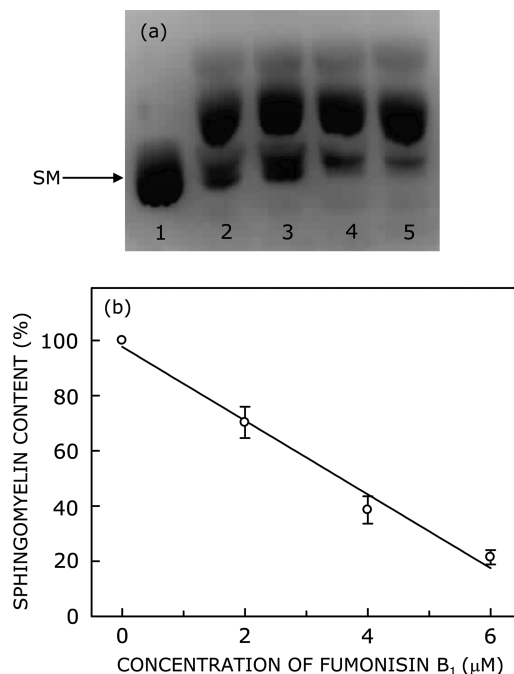
FIGURE 2: Estimation of sphingomyelin content in membranes isolated from control cells and cells treated with increasing concentrations of FB_1. Total lipids were extracted from membranes of cells treated with increasing concentrations of FB_1 and were separated by thin layer chromatography (a). Lanes contained lipids extracted from membranes isolated from control cells (lane 2) and cells treated with 2 (lane 3), 4 (lane 4), and 6 (lane 5) μM FB_1. The arrow represents the position of sphingomyelin identified using a standard in lane 1. Sphingomyelin contents were quantified by estimation of phosphate content and are shown in panel (b). Values are expressed as percentages of the sphingomyelin content of membranes from control cells (without any treatment). Data represent means ± the standard error of at least three independent experiments. See Experimental Procedures for other details.

catalyzes acylation of sphinganine in *de novo* biosynthesis of sphingolipids and the reutilization of sphingosine derived from sphingolipid turnover (*35*). The structures of sphingosine, sphinganine, and FB_1 are shown in Figure 1. Besides disrupting sphingolipid metabolism, FB_1 is known to induce oxidative stress leading to cytotoxicity when used at high concentrations (*36*). It is therefore important to ensure that FB_1 concentrations used are below the concentration range in which cytotoxic effects are predominant. It has previously been shown that treatment up to 50 μM FB_1 does not result in cell death (*37, 38*). We therefore chose to use low concentrations of FB_1, and the concentration of FB_1 used in this work never exceeded 6 μM. Total lipids were extracted from membranes prepared from control and FB_1-treated cells and were separated on TLC plates (shown in Figure 2a). Sphingomyelin bands were scraped from TLC plates, and their phosphate contents were estimated as described in Experimental Procedures and shown in Figure 2b. The sphingomyelin content shows a progressive reduction with increasing FB_1 concentration. Figure 2b shows that ~80% of sphingomyelin is depleted in CHO-5-HT_{1A}R cells treated with 6 μM FB_1. Importantly, the membrane cholesterol content of cells treated with FB_1 remains unaltered, even when the highest concentration of FB_1 is used (not shown).

Specific Ligand Binding of the Human Serotonin$_{1A}$ Receptor Is Reduced upon Metabolic Depletion of Sphingolipids. To monitor the effect of metabolic depletion of sphingolipids on the ligand binding activity of the serotonin$_{1A}$
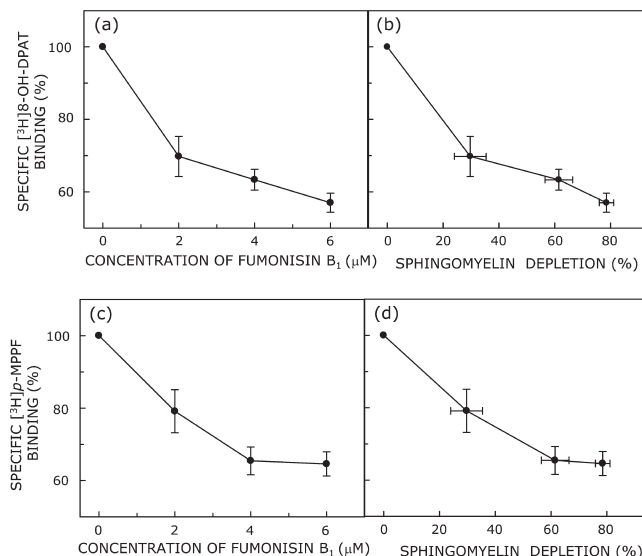

FIGURE 3: Effect of metabolic depletion of sphingolipids on specific ligand binding of the human serotonin$_{1A}$ receptor. CHO-5-HT_{1A}R cells were treated with increasing concentrations of FB_1, and specific [³H]-8-OH-DPAT binding to the serotonin$_{1A}$ receptor in membranes isolated from these cells was measured (a). The change in specific [³H]-8-OH-DPAT binding is plotted with increasing sphingomyelin depletion (b). Data shown are means ± the standard error of at least three independent experiments. Effects of increasing concentrations of FB_1 and accompanying sphingomyelin depletion on specific [³H]-*p*-MPPF binding to the serotonin$_{1A}$ receptor are shown in panels (c) and (d), respectively. Values are expressed as percentages of specific binding for control cell membranes without FB_1 treatment. Data shown are means ± the standard error of at least three independent experiments. Data for the extent of sphingomyelin depletion with increasing concentrations of FB_1 (b and d) are from Figure 2b. See Experimental Procedures for other details.

receptor, CHO-5-HT_{1A}R cells were treated with varying concentrations of FB_1 and ligand binding was assessed. For this, we measured binding of the selective serotonin$_{1A}$ receptor agonist [³H]-8-OH-DPAT and antagonist [³H]-*p*-MPPF to cell membranes prepared from CHO-5-HT_{1A}R cells under control (without FB_1 treatment) and FB_1-treated conditions. Figure 3 shows the decrease in the level of specific binding of the serotonin$_{1A}$ receptor agonist [³H]-8-OH-DPAT with increasing concentrations of FB_1 (Figure 3a), and the accompanying reduction in membrane sphingomyelin levels (Figure 3b). The level of specific agonist binding is reduced to ~57% of the original value upon metabolic depletion of ~80% sphingomyelin. The effects of increasing concentrations of FB_1 and accompanying sphingomyelin depletion on specific [³H]-*p*-MPPF binding to the serotonin$_{1A}$ receptor are shown in panels (c) and (d), respectively. Figure 3c shows that the level of specific binding of the antagonist [³H]-*p*-MPPF is decreased upon FB_1 treatment and is reduced to ~65% of its original value upon treatment with 6 μM FB_1. Figure 3 shows that the reduction in the level of ligand binding is more pronounced up to an ~30% loss of sphingomyelin (corresponding to 2 μM FB_1). It has been previously reported that neurotransmitters such as serotonin could bind to gangliosides in cell membranes (*39*). To rule out the possibility that 8-OH-DPAT could bind to gangliosides, we monitored the binding of [³H]-8-OH-DPAT to untransfected CHO cells (*i.e.*, CHO cells without the serotonin$_{1A}$ receptor). Our results show that [³H]-8-OH-DPAT does not significantly bind to untransfected CHO cell membranes (data not shown). Taken together, these results show that the reduction in membrane

sphingomyelin content in CHO-5-HT$_{1A}$R cells by metabolic depletion using FB$_1$ results in the loss of the serotonin$_{1A}$ receptor ligand binding ability.

The Ligand-Dependent Downstream Signaling Efficiency of the Human Serotonin$_{1A}$ Receptor Is Reduced upon Metabolic Depletion of Sphingolipids. Most of the seven-transmembrane domain receptors are coupled to G-proteins, and therefore, guanine nucleotides are known to modulate ligand binding. The serotonin$_{1A}$ receptor agonists such as 8-OH-DPAT are known to specifically activate the G$_i$/G$_o$ class of G-proteins in CHO cells (*40*). Agonist binding to such receptors therefore displays sensitivity to agents such as GTP-γ-S, a nonhydrolyzable analogue of GTP, that uncouple the normal cycle of guanine nucleotide exchange at the Gα subunit caused by receptor activation. We have previously shown that in the presence of GTP-γ-S, serotonin$_{1A}$ receptors undergo an affinity transition, from a high-affinity G-protein-coupled state to a low-affinity G-protein-uncoupled state (*41*). In agreement with these results, Figure 4a shows a characteristic reduction in the level of binding of the agonist [^{3}H]-8-OH-DPAT in the presence of increasing concentrations of GTP-γ-S with an estimated half-maximal inhibition concentration (IC$_{50}$) of 3.41 nM for control cells. The inhibition curve in the case of cells treated with 6 μM FB$_1$ displays a significant shift toward higher concentrations of GTP-γ-S with an increased IC$_{50}$ value of 19.75 nM (~5-fold; *p* < 0.05). This implies that the agonist binding to the serotonin$_{1A}$ receptor upon metabolic depletion of sphingolipids is less sensitive to GTP-γ-S, indicating that the G-protein coupling efficiency is reduced under these conditions. This indicates a possible perturbation of receptor−G-protein interaction upon metabolic depletion of sphingolipids.

In addition to ligand binding properties, we monitored the function of serotonin$_{1A}$ receptors in CHO-5-HT$_{1A}$R cells by measuring their ability to catalyze downstream signal transduction processes upon stimulation with the specific agonist, 8-OH-DPAT. Serotonin$_{1A}$ receptor agonists such as 8-OH-DPAT are known to specifically activate the G$_i$/G$_o$ class of G-proteins in CHO cells and subsequently reduce cAMP levels in cells (*40, 42*). As shown in Figure 4b, the forskolin-stimulated increase in cAMP levels is inhibited by 8-OH-DPAT with a half-maximal inhibition concentration (IC$_{50}$) of 9.49 nM in control cells. In cells treated with 6 μM FB$_1$, the IC$_{50}$ value is increased to a significant extent (~2.5-fold; *p* < 0.05) to 23.93 nM. This reveals that the downstream signaling efficiency of the human serotonin$_{1A}$ receptor is reduced upon metabolic sphingolipid depletion.

The Overall Membrane Order Remains Largely Invariant upon Metabolic Depletion of Sphingolipids. To monitor any possible change in overall membrane order upon FB$_1$ treatment, we measured the fluorescence anisotropy of the fluorescent probe DPH. Fluorescence anisotropy measured using probes such as DPH is correlated to the rotational diffusion of membrane-embedded probes (*33*), which is sensitive to the packing of lipid fatty acyl chains. This is due to the fact that fluorescence anisotropy depends on the degree to which the probe is able to reorient after excitation, and probe reorientation is a function of local lipid packing. DPH, a rodlike hydrophobic molecule, partitions into the interior (fatty acyl chain region) of the bilayer. Figure 5 shows the effect of increasing concentrations of FB$_1$ on the fluorescence anisotropy of the membrane probe DPH incorporated into CHO-5-HT$_{1A}$R cell membranes. The fluorescence anisotropy of DPH appears to decrease slightly (~14%) upon treatment with 6 μM FB$_1$, compared to the
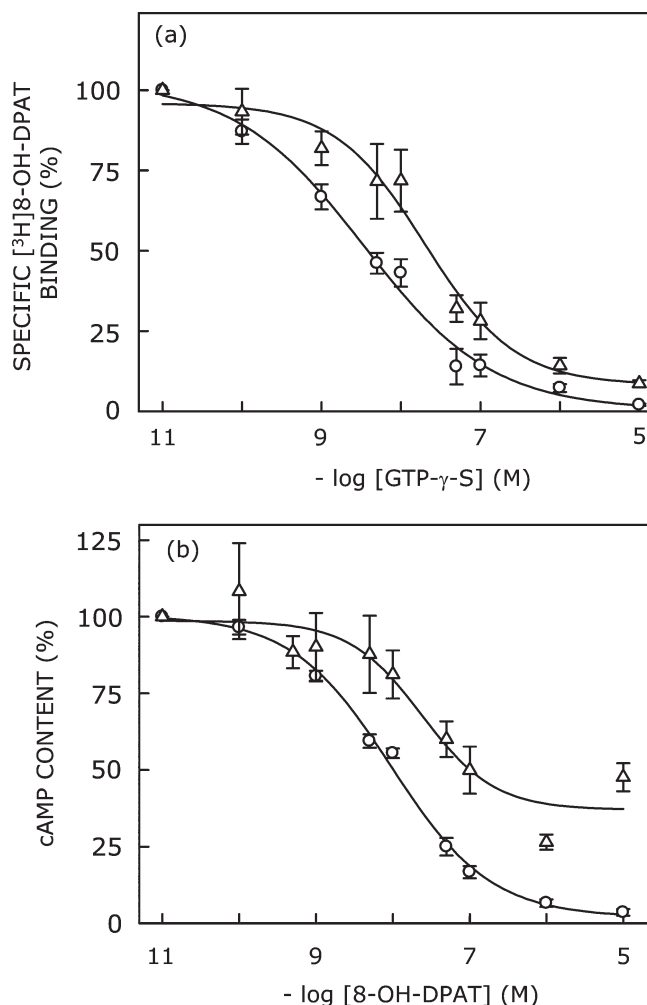

FIGURE 4: Reduced downstream signaling of the human serotonin$_{1A}$ receptor upon metabolic depletion of sphingolipids. The downstream signaling efficiency of the serotonin$_{1A}$ receptor was monitored by the sensitivity of [^{3}H]-8-OH-DPAT binding to GTP-γ-S and cyclic AMP levels. (a) Effect of increasing concentrations of GTP-γ-S on specific binding of the agonist [^{3}H]-8-OH-DPAT to serotonin$_{1A}$ receptors in control cells (O) and cells treated with 6 μM FB$_1$ ($\triangle$). Values are expressed as percentages of specific binding obtained at the lowest concentration of GTP-γ-S. Curves are nonlinear regression fits to the experimental data using eq 1. Data points represent means ± the standard error of duplicate points from at least three independent experiments. (b) Estimation of cAMP levels in CHO-5-HT$_{1A}$R cells. The ability of the specific agonist (8-OH-DPAT) to reduce the forskolin-stimulated increase in cAMP levels in control cells (O) and cells treated with 6 μM FB$_1$ ($\triangle$) was assessed. cAMP levels in cells were estimated as described in Experimental Procedures. Curves shown are nonlinear regression fits to the experimental data using eq 1'. Data are normalized to cAMP levels present at the lowest concentration of 8-OH-DPAT used and represent means ± the standard error of duplicate points from at least three independent experiments. See Experimental Procedures for other details.

corresponding anisotropy in membranes prepared from control cells (without FB$_1$ treatment). The slight reduction in overall membrane order could be due to the disruption of ordered sphingomyelin-rich domains since sphingomyelin and ceramide have previously been reported to partition into ordered domains (*43*). These results therefore suggest that metabolic depletion of sphingolipids does not appreciably alter the global membrane order.

Replenishment of Sphingolipids Using Sphingosine Restores the Membrane Sphingomyelin Content and Ligand
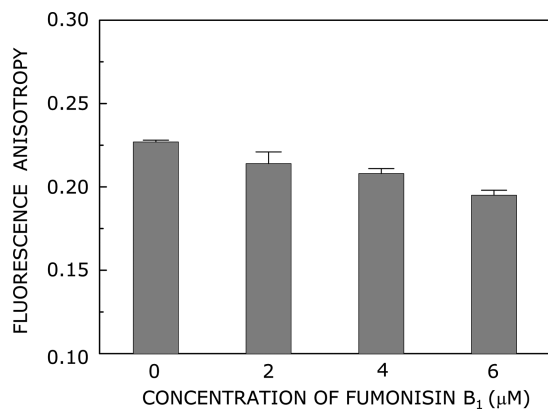
FIGURE 5: Measurement of membrane order upon metabolic depletion of sphingolipids. The overall (average) membrane order was estimated in control cell membranes and membranes of cells treated with FB_1 by measurement of fluorescence anisotropy of the membrane probe DPH. Fluorescence anisotropy measurements were conducted with membranes containing 50 nmol of phospholipid at a probe:phospholipid ratio of ~1:100 (molar) at room temperature (~23 °C). Data represent means ± the standard error of duplicate points from at least three independent experiments. See Experimental Procedures for other details.

Binding Function of the Human Serotonin$_{1A}$ Receptor. To monitor the reversibility of the effect of sphingolipids on the function of the human serotonin$_{1A}$ receptor, we treated CHO-5-HT$_{1A}$R cells with sphingosine, and sphingomyelin content and ligand binding function were measured. Figure 6 shows that pretreatment of CHO-5-HT$_{1A}$R cells with FB_1 followed by treatment with sphingosine results in restoration of sphingomyelin levels. The corresponding changes in the ligand binding function are shown in Figure 7. Total lipids were extracted from control, FB_1-treated, and sphingosine-treated cells that were pretreated with FB_1. Total lipids were separated on TLC plates and are shown in Figure 6a. Sphingomyelin bands were scraped from chromatographic plates, and their phosphate contents were estimated as described in Experimental Procedures and shown in Figure 6b. The figure shows that sphingomyelin content was increased to ~113% in control and ~160% in FB_1-treated cells upon treatment with sphingosine. Figure 7 shows the corresponding restoration of specific binding of the agonist [³H]-8-OH-DPAT upon sphingolipid replenishment. The level of specific agonist binding is reduced to ~60% of the original value upon FB_1 treatment and is restored to its normal level (~117%) upon treatment with sphingosine. Taken together, these results show that the reduction in the ligand binding function of the serotonin$_{1A}$ receptor by metabolic depletion of sphingolipids using FB_1 is reversible.

Cellular Morphology and Overall Fluorescence Distribution of EYFP-Tagged Serotonin$_{1A}$ Receptors Remain Unaltered upon Metabolic Depletion of Sphingolipids. We have earlier shown that fusion of the EYFP motif to the serotonin$_{1A}$ receptor does not affect the ligand binding property, G-protein coupling, or signaling function of the receptor (34). CHO cells stably expressing 5-HT$_{1A}$R-EYFP therefore represent a reliable system for exploring the membrane organization and dynamics of the serotonin$_{1A}$ receptor. The fluorescence distribution of 5-HT$_{1A}$R-EYFP in CHO-5-HT$_{1A}$R-EYFP cells was recorded in control cells and cells treated with 6 μM FB_1 (shown in Figure 8). Analyses of several independent images acquired with control and FB_1-treated cells do not indicate a significant redistribution of the fluorescence of 5-HT$_{1A}$R-EYFP.
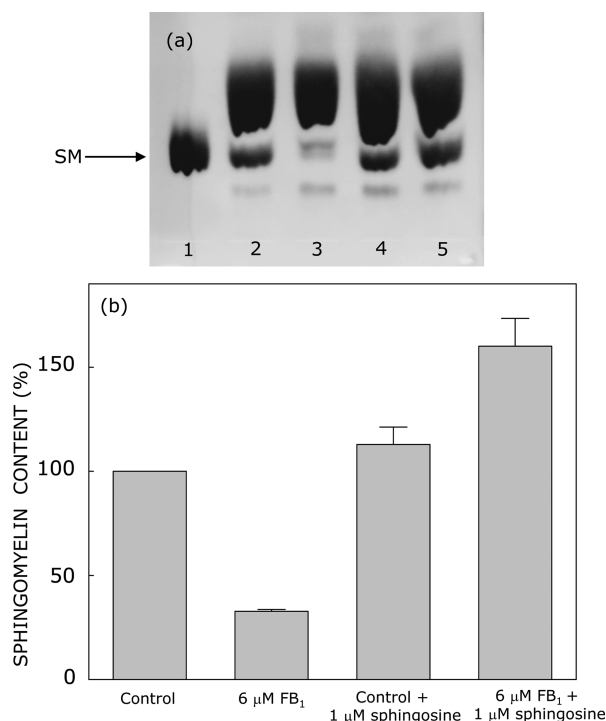

FIGURE 6: Replenishment of sphingolipids using sphingosine. Following treatment with 6 μM FB_1, CHO-5-HT$_{1A}$R cells were grown for 24 h with 1 μM sphingosine in D-MEM/F-12 (1:1) medium (supplemented with 2.4 g/L sodium bicarbonate, 10% fetal calf serum, 60 μg/mL penicillin, 50 μg/mL streptomycin, and 50 μg/mL gentamycin sulfate) in a humidified atmosphere with 5% CO_2 at 37 °C. Total lipids were extracted from cells and were separated by thin layer chromatography as shown in panel (a). Lanes contained lipids extracted from control cells (lane 2) and cells treated with 6 μM FB_1 (lane 3), control cells incubated with 1 μM sphingosine (lane 4), and FB_1-treated cells incubated with 1 μM sphingosine (lane 5). The arrow represents the position of sphingomyelin identified using a standard in lane 1. Sphingomyelin contents were quantified by estimation of phosphate content (b). Values are expressed as percentages of sphingomyelin contents of control cells (without any treatment). Data represent means ± the standard error of at least three independent experiments. See Experimental Procedures for other details.
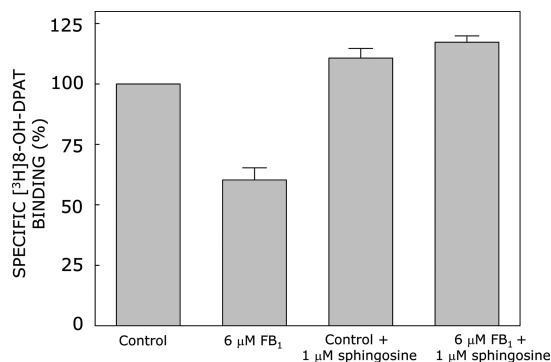

FIGURE 7: Effect of replenishment of sphingolipids on specific agonist binding of the human serotonin$_{1A}$ receptor. Changes in the specific binding of the agonist [³H]-8-OH-DPAT to serotonin$_{1A}$ receptors under control, 6 μM FB_1-treated, and sphingolipid-replenished conditions are shown. See Experimental Procedures for other details.

The Membrane Expression Level of the Human Serotonin$_{1A}$ Receptor Is Not Reduced upon FB_1 Treatment. The impaired ligand binding activity and signaling of the human serotonin$_{1A}$ receptor observed upon FB_1 treatment could be due
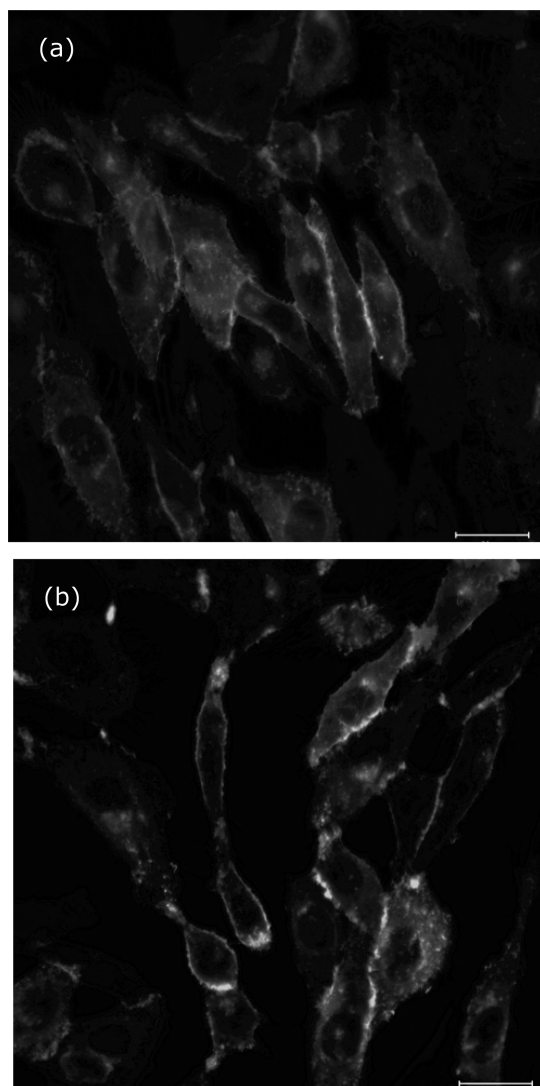
FIGURE 8: Cellular morphology and overall distribution of 5-HT$_{1A}$R-EYFP that remain unaltered in control cells and cells treated with 6 μM FB$_1$. Panels (a) and (b) show typical fluorescence distributions of 5-HT$_{1A}$R-EYFP in CHO-5-HT$_{1A}$R-EYFP cells under control (a) and 6 μM FB$_1$-treated (b) conditions. Fluorescence images of cells grown on coverslips and placed in the Bioptechs FCS2 closed chamber system were acquired at 37 °C in the presence of HEPES-Hanks buffer. The images represent midplane confocal sections of cells under conditions described in Experimental Procedures. The scale bar represents 20 μm. See Experimental Procedures for other details.

to reduced expression levels of serotonin$_{1A}$ receptors. To explore this possibility, we performed Western blot analysis of 5-HT$_{1A}$R-EYFP in cell membranes prepared from control, FB$_1$-treated, and sphingolipid-replenished CHO-5-HT$_{1A}$R-EYFP cells (see Figure 9). For these experiments, we chose to use the receptor tagged with EYFP (5-HT$_{1A}$R-EYFP) since no monoclonal antibodies for the serotonin$_{1A}$ receptor are yet available, and the polyclonal antibodies have been reported to give variable results on Western blots (44). As mentioned earlier, we have previously shown that fusion of the EYFP motif to the serotonin$_{1A}$ receptor does not affect the ligand binding property, G-protein coupling, or signaling of the receptor (34). Figure 9 shows that the levels of the serotonin$_{1A}$ receptor in membranes are not reduced upon FB$_1$ treatment. The receptor level is slightly increased ($\sim$1.2-fold compared to control) following FB$_1$ treatment. This increase, however, was found to be not significant
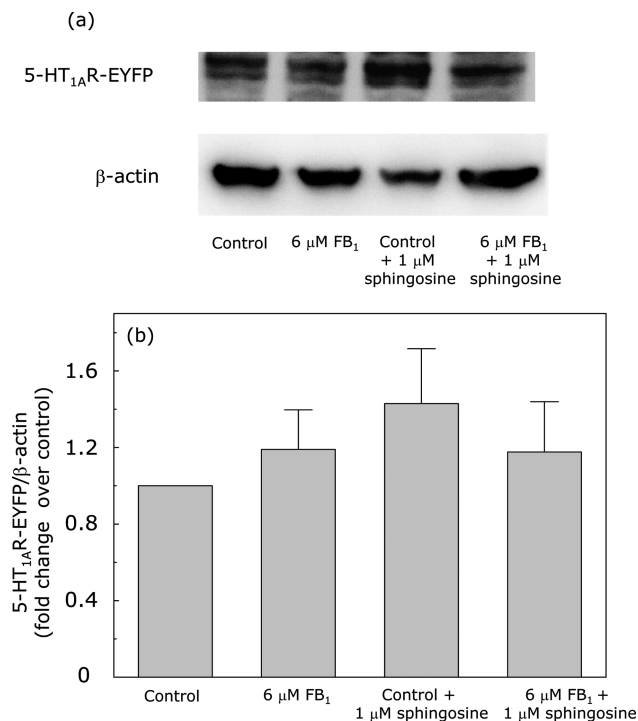

FIGURE 9: Expression level of the human serotonin$_{1A}$ receptor which is not reduced in membranes upon FB$_1$ treatment. Western blot analysis of 5-HT$_{1A}$R-EYFP in membranes prepared from CHO-5-HT$_{1A}$R-EYFP cells without any treatment (control), treated with 6 μM FB$_1$, and following incubation with sphingosine to achieve replenishment of sphingolipids (for both control and 6 μM FB$_1$-treated cells). Panel (a) shows the human serotonin$_{1A}$ receptor tagged with EYFP with corresponding β-actin probed with antibodies directed against GFP and β-actin. Panel (b) shows the quantitation of 5-HT$_{1A}$R-EYFP and β-actin levels using densitometry. 5-HT$_{1A}$R-EYFP levels were normalized to the β-actin of the corresponding sample. Data are shown as the fold change of 5-HT$_{1A}$R-EYFP over control and represent means ± the standard error of at least four independent experiments. See Experimental Procedures for other details.

($p > 0.05$). Figure 9 also shows that the receptor level exhibits no reduction upon sphingolipid replenishment.

DISCUSSION

Lipid−protein interactions play a crucial role in maintaining the structure and function of integral membrane proteins and receptors (45−47). A possible role of lipids in a variety of neurological disorders is well-documented (48). For example, several epidemiological studies indicate a possible role of lipids in a variety of neurological disorders that have been shown to involve deregulated lipid metabolism (49). A large portion of any given transmembrane receptor remains in contact with the membrane lipid environment. This raises the obvious possibility that the membrane could be an important modulator of receptor structure and function. Monitoring such lipid−receptor interactions is of particular importance because a cell has the ability to vary its membrane lipid composition in response to a variety of stress and stimuli, thereby changing the environment and the activity of the receptors in its membrane. Considering the diverse array of lipids in natural membranes, it is believed that physiologically relevant processes occurring in membranes involve an intense coordination of multiple lipid−protein interactions. Importantly, sphingolipids are shown to modulate the membrane protein/receptor function (50, 51).

Sphingomyelin typically amounts to 2–15% of the total phospholipids of mammalian cells (*52*), and even higher levels of sphingomyelin are found in the peripheral nerve and brain tissue (*15*). Its subcellular localization is mainly in the plasma membrane (*53*). Metabolic turnover of sphingomyelin produces derivatives, such as ceramide, sphingosine, and sphingosine 1-phosphate, which have a crucial role in signal transduction events (*54*). The metabolic turnover of sphingomyelin therefore is involved in the regulation of signal transduction (*52*). In view of the importance of sphingolipids in relation to membrane domains (*43*), the interaction of sphingolipids with membrane receptors represents an important determinant in functional studies of such receptors.

Sphingolipids are being increasingly implicated in the pathogenesis of several disorders such as cancer and metabolic and neurological disorders (*55*). In this work, we have modulated sphingolipid levels in CHO-5-HT$_{1A}$R cells by metabolically inhibiting the biosynthesis of sphingolipids using FB$_1$. FB$_1$ acts as a competitive inhibitor to ceramide synthase, which acylates sphinganine to ceramide. Since FB$_1$ has been reported to induce neurodegeneration (*56*), thereby leading to changes in neurotransmission, exploring the function of an important neurotransmitter receptor under these conditions assumes relevance. We explored the function of the human serotonin$_{1A}$ receptor under these conditions by monitoring ligand binding, G-protein coupling, and downstream signaling of the receptor. Our results show that the function of the serotonin$_{1A}$ receptor is impaired upon metabolic depletion of sphingolipids, although the membrane receptor level does not display any reduction. These results are significant since FB$_1$ induces a number of diseases (see above) and could possibly even impair neurotransmission. Interestingly, our results demonstrate that the effect of sphingolipids on the ligand binding function of the serotonin$_{1A}$ receptor caused by metabolic depletion of sphingolipids is reversible. It has been previously reported that depletion of sphingolipids results in the inhibition of internalization and trafficking of the cholecystokinin receptor (*57*) and trafficking of the nicotinic acetylcholine receptor (*58*). Our results show that there is no overall change in the membrane expression and distribution of the serotonin$_{1A}$ receptor upon sphingolipid depletion (Figures 8 and 9). It is important to mention here that the possibility that FB$_1$ may exert effects other than inhibition of sphingolipid metabolism must always be considered, and an earlier report has described the inhibition of protein phosphatases by FB$_1$ (*59*). However, such effects are observed at much higher concentrations, several orders of magnitude higher than the concentrations used by us.

The effect of sphingolipids on the conformation and function of membrane proteins could be due to specific interaction, possibly induced by the proposed "sphingolipid-binding domain" (SBD) (*39*, *50*). For example, the nerve growth factor receptor tyrosine kinase has been shown to interact directly with gangliosides (*60*). It is therefore possible that the serotonin$_{1A}$ receptor enjoys specific interaction with membrane sphingolipids. We have previously shown that membrane cholesterol is necessary for the function of the serotonin$_{1A}$ receptor (*22*, *47*, *61*). We have recently proposed that cholesterol may occupy "nonannular" binding sites around the serotonin$_{1A}$ receptor (*62*). Nonannular sites are characterized by a lack of accessibility to the annular lipids; i.e., these sites cannot be displaced by competition with annular lipids. The locations of the nonannular sites are believed to be either inter- or intramolecular protein interfaces, characterized as deep clefts (or cavities) on the protein surface.

Do sphingolipids occupy nonannular sites around the receptor? This is relevant since it has been proposed that cholesterol may adjust the orientation of sphingolipids in the membrane interface (*50*, *63*). Future work involving realistic atomistic simulations could further refine this model. Taken together, our results show that sphingolipids have an important role in maintaining the function of the serotonin$_{1A}$ receptor and could be relevant in understanding the role of the membrane lipid environment on the activity and signal transduction of other G-protein-coupled receptors.

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