Chapter 4

Metabolic depletion of sphingolipids impairs ligand binding and signaling functions of the human serotonin$_{1A}$ receptor
4.1. Introduction

Sphingolipids are essential and indispensable components of eukaryotic cell membranes and constitute 10-20% of the total membrane lipids (Holthius et al., 2001). Sphingolipids are thought to be involved 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 second messengers such as sphingosine, ceramide and sphingosine 1-phosphate (Merrill et al., 1996). 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 occur in laterally segregated lipid domains (sometimes termed as ‘lipid rafts’) (Brown, 1998; Masserini and Ravasi, 2001; Mukherjee and Maxfield, 2004; see chapter 1).

Ceramide is at the center of sphingolipid metabolism and has been recognized as a critical second messenger (Hannun and Obeid, 2002). Ceramide levels can reach up to 10 mol% of the total phospholipids (Hannun, 1996) emphasizing its role as a signaling molecule. Furthermore, the formation of ceramide upon stimulation occurs in restricted cellular sites, and the local concentration of ceramide has been estimated to exceed 25 mol% (Holopainen et al., 2000). The pool of ceramide is maintained by de novo synthesis. The cellular levels of ceramide and sphingomyelin can be modulated using compounds such as fumonisins. Fumonisins have been extensively used to explore functions of ceramides, sphingomyelin and complex sphingolipids (Desai et al., 2002). Fumonisins are a group of naturally occurring mycotoxins, which are ubiquitous contaminants of corn and other grain products, produced by Fusarium verticilloides and several other Fusarium species (Gelderblom et al., 1988; Marasas, 1996). There are at least 14 known fumonisins of which fumonisin B₁ (FB₁) is the most abundant (Dragan et al., 2001). FB₁ is structurally similar to sphingoid bases such as sphinganine and sphingosine (see Figure 4.1), which are
intermediates in sphingolipid metabolism, and an important site of inhibition is the reaction catalyzed by sphinganine N-acetyltransferase (ceramide synthase) (Wang et al., 1991). Consumption of FB₁ through contaminated corn has been reported to induce neurotoxicity (Desai et al., 2002; Gelderblom et al., 1992), and esophageal and liver cancer in humans (Soriano et al., 2005). Although little is known about the molecular mechanism of action by which these mycotoxins induce carcinogenic effects, disruption of the sphingolipid metabolism appears to be a major factor. It has been previously demonstrated that inhibition of sphingolipid biosynthesis using FB₁ results in depletion of cellular (glyco)sphingolipids and significantly affects axonal growth, suggesting that sphingolipids may play a vital role in regulating neuronal development (Harel and Futerman, 1993). 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 (Posee de Chaves, 2006), which could be due to impaired neurotransmission. Modulating sphingolipid levels and monitoring the function of an important neurotransmitter receptor therefore assumes relevance.

The serotonin₁₅ receptor is an important neurotransmitter G-protein coupled receptor and serves as an important target in the development of therapeutic agents for neuropsychiatric disorders such as anxiety and depression. Keeping in mind the pharmacological relevance of the serotonin₁₅ receptor, its interaction with the surrounding lipid environment assumes greater significance in modulating its function in healthy and diseased states. This chapter describes about the work in which sphingolipid levels in CHO cells stably expressing the human serotonin₁₅ receptor (CHO-5-HT₁₅AR) were modulated by metabolically inhibiting the biosynthesis of sphingolipids. In order to achieve this, FB₁ (a specific metabolic inhibitor of ceramide synthase) was utilized. FB₁ treatment results in reduction of sphingomyelin levels. The function of the human serotonin₁₅ receptor under these conditions was explored by monitoring ligand binding, G-protein coupling and
downstream signaling of the receptor along with lateral mobility measurements using Fluorescence Recovery After Photobleaching (FRAP). Our results show that the function of the serotonin1A receptor is impaired upon metabolic depletion of sphingomyelin. These results are significant since FB1 induces a number of diseases (see above) and could possibly even impair neurotransmission. Importantly, our results provide evidence, that sphingolipids are necessary for ligand binding and downstream signaling of the human serotonin1A receptor. In addition, our results demonstrate that the effect of sphingomyelin on the ligand binding function of the serotonin1A receptor caused by metabolic depletion of sphingolipids is reversible.

4.2. Materials and methods

Materials

DMPC, fumonisins B1, EDTA, MgCl2, MnCl2, 8-OH-DPAT, penicillin, streptomycin, gentamycin sulfate, polyethyleneimine, PMSF, p-MPPI, primuline, serotonin, sphingosine, sodium bicarbonate, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). DMEM/F-12 [Dulbecco's modified Eagle 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). [3H]8-OH-DPAT (sp. activity = 135.0 Ci/mmol) and [3H]p-MPPF (sp. activity = 70.5 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA). The cyclic [3H]AMP assay kit was purchased from Amersham Biosciences (Buckinghamshire, U.K.). GF/B glass microfiber filters were from Whatman International (Kent, U.K.). Pre-coated silica gel 60 thin layer chromatography plates were
from Merck (Merck, 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 FB₁ treatment**

CHO cells stably expressing the human serotonin₁A receptor (termed as CHO-5-HT₁AR) and stably expressing the human serotonin₁A receptor tagged to enhanced yellow fluorescent protein (termed as CHO-5-HT₁AR-EYFP) were maintained in D-MEM/F-12 (1:1) supplemented with 2.4 g/l of sodium bicarbonate, 10% fetal calf serum, 60 µg/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml gentamycin sulfate, and 200 µg/ml geneticin in a humidified atmosphere with 5% CO₂ at 37 °C. Stock solutions (1 mM) of FB₁ were prepared in water and added to cells grown for 24 h (final concentration of FB₁ was 2-6 µM) and incubated in 5% serum for 63-66 h. Control cells were grown under similar conditions without FB₁ treatment.

**Cell membrane preparation**

Cell membranes were prepared as described earlier (Kalipatnapu et al., 2004a; Paila and Chattopadhyay, 2006) and in section 3.2. Total protein concentration in the isolated membranes was determined using the BCA assay (Smith et al., 1985).

**Estimation of sphingomyelin by thin layer chromatography**

Total lipid extraction from membranes of control and FB₁ treated cells was carried out according to Bligh and Dyer (1959). Lipid extracts were dried under a stream of nitrogen at 45 °C. The dried extracts were resuspended in a mixture of chloroform/methanol (1:1, v/v). Total lipid extracts were resolved by thin layer chromatography (TLC) using chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v/v) as the solvent system (Ito et al., 2002). The separated lipids were visualized by spraying a
fluorescent stain, primuline (Skipski, 1975). Sphingomyelin standard was used to identify sphingomyelin bands on the thin layer chromatogram run with total lipid extracts obtained from membranes of control and FB1 treated cells. The sphingomyelin bands were scraped from the TLC plates, and lipids were re-extracted with chloroform/methanol (1:1, v/v) from samples, and the phosphate contents were estimated and normalized to the phosphate content obtained from native (control) cell membranes.

*Estimation of inorganic phosphate*

The concentration of lipid phosphate was as described in section 2.2.

*Radioligand binding assays*

Receptor binding assays were carried out as described in section 2.2 with ~50 μg total protein.

*GTP-γ-S sensitivity assay*

In order to estimate the efficiency of G-protein coupling, GTP-γ-S sensitivity assays were carried out as described in section 2.2.

*Estimation of cyclic AMP content in cells*

The ability of ligands to affect the forskolin-stimulated increase in cAMP levels in CHO-5-HT1A cells was assessed as described in section 3.2.

*Fluorescence anisotropy measurements*

Fluorescence anisotropy experiments were carried out using the fluorescent probe DPH with membranes prepared from cells that were treated with varying concentrations of FB1, containing 50 nmol of total phospholipids suspended in 1.5 ml of 50 mM Tris, pH 7.4 buffer, as described earlier (Paila et al., 2005) and in section 3.2.
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Metabolic replenishment of sphingolipids using sphingosine

After treatment with 6 μM FB_{1}, CHO-5-HT_{1A}R cells were grown for 24 hs in D-MEM/F-12 (1:1) supplemented with 1 μM sphingosine, 2.4 g/l of sodium bicarbonate, 10% fetal calf serum, 60 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamycin sulfate, and 200 μg/ml geneticin in a humidified atmosphere with 5% CO_{2} at 37 °C in order to achieve replenishment of sphingolipids.

Confocal microscopy and live cell imaging

In order to visualize the serotonin_{1A} receptor, CHO-K1 cells stably expressing the serotonin_{1A} receptor tagged to enhanced yellow fluorescent protein (referred to as CHO-5-HT_{1A}R-EYFP) were used (Pucadyil et al., 2004a). CHO-5-HT_{1A}R-EYFP cells were plated at a density of 5 x 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, pH 7.4 buffer, and mounted on an FCS2 closed temperature controlled Bioptechs chamber (Butler, PA). The chamber was gently perfused with 10 ml of the same buffer and was allowed to attain 37 °C, which took ~10 min. Images were acquired on an inverted Zeiss LSM 510 Meta confocal microscope (Jena, Germany), with a 63x, 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.

Fluorescence recovery after photobleaching (FRAP) measurements and statistical analysis

FRAP experiments were carried out at room temperature (~23 °C) on cells that were grown in D-MEM/F-12 medium containing 5% serum with or without FB_{1} treatment on Lab-Tek chambered coverglass (Nunc, Denmark). Fluorescence images of cells grown on Lab-Tek chambers were acquired in the presence of PBS buffer pH 7.4, containing 0.5 mM MgCl_{2} and 1 mM CaCl_{2}. Images were acquired on an inverted Zeiss LSM 510 Meta confocal microscope as described above, and recorded at a 225 μm pinhole resolution,
giving an optimal z-slice thickness of 1.7 \mu m. A circular region of interest (ROI), with a radius of 1.4 \mu m was chosen as the bleach ROI. The time interval between successive scans was \sim 0.53 \text{s.} Analysis with a control ROI drawn a fair distance away from the bleach ROI indicated no significant bleach while fluorescence recovery was monitored. Data representing the mean fluorescence intensity of the bleached ROI were background subtracted using an ROI placed outside the cell boundary and were analyzed to determine the diffusion coefficient \(D\). FRAP recovery plots were analyzed on the basis of the equation for a uniform disk illumination condition (Soumpasis, 1983):

\[
F(t) = [F(\infty) - F(0)] \left[ \exp(-2\tau_d/t) \left( I_0(2\tau_d/t) + I_1(2\tau_d/t) \right) \right] + F(0) \tag{5}
\]

where \(F(t)\) is the mean background corrected and normalized fluorescence intensity at time \(t\) in the bleached ROI, \(F(\infty)\) is the recovered fluorescence at time \(t = \infty\), \(F(0)\) is the bleached fluorescence intensity set at time \(t = 0\), and \(\tau_d\) is the characteristic diffusion time. \(I_0\) and \(I_1\) are modified Bessel functions. Diffusion coefficient \(D\) is determined from the equation:

\[
D = \frac{\omega^2}{4\tau_d} \tag{6}
\]

where \(\omega\) is the actual radius of the bleached ROI. Mobile fraction estimates of the fluorescence recovery were obtained from the equation:

\[
\text{mobile fraction} = \frac{[F(\infty) - F(0)]}{[1 - F(0)]} \tag{7}
\]

where the mean background corrected and normalized prebleach fluorescence intensity is equal to unity. Nonlinear curve fitting of the fluorescence recovery data to equation 5 was carried out using the Graphpad Prism software version 4.00 (San Diego, CA). Frequency distribution plot and statistical analysis were performed using Microcal Origin software version 5.0 (OriginLab Corp., Northampton, MA).

*Western blot analysis*

Cell membranes were prepared from CHO-5-HT\textsubscript{1A}-EYFP (control), FB\textsubscript{1} treated and FB\textsubscript{1} treatment followed by sphingosine-treated cells as previously described earlier (section 3.2; Paila and Chattopadhyay, 2006) with an addition of 1:20 dilution of freshly
added protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Western blot analysis was performed as described in section 3.2.

4.3. Results

Quantification of sphingomyelin upon metabolic depletion using FB1

CHO cells stably expressing the human serotonin1A receptor (CHO-5-HT1AR) were treated with FB1, in order to achieve the metabolic depletion of sphingolipids. FB1 is a potent and competitive inhibitor of ceramide synthase, the enzyme that catalyzes the acylation of sphinganine in de novo biosynthesis of sphingolipids and the reutilization of sphingosine derived from sphingolipid turnover (Merrill et al., 1996). The structures of sphingosine, sphinganine and FB1 are shown in Figure 4.1. Besides disrupting sphingolipid metabolism, FB1 is known to induce oxidative stress leading to cytotoxicity when used at high concentrations (Kouadio et al., 2005). It is therefore important to ensure that the FB1 concentrations used are below the concentration range in which cytotoxic effects are predominant. It has previously been shown that treatment up to 50 μM FB1 does not result in cell death (Yu et al., 2001; Sjögren and Svenningsson, 2007). We therefore chose to use low concentrations of FB1 and the concentration of FB1 used in the present work never exceeded 6 μM. Total lipids were extracted from membranes prepared from control and FB1 treated cells, and were separated on TLC plates (shown in Figure 4.2a). Sphingomyelin bands were scraped from chromatographic plates and their phosphate contents were estimated as described in section 4.2 and shown in Figure 4.2b. The sphingomyelin content shows a progressive reduction with increasing FB1 concentration. Figure 4.2b shows that ~80% of sphingomyelin is metabolically depleted in membranes of CHO-5-HT1AR following treatment with 6 μM FB1.
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Figure 4.1 Chemical structures of sphingosine, sphinganine and fumonisin B$_1$. 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 (Merrill et al., 1996). See text for more details.
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Figure 4.2. Estimation of sphingomyelin content in membranes isolated from control cells and cells treated with varying concentrations of $\text{FB}_1$. Total lipids were extracted from membranes of control cells and cells treated with varying concentrations of $\text{FB}_1$ and were separated by thin layer chromatography as shown in (a). The lanes represent lipids extracted from membranes from control cells (lane 2), and membranes isolated from cells treated with 2 (lane 3), 4 (lane 4) and 6 (lane 5) $\mu$M of $\text{FB}_1$. The arrow represents position of sphingomyelin on the thin layer chromatogram identified using a standard in lane 1. Sphingomyelin contents were quantified by estimation of phosphate content and are shown in (b). Values are expressed as percentages of the sphingomyelin content of membranes of control cells (without any treatment). Data represent means ± SE of at least three independent experiments. See section 4.2 for other details.
Specific ligand binding of the human serotonin_{1A} receptor is reduced upon metabolic depletion of sphingolipids

In order to monitor the effect of metabolic depletion of sphingolipids on the ligand binding activity of the serotonin_{1A} receptor, CHO-5-HT_{1A}R cells were treated with varying concentrations of FB_{1} and ligand binding was measured. For this, we measured binding of the selective serotonin_{1A} receptor agonist [{}^{3}\text{H}]8-OH-DPAT and antagonist [{}^{3}\text{H}]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 4.3 shows the decrease in specific binding of the selective serotonin_{1A} receptor agonist [{}^{3}\text{H}]8-OH-DPAT with increasing concentrations of FB_{1} (Figure 4.3a), and the accompanying reduction in membrane sphingomyelin levels (Figure 4.3b). The 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 [{}^{3}\text{H}]p-MPPF binding to the serotonin_{1A} receptor are shown in panels (c) and (d), respectively. Figure 4.3c shows that specific binding of the selective antagonist [{}^{3}\text{H}]p-MPPF is decreased with FB_{1} treatment, and is reduced to ~65% of its original value upon treatment with 6 \mu M FB_{1}. Figure 4.3 shows that the reduction in ligand binding is somewhat drastic till ~30% sphingomyelin is lost (corresponding to 2 \mu M FB_{1}). 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.

Ligand-dependent downstream signaling efficiency of the human serotonin_{1A} receptor is reduced upon metabolic depletion of sphingolipids

Figure 4.4a shows a characteristic reduction in binding of the agonist [{}^{3}\text{H}]8-OH-DPAT in the presence of a range of concentrations of GTP-\gamma-S with an estimated half maximal inhibition concentration (IC_{50}) of 3.41 nM for control cells. The inhibition curve in case of cells treated with 6 \mu M FB_{1} displays a significant (~5-fold) shift toward higher
Figure 4.3. Effect of metabolic depletion of sphingolipids on specific ligand binding of the human serotonin\textsubscript{1A} receptor. CHO-5-HT\textsubscript{1A}R cells were treated with varying concentrations of FB\textsubscript{1} and specific [\textsuperscript{3}H]8-OH-DPAT binding to the serotonin\textsubscript{1A} receptor in membranes isolated from these cells was measured and shown in (a). The change in specific [\textsuperscript{3}H]8-OH-DPAT binding is plotted with increasing sphingomyelin depletion in (b). Values are expressed as percentages of specific binding for control cell membranes without FB\textsubscript{1} treatment. Data shown are means ± SE of at least three independent experiments. The effects of increasing concentrations of FB\textsubscript{1} and accompanying sphingomyelin depletion on specific [\textsuperscript{3}H]p-MPPF binding to the serotonin\textsubscript{1A} receptor are shown in panels (c) and (d), respectively. Values are expressed as percentages of specific binding for control cell membranes without FB\textsubscript{1} treatment. Data shown are means ± SE of at least three independent experiments. Data for the extent of sphingomyelin depletion with increasing concentrations of FB\textsubscript{1} are taken from Figure 4.2b. See section 4.2 for other details.
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concentrations of GTP-γ-S with an increased IC₅₀ value of 19.75 nM. This implies that the agonist binding to the serotonin₁₆ 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 serotonin₁₆ receptors in CHO-5-HT₁₆R cells by measuring its ability to catalyze downstream signal transduction processes upon stimulation with the specific agonist, 8-OH-DPAT. Serotonin₁₆ receptor agonists such as 8-OH-DPAT are known to specifically activate the G₄/G₆ class of G-proteins in CHO cells, which subsequently reduce cAMP levels in cells (Raymond et al., 1993). As shown in Figure 4.4b, the forskolin-stimulated increase in cAMP levels is inhibited by 8-OH-DPAT with a half maximal inhibition concentration (IC₅₀) of 9.49 nM in control cells. In cells treated with 6 µM FB₁, the IC₅₀ value is increased to a significant extent (~2.5-fold) to 23.93 nM. This points out that the downstream signaling efficiency of the human serotonin₁₆ receptor is reduced under sphingolipid depleted condition.

The overall membrane order remains largely invariant upon metabolic depletion of sphingolipids

In order to monitor any possible change in overall membrane order upon FB₁ treatment, we measured the fluorescence anisotropy of the fluorescent probe DPH. Figure 4.5 shows the effect of increasing concentrations of FB₁ on the fluorescence anisotropy of the membrane probe DPH incorporated into CHO-5-HT₁₆R cell membranes. The fluorescence anisotropy of DPH appears to decrease slightly (~14%) upon treatment with 6 µM FB₁, compared to the fluorescence anisotropy value in membranes prepared from control cells (without FB₁ treatment). The slight reduction in overall membrane order could be due to disruption of ordered sphingomyelin-rich domains since sphingomyelin and
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Figure 4.4. Reduced downstream signaling of the human serotonin1A receptor upon metabolic depletion of sphingolipids. The downstream signaling efficiency of the serotonin1A receptor was monitored by the sensitivity of [3H]8-OH-DPAT binding to GTP-γ-S and cyclic AMP levels. Panel (a) shows the effect of increasing concentrations of GTP-γ-S on specific binding of the agonist [3H]8-OH-DPAT to serotonin1A receptors in control cells (O) and in cells treated with 6 μM FB1 (Δ). Values are expressed as percentages of specific binding obtained at the lowest concentration of GTP-γ-S. The curves are non-linear regression fits to the experimental data using equation 1. The data points represent means ± SE of duplicate points from at least three independent experiments. See section 4.2 for other details. Panel (b) shows the estimation of cAMP levels in CHO-5-HT1A-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 in cells treated with 6 μM FB1 (Δ) was assessed. cAMP levels in cells were estimated as described in section 4.2. The curves are non-linear regression fits to the experimental data using equation 1. The data are normalized to cAMP levels present in the lowest concentration of 8-OH-DPAT used in the experiment and represent the means ± SE of duplicate points from at least three independent experiments. See section 4.2 for other details.
Figure 4.5. Measurement of overall membrane order upon metabolic depletion of sphingolipids. The overall (average) membrane order was estimated in control cell membranes and in membranes of cells treated with varying concentrations of FB$_1$, using fluorescence anisotropy of the membrane probe DPH. Fluorescence anisotropy measurements were carried out with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (~23 °C). The data represent means ± SE of duplicate points from at least three independent experiments. See section 4.2 for other details.

ceramide have previously been reported to partition into ordered domains (Ramstedt and Slotte, 2006). These results therefore suggest that the overall (global) membrane order does not change by a considerable extent upon metabolic depletion of sphingolipids.

Replenishment of sphingolipids using sphingosine restores the membrane sphingomyelin content and ligand binding function of the human serotonin$_{1A}$ receptor

In order to monitor the reversibility of the effect of sphingolipids on the function of the human serotonin$_{1A}$ receptor, CHO-5-HT$_{1A}$R cells were treated with sphingosine and the sphingomyelin content and ligand binding function of the receptor were measured. Figure 4.6 shows that pre-treatment of CHO-5-HT$_{1A}$R cells with FB$_1$ followed by treatment with sphingosine, results in restoration of sphingomyelin levels to normal and the corresponding
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Figure 4.6. Replenishment of sphingolipids using sphingosine. After treatment with 6 \mu M FB_{1}, CHO-5-HT_{1A}R cells were grown for 24 hs with 1 \mu M sphingosine in D-MEM/F-12 (1:1) medium (supplemented with 2.4 g/l of sodium bicarbonate, 10% fetal calf serum, 60 \mu g/ml penicillin, 50 \mu g/ml streptomycin, 50 \mu g/ml gentamycin sulfate, and 200 \mu g/ml geneticin) 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 (a). The lanes represent lipids extracted from membranes from control cells (lane 2), and membranes isolated from cells treated with 6 \mu M of FB_{1} (lane 3), membranes isolated from control cells incubated with 1 \mu M sphingosine (lane 4) and membranes isolated from cells after treatment with 6 \mu M of FB_{1} and incubated with 1 \mu M sphingosine (lane 5). The arrow represents position of sphingomyelin on the thin layer chromatogram identified using a standard in lane 1. Sphingomyelin contents were quantified by estimation of phosphate content and are shown in (b). Values are expressed as percentages of the sphingomyelin content of membranes of control cells (without any treatment). Data represent means ± SE of at least three independent experiments. See section 4.2 for other details.
changes in the ligand binding function are shown in Figure 4.7. Total lipids were extracted from membranes prepared from control, FB1 treated and sphingosine treated cells, which were pre-treated with FB1. Total lipids were separated on TLC plates and are shown in Figure 4.6a. Sphingomyelin bands were scraped from chromatographic plates and their phosphate contents were estimated as described in section 4.2 and shown in Figure 4.6b. This Figure shows that ~70% of sphingomyelin is metabolically depleted in membranes of CHO-5-HT1A R cells following treatment with 6 \( \mu \)M FB1. The membrane sphingomyelin content was increased to ~115% in control and to ~160% in FB1 treated cells upon treatment with sphingosine (Figure 4.6).

Figure 4.7 demonstrates the restoration in specific binding of the selective serotonin1A receptor agonist \( ^{3}H \)8-OH-DPAT upon sphingolipid replenishment. The specific agonist binding is reduced to ~60% of the original value upon FB1 treatment and is restored to its normal level upon treatment with sphingosine. Figure 4.7 shows that the ligand binding function of the receptor is increased to ~117% upon sphingolipid replenishment. Taken together, these results show that the reduction in the ligand binding function of the serotonin1A receptor by metabolic depletion using FB1 is reversible.

*The membrane expression level of the human serotonin1A receptor is not reduced upon FB1 treatment*

The impaired ligand binding activity and signaling of the human serotonin1A receptor observed upon FB1 treatment could be due to reduced expression levels of serotonin1A receptors. In order to explore this possibility, we performed Western blot analysis of 5-HT1A R-EYFP in cell membranes prepared from control, FB1 treated and sphingolipid replenished CHO-5-HT1A R-EYFP cells (see Figure 4.8). For these experiments, we chose to use the receptor tagged to EYFP (5-HT1A R-EYFP) since no monoclonal antibodies for the serotonin1A receptor are available yet, and the polyclonal antibodies have been reported to give variable results on Western blots (Zhou *et al.*, 1999).
Figure 4.7. The changes in the specific binding of the agonist $[^3H]8$-OH-DPAT to serotonin$\textsubscript{1A}$ receptors under control, 6 μM FB$_1$ treated and sphingolipid replenished conditions. See section 4.2 for other details.

We have earlier shown that EYFP fusion to the serotonin$\textsubscript{1A}$ receptor does not affect the ligand binding properties, G-protein coupling and signaling functions of the receptor (Pucadyil et al., 2004a). Figure 4.8b shows that the levels of the serotonin$\textsubscript{1A}$ receptor in membranes are not reduced upon FB$_1$ treatment. The receptor level is slightly increased (~1.2-fold compared to control) following FB$_1$ treatment. It has been previously reported that serotonin$\textsubscript{1A}$ receptor levels in CHO cells could increase upon induction of stress (Singh et al., 1996b).

**Cellular morphology and overall fluorescence distribution of EYFP tagged serotonin$\textsubscript{1A}$ receptors remain unaltered upon metabolic depletion of sphingolipids**

Our group has earlier shown that EYFP fusion to the serotonin$\textsubscript{1A}$ receptor does not affect the ligand binding properties, G-protein coupling and signaling functions of the receptor (Pucadyil et al., 2004a). CHO-K1 cells stably expressing the 5-HT$_{1A}$R-EYFP therefore represent a reliable system to explore membrane organization and dynamics of the
Figure 4.8. The expression level of the human serotonin$_{1A}$ receptor 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 (control) cells, CHO-5-HT$_{1A}$R-EYFP cells treated with 6 µM FB$_1$ and after incubating with sphingosine to achieve replenishment of sphingolipids. Panel (a) shows the human serotonin$_{1A}$ receptor tagged to 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 β-actin of the corresponding sample. Data are shown as fold increase of 5-HT$_{1A}$R-EYFP over control and represent means ± SE of at least four independent experiments. See section 4.2 for other details.
Figure 4.9. Cellular morphology and the overall distribution of 5-HT<sub>1A</sub>R-EYFP remain unaltered in control and cells treated with 6 μM FB<sub>1</sub>. Panels (a) and (b) show typical fluorescence distribution of 5-HT<sub>1A</sub>R-EYFP in CHO-5-HT<sub>1A</sub>R-EYFP cells under control (a) and 6 μM FB<sub>1</sub> 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 mid-plane confocal sections of the cells under conditions as described in section 4.2. The scale bar represents 20 μm. See section 4.2 for other details.
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CHO-5-HT₁AR-EYFP cells do not indicate a significant redistribution of fluorescence of the 5-HT₁AR-EYFP. These results set up the background for the experiments described below to assess diffusion characteristics of the receptor by FRAP as they indicate that the analysis of fluorescence recovery is not complicated by any significant alteration in the distribution of receptors due to FB₁ treatment during these experiments.

Fluorescence recovery after photobleaching analysis of 5-HT₁AR-EYFP

Fluorescence recovery after photobleaching involves generating a concentration gradient of fluorescent molecules by irreversibly photobleaching a fraction of fluorophores in the sample region. The dissipation of this gradient with time owing to diffusion of fluorophores into the bleached region from the unbleached regions in the membrane is an indicator of the mobility of the fluorophores in the membrane. A representative panel of images showing the recovery of fluorescence intensity after photobleaching is shown in Figure 4.10 (panels (a-d). A representative fluorescence recovery plot with regression fits to the data is shown in Figure 4.10 (lower panel). A large data set was collected keeping in mind the inherent statistical variation in biological membranes. The frequency distribution histograms of diffusion coefficients and mobile fractions of 5-HT₁AR-EYFP in control cells and cells treated with 6 µM FB₁ are shown in Figure 4.11 (panels a-d). The diffusion coefficient of 5-HT₁AR-EYFP did not exhibit any significant change upon FB₁ treatment (Figure 4.11, panels a and b). The distribution of diffusion coefficients remain essentially unimodal with comparable standard deviations indicating the presence of a predominantly single mobile population (at least in the time scale of FRAP measurement), which does not display any significant change upon FB₁ treatment. Interestingly, the mobile fraction of 5-HT₁AR-EYFP shows a significant (~7%, \( p<0.001 \)) increase in its mean value upon FB₁ treatment (see panels c and d), although the distribution remains essentially unimodal.
Figure 4.10. Representative images of recovery of fluorescence intensity after photobleaching of 5-HT$_{1A}$R-EYFP in CHO-5-HT$_{1A}$R-EYFP cells. Fluorescence images of cells represent confocal sections of the cell periphery and were acquired at room temperature (~23°C). Panels A-D show fluorescence intensity monitored at various time points corresponding to prebleach, bleach, immediate postbleach and complete recovery, respectively. The scale bar represents 10 µm. The fluorescence recovery plot (see lower panel) shows a single set of normalized data, fitted to equation 5. The recovery of 5-HT$_{1A}$R-EYFP (○), and background (●) fluorescence intensities are shown. Note that the background fluorescence intensity (●) monitored for the same time period indicates no significant photobleaching of the field due to repeated imaging. The prebleach intensities are shown at time t < 0. See section 4.2 for other details.
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Figure 4.11. Frequency distribution histograms of the diffusion coefficient and mobile fraction of 5-HT$_{1A}$R-EYFP determined by FRAP. The frequency distribution histograms are obtained by fitting the normalized recovery data of individual experiments to equation 5. Panels (a) and (b) show the diffusion coefficient histograms for control (untreated) cells, and 6 μM FB$_1$ treated cells, respectively. Panels (c) and (d) show the mobile fraction histograms for control (untreated) cells, and 6 μM FB$_1$ treated cells, respectively. The means ± SE are shown in all cases. N represents the number of independent experiments performed in each case. See section 4.2 for other details.
4.4. Discussion

Sphingomyelin typically amounts to 2-15% of the total phospholipids of mammalian cells (Koval and Pagano, 1991) and even higher levels of sphingomyelin are found in the peripheral nerve and brain tissue (Soriano et al., 2005). Its subcellular localization is mainly in the plasma membrane. Metabolic turnover of sphingomyelin produces derivatives such as ceramide, sphingosine, sphingosine 1-phosphate which have crucial role in signal transduction events (Futerman and Hannun, 2004). The metabolic turnover of sphingomyelin therefore is involved in the regulation of signal transduction (Koval and Pagano, 1991). In view of the importance of sphingolipids in relation to membrane domains (Ramstedt and Slotte, 2006), 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, metabolic and neurological disorders (Zeidan and Hannun, 2007). In this work, we have modulated sphingolipid levels in CHO-5-HT\textsubscript{1A}AR cells by metabolically inhibiting the biosynthesis of sphingolipids using FB\textsubscript{1}. FB\textsubscript{1} acts as a competitive inhibitor to ceramide synthase, which acylates sphinganine to ceramide. Since FB\textsubscript{1} has been reported to induce neurodegeneration (Osuchowski et al., 2005), 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\textsubscript{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\textsubscript{1A} receptor is impaired upon metabolic depletion of sphingolipids. Importantly, our results show that the receptor level is not reduced under this condition. This implies that the fraction of functional receptors is reduced upon FB\textsubscript{1} treatment resulting in a higher fraction of non-functional receptors. Sphingolipids were replenished using sphingosine in cells pre-treated with FB\textsubscript{1} and it was found that the
sphingomyelin content and ligand binding activity of the human serotonin_{1A} receptor could be recovered (Figures 4.6 and 4.7). In addition, we observe a small but significant increase in mobile fraction of the receptor upon sphingolipid depletion although the overall membrane order does not get altered significantly. 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} (Fukuda et al., 1996). However, such effects are observed at much higher concentrations, several orders of magnitude higher than the concentrations used by us.

The implication of membrane organization on the signaling functions of G-protein coupled receptors represents an interesting aspect. The role of membrane domains in the organization and function of the serotonin_{1A} receptor assumes relevance against this backdrop. This issue was previously addressed in our laboratory by employing the biochemical criterion of detergent insolubility. Utilizing a novel green fluorescent protein-based assay, we reported that a small yet significant fraction (~26%) of the serotonin_{1A} receptor exhibits detergent (Triton X-100) insolubility (Kalipatnapu and Chattopadhyay, 2004a, 2005b). These results are further supported by previous findings from our laboratory using a detergent-free approach (Kalipatnapu and Chattopadhyay, 2007b). Importantly, the detergent (Triton X-100) insoluble fraction of the serotonin_{1A} receptor has recently been estimated to be ~30% using density gradient centrifugation (Renner et al., 2007). The close agreement in the estimate of fraction of receptors in such membrane domains, using very different approaches, points out the existence of a significant population of the receptor in such domains. It is interesting to postulate the nature and composition of such putative domains. Our laboratory has previously shown that membrane cholesterol is required for ligand binding and G-protein coupling of the serotonin_{1A} receptor (Pucadyil and Chattopadhyay, 2006; Pucadyil and Chattopadhyay, 2004a). Our present results demonstrate that sphingolipids are necessary for maintaining the function of the serotonin_{1A}
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receptor. Interestingly, we observe no significant alteration in membrane cholesterol levels upon metabolic depletion of sphingolipids using FB$_1$. However, it is difficult to correlate functional characteristics with membrane organization of the receptor in a direct fashion.

The effect of sphingolipids on the conformation and function of membrane proteins could be due to specific interaction (Fantini, 2003). For example, the nerve growth factor receptor tyrosine kinase has been shown to interact directly with gangliosides (Mutoh et al., 1995). Alternatively, it could be due to modulations in the membrane physical properties induced by sphingolipids or, due to a combination of both factors. Although our results show a slight reduction in overall membrane order upon sphingolipid depletion, specific interaction of sphingolipids with the serotonin$_{1A}$ receptor cannot be ruled out. Interestingly, our results show that the diffusion coefficient of the receptor exhibits no significant change with sphingolipid depletion, while the mobile fraction of the receptor increases by a small yet significant extent. Such a change in the mobile fraction could be attributed to the perturbation of receptor-sphingolipid assembly that preexisted in the membrane prior to sphingolipid depletion. This proposition is supported by the recently reported copatching of a fraction (~30%) of the serotonin$_{1A}$ receptor with GM1 (Renner et al., 2007). The population of receptor in such an assembly could appear immobile in the time scale of FRAP measurements, perhaps limited by the rate of diffusion of the entire assembly. Upon sphingolipid depletion, some of these receptors may be freed resulting in an increase in mobile fraction.

Taken together, these 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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