Role of glycosphingolipids in the function of human serotonin_{1A} receptors

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Abstract
Glycosphingolipids are essential components of eukaryotic cell membranes and are involved in the regulation of cell growth, differentiation, and neoplastic transformation. In this work, we have modulated glycosphingolipid levels in CHO cells stably expressing the human serotonin_{1A} receptor by inhibiting the activity of glucosylceramide synthase using (±)-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), a commonly used inhibitor of the enzyme. Serotonin_{1A} receptors belong to the family of G-protein-coupled receptors and are implicated in the generation and modulation of various cognitive, behavioral, and developmental functions. We explored the function of the serotonin_{1A} receptor under glycosphingolipid-depleted condition by monitoring ligand-binding activity and G-protein coupling of the receptor. Our results show that ligand binding of the receptor is impaired under these conditions although the efficiency of G-protein coupling remains unaltered. The expression of the receptor at the cell membrane appears to be reduced. Interestingly, our results show that the effect of glycosphingolipids on ligand binding caused by metabolic depletion of these lipids is reversible. These novel results demonstrate that glycosphingolipids are necessary for the function of the serotonin_{1A} receptor. We discuss possible mechanisms of specific interaction of glycosphingolipids with the serotonin_{1A} receptor that could involve the proposed ‘sphingolipid-binding domain’.

Keywords: glucosylceramide synthase, glycosphingolipids, G-protein-coupled receptor, PDMP, SBD, serotonin_{1A} receptors.

The serotonin₁₅ receptor is an important neurotransmitter receptor and belongs to the G-protein-coupled receptor (GPCR) superfamily and is the most extensively studied among serotonin receptors for a number of reasons (Pucadyil et al. 2005; Kalipatnapu and Chattopadhyay 2007). The serotonin₁₅ receptor plays 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 (Müller 2005; Kalipatnapu and Chattopadhyay 2007). We utilized a synthetic analog of ceramide and is a competitive inhibitor of glucosylceramide synthase. Sphingosine is not a direct intermediate of the biosynthetic pathway, but can be utilized to generate ceramide as shown in panel (a). Chemical structures of ceramide, PDMP and sphingosine are shown in panel (b). See text for more details.

In this work, we have modulated glycosphingolipid levels in Chinese Hamster Ovary (CHO) cells stably expressing the human serotonin₁₅ receptor (CHO-5-HT₁₅R) by inhibiting the activity of glucosylceramide synthase, the first enzyme in the biosynthesis of glycosphingolipids (see Fig. 1a). This enzyme catalyzes the glucosylation of ceramide in biosynthesis of glycosphingolipids and deletion of this enzyme in the brain has been reported to cause severe neural defects (Jennemann et al. 2005). We utilized (±)-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), the most extensively used inhibitor of glucosylceramide synthase, which is a structural analog of ceramide to modulate cellular glycosphingolipid level (Fig. 1; Inokuchi and Radin 1987). We analyzed the function of the human serotonin₁₅ receptor under these conditions by monitoring ligand binding and G-protein coupling of the receptor. Our results show that the function of the serotonin₁₅ receptor is impaired upon metabolic depletion of glycosphingolipids. Importantly, we show here that the effect of metabolic depletion of glycosphingolipids on the ligand binding of serotonin₁₅ receptors is restored upon metabolic replenishment.

Materials and methods

Cell culture and PDMP treatment

CHO cells stably expressing the human serotonin₁₅ receptor (termed as CHO-5-HT₁₅R) and CHO cells stably expressing the
human serotonin_1A receptor tagged with enhanced yellow fluorescent protein (termed as CHO-5-HT_1A-R-EYFP) were maintained in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (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 gentamicin sulfate, (termed as D-MEM/F-12 complete medium), and 200 µg/mL geneticin (300 µg/mL in case of CHO-5-HT_1A-R-EYFP) in a humidified atmosphere with 5% CO_2 at 37°C. Drug treatment was performed using 1% Nutridoma-SP, 0.33 mg/mL oleic acid albumin, 0.1% fetal calf serum, 12 µg/mL penicillin, 10 µg/mL streptomycin, and 10 µg/mL gentamicin sulfate. Stock solutions (10 mM) of PDMP and (22.8 mM) NB-DNJ were prepared in water. The final concentrations of PDMP used were 20 and 30 µM (500 µM in case of NB-DNJ). Cells were grown for 24 h in D-MEM/F-12 complete medium and then shifted to Nutridoma-BO medium containing PDMP (or NB-DNJ) for 48 h, in a humidified atmosphere with 5% CO_2 at 37°C. Control cells were grown for 24 h in D-MEM/F-12 complete medium and then changed to Nutridoma-BO (lipid-deficient) medium for 48 h.

Cell membrane preparation

Cell membranes were prepared as described earlier (Kalipatnapu et al. 2004). Total protein concentration in the isolated membranes was determined using the bicinchoninic acid (BCA) assay (Smith et al. 1985).

Estimation of phospholipids and cholesterol

The concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid (McClare 1971) using Na_2HPO_4 as standard. Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings. Cholesterol was estimated using the Amplex Red cholesterol assay kit (Amundson and Zhou 1999).

Radioligand binding assays

Receptor binding assays were carried out as described earlier (Kalipatnapu et al. 2004), with ~50 µg total protein. The concentration of [3H]8-OH-DPAT in each assay tube was 0.29 nM.

Metabolic replenishment of glycosphingolipids

Following treatment with 30 µM PDMP for 48 h in Nutridoma-BO medium as described above, CHO-5-HT_1A-R cells were grown for 24 h in D-MEM/F-12 complete medium supplemented with 1 µM sphingosine in a humidified atmosphere with 5% CO_2 at 37°C to achieve metabolic replenishment of sphingolipids.

Statistical analysis

Significance levels were estimated using Student’s two-tailed unpaired t-test using Graphpad Prism software version 4.0 (San Diego, CA, USA).

Details of materials, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) viability assay, saturation radioligand binding assay, GTP-γ-S sensitivity assay, western blot analysis, and fluorescence anisotropy measurements are provided in the Supporting Information.

Results

Cell viability upon PDMP treatment

PDMP has been shown to reduce the level of glycosphingolipids by inhibiting glucosylceramide synthase (Shayman et al. 1990; Nagafuku et al. 2003). To assess the effect of PDMP on cell viability, CHO cells stably expressing the human serotonin_1A receptor were tested for viability using MTT viability assay following PDMP treatment. MTT assay is a cell proliferation assay and provides estimate of the cell growth rate and viability of the cells. No cell death was observed when the concentration of PDMP used was 30 µM. However, cell growth rate was reduced by ~33% with 30 µM PDMP (see Fig. S1). We therefore decided to use 30 µM as the highest concentration of PDMP in our experiments. In addition, PDMP could exert effects other than inhibition of glycosphingolipid metabolism. For example, PDMP has been reported to alter cellular cholesterol homeostasis (Makino et al. 2006). However, PDMP inhibits cholesterol esterification only in the presence of low-density lipoprotein (LDL) (Makino et al. 2006). Figure S2 shows that cholesterol levels were invariant in CHO-5-HT_1A-R cells upon PDMP treatment. PDMP therefore does not affect cholesterol homeostasis in our experimental conditions as we used serum-free NBO medium (i.e., in the absence of LDL).

Specific ligand binding is reduced upon metabolic depletion of glycosphingolipids

To monitor the effect of metabolic depletion of glycosphingolipids on the ligand-binding activity of the serotonin_1A receptor, binding of the selective agonist [3H]8-OH-DPAT to the serotonin_1A receptor was measured in cell membranes prepared from control and PDMP-treated CHO-5-HT_1A-R cells. Fig. 2a shows the reduction in [3H]8-OH-DPAT binding with increasing concentrations of PDMP. The figure shows that specific [3H]8-OH-DPAT binding is reduced to ~84% of the original value when PDMP concentration used was 20 µM. The corresponding value of specific agonist binding is ~51% when a higher concentration (30 µM) of PDMP was used. Importantly, treatment with NB-DNJ, another specific inhibitor of glycosphingolipid biosynthesis (Platt et al. 1994), also resulted in reduction (~22%) in specific agonist binding (see Fig. S3). These results suggest that the reduction in specific ligand binding is primarily because of metabolic depletion of glycosphingolipids, and is independent of the inhibitor used to modulate glycosphingolipid levels.

The reduction in the specific agonist [3H]8-OH-DPAT binding to serotonin_1A receptors (Fig. 2a) could be either because of reduction in affinity of the receptor to the ligand or loss in ligand-binding sites, or both. Saturation binding analysis of [3H]8-OH-DPAT to serotonin_1A receptors is shown in Fig. 2b and Table 1. The results of saturation
binding analysis showed that the reduction in ligand binding can primarily be attributed to a reduction in the number of total binding sites with no significant change in the affinity of ligand binding (Table 1). The table shows that there is a significant reduction (~38%, p < 0.05) in the maximum number of binding sites ($B_{\text{max}}$) when CHO-5-HT1AR cells were treated with PDMP. This indicates that metabolic depletion of glycosphingolipids leads to a reduction in functional receptors without altering receptor affinity.

**G-protein coupling is unaltered upon metabolic depletion of glycosphingolipids**

Seven transmembrane domain receptors are generally coupled to G-proteins, and therefore, guanine nucleotides are known to modulate ligand binding. The serotonin1A receptor agonists (such as 8-OH-DPAT) specifically activate the $G_i/G_o$ class of G-proteins and subsequently dissociate G-proteins, as a result of GTP to GDP exchange at $\alpha$ subunit in CHO cells (Raymond et al. 1993). Agonist binding to such receptors therefore exhibits sensitivity to non-hydrolyzable analogs of GTP such as GTP-$\gamma$-S that uncouples the normal cycle of guanine nucleotide exchange at the $\alpha$ subunit triggered by receptor activation. We have previously shown that serotonin1A receptors undergo an affinity transition from a high-affinity G-protein coupled to a low-affinity G-protein uncoupled state in the presence of GTP-$\gamma$-S (Harikumar and Chattopadhyay 1999). Fig. 3 and Table 2 show a characteristic reduction in binding of the agonist [$^3$H]8-OH-DPAT in presence of GTP-$\gamma$-S with an estimated half-maximal inhibition concentration ($IC_{50}$) of 6.20 nM for control cells. The corresponding $IC_{50}$ value exhibits an increase to 7.53 nM when cells were treated with 30 $\mu$M PDMP (Fig. 3 and Table 2). However, the change in $IC_{50}$ value was found to be

![Figure 2](image.png)

**Fig. 2** (a) Effect of metabolic depletion of glycosphingolipids on specific ligand binding of the human serotonin1A receptor. CHO-5-HT1AR cells were treated with PDMP and specific [$^3$H]8-OH-DPAT binding to the serotonin1A receptor was measured in membranes isolated from these cells. Values are expressed as percentages of specific binding for control cell membranes without PDMP treatment. Data shown are means ± SE of at least three independent experiments. (b) Saturation binding analysis of specific [$^3$H]8-OH-DPAT binding to serotonin1A receptors from CHO-5-HT1AR cell membranes upon glycosphingolipid depletion. CHO-5-HT1AR cells were treated with 30 $\mu$M PDMP and specific [$^3$H]8-OH-DPAT binding to serotonin1A receptors was measured with increasing concentrations of free [$^3$H]8-OH-DPAT. Representative binding plots are shown in case of membranes isolated from control (○) and PDMP-treated (●) cells. See Materials and methods and Table 1 for other details.

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>$K_d$ (nM)</th>
<th>$B_{\text{max}}$ (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.53 ± 0.43</td>
<td>1.0 ± 0.11</td>
</tr>
<tr>
<td>PDMP (30 $\mu$M)</td>
<td>0.54 ± 0.12</td>
<td>0.62 ± 0.08$^b$</td>
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$^b$Corresponds to $p < 0.05$.

![Figure 3](image.png)

**Fig. 3** Effect of metabolic depletion of glycosphingolipids on G-protein coupling of the human serotonin1A receptor. G-protein coupling efficiency of the serotonin1A receptor was monitored by the sensitivity of specific [$^3$H]8-OH-DPAT binding in presence of GTP-$\gamma$-S, a non-hydrolyzable analog of GTP. The figure shows the effect of increasing concentrations of GTP-$\gamma$-S on the specific binding of the agonist [$^3$H]8-OH-DPAT to serotonin1A receptors in membranes isolated from control (○) and PDMP-treated (●) cells. The concentration of PDMP used was 30 $\mu$M. Values are expressed as percentages of specific binding obtained at the lowest concentration of GTP-$\gamma$-S. Curves are non-linear regression fits to the experimental data using eqn. S2. Data points represent means ± SE of duplicate points from at least three independent experiments. See Materials and methods and Table 2 for other details.

GSLs and function of the human serotonin1A receptors

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In addition, metabolic depletion of glycosphingolipids in the cell membrane is reduced to ~67% (p < 0.05) of control value upon PDMP treatment, possibly because of impairment of biogenesis and trafficking. Interestingly, such impaired trafficking upon PDMP treatment has previously been reported for the nicotinic acetylcholine receptor (Baier and Barrantes 2007). These results indicate that the observed impairment in ligand binding of the serotonin1A receptor upon glycosphingolipid depletion is partly because of reduction in receptor expression level in the membrane.

Overall membrane order remains unaltered upon metabolic depletion of glycosphingolipids

Alteration in membrane physical properties could lead to change in ligand binding (Gimpl et al. 1997; Prasad et al. 2009). In addition, metabolic depletion of glycosphingolipids could also result in perturbation of membrane domains containing these lipids (as mentioned earlier), thereby possibly changing membrane order. To monitor any possible change in overall membrane order upon PDMP treatment, we measured anisotropy of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) in membranes from control and PDMP-treated cells. Fluorescence anisotropy measured using probes such as DPH is correlated to the rotational diffusion of membrane-embedded probes (Lakowicz 2006), which is sensitive to the packing of lipid fatty acyl chains. DPH, a rod-like hydrophobic molecule, partitions into the interior hydrophobic region of the membrane. Figure 5 shows that fluorescence anisotropy of DPH does not exhibit any significant change upon metabolic depletion of glycosphingolipids indicating that the overall membrane order is not altered. These results suggest that the observed decrease in ligand binding of the serotonin1A receptor is not brought about by any change in overall membrane order (i.e., general effect). These results also show that PDMP does not change membrane order, at least in the concentration used by us. Specific interactions between glycosphingolipids and the serotonin1A receptor could therefore play an important role in the function of the serotonin1A receptor.

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Table 2 Effect of metabolic glycosphingolipid depletion on the efficiency of G-protein coupling

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>IC50 (nM)</th>
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<tbody>
<tr>
<td>Control</td>
<td>6.20 ± 1.48</td>
</tr>
<tr>
<td>PDMP (30 µM)</td>
<td>7.53 ± 2.73</td>
</tr>
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</table>

*The sensitivity of specific [3H]8-OH-DPAT binding to the receptor was measured by calculating the IC50 for inhibition of [3H]8-OH-DPAT binding in the presence of a range of concentrations of GTP-γ-S. Inhibition curves were analyzed using the four-parameter logistic function. Data represent means ± SE of four independent experiments. See Materials and methods for other details.

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**Receptor expression level is reduced upon metabolic depletion of glycosphingolipids**

The reduction in ligand binding of the serotonin1A receptor observed upon PDMP treatment (Fig. 2a) could be because of decrease in the expression levels of serotonin1A receptors in the cell membrane. We carried out western blot analysis of 5-HT1A-R-EYFP in cell membranes prepared from control and PDMP-treated CHO-5-HT1A-R-EYFP cells (see Fig. 4) to monitor the receptor expression level upon glycosphingolipid depletion. We chose to use the receptor tagged to EYFP (5-HT1A-R-EYFP) as monoclonal antibodies for the serotonin1A receptor are not available, and polyclonal antibodies have been reported to give variable results on Western blots (Zhou et al. 1999). We have previously shown that EYFP fusion to the serotonin1A receptor does not affect ligand binding, G-protein coupling, and signaling of the receptor (Pucadyil et al. 2004). Importantly, CHO-5-HT1A-R-EYFP cells exhibit reduction in specific binding of the agonist [3H]8-OH-DPAT to serotonin1A receptors upon PDMP treatment, similar to what is observed with CHO-5-HT1A-R cells (see Fig. 2a and Fig. S4). Figure 4 shows that the receptor level in the cell membrane is reduced to ~67% (p < 0.05) of control value upon PDMP treatment, possibly because of impairment of biogenesis and trafficking. Interestingly, such impaired trafficking upon PDMP treatment has previously been reported for the nicotinic acetylcholine receptor (Baier and Barrantes 2007). These results indicate that the observed impairment in ligand binding of the serotonin1A receptor upon glycosphingolipid depletion is partly because of reduction in receptor expression level in the membrane.

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Discussion

The serotonin1A receptor is an important member of the GPCR superfamily. The GPCR superfamily is the largest and most diverse protein family in mammals, involved in signal transduction across membranes (Pierce et al. 2002; Rosenbaum et al. 2009). GPCRs are seven transmembrane domain proteins and include > 800 members which are encoded by ~5% of human genes (Zhang et al. 2006). GPCRs regulate physiological responses to a diverse array of stimuli, and mediate multiple physiological processes. As a result, GPCRs have emerged as major targets for the development of novel drug candidates in all clinical areas (Heilker et al. 2009). It is estimated that ~50% of clinically prescribed drugs act as either agonists or antagonists of GPCRs (Schlyer and Horuk 2006).

As GPCRs are integral membrane proteins with multiple transmembrane domains, the interaction of membrane lipids with receptors represent a crucial factor in maintaining their structure and function. Lipid–protein interactions are particularly relevant in case of GPCRs as they undergo conformational changes for carrying out their function (Deupi and Kobilka 2010; Unal and Karnik 2012). This is supported by the recent crystal structure of the β2-adrenergic receptor, which shows specific cholesterol-binding sites in the receptor (Cherezov et al. 2007; Hanson et al. 2008). It has been recently reported that the interaction between GPCRs and G-proteins could be modulated by membrane lipids (Inagaki et al. 2012). Importantly, the membrane lipid environment of GPCRs has been implicated in disease progression during aging (Alemany et al. 2007). In this emerging scenario, the interaction of the serotonin1A receptor with surrounding membrane lipids such as glycosphingolipids assumes significance. Interestingly, glycosphingolipids have previously been shown to modulate the function of membrane receptors (Wang et al. 2001).

In this work, we monitored ligand binding and G-protein coupling of the serotonin1A receptor stably expressed in CHO cells under condition of metabolic glycosphingolipid replenishment using sphingosine on specific agonist binding of the human serotonin1A receptor. Following treatment with 30 μM PDMP in Nutridoma-BO (lipid-deficient) medium, CHO-5-HT1AR cells were grown for 24 h in D-MEM/F-12 complete medium supplemented with 1 μM sphingosine in a humidified atmosphere with 5% CO2 at 37°C. Changes in the specific binding of the agonist 3H]B-OH-DPAT to serotonin1A receptors in control, 30 μM PDMP-treated and glycosphingolipid-replenished conditions are shown (*corresponds to a p < 0.05 for the difference between PDMP-treated and glycosphingolipid-replenished conditions). Data represent means ± SE of at least three independent experiments. See Materials and methods for other details.

Replenishment of glycosphingolipids restores ligand binding

To monitor the reversibility of the effect of glycosphingolipids on the function of the serotonin1A receptor, we supplemented CHO-5-HT1AR cells with sphingosine and monitored ligand binding. Sphingosine is a catabolic intermediate of sphingolipids and can enter sphingolipid biosynthetic pathway via ceramide as shown in Fig. 1a. Sphingosine has previously been shown to restore sphingolipid levels in sphingolipid mutant CHO cells and cells treated with sphingolipid inhibitor (Fukasawa et al. 2000; Paila et al. 2010). Figure 6 shows that pre-treatment of CHO-5-HT1AR cells with PDMP in serum-free NBO (lipid-deficient) medium followed by replenishment with 1 μM sphingosine in D-MEM/F-12 complete medium restored ligand binding of the serotonin1A receptor to a considerable extent. The specific agonist binding was reduced to ~51% of the original value on PDMP treatment and was restored to ~78% on replenishment with sphingosine. Taken together, these results show that the reduction in ligand binding of the serotonin1A receptor by metabolic depletion of glycosphingolipids is predominantly reversible.

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depletion using PDMP. Our results show that ligand binding of the receptor is impaired under these conditions, although the efficiency of G-protein coupling appears unaltered. We also observed lowered expression of the receptor at the cell membrane under these conditions that could partly account for the reduction in ligand binding. Interestingly, our results show that the effect of glycosphingolipids on ligand binding caused by metabolic depletion of these lipids is reversible to a considerable extent.

The effect of glycosphingolipids on the conformation and function of membrane proteins could be because of specific interaction. For example, the nerve growth factor receptor tyrosine kinase has been shown to interact directly with gangliosides (Mutoh et al. 1995). It has been previously reported that proteins that interact with glycosphingolipids appear to have a characteristic amino acid sequence, termed the ‘sphingolipid-binding domain’ (SBD) (Mahfoud et al. 2002b; Fantini 2003; Chakrabandhu et al. 2008; Hebbar et al. 2008; Fantini and Barrantes 2009). We recently reported, using an algorithm (Chakrabandhu et al. 2008) based on the systematic presence of key amino acids belonging to hairpin structures that the human serotonin1A receptor contains a putative SBD motif (LNKWTLGQVTC, corresponding to residues 99–109) (Chattopadhyay et al. 2012). In addition, we showed that the SBD motif appears to be an inherent feature of serotonin1A receptors and is conserved over natural evolution across various phyla (Chattopadhyay et al. 2012). The apparent glycosphingolipid sensitivity of the receptor function reported here could be because of specific interaction of the SBD motif with membrane glycosphingolipids. Our future efforts will focus on mutating this region in the receptor and examining glycosphingolipid sensitivity. Interestingly, specific interaction between a single sphingolipid species and transmembrane domain of a receptor has been recently reported (Contreras et al. 2012).

We have previously shown that membrane cholesterol is necessary for the function of the serotonin1A receptor (Pucadyil and Chattopadhyay 2004, 2006; Paila et al. 2008; Singh et al. 2009; Paila and Chattopadhyay 2010; Shrivastava et al. 2010). We recently reported the presence of cholesterol recognition/interaction amino acid consensus (CRAC) motifs in the serotonin1A receptor (Jafurulla et al. 2011). The CRAC motif represents a characteristic structural feature of proteins that are believed to result in preferential association with cholesterol (Li and Papadopoulos 1998; Epand 2006). The serotonin1A receptor sequence contains CRAC motifs consisting of 12 amino acids in putative transmembrane helices II (residues 90–101), V (residues 208–219), and VII (residues 394–405). Interestingly, the SBD motif proposed for the serotonin1A receptor (Chattopadhyay et al. 2012) overlaps with the CRAC motif proposed for the receptor (residues 99–101). This is significant in the context of the reported cholesterol-dependent sphingolipid membrane microdomains (Hebbar et al. 2008). In case of the serotonin1A receptor, both cholesterol and sphingolipids are necessary for receptor function and therefore an interplay between these membrane lipids would be relevant. In summary, our results show that glycosphingolipids have a crucial role in maintaining the function of the serotonin1A receptor. These results could be useful in understanding the role of the membrane lipid environment on the function of the serotonin1A receptor in particular, and GPCRs in general.

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Conflict of interest

Authors declare no conflict of interest.

Supporting information

Additional supporting information may be found in the online version of this article:
Appendix S1. Materials and methods.
Figure S1. Effect of PDMP on cell viability.
Figure S2. Cholesterol content in membranes isolated from control and PDMP-treated cells.
Figure S3. Effect of NB-DNJ-mediated metabolic depletion of glycosphingolipids on specific ligand binding of the human serotonin1A receptor.
Figure S4. Effect of metabolic depletion of glycosphingolipids on specific ligand binding of the human serotonin1A receptor tagged to EYFP.

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SUPPORTING INFORMATION

Role of Glycosphingolipids in the Function of Human Serotonin$_{1A}$ Receptors

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Materials and methods

Materials

DMPC, PDMP, NB-DNJ, EDTA, MgCl₂, MnCl₂, 8-OH-DPAT, penicillin, streptomycin, gentamycin sulfate, polyethyleneimine, PMSF, serotonin, sodium bicarbonate, oleic acid albumin, Tris and MTT were obtained from Sigma Chemical Co. (St. Louis, MO, USA). D-MEM/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, USA). GTP-γ-S and Nutridoma-SP were from Roche Applied Science (Mannheim, Germany). Primary antibodies against GFP were from Abcam (Cambridge, UK) and antibodies against β-actin were from Chemicon International (Temecula, CA, USA). Chemiluminescence detection reagents and secondary antibodies (anti-rabbit antibody for 5-HT₁AR-EYFP and anti-mouse antibody for β-actin conjugated to horseradish peroxidase) were from Amersham (Amersham Biosciences, Buckinghamshire, UK). BCA reagent for protein estimation was from Pierce (Rockford, IL, USA). [³H]8-OH-DPAT (sp. activity 135.0 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA, USA). GF/B glass microfiber filters were from Whatman International (Kent, UK). All other chemicals and solvents used were of the highest available purity. Water was purified through a Millipore (Bedford, MA, USA) Milli-Q system and used throughout.

MTT viability assay

In order to determine appropriate concentrations of PDMP, a dose-response for cell viability was monitored using the MTT assay. Equal number of cells (~1 x 10⁴) were seeded in 96 well plate and treatments were carried out as described above. Treatment with PDMP (up to 50 μM) was carried out for 48 h in Nutridoma-BO medium. MTT was dissolved in PBS and added to cells to a final concentration of 0.3 mg/ml. Cells were incubated at 37 °C for 1 h. Formazan crystals formed upon reduction of MTT salt by mitochondrial enzymes in live cells (Vistica et al. 1991) are insoluble in aqueous medium. Cells were centrifuged in 96 well plate
and subsequently dissolved in DMSO after discarding the medium. The color obtained was measured by absorbance at 550 nm in a SpectraMax 190 absorbance microplate reader (Molecular Devices).

**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 earlier (Kalipatnapu *et al.* 2004). The concentrations of GTP-γ-S leading to 50% inhibition of specific agonist binding (IC$_{50}$) were calculated by non-linear regression fitting of the data to a four parameter logistic function (Higashijima *et al.* 1987):

$$B = \frac{a}{1 + \left(\frac{x}{I}\right)^s} + b$$

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.

**Saturation radioligand binding assay**

Saturation binding assays were carried out with increasing concentrations (0.1–7.5 nM) of the radiolabeled agonist $[^3H]8$-OH-DPAT as described previously (Kalipatnapu *et al.* 2004). Non-specific binding was measured in the presence of 10 μM serotonin for agonist binding. The concentration of the bound radioligand (RL*) was calculated from the equation:

$$RL^* = 10^{-9} \times \frac{B}{(V \times SA \times 2220)} M$$

where $B$ is the bound radioactivity in disintegrations per minute (dpm) (*i.e.*, total dpm–non-specific dpm), $V$ is the assay volume in ml, and $SA$ is the specific activity of the radioligand. Data could be fitted best to a one-site ligand binding equation. The dissociation constant (K$_d$) and maximum binding sites (B$_{max}$) were calculated by non-linear regression analysis of binding data using Graphpad Prism software version 4.0 (San Diego, CA, USA). Data obtained after
regression analysis were used to plot graphs with the GRAFIT program version 3.09b (Erithacus Software, Surrey, UK).

**Western blot analysis**

Western blot was performed as described previously (Shrivastava et al. 2010). Briefly 60 μg of total protein from each sample was run on SDS PAGE and transferred to nitrocellulose membrane using semi-dry transfer apparatus. To monitor the expression of 5-HT\textsubscript{1A}R-EYFP, blots were probed with antibodies raised against GFP (1:1500 dilution in PBS/Tween 20), incubated for 90 min at room temperature (~23 °C). To monitor the levels of β-actin, which acts as a loading control, membranes were probed with antibodies raised against β-actin (diluted 1:3000 in PBS/Tween 20), incubated for 90 min at room temperature (~23 °C). Membranes were washed with PBS/Tween 20 (washing buffer) for 15 min and the washing buffer was changed every 5 min. Membranes were then incubated with 1:4000 dilution of respective secondary antibodies in PBS/Tween 20 for 45 min at room temperature (~23 °C). Membranes were then washed and developed using the enhanced chemiluminescence detection reagents. 5-HT\textsubscript{1A}R-EYFP and β-actin were detected using the chemiluminescence detection system (Chemi-Smart 5000, Vilber Lourmat, Germany). 5-HT\textsubscript{1A}R-EYFP and β-actin levels were quantitated using Bio-Profile (Bio-1D+, version 11.9).

**Fluorescence anisotropy measurements**

Fluorescence anisotropy experiments were carried out using the fluorescent probe DPH with membranes prepared from control and PDMP-treated cells, containing 50 nmol of total phospholipids suspended in 1.5 ml of 50 mM Tris, pH 7.4 buffer, as described earlier (Singh et al. 2007). Steady state fluorescence was measured in a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvette at room temperature (~23 °C). Excitation and emission wavelengths were set at 358 and 430 nm. Excitation and emission slits with nominal bandpasses of 1.5 nm and 20 nm were used. The optical density of the samples measured at 358 nm was less
than 0.10. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy (r) values were calculated from the equation (Lakowicz 2006):

\[
    r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \tag{3}
\]

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

**Metabolic replenishment of glycosphingolipids**

Following treatment with 30 \(\mu\)M PDMP for 48 h in Nutridoma-BO medium as described above, CHO-5-HT\(_{1A}\)R cells were grown for 24 h in D-MEM/F-12 complete medium supplemented with 1 \(\mu\)M sphingosine in a humidified atmosphere with 5% CO\(_2\) at 37 °C in order to achieve metabolic replenishment of sphingolipids.

**Statistical analysis**

Significance levels were estimated using Student’s two-tailed unpaired \(t\)-test using Graphpad Prism software version 4.0 (San Diego, CA, USA).
References


Figure Legends

Fig. S1 Effect of PDMP on cell viability. CHO-5-HT₁AR cells were assayed for viability by a standard MTT assay after treating cells with increasing concentrations of PDMP (up to 50 μM) for 48 h. Values are expressed as percentages of viability for control cells (in absence of PDMP). Data represent means ± SE of at least three independent experiments. See Materials and methods for other details.

Fig. S2 Cholesterol content in membranes isolated from control and PDMP-treated cells. The concentration of PDMP used was 30 μM. Data represent means ± SE of duplicate points from at least three independent experiments. See Materials and methods for other details.

Fig. S3 Effect of NB-DNJ-mediated metabolic depletion of glycosphingolipids on specific ligand binding of the human serotonin₁A receptor. CHO-5-HT₁AR-EYFP cells were treated with NB-DNJ and specific $[^3H]$8-OH-DPAT binding to the serotonin₁A receptor was measured in membranes isolated from these cells. Values are expressed as percentages of specific binding for control cell membranes without NB-DNJ treatment. Data shown are means ± SE of three independent experiments. (*corresponds to $p < 0.05$ for the difference between NB-DNJ-treated and control conditions). See Materials and methods for other details.

Fig. S4 Effect of metabolic depletion of glycosphingolipids on specific ligand binding of the human serotonin₁A receptor tagged to EYFP. CHO-5-HT₁AR-EYFP cells were treated with 30 μM PDMP and specific $[^3H]$8-OH-DPAT binding to the serotonin₁A receptor was measured in membranes isolated from these cells. Values are expressed as percentages of specific binding for control cell membranes without PDMP treatment. Data shown are means ± SE of at least three independent experiments. See Materials and methods for other details.
Supporting Information

Singh et al.

Figure S1

CONCENTRATION OF PDMP (µM)

CELL VIABILITY (%)
Figure S2
Singh et al.

Supporting Information

<table>
<thead>
<tr>
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<th>Control</th>
<th>PDMP</th>
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<td>CHOLESTEROL CONTENT (%)</td>
<td>100</td>
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Error bars indicate standard error of the mean.
Figure S3
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Supporting Information
Figure S4
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**Figure S4**

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The figure shows a bar graph titled "SPECIFIC [3H]8-OH-DPAT BINDING (%)". The x-axis represents "Control" and "PDMP", while the y-axis ranges from 0 to 100. The graph indicates a significant difference in specific binding between Control and PDMP conditions, with Control showing a much higher value than PDMP.