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ORIGINAL ARTICLE

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.

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Glycosphingolipids (GSLs) are essential components of eukaryotic cell membranes constituting ~5% of the total membrane lipids (Fukasawa *et al.* 2000). They are major components of neuronal membranes where they constitute up to 30% of the total lipid content. Glycosphingolipids are more abundant in the plasma membrane and are found to be predominantly distributed in the outer leaflet of the plasma membrane (Hoekstra and Kok 1992). The distribution of glycosphingolipids in the membrane appears to be heterogeneous, and it has been postulated that glycosphingolipids and cholesterol occur in laterally segregated lipid domains (Brown 1998; Masserini and Ravasi 2001; Prinetti *et al.* 2009). Many of these domains are believed to be important for the maintenance of membrane structure and function, although characterizing the spatiotemporal resolution of these domains has proven to be challenging (Jacobson *et al.* 2007; Ganguly and Chattopadhyay 2010). These specialized regions (sometimes termed as 'lipid rafts') contribute to variable patchiness of the membrane, and are believed to be enriched in specific lipids and proteins. They facilitate processes such as cellular trafficking and sorting (Simons and Meer 1988), signal transduction (Simons and Toomre 2000) and pathogen entry (Riethmüller *et al.* 2006; Pucadyil and Chattopadhyay 2007).

Glycosphingolipids are synthesized in the Golgi complex where the first step of glycosphingolipid synthesis (*i.e.*,

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Abbreviations used: 5-HT$_{1A}$ receptor, 5-hydroxytryptamine-1A receptor; 5-HT$_{1A}$R-EYFP, 5-hydroxytryptamine$_{1A}$ receptor tagged to enhanced yellow fluorescent protein; 8-OH-DPAT, 8-hydroxy-2(di-*N*-propylamino)tetralin; BCA, bicinchoninic acid; CRAC, cholesterol recognition/interaction amino acid consensus; DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; GPCR, G-protein-coupled receptor; GSLs, glycosphingolipids; GTP-γ-S, guanosine-5′-*O*-(3-thiotriphosphate); LDL, low-density lipoprotein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NB-DNJ, *N*-butyldeoxynojirimycin; PDMP, (±)-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PMSF, phenylmethylsulfonyl fluoride; SBD, sphingolipid-binding domain.

glucosylation of ceramide into glucosylceramide) is catalyzed by the enzyme glucosylceramide synthase (also called as glucosyltransferase) by transferring glucose moiety from UDP-glucose to ceramide. Glucosylceramide is the simplest glycosphingolipid and precursor of hundreds of complex glycosphingolipids such as gangliosides. Gangliosides belong to an important and specialized subclass of glycosphingolipids containing sialic acid moiety. Glycosphingolipids are 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 (Lahiri and Futerman 2007). Interestingly, glycosphingolipids help and promote the entry of human immunodeficiency virus type I (Hug *et al.* 2000; Mahfoud *et al.* 2002a) and are shown to act as receptors for pore-forming toxins produced by *Bacillus thuringiensis* (Griffitts *et al.* 2005). Knockout studies in mice have demonstrated that the synthesis of glycosphingolipids is essential for embryonic development (Yamashita *et al.* 1999). In addition, glycosphingolipids have been demonstrated to regulate apoptosis, survival, and regeneration of cells (Bektas and Spiegel 2004). Importantly, the emerging role of glycosphingolipids in the development and progression of several neurological diseases such as Alzheimer's disease is well documented (Ariga *et al.* 2008). Modulating glycosphingolipid levels and monitoring the function of an important neurotransmitter receptor therefore assume relevance.

The serotonin$_{1A}$ 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 serotonin$_{1A}$ 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 *et al.* 2007). Agonists (Blier and Ward 2003) and antagonists (Griebel 1999) of the serotonin$_{1A}$ receptor therefore represent major classes of molecules with potential therapeutic effects in anxiety- or stress-related disorders. Interestingly, mutant (knockout) mice lacking the serotonin$_{1A}$ receptor display 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 (Gardier 2009).

In this work, we have modulated glycosphingolipid levels in Chinese Hamster Ovary (CHO) cells stably expressing the human serotonin$_{1A}$ receptor (CHO-5-HT$_{1A}$R) 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

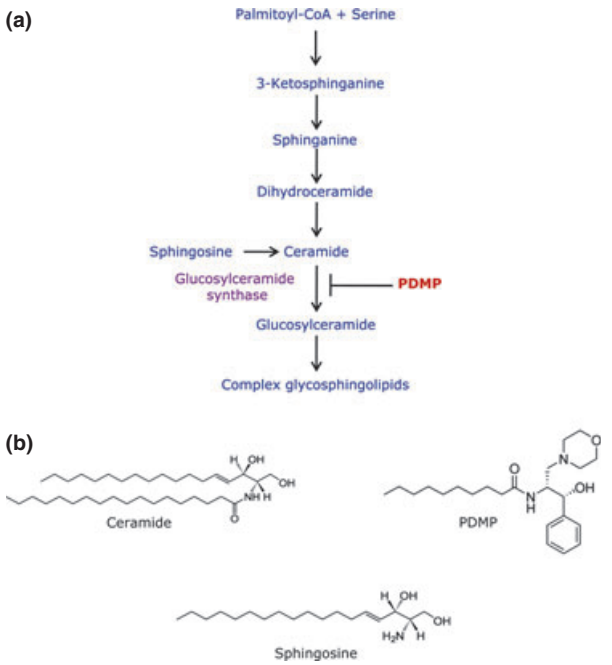

Fig. 1 Biosynthetic pathway of glycosphingolipids and chemical structures of ceramide, PDMP and sphingosine. PDMP is an inhibitor of glucosylceramide synthase, the first enzyme in the biosynthesis of glycosphingolipids. Glucosylceramide synthase catalyzes the glucosylation of ceramide in biosynthesis of glycosphingolipids. Panel (a) shows the biosynthetic pathway of glycosphingolipids. PDMP is 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.

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 serotonin$_{1A}$ receptor under these conditions by monitoring ligand binding and G-protein coupling of the receptor. Our results show that the function of the serotonin$_{1A}$ 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 serotonin$_{1A}$ receptors is restored upon metabolic replenishment.

Materials and methods

Cell culture and PDMP treatment

CHO cells stably expressing the human serotonin$_{1A}$ receptor (termed as CHO-5-HT$_{1A}$R) 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 gentamycin 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. Nutridoma-BO (lipid-deficient) medium was prepared 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 gentamycin 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 [^{3}H]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 [^{3}H]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 [^{3}H]8-OH-DPAT binding with increasing concentrations of PDMP. The figure shows that specific [^{3}H]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 [^{3}H]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 [^{3}H]8-OH-DPAT to serotonin$_{1A}$ receptors is shown in Fig. 2b and Table 1. The results of saturation

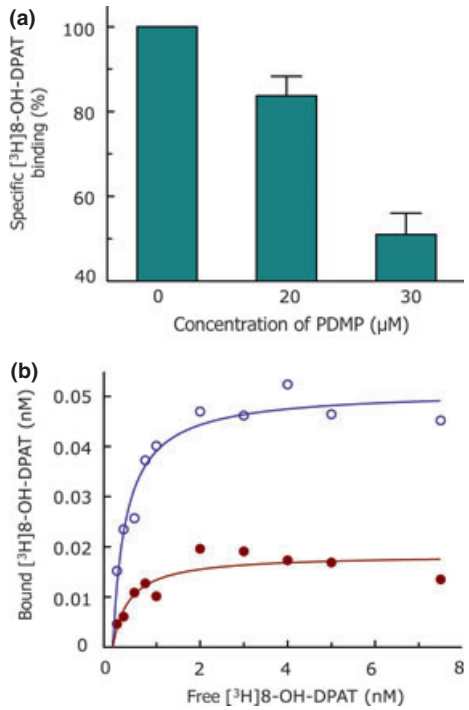

Fig. 2 (a) Effect of metabolic depletion of glycosphingolipids on specific ligand binding of the human serotonin$_{1A}$ receptor. CHO-5-HT$_{1A}$R cells were treated with PDMP and specific [^{3}H]8-OH-DPAT binding to the serotonin$_{1A}$ receptor was measured in membranes isolated from these cells. Values 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 serotonin$_{1A}$ receptors from CHO-5-HT$_{1A}$R cell membranes upon glycosphingolipid depletion. CHO-5-HT$_{1A}$R cells were treated with 30 μM PDMP and specific [^{3}H]8-OH-DPAT binding to serotonin$_{1A}$ 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.

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_{max}) when CHO-5-HT$_{1A}$R 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 serotonin$_{1A}$ receptor agonists (such as 8-OH-DPAT) specifically activate the G$_i$/G$_o$ class of

Table 1 Effect of metabolic glycosphingolipid depletion on specific [^{3}H]8-OH-DPAT binding[a]

Experimental condition	K_d (nM)	B_{max} (pmol/mg)
Control	0.53 ± 0.43	1.0 ± 0.11
PDMP (30 μM)	0.54 ± 0.12	0.62 ± 0.08[b]

[a]Binding parameters were calculated by analyzing saturation binding isotherms with a range of [^{3}H]8-OH-DPAT concentrations using Graphpad Prism software. Data shown represent means ± SE of four independent experiments. See Materials and methods for other details.
[b]Corresponds to $p < 0.05$.

G-proteins and subsequently dissociate G-proteins, as a result of GTP to GDP exchange at Gα 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-γ-S that uncouples the normal cycle of guanine nucleotide exchange at the Gα subunit triggered by receptor activation. We have previously shown that serotonin$_{1A}$ 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-γ-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-γ-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 μM PDMP (Fig. 3 and Table 2). However, the change in IC_{50} value was found to be

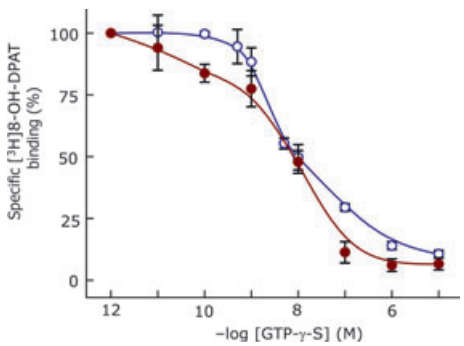

Fig. 3 Effect of metabolic depletion of glycosphingolipids on G-protein coupling of the human serotonin$_{1A}$ receptor. G-protein coupling efficiency of the serotonin$_{1A}$ receptor was monitored by the sensitivity of specific [^{3}H]8-OH-DPAT binding in presence of GTP-γ-S, a non-hydrolyzable analog of GTP. The figure shows the effect of increasing concentrations of GTP-γ-S on the specific binding of the agonist [^{3}H]8-OH-DPAT to serotonin$_{1A}$ receptors in membranes isolated from control (○) and PDMP-treated (●) cells. The concentration of PDMP used was 30 μM. Values are expressed as percentages of specific binding obtained at the lowest concentration of GTP-γ-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.

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

Experimental condition	IC_{50} (nM)
Control	6.20 ± 1.48
PDMP (30 μM)	7.53 ± 2.73

[c]The sensitivity of specific [^{3}H]8-OH-DPAT binding to the receptor was measured by calculating the IC_{50} for inhibition of [^{3}H]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.

not significant. This shows that G-protein coupling is not affected upon metabolic glycosphingolipid depletion by PDMP.

Receptor expression level is reduced upon metabolic depletion of glycosphingolipids

The reduction in ligand binding of the serotonin$_{1A}$ receptor observed upon PDMP treatment (Fig. 2a) could be because of decrease in the expression levels of serotonin$_{1A}$ receptors in the cell membrane. We carried out western blot analysis of 5-HT$_{1A}$R-EYFP in cell membranes prepared from control and PDMP-treated CHO-5-HT$_{1A}$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-HT$_{1A}$R-EYFP) as monoclonal antibodies for the serotonin$_{1A}$ 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 serotonin$_{1A}$ receptor does not affect ligand binding, G-protein coupling, and signaling of the receptor (Pucadyil *et al.* 2004). Importantly, CHO-5-HT$_{1A}$R-EYFP cells exhibit reduction in specific binding of the agonist [^{3}H] 8-OH-DPAT to serotonin$_{1A}$ receptors upon PDMP treatment, similar to what is observed with CHO-5-HT$_{1A}$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 serotonin$_{1A}$ 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

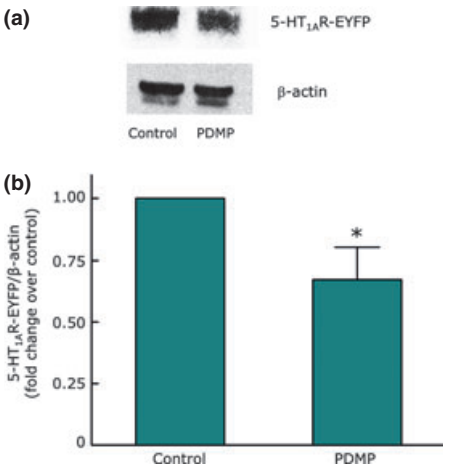

Fig. 4 Effect of metabolic depletion of glycosphingolipids on the expression level of the human serotonin$_{1A}$ receptor in membranes. Western blot analysis of 5-hydroxytryptamine$_{1A}$ receptor tagged to enhanced yellow fluorescent protein (5-HT$_{1A}$R-EYFP) in membranes prepared from control and PDMP-treated CHO-5-HT$_{1A}$R-EYFP cells are shown. 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. The concentration of PDMP used was 30 μM. 5-HT$_{1A}$R-EYFP levels were normalized to β-actin of the corresponding sample. Data are shown as fold change of 5-HT$_{1A}$R-EYFP over control and represent means ± SE of at least three independent experiments (*corresponds to $p < 0.05$ for the difference between PDMP-treated and control conditions). See Materials and methods for other details.

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 serotonin$_{1A}$ 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 serotonin$_{1A}$ receptor could therefore play an important role in the function of the serotonin$_{1A}$ receptor.

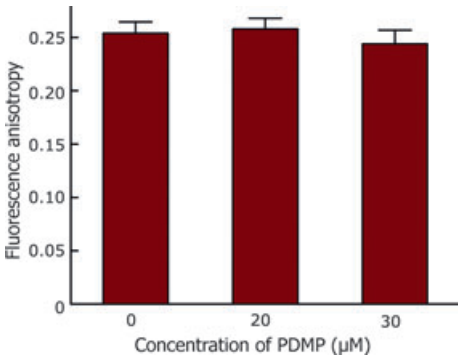

Fig. 5 Effect of metabolic depletion of glycosphingolipids on membrane order. The overall (average) membrane order was estimated in membranes isolated from control and PDMP-treated cells by measurement of fluorescence anisotropy of the membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH). Fluorescence 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). Data represent means ± SE of duplicate points from at least three independent experiments. See Materials and methods for other details.

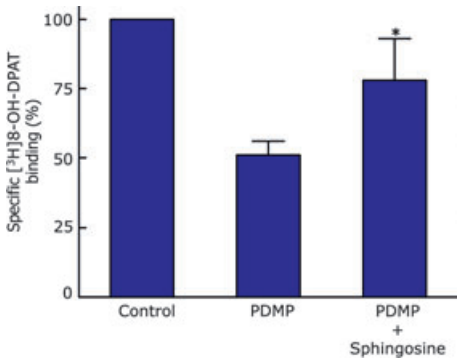

Fig. 6 Effect of replenishment of glycosphingolipids using sphingosine on specific agonist binding of the human serotonin$_{1A}$ receptor. Following treatment with 30 μM PDMP in Nutridoma-BO (lipid-deficient) medium, CHO-5-HT$_{1A}$R cells were grown for 24 h in D-MEM/F-12 complete medium supplemented with 1 μM sphingosine in a humidified atmosphere with 5% CO_2 at 37°C. Changes in the specific binding of the agonist [^{3}H]8-OH-DPAT to serotonin$_{1A}$ 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 serotonin$_{1A}$ receptor, we supplemented CHO-5-HT$_{1A}$R 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-HT$_{1A}$R 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 serotonin$_{1A}$ 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 serotonin$_{1A}$ receptor by metabolic depletion of glycosphingolipids is predominantly reversible.

Discussion

The serotonin$_{1A}$ 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 β_2-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 serotonin$_{1A}$ 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 serotonin$_{1A}$ receptor stably expressed in CHO cells under condition of metabolic glycosphingolipid

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 serotonin$_{1A}$ 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 serotonin$_{1A}$ 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 serotonin$_{1A}$ 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 serotonin$_{1A}$ 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 serotonin$_{1A}$ 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 serotonin$_{1A}$ 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 serotonin$_{1A}$ 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 serotonin$_{1A}$ receptor. These results could be useful in understanding the role of the membrane lipid environment on the function of the serotonin$_{1A}$ 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, $MgCl_2$, $MnCl_2$, 8-OH-DPAT, penicillin, streptomycin, gentamycin sulfate, polyethylenimine, 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-HT_{1A}R-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). [^{3}H]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 10^4) 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 ºC 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):

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

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.

Saturation radioligand binding assay

Saturation binding assays were carried out with increasing concentrations (0.1–7.5 nM) of the radiolabeled agonist [^{3}H]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:

$$RL^* = 10^{-9} \times B/(V \times SA \times 2220)\ M \qquad (2)$$

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_{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_{1A}R-EYFP and β-actin were detected using the chemiluminescence detection system (Chemi-Smart 5000, Vilber Lourmat, Germany). 5-HT_{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):

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \qquad (3)$$

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 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 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).

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Figure Legends

Fig. S1 Effect of PDMP on cell viability. CHO-5-HT_{1A}R 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 serotonin$_{1A}$ receptor. CHO-5-HT_{1A}R-EYFP cells were treated with NB-DNJ and specific [^{3}H]8-OH-DPAT binding to the serotonin$_{1A}$ 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 serotonin$_{1A}$ receptor tagged to EYFP. CHO-5-HT_{1A}R-EYFP cells were treated with 30 μM PDMP and specific [^{3}H]8-OH-DPAT binding to the serotonin$_{1A}$ 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.

Figure S1
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Supporting Information

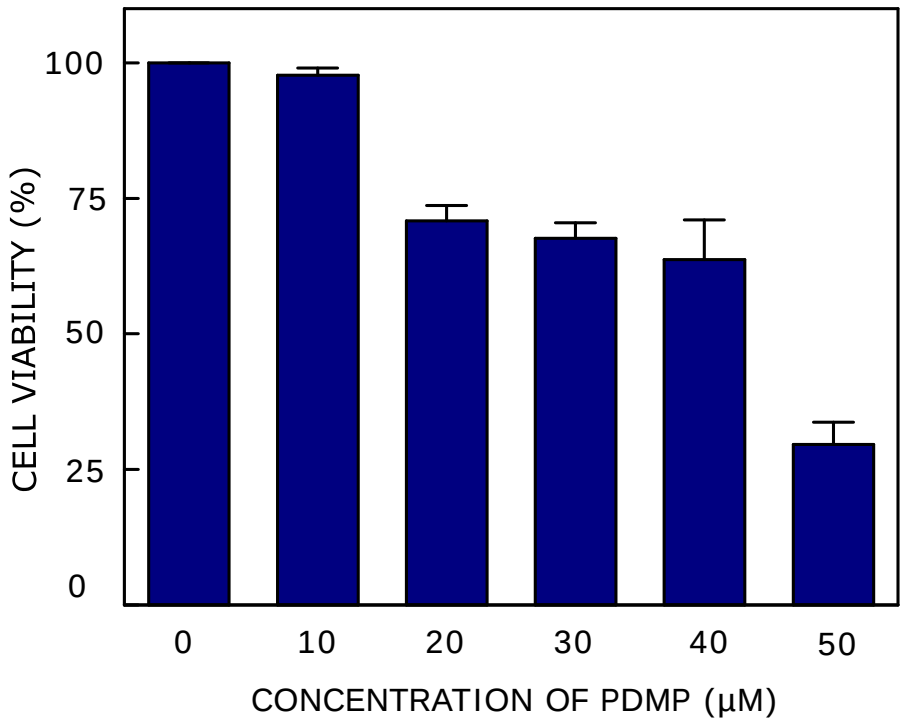


Figure S2
Singh *et al.*

Supporting Information

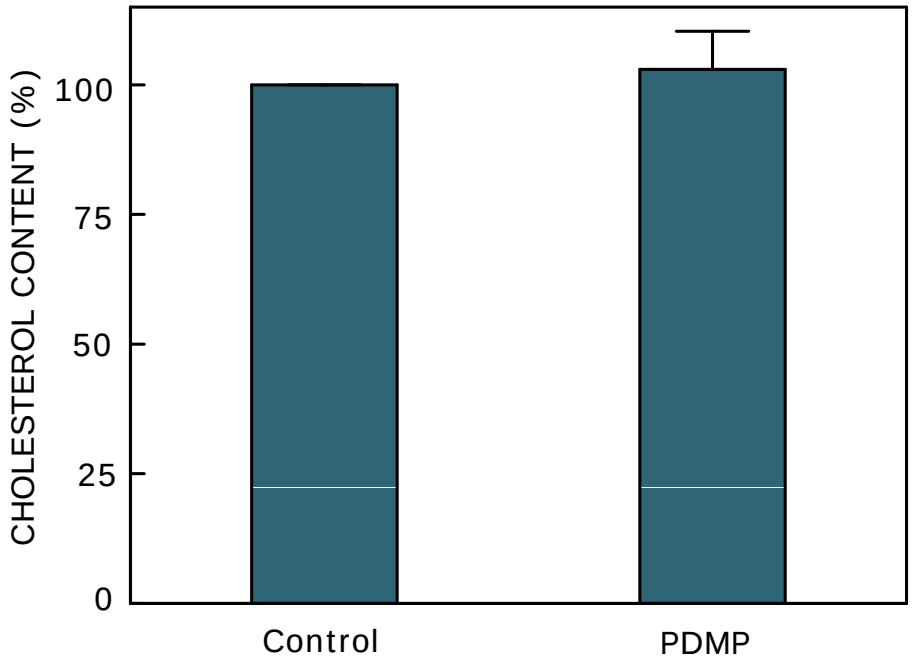
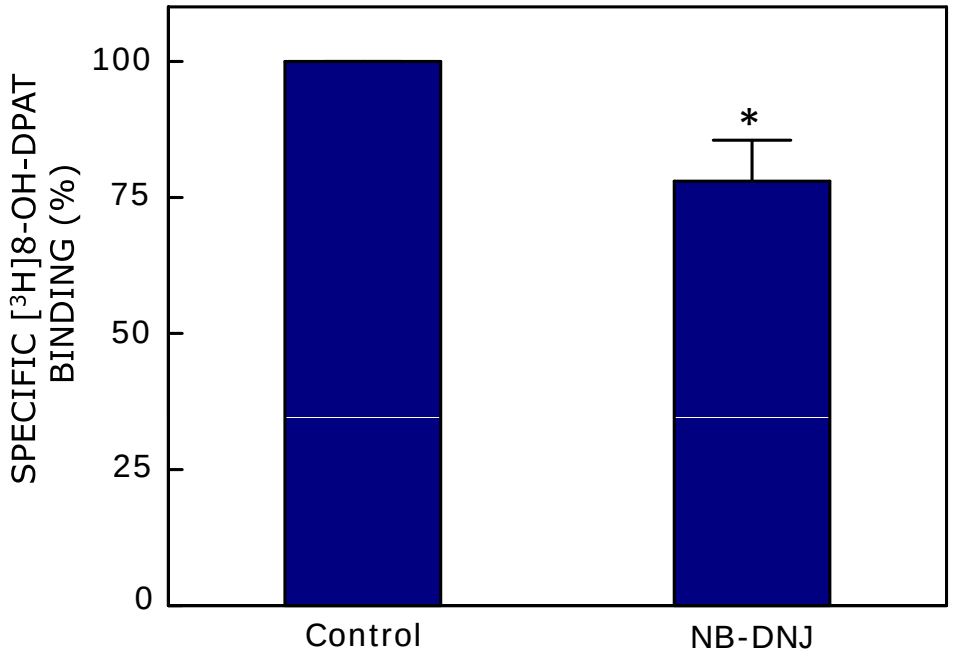


Figure S3
Singh *et al.*

Supporting Information



Supporting Information