Role of Membrane Lipids in the Organization and Function of the Serotonin$_{1A}$ Receptor

Thesis

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Certificate

The research work embodied in this thesis has been carried out under the guidance of Prof. Amitabha Chattopadhyay at the Centre for Cellular and Molecular Biology, Hyderabad. This work is original and has not been submitted in part or full for any other degree or diploma to any other university.

Signature of the Supervisor

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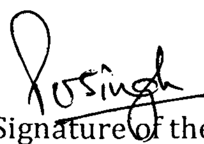

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Table of Contents

Abbreviations i
Synopsis ii
Publications ix
Abstracts xi

Chapter 1. Introduction

1.1. The Cell Membrane 2
1.2. Cholesterol 3
1.3. Sphingolipids 9
1.4. G-protein Coupled Receptors (GPCRs) 14
1.5. Role of Membrane Lipids in the Function of GPCRs 21
1.6. Nonannular Lipids in the Function of Membrane Proteins 25
1.7. Cholesterol Binding Motif(s) in GPCRs 28

Chapter 2. Differential effects of cholesterol and 7-dehydrocholesterol on the ligand binding activity of the hippocampal serotonin$_{1A}$ receptor: Implications in SLOS

2.1. Introduction 34
2.2. Materials and methods 36
2.3. Results and discussion 40

Chapter 3. Differential effects of cholesterol and 7-dehydrocholesterol on the ligand binding function of the hippocampal serotonin$_{1A}$ receptor: Implications in desmosterolosis

3.1. Introduction 46
3.2. Materials and methods 47
3.3. Results and discussion 50

Chapter 4. Desmosterol replaces cholesterol for ligand binding function of the serotonin$_{1A}$ receptor in solubilized hippocampal membranes

4.1. Introduction 58
4.2. Materials and methods....60
4.3. Results and discussion....62

Chapter 5. Organization and dynamics of hippocampal membranes in a depth-dependent manner by electron spin resonance spectroscopy

5.1. Introduction 75
5.2. Materials and methods....77
5.3. Results and discussion....81

Chapter 6. Sphingomyelin headgroup is necessary for the serotonin$_{1A}$ receptor function

6.1. Introduction....97
6.2. Materials and methods....98
6.3. Results 100
6.4. Discussion....105

Chapter 7. Metabolic depletion of glycosphingolipid impairs the human serotonin$_{1A}$ receptor function

7.1. Introduction 108
7.2. Materials and methods....111
7.3. Results....115
7.4. Discussion....127

Chapter 8. Conclusion and future perspectives

8.1. Conclusion....132
8.2. Future perspectives....136

References 140

Abbreviations

5-HT: 5-hydroxytryptamine
5-HT_{1A} receptor: 5-hydroxytryptamine-1A receptor
5-HT_{1A}R-EYFP: 5-hydroxytryptamine-1A receptor tagged to enhanced yellow fluorescent protein
7-DHC, 7-dehydrocholesterol
7-DHCR, 3β-hydroxy-steroid-Δ^7-reductase
8-OH-DPAT, 8-hydroxy-2(di-N-propylamino)tetralin
BCA, bicinchoninic acid
CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CRAC, cholesterol recognition/interaction amino acid consensus
cAMP: adenosine 3′,5′-cyclic monophosphate
CCK: cholecystokinin
CCM: cholesterol consensus motif
DHCR24, 3β-hydroxy-steroid-Δ^{24}-reductase
DMPC, dimyristoyl-sn-glycero-3-phosphocholine
DPH, 1,6-diphenyl-1,3,5-hexatriene
ESR, electron spin resonance
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
MβCD, methyl-β-cyclodextrin
NLLS, nonlinear least squares
PDMP, (±)-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol
PEG, polyethylene glycol
PMSF, phenylmethylsulfonyl fluoride
SBD, sphingolipid-binding domain
SLOS, Smith-Lemli-Opitz syndrome
Tris: *tris*-(hydroxymethyl)aminomethane

Synopsis

Biological membranes are complex non-covalent assemblies of a diverse variety of lipids and proteins that allow cellular compartmentalization. The cell membrane imparts identity to a cell and is proposed to be utmost requirement for a living organism. The cell membrane separates the inner cellular environment from extracellular space and plays crucial role in communication between cells and their environments. Membranes are selectively permeable and various physiologically important reactions such as transport, signaling, trafficking and host-pathogen interactions occur at the membrane. Lipid and protein are two key components of the membrane. A significant portion of integral proteins is embedded in the membrane. Biophysical properties of lipids and membranes could therefore influence the protein function. Lipid-protein interactions in membranes are therefore of prime importance in assembly, stability, and function of membrane proteins. Cholesterol and sphingolipids are important lipids in this context since they are known to regulate the function of membrane proteins. Cholesterol and sphingolipids are often found distributed non-randomly in domains in biological and model membranes. Many of these domains (sometimes termed as 'lipid rafts') are thought to be important for the maintenance of membrane structure and function, although characterizing the spatiotemporal resolution of these domains has proven to be challenging. Specifically, cholesterol is known to play a vital role in the function of neuronal receptors, thereby affecting neurotransmission and giving rise to mood and anxiety disorders.

GPCRs are prototypical members of the family of seven transmembrane domain proteins and include ~1000 members which are encoded by ~5% of the human genes. GPCRs couple to heterotrimeric G-proteins and transmit signals

across the plasma membrane *via* their interactions with heterotrimeric G-proteins present on the cytoplasmic side of the cell membrane, thereby providing a mechanism of communication between the exterior and the interior of the cell. GPCRs mediate multiple physiological processes such as neurotransmission, cellular metabolism, secretion, cellular differentiation, growth, inflammatory and immune responses. GPCRs have therefore emerged as major targets for the development of novel drug candidates in all clinical areas. It is estimated that ~50% of all clinically prescribed drugs act as either agonists or antagonists of GPCRs which indicates their immense therapeutic potential.

The serotonin$_{1A}$ receptor is an important neurotransmitter receptor and is the most extensively studied of the serotonin receptors. The serotonin$_{1A}$ receptor is the first among all types of serotonin receptors to be cloned as an intronless genomic clone (G-21) of the human genome which cross-hybridized with a full length β-adrenergic receptor probe at reduced stringency. Sequence analysis of this genomic clone (later identified as the serotonin$_{1A}$ receptor gene) showed ~48% amino acid similarity with the β_2-adrenergic receptor in the transmembrane domain. The human gene for the receptor encodes a protein of 422 amino acids. Serotonergic signaling plays a key role in the generation and modulation of various cognitive, developmental and behavioral functions. The serotonin$_{1A}$ receptor agonists and antagonists have been shown to possess potential therapeutic effects in anxiety- or stress-related disorders. The serotonin$_{1A}$ receptor therefore is involved in a multitude of physiological processes, and an important drug target.

Cholesterol plays a fundamental role in the function and organization of membrane proteins and receptors. The effect of cholesterol on the function of integral membrane proteins and receptors has been a subject of intense investigation (reviewed in chapter 1). For example, it has been proposed that cholesterol modulates the function of GPCRs through (i) a direct/specific

interaction with the GPCR, which could induce a conformational change in the receptor, and/or (ii) an indirect way by altering the membrane physical properties in which the receptor is embedded. The focus of the work presented in this thesis is to understand the specificity of lipid-protein interactions that could modulate the function of membrane proteins. In this regard, the function of the serotonin$_{1A}$ receptor has been monitored upon modulation of membrane lipids such as cholesterol and sphingolipids. In addition, membrane organization and dynamics have been explored upon modulating membrane cholesterol and protein content. This synopsis provides a brief outline of these studies.

Effects of cholesterol and its immediate biosynthetic precursors on the ligand binding function of the hippocampal serotonin$_{1A}$ receptor: Implications in SLOS and desmosterolosis

Our laboratory has previously shown the necessity of membrane cholesterol for ligand binding function of the hippocampal serotonin$_{1A}$ receptor. Cholesterol is a unique molecule in terms of high level of in-built stringency, fine tuned by natural evolution for its ability to optimize physical properties of eukaryotic cell membranes in relation to biological functions (reviewed in chapter 1). In order to test the stringency of cholesterol requirement for ligand binding to serotonin$_{1A}$ receptors, cholesterol was depleted from hippocampal membranes followed by replenishment with 7-dehydrocholesterol (chapter 2) or desmosterol (chapter 3). Desmosterol and 7-dehydrocholesterol are the immediate precursors of cholesterol in the Bloch and Kandutsch-Russell pathway of cholesterol biosynthesis. Desmosterol and 7-dehydrocholesterol are positional isomers which carry an extra double bond at 24th and 7th position, respectively. Results thus obtained show that replenishment with desmosterol or 7-dehydrocholesterol does not restore ligand binding function of the hippocampal serotonin$_{1A}$ receptor. This

is despite of similar membrane organization (order) in sterol-replenished membranes, as monitored by fluorescence anisotropy measurements. The requirement for restoration of ligand binding therefore appears to be more stringent than that for the recovery of overall membrane order. These novel results have potential implications in understanding the interaction of membrane lipids with this important neuronal receptor under pathogenic conditions such as SLOS and desmosterolosis.

Desmosterol replaces cholesterol for ligand binding function of the serotonin$_{1A}$ receptor in solubilized hippocampal membranes

Solubilization of the hippocampal serotonin$_{1A}$ receptor by CHAPS is accompanied by loss of cholesterol that results in a reduction in specific agonist binding function. Replenishment of cholesterol to solubilized membranes restores membrane cholesterol content and significantly recovers specific agonist binding. Since lipid-protein interactions can be suitably monitored with purified membrane protein. Stringency of cholesterol requirement for receptor function was tested by replenishing solubilized membranes with desmosterol (as described in chapter 4). Solubilized membranes represent serotonin$_{1A}$ receptor in relatively purified (enriched) form after loss of other proteins and lipids. Desmosterol is the immediate biosynthetic precursor of cholesterol in the Bloch pathway differing only in an extra double bond at the 24th position. These results show that replenishment with desmosterol restores ligand binding of serotonin$_{1A}$ receptors in solubilized membranes. This is consistent with earlier results showing that desmosterol can replace cholesterol in a large number of cases. However, these results appear to be contradictory to earlier findings, performed by sterol manipulation utilizing methyl-β-cyclodextrin (chapter 3), where replacement of cholesterol with desmosterol does not restore specific ligand binding of the

hippocampal serotonin$_{1A}$ receptor. The possible molecular mechanism of these differences has been discussed in terms of nonannular lipid binding sites around the receptor.

Organization and dynamics of hippocampal membranes in a depth-dependent manner by electron spin resonance spectroscopy

Organization and dynamics of neuronal membranes represent crucial determinants for the function of neuronal receptors and signal transduction. Previous work from our laboratory has established hippocampal membranes as a convenient natural source for studying neuronal receptors. In chapter 5, organization and dynamics of hippocampal membranes and their modulation by cholesterol and protein content is monitored utilizing location (depth)-specific spin-labeled phospholipids by ESR spectroscopy. The choice of ESR spectroscopy is appropriate due to slow diffusion encountered in crowded environments of neuronal membranes. Based on nonlinear least squares analysis of ESR spectra, it is observed that cholesterol increases hippocampal membrane order. On the other hand, membrane proteins were found to increase lipid dynamics resulting in disordered membranes. These results are relevant in understanding the complex organization and dynamics of hippocampal membranes and could have implications in neuronal diseases characterized by defective cholesterol metabolism such as SLOS and desmosterolosis.

Sphingomyelin headgroup is necessary for the serotonin$_{1A}$ receptor function

Sphingolipids are essential and indispensable components of eukaryotic cell membranes and constitute 10-20% of the total membrane lipids. Sphingomyelin is the most abundant sphingolipid and comprises up to 25% of the

total lipids of neuronal tissues (reviewed in chapter 1). In chapter 6, ligand binding function of the serotonin$_{1A}$ receptor is examined upon hydrolyzing the phosphocholine headgroup of sphingomyelin using sphingomyelinase. The serotonin$_{1A}$ receptor is a seven transmembrane G-protein coupled receptor and involved in the generation and modulation of various cognitive, behavioral and developmental functions. These results show that the specific agonist binding to serotonin$_{1A}$ receptors exhibits ~43% reduction upon ~70% hydrolysis of sphingomyelin. Interestingly, overall membrane order does not display any significant change upon phosphocholine headgroup hydrolysis. These results therefore indicate that sphingomyelin headgroup is neccessary for function of the serotonin$_{1A}$ receptor. Removal of phosphocholine headgroup inhibits the serotonin$_{1A}$ receptor ligand binding function, probably by abolishing sphingomyelin-receptor interactions. The ligand binding function of the serotonin$_{1A}$ receptor therefore is a sphingomyelin headgroup dependent phenomenon which cannot be attributed to change in the membrane order. In light of the effect of sphingomyelin metabolic depletion on the serotonin$_{1A}$ receptor function, these results further refine the specific importance of sphingomyelin for the serotonin$_{1A}$ receptor function. Results obtained here are relevant in the overall context of the influence of the membrane lipid environment on the function of the serotonin$_{1A}$ receptor in particular, and other G-protein coupled receptors in general.

Metabolic depletion of glycosphingolipid impairs the human serotonin$_{1A}$ receptor function

Glycosphingolipids are essential components of eukaryotic cell membranes and are involved in the regulation of cell growth, differentiation, and neoplastic transformation. In this work, glycosphingolipid levels in CHO cells stably

expressing the human serotonin$_{1A}$ receptor were modulated by inhibiting the activity of glucosylceramide synthase using 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. Functions of the serotonin$_{1A}$ receptor were monitored under glycosphingolipid-depleted condition by analyzing ligand binding and G-protein coupling of the receptor in chapter 7. These 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, these 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. Possible mechanisms of specific interaction of glycosphingolipids with the serotonin$_{1A}$ receptor was discussed that could involve the proposed 'sphingolipid binding domain'.

In Chapter 8, we have concluded the findings of chapters 2-7 and discussed the future directions that could enhance understanding of lipid-protein interactions in the function of the serotonin$_{1A}$ receptor, in particular and other GPCRs and membrane proteins in general.

Publications

1. **Singh, P.**, Paila, Y.D. and Chattopadhyay, A. (2007) Differential Effects of Cholesterol and 7-Dehydrocholesterol on the Ligand Binding Activity of the Hippocampal Serotonin$_{1A}$ Receptor: Implications in SLOS. *Biochem. Biophys. Res. Commun.* 358: 495-499.

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3. **Singh, P.**, Saxena, R., Paila, Y.D., Jafurulla, M. and Chattopadhyay, A. (2009) Differential Effects of Cholesterol and Desmosterol on the Ligand Binding Function of the Hippocampal Serotonin$_{1A}$ Receptor: Implications in Desmosterolosis. *Biochim. Biophys. Acta* 1788: 2169-2173.

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*5. Prasad, R., **Singh, P.** and Chattopadhyay, A. (2009) Effect of Capsaicin on Ligand Binding Activity of the Hippocampal Serotonin$_{1A}$ Receptor. *Glycoconj. J.* 26: 733-738.

6. **Singh, P.**, Jafurulla, M., Paila, Y.D. and Chattopadhyay, A. (2011) Desmosterol Replaces Cholesterol for Ligand Binding Function of the Serotonin$_{1A}$ Receptor in Solubilized Membranes: Support for 'Nonannular' Binding Sites for Cholesterol? *Biochim. Biophys. Acta* 1808: 2428-2434.

7. **Singh, P.** and Chattopadhyay A. (2012) Removal of Sphingomyelin Headgroup Inhibits the Ligand Binding Function of Hippocampal Serotonin$_{1A}$ Receptors. *Biochem. Biophys. Res. Commun.* 419: 321-325.

8. **Singh, P.**[1], Tarafdar, P.K.[1], Swamy M.J. and Chattopadhyay, A. (2012) Organization and Dynamics of Hippocampal Membranes in a Depth-dependent Manner: An Electron Spin Resonance Study. *J. Phys. Chem. B* 116: 2999-3006.

*9. Chattopadhyay, A., Paila, Y.D., Tiwari, S., Shrivastava, S., **Singh, P.** and Fantini, J. (2012) Sphingolipid Binding Domain in the Serotonin$_{1A}$ Receptor. *Adv. Exp. Med. Biol.* (in press).

10. **Singh, P.**, Paila, Y.D. and Chattopadhyay, A. (2012) Role of Glycosphingolipids in the Function of Human Serotonin$_{1A}$ Receptors. (manuscript submitted).

*11. **Singh, P.**[1], Saxena, R. [1], Srinivas, G., Pande, G. and Chattopadhyay, A. (2012) Role of Cholesterol Biosynthesis and Homeostasis in Cell Cycle Progression. (manuscript in preparation).

*12. **Singh, P.**, Haldar, S. and Chattopadhyay, A. (2012) Differential Effect of Cholesterol and 7-Dehydrocholesterol on the Dipole Potential in Hippocampal Membranes: Dipole Potential Correlates to the Serotonin$_{1A}$ Receptor Function. (manuscript in preparation).

(*Not included in the thesis; [1]Equal contribution)

Abstracts

1. Singh, P., Paila, Y.D., and Chattopadhyay, A. (2006) "*Effect of Replacement of Membrane Cholesterol by 7-Dehydrocholesterol on Ligand-binding activity of Serotonin$_{1A}$ Receptor: Implications in SLOS*" poster presented at 75th Society for Biological Chemist at Jawaharlal Nehru University, New Delhi, India. Abstract no: P.III-21.

2. Singh, P., Haldar, S., and Chattopadhyay, A. (2007) "*Monitoring the Dipole Potential in Bovine Hippocampal Membranes under Conditions of Sterol Alteration*" poster presented at National Symposium on Biophysics: Biophysics in Medicine and Biology at Panjab university, Chandigarh, India. Abstract no: PP-03.

3. Singh, P., Paila, Y.D., and Chattopadhyay, A. (2008) "*Effect of Metabolic Depletion of Glycosphingolipids on the Function of the Human Serotonin$_{1A}$ Receptor*" poster presented at 8th International Symposium on Biochemical Roles of Eukaryotic Cell Surface Macromolecules at Centre for Cellular and Molecular Biology, Hyderabad, India. Abstract no: P-19.

4. Prasad, R., **Singh, P.**, and Chattopadhyay, A. (2008) "*Monitoring the effect of Modulation of Membrane Material Properties on Ligand-binding Activity of the Serotonin$_{1A}$ Receptor*" poster presented at 8th International Symposium on Biochemical Roles of Eukaryotic Cell Surface Macromolecules at Centre for Cellular and Molecular Biology, Hyderabad, India. Abstract no: P-15.

5. Singh, P., Tarafdar, P.K., Swamy M.J., and Chattopadhyay, A. (2009) "*Exploring the Organization and Dynamics of Hippocampal Membranes in a Depth-dependent Manner: An Electron Spin Resonance Study*" poster presented at National Symposium on Cellular and Molecular Biophysics at Centre for Cellular and Molecular Biology, Hyderabad India. Abstract no: P-84.

6. Singh, P., Tarafdar, P.K., Swamy M.J., and Chattopadhyay, A. (2009) "*Exploring the Organization and Dynamics of Hippocampal Membranes in a Depth-dependent Manner: An Electron Spin Resonance Study*" poster presented at the "Joint FEBS-EMBO Advanced Lecture Course" on "*Molecular and Cellular Membrane Biology*", held at Cargese, Corsica, France.

7. Singh, P., Saxena, R., Srinivas, G., Pande, G., and Chattopadhyay, A. (2009) "*Monitoring the Role of Cholesterol Biosynthesis and Homeostasis in Cell Cycle Progression*" at XXXIII All India Cell Biology Conference and

International Workshop on Cell Cycle Regulation at University of Hyderabad. Abstract No. P-139.

8. **Singh, P.**, Saxena, R., Srinivas, G., Pande, G., and Chattopadhyay, A. (2010) *"Role of Cholesterol Biosynthesis and Homeostasis in Cell Cycle Progression"* at 3rd lipid symposium at National University of Singapore, Singapore.

9. Chattopadhyay, A., Paila,Y.D., Tiwari, S. **Singh, P.**, Shrivastava, S., and Fantini, J. (2011) *"Sphingolipid Binding Domain in the Serotonin$_{1A}$ Receptor"* at 9th International Symposium on Biochemical Roles of Eukaryotic Cell Surface Macromolecules at Estuary Island Resort, Poovar, Trivandrum, Kerala, India.

10. Saxena, R., **Singh, P.**, Srinivas, G., Pande, G., and Chattopadhyay, A (2011) *"Role of Cholesterol Biosynthesis and Homeostasis in Cell Cycle Progression"* poster presented at "Joint FEBS-EMBO Advanced Lecture Course on Biomembrane Dynamics: from Molecules to Cells" at the Institut D'etudes Scientifiques de Cargèse, Cargèse, Corsica, France.

11. Saxena, R., **Singh, P.**, Srinivas, G., Pande, G., and Chattopadhyay, A. (2011) *"Role of Cholesterol Biosynthesis and Homeostasis in Cell Cycle Progression"* poster presented at XXXIV all India cell biology conference and symposium on "Quantitative biology of the cell" at NISER, Bhubaneswar.

Chapter 1

Introduction

1.1. The Cell Membrane

The cell membrane imparts identity to a cell and is the prime requirement for living organisms (Oparin, 1961; Luisi, 1998). The cell membrane separates the inner cellular environment from the extracellular space and plays a crucial role in communication between cells and their environments. Membranes are selectively permeable and various physiologically important reactions such as transport, signaling, trafficking and host-pathogen interactions occur at the membrane. Biological membranes therefore act as security check as well as scaffold for the initiation of diverse physiological activities in a cell. Cell membranes are highly organized molecular assembly of lipids and proteins, largely confined to two dimensions, and exhibit considerable degree of anisotropy along the axis perpendicular to the membrane. The relative ratio of proteins and lipids determine the structure and biological function of the membrane. Membrane proteins are categorized as peripheral and transmembrane on the basis of their strength of association with the membrane. They are mainly required to communicate between cell exterior to interior, whereas lipids are involved in a variety of functions inside a cell besides their crucial role in the membrane structure. They act as signaling reservoir (Wymann and Schneiter, 2008), receptor for different pathogens (Simons and Ehehalt, 2002; Riethmüller *et al.*, 2006; Pucadyil and Chattopadhyay, 2007) and modulate the function of different proteins associated with the membrane (Lee, 2004; 2011). Cholesterol and sphingolipids are two important constituent lipids of biological membranes and are shown to be required for proper membrane function (Brown, 1998; Ramstedt and Slotte, 2006). Cholesterol and sphingolipids are known to interact in membranes leading to the formation of ordered domains. Many of these domains (sometimes termed as 'lipid rafts') are believed to be important for the maintenance of membrane structure

and function. These domains are thought to be transiently stable and have been implicated in crucial cellular processes such as signaling (Simons and Toomre, 2000), membrane trafficking, sorting (Simons and van Meer, 1988) and the entry of pathogens (Simons and Ehehalt, 2002; Riethmüller *et al.*, 2006; Pucadyil and Chattopadhyay, 2007).

1.2. Cholesterol

Cholesterol is an essential and representative lipid in higher eukaryotic cellular membranes and is crucial in membrane organization, dynamics, function, and sorting (Liscum and Underwood, 1995; Simons and Ikonen, 2000; Mouritsen and Zuckermann, 2004). Cholesterol is a predominantly hydrophobic molecule comprising a near planar tetracyclic fused steroid ring and a flexible isooctyl hydrocarbon tail (see Fig. 1.1a). The 3β-hydroxyl moiety provides cholesterol its amphiphilic character and helps cholesterol to orient and anchor in the membrane (Villalaín, 1996). The tetracyclic nucleus and isooctyl side chain create the bulky wedge-type shape of the molecule. Interestingly, the planar tetracyclic ring arrangement of cholesterol is asymmetric about the ring plane. The sterol ring has a flat and smooth side with no substituents (the α face) and an uneven side with methyl substitutions (the β face; see Fig. 1.1b). The smooth α face of cholesterol nucleus helps in favorable van der Waals interaction with the saturated fatty acyl chains of phospholipids (Lange and Steck, 2008). The α face of cholesterol contains only axial hydrogen atoms. The absence of any bulky group in this face facilitates close contact between the sterol nucleus and phospholipid chains. The bumpiness of the β face of cholesterol molecule is due to the protruding methyl groups at positions C_{18}, C_{19} and C_{21}.

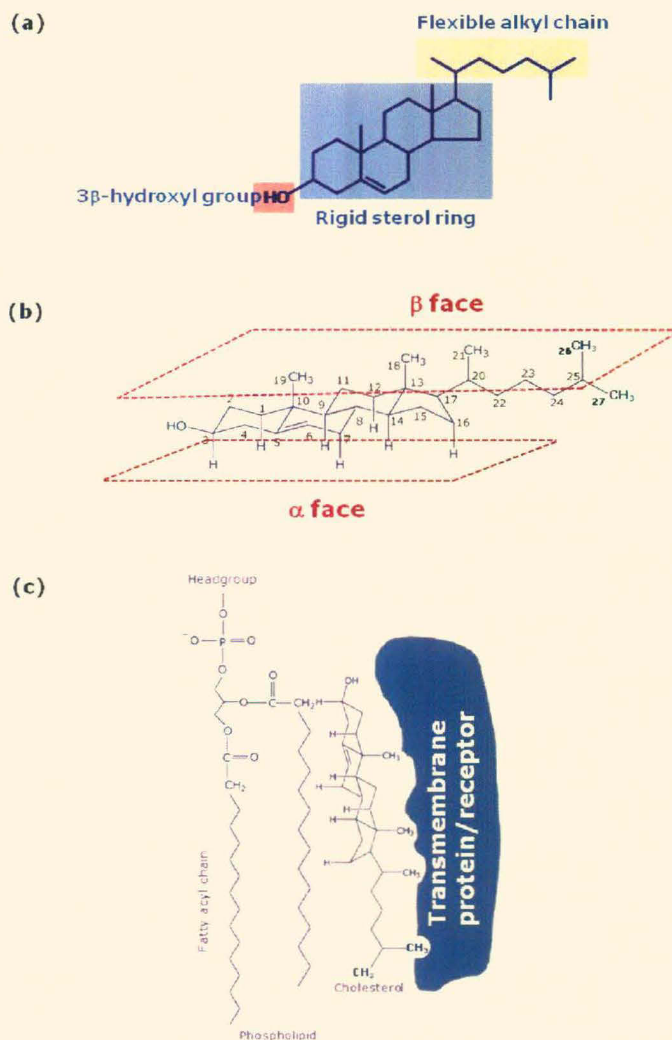

Figure 1.1. *Chemical structure and membrane orientation of cholesterol.* (a) *Structure of cholesterol showing the individual rings.* Three structurally distinct regions are shown as shaded boxes: the 3β-hydroxyl group, the rigid steroid ring, and the flexible alkyl chain. (b) *Two faces of cholesterol.* Cholesterol is characterized by a flat and smooth α face, and a rough β face. The α face of cholesterol contains only axial hydrogen atoms. The roughness of the β face is due to the protruding bulky methyl groups. (c) *Schematic orientation of cholesterol in relation to a phospholipid molecule in a lipid bilayer.* The smooth α face of the sterol nucleus helps in favorable van der Waals interaction with the saturated fatty acyl chains of phospholipids. The α and β faces of cholesterol can simultaneously interact with a saturated fatty acyl chain of phospholipids and uneven transmembrane domain of an integral membrane protein, respectively. See section 1.2 for details. Adapted from Paila and Chattopadhyay, 2010.

The molecular structure of cholesterol is exceedingly fine-tuned over a very long time scale of natural evolution. It has been shown recently by atomic scale molecular dynamics simulations (Róg *et al.*, 2007; Pöyry *et al.*, 2008) that removal of methyl groups from cholesterol results in altered tilt angle which affects its ordering and condensing effects on the membrane. Molecular simulation approaches have earlier shown that the α face of cholesterol promoted a stronger ordering effect on saturated alkyl chains compared to the β face (Róg and Pasenkiewicz-Gierula, 2001). In addition, molecular dynamics simulation has shown that cholesterol orients its smooth α face toward saturated chains and its uneven β face toward unsaturated chains of phospholipids (Pandit *et al.*, 2004), or with a bumpy transmembrane domain of an integral membrane protein (see Fig. 1.1c). Cholesterol is oriented in the membrane bilayer with its long axis perpendicular to the plane of the membrane (Fig. 1.1c). Its polar hydroxyl group encounters the aqueous environment and the hydrophobic steroid ring is oriented parallel to the fatty acyl chains of the phospholipids (Yeagle, 1985). It has been previously shown using x-ray and neutron diffraction that cholesterol is aligned in bilayers with its 3β-hydroxyl group in the proximity of the ester bonds of phospholipids and its tetracyclic ring buried in the bilayer interior, in close contact with a part of the phospholipid fatty acyl chains (Villalaín, 1996; Bittman, 1997).

Cholesterol is often found distributed nonrandomly in domains in biological and model membranes (Liscum and Underwood, 1995; Schroeder *et al.*, 1995; Simons and Ikonen, 1997, 2000; Xu and London, 2000; Mukherjee and Maxfield, 2004, Chaudhuri and Chattopadhyay, 2011). The 3β-hydroxyl group of cholesterol is located below the aqueous interface in the membrane and the steroid moiety rotates rapidly about the long molecular axis that wobbles through a narrow range of angles slightly tilted relative to the bilayer normal (Marsan *et al.*, 1999). Cholesterol is known to interact strongly with sphingolipids, thereby

leading to the formation of ordered domains that are believed to be important for the maintenance of membrane structure and function. In addition, the interaction of cholesterol with polyunsaturated fatty acid (PUFA)-containing phospholipids has recently been investigated. A variety of biophysical measurements have revealed that cholesterol has a strong dislike to PUFA-rich membranes that drives the formation of PUFA-rich/sterol-poor domains and saturated lipid/sterol-rich domains (Harroun *et al.*, 2006; Kucerka *et al.*, 2009; Huster, 1998; Niu *et al.*, 2002). Importantly, the nervous system is rich in PUFA containing phospholipids, where they are required for neurological functions. It has been hypothesized that the poor affinity of cholesterol for PUFA containing phospholipids plays a crucial biological role (Harroun *et al.*, 2006; Stillwell and Wassall, 2003; Wassall *et al.*, 2004, Huster *et al.*, 1998, Mitchell and Litman, 1998). In general, membrane domains assume significance in cell biology since physiologically important functions such as membrane sorting and trafficking (Simons and van Meer, 1988), signal transduction processes (Simons and Toomre, 2000), and the entry of pathogens (Simons and Ehehalt, 2002; Riethmüller *et al.*, 2006; Pucadyil and Chattopadhyay, 2007) have been attributed to these domains. Importantly, cholesterol plays a vital role in the function and organization of membrane proteins and receptors (Burger *et al.*, 2000; Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay 2010).

Cholesterol is the end product of the long and multi-step sterol biosynthetic pathway. There are two major pathways for cholesterol biosynthesis: the Bloch (Bloch, 1983) and Kandutsch-Russell (Kandutsch and Russell, 1960) pathway. Konrad Bloch speculated that the sterol biosynthetic pathway parallels sterol evolution (the 'Bloch hypothesis').

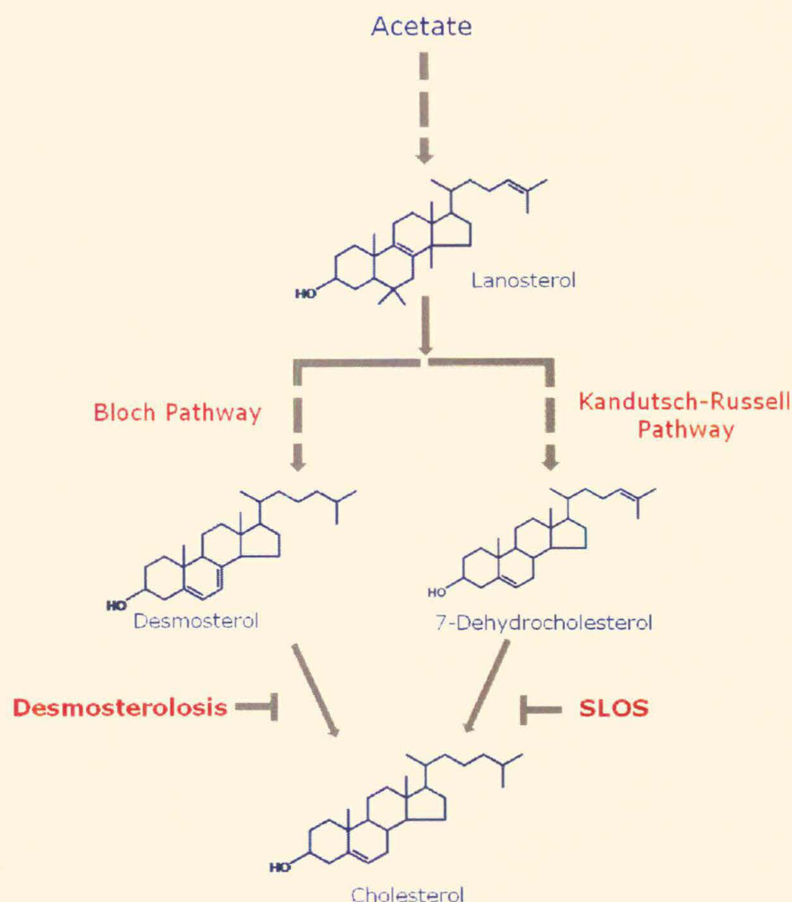

Figure 1.2. *Schematic of cholesterol biosynthesis.* Cholesterol biosynthesis occurs *via* the Kandutsch-Russell and Bloch pathways as shown in the figure. These pathways have common initial steps starting from acetate and branch out at lanosterol. 7-Dehdrocholesterol (7-DHC) and desmosterol are immediate precursors of cholesterol biosynthesis in the Kandutsch-Russell and the Bloch pathway. 7-DHC and desmosterol are positional isomers and differ with cholesterol only in an extra double bond at the 7th position in the sterol ring and at the 24th position in the flexible alkyl side chain of the sterol, respectively. Importantly, 3β-hydroxy-steroid-Δ^7-reductase (7-DHCR) catalyzes the conversion of 7-DHC to cholesterol in the last step of the Kandutsch-Russell pathway. On the other hand, 3β-hydroxy-steroid-Δ^{24}-reductase (24-DHCR) is responsible for the reduction of desmosterol into cholesterol (last step of the Bloch pathway). Malfunctioning of 7-DHCR and 24-DHCR results in the accumulation in 7-DHC and desmosterol leading to Smith-Lemli-Opitz Syndrome (SLOS) and desmosterolosis, respectively. See section 1.2 for more details. Adapted and modified from Shrivastava and Chattopadhyay, 2012.

According to this hypothesis, cholesterol has been selected over a very long time scale of natural evolution for its ability to optimize certain properties of eukaryotic cell membranes with regard to biological functions (Bloch, 1983). Cholesterol precursors should therefore have properties that gradually support cellular function of higher organisms as they progress along the pathway toward cholesterol. Defects in cholesterol biosynthetic pathway have been identified with several inherited metabolic disorders such as Smith-Lemli- Opitz Syndrome (SLOS) and desmosterolosis (Waterham, 2006).

There is accumulation of 7-dehydrocholesterol (7-DHC) and desmosterol in SLOS and desmosterolosis, respectively. 7-DHC and desmosterol are positional isomers, they contain one extra double bond in comparison to cholesterol (see Fig. 1.2). 7-DHC is an immediate biosynthetic precursor of cholesterol in the Kandutsch-Russell pathway, and is reduced to cholesterol in the final step by the enzyme, 3β-hydroxy-steroid-Δ^7-reductase (7-DHCR). 7-DHC differs with cholesterol only in an extra double bond at the 7th position in the sterol ring (see Fig. 1.2). Similarly, desmosterol, the immediate biosynthetic precursor of cholesterol in the Bloch pathway, contains an extra double bond at the 24th position in the flexible alkyl side chain (Fig. 1.2). Desmosterol is converted to cholesterol in the final step of the pathway by the enzyme, 3β-hydroxy-steroid-Δ^{24}-reductase (24-DHCR).

SLOS (Smith *et al.*, 1964) is an autosomal recessive disorder characterized clinically by mental retardation, physical deformities, failure to thrive and multiple congenital anomalies (Waterham and Wanders, 2000; Yu and Patel 2005, Porter and Herman, 2011). SLOS is caused by mutations in the gene encoding 7-DHCR (Irons *et al.*, 1993; Tint *et al.*, 1994). To date, close to 100 different mutations in the DHCR7 gene have been identified which lead to the disease (Jira *et al.*, 2003). SLOS is ranked as one of the most serious recessive genetic conditions (Yu and

Patel 2005; Battaile and Steiner, 2000). Reduced levels of plasma cholesterol along with elevated levels of 7-DHC and the ratio of their concentrations to that of cholesterol are representative parameters for diagnosis of SLOS (Tint *et al.*, 1995). Malfunctioning of 24-DHCR due to mutations in Dhcr24 gene lead to desmosterolosis (Clayton *et al.*, 1996; FitzPatrick *et al.*, 1998; Fliesler *et al.*, 2000; Waterham *et al.*, 2001; Andersson *et al.*, 2002). Desmosterolosis is an autosomal, recessive congenital disease and has been characterized by multiple anomalies. Desmosterolosis is diagnosed with elevated levels of desmosterol and reduced levels of cholesterol in plasma, tissues and cells. The disease is characterized by distinct facial anomalies, underdeveloped genital organs and abnormalities in brain development and function, leading to serious developmental and neurological dysfunctions.

1.3. Sphingolipids

Sphingolipids are essential and indispensable components of eukaryotic cell membranes and constitute ~10-20% of the total membrane lipids (Holthius *et al.*, 2001). Sphingolipids serve as structural components and are highly abundant in the extracellular face of the plasma membrane, where they participate in cell-cell communication and host pathogen interactions (Lopez and Schnaar, 2009; Tsai *et al.*, 2003). 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 are recognized as diverse and dynamic regulators of a multitude of cellular processes. In addition, sphingolipids play important role in the structural organization of membranes. Sphingolipids differ in their headgroup structures and

acyl chain compositions and generally partition into ordered domains (Wang and Silvius, 2003).

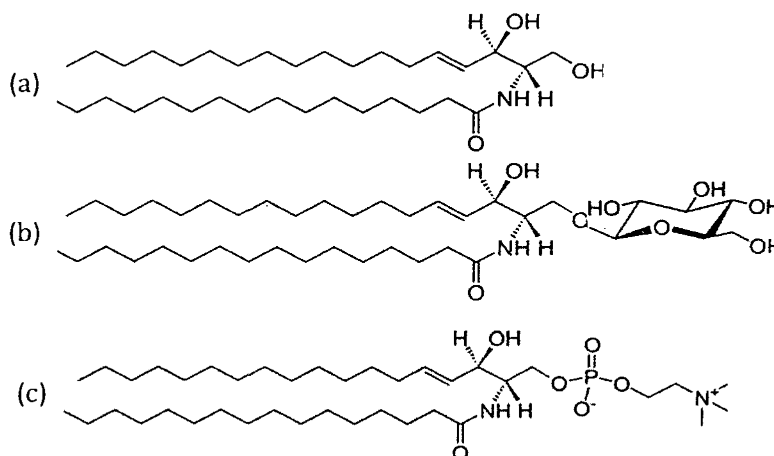

Figure 1.3. *Chemical structure of (a) ceramide, (b) glucosylceramide and (c) sphingomyelin.* Ceramide is composed of sphingosine and fatty acid. Ceramide is the precursor for complex sphingolipids such as glycosphingolipids and sphingomyelin. Glucosylceramide carries one glucose moiety as headgroup. Glucosylceramide is the simplest glycosphingolipid and is employed to generate complex glycosphingolipids such as gangliosides. Sphingomyelin carries a phosphate and the choline as the headgroup on ceramide moiety. Sphingomyelins are the non-glycerophospholipids in the cell. See section 1.3 for more details.

The polar headgroup of sphingolipids varies as follows: free hydroxyl for ceramide, phosphocholine for sphingomyelin and complex oligosaccharides for the complex glycosphingolipids. Ceramide moiety with the long chain base and long saturated N-acyl chains provides the hydrophobic backbone to sphingolipids. Combination of headgroup and acyl chain composition therefore determines the partitioning of these lipids. It is becoming increasingly evident that diverse sphingolipids exhibit different segregation patterns in the lateral dimension in membrane bilayer. Sphingolipids such as glycosphingolipid and sphingomyelin are of particular importance in regulating various physiological functions (Merrill *et al.*, 1996; Lahiri and Futerman, 2007; Sillence, 2007).

1.3.1. Glycosphingolipid

Glycosphingolipids (GSLs) are essential components of eukaryotic cell membranes and constitute ~5% of the total membrane lipids (Fukasawa *et al.*, 2000) and are major components of neuronal membranes where they constitute up to 30% of the total lipid content (Hoekstra and Kok, 1992). Glycosphingolipids are the reservoir for many signaling components and also play important role in the architecture of membranes. Interfacial region of GSLs contains chemical groups that can function both as hydrogen bond (H-bond) donor and acceptor while glycerolipids have only H-bond accepting properties in their interfacial region. In addition, GSLs can participate in H-bonding through their sugar headgroups. In combination with the higher H-bonding propensity, the saturated fatty acyl chains results in tighter packing (Sillence, 2007). It has previously been demonstrated that depletion of cellular glycosphingolipids significantly affects axonal growth, suggesting that sphingolipids may play a vital role in regulating neuronal development (Harel and Futerman, 1993). Glycosphingolipids are synthesized in the golgi complex where glucosylation of ceramide into glucosylceramide (*i.e.*, first step of glycosphingolipid synthesis) 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 the precursor of hundreds of complex glycosphingolipids such as gangliosides. Gangliosides belong to an important and specialized subclass of glycosphingolipids containing sialic acid (N-acetylneuraminic acid) headgroup. Glycosphingolipids are shown to be involved in the regulation of cell growth, differentiation, and neoplastic transformation by participating in cell-cell communication, and possible interactions with receptors and signaling systems (Lahiri and Futerman, 2007).

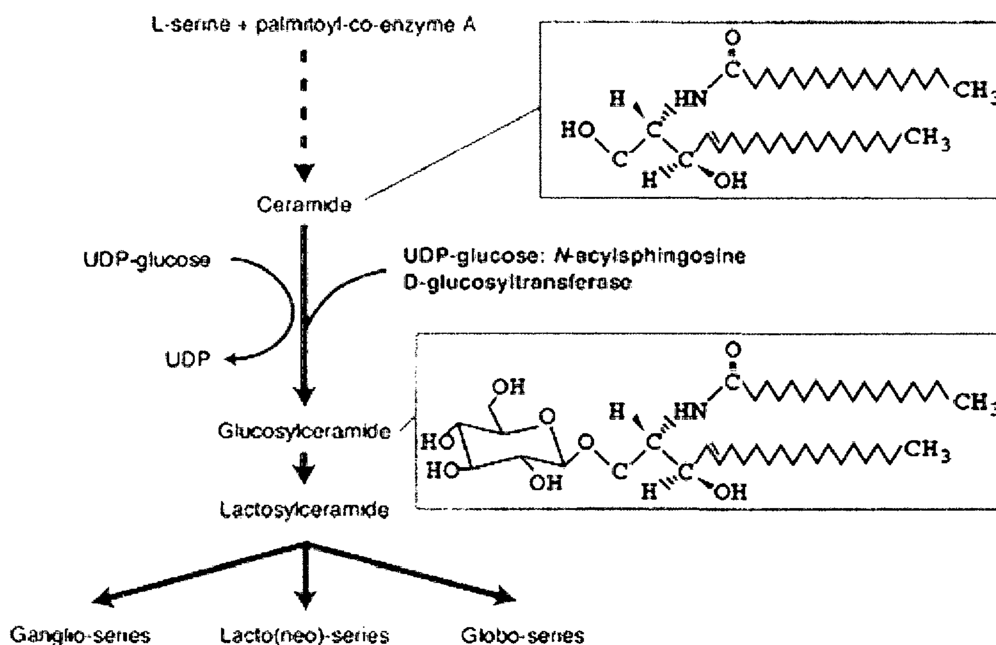

Figure 1.4. *Biosynthesis of glycosphingolipids (GSLs).* In the first step of GSL biosynthesis, glucose is transferred to ceramide as uridine diphosphate glucose (UDP-glucose) to generate glucosylceramide. This step is catalyzed by the enzyme glucosylceramide synthase. Glucosylceramide is the simplest GSL and is a precursor for different complex GSLs. See section 1.3 for more details. Adapted and modified from Platt and Butters, 2000.

Moreover, glycosphingolipids are also shown to promote pathogen entry such as HIV-I (Hug *et al.*, 2000; Mahfoud *et al.*, 2002a). Importantly, knockout studies in mice have demonstrated that the synthesis of glycosphingolipids is essential for embryonic development and deficiency of the glucosylceramide synthase enzyme responsible for the synthesis of glucosylceramide was found to be embryonically lethal (Yamashita *et al.*, 2005). Besides, glycosphingolipids have been demonstrated to regulate apoptosis, survival and regeneration of cells in the nervous system (Hakomori, 2002; Bektas and Spiegel, 2004). In addition, the role of glycosphingolipids in the development and progression of several neurological

diseases such as Alzheimer's disease is well documented (Ariga *et al.,* 2008) which could be due to impaired neurotransmission.

1.3.2. Sphingomyelin

Sphingomyelin is the most abundant sphingolipid and comprises upto 25% of the total lipids of peripheral nerve and brain tissues (Soriano *et al.*, 2005). In comparison to intracellular membranes, the plasma membrane is rich in sphingomyelin. Sphingomyelins are regarded as reservoirs for second messengers such as sphingosine, ceramide and sphingosine 1-phosphate (Merrill *et al.*, 1996). The breakdown of sphingomyelin is regulated by the action of sphingomyelinase enzymes (Goñi and Alonso, 2002; Marchesini and Hannun, 2004). Sphingomyelinases are activated by a variety of stimuli, including inflammatory cytokines, growth factors, G-protein coupled receptors and cellular stress. Ceramide is at the center of sphingolipid metabolism and has been recognized as a critical second messenger (Hannun and Obeid, 2002). Sphingomyelins are hydrolyzed by sphingomyelinases into ceramide and phosphocholine (Chatterjee, 1999; Goñi and Alonso, 2002). Activation of Sphingomyelinase leads to the breakdown of sphingomyelin to ceramide, which can be either phosphorylated by ceramide kinase to form ceramide-1-phosphate or degraded further by ceramidase to produce sphingosine (Hannun and Bell, 1993). Similarly, sphingosine can be phosphorylated by sphingosine kinases to sphingosine 1-phosphate. Sphingomyelinase therefore are proposed to regulate cell signaling by modulating cellular ceramide levels (Grassme´ *et al.*, 2007). Ceramide levels can reach upto 10 mol% of the total phospholipids (Hannun, 1996) emphasizing its role as a signaling molecule. The pool of ceramide is maintained by *de novo* synthesis (Nixon, 2009).

1.4. G-protein coupled receptors (GPCRs)

G-protein-coupled receptors (GPCRs) constitute a superfamily of the largest class of transmembrane proteins. They transmit information across the cell membrane from the extracellular environment to the interior of cells, thereby providing a mechanism of communication between the exterior and the interior of the cell (Pierce *et al.*, 2002; Kroeze *et al.*, 2003; Perez, 2003). Cellular signaling by GPCRs involves their activation upon binding to ligands and the subsequent transduction of signals to the interior of the cell through concerted changes in their transmembrane domain structure (Gether, 2000). GPCRs are prototypical members of the family of seven transmembrane domain proteins and include ~1000 members which are encoded by ~5% of the total human genes (Zhang *et al.*, 2006). They are involved in the generation of cellular responses to a diverse array of stimuli that include biogenic amines, peptides, glycoproteins, lipids, nucleotides, and even photons. As a consequence, these receptors mediate multiple physiological processes such as neurotransmission, cellular metabolism, secretion, cellular differentiation, growth, inflammatory and immune responses. GPCRs have therefore emerged as major targets for the development of novel drug candidates in all clinical areas (Schlyer and Horuk, 2006; Jacoby *et al.*., 2006; Insel *et al.*, 2007). It is estimated that ~50% of all clinically prescribed drugs act as either agonists or antagonists of GPCRs which indicates their immense therapeutic potential (Karnik *et al.*, 2003). Interestingly, although GPCRs represent ~50% of current drug targets, only a small fraction of all GPCRs are presently targeted by drugs (Lin and Civelli, 2004). In fact, as many as half (~500) of the GPCR currently remain orphan presenting a remarkable therapeutic prospective for the development of novel drugs which can target these receptors (Lefkowitz, 2007).

GPCRs couple to heterotrimeric G-proteins and transmit signals across the plasma membrane *via* their interactions with G-proteins present on the cytoplasmic side of the cell membrane (Neer, 1995; Hamm, 2001). Heterotrimeric G-proteins are composed of α, β and γ subunits, with molecular masses of ~39-45, 35-39, and 6-8 kDa, respectively. The α subunit is bound to guanosine diphosphate (GDP) in inactive state of G-proteins. Upon activation of GPCR by agonist, the GDP is exchanged for guanosine triphosphate (GTP) and the α subunit dissociates from the βγ subunits. Both entities can then stimulate downstream effectors and initiate the signaling cascade inside the cell. The G-protein is inactivated when GTP is hydrolyzed back to GDP. Although α subunit of G-proteins possesses inherent GTPase activity to hydrolyze GTP, the process is very slow and inefficient to propagate GPCR signaling *in vivo*. The hydrolysis of GTP inside the cell is enhanced by a family of regulator of G-protein signaling (RGS) proteins. RGS proteins can modulate the GTPase activity of α subunit of the G-proteins *in vivo*. They can increase the rate of GTP hydrolysis ~1000 fold (Hollinger and Hepler, 2002; Ross and Wilkie, 2000; Lan *et al.*, 1998).

More than 28 α, 5 β, and 12 γ subunits of G-proteins have been described (Cabrera-Vera *et al.*, 2003). Heterotrimeric G-proteins can be divided into four families based on the degree of primary sequence similarities of their α subunits: G_s (G_s and G_{olf}), G_i (G_{tr}, G_{tc}, G_g, G_{i1-3}, G_o, and G_z), G_q (G_q, G_{11}, G_{14}, and $G_{15/16}$), and G_{12} (G_{12} and G_{13}). These heterotrimeric G-proteins follow the same scheme of activation/inactivation cycle allowing reversible and specific transmission of signals into cells. The G-protein heterotrimer is maintained in an inactive state by mutual association in a complex, with the α subunit bound to a GDP moiety.

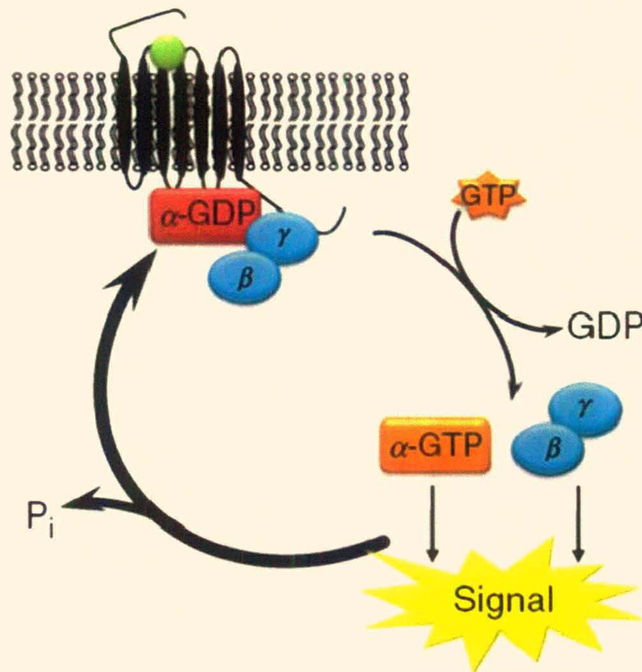

Figure 1.5. *Scheme of G-protein activation.* Binding of an agonist to the GPCR brings about changes in the GPCR structure causing the changes in G-protein structure in turn. Conformational changes in the G-protein facilitate the exchange of GDP for GTP on the α subunit. Activation of G-proteins leads to dissociation of the α and βγ subunits, which eventually initiate downstream signaling in cell. GTP is hydrolyzed back to GDP due to inherent GTPase activity of α subunit of G-proteins. Hydrolysis of GTP is enhanced by the action of RGS proteins. See section 1.4 for more details. Adapted and modified from Sjögren *et al.*, 2010.

Although a GDP-bound α subunit is able to bind to the receptor without βγ, its association with the receptor is greatly enhanced by the presence of βγ. Upon binding to the agonist, the receptor undergoes a conformational change resulting in enhanced affinity for the G-proteins (Rasmussen *et al.*, 2011). The conformational change in the receptor acts as a switch to release GDP from the Gα subunit. Since the concentration of GTP is much higher than GDP under physiological conditions, GTP immediately replaces GDP. The activated state lasts until GTP is hydrolyzed to GDP by the intrinsic GTPase activity of Gα subunit. Exchange of GDP for GTP on the α subunit leads to dissociation/reorganization (Frank *et al.*, 2005) of the

heterotrimeric G-protein complex that facilitates transduction of signals to effector molecules such as adenylyl cyclase, phospholipases, and ion channels (Pierce *et al.*, 2002). The multiple components of GPCR signal transduction such as different types of receptors and G-protein subunits provide cells with enough flexibility to customize their responses to a diverse array of ligands such as hormones, neurotransmitters, and pharmacological agonists. In addition, numerous evidences suggests that GPCRs are capable of transducing signals across the plasma membrane through alternative mechanisms such as by activating Jak2 kinase, phospholipase Cγ, or protein kinase C *via* direct interaction with the receptor (Ji *et al.*, 1998; Hall *et al.*, 1999).

The membrane organization of GPCRs assumes significance in the light of their role in health and disease. Interestingly, the efficiency of signal transduction processes carried out by GPCRs appears to be influenced by the local composition and organization of lipids within the plasma membrane (Ostrom and Insel, 2004). It has been proposed that the G-protein coupled receptors are not uniformly present on the plasma membrane but are concentrated in specific membrane microdomains (Ostrom *et al.*, 2000; Ostrom, 2002). It has been shown that some of these domains are enriched in cholesterol (Ostrom and Insel, 2004). For example, it has been reported that serotonin$_{2A}$ receptors are localized in cholesterol-enriched membrane microdomains (caveolae) and serotonergic signaling induced by serotonin$_{2A}$ receptors depends on the membrane cholesterol content (Dreja *et al.*, 2002) and on caveolin-1, a scaffolding protein found in caveolae (Bhatnagar *et al.*, 2004). Localization of GPCRs into domains has given rise to new challenges and complexities in receptor signaling since signaling has to be understood in context of the three dimensional organization of various signal transduction components which include receptors and G-proteins (Ostrom *et al.*, 2000).

1.4.1. The Serotonin$_{1A}$ Receptor: A representative Member of the GPCR Family

Serotonin is one of the ancient signaling molecules, derived from essential amino acid tryptophan. Chemically, serotonin is called as 5-hydroxytryptamine and belongs to the class of monoamine neurotransmitters. Serotonin is found across the phyla from single-celled eukaryote to humans (Csabad, 1993; Greczek-Stachura, 2002). Serotonin receptors are found in a very diverse range of organisms up the evolutionary tree from *C. elegans* to *H. sapiens*. They have been classified into at least 14 subtypes on the basis of their pharmacological responses to specific ligands, sequence similarities and second messenger coupling pathways (Hoyer *et al.*, 2002). The serotonin$_{1A}$ (5-HT$_{1A}$) receptor is an important neurotransmitter receptor and is the most extensively studied of the serotonin receptors for a number of reasons (Pucadyil *et al.*, 2005a; Kalipatnapu and Chattopadhyay, 2007a,b). The serotonin$_{1A}$ receptor is the first among all types of serotonin receptors to be cloned as an intronless genomic clone (G-21) of the human genome which cross-hybridized with a full length β-adrenergic receptor probe at reduced stringency (Kobilka *et al.*, 1987; Pucadyil *et al.*, 2005a). Sequence analysis of this genomic clone (later identified as the serotonin$_{1A}$ receptor gene) showed ~43% amino acid similarity with the β$_2$-adrenergic receptor in the transmembrane domain. The serotonin$_{1A}$ receptor was therefore initially discovered as an 'orphan' receptor and was identified ('deorphanized') later (Fargin *et al.*, 1988). In fact, serotonin$_{1A}$ receptor was the first G-protein coupled receptor to be 'deorphanized'. The human gene for the receptor encodes a protein of 422 amino acids (see Fig. 1.6) and is characterized by molecular weight of ~46 kDa and an isoelectric point of 8.8. Considering the presence of three consensus sequences for N-linked glycosylation on the amino terminus, and the homology of

the receptor with β-adrenergic receptor, it is predicted that the receptor is oriented in the plasma membrane with the amino (N-) terminus facing the extracellular region and the carboxy (C-) terminus facing the intracellular cytoplasmic region (Raymond *et al.,* 1999; Pucadyil *et al.,* 2005a; Kalipatnapu and Chattopadhyay, 2007a,b; see Fig. 1.6). From hydropathy plots, serotonin$_{1A}$ receptors are predicted to contain seven hydrophobic stretches that could possibly be membrane spanning α-helices. The transmembrane domains (TM1-TM7) of the receptor are connected by hydrophilic sequences of three extracellular loops (EL1, EL2, EL3) and three intracellular loops (IL1, IL2, IL3) (Paila and Chattopadhyay, 2010; Paila *et al.*, 2011). Such an arrangement is a characteristic of the G-protein coupled receptor superfamily (Gether and Kobilka 1998). Although the structure of the serotonin$_{1A}$ receptor has not yet been determined, mutagenesis studies have helped in identifying amino acid residues important for ligand binding and G-protein coupling of the serotonin$_{1A}$ receptor (reviewed in Pucadyil *et al.,* 2005a). Among the predicted structural features of the serotonin$_{1A}$ receptor, palmitoylation status of the receptor has been confirmed in a recent report (Papoucheva *et al.,* 2004). An interesting aspect of this study is that palmitoylation of the serotonin$_{1A}$ receptor was found to be stable and independent of stimulation by the agonist. This is rare for GPCRs which undergo repeated cycles of palmitoylation and depalmitoylation (Milligan *et al.,* 1995). Importantly, palmitoylation of the serotonin$_{1A}$ receptor is shown to be crucial for its apparent raft localization that in turn appears to regulate receptor-mediated signaling (Renner *et al.* 2007). It has therefore been proposed that stable palmitoylation of the receptor could play an important role in maintaining the receptor structure and function (Papoucheva *et al.,* 2004, Renner *et al.,* 2007, Kvachnina *et al.,* 2009).

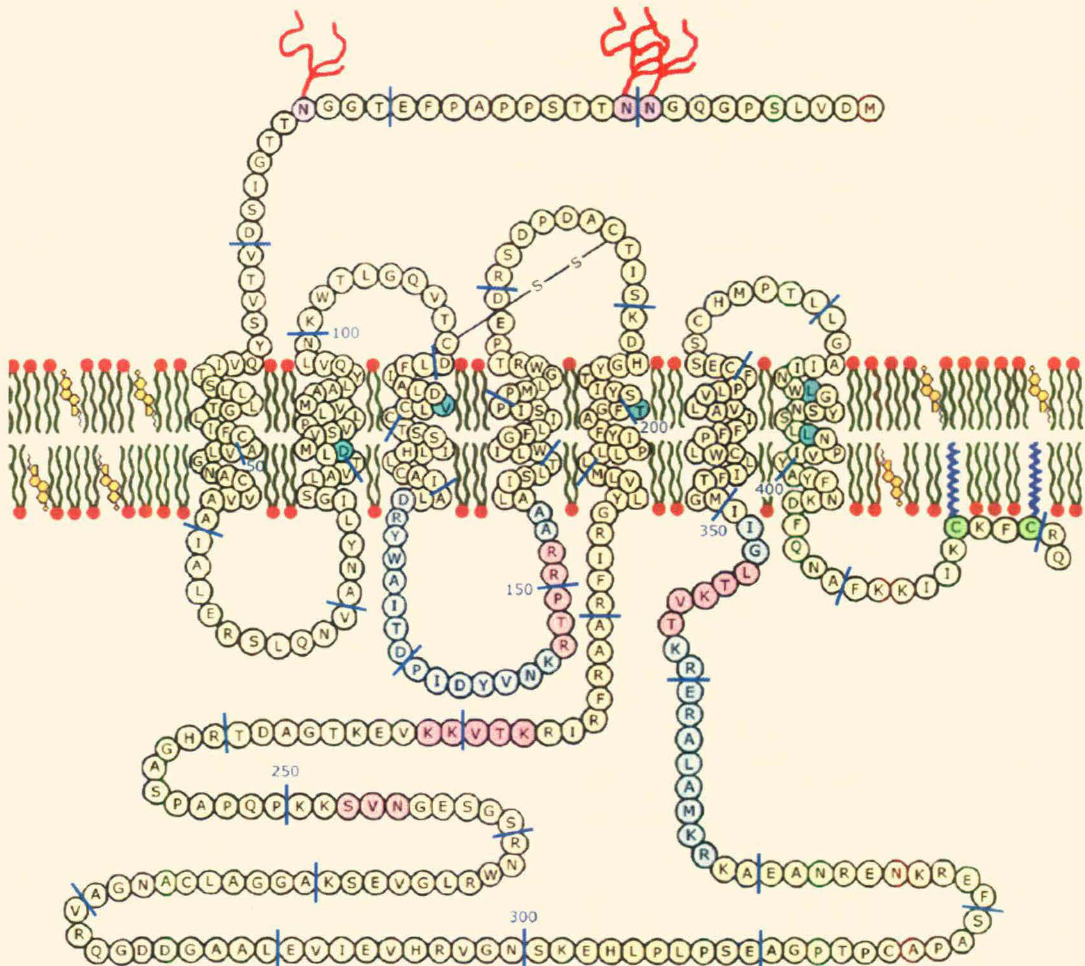

Figure 1.6. *A schematic representation of the membrane embedded human serotonin$_{1A}$ receptor showing its topological and other structural features.* The membrane is shown as a bilayer of phospholipids and cholesterol, representing typical eukaryotic membranes. Seven transmembrane stretches, each composed of ~22 amino acids, are depicted as putative α-helices. The amino acids in the receptor sequence are shown as circles and are marked for convenience. The potential sites (shown in lavender) for N-linked glycosylation (depicted as branching trees in red) on the amino terminus are shown. A putative disulfide bond between Cys^{109} and Cys^{187} is shown. The transmembrane domains contain residues (shown in cyan) that are important for ligand binding. The putative cholesterol binding site is highlighted (in orange). The receptor is stably palmitoylated (shown in blue) at residues Cys^{417} and/or Cys^{420} (shown in green). Light blue circles represent contact sites for G-proteins. Light pink circles represent sites for protein kinase mediated phosphorylation. Further structural details of the receptor are available in Pucadyil *et al.*, 2005a; Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2009. Adapted from Paila and Chattopadhyay, 2009.

Serotonergic signaling plays a key role in the generation and modulation of various cognitive, developmental and behavioral functions. The serotonin$_{1A}$ receptor agonists and antagonists have been shown to possess potential therapeutic effects in anxiety- or stress-related disorders (Griebel, 1999; Pucadyil *et al.,* 2005a). The serotonin$_{1A}$ receptor therefore serves as an important target in the development of therapeutic agents for neuropsychiatric disorders such as anxiety and depression. Interestingly, mutant (knockout) mice lacking the serotonin$_{1A}$ receptor exhibit enhanced anxiety-related behavior, and represent an important animal model for genetic vulnerability to complex traits such as anxiety disorders and aggression in higher animals (Toth, 2003; Gardier, 2009). Taken together, the serotonin$_{1A}$ receptor is involved in a multitude of physiological processes, therefore an important drug target.

1.5. Role of Membrane Lipids in the Function of GPCRs

GPCRs are integral membrane proteins with a significant portion of the protein embedded in the membrane. In the case of rhodopsin, molecular dynamics simulation studies have estimated that the lipid-protein interface corresponds to ~38% of the total surface area of the receptor (Huber *et al.*, 2004). This raises the strong possibility that the membrane lipid environment could be an important modulator of receptor structure and function (Lee, 2004). The importance of a membrane lipid environment for optimal function of membrane proteins in general, and GPCRs in particular, is evident from the adverse effects of delipidation on receptor function (Kirilovsky and Schramm, 1983; Jones *et al.*, 1988).

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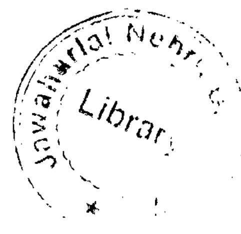


1.5.1. Effect of Membrane Cholesterol on the Function of GPCRs

Membrane cholesterol has been shown to modulate the function of a number of GPCRs. From the available data on the role of cholesterol on GPCR function (see Table 1), it appears that there is a lack of consensus in the manner in which cholesterol modulates receptor function. For example, while cholesterol is found to be essential for the proper function of several GPCRs, the function of rhodopsin and cannabinoid receptors has been shown to be inhibited in the presence of cholesterol. The mechanism underlying the effect of cholesterol on the structure and function of integral membrane proteins and receptors is complex and no general consensus has evolved yet (Burger *et al.*, 2000; Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2009). It has been proposed that cholesterol can modulate the function of GPCRs in two ways: (i) by a direct/specific interaction with the GPCR, which could induce a conformational change in the receptor (Gimpl *et al.*, 2002a,b), or (ii) through an indirect way by altering the membrane physical properties in which the receptor is embedded (Ohvo-Rekilä *et al.*, 2002; Lee, 2004) or due to a combination of both. There could be yet another fashion in which membrane cholesterol could affect structure and function of membrane proteins. This mechanism invokes the concept of 'nonannular' binding sites of membrane lipids (Lee *et al.*, 1982; Simmonds *et al.*, 1982). It has recently been proposed that cholesterol binding sites in GPCRs could represent nonannular binding sites (Paila *et al.*, 2009, see later).

Table 1.1

Membrane Cholesterol and GPCR Function

GPCR	References
Rhodopsin	(Straume and Litman, 1988; Mitchell *et al.*, 1990; Albert and Boesze-Battaglia, 2005)
Cholecystokinin (CCK)	(Gimpl *et al.*, 1997; Burger *et al.*, 2000; Harikumar *et al.*, 2005)
Galanin (GAL2)	(Pang *et al.*, 1999)
$Serotonin_{1A}$ ($5\text{-}HT_{1A}$)	(Pucadyil and Chattopadhyay, 2004b, 2005, 2006; Paila *et al.*, 2005, 2008)
$Serotonin_7$ ($5\text{-}HT_7$)	(Sjögren *et al.*, 2006)
Metabotropic glutamate[a]	(Eroglu *et al.*, 2002, 2003)
δ Opioid	(Huang *et al.*, 2007; Levitt *et al.*, 2009)
κ Opioid	(Xu *et al.*, 2006)
μ Opioid	(Lagane *et al.*, 2000; Levitt *et al.*, 2009)
Oxytocin	(Gimpl *et al.*, 1995, 1997, 2002b; Fahrenholz *et al.*, 1995; Klein *et al.*, 1995)
β_2-adrenergic	(Kirilovsky and Schramm, 1983; Kirilovsky *et al.*, 1987; Ben-Arie *et al.*, 1988)
Chemokine (CXCR4, CCR5)	(Nguyen and Taub, 2002a,b, 2003)
Neurokinin (NK1)	(Monastyrskaya *et al.*, 2005)
Cannabinoid (CB1)	(Bari *et al.*, 2005a,b)
M_2 Muscarinic	(Colozo *et al.*, 2007)

[a]These studies were carried out in the *Drosophila* eye where the major sterol present is ergosterol. Adapted from Paila and Chattopadhyay, 2010.

1.5.2. Role of Sphingolipids in the Function of GPCRs

Sphingolipids play a crucial role in the structure and function of membrane proteins (Fantini, 2003). Together with cholesterol, they participate in generation of laterally segregated ordered domains in the membrane. It has been proposed that the G-protein coupled receptors are not uniformly present on the plasma membrane but are concentrated in specific membrane microdomains (Ostrom *et al.*, 2000; Ostrom, 2002). Although many GPCRs have been shown to partition in membrane domains enriched with sphingolipid and cholesterol (Chini and Parenti, 2004), their degree of association varies. Some GPCRs preferentially partition in such specialized domains (~90% of the gonadotrophin-releasing hormone receptor) (Navratil *et al.*, 2003), whereas others are present in small amount (~10% of the oxytocin receptor) (Gimpl and Fahrenholz 2002, Guzzi *et al.* 2002). There appear to be various molecular determinants affecting the affinity of GPCRs for lipid rafts/caveolae. In addition, as some GPCRs can move inside or outside lipid rafts/caveolae upon activation, these determinants must be subject to dynamic modulation by one or more effectors (Chini and Parenti, 2004). The serotonin receptor family represents the largest family of G-protein coupled neurotransmitter receptors. Various serotonin receptors such as serotonin$_{1A}$ receptors (Renner *et al.*, 2007), serotonin$_{2A}$ receptors (Dreja *et al.*, 2002), and serotonin$_7$ receptors (Sjögren *et al.*, 2007) have been shown to distribute themselves in detergent resistant fractions to different degrees. The double palmitoylation of serotonin$_4$ receptor raises exciting possibility of its recruitment in ordered domains (Ponimaskin *et al.*, 2002), as shown for the serotonin$_{1A}$ receptor (Renner *et al.*, 2007). Interestingly, serotonin$_6$ receptors are shown to interact with Fyn (Yun *et al.*, 2007), a member of the Src family of non-receptor protein-tyrosine kinase known to be enriched in lipid ordered domains (Harder *et al.*,

1998). A direct link between sphingolipid and G-protein coupled receptor has been established recently where metabolic depletion of sphingolipids led to impairment of the function and organization of the serotonin$_{1A}$ receptor (Paila *et al.*, 2010; Ganguly *et al.*, 2011).

1.6. Nonannular Lipids in the Function of Membrane Proteins

It has been proposed for the nicotinic acetylcholine receptor (which requires cholesterol for its function) that cholesterol could be present at the 'nonannular' sites of the receptor (Jones and McNamee, 1988). Early evidence for the presence of nonannular lipids was obtained from experiments monitoring effects of cholesterol and fatty acids on Ca^{2+}/Mg^{2+}-ATPase (Lee *et al.*, 1982; Simmonds *et al.*, 1982). Integral membrane proteins are surrounded by a shell or annulus of lipid molecules, which mimics the immediate layer of solvent surrounding soluble proteins (Jost *et al.*, 1973; Lee, 2003). These are termed 'annular' lipids surrounding the membrane protein. After several years of moderate controversy surrounding the interpretation of spectroscopic data, it later became clear that the annular lipids are exchangeable with bulk lipids (Devaux and Seigneuret, 1985). The rate of exchange of lipids between the annular lipid shell and the bulk lipid phase was shown to be approximately an order of magnitude slower than the rate of exchange of bulk lipids, resulting from translational diffusion of lipids in the plane of the membrane. It therefore appears that exchange between annular and bulk lipids, is relatively slow since lipid-protein interaction is favorable compared to lipid-lipid interaction. However, the difference in interaction energy is modest, consistent with the observation that lipid-protein binding constants (affinity) depend weakly on lipid structure (Lee, 2003). Interestingly, the two different types of lipid environments (annular and bulk) can

be readily detected using electron spin resonance (ESR) spectroscopy (Marsh, 1990). In addition to the annular lipids, there is evidence for other lipid molecules in the immediate vicinity of integral membrane proteins. These are termed as 'nonannular' lipids. Nonannular sites are characterized by lack of accessibility to the annular lipids, *i.e.*, these sites cannot be displaced by competition with annular lipids. This is evident from analysis of fluorescence quenching of intrinsic tryptophans of membrane proteins by phospholipids or cholesterol covalently labeled with bromine (Simmonds *et al.*, 1982; Jones and McNamee, 1988), which acts as a quencher due to the presence of the heavy bromine atom (Chattopadhyay, 1992).

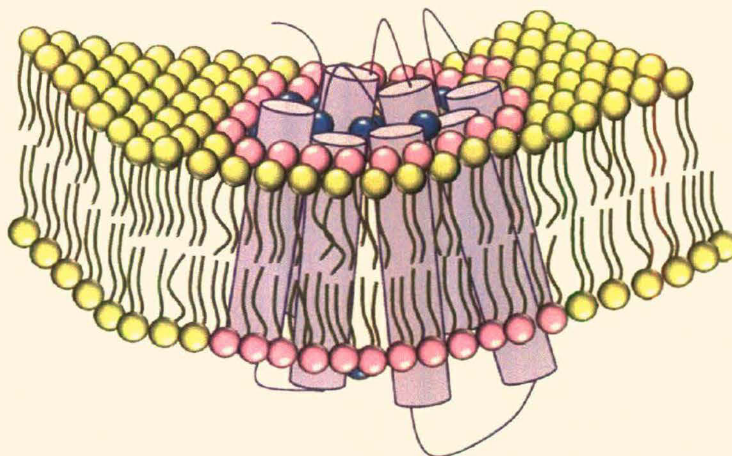


Figure 1.7. *A schematic representation of a membrane embedded seven transmembrane domain protein showing various classes of lipids in the vicinity of the protein.* Annular lipids (shown in pink) represent the shell (or annulus) of lipid molecules which mimics the immediate layer of solvent surrounding soluble proteins. The annular lipids are in dynamic equilibrium (exchangeable) with bulk lipids (shown in green). The rate of exchange of lipids between the annular lipid shell and the bulk lipid phase is approximately an order of magnitude slower than that of exchange of bulk lipids. See sections 1.5 and 1.6 for more details.

These results signify that nonannular lipid binding sites remain vacant even in the presence of annular lipids around the protein (Marius *et al.*, 2008).

Nonannular lipids (shown in blue) are characterized by lack of accessibility to annular lipids. See text for details. The exchange of lipid molecules between nonannular sites and bulk lipids proposed to be relatively slow compared to the exchange between annular sites and bulk lipids, and binding to the nonannular sites is considered to be more specific compared to annular binding sites (Lee, 2003). The location of the postulated nonannular sites merits comment. It has been suggested that the possible locations for the nonannular sites could be either inter or intramolecular (interhelical) protein interfaces, characterized as deep clefts (or cavities) on the protein surface (Simmonds *et al.*, 1982; Marius *et al.*, 2008) (see Fig. 1.7). For example, in the crystal structure of the potassium channel KcsA from *S. lividans*, a negatively charged lipid molecule was found to be bound as 'anionic nonannular' lipid at each of the protein-protein interface in the homotetrameric structure (Marius *et al.*, 2005). These nonannular sites show high selectivity for anionic lipids over zwitterionic lipids, and it has been proposed that the change in the nature of the nonannular lipid leads to a change in packing at the protein-protein interface which modulates the open channel probability and conductance. Interestingly, the relationship between open channel probability of KcsA and negative phospholipid content exhibits cooperativity. This is consistent with a model in which the nonannular sites in the KcsA homotetramer have to be occupied by anionic lipids for the channel to remain open (Marius *et al.*, 2008). This example demonstrates the crucial requirement of nonannular lipids in the function of membrane proteins and the stringency associated with regard to specificity of nonannular lipids.

1.7. Cholesterol Binding Motif(s) in GPCRs

As mentioned earlier, there could be two possible mechanisms by which membrane cholesterol could influence the structure and function of GPCRs (Gimpl *et al.*, 2002a,b): (i) through a direct/specific interaction with GPCRs, or (ii) through an indirect way by altering membrane physical properties in which the receptor is embedded, or due to a combination of both. Interestingly, recently reported crystal structures of GPCRs have shown structural evidence of cholesterol binding sites (Gimpl *et al.*, 2002b; Lee, 2004). Several structural features of proteins that are believed to result in preferential association with cholesterol have been recognized (Ohvo-Rekilä *et al.*, 2002; Jones and McNamee, 1988). In many cases, proteins that interact with cholesterol have a characteristic amino acid sequences, termed the cholesterol recognition/interaction amino acid consensus (CRAC) sequence and cholesterol consensus motif (CCM). The CRAC sequence is defined by the presence of the pattern $-L/V-(X)_{1-5}-Y-(X)_{1-5}-R/K-$, in which $(X)_{1-5}$ represents between one and five residues of any amino acid (Ohvo-Rekilä *et al.*, 2002; Simmonds *et al.*, 1982). This motif has been shown to be present in caveolin-1 (Lee *et al.*, 1982), the peripheral-type benzodiazepine receptor (Simmonds *et al.*, 1982; Devaux and Seigneuret, 1985), the HIV-1 transmembrane protein gp41 (Jost *et al.*, 1973), and the mammalian seminal plasma protein PDC-109 (Lee, 2003). On the other hand, CCM has been recently reported in the crystal structure of β_2-adrenergic receptor. Three amino acids in transmembrane helix IV, along with an amino acid in transmembrane helix II, have been shown to constitute a cholesterol consensus motif (CCM) in the crystal structure of the β_2-adrenergic receptor. The aromatic $Trp158^{4.50}$ (according to the Ballesteros–Weinstein numbering system (Ballesteros and Weinstein, 1995) is conserved to a high degree (~94%) among class A GPCRs and appears to contribute the most significant interaction with ring D of cholesterol

(Hanson *et al.*, 2008). In this structure, the hydrophobic residue Ile154$^{4.46}$ would interact with rings A and B of cholesterol and is largely conserved (~60%) in class A GPCRs. The aromatic residue Tyr70$^{2.41}$ in transmembrane helix II could interact with ring A of cholesterol and with Arg151$^{4.43}$ of transmembrane helix IV through hydrogen bonding. The criterion of specific residues in CCM could be somewhat broadened by conservative substitutions of amino acids.

In the overall context of cholesterol sensitivity of GPCR function (Simons and Ikonen, 1997, 2000; Schroeder *et al.*, 1995), we have shown that the serotonin$_{1A}$ receptor sequence contains the cholesterol binding motifs such as CCM and CRAC. These findings substantiate the earlier results showing cholesterol dependence of the serotonin$_{1A}$ receptor function (Pucadyil and Chattopadhyay, 2006). The cholesterol binding site (s) of the serotonin$_{1A}$ receptor was found to be conserved over various phyla. The sequences of rhodopsin and the serotonin$_{1A}$ receptor contain three CRAC motifs, the β_2-adrenergic receptor sequence contains two CRAC motifs and cannbinoid receptor harbors one CRAC motif. Rhodopsin sequence contains CRAC motifs in putative transmembrane helices I (residues 57-66), III (residues 131-141) and VII (residues 304-311), while the serotonin$_{1A}$ receptor sequence is characterized by CRAC motifs in putative transmembrane helices II (residues 90-101), V (residues 208-219) and VII (residues 394-405). The β_2-adrenergic receptor sequence, on the other hand, exhibits CRAC motifs in putative transmembrane helices V (residues 213-221) and VII (residues 324-328) (Jafurulla *et al.*, 2011). The cannabinoid receptor contains CRAC motif in putative transmembrane helix VII (residues 392-402 in cannabinoid receptor type-1; residues 294 to 304 in cannabinoid receptor type-2) (Oddi *et al.*, 2011). It is apparent from this alignment that the cholesterol consensus motif, which includes Tyr73 in the putative transmembrane helix II and Arg151, Ile157 and Trp161 in the putative transmembrane helix IV, is conserved in most species. The serotonin$_{1A}$

receptor is an important member of the GPCR superfamily and is estimated to have differentiated ~650 million years ago from the serotonin$_1$ receptor subfamily in the time period during which vertebrates diverged from invertebrates (Peroutka and Howell, 1994). In addition to the presence of CCM and CRAC motifs in the human serotonin$_{1A}$ receptor, cholesterol binding sites are found to be present in serotonin$_{1A}$ receptor over different phyla. Cholesterol binding motifs therefore represent an inherent characteristic feature of serotonin$_{1A}$ receptors which are conserved during the course of natural evolution. It is interesting to note here that cholesterol binding sites appear to be present even in organisms which are not capable of biosynthesis of cholesterol. Organisms which lack cholesterol biosynthesis could nevertheless acquire cholesterol through diet (Bloch, 1983). Organisms such as insects possess sterols that are different from cholesterol which have diverged from cholesterol during the sterol evolution pathway (Clark and Bloch, 1959). The presence of CCM and CRAC motifs in these organisms could be due to binding of closely related sterols or dietary cholesterol to these motifs (Paila *et al.*, 2009; Jafurulla *et al.*, 2011).

The mechanism underlying the effect of cholesterol on the structure and function of integral membrane proteins and receptors is complex and no general consensus has evolved yet (Simons and Ikonen, 1997, 2000; Schroeder *et al.*, 1995). It has recently been suggested that cholesterol-dependent modulation of GPCR function could be due to (i) a specific interaction with the GPCR, or (ii) through an indirect way by altering the membrane physical properties in which the receptor is embedded (Ohvo-Rekilä *et al.* 2002; Lee, 2004) or as a result of a combination of both. In view of these observations, presence of CCM and CRAC motifs in GPCRs lends support to specific interaction of cholesterol with GPCRs. This is further corroborated by recent results from receptor modeling studies in which our group showed that the serotonin$_{1A}$ receptor is more compact in the presence of

cholesterol (Paila *et al.* 2011). More importantly, cholesterol has been shown to improve stability of GPCRs such as the β_2-adrenergic receptor (Yao and Kobilka, 2005), and appears to be a necessary component for crystallization of the receptor since it facilitates receptor-receptor interaction and consequent oligomerization (Cherezov *et al.*, 2007). Since a possible location of the nonannular sites is interprotein interfaces (Simmonds *et al.*, 1982; Jones and McNamee, 1988), it is possible that cholesterol molecules located between individual receptor molecules occupy nonannular sites and modulate receptor structure and function.

Work in the last decade from our laboratory has comprehensively demonstrated the requirement of membrane cholesterol in the function of the serotonin$_{1A}$ receptor (Pucadyil and Chattopadhyay, 2004b, 2006). We demonstrated that depletion of membrane cholesterol had led to reduction in the ligand binding function of the hippocampal serotonin$_{1A}$ receptor (Pucadyil and Chattopadhyay, 2004b). Membrane cholesterol depletion was achieved by the use of methyl-β-cyclodextrin (MβCD) which physically depletes cholesterol from membranes. Importantly, ligand binding function of the serotonin$_{1A}$ receptor was restored upon replenishment with cholesterol using MβCD-cholesterol complex. These results showed that cholesterol is essential for the function of the serotonin$_{1A}$ receptor. In order to explore the specificity of cholesterol requirement for the function of the serotonin$_{1A}$ receptor, membrane cholesterol in the hippocampal membranes was replaced with 7-dehydrocholesterol (7-DHC) and desmosterol. 7-DHC and desmosterol are immediate precursors of cholesterol in the Kandutsch-Russell and Bloch pathways, respectively. 7-DHC and desmosterol are positional isomers which differ from cholesterol in only one additional unsaturation at 7th position in the sterol ring and 24th position in flexible acyl chain, respectively. The stringency of cholesterol requirement for the function of the serotonin$_{1A}$ receptor was examined in the first part of the thesis, by replacing

cholesterol with 7-DHC and desmosterol in hippocampal membranes. This work is followed by monitoring the membrane organization and dynamics of native and lipid extract of hippocampal membranes under the conditions of varying cholesterol and protein content. For this, spin labeled phospholipids at two different locations (depths) were incorporated to probe acyl chain dynamics of these membranes in a depth-dependent manner by electron spin resonance (ESR) specroscopy. We have recently shown that metabolic depletion of total sphingolipid in CHO cells, stably expressing the human serotonin$_{1A}$ receptor, impaired the function of serotonin$_{1A}$ receptors (Paila *et al.*, 2010). In addition, the structural importance of sphingomyelin headgroup for the ligand binding function of the serotonin$_{1A}$ receptor was explored in hippocampal membranes. In order to further explore the specificity of sphingolipid requirement, function of the human serotonin$_{1A}$ receptor was studied upon depleting glycosphingolipids metabolically. These results are discussed in the latter part of the thesis.

Chapter 2

Differential effects of cholesterol and 7-dehydrocholesterol on the ligand binding activity of the hippocampal serotonin$_{1A}$ receptor: Implications in SLOS

2.1. Introduction

Seven transmembrane domain G-protein coupled receptors (GPCRs) constitute one of the largest family of proteins in mammals and account for ~5% of the total proteins coded by the human genes (Zhang *et al.*, 2006). GPCRs represent major targets for the development of novel drug candidates in all clinical areas (Hopkins and Groom, 2002). The serotonin$_{1A}$ receptor is an important GPCR and is involved in a variety of cognitive, behavioral, and developmental functions as described in section 1.4. The serotonin$_{1A}$ receptor agonists and antagonists represent major classes of molecules with potential therapeutic effects in anxiety- or stress-related disorders. Interestingly, mice lacking the serotonin$_{1A}$ receptor exhibit enhanced anxiety-related behavior (Julius, 1998) and represent an important animal model for genetic vulnerability to conditions such as anxiety disorders and aggression (Toth, 2003).

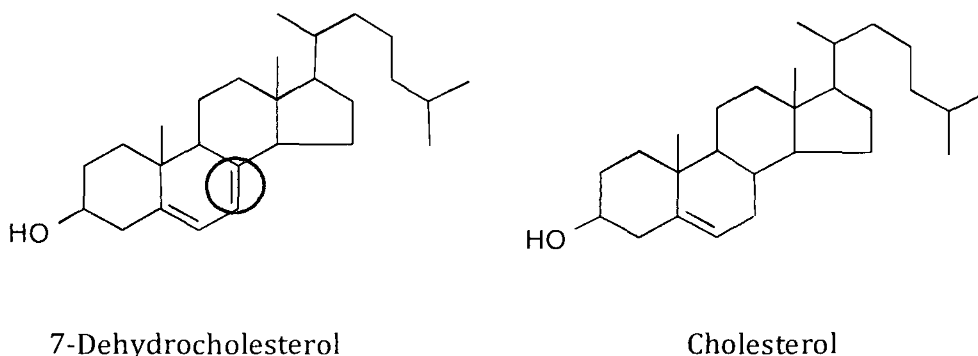


Figure 2.1. *Chemical structures of cholesterol and 7-dehydrocholesterol.* The principal route of cholesterol synthesis in humans is the Kandutsch-Russell pathway (Kandustch and Russell, 1960). In this pathway, the immediate precursor of cholesterol is 7-dehydrocholesterol which differs only in its unsaturation at 7th position in the sterol ring (highlighted in its chemical structure). Elevated levels of 7-dehydrocholesterol have been characterized as a diagnostic parameter of the Smith-Lemli-Opitz syndrome. See section 1.2 for more details.

Cholesterol is an abundant and essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function and sorting (Simons and Ikonen, 2000). Interestingly, the central nervous system which accounts for only 2% of the body mass contains ~25% of free cholesterol present in the whole body (Chattopadhyay and Paila, 2007). Brain cholesterol is synthesized *in situ* and is developmentally regulated. As a result, a number of neurological diseases share a common etiology of defective cholesterol metabolism in the brain (Porter, 2002). In the Smith-Lemli-Opitz syndrome (SLOS), for example, the marked abnormalities in brain development and function leading to serious neurological and mental dysfunctions have their origin in the fact that the major input of brain cholesterol comes from the *in situ* synthesis and such synthesis is defective in this syndrome (Waterham and Wanders, 2000). SLOS is caused by mutations in 3β-hydroxy-steroid-Δ^7-reductase (7-DHCR), an enzyme required in the final step of cholesterol biosynthesis. Elevated plasma levels of 7- and 8-dehydrocholesterol and the ratio of their contents to that of cholesterol are representative parameters for diagnosis of SLOS. The effect of alteration in the cholesterol content of neuronal membranes on membrane dynamics and protein/receptor function therefore represents an important determinant in the analysis of neurogenesis and several neuropathologies.

It has previously been shown that membrane cholesterol is essential for ligand binding activity of the hippocampal serotonin$_{1A}$ receptor (Pucadyil and Chattopadhyay, 2004b, 2006). In order to further examine the stringency of cholesterol requirement for ligand binding activity of serotonin$_{1A}$ receptors, cholesterol is replaced with 7-DHC, an immediate biosynthetic precursor of cholesterol differing only in its unsaturation at 7th position in the sterol ring (see Fig. 2.1).

2.2. Materials and methods

Materials

Cholesterol, 7-DHC, MβCD, DMPC, DPH, EDTA, EGTA, $MgCl_2$, $MnCl_2$, iodoacetamide, PMSF, serotonin, sucrose, polyethylenimine, sodium azide, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). BCA reagent for protein estimation was from Pierce (Rockford, IL). [^{3}H]8-OH-DPAT (sp. activity 135 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). GF/B glass microfiber filters were from Whatman International (Kent, UK). All solvents used were of analytical grade. Precoated silica gel 60 thin layer chromatography plates were from Merck (Darmstadt, Germany). All other chemicals used were of the highest purity available. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. Fresh bovine brains were obtained from a local slaughterhouse within 10 min of death and the hippocampal region was carefully dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at -70 °C till further use.

Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described earlier (Pucadyil and Chattopadhyay, 2004b). Protein concentration was determined using the BCA reagent with bovine serum albumin as a standard (Smith *et al.*, 1985).

Radioligand binding assays

Receptor binding assays were carried out as described earlier (Pucadyil and Chattopadhyay, 2004b) using 0.5 mg total protein. Briefly, tubes in duplicate with 0.5 mg protein in a total volume of 1 ml of buffer (50 mM Tris, 1 mM EDTA, 10

mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.4) were incubated with the radiolabeled agonist [^{3}H]8-OH-DPAT (final concentration in assay tube being 0.29 nM) for 1 h at 25 °C. Nonspecific binding was determined by performing the assay in the presence of 10 µM serotonin. The binding reaction was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B 2.5 cm diameter glass microfiber filters (1.0 µm pore size), which were presoaked in 0.15% polyethylenimine for 1 h. Filters were then washed three times with 3 ml of cold water (4 °C), dried and the retained radioactivity was measured in a Packard Tri-Carb 1500 liquid scintillation counter using 5 ml of scintillation fluid.

Cholesterol depletion of native membranes

Native hippocampal membranes were depleted of cholesterol using MβCD as described previously (Pucadyil and Chattopadhyay, 2004b). Briefly, membranes resuspended at a protein concentration of 2 mg/ml were treated with 40 mM MβCD in 50 mM Tris, pH 7.4 buffer at 25 °C with constant shaking for 1 h. Membranes were then spun down at 50,000 x g for 5 min at 4 °C, washed with 50 mM Tris, pH 7.4 buffer and resuspended in the same buffer.

7-Dehydrocholesterol and cholesterol replenishment of cholesterol-depleted membranes

Cholesterol-depleted hippocampal membranes were replenished with 7-dehydrocholesterol (7-DHC) or cholesterol using either 7-DHC-MβCD or cholesterol-MβCD complex which are soluble in water. The complex was prepared by dissolving the required amounts of 7-DHC or cholesterol and MβCD in a ratio of 1:10 (mol/mol) in 50 mM Tris, pH 7.4 buffer by constant shaking at 25 °C. Stock solutions (typically 2 mM 7-DHC (or cholesterol): 20 mM MβCD) of this complex were freshly prepared before each experiment. 7-DHC and cholesterol

replenishment were carried out at a protein concentration of 2 mg/ml by incubating the cholesterol-depleted membranes with 1 mM 7-DHC (or cholesterol): 10 mM MβCD complex for 1 h in 50 mM Tris, pH 7.4 buffer at 25 °C under constant shaking. Membranes were then spun down at 50,000 x g for 5 min at 4 °C, washed with 50 mM Tris, pH 7.4 buffer and resuspended in the same buffer.

Estimation of 7-DHC and cholesterol by thin layer chromatography

Lipid extraction from native, cholesterol-depleted, and membranes replenished with 7-DHC or cholesterol after cholesterol depletion using 40 mM MβCD was carried out according to Bligh and Dyer (Bligh and Dyer, 1959). The 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). 7-DHC and cholesterol were resolved by thin layer chromatography (TLC). TLC plates were impregnated with 3% (w/v) silver nitrate solution in 97% methanol, allowed to dry briefly and activated at 120 °C for 15 min. Total lipid extracts were separated using n-heptane/ethylacetate (2:1, v/v) as the solvent system (Aufenanger *et al.*, 1986). The separated lipids were visualized by charring with a solution containing cupric sulfate (10%, w/v) and phosphoric acid (8%, v/v) at 150 °C. 7-DHC and cholesterol were used as standards to identify 7-DHC and cholesterol bands on the thin layer chromatogram run with lipid extracts from native, cholesterol-depleted, and 7-DHC or cholesterol replenished hippocampal membranes. The TLC plates were scanned and lipid band intensities were analyzed using the Adobe Photoshop software version 5.0 (Adobe Systems, San Jose, CA). Intensities of the sterols (7-DHC and cholesterol) from all samples on the TLC plate were normalized to the intensity of the cholesterol band obtained from the native membrane.

Estimation of phospholipids

Concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid (McClare, 1971) using Na_2HPO_4 as standard. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

Fluorescence anisotropy measurements

Fluorescence anisotropy measurements were carried out with fluorescent membrane probe DPH with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvette at room temperature (23 °C) as described earlier (Paila *et al.*, 2005). Excitation and emission wavelengths were set at 358 and 430 nm. Excitation and emission slits with nominal bandpasses of 1.5 and 20 nm were used. The optical density of the samples measured at 358 nm was 0.15 ± 0.01. The anisotropy values remained identical even after dilution of membrane samples, indicating the absence of any scattering artifact (Lentz *et al.*, 1979). Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy values were calculated from the equation (Lakowicz, 2006):

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \qquad (2.1)$$

Where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is equal to I_{HV}/I_{HH}. All experiments were done with multiple sets of samples and average values of fluorescence anisotropy are shown in Fig. 2.5.

2.3. Results and discussion

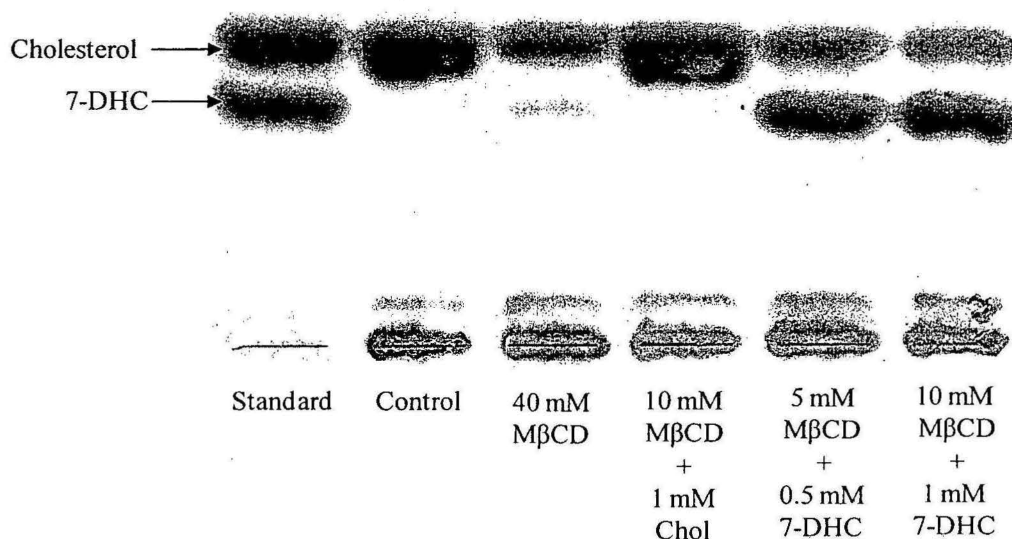


Figure 2.2. *Separation of sterol content of native and cholesterol-depleted hippocampal membranes, and cholesterol-depleted membranes replenished with varying concentrations of sterols (7-DHC and cholesterol).* Total lipids were extracted from native hippocampal membranes, membranes treated with 40 mM MβCD, and after replenishment with varying concentrations of sterols. Thereafter sterols were separated by thin layer chromatography. The lanes represent lipids extracted from native membranes (control), membranes treated with 40 mM MβCD, and membranes treated with 40 mM MβCD followed by replenishment with 1 mM cholesterol, 0.5 mM of 7-DHC and 1 mM 7-DHC. The arrows represent positions of cholesterol and 7-DHC on the thin layer chromatogram identified using standards in lane 1. See section 2.2 for other details.

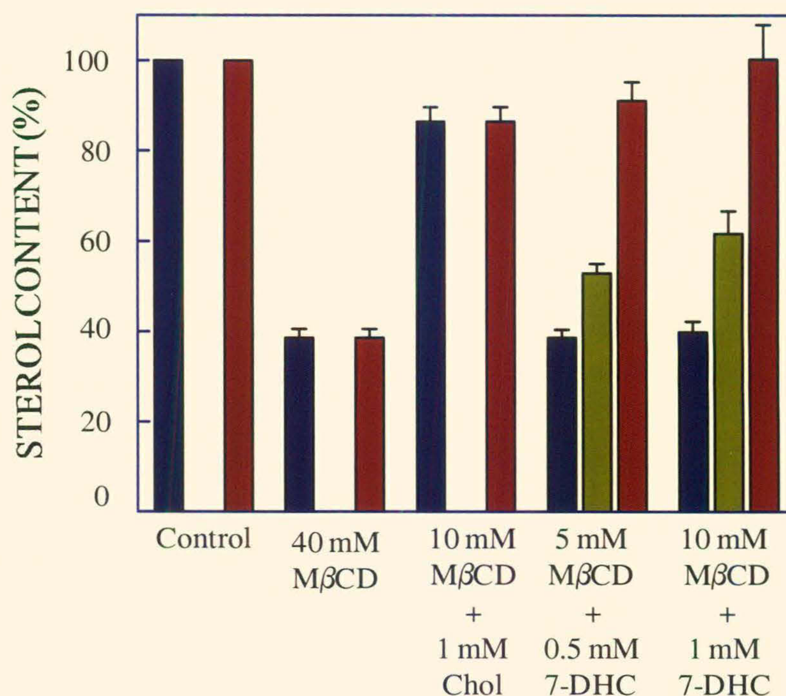


Figure 2.3. *Estimation of sterol content of native, cholesterol-depleted and sterol (7-DHC and cholesterol) replenished hippocampal membranes.* Cholesterol (blue color bars), 7-DHC (mustard color bars) and total sterol (maroon color bars) were quantified by densiometric analysis of the thin layer chromatogram. Values are expressed as percentages of the cholesterol content of native membranes without any treatment and total sterol content of membranes were obtained by the addition of 7-DHC and cholesterol contents. Data represent means ± SE of at least three independent experiments. See section 2.2 for other details.

Cholesterol depletion of native hippocampal membranes was carried out using the water-soluble compound MβCD which has previously been shown to selectively and efficiently extract cholesterol from membranes (Pucadyil and Chattopadhyay, 2004b). Figs. 2.2 and 2.3 show the extents of replenishment of 7-DHC and cholesterol into hippocampal membranes treated with 40 mM MβCD. Treatment of membranes with 40 mM MβCD results in ~61% reduction in the cholesterol content (see Fig. 2.3). This is accompanied by a corresponding reduction (~56%) in the specific [^{3}H]8-OH-DPAT binding (see Fig. 2.4).

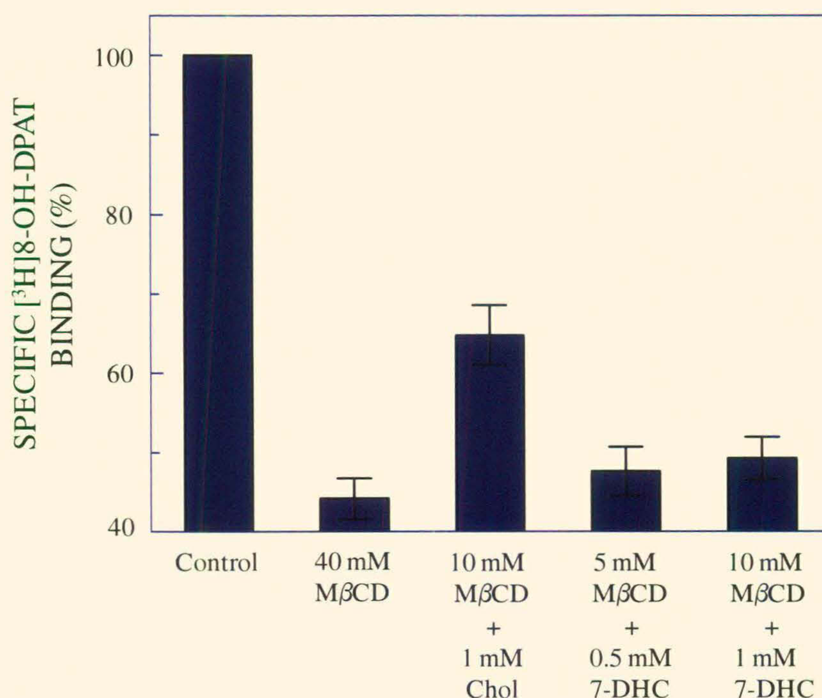


Figure 2.4. *Effect of replenishment of 7-DHC and cholesterol into cholesterol-depleted membranes on the specific binding of the [^{3}H]8-OH-DPAT to the hippocampal serotonin$_{1A}$ receptor.* Native membranes were treated with 40 mM MβCD and were replenished with varying concentrations of 7-DHC and cholesterol. Values are expressed as percentages of the specific binding obtained in native membranes. The data shown are means ± SE from at least five independent experiments. See section 2.2 for other details.

Replenishment with 1 mM cholesterol resulted in recovery of specific agonist binding to ~65% of native membranes (Fig. 2.4) even when ~87% of the cholesterol could be replenished (Fig. 2.3). In order to monitor whether replenishment with 7-DHC could restore the specific [^{3}H]8-OH-DPAT binding, cholesterol-depleted membranes were replenished with 0.5 and 1 mM 7-DHC. Importantly, the total sterol content (7-DHC + cholesterol) appears to be comparable to native membranes, especially when loading was carried out with 1 mM 7-DHC (Fig. 2.3). Interestingly, even when loading was carried out with 1 mM 7-DHC, the specific agonist binding could not be restored (Fig. 2.4) in spite of the

fact that the extent of loading of 7-DHC was similar to what was obtained with 1 mM cholesterol (Fig. 2.3). It appears that 7-DHC is not able to restore the specific agonist binding activity of the hippocampal serotonin$_{1A}$ receptor.

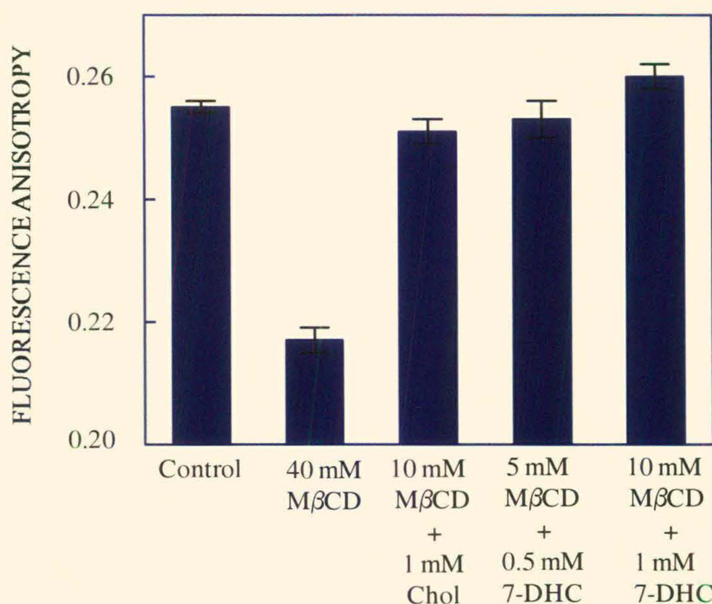


Figure 2.5. *Effect of replenishment of 7-DHC and cholesterol into cholesterol-depleted membranes on fluorescence anisotropy of the membrane probe DPH.* Cholesterol depletion was carried out using 40 mM MβCD. Fluorescence anisotropy experiments were performed with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (23 °C). The values represent the means ± SE of duplicate points from at least three independent experiments. See section 2.2 for other details.

The observed difference between cholesterol and 7-DHC, in terms of being able to restore the ligand binding activity (Fig. 2.4), could be due to an alteration in overall membrane organization (order). In order to monitor whether there is a change in overall membrane order when cholesterol-depleted membranes were replenished with cholesterol or 7-DHC, fluorescence anisotropy measurements were carried out with the membrane probe, DPH. 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 chains. Fig. 2.5 shows that the fluorescence anisotropy of DPH exhibits a significant reduction upon cholesterol depletion from native membranes. Interestingly, when cholesterol-depleted membranes were replenished with either cholesterol or 7-DHC, the fluorescence anisotropy was found to be similar to that of native (control) membranes. This indicates that the overall membrane order is unaltered upon replenishment. The requirement for restoration of ligand binding activity is therefore more stringent than the requirement for the recovery of overall membrane order.

It has earlier been reported that membrane cholesterol is required in maintaining ligand binding activity of the hippocampal serotonin$_{1A}$ receptor (Pucadyil and Chattopadhyay, 2006). In order to test the stringency of cholesterol requirement for the function of hippocampal serotonin$_{1A}$ receptors. In this work, cholesterol was replaced with 7-DHC, which differs with cholesterol in only difference one extra double bond at the 7th position. These results show that 7-DHC does not support the ligand binding activity of the hippocampal serotonin$_{1A}$ receptor. This is in spite of the fact that replenishment with 7-DHC restores overall membrane order to that of native membranes. It has previously been shown that membrane cholesterol oxidation leads to inhibition of the ligand binding activity of the hippocampal serotonin$_{1A}$ receptor without changing membrane order (Pucadyil *et al.*, 2005a). Taken together, these results indicate that the molecular basis for the requirement of membrane cholesterol in maintaining the ligand binding activity of serotonin$_{1A}$ receptors could be specific interaction, although global bilayer effects may not be ruled out. These results have potential implications in understanding the interaction of membrane lipids with this important neuronal receptor under SLOS-like condition in which 7-DHC accumulates due to mutations in the *DHCR7* gene.

Chapter 3

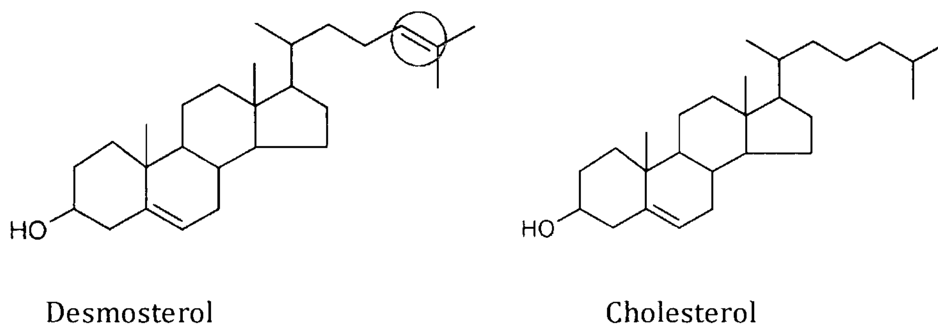
Differential effects of cholesterol and desmosterol on the ligand binding function of the hippocampal serotonin$_{1A}$ receptor: Implications in Desmosterolosis

3.1. Introduction

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function, and sorting as described in section 1.2. Cholesterol is the end product of the long and multistep sterol biosynthetic pathway. Konrad Bloch speculated that the sterol biosynthetic pathway parallels sterol evolution (the 'Bloch hypothesis'). According to the Bloch hypothesis, cholesterol has been selected over a very long time scale of natural evolution for its ability to optimize certain physical properties of eukaryotic cell membranes with regard to biological functions (Bloch, 1983). Defects in the cholesterol biosynthetic pathway have been identified with several inherited metabolic disorders such as desmosterolosis (Clayton *et al.*, 1996; FitzPatrick *et al.*, 1998; Fliesler *et al.*, 2000; Waterham *et al.*, 2001; Andersson *et al.*, 2002). Desmosterolosis is an autosomal, recessive congenital disease and is characterized by multiple anomalies. It is caused by mutations in 3β-hydroxy-steroid-Δ^{24}-reductase (DHCR24), an enzyme required in the final step of the Bloch pathway of cholesterol biosynthesis. Desmosterolosis is diagnosed with elevated levels of desmosterol and reduced levels of cholesterol in plasma, tissues and cells. The disease is characterized by distinct facial anomalies, underdeveloped genital organs and abnormalities in brain development and function, leading to serious developmental and neurological dysfunctions.

Desmosterol, an immediate biosynthetic precursor of cholesterol in the Bloch pathway of cholesterol biosynthesis, differs with cholesterol only in a double bond at the 24th position in the flexible alkyl side chain (see Fig. 3.1). Desmosterol is converted to cholesterol in the final step of the Bloch pathway by the enzyme DHCR24. Importantly, it has been recently demonstrated that *Dhcr24* gene knockout (*Dhcr24$^{-/-}$*) mice develop lethal dermopathy with differentiation

and maturation defects in the epidermis. *Dhcr24$^{-/-}$* mice were reported to be dead within a few hours after birth (Mirza *et al.*, 2006).



Desmosterol Cholesterol

Figure 3.1. *Chemical structures of desmosterol and cholesterol.* Desmosterol is the immediate precursor of cholesterol in the Bloch pathway, one of the two routes of cholesterol biosynthesis in humans. It differs with cholesterol only in a double bond at the 24th position in the flexible alkyl side chain (highlighted in its chemical structure). Elevated levels of desmosterol have been characterized as a diagnostic parameter of desmosterolosis. See section 1.2 for more details.

It has been shown earlier that membrane cholesterol is essential for ligand binding function of the hippocampal serotonin$_{1A}$ receptor (Pucadyil and Chattopadhyay, 2004b, 2006). In order to explore the stringency of cholesterol requirement for the ligand binding function of the hippocampal serotonin$_{1A}$ receptor, cholesterol was replaced with desmosterol. Alteration in the cholesterol content of neuronal membranes on membrane dynamics and protein/receptor function represents an important determinant in the analysis of neurogenesis and several neuropathologies.

3.2. Materials and methods

Materials

Desmosterol was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were obtained from the sources described in section 2.2.

Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described in section 2.2.

Radioligand binding assays

Receptor binding assays were carried out as described in section 2.2.

Cholesterol depletion of native membranes

Native hippocampal membranes were depleted of cholesterol using MβCD as described in section 2.2.

Desmosterol and cholesterol replenishment to cholesterol-depleted membranes

Cholesterol-depleted hippocampal membranes were replenished with cholesterol or desmosterol using either cholesterol-MβCD or desmosterol-MβCD complex which are soluble in water. The sterol-MβCD complex was prepared as described in section 2.2. The complex was prepared by dissolving the required amounts of desmosterol or cholesterol and MβCD in a ratio of 1:10 (mol/mol), respectively, in buffer C by constant shaking at 25 °C. Stock solutions (typically 2 mM desmosterol (or cholesterol):20 mM MβCD) of this complex were freshly prepared before each experiment. Desmosterol and cholesterol replenishment were carried out at a protein concentration of 2 mg/ml by incubating the cholesterol-depleted membranes with 1 mM desmosterol (or cholesterol):10 mM MβCD complex for 1 h in buffer C at 25 °C under constant shaking. Membranes were then spun down at 50,000 x g for 5 min at 4 °C, washed with buffer C and resuspended in the same buffer.

Estimation of desmosterol and cholesterol by thin layer chromatography

Total lipids were extracted from cholesterol-depleted and sterol-replenished membranes as described in section 2.2. Sterol contents of membranes under various treatment conditions were estimated by TLC. Precoated silica gel TLC plates were impregnated with 3% (w/v) silver nitrate solution in 97% methanol, allowed to dry briefly and activated at 120 °C for 15 min. Sterols were resolved with chloroform/diethyl ether (95:5, v/v) as the solvent system. In order to achieve maximum separation, TLC was run three times in the same solvent and the chromatogram was dried after each run. The separated lipids were visualized by charring with a solution containing cupric sulfate (10%, w/v) and phosphoric acid (8%, v/v) at 150 °C. Desmosterol and cholesterol bands were identified with the help of standards (Fig. 3.2A). The TLC plates were scanned and sterol band intensities were analyzed as described in section 2.2.

Estimation of phospholipids

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

Fluorescence anisotropy measurements

Fluorescence anisotropy experiments were carried out using the fluorescent probe DPH as described in section 2.2.

Statistical analysis

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

3.3. Results and discussion

Fig. 3.2B shows that the cholesterol content in hippocampal membranes exhibits ~70% reduction upon treatment with MβCD. This is accompanied by a corresponding reduction (~51%) in specific [^{3}H]8-OH-DPAT binding (see Fig. 3.3). Replenishment with cholesterol resulted in recovery of specific [^{3}H]8-OH-DPAT binding to ~70% of native membranes when ~57% of cholesterol could be replenished (Fig. 3.2B). In order to examine whether replenishment with desmosterol could restore specific ligand binding, cholesterol-depleted membranes were replenished with desmosterol. Interestingly, Fig. 3.3 shows that specific [^{3}H]8-OH-DPAT binding could not be restored significantly when replenishment was carried out with desmosterol. This is in spite of the fact that the extent of replenishment of desmosterol (~55%) was comparable to that obtained with cholesterol (Fig. 3.2B). This can be concluded that desmosterol is not capable in restoring specific ligand binding activity of the hippocampal serotonin$_{1A}$ receptor. It should be noted here that the total sterol content (desmosterol + cholesterol) appears to be comparable in cholesterol- and desmosterol-replenished membranes (Fig. 3.2B). Importantly, the phospholipid content remains unaltered under these conditions. The differences between cholesterol and desmosterol, in terms of the sterol's ability to restore specific ligand binding could be due to alteration in overall membrane organization (order).

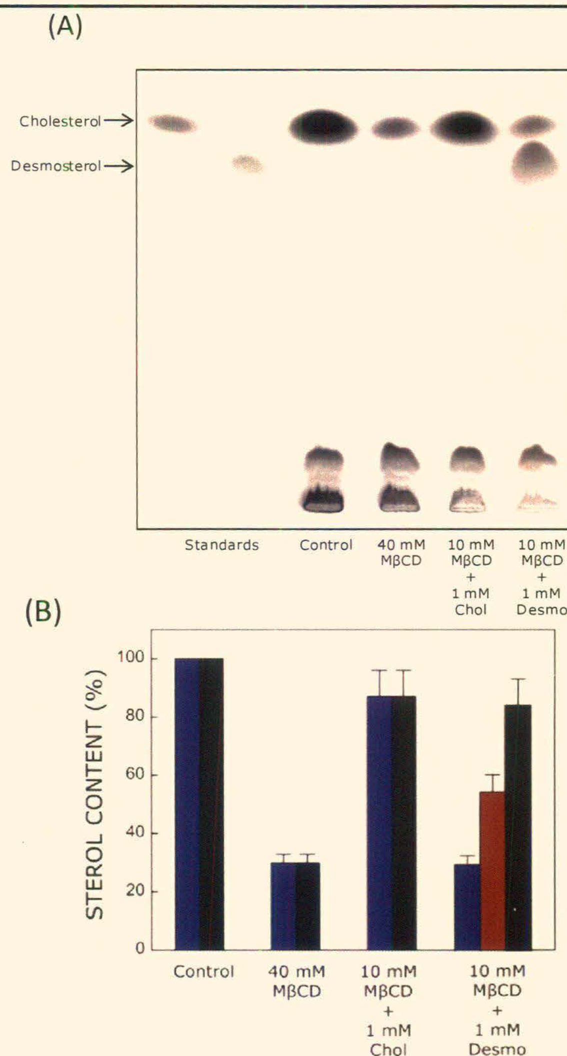


Figure 3.2. *Estimation of sterol content of native and cholesterol-depleted hippocampal membranes, and cholesterol-depleted membranes replenished with cholesterol or desmosterol.* Total lipids were extracted from membranes and sterols were separated using TLC as shown in (A). The chromatogram shows sterols from native membranes, membranes treated with MβCD, and cholesterol-depleted membranes upon replenishment with either cholesterol or desmosterol. The arrows represent positions of cholesterol and desmosterol on the thin layer chromatogram identified using standards in lanes 1 and 2, respectively. Cholesterol (blue bars), desmosterol (maroon bars) and total sterol (black bars) were quantified by densiometric analysis of the chromatogram and are shown in (B). Values are expressed as percentages of the cholesterol content of native membranes without any treatment and total sterol content of membranes were obtained by the addition of cholesterol and desmosterol contents. Data represent means ± SE of three independent experiments. See section 3.2 for more details.

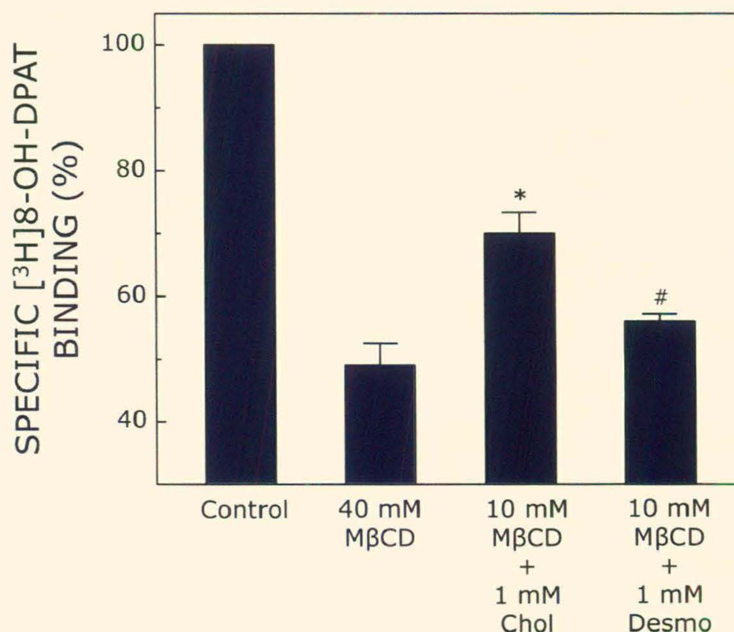


Figure 3.3. *Effect of replenishment of cholesterol and desmosterol into cholesterol-depleted membranes on specific binding of [^{3}H]8-OH-DPAT to the hippocampal serotonin$_{1A}$ receptor.* Native membranes were treated with MβCD and were replenished with either cholesterol or desmosterol. Values are expressed as percentages of specific binding obtained in native membranes. Data shown are means ± SE from five independent experiments. (*corresponds to significant (p = 0.001) and #represents not significant difference (p = 0.127) between specific [^{3}H]8-OH-DPAT binding of cholesterol- and desmosterol replenished membranes, respectively, in comparison to MβCD treated membranes (cholesterol-depleted membranes)). See section 3.2 for more details.

In order to monitor the overall membrane order, fluorescence anisotropy measurements were carried out with the membrane probe, diphenylhexatriene (DPH). DPH is a rod-like molecule and partitions into the interior of the bilayer. 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 acyl chains. Fig. 3.4 shows that the fluorescence anisotropy of DPH exhibits a significant reduction upon cholesterol depletion from native membranes. Interestingly, fluorescence anisotropy is restored to the same

extent upon cholesterol and desmosterol replenishment. This points out that the differential effect of sterols on specific ligand binding is not due to difference in membrane organization (order).

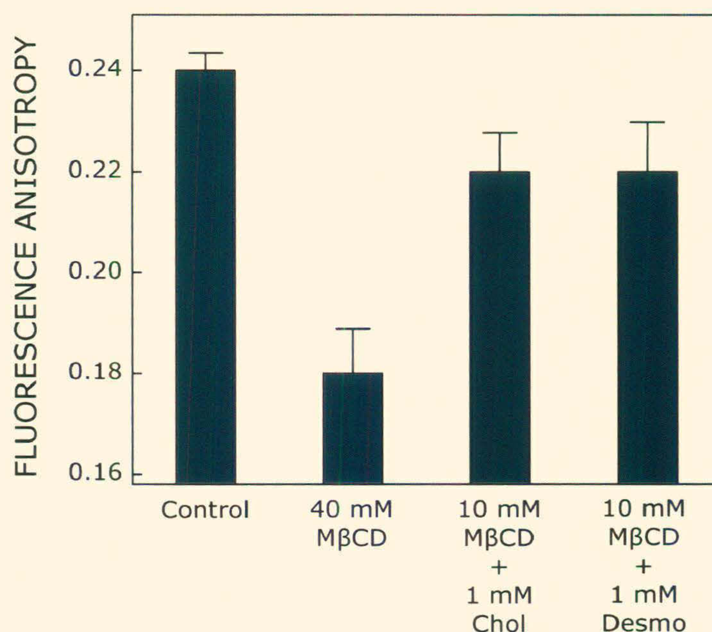


Figure 3.4. *Effect of replenishment of cholesterol and desmosterol to cholesterol-depleted membranes on fluorescence anisotropy of the membrane probe DPH.* Cholesterol depletion was achieved using MβCD. Fluorescence anisotropy experiments were performed with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (~23 °C). The values represent means ± SE of duplicate points from three independent experiments. See section 3.2 for more details.

The necessity of membrane cholesterol for ligand binding function of the hippocampal serotonin$_{1A}$ receptor has previously been established (Pucadyil and Chattopadhyay, 2004b; Paila *et al.*, 2005). Interestingly, serotonin$_{7a}$ receptors have subsequently been shown to exhibit cholesterol dependence of function (Sjögren *et al.*, 2006) (for a comprehensive list of GPCRs whose function depend on membrane cholesterol, see Table 1.1). In this work, cholesterol has been

Chapter 5

Organization and dynamics of hippocampal membranes in a depth-dependent manner by electron spin resonance spectroscopy

replaced with desmosterol to test the stringency of the requirement of membrane cholesterol in maintaining the function of the hippocampal serotonin$_{1A}$ receptor. Desmosterol differs with cholesterol only in a double bond at the 24th position. These results demonstrate that desmosterol is not capable of supporting the ligand binding function of the serotonin$_{1A}$ receptor. These results are interesting in the light of the fact that replenishment with cholesterol and desmosterol resulted in comparable overall membrane order. Moreover, earlier biophysical studies have shown that the effect of cholesterol and desmosterol on membrane organization and dynamics is similar (Shrivastava *et al.,* 2008; Huster *et al.*, 2005). This reinforces the fact that the requirement for ligand binding is more stringent than that of overall membrane organization. Interestingly, it has been reported that *Dhcr24* gene knockout (*Dhcr24$^{-/-}$*) mice (*i.e.*, mice with predominatly desmosterol and almost no cholesterol) develop a number of abnormalities from sterility (Wechsler *et al.*, 2003) to lethal dermopathy (Mirza *et al.*, 2006).

These results are reminiscent of observations of chapter 2, in which 7-dehydrocholesterol (7-DHC, an immediate biosynthetic precursor of cholesterol in the Kandutsch-Russell pathway, differing with cholesterol only in a double bond at the 7th position) does not support the function of the serotonin$_{1A}$ receptor (Chapter 2; Singh *et al.*, 2007; Paila *et al.*, 2008). This is in support of the proposition that cholesterol represents a molecule with high level of in-built stringency, fine tuned by millions of years of natural evolution for its ability to optimize physical properties of eukaryotic cell membranes in relation to biological functions (Bloch, 1983; Shrivastava *et al.,* 2008). The molecular mechanism underlying the effect of cholesterol on the structure and function of integral membrane proteins is not always clear (Pucadyil and Chattopadhyay, 2006; Burger *et al.*, 2000). It has been proposed that such effects could occur either due to a specific molecular interaction with membrane proteins leading to a

conformational change in the receptor (Gimpl *et al.*, 2002a,b), or due to alterations in the membrane physical properties induced by the presence of cholesterol (Ohvo-Rekila *et al.*, 2002; Lee, 2004), or due to a combination of both factors. It has previously been reported that oxidation of membrane cholesterol results in inhibition of the ligand binding function of the hippocampal serotonin$_{1A}$ receptor without any alteration in overall membrane order (Pucadyil *et al.*, 2005b). In addition, it has recently been shown that 7-DHC, an immediate biosynthetic precursor of cholesterol with an extra double bond, does not support the function of the serotonin$_{1A}$ receptor, even when there is no change in overall membrane order (Chapter 2; Singh *et al.*, 2007; Paila *et al.*, 2008). Taken together, these results show that the requirement of membrane cholesterol in maintaining the ligand binding function of the serotonin$_{1A}$ receptors could be attributed to specific interaction, although global bilayer effects may not be completely ruled out (Prasad *et al.*, 2009).

It has recently been proposed that membrane cholesterol could occupy 'nonannular' binding sites in GPCRs (Paila *et al.*, 2009). Nonannular sites are characterized by lack of accessibility to the annular lipids, *i.e.*, these sites cannot be displaced by competition with annular lipids (Simmonds *et al.*, 1982). The binding to the nonannular sites is believed to be more specific compared to annular binding sites (Lee, 2003). The possible locations for the nonannular sites has been postulated to be either inter or intramolecular (interhelical) protein interfaces, characterized as deep clefts (or cavities) on the protein surface (Simmonds *et al.*, 1982; Marius *et al.*, 2008). It is therefore possible that the inability of desmosterol to support the ligand binding activity of the serotonin$_{1A}$ receptor could be due to its relative inability (in comparison to cholesterol) to be accommodated at the nonannular sites in native hippocampal membranes. It is not possible at this point in time to predict the relative ease of accommodating

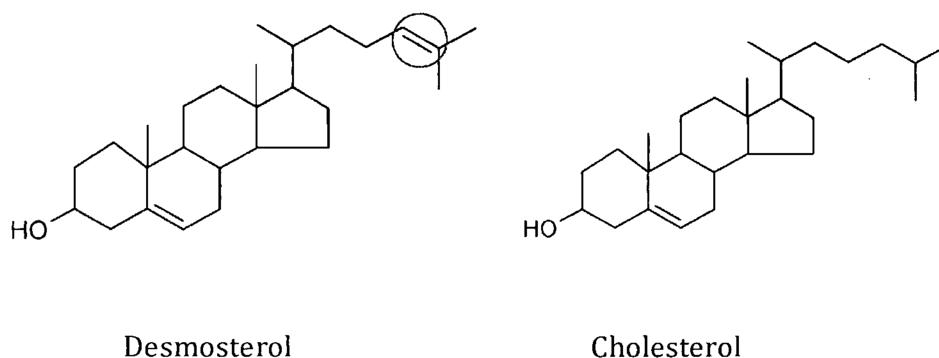
closely related sterols to the nonannular binding sites of membrane receptors. A combination of x-ray crystallographic structures and molecular modeling could provide some useful insight in addressing the issue of lipid shape and binding to nonannular sites. In summary, these results have potential implications in understanding the interaction of membrane cholesterol with the serotonin$_{1A}$ receptor, in general and under desmosterolosis-like condition in particular.

Chapter 4

Desmosterol replaces cholesterol for ligand binding function of the $serotonin_{1A}$ receptor in solubilized hippocampal membranes

4.1. Introduction

Cholesterol is an essential and representative lipid in higher eukaryotic cellular membranes and is crucial in membrane organization, dynamics, function, and sorting as described in section 1.2. Cholesterol is the end product of a long, multi-step and exceedingly fine-tuned sterol biosynthetic pathway that parallels sterol evolution (Bloch, 1983, 1994). Konrad Bloch speculated that the sterol biosynthetic pathway parallels sterol evolution (the 'Bloch hypothesis'). According to this hypothesis, cholesterol has been selected over a very long time scale of natural evolution for its ability to optimize certain physical properties of eukaryotic cell membranes with regard to biological functions.



Desmosterol Cholesterol

Figure 4.1. *Chemical structures of desmosterol and cholesterol.* Desmosterol is the immediate precursor of cholesterol in the Bloch pathway, one of the two routes of cholesterol biosynthesis. It differs with cholesterol only in a double bond at the 24th position in the flexible alkyl side chain (highlighted in its chemical structure). See section 1.2 for more details.

Desmosterol is the immediate precursor of cholesterol in the Bloch pathway of cholesterol biosynthesis and differs with cholesterol only in a double bond at the 24th position in its flexible alkyl side chain (see Fig. 4.1). Desmosterol is converted to cholesterol in the final step of the Bloch pathway by the enzyme

3β-hydroxy-steroid-Δ^{24}-reductase (DHCR24). Interestingly, it has been previously reported that *Dhcr24* gene knockout (*Dhcr24*$^{-/-}$) mice, which contain desmosterol instead of cholesterol, are viable and exhibit a mild phenotype (Wechsler *et al.*, 2003).

The serotonin$_{1A}$ receptor is an important member of the GPCR family and is implicated in the generation and modulation of various cognitive, behavioral and developmental functions. As a result, the serotonin$_{1A}$ receptor serves as an important target in the development of therapeutic agents for neuropsychiatric disorders such as anxiety and depression. In view of the increasing pharmacological relevance of the serotonin$_{1A}$ receptor, a transmembrane protein, its interaction with the surrounding membrane lipids assumes significance in modulating the function of the receptor in healthy and diseased states (Paila *et al.*, 2008). In this context, previous work from our laboratory comprehensively demonstrated the requirement of membrane cholesterol in the function of the serotonin$_{1A}$ receptor (Paila *et al.*, 2008; Pucadyil and Chattopadhyay, 2004b; Shrivastava *et al.*, 2010; reviewed in (Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2010)).

Lipid-protein interactions can be suitably monitored if the membrane protein in question is purified. An essential criterion for purification of an integral membrane protein is that the protein must be carefully removed from the native membrane and dispersed individually in solution. This is most effectively accomplished using amphiphilic detergents and the process is known as solubilization (Kalipatnapu and Chattopadhyay, 2005; Seddon *et al.*, 2004). Solubilization of membrane proteins is a process in which the proteins and lipids that are held together in native membranes, are suitably dissociated in a buffered detergent solution. The controlled dissociation of the membrane results in the formation of small protein and lipid clusters that remain dissolved in the aqueous

solution. The serotonin$_{1A}$ receptor was earlier partially purified by solubilizing the hippocampal membranes in a functionally active form (Chattopadhyay *et al.*, 2002) using CHAPS, a mild non-denaturing and zwitterionic detergent. Solubilized membranes represent serotonin$_{1A}$ receptor in relatively purified (enriched) form after loss of other proteins and lipids. In the absence of purified receptors (*none of the G-protein coupled serotonin receptor has been purified from natural sources yet*), functionally active solubilized receptors represent a suitable system to monitor lipid-protein interactions. Solubilization often leads to delipidation, *i.e.*, loss of membrane lipids. This results in considerable loss of activity of the solubilized protein or receptor, since lipid-protein interactions play a crucial role in maintaining the structure and function of integral membrane proteins and receptors (Lee, 2004). Interestingly, it has been previously shown that solubilization of the serotonin$_{1A}$ receptor by CHAPS leads to reduction in membrane cholesterol and ligand binding function (Banerjee *et al.*, 1995; Chattopadhyay *et al.*, 2005). Importantly, ligand binding function of the serotonin$_{1A}$ receptor could be restored upon replenishment of solubilized membranes with cholesterol (Chattopadhyay *et al.*, 2005). In order to examine the stringency of sterol structure necessary for ligand binding function of serotonin$_{1A}$ receptors, ligand binding function of the solubilized hippocampal serotonin$_{1A}$ receptor was analyzed upon desmosterol-replenishment.

4.2. Materials and methods

Materials

Desmosterol, CHAPS and PEG were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were obtained from the sources described in section 2.2.

Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described in section 2.2.

Solubilization of native membranes

Native hippocampal membranes were solubilized as described earlier using CHAPS (Chattopadhyay *et al.*, 2002, 2007). CHAPS-solubilized membrane was precipitated using PEG in order to remove NaCl from the solubilized extract, since agonist binding of the serotonin$_{1A}$ receptor is inhibited by NaCl (Harikumar and Chattopadhyay, 1998). This procedure is also believed to remove detergent. The PEG-precipitated CHAPS-solubilized membrane (referred to as solubilized membranes in this chapter) was suspended in buffer C and used immediately either for radioligand binding assays or for sterol replenishment.

Desmosterol and cholesterol replenishment of solubilized membranes

Solubilized membranes were replenished with desmosterol or cholesterol using water soluble desmosterol-MβCD or cholesterol-MβCD complex as described in section 3.2.

Radioligand binding assays

Receptor binding assays were carried out as described in section 2.2.

Estimation of desmosterol and cholesterol by thin layer chromatography

Estimation of desmosterol and cholesterol from native and solubilized membranes, and cholesterol/desmosterol-replenished solubilized membranes by thin layer chromatography as described in section 3.2.

Estimation of phospholipids

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

Fluorescence anisotropy measurements

Fluorescence anisotropy measurements were carried out as described in section 2.2. All experiments were done with multiple sets of samples and average values of fluorescence anisotropy are shown in Fig. 4.5.

Statistical analysis

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

4.3. Results and discussion

Effective solubilization and purification of a membrane protein in a functionally active form represent important steps in understanding the structure-function relationship of a given protein (Kalipatnapu and Chattopadhyay, 2005a). However, solubilization of a membrane protein with retention of activity poses a formidable challenge since many detergents irreversibly denature membrane proteins (Garavito and Ferguson-Miller, 2001). It should be mentioned here that the conditions used by us for solubilization of the serotonin$_{1A}$ receptor are fine-tuned and highly optimized. For example, it is known that treatment of membranes with high concentration of CHAPS results in dissociation and depletion of βγ dimer of trimeric G-proteins (Jones and Garrison, 1999; Waldhoer *et al.*, 1999; Bayewitch *et al.*, 2000). Use of CHAPS at high concentration may

therefore be detrimental for solubilizing G-protein coupled receptors in a functionally active form. Keeping this in mind, an efficient strategy was devised by using CHAPS at a low (pre-micellar) concentration in presence of NaCl followed by PEG precipitation. The advantage of using low concentration of CHAPS is that the receptor-G protein coupling remains unperturbed. In addition, PEG precipitation helps in efficiently removing detergent and salt from solubilized membranes (Kremenetzky and Atlas, 1984; Aguilar and Ochoa, 1986; Aguilar *et al.*, 1987; Medrano *et al.*, 1989; Gal *et al.*, 1983). Taken together, hippocampal membranes solubilized this way represent one of the best membrane system available for exploring lipid-protein interactions.

Solubilization of native membranes results in loss of cholesterol and treatment with MβCD-sterol complex is an effective approach to replenish membranes with sterol (Singh *et al.*, 2007). Fig. 4.2 shows the chromatogram displaying sterol contents in native (control), solubilized and solubilized membranes replenished with either desmosterol or cholesterol. Cholesterol content in native hippocampal membranes exhibits ~18% reduction upon solubilization (see Fig. 4.3). This is accompanied by a corresponding reduction (~36%) in specific [^{3}H]8-OH-DPAT binding to the serotonin$_{1A}$ receptor (see Fig. 4.4). Subsequent treatment with MβCD-cholesterol complex increased the cholesterol content to ~114% of control membranes (Fig. 4.3). This resulted in recovery (~99% of native membranes) of specific [^{3}H]8-OH-DPAT binding (Fig. 4.4). In order to examine whether replenishment with desmosterol could restore specific [^{3}H]8-OH-DPAT binding, solubilized membranes were treated with MβCD-desmosterol complex. Fig. 4.3 shows that the extent of replenishment of desmosterol was comparable such that the total sterol (cholesterol + desmosterol) content of desmosterol-replenished membranes was ~122%.

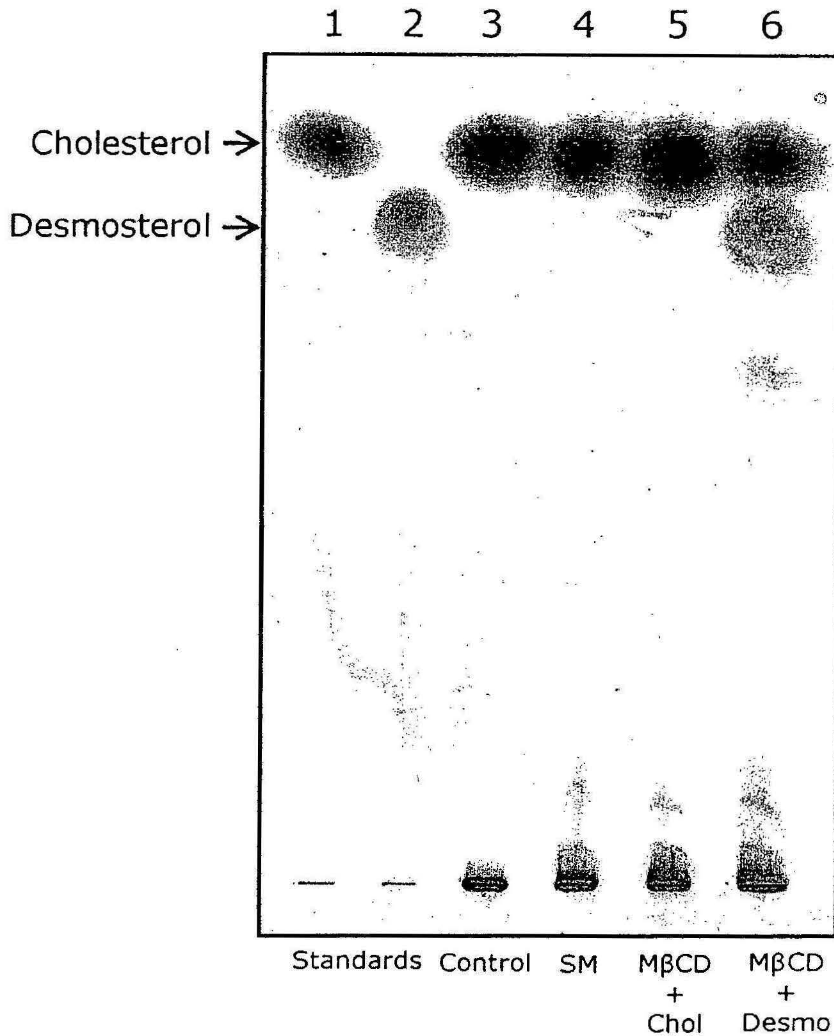


Figure 4.2. *Separation of sterol content of native and PEG-precipitated CHAPS-solubilized hippocampal membranes (solubilized membranes, SM), and cholesterol/desmosterol-replenished solubilized membranes.* Total lipids were extracted from membranes and sterols were separated using TLC. The chromatogram shows sterols from native membranes (lane 3), solubilized membranes (lane 4), and solubilized membranes replenished with either cholesterol (lane 5) or desmosterol (lane 6). The arrows represent positions of cholesterol and desmosterol on the thin layer chromatogram identified using standards in lanes 1 and 2, respectively. See sections 2.2 and 4.2 for more details.

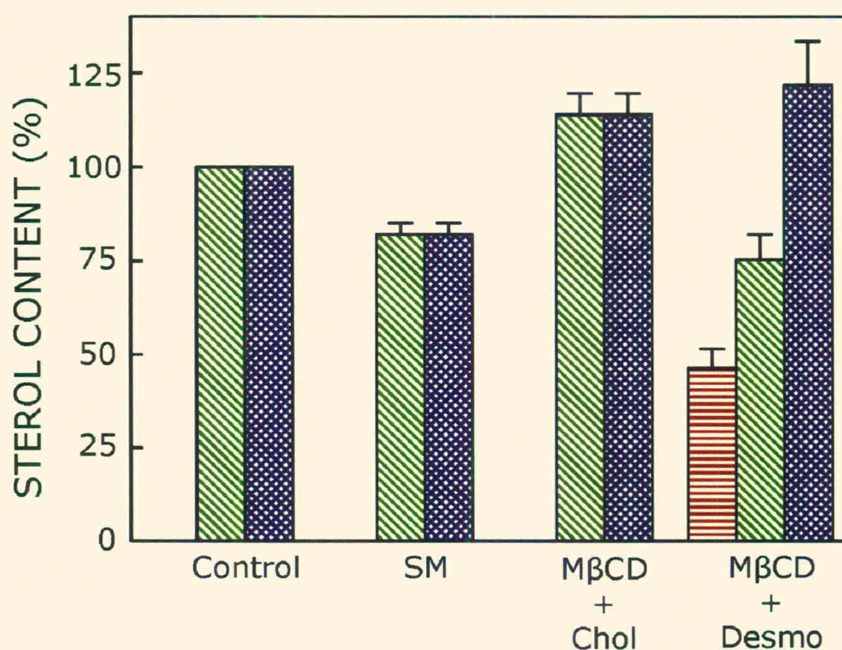


Figure 4.3. *Estimation of sterol content of native and PEG-precipitated CHAPS-solubilized hippocampal membranes (solubilized membranes, SM), and cholesterol/desmosterol-replenished solubilized membranes.* Cholesterol (hatched bar), desmosterol (horizontal lined bar) and total sterol (crisscrossed bar) were quantified by densiometric analysis of the chromatogram. Values are expressed as percentages of the cholesterol content of native membranes without any treatment and total sterol content of membranes was obtained by the addition of cholesterol and desmosterol contents. Data represent means ± SE of three independent experiments. See sections 2.2 and 4.2 for more details.

The phospholipid content was found to be unaltered under sterol replenishment conditions. Interestingly, Fig. 4.4 shows that specific [^{3}H]8-OH-DPAT binding was completely restored (to ~100% of native membranes), when replenishment was carried out with desmosterol. These results therefore demonstrate that *desmosterol can replace cholesterol for specific ligand binding function of the hippocampal serotonin$_{1A}$ receptor in solubilized membranes.* These results could have important implications in the overall context of sterol binding sites in the serotonin$_{1A}$ receptor (see later).

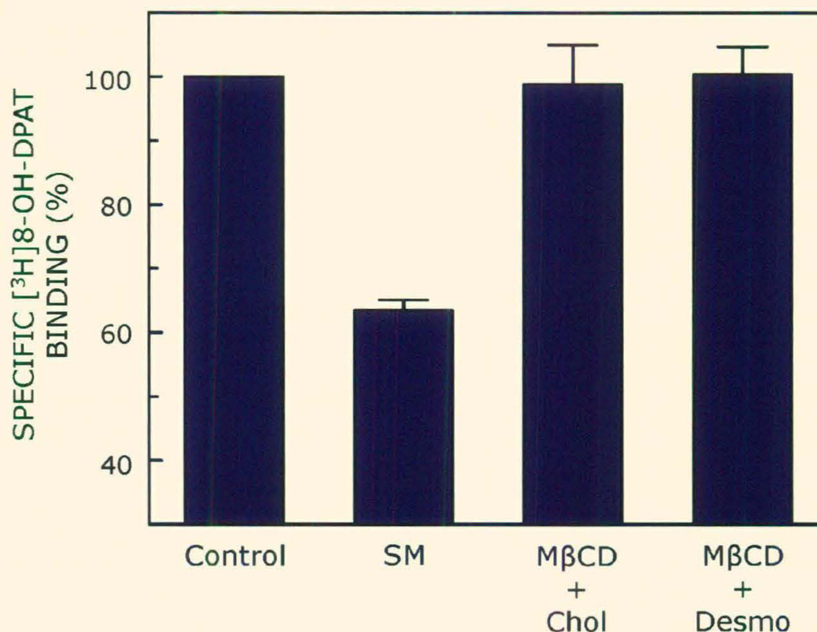


Figure 4.4. *Effect of replenishment of desmosterol or cholesterol into solubilized membranes on specific binding of [^{3}H]8-OH-DPAT to the hippocampal serotonin$_{1A}$ receptor.* Solubilized membranes were replenished with desmosterol or cholesterol, using 1 mM desmosterol (or cholesterol):10 mM MβCD complex. Values are expressed as percentages of specific binding obtained in native membranes. Data shown are means ± SE from at least five independent experiments. See sections 2.2 and 4.2 for more details.

In order to monitor the overall membrane order under these conditions, fluorescence anisotropy measurements were carried out with the membrane probe DPH. DPH is a rod-like molecule and partitions into the interior of the bilayer. This partitioning has previously been shown to be independent of the phase state of the membrane (London and Feigenson, 1981). Fluorescence anisotropy is correlated to the rotational diffusion of membrane embedded probes such as DPH (Lakowicz, 2006), which is sensitive to the packing of lipid acyl chains. Fig. 4.5 shows that the fluorescence anisotropy of DPH does not exhibit a significant change in solubilized and desmosterol- or cholesterol-replenished

solubilized hippocampal membranes. The relative invariance of the fluorescence anisotropy of DPH could be due to minor changes in sterol/phospholipid ratio under these conditions (Garda and Brenner, 1985). This points out that the overall membrane order during solubilization and upon sterol replenishment remains invariant within experimental error. The function of a growing number of GPCRs depends on membrane cholesterol (a comprehensive list is provided in Table 1.1).

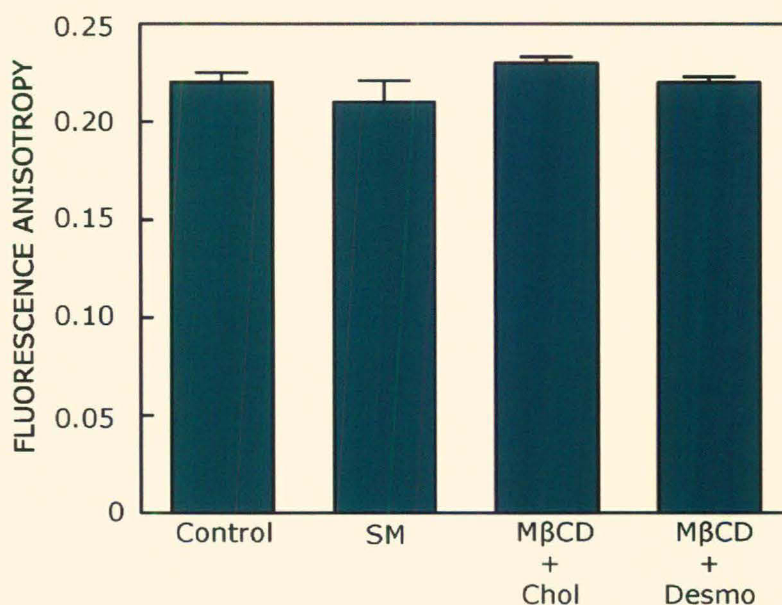


Figure 4.5. *Effect of replenishment of cholesterol and desmosterol into solubilized membranes on steady state fluorescence anisotropy of the membrane probe DPH.* Solubilized membranes were replenished with desmosterol or cholesterol, using 1 mM desmosterol (or cholesterol):10 mM MβCD complex. Fluorescence anisotropy measurements were performed with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (~23 °C). Values represent means ± SE of duplicate points from three independent experiments. See sections 2.2 and 4.2 for more details.

Earlier work from our laboratory demonstrated the necessity of membrane cholesterol in maintaining the ligand binding function of the hippocampal serotonin$_{1A}$ receptor (Pucadyil and Chattopadhyay, 2004b, 2005;

Paila *et al.*, 2005). In the present work, cholesterol has been replaced with desmosterol to test the stringency of the requirement of membrane cholesterol in maintaining the function of the *solubilized* hippocampal serotonin$_{1A}$ receptor. As mentioned earlier, desmosterol differs with cholesterol only in a double bond at the 24th position in the flexible acyl side chain. These results demonstrate that desmosterol is capable of supporting the ligand binding function of the solubilized serotonin$_{1A}$ receptor.

Previous biophysical measurements have shown that the effects of cholesterol and desmosterol on membrane organization and dynamics are similar (Shrivastava *et al.*, 2008; Huster *et al.*, 2005; Simonsen *et al.*, 2009). Desmosterol has previously been shown to substitute for cholesterol in mutant mouse L-cell fibroblasts (Rothblat *et al.*, 1970). In addition, it has been shown that in absence of cholesterol, desmosterol alone can support cell proliferation in a murine macrophage-like cells (Rodríguez-Acebes *et al.*, 2009). More importantly, results from studies using *Dhcr24* gene knockout (*Dhcr24*$^{-/-}$) mice (Wechsler *et al.*, 2003) or cells derived from them (Lu *et al.*, 2006; Heverin *et al.*, 2007) show that desmosterol can act as a suitable replacement for cholesterol. For example, the *Dhcr24* gene knockout mice are viable and exhibit a mild phenotype although they are smaller in size and are sterile (Wechsler *et al.*, 2003). However, certain degree of caution should be exercised while interpreting these results. The *Dhcr24*$^{-/-}$ mice are not totally cholesterol-free (strictly speaking, these are low cholesterol, high desmosterol *Dhcr24*$^{-/-}$ knockout mice) due to availability of maternal cholesterol in mice during embryogenesis (Wechsler *et al.*, 2003) since maternal cholesterol can cross the placenta in rodents (Brown, 2004). These results showing that desmosterol is capable of supporting the ligand binding function of the solubilized serotonin$_{1A}$ receptor is in overall agreement with the above reports.

Interestingly, it has previously been shown that desmosterol is not capable of supporting the ligand binding function of the hippocampal serotonin$_{1A}$ receptor *when sterol (cholesterol) depletion and replenishment are carried out using MβCD and MβCD-sterol complex, respectively* (Chapter 3; Singh *et al.*, 2009). The present results appear to be contradictory to earlier results. An analysis of the reason for this apparent discrepancy could provide novel information about the nature of cholesterol binding sites around the serotonin$_{1A}$ receptor. The key difference in these instances is the way sterol manipulations were carried out in the membrane. While in the earlier work, MβCD was used for such sterol manipulations (Chapter 3; Singh *et al.*, 2009), we employed the strategy of solubilization and effectively utilized the lipid loss associated with solubilization (Banerjee *et al.*, 1995; Chattopadhyay *et al.*, 2005) for achieving sterol depletion in this work. It has recently been proposed that cholesterol binding sites in GPCRs could represent nonannular binding sites (Paila *et al.*, 2009). Integral membrane proteins are surrounded by a shell or annulus of lipid molecules, which mimics the immediate layer of solvent surrounding soluble proteins (Lee, 2003). These are termed 'annular' lipids surrounding the membrane protein. The annular lipids are exchangeable with bulk lipids (Devaux and Seigneuret, 1985), although this exchange rate is slower compared to exchange rate between bulk lipids. In addition to the annular lipids, there is evidence for other lipid molecules in the immediate vicinity of integral membrane proteins. These are termed as 'nonannular' lipids (see Fig. 4.6a). Cholesterol has previously been proposed to be localized in nonannular sites in Ca^{2+}/Mg^{2+}-ATPase (Simmonds *et al.*, 1982), the nicotinic acetylcholine receptor (Jones and McNamee, 1988), and anionic phospholipids have been reported to occupy nonannular sites in the potassium channel KcsA from *S. lividans* (Marius *et al.*, 2008) and gap junction hemichannel connexin 26 (Hung and Yarovsky, 2011).

Nonannular sites are characterized by lack of accessibility to the annular lipids, *i.e.*, these sites cannot be displaced by competition with annular lipids. Binding to the nonannular sites is considered to be more specific compared to annular binding sites (Lee, 2003). As a result, nonannular lipid binding sites remain vacant even in the presence of annular lipids around the protein (Marius *et al.*, 2008). It has been suggested that the possible locations for the nonannular sites could be either inter or intramolecular (interhelical) protein interfaces, characterized as deep clefts (or cavities) on the protein surface (Simmonds *et al.*, 1982; Marius *et al.*, 2008).

Nonannular lipids are often believed to be preserved in the high resolution crystal structure of membrane proteins, *i.e.*, they survive the crystallization conditions (Lee, 2003, 2011). Lipid molecules resolved in high resolution crystal structures of membrane proteins are therefore likely to be nonannular lipids (Lee, 2004). It has recently been suggested, based on the reported crystal structure of the β_2-adrenergic receptor (Cherezov *et al.*, 2007; Hanson *et al.*, 2008), that cholesterol molecules located at the interhelical/interprotein regions of the receptor could represent nonannular lipids (Paila *et al.*, 2009). Based on the high degree of sequence similarity (~48%) between transmembrane regions of the serotonin$_{1A}$ receptor and β_2-adrenergic receptor (Paila *et al.*, 2011), it is proposed that the cholesterol-dependent activity of the serotonin$_{1A}$ receptor is partly due to the presence of tightly bound cholesterol molecules at the nonannular site(s). In fact, it has recently been reported from receptor modeling studies that the serotonin$_{1A}$ receptor is more compact in the presence of tightly bound cholesterol (Paila *et al.*, 2011).

In addition, cholesterol recognition/interaction amino acid consensus (CRAC) motifs in the serotonin$_{1A}$ receptor has recently identified (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). As mentioned above, it has been previously postulated that the possible locations for the nonannular sites could be either inter or intramolecular (interhelical) protein interfaces, characterized as deep clefts (or cavities) on the protein surface (Simmonds *et al.*, 1982; Jones and McNamee, 1988). Interestingly, it has been suggested that cholesterol binding by CRAC motif is induced by a similar cleft located at the membrane interfacial region (Jamin *et al.*, 2005). Taken together, nonannular cholesterol appears to be crucial for maintaining the function of the serotonin$_{1A}$ receptor. The difference in results obtained in the above two cases lies in the ability of desmosterol to occupy nonannular sites when added as MβCD-desmosterol complex following solubilization since solubilization allows a more robust reorganization of membranes (see Fig. 4.6b). This is because membrane lipids and proteins are loosely packed in solubilized membranes compared to MβCD-treated native membranes. Solubilized membranes are loose, metastable and are composed of heterogeneous complexes of detergent, lipid and protein, forming mixed micelles (Kalipatnapu and Chattopadhyay, 2005a). Such loose packing of solubilized membranes was previously attributed to enhanced susceptibility of the serotonin$_{1A}$ receptor to ethanol (Harikumar and Chattopadhyay, 1998). On the other hand, MβCD is known to preferentially deplete membrane cholesterol from regions of the membrane where cholesterol is present in relatively fluid (liquid disordered) phase and not from the ordered cholesterol-rich domains (Ilangumaran and Hoessli, 1998; Beseničar *et al.*, 2008; Sanchez *et al.*, 2011). Membrane reorganization as a result of such cholesterol depletion by MβCD therefore may not be sufficient (robust) to replace the nonannular sites with desmosterol, a necessary step for recovery of activity.

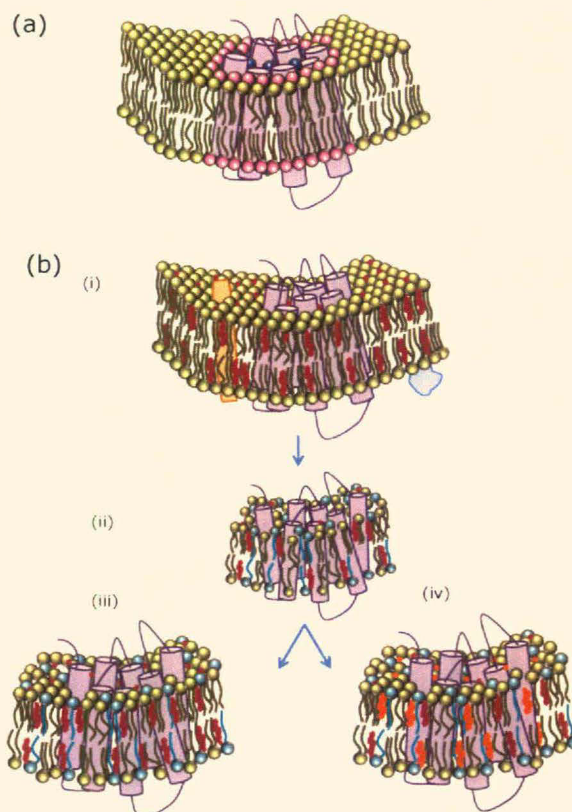


Figure 4.6. (a) *A schematic representation of a membrane embedded seven transmembrane domain receptor showing various classes of lipids in the vicinity of the receptor.* Annular lipids (shown in pink) represent the shell (or annulus) of lipid molecules around the receptor. Nonannular lipids (shown in blue) are characterized by lack of accessibility to annular lipids. Bulk lipids are shown in green. *(b) A schematic representation depicting (i) native membranes, (ii) solubilized membranes, (iii) cholesterol-replenished solubilized membranes, and (iv) desmosterol-replenished solubilized membranes.* Native membranes in (i) show phospholipids (green), cholesterol (maroon), the serotonin$_{1A}$ receptor (purple), other integral (light brown) and peripheral (light blue) membrane proteins. (ii) Solubilization of native membranes with the zwitterionic detergent CHAPS (single tailed molecule in cyan), results in loss of phospholipids, and integral and peripheral membrane proteins, leading to partial purification of serotonin$_{1A}$ receptors. Solubilization also causes loss of cholesterol bound to the serotonin$_{1A}$ receptor and appears to affect the 'nonannular' binding sites for cholesterol. Replenishment of solubilized membranes with (iii) cholesterol and (iv) desmosterol (shown in orange) appears to restore nonannular lipid sites resulting in recovery of specific ligand binding to the serotonin$_{1A}$ receptor. Note that the membrane components are loosely packed in the solubilized membrane. See section 4.3 for more details.

Solubilization and subsequent sterol replenishment may just allow this important step, thereby resulting in recovery of activity (Fig. 4.4).

In this work, it has been shown that desmosterol is capable of supporting the ligand binding function of the solubilized serotonin$_{1A}$ receptor, provided sterol manipulation is carried out on solubilized membranes. These results are consistent with previous reports, spanning biophysical studies using model membranes (Shrivastava *et al.*, 2008; Huster *et al.*, 2005; Simonsen *et al.*, 2009) to animal models (Wechsler *et al.*, 2003; Lu *et al.*, 2006; Heverin *et al.*, 2007), showing that desmosterol can replace cholesterol in a large number of cases. It is further concluded that caution should be exercised while interpreting results from sterol replacement experiments, in view of the complexities involved.

5.1. Introduction

Biological membranes are complex non-covalent assemblies of a diverse variety of lipids and proteins that allow cellular compartmentalization, thereby imparting an identity to the cell. The lipid composition of cells that makes up the nervous system is unique and has been correlated with increased complexity in the organization of the nervous system during evolution (Sastry, 1985). The nervous system characteristically contains a very high concentration of lipids, and displays remarkable lipid diversity (Wenk, 2005). Cholesterol is an important lipid in this context since it is known to regulate the function of neuronal receptors (Allen *et al.*, 2007; Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2010), thereby affecting neurotransmission and giving rise to mood and anxiety disorders (Papakostas *et al.*, 2004). Cholesterol is often found distributed non-randomly in domains in biological and model membranes as discussed in section 1.2. The idea of such specialized membrane domains assumes significance in cell biology since physiologically important functions such as membrane sorting and trafficking, signal transduction processes, and the entry of pathogens have been attributed to these domains (Simons and Toomre, 2000; Pucadyil and Chattopadhyay, 2007). Interestingly, a number of neurological diseases share a common etiology of defective cholesterol metabolism in the brain (Porter and Herman, 2011), yet the organization and dynamics of neuronal membranes as a consequence of alterations in membrane cholesterol is poorly understood (Chattopadhyay and Paila, 2007; Beasley *et al.*, 2005; Korade and Kenworthy, 2008).

Native hippocampal membranes have been established as a convenient natural source for exploring the interaction of the serotonin$_{1A}$ receptor with membrane lipids (Harikumar and Chattopadhyay, 1998; Pucadyil and Chattopadhyay, 2004b). Interestingly, requirement of membrane cholesterol in modulating ligand binding function of the serotonin$_{1A}$ receptor has been

established utilizing a variety of approaches (Pucadyil and Chattopadhyay, 2004b, 2006; Paila and Chattopadhyay, 2010; Paila *et al.*, 2008; Chapter 3; Singh *et al.*, 2009; Shrivastava *et al.*, 2010). In order to correlate these cholesterol-dependent functional changes with alterations in membrane organization and dynamics, fluorescence-based approaches were employed previously (Mukherjee and Chattopadhyay, 2005; Mukherjee *et al.*, 2006; Saxena *et al.*, 2008). However, fluorescence spectroscopic approaches can only provide information in a relatively fast (~ns) time scale. A comprehensive understanding of the organization and dynamics of biological membranes requires a wide range of spatiotemporal scales (Jacobson *et al.*, 2007; Ganguly and Chattopadhyay, 2010). In this work, organization and dynamics of hippocampal membranes was explored upon modulating cholesterol and protein content, utilizing approaches based on electron spin resonance (ESR). ESR provides information in a relatively slow time scale and therefore would involve more averaging (compared to fluorescence spectroscopy), which could be crucial in a slow diffusing system such as natural membranes of neuronal origin (Nakada *et al.*, 2003; Takamori *et al.*, 2006). In addition, since the membrane is considered to be a two-dimensional anisotropic asymmetric fluid, any possible change in membrane organization and dynamics may not be uniform and restricted to a unique location in the membrane. For example, it has previously been shown that stress such as heat shock can induce anisotropic changes in membrane organization, *i.e.*, the change in membrane organization is different when monitored in different positions (depths) in adult rat liver cell plasma membranes (Revathi *et al.*, 1994).

The organization and dynamics of hippocampal membranes were explored upon modulating their cholesterol and protein content utilizing depth-specific spin-labeled phospholipids, 5- and 14-PC (see Fig. 5.1). 5- and 14-PC contain the stable doxyl nitroxide spin label on the 5th and 14th carbon atoms of the *sn*-2 acyl chain of the phospholipid, respectively. While 5-PC provides

information on the order and dynamics at the membrane interface, 14-PC reports on the mobility and dynamics near the more isotropic center of the bilayer (Chattopadhyay and London, 1987; Abrams and London, 1992). The results obtained indicate that both cholesterol and proteins modulate hippocampal membrane dynamics. While cholesterol increases membrane order, membrane proteins appear to increase membrane lipid dynamics by disturbing the membrane order.

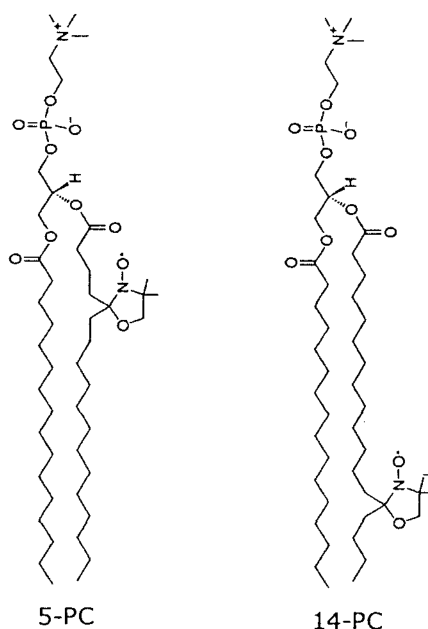


Figure 5.1. *Chemical structure of spin labeled phospholipids.*

5.2. Materials and methods

Materials

Amplex Red cholesterol assay kit was from Molecular Probes (Eugene, OR). 1-Palmitoyl-2-(5-doxyl) stearoyl-*sn*-glycero-3-phosphocholine (5-PC), and 1-palmitoyl-2-(14-doxyl) stearoyl-*sn*-glycero-3-phosphocholine (14-PC), were

purchased from Avanti Polar Lipids (Alabaster, AL). All other chemicals used were from the sources described in section 2.2.

Preparation of native hippocampal membranes

Native hippocampal membranes were prepared from frozen hippocampal tissue as described in section 2.2.

Cholesterol depletion of native membranes

Native hippocampal membranes were depleted of cholesterol using MβCD as described previously (Pucadyil and Chattopadhyay, 2004b). Briefly, membranes with a total protein concentration of ~2 mg/ml were treated with different concentrations of MβCD in 50 mM Tris buffer (pH 7.4) at 25 °C in a temperature controlled water bath with constant shaking for 1 h. Membranes were then spun down at 50,000×*g* for 5 min, washed once with Tris buffer and resuspended in the same buffer. Cholesterol was estimated using the Amplex Red cholesterol assay kit (Amundson and Zhou, 1999).

Lipid extraction from native and cholesterol-depleted membranes

Lipid extraction was carried out according to the method of Bligh and Dyer (Bligh and Dyer, 1959) from native hippocampal membranes with some modifications. In order to yield efficient extraction of total lipids, hippocampal membranes were successively treated with varying ratios of methanol-chloroform (2:1, 1:1, and 1:2, v/v). Subsequently, water-chloroform (1:1, v/v) was added and organic and aqueous phases were separated upon centrifuging the samples at low speed. In order to retrieve total lipids, the lower layer of organic phase was isolated and dried off under nitrogen at ~45 °C. After further drying under a high vacuum for 6 h, the lipid extract was dissolved in a mixture of chloroform-methanol (1:1, v/v).

Estimation of phospholipids

Concentration of lipid phosphate was determined as described in section 2.2. The phospholipid content of native membranes is typically ~960 nmol/mg of total protein (Pucadyil and Chattopadhyay, 2004a).

Sample preparation

Spin-labeled lipids from a methanolic stock solution were added to hippocampal membranes containing ~2.5 mg total protein (~2.5 μmol phospholipids). The amount of spin label added was such that the final probe concentration was ~1 mol% with respect to the total phospholipid content. This ensures optimal ESR signal with negligible membrane perturbation. In case of lipid extracts, ~2.5 μmol of total phospholipids in chloroform-methanol (1:1, v/v) were mixed well with 25 nmol of the spin-labeled phospholipid (5-PC or 14-PC) and dried under a stream of nitrogen while being warmed gently (~45 °C). The residual solvent was removed by drying under vacuum for 6 h. The lipid film obtained was then hydrated by adding 1 ml of 50 mM Tris, pH 7.4 buffer containing 1 mM EDTA at ~70 °C while being intermittently vortexed for 3 min to disperse the lipid and form homogeneous multilamellar vesicles (MLVs). MLVs were kept at ~70 °C for an additional hour to ensure proper swelling as the vesicles were formed. Such high temperatures are necessary for hydrating the samples due to the presence of lipids with high melting temperature in neuronal tissues (Koynova and Caffrey, 1995). After vortexing, samples were transferred into a glass capillary (1 mm inside diameter (ID)), sealed at one end, and pelleted by centrifugation in a benchtop centrifuge. The excess supernatant was removed and the capillary was sealed and stored at 4 °C. Samples were always prepared on the day of the measurement and were never stored for more than 24 h before measurement.

ESR spectroscopy

ESR spectra were recorded on a JEOL JES-FA 200 ESR spectrometer operating at 9 GHz. Samples in 1 mm ID glass capillaries, prepared as described above, were placed in a standard quartz ESR tube. The following instrumental settings were used for all measurements: scan width, 100 G; scan time, 4 min; number of accumulations, 4; time constant, 1 s; modulation width, 2 G; microwave power, 5 mW. Spectra were recorded at 25 $\pm$ 0.2 ºC and temperature was maintained constant during the measurement with a temperature-controller attached to the ESR spectrometer. Temperature was measured by a thermocouple placed close to the sample tube.

Nonlinear least squares analysis of ESR spectra

ESR spectra of 5- and 14-PC dispersed in hippocampal membranes and liposomes of lipid extract were analyzed by a nonlinear least squares (NLLS) method, based on the stochastic Liouville equation (Meirovitch *et al.*, 1984; Schneider and Freed, 1989). Simulations were carried out using the latest version of the ESR fitting program (Budil *et al.*, 1996) configured to run on a computer running on Windows operating system. The program package (PC.NEW) is available from the Advanced Centre for ESR Technology (ACERT) atCornellUniversity(Ithaca,NY;http://www.acert.cornell.edu/index_files/acert_resources.php). In this analysis, values of the hyperfine tensors (A_{xx}, A_{yy}, A_{zz}) and g-tensor (g_{xx}, g_{yy}, g_{zz}) are kept fixed for each simulation. These parameters are slightly modified, if fits obtained are not satisfactory and the simulation is repeated until satisfactory fits are obtained. The optimized values are shown in Table 5.1 and were used for all spectra of a particular spin-labeled phospholipid. The values of the rotational diffusion coefficients and the terms that describe Gaussian inhomogeneous broadening and coefficients for orienting potential were varied (Budil *et al.*, 1996). The data points were reduced to 1024 by

averaging the original data points from spectra. The input parameters of a run file were tuned in order to generate a good fit. NLLS analysis of a spectrum provides the values of two important parameters that describe the dynamic order of the membrane lipids. These are: (i) the rotational diffusion coefficient ($R_\perp$) of the nitroxide radical around the axis perpendicular to the mean symmetry axis for rotation (it represents the principal component of the rotational diffusion tensor of the nitroxide radical (Ge *et al.*, 1994)); and (ii) the order parameter (S_0), which is a measure of the angular extent of the rotational diffusion of the nitroxide moiety relative to the membrane normal.

Table 5.1

Values of g and A Tensor Components used for Simulations

Spin label	g_{xx}	g_{yy}	g_{zz}	A_{xx} (G)	A_{yy} (G)	A_{zz} (G)
5-PC	2.0080	2.0058	2.0024	5.5	5.5	33.3
14-PC	2.0087	2.0059	2.0024	5.4	5.4	33.0

5.3. Results and discussion

MβCD is a water-soluble compound and has previously been shown to selectively and efficiently extract cholesterol from hippocampal membranes by including it in a central nonpolar cavity (Pucadyil and Chattopadhyay, 2004b). Fig. 5.2A shows that the cholesterol content of hippocampal membranes exhibits a progressive reduction upon treatment with increasing concentrations of MβCD. When hippocampal membranes were treated with 10 mM MβCD, cholesterol content was reduced to ~78% of the original value. This effect levels off at higher concentrations of MβCD, with the cholesterol concentration

being reduced to ~17% of the original value when 40 mM MβCD was used (see Fig. 5.2A). Importantly, there is no appreciable alteration in the membrane phospholipid levels under these conditions (see Fig. 5.2B), thereby ensuring the specific nature of cholesterol removal.

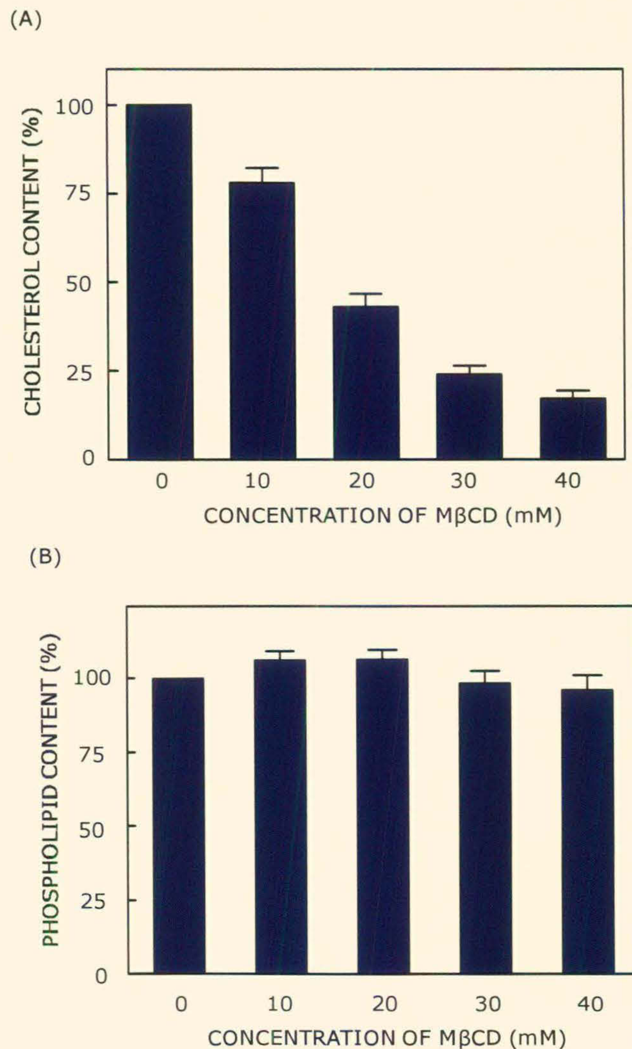


Figure 5.2. *Lipid contents of hippocampal membranes upon cholesterol depletion.* Hippocampal membranes were treated with increasing concentration of MβCD, followed by (A) cholesterol and (B) phospholipid estimation. Values are expressed as percentages of cholesterol and phospholipid contents in control (without MβCD treatment) hippocampal membranes. Data represent means ± SE of at least four independent measurements. See section 5.2 for other details.

In order to monitor the dynamic gradient in hippocampal membranes and its variation with cholesterol and protein content, spin-labeled

phospholipids containing spin label (*i.e.*, paramagnetic nitroxide moiety) at different depths were utilized. While the spin label group is located at the membrane interface region in 5-PC (~12 Å from the center of the bilayer), the position of the label is much deeper in the hydrocarbon region of the membrane in case of 14-PC (~4 Å from the center of the bilayer) (Chattopadhyay and London, 1987; Abrams and London, 1992).

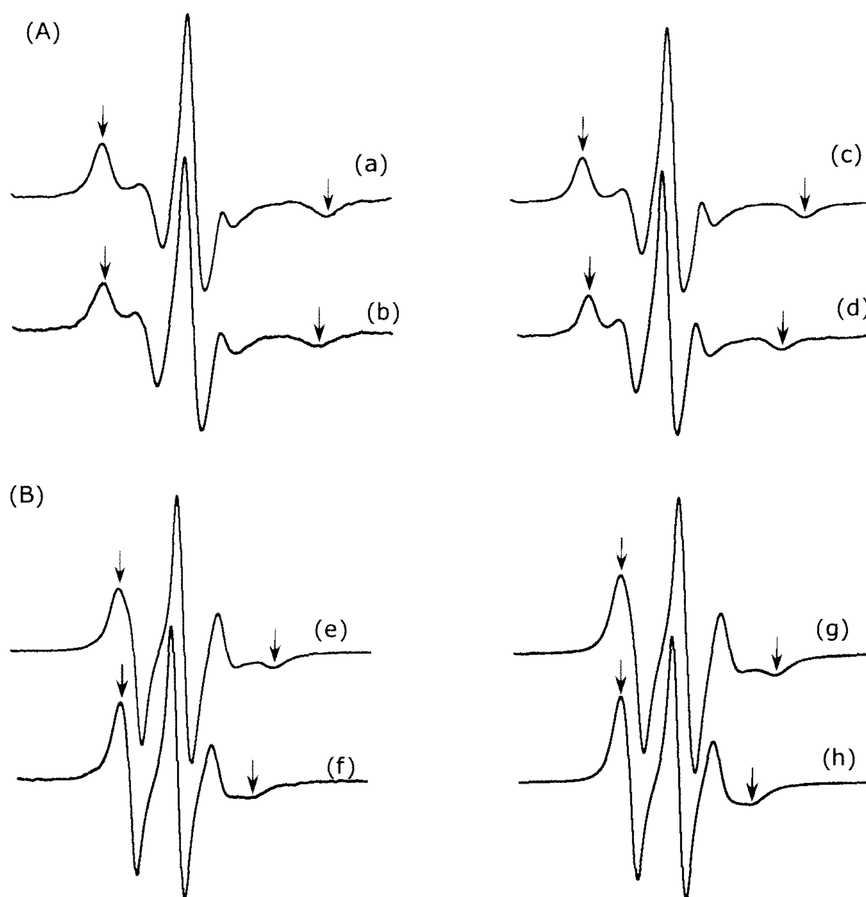


Figure 5.3. *Typical ESR spectra of (A) 5-PC and (B) 14-PC incorporated in hippocampal membranes and liposomes of lipid extract.* Spectra shown correspond to control (a and e) and cholesterol-depleted (b and f) hippocampal membranes. ESR spectra corresponding to liposomes of control (c and g) and cholesterol-depleted (d and h) lipid extract are also shown. Cholesterol depletion was carried out with 40 mM MβCD. See section 5.2 for other details.

ESR spectra of 5-PC incorporated into native hippocampal membranes (a) and cholesterol-depleted membranes (b, using 40 mM MβCD) are shown in Fig. 5.3A. The spectra of 5-PC incorporated into liposomes of lipid extract from

hippocampal membranes (c) and cholesterol-depleted hippocampal membranes ((d), using 40 mM MβCD) are also shown in Fig. 5.3A. Corresponding ESR spectra of 14-PC under similar conditions (e-h) are shown in Fig. 5.3B. It is apparent from the spectra that the positions of the first maximum and the last minimum display a slight shift toward each other upon cholesterol depletion in each case (indicated by arrows), although the overall shape of the spectra remains similar. This results in a reduction in outer maximum hyperfine splitting ($2A_{max}$) values for both 5- and 14-PC.

The outer maximum hyperfine splitting is a sensitive parameter in ESR spectroscopy and contains information on motional dynamics and environmental polarity sensed by the spin label (Görrissen *et al.*, 1986; Swamy *et al.*, 2000). While increase in motional dynamics results in a reduction in hyperfine splitting ($2A_{max}$), increase in polarity leads to an increase in hyperfine splitting. The value of $2A_{max}$ exhibits a reduction from 59.0 to 56.2 G for native hippocampal membranes upon cholesterol depletion in case of 5-PC (see (a) and (b) in Fig. 5.3A). The corresponding reduction in $2A_{max}$ for liposomes of lipid extract is from 58.1 to 54.6 G (see (c) and (d) in Fig. 5.3A). On the other hand, the value of $2A_{max}$ displays a reduction from 41.2 to 37.6 G for native membranes upon cholesterol depletion when 14-PC was used (see (e) and (f) in Fig. 5.3B). The corresponding reduction in $2A_{max}$ in case of liposomes of lipid extract is from 41.1 to 37.1 G (see (g) and (h) in Fig. 5.3B) when dynamics upon cholesterol depletion (Mukherjee and Chattopadhyay, 2005; Mukherjee *et al.*, 2006; Saxena *et al.*, 2008; Subczynski *et al.*, 1994). It has previously been shown, using pyrene vibronic band intensity ratio, that the apparent polarity of hippocampal membranes exhibits an increase upon cholesterol depletion (Saxena *et al.*, 2008). This suggests that the observed reduction in $2A_{max}$ would have been larger if there were no change in polarity. A comprehensive table of $2A_{max}$ values in native membranes and liposomes of lipid extract with progressive cholesterol depletion is shown in Table 5.2. In order to analyze the observed changes in

ESR spectra of the spin-labeled lipids under these conditions in a comprehensive and rigorous manner, spectral simulations of the observed ESR spectra using the NLLS approach were carried out.

Table 5.2

Outer Maximum Hyperfine Splitting ($2A_{max}$) values of ESR Spectra in Hippocampal Membranes and Liposomes of Lipid Extract*

Spin label	MβCD (mM)	Cholesterol content (%)	2Amax	
			Native	Lipid extract
5-PC	0	100	59.0 ± 0.6	58.1 ± 0.1
	10	78	58.5 ± 0.6	57.3 ± 0.3
	20	43	57.0 ± 0.7	55.9 ± 0.5
	30	24	56.1 ± 0.6	54.8 ± 0.4
	40	17	56.2 ± 0.7	54.6 ± 0.1
14-PC	0	100	41.2 ± 0.4	41.1 ± 0.3
	10	78	40.5 ± 0.1	40.6 ± 0.2
	20	43	39.5 ± 0.3	39.5 ± 0.4
	30	24	38.1 ± 0.1	38.0 ± 0.2
	40	17	37.6 ± 0.3	37.1 ± 0.4

*Values of the parameters shown correspond to means ± SD obtained from three independent measurements. All measurements were carried out at room temperature (~25 °C). See sections 5.1 and 5.2 for other details.

Analysis of ESR spectra of spin labels in such slow environments poses considerable challenge since the relationship between ESR spectral features and

physical parameters of interest is not direct. The NLLS approach provides a convenient and useful tool for the analysis of ESR spectra in motionally restricted (slow) environment typically experienced by spin-labeled lipids in natural membranes (Meirovitch *et al.*, 1984; Schneider and Freed, 1989; Budil *et al.*, 1996; Swamy *et al.*, 2006). The NLLS analysis of ESR spectra of spin-labeled phospholipids with the nitroxide group attached to different positions in the acyl chain would provide valuable information on membrane order and dynamics in a depth-dependent manner.

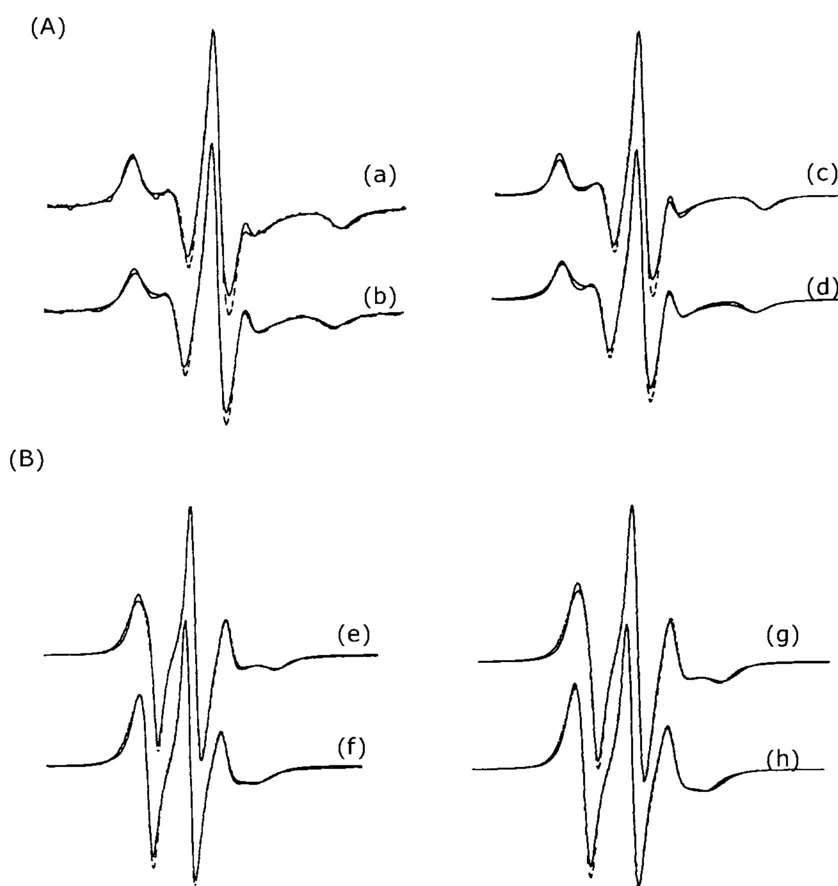


Figure 5.4. *Typical ESR spectra of (A) 5-PC and (B) 14-PC incorporated in hippocampal membranes and liposomes of lipid extract (solid line).* The simulations by NLLS analysis are also shown in each case (dotted line). Spectra and simulations are shown corresponding to control (a and e) and cholesterol-depleted (b and f) hippocampal membranes, and liposomes of control (c and g) and cholesterol-depleted (d and h) lipid extract. Cholesterol depletion was carried out with 40 mM MβCD. See section 5.2 for other details.

Table 5.3

NLLS Analysis of ESR Spectra in Hippocampal Membranes*

Spin label	MβCD (mM)	Cholesterol content (%)	S_0	$R_\perp$ (s^{-1}) ($\times 10^{-7}$)
5-PC	0	100	0.37 ± 0.03	7.44 ± 0.02
	10	78	0.36 ± 0.04	7.44 ± 0.02
	20	43	0.35± 0.07	7.41 ± 0.05
	30	24	0.34 ± 0.00	7.51 ± 0.01
	40	17	0.34 ± 0.00	7.53 ± 0.01
14-PC	0	100	0.36 ± 0.00	8.30 ± 0.00
	10	78	0.34 ± 0.01	8.25 ± 0.04
	20	43	0.31 ± 0.00	8.18 ± 0.02
	30	24	0.29 ± 0.00	8.15 ± 0.02
	40	17	0.28 ± 0.01	8.16 ± 0.02

*Values of the parameters shown correspond to means ± SD obtained from three independent measurements. All measurements were carried out at room temperature (~25 °C). See sections 5.1 and 5.2 for other details.

The best-fit of the simulated ESR spectra of 5- and 14-PC in native hippocampal membranes and cholesterol-depleted membranes are shown in Fig. 5.4A and B. It is apparent from Fig. 5.4 that the simulated spectra are in excellent agreement with the recorded spectra. The simulated spectra of 5- and 14-PC in hippocampal membranes and liposomes of lipid extract could be fitted well with a single component (simulations with two components produced

inconsistent results). The simulation parameters represent the time-averaged values corresponding to dynamically heterogeneous lipid populations. Fits of comparable quality were also achieved for ESR spectra of 5- and 14-PC in cholesterol-depleted conditions in hippocampal membranes and liposomes of lipid extract (see Fig. 5.4). The parameters derived from the best-fit simulations corresponding to 5- and 14-PC incorporated in hippocampal membranes and liposomes of lipid extract are listed in Tables 5.3 and 5.4, respectively.

Table 5.4

NLLS Analysis of ESR Spectra in Liposomes of Lipid Extract*

Spin label	MβCD (mM)	Cholesterol content (%)	S_0	$R_{\perp}$ (s^{-1}) ($\times 10^{-7}$)
5-PC	0	100	0.48 ± 0.01	7.47 ± 0.01
	10	78	0.46 ± 0.02	7.53 ± 0.01
	20	43	0.43± 0.00	7.60 ± 0.01
	30	24	0.42 ± 0.02	7.65 ± 0.02
	40	17	0.42 ± 0.01	7.66 ± 0.02
14-PC	0	100	0.37 ± 0.02	8.51 ± 0.03
	10	78	0.37 ± 0.01	8.69 ± 0.34
	20	43	0.32 ± 0.00	8.44 ± 0.03
	30	24	0.30 ± 0.00	8.40 ± 0.02
	40	17	0.27 ± 0.01	8.37 ± 0.03

*Values of the parameters shown correspond to means ± SD obtained from three independent measurements. All measurements were carried out at room temperature (~25 °C). See sections 5.1 and 5.2 for other details.

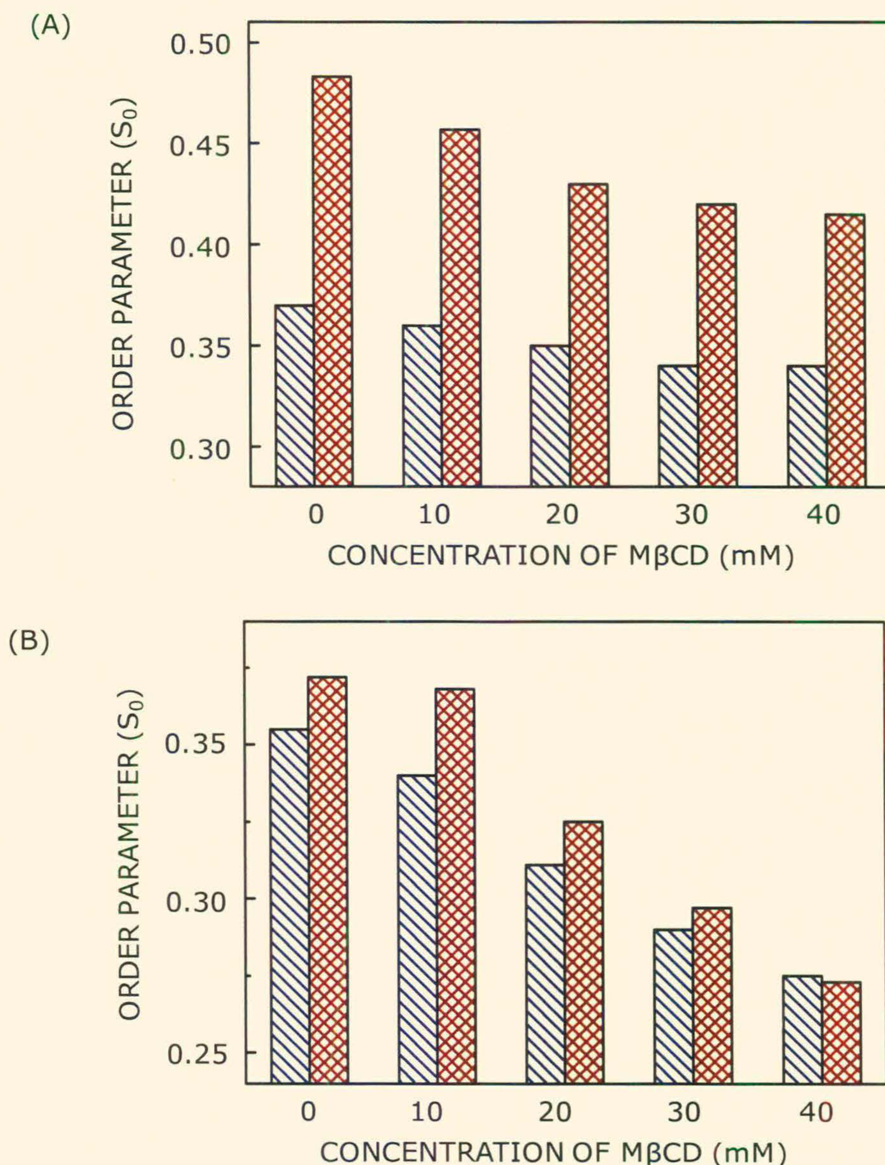


Figure 5.5. *Order parameter (S_0) of (A) 5-PC and (B) 14-PC incorporated in hippocampal membranes (hatched bar) and liposomes of lipid extract (crisscrossed bar) upon increasing cholesterol depletion.* The order parameter provides a measure of the angular extent of the rotational diffusion of the nitroxide moiety relative to the bilayer normal. See Tables 5.3 and 5.4, and section 5.2 for other details.

Fig. 5.5 shows the best-fit values of order parameter (S_0) of 5- and 14-PC in hippocampal membranes and liposomes of lipid extract. The order

parameter exhibits progressive reduction with decreasing cholesterol content in all cases. The extent of reduction in order parameter appears more pronounced in case of liposomes of lipid extract. For example, the maximum reduction observed in the order parameter for hippocampal membranes is ~8% when 5-PC was used, while the corresponding value for liposomes of lipid extract is ~13%. This overall trend is valid even when membrane order is monitored at deeper location using 14-PC. The extents of reduction observed in order parameter in this case are ~22% and ~27% for native membranes and liposomes of lipid extracts, respectively. This indicates that both native membranes and liposomes of lipid extract become disordered upon cholesterol depletion. These results are in agreement with previous reports utilizing fluorescence spectroscopic approaches that cholesterol depletion leads to a reduction in membrane order in native hippocampal membranes (Pucadyil and Chattopadhyay, 2004b; Mukherjee and Chattopadhyay, 2005). This implies that the reduction in membrane order is not limited to a particular time scale, but covers a broad range of time scale (~ns to μs). The observed depth-dependence of the extent of reduction in membrane order parameter upon cholesterol depletion merits comment. The change in order parameter is considerably high when 14-PC was used to monitor membrane dynamics. These results imply that the deeper hydrocarbon region of the membrane is more sensitive to changes in membrane organization and dynamics due to cholesterol depletion than the interfacial region (Bittman, 1997; Benninger *et al.*, 2005), in agreement with earlier reports.

It is interesting to note that the order parameter of 5-PC in liposomes (0.48) is considerably higher compared to that of native membranes (0.37). This points out the difference in membrane packing (and therefore membrane order) in these two cases, possibly due to the bumpiness induced by membrane proteins (~75% of total proteins in hippocampal membranes are estimated to be integral membrane proteins). Similar observations have been made

regarding the effect of proteins on the lipid acyl chain packing and order parameter of lipid spin labels incorporated into the plasma membranes of live RBL-2H3 mast cells as well as plasma membrane vesicles derived from them (Swamy *et al.*, 2006; Ge *et al.*, 2003). Interestingly, this difference in order parameter between liposomes of lipid extract and native membranes is absent when 14-PC was used. This indicates that membrane proteins exert considerable influence on membrane order in the membrane interfacial region. This effect decreases in deeper regions of the membrane, possibly due to the mobility gradient that exists along the length of the fatty acyl chain (Chattopadhyay, 2003).

An order parameter of 0.37 was observed for 5-PC in native membranes at 25 °C (Fig. 5.5A). This value is slightly lower than the order parameter obtained for 5-PC (0.42) in plasma membrane vesicles derived from the RBL-2H3 mast cells at 23 °C (Ge *et al.*, 2003), and significantly lower than the order parameter of 5-PC in sphingomyelin model membranes at 20 °C in the gel phase (0.46) and in detergent-resistant membranes at 22 °C (0.52) (Ge *et al.*, 1999, 2003). Interestingly, the order parameter of 5-PC in native hippocampal membranes is comparable to the order parameter of 0.38 obtained for the same spin label in the liquid-ordered component of live RBL-2H3 cells at 25 °C (Swamy *et al.*, 2006). This is in agreement with our previous results on native hippocampal membranes using fluorescence approaches. For example, it has previously been reported that the fluorescence polarization of the popular membrane probe DPH (1,6-diphenyl-1,3,5- hexatriene) in native hippocampal membranes is ~0.33 at 25 °C (Pucadyil and Chattopadhyay, 2004b), a value that is characteristic of liquid-ordered phase in membranes (Schroeder *et al.*, 1994; Brown and London, 1998). In addition, earlier report shows that native hippocampal membranes display a characteristic wavelength dependence of Laurdan generalized polarization (GP), reminiscent of the liquid-ordered phase (Mukherjee and Chattopadhyay, 2005).

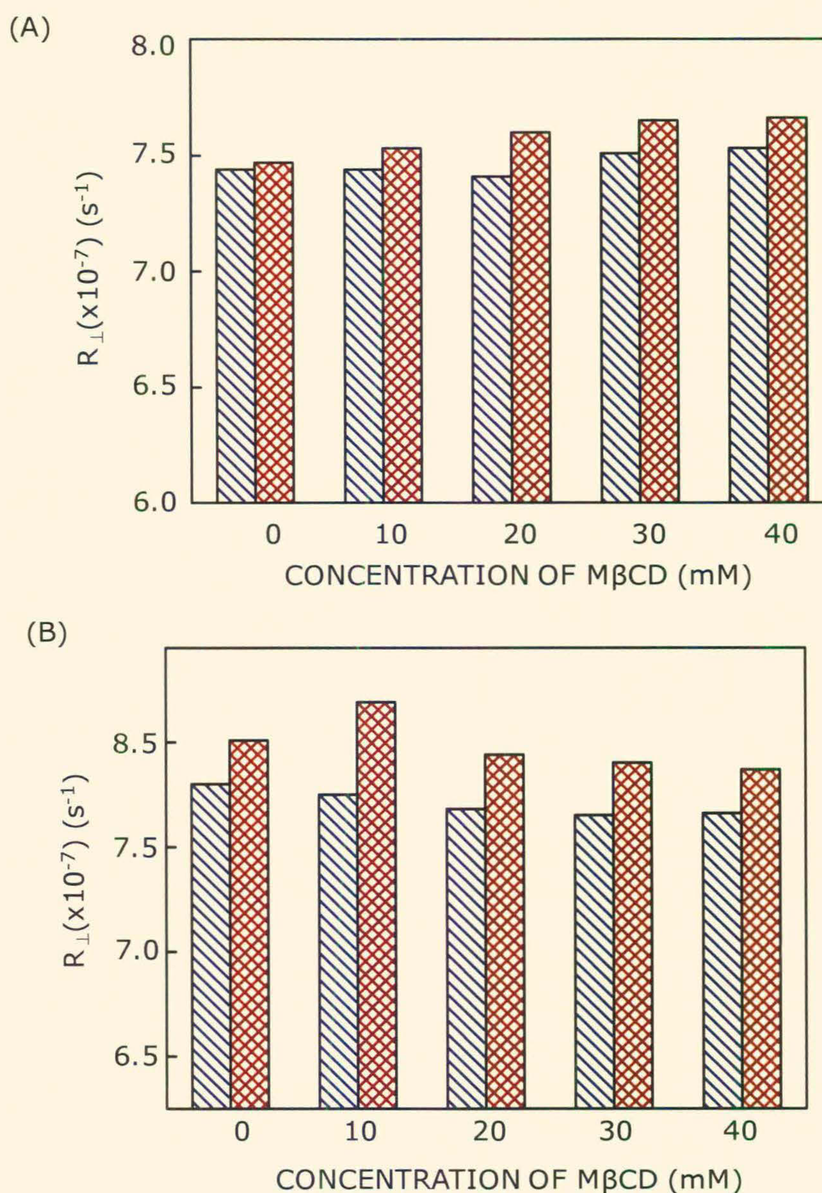


Figure 5.6. *Rotational diffusion coefficient ($R_\perp$) of (A) 5-PC and (B) 14-PC incorporated in hippocampal membranes (hatched bar) and liposomes of lipid extract (crisscrossed bar) upon increasing cholesterol depletion.* $R_\perp$ represents the rotational diffusion coefficient of the nitroxide radical around the axis perpendicular to the mean symmetry axis for rotation. See Tables 5.3 and 5.4, and See section 5.2 for other details.

The apparent liquid-ordered nature of native membranes could be attributed to high levels (~31 mol%) of cholesterol (Mukherjee and Chattopadhyay, 2005; Pucadyil and Chattopadhyay, 2004a) in hippocampal

membranes, since liquid-ordered phase membranes typically contain high amounts of cholesterol (Mouritsen, 2010; Brown and London, 1998). The best-fit values of the rotational diffusion coefficient ($R_\perp$) of 5- and 14-PC in hippocampal membranes and liposomes of lipid extract under different conditions are shown in Tables 5.3 and 5.4 and summarized in Fig. 5.6. The value of $R_\perp$ in liposomes is found to be higher compared to that of native membranes in all cases. It is also apparent from Fig. 5.6 that $R_\perp$ displays much less sensitivity under conditions of cholesterol depletion. A value of 7.44×10^7 s^{-1} as $R_\perp$ was observed for 5-PC in native membranes at 25 °C (Fig. 5.6A and Table 5.4). This value is clearly higher than the reported $R_\perp$ of 5-PC in detergent-resistant membranes of RBL-2H3 cells at 22 °C (4.07×10^7 s^{-1}) and considerably higher than the corresponding value in sphingomyelin model membranes at 20 °C in the gel phase (1.62×10^7 s^{-1}) (Ge *et al.*, 1999). The value of $R_\perp$ of 5-PC in native membranes is comparable to the corresponding value (7.41×10^7 s^{-1}) in the less abundant liquid-crystalline-like component of plasma membrane vesicles derived from RBL-2H3 cells at 22 °C (Ge *et al.*, 2003).

Although the membrane lipid composition of bovine hippocampus is not known, the phospholipid composition of rat hippocampus has been reported (Ulmann *et al.*, 2001; Murthy *et al.*, 2002; Wen and Kim, 2004). Analysis of the phospholipid composition of the rat hippocampus shows phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine as the predominant headgroups, while the fatty acid composition shows enrichment with 16:0, 18:0, 18:1, 18:2, 20:4, and 22:6 fatty acids. In addition, plasmalogens have been reported in rat hippocampus. In this work, organization and dynamics of hippocampal membranes was monitored upon modulating their cholesterol and protein content utilizing depth-specific spin-labeled phospholipids. These results show that while cholesterol increases hippocampal membrane order, membrane proteins increase lipid dynamics resulting in disordered membranes by disturbing the membrane order (see Fig.

5.7). It is noteworthy to mention here that these results are obtained using ESR spectroscopy that provides information in a relatively slow time scale, appropriate for a slow diffusing system such as crowded neuronal membranes (Nakada *et al.*, 2003; Takamori *et al.*, 2006).

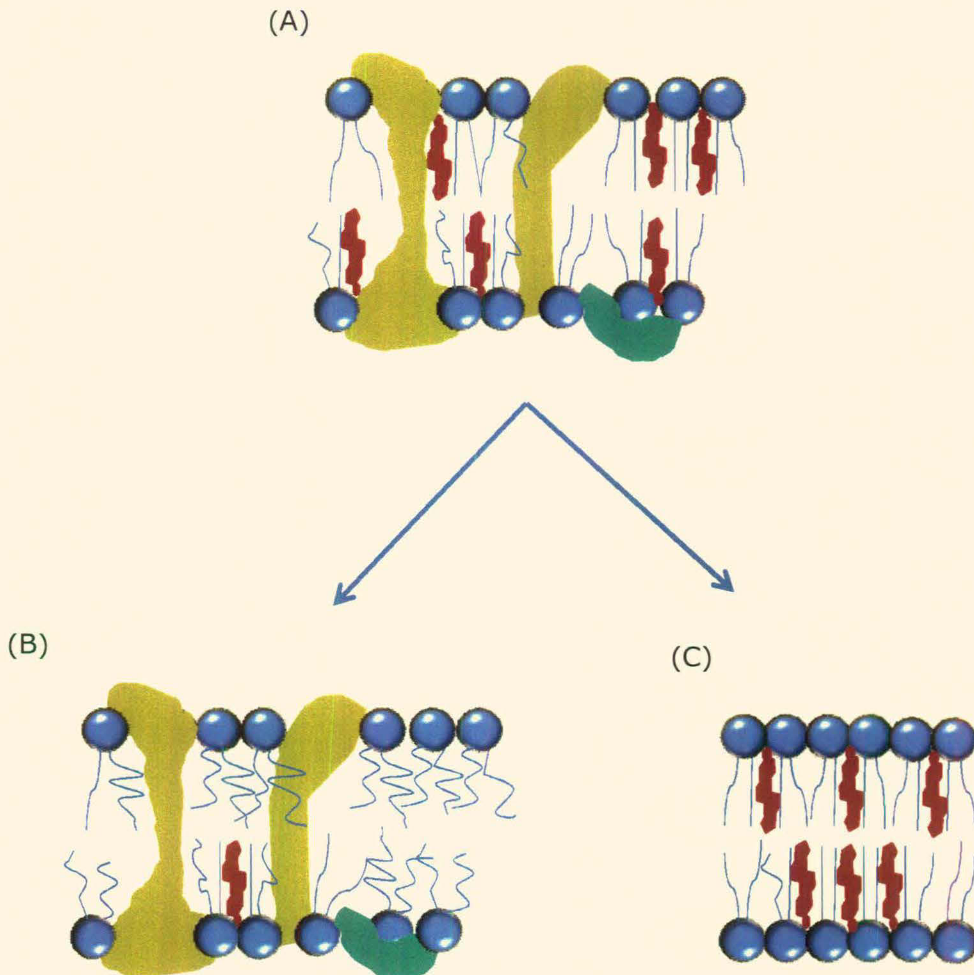


Figure 5.7. *A schematic representation of the membrane organization in (A) native, (B) cholesterol-depleted, and (C) liposomes of lipid extract of hippocampal membranes.* Phospholipids are shown in blue, cholesterol in maroon, and peripheral and integral membrane proteins in light green and mustard color, respectively. (A) The interaction between fatty acyl chains of phospholipids with the rigid sterol ring of cholesterol increases membrane order. (B) Cholesterol depletion increases the degree of segmental motion of fatty acyl chains of phospholipids leading to relatively less ordered membranes. (C) Increase in order accompanied by removal of membrane proteins: liposomes of lipid extract displays increased order relative to native membranes under similar condition. See Fig. 5.5 and section 5.3 for more details.

Knowledge of membrane order and dynamics would help in analyzing functional data generated by modulation of membrane lipid composition (Pucadyil and Chattopadhyay, 2004b, 2006). The interaction between cholesterol and other molecular components in neuronal membranes (such as receptors and lipids) assumes relevance for understanding brain function.

The organization and dynamics of cellular membranes in the nervous system is therefore significant for a comprehensive understanding of the functional roles played by the membrane-bound neuronal receptors which represent crucial components in signal transduction in the nervous system. Taken together, these results comprehensively show depth-dependent changes in the organization and dynamics of hippocampal membranes and its modulation by cholesterol and protein content using ESR (characterized by relatively slow time scale) of depth-specific spin-labeled phospholipids. Membrane organization and dynamics represent important determinants in protein-protein interactions in cell membranes and have significant impact on the overall efficiency of the signal transduction process (Calvert *et al.*, 2001; Pucadyil *et al.*, 2004a; Ganguly *et al.*, 2008). Interestingly, membrane organization under low cholesterol condition is relevant since reduced membrane cholesterol results in manifestation of several physiological effects. For example, it has been previously shown that cholesterol depletion affects sorting (Hansen *et al.*, 2000), distribution (Pike and Casey, 2002), endocytosis (Subtil *et al.*, 1999) and trafficking (Pediconi *et al.*, 2004) of membrane proteins. Importantly, we recently reported that chronic cholesterol depletion impairs the function of the serotonin$_{1A}$ receptor, which could have important implications in mood disorders (Shrivastava *et al.*, 2010). In a broader perspective, these results are significant in understanding the complex spatiotemporal organization of neuronal membranes, and could have functional implications in neuronal diseases such as the Smith-Lemli-Opitz syndrome (Porter and Herman, 2011; Paila *et al.*, 2008; Porter, 2008) characterized by low cholesterol condition due to defective cholesterol biosynthesis.

Chapter 6

Sphingomyelin headgroup is necessary for the serotonin$_{1A}$ receptor function

6.1. Introduction

Sphingolipids are essential components of eukaryotic cell membranes and constitute ~10-20% of the total membrane lipids as described in section 1.3. Sphingolipids such as sphingomyelin are regarded as reservoirs for bioactive second messengers such as ceramide and sphingosine 1-phosphate (Bartke and Hannun, 2009; Posse de Chaves, 2006). The distribution of sphingomyelin in the cellular plasma membrane appears heterogeneous and patchy (Ramstedt and Slotte, 2006). Importantly, sphingomyelins have been shown to be involved in the development and progression of several neurological diseases such as Alzheimer's disease (Posse de Chaves, 2006; Posse de Chaves and Sipione, 2010) which could be due to impaired neurotransmission.

The serotonin$_{1A}$ receptor is an important neurotransmitter receptor serves as an important target in the development of therapeutic agents for neuropsychiatric disorders such as anxiety and depression. In the context of increasing pharmacological significance of the serotonin$_{1A}$ receptor (Lacivita *et al.*, 2008; Gogos *et al.*, 2008), its interaction with the surrounding lipids such as sphingomyelin assumes relevance. In this overall context and keeping in mind the relevance of sphingolipids in the nervous system (Posse de Chaves, 2006), importance of the sphingomyelin headgroup was explored on ligand binding function of the serotonin$_{1A}$ receptor in native hippocampal membranes.

6.2. Materials and methods

Materials

Sphingomyelinase (EC 3.1.4.12) from *Bacillus cereus* was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest purity available as described in section 2.2.

Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described in section 2.2.

Treatment of native membranes with sphingomyelinase

Native membranes were resuspended in 50 mM Tris, pH 7.4 buffer at a protein concentration of 2 mg/ml and treated with sphingomyelinase (aliquoted from a stock solution of 200 U/ml in 10 mM Tris, pH 7.4 buffer) at 25 °C with constant shaking for 1 h. Membranes were then spun down at 50,000xg for 10 min at 4 °C and resuspended in the same buffer.

Radioligand binding assays

Receptor binding assays were carried out as described in section 2.2.

Estimation of sphingomyelin by thin layer chromatography

Total lipids were extracted from control and sphingomyelinase-treated membranes according to Bligh and Dyer (Bligh and Dyer, 1959). The lipid extracts were dried under a stream of nitrogen at 45 °C. The dried extracts were dissolved in a chloroform/methanol mixture (1:1, v/v). Total lipid extracts were resolved by thin layer chromatography using a chloroform/methanol/water (65:25:4, v/v/v) as the solvent system (Jafurulla *et al.*, 2008). The separated lipids were visualized

under ultraviolet light by spraying a fluorescent solution of 0.01% (w/v) primuline prepared in acetone (van Echten-Deckert, 2000). A sphingomyelin standard was used to identify its position on TLC plates run with total lipid extracts obtained from control and sphingomyelinase-treated membranes. Sphingomyelin bands were scraped from TLC plates, lipids were re-extracted with a chloroform/methanol mixture (1:1, v/v) from samples, and the phosphate content was estimated and normalized to the phosphate content obtained from control samples.

Estimation of cholesterol content

Cholesterol content in membranes treated with different concentration of sphingomyelinase was estimated as described in section 5.2.

Estimation of phospholipid content

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

Fluorescence anisotropy measurements

Fluorescence anisotropy experiments were carried out using the fluorescent probe DPH as described in section 2.2.

Statistical analysis

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

6.3. Results

Sphingomyelinases are water soluble enzymes that act at the membrane interface and specifically hydrolyze sphingomyelin into ceramide and phosphocholine (Chatterjee, 1999; Goñi and Alonso, 2002; see Fig. 6.1). Phosphocholine, a water soluble moiety, is liberated from sphingomyelin leaving a hydrophobic ceramide backbone of sphingomyelin in the membrane. The phosphocholine moiety appears to act as an anchor for sphingomyelin at the membrane interface. Importantly, sphingomyelinases act as regulators of cell signaling by modulating cellular ceramide levels (Ramstedt and Slotte, 2006; Grassme´ *et al.*, 2007), and can be stimulated with naturally occurring ligands or under stress conditions (Posse de Chaves, 2006; Krönke, 1999).

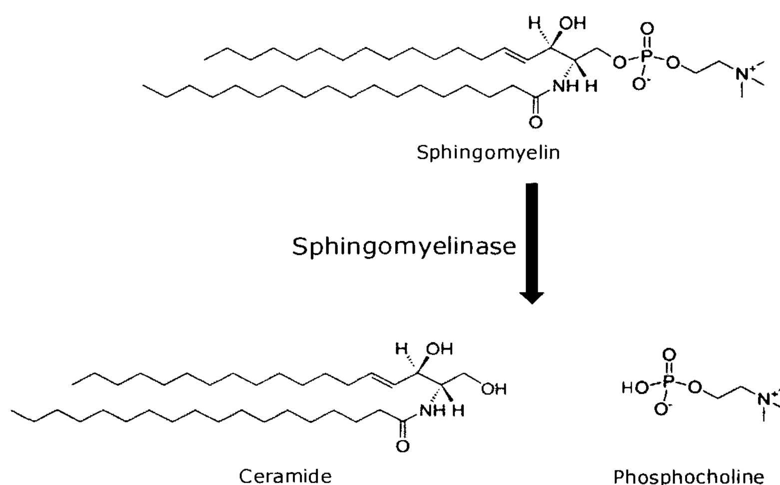


Figure 6.1. *A schematic representation of sphingomyelin hydrolysis catalyzed by sphingomyelinase, with chemical structures of sphingomyelin (ceramide phosphocholine), ceramide and phosphocholine.* Sphingomyelinase, an important hydrolytic enzyme, is involved in sphingolipid metabolism. It catalyzes the hydrolysis of sphingomyelin into ceramide and phosphocholine. See section 6.2 for details.

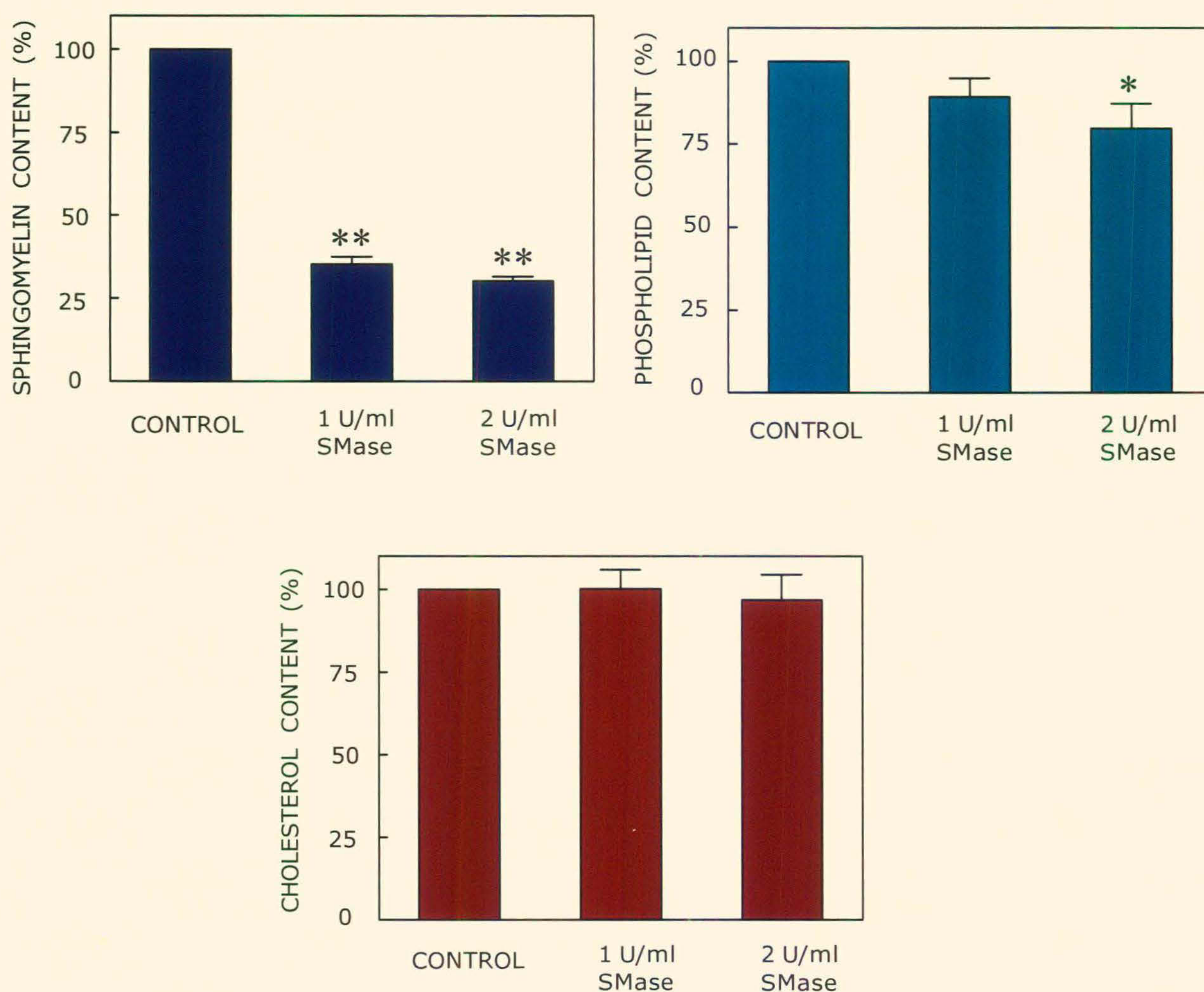


Figure 6.2. *Estimation of lipid content of control and sphingomyelinase-treated hippocampal membranes.* Total lipids extracted from control and sphingomyelinase-treated membranes were separated by thin layer chromatography and spingomyelin was recovered from the chromatogram. (A) Sphingomyelin and (B) total phospholipid contents were determined by phosphate assay subsequent to total digestion by perchloric acid using Na_2HPO4 as standard. (C) Cholesterol content from control and sphingomyelinase-treated membranes was estimated using Amplex red assay kit. Values are expressed as percentages of the corresponding lipid content in control (untreated) membranes. Data shown are means ± SE of four independent experiments (*corresponds to a p-value < 0.05, **corresponds to a p-value < 0.0001). See sections 2.2 and 5.2 for other details.

Fig. 6.2A shows that treatment of hippocampal membranes with sphingomyelinase results in the reduction of sphingomyelin content. Treatment of hippocampal membranes with 1 U/ml sphingomyelinase hydrolyzes ~65% sphingomyelin, whereas increasing the concentration of the enzyme to 2 U/ml resulted in ~70% hydrolysis of sphingomyelin. The extent of sphingomyelin hydrolysis therefore appears to level off under these conditions. Since sphingomyelin contains phosphocholine headgroup, it contributes to the total phospholipid content of hippocampal membranes. Fig. 6.2B shows that the total phospholipid content in sphingomyelinase-treated membranes shows a reduction by ~19% corresponding to ~70% reduction in sphingomyelin content due to hydrolysis with 2 U/ml sphingomyelinase (Fig. 6.2A).

It has previously been reported that bovine hippocampal membranes contain ~68% phospholipids and ~32% cholesterol (Pucadyil, and Chattopadhyay, 2004a). Sphingomyelin therefore accounts for ~18% of the total lipids in hippocampal membranes. These values are in very good agreement with literature reports of sphingomyelin content in neuronal membranes (Holthuis *et al.*, 2001; Soriano *et al.*, 2005). Sphingomyelin is anchored through phosphocholine moiety toward the membrane interface and is believed to interact with cholesterol through hydrogen-bonding between the hydroxyl group of cholesterol and the amide group of sphingomyelin (Brown, 1998). Ceramide, generated by the action of sphingomyelinase, tends to form ceramide-enriched microdomains (Grassme´ *et al.*, 2007). To examine the effect of sphingomyelinase on membrane cholesterol content, cholesterol content in sphingomyelinase-treated membranes was estimated. Fig. 6.2C shows that cholesterol content does not exhibit any significant change upon sphingomyelinase treatment. In order to explore the effect of sphingomyelinase on ligand binding function of serotonin$_{1A}$ receptors in hippocampal membranes, specific binding of the agonist [^{3}H]8-OH-DPAT to the

receptor was monitored. Fig. 6.3 shows the change in specific binding of the agonist [^{3}H]8-OH-DPAT to the serotonin$_{1A}$ receptor upon sphingomyelinase treatment. Interestingly, specific agonist binding shows a reduction with increasing concentration of sphingomyelinase.

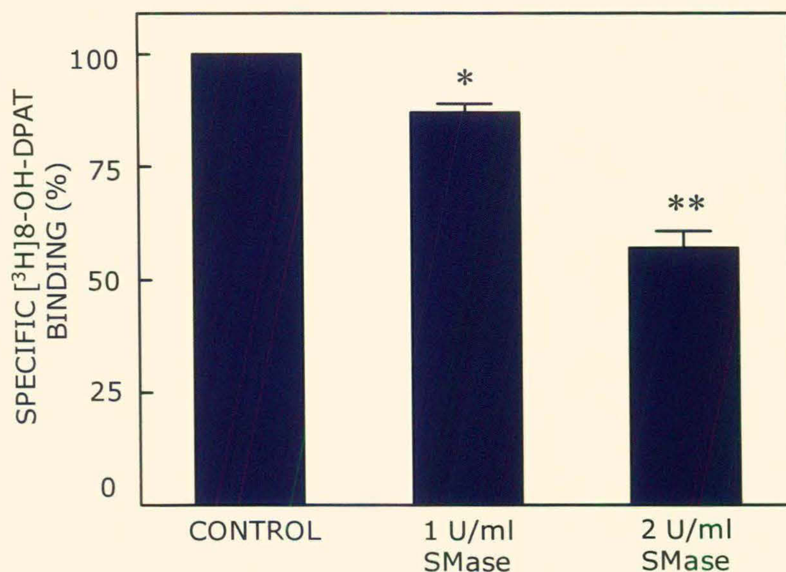


Figure 6.3. *Specific binding of the agonist [^{3}H]8-OH-DPAT to serotonin$_{1A}$ receptors in control and sphingomyelinase-treated hippocampal membranes.* Values are expressed as percentages of specific agonist binding obtained in control membranes. Data represent means ± SE of duplicate points from five independent measurements (*corresponds to a p-value < 0.001, **corresponds to a p-value < 0.0001). See sections 2.2 and 6.2 for other details.

Fig. 6.3 shows that specific agonist binding is reduced by ~13% when 1 U/ml of the enzyme was used. The reduction in specific agonist binding increases to ~43% when an enzyme concentration of 2 U/ml was used. These results show that removal of the phosphocholine headgroup from sphingomyelin (resulting in the formation of ceramide) inhibits the ligand binding function of hippocampal serotonin$_{1A}$ receptors. These observations are relevant in the light of the recent observations that metabolic depletion of sphingolipids impairs the serotonin$_{1A}$

receptor function (Paila *et al.*, 2010). These results show the structural importance of sphingomyelin for serotonin$_{1A}$ receptor function. The observed reduction in specific agonist binding induced by change in sphingomyelin (or ceramide) level could possibly be due to an alteration in membrane order.

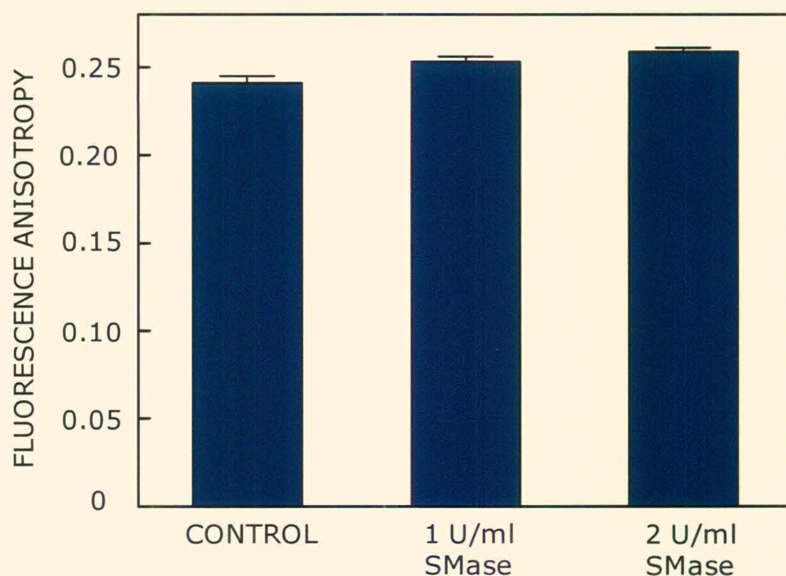


Figure 6.4. *Fluorescence anisotropy of the membrane probe DPH in control and sphingomyelinase-treated hippocampal membranes.* Fluorescence anisotropy measurements were carried out with membranes containing 50 nmol phospholipid at a probe to phospholipid ratio of 1:100 (mol/mol) at room temperature (~23 °C). The excitation wavelength was 358 nm and emission was monitored at 430 nm. Data shown are means ± SE of duplicate points from three independent experiments. See sections 2.2 and 6.2 for other details.

This possibility arises since treatment of hippocampal membranes with sphingomyelinase would generate ceramide that could alter the packing of lipid acyl chain in the membrane (Grassme´ *et al.*, 2007). In order to examine whether there is a change in membrane order upon sphingomyelinase treatment, fluorescence anisotropy measurements were carried out using the membrane probe DPH. Fluorescence anisotropy of probes such as DPH is correlated to the rotational diffusion of membrane embedded probes (Lakowicz, 2006), which is

sensitive to the packing of lipid acyl chains. This is due to the fact that fluorescence anisotropy depends on the degree to which the probe is able to reorient after excitation, and probe reorientation is a function of local lipid packing. DPH, a rod-like hydrophobic molecule, partitions into the interior (fatty acyl chain region) of the bilayer. Fig. 6.4 shows fluorescence anisotropy of the membrane probe DPH incorporated in hippocampal membranes upon increasing concentrations of sphingomyelinase. Fluorescence anisotropy appears to increase slightly (~7%) upon treatment with 2 U/ml sphingomyelinase, compared to the corresponding anisotropy in control (untreated) hippocampal membranes. These results therefore suggest that removal of the sphingomyelin headgroup does not significantly alter the overall membrane order. Taken together, the reduction in the ligand binding function of the serotonin$_{1A}$ receptor upon sphingomyelin hydrolysis is not accompanied by appreciable change in membrane order. These results indicate that phosphocholine headgroup of sphingomyelin could interact with the serotonin$_{1A}$ receptor, thereby influencing ligand binding function of the receptor.

6.4. Discussion

In order to examine the structural importance of sphingomyelin in serotonin$_{1A}$ receptor function, ligand (agonist) binding function of the hippocampal serotonin$_{1A}$ receptor was monitored upon hydrolyzing sphingomyelin to ceramide and phosphocholine with sphingomyelinase. These results show that specific agonist binding to the serotonin$_{1A}$ receptor is reduced upon sphingomyelinase treatment without any appreciable change in overall membrane order. This could be due to the reduction in membrane sphingomyelin content or the resultant increase in ceramide content, or both. It has recently been shown that metabolic

depletion of sphingolipids impairs the serotonin$_{1A}$ receptor function and leads to an enhancement of receptor mobility (Paila *et al.*, 2010; Ganguly *et al.*, 2011). These results, along with these earlier observations, comprehensively demonstrate the importance of sphingolipids in the function of the serotonin$_{1A}$ receptor. It has been previously proposed that G-protein coupled receptors are not uniformly present on the plasma membrane but are concentrated in specific membrane microdomains, some of which are presumably enriched in sphingomyelin and cholesterol (Ostrom and Insel, 2004). In this context, analysis of membrane protein function under conditions that affect sphingomyelin and cholesterol distribution in membranes assumes significance. Importantly, it was earlier demonstrated that physical depletion of cholesterol from hippocampal membranes using methyl-β-cyclodextrin resulted in loss of ligand binding of the serotonin$_{1A}$ receptor (Pucadyil and Chattopadhyay, 2004b). Removal of phosphocholine headgroup of sphingomyelin could disrupt sphingomyelin-cholesterol interactions (Brown, 1998), leading to membrane reorganization. These results constitute the first report on the effect of enzymatic hydrolysis of sphingomyelin on the ligand binding function of this important neurotransmitter receptor in native hippocampal membranes. These results assume relevance in the overall context of the influence of the membrane lipid environment on the function of the serotonin$_{1A}$ receptor in particular, and other G-protein coupled receptors in general (Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2010).

Chapter 7

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

7.1. Introduction

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. These specialized regions (sometimes termed as 'lipid rafts') contribute to variable patchiness of the membrane, and facilitate various cellular processes as described in chapter 1.

Glycosphingolipids are synthesized in the Golgi complex where the first step of glycosphingolipid synthesis (*i.e.,* 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 (See Fig. 1.4). 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 G-protein coupled receptor (GPCR) and is known to play a key role in the generation and modulation of various physiological processes as described in section 1.4. In this work, glycosphingolipid levels were modulated in 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. 7.1). 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). PDMP, the most extensively used inhibitor of glucosylceramide synthase, was utilized for this study. PDMP is a structural analog of ceramide to modulate cellular glycosphingolipid level (Fig. 7.1; Inokuchi and Radin, 1987). Functions of the human serotonin$_{1A}$ receptor under these conditions were analyzed by monitoring ligand binding and G-protein coupling of the receptor. These results show that the function of the serotonin$_{1A}$ receptor is impaired upon metabolic depletion of glycosphingolipids. Importantly, the effect of metabolic depletion of glycosphingolipids on the ligand binding of serotonin$_{1A}$ receptors is restored upon metabolic replenishment.

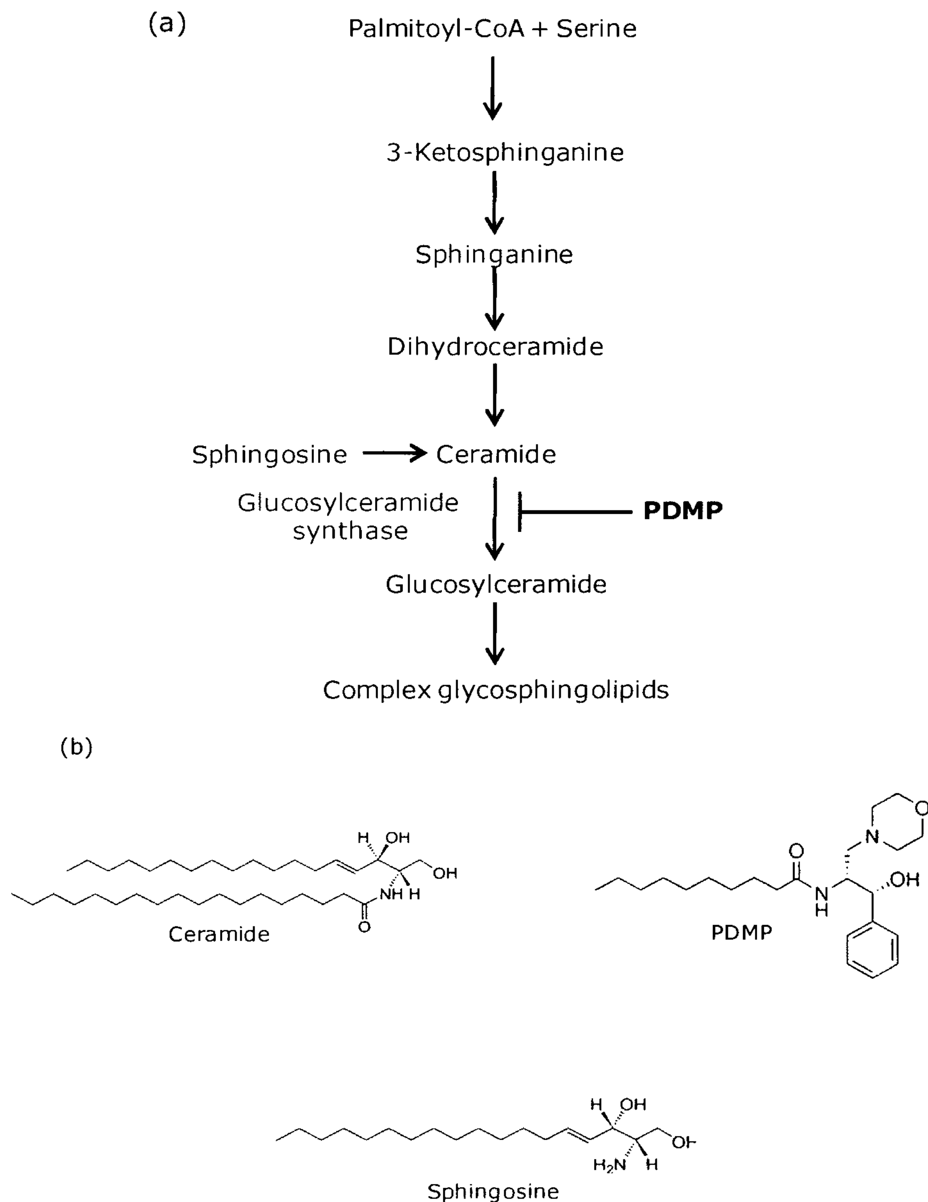


Figure 7.1. *Biosynthetic pathway of glycosphingolipids and chemical structures of ceramide, PDMP and sphingosine.* PDMP is an inhibitor of glucosylceramide synthase, the first enzyme in the biosynthesis of glycosphingolipids. Glucosylceramide synthase catalyzes the glucosylation of ceramide in biosynthesis 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 can be utilized to generate ceramide as shown in panel (a). Chemical structures of ceramide, PDMP and sphingosine are shown in panel (b). See sections 1.3 and 7.1 for more details.

7.2. Materials and methods

Materials

PDMP, oleic acid albumin 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). All other chemicals used were of the highest purity available as described in section 2.2.

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 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 were prepared in water. The final concentrations of PDMP used were 20 and 30 μM. In case of PDMP treatment, cells were grown for 24 h in D-MEM/F-12 complete medium and then shifted to Nutridoma-BO medium containing PDMP 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.

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 °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).

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 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. 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 with ~50 μg total protein as described in section 2.2. The concentration of $[^3H]$8-OH-DPAT in each assay tube was 0.29 nM.

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):

$$B = [a / (1 + (x / I)^s)] + b \qquad (7.1)$$

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:

$$RL^* = 10^{-9} \times B/(V \times SA \times 2220)\ M \qquad (7.2)$$

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 again 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 as described in section 2.2. The optical density of the samples measured at 358 nm was less than 0.10.

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).

7.3. Results

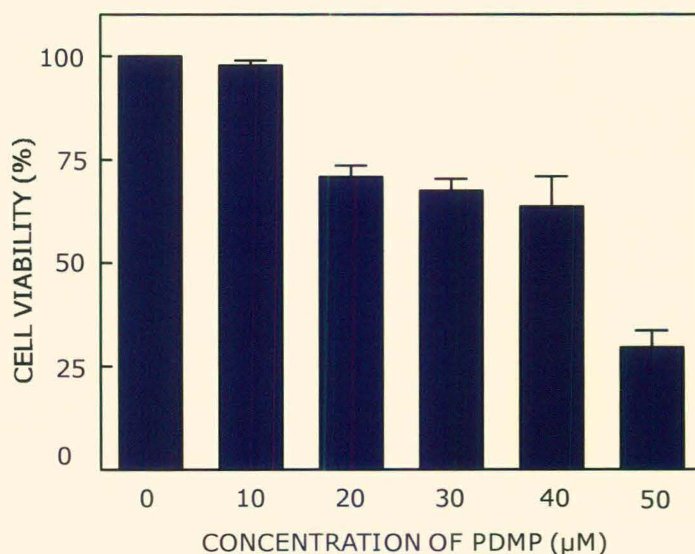


Figure 7.2. *Effect of PDMP on cell viability.* CHO-5-HT$_{1A}$R cells were assayed for viability by MTT assay after treating cells with increasing concentrations of PDMP (up to 50 μM) for 48 h. Values are expressed as percentages of viability for control cells (in absence of PDMP). Data represent means ± SE of at least three independent experiments. See section 7.2 for other details.

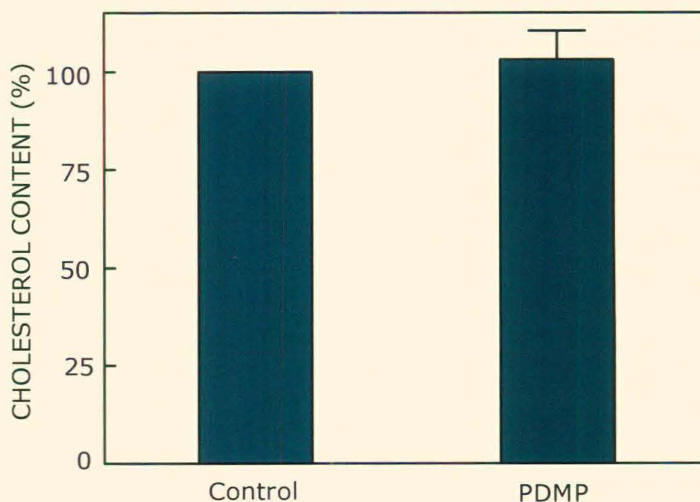


Figure 7.3. *Cholesterol content in membranes isolated from control and PDMP-treated cells.* The concentration of PDMP used was 30 μM. Data represent means ± SE of duplicate points from at least three independent experiments. See section 7.2 for other details.

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). In order to assess the effect of PDMP on cell viability, CHO cells stably expressing the human serotonin$_{1A}$ receptor were tested for viability using MTT assay following PDMP treatment. MTT assay is a cell proliferation assay and provides estimate of the cell growth rate and viability of the cells. As shown in Fig. 7.2, treatment with 50 μM PDMP for 48 h reduced the cell number by ~70% with significant cell death. 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 (Fig. 7.2). Highest concentration of PDMP was used therefore decided to be 30 μM in all the experiments.

Cellular cholesterol content upon PDMP treatment

PDMP could exert effects other than inhibition of glycosphingolipid metabolism. For example, it has been reported earlier that PDMP alters cellular cholesterol homeostasis (Makino *et al.*, 2006). However, PDMP was observed to affect cholesterol esterification only in the presence of LDL (Makino *et al.*, 2006). As shown in Fig. 7.3, cholesterol levels were invariant in CHO-5-HT$_{1A}$R cells upon PDMP treatment showing that PDMP does not affect cholesterol homeostasis in present experimental conditions due to the absence of LDL (*i.e.*, serum free NBO medium).

Specific ligand binding is reduced upon metabolic depletion of glycosphingolipids

In order 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. 7.4 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.

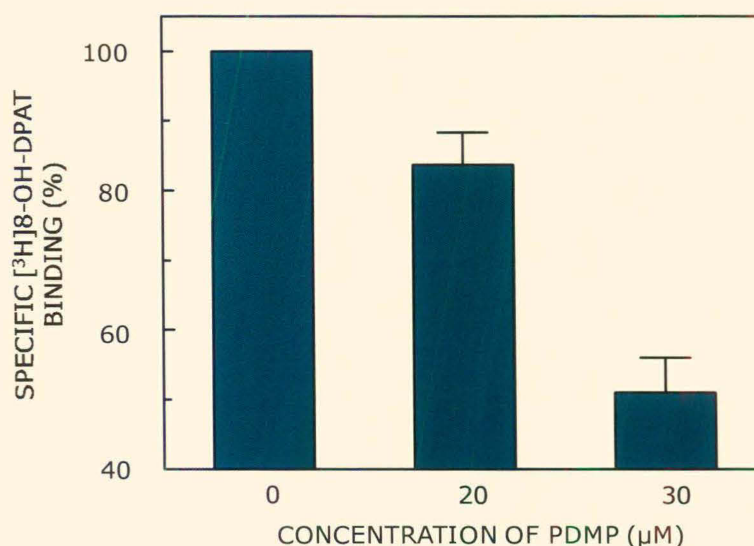


Figure 7.4. *Effect of metabolic depletion of glycosphingolipids on specific ligand binding of the human serotonin$_{1A}$ receptor.* CHO-5-HT$_{1A}$R cells were treated with PDMP and specific [^{3}H]8-OH-DPAT binding to the serotonin$_{1A}$ receptor was measured in membranes isolated from these cells. 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. See sections 2.2 and 7.2 for other details.

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 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.

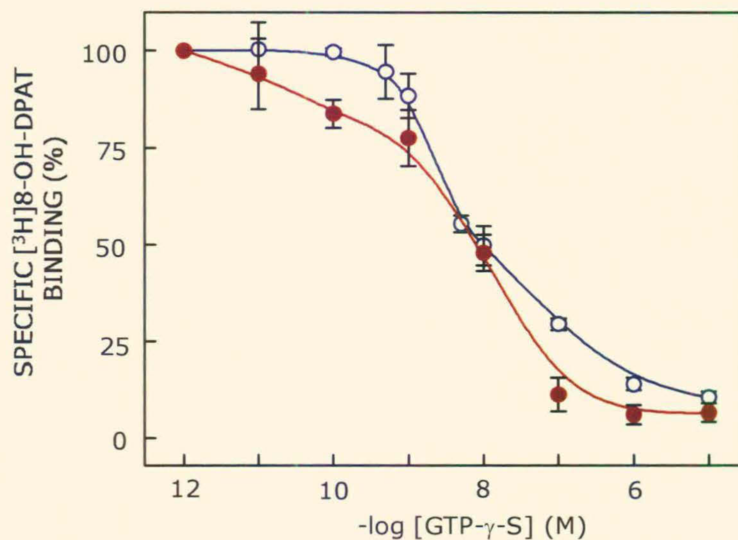


Figure 7.5. *Effect of metabolic depletion of glycosphingolipids on G-protein coupling of the human serotonin$_{1A}$ receptor.* G-protein coupling efficiency of the serotonin$_{1A}$ receptor was monitored by the sensitivity of specific [^{3}H]8-OH-DPAT binding in presence of GTP-γ-S, a non-hydrolyzable 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 nonlinear regression fits to the experimental data using eqn 7.1. Data points represent means $\pm$ SE of duplicate points from at least three independent experiments. See section 7.2 and Table 7.1 for other details.

It has previously been 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).

Table 7.1

Effect of Metabolic Glycosphingolipid Depletion on the Efficiency of G-protein Coupling[a]

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

[a]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 section 7.2 for other details.

Fig. 7.5 and Table 7.1 shows 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 inhibition curve (Fig. 7.5) does not exhibit appreciable shift when cells were treated with 30 μM PDMP and the corresponding IC_{50} value is 7.53 nM. The change in IC_{50} value was found to be not significant. This shows that G-protein coupling is not affected upon metabolic glycosphingolipid depletion by PDMP.

Saturation binding analysis

The reduction in the specific agonist [^{3}H]8-OH-DPAT binding to serotonin$_{1A}$ receptors (Fig. 7.4) could be either due to reduction in affinity of the receptor to the ligand or loss in ligand binding sites, or both.

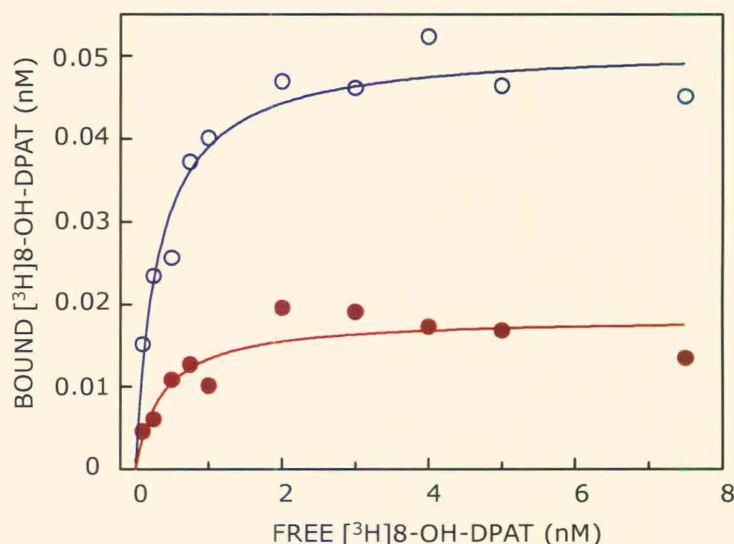


Figure 7.6. *Saturation binding analysis of specific [^{3}H]8-OH-DPAT binding to serotonin$_{1A}$ receptors from CHO-5-HT$_{1A}$R cell membranes upon glycosphingolipid depletion.* CHO-5-HT$_{1A}$R cells were treated with 30 μM PDMP and specific [^{3}H]8-OH-DPAT binding to serotonin$_{1A}$ receptors was 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 section 7.2 and Table 7.2 for other details.

Table 7.2

Effect of Metabolic Glycosphingolipid Depletion on the Specific [^{3}H]8-OH-DPAT Binding[b]

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[c]

[b]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 section 7.2 for other details.

[c]Corresponds to $p < 0.05$

Saturation binding analysis of [^{3}H]8-OH-DPAT to serotonin$_{1A}$ receptors is shown in Fig. 7.6 and Table 7.2. 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 7.2). 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.

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. 7.4) could be due to decrease in the expression levels of serotonin$_{1A}$ receptors in the cell membrane. Western blot analysis of 5-HT$_{1A}$R-EYFP was carried out in cell membranes prepared from control and PDMP-treated CHO-5-HT$_{1A}$R-EYFP cells (see Fig. 7.7) in order to monitor the receptor expression level upon glycosphingolipid depletion. Receptor tagged to EYFP (5-HT$_{1A}$R-EYFP) was chosen since 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). It has previously been 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.*, 2004a). 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 Figs. 7.4 and 7.8).

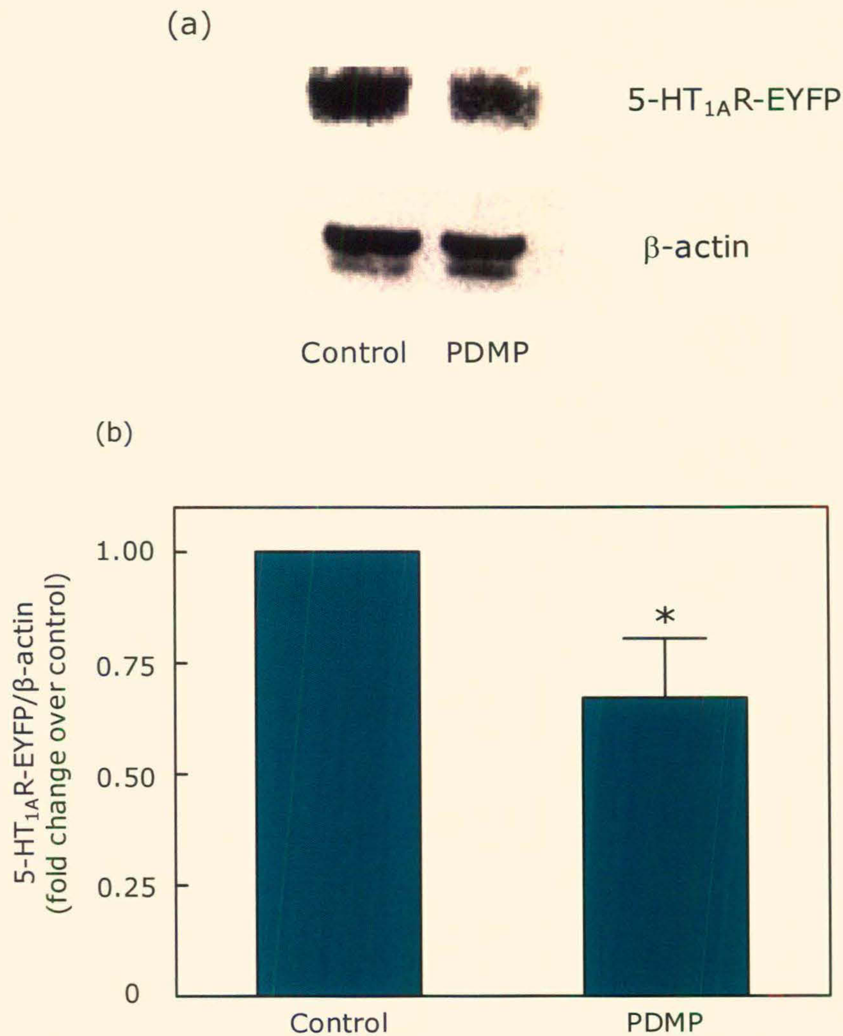


Figure 7.7. *Effect of metabolic depletion of glycosphingolipids on the expression level of the human serotonin$_{1A}$ receptor in membranes.* Western blot analysis of 5-HT$_{1A}$R-EYFP in membranes prepared from control and PDMP-treated CHO-5-HT$_{1A}$R-EYFP cells are shown. Panel (a) shows the human 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 section 7.2 for other details.

Fig. 7.7 shows that the receptor level in the cell membrane is reduced to ~67% ($p < 0.05$) of control value upon PDMP treatment, possibly due to 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).

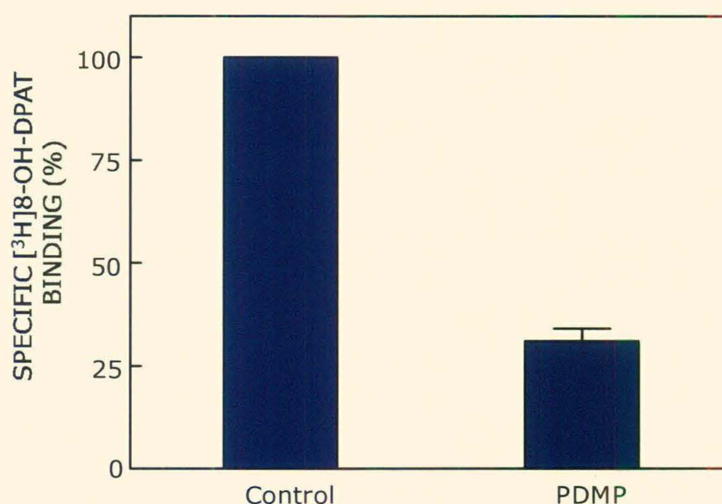


Figure 7.8. *Effect of metabolic depletion of glycosphingolipids on specific ligand binding of the human serotonin$_{1A}$ receptor tagged to EYFP.* CHO-5-HT$_{1A}$R-EYFP cells were treated with 30 μM PDMP and specific [^{3}H]8-OH-DPAT binding to the serotonin$_{1A}$ receptor was measured in membranes 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 sections 2.2 and 7.2 for other details.

Overall membrane order remains unaltered upon metabolic depletion of glycosphingolipids

These results indicate that the observed impairment in ligand binding of the serotonin$_{1A}$ receptor upon glycosphingolipid depletion is partly due to reduction in receptor expression level in the membrane. Alteration in membrane physical properties could lead to change in ligand binding (Gimpl *et al.*, 1997; Prasad *et al.*, 2009).

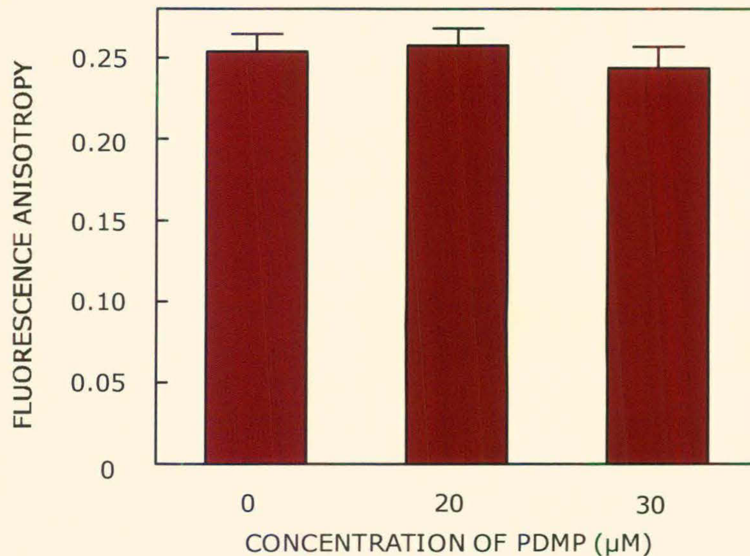


Figure 7.9. *Effect of metabolic depletion of glycosphingolipids on membrane order.* The overall (average) membrane order was estimated in membranes isolated from control and PDMP-treated cells by measurement of fluorescence anisotropy of the membrane probe DPH. Data represent means ± SE of duplicate points from at least three independent experiments. See section 2.2 for other details.

To monitor any possible change in overall membrane order upon PDMP treatment, anisotropy of the fluorescent probe DPH was determined 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. Fig. 7.9 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). Specific interactions between glycosphingolipids and

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

Replenishment of glycosphingolipids restores ligand binding

In order to monitor the reversibility of the effect of glycosphingolipids on the function of the serotonin$_{1A}$ receptor, CHO-5-HT$_{1A}$R cells were supplemented with sphingosine and ligand binding was monitored. Sphingosine is a catabolic intermediate of sphingolipids and can enter sphingolipid biosynthetic pathway *via* ceramide as shown in Fig. 7.1a.

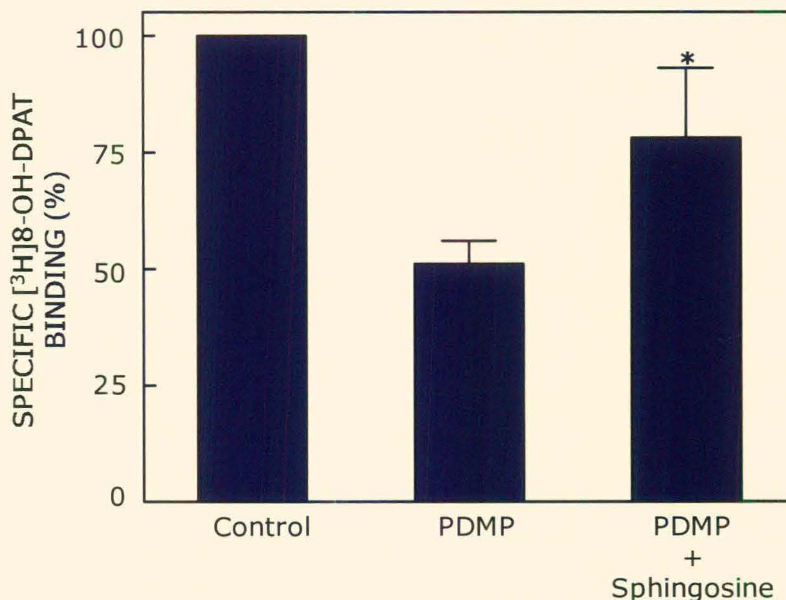


Figure 7.10. *Effect of replenishment of glycosphingolipids using sphingosine on specific agonist binding of the human serotonin$_{1A}$ receptor.* Following treatment with 30 μM PDMP in Nutridoma-BO (lipid deficient) medium, CHO-5-HT$_{1A}$R cells were grown for 24 h in D-MEM/F-12 complete medium supplemented 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 section 7.2 for other details.

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). Fig. 7.10 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 upon PDMP treatment and was restored to ~78% upon 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.

7.4. 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).

Since 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 since 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, ligand binding and G-protein coupling of the serotonin$_{1A}$ receptor was explored in CHO cells under condition of metabolic glycosphingolipid depletion using PDMP. These results show that ligand binding of the receptor is impaired under these conditions although the efficiency of G-protein coupling appears unaltered. Receptor expression level was found to be reduced at the cell membrane under these conditions that could partly account for the reduction in ligand binding. Interestingly, these 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 due to 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; Fantini and Barrantes, 2009; Chakrabandhu *et al.*, 2008; Hebbar *et al.*, 2008). It has recently been 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, SBD motif has been shown 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 due to specific interaction of the SBD motif with membrane glycosphingolipids. Interestingly, specific interaction between a single sphingolipid species and transmembrane domain of a receptor has been recently reported (Contreras *et al.*, 2012).

It has previously been shown that membrane cholesterol is necessary for the function of the serotonin$_{1A}$ receptor (Pucadyil and Chattopadhyay, 2004b, 2006; Paila *et al.*, 2008; Singh *et al.*, 2009; Shrivastava *et al.*, 2010; Paila and Chattopadhyay, 2010). The presence of cholesterol recognition/interaction amino acid consensus (CRAC) motifs in the serotonin$_{1A}$ receptor has recently been reported (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 interplay between these membrane lipids would be relevant. In summary, these 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.

Chapter 8

Conclusion and future perspectives

8.1. Conclusion

Seminal work in the last decade from our laboratory has established the cholesterol dependence on the function of serotonin$_{1A}$ receptors. Numerous approaches were employed to understand the molecular interactions between cholesterol and the serotonin$_{1A}$ receptor required for the proper function of the receptor. Our group showed that depletion of membrane cholesterol using MβCD led to reduction in the ligand binding function of serotonin$_{1A}$ receptors (Pucadyil and Chattopadhyay, 2004b, 2005). Importantly, the receptor function was restored upon replenishing membrane cholesterol (Pucadyil and Chattopadhyay, 2004b, 2005). Modulation of the function of GPCR upon cholesterol depletion could be attributed to alteration in the receptor structure mediated through (i) a direct/specific interaction with GPCRs, or (ii) an indirect way by altering membrane physical properties in which the receptor is embedded, or due to a combination of both (Gimpl *et al.*, 2002a,b). In order to test the stringency of cholesterol requirement for function of the serotonin$_{1A}$ receptor, we replenished cholesterol-depleted membranes with 7-DHC (Chapter 2, Singh *et al.*, 2007) and desmosterol (Chapter 3, Singh *et al.*, 2009). 7-DHC and desmosterol are positional isomers. They differ from cholesterol in only one extra double bond at 7th position in the sterol ring (7-DHC) and 24th position in flexible acyl chain (desmosterol). 7-DHC and desmosterol are the immediate precursors of cholesterol in the Kandutsch-Russell and Bloch pathways of cholesterol biosynthesis. Our results showed that 7-DHC and desmosterol do not support the ligand binding function of the serotonin$_{1A}$ receptor whereas overall membrane order is restored upon replenishment with these sterols (Chapter 2, 3; Singh *et al.*, 2007, 2009). These observations therefore demonstrate that cholesterol is specifically required for the serotonin$_{1A}$ receptor function.

Our laboratory previously monitored the serotonin$_{1A}$ receptor function upon sequestering membrane cholesterol using (ii) the sterol-complexing agent digitonin (Paila *et al.*, 2005), and (iii) the sterol-binding antifungal polyene antibiotic nystatin (Pucadyil *et al.*, 2004b). While MβCD physically depletes cholesterol from membranes, treatment with these agents makes membrane cholesterol 'unavailable' without physically depleting it. The function of serotonin$_{1A}$ receptors upon cholesterol sequestration was affected in the same manner as with cholesterol depletion by MβCD. These results therefore suggest that it is not merely physical presence of cholesterol but its 'availability', is required for the receptor function. Interestingly, oxidation of membrane cholesterol led to inhibition of the ligand binding activity of the serotonin$_{1A}$ receptor without altering overall membrane order (Pucadyil *et al.*, 2005b). Cholesterol oxidase catalyzes the oxidation of cholesterol to more hydrophobic cholestenone. In additon, function of the human serotonin$_{1A}$ receptor was shown to be reduced in cellular model of the Smith-Lemli-Opitz Syndrome (SLOS) (Paila *et al.*, 2008). In SLOS, cholesterol biosynthesis is dysregulated at the last step in the Kandutsch-Russell pathway leading to accumulation of 7-DHC in the cell. Taken together, these results support the specificity of cholesterol in the serotonin$_{1A}$ receptor function.

Our group earlier demonstrated that solubilization of the hippocampal serotonin$_{1A}$ receptor results in a reduction in specific ligand binding activity and overall membrane order (Chattopadhyay *et al.*, 2007). Solubilization of hippocampal serotonin$_{1A}$ receptor is accompanied by loss of membrane cholesterol. Replenishment of cholesterol to solubilized membranes significantly restores specific ligand binding activity and overall membrane order. Interestingly, similar to MβCD-treated hippocampal membranes, replenishment of solubilized hippocampal membranes with 7-DHC does not restore the serotonin$_{1A}$ receptor function. On the other hand, replenishment of

solubilized hippocampal membranes with desmosterol restores the function of serotonin$_{1A}$ receptor (Chapter 4, Singh *et al.*, 2011). These results are somewhat contradictory to our earlier observations (Chapter 3, Singh *et al.*, 2009), which appears to be due to ability of desmosterol to occupy nonannular sites in solubilized membranes. Unlike MβCD-treatment, solubilization leads to robust reorganization of hippocampal membranes that appears to be necessary for access of nonannular sites for desmosterol (Chapter 4, Singh *et al.*, 2011). Moreover, our group has recently identified cholesterol recognition/interaction amino acid consensus (CRAC) sequence 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). Interestingly, it has been suggested that cholesterol binding by CRAC motif is induced by a similar cleft located at the membrane interfacial region (Jamin *et al.*, 2005). Our group recently proposed that cholesterol binding sites in GPCRs could represent nonannular binding sites (Paila *et al.*, 2009). Taken together, these results indicate that nonannular cholesterol appears to be crucial for maintaining function of the serotonin$_{1A}$ receptor.

In order to further understand the role of cholesterol in hippocampal membranes, we explored the organization and dynamics of hippocampal membranes under varying membrane cholesterol and protein content in a depth dependent-manner by ESR spectroscopy. Our results showed that cholesterol increases membrane order at deeper hydrocarbon region as compared to interface in hippocampal membranes, whereas membrane proteins introduce disorder at the membrane interface with less effect at deeper hydrocarbon region (Chapter 5; Singh *et al.*, 2012). In addition, the structural specificity of sphingolipids for function of the human serotonin$_{1A}$ receptor was studied upon (i) removal of sphingomyelin headgroup (Chapter 6; Singh and

Chattopadhyay, 2012). (ii) metabolic depletion of glycosphingolipids (Chapter 7; Singh *et al.*, manuscript submitted). Sphingolipids, particularly glycosphingolipids and sphingomyelins are known to generate ordered domains together with cholesterol in membranes. Our group has recently demonstrated that metabolic depletion of total sphingolipids in CHO cells (stably expressing the human serotonin$_{1A}$ receptor) impaired the function of serotonin$_{1A}$ receptors (Paila *et al.*, 2010). Metabolic depletion of total glycosphingolipids and removal of sphingomyelin headgroup resulted in reduction in ligand binding and G-protein coupling of the serotonin$_{1A}$ receptor. These results show that sphingolipids are specifically required to support receptor function. Altogether, cholesterol and sphingolipids are specifically required for the receptor function. These observations are further corroborated with the fact that ~30% of serotonin$_{1A}$ receptor partitions in sphingolipid-cholesterol rich ordered domains (Renner *et al.*, 2007; Kalipatnapu and Chattopadhyay, 2004). In summary, our studies establish that lipid-protein interactions are crucial for different physiological functions such as ligand binding, G-protein coupling and signaling. Cholesterol and sphingolipid structures are highly fine-tuned and optimized to carry out their proper function in membranes. Even small tinkering in the structure (*i.e.*, extra double bond in case of 7-DHC and desmosterol, and lipid headgroup in case of glycosphingolipid and sphingomyelin) leads to serious physiological implications resulting in disorders such as SLOS, desmosterolosis and stress-like conditions.

8.2. Future Perspectives

8.2.1. Role of Cholesterol Homeostasis in Regulating the Function of Serotonin$_{1A}$ Receptors

Cholesterol is obtained in humans through diet and by *de novo* synthesis from acetate. It is present in free and esterified forms in cells. Most of the free form of cholesterol is located in the plasma membrane whereas excess of it is esterified by acylCoA:cholesterolacyltransferase (ACAT) enzyme and stored in the form of lipid droplets. Cholesterol homeostasis therefore is maintained by the activity of ACAT inside the cell (Ikonen, 2008). Levels of esterified and free cholesterol can be modulated with the help of ACAT inhibitor, Sandoz 58-035. Previous work from our laboratory has established that cholesterol is essential for the function of serotoin$_{1A}$ receptors. It would be interesting to explore the effect of alteration in cholesterol homeostasis on the serotonin$_{1A}$ receptor function.

8.2.2. Effect of Haloperidol on the Serotonin$_{1A}$ Receptor Biology

Serotonin$_{1A}$ receptors are implicated in many psychological disorders and agonists of them are employed in the treatment of certain anxiety-like behaviour (Griebel, 1999). Importantly, our laboratory has established the necessity of cholesterol for proper functioning of the serotonin$_{1A}$ receptor (Paila *et al.*, 2010). Haloperidol is an antipsychotic drug widely used in the treatment of psychological disorders such as schizophrenia and delirium. Its antipsychotic activity is thought to be partly due to its dopamine D_2 receptor antagonist properties (Miyamoto *et al.*, 2005). In addition, it has recently been shown to disrupt cholesterol-rich domains by inhibiting cholesterol biosynthesis (Sánchez-Wandelmer *et al.*, 2009, 2010). Studying the function of the serotonin$_{1A}$ receptor upon treatment with haloperidol therefore assumes

relevance. These studies would provide an insight on the regulation of the serotonin$_{1A}$ receptor function under psychotherapy and would also help in understanding the mechanism of action of haloperidol. These results would help in delineating the molecular link between serotonin$_{1A}$ receptor function and psychosis and would provide novel insight into the behavioural pattern of patients under rehabilitation.

8.2.3. Signaling and Lateral Dynamics of the Serotonin$_{1A}$ Receptor in Different Stages of Cell Cycle

Cell cycle is a ubiquitous and complex process essential for the growth and development of multicellular organisms. Interestingly, serotonin$_{1A}$ receptors are implicated in developmental disorders such as SLOS and desmosterolosis (Singh *et al.*, 2007, 2009; Paila *et al.*, 2008). Importantly, lateral dynamics of the serotonin$_{1A}$ receptor has recently been shown to correlate strongly with signaling of the receptor (Ganguly *et al.*, 2008). In addition, serotonergic signaling is shown to influence cell proliferation (Sharma *et al.*, 1999; Radley and Jacobs, 2002; Banasr *et al.*, 2004). It would therefore be intriguing to monitor the signaling and lateral dynamics of the serotonin$_{1A}$ receptor in cell cycle dependent manner.

8.2.4. Dynamics of Cysteine Mutants of Serotonin$_{1A}$ Receptors

As discussed earlier, among the predicted structural features of the serotonin$_{1A}$ receptor, palmitoylation status of the receptor has been confirmed in a recent report (Papoucheva *et al.*, 2004). Serotonin receptors are palmitoylated at two cysteine residues, 417 and 420 at the C-terminus of the receptor. An interesting aspect of this study is that palmitoylation of the serotonin$_{1A}$ receptor was found to be stable and independent of stimulation by

the agonist (Milligan *et al.*, 1995). Importantly, palmitoylation of the serotonin$_{1A}$ receptor is shown to be crucial for its localization in ordered membrane domains that in turn appears to regulate receptor-mediated signaling (Renner *et al.*, 2007). As mentioned earlier, lateral dynamics of the serotonin$_{1A}$ receptor correlates strongly with signaling of the serotonin$_{1A}$ receptor (Ganguly *et al.*, 2008). It would therefore be interesting to investigate the dynamics of cysteine mutants of the serotonin$_{1A}$ receptor, as their localization and function are shown to be impaired.

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