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ORIGINAL PAPER

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

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Abstract (1) The serotonin$_{1A}$ receptor is a G–protein coupled receptor involved in several cognitive, behavioral, and developmental functions. It binds the neurotransmitter serotonin and signals across the membrane through its interactions with heterotrimeric G–proteins. (2) Lipid–protein interactions in membranes play an important role in the assembly, stability, and function of membrane proteins. The role of membrane environment in serotonin$_{1A}$ receptor function is beginning to be addressed by exploring the consequences of lipid manipulations on the ligand binding and G–protein coupling of serotonin$_{1A}$ receptors, the ability to functionally solubilize the serotonin$_{1A}$ receptor, and the factors influencing the membrane organization of the serotonin$_{1A}$ receptor. (3) Recent developments involving the application of detergent–based and detergent–free approaches to understand the membrane organization of the serotonin$_{1A}$ receptor under conditions of ligand activation and modulation of membrane lipid content, with an emphasis on membrane cholesterol, are described.

Keywords Serotonin$_{1A}$ receptor · G-protein coupling · Solubilization · Membrane lipid environment · Cholesterol · Detergent insolubility · Detergent-free approach

Introduction

Biological membranes are complex non–covalent assemblies of a diverse variety of lipids and proteins. They impart an identity to the cell and its organelles and provide an

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appropriate milieu for the function of membrane proteins involved in signaling across the membrane, cell–cell recognition, and membrane transport. Since a significant portion of integral membrane proteins remains in contact with the membrane (Lee 2003), and reaction centers in them are often buried within the membrane, the function of membrane proteins often depends on the surrounding membrane environment. Lipid–protein interactions in membranes have attracted a lot of attention in relation to the role of such interactions in assembly, stability, and function of membrane proteins (Lee 2003, 2004; Palsdottir and Hunte 2004). These effects have been attributed either to specific interactions of lipids with residues in proteins or to bulk properties of membranes. Considering the diverse array of lipids in natural membranes, it is believed that physiologically relevant processes occurring in membranes involve an intense coordination of multiple lipid–protein interactions. Since the organization and dynamics of membranes have considerable impact on membrane protein structure and function (Burger et al. 2000; Pucadyil and Chattopadhyay 2006), the development and characterization of experimental tools to analyze these aspects of membranes assume significance.

It is estimated that membrane proteins make up ~30% of the total coding sequences in the human genome (Liu et al. 2002; Wallin and von Heijne 1998). The G–protein coupled receptors (GPCRs) are the largest class of molecules involved in signal transduction across membranes (Pierce et al. 2002), and represent a major fraction of membrane proteins. GPCRs are prototypical members of the family of seven transmembrane domain proteins and include >800 members which together constitute ~2% of the human genome (Fredriksson et al. 2003). They respond to a diverse variety of ligands and mediate multiple physiological processes and have therefore emerged as major targets for the development of novel drug candidates in all clinical areas (Nature reviews drug discovery GPCR questionnaire participants 2004; Insel et al. 2007). The serotonin$_{1A}$ receptor is a G–protein coupled receptor involved in several cognitive, behavioral, and developmental functions. It binds the intrinsically fluorescent (Chattopadhyay et al. 1996) neurotransmitter serotonin (5–HT or 5–hydroxytryptamine) and signals across the membrane through its interaction with heterotrimeric guanine nucleotide binding regulatory proteins (G–proteins; Clapham 1996; Milligan and Kostenis 2006) which are membrane associated signaling molecules on the cytoplasmic side of the membrane. This review describes recent developments contributing to the understanding that the membrane is an important modulator of the organization and function of the serotonin$_{1A}$ receptor.

The Serotonin$_{1a}$ Receptor: A Key Component in Serotonergic Signaling

The serotonin$_{1A}$ (5–HT$_{1A}$) receptor is an important member of the large family of serotonin receptors (Pucadyil et al. 2005a). Serotonin receptors have been classified into at least 14 subtypes on the basis of their pharmacological responses to specific ligands, sequence similarities at the gene and amino acid levels, gene organization, and second messenger coupling pathways (Hoyer et al. 2002). The serotonin$_{1A}$ receptor is the first among all the 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). Sequence analysis of this genomic clone (to be later identified as the serotonin$_{1A}$ receptor gene) indicated 43%

amino acid homology with the β_2–adrenergic receptor in the transmembrane domain (in a recent review, it has been stated that the serotonin$_{1A}$ receptor was the first orphan GPCR and it was also the first orphan to be ''deorphanized'' (Lefkowitz 2007)). While the gene was shown to be localized in chromosome 5 of the human genome and speculated to code for a potential member of the GPCR superfamily (Kobilka et al. 1987), its identity as a serotonin receptor was discovered only later (Fargin et al. 1988). Membranes prepared from COS–1 cells transiently transfected with G–21 showed typical ligand binding characteristics of the serotonin$_{1A}$ receptor. Subsequently, genes for the rat and mouse serotonin$_{1A}$ receptors have been cloned, and their amino acid sequences deduced (Albert et al. 1990; Charest et al. 1993). These developments facilitated stable expression and characterization of the receptor in a number of neural and non–neural cell lines (Banerjee et al. 1993; Newman–Tancredi et al. 1997; Kalipatnapu et al. 2004b; Paila and Chattopadhyay 2006). Furthermore, it was the first serotonin receptor for which polyclonal antibodies were obtained (Fargin et al. 1988; Pucadyil et al. 2005a) allowing their visualization at the subcellular level in various regions of the brain.

In addition, the availability of a selective ligand 8–OH–DPAT (8–hydroxy–2–(di–*N*–propylamino)tetralin) (Arvidsson et al. 1981; Gozlan et al. 1983), which acts as an agonist for the serotonin$_{1A}$ receptor, allowed extensive characterization of the serotonin$_{1A}$ receptor. 8–OH–DPAT (see Fig. 1 for chemical structure) displays high affinity (K_d = 0.3–1.8 nM) for the serotonin$_{1A}$ receptor isolated from various sources. It displays a typical sensitivity to GTP–γ–S, a non–hydrolyzable analogue of GTP, indicating that this ligand binds to the sub–population of receptors which are coupled to G–proteins (Harikumar and Chattopadhyay 1999; Javadekar–Subhedar and Chattopadhyay 2004; Kalipatnapu et al. 2004a). Selective antagonists for the serotonin$_{1A}$ receptor such as *p*–MPPI and WAY–100635 have been developed which display several fold selectivity for the serotonin$_{1A}$ receptor over other neurotransmitter receptors (see Caliendo et al. 2005 for a comprehensive review of ligands to the serotonin$_{1A}$ receptor). The selective antagonist for the serotonin$_{1A}$ receptor, *p*–MPPI, and its fluorinated analogue *p*–MPPF (see Fig. 1) (Kung et al. 1994, 1995) bind specifically to the serotonin$_{1A}$ receptor with high affinity (Kung et al. 1994; Harikumar and Chattopadhyay 2001; Kalipatnapu et al. 2004b). Moreover, binding of *p*–MPPF remains unaffected in

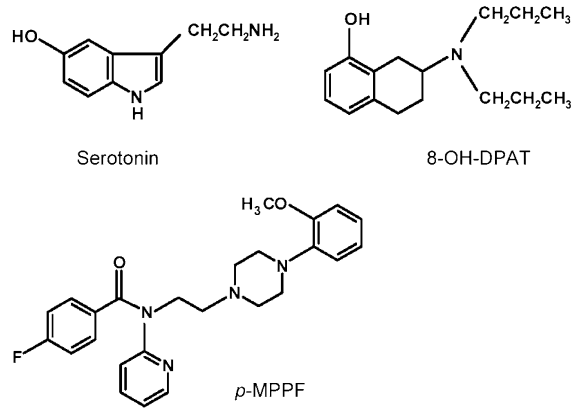

Fig. 1 Chemical structures of ligands that bind to the serotonin$_{1A}$ receptor

presence of GTP–γ–S indicating that they belong to the category of neutral antagonists, i.e., their binding does not require G–proteins to interact with the receptors (Harikumar and Chattopadhyay 1999). This differential discrimination of the agonist and antagonist binding to serotonin$_{1A}$ receptors could manifest in the lower adverse effects on the antagonist binding of serotonin$_{1A}$ receptors compared to the agonist binding at high–temperature which is likely to inactivate the G–proteins (Javadekar–Subhedar and Chattopadhyay 2004). Site–directed mutagenesis studies have indicated the amino acid residues of the serotonin$_{1A}$ receptor which are crucial for binding to various ligands (see Pucadyil et al. 2005a). In addition, experiments involving the effect of metal ions and alcohols have provided important information on the nature of the ligand–binding sites of the receptor. The agonist 8–OH–DPAT and the antagonist *p*–MPPF binding have been shown to be modulated by metal ions indicating that the ligand binding could be well–regulated by the ionic environment (Harikumar and Chattopadhyay, 1998a, 2001). It has been proposed that the agonist– and antagonist–binding sites could be overlapping but not identical in the bovine hippocampal serotonin$_{1A}$ receptor, an aspect that is apparent from the effects of ethanol (Harikumar and Chattopadhyay 1998b, 2000), and modifications of disulfide and sulfhydryl groups (Harikumar et al. 2000) on the agonist and antagonist binding of the serotonin$_{1A}$ receptor. Results from these experiments have suggested that the antagonist–binding site in the hippocampal serotonin$_{1A}$ receptor is localized in a more polar environment (perhaps at a shallower location in the membrane) than the agonist–binding site, which is known to be formed by residues present in the transmembrane domains of the receptor.

The human serotonin$_{1A}$ receptor is composed of 422 amino acids with a core molecular weight of ~46,000 (Raymond et al. 1999; Pucadyil et al. 2005a). 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 terminus facing the extracellular region and the carboxy terminus facing the intracellular cytoplasmic region (Raymond et al. 1999; Pucadyil et al. 2005a; see Fig. 2). The transmembrane domains (TM1–TM7) of the receptor are connected by hydrophilic sequences of three extracellular loops (EC1, EC2, EC3) and three intracellular loops (IC1, IC2, IC3). Such an arrangement is typical of the G–protein coupled receptor superfamily (Gether and Kobilka 1998). Although the structure of the serotonin$_{1A}$ receptor has not yet been experimentally determined, mutagenesis studies have helped in identifying amino acid residues important for ligand binding and G–protein coupling of the serotonin$_{1A}$ receptor (discussed 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). Palmitoylation of Cys–417 and Cys–420 of the heterologously expressed rat serotonin$_{1A}$ receptor, and its requirement in G–protein coupling and signaling of the serotonin$_{1A}$ receptor have been demonstrated in this report. 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 unusual for GPCRs which undergo repeated cycles of palmitoylation and depalmitoylation (Milligan et al. 1995). It has therefore been proposed that stable palmitoylation of the receptor could play an important role in maintaining the receptor structure (Papoucheva et al. 2004).

The serotonin$_{1A}$ receptor has recently been shown to have a role in neural development (del Olmo et al. 1998), and protection of stressed neuronal cells undergoing degeneration and apoptosis (Singh et al. 1996). Treatment using agonists

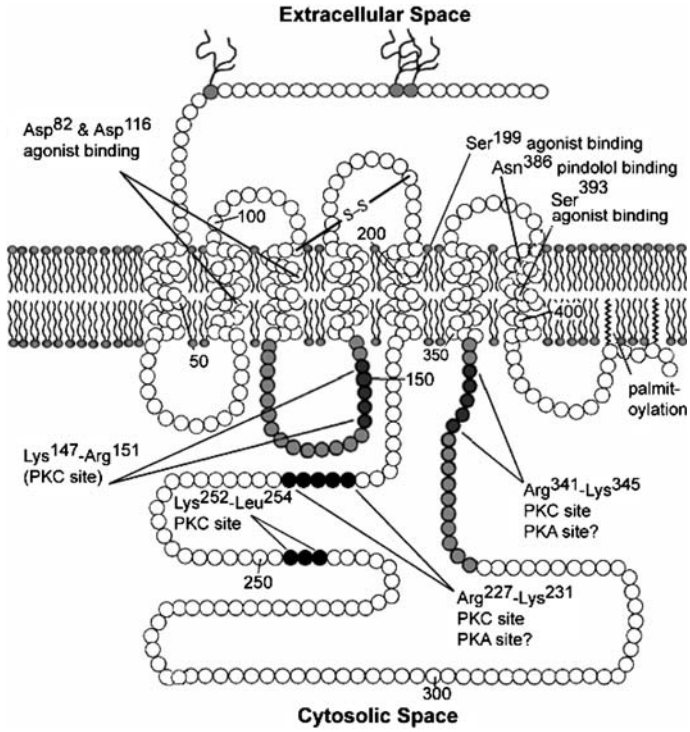

Fig. 2 A schematic representation of the membrane embedded human serotonin$_{1A}$ receptor showing its predicted topological and other structural features. The membrane is shown as a bilayer of two leaflets of lipids. The amino acids in the receptor sequence are shown as circles and are marked after every 50 residues for convenience. Seven transmembrane regions, each composed of 20–26 amino acids, are depicted as α–helices. There are three potential sites of *N*–linked glycosylation on the amino terminus (depicted as branching trees). A putative disulfide bond between Cys–109 and Cys–187 is shown. Transmembrane (TM) domains contain residues (which are marked) that are important for ligand binding. Putative palmitoylation sites are Cys–417 and/or Cys–420. Light gray circles represent contact sites for G–proteins. Black circles represent sites for protein kinase mediated phosphorylation. Adapted from Pucadyil et al. (2005a)

for the serotonin$_{1A}$ receptor constitutes a potentially useful approach in case of children with developmental disorders (Azmitia 2001). The serotonin$_{1A}$ receptor agonists and antagonists represent a major class of molecules with potential therapeutic effects in anxiety– or stress–related disorders (Pucadyil et al. 2005a). 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. Interestingly, mutant (knockout) mice lacking the serotonin$_{1A}$ receptor generated a few years back exhibit enhanced anxiety–related behavior (described in Julius 1998), and therefore the serotonin$_{1A}$ receptor knockout mouse serves as an excellent model system to understand anxiety–related behavior in higher animals (Toth 2003).

On the clinical front, serotonin$_{1A}$ receptor levels have been shown to be altered in schizophrenia, and in patients suffering from major depression (Pucadyil et al. 2005a). Interestingly, a recent observation has associated genetic polymorphisms at the upstream repressor region of the serotonin$_{1A}$ receptor gene to major depression and suicide in humans (Lemonde et al. 2003) linking its expression status to these clinical

syndromes. The selective serotonin$_{1A}$ receptor agonist 8–OH–DPAT has recently been shown to inhibit growth of *Plasmodium falciparum* (reviewed in Chattopadhyay and Kalipatnapu 2004) opening novel possibilities in antimalarial drug research. Besides, serotonin$_{1A}$ receptors are implicated in feeding, regulation of blood pressure, temperature, and working memory (Pucadyil et al. 2005a). Taken together, the serotonin$_{1A}$ receptor is a central player in a multitude of physiological processes, and an important drug target.

Membrane Biology of the Serotonin$_{1a}$ Receptor

The serotonin$_{1A}$ receptor is relatively abundant in the hippocampus of the brain (Palacios et al. 1990). Since the structure, organization and function of integral membrane proteins crucially depend on the membrane lipid composition and environment, native membranes prepared from bovine hippocampus represent an ideal natural source for the serotonin$_{1A}$ receptor. The biophysical (Mukherjee and Chattopadhyay 2005; Mukherjee et al. 2006) and biochemical (Pucadyil and Chattopadhyay 2004a) properties of such membranes have been well characterized. Further, mammalian cells in culture heterologously expressing serotonin$_{1A}$ receptors present a useful source of the receptor. Together, these systems have led to characterization of ligand binding, G–protein coupling, and signaling functions of the serotonin$_{1A}$ receptor, and more importantly, have provided novel information on the role of membrane environment in the function of this integral membrane protein. A brief overview of these studies is provided below.

Modulation of Ligand Binding and G-protein Coupling Functions of the Serotonin$_{1A}$ Receptor by the Membrane Environment

A large portion of any given transmembrane receptor remains in contact with the membrane lipid environment. This raises the obvious possibility that the membrane could be an important modulator of receptor structure and function (Burger et al. 2000). The receptor function could be influenced either by the bulk properties of the membrane or by specific interactions with membrane components. One of the approaches to comprehend the role of membrane environment is to monitor the receptor function by perturbing the membrane composition and/or properties. An example would be the use of alcohols or anesthetics which are thought to modulate membrane protein function either indirectly by changing the bulk properties of the membrane, or directly by binding to specific sites on membrane proteins. Local anesthetics of the tertiary amine group when used at clinically relevant concentrations have been found to inhibit specific agonist and antagonist binding of the serotonin$_{1A}$ receptor (Kalipatnapu and Chattopadhyay 2004a). In addition, local anesthetics were found to reduce the extent of interaction of the receptor with G–proteins. These results, along with fluorescence polarization studies with probes located at different depths in the membrane and ligand binding carried out after a significant alteration in the lipid composition of the membranes (i.e., cholesterol depletion), suggest interaction between the receptor and the local anesthetics as a probable mechanism of the action of local anesthetics.

In view of the significance of lipid–protein interactions in the assembly, stability and function of membrane proteins (Lee 2004; Palsdottir and Hunte 2004), understanding

organization of membranes and its relation to membrane protein function assumes significance. Monitoring lipid–protein interactions and determining specific lipid requirements of a membrane protein represent challenging tasks since very few membrane proteins have been purified to homogeneity. As a result, specific lipid requirements for membrane protein function have been reported in very few cases. Examples of membrane proteins whose function is shown to be affected by specific lipids include β–hydroxybutyrate dehydrogenase for the choline headgroup of phophatidylcholine, P–glycoprotein for lipids such as PC and PE, and the Ca^{2+}–ATPase for PE and cholesterol (reviewed in Opekarova and Tanner 2003). Further, neutral and anionic phospholipids have been shown to modulate the nicotinic acetylcholine receptor activity (Barrantes 2004).

In comparison to limited reports on specific lipid–protein interactions in purified systems, more information is beginning to be available for modulation of receptor function by membrane lipids in natural membranes. In particular, the role of cholesterol, an essential lipid in eukaryotic membranes, in the function of several membrane proteins and receptors from native and heterologous systems has been well addressed (Burger et al. 2000; Pucadyil and Chattopadhyay 2006). In a pioneering study, the modulatory role of cholesterol on the ligand binding activity and G–protein coupling of the bovine hippocampal serotonin$_{1A}$ receptor was shown by depleting cholesterol from native membranes using methyl–β–cyclodextrin (Pucadyil and Chattopadhyay 2004b). Removal of cholesterol from hippocampal membranes was found to reduce specific ligand binding and G–protein coupling of serotonin$_{1A}$ receptors. Importantly, replenishment of membranes with cholesterol led to recovery of ligand binding activity (Pucadyil and Chattopadhyay 2004b, 2005, 2006). The importance of receptor–cholesterol interaction in the function of the serotonin$_{1A}$ receptor is further emphasized by the observation that ligand binding function of the serotonin$_{1A}$ receptor could be modulated even by sequestering membrane cholesterol with agents such as digitonin (Paila et al. 2005) or nystatin (Pucadyil et al. 2004a). Making membrane cholesterol unavailable to the receptor therefore is found to affect the function of the serotonin$_{1A}$ receptor. Oxidation of membrane cholesterol significantly inhibits the specific binding of the agonist and antagonist to serotonin$_{1A}$ receptors (Pucadyil et al. 2005b), and replacement of membrane cholesterol with 7–dehydrocholesterol is found to be ineffective in restoring the ligand binding of the serotonin$_{1A}$ receptor (Singh et al. 2007). Taken together, these observations further emphasize the requirement of cholesterol in serotonin$_{1A}$ receptor function, and point to a possible specificity in the interaction of cholesterol with the serotonin$_{1A}$ receptor.

The results on the role of cholesterol in the serotonin$_{1A}$ receptor function could have significant implications in understanding the influence of the membrane lipid environment on the activity and signal transduction of other G–protein coupled transmembrane receptors. The clinical significance of membrane cholesterol levels resulting in receptor dysfunction has been aptly exemplified in the case of cholecystokinin (CCK) receptors (Xiao et al. 2000). Thus, agonist binding is reduced and G–protein coupling affected for CCK receptors isolated from muscle tissues in human gallbladders with cholesterol stones. These effects are reversed upon treatment with cholesterol–free liposomes. In the Smith–Lemli–Opitz syndrome, 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). Interestingly, certain types of mood and anxiety disorders are characterized by

symptoms that are similar to those which appear upon disruption of serotonergic signaling (Papakostas et al. 2004). The interaction between cholesterol and other molecular components (such as receptors) in neuronal membranes such as the bovine hippocampal membranes therefore assumes relevance for a comprehensive understanding of brain function (Chattopadhyay and Paila 2007).

Functional Solubilization of Serotonin$_{1A}$ Receptors

Membrane protein purification represents an area of considerable challenge in contemporary membrane biology. Studies carried out on purified and reconstituted membrane receptors have considerably advanced our knowledge of the molecular aspects of receptor function (Gether 2000). It is noteworthy that none of the subtypes of G–protein coupled serotonin receptors have yet been purified to homogeneity from natural sources. An essential criterion for purification of an integral membrane protein is that the protein must be carefully removed from the native membrane and individually dispersed in solution. This process is known as solubilization and is most effectively accomplished using amphiphilic detergents (Garavito and Ferguson–Miller 2001; Kalipatnapu and Chattopadhyay 2005a). Solubilization of a membrane protein is a process in which the proteins and lipids that are held together in the native membrane 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. Effective solubilization and purification of G–protein coupled receptors in a functionally active form represent important steps in understanding structure–function relationship and pharmacological characterization of a specific receptor. Yet, 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). This is the main reason for the rather modest list of membrane proteins which have been solubilized with retention of function.

Critical factors affecting solubilization include appropriate choice of detergent and the concentration at which it is used. Detergents self associate to form non–covalent aggregates (micelles) above a narrow range of concentration referred to as the critical micelle concentration (CMC). While detergents can be most effective when used beyond their CMC, loss of function of the protein of interest could occur at such high concentrations. However, the phenomenon of reduction in the CMC of a charged detergent upon addition of salts can be exploited to achieve functional solubilization of membrane proteins. The resultant 'effective CMC' of the detergent takes into account contributions from other components in the system (such as lipids, proteins, ionic strength, pH, temperature) and its determination can be useful in optimizing solubilization conditions (Chattopadhyay and Harikumar 1996). A low ('pre–micellar') concentration of the mild and non–denaturing, zwitterionic detergent CHAPS (3–[(3–cholamidopropyl)–dimethylammonio]–1–propanesulfonate) has been used for solubilizing serotonin$_{1A}$ receptors in presence of salt followed by polyethylene glycol precipitation to remove the salt (Chattopadhyay and Harikumar 1996; Chattopadhyay et al. 2002, 2004). This has resulted in efficient solubilization of serotonin$_{1A}$ receptors with a high ligand binding affinity and ability to couple to G–proteins. As high concentrations of CHAPS are known to cause dissociation of G–protein subunits from the membrane (Jones and Garrison 1999; Kalipatnapu and Chattopadhyay 2005a), the use of salt to effectively lower the concentration required to achieve optimal

solubilization of the serotonin$_{1A}$ receptor therefore represents an elegant approach. Efficient solubilization of the receptor from the native source with high ligand binding affinity and intact signal transduction components may constitute the first step in the molecular characterization of this G–protein coupled receptor.

The choice of the detergent CHAPS and its ability to solubilize serotonin$_{1A}$ receptors from bovine hippocampal membranes, which is not achieved optimally using other detergents (Harikumar and Chattopadhyay, unpublished observations), brings to light the importance of membrane lipids in maintaining the function of membrane proteins. In fact, it has earlier been shown that different classes of detergents used for solubilization of membrane receptors result in differential solubilization of lipids and proteins (Banerjee et al. 1995) since some detergents even extract some of the 'annular' lipids necessary for preserving the function of the receptor (Jones et al. 1988). This could result in a solubilized but non–functional receptor. The importance of the immediate lipid environment of the membrane protein therefore has to be kept in mind while choosing the appropriate detergent for optimal solubilization with retention of function.

One of the basic demonstrations of the importance of membrane environment in membrane protein function is the decrease in membrane protein activity upon delipidation of membranes (Jones et al. 1988; Chattopadhyay et al. 2005), a common consequence of the process of solubilization. Considering the significance of lipid–protein interactions in maintaining the structure and function of biological membranes (Lee 2004; Palsdottir and Hunte 2004), it is conceivable that replacement of a specific lipid environment with detergent or detergent–lipid complex during solubilization could affect the function of a membrane protein. For example, displacement of lipids from the receptor has been shown to be an integral feature of detergent–induced inactivation in case of the nicotinic acetylcholine receptor (Jones et al. 1988). The phenomenon of delipidation and its consequences on activity of solubilized membrane proteins have previously been utilized to gain insight into the specific lipid requirements of membrane proteins (Jones et al. 1988; Kalipatnapu and Chattopadhyay 2005a). It is possible that the ability of a detergent to solubilize a membrane protein in its functional state depends on cosolubilization of certain membrane lipids. While CHAPS can efficiently solubilize serotonin$_{1A}$ receptors from bovine hippocampus in a functionally active form (Chattopadhyay and Harikumar 1996; Chattopadhyay et al. 2002), a fraction of functional receptors is lost during solubilization. This could either be due to inability of the detergent to solubilize those receptors or could be a consequence of delipidation of the receptor. Solubilization of the hippocampal serotonin$_{1A}$ receptors by CHAPS has been shown to be accompanied by loss of membrane cholesterol (Banerjee et al. 1995; Chattopadhyay et al. 2005). Since the role of cholesterol in modulation of ligand binding and G–protein coupling of the hippocampal serotonin$_{1A}$ receptor has been demonstrated earlier (Pucadyil and Chattopadhyay 2004b; Paila et al. 2005; Pucadyil and Chattopadhyay 2006), it is possible that the apparent loss in activity of the solubilized receptor could be due to loss of cholesterol. This proposal has recently been tested by incorporating cholesterol in bovine hippocampal membranes solubilized in presence of CHAPS and NaCl. Interestingly, replenishment of membrane cholesterol to solubilized bovine hippocampal membranes resulted in an increase in ligand binding of the serotonin$_{1A}$ receptor (Chattopadhyay et al. 2005). This further reinforces the importance of the membrane lipid environment in general, and membrane cholesterol in particular, in the function of the serotonin$_{1A}$ receptor.

Monitoring Membrane Organization of the Serotonin$_{1A}$ Receptor by Detergent-Based and Detergent-Free Approaches

The understanding of how lipids and proteins are organized in cellular membranes has undergone significant changes beginning with the Singer and Nicolson's fluid mosaic model (see Edidin 2003 for a historical perspective). The fluid mosaic model for cell membranes (Singer and Nicolson 1972) visualized a largely fluid membrane bilayer in which proteins are embedded. This model proposed a dynamic bilayer with free translational diffusion of lipids and proteins and possible interactions between them, and a restricted movement of the membrane components across the bilayer which would preserve asymmetry of the bilayer. Some of the tenets set by this model were later modified with results from several laboratories (Jacobson et al. 1995; Edidin 2003) favoring non–random organization of lipids and proteins, i.e., heterogeneities (domains) in the membrane. Current understanding of membranes involves membrane domains with defined lipid and protein compositions, although resolving the spatiotemporal resolution of these domains is proving to be challenging (Mukherjee and Maxfield 2004; Jacobson et al. 2007). These domains, sometimes referred to as 'rafts', are believed to serve as platforms for signaling by concentrating certain lipids (such as cholesterol and sphingolipids) and proteins while excluding others (Simons and Ikonen 1997; Mukherjee and Maxfield 2004; Jacobson et al. 2007). Organization of membranes into domains could play a key role in a number of processes such as membrane trafficking, sorting, signal transduction, and pathogen entry (Simons and Toomre 2000; van der Goot and Harder 2001; Mukherjee and Maxfield 2004; Pucadyil and Chattopadhyay 2007a).

The implication of membrane organization on the signaling functions of membrane proteins in general, and on G–protein coupled receptors in particular, represents an interesting aspect. The classical view of receptor–G–protein function in cells proposes free diffusion of molecules on the cell surface and that their interaction would depend on random collisions, although the actual sites of interaction are specific (Neubig 1994). The specific and rapid signaling responses characteristic of GPCR activation appear to be difficult to explain, based on uniform distribution of the receptors, G–proteins, and effectors—one or more of which could even be low in abundance on the cell surface (Huang et al. 1997; Ostrom and Insel 2004). This leads to the possibility that receptor–G–protein interactions may be dependent on their organization in membranes and not solely on the binding sites present on the interacting proteins. Spatiotemporal organization and dynamic confinement of receptors and effector molecules on the plasma membrane microdomains is now therefore believed to be an important determinant in GPCR signaling (Neubig 1994; Hur and Kim 2002).

The role of membrane domains in the organization and function of the G–protein coupled serotonin$_{1A}$ receptor assumes relevance against this backdrop. This issue has been recently addressed employing the biochemical criterion of detergent insolubility. Resistance to solubilization by mild non–ionic detergents such as Triton X–100 at low temperature has emerged as an extensively used biochemical tool to identify, isolate and characterize certain types of membrane domains (Brown and Rose 1992; Brown and London 1998; Chamberlain 2004). The tight acyl chain packing of sphingolipids and saturated lipids is thought to confer detergent resistance to membrane regions enriched in these lipids and to the proteins which reside in them. Insolubility in cold Triton X–100 has therefore been increasingly used as a hallmark of the presence of 'rafts', the class of membrane domains enriched in sphingolipids and cholesterol (Brown and London 1998;

Chamberlain 2004). Several GPI–anchored proteins, few transmembrane proteins and certain G–proteins have been found to reside in detergent resistant membrane domains, popularly referred to as DRMs (Brown and Rose 1992; Brown and London 1998; Chamberlain 2004).

Detergent insolubility of the serotonin$_{1A}$ receptor has been monitored by a fluorescence–based approach using the serotonin$_{1A}$ receptor fused to the enhanced yellow fluorescent protein (EYFP) stably expressed in CHO cells (Kalipatnapu and Chattopadhyay 2004b). Importantly, the ligand binding properties of the serotonin$_{1A}$ receptor were found to be unaltered upon EYFP fusion (Pucadyil et al. 2004b). Detergent insolubility of serotonin$_{1A}$ receptors was assessed by treatment of cells in culture with cold Triton X–100 followed by quantitation of the residual fluorescence of the receptor (Kalipatnapu and Chattopadhyay 2004b, see Fig. 3). These results indicate that a small fraction of serotonin$_{1A}$ receptors is insoluble in the detergent as monitored by the residual fluorescence upon detergent treatment. In order to validate this fluorescence microscopic approach toward determination of detergent insolubility of membrane components, specific lipid (phase–sensitive dialkylindocarbocyanine (DiI) probes) and protein (transferrin receptor) markers were used whose organization in membranes and ability to be extracted by cold non–ionic detergents have been well documented (Mayor and Maxfield 1995; Mukherjee et al. 1998). The DiI series of lipid analogues have been shown to exhibit preferential phase partitioning into biological and model membranes of varying degrees of order (fluidity) depending on the relative headgroup to tail cross–sectional areas and the chain length (Klausner and Wolf 1980; Spink et al. 1990; Mukherjee et al. 1998). For example, DiIC$_{16}$ with its two 16–carbon saturated alkyl chains preferentially partitions into relatively rigid (highly ordered) domains, whereas *FAST* DiI which has two 18–carbon chains with two *cis* double bonds in each chain preferentially partitions into fluid domains in membranes (Mukherjee et al. 1998). Results obtained from these experiments showed that this method is capable of distinguishing ordered domains labeled by DiIC$_{16}$ (1,1′–dihexadecyl–3,3,3′,3′–tetramethylindocarbocyanine perchlorate) from the fluid regions of the

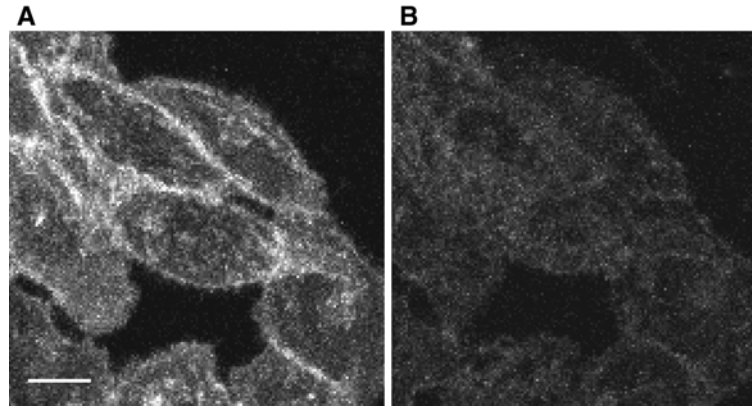

Fig. 3 Detergent insolubility of the serotonin$_{1A}$ receptor fused to EYFP. Cells expressing serotonin$_{1A}$–EYFP receptors are shown (**A**) before and (**B**) after treatment with cold Triton X–100 (0.05%, w/v) for 10 min. The images represent combined mid–plane confocal sections of the same group of cells before and after detergent extraction. The scale bar represents 10 lm. Reproduced from Kalipatnapu and Chattopadhyay (2004b)

membrane characterized by *FAST* DiI (1,1′–dilinoleyl–3,3,3′,3′–tetramethylindocarbocyanine perchlorate) labeling (Kalipatnapu and Chattopadhyay 2004b). These results, along with the observation of low detergent insolubility of transferrin receptor, validated the novel observation of detergent insolubility of the serotonin$_{1A}$ receptor in particular and GFP fluorescence–based approach in general (Kalipatnapu and Chattopadhyay 2004b). These experiments represent one of the first attempts to address membrane organization of the serotonin$_{1A}$ receptor. Importantly, the fluorescence–based approach to monitor detergent insolubility can be potentially useful in exploring membrane organization of other G–protein coupled receptors.

Detergent insolubility has been a principal tool in the isolation and characterization of membrane domains. However, the issue of whether use of detergent merely helps in isolation of membrane domains, or induces their formation continues to be a cause for concern (Heerklotz 2002; Edidin 2003). Moreover, weak but essential interactions of proteins with membrane domains may be difficult to identify in the presence of detergents. In order to avoid the limitations of detergent–based methods, biochemical approaches which do not require detergents for the isolation of membrane domains have been proposed (Smart et al. 1995; Song et al. 1996; Luria et al. 2002; Macdonald and Pike 2005). These approaches involve milder treatments such as mild sonication and/or extraction with sodium carbonate. The membrane organization of the serotonin$_{1A}$ receptor has been probed employing a detergent–free method. This method (Luria et al. 2002) has previously been shown to give rise to membrane fractions which correspond to those isolated employing detergents such as Triton X–100. Results from these experiments indicated a distinct enrichment of the serotonin$_{1A}$ receptor in the heavy membrane fraction over that of the light membrane fraction as monitored by ligand binding assays (Kalipatnapu and Chattopadhyay 2007, see Fig. 4). The light fraction isolated by this detergent–free method has previously been shown to resemble detergent–resistant membranes in terms of its lipid and protein composition (Luria et al. 2002). The earlier findings on detergent insolubility of the serotonin$_{1A}$ receptor fused to EYFP suggest a small fraction of the receptor to be insoluble in Triton X–100 (Kalipatnapu and Chattopadhyay 2004b), indicating a relatively large fraction to be soluble in the detergent. This is probably reflected in the higher ligand binding in case of the heavy membrane fraction compared to the light fraction isolated by the detergent–free method. Further, a similar overall trend was observed whether hippocampal membranes, or a heterologous expression system were used for the analysis (Kalipatnapu and Chattopadhyay 2007).

The results from detergent–free approach therefore correlate well with the findings on the detergent insolubility of the serotonin$_{1A}$ receptor fused to EYFP. An overall analysis of reports where detergent–based and detergent–free methods of membrane domain isolation have been compared earlier in the literature presents a somewhat varied picture. Domains isolated by these two approaches have been shown to display overlapping characteristics involving lipid and protein composition, and physical properties (Luria et al. 2002; Gaus et al. 2005). On the other hand, there are reports suggesting membrane domains prepared in the presence or absence of detergents could have different constituent lipids and proteins. For example, the epidermal growth factor receptor is found to be soluble in Triton X–100, but found to localize in membrane domains when assessed using a detergent–free method (Pike et al. 2005). In this context, it is only appropriate that the membrane localization of the serotonin$_{1A}$ receptor be monitored using both detergent–based as well as detergent–free approaches. The fact that similar observations on the membrane localization of the serotonin$_{1A}$ receptor are

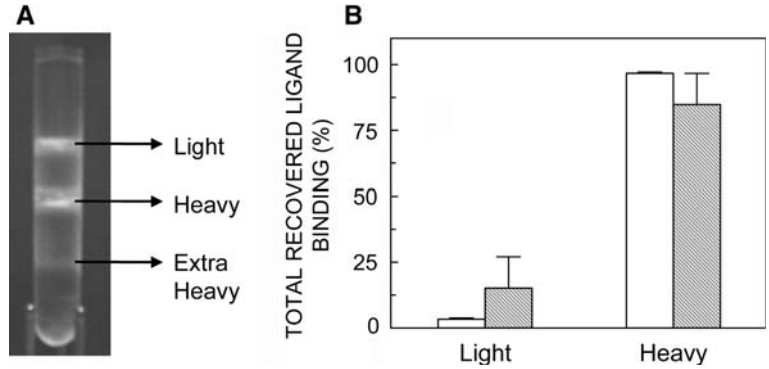

Fig. 4 Isolation and analysis of membrane fractions from bovine hippocampal membranes using a detergent–free method to isolate membrane domains. Panel A shows the typical pattern of isolation of light, heavy, and extra heavy membrane fractions from hippocampal membranes on a sucrose density gradient using the detergent–free method of Luria et al. (2002). The membrane fraction at 10–22.5% sucrose interface is designated as 'light', at 22.5–35% sucrose interface as 'heavy', and a faintly visible fraction at 35–40% sucrose interface as 'extra heavy'. The light and heavy membrane fractions have been shown to be derived primarily from the plasma membrane, whereas the extra heavy fraction is shown to be mainly from intracellular components (Monneron and d'Alayer 1978; Luria et al. 2002). Comparison of ligand binding to serotonin$_{1A}$ receptors from the light and heavy membrane fractions isolated using a detergent–free method from native hippocampal membranes is shown in panel B. The white bars represent the binding of the agonist [^{3}H]8–OH–DPAT and the shaded bars that of the antagonist [^{3}H]*p*–MPPF. Data obtained from radioligand binding assays have been represented as a percentage of the total recovered ligand binding obtained from the light and heavy membrane fractions in order to appreciate the distribution of serotonin$_{1A}$ receptors among the light and heavy membrane fractions. The data points represent means ± SD of duplicate points from three independent experiments. Adapted and modified from Kalipatnapu and Chattopadhyay (2007)

made using the detergent–free approach further support the GFP–fluorescence–based approach to monitor detergent insolubility of the receptor.

The detergent insolubility of the serotonin$_{1A}$ receptor fused to EYFP (5–HT$_{1A}$R–-EYFP) has been monitored utilizing the GFP fluorescence–based approach under conditions of reduced membrane cholesterol and upon activation by ligand (Kalipatnapu and Chattopadhyay 2005b). Based on these experiments, the detergent insolubility of the serotonin$_{1A}$ receptor was found to increase upon depletion of membrane cholesterol, and no significant change in its detergent insolubility was observed upon activation by its endogenous ligand serotonin. Cholesterol is often found to be distributed non–randomly in domains or pools in biological and model membranes (Liscum and Underwood 1995; Simons and Ikonen 2000; Rukmini et al. 2001). Based on the proposed role of cholesterol in maintaining the domain organization of membranes, depletion of cholesterol is believed to cause disruption of such domains resulting in an increased extraction of proteins residing in the domain (Edidin 2001). Several examples are known where decreased detergent insolubility of membrane proteins has been observed upon depletion of membrane cholesterol (Field et al. 1995; Harder et al. 1998). In addition, the lateral mobility of certain proteins generally found in detergent resistant membrane domains has been reported to be increased upon lowering membrane cholesterol content (Pralle et al. 2000; Shvartsman et al. 2003), further supporting this model. Interestingly, in contrast to these observations, there are reports indicating a decrease in lateral mobility of membrane components upon lowering membrane cholesterol levels (Hao et al. 2001; Vrljic et al. 2005). This is consistent with

the observation that cholesterol depletion from lipid vesicles originally present in a uniform liquid phase leads to separation of phases as monitored by the distribution of fluorescent lipid probes (Veatch and Keller 2003). Similarly, reduction in membrane cholesterol content was shown to induce formation of micrometer–scale domains on the cell surface visualized by fluorescent lipid probes with preferential phase partitioning properties (Hao et al. 2001). These results, along with evidence from model membrane studies (Veatch and Keller 2003), have given rise to the proposal that cholesterol, while maintaining domain organization in membranes, could also be involved in reducing immiscibility of domains. Hence, reduction in cholesterol levels may induce domain segregation (Mukherjee and Maxfield 2004). In such a scenario, cholesterol depletion could lead to segregation of ordered domains on the cell surface, into which a slightly greater fraction of 5–HT_{1A}R–EYFP may be included, resulting in an increase in the relative detergent insoluble fraction of the 5–HT_{1A}R–EYFP. The increase in detergent insolubility of the $serotonin_{1A}$ receptor under conditions of reduced cholesterol can therefore be interpreted based on this model of formation of large sized ordered domains upon cholesterol depletion. This interpretation is supported by a recent report in which cholesterol depletion was found to induce dynamic confinement of the $serotonin_{1A}$ receptor on the plasma membrane, monitored by fluorescence recovery after photobleaching (FRAP) measurements using variable bleach spot radii (Pucadyil and Chattopadhyay 2007b). These results on detergent insolubility and diffusion parameters of the $serotonin_{1A}$ receptor during normal and cholesterol–depleted conditions provide novel information on the membrane organization of the $serotonin_{1A}$ receptor.

The localization of G–protein coupled receptors in membrane domains has attracted a lot of attention in recent years due to its possible implications in the signaling functions of the receptors (Ostrom and Insel 2004; Chini and Parenti 2004). For example, coupling efficacy of β_1 and β_2–adrenergic receptors (β_1AR and β_2AR) and prostaglandin E2 receptors to adenylate cyclase (AC6) correlates with their colocalization or lack of it with AC6 in caveolae (Ostrom et al. 2001). Upon exposure to agonist, β_2AR, but not β_1AR, is found to translocate out of caveolin–rich fractions (Rybin et al. 2000). Such an agonist–dependent spatial segregation of the receptor and effector on the cell surface could explain lower efficacy of β_2AR coupling to its effector AC6 compared to β_1AR (Ostrom et al. 2001). Similar agonist dependent association of receptors and cognate G–proteins has been shown in the case of bradykinin receptors (de Weerd and Leeb–Lundberg 1997). In case of the $serotonin_{1A}$ receptor, it appears that there is no specific change in the membrane organization of the receptor when activated by serotonin as assessed by the phenomenon of detergent insolubility (Kalipatnapu and Chattopadhyay 2005b). In addition, stimulation by serotonin has not been found to result in any significant difference in the fluorescence distribution of $serotonin_{1A}$ receptor fused to EYFP (Pucadyil et al. 2004b). However, a significant increase in the lateral mobility of the $serotonin_{1A}$ receptor fused to EYFP has been shown using fluorescence recovery after photobleaching (FRAP) (Pucadyil et al. 2004b; Pucadyil and Chattopadhyay 2007c). Based on all these results, it appears that while the membrane dynamics (diffusion) of the $serotonin_{1A}$ receptor could be modulated in the presence of serotonin, fluorescence distribution and detergent insolubility measurements do not indicate any apparent cell surface reorganization of the receptor when stimulated by serotonin.

Conclusion

The serotonin$_{1A}$ receptor is an important representative of the G–protein coupled receptor family involved in a multitude of physiological functions. Although the pharmacological and signaling features of the serotonin$_{1A}$ receptor have been extensively studied, aspects related to the membrane organization and function of this integral membrane protein have not been addressed until the last few years. As in the case of many other membrane proteins, low expression levels of the serotonin$_{1A}$ receptor in natural membranes, and inherent difficulties in purifying membrane proteins have posed considerable challenges in addressing various issues related to membrane biology of the serotonin$_{1A}$ receptor. Nonetheless, natural membranes and cultured cells heterologously expressing the serotonin$_{1A}$ receptor together have made it possible to address important aspects related to membrane organization and function of the serotonin$_{1A}$ receptor. Some of these recent exciting developments involving the membrane localization of the serotonin$_{1A}$ receptor and the importance of membrane lipids such as cholesterol in the receptor function have been described in this review. It has recently been possible to purify the serotonin$_{1A}$ receptor from *Xenopus laevis* employing a novel expression strategy (Zhang et al. 2005) further opening the field. A comprehensive understanding of the serotonin$_{1A}$ receptor function in relation to its membrane lipid environment is important in view of the enormous implications of the serotonin$_{1A}$ receptor function in human health (Julius 1998), and the observation that several diagnosed brain diseases are attributed to altered lipid–protein interactions (Pavlidis et al. 1994; Chattopadhyay and Paila 2007).

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