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Review

Role of cholesterol in the function and organization of G-protein coupled receptors

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Abstract

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics and function. The modulatory role of cholesterol in the function of a number of membrane proteins is well established. This effect has been proposed to occur either due to a specific molecular interaction between cholesterol and membrane proteins or due to alterations in the membrane physical properties induced by the presence of cholesterol. The contemporary view regarding heterogeneity in cholesterol distribution in membrane domains that sequester certain types of membrane proteins while excluding others has further contributed to its significance in membrane protein function. The seven transmembrane domain G-protein coupled receptors (GPCRs) are among the largest protein families in mammals and represent ~2% of the total proteins coded by the human genome. Signal transduction events mediated by this class of proteins are the primary means by which cells communicate with and respond to their external environment. GPCRs therefore represent major targets for the development of novel drug candidates in all clinical areas. In view of their importance in cellular signaling, the interaction of cholesterol with such receptors represents an important determinant in functional studies of such receptors. This review focuses on the effect of cholesterol on the membrane organization and function of

Abbreviations: 8-OH-DPAT, 8-hydroxy-2(di-*N*-propylamino)tetralin; AIDS, acquired immunodeficiency syndrome; CCR5, CC-chemokine receptor-5; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CHAPSO, 3-[(cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; CHO, Chinese hamster ovary; CMC, critical micelle concentration; CXCR4, CXC-chemokine receptor-4; DPH, 1,6-diphenyl-1,3,5-hexatriene; EC, extracellular; EYFP, enhanced yellow fluorescent protein; FAD, flavin adenine dinucleotide; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; GABA, γ-amino butyric acid; GDP, guanosine-5′-diphosphate; GnRH, gonadotropin-releasing hormone; GPCR, G-protein coupled receptor; GRAFS, glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin system of classification of GPCRs; GTP, guanosine-5′-triphosphate; GTP-γ-S, guanosine-5′-*O*-(3-thiotriphosphate); HCMV, human cytomegalovirus; HIV-1, human immunodeficiency virus-1; HMG-CoA, hydroxymethylglutaryl-coenzyme A; IC, intracellular; KSHV, Kaposi's sarcoma associated herpesvirus; L_d, liquid-crystalline; LDL, low-density lipoprotein; LH, leutinizing hormone; L_o, liquid-ordered; MβCD, methyl-β-cyclodextrin; mGluR, metabotropic glutamate receptor; nAChR, nicotinic acetylcholine receptor; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; *p*-MPPF, 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine; POPC, palmitoyloleoylphosphatidylcholine; PS, phosphatidylserine; SCAP, SREBP cleavage activating protein; SM, sphingomyelin; S_o, solid-ordered; SREBP, sterol regulatory element binding protein; TM, transmembrane; T_m, melting temperature defined as the temperature of maximum change in the heat capacity during the main gel to liquid crystalline phase transition; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene; TSH, thyroid stimulating hormone.

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GPCRs from a variety of sources, with an emphasis on the more contemporary role of cholesterol in maintaining a domain-like organization of such receptors on the cell surface. Importantly, the recently reported role of cholesterol in the function and organization of the neuronal serotonin$_{1A}$ receptor, a representative of the GPCR family which is present endogenously in the hippocampal region of the brain, will be highlighted.

Keywords: G-protein coupled receptors; Lipid–protein interactions; Cholesterol; Serotonin$_{1A}$ receptors

Contents

1. Introduction

Biological membranes are formed by the spontaneous assembly of amphipathic lipid molecules in an aqueous medium [1]. They constitute an environment that displays a remarkable degree of functional and compositional heterogeneity [2]. Biological membranes represent an ideal milieu for the proper function of a diverse set of membrane proteins [3–5]. Membrane proteins mediate a wide range of essential cellular processes such as cellular signaling, uptake of nutrients, and preserving a unique molecular identity of a specific cell type. Work spanning several years from a number of groups has contributed to our understanding regarding the requirement of specific lipids or the membrane environment in the topology, structure and function of membrane proteins [3,6–8]. As a class, membrane proteins make up ~30% of the total coding sequences in the human genome [9,10]. Importantly, they represent prime candidates for the generation of novel drugs in all clinical areas due to their involvement in a wide variety of cellular processes [11]. Insights into the structure of membrane proteins and specific lipid–protein interactions required for their function

are therefore of considerable interest and commercial value. Seven transmembrane domain GPCRs are important members of this class of proteins [12,13]. 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, and mediate multiple physiological processes such as neurotransmission, cellular metabolism, secretion, cellular differentiation, growth, inflammatory and immune responses. This review focuses on the role of the membrane lipid environment in the function of this important class of membrane proteins. We highlight the importance of lipid–protein interactions, especially cholesterol–receptor interactions, involved in the function of GPCRs from a variety of sources, with the recently reported role of cholesterol in the function of the neuronal serotonin$_{1A}$ receptor, present endogenously in the hippocampus, reviewed as a specific example.

2. Role of cholesterol in the function and organization of biological membranes

2.1. Structure, function, and organization of membrane cholesterol

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function and sorting [14–16]. Cholesterol is a largely hydrophobic molecule. The only polar group in cholesterol is the 3β-hydroxyl moiety (see Fig. 1). The remainder of the molecule is hydrophobic which comprises of a planar tetracyclic fused steroid ring and a flexible isooctyl hydrocarbon tail. The 3β-hydroxyl moiety of cholesterol gives the molecule its amphiphilic character. This makes cholesterol surface-active, causing it to orient in a phospholipid bilayer with its long axis perpendicular to the plane of the membrane (see Fig. 1). Interestingly, there is some evidence to suggest that tail-to-tail cholesterol dimers spanning the two leaflets of the membrane bilayer can be formed under certain conditions [17–22].

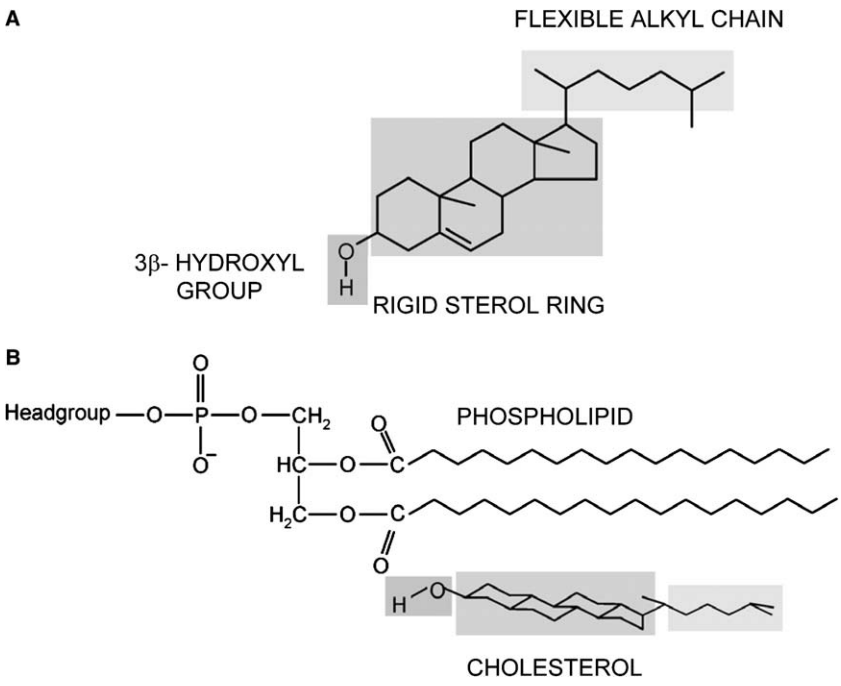

Fig. 1. Panel A shows three structurally distinct regions of cholesterol – the 3β-hydroxyl group, the rigid steroid ring, and the flexible alkyl chain (shown as shaded boxes). The 3β-hydroxyl moiety is the only polar group in cholesterol and serves to anchor cholesterol in the membrane. Panel B shows the orientation of cholesterol in relation to a phospholipid molecule in a lipid bilayer. The rigid and near planar steroid ring contributes to the ordering effect of cholesterol in phospholipid bilayers due to the restriction in motion imposed by it to adjacent phospholipid fatty acyl chains. The flexible alkyl chain extends into the hydrophobic core of the membrane. Adapted and modified from Ref. [24].

Cholesterol has a wide variety of effects on the physical properties of membranes [16,23,347]. On account of the inflexible and approximately planar steroid ring of cholesterol, the phospholipid fatty acyl chains from carbon atoms 2–10 that lie adjacent to cholesterol experience restriction in motion. The presence of cholesterol therefore induces order in membranes. However, this effect is apparent only in membranes that are inherently in a state of disorder (for example, in the liquid-crystalline (L_d) membrane phase formed above the temperature of maximum change in the heat capacity during the main gel to liquid crystalline phase transition (T_m) of the constituent phospholipid in a model membrane). In contrast, the inflexible and near planar steroid ring in cholesterol tends to disrupt the tight acyl chain packing of phospholipid fatty acyl chains when present in membranes that exhibit a high degree of order (like in the solid-ordered (S_o) membrane phase formed below the T_m of the constituent phospholipid) [23,24]. This dual effect of cholesterol effectively abolishes the temperature-dependent phase transition occurring in phospholipid membranes [347]. When present at high concentrations, cholesterol is known to induce the formation of a liquid-ordered (L_o) phase in membranes. Such a phase is characterized by high packing density of fatty acyl chains, as in an ordered gel-like phase (S_o) [25,26] but characterized by rapid lateral diffusion [27] as in a liquid-crystalline (L_d) phase. In model membranes, cholesterol has been shown to preferentially interact with phospholipids that possess saturated fatty acyl chains than with those having unsaturated fatty acyl chains [28–30]. The reason for this effect is believed to be due to the tighter packing and greater van der Waal's interaction between the near planar steroid ring of cholesterol and saturated fatty acyl chains of phospholipids. Such a preferential interaction has been considered the basis for cholesterol containing model membranes formed of saturated and unsaturated phospholipids to exhibit coexisting membrane phases – a sterol-rich, saturated phospholipid phase or condensed complex, and a sterol-poor, unsaturated phospholipid phase [31,32]. These coexisting phases are large enough to be visualized by fluorescence microscopy [31–34]. Moreover, cholesterol is reported to preferentially interact with lipids such as sphingolipids that are naturally abundant in long and saturated fatty acyl chains [35]. In addition to greater van der Waal's interaction between the saturated fatty acyl chains and the near planar ring of cholesterol, such interaction is favored due to hydrogen bond interactions between the 3β-hydroxyl group of cholesterol and the amino group of ceramide in sphingolipids.

2.2. Cholesterol and membrane domains

Cholesterol is often found distributed non-randomly in domains or pools in biological and model membranes [14,15,21,36–39]. Many of these domains are believed to be important for the maintenance of membrane structure and function. Stable macroscopic domains enriched in cholesterol have been identified in the axonal regions of neurons [40], on the apical membranes of epithelial cells [41], and in ocular lens fiber cells [22]. In addition, cholesterol is found distributed heterogeneously among various intracellular membranes. The lowest cholesterol concentration is found in the membranes of the endoplasmic reticulum, which interestingly, is the site of cholesterol biosynthesis [42]. The highest concentration (~90% of the total cellular cholesterol) is found in the plasma membrane [43,44]. The physical forces that induce segregation of cholesterol into domains in model membranes, namely their preferential interaction with sphingolipids and/or phospholipids with saturated fatty acyl chains, are believed to be the cause for its segregation in biological membranes as well [35,45]. In biological membranes, a non-random distribution of cholesterol has been unequivocally established due to the identification and isolation of membrane regions such as caveolae [46]. These flask-shaped, cholesterol-enriched structures are present in cells expressing isoforms of the cholesterol-binding protein caveolin. Caveolae are proposed to function as an alternative route of endocytosis. The existence of yet another type of membrane domain enriched in cholesterol termed as ''lipid rafts'' has been proposed [38]. The liquid-ordered phase seen in model membranes has been equated to lipid-raft like domains in natural membranes [47,48]. The idea of such specialized membrane domains assumes importance in cell biology since physiologically important functions such as membrane sorting and trafficking [41] and signal transduction processes [49], in addition to the entry of pathogens [50,51], have been attributed to these domains. This is believed to be due to their unique lipid and protein composition and their potential to sequester receptors and signaling molecules in the plane of the membrane. These domains have been operationally defined based on the biochemical criterion of differential insolubility in mild, non-ionic detergents like Triton

X-100 [47,52], a phenomenon that is attributed to the inability of non-ionic detergents to penetrate and hence solubilize membranes composed of lipids with a high melting temperature (T_m) such as sphingolipids and lipids with saturated fatty acyl chains [53,54] and/or their preferential interaction with cholesterol [39,55]. Whether detergents isolate pre-existing membrane domains or artifactually create them remains an open question [56,57,348]. Nevertheless, the existence of such lateral heterogeneities in cell membranes has been proposed based on read-outs from several biophysical techniques that monitor membrane dynamics and/or distribution of membrane components on a scale far lower than the resolution of visible light microscopy [58–61].

The heterogenous distribution of cholesterol in membrane domains effectively results in the presence of diverse membrane environments containing varying amounts of cholesterol. If membrane protein function is modulated by the cholesterol content in membranes (see later), a cellular mechanism that regulates membrane protein function by utilizing such diversity in the membrane environment represents an interesting possibility. Such a possibility has been put to test by analyzing the consequences of a selective alteration in the cholesterol content on membrane protein function in natural membranes. These studies have been possible due to the availability of a variety of means to alter the physical content and/or availability of cholesterol in membranes.

2.3. Approaches to alter cholesterol content and availability in membranes

The development of techniques to manipulate the content and availability of cholesterol in membranes has perhaps largely been due to its significance to human health and disease that has sustained extensive research on this lipid [62–64]. Such technologies include the use of water soluble carriers that efficiently remove cholesterol from membranes, cholesterol-binding compounds that sequester it in the membrane, cholesterol-modifying enzymes, and biosynthetic inhibitors of cholesterol.

2.3.1. Sterol-specific carriers as acute modulators of cholesterol content

Cyclodextrins are efficient carriers of cholesterol and have been utilized to achieve acute modulation of cholesterol content in membranes. They are among the most effective catalysts to mediate cholesterol efflux from membranes [65]. Cyclodextrins in general exhibit a broad specificity for extracting membrane lipids [66]. However, the oligomer with seven residues (β-cyclodextrin) of methylated-glucose, methyl-β-cyclodextrin (MβCD), displays a relatively higher specificity for cholesterol and other steroids compared to phospholipids (see Fig. 2) [67–69]. It can entrap cholesterol in its inner cavity and render it soluble in an aqueous medium [70]. The stoichiometry of the cholesterol:cyclodextrin complex thus formed has been reported to be 1:2 (mol/mol) [71]. Importantly, a careful selection of the range of MβCD concentrations used in experiments can bring about a specific depletion of cholesterol from natural membranes (see Fig. 2) [72,73]. Cyclodextrins are polar molecules and therefore do not partition into membranes. Due to their polarity, their separation from membranes suspended in buffers can be carried out with a routine centrifugation spin. The small dimensions of cyclodextrins compared to other carriers such as liposomes [65] or lipoprotein particles [70] allow it to approach closer to target membranes and hence lead to efficient efflux of cholesterol [74–76]. The close proximity allows the spontaneous desorption of cholesterol from the membrane, without cholesterol having to encounter the energetically unfavorable aqueous environment. The cyclodextrin-mediated cholesterol efflux process therefore has a lower activation energy compared to that mediated by liposomes or lipoproteins [75]. For reasons that make it efficient in specific removal of cholesterol from membranes, cyclodextrins can be employed as carriers to replenish or enrich membranes with cholesterol and its analogues [77]. Taken together, these attributes have popularized the use of cyclodextrins in understanding molecular details regarding the behavior of cholesterol in membranes. These include monitoring the transbilayer distribution and kinetics of flip-flop of sterols [78,79], estimating cholesterol partition coefficients in multi-component membrane systems [29,78], monitoring cholesterol-dependent alterations in protein structure and function [77,80], analyzing the cellular distribution of cholesterol using the fluorescent analogue dehydroergosterol [81], and demonstrating heterogeneity in cholesterol distribution in natural membranes based on kinetics of cholesterol-efflux [82]. Cyclodextrins have earlier been used extensively in pharmaceutical formulations to aid in the delivery and release of pharmaceutically

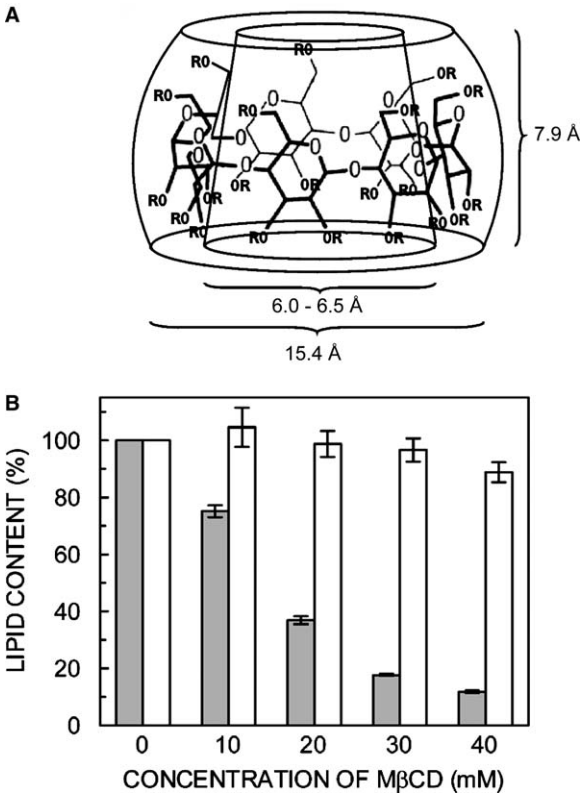

Fig. 2. The chemical structure of a β-cyclodextrin (containing 7 glucose residues) molecule is shown in panel A. Cyclodextrins can solubilize a variety of hydrophobic compounds by trapping them in their inner cavity. The specificity of this process depends on the structure of their inner cavity which can be modified by substitution of the hydroxyl groups (indicated as R in the figure) in each glucose residue. The commonly used cholesterol-depleting compound methyl-β-cyclodextrin (MβCD) has R as a methyl group, with an average methyl group substitution of 10.5–14.7 per molecule of β-cyclodextrin. Structure adapted and modified from http://www.cyclodex.com. The effect of increasing concentrations of MβCD on the percentage of cholesterol (gray bars) and total phospholipid (white bars) contents of native hippocampal membranes is shown in panel B. Increasing concentrations of MβCD leads to a progressive reduction in the cholesterol content while the total phospholipid content remains unaltered thereby indicating the specificity of cholesterol depletion mediated by MβCD. Adapted from Ref. [72].

active hydrophobic compounds [83]. Their cholesterol-modulating ability has found newer therapeutic applications. Thus, cyclodextrin-like molecules have been shown to be pharmacologically effective in treating unstable atherosclerotic plaques in macrophage foam cells [76]. In addition, oral administration of β-cyclodextrins has been shown to cause hypolipidemic effects in genetically hypercholesterolimic rats [84]. Moreover, topical application of β-cyclodextrin has been shown to block the transmission of cell-associated human immunodeficiency virus-1 (HIV-1) due to its ability to deplete cholesterol [85].

2.3.2. Cholesterol-binding agents which modulate cholesterol availability

Compounds that physically bind to cholesterol and sequester it in the membrane have been utilized to effectively reduce the availability of cholesterol. When used at appropriate concentrations, these agents partition into the membrane and sequester cholesterol. The sterol-complexing agent digitonin is a typical example of this class of molecules [86–88]. Digitonin is a plant glycoalkaloid saponin detergent obtained from *Digitalis purpurea* (see Fig. 3). It forms water-insoluble 1:1 complexes (termed 'digitonides') with cholesterol and other steroids which possess a planar sterol ring, a 3β-hydroxy-Δ^5 configuration and a hydrophobic side chain at C_{17} [89]. Digitonin treatment has been shown to result in the formation of cholesterol-digitonin rich domains in the membrane [90], thus reducing the availability of free cholesterol capable of interacting with membrane constituents such as receptors. This property of digitonin has resulted in its use as an agent to distinguish

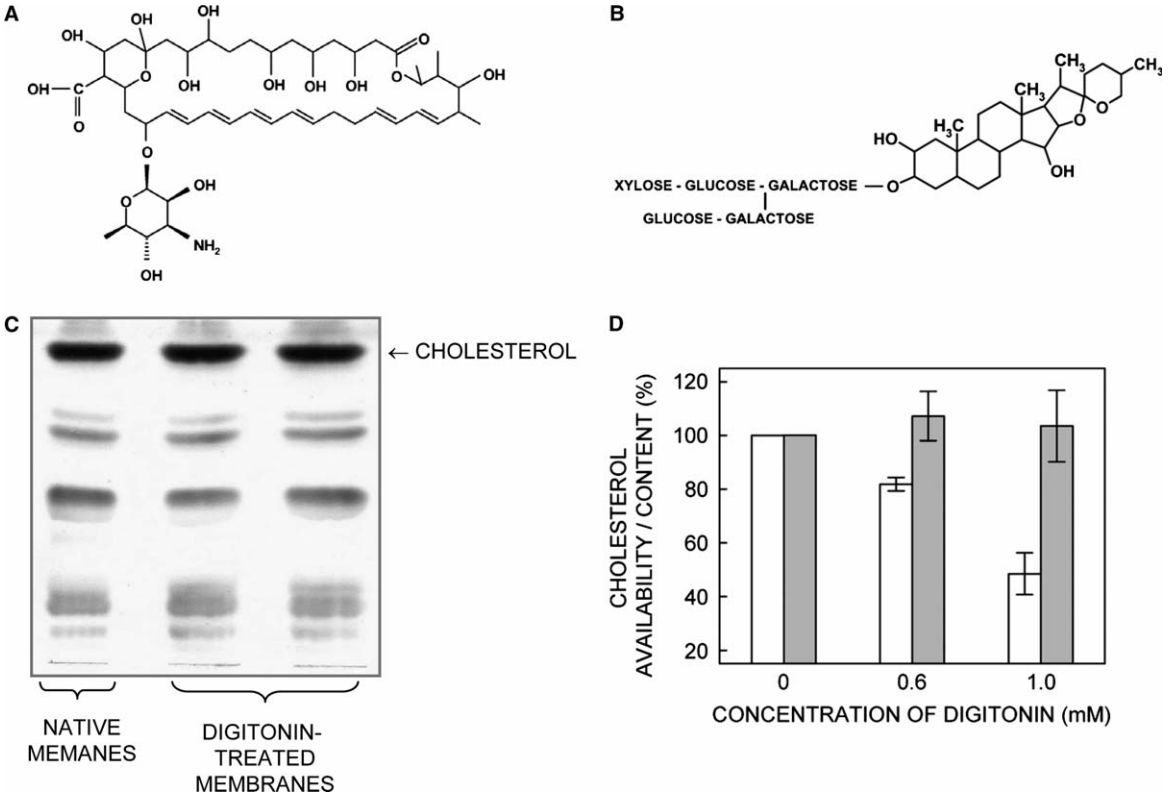

Fig. 3. The chemical structures of two sterol-binding agents, nystatin and digitonin, are shown in panels A and B, respectively. The cholesterol-complexing effect of digitonin (shown in panels C and D) was determined by comparing the total vs. accessible cholesterol in digitonin-treated native hippocampal membranes. Total lipids extracted from digitonin-treated native membranes were separated on a thin layer chromatogram (panel C). The total cholesterol content was estimated by a densitometric analysis of the thin layer chromatogram (gray bars in panel D). The accessible cholesterol content in membranes was determined by an enzyme-based fluorimetric cholesterol assay (white bars in panel D). The difference in the total and accessible cholesterol is an indication of the cholesterol-complexing effect of digitonin. Adapted from Ref. [88].

between cholesterol-rich and -poor membranes in the ultrastructure analysis of cell membranes [91], and to biochemically detect heterogeneity in cholesterol distribution in membranes [92].

The sterol-binding antifungal polyene antibiotic nystatin [93–95] is another compound that has been used to sequester membrane sterols (see Fig. 3). Nystatin is a membrane-active polyene antibiotic that effectively partitions into membranes [96]. It has been proposed that nystatin forms a 1:1 (mol/mol) complex with membrane cholesterol and forms channels in the membrane [97]. The nature and specificity of nystatin–sterol interaction and the orientation of nystatin in membranes containing different sterols has been analyzed quite extensively [95,98]. Studies on ion permeability induced by the presence of nystatin–sterol pore complexes in model membranes suggest that the action of nystatin is considerably enhanced in the presence of membrane sterols. This is evident from the ~10-fold higher amounts of nystatin required to bring about a similar increase in ion permeability in membranes lacking sterols [95]. In addition, other compounds like the polyene antibiotics filipin and amphotericin B that bind and sequester cholesterol in the membrane have been described in the literature [95].

2.3.3. Cholesterol chemistry altered by enzymatic oxidation

Cholesterol oxidase is a water-soluble enzyme that acts at the membrane interface to catalyze the oxidation of cholesterol to cholestenone (cholest-4-en-3-one) (see Fig. 4) [99]. The oxidation of cholesterol perturbs the functionally important hydroxyl group in the molecule thereby altering its potential to participate in specific molecular interactions with sphingomyelin or phospholipids with saturated fatty acyl chains [39,100,101]. The ability of cholesterol to form domains that exist in the liquid-ordered (L_o) state is reportedly affected by cholesterol

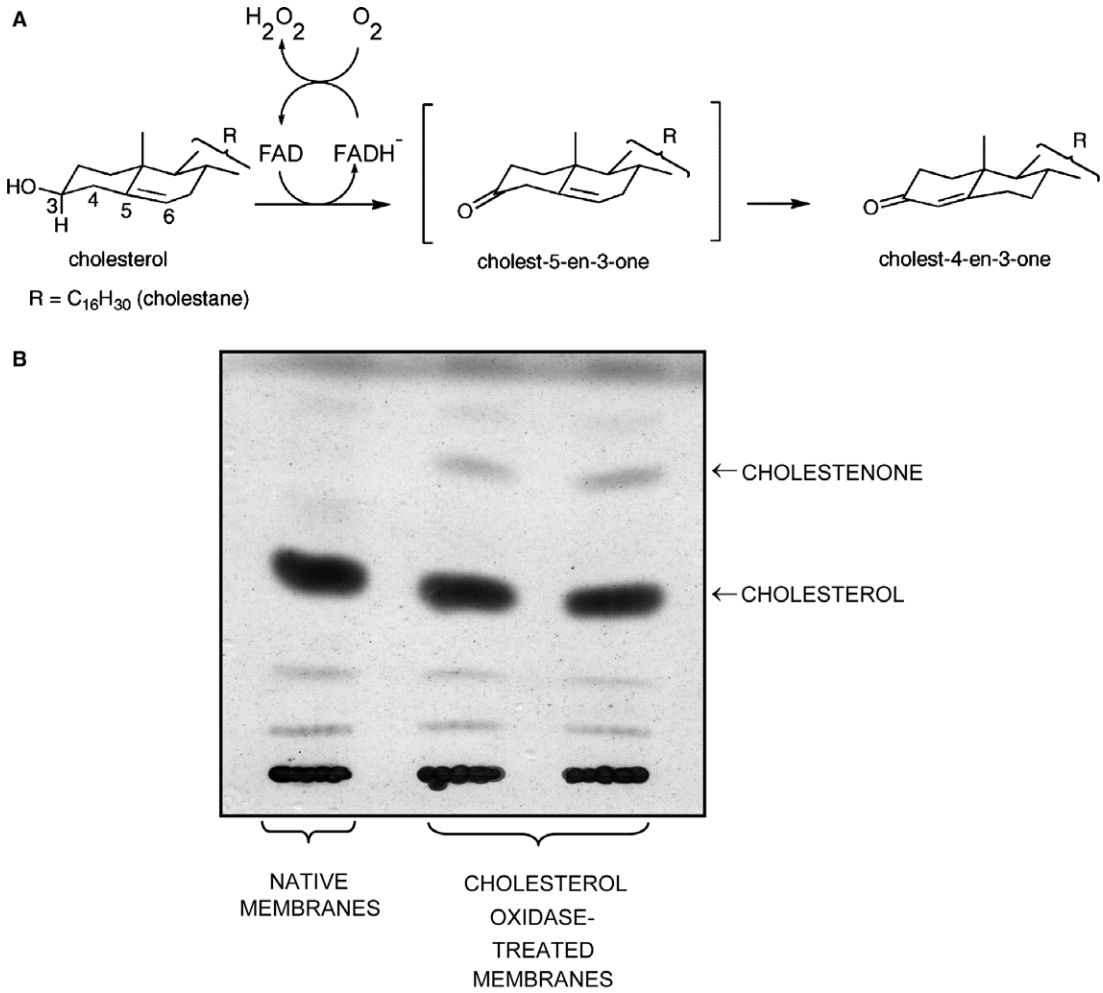

Fig. 4. The scheme of the reaction catalyzed by cholesterol oxidase is shown in panel A. Cholesterol oxidase catalyzes the two-step conversion of cholesterol to cholest-4-en-3-one using a flavine adenine dinucleotide (FAD) co-factor. The first step of the reaction is cholesterol oxidation utilizing a tightly bound FAD molecule in the enzyme to produce H_2O_2 and cholest-5-en-3-one. The second step involves isomerization of cholest-5-en-3-one to produce the final product cholest-4-en-3-one. Adapted and modified from Ref. [104]. Panel B indicates a thin layer chromatogram of total lipids extracted from native and cholesterol-oxidase treated hippocampal membranes that show the presence of cholestenone in cholesterol oxidase-treated membranes. Adapted from Ref. [331].

oxidation [39,101]. Importantly, compared to physical depletion of cholesterol from membranes, cholesterol oxidation appears to exert a milder influence on the membrane, i.e., induces relatively less perturbation to membrane physical properties. Previous reports on the effect of cholesterol oxidase treatment on the physical properties of natural membranes as monitored using fluorescence polarization of the membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (see Section 5.3) have indicated a reduction in membrane order only upon oxidation of a large fraction (>35%) of membrane cholesterol [77,102]. The reaction catalyzed by cholesterol oxidase appears to be influenced by the cholesterol organization in the membrane [103], lipid composition [100], and physical state [104] of the membrane. Interestingly, the restricted ability of the enzyme to oxidize cholesterol in natural membranes has been attributed to its involvement in hydrogen bonding with lipids such as sphingomyelin in domains in the membrane that renders the 3β-hydroxyl of cholesterol unavailable for oxidation [105].

2.3.4. Cholesterol biosynthesis inhibitors as chronic modulators of cholesterol content

Cholesterol biosynthesis inhibitors have been widely used to reduce cholesterol levels in metabolically active cells [106]. These inhibitors are collectively termed statins and constitute a number of structurally homologous

molecules that inhibit the hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, an enzyme that catalyzes the rate-limiting synthesis of mevalonate from HMG-CoA in the cholesterol biosynthesis pathway. Lovastatin is a fermentation product from *Aspergillus niger* [107], simvastatin is a semisynthetic derivative of lovastatin, and pravastatin is derived from compactin, a natural product and the first discovered statin from *Penicillium citrinum* [108]. Statins mediate inhibition of HMG-CoA reductase by competing with the endogenous substrate HMG-CoA to bind at the active site of the enzyme [109].

Statins are clinically proven agents in reducing cholesterol levels in humans [64]. At the whole body level, statins inhibit HMG-CoA reductase leading to a decrease in mevalonate levels and the regulatory pool of cholesterol in hepatic tissues [110]. This results in an upregulation of low-density lipoprotein (LDL) receptors in hepatic tissues [111,112] that enhances the uptake of circulating cholesterol-loaded LDL particles from peripheral tissues, eventually resulting in a decrease in serum cholesterol levels. In the case of cells grown in culture, chronic treatment with statins in the absence of an exogenous supply of cholesterol, such as serum-derived lipoprotein particles, effectively reduces the cellular cholesterol content (see, e.g. [113,114]).

2.4. Influence of cholesterol on membrane protein function

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 [8]. A study of such lipid–receptor interactions is of particular importance because a cell has the ability of varying the lipid composition of its membrane in response to a variety of stress and stimuli, thus changing the environment and the activity of receptors in its membrane. In view of the importance of cholesterol in modulating membrane physical properties, and the heterogeneity in its distribution in domains or kinetic pools that imply a variation in its local concentration (see above), the interaction of cholesterol with membrane proteins represents an important determinant in functional studies of such proteins. Consequently, the analysis of the influence of cholesterol on the structure and function of integral membrane proteins has been a subject of intense investigation [115]. Cholesterol has been reported to modulate conformation and hence the function of integral membrane proteins either through a specific and localized molecular interaction [116], due to alterations in the membrane physical properties induced by the presence of cholesterol [8,23,35], or due to a combination of both factors.

A specific cholesterol–membrane protein interaction has been demonstrated in the case of the sterol regulatory element binding protein (SREBP) cleavage-activating protein (SCAP), a large polytopic membrane protein that is involved in sensing the cholesterol content in the endoplasmic reticulum membranes thereby regulating the level of cholesterol in the cell [117]. Interestingly, endoplasmic reticulum membranes contain very low amounts of cholesterol [42], and therefore represent an ideal membrane environment wherein such proteins display cholesterol-sensing functions through specific cholesterol–protein interactions. The transmembrane region of the SCAP protein contains a specific sterol-sensing domain [118]. The cholesterol-sensing function of SCAP is mechanistically explained by the direct binding of cholesterol to this domain in the protein thereby inducing a conformational change in SCAP [80,119]. The interaction between SCAP and cholesterol is most likely a specific one since the conformational change induced in SCAP is highly specific to the sterol structure and mutants of SCAP that are insensitive to alterations in cellular cholesterol levels do not undergo a sterol-dependent conformational change [80].

On the other hand, cholesterol can modulate membrane protein function by an indirect mechanism. Cholesterol–receptor interactions have been extensively investigated in the case of the ligand-gated ion channel nicotinic acetylcholine receptor (nAChR), a multimeric protein of five subunits ($\alpha_2\beta\gamma\delta$) [120]. Each subunit contains four membrane-spanning segments M1–M4. The M2 segment from each subunit lines the channel pore. The M4 segment is the most hydrophobic and is believed to interact with membrane lipids [121]. The nAChR is located in the post-synaptic membranes of the neuromuscular junction that is enriched in cholesterol [120]. Cholesterol has been demonstrated to modulate channel conductivity of nAChR [122,123]. However, molecules that are different in structure from cholesterol such as vitamin D3 can substitute cholesterol in mediating this effect thereby indicating that the interaction between the receptor and cholesterol occurs at a low level of stringency [124]. Nevertheless, proximity between cholesterol and the receptor was deduced from a series of experiments designed to quench the fluorescence of tryptophan residues in the receptor using

spin-labeled cholesterol and phospholipid analogues [125]. This study proposed the presence of an annulus of specific lipids around the receptor such that lipid exchange from the annulus takes place in a different (slower) timescale compared to bulk membrane lipids. The evidence for a near-neighbor interaction between cholesterol and the receptor has led to several hypotheses on how membrane cholesterol modulates receptor function. It is thought that receptor activation induces the opening of a cholesterol-docking site, which stabilizes the protein in an active conformation [126]. Other examples where the influence of cholesterol on membrane protein functions have been assessed include the ligand-gated channel γ-aminobutyric acid type A ($GABA_A$) receptor which appears to require cholesterol at levels found in natural membranes for optimal function [127–129]. In addition, studies carried out with purified and reconstituted transferrin receptors have suggested a role of cholesterol in modulating cooperativity of transferrin binding to the receptor [130].

These examples highlight the different ways in which cholesterol can exert its effect on the function of membrane proteins. Importantly, they convey the importance of this lipid to gain a comprehensive understanding of membrane protein function. The following sections focus on the role of cholesterol in the function and organization of GPCRs, an important class of membrane proteins.

3. G-protein coupled receptors as representative membrane proteins

3.1. Diversity of G-protein coupled receptors

Genome-wide analyses of integral membrane proteins indicate a larger representation of proteins with seven transmembrane domains than others in the human genome [9]. GPCRs are prototypical members of the family of seven transmembrane domain proteins and represent the largest class of molecules involved in signal transduction across the plasma membrane [13]. They include >800 members and constitute ~2% of the human genome [131]. Recent analysis of the entire superfamily of GPCRs in the human genome indicates the presence of five main families, namely the glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin families (collectively referred to as the GRAFS classification system) with the members of each family displaying a common evolutionary origin [131]. The GRAFS classification system is more representative of the entire repertoire of GPCRs coded by a single mammalian species than the previously used A–E classification system which analyzed GPCRs based on the occurrence of seven transmembrane receptors from several species [132,133]. The rhodopsin family constitutes the largest number of GPCRs with 701 receptors of the total of ~800 GPCRs present in the human genome. The members of this family possess several characteristics such as the Asn-Ser-X-X-Asn-Pro-X-X-Tyr motif in the transmembrane domain 7 of the receptor. This motif is involved in maintaining receptors in an inactive conformation through hydrogen bonding interactions with residues in the transmembrane domains 1, 2 and 3 of the receptor. These receptors also possess the Asp/Glu-Arg-Tyr/Phe motif at the interface of transmembrane domain 3 and intracellular loop 2 that is involved in activation of G-proteins (see Fig. 5). The ligands for most of the rhodopsin family of receptors bind within a cavity between the transmembrane regions [134]. These include odorants, prostaglandins, and small biogenic amines such as serotonin and melatonin. The glutamate family comprises of 15 members that includes the metabotropic glutamate and GABA receptors. The ligand recognition domain in the metabotropic glutamate is found in the extracellular N terminus domain. The frizzled/taste 2 group contains 24 members. This group includes two distinct clusters, the frizzled receptors and taste 2 receptors. The frizzled receptors bind the glycoprotein Wnt and control cell fate, proliferation, and polarity during vertebrate development. The taste 2 receptors clearly show the presence of seven hydrophobic regions in a hydrophobicity plot but have a very short N terminus that is unlikely to contain a ligand-binding domain. The secretin family comprises of 15 members and bind large homologous peptides that most often act in a paracrine manner. The adhesion receptor family consists of 24 members that contain GPCR-like transmembrane-spanning regions fused together with one or several functional domains with adhesion-like motifs in the N terminus, such as EGF-like repeats, mucin-like regions, and conserved cysteine-rich motifs.

3.2. Functional significance and structural characteristics of G-protein coupled receptors

GPCRs primarily transmit signals across the plasma membrane via their interactions with heterotrimeric G-proteins present on the cytoplasmic side of the cell membrane [135,136]. Heterotrimeric G-proteins are

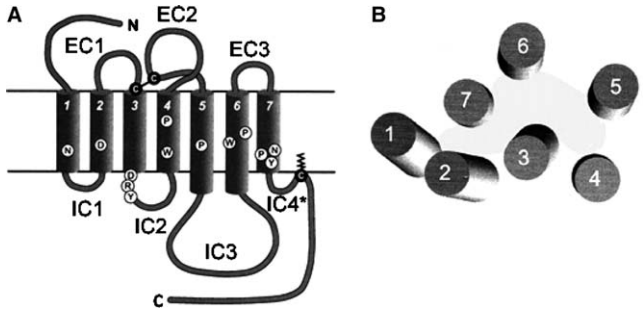

Fig. 5. Panel A is a schematic representation of the topology of the rhodopsin class of GPCRs. These receptors are characterized by a series of highly conserved key residues (black letter in white circles). In most of these receptors, a disulfide bridge connects the first (EC1) and second (EC2) extracellular loops. In addition, a majority of the receptors have one or two palmitoylated cysteine residues in the carboxy-terminal tail causing the formation of a putative fourth intracellular loop which is referred to as IC4*. The conserved Asp-Arg-Tyr (DRY) motif at the bottom of the third transmembrane domain (TM 3) serves to activate G-proteins. Panel B shows the arrangement of the transmembrane helices of rhodopsin in the membrane, as seen from the extracellular side with each helix represented by a cylinder. The orientation and position of the helices are according to the projection maps of frog rhodopsin as described in Ref. [145]. The helices are organized sequentially in a counterclockwise fashion with the transmembrane helix 3 being almost in the center of the molecule. Adapted and modified from Ref. [149].

composed of α, β, and γ subunits, with molecular masses of ~39–45, 35–39, and 6–8 kDa, respectively. To date, at least 28 distinct G-protein α subunits, 5 different β, and 12 different γ subunits have been described [137]. Heterotrimeric G-proteins can be divided into 4 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, $G_{i1\text{-}3}$, 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. 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. On binding the agonist, the receptor undergoes a conformational change resulting in increased affinity for the G-protein. 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 subunits. Exchange of GDP for GTP on the α subunit leads to dissociation/reorganization [138] of the heterotrimeric G-protein complex that facilitates transduction of signals to effector molecules such as adenylyl cyclase, phospholipases, and ion channels [13]. 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, recent evidence 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 [139,140].

GPCRs share a few common structural features such as the presence of seven membrane-spanning putative α-helical segments connected by alternate intracellular and extracellular loops, an extracellular N-terminus and a cytoplasmic C-terminus (see Fig. 5). Due to the inherent difficulties in crystallizing large membrane proteins [141,142], high-resolution crystallographic details of most GPCRs still remain unknown. Such details have been obtained only for one GPCR, rhodopsin, due to its occurrence at high density in native membranes that has made it possible to obtain large amounts of pure receptor for crystallization. The overall organization of the transmembrane segments of rhodopsin was provided using electron cryo-microscopy of two-dimensional crystals of bovine and frog rhodopsin that provided structural details at 7.5–9 Å resolution [143–145]. Projection maps of rhodopsin generated using this technique provided the first insight into how the predicted transmembrane segments were organized relative to each other in rhodopsin. Such maps indicated the presence of transmembrane domains that were tilted with respect to the plane of the membrane (see Fig. 5). The projection maps were interpreted to suggest that the transmembrane domains represent helices that are organized sequentially in a counterclockwise manner as seen from the extracellular side, with the

transmembrane domain 3 being almost in the center of the molecule. These interpretations have been validated with the recently reported structure of rhodopsin solved at 2.8 Å resolution using X-ray crystallography [146–148]. The high-resolution structure of rhodopsin has provided the basis for understanding structural details in other GPCRs, either by molecular modeling studies or using experimental approaches involving fluorescence resonance energy transfer and electron paramagnetic resonance spectroscopy with site-specific labeling of GPCRs with appropriate probes [149].

The binding of specific ligands activates GPCRs by inducing or stabilizing a conformational state of the receptor, which can activate heterotrimeric G-proteins. Site-directed mutagenesis experiments of GPCRs have enormously contributed to our knowledge on the mechanisms by which receptors activate G-proteins [149–151]. Most GPCRs are known to display an agonist-independent basal activity that can stimulate G-proteins. Interestingly, discrete mutations can enhance this basal, agonist-independent activity resulting in a constitutively active GPCR whose signaling functions are no longer regulated by specific ligands. The majority of mutations that resulted in constitutive activity were initially localized to residues in the C-terminus of the third intracellular loop in GPCRs. However, more recent results indicate that mutations in practically any domain of the receptor, even in extracellular loops, results in constitutive activity. It therefore appears that GPCRs are maintained in a preferentially inactive state through constrained intramolecular interactions and agonists function by destabilizing such interactions. Specific examples that serve to illustrate this point are the generation of constitutively active mutants of the α_{1B}-adrenergic receptor created by substitution of the Ala^{293} residue in the C-terminal of the third intracellular loop with any other naturally occurring residue [152], and that the constitutively active mutants of the β_2-adrenergic receptor display enhanced conformational flexibility and are structurally more unstable [153–155]. In combination, these results imply that the constitutive activity is due to a greater probability for such mutants to undergo conversion between conformations capable of activating G-proteins independent of the presence of agonists. A possible mechanism for the agonist-mediated receptor activation could involve the destabilization of constrained intramolecular interaction. Several studies point toward the importance of protonation of key residues in the agonist-dependent activation of GPCRs. Among the rhodopsin family of GPCRs, protonation of the Asp/Glu residue in the highly conserved Asp/Glu-Arg-Tyr/Phe motif at the interface of transmembrane domain 3 and intracellular loop 2 (see Fig. 5) has been shown to be involved in receptor activation [156]. In addition, charge-neutralizing mutations that mimic the protonated state of the Asp/Glu, cause dramatic constitutive activation of GPCRs [154]. The protonation of the Asp/Glu residues is believed to result in long-range conformational changes in the receptor molecule leading to the exposure of receptor sites that interact with G-proteins. The conformational changes occurring in GPCRs upon activation monitored using sulfhydryl-reactive spin or fluorescent probes incorporated into cysteine-substituted mutants have enabled measurement of changes in relative distance between transmembrane segments. Such measurements have provided evidence for a significant rigid-body movement of transmembrane segments upon agonist-induced activation [149,157,158].

Functional analyses of GPCRs have relied on receptor-specific radioligand binding experiments and have monitored the influence of guanine nucleotides on radioligand binding to receptors. In addition, the ability of ligands to stimulate signal transduction pathways via receptors has been monitored. The ternary complex model describes signal transduction from activated receptors to G-proteins [159,160]. This model proposes that GPCRs can independently exist in two conformations, the ground state (R) and the partially activated state (R^*), which are in equilibrium with each other. Mutations that result in constitutive activity shift this equilibrium towards R^*. The R^* state is capable of interacting with G-proteins. Agonists have preferential affinity for and stabilize the R^* state and subsequently promote G-protein/receptor interaction thus forming the ternary complex of agonist-activated receptor R^*-G-protein. Ligands that stabilize the ground state R and therefore decrease the number of spontaneously active R^* receptors act as inverse agonists, whereas neutral antagonists display similar affinities for both R and R^*. As a consequence, these models predict that the affinity of ligands binding to receptors is dependent on the basal R/R^* ratio. The ternary complex model provides a framework to understand how radioligand analysis with agonists and inverse agonists can help discriminate between G-protein coupled and uncoupled forms of the receptor. Agonists bind to receptors with high affinity in the absence of guanine nucleotides but in the presence of G-proteins. The presence of high concentrations of GTP, or its non-hydrolyzable analogue GTP-γ-S (that reduce G-protein/receptor interaction), tend to reduce the binding affinity of agonists with a concomitant increase in the affinity of

inverse agonists. The binding affinity of neutral antagonists however remains unchanged in presence of guanine nucleotides. Since the relative concentrations of G-protein coupled and uncoupled forms of the receptor determine signaling functions, radioligand binding analysis provides a convenient approach to analyze GPCR function.

3.3. Relevance of G-protein coupled receptor functions in health and disease

The superfamily of GPCRs dictate physiological responses to a diverse array of stimuli that include endogenous ligands such as biogenic amines, peptides, glycoproteins, lipids, nucleotides, Ca^{2+} ions and various exogenous ligands for sensory perception such as odorants, pheromones, and even photons. Their involvement in multiple physiological processes such as neurotransmission, cellular metabolism, secretion, cellular differentiation, growth, inflammatory and immune responses is therefore obvious [13,140]. Importantly, 30–50% of clinically administered drugs act as either agonists or antagonists at GPCRs with several ligands of GPCRs among the top 100 globally selling drugs thus pointing out their immense therapeutic potential [161,162]. A number of hereditary diseases are attributed to mutations occurring in GPCRs as a consequence of their abundance and functional significance. These include diseases such as color blindness and retinitis pigmentosa associated with X-chromosome rearrangements and missense mutations in the opsin proteins, respectively, nephrogenic diabetes insipidus linked to loss of function mutations in the vasopressin V_2 receptors, hyperfunctioning thyroid adenomas associated with missense mutations in the thyroid stimulating hormone (TSH) receptor, and familial hypocalciuric hypercalcaemia and neonatal severe hyperparathyroidism occurring due to missense mutations in the Ca^{2+}-sensing receptor [161,163]. Interestingly, several spontaneously occurring constitutively active GPCR mutants have been associated with human diseases [150,151]. These include naturally occurring constitutively active mutants of rhodopsin in autosomal dominant retinitis pigmentosa [164], of the TSH receptor in hereditary thyroid adenomas [165], and of the leutinizing hormone (LH) receptor leading to familial male precocious puberty [166].

In addition, the relevance of GPCRs to human health and disease is highlighted by their involvement in the entry of several viruses and intracellular pathogens [167]. The role of the chemokine receptors in the acquired immunodeficiency syndrome (AIDS) pandemic caused by HIV is well documented [168]. A critical step in the entry of the HIV into target cells in vivo is the utilization of two GPCRs, CXC-chemokine receptor-4 (CXCR4) and CC-chemokine receptor-5 (CCR5), as co-receptors. Indeed, individuals who are homozygous for a mutant allele of CCR5 are resistant to HIV infection, whereas heterozygous individuals show delayed progression to AIDS [169,170]. Several viral genomes code for variants of endogenous GPCRs which are believed to have been hijacked from their host genomes. Emerging evidence indicates that these virally encoded GPCRs and their regulated signaling pathways have an essential role in viral pathogenesis and might therefore represent new targets for therapeutic intervention in virally induced diseases. An interesting example is the presence of a GPCR in the genome of the Kaposi's sarcoma associated herpesvirus (KSHV), the viral etiologic agent of Kaposi's sarcoma which is the most frequent type of tumour occurring in HIV infected patients. This viral GPCR possesses transforming and pro-angiogenic activity and is a mutant of an endogenous GPCR. The KSHV GPCR is highly related to the CXC family of chemokine receptors, in particular, to the CXCR1 and CXCR2 receptors for interleukin-8 (IL-8), but is a constitutively active receptor owing to the presence of a mutation ($Asp^{142}Val$) within its Asp-Arg-Tyr motif that provides the much needed transforming and pro-angiogenic activity [171,172]. In addition, the human cytomegalovirus (HCMV) that causes severe disease in immunocompromised individuals and remains the leading cause of congenital viral infection in humans, encodes four GPCRs – US27, US28, UL33 and UL78. US28, which can function as a co-factor for HIV-1 entry, is unrelated to CXCR4 and CCR5, but instead shows high homology to the CCR1 and CCR2 chemokine receptors. Interestingly, US28 is a constitutively active receptor that can be further stimulated by certain chemokines [173]. Due to its high affinity for certain chemokines, the US28 receptor sequesters these chemokines thereby depleting them from the site of infection. HCMV utilizes this mechanism to evade the host immune system to maintain its latent presence within the host [174]. In addition, the absolute requirement of serotonin receptors for infection by the human polyomavirus [175] and the modulatory role of β_2-adrenergic receptor signaling in erythrocytes during malarial infection [176] further highlight the importance of GPCRs in human health and disease.

3.4. Membrane biology of G-protein coupled receptors

The major paradigm in GPCR signal transduction is that their stimulation leads to the recruitment and activation of heterotrimeric G-proteins [135,136]. These initial events, which are fundamental to all types of GPCR signaling, occur at the plasma membrane via protein–protein interactions. An important consequence of this is that the structure, organization and dynamics of the receptor, largely determined by the membrane environment the receptor is present in, represents an important determinant in its interaction with G-proteins, and has significant impact on the overall efficiency of the signal transduction process. 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 [177]. This raises the obvious possibility that the membrane could be an important modulator of receptor structure and function [8]. The importance of a membrane-like environment for optimal function of membrane proteins in general, and GPCRs in particular, is evident from the adverse effects of delipidation of membranes on receptor function (see, e.g. [178–180]). Importantly, like any other integral membrane protein, purification of GPCRs requires their suitable dissociation in a buffered detergent solution from the membrane by a process termed solubilization [181–183]. Effective solubilization of GPCRs with retention of functional activity is to a large extent dependent on the choice of detergents, since the treatment of membranes with detergents invariably leads to the enrichment and/or depletion of certain classes of lipids [184,185]. The significance of specific lipids in the function of GPCRs has been demonstrated in a variety of cases. These include the requirement of phospholipids with polyunsaturated fatty acids such docosahexaenoic acid in efficient signaling of rhodopsin [186], and the requirement of cholesterol in the optimal functioning of several GPCRs that will be described in greater detail in the following sections. In addition, the presence of lipids such as phosphatidylethanolamine (PE) that adopt a non-lamellar phase, appear to modulate the activation of GPCRs [187] and the recruitment and association of heterotrimeric G-proteins with receptors [188,189].

Lipid–protein interactions of the kind mentioned above involve short-range and transient interactions of GPCRs with lipids present in their immediate membrane environment. These constitute non-covalent interactions and are believed to modulate receptor function provided the presence of these specific lipids is felt by the receptor (in a time-averaged manner) while it executes its functions. In contrast, covalent modification of GPCRs by attachment of lipid-like moieties represents more stable interactions. GPCRs are subject to covalent lipid modifications such as the attachment of palmitic acid [190]. In general, palmitoylation represents a post-translational modification of reactive cysteines in membrane proteins. Palmitoylation reactions are catalyzed by putative palmitoyl transferases which possibly involve an activated palmitoyl CoA moiety as a substrate [191]. Rhodopsin was the first GPCR that was reported to be palmitoylated [192]. This modification was reported to occur at residues Cys^{322} and Cys^{323} present in the carboxy terminus of the protein [193]. The location of these residues in rhodopsin, and the general membrane-anchoring role of fatty acyl chains present in several peripherally associated proteins, led to the proposal that palmitoylation induces a major alteration in the topology of the protein, i.e., formation of a fourth intracellular loop. The existence of such an intracellular loop has been confirmed by fluorescence quenching studies with fluorescently labeled fatty acids incorporated into rhodopsin [194]. Subsequently, palmitoylation has been detected in several GPCRs including the β_2-adrenergic receptor, vassopressin V_2 receptor, and the CCR5 chemokine receptor [190], suggesting that this modification represents a general feature among members of the GPCR superfamily. However, an increasing body of the literature points to the occurrence of palmitoylation at sites in the protein other than in the carboxy terminus (see, e.g. [195]). The palmitoyl moiety in GPCRs has been proposed to play a regulatory role in receptor function. For instance, constitutive palmitoylation of GPCRs is thought to play a role in the processing and targeting of the protein to the cell surface, whereas dynamic palmitoylation may either serve to target the GPCR into certain types of membrane domains, or regulate its association with signaling molecules on the plasma membrane through the formation of the fourth intracellular loop.

The lateral organization and dynamics of the receptor in membranes and its implications in cellular signaling perhaps represent the most interesting aspects in the membrane biology of GPCRs. The fluid mosaic model for cell membranes envisaged a largely fluid membrane bilayer in which proteins and lipids exhibit free translational diffusion, while transverse movement of membrane components was restricted across the bilayer

to preserve asymmetry of the bilayer [196]. One of the significant findings that led to the proposition of the fluid mosaic model was the experimental demonstration that the GPCR rhodopsin exhibits translational diffusion in the plane of the membrane [197,198]. These experiments exploited the presence of a covalently attached natural chromophore in rhodopsin – the retinal moiety, which made it possible to monitor rhodopsin translational dynamics in the membrane based on recovery in absorption intensity after photobleaching of the chromophore. Some of the tenets of the fluid mosaic model were later modified to account for the observation of non-random organization of lipids and proteins in the membrane that began to emerge from several laboratories (see for e.g. [199]). Current concepts of cell membrane structure envisages the presence of several diverse microenvironments enriched in certain types of lipids and proteins which serve to regulate several cellular processes such as trafficking and sorting of lipids and proteins, and signal transduction [38,200,201].

The diversity in the membrane environment has significant implications on the manner in which cellular signaling processes involving GPCRs are regulated. The significance of receptor lateral diffusion on the plasma membrane in the signaling functions of GPCRs forms the basis of the mobile receptor hypothesis [202]. This model proposes that receptor–effector interactions at the plasma membrane are controlled by lateral mobility of the interacting components. Evidence for this comes from a number of reports that correlate receptor signaling to the membrane dynamics of the individual components involved in such signaling. These include experimental evidences such as: (i) the dependence of the vasopressin V_2 receptor to activate adenylyl cyclase through G-proteins on the fraction of receptors that are mobile on the cell surface [203], (ii) dependence of the agonist-stimulated adenylyl cyclase signal transduction process on the mobile fractions of proteins in reticulocyte plasma membranes [204], (iii) the correlation between lateral diffusion of rhodopsin in the membrane and light-stimulated G-protein activation [205], and, (iv) theoretical calculations from simulation studies where the efficacy of cellular signaling could be modeled more accurately based on the diffusion limited collisional encounter of receptors and G-proteins rather than mere density of receptors and G-protein in a given membrane [206]. This model has evolved taking into consideration more recent observations on the nature and specificity of GPCR signal transduction events along with current understanding of the organization of cell membranes. Recent evidence indicates a spatiotemporally organized system of receptors and G-proteins in membranes rather than a freely diffusible system that is responsible for rapid and specific propagation of extracellular stimuli to intracellular signaling molecules [207,208]. 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 in low abundance, on the cell surface [209]. Based on these observations, it has been proposed that GPCRs are not uniformly present on the plasma membrane but are concentrated in stable cholesterol-enriched membrane microdomains such as caveolae, and non-ionic detergent-insoluble membrane microdomains putatively referred to as ''lipid rafts'' (see Section 2.2) [209]. For example, the efficient interaction of β_1- and β_2-adrenergic receptors with adenylyl cyclase (compared to prostaglandin E2 receptors) appears to correlate with the localization of β_1- and β_2-adrenergic receptors and adenylyl cyclase (and absence of prostaglandin E2 receptors) together in caveolae [210]. Overexpression of adenylyl cyclase selectively enhances β-adrenergic receptor-mediated stimulation of adenylyl cyclase activity, but not that mediated by prostaglandin E2 receptors. Furthermore, β_1-adrenergic receptors are found to stimulate adenylyl cyclase more efficiently than β_2-adrenergic receptors. Although both β_1- and β_2-adrenergic receptors are initially localized in caveolae along with adenylyl cyclase, the latter signal to adenylyl cyclase with lower efficiency due to their translocation out of caveolae upon agonist-stimulation [211]. In addition, constitutive localization of the gonadotrophin-releasing hormone (GnRH) receptor into low-density, non-ionic detergent-insoluble membrane fractions appears to be necessary for its signaling functions, namely activation of the extracellular signal-related kinase (ERK) [212]. Interestingly, stimulation of the GnRH receptor by its agonist has earlier been reported to reduce its lateral diffusion in the membrane as monitored using fluorescence recovery after photobleaching (FRAP) experiments [213], and induce homodimerization as monitored using fluorescence resonance energy transfer (FRET) techniques [214]. On the other hand, targeting of the oxytocin receptor, which is predominantly excluded from caveolae [215], to such membrane microdomains by its fusion with caveolin can turn the receptor-mediated inhibition of cell growth into a proliferative response [216]. Taken together, the spatiotemporal segregation of GPCRs and their effectors into microdomains has given rise to new challenges and complexities in receptor signaling since signaling now has to be understood in context of the three-dimensional organization of various signaling components which include receptors and G-proteins.

Several mechanisms for the steady-state localization or activation-dependent translocation of GPCRs into specific membrane microdomains have been proposed. These include extrinsic factors such as accessory protein-based sorting mechanisms in cells, and intrinsic factors such as covalent lipid-modifications of GPCRs (see above) and structural alterations in the transmembrane domain structure of the receptor that favor its partitioning into certain types of membrane domains [217]. While the possibility for extrinsic sorting mechanisms always exists in a metabolically active cell, the sorting of GPCRs into domains based on intrinsic receptor-dependent mechanisms represents a more interesting phenomenon. As described earlier, cholesterol and sphingolipid-enriched membrane domains are thought to represent an ordered membrane phase due to tighter packing of the lipids [47,218]. Due to lipid acyl chain packing considerations, several lines of evidence suggest that the covalent modification of peripheral membrane proteins or peptides with two saturated fatty acyl chains such as myristate or palmitate favor their partitioning into ordered membrane microdomains, while modifications with branched isoprenyl or unsaturated chains exclude them from such regions [219–222]. Although fatty acylation appears to be important in determining the location of peripherally attached peptides and proteins into ordered membrane domains, sorting of integral membrane proteins such as GPCRs based on such modifications may not be sufficient considering the contributions from the relatively large transmembrane regions in such proteins [217]. An alternative mechanism that could explain the sorting of GPCRs into membrane microdomains is based on the hydrophobic matching hypothesis [223]. A recent study on the purified human δ-opioid receptor reconstituted in planar supported bilayers containing two coexisting membrane phases, a thinner palmitoyloleoylphosphatidylcholine (POPC)-rich phase along with a thicker sphingomyelin (SM)-rich phase, suggests that alterations in the transmembrane domain structure of the receptor upon activation could represent a sufficient determinant in its sorting into either of these membrane phases [224]. In the absence of the agonist, the δ-opioid receptor is found to preferentially localize in the thinner POPC-rich phase. However, activation with the agonist leads to partitioning of the receptor into the thicker SM-rich phase. The phenomenon of activation-dependent translocation of the receptor in such a simple system has been explained on the basis of the hydrophobic matching hypothesis, where the increase in the hydrophobic thickness of the receptor upon activation [225] energetically favors the partitioning of the receptor into the thicker SM-rich phase [224].

3.5. Role of cholesterol in the function of G-protein coupled receptors

Cholesterol–receptor interactions have been studied extensively in the case of rhodopsin. Rhodopsin functions as a photon stimulated switch which upon activation leads to the closure of ion channels causing the retinal rod cells to become hyperpolarized. The availability of membrane preparations from rod outer-segments that are highly enriched in the receptor has enabled its purification, reconstitution and crystallization thus making it the only GPCR whose structure has been determined to atomic resolution [146]. The light activated receptor exists in equilibrium with various intermediates collectively called metarhodopsins. The state of equilibrium is sensitive to the presence of cholesterol in the membrane. An increase in the amount of cholesterol in the membrane shifts this equilibrium toward the inactive conformation of the protein [226–228]. The inhibitory effect of cholesterol on rhodopsin function has been explained by direct as well as indirect modes of action. Direct interaction between rhodopsin and cholesterol has been investigated using fluorescence resonance energy transfer between the tryptophan residues in the receptor and a fluorescent cholesterol analogue, cholestatrienol [229]. Fluorescence resonance energy transfer was not observed between ergosterol and tryptophan indicating a specific interaction between rhodopsin and cholestatrienol. In addition, this study postulated the presence of one sterol molecule per molecule of receptor present at the lipid–protein interface. On the other hand, the indirect mode of action has been rationalized based on the free-volume theory of membranes that relates the alteration in membrane physical properties due to the presence of cholesterol to receptor function [227]. The conversion of the photointermediates, metarhodopsin I to metarhodopsin II, upon exposure to light involves an expansion of the protein in the plane of the bilayer [230], which occupies the available partial free volume from the surrounding bilayer. The presence of cholesterol in the membrane has been reported to inhibit the formation of metarhodopsin II due to its role in reducing the partial free volume in the membrane [231]. Importantly, fluorescence energy transfer approaches have indicated an inherent property of rhodopsin to partition out of cholesterol-rich regions of the membrane [232]. These results have recently been reinforced

by molecular dynamics simulation experiments with rhodopsin in a membrane containing a mixture of cholesterol and polyunsaturated phospholipids [233]. The modulatory effect of cholesterol on rhodopsin function has significant physiological consequences. Membranes of the rod outer-segment are heterogeneous with respect to their cholesterol content. This heterogeneity is developmentally regulated and is maintained during the entire life span of the individual. Newly formed rod outer-segment disks have a high (~30 mol%) cholesterol content and as they mature, their cholesterol content drops to ~5 mol% [92]. Maturation of rod outer-segment disks involves a concomitant increase in the phosphatidylethanolamine (PE) content at the expense of phosphatidylcholine (PC) [234]. PE-rich membranes provide a thermodynamically unfavorable environment for cholesterol and hence contribute to establishing a gradient of cholesterol in the rod outer-segment disks [235]. While rhodopsin is most active in membranes with low cholesterol content, the protein is prone to greater thermal denaturation in these membranes [236]. Hence, cholesterol has a dual effect on rhodopsin – while it reduces receptor function, it also increases the stability and hence the lifespan of the receptor in the membrane.

In addition, cholesterol–receptor interactions have been monitored in case of the β-adrenergic receptor. This receptor has been solubilized and reconstituted in the presence of specific lipids [237,238]. Studies with such purified and reconstituted receptor preparations have revealed that it requires membrane cholesterol for efficient ligand binding, receptor/G-protein interaction, and downstream signal transduction [180,239]. In addition, recent reports indicate that the function of the metabotropic glutamate receptor (mGluR) in the *Drosophila* eye depends on the membrane content of ergosterol, the predominant sterol in insects [240,241]. Interestingly, unlike receptors that bind to small biogenic amines, the ligand-binding pocket in mGluRs comprises of a large extracellular domain and is several angstroms away from the membrane. Yet, the presence of ergosterol in reconstituted membranes has been found to be a prerequisite for ligand binding to these receptors.

Several GPCRs that bind peptides have been analyzed in terms of cholesterol–receptor interactions. Experiments carried out with natural membranes expressing the oxytocin receptor [77,215,242,243] or with solubilized receptor preparations [242,244] indicate that cholesterol is required for the high affinity ligand-binding function of the receptor. A novel aspect of some of these experiments is the use of cyclodextrin-like compounds to manipulate cholesterol levels in native membranes. Thus, depletion of cholesterol using MβCD from membranes expressing the oxytocin receptor reduces the oxytocin binding affinity of the receptor by ~87-fold, causing a transition from a high affinity to a low affinity state of the receptor [242]. Replenishment of cholesterol to cholesterol-depleted membranes tends to reverse this effect. In order to assess the specific structural features of cholesterol that are required to maintain the high-affinity state of the oxytocin receptor, cyclodextrins were used to replenish cholesterol-depleted membranes with a broad range of sterol analogues that were subtly different from cholesterol either in the head group, the steroid ring, or in the hydrocarbon tail [77,242]. Interestingly, high affinity ligand-binding function of the oxytocin receptor could be restored only with certain analogues, thus pointing to a specific structural feature in cholesterol to support receptor function. A comparison of the effects of cholesterol depletion on the oxytocin receptor with that on a related GPCR, the cholecystokinin receptor, tested whether the influence of cholesterol on membrane protein function is exerted by a specific direct association between cholesterol and the receptor, or by the ability of cholesterol to modulate membrane physical properties [77]. Based on the effect of cholesterol depletion on ligand binding to these receptors, and its effect on membrane order analyzed by monitoring the fluorescence polarization of the membrane embedded probe diphenylhexatriene (DPH) (see Section 5.3), it was concluded that the oxytocin receptor exhibits higher specificity in interacting with cholesterol than the cholecystokinin receptor. Although cholesterol depletion reduces ligand binding to the cholecystokinin receptor, this effect could be reversed with most analogues of cholesterol that could restore membrane order. Similar effects on the oxytocin receptor could however be demonstrated only with certain analogues that structurally resembled cholesterol in some critical features. This data provides support for a specific molecular interaction between the oxytocin receptor and cholesterol. Further, molecular modeling studies have indicated a putative docking site for cholesterol in the oxytocin receptor that is absent in the cholecystokinin receptor [245]. In addition, cholesterol has been reported to maintain the thermal stability of the oxytocin receptor in solubilized membrane preparations [243]. A recent report has compared the role of cholesterol in the function of the earlier described cholecystokinin receptor and the secretin receptor [246]. It was found that while the ligand binding and cellular

signaling functions of the cholecystokinin receptor were reduced upon cholesterol depletion, similar effects were not observed with the secretin receptor. In this case, cholesterol depletion was carried out by treating cells individually expressing the cholecystokinin and secretin receptors either with MβCD, or by culturing them in lipoprotein-deficient serum in the presence of statins (see Section 2.3). Importantly, this study further analyzed the role of cholesterol in the conformation of the ligand binding site in the cholecystokinin receptor in a native membrane environment. This was achieved by monitoring the fluorescence properties of cholecystokinin analogues that were previously shown to be sensitive indicators of the environment around the ligand binding site in the receptor [247]. It was found that cholesterol depletion of membranes containing the cholecystokinin receptor results in a reduction in the fluorescence anisotropy and lifetime of these analogues, suggesting a possible alteration in the structure of the ligand binding site in the receptor [246].

On a more global scale, the diffusion properties of the oxytocin receptor on the surface of living cells in response to cholesterol depletion were monitored using FRAP [245]. The alteration in the diffusion properties of the oxytocin receptor in response to cholesterol depletion was interpreted to be caused due to phase separation within the lipid bilayer and/or alteration in the cytoskeleton. Interestingly, although the ligand binding function of oxytocin receptors shows sensitivity to cholesterol, these receptors are largely excluded from membrane domains such as caveolae that are enriched in cholesterol [215]. This example clearly indicates that functions of GPCRs can be modulated by membrane cholesterol in spite of their absence in cholesterol-enriched membrane domains.

Experiments carried out with heterologously expressed galanin receptors have indicated the requirement of cholesterol in the ligand binding and intracellular signaling functions of these receptors [248]. The role of membrane cholesterol in modulating ligand binding to the galanin receptor was examined by treating membranes with MβCD or by culturing cells expressing the receptor in lipoprotein-deficient serum. These studies revealed a marked reduction in galanin binding to the receptor in cholesterol-deficient membranes. Importantly, addition of cholesterol back to cholesterol-depleted membranes restored galanin binding to normal levels. This interaction appears to be specific to cholesterol as only a limited number of cholesterol analogues were able to rescue galanin binding. In addition, treatment of membranes with filipin, a cholesterol-binding agent (see Section 2.3), or with cholesterol oxidase (see Section 2.3) markedly reduced galanin binding. Moreover, this effect was found to be independent of receptor/G-protein interaction based on the sensitivity of ligand binding to GTP-γ-S, the non-hydrolyzable analogue of GTP, in normal and in cholesterol-depleted membranes.

The chemokine receptors are a good example where the role of cholesterol in receptor function and dynamics have been extensively studied. The chemokine receptors CXCR4 and CCR5 require membrane cholesterol for their ligand binding and signaling functions [249–251]. Thus, ligand binding and ligand-dependent intracellular signaling responses are reduced by depletion or oxidation of cholesterol. Further, by monitoring the accessibility of monoclonal antibodies to distinct epitopes on the receptor, these studies demonstrate that depletion and/or oxidation of membrane cholesterol can induce significant conformational changes in GPCRs. Since cholesterol depletion has earlier been found to reduce infection by HIV into cells that display these receptors [252], these studies have been extended to further understand the requirement of cholesterol in the CXCR4 and CCR5 receptor organization and dynamics during the process of HIV infection. In target T cells, binding of the HIV envelope proteins to CXCR4 and CCR5 receptors results in polarization and redistribution of cellular components that are critical to the process of cellular activation and migration. Importantly, this process has been found to be dependent on the presence of cellular cholesterol [253,254], thereby suggesting a mechanism by which cholesterol depletion reduces HIV infection both on a local scale by reducing ligand binding to the CXCR4 and CCR5 receptors, and on a global scale by altering the receptor-activation dependent cell surface reorganization of these receptors.

The examples mentioned above signify the importance of cholesterol–protein interactions in GPCR function. In addition, they indicate that even among the members of the GPCR family, there appears to be a lack of consensus in the manner in which cholesterol modulates receptor function. While cholesterol in essential for the proper function of several GPCRs, rhodopsin function is inhibited by the presence of cholesterol. This points out the necessity for a detailed analysis of the effects of cholesterol on the specific receptor system to be studied. The following sections highlight the requirement of cholesterol in the function of the serotonin$_{1A}$ receptor, an important member of the GPCR superfamily.

4. Serotonin$_{1A}$ receptor as a representative member of the G-protein coupled receptor family

4.1. Molecular and structural characteristics of serotonin$_{1A}$ receptors

Serotonin is a biogenic amine present in a variety of organisms ranging from worms to humans [255]. It acts as a neurotransmitter and is found in a wide variety of sites in the central and peripheral nervous system [256]. Serotonin exerts its diverse actions by binding to distinct cell surface receptors which have been classified into various groups 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 [257,258]. Based on an analysis of the extent of amino acid homology between various species, it has been estimated that the primordial serotonin receptor must have evolved more than 800 million years ago [259]. The development of pharmacological ligands with enhanced specificity along with the molecular cloning of several of these receptors and subsequent heterologous expression have unambiguously confirmed the existence of at least 14 subtypes of serotonin receptors [258]. Most of the serotonin receptors, except the serotonin$_3$ receptor, belong to the GPCR family. The serotonin$_{1A}$ receptor is an important member of this large family of receptors 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 [259].

The serotonin$_{1A}$ receptor belongs to the rhodopsin family of GPCRs [131,260,261]. It was one of the first GPCRs for which the gene was cloned [262,263]. The gene is intronless and its mRNA is expressed mainly in the brain, spleen, neonatal kidney and gut. The human gene encodes a predicted protein of 422 amino acids. Although no high-resolution structure is available for the serotonin$_{1A}$ receptor, hydropathy plots of the amino acid sequences predict the presence of seven putative transmembrane domains, each of ~25 residues in length, which are thought to represent membrane-spanning α-helices (see Fig. 6) [260,261]. Based on the presence of three consensus N-linked glycosylation residues in the amino terminus and on its homology to the β_2-adrenoceptor, the serotonin$_{1A}$ receptor is predicted to have a topology where the amino terminus is oriented facing the extracellular space. According to this topology, the hydrophilic sequences connecting the transmembrane helices would form three intracellular (IC) and three extracellular (EC) loops in the protein with respect to the plasma membrane (Fig. 6). Receptors that bind to biogenic amines (such as the serotonin$_{1A}$ receptor) are known to possess binding sites at the transmembrane helix-lined pocket within the membrane [134]. Ligands that act as agonists, bind to this pocket and induce a conformational change in the transmembrane helices. This change acts as a switch to activate G-proteins bound to the second and third intracellular loops (IC2 and IC3) of the receptor. Similar to other GPCRs, the serotonin$_{1A}$ receptor includes a fourth putative cytoplasmic loop that is formed on account of post-translational and stable palmitoylation at the two conserved residues Cys^{417} and Cys^{420} in the carboxy terminus of the receptor. The importance of this post-translational modification is highlighted by the fact that palmitoylation deficient mutants, i.e., those that lack the two conserved cysteine residues, of the serotonin$_{1A}$ receptor are unable to interact with G-proteins and transduce signals upon binding to activating ligands [264]. A putative disulfide bond exists between Cys^{186} and Cys^{109} in the second extracellular domain (see Fig. 6). In comparison with the β_2-adrenoceptor [265,266], this disulfide bond may stabilize receptor conformation and possibly explain the inhibitory action of disulfide reducing and modifying agents on the ligand binding to the serotonin$_{1A}$ receptor [267,268].

4.2. Serotonin$_{1A}$ receptor as a key component of serotonergic signaling

Serotonergic signaling appears to play a key role in the generation and modulation of various cognitive and behavioral functions such as sleep, mood, pain, addiction, locomotion, sexual activity, depression, anxiety, alcohol abuse, aggression and learning [269–274]. Serotonergic signaling pathways play crucial roles in brain development processes such as neurogenesis and axonal branching during various stages of development [275–277]. Disruptions in serotonergic systems have been implicated in the etiology of mental disorders such as schizophrenia, migraine, depression, suicidal behavior, infantile autism, eating disorders, and obsessive compulsive disorder [272,273,278–281]. In addition, novel roles for serotonin and its receptors in heart disease

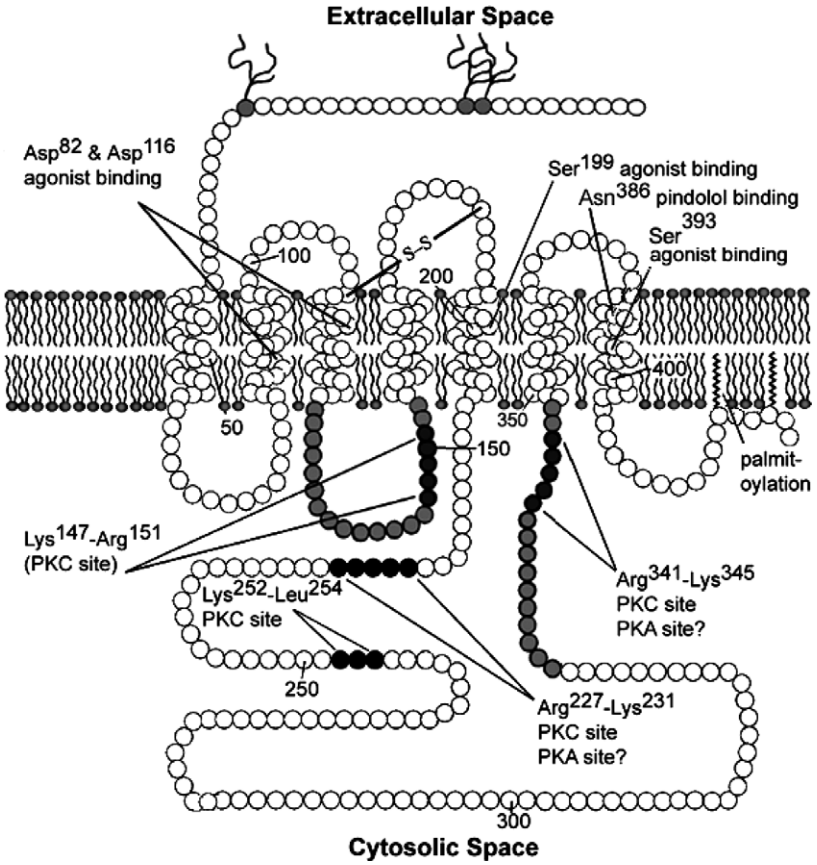

Fig. 6. A schematic representation of the membrane-embedded human serotonin$_{1A}$ receptor is shown with its topological and other structural features. The membrane is shown as a bilayer of two leaflets of lipids. The amino acids in the receptor sequence are shown as circles and are marked after every 50 residues for convenience. Seven transmembrane stretches, 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 disulfide bond putatively exists between Cys^{109} and Cys^{187}. Transmembrane (TM) domains contain residues (which are marked) that are important for ligand binding (see Ref. [261] for further details). The receptor is stably palmitoylated at residues Cys^{417} and/or Cys^{420}. Light gray circles represent contact sites for G-proteins. Black circles represent sites for protein kinase C (PKC) and/or protein kinase A (PKA) mediated phosphorylation. Adapted from Ref. [261].

[282], asthma [283], phagocytosis [284], as a candidate receptor for virus entry [175], and as a therapeutic target in malaria [285] have been recently reported.

The serotonin$_{1A}$ receptor has been shown to have a role in neural development [275,276] and protection of stressed neuronal cells undergoing degeneration and apoptosis [286]. Treatment using agonists for the serotonin$_{1A}$ receptor constitutes a potentially useful approach in the case of children with developmental disorders [287]. The serotonin$_{1A}$ receptor agonists [288] and antagonists [289] represent a major class of molecules with potential therapeutic effects in anxiety- or stress-related disorders. As a result, the serotonin$_{1A}$ receptor serves as an important target in the development of therapeutic agents to treat neuropsychiatric disorders such as anxiety and depression. On the clinical front, serotonin$_{1A}$ receptor expression levels are altered in schizophrenia [290], and in patients suffering from major depression [291]. A recent observation associates genetic polymorphisms at the upstream repressor region of the serotonin$_{1A}$ receptor gene to major depression and suicide in humans [292] linking its expression status to these clinical syndromes. Further, the antagonist binding and signaling function of serotonin$_{1A}$ receptors are attenuated in brains of suicide victims [293]. Interestingly, mice lacking the serotonin$_{1A}$ receptor generated a few years back exhibit enhanced anxiety-related behavior [272,278,279] and represent an important animal model for genetic vulnerability to conditions such as anxiety disorders and aggression [294].

5. Membrane biology of serotonin$_{1A}$ receptors

5.1. Lipid–protein interactions in serotonin$_{1A}$ receptors

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 system for studying the serotonin$_{1A}$ receptor function [183,261]. The receptor is relatively abundant in this tissue. Studies carried out using this system have led to characterization of the receptor and more importantly, have provided novel information on the interaction of the receptor with membrane lipids in its native environment.

The absence of a specific receptor for alcohols such as ethanol has led to the idea that alcohols exert their effects primarily by altering the membrane physical properties that in turn modulate membrane protein function [295]. Serotonergic signaling has been shown to play an important role in the regulation of alcohol intake, preference and dependence. A number of studies have indicated the involvement of serotonergic neurotransmission in alcohol tolerance and dependence [296,297]. The demonstration of an inhibition in the specific binding of the agonist 8-hydroxy-2(di-*N*-propylamino)tetralin (8-OH-DPAT) and the antagonist 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine (*p*-MPPF) to bovine hippocampal serotonin$_{1A}$ receptors in the presence of a homologous series of alcohols assumes significance in this regard [298,299]. Further, experiments involving effects of ethanol [298,299], and modifications of disulfide and sulfhydryl groups by agents that differ in their hydrophobicity [268] suggest that the antagonist binding site in the hippocampal serotonin$_{1A}$ receptors is localized in a more polar environment (perhaps shallower in relation to the membrane) than the agonist binding site, which is known to comprise of residues present in the transmembrane domains in the receptor [261]. In addition, GPCRs represent strong candidates for the action of local anesthetics since anesthetics have been shown to affect G-protein signal transduction pathways [300,301]. Importantly, tertiary amine local anesthetics have been shown to interact with the serotonin$_{1A}$ receptor by inhibiting specific agonist and antagonist binding when used at clinically relevant concentrations [302]. Further analysis of the effects of local anesthetics at such concentrations on the fluorescence polarization of probes located at different depths in the membrane, in combination with ligand binding carried out after a significant alteration in the membrane lipid (cholesterol) composition, favors the model of a direct interaction between the receptor and the local anesthetics as the predominant mechanism for inhibition in receptor function.

Since agonists like 8-OH-DPAT bind to receptors coupled to G-proteins whereas antagonists like *p*-MPPF bind to both G-protein coupled and uncoupled forms of the receptor [303–306], their relative binding abilities can be used to differentially discriminate the extent of interaction between the receptor and G-proteins [305,306]. The striking differences in agonist and antagonist binding to the serotonin$_{1A}$ receptors from bovine hippocampal membranes upon exposure to high temperatures has been explained on this basis [307]. Incubation of bovine hippocampal membranes to high temperatures irreversibly affects agonist binding to serotonin$_{1A}$ receptors. However, the antagonist binding remains relatively unaffected. Since integral membrane proteins are considered to possess high thermal stability [308], these results indicate that high temperature leads to inactivation of the peripherally associated G-proteins thereby rendering the agonist binding more sensitive to such treatments.

Membrane protein purification represents an area of considerable challenge in contemporary molecular biology. Studies carried out on purified and reconstituted membrane receptors have considerably advanced our knowledge of the molecular aspects of receptor function [149]. It is noteworthy that none of the subtypes of G-protein coupled serotonin receptors have yet been purified to homogeneity from a natural source. 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 [182,183,309]. Effective solubilization and purification of GPCRs in a functionally active form represent important steps in understanding structure–function relationships 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 [310]. 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 detergent concentrations. However, the ability of salts to reduce the CMC of charged detergents 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 [311]. A low (premicellar) concentration of the mild and non-denaturing, zwitterionic detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (see Fig. 9) has been used for solubilizing the serotonin$_{1A}$ receptor in the presence of salt followed by polyethylene glycol precipitation to remove the salt [311–313]. This results in efficient solubilization of serotonin$_{1A}$ receptors from native as well as heterologous expression systems with a high ligand binding affinity and ability to couple to G-proteins. Since high concentrations of CHAPS can cause dissociation of G-protein subunits from the membrane [314,315], the use of salt to effectively lower the concentrations required to achieve optimal solubilization of the serotonin$_{1A}$ receptor thus represents an elegant approach in the solubilization of the receptor with high ligand binding affinity and intact signal transduction components. 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. It has earlier been shown that different classes of detergents used for solubilization of membrane receptors result in differential solubilization of lipids and proteins since some detergents even extract some of the 'annular' lipids necessary for preserving the function of the receptor. This could result in a solubilized yet 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. In fact, the first detailed analysis of the requirement of specific lipids in the function of serotonin$_{1A}$ receptors was carried out by analyzing the ability of different detergents to co-solubilize active serotonin$_{1A}$ receptors and specific lipids from sheep brain membranes [185,316,317]. Interestingly, detergents such as CHAPS and 3-[(cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) which are most efficient in extracting functionally active serotonin$_{1A}$ receptors also displayed an enhanced ability in solubilizing the most amount of lipids from the membrane. Importantly, solubilization by CHAPS and CHAPSO led to the enrichment of PE, PC, phosphatidylinositol (PI) and phosphatidylserine (PS), and a depletion of sphingomyelin (SM), galactosylceramide, and cholesterol. In addition, the co-solubilized lipids displayed a markedly higher presence of saturated fatty acyl chains. These results were interpreted to reflect the localization of the serotonin$_{1A}$ receptor in specific regions of the membrane which displayed a unique lipid composition and could be solubilized by certain detergents such as CHAPS and CHAPSO. In addition, the role of an optimal membrane environment due to the presence of specific phospholipids, which exhibit a higher proportion of saturated fatty acids in the serotonin$_{1A}$ receptor function was proposed [317]. These studies serve to highlight the importance of the membrane environment in general and specific lipids in particular, in the serotonin$_{1A}$ receptor function.

5.2. Membrane dynamics of serotonin$_{1A}$ receptors

In light of the proposed significance of lateral diffusion of GPCRs in determining their interaction with G-proteins (see Section 3.4), the serotonin$_{1A}$ receptor has recently been used as a model GPCR to analyze the significance of receptor/G-protein interaction on the membrane dynamics of GPCRs [318,319]. Importantly, these results for the first time provide convincing evidence that the cell surface dynamics of a GPCR is dependent on its interaction with G-proteins. The fluorescence of the human serotonin$_{1A}$ receptor tagged to the enhanced yellow fluorescent protein (EYFP) has been utilized to analyze its cell surface dynamics using FRAP (see Fig. 7). This technique involves generating a concentration gradient of fluorescent molecules by irreversibly photobleaching a fraction of fluorophores in the observation region. The dissipation of this gradient with time owing to diffusion of fluorophores into the bleached region from the unbleached regions in the membrane is an indicator of the mobility of the fluorophores in the membrane [320,321]. Such an analysis carried out on serotonin$_{1A}$ receptors tagged to EYFP indicates that the

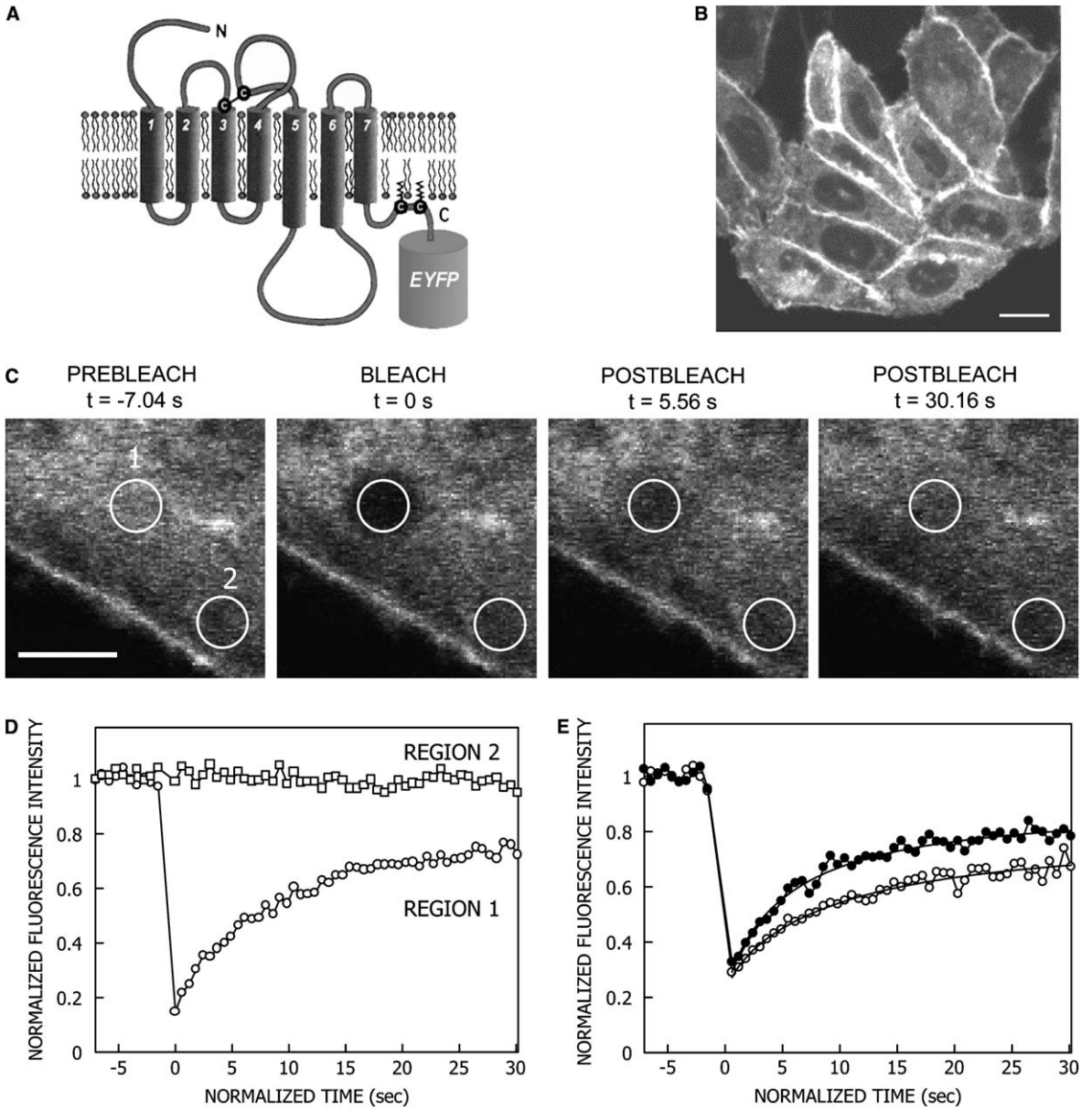

Fig. 7. Panel A is a schematic diagram indicating the overall topology of the serotonin$_{1A}$ receptor and the site of the EYFP tag on the receptor. Typical fluorescence distribution of the serotonin$_{1A}$ receptor-EYFP fusion protein stably expressed in CHO cells is shown in panel B. The image in panel B represents a mid-plane confocal section of this group of cells. The scale bar represents 10 μm. Panel C depicts confocal fluorescence images corresponding to the base of the same cell shown before and after photobleaching for the indicated duration of time in a typical FRAP experiment. The scale bar represents 5 μm. The prebleach image is shown at time $t < 0$ and the bleach event is shown at time $t = 0$. Normalized fluorescence intensity in regions 1 (bleach region) and 2 (control region) of the images in panel C are shown for the entire duration of the FRAP experiment in panel D. The constant fluorescence intensity in region 2 in the plot in panel D indicates no significant photobleaching of the field due to repeated imaging. The prebleach intensities are shown at time, $t < 0$. Typical fluorescence recovery plots of the serotonin$_{1A}$ receptor-EYFP fusion protein in cells in the absence (–○–) or presence (–●–) of AlF_4^-, a receptor-independent activator of G-proteins, are shown in panel E. The faster recovery of fluorescence in the presence of AlF_4^- indicates an increase in the lateral diffusion of the receptor upon G-protein activation. Adapted from Ref. [319].

mobility of the receptor is dependent on its interaction with G-proteins. Prior incubation with agents that activate G-proteins through receptor-dependent and -independent pathways increased receptor mobility on the plasma membrane.

The G-protein heterotrimer is a large protein complex with an average molecular mass of ~88 kDa, which would be ~1.2 times the mass of the receptor tagged to EYFP. It is therefore possible that their association with the receptor would reduce the mobility of the receptor. Receptor-dependent and -independent activation of G-proteins stimulates the exchange of a GTP for the existing GDP molecule at the Gα subunit of G-proteins, resulting in the dissociation of G-protein heterotrimer complex from the receptor. The proposal that the association of G-proteins to the receptor reduces its mobility is further validated by the observation that treatment of cells with pertussis toxin which reduces receptor and G-protein interaction also causes an increase in receptor mobility [318]. Diffusion behavior of several integral membrane proteins indicates that the cytoskeleton underlying the plasma membrane can act as a barrier to free diffusion of these proteins. Likewise, the presence of the bulky heterotrimeric G-protein complex associated with the receptor (since G-proteins, when bound to membrane receptors, could be considered as equivalent to cytoplasmic domains of membrane proteins) could further reduce (over the differences arising due to molecular mass of G-proteins) receptor diffusion, which would be partially relieved when the G-protein dissociates from the receptor. Another possibility could be that the increase in receptor diffusion could reflect changes in the oligomeric state of the receptor, as has been shown for the δ-opioid receptor [322] and the cholecystokinin receptor [323], or their partitioning into or out of domains proposed to exist on the cell surface (see Section 3.4) [207,209]. The demonstration of G-protein-dependent cell surface dynamics of the serotonin$_{1A}$ receptor provides novel insight into signal transduction involving this receptor in particular, and other GPCRs in general. Due to the similarity in the initial events of signal transduction involving GPCRs, it is possible that the increase in receptor mobility upon G-protein activation could take place in case of other GPCRs as well. Analysis of GPCR mobility therefore could be a sensitive and powerful approach to assess receptor/G-protein interaction in intact cells.

5.3. Role of cholesterol in the function of serotonin$_{1A}$ receptors

As mentioned earlier, cholesterol is an essential constituent of eukaryotic membranes and is important for the maintenance of membrane protein function, especially in the nervous system. The central nervous system which accounts for only 2% of the body mass contains ~25% of free cholesterol present in the whole body [324,325]. Although the brain is an organ that is highly enriched in cholesterol, the organization and dynamics of brain cholesterol is still poorly understood [326]. Brain cholesterol is synthesized in situ and is developmentally regulated [324,325]. Cholesterol organization, traffic, and dynamics in the brain is stringently controlled since the input of cholesterol into the central nervous system is almost exclusively from in situ synthesis as there is no available evidence for the transfer of cholesterol from blood stream to brain [324]. As a result of this, a number of neurological diseases share a common etiology of defective cholesterol metabolism in the brain [63]. 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 in situ synthesis and such synthesis is defective in this syndrome [327]. Interestingly, some of these diseases show symptoms that are similar to those which appear upon disruption of serotonergic signaling [328]. A detailed analysis of the effects of modulating the membrane cholesterol content on the function of serotonin receptors could provide strong and direct evidence to support this possibility.

The modulatory role of cholesterol on the ligand binding activity and receptor/G-protein interaction of the bovine hippocampal serotonin$_{1A}$ receptor has recently been shown by depleting cholesterol from native membranes using MβCD [72]. Specific removal of cholesterol from hippocampal membranes using MβCD resulted in a concentration-dependent reduction in specific binding of the agonist 8-OH-DPAT to serotonin$_{1A}$ receptors. This is accompanied by alterations in binding affinity and sites obtained from analysis of saturation binding data. Importantly, cholesterol depletion affects interaction between the receptor and G-proteins when monitored by analyzing the sensitivity of the agonist binding to GTP-γ-S, a non-hydrolyzable analogue of GTP (see Fig. 8). Thus, the serotonin$_{1A}$ receptor in cholesterol-depleted membranes displays a ~2.5-fold lower sensitivity to GTP-γ-S indicating a reduced extent of receptor/G-protein interaction. Replenishment of cholesterol-depleted membranes with cholesterol led to recovery of the agonist binding activity to a considerable extent indicating the specificity of the effect of cholesterol depletion. These results provide evidence that

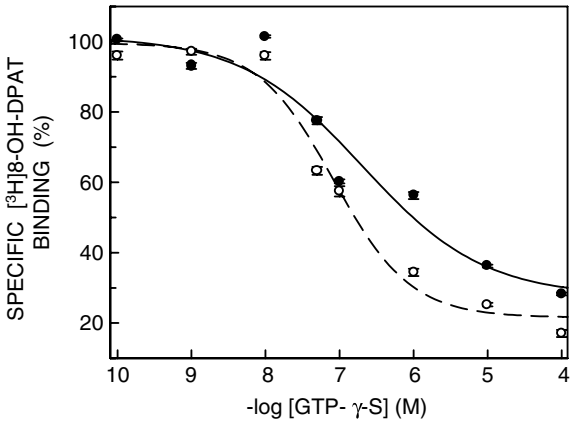

Fig. 8. The sensitivity of binding of the specific radiolabeled agonist [^{3}H]8-OH-DPAT to serotonin$_{1A}$ receptors in native membranes to GTP-γ-S, a non-hydrolyzable analogue of GTP, is a measure of the extent of receptor/G-protein interaction (see Ref. [305]). The plots show the effect of increasing concentrations of GTP-γ-S on the percentage specific [^{3}H]8-OH-DPAT binding to native (–○–) and MβCD-treated (–●–) membranes. The higher concentration of GTP-γ-S required to reduce the [^{3}H]8-OH-DPAT binding by 50% of its initial value (IC_{50}) in MβCD-treated membranes indicates that cholesterol depletion reduces the interaction between the serotonin$_{1A}$ receptor and G-proteins. Adapted from Ref. [72].

cholesterol is necessary for ligand binding and receptor/G-protein interaction of this important neurotransmitter receptor. Analyses of the effects of cholesterol depletion on the antagonist *p*-MPPF binding to the receptor provide further insight into the modulatory role of cholesterol on receptor function. The specific depletion of cholesterol significantly reduces the binding of this antagonist to the serotonin$_{1A}$ receptor [329]. Since the binding of the antagonist *p*-MPPF does not depend on a functional interaction between the serotonin$_{1A}$ receptor and G-protein [305,306], these results imply that membrane cholesterol modulates serotonin$_{1A}$ receptor function irrespective of its ability to interact with G-proteins. The effects of cholesterol depletion on antagonist binding to the receptor are predominantly reversed upon cholesterol replenishment of cholesterol-depleted membranes. The effects of cholesterol depletion on the serotonin$_{1A}$ receptor ligand binding function therefore suggest a possible alteration in the structure/organization of the receptor in the membrane that effectively reduces interaction between the receptor and G-protein. In addition, these results demonstrate that pharmacologically well-characterized ligands, capable of distinguishing alternate forms of the receptor (G-protein coupled and/or uncoupled), could be useful to delineate possible mechanisms by which membrane lipids modulate the function of GPCRs in general.

A comprehensive analysis of the requirement of cholesterol in the serotonin$_{1A}$ receptor function has been carried out using techniques that independently alter cholesterol content and/or availability in the membrane. These approaches tested the proposal that if cholesterol is necessary for ligand binding to the serotonin$_{1A}$ receptor, modulating cholesterol content and/or availability by other means could lead to similar effects as that observed using MβCD. The membrane active, sterol-complexing agent nystatin (see Section 2.3) differentially affects ligand binding to the serotonin$_{1A}$ receptor [330]. Interestingly, the treatment of hippocampal membranes with nystatin leads to a reduction in the antagonist binding whereas the agonist binding remains largely unaffected. On the other hand, the widely used cholesterol-complexing detergent digitonin (see Section 2.3) causes a marked reduction in both the agonist- and antagonist-binding function of the serotonin$_{1A}$ receptor [88]. In this work, digitonin was used at concentrations that do not lead to membrane solubilization. Furthermore, the cholesterol-complexing ability of digitonin was confirmed by using a combination of thin-layer chromatography to determine the total cholesterol content and an enzyme-based cholesterol assay to determine the available cholesterol content in the membrane (see Fig. 3). These results demonstrate interesting differences in the manner in which cholesterol modulates ligand-binding activity of the serotonin$_{1A}$ receptor depending on the exact approach used to perturb membrane cholesterol (i.e., by depletion with agents such as MβCD or complexation with nystatin and digitonin).

The role of cholesterol in the serotonin$_{1A}$ receptor function was further analyzed by monitoring the consequences of cholesterol oxidation (see Section 2.3) [331]. These studies revealed a remarkable sensitivity of

ligand binding to the receptor due to oxidation of cholesterol. Thus, a ~30% oxidation of cholesterol results in a dramatic reduction in the agonist (8-OH-DPAT) and antagonist (*p*-MPPF) binding to the serotonin$_{1A}$ receptor. As described earlier (see Section 2.3), the oxidation of cholesterol perturbs the functionally important hydroxyl group in the molecule thereby altering its potential to participate in specific molecular interactions with sphingomyelin or lipids with saturated fatty acyl chains [39,100,101]. Importantly, compared to physical depletion of cholesterol from membranes using MβCD, cholesterol oxidation appears to be milder in that it induces relatively less perturbation to membrane physical properties. The analysis of the ligand binding function of the serotonin$_{1A}$ receptor under conditions of cholesterol oxidation therefore provides useful information regarding the specificity of the receptor/cholesterol interaction in a native-like, yet cholesterol-deficient, membrane environment.

It has been proposed that the effects of cholesterol on the function of GPCRs could occur due to alterations either in the membrane physical properties or in specific local molecular interaction between cholesterol and the receptor [115]. Cholesterol is reported to modulate rhodopsin functions through an indirect mode by altering physical properties of the membrane (see Section 3.5). On the other hand, the requirement of cholesterol to support the oxytocin receptor ligand binding function is attributed to a specific interaction between cholesterol and the receptor. Whether the reduction in ligand binding to the serotonin$_{1A}$ receptor upon alteration in cholesterol content and/or availability results from an alteration in receptor conformation due to a change in the membrane physical properties or due to the loss of specific cholesterol–protein interaction poses an interesting question. The changes occurring in the membrane physical properties upon altering the availability and/or content of cholesterol were analyzed in order to delineate the role of cholesterol in the serotonin$_{1A}$ receptor function. More specifically, the changes occurring in membrane order were monitored by steady-state fluorescence polarization of membrane probes which are incorporated at different locations (depths) in the membrane. The fluorescent probe DPH and its derivatives represent popular membrane probes for monitoring organization and dynamics in membranes [332]. Fluorescence polarization is correlated to the rotational diffusion [333] of membrane-embedded probes, which is sensitive to the packing of fatty acyl chains and cholesterol. Since the membrane is considered to be a two-dimensional anisotropic fluid, any possible change in membrane order may not be uniform and restricted to a unique location in the membrane. It is therefore important to monitor the change in membrane order at multiple regions in the membrane to obtain a comprehensive understanding of any change in membrane (lipid) dynamics induced by cholesterol depletion. DPH, which is a rod-like molecule, partitions into the interior of the bilayer. However, its precise orientation in the membrane interior is not known. The derivative of DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) has a cationic moiety attached to the *para* position of one of the phenyl rings [334]. While DPH is known to partition into the hydrophobic core of the membrane, the amphipathic TMA-DPH is oriented in the membrane bilayer with its positive charge localized at the lipid–water interface [335]. Its DPH moiety is localized at ~11 Å from the center of the bilayer and reports the interfacial region of the membrane [336]. In contrast to this, the average location of DPH has been shown to be ~8 Å from the center of the bilayer [336].

Fluorescence polarization experiments with these probes incorporated in hippocampal membranes indicate that the physical depletion of cholesterol using MβCD leads to a reduction in membrane order [72]. Importantly, membrane order in the deeper regions of the membrane (sampled by DPH) is more sensitive to cholesterol depletion compared to the shallower regions (sampled by TMA-DPH). The effects of the membrane active compounds nystatin and digitonin on membrane order are different. While the presence of nystatin reduced the fluorescence polarization of both these probes to a similar extent [330], digitonin does not significantly affect fluorescence polarization of these probes [88]. Nevertheless, digitonin reduces the ligand binding of the serotonin$_{1A}$ receptor to a dramatic extent. Furthermore, the oxidation of cholesterol by cholesterol oxidase, which leads to a reduction in ligand binding to the serotonin$_{1A}$ receptor, does not significantly alter fluorescence polarization of both these probes [331]. Taken together, these results indicate a lack of correlation between effects on ligand binding function of the serotonin$_{1A}$ receptor and changes in the membrane order as monitored using the fluorescence polarization of membrane embedded probes. It is therefore possible that the effects of an altered cholesterol content and/or availability on the serotonin$_{1A}$ receptor ligand binding function are a consequence of disruption of a specific local interaction between cholesterol and the receptor.

Furthermore, the requirement of cholesterol in the function of serotonin$_{1A}$ receptors has been demonstrated in solubilized membrane preparations. As mentioned earlier, the detergent CHAPS has been found

to be efficient in solubilizing functionally active serotonin$_{1A}$ receptors from native [185,311,312,316,317], and heterologous expression systems [313]. It is interesting to note that membrane solubilization by CHAPS leads to depletion of cholesterol from the membrane (see Fig. 9) [184,337]. Importantly, CHAPS-solubilized serotonin$_{1A}$ receptors consistently display reduced ligand binding activity and a reduced capacity to interact with G-proteins [312]. Whether the reduced function of the serotonin$_{1A}$ receptor in CHAPS-solubilized membrane preparations is due to the loss of cholesterol from these membranes was analyzed by replenishing the solubilized membrane preparations with cholesterol complexed with MβCD. Significantly, cholesterol replenishment of solubilized membranes leads to an increase in ligand binding and receptor/G-protein interaction of the serotonin$_{1A}$ receptor [337]. These results provide additional support for the requirement of membrane cholesterol in the serotonin$_{1A}$ receptor function, and have contributed in devising a more efficient solubilization procedure for the serotonin$_{1A}$ receptor.

The most important aspect of these results is that alteration in the content and/or availability of membrane cholesterol can induce significant changes in the activity and receptor/G-protein interaction of the serotonin$_{1A}$ receptor. Whether such manipulations in membrane cholesterol content could be induced in vivo represents a challenging question. The turnover of brain cholesterol is very low, with a half-life of ~6 months [324]. As a result, the cerebrospinal fluid levels of cholesterol are ~40–50-fold lower than the plasma cholesterol [128]. Due to the presence of the blood brain barrier, alterations in serum levels of cholesterol are believed not to affect the total cholesterol level in the central nervous system. However, under such conditions, the neuronal plasma membrane fractions have not been studied adequately. In addition, regions in the central nervous system (such as the hypothalamic area) that are somewhat weakly protected by the blood brain barrier may be sensitive to fluctuations in the plasma levels of cholesterol. Interestingly, chronic in vivo administration of statins specifically reduces brain cholesterol levels leaving serum cholesterol levels unaffected [338,339]. It is possible that the

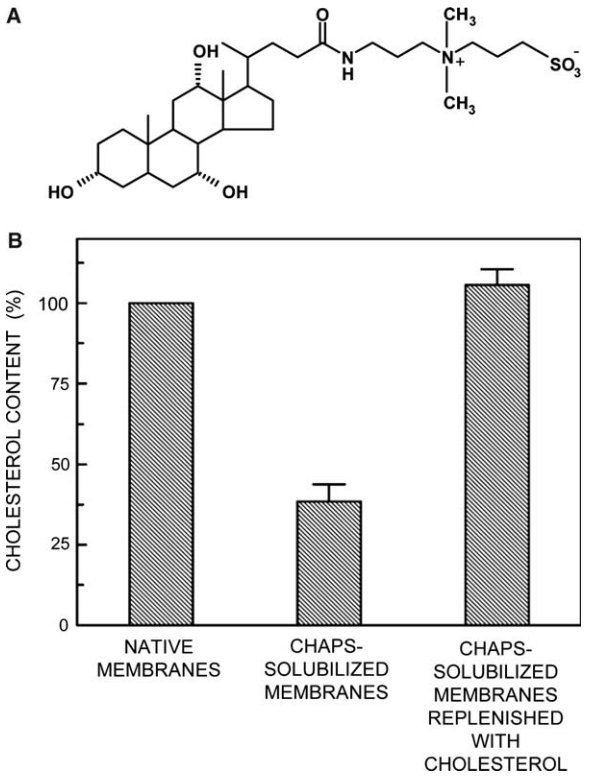

Fig. 9. Panel A shows the chemical structure of the detergent CHAPS. The cholesterol contents in native, CHAPS-solubilized, and cholesterol-replenished CHAPS-solubilized hippocampal membranes are shown in panel B. Thus, membrane solubilization by CHAPS results in a reduction in the cholesterol content that can be replenished using a water soluble MβCD-cholesterol complex. The figure in panel B is adapted from Ref. [337].

more severe deficiency in cholesterol levels in the brain occurs on account of the lower turnover of cholesterol in this tissue. Interestingly, low serum cholesterol concentration has been correlated with an increase in the prevalence of suicide in humans [340] and is partly attributed to an altered serotonin metabolism [341]. Moreover, a reduction in the membrane cholesterol content in post-mortem brain tissues from patients suffering from mood disorders has recently been reported [342]. In light of the requirement of membrane cholesterol in the serotonin$_{1A}$ receptor function, the role of brain membrane cholesterol in the etiology of psychological disorders that are correlated with an altered cholesterol metabolism requires further investigation.

5.4. Cholesterol-dependent organization and dynamics of the serotonin$_{1A}$ receptor in the plasma membrane

The distribution of the serotonin$_{1A}$ receptor on the plasma membrane and its relationship with cholesterol-rich membrane domains (see Sections 2.2 and 3.4) that are implicated in regulating cellular signaling functions is currently being addressed. Insolubility of membrane constituents in non-ionic detergents has proved to be a useful tool to characterize membrane domains [52,53,343]. These domains have been operationally defined using the criterion of insolubility in non-ionic detergents such as Triton X-100 at 4 °C. Importantly, detergent insolubility of serotonin$_{1A}$ receptors has been recently assessed by a novel approach based on the treatment of cells stably expressing the serotonin$_{1A}$ receptor tagged to EYFP in culture with cold Triton X-100, followed by quantitation of the residual fluorescence of the serotonin$_{1A}$ receptor tagged to EYFP (see Fig. 10) [344]. This constitutes the first report that monitors the membrane organization of the serotonin$_{1A}$ receptor by monitoring its insolubility in non-ionic detergents. This fluorescence microscopic approach toward determination of detergent insolubility of membrane components has been validated with the use of specific lipid and protein markers, whose organization in membranes and ability to be extracted by cold non-ionic detergents have been well documented. Results from such an approach indicate that a large fraction of serotonin$_{1A}$ receptors is soluble in the detergent. Analyzing the detergent insolubility status of the serotonin$_{1A}$ receptor upon agonist-dependent activation and upon alteration in the membrane cholesterol content opens up new areas in receptor signaling and membrane domain organization of serotonin$_{1A}$ receptors [345]. Significantly, we observe that the cell surface dynamics of the serotonin$_{1A}$ receptor is modulated upon agonist-dependent activation [318] and upon cholesterol depletion [346]. Whether the differences in the diffusion properties of the receptor in cholesterol-depleted membranes result from the movement of receptors into or out of such domains enriched in cholesterol represents an interesting possibility and is currently being addressed in our laboratory. Taken together, these results bring out several interesting possibilities on the function and organization of serotonin$_{1A}$ receptors in the general context of lipid–protein interactions. These studies assume greater importance on account of the enormous implications of serotonin$_{1A}$ receptor function in human health and the observation that several diagnosed brain diseases are attributed to an altered lipid–protein interaction.

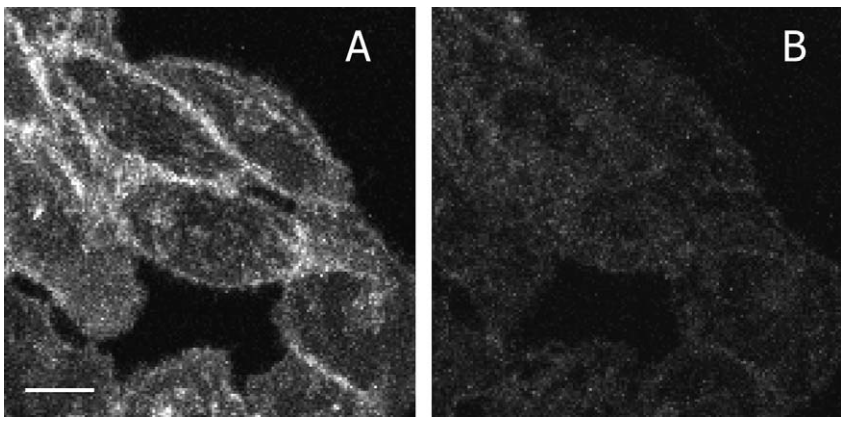

Fig. 10. Cells stably expressing the serotonin$_{1A}$ receptor-EYFP fusion protein are shown (A) before and (B) after treatment with 0.05% (w/v) cold Triton X-100 for 10 min. The images represent combined mid-plane confocal sections of the same group of cells before and after detergent extraction. The scale bar represents 10 μm. Adapted from Ref. [344].

6. Conclusions and future perspectives

We have highlighted the importance of lipid–protein interactions, especially cholesterol–receptor interactions, involved in the function of GPCRs from a variety of sources. More specifically, we have reviewed the recently reported role of cholesterol in the function of the serotonin$_{1A}$ receptor, an important member of the GPCR superfamily. Research on the serotonin$_{1A}$ receptor has presently reached an interesting stage. The early development of receptor-specific ligands, and generation of mice models that lack the serotonin$_{1A}$ receptor have generated important information regarding the pharmacology of this receptor and its importance to neuronal physiology. Along with this, more recent information regarding the membrane organization and dynamics of the serotonin$_{1A}$ receptor and the requirement of specific lipids such as cholesterol in its ligand binding and signaling functions have proved to be essential in developing a comprehensive framework to understand the function of the serotonin$_{1A}$ receptor. These advances in understanding the receptor function are noteworthy since the serotonin$_{1A}$ receptor has not yet been purified to homogeneity. As a consequence, inputs from structural biology (crystallography and NMR spectroscopy) have been sorely missing for this important receptor. The challenge lies in better defining lipid–protein interactions involving this receptor. While the requirement of cholesterol in serotonin$_{1A}$ receptor function is now known, the application of fluorescence-based and photolabeling techniques with cholesterol analogues to investigate a possible direct interaction between the serotonin$_{1A}$ receptor and cholesterol would tremendously help in elucidating the role of cholesterol in the function of this important member of the GPCR family.

The realization that lipids such as cholesterol influence the function of GPCRs has remarkably transformed our idea regarding the behavior of this important class of membrane proteins. In a broader sense, the diversity of lipids found in natural membranes combined with the ability of cells to modulate their membrane lipid composition under conditions of a variety of stress vastly increase the potential by which lipids can exert their influence on receptor function. The development of newer and more sensitive technologies that determine these interactions and their influence on receptor function in a more native-like membrane environment would provide a more comprehensive understanding of GPCR function.

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