Received: 30 September 2019 | Revised: 28 December 2019 | Accepted: 9 January 2020

DOI: 10.1002/wsbm.1481

ADVANCED REVIEW

WIREs SYSTEMS BIOLOGY AND MEDICINE WILEY

Cholesterol interaction motifs in G protein-coupled receptors: Slippery hot spots?

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Funding information
Science and Engineering Research Board

Abstract

G protein-coupled receptors (GPCRs) are cell membrane associated signaling hubs that orchestrate a multitude of cellular functions upon binding to a diverse variety of extracellular ligands. Since GPCRs are integral membrane proteins with seven-transmembrane domain architecture, their function, organization and dynamics are intimately regulated by membrane lipids, such as cholesterol. Cholesterol is an extensively studied lipids in terms of its effects on GPCR structure and function. One of the possible mechanisms underlying modulation of GPCR function by cholesterol is *via* specific interaction of GPCRs with membrane cholesterol. These interactions of GPCRs with membrane cholesterol are often attributed to structural features of GPCRs that could facilitate their preferential association with cholesterol. In this backdrop, cholesterol interaction motifs represent putative interaction sites on GPCRs that could facilitate cholesterol-sensitive function of these receptors. In this review, we provide an overview of cholesterol interaction motifs found in GPCRs, which have been identified through a combination of crystallography, bioinformatics analysis, and functional studies. In addition, we will highlight, using specific examples, why *mere* presence of a cholesterol interaction motif at a given site may not directly implicate its role in interaction with membrane cholesterol. We therefore believe that experimental approaches, followed by functional analysis of cholesterol sensitivity of GPCRs, would provide a better understanding of the role played by these motifs in cholesterol-sensitive function. We envision that a comprehensive knowledge of cholesterol interaction sites in GPCRs would allow us to develop a better understanding of GPCR structure-function paradigm, and could be useful in future therapeutics.

ABBREVIATIONS: GPCR, G protein-coupled receptor; TM, transmembrane helix; CCM, cholesterol consensus motif; CRAC, cholesterol recognition/interaction amino acid consensus; ECL, extracellular loop; PBR, peripheral-type benzodiazepine receptor; P450scc, cholesterol side-chain cleavage enzyme; SBD, sphingolipid-binding domain; SBM, sphingolipid-binding motif; ICL3, intracellular loop 3; 5-HT_{1A}R, serotonin$_{1A}$ receptor; A_{2A}R, adenosine$_{2A}$ receptor; α_{1A}AR, α_{1A} adrenergic receptor; CB_1, type-1 cannabinoid receptor; CB_2, type-2 cannabinoid receptor; $GABA_B$, γ-aminobutyric acid B receptor; CXCR4, CXC chemokine receptor 4; β_2AR, β_2-adrenergic receptor; CCK1, type-1 cholecystokinin receptor; DRD_1, D1 dopamine receptor; m_2AChR, muscarinic acetylcholine receptor 2; NTS_1R, type-1 neurotensin receptor; OPN1MW, medium-wave-sensitive opsin 1; OXTR, oxytocin receptor; S1PR, sphingosine 1-phosphate receptor; T2R4, bitter taste receptor 4.

Based on a presentation to the 13th International Conference on Pathways, Networks and Systems Medicine. *www.aegeanconferences.org*

This article is categorized under:
Models of Systems Properties and Processes > Mechanistic Models
Analytical and Computational Methods > Computational Methods
Laboratory Methods and Technologies > Macromolecular Interactions, Methods

KEYWORDS

GPCR, cholesterol interaction motifs, cholesterol recognition/interaction amino acid consensus, cholesterol consensus motif

1 | INTRODUCTION

G protein-coupled receptors (GPCRs) are the largest superfamily of integral membrane proteins that act as versatile signaling hubs and respond to a wide array of external stimuli like photons, odorants, ions, neurotransmitters, and hormones (Chattopadhyay, 2014; Erlandson, McMahon, & Kruse, 2018; Rosenbaum, Rasmussen, & Kobilka, 2009). Since GPCRs cross the membrane seven times (due to their seven transmembrane domain architecture), they are intimately associated with their immediate membrane environment. There is extensive literature on the role of membrane lipids in GPCR biology, encompassing structural, biochemical, biophysical and computational approaches. In particular, membrane cholesterol has been shown to be a crucial modulator of GPCR organization, dynamics, oligomerization, and function (Burger, Gimpl, & Fahrenholz, 2000; Chattopadhyay, 2014; Chini & Parenti, 2009; Fantini, Epand, & Barrantes, 2019; Gahbauer & Böckmann, 2016; Gimpl, 2016; Jafurulla & Chattopadhyay, 2013; Kiriakidi et al., 2019; Mondal, Khelashvili, Johner, & Weinstein, 2014; Oates & Watts, 2011; Paila & Chattopadhyay, 2010; Pucadyil & Chattopadhyay, 2006; Sengupta & Chattopadhyay, 2015; Sengupta, Prasanna, Mohole, & Chattopadhyay, 2018). Cholesterol (Figure 1) is an indispensable constituent of cellular membranes of all higher eukaryotes and is crucial in membrane organization (Mouritsen & Zuckermann, 2004), dynamics (Grouleff, Irudayam, Skeby, & Schiøtt, 2015), function (Simons & Ikonen, 2000), sorting (Liscum & Underwood, 1995) of membrane proteins and entry of intracellular pathogens (Kumar, Jafurulla, & Chattopadhyay, 2016).

Membrane cholesterol has been shown to affect ligand binding, G-protein coupling and intracellular signaling of GPCRs. The possible mechanism underlying the modulation of GPCR function by cholesterol could be *via* specific interaction of GPCRs with membrane cholesterol, or cholesterol-induced changes in global bilayer properties, or a combination of both mechanisms (recently reviewed in Jafurulla, Kumar, Rao, & Chattopadhyay, 2019). Specific interaction of GPCRs with membrane cholesterol are attributed to structural features of these receptors that could facilitate their preferential association with membrane cholesterol. In this backdrop, cholesterol interaction motifs represent putative interaction sites in GPCRs that could facilitate cholesterol-sensitive function of these receptors. In general, cholesterol interaction motifs in membrane receptors are proposed to interact with cholesterol *via* aromatic amino acid residues that have been suggested to interact with ring D of the fused steroid ring of cholesterol (Figure 1; Hanson et al., 2008). On the other hand, the polar 3β-hydroxyl group of cholesterol has been proposed to be involved in electrostatic interactions with positively charged residues in cholesterol interaction motifs (Figure 1; Jamin et al., 2005; Epand et al., 2006).

Interestingly, cholesterol or closely related cholesterol derivatives can modulate the function of certain GPCRs by binding deep in the seven-transmembrane pocket, thereby acting like conventional GPCR ligands. For example, oxygenated

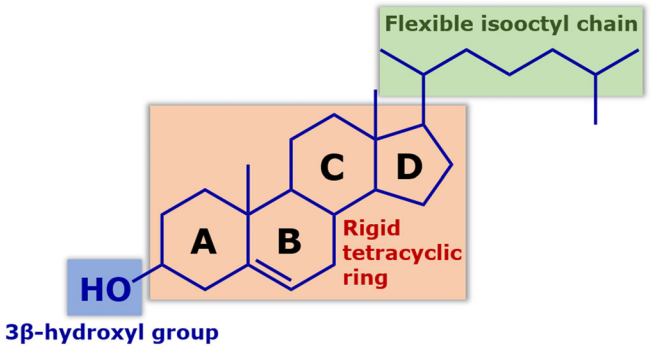

FIGURE 1 Chemical structure of cholesterol. Chemical structure of cholesterol with its three structurally distinct regions (shown as shaded boxes): the polar 3β-hydroxyl group, the rigid tetracyclic fused ring (shown as A–D), and the flexible isooctyl side chain

cholesterol derivatives that are emerging as a physiologically important group of sterols (oxysterol), is believed to follow this mode of binding mode at the Epstein-Barr virus-induced G protein-coupled receptor 2 (Benned-Jensen et al., 2012) and the chemokine receptor CXCR2 (Sensi et al., 2014). A recent study, employing simulation and experimental approaches, has proposed that membrane cholesterol could enter the deep orthosteric ligand binding pocket in the adenosine A_{2A} receptor (Guixà-González et al., 2017). One of the most compelling functional correlates of cholesterol interaction with GPCRs was shown in the recently reported structure of the sterol binding frizzled (class F) GPCR, smoothened (Smo) (Byrne et al., 2016; Huang et al., 2018). Cholesterol acts as the endogenous activator of the hedgehog pathway by inducing conformational changes in the Smo receptor. The structure of Smo showed a bound cholesterol molecule to the extracellular cysteine-rich domain of the receptor which is crucial for transduction of hedgehog signals (Deshpande et al., 2019).

In this review, we provide a broad overview of cholesterol binding/interaction motifs in GPCRs, that have been characterized through a combination of crystallography, bioinformatics analysis, and functional studies. This will be followed by specific examples of representative cholesterol binding/interaction motifs in key GPCRs that are known to display cholesterol-sensitive function. Importantly, we would highlight that it is advisable to exercise caution before attributing cholesterol sensitivity of receptor function to *mere* presence of these motifs.

2 | CHOLESTEROL MOLECULES CLOSELY ASSOCIATED WITH GPCRS IN CRYSTAL STRUCTURES

An interesting common feature observed in high-resolution crystal structures of GPCRs is the close association of bound cholesterol molecule(s) to the receptor. One of the first examples of such bound cholesterol was observed in the crystal structure of the β_2-adrenergic receptor (Cherezov et al., 2007; Rosenbaum et al., 2007), in which three cholesterol molecules were found for each monomer at the dimeric interface of the receptor (Figure 2a). Subsequently, many such examples of bound cholesterol molecules were found for a range of GPCRs with ~1–4 closely associated cholesterol molecules per receptor monomer (for a comprehensive list of GPCR crystal structures with bound cholesterol, please refer to Jafurulla et al., 2019). However, a caveat here is that GPCRs are crystallized in the lipidic cubic phase containing cholesterol hemisuccinate, and therefore the physiological significance of receptor-bound cholesterol molecules could be somewhat tenuous (Khelashvili et al., 2012). In addition, cholesterol hemisuccinate is often used to replace cholesterol in crystallization of membrane proteins. Recent evidences suggest that it may not mimic cholesterol very well and could behave somewhat differently than cholesterol (Kulig et al., 2014, 2015). Interestingly, lipid molecules that are co-crystallized with membrane proteins (and therefore remain preserved even in the crystal structure) are often localized in protein–protein interfaces in oligomeric proteins and belong to the class of "nonannular" (or sometimes termed as "co-factor") lipids (Lee, 2003, 2005; Paila, Tiwari, & Chattopadhyay, 2009). In addition, the absence of a specific structural motif for a distinct cholesterol interaction site could be an issue in understanding the biological significance of such bound cholesterol molecules (Hanson et al., 2008).

In a subsequent crystal structure of the human β_2-adrenergic receptor, two cholesterol molecules (cholesterol 1 and 2) were found in a specific cholesterol binding site formed by transmembrane helices I–IV (TM I–IV; Hanson et al., 2008, see Figure 2b), which were not positioned in the crystal packing interface of the receptor monomers (Cherezov et al., 2007). The binding site in the crystal structure of the receptor consists of four key amino acid residues across two different transmembrane helices (TM II and IV), which were instrumental in defining a plausible cholesterol binding site in GPCRs. This site was defined as the *strict* cholesterol consensus motif (CCM) (Hanson et al., 2008). The shallow cleft formed by TM I–IV of the β_2-adrenergic receptor could accommodate two cholesterol molecules, although the extent of interaction was less between cholesterol 2 and the receptor (Figure 2b). The aromatic tryptophan residue at position 4.50 (according to the Ballesteros–Weinstein numbering scheme; Ballesteros & Weinstein, 1995) is the most conserved (~94%) residue in TM IV of class A GPCRs (Figure 2b) and appears to contribute to the most crucial interaction with the ring D of the fused steroid ring of cholesterol 1 through CH-π interaction. In this structure, the hydrophobic isoleucine residue (position 4.46, ~60% homology conserved) interacts with rings A and B of the fused steroid ring of cholesterol. An additional aromatic residue from TM II (tyrosine 2.41 in β_2-adrenergic receptor) forms van der Waals interactions with ring A of cholesterol 1 and hydrogen bonds with a positively charged arginine residue (position 4.43). The tetrad of these amino acid residues in a spatially defined region constitutes a *strict* CCM. Importantly, the criterion for specific residues in CCM (as observed in case of β_2-adrenergic receptors) could be somewhat extended by conservative replacement of amino acids with similar physicochemical properties. In addition, the positively charged residue at an analogous position to arginine 4.43 in the β_2-adrenergic receptor is only ~22% conserved in class A GPCRs (Hanson et al., 2008). However, due to the nonspecific nature of electrostatic interactions at the membrane interface, proximal

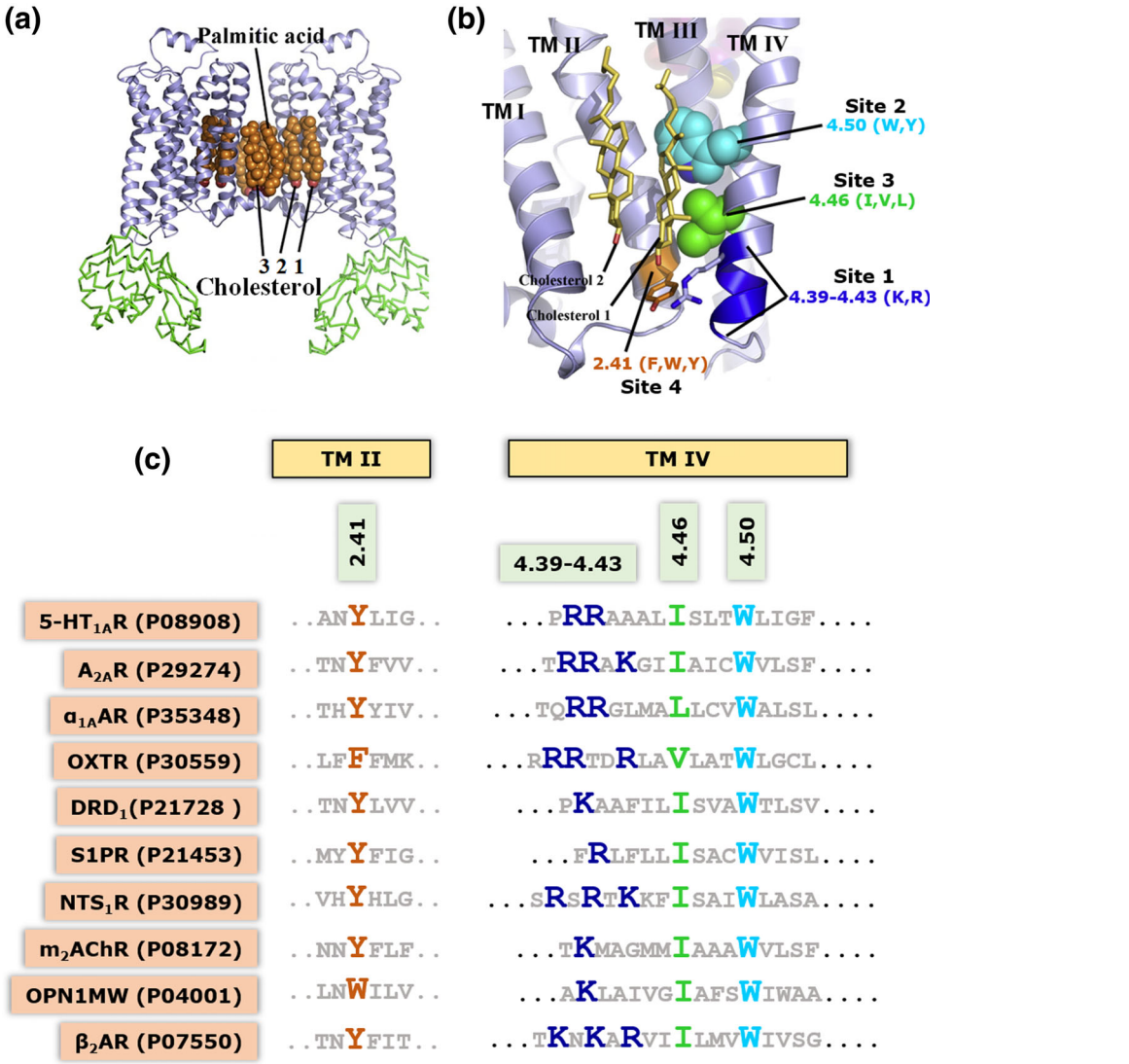

FIGURE 2 Closely associated cholesterol molecules in G protein-coupled receptor crystal structures. (a) Crystal structure of the human β_2-adrenergic receptor (Cherezov et al., 2007; Rosenbaum et al., 2007; PDB ID: 2RH1). The receptor monomers (shown in blue) pack in a parallel orientation with three cholesterol molecules (shown in orange) bound to each monomer and a palmitic acid alkyl chain is located between cholesterol 2 and 3. The receptor construct was modified for enhanced crystallizability by incorporation of T4-lysozyme between helices V and VI (shown in green) (Reprinted with permission from Hanson et al. (2008). Copyright 2008 Elsevier Ltd.). (b) The key amino acid residues in the *strict* cholesterol consensus motif (CCM) from the crystal structure of the human β_2-adrenergic receptor (Hanson et al., 2008; PDB ID: 3D4S). Two bound cholesterol molecules are shown in yellow and the side chain positions of the crucial amino acids in the CCM are highlighted. Site 1 (blue) at the cytoplasmic end of transmembrane helix IV (TM IV) spanning positions 4.39–4.43 fulfills the CCM requirement, if one or more of these positions contain a basic amino acid residue (arginine or lysine). Site 2 (cyan) at position 4.50 on TM IV contributes to CH-π interactions (represented as space-filling cyan side-chain atoms) and is the most conserved site with tryptophan occupying the position in ~94% of class A GPCRs. The other allowed amino acid in this position is tyrosine. Site 3 (represented as space-filling side-chain atoms in green) at position 4.46 on TM IV contributes *via* van der Waals interaction to cholesterol binding and fulfills the CCM requirement, if isoleucine, leucine, or valine is present in this position. Site 4 (maroon) on TM II at position 2.41 can be either tryptophan or phenylalanine or tyrosine. Sites 1–3 together defines CCM, whereas the presence of site 4 along with other three sites defines the four component *strict* CCM (Reprinted with permission from Hanson et al. (2008). Copyright 2008 Elsevier Ltd.). (c) A representative list of GPCRs with their CCM motifs. The positions of the aromatic amino acid residues (tryptophan or tyrosine or phenylalanine) in TM II and TM IV are highlighted in maroon and cyan, respectively. The basic amino acid residues (arginine or lysine) at the cytoplasmic end of TM IV are highlighted in blue and the central aliphatic amino acid residues (isoleucine, leucine, or valine) in TM IV are highlighted in green. The numbers above the amino acid sequence represent the Ballesteros–Weinstein numbering scheme for GPCRs. The corresponding protein accession numbers are indicated in parentheses

lysine or arginine residues are believed to be capable of interacting with the cholesterol hydroxyl group. Interestingly, a similar four-component cholesterol binding site is found in ~21% of human class A GPCRs (Hanson et al., 2008), and representative examples are shown in Figure 2c. Importantly, most of the GPCRs listed in Figure 2c display cholesterol sensitivity in their function (Cheema & Fisher, 2008; Gimpl, Burger, & Fahrenholz, 1997; Lam, Nahirney, & Duszyk, 2009; Lei, Morris, Smith, & Schwinn, 2009; Michal, Rudajev, El-Fakahany, & Doležal, 2009; Pontier et al., 2008; Pucadyil & Chattopadhyay, 2004; Yu et al., 2014). Using coarse-grain molecular dynamics simulations, we have previously shown high cholesterol occupancy at the *strict* CCM site formed by TM II and IV of the β_2-adrenergic receptor (Prasanna, Chattopadhyay, & Sengupta, 2014). In addition, we identified an evolutionarily conserved CCM in the serotonin$_{1A}$ receptor, a crucial neurotransmitter GPCR (Paila et al., 2009). However, the *mere* presence of cholesterol interaction motifs does not necessarily translate to cholesterol-sensitive function of GPCRs. For example, the neurotensin type-1 and secretin receptors have CCM in their TM IV (Hanson et al., 2008). However, both these GPCRs do not exhibit any appreciable change in downstream signaling response upon cholesterol depletion relative to untreated cells (Harikumar et al., 2005; Oates et al., 2012).

3 | IDENTIFICATION OF CHOLESTEROL RECOGNITION/INTERACTION AMINO ACID CONSENSUS MOTIF

Cholesterol recognition/interaction amino acid consensus (CRAC) motif is one of the most well documented linear sequence motifs implicated in the interaction of cholesterol with membrane proteins (Epand, 2006; Fantini & Barrantes, 2013; Fantini, Di Scala, Baier, & Barrantes, 2016a; Jafurulla et al., 2019; Li & Papadopoulos, 1998). The CRAC motif is defined by the presence of a linear sequence of amino acids from the N-terminal to C-terminal direction as: a branched apolar leucine or valine residue, followed by a segment of one to five residues of any amino acid, an aromatic tyrosine residue, another segment of one to five residues of any amino acid, and finally a basic lysine or arginine residue. The motif is commonly defined by the one letter amino acid code as (L/V) $-(X)_{1-5}-$ Y $-(X)_{1-5}-$ (R/K) (Figure 3a, Li & Papadopoulos, 1998). This motif was first identified in the peripheral-type benzodiazepine receptor (PBR), a mitochondrial outer membrane protein involved in the regulation of cholesterol transport across mitochondrial membranes (Li & Papadopoulos, 1998). By performing deletions and site-directed mutagenesis in the cytoplasmic C-terminus region of PBR, key amino acid residues involved in cholesterol transport function were identified and the sequence of the CRAC motif was postulated. Interestingly, this amino acid consensus pattern was found in a diverse range of proteins (Li & Papadopoulos, 1998) that were known to interact with cholesterol, such as the cytochrome P450scc (Su et al., 1990), mouse apolipoprotein A-I (Boyle & Marotti, 1992), mouse caveolin 1 (Murata et al., 1995), and *Streptomyces* cholesterol oxidase (Ishizaki, Hirayama, Shinkawa, Nimi, & Murooka, 1989) (see Figure 3b). Moreover, single mutations of key amino acid residues in the CRAC motif highlighted the absolute requirement for these characteristic amino acids in regulating receptor interaction with cholesterol (Epand, 2006). In most of these cases, mutations in the amino acid residues of CRAC motif drastically reduce (or abolish) interaction with cholesterol (or cholesterol-sensitive function). For example, in the case of PBR, the central aromatic residue (which is necessarily tyrosine) could not be replaced with other aromatic residues (Jamin et al., 2005), and in HIV-1 transmembrane protein gp41, the N-terminal leucine residue could not be substituted even by isomeric residues such as isoleucine (Epand et al., 2006), to retain the cholesterol sensitivity of the receptor function.

One of the most well studied proteins with respect to CRAC motif is caveolin-1. Caveolin-1 represents the best documented example of membrane protein segregation exclusively to domains that are enriched in sphingomyelin and cholesterol (Örtegren et al., 2004; Smart & Anderson, 2002). The peptide fragment comprising residues 82–101 in caveolin-1 were necessary and sufficient for its membrane binding activity (Schlegel, Schwab, Scherer, & Lisanti, 1999) and recruitment of NBD-labeled cholesterol (Wanaski, Ng, & Glaser, 2003). This was shown by mutation of key amino acids in this region that abolished membrane binding activity and recruitment of NBD-labeled cholesterol. Interestingly, this peptide fragment of caveolin-1 contains the sequence VTKYWFYR (residues 94–101; see Figure 3b) that resembles a CRAC motif. A smaller fragment of this peptide segment that does not contain a classic CRAC motif, KYWFYR, was found to sequester the protein nonspecifically to membranes without targeting it to cholesterol-rich domains (Woodman, Schlegel, Cohen, & Lisanti, 2002). In addition, the truncated peptide did not promote cholesterol-rich domain formation in liposomes and lacked preferential interaction with cholesterol (Epand, Sayer, & Epand, 2003). On the other hand, peptide containing the full length CRAC motif was shown to segregate cholesterol into domains (Epand, Sayer, & Epand, 2005).

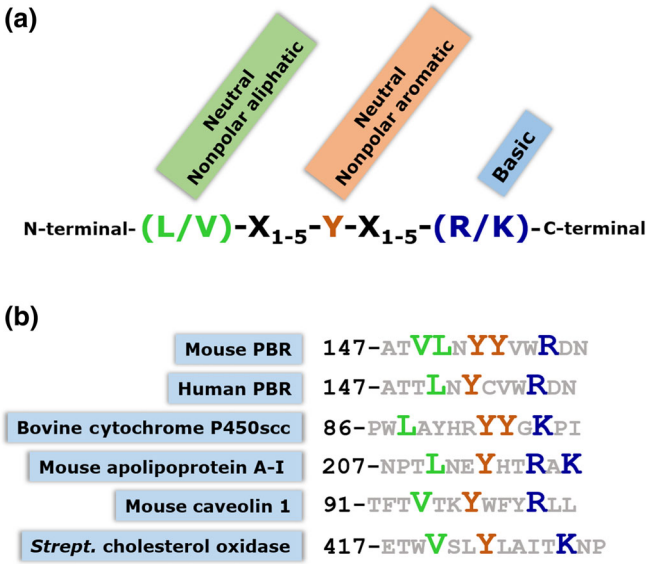

FIGURE 3 Identification of cholesterol recognition/interaction amino acid consensus (CRAC) motifs in proteins. (a) The key elements of the CRAC motif. CRAC is a short linear motif that fulfills the following sequence algorithm from the N-terminus to C-terminus: a branched apolar leucine or valine residue, followed by a segment containing 1–5 of any amino acid residues, an aromatic residue that is specifically tyrosine, followed by a stretch of 1–5 of any amino acid residues, and finally a basic lysine or arginine residue at the C-terminus. (b) CRAC motifs in representative proteins that have been shown to interact with cholesterol. The numbers corresponding to the starting amino acid position in the respective sequences are mentioned before the CRAC motif for each protein. The positions of the central aromatic amino acid residues (tyrosine) are highlighted in maroon. The basic amino acid residues (arginine or lysine) at the C-termini are highlighted in blue and the nonpolar branched aliphatic amino acid residues (leucine or valine) at the N-termini are highlighted in green. The sequences containing the CRAC motifs are taken from Li and Papadopoulos (1998)

4 | NONSPECIFIC NATURE OF THE CRAC MOTIF

While the CRAC motif is characterized by the lack of a strict amino acid sequence and length, it could mediate interaction with cholesterol, a specific lipid with a strictly defined structure with no scope for any variation. This apparent anomaly merits comment. According to the CRAC algorithm, originally proposed by Li and Papadopoulos (Li & Papadopoulos, 1998), a CRAC motif can have a length anywhere from 5 to 13 amino acid residues. For the longest possible CRAC motif (with X = 5 in both places, see Figure 3a), there are 10 amino acid positions that can be occupied by any one of the twenty naturally occurring amino acids. In principle, this algorithm alone can create 20^{10} (~10^{13}) possible CRAC motif sequences and if one includes the variability in length of this motif (i.e., X varying between 1 and 5 in both places), the total number of possible motifs gets even larger. In molecular terms, it is highly improbable that such a large number of possible sequences could be equally efficient in interacting with cholesterol. For example, previous analysis of bacterial genomes (*Streptococcus agalactiae*, *Staphylococcus aureus*, and *Escherichia coli*) showed the presence of ~3 CRAC motifs/protein in diverse classes of proteins that do not interact with cholesterol (Palmer, 2004). We therefore believe that experimental approaches, such as site-directed mutation of the amino acid residues in CRAC motifs implicated in such interactions, followed by functional analysis of cholesterol sensitivity of the receptor, would provide a better understanding of the role of these motifs in regulating cholesterol-sensitive function.

5 | CRAC MOTIFS IN G PROTEIN-COUPLED RECEPTORS

In the overall context of cholesterol sensitivity of GPCR function, we reported, for the first time, the presence of CRAC motifs in GPCRs (namely the serotonin$_{1A}$ receptor, the β_2-adrenergic receptor and rhodopsin) (Figure 4a; Jafurulla, Tiwari, and Chattopadhyay (2011). Interestingly, all these GPCRs are known to display characteristic cholesterol-sensitive function (Niu, Mitchell, & Litman, 2002; Pontier et al., 2008; Pucadyil & Chattopadhyay, 2004). In case of the serotonin$_{1A}$ receptor, membrane cholesterol was necessary for agonist binding and subsequently G-protein coupling of

the receptor (Pucadyil & Chattopadhyay, 2004). For rhodopsin, reduced levels of membrane cholesterol led to higher levels of active metarhodopsin II conformation that leads to G-protein activation (Niu et al., 2002). Further, for β_2-adrenergic receptor, increase in cAMP signaling efficacy was enhanced by depletion of membrane cholesterol (Pontier et al., 2008).

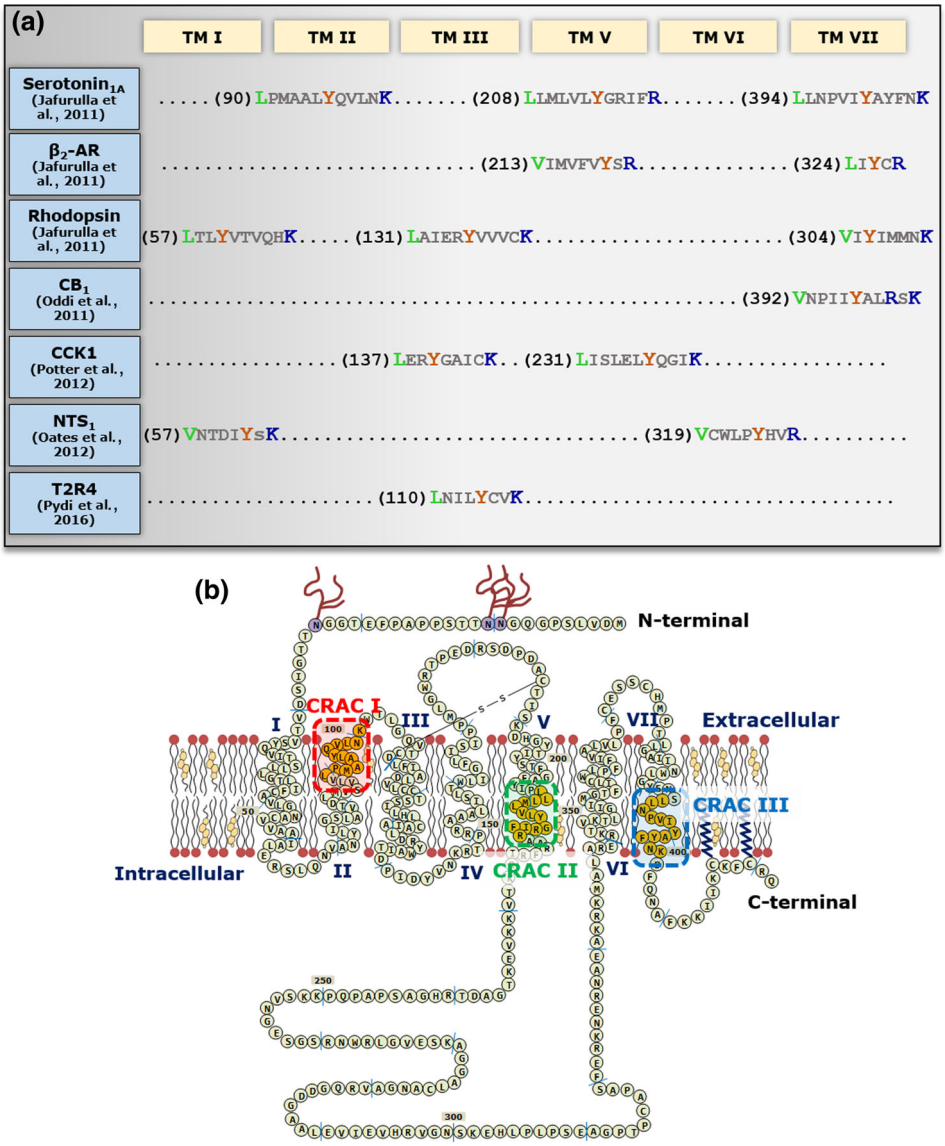

FIGURE 4 Cholesterol recognition/interaction amino acid consensus (CRAC) motifs in G protein-coupled receptors. (a) CRAC motifs in representative GPCRs. The numbers corresponding to the starting amino acid position in the respective sequences are mentioned in parentheses before the sequences. The putative positions of the CRAC motifs mapped to individual helices in these GPCRs are indicated at the top. The central aromatic amino acid residues (tyrosine) of the CRAC motifs are highlighted in maroon. The basic amino acid residues (arginine or lysine) at the C-termini are highlighted in blue and the nonpolar branched aliphatic amino acid residues (leucine or valine) at the N-termini are highlighted in green. (b) A schematic representation depicting the topological features and amino acid sequence of the human serotonin$_{1A}$ receptor embedded in a membrane bilayer consisting of phospholipids and cholesterol. The putative positions of the transmembrane helices of the human serotonin$_{1A}$ receptor was predicted using the crystal structure of the human serotonin$_{1B}$ receptor (PDB ID: 6G79) and the amino acids in the receptor sequence are shown as circles. The receptor has seven transmembrane stretches, each composed of ~22 amino acids, that are depicted as putative α-helices and are marked as I–VII. Since there are no crystal structures available for the serotonin$_{1A}$ receptor, the exact boundary between the membrane and the aqueous phase is not known and therefore location of the amino acid residues relative to the membrane bilayer is putative. The serotonin$_{1A}$ receptor consists of three CRAC motifs (highlighted in yellow) in TM II (CRAC I, boxed in red), TM V (CRAC II, boxed in green), and TM VII (CRAC III, boxed in blue). (Reprinted with permission from Jafurulla et al. (2011). Copyright 2010 Elsevier Inc.)

Our analysis revealed that the sequences of the serotonin$_{1A}$ receptor and rhodopsin have three CRAC motifs (see Figure 4a). While all the motifs in the serotonin$_{1A}$ receptor are characterized by 12 residues, the number of amino acids in CRAC motifs of rhodopsin display variation, ranging from 8 to 11 (Figure 4a). In contrast, the β_2-adrenergic receptor sequence exhibits two CRAC motifs of varying number of residues (9 and 5) (Figure 4a). The serotonin$_{1A}$ receptor is the most well-studied GPCR in terms of cholesterol sensitivity in the organization, dynamics, oligomerization and function of the receptor (Chattopadhyay, 2014; Jafurulla & Chattopadhyay, 2013; Pucadyil & Chattopadhyay, 2006; Sengupta, Kumar, & Chattopadhyay, 2017). Figure 4b highlights three CRAC motifs (CRAC I–III) that are present in the serotonin$_{1A}$ receptor, in the overall context of the topology of the receptor. These motifs are present in putative TM II (residues 90–101), V (residues 208–219) and VII (residues 394–405) (see Figure 4b). In case of rhodopsin, the CRAC motifs are present in TM I, III and VII (Figure 4a), while for the β_2-adrenergic receptor, TM V and VII harbor the CRAC motifs (Figure 4a). We further showed that the CRAC motif(s) in serotonin$_{1A}$ receptors are conserved during the course of natural evolution in a diverse range of taxa that include amphibians, fish and other marine species, extending up to mammals (Jafurulla et al., 2011).

6 | DOES PRESENCE OF A CRAC MOTIF IMPLY CHOLESTEROL-SENSITIVE RECEPTOR FUNCTION?

Subsequently, presence of CRAC motif was reported for type-1 cannabinoid receptor (CB_1) (Oddi et al., 2011), which belongs to the class of GPCRs involved in neurodegenerative and neuroinflammatory disorders (Bisogno & Di Marzo, 2010). The human CB_1 receptor is known to show cholesterol-sensitive function in terms of ligand (endocannabinoid anandamide) binding, coupling to G-proteins and downstream activation of MAP kinase (Bari et al., 2005a; Bari et al., 2005b) and has one CRAC motif in its TM VII (residues 392–402, Figure 4a). The functional implication of specific amino acid residues in the CRAC motif in sensing membrane cholesterol was later shown from the loss of cholesterol sensitivity of the CB_1 receptor upon mutation of a key lysine residue in this sequence (Oddi et al., 2011). Interestingly, the type-2 cannabinoid receptor (CB_2), that does not display cholesterol-sensitive function (Bari et al., 2006; Oddi et al., 2011), has a glycine residue instead of lysine in its CRAC motif, corresponding to a single mutation in CB_1 receptor (Oddi et al., 2011). Along similar lines, functional implications (ligand binding and rise in intracellular calcium concentration) in cholesterol sensitivity *via* CRAC motifs are shown for the type 1 cholecystokinin receptors (CCK1) (Potter, Harikumar, Wu, & Miller, 2012). In contrast, a close subtype of this receptor, CCK2, having two CRAC motifs in TM III and V (Potter et al., 2012), was shown to be functionally insensitive to membrane cholesterol content (Potter et al., 2012). We recently showed that in case of bitter taste receptor 4 (T2R4), lysine 117 (an important CRAC residue, Figure 4a) is crucial for cholesterol sensitivity of T2R4 receptors (Pydi et al., 2016). In general, the presence of CRAC motifs in transmembrane domains of GPCRs suggests the possibility of cholesterol interaction with the receptor. However, it is advisable to exercise caution before attributing cholesterol sensitivity in receptor function to the presence of these motifs (see below).

7 | CHOLESTEROL INTERACTION HOT SPOTS IN GPCRS: WEAK, DYNAMIC YET ESSENTIAL

Although several cholesterol interaction/binding sites have been reported in GPCRs, their role in preferential cholesterol interaction constitutes an emerging area of research (Genheden, Essex, & Lee, 2017; Lee, 2019; Rouviere, Arnarez, Yang, & Lyman, 2017; Sengupta & Chattopadhyay, 2012). Atomistic molecular dynamics simulations have been successful in demonstrating the preferential interaction of membrane cholesterol with certain sites on GPCRs, such as the serotonin$_{1A}$ receptor (Patra et al., 2015), the β_2-adrenergic receptor (Cang et al., 2013), the A_{2A} adenosine receptor (Guixà-González et al., 2017; Lee & Lyman, 2012) and rhodopsin (Khelashvili, Grossfield, Feller, Pitman, & Weinstein, 2009). In this context, we performed long time scale (microseconds) coarse-grain molecular dynamics simulations using homology model of the serotonin$_{1A}$ receptor (since its atomic resolution structure has not been solved by x-ray crystallography, cryo electron microscopy or solid state NMR) to show that membrane cholesterol interacts preferentially with certain regions of transmembrane helices in the receptor (Figure 5; Sengupta & Chattopadhyay, 2012). To quantify specific interaction events at each residue in the receptor, we previously estimated the maximum occupancy time, that is, the maximum time a given cholesterol molecule was bound at each residue of the receptor, during the course of the

simulation (Figure 5). The value was normalized for all simulation lengths, such that the largest value of maximum occupancy time is 1 in all cases. The sites on the receptor that showed high maximum occupancy time of cholesterol were in the outer (extracellular) leaflet of TM II and VII, and inner (intracellular) leaflet of TM I and V. Interestingly, we observed high cholesterol occupancy (cholesterol hot spot) at one of the previously identified (Jafurulla et al., 2011) CRAC motif site (CRAC II, highlighted in Figure 5a), thereby suggesting its role as a cholesterol interaction motif in the serotonin$_{1A}$ receptor. A characteristic feature of these cholesterol interaction (occupancy) sites is the considerable dynamics displayed by cholesterol molecules that ranges from ns to μs time scale. The energy landscape of cholesterol association with GPCRs can be represented as a series of shallow minima, interconnected by low energy (~kT) barriers

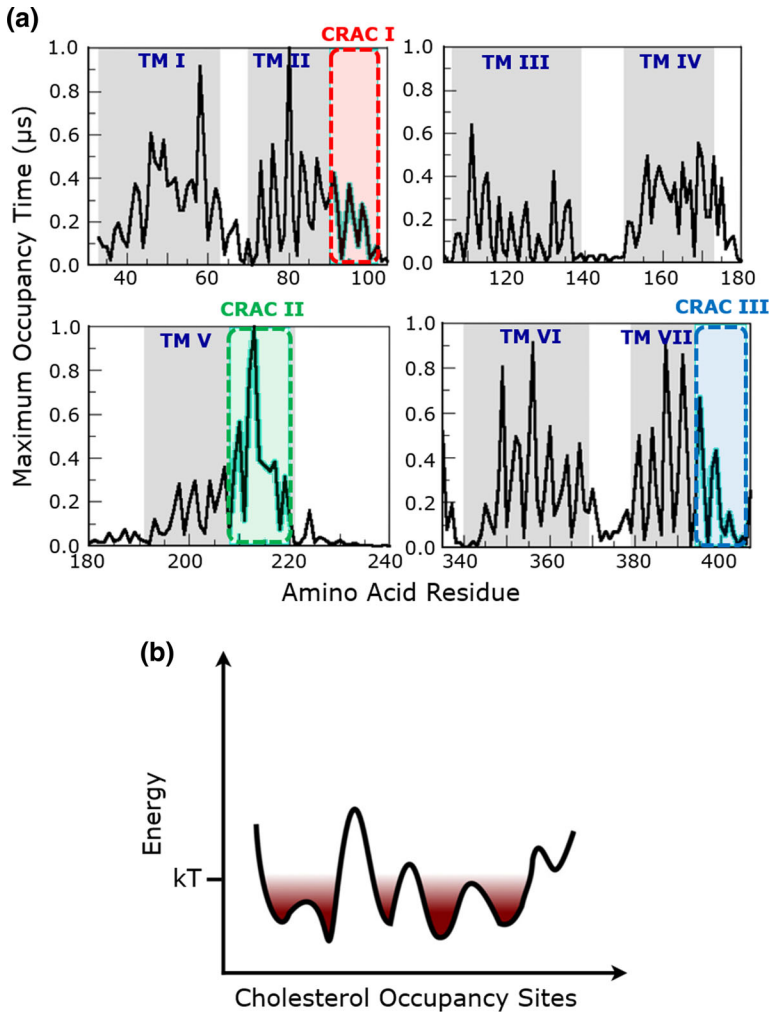

FIGURE 5 Cholesterol interaction hot spots in the serotonin$_{1A}$ receptor. (a) Residue-wise maximum occupancy of cholesterol bound to the serotonin$_{1A}$ receptor, obtained by coarse-grain molecular dynamics simulations. Maximum occupancy time (defined as the maximum time a given cholesterol molecule is found at a particular residue during the course of the simulation; see text for details) of cholesterol at each amino acid of the serotonin$_{1A}$ receptor was averaged and normalized over simulations carried out at varying concentrations of cholesterol. The transmembrane helices are represented as gray bands, and CRAC motifs are highlighted with the same color coding as in Figure 4a. The high cholesterol occupancy observed at the CRAC motif on transmembrane helix V (CRAC II) is noteworthy (Reprinted with permission from Sengupta and Chattopadhyay (2012). Copyright 2012 American Chemical Society). (b) A schematic energy landscape corresponding to cholesterol interaction sites in GPCRs. The interaction of cholesterol with GPCRs is weak, yet dynamic with varying occupancy times ranging from ns to μs time scale. This aspect of the interaction of cholesterol with GPCRs is reflected in the energy landscape of cholesterol interaction which is represented as a series of shallow minima interconnected by low energy barriers. The abscissa can be thought to correspond to individual occupancy sites represented by single residues or by a sub-space at the receptor surface (such as cholesterol consensus motif [CCM] or CRAC sites). The occupancy sites are most likely to be accessed *via* an exchange with the annular lipids and less often by direct site hopping of cholesterol. Note that the energy barriers and the minima could be modulated by other membrane lipids such as sphingolipids (Reprinted with permission from Sengupta and Chattopadhyay (2015). Copyright 2015 Elsevier B.V.)

(see Figure 5b for a schematic representation; Sengupta & Chattopadhyay, 2015). As the figure shows, cholesterol occupancy sites could be either represented by individual residues or by a sub-space at the receptor surface (such as the CCM/CRAC motif). Interestingly, we did not observe high cholesterol occupancy at CRAC motifs I and III, suggesting that *mere* presence of cholesterol interaction motif at a given site may not directly reflect its role in interaction with cholesterol. However, a caveat of this inference is that coarse-grain simulations does not provide information at atomistic resolution and therefore cholesterol occupancy (or lack of it) at CRAC motifs could suffer from this limitation. Having said that, we would like to mention here that we were able to reproduce, using coarse-grain simulations, cholesterol interaction sites in β_2-adrenergic receptor from reported crystal structures and from long time scale atomistic simulations (Prasanna et al., 2014).

In a cautionary note, we would like to add here that due to their inherent dynamic nature, the CRAC motifs should not be considered in the same lights as in some of the classical motifs (such as zinc finger motif and Greek key motif). In other words, one needs to make this adjustment when considering CRAC motifs. This point could be further elucidated from a closer look at the CRAC motif in the transmembrane helix V of the human type 3 somatostatin receptor (Fantini & Barrantes, 2013). In this motif, in the transmembrane helix V of the human type 3 somatostatin receptor, the central tyrosine residue is observed not to interact with cholesterol from molecular modeling studies. It therefore appears that cholesterol can exhibit a slightly different fit around the CRAC motif to adjust its overall shape in relation to the three-dimensional structure of the transmembrane domain.

8 | OVERLAP OF LIPID INTERACTION MOTIFS IN THE SEROTONIN$_{1A}$ RECEPTOR

The distribution of both cholesterol and sphingolipids is heterogeneous in the membrane bilayer. It has been postulated that sphingolipids and cholesterol are often localized in laterally segregated lipid domains (Brown, 1998; Masserini & Ravasi, 2001; Ramstedt & Slotte, 2006). The function of several transmembrane proteins, including GPCRs, has been shown to be dependent on sphingolipids (Jafurulla & Chattopadhyay, 2015; Slotte, 2013). It was previously shown that proteins known to interact with (glyco)sphingolipids, have a characteristic amino acid sequence, termed the sphingolipid-binding domain (SBD; Mahfoud et al., 2002; Fantini, 2003; Fantini, Garmy, & Yahi, 2006; Fantini & Barrantes, 2009). In addition, a specific binding motif for sphingomyelin, termed the sphingolipid-binding motif (SBM) has been previously proposed to exist in the transmembrane protein p24 (a component of the COPI transport machinery) (Contreras et al., 2012). In order to explore whether the previously observed sphingolipid-sensitive function of the serotonin$_{1A}$ receptor (Jafurulla, Pucadyil, & Chattopadhyay, 2008; Paila, Ganguly, & Chattopadhyay, 2010) could originate from direct interaction of sphingolipids with specific sequences present in the receptor, we identified signatures of SBD and SBM in the first extracellular loop (ECL1) and TM V of the human serotonin$_{1A}$ receptor, respectively (highlighted in cyan in Figure 6; Chattopadhyay et al., 2012; Shrivastava, Jafurulla, Tiwari, & Chattopadhyay, 2018). Interestingly, both of these sphingolipid binding/interaction motifs have some sequence overlap with the CRAC motifs that we identified earlier (Jafurulla et al., 2011). In case of SBD, residues 99–101 overlap with CRAC motif I and residues 209–213 of SBM overlap with CRAC motif II (Figure 6). We believe that such overlapping motifs which could simultaneously interact with cholesterol and sphingolipid could be relevant in the context of previously reported cholesterol-dependent sphingolipid membrane microdomains (Hebbar et al., 2008). Interestingly, we recently demonstrated, using coarse-grain molecular dynamics simulations, that various residues of GM1 (the most common glycosphingolipid that is typically ~2–5% of total membrane lipids) headgroup predominantly interacts with the proposed SBD site (Chattopadhyay et al., 2012) in the extracellular loop 1 of the serotonin$_{1A}$ receptor in a cholesterol-dependent manner (Prasanna, Jafurulla, Sengupta, & Chattopadhyay, 2016a).

9 | DIVERSIFICATION OF CHOLESTEROL BINDING/INTERACTION MOTIFS

The specific sequence arrangement of amino acid residues in a CRAC motif restricts its occurrence in the extracellular leaflet of odd numbered helices in GPCRs, since the presence of a basic amino acid residue in the hydrophobic core of the membrane bilayer would be energetically unfavorable. The search for new cholesterol interaction motifs in the

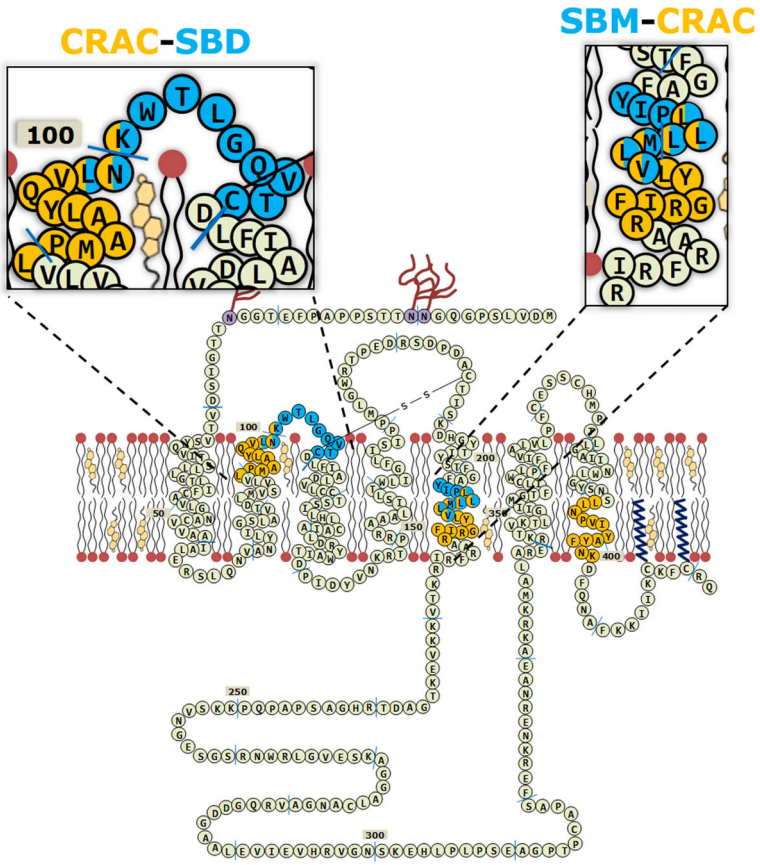

FIGURE 6 A schematic representation of the membrane-embedded human serotonin$_{1A}$ receptor highlighting overlapping lipid binding/interaction motifs. The amino acids in the receptor sequence are shown as circles. The sphingolipid binding domain (SBD) and sphingolipid binding motif (SBM) in TM II and TM V, respectively, are highlighted in cyan. Enlarged representations of TM II and TM V of the human serotonin$_{1A}$ receptor showing the overlap of SBD and SBM (highlighted in cyan) with CRAC motif I and II (highlighted in yellow), respectively. Residues common to both SBM/SBD and CRAC motifs are shown in a combination of cyan and yellow. (Reprinted with permission from Shrivastava et al. (2018). Copyright 2018 Springer Nature Singapore Pte Ltd.)

extracellular leaflet of odd numbered helices in GPCRs and class I membrane proteins (whose N-terminus is extracellular) led to the discovery of a new class of cholesterol interaction motifs that is very similar to the CRAC motif but is oriented in an opposite direction along the polypeptide chain (Baier, Fantini, & Barrantes, 2011). This is termed the CARC motif ("inverted CRAC"), which unlike CRAC (which by definition has a specific requirement for tyrosine), could have tyrosine, phenylalanine or tryptophan as the central aromatic residue (Figure 7a; Fantini et al., 2019). The CARC motif was first identified in the nicotinic acetylcholine receptor (Baier et al., 2011) and was found in several key GPCRs such as rhodopsin, β_2-adrenergic, GABA$_B$, adenosine A$_1$, serotonin$_7$, and chemokine CXCR4 receptor (Baier et al., 2011; Fantini et al., 2016a; Fantini et al., 2016b), most of which display cholesterol-sensitive function. The biochemical "rules" that apply to a CRAC-cholesterol interaction, remain valid for CARC motif, since both these motifs are characterized by a triad of specific amino acids with a central aromatic amino acid flanked by basic and branched apolar amino acid residues at each end (Figure 7a). In most of the cases, in a CRAC motif, the tyrosine residue cannot be replaced by other aromatic residues such as phenylalanine or tryptophan (Epand, 2006; Epand et al., 2006; Jamin et al., 2005). However, analysis of interaction energy between cholesterol and CRAC motif through molecular docking studies suggests that, at least in some cases, the aromatic ring of phenylalanine could sustain CH-π stacking interactions (Baier et al., 2011). This constitutes another putative cholesterol interaction motif, namely the CRAC-like motif, where the central aromatic residue is phenylalanine (Figure 7a).

An interesting feature of the CARC and CRAC definition is that these motifs are both vectorial (from N-terminus to C-terminus) and symmetric when placed in a continuous stretch of sequence (i.e., [CARC]basic-aromatic-apolar branched—apolar branched-aromatic-basic[CRAC]). It is therefore possible that the same transmembrane domain contains a CARC and a CRAC motif in a linear fashion. Due to the nature of amino acids that defines the terminal residues of both these motifs, odd numbered helices in GPCRs (TM I, III, V, and VII) would have a CARC–CRAC topology, whereas, even number helices would have a CRAC–CARC topology (from outer to inner leaflet of the membrane, Figure 7b). The simultaneous presence of such CARC and CRAC motifs within a transmembrane helix has been proposed to form a "mirror code" for protein-cholesterol interaction (Fantini et al., 2016a; Fantini et al., 2016b). The CARC/CRAC "mirror code" has been found in transmembrane helices of a broad range of membrane proteins, including GPCRs such as the GABA$_B$ receptor (TM I), metabotropic glutamate receptor 5 (TM VII), adenosine A$_1$ receptor

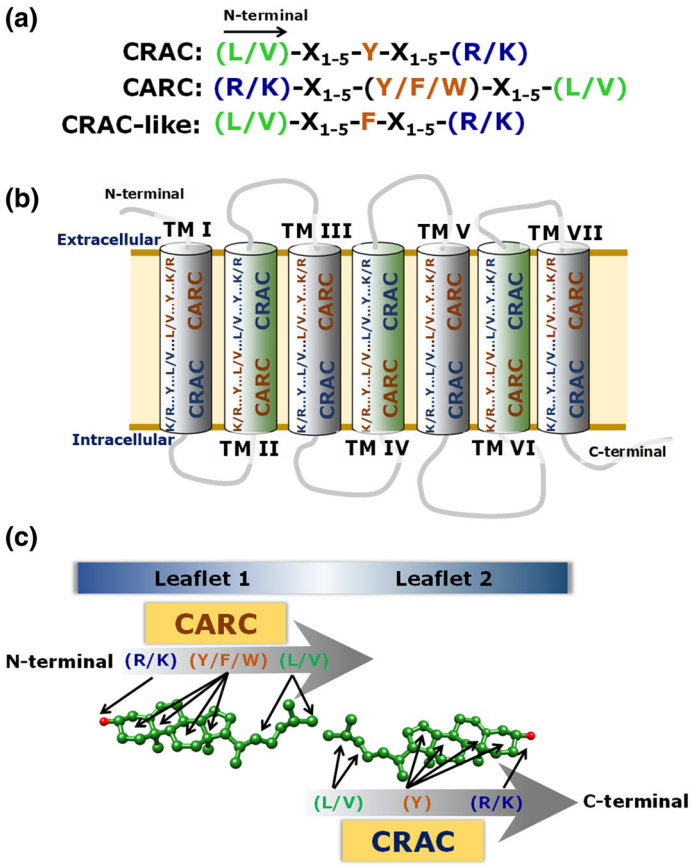

FIGURE 7 Diversification of cholesterol interaction motifs. (a) The CARC motif is similar to the CRAC sequence, but exhibits the opposite orientation ("inverted CRAC") along the polypeptide chain. For CARC motif, the central residue is still aromatic, but unlike CRAC which by definition has a specific requirement for tyrosine, the CARC motif could have tyrosine, phenylalanine or tryptophan as a central aromatic residue. In case of CRAC motif, the aromatic ring of phenylalanine could sustain the interaction with cholesterol when tyrosine is not available. This constitutes the CRAC-like motif, where the central aromatic residue is phenylalanine. (b) For GPCRs (where the N-terminus is extracellular), in TM I, III, V, and VII, the CARC motif is located in the outer leaflet and the CRAC domain is in the inner leaflet. In case of TM II, IV, and VI, the arrangement still holds but in this case CARC is located in the inner leaflet and CRAC in the outer leaflet. (c) The simultaneous presence of CRAC and CARC motifs within the same transmembrane helix constitutes a "mirror code" that could accommodate two cholesterol molecules (shown in green) in a typical tail-to-tail orientation, one bound to CRAC and the other to CARC. (Reprinted with permission from Fantini et al., (2016b). Copyright 2016 Springer Nature Ltd.)

(TM VII), and oxytocin receptor (TM V; Fantini et al. 2016a; Fantini et al., 2016b). It is interesting to speculate that such mirror motifs could enable interaction of transmembrane helices with a pair of cholesterol molecules, resembling a transbilayer tail-to-tail dimer observed in low cholesterol concentrations in the membrane (Figure 7c; Mukherjee & Chattopadhyay, 1996; Rukmini, Rawat, Biswas, & Chattopadhyay, 2001; Chaudhuri & Chattopadhyay, 2011). This could be relevant in understanding GPCR-cholesterol interaction in membranes that have very low cholesterol content *in vivo*, such as the endoplasmic reticulum (Menon, 2018), which is also the site for GPCR synthesis (Dong, Filipeanu, Duvernay, & Wu, 2007).

10 | COLLAGE OF CHOLESTEROL INTERACTION MOTIFS: AN EVOLUTIONARY PERSPECTIVE

GPCRs in general and the serotonin$_{1A}$ receptor in particular are found across diverse vertebrate species (Peroutka & Howell, 1994) that have very different membrane lipid compositions. For example, the composition of membrane lipids (such as cholesterol) in endotherm (warm-blooded) and ectotherm (cold-blooded) species is very different (Harayama & Riezman, 2018; Hassett & Crockett, 2009; Sackmann, 1995; van Meer, Voelker, & Feigenson, 2008). In this context, an interesting question to ask is how does a predominantly conserved sequence of any GPCR, function in very diverse membrane lipid environments? To address this, in a recent work, we analyzed, using multiple sequence alignment, cholesterol interaction sites in TM V and the adjacent intracellular loop 3 (ICL3) fragment of the serotonin$_{1A}$ receptor from a diverse range of vertebrates (Figure 8a) and explored its evolutionary implications (Fatakia, Sarkar, & Chattopadhyay, 2019). We observed that the TM V and ICL3 contain a conserved "collage" of four categories of cholesterol interaction motifs that could accommodate up to 20 distinct possible cholesterol interaction configurations (8 CRAC, 4 CRAC-like, 1 CARC, and 7 CCM motifs) (Figure 8b), that could allow the serotonin$_{1A}$ receptor to differentially interact with membrane cholesterol. In light of the diversity of cellular cholesterol content across different species (Dinh et al., 2011; Yin et al., 2012), we believe that a multiplicity of cholesterol interaction sites could enable GPCRs to interact differentially with membrane cholesterol in a cell type specific manner. Interestingly, this is in agreement with our previous work

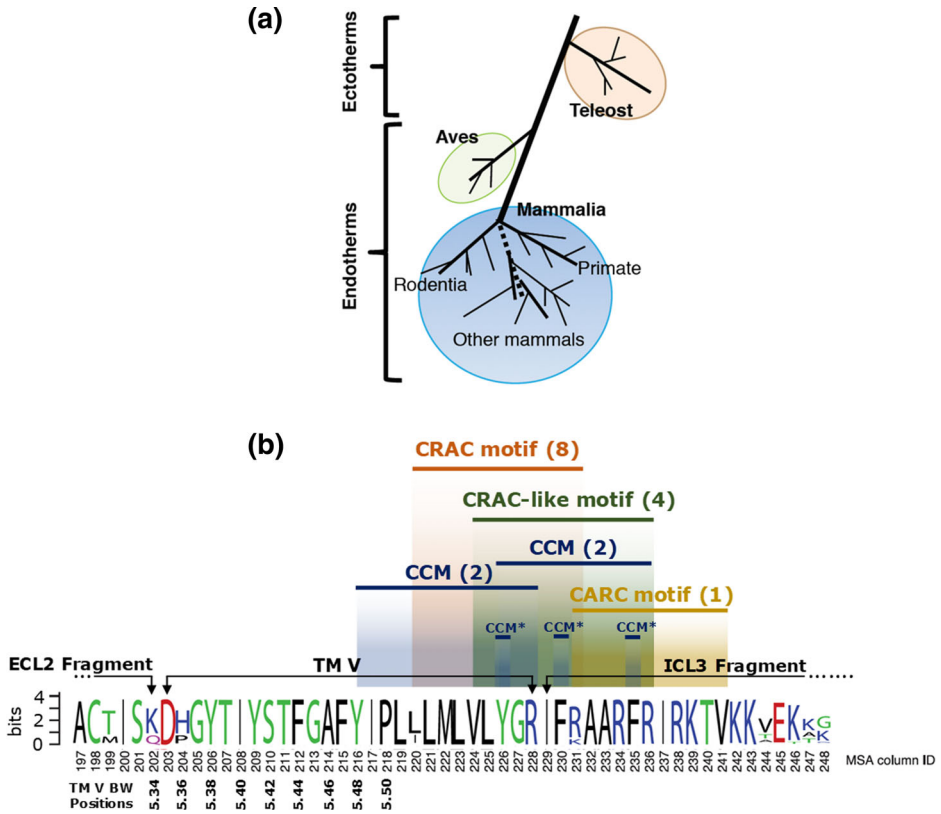

FIGURE 8 An evolutionarily conserved collage of four categories of cholesterol interaction motifs associated with TM V and the adjacent intracellular loop 3 fragment of the vertebrate serotonin$_{1A}$ receptor. (a) A schematic representation of phylogenetic clades: Teleost, Aves, and Mammalia (includes Primate and Rodentia subclades), used for this analysis. Aves and Mammalia represent endotherms, and Teleostei represent ectotherms. The dashed line in the Mammalia clade represents the species that are not categorized into subclades. (b) A collage of putative cholesterol interaction motifs overlaid on the sequence logo of serotonin$_{1A}$ receptor TM V and intracellular loop 3 (ICL3) fragment. Multiple sequence alignment (MSA) positions in TM V are represented by Ballesteros–Weinstein (BW) indices from 5.34 to 5.50. Position 5.50 represents the position of the evolutionarily conserved proline. In addition to TM V and ICL3, the juxtamembrane regions from extracellular loop 2 (ECL2) is shown. Colored boxes represent various cholesterol-sensitive motifs (CRAC, CRAC-like, CARC, and cholesterol consensus motif [CCM] motifs), and numbers in parentheses represent the total number of configurations possible for each motif. The boxes labeled as CCM* may complement an existing CCM in its spatial proximity to constitute a *strict* CCM (Reprinted with permission from Fatakia et al. (2019). Copyright 2019 Elsevier B.V.)

where we showed, by molecular dynamics simulations, that the serotonin$_{1A}$ receptor exhibits cholesterol-dependent conformational plasticity and flexibility (Prasanna, Sengupta, & Chattopadhyay, 2016b). We envision that evolutionary conservation in the interplay among membrane lipids such as cholesterol and signaling hubs like GPCRs, could be crucial in determining cellular signaling in different species.

11 | CONCLUSION AND ROAD AHEAD

While cholesterol binding/interaction motifs definitely shaped our thought process on the role of membrane cholesterol in GPCR function, it appears that there is some amount of ambiguity associated with cholesterol interaction motifs, cholesterol occupancy in these motifs and GPCR function. Clearly, future GPCR research should concentrate on building experimental and computational tools that would enable to link cholesterol interaction motifs with GPCR function in a more robust and balanced way. Importantly, the role of overall (global) structural background needs to be addressed. Mechanistic insight of cholesterol interaction sites in GPCRs would allow us to develop a better paradigm of GPCR structure-function, and could be useful in future drug discovery.

ACKNOWLEDGMENTS

A.C. gratefully acknowledges support from SERB Distinguished Fellowship (Department of Science and Technology, Govt. of India). P.S. thanks the Council of Scientific and Industrial Research for the award of a Shyama Prasad Mukherjee Fellowship. A.C. is a Distinguished Visiting Professor at the Indian Institute of Technology Bombay (Mumbai), and Adjunct Professor at the RMIT University (Melbourne, Australia), Tata Institute of Fundamental Research (Mumbai) and Indian Institute of Science Education and Research (Kolkata), and an Honorary Professor at the Jawaharlal Nehru Centre for Advanced Scientific Research (Bengaluru). Some of the work described in this article was carried out by present and former members of A.C.'s research group whose contributions are gratefully acknowledged. We thank members of the Chattopadhyay laboratory for critically reading the manuscript.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Parijat Sarkar: Writing-original draft; writing-review and editing. **Amitabha Chattopadhyay**: Conceptualization; writing-original draft; writing-review and editing.

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How to cite this article: Sarkar P, Chattopadhyay A. Cholesterol interaction motifs in G protein-coupled receptors: Slippery hot spots? *WIREs Syst Biol Med*. 2020;e1481. https://doi.org/10.1002/wsbm.1481