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A collage of cholesterol interaction motifs in the serotonin$_{1A}$ receptor: An evolutionary implication for differential cholesterol interaction

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ABSTRACT

The serotonin$_{1A}$ receptor is a representative member of the G protein-coupled receptor (GPCR) superfamily and acts as an important drug target. In our previous work, we comprehensively demonstrated that membrane cholesterol is necessary in the organization, dynamics and function of the serotonin$_{1A}$ receptor. In this context, analysis of high-resolution GPCR crystal structures in general and *in silico* studies of the serotonin$_{1A}$ receptor in particular, have suggested the presence of cholesterol interaction sites (hotspots) in various regions of the receptor. In this work, we have identified an evolutionarily conserved collage of four categories of cholesterol interaction motifs associated with transmembrane helix V and the adjacent intracellular loop 3 fragment of the vertebrate serotonin$_{1A}$ receptor. This collage of motifs represents a total of twenty diverse context-dependent cholesterol interaction configurations. We envision that the gamut of cholesterol interaction sites, characterized by sequence plasticity in cholesterol interaction, could be relevant in receptor-cholesterol interaction in membranes of varying cholesterol content and organization, as found in diverse cell types. We conclude that an evolutionarily conserved mechanism of GPCR-cholesterol interaction allows the serotonin$_{1A}$ receptor to adapt to diverse membrane cholesterol levels during natural evolution.

1. Introduction

G protein-coupled receptors (GPCRs) are transmembrane proteins that transduce diverse stimuli from the extracellular region across the cell membrane into the cellular interior (Pierce et al., 2002; Rosenbaum et al., 2009; Chattopadhyay, 2014). They are activated by a range of extracellular ligands which induce the transduction of signals into the cellular interior via subtle yet concerted conformational changes in their transmembrane and extramembranous domains (Weis and Kobilka, 2018). GPCRs are intrinsic membrane proteins and remain embedded in their native membrane environment, and cross the membrane several times through seven α-helical transmembrane passes (denoted as TM I-TM VII). The human genome encodes ~800 distinct GPCRs (Allen and Roth, 2011), which represent the largest superfamily of drug targets, since ~40% of currently marketable drugs target GPCRs (Hauser et al., 2017; Sriram and Insel, 2018).

The serotonin$_{1A}$ receptor (Pucadyil et al., 2005; Kalipatnapu and Chattopadhyay, 2007; Müller et al., 2007) belongs to the subfamily of serotonin receptors (Hen, 1992; Nichols and Nichols, 2008), which are categorized under the family of class A GPCRs (Fredriksson et al., 2003; Surgand et al., 2006; Venkatakrishnan et al., 2013). The mammalian serotonin$_{1A}$ receptor is predominantly expressed in the brain (Regard et al., 2008). The serotonin$_{1A}$ receptor is involved in the generation and modulation of various cognitive, behavioral and developmental functions (Gordon and Hen, 2004; Belmaker and Agam, 2008) and serves as an important drug target for disorders of the central nervous system to cancer (Lacivita et al., 2008; Fiorino et al., 2014). It is noteworthy to mention here that the serotonin$_{1A}$ receptor has been shown to be involved in a number of cross talks with other GPCRs (Cussac et al., 2012; Renner et al., 2012).

Using a variety of experimental and computational approaches, we have previously reported the requirement of membrane lipids, such as cholesterol (Pucadyil and Chattopadhyay, 2006; Paila and Chattopadhyay, 2010; Jafurulla and Chattopadhyay, 2013; Sengupta and Chattopadhyay, 2015; Sengupta et al., 2018), and sphingolipids (Jafurulla and Chattopadhyay, 2013, 2015) in the organization, dynamics and function of the serotonin$_{1A}$ receptor. Interestingly, one of the first resolved GPCR crystal structures, that of the human β_2-adrenergic receptor, reported cholesterol bound to transmembrane helices II and IV (TM II and IV) (Hanson et al., 2008). The high resolution

Abbreviations: CCM, cholesterol consensus motif; CRAC, cholesterol recognition/interaction amino acid consensus; ECL2, extracellular loop 2; GPCR, G protein-coupled receptor; ICL3, intracellular loop 3; MSA, multiple sequence alignment; TM V, transmembrane helix V

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structure revealed cholesterol between TM II and IV, conforming to a three-dimensional (3D) motif, which has been referred to as the *strict* Cholesterol Consensus Motif (CCM) (Hanson et al., 2008). Interestingly, *in silico* studies based on the same structure showed evolutionarily similar (homologous) motifs among class A GPCRs, including the serotonin$_{1A}$ receptor (Hanson et al., 2008).

The Cholesterol Recognition/interaction Amino acid Consensus (CRAC) motif is one of the most well studied sequence motifs implicated in the interaction of membrane receptors with cholesterol. The CRAC motif has been proposed to interact with cholesterol in a number of membrane proteins (Li and Papadopoulos, 1998; Epand et al., 2005; Epand, 2006; Fantini et al., 2016a). In the context of GPCRs, we reported, for the first time, the presence of CRAC motifs in three representative class A GPCRs (rhodopsin, β_2-adrenergic receptor and serotonin$_{1A}$ receptor), all of which display cholesterol-sensitive function (Jafurulla et al., 2011). In fact, the functional importance of cholesterol-based interactions with serotonin$_{1A}$ receptors was further apparent by three conserved CRAC motifs (in TM II, V and VII) across vertebrates (Jafurulla et al., 2011). Using coarse-grain molecular dynamics simulations, we further showed that the TM V CRAC motif in the human serotonin$_{1A}$ receptor represents a 'hotspot' for cholesterol interaction, *i.e.*, cholesterol displayed maximum occupancy at this site (Sengupta and Chattopadhyay, 2012).

The subtype of serotonin$_{1A}$ receptors is ancient in origin (Sarkar et al., 2018). Based on analysis of the extent of amino acid sequence similarity between orthologous receptors of various species, it has been estimated that the primordial serotonin receptor must have evolved ~800 million years ago (Peroutka and Howell, 1994). 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 ancestral serotonin$_1$ receptor subfamily during the period in which vertebrates diverged from invertebrates (Peroutka and Howell, 1994). As such, the serotonin$_{1A}$ receptor exists in diverse organisms widely varying in their relative phylogenetic position in the evolutionary tree (Hen, 1992). These organisms are characterized by varying levels of membrane cholesterol content (Dinh et al., 2011; Yin et al., 2012). Although the mammalian serotonin$_{1A}$ receptor is predominantly expressed in the brain, a range of receptor levels (two-fold to over ten-fold difference in expression level) have been detected in various regions of the brain such as the cerebellum, cortex and hippocampus (Regard et al., 2008). Interestingly, it is also known that membrane cholesterol levels vary among brain compartments in an age-dependent fashion (Zhang et al., 1996). How does the serotonin$_{1A}$ receptor function in such varying cholesterol "environment" in the membrane and how receptor-cholesterol interaction is modulated under these circumstances constitute an important problem. In this work, we addressed this issue by identifying an evolutionarily conserved collage of cholesterol interaction motifs on the receptor. These consist of four different categories of cholesterol-sensitive motifs, which span the serotonin$_{1A}$ receptor TM V and its juxtamembrane region with the intracellular loop 3 (ICL3). Our results show that this collage of cholesterol interaction motifs encompasses receptor-cholesterol interactions in twenty possible ways, in a context-dependent manner. Keeping in mind the diversity of cellular cholesterol content (Sackmann, 1995; van Meer et al., 2008; Harayama and Riezman, 2018), we speculate that a multiplicity of cholesterol interaction sites could be useful in enabling the receptor to interact differentially with membrane cholesterol in a cell type specific manner. In addition, our results highlight the presence of species-specific cholesterol interacting motifs that could enable the serotonin$_{1A}$ receptor to adapt to diverse membrane cholesterol levels during natural evolution.

2. Materials and methods

2.1. Amino acid sequence records of vertebrate serotonin$_{1A}$ receptors

The protein sequence records were retrieved from the protein database (NCBI Resource Coordinators, 2016) of the National Center for Biotechnology Information (NCBI), National Institutes of Health, Bethesda. A list of orthologous sequence records, with the NCBI Accession identifiers and species, is shown in Table S1 (see Supplementary Material). We used protein sequence records from 19 Teleost (phylogenetic clade representing bony fish), 8 Aves (phylogenetic clade representing birds) and 43 Mammalia (phylogenetic clade representing mammals) species (Fig. 1a). The records representing Mammalia included Primates (16 species) and Rodentia (12 species) sub-clades, along with other mammalian species (Fig. 1a). We omitted records tagged as "partial" or "hypothetical". Any other record where "X" represented a putative amino acid residue was also omitted. We ensured that representative phylogenetic sub-clades contained at least three species.

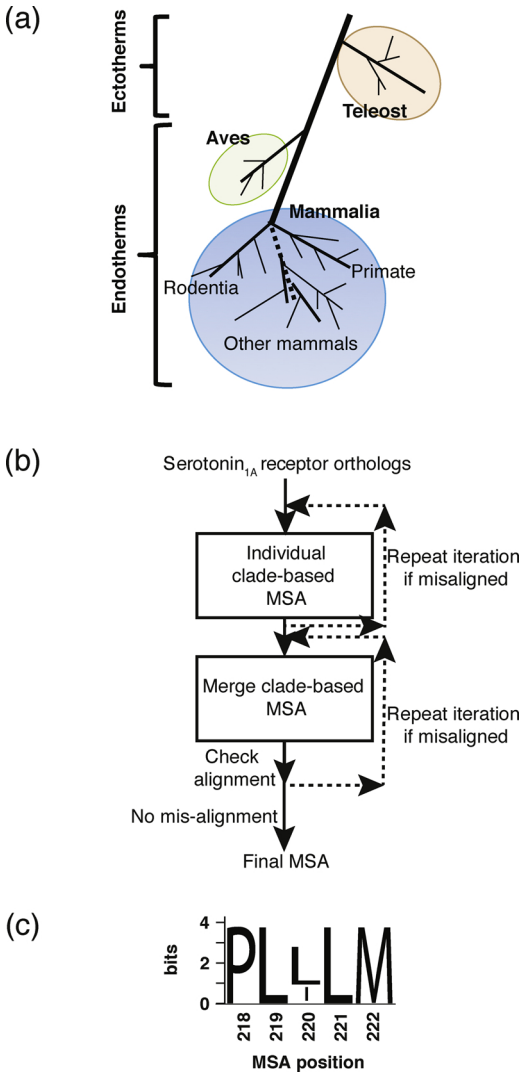

Fig. 1. An overview of our evolutionary study representing the phylogenetic diversity of sequence data and a workflow for multiple sequence alignment. (a) A schematic representation of phylogenetic clades: Teleost, Aves, and Mammalia (includes Primate and Rodentia sub-clade), used for this study. Aves and Mammalia represent endotherms, and Teleostei represent ectotherms. The dashed line in the Mammalia clade represents the species we were unable to categorize into sub-clades. (b) The workflow used to compute the multiple sequence alignment of serotonin$_{1A}$ receptor orthologs. (c) A prototype of a typical logo plot that is used to represent the aligned TM V and ICL3 fragment. The height of the representative amino acid, at each MSA position, is proportional to the logarithm (base 2) of the occurrence frequency of that residue. The ordinate denotes information content in bits. See Section 2 for more details.

2.2. Multiple sequence alignment of vertebrate serotonin$_{1A}$ receptors

ClustalX (Thompson et al., 2002) was used to compute the multiple sequence alignment (MSA) of the full-length serotonin$_{1A}$ receptor sequence for each phylogenetic clade/sub-clade. In order to constrain a gapless alignment of the 7TM MSA, we used a previously published seed alignment of human class A GPCR 7TMs (Surgand et al., 2006), which was used to report the conserved (Surgand et al., 2006) and coevolving (Fatakia et al., 2009) residues in the ligand binding pocket of class A GPCRs. Subsequently, we aggregated the full alignment by combining MSA from two or more clades (Fig. 1b). A penalty of 0.2 units for gap extension and 10 units for generating a new gap was retained for each clade-based alignment, using the Gonnet substitution matrix (default ClustalX setting). However, to prevent any unintentional bias in computing distant sequences, the gap generation penalty was doubled (from the default value) to 20 units. We iterated these steps to ensure an unambiguous alignment (Fig. 1b).

2.3. Delineating aligned amino acid residues in the multiple sequence alignment

The 52 aligned positions from the MSA of serotonin$_{1A}$ receptor TM V and its juxtamembrane region are labeled 197 to 248 (Fig. 2). These positions indicate the MSA fragment of the vertebrate serotonin$_{1A}$ receptor that include (i) C-terminal fragment of the extracellular loop 2 (ECL2), (ii) TM V, and (iii) N-terminal fragment of ICL3. In this work, we defined the TM V fragment based on previous work (Surgand et al., 2006; Fatakia et al., 2009). We used the Ballesteros Weinstein indexing scheme (Ballesteros and Weinstein, 1995) to delineate all MSA positions in TM V. These positions are labeled with respect to the evolutionarily conserved Proline residue (Proline 207 in human serotonin$_{1A}$ receptor sequence, MSA position 218 in Fig. 3), which is represented as 5.50. The aligned sequence fragment corresponds to amino acid residues 186–235 in the human serotonin$_{1A}$ receptor.

2.4. Conserved amino acid motifs sensitive to membrane cholesterol

Putative cholesterol interaction motifs were identified from evolutionarily conserved amino acid sequence patterns derived from the MSA. We explored the following motifs:

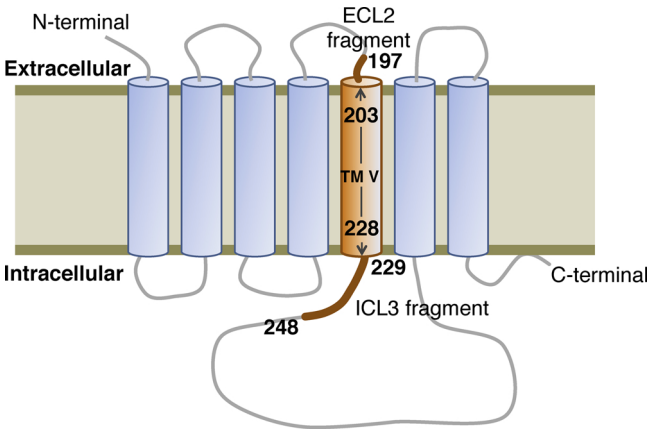

Fig. 2. A schematic representation of the overall topological features of the serotonin$_{1A}$ receptor. The regions of interest for this work, transmembrane helix V (TM V, that consists of MSA positions 203–228) and its intracellular juxtamembranous region (MSA positions 229–248, termed as ICL3 fragment in this work) of the N-terminal region of ICL3, are highlighted and shown in brown. The ECL2 fragment (MSA positions 197–202) is also shown. This corresponds to residues 186–235 in the human serotonin$_{1A}$ receptor. The horizontal lines represent the boundaries of the membrane bilayer. It should be noted that the ICL3 region of the serotonin$_{1A}$ receptor is one of the longest among class A GPCRs.

2.4.1. Cholesterol recognition and interaction motif: CRAC and CRAC-like motif

The CRAC motif is characterized (from the N- to C-terminal) by the amino acid sequence algorithm [L/V]-X$_{1-5}$-Y-X$_{1-5}$-[K/R] (Jafurulla et al., 2011). Here, X$_{1-5}$ could represent up to five amino acid residues in the peptide fragment. An evolutionarily related cholesterol recognition/interaction motif, where the nonpolar aromatic Phenylalanine (F) replaces the Tyrosine (Y) residue, represents a CRAC-like motif (Baier et al., 2011). The representative sequence algorithm for the CRAC-like motif is [L/V]-X$_{1-5}$-F-X$_{1-5}$-[K/R].

2.4.2. Cholesterol recognition and interaction motif: CARC and CARC-like motif

The inverted sequence fragments of the CRAC and CRAC-like motifs have been proposed as alternate cholesterol recognition/interaction motifs (Baier et al., 2011). These evolutionary analogs, which are referred to as CARC and CARC-like motifs are represented as [K/R]-X$_{1-5}$-Y-X$_{1-5}$-[L/V] and [K/R]-X$_{1-5}$-F-X$_{1-5}$-[L/V], respectively (Baier et al., 2011).

2.4.3. Cholesterol consensus motif

The amino acid sequence pattern [K/R]-X$_{2/6}$-[I/V/L]-X$_3$-[W/Y] (from inner to outer leaflet) has been identified as CCM in the TM IV of various class A GPCRs (Hanson et al., 2008), including the serotonin$_{1A}$ receptor (Paila et al., 2009). As reported previously, the intracellular juxtamembrane region (proximal to TM V) in serotonin$_{1A}$ receptor could manifest as an amphipathic α-helical conformation (Turner et al., 2007; Pal et al., 2018), and therefore this region could be amenable to a CCM. In this work, we explored other homologous CCMs in TM V and its intracellular juxtamembrane region.

2.4.4. Strict cholesterol consensus motif

A CCM that interacts with cholesterol, may be in proximity with a nonpolar aromatic amino acid residue: Phenylalanine, Tyrosine, or Tryptophan (F/Y/W), which is from another α-helix toward the inner leaflet of the membrane. When the two interacting α-helices are in close spatial proximity, the consolidated conformation could conform to a *strict* CCM (Hanson et al., 2008). We examined these amino acid residues from TM V and its intracellular juxtamembrane region, in case it is in spatial proximity with another CCM, giving rise to a putative conformation analogous to a *strict* CCM.

2.5. Sequence logos that highlight evolutionarily conserved motifs

A graphical illustration of the sequence conservation/diversity in vertebrate serotonin$_{1A}$ receptor TM V and its juxtamembrane region was generated using the Protter-visualize proteoforms (Omasits et al., 2014) for the corresponding MSA. The amino acid sequence logo was reconstructed using the WebLogo webserver (http://weblogo.berkeley.edu/) (Crooks et al., 2004). A prototype is shown in Fig. 1c.

3. Results

The vertebrate serotonin$_{1A}$ receptor is found across diverse species (Peroutka and Howell, 1994). Interestingly, it is well documented that the composition of membrane lipids (such as cholesterol) in endotherm (warm-blooded) (Sackmann, 1995; van Meer et al., 2008; Harayama and Riezman, 2018) and ectotherm (cold-blooded) (Hassett and Crockett, 2009) species vary a lot. In this work, we computationally addressed the evolution of the amino acid sequence of TM V and its juxtamembrane ICL3 fragment of the vertebrate serotonin$_{1A}$ receptor. Fig. 2 shows a schematic representation of the overall topology of the serotonin$_{1A}$ receptor with focus on the regions we have highlighted in this work. The overall focus of our study is to explore how membrane

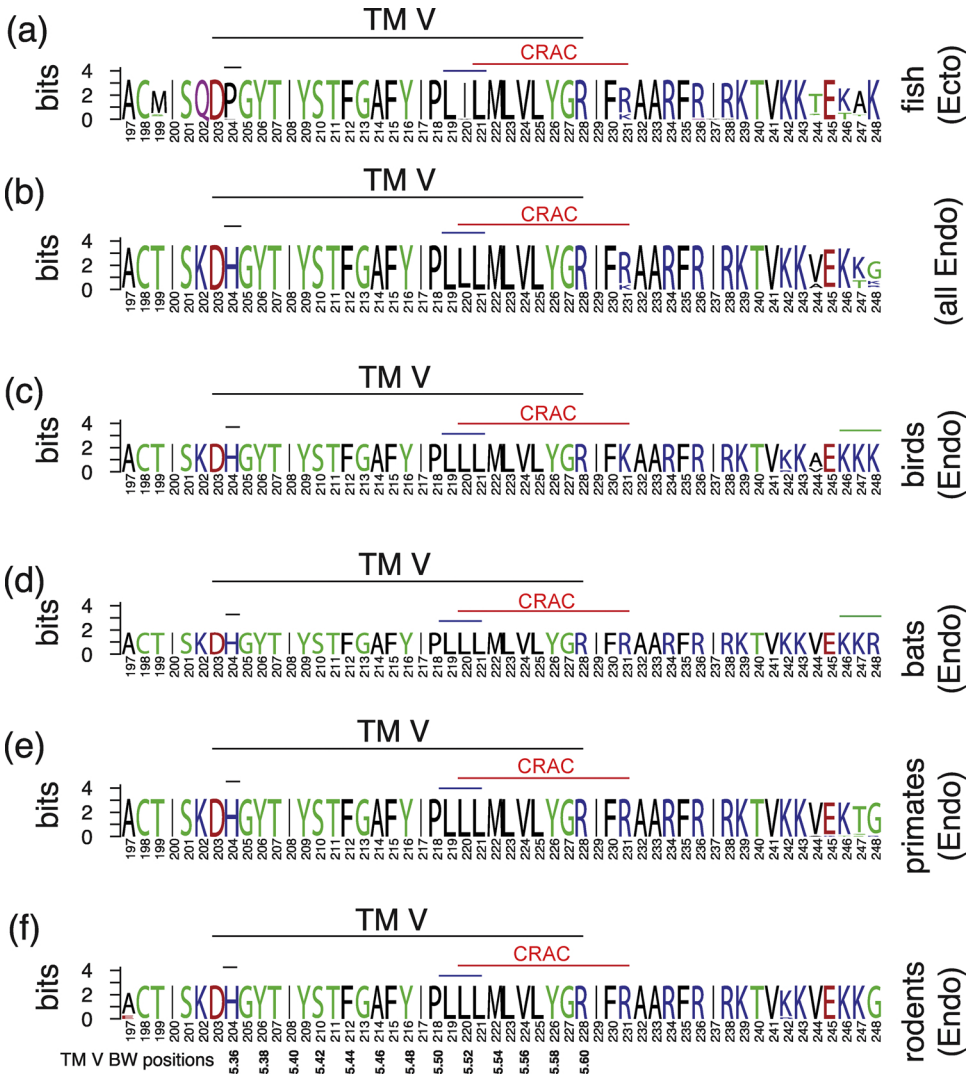

Fig. 3. Sequence logos from vertebrate phylogenetic clades for TM V and ICL3 fragment. The sequence logo from the MSA of orthologous sertotonin$_{1A}$ receptors is categorized into various phylogenetic clades and sub-clades. Panels (a) ectotherms (Ecto) and (b) endotherms (Endo) comprise the full dataset. From the set of endotherms, sequence logos were generated for (c) birds (Aves), (d) bats, (e) primates and (f) rodents. MSA positions 197–248 are shown under the logos, and the putative positions from TM V have been indicated by the Ballesteros Weinstein indexing scheme (Ballesteros and Weinstein, 1995) (see below panel (f)). The MSA position 204 represents a Proline residue among Teleost species, which is substituted by a Histidine residue in endotherms (Aves and Mammalia). MSA positions 220–231 (shown as a red overline) represent a CRAC motif as described by Jafurulla et al., 2011. Partially overlapping the CRAC motif is the triple Leucine motif (LLL), consisting of MSA positions 219–221 (shown as a blue line) is conserved among endotherms shown in panels (b)-(f). Most of the non-conserved amino acid residues are observed beyond positions 243 in ICL3, as shown in panels (a) and (b). Clade-specific conserved motifs with triple basic amino acid residues, KKK and KKR, are shown at MSA positions 246–248 (shown as a green line) in birds and bats, respectively. All species used in our study are listed in Table S1. See Section 2 for more details.

cholesterol could interact with serotonin$_{1A}$ receptor, in the backdrop of its native membrane lipid environment. Toward this goal, we computed the conserved amino acid residues (see Section 2.2. and Fig. 1b) in diverse ectotherm and endotherm species (as outlined in Fig. 1a), as these residues could influence the structure and function of the vertebrate serotonin$_{1A}$ receptor.

3.1. Amino acid sequence of TM V is largely conserved among endotherms

We have represented TM V as a gapless MSA with 26 positions of evolutionarily related amino acid residues (MSA positions 203–228 in Fig. 3). Panels in Fig. 3 represent phylogenetic clade-specific sequence logos derived from the original MSA (see Section 2.5). The Teleost (bony fish) clade represents ectotherm species, and the representative sequence logo for the MSA is shown in Fig. 3a. The corresponding sequence logo derived from representative endotherm species is shown in Fig. 3b. Subsequently, we have shown the sequence logos derived from the various representative clades/sub-clades of endotherms such as birds (Fig. 3c), bats (Fig. 3d), primates (Fig. 3e) and rodents (Fig. 3f). We observe that the serotonin$_{1A}$ receptor TM V has remained largely conserved during natural evolution of vertebrates. We found two MSA positions (204 and 220) in TM V that are not identically conserved during vertebrate evolution (see Fig. 3a,b). The MSA position 204 has a Proline residue in ectotherm species that gets substituted by a conserved Histidine residue in endotherms (overlined as a black line in Fig. 3). Histidine is substituted by Glutamine residue in the *Trichechus manatus latirostris* (Florida Manatee), but remains conserved among the other fifty endotherm species that we studied (not shown). It is therefore apparent that the Histidine residue is predominantly conserved among all endotherms, and could be important for the structure and function of TM V in the membrane milieu. A close examination of the TM V sequence in Fig. 3 reveals that a second non-conserved amino acid residue is present at MSA position 220. Most endotherm species have a nearly conserved Leucine at position 220, while this Leucine is substituted by Isoleucine in most ectotherm species. In endotherms, the Leucine at MSA position 220 is flanked by two other conserved Leucines at MSA positions 219 and 221 giving rise to a LLL motif (LIL motif in ectotherms, shown by a blue overline in Fig. 3). Consequently, we highlight that the Histidine at MSA position 204 and triple-Leucine (LLL) motif could be an endotherm-specific adaptation for TM V, since they are largely conserved across endotherms, but not in ectotherms (Fig. 3a *vs.* 3b).

Flanking the L(L/I)L motif in the vertebrate serotonin$_{1A}$ receptor TM V is a Proline residue on one side and a Methionine on the other side, giving rise to PL(L/I)LM motif (MSA positions 218–222, Fig. 3). A recent *in silico* study, which was motivated by extensive studies of TM V from A_{2A}R adenosine receptor (Thévenin and Lazarova, 2008), has revealed a broader role of the PXXXM motif in the oligomerization of four class A GPCRs (Altwaijry et al., 2017). In addition to the PXXXM motif, flanking Tyrosine residues from TM V (YIPL(L/I)LMLVLY), has been previously implicated in stabilizing polyleucine-based transmembrane helices (Zhou et al., 2001). It is plausible that both LLL/LIL and PXXXM

motifs could influence inter-TM interactions, as their occurrence during endotherm evolution is noteworthy.

3.2. Evolution of the ICL3 fragment

The intracellular juxtamembrane region of TM V from the serotonin$_{1A}$ receptor, which is also the N-terminal region of ICL3, conforms to an amphipathic α-helical secondary structure with high- and moderate-affinity calmodulin binding sites (Turner et al., 2007). The TM V intracellular juxtamembrane region is implicated in G-protein (G_i) coupling (Malmberg and Strange, 2000; Ortiz et al., 2000), phosphorylation (Lembo and Albert, 1995) and desensitization (Raymond et al., 1999). Consequently, we monitored cholesterol interaction motifs spanning the intracellular juxtamembrane region of TM V (see MSA positions 229–248 in Fig. 3), a region we will term as ICL3 fragment in the rest of the paper.

We observed that the ICL3 fragment is largely conserved during vertebrate evolution (Figs. 3 and 4). More importantly, Arginine, an amino acid that has been suggested to "snorkel" the cytosolic domain enveloping the inner leaflet of the membrane (Strandberg and Killian, 2003; Chamberlain et al., 2004; Baier et al., 2011), is largely conserved at four different positions (MSA positions 231, 234, 236 and 238, see Figs. 3 and 4). It is plausible that Arginine residues may have been preferentially selected during vertebrate evolution to preserve the receptor structure and function (Chamberlain et al., 2004; Saha et al., 2017). We also highlighted two triple basic amino acid motifs comprising of Arginine or Lysine residues. KKK and KKR motifs are exclusively conserved among birds and bats, respectively (Fig. 3c,d, MSA positions 246–248, shown as green overline), but not among any other phylogenetic clade/sub-clade.

In addition, we observed non-conserved amino acid residues beyond MSA position 243, which indicates a species-specific structural and functional role of the serotonin$_{1A}$ receptor ICL3 fragment in the native membrane environment. Interestingly, it has been previously reported that the serotonin$_{1A}$ receptor sequence is under strong evolutionary selection pressure during primate evolution (Anbazhagan et al., 2010). However, various nonsynonymous amino acid substitutions, insertions and deletions have resulted in ICL3 sequence variation across distant vertebrate species. Consequently, for residues beyond MSA position 243, we found amino acid sequence conservation among closely related species but not within their entire clade/sub-clade (Fig. 3).

3.3. Evolutionarily conserved putative cholesterol recognition motifs on TM V and ICL3 fragment

An interesting question to ask is how does a predominantly conserved sequence fragment (corresponding to TM V) function in very diverse membrane lipid environments? This prompted us to explore a possible unique role of TM V in the context of diverse membrane lipid compositions across vertebrate species.

In this work, we have identified evolutionarily conserved amino acid residues, which could play a salient role in the interaction of the serotonin$_{1A}$ receptor with membrane lipids. To the best of our knowledge, we report, for the first time, three categories of cholesterol interaction motifs in vertebrate serotonin$_{1A}$ receptor TM V and ICL3 fragment, in addition to the CRAC motif that we reported earlier (Jafurulla et al., 2011). These additional cholesterol interaction motifs are: (i) CRAC-like, (ii) CARC-like and (iii) CCM. *We show here, that between these three additional cholesterol interaction motifs and the original CRAC motif, a collage of twenty distinct cholesterol interaction configurations (see below) are preserved across vertebrate evolution spanning ~650 million years. The residues that belong to this set of cholesterol interaction configurations, therefore constitute an array of molecular determinants, that may impinge on receptor structure and function from an evolutionary perspective.* We will now describe each of these motifs below.

3.3.1. Conserved CRAC motif across Mammalia is not identical but homologous to CRAC motif among Aves

We previously identified an evolutionarily conserved CRAC motif on TM V of the serotonin$_{1A}$ receptor (Jafurulla et al., 2011). As mentioned earlier, the typical CRAC motif sequence is defined, from N-terminal to C-terminal, by the sequence fragment [L/V]-X$_{1-5}$-Y-X$_{1-5}$-[K/R]. The corresponding sequence fragment from the human serotonin$_{1A}$ receptor is **L**LMLVL**Y**GRIF**R** (residues that fulfill the CRAC algorithm are shown in bold and underlined, corresponding to MSA positions 220–231, shown by a red overline in Fig. 3). We previously showed that this CRAC motif is conserved across Teleost and Mammalia (Jafurulla et al., 2011). However, the invariance of this motif among Aves has not been explored before. In this work, we identify a homologous, but not identical, CRAC motif among Aves. We observe that the Arginine residue (MSA position 231 in Fig. 3) is substituted by a Lysine residue in the corresponding sequence fragment across Aves, and therefore the Avian TM V CRAC motif is **L**LMLVL**Y**GRIF**K** (MSA positions 220–231 in

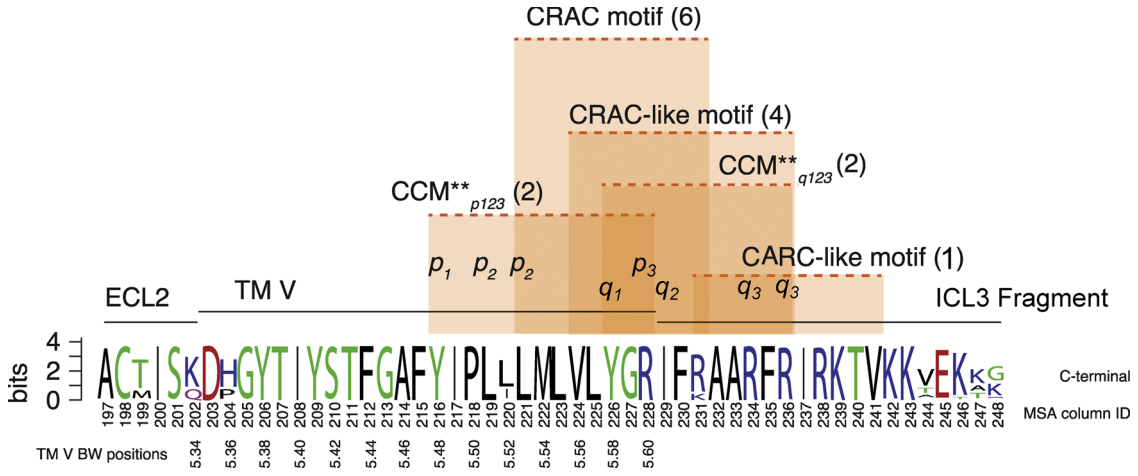

Fig. 4. A collage of putative cholesterol interaction motifs overlaid on the sequence logo of serotonin$_{1A}$ receptor TM V and ICL3 fragment. MSA positions in TM V are represented by Ballesteros Weinstein indices from 5.34-5.60. Position 5.50 represents the position of the evolutionarily conserved Proline. In addition to TM V, the juxtamembrane regions from ECL2 and ICL3 are included. Orange boxes represent various cholesterol-sensitive motifs (CRAC, CRAC-like, CARC-like and CCM motifs), and numbers in parentheses represent the total number of possible configurations present (See Table 1). Each of the CCM motifs is defined by two sets of three amino acid residues. Positions labeled by (p_1,p_2,p_2,p_3) and (q_1,q_2,q_3,q_3) define these CCM configurations. A repetitive index (*e.g.*, p_2,p_2) suggests two possible ways for defining position p_2. Patterns (q_1,q_2,q_3,q_3) for CCM**$_{q123}$ are represented as configurations 14 and 15 in Table 1. Similarly, patterns (p_1,p_2,p_2,p_3) for CCM**$_{p123}$ are represented as configurations 16 and 17 in Table 1. Dashed lines suggest overlapping configurations.

Table 1
Configurations from Cholesterol Interaction Motifs in TM V and ICL3 Fragment of the Serotonin$_{1A}$ Receptor.

Cholesterol Interacting Motifs	Configuration #	MSA Position	TM V													ICL3 Fragment																
			216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241
		All Species →	Y	I	P	L	I	L	M	L	V	L	Y	G	R	I	F	R/K	A	A	R	F	R	I	R	K	T	V
		Endo* →					L																					
CRAC	1						Δ						□		●													
	2							▲					□		●													
	3									▲			□		●													
	4										▲		□		●													
	5						Δ						□					●										
	6							▲					□					●										
	7									▲			□					●										
	8										▲		□					●										
CRAC-like	9										▲						□				●							
	10											▲					□				●							
	11										▲						□						●					
	12											▲					□						●					
CARC-like	13																	●				□						▲
CCM**	14												□			▲					●							
CCM**	15												□			▲							●					
CCM**	16		□				▲								●													
CCM**	17		□					▲							●													
CCM*	18												α															
CCM*	19																β											
CCM*	20																					γ						

Most endotherm (Endo*) species have an identical fragment sequence, except that Isoleucine (**I**) is substituted by Leucine (**L**). Configuration 5 represents the CRAC motif that has been previously reported in GPCRs from our group (Jafurulla et al., 2011). Configurations 1 and 5 are only tenable in mammals and birds, configurations 2–4 and 6–8 are tenable across all vertebrates. The CCM configurations (CCM**, 14–17) have one more (or less) amino acid and therefore do not conform to the prototypical CCM. CCM* (configurations 18–20) represents contribution from a sole amino acid residue, to complement a pre-existing CCM, and may conform to a *strict* CCM.

··· ICL3 amino acid sequence continuity.

▲ Aliphatic amino acid residues (Leucine/Valine/Isoleucine) in a cholesterol recognition motif.

Δ Leucine residue in a cholesterol recognition motif across most endotherms.

□ Aromatic amino acid residues (Tyrosine/Phenylalanine) in a cholesterol recognition motif.

● Basic amino acid residues (Lysine/Arginine) in a cholesterol recognition motif.

* Speculative and does not conform to exact specifications of a CCM (CCM** and CCM*).

α,β,γ Position of the fourth amino acid involved with proximal CCM (other than TM V) to conform as a *strict* CCM.

Fig. 3c). Most importantly, this clade-specific substitution does not disturb the overall homology of CRAC across vertebrates.

3.3.2. TM V has at least six possible configurations of the CRAC motif in vertebrates

We observed that due to the degeneracy of amino acid sequence in the definition of a CRAC motif (Leucine or Valine in N-terminal and Arginine or Lysine in C-terminal, see Section 2.4.1.), the sequence fragment **L**M**L**V**L**Y**G**R**I**F**(R/K)** from TM V could give rise to at least six possible CRAC configurations (Table 1, configurations #2–4, 6–8; MSA positions 221–231 in Fig. 4). This sequence provides some amount of plasticity in cholesterol interaction with TM V of the serotonin$_{1A}$ receptor. Interestingly, we find that the six overlapping CRAC configurations are retained during vertebrate evolution (see Table 1, configurations #2–4, 6–8). For sequence fragments from ectotherms, there is an Isoleucine residue at MSA position 220 (Fig. 3a, ^{220}**I**LMLVLYGRIF (R/K)) that does not algorithmically code for a CRAC motif. Interestingly, among most endotherm species, the same Isoleucine has been substituted by Leucine (MSA position 220 in Fig. 3b). As Leucine algorithmically codes for a N-terminal residue in a CRAC motif, two additional CRAC configurations are possible in most endotherm species based on the sequence fragment ^{220}**L**LMLVL**Y**G**R**IF**(R/K)** (Table 1, configurations #1,5). In summary, eight partly overlapping CRAC configurations exist in most endotherms (Table 1, configurations #1-8), six of which are conserved across ectotherm and endotherm vertebrates (Table 1, configurations #2–4, 6–8 and Fig. 4).

3.3.3. TM V and ICL3 fragment have a CRAC-like motif

We have identified a conserved CRAC-like motif in the serotonin$_{1A}$ receptor spanning a portion of TM V and ICL3 fragment. The sequence algorithm of a CRAC-like motif is identical to CRAC motif, except that the central Tyrosine residue is replaced by another aromatic residue Phenylalanine (Baier et al., 2011). The corresponding sequence fragment for CRAC-like motif from the serotonin$_{1A}$ receptor can be represented as **VL**YGRI**F**(R/K)AA**R**F**R** (MSA positions 224–236 in Fig. 4); this CRAC-like motif has four possible configurations due to degeneracy in the positioning of cholesterol-sensitive residues in its sequence, each of which have remained conserved during vertebrate evolution (see Table 1, configurations #9-12, and Fig. 4). It is therefore plausible that any one of these four configurations may putatively interact with cholesterol.

3.3.4. ICL3 fragment has a unique CARC-like motif

We observed that the CRAC and CRAC-like motif is followed by a conserved CARC-like motif (see Table 1, configuration #13, and Fig. 4). The CARC-like motif exhibits an opposite orientation of the CRAC motif along the polypeptide chain (Baier et al., 2011). The corresponding sequence of the CARC-like motif in ICL3 fragment from the serotonin$_{1A}$ receptor is (**R/K**)AAR**F**RIRKT**V** (MSA positions 231–241 in Fig. 4). We observed that a basic amino acid residue (Lysine or Arginine) is retained at MSA position 231 throughout vertebrate evolution preserving this CARC-like motif (Fig. 3). Importantly, we report that this residue overlaps in four distinct CRAC configurations (Table 1, configurations

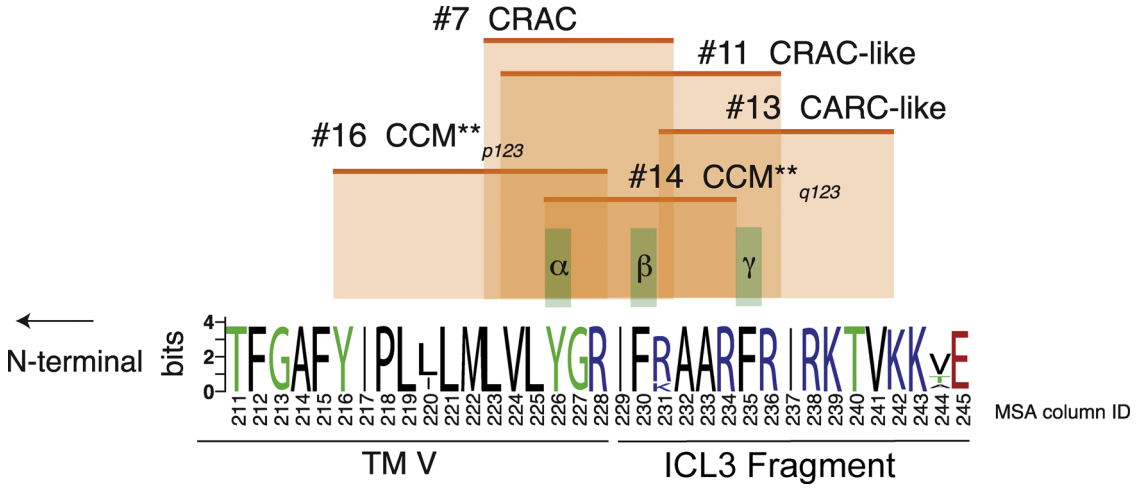

Fig. 5. A milieu of representative cholesterol interaction configurations from TM V and ICL3 fragment. Overlaid on the TM V sequence logo are orange boxes representing putative configurations for cholesterol interaction. The schematic has a list of five representative configurations (#s 7 (CRAC), 11 (CRAC-like), 13 (CARC-like), 14 (CCM) and 16 (CCM**)) for cholesterol interaction (see Table 1), each of which involves three amino acid residues. The green boxes labeled α, β and γ denote Tyrosine (MSA position 226), Phenylalanine (position 230), and Phenylalanine (position 235), respectively. Any one of these residues (configurations #18, 19, 20 in Table 1) may complement an existing CCM in its spatial proximity to constitute a *strict* CCM. See text for more details.

#5-8) and one CARC-like configuration (Table 1, configuration #13). Interestingly, each of these conserved CRAC and CARC-like paired configuration is poised for simultaneous interaction with a pair of cholesterol molecules. In this context, it would be interesting to explore whether these paired configurations (in TM V and ICL3 fragment) could potentially interact with two cholesterol molecules in a transbilayer tail-to-tail dimer of cholesterol (Harris et al., 1995; Mukherjee and Chattopadhyay, 1996; Rukmini et al., 2001; Chaudhuri and Chattopadhyay, 2011). This CRAC and CARC-like paired configuration represents an evolutionary homolog of a previously identified CARC-CRAC mirrored motif involving two leaflets of the membrane bilayer (Fantini et al., 2016b; Di Scala et al., 2017).

3.3.5. TM V and ICL3 fragment have overlapping cholesterol consensus motifs

As the ICL3 fragment may conform to an amphipathic α-helix (Turner et al., 2007; see Section 3.2), it is therefore plausible that its conformation could be amenable to additional cholesterol interactions. It has been proposed that cholesterol interaction sequence should at least contain one aromatic amino acid that could interact with ring D of cholesterol and a basic residue capable of participating in electrostatic interactions with the hydroxyl group of cholesterol (Epand, 2006; Hanson et al., 2008; Fantini and Barrantes, 2013). In the crystal structure of the β_2-adrenergic receptor, three amino acids in TM IV, along with an amino acid in TM II, have been shown to constitute a new class of cholesterol interacting motif, namely, the *strict* CCM (Hanson et al., 2008). The criterion of specific residues in CCM could be somewhat broadened by conservative amino acid substitution, as the relative positions of crucial amino acids in CCM are not stringent (Hanson et al., 2008). Consequently, we looked for patterns from TM V and ICL3 sequence fragment, which may qualitatively conform to CCM configurations.

We subsequently report four configurations that may qualitatively conform to CCM algorithm (see Section 2.4.3). We identified two overlapping sequence fragments from TM V and ICL3 fragment from the serotonin$_{1A}$ receptor sequence, which qualitatively resemble CCM motifs. The two relevant sequence fragments are: **Y**IPL(**I**/**L**)**L**MLVLYG**R** and **Y**GR**I**F(R/K)AA**R**F**R** (see MSA positions 216–228 and 226–236, respectively, in Fig. 4). Let us first consider the fragment **Y**GR**I**F(R/K)AA**R**F**R**. From this fragment, two possible configurations, that may qualitatively resemble CCM (termed CCM**$_{q123}$ in Fig. 4), are shown in Table 1 (configurations #14,15). A typical CCM would require strictly three residues between Tyrosine and Isoleucine (see Section 2.4.3.). However, in this case, the CCM algorithm is disrupted by the absence of a third amino acid between Tyrosine and Isoleucine residues at MSA positions 226 and 229, respectively, as shown in Fig. 4. The second peptide fragment **Y**IPL(**I**/**L**)**L**MLVLYG**R** may qualitatively resemble two additional putative cholesterol interaction configurations (Table 1, configurations #16,17 and termed CCM**$_{p123}$ in Fig. 4). In this case, the CCM algorithm is violated by the presence of an additional amino acid, a conserved Glycine (MSA position 227 in Fig. 4). It has been suggested that due to the nonspecific nature of electrostatic interactions in the interfacial region of the membrane, Arginine or Lysine conforming to a CCM, or neighboring it, could interact with the cholesterol hydroxyl group (Hanson et al., 2008). Taken together, configurations analogous to CCM could exist as transient 3D conformations in TM V and ICL3 fragment, depending on membrane cholesterol content.

3.3.6. A strict cholesterol consensus motif involving TM IV with TM V and ICL3 fragment

A *strict* CCM constitutes a 3D conformation that interacts with cholesterol between two spatially proximal α-helices, toward the inner leaflet of the cell membrane (Hanson et al., 2008). We have previously shown that the serotonin$_{1A}$ receptor TM IV and II form a conserved *strict* CCM (Paila et al., 2009). Interestingly, on transitioning from an inactive to an active state, the orientations of TMs V and VI are altered (Reiter et al., 2012; Wacker et al., 2013), thereby indicating that the spatial proximity of TMs II and IV may not always be conserved. In a 3D conformation where CCM from TM II is not in the vicinity of TM IV, and yet interacts with cholesterol, a nonpolar aromatic amino acid residue (Tyrosine from TM V, or Phenylalanine from ICL3 fragment) could become accessible, and conform to the *strict* CCM involving four residues (see Table 1, configurations #18-20, denoted as α, β and γ in Fig. 5).

4. Discussion

Taken together, in this work, we have identified a conserved collage of cholesterol interaction motifs (see Fig. 5 for representative configurations from each of CRAC, CRAC-like, CARC-like and CCM) associated with TM V and ICL3 fragment of the serotonin$_{1A}$ receptor. This collage of motifs represents a total of twenty diverse cholesterol interaction configurations (listed in Table 1). What does this finding mean in terms of evolution and function of the serotonin$_{1A}$ receptor in a variety

of tissue types, differing in their cholesterol content? This forms the basis of the relevance of our results.

Physiological variations in cholesterol content could have multiple origins. For example, although the central nervous system constitutes ~2% of the body mass, it accounts for ~25% of the cholesterol content in the body (Dietschy and Turley, 2001; Chattopadhyay and Paila, 2007). In addition, cellular cholesterol content is age-dependent and developmentally regulated (Martin et al., 2010; Smiljanic et al., 2013). A hallmark of cellular cholesterol is that it is heterogeneously distributed and is present in various thermodynamic and kinetic pools (domains) across the cellular milieu (Schroeder et al., 1995; Simons and Ikonen, 2000; Xu and London, 2000; Rukmini et al., 2001; Mukherjee and Maxfield, 2004; Steck and Lange, 2018). Variation of cholesterol content in various tissue types and species (Dinh et al., 2011; Yin et al., 2012) therefore could be further complicated by the distribution of cholesterol among various cellular pools among species. Such variations are challenging to measure experimentally (Steck and Lange, 2018), yet represent an intriguing conceptual paradigm for a comprehensive understanding of lipid-protein interaction and cellular signaling during evolution. This assumes further significance in light of the fact that residues in the receptor could differ in terms of accessibility (latency) since some of them could be buried and others accessible to varying extents.

It is noteworthy that mere presence of CCM, CRAC(-like) or CARC(-like) motifs in proteins do not necessarily translate to cholesterol-based interactions. For example, the neurotensin receptor 1, a class A GPCR that is predominantly expressed in the brain (Regard et al., 2008), does not exhibit cholesterol sensitivity for its function although the receptor has CCM in its sequence (Oates et al., 2012). An earlier study of bacterial genomes (*Streptococcus agalactiae, Staphylococcus aureus and Escherichia coli*) reported an average occurrence rate of 2–3 CRAC motifs in diverse proteins that do not interact with cholesterol (Palmer, 2004). We believe that experimental approaches, such as site-directed mutational analysis of the amino acid residues involved in such interactions, followed by functional analyses of the protein, is likely to provide a better understanding of specific lipid dependence of the protein function (Epand, 2006; Chattopadhyay, 2014; Levitan et al., 2014; Fantini et al., 2016b; Di Scala et al., 2017; Jafurulla et al., 2019).

In this overall context and based on our analysis, we propose that a multiplicity of cholesterol interaction sites could be useful in enabling the serotonin$_{1A}$ receptor to interact differentially with membrane cholesterol in a cell type specific manner in light of the diversity of cellular cholesterol content. Our proposition is in agreement with our previous work where we showed, by molecular dynamics simulations, that the serotonin$_{1A}$ receptor exhibits cholesterol-dependent conformational plasticity (Prasanna et al., 2016). We envision that evolutionarily conserved mode of interplay among crucial membrane lipids such as cholesterol and signaling hubs like GPCRs, could act as a powerful driver of cellular signaling across diverse species.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chemphyslip.2019.02.010.

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