Abstract
The interaction of G protein-coupled receptors (GPCRs) with cholesterol is a hallmark of their function, organization, and structural dynamics. Several cholesterol interaction sites, such as the cholesterol recognition amino acid consensus (CRAC) and cholesterol consensus motif (CCM), have been mapped from crystallography, bioinformatics, and simulation studies. In this article, we characterize common descriptors for cholesterol interaction sites in the serotonin 1A receptor from a series of coarse-grain simulations. We have identified a novel interaction mode for cholesterol in which the cholesterol polar headgroup interacts with aromatic amino acid residues, such as tryptophan and tyrosine. The cholesterol rings interact with both aromatic residues and nonpolar residues, thereby constituting a signature aromatic interaction site. In addition, we report a similar binding mode in the crystal structures of the serotonin 2B receptor, suggesting that this binding mode could be a general feature of the serotonin receptor family. Interestingly, this signature aromatic interaction site is present along with one of the CRAC motifs in the serotonin 1A receptor. Our results represent an important step toward mapping out the diversity of cholesterol-GPCR interaction sites.

Keywords
GPCR · MARTINI coarse-grain simulation · Signature aromatic cholesterol interaction site · Serotonin receptors · Cholesterol interaction site · Cholesterol occupancy

11.1 Introduction
G protein-coupled receptors (GPCRs) represent a unique class of membrane proteins that mediate multiple physiological processes (Pierce et al. 2002; Rosenbaum et al. 2009). These receptors are comprised of a conserved topology of seven transmembrane helices interconnected by extracellular and intracellular loops (Venkatakrishnan et al. 2013). Ligands such as neurotransmitters and hormones bind to these receptors and transduce signals inside the cell (Pierce et al. 2002; Rosenbaum et al. 2009). An important feature of GPCR functional dynamics is the effect of membrane composition on receptor structure and
function (Chattopadhyay 2014). For instance, membrane cholesterol has been shown to modulate the structure and function of several GPCRs in a context-dependent fashion (Burger et al. 2000; Pucadyil and Chattopadhyay 2006; Paila and Chattopadhyay 2010; Oates and Watts 2011). To exert its effect on receptor function, cholesterol has been suggested to act by both direct interactions with the receptor and/or indirectly by modulating membrane properties (Paila and Chattopadhyay 2009). Interestingly, the crystal structures of several GPCRs, such as β2-adrenergic receptor (Hanson et al. 2008), adenosine2A receptor (Liu et al. 2012), and serotonin2B receptor (Wacker et al. 2013), have been able to resolve bound cholesterol molecules, suggesting a direct effect. Similarly, direct interactions of cholesterol have been observed in simulations of several GPCRs (Lee and Lyman 2012; Cang et al. 2013; Prasanna et al. 2014).

One of the most comprehensive cholesterol dependencies has been reported in the serotonin1A receptor, a representative GPCR that is abundant in the hippocampal region of the brain (Chattopadhyay 2014). It is activated by serotonin (a neurotransmitter) and plays a key role in physiological responses such as anxiety, depression, and mood (Pucadyil et al. 2005; Kalipatnapu and Chattopadhyay 2007; Lacivita et al. 2008; Fiorino et al. 2014). We have previously shown that depletion of cholesterol affects the stability, function, and dynamics of the serotonin1A receptor (Pucadyil and Chattopadhyay 2004, 2007; Saxena and Chattopadhyay 2011, 2012; Jafurulla and Chattopadhyay 2013). In addition, cholesterol was observed to modulate receptor organization by modulating the population of the different oligomers (Ganguly et al. 2011; Paila et al. 2011a; Chakraborty et al. 2018). To dissect the molecular basis of these cholesterol effects, molecular dynamics simulations were performed using both atomistic and coarse-grain force fields (Sengupta and Chattopadhyay 2012; Patra et al. 2015; Prasanna et al. 2016). We were able to distinguish direct interactions of cholesterol with the receptor at certain “hot spots”. From a series of coarse-grain and atomistic simulations, we have identified multiple cholesterol interaction sites with an occupancy time of nanoseconds to microseconds (Sengupta and Chattopadhyay 2012; Patra et al. 2015; Prasanna et al. 2016). Indirect effects related to membrane thickness changes in the bulk membrane as well as around the receptor have been observed, but their significance is yet to be fully analyzed (Prasanna et al. 2016).

In order to understand the nature of direct GPCR-cholesterol interactions, several cholesterol binding motifs have been reported in GPCRs (Rouviere et al. 2017). These include the cholesterol consensus motif (CCM) (Hanson et al. 2008) and the cholesterol recognition amino acid consensus (CRAC) motif (Jafurulla et al. 2011). The presence of the CCM site was earlier reported in crystal structure of β2-adrenergic receptor (Hanson et al. 2008). The motif is located between transmembrane helices II and IV and consists of a charged residue R or K followed by W-Y, I-L-V and F-Y residues. An important motif that we previously characterized in GPCRs is the CRAC motif, comprising of hydrophobic and aromatic residues followed by a charged residue (arginine or lysine) (Jafurulla et al. 2011). The CRAC motif is defined by the presence of the pattern −L/V-(X)1−5-Y-(X)1−5-R/K−, in which (X)1−5 represents between one and five residues of any amino acid. This motif is present on the transmembrane region of the helix V of the serotonin1A receptor (along with transmembrane regions of helix II and VII). A mirror image of the CRAC motif, known as the CARC motif, has also been reported. In this motif, the terminal residues of the CRAC motif are reversed, with K/R on the N-terminus and L/V on the C-terminus of the helix (Baier et al. 2011). The CRAC and CARC sites have been mainly identified from bioinformatics and molecular dynamics studies and are yet to be experimentally validated. In all reported cholesterol sites, the hydroxyl group of cholesterol interacts with a charged amino acid residue. Despite these structural and sequence cholesterol motifs, not all crystallographically resolved sites or those identified from molecular dynamics simulations have been characterized. For instance, we have
identified several cholesterol interaction sites in the serotonin$_{1A}$ receptor (Sengupta and Chattopadhyay 2012; Prasanna et al. 2016), only one of which correlates to a CRAC site. Other sites that were observed in simulations remain uncharacterized, and the need arises to determine the molecular signatures of these sites.

In this work, we have identified common descriptors of cholesterol dynamics in the serotonin$_{1A}$ receptor using coarse-grain simulations. The work extends from our previous work in which we have analyzed the effect of cholesterol on receptor dimerization (Prasanna et al. 2016). We have performed ten simulations of the receptor embedded in POPC/cholesterol bilayers with 30% cholesterol, as a representative model membrane for eukaryotic cell membranes. In order to extract common features of cholesterol binding, we have calculated the interaction propensities of different parts of the cholesterol molecule (such as hydroxyl group and sterol ring) and have determined a pattern of amino acids that are preferred by each coarse-grain bead of cholesterol molecule. A new interaction mode in which the cholesterol headgroup interacts with an aromatic residue is discussed. These results would contribute to our overall understanding of cholesterol-GPCR interaction.

### 11.2 Methods

**System Setup:** The homology model of the serotonin$_{1A}$ receptor was taken from our previous work (Paila et al. 2011b; Sengupta and Chattopadhyay 2012; Prasanna et al. 2016). This model was built based on the crystal structure of the β$_2$-adrenergic receptor (PDB: 2RH1; Cherezov et al. 2007) and template sequence of the serotonin$_{1A}$ receptor. The receptor monomer was embedded in a pre-equilibrated POPC/cholesterol bilayer with 30% cholesterol. The average cholesterol concentration in a eukaryotic cell is about 30% (Van Meer and de Kroon 2011). Simulations were performed with the MARTINI coarse-grain force field (Marrink et al. 2007; Monticelli et al. 2008) using the GROMACS simulation package (Van Der Spoel et al. 2005). Ten replicates of this system were simulated for 10 µs each. The temperature coupling was carried out using v-rescale thermostat at 300 K for each component of the system separately, with a coupling constant of 0.1 ps (Bussi et al. 2007). Pressure was maintained at 1 bar semi-isotropically in the plane of the bilayer and perpendicularly to the bilayer using the Berendsen barostat, with a coupling constant of 0.5 ps and a compressibility of 3 × 10$^{-5}$ bar$^{-1}$ (Berendsen et al. 1984). A time step of 20 fs was used (Marrink et al. 2007).

**Analysis:** The maximum occupancy time was calculated as the maximum time a cholesterol molecule is bound at a specific location during the course of simulation (Sengupta and Chattopadhyay 2012). The MARTINI model for cholesterol consists of eight beads, with a ROH bead mapping to the hydroxyl group, R1–R5 beads modeling the rings, and C1 and C2 representing the aliphatic tail. For each of these beads, the residue-wise-specific occupancy was calculated using a cutoff of 0.6 nm. For each of the 20 amino acids, occupancy was calculated as the maximum occupancy time for cholesterol around a given residue type and normalized to the number of instances it appears in the receptor. The occupancy time for each amino acid was averaged and normalized across ten sets of simulations. The maximum occupancy time for cholesterol around transmembrane region of each helix was calculated as described earlier (Prasanna et al. 2016). The transmembrane domain was divided into the upper (extracellular) or lower (intracellular) leaflets depending on its location in the bilayer. Representative images of the cholesterol binding modes were rendered with VMD (Humphrey et al. 1996).

### 11.3 Results

A series of coarse-grain simulations were performed with the serotonin$_{1A}$ receptor monomer embedded in POPC/cholesterol bilayers with 30% cholesterol. To map out the common descriptors of cholesterol interaction sites, we calculated the interactions of cholesterol beads with each amino acid residue. As described in
the Methods section, the coarse-grain model for cholesterol consists of eight beads, with a ROH bead mapping to the hydroxyl group, R1–R5 beads modeling the rings, and C1 and C2 representing the aliphatic tail. From the residue-wise occupancies, we estimated the binding propensity of cholesterol to each amino acid residue type. Using this approach, we have been able to identify the molecular signatures of a novel cholesterol binding mode.

### 11.3.1 Common Descriptors for Cholesterol Interaction Sites

The maximum occupancy time of cholesterol (considering each coarse-grain bead of cholesterol individually) was calculated for each residue in the serotonin1A receptor. The values were averaged for each residue type and normalized by the frequency of that particular amino acid. The normalized occupancy time for each of the cholesterol beads is shown in Fig. 11.1. The polar ROH bead of cholesterol (representing the hydroxyl group) is observed to mainly interact with the aromatic amino acids Y, W, and F (maximum occupancy ≥60%). The positively charged amino acid R exhibits a lower interaction (60% ≥ maximum occupancy ≥50%). This is surprising since all known cholesterol binding motifs involve a positively charged residue such as R or K interacting with the polar headgroup. Further, the ROH bead is observed to interact with almost all residues (but with a low occupancy), indicating high dynamics.

The coarse-grain beads, R1–R5, represent the sterol rings in cholesterol. These beads show a high interaction with the residues L and M. However the ring beads closer to the polar headgroup (R1 and R2) also show high interactions with the aromatic residues similar to the ROH bead. Interestingly, the R2 and R4 beads map to the “smooth” face of the cholesterol but are nonetheless seen to interact strongly with the receptor. We believe that it represents a limitation of the coarse-grain model since the methyl groups on the cholesterol ring are difficult to represent in such a mapping. The hydrocarbon tail of the cholesterol molecule is mapped to two coarse-grain beads, C1 and C2. These beads show a strong interaction with several residues including the aromatic residues and the nonpolar residues, L and I.

Based on the residue-wise occupancies, we propose a new descriptor for cholesterol interactions in the serotonin1A receptor. A schematic representation of these amino acid descriptors is shown in Fig. 11.2. The residues showing a high occupancy time for each cholesterol bead (≥ 50%) are considered, and the height of the residue in the schematic depicts the relative value of the occupancy times. The most common residues that interact with the polar group of cholesterol are the aromatic residues, while the remaining beads show increased association with nonpolar residues. We would like to point out that these residue descriptors represent a spatial pattern of these residues, rather than sequence motif. The signature aromatic binding mode is easily distinguished in the figure and represents a previously uncharacterized interaction site that could have implications for cholesterol-receptor interactions.

### 11.3.2 The Signature Aromatic Binding Mode in the Serotonin1A Receptor

In the next step, we evaluated cholesterol interactions at the receptor in order to map these descriptors to individual cholesterol interaction sites. To identify the individual sites with high cholesterol occupancy, the maximum occupancy time was calculated for each helix in the upper and lower leaflets, considering cholesterol as a whole (Fig. 11.3). The values match well to our previous reports (Sengupta and Chattopadhyay 2012; Prasanna et al. 2016), despite the high stochasticity in the interaction sites. As expected, a large occupancy is observed at transmembrane helix V, which contains the CRAC site. However, multiple other sites are mapped as well, similar to our previous work (Sengupta and Chattopadhyay 2012; Prasanna et al. 2016). In the upper leaflet, we observe the highest occupancy time for the cholesterol around transmembrane helices IV and
VI. In the lower leaflet, the maximum cholesterol occupancy was observed around transmembrane helices I, IV, and V.

We mapped four of these high occupancy cholesterol sites to the signature aromatic binding mode. A schematic representation of these four sites is shown in Fig. 11.1. The maximum occupancy time of each cholesterol bead at each residue type in the serotonin1A receptor, averaged and normalized over simulation time and number of residues, is illustrated. A value of 1 indicates that the cholesterol bead is always present at that site, and a value of 0 indicates that there are no interactions during the simulation. See Methods for more details.
sites is shown in Fig. 11.4. The top panel represents the binding mode present in the upper leaflet of the membrane (transmembrane helices IV and VI) and the lower panel the binding mode in the lower leaflet (transmembrane helices I and V). The four sites are quite varied in their sequence and structure, although all interact with the cholesterol headgroup via an aromatic residue. Interestingly, in transmembrane helix V, the signature aromatic site lies next to the previously identified CRAC site. The fast cholesterol dynamics coupled with site hopping had previously made it difficult to identify the signature aromatic residue binding mode. At the site

Fig. 11.2 A schematic representation of the amino acid preferences for each bead of cholesterol molecule. Only the residues showing a high occupancy time for each cholesterol bead (≥ 50%) are considered (see Fig. 11.1), and the height and color of the residue in the schematic depict the relative order of the occupancies. For instance, residues W and Y with a high occupancy at cholesterol headgroup are shown as the largest and darkest alphabet. The cholesterol molecule is shown in green with the underlying coarse-grain mapping scheme, and the headgroup bead (ROH) is colored orange. See Methods for more details.

Fig. 11.3 Maximum occupancy time of cholesterol molecule around the serotonin1A receptor that are averaged and normalized over ten sets of simulations. The occupancy time is shown for the transmembrane helices corresponding to the extracellular or upper (light blue) and intracellular or lower (dark blue) leaflets separately.
Fig. 11.4 Representative snapshots of cholesterol interaction with the serotonin1A receptor at the signature aromatic binding modes observed in simulations. The top panel represents the binding mode present in the upper leaflet of the membrane (transmembrane helices IV and VI) and the lower panel the binding mode in the lower leaflet (transmembrane helices I and V). The transmembrane helix and the main interacting residues are shown in blue and the remaining part of the receptor in gray. The cholesterol molecules are shown in green, and the headgroup bead (ROH) is colored orange.
corresponding to transmembrane helix I, the aromatic residue is actually a part of the extracellular loop and not of transmembrane helix I. Due to the presence of multiple aromatic residues in transmembrane helix IV, the cholesterol molecule can adopt horizontal conformations. As a result, this binding mode of cholesterol cannot be mapped to a single sequence motif but represents a spatial pattern of residues. Overall, the signature aromatic binding mode occurs at multiple sites on the serotonin\textsubscript{1A} receptor and represents a novel cholesterol binding mode.

11.3.3 A Common Binding Mode for Serotonin Receptors

To analyze whether the signature aromatic binding mode is a general cholesterol binding mode, we analyzed the crystal structures of the serotonin receptor family. Two crystal structures of the serotonin\textsubscript{2B} receptor (PDB ID: 4IB4 and 5TVN) were resolved along with bound cholesterol (see Fig. 11.5). In both these structures, a cholesterol molecule is observed to interact at transmembrane helix I of the receptor. However, hydroxyl group is oriented toward the residue Y399 on helix VIII. In a similar site in the serotonin\textsubscript{1A} receptor, the aromatic residue was present on intracellular loop 1. The aliphatic amino acids L and I on transmembrane helix I interact with the sterol rings of cholesterol. The two binding modes in the crystal structures differ in the orientation of cholesterol, confirming the dynamics of cholesterol at these interaction sites. Taken together, the data suggests that cholesterol could interact with the serotonin receptor family by a signature aromatic binding mode. This signature binding mode does not represent an exclusive site but is present together with previously identified motifs such as the CRAC motif.

11.4 Discussion

The identification of cholesterol interaction sites has received attention due to the central role of cholesterol in the function and structural dynamics of GPCRs. The crystal structure of the \( \beta_2 \)-adrenergic receptor revealed a cholesterol bound at the cleft of transmembrane helices II and IV that was named the CCM motif (Hanson et al. 2008). Another cholesterol binding site, the CRAC motif was identified in the serotonin\textsubscript{1A} receptor based on similarity to other cholesterol binding proteins (Jafurulla et al. 2011). In addition, molecular dynamics simulations using both atomistic and coarse-grain force fields were able to identify multiple cholesterol interaction sites on several GPCRs (Lee and Lyman 2012; Sengupta and Chattopadhyay 2012; Cang et al. 2013; Prasanna et al. 2014, 2016; Patra et al. 2015). Although cholesterol has been found to be associated with several GPCRs in their crystal structures, the mapping of the location of cholesterol molecules in these crystal structures to proposed cholesterol binding motifs has proved to be less than straightforward. We identify here a signature aromatic cholesterol interaction mode, in which the headgroup of the cholesterol molecule interacts with an aromatic residue. This interaction mode is observed in serotonin\textsubscript{1A} and serotonin\textsubscript{2B} receptors and could represent a common signature in the serotonin receptor family.

An interesting observation from our work is that cholesterol is highly dynamic when interacting at the signature aromatic binding mode. It is possible that this interaction mode represents a site with less favorable interaction energy but is entropically favored due to the high site dynamics. In one of the binding modes identified here (on transmembrane helix IV), cholesterol adopts an orientation parallel to the membrane surface, reminiscent of a site identified in the \( \beta_2 \)-adrenergic receptor (Prasanna et al. 2014). Further analysis is required to determine whether this signature interaction mode is specific to the serotonin receptor family or could be generalized to other GPCRs. In general, this binding mode of cholesterol cannot be mapped to a single sequence motif but represents a spatial pattern of residues. To comprehensively map these cholesterol determinants into a spatial 3D motif, we would require to identify more such sites from the serotonin receptor family and other related GPCRs.
In conclusion, we propose a new signature cholesterol binding mode in which the cholesterol headgroup interacts with an aromatic residue. Increased cholesterol dynamics is observed at this site and could be related to reduced interaction energy but an entropically favorable site. The molecular signatures of cholesterol interaction with receptors represent an important step in our overall understanding of GPCR function in health and disease.

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