BRIEF COMMUNICATION

Cholesterol in GPCR Structures: Prevalence and Relevance
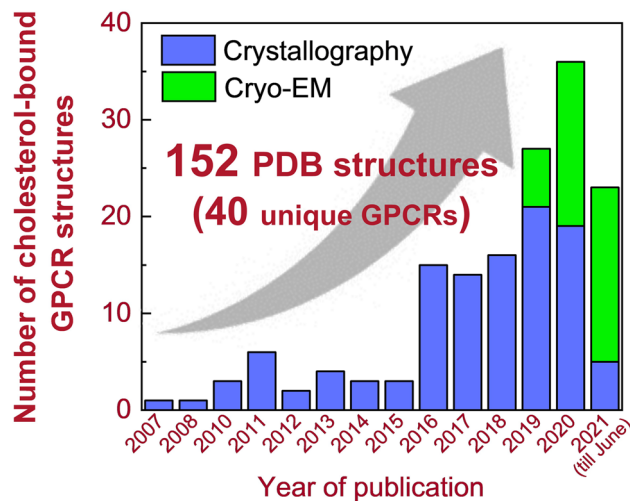
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Abstract
Bound cholesterol molecules are emerging as important hallmarks of GPCR structures. In this commentary, we analyze their statistical prevalence and biological relevance.

Graphic Abstract

Keywords GPCRs · Bound cholesterol · Crystal structure · Cryo-EM · Cholesterol sensitivity

G protein-coupled receptors (GPCRs) are the largest class of membrane proteins, with more than 800 members, that are involved in information transfer from outside the cell to the cellular interior by signal transduction across the cell membrane (Pierce et al. 2002; Zhang et al. 2006; Rosenbaum et al. 2009; Chattopadhyay 2014; Weis and Kobilka 2018). The overall organization of GPCRs consists of seven transmembrane α-helical domains interconnected by alternating intra- and extracellular loops. Signaling by GPCRs initiates as a result of binding to extracellular ligands (such as neurotransmitters, peptides, odorants, hormones, and even photons) that subsequently triggers signal transduction via a set of subtle concerted conformational rearrangements in their transmembrane domains and extramembranous regions (Weis and Kobilka 2018; Filipek 2019; Wingler and Lefkowitz 2020). Interestingly, ligand binding to the cognate GPCR involves structural dynamics (conformational plasticity) that allows different conformations adopted by the receptor (and ligand in some cases) in recognizing and binding the GPCR (Kharche et al. 2021; Torrens-Fontanals et al. 2021). Due to their involvement in activating diverse signaling pathways resulting in a wide range of physiological responses (e.g., neurotransmission, immune response, cellular growth, and differentiation), GPCRs have emerged as major therapeutic targets across all clinical areas (Chan et al. 2019; Insel et al. 2019; Yang et al. 2021). Molecular

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understanding of GPCR activation pathways has emerged from structures of several GPCRs solved using crystallography or cryo-electron microscopy (cryo-EM) in inactive and active states, spectroscopic (fluorescence, ESR and NMR) data, and molecular dynamics simulations (Katritch et al. 2012; Sengupta et al. 2016, 2017; Latorraca et al. 2017; Safdari et al. 2018; Shimada et al. 2019; Grisshammer 2020; Torrens-Fontanals et al. 2020; Wingler and Lefkowitz 2020).

As with many integral membrane proteins, GPCR structure and function are intimately associated with their membrane microenvironment due to the presence of seven membrane spanning domains and dynamic extramembranous regions. There is extensive literature (encompassing biochemical, biophysical, and computational approaches) on the role of membrane lipids in several aspects of GPCR biology. In particular, cholesterol, a major component of eukaryotic cell membranes (Mouritsen and Zuckermann 2004; Kumar and Chattopadhyay 2016), has been shown to be a key determinant of GPCR organization, dynamics, oligomerization, and function (reviewed in Pucadyil and Chattopadhyay 2006; Paila and Chattopadhyay 2010; Oates and Watts 2011; Goddard and Watts 2012; Jafurulla and Chattopadhyay 2013; Chattopadhyay 2014; Sengupta and Chattopadhyay 2015; Gimpl 2016; Sengupta et al. 2018; Jafurulla et al. 2019; Kiriakidi et al. 2019; Jakubík and El-Fakahany 2021). 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 (reviewed in Jafurulla et al. 2019). A recent development is the effect of membrane cholesterol on endocytic route and intracellular trafficking of GPCRs (Kumar and Chattopadhyay 2020, 2021). The specific requirement of cholesterol for the function of GPCRs has gained support from increasing numbers of high-resolution GPCR structures solved by x-ray diffraction and cryo-EM with bound cholesterol molecules (Paila et al. 2009; Jafurulla et al. 2019; Lee 2019; Wang et al. 2019). Over the last decade, crystal and cryo-EM structures of several GPCRs have been resolved with bound cholesterol molecules. Table 1 shows a comprehensive breakdown of GPCR structures reported with bound cholesterol molecules. For example, structures of the β_2-adrenergic receptor (Cherezov et al. 2007; Hanson et al. 2008), A_{2A} adenosine receptor (Liu et al. 2012), and metabotropic glutamate receptor 1 (Wu et al. 2014) displayed bound cholesterol molecules. As we write this Commentary, the number of GPCRs displaying bound cholesterol molecules has gone up significantly (see Table 1). At present, there are 560 structures of GPCRs representing 101 unique receptors deposited in the PDB (https://www.rcsb.org/) (see Fig. 1).

As shown in Fig. 1, ~40% of unique GPCRs (40 out of 101) corresponding to ~27% of PDB structures (152 out of 560) display bound cholesterol molecules. We further observed that cholesterol hemisuccinate (CHS), an amphiphilic analog of cholesterol used to stabilize GPCRs during solubilization, is present in a smaller fraction of these structures (see Fig. 1 and Table 1). As individual GPCRs are characterized with multiple structures (bound to various ligands, depending on their functional state), multiple cholesterol-bound structures have been reported for several GPCRs, with the A_{2A} adenosine receptor displaying maximum number (a total of 39) of cholesterol-bound structures (Table 1). Figure 2 shows the rather diverse distribution of number of bound cholesterol molecule(s) present in each GPCR PDB structure. Overall, the number of bound cholesterol molecule(s) display variation between 1 and 16. Interestingly, the figure reveals that most of the PDB structures (57 out of 152, i.e., ~37%) exhibit only one bound cholesterol molecule. On the other end of the distribution, the heterodimeric $GABA_B$ receptor (PDB ID: 7CUM) and the recently reported *apo*-form of the serotonin$_{1A}$ receptor (PDB ID: 7E2X) display the maximum number of bound cholesterol molecules (16 per receptor dimer and 10 per receptor monomer, respectively) in their structures (see Table 1). While these are interesting observations, the biological relevance of this diversity in bound cholesterol molecules is not apparent at this point. It is envisioned that with more reports of well-resolved GPCR structures with bound cholesterol molecule(s), accompanied with more information on their function, could help generate certain pattern in this large, diverse, and emerging structural database.

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 (Li and Papadopoulos 1998; Epand 2006; Fantini and Barrantes 2013; Fantini et al. 2016; Jafurulla et al. 2019; Sarkar and Chattopadhyay 2020). The CRAC motif consists of a linear sequence of amino acids from the N-terminal to C-terminal direction and follows the order: a branched nonpolar leucine (or valine), followed by 1–5 amino acids (no preference), an aromatic tyrosine residue, another segment of 1–5 amino acids (no preference), and lastly, a basic lysine (or arginine) residue [(L/V)-$(X)_{1-5}$-Y-$(X)_{1-5}$-(R/K)]. We reported the presence of CRAC motifs in several GPCRs (the serotonin$_{1A}$ receptor, the β_2-adrenergic receptor, and rhodopsin) that exhibit cholesterol-sensitive function (Jafurulla et al. 2011). In case of the serotonin$_{1A}$ receptor, our analysis showed the presence of CRAC motifs in TM2, TM5, and TM7. Subsequently, presence of CRAC motif was reported for type-1 cannabinoid (CB_1) receptor (Oddi et al. 2011). Interestingly, coarse-grain molecular dynamics simulations of the serotonin$_{1A}$ receptor showed preferential (dynamic) occupancy of membrane

Table 1 GPCR structures with bound cholesterol

Receptor	PDB ID	#	Ref
A_{2A} adenosine receptor	4EIY	3	Liu et al. (2012) Science 337:232–236
	5IU4, 5IU7, 5IU8, 5IUA	4	Segala et al. (2016) J Med Chem 59:6470–6479
	5IUB	3	
	5JTB	3	Melnikov et al. (2017) Sci Adv 3:e1602952
	5K2A, 5K2B, 5K2C, 5K2D	3	Batyuk et al. (2016) Sci Adv 2:e1600292
	5MZJ, 5N2R	3	Cheng et al. (2017) Structure 25:1275–1285
	5MZP	4	
	5NLX, 5NM2, 5NM4	3	Weinert et al. (2017) Nat Commun 8:542
	5OLG, 5OLV, 5OLZ, 5OM1, 5OM4	4	Rucktooa et al. (2018) Sci Rep 8:41
	5OLH, 5OLO	3	
	5UVI	3	Martin-Garcia et al. (2017) IUCrJ 4:439–454
	5VRA	3	Broecker et al. (2018) Nat Protoc 13:260–292
	6AQF	3	Eddy et al. (2018) Cell 172:68–80
	6GT3	3	Borodovsky et al. (2020) J Immunother Cancer 8:e000417
	6JZH	3	Shimazu et al. (2019) J Appl Cryst 52:1280–1288
	6LPK, 6LPJ, 6LPL	3	Ihara et al. (2020) Sci Rep 10:19305
	6PS7	3	Ishchenko et al. (2019) IUCrJ 6:1106–1119
	6S0Q, 6S0L	3	Nass et al. (2020) IUCrJ 7:965–975
	6WQA	3	Lee et al. (2020) IUCrJ 7:976–984
	6ZDR, 6ZDV	3	Jespers et al. (2020) Angew Chem Int Ed Engl 59:16536–16543
	7ARO	1	Amelia et al. (2021) J Med Chem 64: 3827–3842
α_{2C} adrenergic receptor	6KUW	2*	Chen et al. (2019) Cell Rep 29:2936–2943
Adhesion receptor GPR7	7D76, 7D77	2 CL 1 CHS	Ping et al. (2021) Nature 589:620–626
β_1 adrenergic receptor	2Y00, 2Y01, 2Y02, 2Y04	4 CHS*	Warne et al. (2011) Nature 469:241–244
	2Y03	2 CHS*	
	3ZPQ, 3ZPR	4 CHS*	Christopher et al. (2013) J Med Chem 56:3446–3455
	7BVQ, 7BTS, 7BU6, 7BU7	1	Xu et al. (2021) Cell Res 31:569–579
β_2 adrenergic receptor	2RH1	3	Cherezov et al. (2007) Science 318:1258–1265
	3D4S	2	Hanson et al. (2008) Structure 16:897–905
	3NY8, 3NY9, 3NYA	2	Wacker et al. (2010) J Am Chem Soc 132:11443–11445
	3PDS	1	Rosenbaum et al. (2011) Nature 469:236–240
	5D5A, 5D5B	3	Huang et al. (2016) Acta Crystallogr D Struct Biol 72:93–112
	5D6L	3	Ma et al. (2017) Nat. Protoc 12:1745–1762
	5JQH	2*	Staus et al. (2016) Nature 535:448–452
	5X7D	2	Liu et al. (2017) Nature 548:480–484
	6OBA	2	Liu et al. (2020) Nat Chem Biol 16:749–755
	6PRZ, 6PS0, 6PS1, 6PS2, 6PS3, 6PS4, 6PS5, 6PS6	1	Ishchenko et al. (2019) IUCrJ 6:1106–1119
Angiotensin II type 1 receptor	6OS1, 6OS2	1	Wingler et al. (2020) Science 367:888–892
CC chemokine receptor type 9 (CCR9)	5LWE	1	Oswald et al. (2016) Nature 540:462–465
M_1 muscarinic acetylcholine receptor	5CXV	1 CHS	Thal et al. (2016) Nature 531:335–340
	6OIJ	2 CHS	Maeda et al. (2019) Science 364:552–557
	6WJC	4 CHS	Maeda et al. (2020) Science 369:161–167

Table 1 (continued)

| Receptor	PDB ID	#	Ref
CB$_1$ cannabinoid receptor	5XR8, 5XRA	1	Hua et al. (2017) Nature 547:468–471
	6N4B	2	Kumar et al. (2019) Cell 176:448–458
CB$_2$ cannabinoid receptor	6PT0	4	Xing et al. (2020) Cell 180:645–654
Cholecystokinin 1 receptor	7MBX, 7MBY	1 CHS	Mobbs et al. (2021) PLoS Biol 19:e3001295
Corticotropin-releasing factor 1 receptor	6PB0	5	Ma et al. (2020) Mol Cell 77:669–680
Corticotropin-releasing factor 2 receptor	6PB1	4	Ma et al. (2020) Mol Cell 77:669–680
CXC chemokine receptor 2	6LFM, 6LFO	1	Liu et al. (2020) Nature 585:135–140
Human cytomegalovirus GPCR US28	4XT1	2	Burg et al. (2015) Science 347:1113–1117
	5WB2	2	Miles et al. (2018) eLife 7:e35850
Cysteinyl leukotriene receptor 2	6RZ6, 6RZ7, 6RZ9	1	Gusach et al. (2019) Nat Commun 10:5573
Dopamine receptor 1	7LJC	6	Zhuang et al. (2021) Cell Res 31:593–596
	7LJD	5	
	7CKW, 7CKX, 7CKY, 7CKZ	1	Xiao et al. (2021) Cell 184:943–956
	7JV5, 7JVP	6	Zhuang et al. (2021) Cell 184:931–942
	7JVQ	5	
Endothelin receptor type-B	5X93	1	Shihoya et al. (2017) Nat Struct Mol Biol 24:758–764
Formyl peptide receptor 2	6LW5	2	Chen et al. (2020) Nat Commun 11:1208
	6OMM	5	Zhuang et al. (2020) Nat Commun 11:885
GABA$_B$ receptor	6WIV	10*	Park et al. (2020) Nature 584:304–309
	7CUM	16*	Kim et al. (2020) J Mol Biol 432:5966–5984
	7CA3	3*	
G-protein-coupled bile acid receptor	7CFM	3	Yang et al. (2020) Nature 587:499–504
	7CFN	1	
Growth hormone-releasing hormone receptor	7CZ5	1	Zhou et al. (2020) Nat Commun 11:5205
Metabotropic glutamate receptor 1	4OR2	6*	Wu et al. (2014) Science 344:58–64
Orexin 1 receptor	6V9S	1	Hellmann et al. (2020) Proc Natl Acad Sci USA 117:18059–18067
Serotonin$_{1A}$ receptor	7E2X	10	Xu et al. (2021) Nature 592:469–473
	7E2Y	4	
	7E2Z	3	
Serotonin$_{1D}$ receptor	7E32	1	Xu et al. (2021) Nature 592:469–473
Serotonin$_{2A}$ receptor	6A93, 6A94	2*	Kimura et al. (2019) Nat Struct Mol Biol 26:121–128
	6WGT	2	Kim et al. (2020) Cell 182:1574–1588
	6WH4	2*	
Serotonin$_{2B}$ receptor	4IB4	1	Wacker et al. (2013) Science 340:615–619
	4NC3	1	Liu et al. (2013) Science 342:1521–1524
	5TVN	1	Wacker et al. (2017) Cell 168:377–389
	6DRX, 6DRY, 6DRZ, 6DS0	1	McCorvy et al. (2018) Nat Struct Mol Biol 25:787–796
Ste2 receptor	7AD3	6 CHS*	Velazhahan et al. (2021) Nature 589:148–153
Succinate receptor 1	6IBB	1	Haffke et al. (2019) Nature 574:581–585
δ opioid receptor	6PT2	1	Claff et al. (2019) Sci Adv 5:eaax9115
κ opioid receptor	6B73	2*	Che et al. (2018) Cell 172:55–67
	6VI4	1	Che et al. (2020) Nat Commun 11:1145
μ opioid receptor	4DKL	1	Manglik et al. (2012) Nature 485:321–326
	5C1M	1	Huang et al. (2015) Nature 524:315–321
Oxytocin receptor	6TPK	1	Waltenspühl et al. (2020) Sci Adv 6:eabb5419

Table 1 (continued)

Receptor	PDB ID	#	Ref
P2Y1 receptor	4XNV	1 CL	Zhang et al. (2015) Nature 520:317–321
		3 CHS	
P2Y12 receptor	4NTJ	2	Zhang et al. (2014) Nature 509:115–118
	4PXZ	1	Zhang et al. (2014) Nature 509:119–122
Parathyroid hormone receptor type	6NBF	6	Zhao et al. (2019) Science 364:148–153
	6NBH	7	
	6NBI	3	
Smoothened	5L7D	1	Byrne et al. (2016) Nature 535:517–522
	6OT0	1 EPL	Qi et al. (2019) Nature 571:279–283
	6XBJ, 6XBK, 6XBL	1	Qi et al. (2020) Nat Chem Biol 16:1368–1375
	6XBM	2 EPL	
	6D35	1	Huang et al. (2018) Cell 174:312–324
	6O3C	2	Deshpande et al. (2019) Nature 571:284–288
Thromboxane A2 receptor	6IIU, 6IIV	1	Fan et al. (2019) Nat Chem Biol 15:27–33
VIP1 receptor	6VN7	6	Duan et al. (2020) Nat Commun 11:4121

The list was generated by searching the PDB database (https://www.rcsb.org/) for GPCR structures with cholesterol (and CHS) as a small molecule ligand

#number of cholesterol (or CHS) molecules per PDB structure (unless specified); *per dimer; *CHS* cholesterol hemisuccinate; *CL* cholesterol; *EPL* 24(S),25-epoxycholesterol

Fig. 1 Statistical analysis of cholesterol-bound GPCR structures. Breakdown of the current set of 560 PDB entries from 101 unique GPCRs for presence or absence of cholesterol (and cholesterol hemisuccinate (CHS)) by **a** number of unique GPCRs and **b** total number of PDB entries of GPCRs. The number of GPCRs and PDB entries are further classified based on the presence of only cholesterol, only CHS, or both cholesterol and CHS molecules. The current list of GPCR structures solved by x-ray crystallography and cryo-EM were obtained from GPCRdb (https://gpcrdb.org/structure/) and protein data bank (PDB) (https://www.rcsb.org/). See text for more details
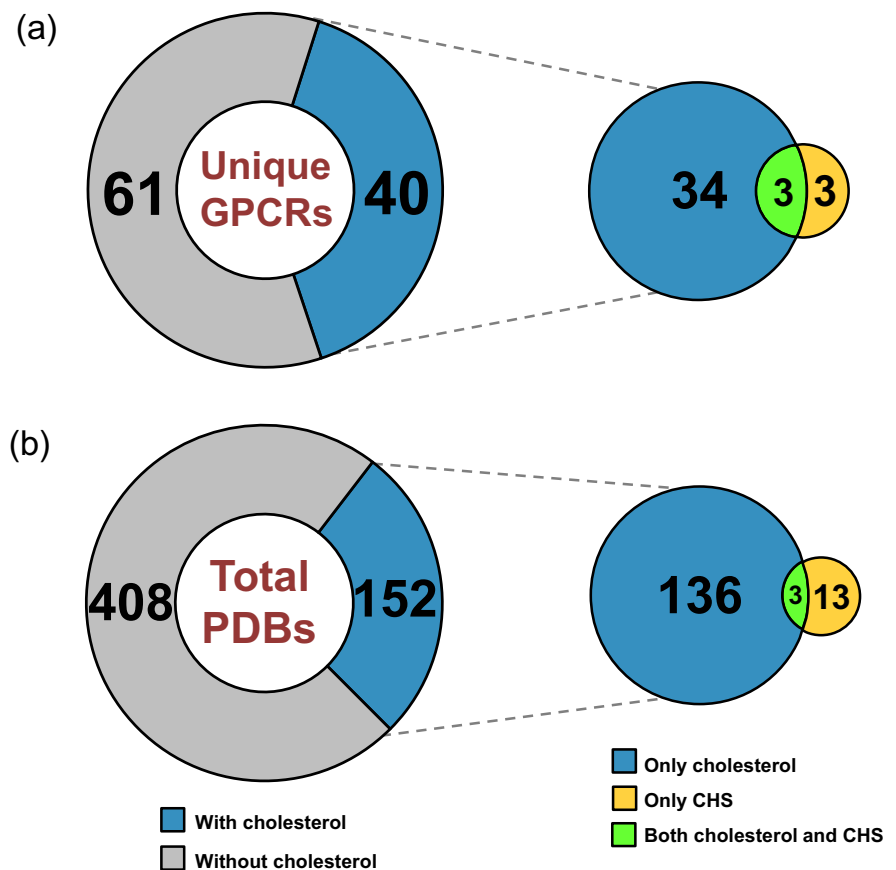
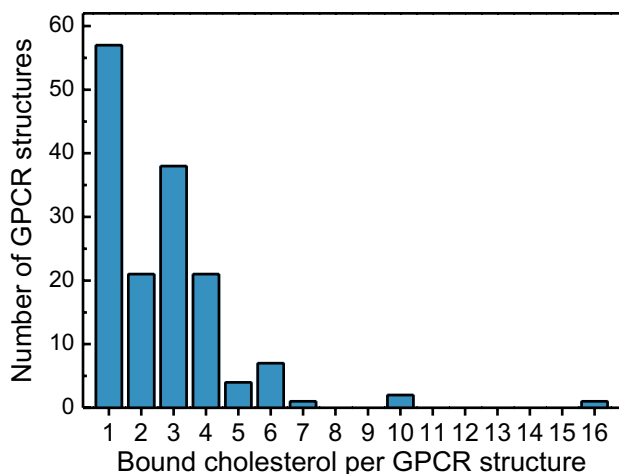**Fig. 2** Variance in number of cholesterol molecules per GPCR structure. Data were generated by searching the protein data bank (PDB) (https://www.rcsb.org/) for GPCR structures with cholesterol or CHS as a small molecule ligand. The current list of GPCR structures solved by x-ray crystallography and cryo-EM were obtained from GPCRdb (https://gpcrdb.org/structure/) and protein data bank (PDB) (https://www.rcsb.org/). See text for more details

cholesterol in some of the CRAC sites in the serotonin$_{1A}$ receptor (Sengupta and Chattopadhyay 2012). Importantly, in a recent work from our laboratory (Kumar et al. 2021), we showed using all-atom molecular dynamics simulations that a cholesterol molecule is found near a CRAC motif in a position almost identical to the one reported in the cryo-EM structure of the serotonin$_{1A}$ receptor (Xu et al. 2021; PDB ID: 7E2X). To provide mechanistic insights into cholesterol sensitivity for the serotonin$_{1A}$ receptor, we further examined the molecular basis of cholesterol sensitivity of the receptor function by mutating various key residues in the CRAC motifs of the receptor and monitoring corresponding functional readout (cAMP signaling) (Kumar et al. 2021). Our results showed that the functional sensitivity of the serotonin$_{1A}$ receptor to membrane cholesterol is lost when the residue K101 in a CRAC motif in TM2 is mutated, indicating the role of K101 as a molecular sensor of membrane cholesterol. To the best of our knowledge, our results constitute one of the first reports that comprehensively demonstrate that cholesterol sensitivity could be knocked out by a single point mutation in a specific cholesterol-binding site. In general, the presence of CRAC motifs in a transmembrane region of GPCRs suggests the possibility of cholesterol interaction with the receptor. However, mere presence of CRAC motifs may not necessarily translate to association of cholesterol around these motifs (Sarkar and Chattopadhyay 2020). For example, a recent analysis using cryo-EM and x-ray structures showed that majority of bound cholesterol molecules on GPCR surfaces reside in locations that lack cholesterol-binding motifs (Taghon et al. 2021).

A logical question that comes to mind is what is the relevance of bound cholesterol molecules in GPCR structures and how they help us in understanding cholesterol-sensitive function of GPCRs (Chattopadhyay 2014). It turns out that an answer to this question is not straightforward and may require more data on structure and function of cholesterol-sensitive GPCRs. We still do not know what fraction of GPCRs exhibit cholesterol-sensitive function since the total number of GPCRs showing cholesterol sensitivity is still low (~ 7% by latest estimate) relative to total number of GPCRs present in our body. Another complexity arises due to a variety of factors associated with GPCR solubilization and crystallization in lipidic cubic phases and the relatively heavy protein engineering carried out on GPCRs for crystallization (Ghosh et al. 2015). This protein engineering also includes mutations for thermal stability. There is also some concern due to the fact that the flexible extramembranous regions (loops) are often stabilized using monoclonal antibody (Day et al. 2007), or replaced with lysozyme (Cherezov et al. 2007; Rosenbaum et al. 2007), or a nanobody (Manglik et al. 2017) during structure determination. This is in spite of the fact that the flexible loops have been shown to be important for GPCR function (Wheatley et al. 2012; Pal and Chattopadhyay 2019; Kharche et al. 2021).

Structural biology of GPCRs often utilizes detergent micelles or lipidic cubic phases for structure determination. GPCR conformation in micelles could differ from the conformation in membranes due to difference in the radius of curvature and thickness of interface of micelles and membrane bilayers (Mukherjee and Chattopadhyay 1994). Lipidic cubic phases have proved to be handy for GPCR crystallization (Caffrey 2015). Yet, the physiological significance of bound cholesterol molecules in GPCR crystal structures in lipidic cubic phases is a matter of discussion (Khelashvili et al. 2012). It could be conceived that bound cholesterol molecules in GPCRs could have their origin in packing in the lipidic cubic phase. In addition, as mentioned in Fig. 1 and Table 1, CHS is sometimes used to mimic/replace natural cholesterol in GPCRs, although the ability of CHS to mimic cholesterol has been debated (Kulig et al. 2014, 2015; Augustyn et al. 2019). We envision that additional experimental data and molecular dynamics simulations would help in providing further clarity in relating bound cholesterol in GPCR structures to their cholesterol-sensitive function although some insight has started emerging from cholesterol-bound structures of GPCRs, e.g., the serotonin$_{1A}$ receptor (Xu et al. 2021) with corresponding cholesterol-sensitive function (Kumar et al. 2021). In addition, recent advancement in the resolution revolution by cryo-EM along with is its ability to capture structural details of molecular assemblies (GPCRs complexed with their transducer such as G-proteins) in physiologically relevant environments, could lead to a better understanding of bound cholesterol

molecules in GPCR structures. We envision that the future appears exciting with further analysis of emerging data on structures of cholesterol-sensitive GPCRs and their function.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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