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Interactions of cholesterol molecules with GPCRs in different states: a comparative analysis of GPCRs' structures

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Highlights

- The number and position of cholesterol molecules in structures of 68 GPCRs in different states are gathered in an interactive table
- A comparative analysis of CLR number and position is performed between different states of GPCR
- Cholesterol molecules bind differently when some GPCRs adopt a ligand-bound state
- The type of ligands and intracellular binders affect the interaction of CLR in several GPCR structures

Abstract

GPCRs form a very large family of transmembrane proteins involved in a wide range of physiological functions. Some GPCRs have their activity affected by the presence of cholesterol either indirectly through changes in the physical properties of the membrane or directly by binding to the receptor. Thanks to recent advances in structural biology, many structures of GPCRs have been solved, 240 of which contain at least one cholesterol molecule or surrogate. This review lists the position of these molecules and provides a comparative analysis of the changes observed in the structures depending on the state of the receptor.

Keywords

GPCRs, structures, cholesterol, interaction, protein/lipid, database

Introduction

In the Fluid Mosaic Model initially described by Singer S.J. and Nicolson G.L. in 1972, transmembrane proteins totally span the lipid bilayer of membranes, which are dynamic and behave "as a two dimensional oriented viscous solution" [1]. This model has been improved with additional heterogeneity discovered in biological membranes [2], which includes the presence of specific lipids such as cholesterol. Cholesterol is not only one of the most abundant lipids in the plasma membrane of animal eukaryotic cells [3], but also a modulator of the physicochemical properties of the lipid bilayer (thickness, fluidity/rigidity, curvature, lateral pressure) [4] and of the activity of various membrane proteins [5] [6] [7] [8]. In the human genome, the largest family of transmembrane proteins is the G protein-coupled receptor (GPCR) family with more than 800 members. For some of them, cholesterol affects their activity by modes of action that can be very different and that rely on dynamics

of receptors and sterol. These modes of action can be: 1) co-localisation of receptors in cholesterol-rich caveolae or raft subsequent to binding of their agonists [9] [10] ; 2) the enrichment of GPCRs in high-affinity states for their ligands in cholesterol-rich domains (example of the oxytocin (OT) receptor [11]); and 3) the preservation of oligomeric states affecting function (example of the P2Y₁₂ receptor [12]).

The molecular mechanisms governing the action of cholesterol on GPCRs [13] are either indirect and related to changes in the physicochemical properties of the lipid bilayer (example of the cholecystokinin receptor CCK₂ that is sensitive to the membrane fluidity [14]); or direct by binding to the receptor as an allosteric ligand (example of OT [15] and 5-TH_{1a} [16]) or as an orthosteric ligand (example of the A_{2A} receptor [17] and the Smoothed (Smo) receptor [18]). The binding of cholesterol molecules to GPCRs is observed not only in computational simulations ([19] [20], but also in structures of some GPCRs. The first structure of a GPCR with cholesterol molecules (CLR) was published in 2007 and involved the β 2-adrenergic receptor (β 2) [21]. The functional role of these CLR molecules is not clearly established but they are at least involved in stabilising the interface between two β 2 receptors in the crystal lattice. Since then, nearly 560 other GPCR structures [22] have been obtained thanks to several major technological advances (chimeric approaches with exogenous domains, thermostabilising mutations, crystallisation in lipid cubic phase, high-resolution cryo-electron microscopy) and they have confirmed the presence of CLR or cholesteryl hemisuccinate (CHS) in 240 structures (Supplementary Table S1). CHS is a more hydrophilic analogue of CLR and is used as a surrogate in preparative biochemical steps [24],[23]. As it is difficult to differentiate between CLR and CHS in the electron density maps, these molecules are referred to as CLR in this article. Among the 240 GPCR structures with CLR, 68 were obtained from different GPCRs, mainly of human origin, which implies a multiplicity of structures for many GPCRs. Thus, several structures of the same GPCR were determined under various ligand conditions and methods, providing structural information to explore the molecular mechanisms of CLR on GPCRs. Interpretation of the role of CLR from structural data remains challenging due to possible artefacts related to the experimental conditions. Thus, CLR molecules can stabilise an interface between receptors in a crystal structure without having any physiological meaning. For example, some structures show GPCR dimers containing CLR molecules at the dimeric interfaces but with an opposite extracellular/intracellular orientation of the protomers that is not physiologically possible (e.g. PDB ID: 7RM5 and 7F83). However, the multiplication of structures obtained under various conditions and the increasing publication of cryo-electron microscopy structures from isolated particles [22] reinforce the hypothesis of direct interactions of CLR molecules with GPCRs, which is still not experimentally confirmed for many of them. These data also clearly demonstrate that the number and position of CLR molecules do not appear to be conserved across receptors despite the presence of CLR binding consensus sites (CRAC and CCM) in many GPCRs [25]. On the contrary, the reproducibility of CLR positions observed in the structures of some GPCRs suggests the presence of specific interaction sites that are also observed as "hot spots" in different molecular dynamics (MD) simulations [19] . Equilibrium MD simulations have been also developed to determine relative affinities between cholesterol and membrane proteins including GPCRs [26] [20].

One well established role of the CLR is the modulation of affinities of GPCRs for their ligands. For example, the OT receptor has a ~10-fold lower affinity for oxytocin in cholesterol-depleted conditions [27]. More recently, reciprocity has also been observed [28] in the stabilisation of CLR molecules when the OT receptor is in a ligand-bound state. The multiplicity of GPCR structures with CLR in the presence and absence of ligands offers the possibility to structurally explore this mechanism in other GPCRs.

The purpose of this mini-review is to perform a comparative structural study of the position of CLR molecules in GPCR structures in different states. It is important to be very cautious about a functional

interpretation of these data, which are intended to initiate or support functional studies [29]. This mini-review uses the data listed on 28 September 2022 in the databases <https://www.rcsb.org> and <https://blanco.biomol.uci.edu/mpstruc/> and covers 459 GPCR structures of 68 distinct GPCRs in class A (345 PDBs, 49 distinct GPCRs), B1 (67 PDBs, 12 distinct GPCRs), B2 (5 PDBs, 2 distinct GPCRs), C (37 PDBs, 4 distinct GPCRs) and D (5 PDBs, 1 GPCR). The table does not include all GPCR structures, but only those of GPCRs with at least one structure containing one or more CLRs. The excel table accompanying this review contains the following columns: 1) class; 2) receptor name; 3) species; 4) PDB code; 5) year of publication; 6) methods for structure resolution; 7) ligands present in the structure (or apo form); 8) intracellular binders; 9) total number of CLRs; 10) position of CLR, CHS or allosteric ligands; 11) oligomerisation status; 12) position of the dimeric interface (transmembrane (TM) or interfacial (H8) helix number); 13) publication reference. Comments are associated with some of the cells to provide further details. To readjust the position of these comments, if necessary, codes of macro are available through this link: <https://contexturesblog.com/archives/2011/02/16/fix-those-wandering-excel-comments/>. The data in the table can be sorted and filtered with the column headings. The position of CLR, CHS or allosteric ligands has been determined manually and simplified in order to facilitate the comparison of positions between different structures. To read the column of the position of CLR, CHS, PAM or NAM in TMDs, the nomenclature is:

- x/y means "x" CLR or CHS molecules in the inner leaflet side of TMDs on "y" molecules in total in the PDB structure.
- "inner" and "outer" indicate the position related to the membrane leaflet side, "inner" being on the cytoplasmic side and "outer" to the extracellular side.
- "TMx" or "TMx-y" means TransMembrane helix number "x" that interacts predominantly with one CLR, or at the interface between helices "x" and "y". Each position starts with "TM..." except for the last amphipathic helix (H8).
- The position of PAM or NAM is indicated in italics.
- Symmetrical positions of molecules in dimeric structures are noted with the superscript ² (e.g. TM5²).
- Positions participating in the dimeric interface are noted with * (e.g. TM5*).

The comparative analysis of these structures is carried out below in a hierarchical manner by receptor class, name and then state (with ligands and/or binders). The questions raised in the analysis of these data are:

- 1) Does ligand binding induce a change in CLR binding in GPCR structures?
- 2) Are there specific CLR positions depending on the nature of the ligand?
- 3) Do intracellular binders (G proteins, nanobodies, ...) have an impact on CLR binding?
- 4) Is there reproducibility in CLR position across different structures of a GPCR suggesting CLR sensitivity?
- 5) Are there mutations affecting functional roles of CLR?

1 Does ligand binding induce a change in CLR binding in GPCR structures?

To answer this question, the structures of GPCRs are compared between the apo (ligand-free) and the ligand-bound forms.

The GPCR family is divided into subclasses with different characteristics that may differentially affect the role of CLR. Class A is the largest class of GPCRs, which are characterised by ligand binding sites in the transmembrane core and an overwhelming majority of structure in monomeric form. Class B is

characterised by a large N-terminal extracellular domain that forms the orthosteric ligand binding site with the transmembrane domain. Class C is characterised by an obligatory dimeric form as well as large N-terminal extracellular domains that are the binding sites of orthosteric ligands. Class D is found only in fungi, which also have sterols in their membranes. Class F is characterised by an extracellular binding domain and a binding site in the transmembrane domain. The functional role of CLR by direct binding is clearly defined in this family, which is not considered in this comparative analysis due to the atypical position of their CLR binding sites in the extracellular domain.

In the table, to date, only 9 out of 49 class A GPCRs have structures in apo form: 5-HT_{1A}, β 1, β 2, BILF1, CCR1, CX₃CR1, ET_B, SUCNR1 and US28. Apart from BILF1, the other 8 GPCRs have structures in a ligand-bound state, allowing a comparative analysis between apo and ligand-bound forms. In class B, 1 out of 14 GPCRs has a structure in apo form: GLP-1, while 3 out of 4 class C GPCRs have 1 or 5 structures in apo form: GABA_B, mGluR₁ and GPR158. In class D, the only representative also has a structure in apo form: Ste2.

Three profiles emerge from the comparison of apo and ligand-bound structures: 1) a significant difference in the number and/or position of CLRs in the presence of ligand suggesting different interactions of CLR molecules correlated with conformational changes of the receptor induced by ligand binding; 2) no difference between apo and ligand-bound forms, suggesting no effect of ligands on CLR interaction; and 3) structural data that do not allow for any conclusion.

1.1 Cases of significant differences in the number and/or position of CLRs.

Table 1 shows an example of comparison of structures of a GPCR in the apo and ligand-bound forms.

Table 1: Example of comparison of CLR positions between the apo and ligand-bound forms of the β 2-adrenergic receptor

Name	PDB code	Ligands	Nbr of CLR	Position of CLR/CHS/PAM/NAM in TMDs	DOI
$\beta 2$	3KJ6	apo	0		10.1038/nature08650
	3PDS	agonist	1	1/1 inner (TM2-4)	10.1038/nature09665
	6E67		0		10.1016/j.cell.2019.04.021
	3P0G		0		10.1038/nature09648
	3SN6		0		10.1038/nature10361
	7BZ2		0		10.1038/s41421-020-0176-9
	6KR8		0		10.1038/s41589-019-0457-5
	6NI3		0		10.1038/s41594-019-0330-y
	6N48	agonist, PAM	0	PAM inner (TM3-4)	10.1126/science.aaw8981
	3NYA	antagonist	2	2/2 inner (TM2, TM2-4)	10.1021/ja105108q
	6PRZ		1	1/1 inner (TM2-4)	10.1107/S2052252519013137
	6PS2		1	1/1 inner (TM2-4)	10.1107/S2052252519013137
	5X7D	antagonist, NAM	2	2/2 inner (TM1-2, TM2-4)	10.1038/nature23652
	6OBA		2	2/2 inner (TM1-2, TM2-4). NAM inner (TM3-5)	10.1038/s41589-020-0549-2
	6PS3	Biased ligand	1	1/1 inner (TM2-4)	10.1107/S2052252519013137
	5D6L	inverse agonist	3	3/3 inner (TM1-H8, TM1-2, TM2-4)	10.1038/nprot.2017.057
	5D5A		3	3/3 inner (TM1-H8, TM1-2, TM2-4)	10.1107/S2059798315021683
	5D5B		3	3/3 inner (TM1-H8, TM1-2, TM2-4)	10.1107/S2059798315021683
	2RH1		3	3/3 inner (TM1, TM2, TM2-4)	10.1126/science.1150577
	3D45		2	2/2 inner (TM2, TM2-4)	10.1016/j.str.2008.05.001
	3NY8		2	2/2 inner (TM2, TM2-4)	10.1021/ja105108q
	3NY9		2	2/2 inner (TM2, TM2-4)	10.1021/ja105108q
	5JQH		1	1/1 inner (TM1-2)	10.1038/nature18636
	6PS0		1	1/1 inner (TM2-4)	10.1107/S2052252519013137
	6PS1		1	1/1 inner (TM2-4)	10.1107/S2052252519013137
	6PS4		1	1/1 inner (TM2-4)	10.1107/S2052252519013137
	6PS5		1	1/1 inner (TM2-4)	10.1107/S2052252519013137
	6PS6		1	1/1 inner (TM2-4)	10.1107/S2052252519013137
	2RAR		0		10.1038/nature06325
	2RAS		0		10.1038/nature06325
	4GBR	0		10.1371/journal.pone.0046039	

5-HT_{1A}: the structure in apo form shows a large number (10) of CLRs surrounding the receptor on both sides of the lipid bilayer and this number decreases significantly to 3 or 4 in the two structures with agonist + Gi proteins that were obtained in the same study. Although these observations refer to only one study, they suggest a dynamic of the interactions between CLR and the 5-HT_{1A} receptor depending on its conformational state. This observation is in agreement with the known sensitivity of the 5-HT_{1A} receptor to CLR [30] [16].

$\beta 2$ (Table 1): 32 structures of the $\beta 2$ -adrenergic receptor have been published in 21 studies. One structure is in apo form (with an intracellular Fab not in the G protein binding site) that is free of CLR molecules. In other structures, 1 to 3 CLR molecules are observed mainly in non-activated structures in the presence of inverse agonists (13/15 structures with CLR) or antagonists +/- NAM (5/5 with CLR).

CX₃CR1: in the 2 structures of the CX₃CR1 receptor that were published in the same study and under the same conditions, the agonist-bound form (+ Gi proteins) lost 1 CLR at the inner TM2-4 position compared to the apo form (+ Gi proteins). Reinforcing the hypothesis of a functional role for the missing CLR, the authors show that the W154A mutation of the inner TM2-4 site abolishes the coupling of the receptor with Gi proteins [31].

SUCNR1: 3 structures of the receptor were determined in 2 different studies. One structure in apo form (+ Nb6) has 1 CLR in the inner position TM3-5. This CLR is no longer present in the structures with antagonist (+ Nb6) in the same study and another one.

US28: 6 structures are from 3 different studies. The structure of the apo form (+Nb7, PDB ID: 5WB1) shows no CLR while 2 structures (PDB ID: 4XT1 and 5WB2) in the activated state have 2 CLRs in

the same positions (outer TM6 and TM7). Interestingly, 3 other structures with the same agonist (CX₃CL1) but in the presence or absence of G_i proteins and in different states (intermediate: PDB ID 7RKM and orthocanonical: PDB ID 7RKN) have either 1 CLR in another position (PDB ID: 7RKM: inner TM2-4), or no CLR at all (PDB ID: 4XT3 and 7RKN). These observations suggest important dynamics of CLR depending on the state of the receptor.

GLP-1: This receptor is the only member of class B with a structure in apo form (+ G_s proteins). The majority of structures (33/37) in the presence of different ligands (agonists, negative allosteric modulator (NAM) or biased ligand) do not have CLR. However, interestingly, the 4 structures containing Compound 2 (positive allosteric modulator (PAM)) have between 5 to 7 CLR, 2 of which interact with the PAM at the inner TM5-6 position. This position is also occupied by various NAMs but the NAM bound state does not induce CLR binding in the structures. This observation suggests not only an important role of this position for the allosteric regulation of GLP-1 but also a different impact on the interaction of the receptor with CLR depending on the nature of the allosteric regulator.

GABA_B: This receptor belongs to class C, whose members are in the obligatory dimeric form. No CLR were observed in the apo form obtained in 2 structures from 2 different studies (PDB ID: 7CUM and 7C7S). A third structure in the apo form (PDB ID: 6WIV) contained 10 CLR, but the receptor adopted a conformation in inactive state. Antagonist binding also induced the presence of a significant number of CLR (9 and 16) in 2 structures from 2 different studies. Three CLR are also bound to the receptors when a PAM molecule is bound (PDB ID: 7CA3). In these 4 structures with CLR, several CLR are positioned at the interface between the 2 transmembrane domains (noted *) suggesting a role for CLR in the interaction between these two domains. The PAM molecule is also located at the interface of the transmembrane domains in a position similar to some CLR (inner TM5-6) but with a different dimeric interface (TM6 with PAM vs. TM5 with antagonist), which is consistent with the movement of the transmembrane domains during the transition to the active state. Note that the distribution of CLR is not symmetrical in 3 out of 4 structures in agreement with the functional differences of the 2 protomers GABA_{B1} and GABA_{B2} [32].

mGluR₁: The metabotropic glutamate receptor mGluR₁ is another member of the class C forming a homodimeric complex. An apo structure (PDB ID: 7DGD) has distant transmembrane domains, characteristic of a non-activated state, and has no CLR. In contrast, 6 CLR are present in a TMD domain that binds a molecule of NAM. These 6 CLR are symmetrically distributed at the interface between the 2 TMDs. Although another structure in the presence of agonist and PAM is available (PDB ID: 7DGE), the TMDs appear separated and does not show any CLR molecule. Further structures are needed to confirm the position and potential role of CLR, particularly at the dimeric interface.

Ste2: This class D receptor is the only member of this class with known structures. The organism that expresses it physiologically (*Saccharomyces cerevisiae*) does not have CLR in its membrane but an analogue, ergosterol [33] that has a functional role for Ste2 [34]. Consequently, it is not surprising to find sterol molecules (CLR or CHS) in Ste2 structures, however their number (up to 34) is among the largest in GPCR structures that are known so far. These CLR or CHS molecules are symmetrically distributed between the protomers in the 5 structures of the Ste2 homodimeric complex. The single apo structure (PDB ID: 7QB9) has 22 sterol molecules mainly present in the outer leaflet and at the interface. In the presence of agonist, this number drops to 12, mainly on the inner side, in 2 structures and then to 6 in the presence of G proteins (MiniGpa1-Ste4-Ste18). Interestingly, antagonist has an opposite effect by increasing the

number of CLR from 22 (apo form) to 34, mainly in the outer region and still without modification of the dimeric interface. These observations suggest not only a role of sterol molecules in the formation of the dimeric complex by interacting at the dimeric interface, but also an important dynamics of sterol molecules around the Ste2 complex as a function of the receptor state.

The above analysis of the structures of 9 GPCR in different conformational states reveals that the number and the positions of CLR could be physiologically impacted by the binding of ligands with potential functional roles. Thus, in addition to the known effect of cholesterol on affinities for ligands, binding of ligands could induce a reorganisation of bound CLR molecules in some GPCRs. Interestingly, several scenarios appear possible such as: 1) an increase of bound CLR molecules in the active state (e.g. US28); or 2) in the inactive state (e.g. β 2, Ste2); or 3) a decrease of bound CLR in the active state (e.g. 5-HT_{1A}, CX₃CR1, Ste2), or 4) in the inactive state (SUCNR1); or 5) a role in the dimeric state (e.g. GABA_B, mGluR₁, Ste2). Consequently, it would be interesting to assess the functional impact of ligands on the CLR-sensitivity of the above mentioned receptors.

1.2 Case of no difference between apo and ligand-bound forms, suggesting no effect of ligands on CLR binding

CCR1: In the 3 structures of this chemokine receptor from the same study, no change in the number of CLR (1) or in the position (inner TM2-4) is observed between the structure in apo form (+ Gi proteins) and the 2 structures in agonist-bound form (+ Gi proteins). These observations suggest that the number and position of the CLR does not change according to the conformational state of CCR1.

The lack of apparent dynamic of CLR-binding related to the ligand bound state of the receptor does not exclude CLR-sensitivity of the receptor for its ligand binding or the activation of downstream effectors.

1.3 Cases of non-conclusive structural data

β 1: 28 structures of the β 1-adrenergic receptor were determined including 1 in the apo form in the dimeric state. A wide variety of conditions were tested (agonists, antagonists, ligand biased, with or without Gi, Gs, β -arrestin proteins, nanobodies) and the diversity of the number of CLR (0 to 2) in inner or outer positions for similar conditions do not allow a clear interpretation of an effect of ligand binding on the number and position of CLR molecules.

GPR158: this orphan receptor has by definition no known ligand, which does not allow a comparative analysis with the 5 structures obtained in the apo form. However, 8 to 22 molecules are present in 3 structures suggesting a role of CLR for this receptor.

ET_B: 6 structures of this endothelin receptor have been determined under various conditions (apo or agonist-bound, antagonist or inverse agonist). However, only 1 out of 2 antagonist structures has 1 CLR in the TM1 outer position. Further data are needed to confirm CLR binding in the structure of this receptor and to determine a role for ligand-induced conformational changes.

2 Are there specific CLR positions depending on the nature of the ligand?

To answer this question while avoiding biases related to differences in experimental conditions, structures are compared for the same receptor in the same study with the same method but with different types of ligand (agonists, antagonists, ...).

Table 2: Example of comparison of the position of CLRs in the A_{2A} receptor structures according to the type of bound-ligands

Name	PDB code	Ligands	Nbr of CLR	Position of CLR/CHS/PAM/NAM in TMDs	DOI
A _{2A}	7ARO	agonist	1	0/1 inner. Outer (TM5-6)	10.1021/acs.jmedchem.0c01856
	5WF5		0		10.1016/j.str.2017.12.013
	5WF6		0		10.1016/j.str.2017.12.013
	2YDO		0		10.1038/nature10136
	2YDV		0		10.1038/nature10136
	5G53		0		10.1038/nature18966
	7EZC		0		10.1107/S2052252522001907
	4UHR		0		10.1124/mol.114.097360
	3QAK		0		10.1126/science.1202793
	6GDG	0		10.7554/eLife.35946	
	7RMS	antagonist	4	0/4 inner. Outer (TM2-3, TM5-6, TM6, TM6)	10.1073/pnas.2106041118
	50LV		4	0/4 inner. Outer (TM2-3, TM3-4, TM6, TM6)	10.1038/s41598-017-18570-w
	50LZ		4	0/4 inner. Outer (TM2-3, TM3-4, TM6, TM6)	10.1038/s41598-017-18570-w
	50M1		4	0/4 inner. Outer (TM2-3, TM3-4, TM6, TM6)	10.1038/s41598-017-18570-w
	50M4		4	0/4 inner. Outer (TM2-3, TM3-4, TM6, TM6)	10.1038/s41598-017-18570-w
	50LG		4	0/4 inner. Outer (TM2-3, TM6, TM6, TM7)	10.1038/s41598-017-18570-w
	5MZP		4	0/4 inner. Outer (TM2-3, TM3-4, TM6, TM6)	10.1016/j.str.2017.06.012
	5IU4		4	0/4 inner. Outer (TM2-3, TM3-4, TM6, TM6)	10.1021/acs.jmedchem.6b00653
	5IU7		4	0/4 inner. Outer (TM2-3, TM3-4, TM6, TM6)	10.1021/acs.jmedchem.6b00653
	5IU8		4	0/4 inner. Outer (TM2-3, TM3-4, TM6, TM6)	10.1021/acs.jmedchem.6b00653
	5IUA		4	0/4 inner. Outer (TM2-3, TM3-4, TM6, TM6)	10.1021/acs.jmedchem.6b00653
	6LPJ		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1038/s41598-020-76277-x
	6LPK		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1038/s41598-020-76277-x
	6LPL		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1038/s41598-020-76277-x
	5VRA		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1038/nprot.2017.135
	5NM2		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1038/s41467-017-00630-4
	8CU6		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1021/acs.jmedchem.2c00462
	8CU7		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1021/acs.jmedchem.2c00462
	5IUB		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1021/acs.jmedchem.6b00653
	5MJZ		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1016/j.str.2017.06.012
	5N2R		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1016/j.str.2017.06.012
	6ZDR		3	0/3 inner. Outer (TM6, TM6)	10.1002/anie.202003788
	6ZDV		3	0/3 inner. Outer (TM6, TM6)	10.1002/anie.202003788
	7PX4		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1002/anie.202115545
	7PYR		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1002/anie.202115545
	6AQF		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1016/j.cell.2017.12.004
	8DU3		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1016/j.ejmech.2022.114620
	50LH		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1038/s41598-017-18570-w
	5OLO		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1038/s41598-017-18570-w
	6JZH		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1107/S1600576719012846
	5UVI		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1107/S205225251700570X
	6PS7		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1107/S2052252519013137
	6S0L		3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1107/S2052252520011379
	6S0Q	3	0/3 inner. Outer (TM3, TM6, TM6)	10.1107/S2052252520011379	
	6WQA	3	0/3 inner. Outer (TM3, TM6)	10.1107/S2052252520012701	
	5K2A	3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1126/sciadv.1600292	
5K2B	3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1126/sciadv.1600292		
5K2C	3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1126/sciadv.1600292		
5K2D	3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1126/sciadv.1600292		
5JTB	3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1126/sciadv.1602952		
4E1Y	3	0/3 inner. Outer (TM3, TM6, TM6)	10.1126/science.1219218		
6GT3	3	0/3 inner. Outer (TM2-3, TM6, TM6)	10.1136/jitc-2019-000417		
5NM4	2	0/2 inner. Outer (TM2-3, TM6)	10.1038/s41467-017-00630-4		
3PWH	0		10.1016/j.str.2011.06.014		
3REY	0		10.1016/j.str.2011.06.014		
3RFM	0		10.1016/j.str.2011.06.014		
5NLX	0		10.1038/s41467-017-00630-4		
7T32	0		10.1038/s41467-022-32125-2		
3EML	0		10.1126/science.1164772		
3VG9	antagonist, NAM	0		10.1038/nature10750	
3VGA		0		10.1038/nature10750	

2.1 Cases of significant differences in the number and/or position of CLR in the presence of ligands from different type.

5-HT_{2A}: Two different studies solved the crystallographic structures of the 5-HT_{2A} receptor in the presence of different ligands and demonstrated a variation in the number and position of CLR depending on the bound-ligand. Thus, depending on the agonist, 1-3 CLRs are present (PDB ID: 6WGT, 7WC4, 7WC5, 7WC6, 7WC7), while 4 CLRs are present with an antagonist (PDB ID: 7WC8) and 1 CLR in the presence of a biased ligand (PDB ID: 7WC9) or an inverse agonist (PDB ID: 6WH4).

A_{2A} (Table 2) is particular because, although no article brings together structures with ligands of different types in a single study, this receptor has a sufficiently large number of structures (54) to carry out a comparative study. In the presence of agonists, 9 structures were obtained and showed no CLR. In the presence of different antagonists, 38 structures out of 43 have 3 to 4 CLRs in similar positions (Outer TM2-3, TM3-4, TM6 and sometimes TM6). These observations suggest a specific dynamics of CLR molecules correlated with the type of the ligand, with antagonists increasing the number of bound CLRs, without specificity for the ligand itself.

β₂: 2 studies gather structures with ligands of different types (mainly inverse agonists and antagonists) [35] [36] and show preservation of the position of 1 CLR (inner TM2-4). The presence of this CLR is found in 8 other structures out of 10 in the presence of inverse agonists or antagonists (9 different studies), whereas the trend is reversed in the presence of agonists with an absence of CLR in 7 structures out of 8 from 8 different studies.

CXCR2: In the same study, 3 structures of CXCR2 were determined by cryo-EM, two in the presence of agonists (mono- and dimeric CXCL8, PDB ID: 6LFO and 6LFM, respectively) + Gi proteins and one in the presence of an antagonist that binds in the cytoplasmic interface of TMD. These structures show that the CLR present in the structures with agonists in the inner TM2-4 position is no longer present in the structure with antagonist. This would be due to the rotation of TM4, which positions W170 in the CLR binding site and would create a steric hindrance.

TSH: 3 structures under 2 different conditions (agonist + MAP + Gs proteins and antagonist) were determined in the same study. The impact of these two conditions on the number of CLRs is clear since the structure with antagonist (PDB ID: 7XW7) has no CLRs present while 14 and 17 CLRs are present in the structures with agonist + PAM + Gs proteins (PDB ID: 7XW5 and 7XW6). These observations are in agreement with a preferential localisation of the receptor in lipid rafts and the reduction of the activation potency of TSH and ML-109 by double mutations in 3 CLR binding sites (outer TM1, TM7, TM3-4) [37].

GABA_B: Three different studies show a significant difference in the number of CLR depending on the type of ligands [38] [39] [40]. Thus, structures with agonists do not have any CLR, whereas in the presence of an antagonist, 9 to 16 CLRs are present around the dimeric complex. A structure with PAM only has 3 CLRs. A structure in apo form but in an inactive state [41] also has a large number of CLRs (10) as observed in structures with antagonists. It is worth noting that the position of some CLRs and PAMs are asymmetric between the 2 protomers.

Ste2: the same study [42] composed of 4 structures with ligands of different types (2 with agonists, 1 with antagonist and 1 apo) clearly shows that the number of sterol molecules, which is substantial in the apo form (22), evolves in the opposite way in the presence of antagonist (increase up to 34) or agonist (decrease up to 12).

This analysis confirms the observations in the section 1.1 that the active or inactive bound state of some GPCRs could alter the binding of CLR molecules. This could have an impact on the translocation of GPCRs in or out of cholesterol-rich domains depending on their conformational state.

2.2 Case of no difference between ligands of different types

5-HT_{2B}: 1 molecule of CLR is present at the same location (inner TM1-H8) in all structures, not only in one study in the presence of agonists (2) or antagonists (2), but also in 3 other studies in the presence of agonists. These structural observations suggest that different receptor states would not influence the binding or distribution of CLR molecules.

CCK₁: No CLR molecules are observed in 8 of the 10 structures that were determined in the presence of agonists (+/- G proteins) or in the presence of antagonists in 4 different studies. In one of them, the 2 structures with antagonists (PDB ID: 7F8U and 7F8Y) and the structure with agonist (PDB ID: 7F8X) confirm the absence of CLR. Only 1 study in the presence of agonist and Gq or Gs proteins (PDB ID: 7MBX and 7MBY) reveals 1 CLR molecule in the inner position (TM2-4). The absence of CLR in the majority of CCK₁ structures is consistent with an indirect role for CLR observed in CCK₂, which is sensitive to membrane fluidity [15].

2.3 Cases where structural data do not permit the determination of a trend

5-HT_{2C}: A study resolving 2 structures, one with an agonist (PDB ID: 6BQG) and the other one with an antagonist (PDB ID: 6BQH), shows an absence of CLR in both structures. However, another study determined 4 structures with different agonists (+ Gq proteins) and showed the presence of 1 to 2 CLR molecules. These observations do not allow to suggest a role of ligands in the interaction of CLR.

β₁: 2 different studies [43] [44] each determined 3 structures with agonists (+ Nb) and 1 structure with antagonists. Although in the case of 7BVQ the position of the CLR molecule observed in the agonist structures changes position, the second study shows no CLR in all structures. In the other 9 studies, the number and positions of CLR molecules vary too widely to conclude on a differential effect of the type of the ligands on this receptor.

D₁: 13 structures of this receptor were determined mainly with agonists (12) and 1 structure with a biased ligand. The absence of structures with antagonists or inverse agonists, as well as the significant variation in the number (0 to 6) and position of CLR molecules, does not allow to conclude on an effect of the receptor state on the binding of CLR.

NK₁: 4 studies determined 9 structures of this receptor in the presence of agonists, antagonists and biased ligand, but only 1 study combined structures with different types of ligands (agonists and biased ligand). In 3/4 of the studies, no CLR is observed but one study shows the presence of 1 CLR in the inner 2-4 position. Although the absence of CLR is predominant in the structures, CLR dependence is pointed out in the article [45]. The current structural data cannot conclude on the direct role of the CLR on the NK₁ receptor and the impact of the ligand type on its binding.

P2Y₁₂: 1 study determined the structure of this receptor in the presence of agonist (with 1 CLR) and in the presence of antagonist (without CLR) [46]. However, another study showed the presence of 2 CLR molecules in a structure with another antagonist [47]. These data do not allow to conclude on the impact of the antagonist-bound state on the number of CLR molecules.

CaS: 16 structures of this receptor were determined in 5 different studies, 4/5 of them with ligands of different natures (agonists and/or allosteric regulators). The majority of these structures are without CLR (10 structures) or probable densities at the dimeric interface (3 structures). But 1 study shows the presence of 8 CLR molecules in the presence of agonist + PAM while no CLR is observed

in other structures under similar conditions. These data do not allow us to conclude on the role of ligands on the presence or absence of CLR.

3 Do intracellular binders (G proteins, nanobodies, ...) have an impact on CLR binding?

To answer this question while avoiding the same biases as the previous question, structures are compared for the same receptor in the same study with the same method, the same ligand and in presence and absence of intracellular binders or with different binders.

Table 3: example of comparison of the position of CLR in 5-HT₄ receptor structures with different intracellular binders in the same study.

Name	PDB code	Ligands	Intracellular binder	Nbr of CLR	Position of CLR/CHS/PAM/NAM in TMDs	DOI
5-HT ₄	7XT8	agonist	Gs	5	2/5 inner (TM2-4, TM5-6). Outer (TM2-3, TM4-5, TM6-7)	10.1016/j.molcel.2022.05.031
	7XT9		Gs	4	2/5 inner (TM2-4, TM5-6). Outer (TM4-5, TM6-7)	10.1016/j.molcel.2022.05.031
	7XTA		Gi	0		10.1016/j.molcel.2022.05.031

3.1 Cases of significant differences in the number and/or position of CLR.

5-HT₄ (Table 3): 3 structures from the same study [48] in the presence of the same agonist (serotonin) show an absence of CLR in the presence of Gi proteins (PDB ID: 7XTA), but 4 to 5 CLR in the presence of Gs proteins (PDB ID: 7XT8 and 7XT9). These observations suggest a selectivity of CLR interaction according to the nature of the G proteins binding to the 5-HT₄ receptor.

CB₁: 1 study [49] presenting 2 structures with (PDB ID: 7WV9) and without (PDB ID: 7FEE) Gi proteins (in the presence of the same agonist and the same PAM) shows that 2 CLR disappear in the structure in the presence of the Gi proteins, suggesting that the interaction of these proteins would affect the binding of CLR on CB₁.

US28: 1 study [50] resolving 2 structures with and without nanobody (bound in the G protein binding site) and in the presence of the same ligand, have 2 CLR in the outer TM6 and TM7 position when the nanobody is present. These observations suggest that conformational changes on the intracellular side of the TMD may affect the binding of CLR on the extracellular side of the same domain.

These observations suggest that CLR binding can be altered by the interaction of intracellular binders either by decreasing the number of bound CLR (e.g. CB₁) or by increasing it (e.g. US28) or by modifying the number of bound CLR depending on the type of bound G proteins (e.g. 5-HT₄). Interestingly, the altered positions of CLR are not necessary on the "inner" side where the intracellular binders interact, but it can be on the "outer" side as observed in structures of US28.

3.2 Case of an absence of difference in the presence or absence of intracellular binders.

β₁: no CLR are present in structures containing Gs or Gi proteins, nanobodies or β-arrestin and this occurs in 4 different studies [51], [36], [52], [44].

MRGPRX2: In a single study [53], 10 structures show no change in the number (1) and position (inner TM2-4) of the CLR in the presence (8) or absence (2) of Gi proteins (+ different agonists). Another similar study diversifying the G proteins (Gi or Gs) [54] shows an absence of CLR in both conditions. Although the presence of CLR varies between the 2 studies, these observations suggest that this receptor does not alter the dynamics of CLR depending on the binders that bind to it.

GLP-1: Only one study [55] combines structures (3) with (PDB ID: 7LCI) and without (PDB ID: 7LCJ and 7LCK) Gs proteins (+ an identical agonist). No CLR molecules are present in these 2 conditions suggesting an absence of effect of G proteins on CLR binding for this receptor. Nineteen other

structures with agonists including 2 without Gs proteins confirm this absence of CLR in these 2 conditions.

3.3 Cases of non-conclusive structural data

CCK₁: 3 studies were performed in the presence of different G proteins and showed divergent results with identical conditions (same agonist + Gs proteins + Nb35). One CLR is present in 1 study (PDB ID: 7MBX) and none in the other one (PDB ID: 7XOU). These data do not suggest a link between G proteins and CLR.

GPR158: two studies currently available show very different results in identical conditions. Thus, the PDB ID: 7EWR structure in the presence of RGS7-Gbeta5 (ratio2:2:2) does not have any CLR, while the PDB ID: 7SHF structure obtained in the same conditions in another study shows 22 CLR distributed around the dimer. The absence of CLR in structures 7EWP et 7EWR is most probably due to a resolution too low to accurately assign the densities as suggested by the authors[56].

4 Is there reproducibility in the position of CLRs across different structures of a GPCR suggesting sensitivity to CLR?

To answer this question, the redundant positions of CLRs in the structures of the same GPCR with the same type of ligand are compared through different studies.

Table 4: Comparison of the redundant position of CLR in the structures of the 5-HT_{2A} receptor.

Name	PDB code	Ligands	Nbr of CLR	Position of CLR/CHS/PAM/NAM in TMDs	DOI
5-HT _{2A}	7WC4	agonist	3	2/3 inner (TM1-H8, TM1-2). Outer (TM6-7)	10.1126/science.abl8615
	7VOD		3	2/3 inner (TM1-H8, TM1-2). Outer (TM6-7)	10.1038/s41593-021-00971-w
	7VOE		3	2/3 inner (TM1-H8, TM1-2). Outer (TM6-7)	10.1038/s41593-021-00971-w
	6WGT		2	1/2 inner (TM2-4). Outer (TM6-7)	10.1016/j.cell.2020.08.024
	7WC5		2	1/2 inner (TM2-4). Outer (TM6-7)	10.1126/science.abl8615
	7WC6		1	0/1 inner. Outer (TM6-7)	10.1126/science.abl8615
	7WC7	1	0/1 inner. Outer (TM6-7)	10.1126/science.abl8615	
	6WHA	0		10.1016/j.cell.2020.08.024	
	7WC8	antagonist	4	2/4 inner (TM1, TM1-2). Outer (TM4-5, TM6-7)	10.1126/science.abl8615
	6A93		1	1/1 inner (TM2-4)	10.1038/s41594-018-0180-z
	6A94		1	1/1 inner (TM2-4)	10.1038/s41594-018-0180-z
	7WC9	Biased ligand	1	0/1 inner. Outer (TM6-7)	10.1126/science.abl8615
	6WH4	inverse agonist	1	1/1 inner (TM1-8)	10.1016/j.cell.2020.08.024

4.1 Cases of redundancy of the CLR position.

5-HT_{2A} (Table 4): 12 structures out of 13 total (4 studies) have at least 1 CLR with a majority position in outer (TM6-7) in 8 structures with different types of ligands.

5-HT_{2B}: 7 structures out of 7 total (4 studies) all have 1 CLR in the inner position (TM1-H8) with different ligands.

A_{2A}: 41 structures out of 54 (34 studies) have at least 2 CLRs and 31 structures with antagonists have dominant outer positions (TM2-3, TM6, TM6).

β₂: 20 structures out of 32 (20 studies) have at least 1 CLR in the main inner position (TM2-4) with different ligands.

CB₁: 5 out of 10 structures (8 studies) have at least 1 CLR with the position inner (TM2-4) occupied by 1 NAM in one structure (PDB ID: 6KQI).

D₁: 12 structures out of 13 (4 studies) have at least 1 CLR with the main position in inner (TM2-4) in 8 structures with different agonists (+/- PAM) or 1 biased ligand.

GHS: 4 out of 7 facilities (4 studies) have at least 1 CLR always positioned in inner (TM2-4).

GIP: 4 out of 4 structures (2 studies) have at least 6 CLRs in the redundant positions inner (TM2-4, TM5-6) and outer (TM1-7).

Ste₂: 5 out of 5 structures (2 studies) have at least 6 CLRs in the dominant positions inner (1-7) and outer (TM1-2, TM1-7, TM7).

It should be noted that although there is a reproducibility in the position of CLRs in the structures of the above-mentioned receptors, there is also a more or less important divergence between receptors, which creates a more or less specific profile of GPCR/CLR interactions. It is thus often possible to identify a GPCR by observing the position of the CLRs in the structure. This was also observed in MD simulations[57].

4.2 Cases of dominant absence of CLR interaction.

The following GPCRs appear to have limited interaction with CLR:

CCK₁: 8/10 structures without CLR in 3 different studies with different ligands.

δOR: 5/6 structures without CLR in 4 studies with different ligands.

ET_B: 5/6 structures without CLR in 4 studies with different ligands.

MT1: 6/8 structures without CLR in 4 studies with only agonists.

NK₁: 7/9 structures without CLR in 4 studies with different ligands.

Ox₁: 13/14 structures without CLR in 3 studies with antagonists only.

GLP-1: 33/37 structures without CLR in 20 studies with different ligands (except antagonists).

5 Are there mutations affecting functional roles of CLR?

One approach to assess the functional role CLR binding is to mutate the potential binding sites and perform functional assays. To answer the above question, a list of mutants characterized in the references of the structures or in citing references is indicated in the Table 5.

Table 5: Mutations of GPCRs with functional effect related to CLR.

Class	Name	Species	Mutations	Positions	CLR observed at these positions in structures?	Assay and functional effect	DOI
A	5-HT _{1A}	Human	Q97A /I384A, L380A/I384A	Outer TM1-7	Yes (in 7E2X, 7E2Y)	NanoBIT G protein coupling: Higher EC ₅₀	10.1038/s41586-021-03376-8
A	5-HT _{1A}	Human	K101A	ECL1	No (Close to Outer TM1-2 in 7E2X)	cAMP assay: loss of CLR-dependence	10.1126/sciadv.abh2922
A	A _{2A}	Human	S47A, K122A, W129A	Inner TM2-4	No	cAMP assay: S74A: Higher basal activity K122A: Lower basal and agonist-induced activity W129A: Lower basal and agonist-induced activity SPR (Gs protein interaction) S47A: Higher koff W129A: No Gs protein binding	10.3390/molecules27113529
A	β1	Turkey	V129I	Inner TM3-4	No (Close to Inner TM2-4)	ITC: Lower affinity for agonist and Nb80 in presence of CHS	10.1038/s41557-022-01009-9
A	β2	Human	F321A, R328A	Inner TM6-7	No	LCP-Tm assay: F321A: Reduced thermostability induced by CLR. R328A: increased thermostability induced by CLR.	10.1016/j.bbamer.2021.18357
A	CB ₁	Human	K402G	H8	No	Confocal microscopy and FRAP: Lower interaction with CLR and higher immobile fractions	10.1111/j.1471-4159.2010.07041.x
A	CCK ₁	Human	Y140A, W166A, Y237A	Inner TM3-5, TM2-4, TM5	Yes (W166 in 7MBX and 7MBY)	Radioligand binding: Y140A: Higher affinity W166A: Lower affinity Y237A: Lower affinity Ca ²⁺ assay: Y140A: Higher EC ₅₀ W166A: Higher EC ₅₀ Y237A: no change	10.1194/jr.M020065
A	CCK ₁	Human	Y140A	Inner TM3-5	No	Radioligand binding: Higher affinity similar to wt in high CLR environment Ca ²⁺ assay: Higher EC ₅₀ to wt in high CLR content	10.1074/jbc.M114.570200
A	CX ₃ CR1	Human	Site 1: W154A Site 2: F118A/L, V153A/W Site 3: I215A, L234W	Site 1: Inner TM2-4 Site 2: Inner TM3-4 Site 3: Inner TM5	Yes (in 7XBX, 7XBW)	BRET G protein activation: Site 1: Abolished G protein activation Site 2: Abolished or reduced (80% for F118L) G protein activation Site 3: Abolished G protein activation	10.1126/sciadv.abn8048
A	D ₁	Human	Y218S	Inner TM5	Yes (in 7LJC)	Sucrose gradient ultracentrifugation: Present only in "non raft fractions" cAMP assay: Higher basal activity. No response to an agonist	10.1096/fj.201902710RR
A	OT	Human	Y200A/H, W203A/H	Outer TM4-5	Yes (in 6TPK)	HTRF-binding assay: All mutants: no to low binding (<20%) of agonist and antagonist	10.1126/sciadv.abb5419
A	M1	Human	L376I, R365Q	Inner TM6	No	IP and Ca ²⁺ assay: L376I: loss of wash-resistant effect of the agonist xanomeline	10.1016/j.neuropharm.2018.01.027
B1	CRF1	Human	Site1: I232A/W236A Site 2: L251A/W259A	Site1: Inner TM2-4 Site2: Outer TM3-4	Yes (in 6PB0)	cAMP assay: Site1: Higher EC ₅₀ Site2: Higher EC ₅₀	10.1016/j.molcel.2020.01.013
B1	CRF2	Human	Site1: I228A/W232A Site 2: L247A/W255A	Site1: Inner TM2-4 Site2: Outer TM3-4	Yes (in 6PB1)	cAMP assay: Site1: Higher EC ₅₀ Site2: Higher EC ₅₀	10.1016/j.molcel.2020.01.013
C	CaS	Human	F809A/L, L812A, I813A	Inner TM6	Yes (in 7SIL)	IP accumulation assay: Higher EC ₅₀	10.1073/pnas.2115849118

This table demonstrates that site directed and even single point mutations in CLR binding sites observed in structures or other potential binding sites have a functional effect on some GPCRs. It is worth noting that the number of mutagenesis studies is low compared to the number of GPCRs having published structures. The functional characterization of CLR effect is not technically trivial and multiple sites could be involved in the CLR-sensitivity of a GPCR. Interestingly, some studies report functional impact of mutation in CLR binding sites that were not observed in structures, suggesting that these sites were not observed in the experimental conditions of the structural studies, or the CLR-sensitivity related to these mutated sites are not related to CLR binding but to indirect effect.

6 Open questions and future directions

6.1 Concept of functional cholesterol

Analysis of the structural data of the 68 GPCRs in this study shows that molecules of CLR reproducibly bind to certain GPCRs known to be sensitive to CLR (e.g. 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, A_{2A}, β₂, CB₁, D₁, GHS, GIP...) and that the binding of different types of ligands or binders could affect the number and position of CLR molecules. A persistent question concerns the functional role of these CLR molecules. Classical approaches to site-directed mutagenesis of CLR have demonstrated a functional impact, such as on

5-HT_{1A} [58], OT [59], CRF1 and CRF2 [60]. However, these examples are rare and even photolabelling approaches do not identify sites affecting the CLR sensitivity of the OT receptor [61].

In a recent study, we observed that orthosteric ligand binding to the OT receptor preserves the binding of 'functional' CLR molecules upon membrane cholesterol depletion. If this mechanism also exists for other GPCRs, the selective sequestration of functional CLR molecules could facilitate the identification of CLR binding sites that impact receptor function. Among the receptors potentially involved, the present analysis of structural data has highlighted GPCRs showing CLR molecule dynamics upon orthosteric ligand binding. These receptors are: 5-HT_{1A}, CX₃CR1, SUCNR1, US28, GLP-1, GABA_B, mGluR₁ and Ste2 when comparing apo and holo forms, as well as: 5-HT_{2A}, A_{2A}, CXCR2 and TSH when comparing the different types of bound-ligands (agonists, antagonists...).

Another question concerns the molecular mechanisms involved in the modulation of receptor activity by CLR molecules. Among the possible mechanisms, an original model of stabilisation of the 5-HT_{1A} receptor by a partial interaction of a CLR molecule outside the bilayer with the K101 residue is proposed [58]. Class C dimeric GPCRs show significant differences in the number and position of CLRs depending on the state of the receptor, especially at the dimeric interface. These observations suggest that CLR molecules may be involved in the stabilization of the dimeric interface depending on the state of the receptor and/or in the orientation of this interface between TMs or between leaflets. Reinforcing this hypothesis, the asymmetric distribution of CLR molecules in GABA_B structures is consistent with an asymmetric role of the 2 subunits in the function of this receptor.

6.2 Binding sites of allosteric modulators and cholesterol

Among the GPCRs in the attached table, and among the 10 receptors with structures with allosteric regulators (A_{2A}, β₂, CaS, CB₁, CCR9, D₁, GABA_B, GLP-1, mGluR₁ and TSH), 5 bind NAMs or PAMs in the periphery of their transmembrane domain (β₂, CB₁, D₁, GLP-1 and GABA_B). Among the structures comprising allosteric modulators at the periphery of TMDs, 3 bind these modulators in sites occupied by CLR molecules. For example, in the 6KQI structure of the CB₁ receptor, a NAM molecule replaces 1 CLR at the inner TM2-4 position when NAM is added in the presence of agonist. The study [62] suggests that the allosteric regulator Org27569 is replaced by 1 CLR to allow the transition of the receptor from a pre-activated to a fully activated state. In the case of GLP-1, depending on the nature of the PAM, the allosteric regulator occupies a site in the position inner TM6 (PDB ID: 7E14, 7DUQ, 7DUR and 7EVM) or outer TM1-2 (PDB ID: 6VCB) which are sites also occupied by CLRs. For the GABA_B receptor, 1 PAM molecule occupies a position at the dimeric interface (inner TM5-6/TM6-7) in 3 structures (PDB ID: 7C7Q, 6UO8 and 7EB2), a position that is also occupied by 1 CLR molecule in the structures (PDB ID: 7CUM, 6WIV and 7CA3). The co-existence of common binding sites between allosteric regulators and CLR suggests that CLR could play a role as allosteric regulators on these sites if it induces the same conformational changes. The role of these sites on an allosteric action of cholesterol is an avenue that remains to be explored. Conversely, CLR binding sites identified as having a functional role could be pharmacological targets for the development of allosteric regulators by rational structure-oriented drug design. This approach is notably suggested by the authors of the references [63] [64] and well documented in the following review [65].

Interestingly, cholesterol also appears to play a role as "co-factor" in the binding of allosteric regulators. Thus, in a structure of the D₁ receptor (PDB ID: 7X2F), 1 molecule of PAM does not bind to a site previously occupied by CLR, but induces the binding of an additional CLR molecule interacting with itself and the receptor [66]. In the GLP-1R structures with PAM (PDB ID: 7DUQ and 7E14) the ligands are oriented towards 1 CLR (inner TM6) located at a distance of ~5 Å. These observations suggest that some allosteric regulators and CLRs may interact or act together.

6.3 Methodological developments

Thanks to recent technological advances, a large number of GPCR structures (793: <https://gpcrdb.org/structure/statistics> [67]) have been obtained, but due to methodological necessity, these have been obtained on receptors isolated from their natural membrane environment and often with purification steps containing detergents and buffers enriched with CLR or CHS. The development of cryo-electron microscopy allows to approximate natural conditions by using lipid bilayer systems (nanodiscs, bicelles, ... [68]) with natural lipid extracts and has allowed the resolution of complexes with heterotrimeric G-proteins and β -arrestins. However, these conditions do not mimic the full diversity of the fluid mosaic model, especially the CLR-rich domains in which some GPCRs concentrate. The development of whole cell structural technologies such as FIB-SEM is a promising avenue to study the role(s) of CLR on GPCRs in physiological conditions. However, several technological challenges must be overcome in order to reach a sufficiently high resolution to observe CLR molecules and to specifically identify one type of GPCR in a heterogeneous environment composed of other GPCRs.

In conclusion, this review and the accompanying table allowed to observe in the GPCR structures a dynamic of CLR interactions on particular receptors and depending on their state (apo, bound to different types of ligands, bound to intracellular interactants). These observations are indications of the potential role of CLR on particular receptors and on certain states of these receptors. This role needs to be validated functionally and physiologically for many of them. The data available in the accompanying table are certainly not exhaustively analysed and may be of interest for further analysis. Future GPCR structures with CLR and methodological developments will help to consolidate this kind of comparative analysis potentially assisted by AI methods. This information will ultimately help to better understand the molecular mechanisms of binding of CLR molecules on specific GPCRs.

Declaration of Competing Interest

The authors declare no competing interest.

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