



New insights into GPCR coupling and dimerisation from cryo-EM structures

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Abstract


Over the past three years (2020–2022) more structures of GPCRs have been determined than in the previous twenty years (2000–2019), primarily of GPCR complexes that are large enough for structure determination by single-particle cryo-EM. This review will present some structural highlights that have advanced our molecular understanding of promiscuous G protein coupling, how a G protein receptor kinase and β -arrestins couple to GPCRs, and GPCR dimerisation. We will also discuss advances in the use of gene fusions, nanobodies, and F_{ab} fragments to facilitate the structure determination of GPCRs in the inactive state that, on their own, are too small for structure determination by single-particle cryo-EM.


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Introduction

GPCRs play a pivotal role in intercellular signalling throughout the human body and are the targets of 34% of FDA approved drugs [1]. Only a proportion of all GPCRs have been drugged and there is intense scrutiny of other GPCRs to develop novel therapeutics for the treatment of diseases such as diabetes, cancer and neurodegeneration [2]. Structural biology plays a key role in drug development through either providing a

structure suitable for screening *in silico* ultra-large drug libraries [3••] or through providing a mechanistic understanding of fundamental molecular processes such as receptor and G protein activation [4,5]. Here we highlight a few of the fundamental molecular insights that underpin complexities in GPCR pharmacology that have been uncovered by the wealth of structures determined by cryo-EM over the past few years.

Structural mechanisms in promiscuous GPCR-G protein coupling

GPCRs signal through heterotrimeric G proteins and the type of α -subunit determines the downstream signalling cascade affected. There are four major families of G proteins in humans, G_s , $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ that signal through different pathways. Although some GPCRs are specific and activate a single type of G protein, at least 50% of GPCRs activate two or more G proteins [6–8]. Promiscuous coupling activates different G proteins with varying efficacies and kinetics, generating a fingerprint-like signalling profile within the cell [9], thus enhancing the complexity of GPCR signalling and providing new therapeutic opportunities.

Cryo-EM structures of eleven GPCRs have been determined with each GPCR coupled to two or more distinct G proteins: GCGR, β_1 AR, ADGRF1 and 5HT₄R coupled to G_s and $G_{i/o}$ [10–13], NK₁R coupled to G_s and $G_{q/11}$ [14], CCK_AR coupled to G_q , G_{i1} and G_s [15,16], ADGRL3 coupled to G_s , G_i , G_q and G_{12} [17••] and four receptors coupled to $G_{i/o}$ and $G_{q/11}$ (GSHR [18,19], CCK_BR [20], GPR139 [21] and MRGPRX2 [22]). Several trends arise from analysing this set of structures [23].

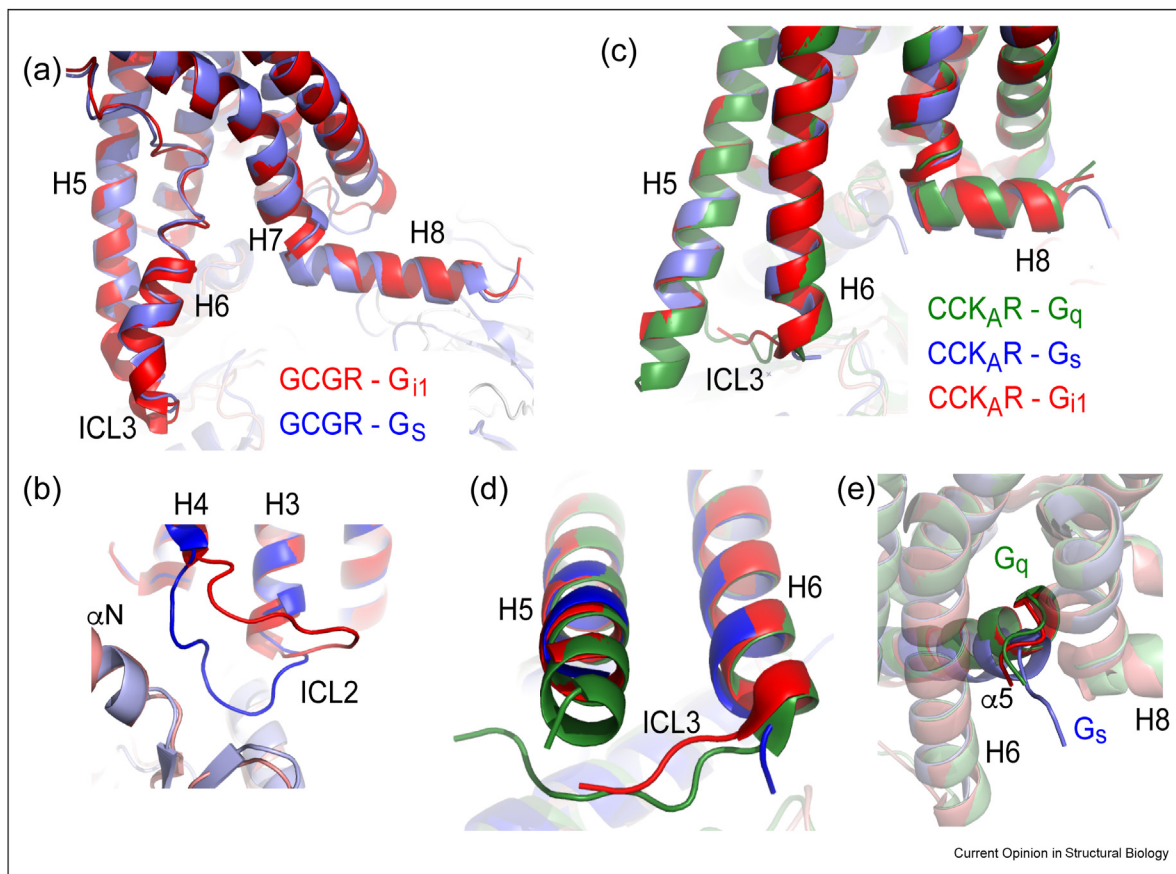
The outward movement of the cytoplasmic end of transmembrane helix TM6 is a hallmark of GPCR activation and is thought to determine the size and shape of the intracellular cleft where the cytoplasmic end of helix α_5 of the G protein α -subunit couples [24]. Structures of many different GPCRs coupled to G proteins suggested initially that the magnitude of TM6 displacement correlated with the type of G protein. A large outward movement of TM6 forms a wide intracellular cleft that is required typically for G_s coupling, whilst smaller movements of TM6 form a narrower cleft

characteristic of $G_{i/o}$ - $G_{q/11}$ coupling [25,26]. However, recent new structures show that this is not always the case when they are the secondary couplers, with G_s sometimes coupling to a narrow cleft and G_i or G_q coupling to a wide cleft. Structures of the same GPCR coupled to either G_s or another G protein suggest that the movement of TM6 is usually the same regardless of the secondary G protein coupled *i.e.* the secondary G protein has to use a similar intracellular cleft for coupling as the primary G protein (Figure 1a–e). For example, the primary coupler to GCGR is G_s and the GCGR- G_s cryo-EM structure shows a wide intracellular cleft; the receptor structure coupled to its secondary coupler $G_{i/o}$ shows an equally wide cleft to when G_s is coupled, and not a narrow cleft as might be expected [10]. Conversely, $CCK_A R$ and $NK_1 R$ couple primarily to G_q and adopt a narrow intracellular cleft upon activation, and the secondary G protein G_s also couples to this narrow cleft. In some instances, such as for $CCK_A R$, this forces the G protein to adopt ‘non-standard’ conformations where the α -subunit shows an unwinding of the ‘wavy hook’ in the α_5 helix C-terminus, which

protrudes outwards from the receptor intracellular cavity (Figure 1e). Primary coupling of $G_{i/o}$ and $G_{q/11}$ results in a similar narrow intracellular cleft, which may explain the high abundance of $G_{i/o}$ - $G_{q/11}$ promiscuous couplings [7].

The intracellular loops (ICLs) of GPCRs are the elements that differ most when coupling to different G proteins. However, there appears to be no correlation with the type of ICL rearrangement and the type of G protein or primary/secondary couplings. ICL3 takes a prominent role in promiscuous G protein coupling in MRGPRX2, 5-HT₄R, ADGRF1, GSHR, GPR139, and $CCK_A R$ where it makes different interactions to different G proteins (Figure 1d.) ICL2 also changes conformation or interactions in most GPCR-G protein complexes (e.g. GCGR and GSHR, Figure 1b), whereas ICL1 differential interactions have only been observed in GCGR. The loop between TM7 and H8 also varies in $\beta_1 AR$ coupled to either G_s or G_i . Such differences in ICLs contribution to promiscuous G protein coupling were supported by mutagenesis and functional assays,

Figure 1



Structural snapshots of promiscuous GPCR-G protein coupling. Structural superposition of the GCGR coupled to G_s (blue) and G_{i1} (red) showing similarities in TM6 (a) and differences in ICL2 (b) [10]. Structural superposition of the CCK_AR coupled to G_q (green), G_{i1} (red) and G_s (blue) showing similarities in TM6 position (c), differences in the ordering of ICL3 depending on the coupled G protein (d) and differences in the engagement mode of the α -subunit C-terminal ‘wavy hook’ for G_s vs G_q (e) [15,16].

where alterations in the CCK_AR ICL3 had a major impact on G_q but not G_S or G_i signalling [15]. Similarly, alterations in the GCGR ICL3 and ICL1 showed a greater impact on G_i compared to G_S signalling [10].

GPCR structures coupled to GRK or arrestin

One mechanism in the cell to terminate GPCR-G protein signalling at the plasma membrane is through receptor phosphorylation by GRKs, recruitment of arrestin via the phosphorylated C-terminus/ICL3 and then clathrin-mediated endocytosis mediated by arrestin-clathrin/AP2 interactions [27]. Arrestin interacts with GPCRs in two distinct ways. Arrestin binds first to the phosphorylated C-terminus/ICL3 of the receptor, causing a conformation change in arrestin that subsequently facilitates coupling of arrestin to the receptor [28–30]. Arrestin couples to GPCRs using the same intracellular cleft that binds the C-terminal $\alpha 5$ helix of the G protein [31] and results in activation of the intracellular ERK1/2 signalling cascade. It is crucial to understand the molecular differences between coupling of G proteins, GRKs and arrestins, because the therapeutic effect and side effects of drugs may arise through different signalling pathways [32]. There is thus intense interest in developing biased ligands that specifically activate/inhibit only one specific pathway.

Structure determination of a GPCR-GRK complex has been difficult, however, stabilisation of the rhodopsin-GRK1 complex by a combination of crosslinking, binding of two F_{abs} and lipids resulted in the first low resolution structures [33••]. The receptor was in its active state, with the N-terminus of GRK1 forming an α -helix that binds to the intracellular cleft like G proteins and arrestin (Figure 2a,d). Comparison between the conformation of rhodopsin when coupled to either GRK, arrestin or the G protein transducin shows that they are virtually identical (RMSDs of 0.9–1.0 Å) and that the binding sites on rhodopsin overlap significantly (Figure 2h). There are eight residues that interact with all three coupled proteins (Val139^{3,54}, Asn145^{34,53}, Phe146^{34,54}, Gln237^{5,72}, Glu249^{6,32}, Val250^{6,33}, Asn310^{8,47}, Gln312^{8,49}) and a further subset of residues (Figure 2h) that interact only with GRK1 (6 residues), visual arrestin (8 residues) or transducin (2 residues).

Seven structures of GPCRs coupled to arrestins have now been determined. The first high-resolution structure of a GPCR-arrestin complex was a crystal structure of constitutively active mutant of human rhodopsin fused to a preactivated form of mouse arrestin 1 (visual arrestin) [34]. A variety of different strategies were required for cryo-EM structure determination of non-visual arrestins coupled to activated receptors, including combinations of the following: fusion with the C-terminus of phosphorylated V₂ receptor, arrestin

mutants, cross-linking, binding of F_{ab}30 to stabilise the active state of arrestin and the use of lipid-mimicking environments. Structures of complexes with arrestin 2 (Arr2; also called β -arrestin1; Figure 2b,c) were determined coupled to NTS₁R [35•,36], β_1 AR [37••], M₂R [38•], V₂R [39•] and 5HT_{2B}R [40••].

G proteins couple to different receptors in a relatively conserved way [25], but in contrast arrestins have shown a wide variation in their binding poses. Significant variations occur in the structure of the finger loop of arrestin inserted into the intracellular cleft of the receptor (Figure 2d) and the angle of interaction between arrestin and the GPCR when viewed both perpendicular to the membrane plane and parallel to the membrane plane (Figure 2e–g).

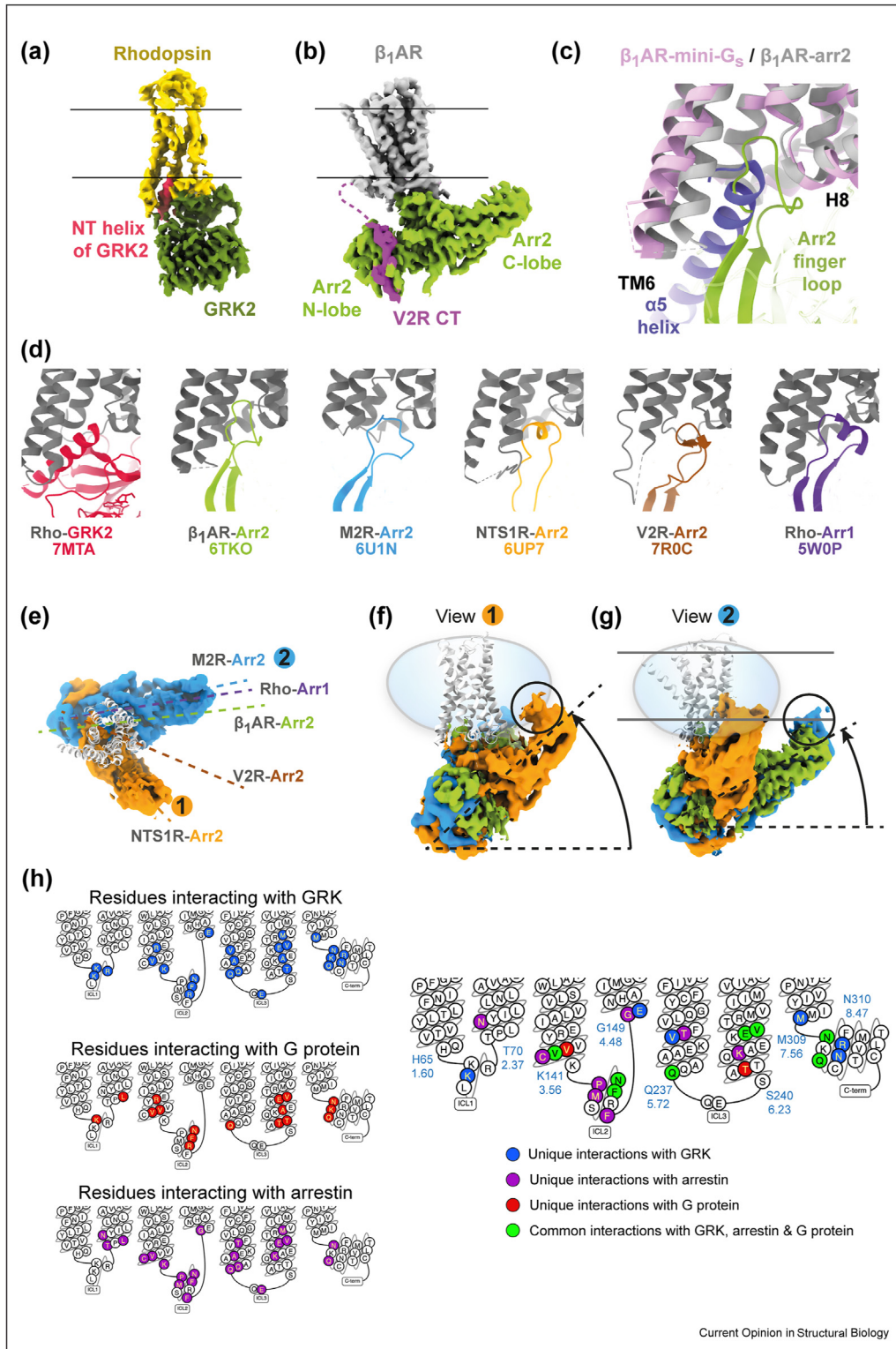
Two GPCR-arrestin structures (β_1 AR [37] and 5HT_{2B}R [40]) have been determined at 3.3 Å resolution where there is good density for the ligand in the orthosteric binding pocket. Comparison with the receptor bound to the same ligand but coupled either to a G protein (5HT_{2B}R) or a G protein-mimetic nanobody (β_1 AR) showed similar weakening of interactions between the ligand and H5, explaining the weaker ligand affinity in the arrestin-coupled state compared to the G protein coupled state. There are also other differences between a G protein-coupled receptor and arrestin-coupled receptor, the most obvious one being the difference in outward movement of H6, although in β_1 AR this is less than in the G protein coupled state whereas for 5HT_{2B}R it is greater than in the G protein coupled state. The differences observed between structures could be used in the development of biased agonists.

GPCR dimers

The existence and functional role of obligate class C and class D GPCR dimers are well-established, both structurally and functionally [41,42]. However, for Class A receptors there is no consensus on whether dimerisation is a ubiquitous mechanism in regulating Class A GPCR function. Some class A GPCRs are accepted to form transient dimers and higher order oligomers, although their physiological role is often uncertain [43,44]. Any structural dimer composed of parallel protomers observed in either X-ray crystal structures [45] or cryo-EM has the potential to be physiologically relevant, but careful validation is required by biochemistry and pharmacology to support this.

Humans possess 22 Class C GPCRs and there are now 76 cryo-EM structures, determined between 2019 and 2022, bound to either antagonist, agonist, positive allosteric modulator (PAM), negative allosteric modulator (NAM), regulator of G protein signalling (RGS) protein and/or G protein. Due to space constraints, we will discuss only those receptors where a

Figure 2



Variations in coupling of arrestins and GRK2 to GPCRs. **(a)** Cryo-EM density (EMDB-23979) of rhodopsin coupled to GRK1 [33]. Density for the F_{ab} required for structure determination has been removed for clarity. **(b)** Cryo-EM density of β_1 AR in a lipid nanodisc coupled to β -arrestin1 (EMDB-10515) [37]. Density for F_{ab30} required for structure determination has been removed for clarity. **(c)** Superposition of β_1 AR coupled to mini- G_s (purple; PDB code 7JJO [72]) and β_1 AR coupled to β -arrestin1 (green; PDB code 6TKO [37]). **(d)** Different conformations of the GRK coupling helix and arrestin finger loop when coupled to different receptors. **(e–g)** Variation in the angle of arrestin coupled to different receptors (see main text for references): **(e)** a view perpendicular to the membrane plane; panels **(f–g)** are views parallel to the membrane plane in positions 1 and 2, respectively, as defined in panel **(e)**. **(h)**

Table 1

Details of GPCR structures discussed in the main text.

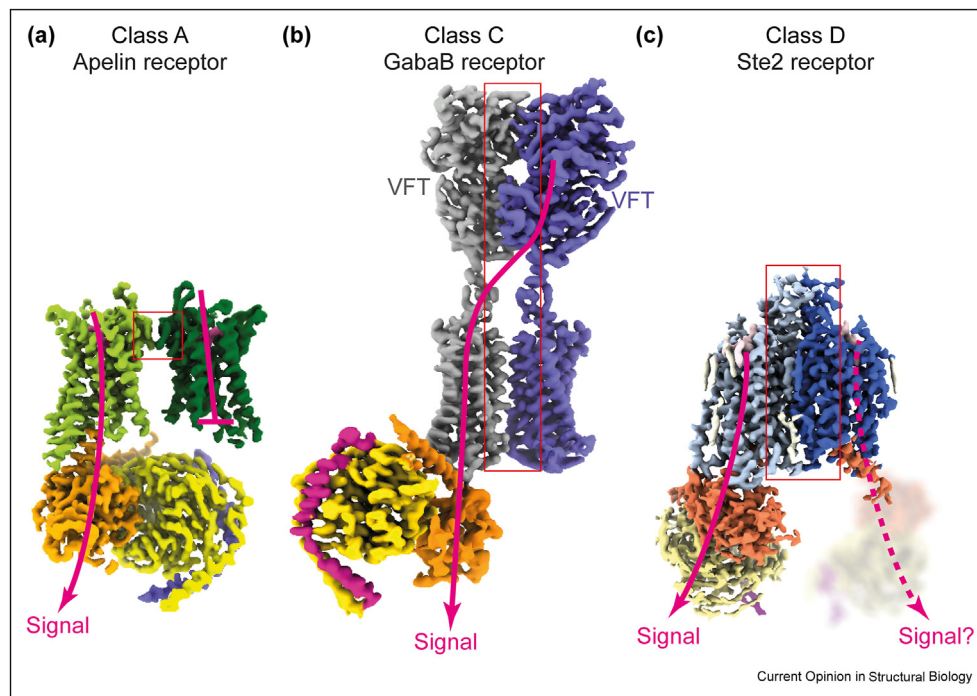
Receptor	Dimer type	Class	PDB	Agonist (Ag), antagonist (Ant), PAM, NAM	G protein family	Stabilising antibodies and fusions	Reference
Apelin	Homo	A	7W0N	Ag	G _i	scFv16 + BRIL	[52●●]
			7W0L	Ag	G _s	scFv16 + BRIL	
Ste2	Homo	D	7AD3	Ag	Gpa1	–	[53●●]
			7QB9	–	–	–	[54●●]
			7QA8	Ant	–	–	
			7QBC	Ag	–	–	
			7QBI	Ag	–	–	
GABA _B	Hetero	C	7EB2	Ag	G _i	scFv16	[47●]
		C	7CA3	PAM	–	–	[49]
		C	7CA5	–	–	–	
		C	7CUM	Ant + NAM	–	–	
		C	6UO8	Ag + PAM	–	–	[50●]
		C	6UO9	Ag	–	–	
		C	6UOA	Ag	–	–	
		C	6VJM	APO	–	–	
		C	7C7S	Ant	–	–	[46●●]
		C	7C7Q	Ag + PAM	G _{i1}	–	
		C	6WIV	–	–	–	[55]
		C	6W2X	Ant + NAM	–	–	[56]
		C	6W2Y	Ant + NAM	–	–	
Metabotropic glutamate receptors	Homo	C	7DGD	–	–	–	[57]
mGlu1	Homo	C	7DGE	Ag	–	Nb43	
mGlu2		C	7E9G	Ag + PAM	G _i	scFv16 + Nb	[48●]
mGlu2	Homo	C	7MTQ	Ant	–	–	[51●]
		C	7MTR	Ago-PAM + Ag	–	–	
		C	7MTS	Ago-PAM	G _i	–	
mGlu2	Homo	C	7EPA	–	–	–	[58●]
		C	7EPB	Ag	–	Nb-RON	
mGlu7	Homo	C	7EPC	–	–	–	
mGlu2mGlu7	Hetero	C	7EPD	–	–	–	
mGlu5	Homo	C	6N52	–	–	–	[59]
		C	6N51	Ag	–	Nb43	
mGlu5-5M	Homo	C	7FD8	Ag	–	–	[60]
		C	7FD9	Ant	–	–	
mGlu3	Homo	C	7WI8	Ant	–	–	[61]
		C	7WI6	Ag + NAM	–	–	
		C	7WIH	Ag	–	–	
mGlu4	Homo	C	7E9H	Ag	G _{i3}	scFv16	[48●]

fully active G protein-coupled state has been determined (Table 1), namely the GABA_B receptor [46●●,47●] and metabotropic glutamate receptors (mGluRs) [48●]. The common feature of Class C dimers is that they are maintained dimeric predominantly through interactions in the extracellular Venus fly trap domain (VFT; Figure 3b) that binds agonists. The agonist-induced conformational change in the VFT is transmitted via a linker region to the transmembrane regions, ultimately resulting in a rotation of one helical bundle with respect to the other. In the GABA_B receptor, this changes the dimer interface from

being formed by predominantly H5-H5 to H6-H6 [46●●,47●] and in the mGluRs from mainly H4-H4 to H6-H6 [48●]. A number of variations between these states have also been described, highlighting the plasticity of these receptors and a number of different solutions for how PAMs can promote the formation of active-like states [46●●,48●,49,50●]. Extensive pharmacological and biochemical studies have determined that only one protomer in the dimer couples to a G protein and that signalling is transmitted from the VFT of one receptor in the dimer to the G protein coupling site on the adjacent dimer [41]. This is recapitulated in

Snake plots of bovine rhodopsin with amino acid residues within 3.9 Å (inclusive) of either GRK, G protein or arrestin coloured appropriately. PDB codes for the complexes are as follows: rhodopsin-GRK, 7MTB [33●●]; rhodopsin-G protein, 6OYA [73]; rhodopsin-arrestin, 5WOP [74]. The panels were made using GPCRdb [75].

Figure 3



Signalling routes in GPCR dimers. **(a)** Cryo-EM density of the apelin receptor (EMDB-32243) shows that there is sufficient room for only one G protein to couple per dimer, and the C-terminus of the adjacent protomer binds in the G protein-coupling cleft in an auto-inhibitory mechanism [52]. The dimer interface is shown by in the GABAB receptor dimer (EMDB-21534) is from the VFT domain of one protomer through the transmembrane helices of the adjacent protomer that can couple to G protein. The structures of two transmembrane helical bundles are not identical and the G protein coupling site forms only in one protomer [46,47]. The dimer interface is shown by the red box. **(c)** The Ste2 dimer (EMDB-11720) contains two protomers of identical conformation that are both capable of coupling to G proteins simultaneously, although one G protein is highly mobile, with the exception of the $\alpha 5$ helix that is ordered where it contacts the receptor [53]. The tilt of the G protein with respect to the receptor is over 50° different from that observed in G protein-coupling to Class A receptors, thus allowing two G proteins to couple simultaneously. The signalling pathways through the receptor are assumed to follow the paradigm of a monomeric receptor, however it is unclear whether both G proteins can signal to the same extent and there could be crosstalk between protomers across the dimer interface [54]. The dimer interface is shown by the red box.

the asymmetric active-state dimer structures where only a single G protein is coupled per dimer, via a coupling site formed through interactions primarily to ICL2, which is distinct to that found in other GPCR families [47,48,51].

In contrast to Class C receptors, the cryo-EM structure of the class D receptor homodimeric GPCR Ste2 (Figure 3c) showed that it couples to two G proteins simultaneously [53]. The density for one G protein was well-resolved, but the density for the adjacent G protein was diffuse and molecular dynamics simulations showed that each G protein underwent phases of mobility, with only one G protein being ordered at any one time. The interface between the two protomers is also dynamic [54], even though it has a very large surface area in the active state (2500 \AA^2) and is composed of interactions between the N-terminus, ECL1 and H1. Cryo-EM structures of five different receptor conformations showed that Ste2 activation upon binding the native agonist α -factor involved an increase in the strength of the interface and a 20 \AA

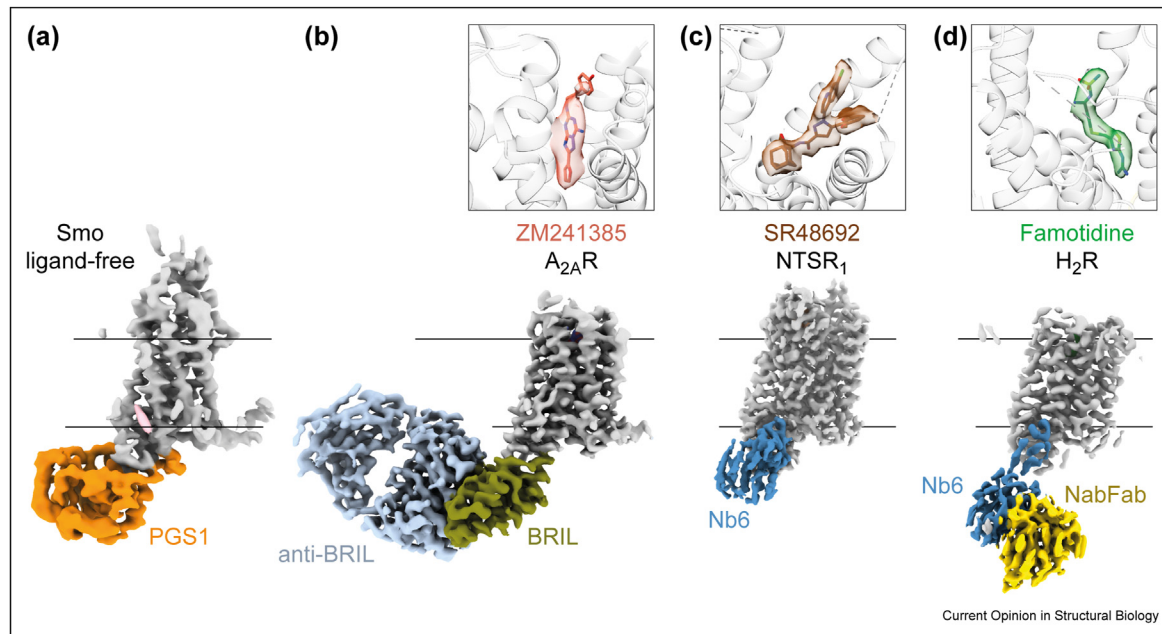
movement of the cytoplasmic end of H7 [54]. The movement of H7 unblocked the G protein coupling site and then formed additional contacts at the dimer interface in a mechanism currently unique to Ste2.

There is currently only one high-resolution structure of a Class A GPCR dimer, the active state of the apelin receptor [52]. This is different from dimers of Class C and Class D receptors as the interface is extremely small (140 \AA^2), comprising residues at the extracellular end of H3 (Figure 3a). Only one of the protomers is coupled to a G protein, and there are no contacts between the G protein and the adjacent protomer. Mutation of a key residue at the dimer interface (F101 A^{3.24}) significantly reduced dimer formation and had a profound effect on the pharmacology of the apelin receptor, increasing basal activity and E_{\max} significantly.

Inactive GPCR structures by cryo-EM

The inactive state of GPCRs may only consist of 35–40 kDa of ordered protein, which is embedded in a detergent micelle typically $\sim 100 \text{ kDa}$ in size and makes

Figure 4



Examples of strategies to determine structures of GPCR inactive states. **(a)** Cryo-EM density of ligand-free Smoothened (EMDB-27062) [69]. **(b)** Cryo-EM density (EMDB-25648) of the adenosine A_{2A} receptor with a BRIL insertion in ICL3 and bound to an anti-BRIL Fab fragment [69]. **(c)** Cryo-EM density (EMDB-26589) of the neurotensin receptor NTSR1 engineered to contain the H5-ICL3-H6 region of MOR and bound to the anti-MOR nanobody Nb6 [67]. **(d)** Cryo-EM density (EMDB-26590) of the histamine H_2 receptor engineered to contain the H5-ICL3-H6 region of MOR, bound to the anti-MOR nanobody Nb6 and the anti-nanobody F_{ab} (NabFab) [67]. Ligand density in the orthosteric binding pocket is shown above each receptor.

processing of cryo-EM images of these small membrane proteins highly challenging. To circumvent this problem, extra mass needs to be added to the receptor that can extend beyond the detergent micelle and facilitate particle alignment during image processing. An obvious solution is to repurpose successful strategies in engineering GPCRs for X-ray structure determination through either binding an antibody F_{ab} fragment [62], nanobody [63] or insert a small soluble protein such as BRIL in ICL3 [64].

One recent approach was to graft a section of H5-ICL3-H6 from the mu opioid receptor (MOR) into a target GPCR and then bind nanobody Nb6 that specifically recognises this region [65,66]. This resulted in sub-3 Å resolution structures of the inactive states of NTS $_1$ R, H_2 R (Figure 4c,d) and somatostatin receptor 2 [67]. Another approach was to insert BRIL in place of ICL3 in Frizzled5 and then use an anti-BRIL F_{ab} /Nb complex to increase the mass further; the structure was determined by single-particle cryo-EM to 3.7 Å resolution, with the low resolution being explained by the flexibility of the GPCR-BRIL fusion points [68]. This methodology was explored further [69] to determine the structure of thermostabilised A_{2A} R-BRIL bound to an anti-BRIL F_{ab} to 3.4 Å resolution (Figure 4b) and a Smoothened ICL3 chimera fused to *Pyrococcus* glycogen synthase (PGS) at 3.7 Å resolution (Figure 4a). A recent innovative strategy to create a

three-point linkage between the heterodimer calcineurin and the β_2 AR facilitated the structure determination of the receptor either in the ligand-free state or bound to antagonist/agonist with overall resolutions between 3.5 and 3.9 Å [70].

Conclusions

The incredible advances in all the technology involved in single particle cryo-EM have made the structure determination of GPCR complexes in all conformational states considerably easier than using X-ray crystallography [71]. There are more advances in the cryo-EM pipeline and so the future holds rich promise for improving the throughput of GPCR structure determination, making it the premier tool for structure-based drug design and the determination of novel GPCR structures. A concerted effort over the coming years will undoubtedly determine structures of all human non-olfactory GPCRs.

Declaration of competing interest

CGT is a shareholder and SAB member of Sosei Heptares. None of the other authors have any conflicts to declare.

Data availability

No data was used for the research described in the article.

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- of special interest
- of outstanding interest

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