TECHNOLOGIES AND TECHNIQUES

Methodological advances: the unsung heroes of the GPCR structural revolution

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Abstract | G protein-coupled receptors (GPCRs) are intricately involved in a diverse array of physiological processes and pathophysiological conditions. They constitute the largest class of drug target in the human genome, which highlights the importance of understanding the molecular basis of their activation, downstream signalling and regulation. In the past few years, considerable progress has been made in our ability to visualize GPCRs and their signalling complexes at the structural level. This is due to a series of methodological developments, improvements in technology and the use of highly innovative approaches, such as protein engineering, new detergents, lipidic cubic phase-based crystallization and microfocus synchrotron beamlines. These advances suggest that an unprecedented amount of structural information will become available in the field of GPCR biology in the coming years.

G protein-coupled receptors (GPCRs), also known as seven-transmembrane (7TM) receptors, are crucial components of numerous cellular signalling cascades[1,2]. There are ~800 GPCRs in the human genome, which makes GPCRs the largest family of cell-surface receptors[3]. GPCRs are classified into six classes based on sequence homology and functional similarities[4]. These are Class A (rhodopsin-like receptors), Class B (the secretin receptor family), Class C (metabotropic glutamate receptors), Class D (fungal mating pheromone receptors), Class E (cyclic AMP receptors) and Class F (Frizzled and Smoothened receptors). Signalling pathways mediated by GPCRs are involved in numerous physiological events such as olfaction, taste, cognition, the regulation of blood pressure, the immune response, behaviour and mood[2,5]. Currently, ~30–40% of marketed drugs target GPCRs, and these drugs include β-blockers, angiotensin receptor blockers, opioid agonists and histamine receptor blockers[6]. Moreover, aberrant GPCR signalling underlies the onset of many disease conditions such as hypertension, diabetes, sepsis, obesity and cancer[5,7]. Although GPCRs share a common overall architecture, they can recognize a diverse array of signals, including photons of light, small molecules, hormones, peptides, lipids and proteins. Furthermore, their mechanisms of activation, signalling and regulation are remarkably conserved and serve as an example of evolutionary convergence[8–10] (FIG. 1). The binding of an agonist ligand to a GPCR stabilizes an active conformation of the receptor, which couples to heterotrimeric G proteins that are composed of Gα, Gβ and Gγ subunits. Subsequently, heterotrimeric G proteins dissociate from the receptor and G protein signalling generates second messengers such as cAMP, inositol phosphates and Ca^{2+}, which trigger different cellular responses. The phosphorylation of activated receptors, primarily by GPCR kinases, enables GPCRs to bind to multifunctional scaffold proteins, called β-arrestins, with high affinity[11–13]. β-arrestins prevent further G proteins from coupling to the activated receptor and desensitize G protein signalling[14]. Receptor-bound β-arrestins can also nucleate the machinery required for clathrin-mediated endocytosis, thereby promoting GPCR internalization[15]. β-arrestins are also independent signal transducers, influencing signalling events, such as the activation of mitogen-activated protein kinases that regulate the cytoskeleton, protein synthesis, cell migration and apoptosis, independent of G proteins[8,16–18].

Until recently, the crystallization of GPCRs and the determination of their structure were considered to be almost impossible tasks. However, since the first structure of a GPCR (that of the human β_2-adrenoceptor) was solved in 2007, remarkable progress has been made in this area[19,20]. Currently, the crystal structures of 26 GPCRs have been determined bound to antagonists, agonists or biased ligands. Perhaps one of the most important structural advances, not only in the area of GPCRs but also

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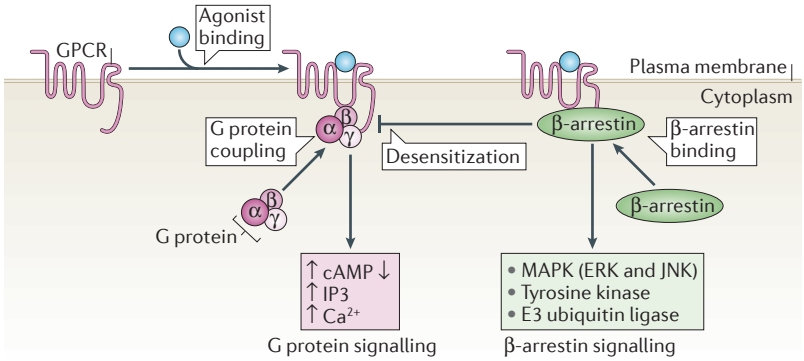

Figure 1 | **A simplified schematic of GPCR signalling.** Binding of an agonist (activating ligand) induces a conformational change in the G protein-coupled receptor (GPCR) to activate it. Activated receptors couple to heterotrimeric G proteins composed of Gα, Gβ and Gγ subunits. Subsequently, the heterotrimeric G proteins dissociate and G protein signalling mediates the generation of second messengers such as cyclic AMP, inositol triphosphate (IP3) and Ca^{2+}. Activated receptors are phosphorylated, primarily in the carboxyl terminus, by GPCR kinases. Phosphorylated receptors recruit β-arrestins, which are multifunctional adaptor proteins that block further G protein–GPCR coupling, potentially through a steric hindrance mechanism (referred to as desensitization). β-arrestins also mediate clathrin-dependent endocytosis of activated GPCRs as well as independent signalling pathways downstream of GPCRs. β-arrestins scaffold mitogen-activated protein kinases (MAPKs; such as extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK)), tyrosine kinases (such as Src) and E3 ubiquitin ligases (such as atrophin-1-interacting protein 4 (AIP4)). The arrows next to cAMP indicate that cAMP levels can go up or down in response to GPCR activation.

in the history of membrane protein crystallography, was the structure of a GPCR–G protein signalling complex[21]. This was followed by the direct visualization of a GPCR–β-arrestin complex using electron microscopy, although this was at a lower resolution than the GPCR–G protein crystal structure[22,23]. In summary, this has been one of the most productive eras in decades of GPCR research.

In this Review, we highlight the methodological advances — at the level of protein engineering, lipid-based crystallization approaches, micro-crystallography and synthetic biology — that enabled these GPCR structures to be determined. We also highlight how GPCRs and their signalling complexes are being used as a model system to push the boundaries of emerging technologies. Finally, we underline how new biophysical and structural approaches that are still in their infancy are likely to change the face of GPCR research and pave the way for novel drug design and discovery.

The challenges of GPCR crystallography

Crystallizing membrane proteins, particularly GPCRs, is challenging; as their native expression is low, it requires the heterologous production of large quantities of recombinant proteins, which must be solubilized and isolated from the membrane in a functional and biochemically stable form. The inherent conformational flexibility of the receptors must also be addressed as it can hinder the formation of well-ordered crystals. Finally, a series of crystallization conditions must be systematically screened to identify those that permit crystallization. Once preliminary crystals are obtained, their diffraction properties must be optimized to collect a high-resolution data set and solve the crystal structure.

Initial efforts to crystallize GPCRs suffered from numerous limitations, including low expression levels of recombinant receptors in hosts, inefficient solubilization and purification of functional receptors, and unstable purified receptors (FIG. 2). Optimized methods for expressing recombinant proteins and protein engineering, as well as the synthesis of new detergents and the development of innovative protein purification strategies, helped to overcome these limitations. Moreover, conventional crystallography methods failed to yield crystals even when suitable samples were available, which led to the need for sophisticated lipid-based crystallization approaches. Furthermore, even very powerful synchrotron-based X-ray sources were not optimal for determining the structure of GPCR microcrystals and required microfocus beamlines for diffraction data collection. Major breakthroughs at every step in the process were necessary to crystallize GPCRs and we describe these below.

Producing enough recombinant protein

An initial challenge encountered in determining GPCR structures was producing enough recombinant protein. In contrast to the photoreceptor rhodopsin, which is packed at high density in the retina, most other GPCRs are present in miniscule amounts in native tissues. To produce these GPCRs in sufficient quantities, several recombinant protein expression hosts were tested, including *Escherichia coli*, yeast species such as *Pichia pastoris* and mammalian cells (using virus-based expression methods)[24–31]. However, the first structure of the β_2-adrenoceptor was obtained using protein purified from baculovirus-mediated expression in insect cells[19,32]. Although the baculovirus expression system itself is not new, using it to produce high levels of β_2-adrenoceptor required optimization. Now, this system is used extensively to yield high-quality recombinant GPCRs for crystallography and has been the approach used for the majority of receptors that have been crystallized so far (see Supplementary information S1 (table)).

More recently, receptors produced by and purified from other expression hosts, such as *P. pastoris* (for example, the histamine type 1 receptor)[33] and *E. coli* (for example, a thermostabilized neurotensin receptor[34]), have also yielded diffraction-quality crystals and led to the determination of structures. Interestingly, tetracycline-inducible expression systems in mammalian cells have also yielded high levels of several GPCRs, although the determination of the structure of a non-rhodopsin GPCR produced in this system remains to be documented[35–38]. These developments highlight that multiple expression systems can yield high-quality protein for crystallography and that a lack of technical expertise in using a specific host may not limit success.

In addition to using the best expression host, the addition of antagonist or inverse agonist ligands to the culture medium also enhances the expression level of functional receptors and increases their stability[39]. For example, the presence of alprenolol, a low-affinity β_2-adrenoceptor-specific antagonist, in the culture medium enhanced the expression level of functional β_2-adrenoceptors[40].

Synchrotron-based X-ray sources
These are powerful X-ray sources that are used to collect high-resolution X-ray diffraction data on three-dimensional crystals. Examples of the synchrotron X-ray sources utilized in G protein-coupled receptor crystallography are the Advanced Photon Source in Chicago (USA) and the European Synchrotron Radiation Facility in Grenoble (France).

Microfocus beamlines
Next-generation X-ray sources at synchrotron facilities that are suitable for the structural analysis of microcrystals (in the size range of 5–20 μm). The most commonly used microfocus beamlines for G protein-coupled receptor crystallography are the ID23 beamline at the European Synchrotron Radiation Facility in Grenoble (France), the I24 beamline at the Diamond Light Source in Oxfordshire (UK) and the 23ID beamline at the Advanced Photon Source in Chicago (USA).

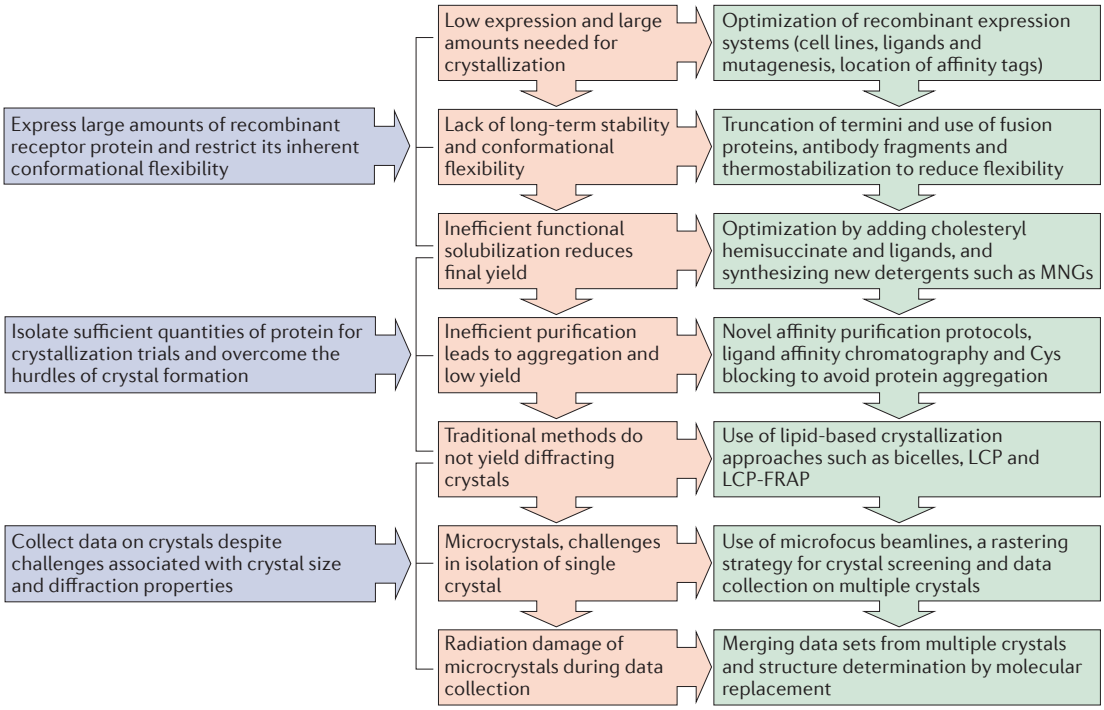

Figure 2 | **Overview of GPCR crystallography, its major challenges and methodological solutions to these challenges.** The crystallization of G protein-coupled receptors (GPCRs) and the determination of their structure are challenging, but have been made possible by the optimization of the protein production, crystallization and structure determination steps in the process. These steps are depicted in blue boxes, with the problems that were associated with them in red boxes and the innovative methodological approaches that overcame them in green boxes. Overcoming these technical challenges was pivotal in the GPCR 'structural revolution'. FRAP, fluorescence recovery after photobleaching; LCP, lipidic cubic phase; MNGs, maltose neopentyl glycols.

The addition of ligands is thought to reduce the constitutive endocytosis of receptors. Often, it is preferable to use an antagonist that has low affinity for the receptor because an agonist might initiate receptor activation and internalization, and in turn adversely affect receptor expression levels. Low-affinity ligands can also be washed off and exchanged with another ligand of choice during subsequent purification steps.

Engineering crystallizable proteins

GPCRs have a high degree of inherent structural and conformational flexibility and, although this is crucial for receptor coupling to multiple effectors and diverse signalling mechanisms, this flexibility restricts the formation of well-ordered crystals that are required for high-resolution diffraction. The amino and carboxyl termini of many GPCRs are structurally dynamic and the third intracellular loop is highly heterogeneous in conformation[41,42]. The flexible N and C termini can be removed by serial truncation while maintaining the ligand-binding properties of the receptors. However, the preparation of highly stable receptor proteins with truncated termini did not yield diffracting crystals, which led to the need for additional approaches for engineering stable GPCRs for crystallography, including the use of antibody fragments, the fusion of receptors with small, easily crystallizable proteins, and GPCR thermostabilization.

Antibody fragments. The third intracellular loop of GPCRs has a flexible conformation and this was thought to be a major bottleneck in obtaining diffracting crystals. To address this, an antibody fragment-mediated crystallization approach was used[19,43]. This approach uses an antibody fragment against a three-dimensional epitope to restrict the conformational flexibility of the target protein, thereby providing a conformationally homogeneous sample for crystallization. Furthermore, membrane proteins purified in detergent micelles often have only small soluble domains (also referred to as the polar surface area) to mediate crystal contacts and this also poses a challenge in their crystallization. The presence of antibody fragments, which are well-folded soluble proteins, also provides an extended polar surface area to facilitate the crystallization of membrane proteins.

This approach was pioneered for obtaining crystals of a bacterial cytochrome bc1 complex[44] and was subsequently used to obtain crystals of several other membrane proteins[45–48]. Several mouse monoclonal antibodies were generated against the β_2-adrenoceptor and characterized for their ability to bind its third intracellular loop[43]. The antigen-binding fragment (Fab) of one of these mouse monoclonal antibodies (referred to as Fab5) facilitated the crystallization of the β_2-adrenoceptor and yielded well-diffracting crystals. This breakthrough resulted in the crystal structure of the first non-rhodopsin GPCR[19] (FIG. 3a). Interestingly, only one other GPCR,

Inverse agonist
Most G protein-coupled receptors when overexpressed display a certain degree of basal or constitutive signalling. Inverse agonists bind to the receptor and reduce its basal or constitutive activity.

Antigen-binding fragment
(Fab). Fab is the region on the antibody that binds antigens and is composed of a heavy chain constant and variable domain, and a light chain constant and variable domain.

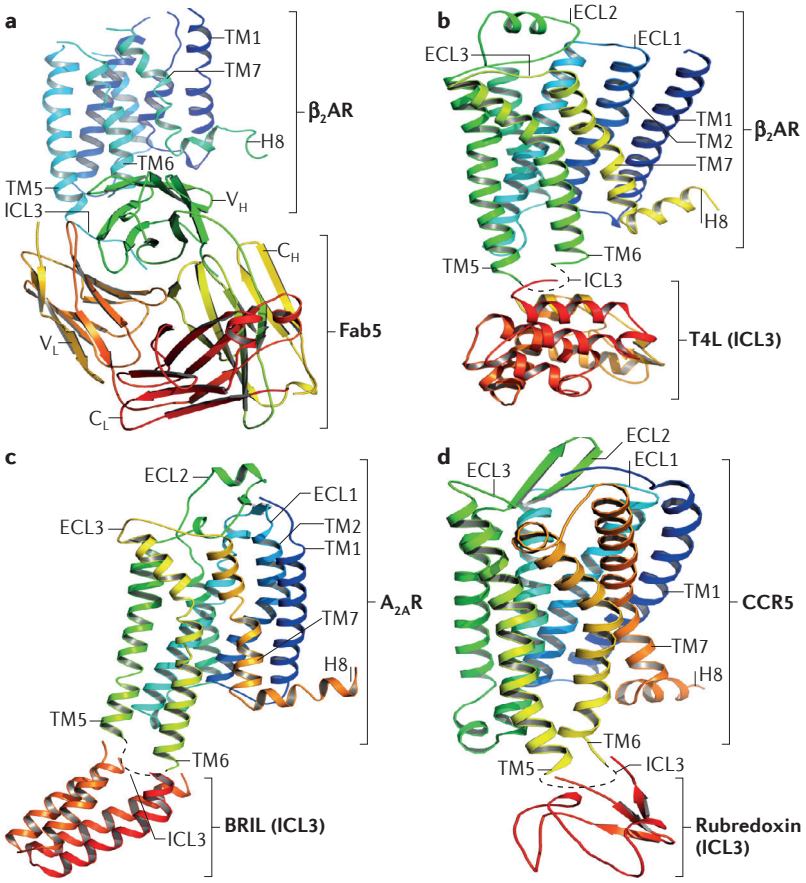

Figure 3 | **Protein engineering approaches to stabilize the third intracellular loop of GPCRs for crystallography.** To determine G protein-coupled receptor (GPCR) structures, large quantities of stable proteins must be produced and conformational flexibility should be minimized. Several approaches have been used to achieve this. **a** | The crystal structure of a β_2-adrenoceptor (β_2AR) bound to an inverse agonist and stabilized by a mouse antigen-binding antibody fragment (Fab), known as Fab5 (RCSB Protein Data Bank (PDB) ID: 2R4R). Fab5 binds to and stabilizes the third intracellular loop (ICL3) of β_2AR. Receptor transmembrane (TM) helices are shown in cyan and blue. Underneath the receptor, the green and orange domains represent the variable heavy (V_H) and variable light (V_L) chains, respectively, of Fab. The yellow and red domains represent the constant regions of the heavy (C_H) and light (C_L) chains, respectively, of Fab. **b** | Fusion of T4 lysozyme (T4L) to the ICL3 of the β_2AR stabilized the receptor and yielded a high-resolution crystal structure (PDB ID: 2RH1). The TM5 and TM6 domains, which are connected by the ICL3 the receptor, are labelled to highlight the fusion position of the T4L. A significant portion of the ICL3 is either deleted or not well resolved in this crystal structure, and is indicated here (and in parts **c** and **d**) by the dashed line. **c** | Fusion of thermostabilized apocytochrome b_{562} (BRIL) to the ICL3 of the adenosine A_{2A} receptor (A_{2A}R) facilitated its crystallization (PDB ID: 4EIY). **d** | Fusion of rubredoxin to the ICL3 of the chemokine receptor CCR5 yielded a high-resolution structure (PDB ID: 4MBS). Fusion of T4L, BRIL and rubredoxin reduce the conformational flexibility of the receptors and provide an additional polar surface area to mediate crystal contacts during crystallization. These figures were generated using PyMOL and presented in an N terminus up and C terminus down orientation. The colour scheme represents the standard output of structural images by PyMOL. For a given structure, the PyMOL output covers a spectrum, starting with blue at the amino terminus and ending with red at the carboxyl terminus. ECL, extracellular loop; H8, helix 8.

the adenosine A_{2A} receptor, has been crystallized in complex with an antibody fragment[49]. Considering the challenges associated with the generation of hybridoma-based monoclonal antibodies (for example, it is time-consuming, expensive and tedious, and requires the preparation of large quantities of fragments), it is not surprising that this approach has not become as popular for GPCR crystallography as the fusion approach that is described below.

Fusion approach. In parallel to the use of antibody fragments, researchers sought to stabilize GPCRs by replacing the flexible third intracellular loop of the β_2-adrenoceptor with bacterial T4 lysozyme (T4L)[32,50]. T4L is a well-folded and highly crystallizable protein that forms diffracting crystals under a range of conditions. More importantly, the N and C termini of T4L are in close proximity, making it suitable to maintain the relative orientation of the third intracellular loop. This innovative approach yielded a crystal structure of the β_2-adrenoceptor with a higher resolution than that obtained using Fab-mediated crystals[32,50] (FIG. 3b). Fusion of T4L to the third intracellular loop not only restricts the conformational flexibility of the loop but also that of the fifth transmembrane (TM5) and sixth transmembrane (TM6) domains, which are connected by the third intracellular loop. Furthermore, T4L fusion also provides an extended polar surface area to mediate crystal contacts and crystallization, similar to antibody fragments. Several GPCRs in addition to the β_2-adrenoceptor have now been crystallized using this approach[33,51–64] (see Supplementary information S1 (table) for a list of GPCRs crystallized using this strategy). The generality of this fusion approach highlights that GPCR transmembrane domains have a common arrangement with a flexible conformation that is required for different signalling outcomes.

Inspired by the generality of the T4L fusion approach, a subsequent study tested a wide variety of potential fusion proteins — including rubredoxin, xylanase, flavodoxin and thermostabilized apocytochrome b_{562} (BRIL) — for their ability to stabilize GPCR structures for crystallization[65]. The fusion of BRIL to the third intracellular loop of the adenosine A_{2A} receptor enhanced the diffraction of the resulting crystal[66] (FIG. 3c). More recently, the chemokine receptor CCR5 was crystallized as a fusion with rubredoxin[67] (FIG. 3d). Interestingly, corticotropin-releasing factor 1 receptor, a Class B GPCR, was crystallized with T4L fused to its second intracellular loop instead of the third intracellular loop[63] (FIG. 4a). It is important to note that as the third intracellular loop of some GPCRs is relatively small, it might be possible to crystallize such receptors with native third intracellular loops. Diffracting crystals of a mutant β_2-adrenoceptor with a truncated third intracellular loop were obtained by fusing its N terminus to T4L[68] (FIG. 4b) and diffracting crystals of the nociception/orphanin FQ receptor were obtained by fusing its N terminus to BRIL[59] (FIG. 4c). Collectively, these studies demonstrate that a tailored fusion strategy can be used to crystallize a range of GPCRs.

Thermostabilization. Another approach to stabilize GPCRs that was pioneered for the crystallization of the β_1-adrenoceptor (FIG. 4d) and has been used for other GPCRs is thermostabilization[69–71]. A series of β_1-adrenoceptor mutants were made and tested in radioligand binding assays for their ability to bind their ligands at increasing temperatures; this resulted in the

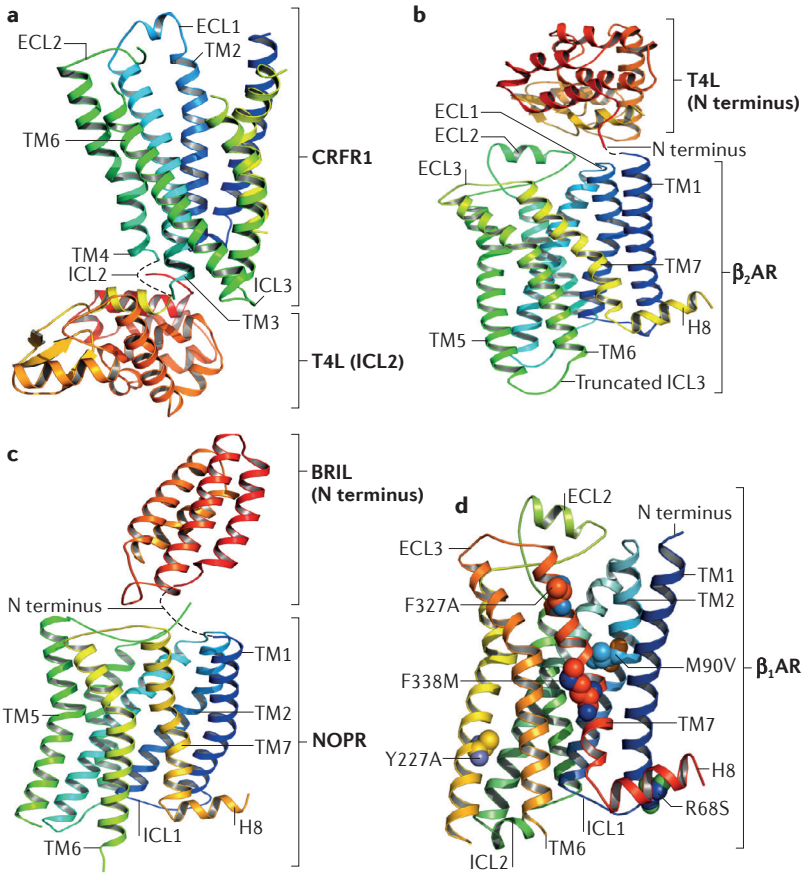

Figure 4 | **Protein engineering approaches to stabilize GPCRs outside the third intracellular loop for crystallography.** In addition to stabilizing the third intracellular loop (ICL3) of G protein-coupled receptors (GPCRs), fusion approaches can stabilize the external surface of the receptor and the second intracellular loop (ICL2). Furthermore, a thermostabilization approach was used to stabilize the overall core of the receptor to facilitate crystallization. **a** | The corticotropin-releasing factor 1 receptor (CRFR1), a Class B GPCR, was thermostabilized and crystallized as a fusion of T4 lysozyme (T4L) in the ICL2 (RCSB Protein Data Bank (PDB) ID: 4K5Y). The third transmembrane (TM3) and TM4 domains are connected via the ICL2, which is indicated by the dashed line as this crystal was not well resolved and cannot be clearly labelled. **b** | Amino-terminal fusion of T4L and truncation of the ICL3 also resulted in diffracting crystals of the β_2-adrenoceptor (β_2AR; PDB ID: 4GBR). The junction between the N terminus of β_2AR and T4L was not well resolved and is indicated by a dashed line. **c** | Fusion of apocytochrome b_{562} (BRIL) to the N terminus of the nociception/orphanin FQ receptor (NOPR) yielded a high-resolution structure (PDB ID: 4EA3). These findings suggest that for receptors that contain a relatively short, and perhaps less flexible, ICL3, N-terminal fusion of T4L or BRIL might be a viable strategy to promote crystallization. The junction between the N terminus of NOPR and BRIL was not well resolved and is indicated by a dashed line. **d** | A thermostabilization approach known as StaR (stabilized receptor) technology was pioneered for the crystallization of the turkey β_1AR (PDB ID: 2VT4) and this technology has been utilized for the crystallization of several other GPCRs. The amino acids that were mutated to generate a crystallizable mutant receptor are highlighted (side chains shown as space fill) and labelled. These figures were generated using the software PyMOL and presented in an N terminus up and C terminus down orientation. The colour scheme represents the standard output of structural images by PyMOL. ECL, extracellular loop; H8, helix 8.

identification of β_1-adrenoceptor mutants with improved thermostability compared with the wild-type receptor. This technology, which is referred to as StaR (stabilized receptor), reduces the conformational flexibility of the receptors and stabilizes them in a particular conformation. StaR has been used to yield well-diffracting crystals for several GPCRs[72]. For some GPCRs — such as the free fatty acid type 1 receptor[73], the neurotensin type 1 receptor[74] and the metabotropic glutamate subtype 5 receptor[62] — thermostabilization was used in combination with T4L fusion to yield high-diffracting crystals. Considering how rapidly the field of GPCR crystallography has evolved in the past few years, it would not be surprising if additional strategies are devised to capture GPCRs in a crystallizable format.

Isolation of recombinant GPCRs

Once a robust expression strategy has been identified and conformational flexibility has been addressed, GPCRs must be extracted from the lipid bilayer in a functional state and then highly purified. Robust extraction and reproducible purification protocols are crucial to obtain high-quality protein samples for structural studies, including crystallization.

Solubilization of GPCRs. The first crystal structure of the β_2-adrenoceptor utilized a receptor purified in dodecyl maltoside (DDM), the most commonly used detergent in membrane protein crystallography. However, the addition of a detergent-soluble cholesterol derivative, called cholesteryl hemisuccinate (CHS), can considerably enhance the functional solubilization and stability of GPCRs[75,76]. A systematic study evaluating the effect of various cholesterol analogues on GPCR stability revealed that CHS increases the size of DDM micelles and presumably forms a bicelle-like lipid detergent environment. This native-like environment appears to maintain solubilized receptors in a functional state for longer periods[76]. The positive effect of CHS on GPCR stability seems to be general and most of the GPCRs crystallized to date were solubilized in a CHS-detergent mixture.

Recently, maltose neopentyl glycols (MNGs), which are a new class of amphiphile, have been developed and characterized for their ability to aid GPCR solubilization[77]. In contrast to DDM, MNGs have two maltose units in their hydrophilic region and two n-decyl chains in their hydrophobic region with a central quaternary carbon, which is likely to yield a less flexible structure. Analysis of β_2-adrenoceptor thermal stability revealed that MNG-3 enhances the melting temperature of the solubilized receptor to a greater degree than DDM[77]. Moreover, the muscarinic acetylcholine type 3 receptor maintains its ability to bind radioligands for longer periods in MNG micelles than in DDM. Several GPCRs have now been purified in MNG micelles and subsequently crystallized in lipidic cubic phase (LCP), and MNG-3 has been pivotal to the successful visualization of active GPCR conformations and GPCR signalling complexes (discussed further below). Several other detergents and amphiphiles have been synthesized and assessed for their suitability in GPCR solubilization and purification, including glucose neopentyl glycol[78], novel tripod amphiphiles[79], steroid-based facial amphiphiles[80,81] and dodecyl trehaloside[81]. As detergents have a crucial role in GPCR crystallography, the synthesis of new detergents is an active area of research that is likely to yield a range of detergents that suit the specific needs of different GPCRs.

Amphiphile
A compound that has both lipophilic and hydrophilic properties.

Target-specific purification of GPCRs. GPCRs expressed at high levels in recombinant systems must be efficiently purified for crystallization. The first crystal structure of a recombinant β_2-adrenoceptor resulted from its purification by FLAG antibody affinity chromatography followed by an alprenolol-based ligand-affinity purification step. This ligand affinity purification scheme, which was developed several decades ago[82], selectively isolates functional receptors (that is, receptors capable of ligand binding) and eliminates misfolded, non-functional receptors, and was thought to be key in yielding diffraction-quality crystals of the β_2-adrenoceptor[83,84]. Most β_2-adrenoceptor and β_1-adrenoceptor crystal structures have used alprenolol-based functional purification. Subsequently, however, several GPCRs, including the β_2-adrenoceptor, were crystallized using epitope tag-based affinity chromatography through either a combination of FLAG antibody affinity chromatography and immobilized metal affinity chromatography (IMAC), or IMAC alone. The neurotensin receptor, which was crystallized using a thermostabilized construct (without T4L fusion), was purified using a combination of neurotensin-based ligand affinity chromatography and cation exchange chromatography steps[34]. Collectively, these studies indicate that although ligand affinity chromatography might have added advantages for the selective isolation of a functional receptor, it may not be essential for generating high-quality protein that is suitable for crystallization.

Maintaining functional stability via high-affinity ligands. In their ligand-free state, GPCRs are thought to be present in different conformations, ranging from inactive to active. Ligand binding drives receptors to adopt one major population, which promotes nucleation (the initial step in protein crystallization) and proper crystal packing (the arrangement of protein layers to yield crystals). A series of ligands were tested for their ability to aid β_2-adrenoceptor crystallization, and carazolol (a high-affinity inverse agonist) bound to the receptor with subnanomolar affinity and with a slow off-rate[43]. Most of the GPCRs crystallized to date were crystallized in complex with a high-affinity ligand. For the successful crystallization of an active conformation of the β_2-adrenoceptor, two parallel approaches aimed at reducing its conformational flexibility through high-affinity ligand binding were used. First, a covalent agonist ligand was designed based on the previous crystal structure of the β_2-adrenoceptor[85]. This covalent agonist irreversibly binds the receptor and, therefore, robustly stabilizes an active conformation. Second, an agonist of extraordinarily high affinity and extremely slow off-rate, BI-167107, was identified from a collection of ligands, again with the intention of being used to stabilize an active receptor conformation for crystallization[85,86]. Recently, the covalent tethering of agonists was extended to other GPCRs to stabilize an active conformation; this approach offers the possibility of stabilizing active conformations in situations when very high-affinity agonists may not be available[87]. Notably, a thermostabilized β_1-adrenoceptor construct and opsin (the ligand-free form of rhodopsin) were crystallized in a ligand-free state[88,89], indicating that crystals for some GPCRs can be obtained in the absence of ligands. This finding has implications for deciphering the structural details of orphan GPCRs for which known ligands are not yet available.

Lipid-based crystallography approaches

Even when high-quality preparations of ligand–GPCR complexes became available, conventional vapour diffusion crystallography, which is typically used for soluble protein and membrane protein crystallization, did not yield crystals or yielded only poorly diffracting crystals (FIG. 5a). Considering the intricate interaction of GPCRs with the lipid bilayer, and previous studies implicating lipid interactions in maintaining the receptor in a functional and stable state, alternative lipid-based crystallization approaches — such as bicelles and LCP — were used to obtain crystals of ligand–GPCR complexes. These approaches require the purified detergent–receptor complex to be reconstituted in well-defined lipid mixtures, which seem to maintain GPCRs in a crystallizable conformation.

Crystallization in bicelles. The use of a bicelle-based crystallization approach resulted in a β_2-adrenoceptor–Fab complex that was suitable for crystal diffraction[19]. The bicelle method, which was first successfully used to produce crystals of the bacteriorhodopsin protein from *Halobacterium salinarum*[90], involves the use of a well-defined lipid–amphiphile mixture[90,91]. Bicelles provide membrane proteins with an environment that is closer to their native surroundings than that achieved using detergent micelles, and therefore might stabilize them (FIG. 5b).

The first non-rhodopsin GPCR crystal was obtained for the β_2-adrenoceptor in complex with Fab5 using bicelles composed of a mixture of DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) and CHAPSO (3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulphonate); these crystals diffracted to ~3.5 Å[19]. However, a significant portion of the β_2-adrenoceptor was not well resolved in these crystals, suggesting that these crystals were of suboptimal quality. Although the resolution can be reasonably improved with further optimization of the bicelle method, the β_2-adrenoceptor is the only reported GPCR that has been crystallized using this approach. This is perhaps due to the success of the LCP strategy.

Crystallization in LCP. LCP, also referred to as *in meso* crystallization, was used to obtain a high-resolution structure of a β_2-adrenoceptor–T4L fusion protein[32]. An LCP is essentially a curved bicontinuous lipid bilayer with separated water channels[92–100]. Incubation of detergent-solubilized proteins with a LCP leads to the exchange of detergent micelles with the lipids and therefore provides a more native-like environment for the receptor (FIG. 5c). The β_2-adrenoceptor–T4L fusion protein was crystallized in a mixture of monoolein and cholesterol, and this has been a primary host lipid combination for the crystallization of GPCRs. However, selecting a suitable lipid for LCP might require a target-specific approach (for example, in the case of protein complexes or oligomeric receptors). Indeed, the

Lipidic cubic phase
(LCP). A novel crystallization approach in which membrane proteins are embedded in a membrane-mimetic lipid environment for crystallization.

Immobilized metal affinity chromatography
(IMAC). This technique refers to a particular type of affinity chromatography that uses coordinate covalent bond formation between specific amino acids in the protein (most often histidines) and immobilized metal ions (most often Ni^{2+}) on a solid support (for example, agarose beads). Ni-nitrilotriacetic acid resin-based protein purification is one of the most commonly used forms of IMAC.

Ligand affinity chromatography
A purification strategy in which a ligand is immobilized on a solid support through chemical modifications and is used to capture a functional receptor through ligand–receptor interactions.

Vapour diffusion crystallography
The most commonly used crystallization method for proteins in which a drop of purified protein solution in buffer and precipitant is equilibrated against a higher concentration of precipitant in a larger reservoir. During the equilibrium process, as the concentration of protein and precipitant increases in the crystallization drop, crystals grow depending on the suitability of the condition.

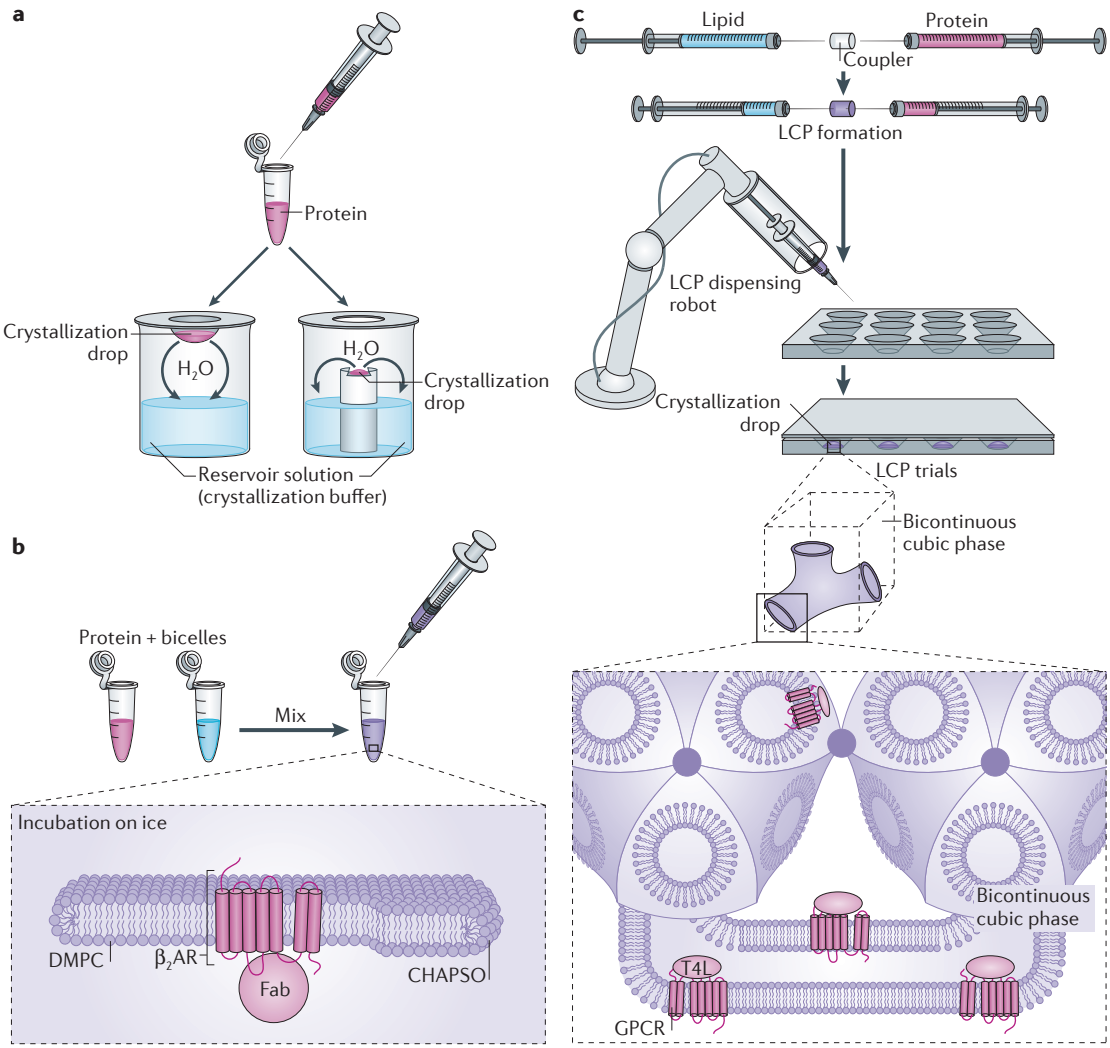

Figure 5 | **Schematic representation of conventional vapour diffusion, bicelle and lipid cubic phase crystallization approaches.** **a** | For typical vapour diffusion crystallization, detergent-solubilized G protein-coupled receptors (GPCRs) are mixed with crystallization buffer and crystallized using a hanging (left) or sitting (right) drop method. Crystallization drops contain a mixture of protein and crystallization buffer. The reservoir solution consists of crystallization buffer that is composed of a primary precipitant (for example, polyethylene glycol), a secondary precipitant (for example, salt ammonium sulphate) and chemical additives (such as heptane-1,2,3-triol) to promote crystallization. **b** | In the bicelle approach, purified GPCRs are mixed with bicelles that are typically composed of DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) and CHAPSO (3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulphonate), followed by crystallization by vapour diffusion. A β_2-adrenoceptor (β_2AR) bound to the ligand carazolol in complex with an antigen-binding fragment (Fab; shown here as an example) used the bicelle approach for crystallization. The presence of bicelles provides a native-like lipid environment for GPCR crystallization. **c** | In the lipid cubic phase (LCP) approach, purified GPCR is first mixed with a lipid–cholesterol mixture (preferably monoolein as the lipid) through a coupled air-tight syringe mixer. During this step, protein is incorporated into the cubic phase and the protein–LCP mixture becomes transparent. Subsequently, this lipid–protein mix is dispensed using a needle dispenser (for example, as part of an automated LCP Mosquito nanodispensing robotic set-up), on to a glass plate followed by the addition of crystallization buffer that is composed of precipitants and additives. Crystallization drops consisting of lipid–protein mix and crystallization buffer are sealed using glass sandwich plates and incubated at 20 °C for crystal formation. LCP provides a native-like lipid environment for GPCRs and potentially adds stability to the purified receptor. The bicontinuous cubic phase represents an optically transparent, highly viscous, solid-like crystalline lipid phase.

crystallization of a larger GPCR–G protein complex used a lipid that forms larger channels for protein diffusion in LCP (discussed further below).

Early attempts at GPCR crystallization in LCP required innovative methods, such as glass sandwich plates to set up crystallization drops, a manually designed automated drop dispensing system for dispensing lipid–protein mixture, and trial-and-error-based crystal harvesting strategies[101,102]. Although LCP-based crystallization is a sophisticated and training-intensive technique, advances in instrumentation are making it more accessible and user friendly. In addition, experts in

LCP are sharing their knowledge across multiple laboratories. The ongoing development of new lipids and their derivatives should provide options for applying LCP to different GPCR oligomers and larger GPCR complexes. The primary precipitant for most GPCRs crystallized in LCP has been a small-molecule polyethylene glycol (see Supplementary information S1 (table)), and the widespread success of this precipitant in this procedure might reflect the conserved domain architecture and common diffusive behaviour of purified GPCRs in LCP.

A key consideration in LCP-based crystallization is the diffusibility of purified proteins in the cubic phase and how this diffusion is affected by precipitants used for crystallization. An assay referred to as LCP–FRAP (fluorescence recovery after photobleaching) was used to study the behaviour of purified GPCRs in LCP[102–104]. In this method, a purified GPCR was fluorescently labelled, incorporated into LCP and studied for recovery of fluorescence after photobleaching. As long-range diffusion of proteins in LCP is important for crystallogenesis, one can quickly monitor the diffusion of the purified receptor in the cubic phase in response to various precipitating agents. This methodology can therefore be used as a pre-screening tool for a set of crystallization conditions that are likely to yield crystals. This technology uses relatively small quantities of samples and can be particularly useful when limited quantities of purified proteins are available and, therefore, only a limited set of conditions can be screened.

It is also important to note that thermostabilized GPCRs — for example, the β_1-adrenoceptor[70], the adenosine A_{2A} receptor[72] and the neurotensin type 1 receptor[74] — can be crystallized in detergent micelles using the conventional vapour diffusion technique. The requirement of receptor thermostabilization for crystallization in detergent micelles further indicates that the lipid environment has a positive effect on the thermostability of purified GPCRs.

Working with GPCR microcrystals

Even when optimized approaches for protein engineering and stabilization were coupled with new methods of crystallization, initial attempts to crystallize GPCRs only yielded microcrystals of 10–20 μm at their longest dimension[32], and this trend continues. In conventional crystallography, single crystals are extracted from crystallization drops, rapidly frozen and the X-ray diffraction data are collected. However, the isolation and freezing of high-quality single crystals of a GPCR is challenging, if not impossible, as the crystals are too small, fragile and present in a viscous lipid–protein mix.

Rastering strategy and microfocus beamlines. To overcome these challenges, GPCR microcrystals are scooped from crystallization drops with the lipid using MicroMount loops and frozen in liquid nitrogen. Attempts to remove the associated lipid reduce the diffraction properties of the crystals, further highlighting the important role of an appropriate lipid environment in obtaining diffraction-quality crystals. In contrast to relatively large protein crystals, for which the X-ray beam is focused on a single crystal for data collection, GPCR microcrystals require a rastering strategy[101]. In rastering, these microcrystals, which are embedded in LCP, are invisible through the beam optics; thus the crystal mounting loops are divided into small areas that are individually analysed for X-ray diffraction. Such rastering enables the rapid identification of areas that harbour crystals and researchers can return to these areas to collect data more systematically. This strategy was first developed manually, with numerous iterations, for β2-adrenoceptor crystals, but it has now been automated and incorporated into the data collection process at some synchrotron X-ray sources. Microcrystals are also sensitive to radiation and lose diffraction after the first few frames of data collection, but several synchrotron sources now have functional, tailored microfocus beamlines in place, and other sources are developing microfocus beamlines.

Using an X-ray-free electron laser. The latest methodological advance applied to GPCR crystallography is X-ray-free electron laser (xFEL), also known as serial femtosecond crystallography. xFEL briefly exposes microcrystals to highly intense and ultrashort X-ray pulses, thus enabling the collection of diffraction data from a single point on the microcrystal. This method was pioneered for studying the structure of photosystem I[105]. Although this method is still in its infancy, the structures of two GPCRs — the human serotonin 5-HT2B receptor and the human Smoothened receptor — were solved using this approach[106,107]. One advantage of xFEL is the ability of microcrystals to escape radiation damage, also referred to as 'diffraction before destruction'. Furthermore, in contrast to traditional X-ray diffraction experiments in which crystals are frozen and analysed under a stream of liquid nitrogen, xFEL data collection is conducted at room temperature. This might be beneficial for sensitive crystals, the freezing of which could be detrimental to their diffraction properties.

Other advantages of xFEL are that it can be used for time-resolved studies such as kinetics, and it is more suitable for soaking experiments compared with typical LCP-based crystallization and diffraction experiments. Furthermore, not only does xFEL enable the collection of data from tiny crystals, but new technical developments such as the LCP injector[108] (in contrast to the initial liquid injector system) reduces the quantity of protein required for X-ray data collection and structure determination. xFEL is available at the Linac Coherent Light Source in the Stanford Linear Accelerator Center, USA, and at the Spring-8 Angstrom Compact Free-electron Laser, Japan. Two additional xFELs — the SwissFEL at the Paul Scherrer Institute, Switzerland, and the European XFEL in Hamburg, Germany — are expected to start operating soon. However, the data processing of xFEL is still evolving and it requires further optimization before it becomes as accessible and automated as conventional crystallography. Considering the rapidly evolving technical advances in xFEL, this approach might become a primary tool for GPCR crystallography.

Soaking experiments
In the context of protein crystallization, soaking experiments refer to the incubation of pre-formed crystals with their ligands, for example, an inhibitor. This method is primarily used to obtain crystal structures of apo (ligand-free) and ligand-bound protein.

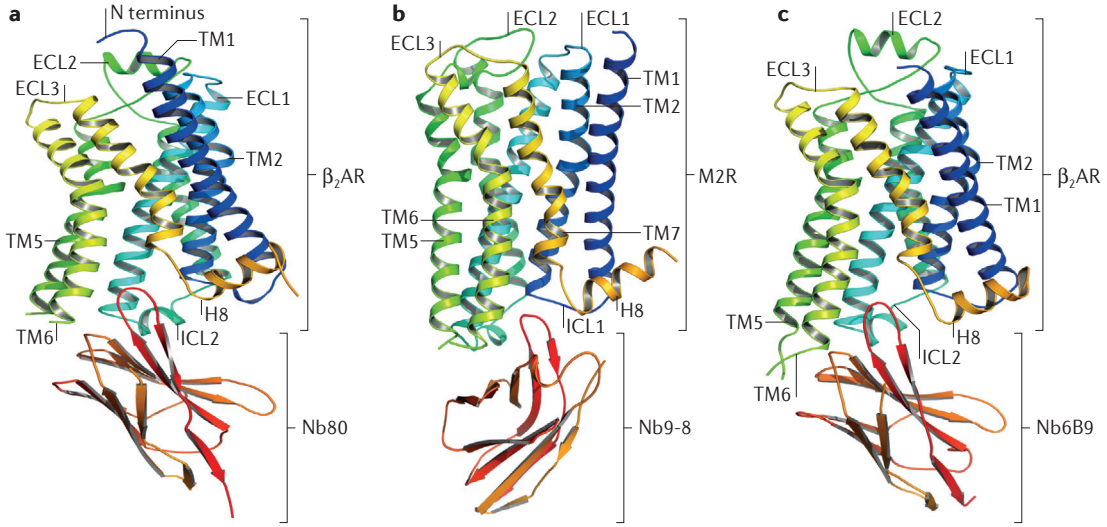

Figure 6 | **Trapping active GPCR conformations using nanobody technology.** The crystallization of G protein-coupled receptors (GPCRs) in a fully activated conformation requires stabilizing chaperones in addition to high-affinity agonist ligands. **a** | A crystal structure of the agonist BI-167107 bound to the β_2-adrenoceptor (β_2AR) in its active state, stabilized by the Nb80 nanobody (RCSB Protein Data Bank (PDB) ID: 3P0G). The Nb80 nanobody was generated by immunizing llamas with purified β_2AR–agonist complexes, and it recognizes and stabilizes an active conformation of β_2AR in biochemical and functional experiments. **b** | A crystal structure of an agonist bound to the muscarinic acetylcholine receptor type 2 (M2R) in complex with the Nb9-8 nanobody (PDB ID: 4MQS). The Nb9-8 nanobody was generated through a combinatorial approach of llama immunization and subsequent *in vitro* yeast display, and the use of Nb9-8 resulted in the crystallographic trapping of an active conformation of the M2R. **c** | A crystal structure of adrenaline (the endogenous agonist) bound to the β_2AR stabilized by a nanobody variant of Nb80 (Nb6B9; PDB ID: 4LDO) that was generated using the combinatorial approach described above for Nb9-8. Crystal structures of active-state GPCRs stabilized by nanobodies show a general pattern of an outward movement of the sixth transmembrane (TM6) domain relative to the central core of the receptor. This pattern mirrors the outward movement of the corresponding helix in the ligand-free active state of opsin. Such movements were predicted on the basis of biophysical studies and have been directly visualized by X-ray crystallography. Note that the presence of a 'G protein-mimetic nanobody' is crucial to capture the fully active conformation of the receptors described here. These figures were generated using the software PyMOL and are presented in an amino terminus up and carboxyl terminus down orientation. The colour scheme represents the standard output of structural images by PyMOL. ECL, extracellular loop; H8, helix 8; ICL, intracellular loop.

Nanobodies: crystallizing active GPCRs

A series of biophysical studies have shown that several conformational changes occur in GPCRs upon agonist-dependent activation[41,42,109,110]. Thus, once the structures of several inactive GPCRs were determined, the next step was to visualize GPCRs in the active conformation and to determine what these conformational changes are. To achieve this, a β_2-adrenoceptor was covalently and irreversibly tethered to an agonist to drive it towards an active conformation[85]. However, even covalent agonist–β_2-adrenoceptor complexes had a structure similar to the inactive receptor[85]. This suggested that trapping GPCRs in an active conformation for crystallography might require stabilizing chaperones. Therefore, purified β_2-adrenoceptor was injected into llamas to generate β_2-adrenoceptor-specific antibodies that might stabilize active β_2-adrenoceptors.

Camelid family members such as camels and llamas produce antibodies that lack a light chain and only consist of two heavy chains. The variable domain of heavy chain-only antibodies is referred to as a nanobody to reflect its small size compared with conventional antibody fragments (for example, Fabs and single-chain fragment variables)[86,111,112]. Nanobodies that were selective for an active state of the β_2-adrenoceptor enabled its crystallization by mimicking the conformation of a G protein to stabilize it in a fully active conformation, as indicated by the significant outward movement of the TM5 and TM6 domains compared with the inactive conformation[86] (FIG. 6a). This observation was in agreement with the crystal structure of opsin, the light-activated version of rhodopsin that undergoes similar structural changes in the transmembrane domain upon activation[89]. Subsequently, the muscarinic acetylcholine type 2 receptor was also crystallized in an active conformation using a nanobody and it exhibited similar activation features to the β_2-adrenoceptor[113] (FIG. 6b). Nanobody technology in conjunction with a yeast display approach also yielded a crystal structure of the β_2-adrenoceptor in complex with its relatively low-affinity endogenous agonist adrenaline[114] (FIG. 6c). These findings highlight the potential of nanobodies in capturing specific conformations of different GPCRs and indicate that nanobodies can be generally used in GPCR crystallography. It is worth noting that opsin and the adenosine A_{2A} receptor have been crystallized in an active conformation without the use of nanobodies or any other stabilizing chaperones[89].

GPCRs can signal through multiple pathways, each of which can be separately targeted with pharmacological compounds[16]. This has led to the concept of biased signalling, which defines the ability of agonists to selectively trigger one of two parallel signalling pathways[8,16]. A concept of conformational sampling has been proposed to underlie the concept of biased signalling, which suggests that multiple active-like receptor conformations are present and that they dictate the subsequent functional outcome[115]. A considerable challenge lies in capturing different active receptor conformations and comparing them to unravel which receptor features determine specific signalling outcomes. Generating monoclonal antibodies to selectively stabilize specific signalling conformations for crystallographic visualization is time-consuming, expensive and challenging. However, the recent development and optimization of nanobody technology and the subsequent *in vitro* screening of nanobodies on immobilized GPCRs is offering a promising alternative to generating monoclonal antibodies[113,114]. In addition, the development and validation of powerful synthetic antibody fragment libraries and target-specific selection strategies are also providing a robust and reliable source for generating antibody fragments to facilitate research into the structural biology of GPCRs[23].

Visualizing signalling complexes

One of the primary objectives in the field of GPCR biology has been to understand the molecular basis of receptor–effector coupling. This involves visualizing the receptor–effector interface and the conformational changes that are associated with this interaction. Considering that receptor–effector assemblies are likely to be transient, technological advances were needed to directly study such signalling complexes, as highlighted by the procedures used to determine the crystal structure of a GPCR–G protein complex and an overall architecture of a GPCR– β-arrestin complex.

Structure of a GPCR–G protein complex. The direct structural visualization of a GPCR–G protein complex — that is, of an activated GPCR — was a major breakthrough in GPCR biology[21,116,117]. A step-by-step analysis of the process used to determine the β_2-adrenoceptor–G protein complex structure reveals the methodological innovation that was required. Although mixing a high concentration of agonist-occupied β_2-adrenoceptors and GDP-bound heterotrimeric G proteins (Gαβγ) resulted in acceptable complex formation, the removal of GDP from the assembled complex, using a non-selective purine pyrophosphatase apyrase, further increased the stability of the complex. Furthermore, exchanging DDM micelles for MNG micelles increased the stability of the complex in solution; in MNG, the complex was stable even below critical micelle concentration levels of MNG. Unfortunately, however, even such stable preparations failed to yield diffracting crystals and in-depth analysis of this complex by single-particle electron microscopy revealed two potential limitations[116]. First, due to large detergent micelles surrounding the receptor, there was a limited polar surface area available to produce crystal contacts. To overcome this issue, an N-terminal fusion of T4L was added to the β_2-adrenoceptor to extend the polar surface area and facilitate crystal contact. Second, a flexible localization of the alpha-helical domain of the Gα subunit ($G\alpha_s$AH), relative to the Ras-like GTPase domain ($G\alpha_s$Ras), was revealed. The addition of foscarnet, a pyrophosphate analogue, restricted the flexibility of the $G\alpha_s$AH domain as assessed by single-particle electron microscopy. As the β_2-adrenoceptor–G protein complex contains a significantly larger hydrophilic region (that is, the G protein) than isolated receptors, a new lipid (MAG 7.7)[93] that was designed to accommodate larger soluble domains was used in LCP instead of monoolein, which is the lipid typically used for receptor crystallography. Despite these adjustments, the resulting complex yielded crystals that only diffracted to ~7 Å, so nanobody technology was subsequently used. Llamas immunized with a crosslinked β_2-adrenoceptor–G protein complex yielded several nanobodies against the complex. One of these nanobodies, Nb35, further stabilized the position of the $G\alpha_s$Ras domain with respect to the Gβγ, as visualized by electron microscopy, and improved the resolution of β_2-adrenoceptor–G protein crystals to ~3 Å[21] (FIG. 7a).

Analysis of the β_2-adrenoceptor–G protein complex structure revealed many functional characteristics. The extracellular surface of the β_2-adrenoceptor in this complex, including the ligand-binding pocket, undergoes only a minor conformational alteration compared with the inactive conformation of this receptor. However, the intracellular surface of the receptor exhibits large conformational changes, including ~14 Å outward movement of the TM6 domain, compared with the inactive β_2-adrenoceptor, which provides a docking interface for $G\alpha_s$. A somewhat surprising finding is the large displacement of the $G\alpha_s$AH domain relative to the $G\alpha_s$Ras domain in this structure. Although there is some biochemical evidence to support such a dramatic domain rearrangement, it would be interesting to learn whether $G\alpha_s$ displays a similar profile when in a complex with other GPCRs. Furthermore, Gβ and Gγ subunits do not directly contact β_2-adrenoceptors and it is unknown whether this is the case for other GPCR–G protein complexes. A crystallographic snapshot of GPCRs in complex with nucleotide-bound heterotrimeric G proteins should provide further details of the G protein cycle. Furthermore, many GPCRs couple to different subtypes of G proteins and the structural characterization of a given receptor in complex with two different subtypes of G proteins would be valuable for understanding the underlying molecular mechanisms of GPCR–G protein signalling. This may require additional methodological developments and technical breakthroughs.

Architecture of a GPCR–β-arrestin complex. The interaction of activated GPCRs with β-arrestins terminates G protein-dependent signalling and also initiates a β-arrestin-dependent signalling pathway that is as diverse as G protein signalling, highlighting the importance of gaining structural insight into GPCR–β-arrestin interactions. However, it was difficult to isolate stable and functional GPCR–β-arrestin complexes because this

Biased signalling
G protein-coupled receptors can signal through two parallel and independent pathways: the G protein-dependent and the β-arrestin-dependent pathways. When a receptor signals preferentially through one of these pathways, it is referred to as biased signalling.

Hydrogen–deuterium exchange mass spectrometry
This technique — in which deuterium in solution is exchanged with the backbone amide hydrogen — is used to study conformational changes and dynamics of proteins. The extent and rate of this exchange, measured by mass spectrometry, reflects the local and overall conformational flexibility and dynamics of the protein. This technique has been used to study the conformational dynamics of the agonist–β_2-adrenoceptor–G protein complex and the agonist–β_2-adrenoceptor–β-arrestin 1 complex.

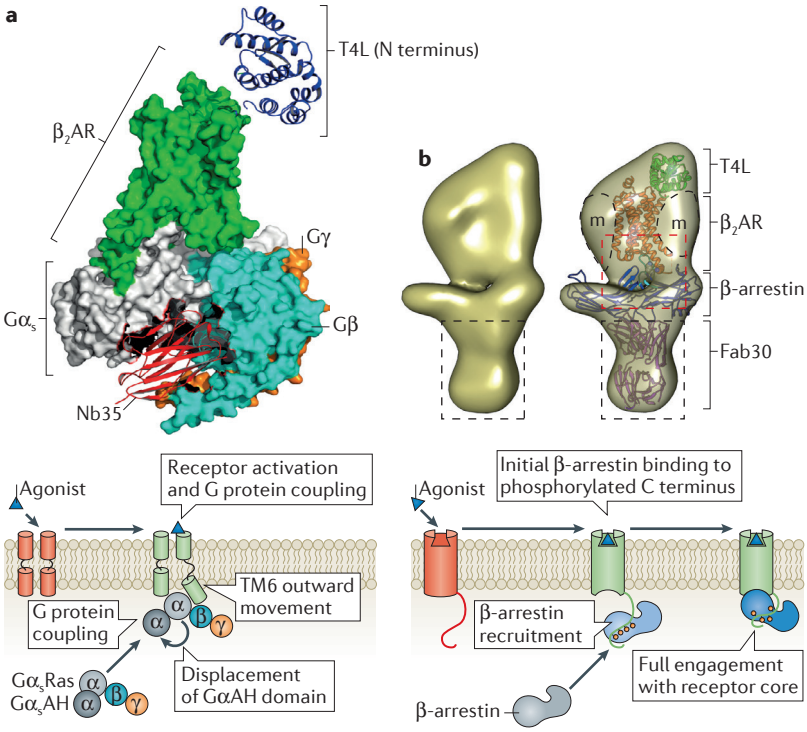

Figure 7 | **Visualizing GPCR signalling complexes.** Recent studies have begun to yield direct structural information on G protein-coupled receptor (GPCR) signalling complexes. **a** | A crystal structure of an agonist–β_2-adrenoceptor (β_2AR)–$G\alpha_s$ protein complex stabilized by a nanobody (Nb35; red ribbon) and extracellular fusion of T4 lysozyme (T4L; blue ribbon; RCSB Protein Data Bank (PDB) ID: 3SN6). The agonist-occupied activated receptor (agonist not shown) exhibits outward movement of the fifth transmembrane (TM5) and TM6 domains, which forms a docking interface for the α5 helix of the $G\alpha_s$ subunit of the heterotrimeric G protein. The alpha-helical domain of the $G\alpha_s$ subunit ($G\alpha_s$AH) shows displacement towards the membrane relative to the Ras-like GTPase domain ($G\alpha_s$Ras). The schematic representation below the crystal structure illustrates the two key features of the β_2AR–G protein complex; outward movement of TM6 that forms a docking interface for $G\alpha_s$, and a major displacement of the $G\alpha_s$AH domain relative to the $G\alpha_s$Ras-like domain. **b** | Architecture of an agonist–β_2AR–β-arrestin complex stabilized by an antigen-binding antibody fragment (Fab30; highlighted by the dashed black rectangle) generated through a combinatorial screening approach. Single-particle electron microscopy was pushed to its limits to image and reconstruct the three-dimensional architecture of this relatively small protein complex ('m' indicates the detergent micelle and the red dashed box indicates the receptor–arrestin interaction interface). This architecture of the agonist–β_2AR–β-arrestin complex reveals a biphasic interaction between activated receptor and β-arrestin, as shown in the schematic. β-arrestin first engages with the phosphorylated carboxyl terminus of activated GPCR and subsequently forms a more intimate interaction with the core of the receptor. The amino domain of β-arrestin seems to be the primary site for receptor interaction and the relatively free C-terminal domain is likely to serve as a major scaffold point for other interaction partners of β-arrestin. Figure part **b** from REF. 22, Nature Publishing Group.

interaction requires receptor phosphorylation; obtaining homogeneously phosphorylated receptors presents several challenges. Attempts to form a complex using purified β_2-adrenoceptors and β-arrestins failed to yield a biochemically stable complex for structural studies, most likely due to unstable β_2-adrenoceptor phosphorylation and the sensitivity of β-arrestins to detergents. A breakthrough was made by using a novel '*in celltro*' method (which combines assembly of the complex in cells followed by purification *in vitro*) to assemble the complex in cells followed by its stabilization by a Fab selective for the active β-arrestin conformation[22,23]. This Fab, referred to as Fab30 — which was generated from a synthetic phage display library — stabilizes the β_2-adrenoceptor–β-arrestin complex in the cell membrane and maintains the integrity of the complex during harsh solubilization and affinity purification steps. In addition, a novel on-column crosslinking protocol was developed to stabilize the biphasic interaction of the β_2-adrenoceptor with the β-arrestin (that is, a β-arrestin conformation that was fully engaged with the receptor through contacts with its C terminus and 7TM core). Single-particle electron microscopy enabled the direct structural visualization of a two-step interaction mechanism for β_2-adrenoceptor–β-arrestin and provided an overall architecture for this signalling complex (FIG. 7b).

This two-step (that is, biphasic) receptor–β-arrestin interaction mechanism was previously proposed based on a series of biophysical studies on the rhodopsin–visual arrestin system[118–120]. It was postulated that arrestin first engages with the phosphorylated C terminus of activated GPCRs and then docks on to the 7TM core of the receptor. The electron microscopy-based study of the β_2-adrenoceptor–β-arrestin complex provided direct evidence for this hypothesis by capturing both an intermediate step in the interaction and a fully engaged conformation of β-arrestin. This structural model was further corroborated by complementary techniques, including hydrogen–deuterium exchange mass spectrometry. Similar to the study that solved the structure of the β_2-adrenoceptor–G protein complex, a high-affinity agonist and MNG-3 amphiphile were crucial in isolating stable preparations of the complex for direct structural visualization. However, it must be noted that the architecture describes a relatively low-resolution assembly of the complex, and a crystal structure of this complex — which is likely to yield additional atomic-level information on this interaction — remains to be visualized. Considering the basic requirement of receptor phosphorylation for β-arrestin recruitment, it is tempting to speculate that the biphasic mechanism might be common to most GPCRs. Moreover, the architecture of the β_2-adrenoceptor–β-arrestin complex reveals that the N-terminal domain of β-arrestin primarily engages with the receptor, leaving the C-terminal domain outside the interaction interface. In view of the robust scaffolding function of β-arrestins in GPCR signalling and regulation, such an arrangement might provide a nucleation point for β-arrestin-interacting proteins. More recently, a crystal structure of activated opsin in complex with the finger loop of visual arrestin (that is, arrestin 1) was determined[121]. This revealed that the finger loop region of arrestin binds to the central core of activated GPCRs, which is in agreement with the GPCR–β-arrestin model generated based on electron microscopy and hydrogen–deuterium exchange mass spectrometry studies.

Conclusion and perspective

The 'structural era' of GPCRs has followed the same pattern as the 'cloning era' of GPCRs, in which the cloning of the first GPCR led to a flurry of GPCRs being cloned. In the 7–8 years since the first non-rhodopsin GPCR was crystallized, 26 GPCR structures have been solved.

Combinatorial biology
An approach in which a large number of variants (for example, of a peptide or protein) are generated as a library and screened to find a variant that binds to the target with high affinity. Phage display is a commonly used combinatorial biology approach.

The structural details of GPCR signalling complexes have begun to provide unprecedented insight into the mechanism of receptor–effector coupling, and studies of GPCR conformational dynamics, which neatly complement static crystallography snapshots, are helping to explain the signalling diversity of GPCRs. Considering the dissemination of technical expertise in the field — including of sophisticated crystallography approaches and combinatorial biology, coupled with the development of next-generation synchrotron beamlines and xFEL — the coming years will bring us closer to fully understanding the molecular basis of GPCR biology at atomic resolution.

It is interesting to note that even though recombinant GPCR production is now a routine procedure and automation is in place for the sophisticated LCP technology, only a few laboratories can successfully generate high-resolution crystal structures of GPCRs. However, considering how knowledge is shared in the field, GPCR structures are likely to be solved in multiple academic laboratories and pharmaceutical enterprises. Although the structural resolution of more GPCRs in inactive and classically active conformations is likely to continue, a major challenge now is to decipher the structural details of biased signalling conformations and non-canonical signalling complexes of different GPCRs; this should provide an effective framework for structure-based novel drug design for numerous human diseases. Furthermore, it would also be interesting to ascertain whether at least some of the methodological advances, such as the T4L fusion strategy and the development of new detergents, might also be useful in the structural characterization of other classes of membrane proteins.

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Competing interests statement
The authors declare no competing interests.

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