REVIEW

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Molecular signatures of G-protein-coupled receptors

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G-protein-coupled receptors (GPCRs) are physiologically important membrane proteins that sense signalling molecules such as hormones and neurotransmitters, and are the targets of several prescribed drugs. Recent exciting developments are providing unprecedented insights into the structure and function of several medically important GPCRs. Here, through a systematic analysis of high-resolution GPCR structures, we uncover a conserved network of non-covalent contacts that defines the GPCR fold. Furthermore, our comparative analysis reveals characteristic features of ligand binding and conformational changes during receptor activation. A holistic understanding that integrates molecular and systems biology of GPCRs holds promise for new therapeutics and personalized medicine.

Signal transduction is a fundamental biological process that is required to maintain cellular homeostasis and to ensure coordinated cellular activity in all organisms. Membrane proteins at the cell surface serve as the communication interface between the cell's external and internal environments. One of the largest and most diverse membrane protein families is the GPCRs, which are encoded by more than 800 genes in the human genome[1]. GPCRs function by detecting a wide spectrum of extracellular signals, including photons, ions, small organic molecules and entire proteins. After ligand binding, GPCRs undergo conformational changes, causing the activation of complex cytosolic signalling networks, resulting in a cellular response. Altering the activities of GPCRs through drugs is already used in the treatment of numerous ailments including cardiac malfunction, asthma and migraines. Given the tremendous diversity of GPCRs, there remains enormous potential for the development of additional drugs to ameliorate neurological disorders, inflammatory diseases, cancer and metabolic imbalances. Thus, determining the structure of GPCRs and understanding the molecular mechanism of receptor activation is not only of fundamental biological interest, but also holds great potential for enhancing human health.

In accordance with the guidelines of the International Union of Basic and Clinical Pharmacology, non-sensory GPCRs (that is, excluding light, odour and taste receptors) can be classified, according to their pharmacological properties, into four main families: class A rhodopsin-like, class B secretin-like, class C metabotropic glutamate/pheromone, and frizzled receptors. In the past 12 years, more than 75 structures of 18 different class A GPCRs have been determined in complex with ligands of varied pharmacology, peptides, antibodies and a G protein (Fig. 1). These structures provide unprecedented insights into the structural and functional diversity of this protein family. Given the recent exciting advances in the field of GPCR structural biology, we are in a unique position to address the following fundamental questions: what are the 'molecular signatures' of the GPCR fold? And what are the molecular changes that the receptor undergoes during activation? The availability of structures of diverse GPCRs now permits a systematic comparative analysis of the GPCR fold. This knowledge, combined with the understanding gained from complementary biophysical, computational, and biochemical studies empowers us to probe the molecular basis of GPCR structure–function relationship comprehensively, and in the process expand the current frontiers of GPCR biology.

In this analysis, we objectively compare known structures and reveal key similarities and differences among diverse GPCRs. We identify a consensus structural scaffold of GPCRs that is constituted by a network of non-covalent contacts between residues on the transmembrane (TM) helices. By systematically analysing structures of the different receptor–ligand complexes, we identify a consensus 'ligand-binding cradle' that constitutes the bottom of the ligand-binding pocket within the TM bundle. Furthermore, our comparative study suggests that the third TM helix (TM3) has a central role as a structural and functional hub. We then synthesize our current understanding of the structural changes during activation by discussing conformational dynamics of the receptor as gleaned from recent biochemical, biophysical and computational studies. Finally, we highlight open challenges in the field and discuss exciting new directions for GPCR research.

Reasons for GPCR crystallography successes

Since 2007, several innovative protein engineering techniques and crystallography methods[2] have resulted in an almost exponential growth in the number of solved structures (Fig. 1a). These include creating receptor–T4 lysozyme[3,4] and receptor–apocytochrome[5] chimaeras, co-crystallization with monoclonal antibody fragments from either mouse[6,7] or camelids[8,9], and thermostabilization of GPCRs by systematic scanning mutagenesis[10–12] or by engineering disulphide bridges[13,14]. Often, it was necessary to truncate flexible regions of the receptor and to use high-affinity/low off-rate ligands to enhance receptor stability. In addition, the use of lipidic cubic phase[15] and new detergents[16] has improved the likelihood of obtaining crystals, and advances in micro-crystallography have allowed obtaining higher resolution diffraction from smaller crystals[17]. Although these methods have been successful, the inherent limitations with such recombinant methods are that (1) post-translational modifications are often removed or not incorporated during protein purification; (2) truncations of loops and amino or carboxy termini provide limited understanding of the structure and function of these regions; and (3) insertion of, for example, T4 lysozyme into intracellular loop 3, addition of thermostabilizing mutations or the use of antibodies may affect the relative mobility of the TM helices, thereby potentially biasing the conformational ensemble of the receptor. Despite the above-mentioned

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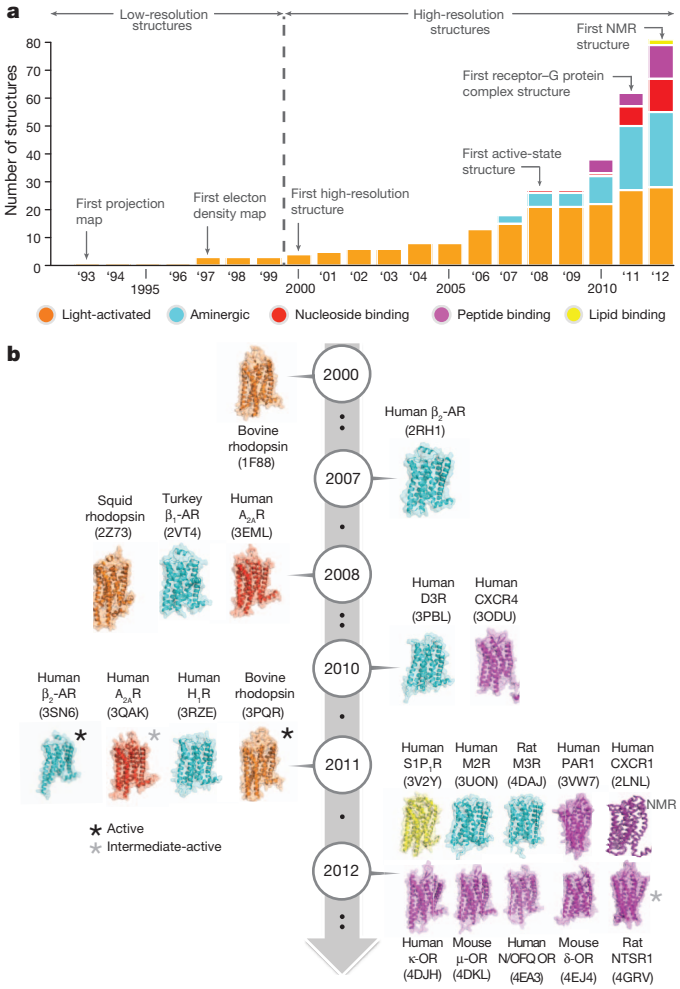

Figure 1 | Time-line of GPCR structures. **a**, Bar chart showing the increase in the number of GPCR structures with time. **b**, Time-line showing representative crystal structures of GPCRs and the year of publication. Active conformations are marked with a black asterisk, and an intermediate-active conformation is marked with a grey asterisk. Protein Data Bank accession numbers are shown in parentheses.

shortcomings, the wide variety of techniques is likely to ensure a steady increase in the number of GPCR structures in the future.

Solved structures of GPCRs

So far, high-resolution structures have been solved for the following class A GPCRs (Supplementary Table 1): (1) rhodopsin (bovine rhodopsin[18] and squid rhodopsin[19]); (2) several members of aminergic GPCRs: β-adrenoceptors (avian β_1-AR (ref. 10) and human β_2-AR (refs 3, 6)), muscarinic acetylcholine receptors (human M2R (ref. 20) and rat M3R (ref. 21)), human H_1 histamine receptor[22], and human D3 dopamine receptor[23]; (3) a nucleoside-binding GPCR: human adenosine A_{2A} receptor ($A_{2A}R$)[24]; (4) several members of the peptide-binding GPCRs: human CXCR4 chemokine receptor[25], opioid receptors (human nociceptin receptor[5] and κ-OR[26] and mouse μ-OR[27] and δ-OR[28]), rat neurotensin receptor (NTSR1)[29] and human protease-activated receptor (PAR1)[30]; and (5) a lipid-binding GPCR: human sphingosine-1 phosphate ($S1P_1$) receptor[31]. The human CXCR1 chemokine receptor is the first GPCR structure that was determined using NMR spectroscopy[32].

The crystal structures of all of the above-mentioned class A GPCRs (except NTSR1) have been obtained in inactive conformations bound to either inverse agonists that reduce basal activity or neutral antagonists that maintain basal activity. Rat NTSR1 (ref. 29), bovine rhodopsin[13,14,33], human β_2-AR (refs 8, 9, 34), avian β_1-AR (refs 35, 36) and human $A_{2A}R$ (refs 11, 37) were crystallized with agonists that induce an increase in biological activity. Of these, only bovine rhodopsin[13,14,33,38], human β_2-AR (refs 8, 9), human $A_{2A}R$ (refs 11, 37) and rat NTSR1 (ref. 29) were obtained in active (or intermediate-active) states. An important landmark in GPCR biology was the determination of the active-state ternary complex of β_2-AR in complex with the heterotrimeric G protein[9].

Molecular signatures of the GPCR fold

The structure of a GPCR can be divided into three parts: (1) the extracellular region, consisting of the N terminus and three extracellular loops (ECL1–ECL3); (2) the TM region, consisting of seven α-helices (TM1–TM7); and (3) the intracellular region, consisting of three intracellular loops (ICL1–ICL3), an intracellular amphipathic helix (H8), and the C terminus (Fig. 2a). In a broad sense, the extracellular region modulates ligand access; the TM region forms the structural core, binds ligands and transduces this information to the intracellular region through conformational changes, and the intracellular region interfaces with cytosolic signalling proteins.

Extracellular region and ligand-binding pocket accessibility

Sequence analysis shows that there is a large diversity in the lengths and sequence compositions of the N terminus[39] and the extracellular loops[40]. The class A GPCR structures reveal two distinct types of extracellular region: those that either occlude the ligand-binding pocket or leave the ligand-binding pocket water-accessible (Fig. 2b). Rhodopsin[18] and the $S1P_1$ receptor[31] have occluded binding pockets, presumably because they both bind hydrophobic ligands that may enter the receptor from the lipid bilayer[41]. The N terminus and ECL2 of rhodopsin fold into β-hairpin loops, and together they form a 'lid' for the ligand-binding pocket. Similarly, the $S1P_1$ receptor contains a three-turn α-helix that packs against ECL2 and ECL3 (ref. 31). In the receptors that bind water-soluble ligands, ECL2 can differ structurally between receptors, but the structures are likely to be conserved in a subfamily-specific manner. ECL2 can contain helices (for example, certain aminergic or adenosine receptors) or sheets (for example, peptide-binding receptors) (Fig. 2a). Even in the absence of defined secondary structural elements in ECL2 (for example, in muscarinic receptors), it still partially folds over the extracellular region and shapes the route for ligand entry into the binding pocket. Indeed, molecular dynamics simulations suggest that ECL2 could be involved in the first steps of ligand recognition and selectivity in the β-ARs[21,42,43]. Furthermore, pharmacological studies have shown that this region is important for ligand-binding kinetics[27]. In contrast to ECL2, ECL1 and ECL3 are relatively short and tend to lack distinct secondary structural elements[40].

A unique feature of the extracellular region is the presence of disulphide bridges that contribute to receptor stability. Although there are several subfamily-specific disulphide bridges, the one between a crucial residue in TM3, $Cys^{3.25}$ (in which the superscript denotes Ballesteros–Weinstein numbering[44]), and ECL2 seems to be highly conserved in most GPCR structures (except $S1P_1$). This TM3–ECL2 disulphide bridge anchors the extracellular side of the helix near the binding site, and limits the extent of the conformational changes of this region during receptor activation. Indeed, reducing the disulphide bridges can influence receptor stability and activity. Furthermore, in several GPCRs, ECL3 contains an additional intra-loop disulphide bridge within a CX_nC motif that possibly influences receptor function by limiting the conformational freedom available to the loop. For instance, a missense mutation (Cys271Arg) in this disulphide bridge in the melanocortin-4 receptor results in receptor malfunction and is linked to obesity.

Conserved structural scaffold in the TM region

The TM helix bundle serves as the communication link between the ligand-binding pocket and the G-protein-coupling region. Although GPCRs share a similar architecture of seven TM helices held together by tertiary contacts, their sequences are diverse. An objective comparison of the structures of diverse GPCRs using a network representation permits

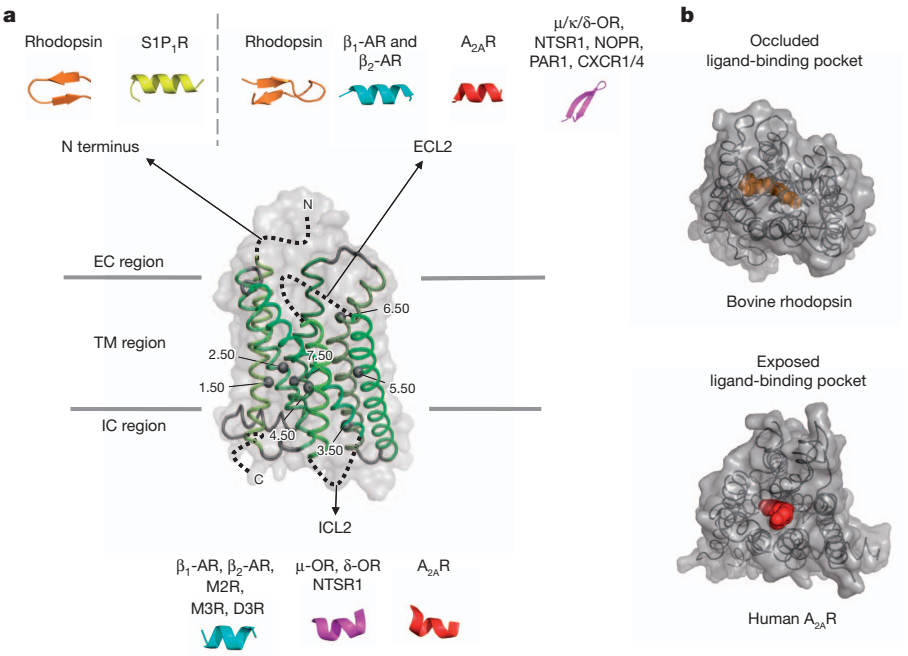

Figure 2 | Diversity in the secondary structure elements of GPCRs in the extracellular and intracellular regions. a, TM helices (TM1–TM7) are shown as cartoon (coloured in a spectrum of green) and surface representation. Numbers denote Ballesteros–Weinstein numbering. In this receptor-independent notation, each residue is identified by two numbers that are separated by a dot (for example, 1.50): the first number ranges from 1 to 7 and corresponds to the TM helix where the residue is located; the second number indicates its position relative to the most conserved residue of the helix, which is assigned the number 50. Residues in the same TM helix that are N- or C-terminal to the '50' residue are assigned a number that decreases or increases sequentially, respectively. For example, a residue just before or after the most highly conserved residue in TM1 will be assigned 1.49 or 1.51, respectively. N and C termini and the segments containing defined secondary structure in the extracellular (EC) and intracellular (IC) region are shown as dotted lines; the type of secondary structure element for the different representative GPCRs are shown in the grey panels. The loop regions lacking an α-helix or a β-sheet in any of the structures are not shown. See Fig. 1 for receptor colour code. **b**, Extracellular region that occludes or exposes the ligand-binding pocket as seen from the extracellular side (top view).

us to investigate whether any tertiary contacts between TM helices are conserved, independent of sequence diversity. A systematic analysis of the different GPCR structures, which includes both active and inactive states, reveals a consensus network of 24 inter-TM contacts mediated by 36 topologically equivalent amino acids (Supplementary Table 2). The topologically equivalent positions are identified through structure-based sequence alignment and are referred to by the Ballesteros–Weinstein numbering scheme (Supplementary Table 3). In this consensus network, the contacts are present in all (or all but one) of the structures, irrespective of their conformational state, and thus are likely to represent structurally important positions in the receptor (Fig. 3). The importance of these positions is highlighted by the fact that mutations in 14 out of 36 positions have been noted to result in either an increase or a loss of receptor activity[45]. With the availability of more high-resolution structures of other GPCRs, one may converge on a unified subset of inter-helical contacts that is maintained in all GPCRs.

The 36 topologically equivalent residues of the structural scaffold include highly conserved residues such as Asn$^{1.50}$, Asp$^{2.50}$, Trp$^{4.50}$ and Pro$^{7.50}$. Nevertheless, we also observe that many topologically equivalent positions can tolerate variability in amino acid substitutions. The identity of some of these 36 positions may be variable, but they all nevertheless predominantly maintain the non-covalent contacts between them. For instance, although a contact between 2.42 and 3.46 is seen in all structures, these residues are different among different receptors: for example, Ile 75 and Leu 131 in bovine rhodopsin and Tyr 97 and Met 152 in the human κ-OR. Thus, the consensus inter-TM contact network seems to provide an evolutionarily conserved structural scaffold of non-covalent contacts for the GPCR fold. It is likely that the tolerance of sequence variability in some of these positions permits diverse sequences to adopt a similar structure, thereby contributing to the evolutionary success of the GPCR fold. These conformation-independent consensus contacts may constitute a rigid platform on which distinct conformation-specific structural changes take place. Importantly, the network approach used here and the consensus set of inter-TM tertiary contacts identified should be valuable for GPCR engineering, *de novo* GPCR modelling and to increase the accuracy of GPCR homology models for various applications[46].

In terms of spatial positioning within the receptor, the consensus inter-TM tertiary contacts are largely localized to the central and cytoplasmic side of the TM bundle and primarily clustered at the interfaces of TM1–TM2, TM3–TM4, TM3–TM5 and TM3–TM6–TM7. Conservation of these contacts across diverse GPCRs may be due to the requirement for receptor biogenesis, protein stability or functionality. For instance, TM1 and TM2 do not undergo any major movement after receptor activation. Because they are the first two TM regions to be translated by the ribosome, the consensus contacts observed here might have an important role in membrane insertion, folding and topogenesis of GPCRs. Indeed, mutagenesis experiments of the neurotensin receptor[47] provide support for this possibility. Similarly, TM3 shares consensus helical packing interfaces with all other TM helices except TM1 and TM7, suggesting a role in maintaining the fold (Fig. 3b). This does not mean that TM3 makes no contacts with TM1 or TM7 but that the contacts between equivalent residues are not maintained across different receptors. Whereas the middle portion of TM3 makes consensus contacts with TM4 and TM6, the portion towards the cytoplasm makes contacts with TM5 and TM2. Thus, TM3 seems to have a key role of 'structural hub' in maintaining the scaffold in all GPCR structures, both in the inactive and active conformational states (Fig. 3b). Intriguingly, TM3 adopts an extreme tilt-angle (~35°), and this unusual geometry may facilitate its role as a structural hub.

Consensus scaffold of class A GPCR ligand–binding pocket

A remarkable feature of the GPCR family is its ability to bind ligands of diverse shapes, sizes and chemical properties. Although all ligands have

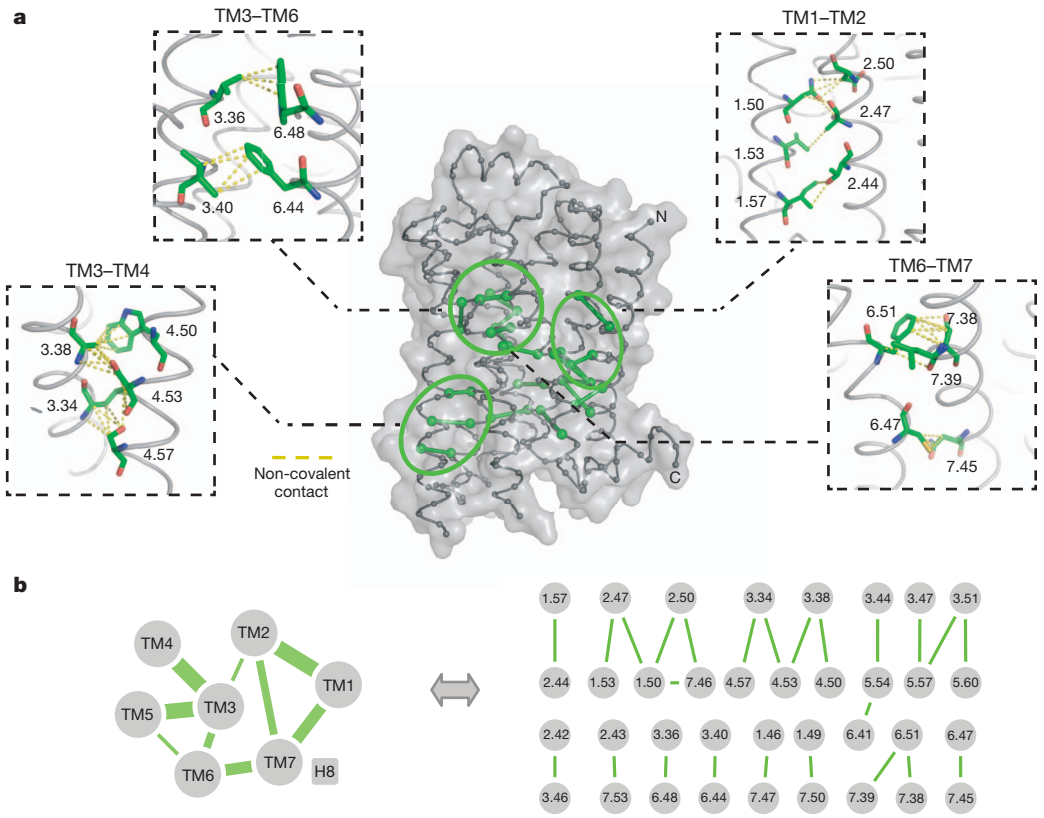

Figure 3 | Consensus scaffold of non-covalent contacts in GPCRs. **a**, Network of 24 inter-helical contacts between 36 topologically equivalent residues is shown on a representative structure of inactive β_1-AR. The spatially clustered contacts between the amino acids are shown in the panels; to maintain visual clarity, contacts between TM3 and TM5 are not shown. Here we define that a pair of residues is in contact if the Euclidean distance between any pair of atoms (side-chain and/or main-chain atoms) is within the van der Waal interaction distance (that is, the sum of the van der Waal radii of the atoms plus 0.6 Å). With the availability of more high-resolution structures of other GPCRs, one may converge on a unified subset of inter-helical contacts that is maintained in all GPCRs. **b**, Schematic representation of the inter-TM tertiary contact network in which TM helices are presented as circles, and H8 is shown as a square. The lines between a pair of circles indicate the presence of consensus contacts, and the thickness of the line is proportional to the number of contacts between the TM helices. In the right panel, the thickness of lines is kept uniform because here a line indicates only the presence of a non-covalent contact and not the number of non-covalent contacts. Numbers denote Ballesteros–Weinstein numbering (see Fig. 2 legend).

been observed to bind in a pocket in the extracellular side of the TM bundle, different ligands penetrate to different depths within this pocket (Fig. 4a). Despite the diversity in the ligands, a systematic comparison of the residues that contact the ligand revealed similarities in the ligand-binding pocket (that is, residues within 4 Å distance of any ligand atom). We observe that except for the ligands of the CXCR4 receptor and NTSR1, most of the ligand-contacting residues are present in the TM helices (Fig. 4b). Topologically equivalent residues from TM3, TM6 and TM7 typically contact the ligand in nearly all receptors. In particular, residues at positions 3.32, 3.33, 3.36, 6.48, 6.51 and 7.39 make consensus contacts with diverse ligands across class A GPCRs. Residues from the other TMs tend to contact specific ligands to different extents. Thus, one can surmise that these key positions in TM3, TM6 and TM7 form a consensus scaffold of the ligand-binding pocket and that variation in the amino acids occupying the topologically equivalent positions contribute to ligand specificity in different receptors. TM1 is not directly involved in contacting the ligand in any structure, suggesting that mutations in TM1 that affect ligand binding are likely to be indirect. In addition to residues that directly contact ligand, water molecules have also been observed to mediate indirect contacts between the ligand and the receptor[11,24,37,48].

The ligand–binding pocket and consensus scaffold interface

Of the positions that form the consensus ligand-binding pocket, two pairs of contacting residues (between 3.36–6.48 and 6.51–7.39) are also present in the consensus inter-TM contact network (Fig. 4b). Indeed, biochemical studies have shown that mutations in these positions affect receptor conformational selectivity[12] and ligand-binding affinity[49]. Furthermore, a non-covalent contact between residues 6.48 and 6.51 in TM6 is also maintained in the structures. Together, residues in these positions appear to form a 'ligand-binding cradle' in the TM bundle in nearly all class A GPCR structures. An important role of the conserved structural scaffold might be to position the ligand-binding region of the TM helices in a precise configuration that forms a pocket. Importantly, this set of consensus ligand-binding positions can further our understanding of the ligand-binding pockets of other GPCRs, the molecular basis of cross-reactivity, the specificity of ligands among subfamilies of receptors, poly-pharmacology and also aid in fragment-based drug discovery.

Functional and structural importance of intracellular regions

Residues in the intracellular region and the cytoplasmic ends of TM regions bind downstream signalling effectors such as G proteins, GPCR kinases and arrestins[9,50]. In the available structures, ICL1 is typically six amino acids long and contains a helical turn, whereas ICL2 generally has either a one- or two-turn α-helix or an unstructured stretch of amino acids (Fig. 2). Interestingly, in all the opioid receptor and muscarinic receptor structures, a conserved Arg in ICL2 forms a salt bridge with Asp$^{3.49}$ of the DRY motif in TM3, thereby tethering ICL2 with the TM core[5]. This is analogous to a conserved Tyr in ICL2 of several aminergic receptors that also forms a hydrogen bond with Asp$^{3.49}$. The importance of this interaction is highlighted by the fact that mutation of Tyr 149 in the ICL2 of avian β_1-AR decreases receptor stability[10], and phosphorylation of Tyr 141 in the ICL2 of human β_2-AR

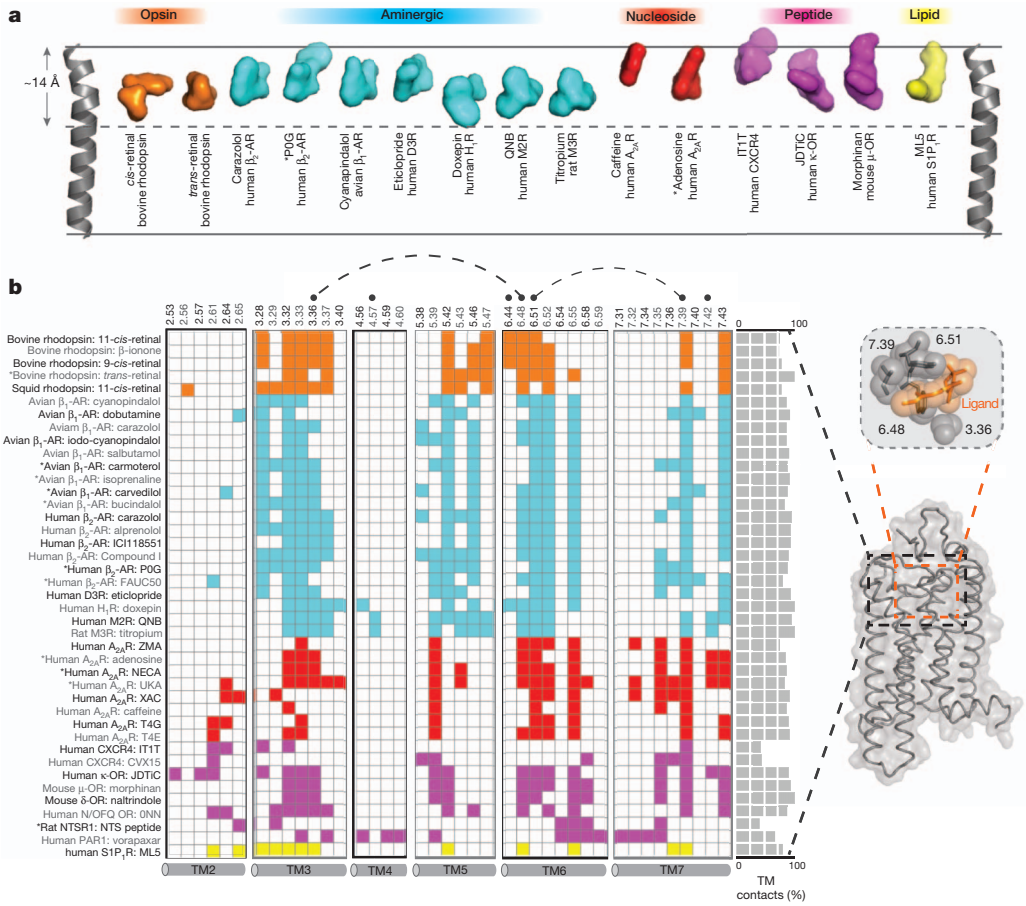

Figure 4 | Ligand-binding pocket in class A GPCRs. a, Comparison of depth of ligand-binding pockets in which TM4 is used as a frame of reference. The depth of ligand penetration into the TM bundle is the deepest for doxepin in the histamine H_1 receptor and shallowest for caffeine in $A_{2A}R$. The label on the top indicates the subfamily of class A GPCRs. **b**, Characterization of ligand-binding pockets of class A GPCRs. Comparison of the TM residues that are present in the ligand-binding pocket is shown as a matrix. Receptor–ligand information is shown as rows, and the Ballesteros–Weinstein numbers of TM residues that contact the ligand are shown as columns. The TM residues that are present in the consensus inter-TM contact network are marked with black dots, and contacts between these residues are shown as dotted lines. Rows marked with an asterisk denote agonist-bound receptor structures. In the matrix, the presence of a contact between the ligand and the TM residue is shown as a coloured box, and the absence of a contact is shown as an empty box. The percentage of TM residue contacts made by the ligand is shown as a bar plot on the right of the matrix (other contacts that are not shown are made with residues in the extracellular region). The backbone representation of rhodopsin is shown as a reference for showing the ligand-binding pocket (black dashed square). The consensus ligand-binding pocket, also referred to as the ligand-binding cradle, is illustrated with retinal as an example (orange dashed box).

facilitates a shift of the receptor conformational equilibrium towards the active state[51]. In the β_2-AR–G-protein complex, ICL2 was observed to interact with the N terminus of the Gα subunit[9]. In addition to the ICLs, a short amphipathic helix (H8), typically three turns long and with palmitoylation sites at its C terminus, is present in several class A GPCR structures with the exception of the CXCR4, NTSR1 and PAR1, in which this region was observed to be unstructured. Although several studies have implicated H8 in G-protein binding, this region is not seen to contact the G protein in the structure of the GPCR heterotrimeric G-protein ternary complex[9]. Future studies on H8 should provide insights into its role in the structure and function of the receptor.

Intrinsically disordered segments in intracellular regions

ICL3 and the C-terminal tail are long, and variable regions[40] and are probably intrinsically disordered in many GPCRs[52]. Such disordered regions typically expose linear peptide motifs that recognize specific partners and allow for regulation of their binding and function[53,54]. Interestingly, a polybasic motif proximal to H8 in many GPCRs has been shown to facilitate G-protein pre-coupling and influence the rate of receptor activation[55]. Furthermore, several residues in the C-terminal tail of β_2-AR (ref. 56) are extensively post-translationally modified, providing support for the existence of a signalling 'bar code'. In β_2-AR, diverse GPCR receptor kinases and insulin receptor tyrosine kinase can phosphorylate the cytoplasmic regions, and the different phosphorylated forms of the receptor show distinct patterns of interaction with β-arrestin, thereby influencing receptor activity and internalization from the membrane[56]. A more thorough investigation of the residues in the disordered regions may provide important insights into the diversity of downstream interaction partners and signalling events.

TM3 is a structural and functional hub

Considering the observations from comparing the structures of diverse GPCRs in the active and inactive states, it emerges that almost every position in TM3 seems to be important for maintaining either the structure or function of GPCRs. Such a role may be facilitated by the unusually large tilt-angle of TM3 with respect to the axis perpendicular to the plane of the lipid bilayer. The residues in TM3 form a consensus network of conformation-independent inter-TM contacts between strategically placed residues in other TM helices, thereby defining the GPCR fold (Fig. 3). The extracellular end of TM3 forms a conserved disulphide bridge with ECL2, and $Asp^{3.49}$ in the cytoplasmic end interacts with ICL2 in several receptors. At the same time, residues in TM3 mediate extensive contacts with ligands of diverse pharmacology and participate in the formation of the 'ligand-binding cradle' (Fig. 4). After

activation, the cytoplasmic end of TM3 forms an important interface for G-protein binding (Fig. 5) and the Arg$^{3.50}$ of the DRY motif directly interacts with a backbone carbonyl of the C terminus of the G protein[9,14]. Importantly, mutations in many of the positions in TM3 cause receptor inactivation or constitutive activation, suggesting that TM3 is a structural and functional hub in GPCRs (Fig. 6).

Molecular changes during receptor activation

Receptor activation involves binding of ligands to the extracellular part of the TM region and the extracellular region, thereby resulting in small conformational changes in the TM core. This ultimately leads to larger structural rearrangements in the cytoplasmic side of the transmembrane–intracellular interface, facilitating the binding of intracellular effectors to the intracellular region. The active state of a GPCR is thus defined as the conformation of the receptor that couples to and stabilizes an effector molecule such as the heterotrimeric G protein[57]. The availability of structures in the intermediate-active and active states has provided important insights into this general mechanism[29,58–60]. However, this 'static' information from crystal structures provides only partial insights into dynamic aspects of activation such as allostery. Several recent studies that interpret biochemical, biophysical and computational techniques in light of the crystal structures are beginning to provide detailed insights into these dynamic processes and the complex equilibrium between the receptor conformational ensembles.

Existence of several conformational states

Our understanding of conformational dynamics and the activation pathway in GPCRs is primarily derived from experiments carried out on bovine rhodopsin and human β_2-AR using various biophysical and biochemical approaches. In rhodopsin, a series of spectroscopically distinct intermediates has been structurally characterized (Supplementary Table 1). For β_2-AR, it has been shown that binding of agonists stabilizes specific sub-states that are typically sparsely populated in the ligand-free receptor[61], and also that ligands with different efficacies are able to shift the equilibrium between receptor conformations to different extents[62]. The existence of an intermediate receptor conformation is also supported by atomistic molecular dynamics simulations of β_2-AR (ref. 63). After stabilization of these lowly populated sub-states by ligand binding, activation proceeds through a series of discrete conformational intermediates[58–60] that ultimately lead to larger structural rearrangements near the transmembrane–intracellular interface.

Conformational changes can be investigated using quantitative mass spectrometry that measures the reactivity of side chains of individual amino acids to particular chemicals. In β_2-AR, it was shown that when receptors bind functionally similar ligands, there were distinct patterns of reactivity of the probe with different amino acids[64]. This supports the view that after binding, a considerable variability in receptor conformation is induced or stabilized by similar ligands. Concordantly, when β_2-AR was studied by hydrogen-deuterium exchange mass spectrometry, it was shown that inverse agonists are more stabilizing whereas agonists induce the largest degree of conformational mobility[65]. Although these findings are consistent with the X-ray structures of the active states, some of the structures of β-ARs bound to agonists were observed to be in the inactive conformation[34–36]. This suggests that agonist binding alone may not be sufficient to stabilize fully active states (as defined by the structure of the β_2-AR–G_s complex[9]), and the conformation adopted by an agonist-bound receptor depends on the energy landscape for each specific receptor. Only after binding of a G protein, arrestin or conformation-specific antibodies will the fully active state of the receptor become the dominant state. An exception is that it was possible to obtain a ligand-free structure of opsin with an activated conformation in the presence of lipids at low pH[66].

Sequence analysis of GPCR family members has identified a network of co-evolving residues that maybe important for allosteric communication[67]. Furthermore, molecular dynamic simulations of β_2-AR also suggest that a loosely coupled allosteric network links small perturbations at the ligand-binding site to large conformational changes at the intracellular G-protein-binding site, and provide insights into metastable states that may be difficult to study experimentally[63]. Along these lines, computational studies have shown that ligands with different efficacies modulate the free-energy landscape of the receptor by shifting the conformational equilibrium towards active or inactive conformations depending on their elicited physiological response[68,69]. Thus it seems that there may not be a single active state and that ligands can stabilize distinct conformations, thereby giving rise to diverse downstream responses.

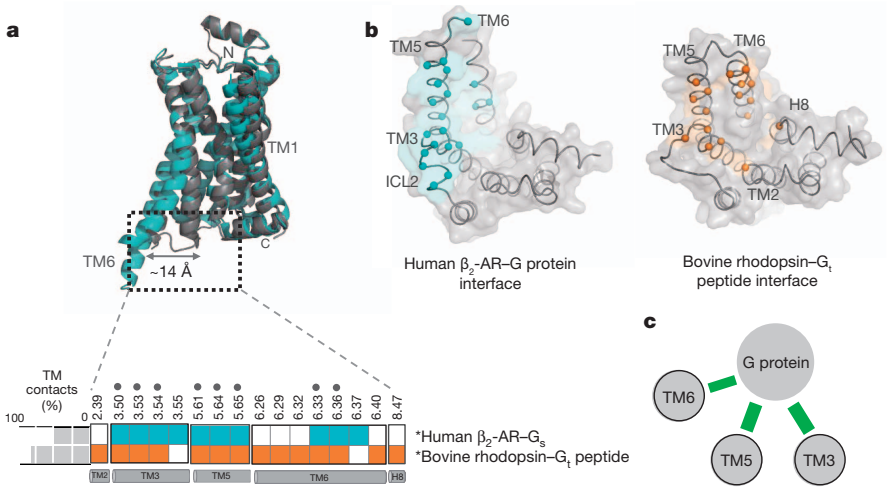

Figure 5 | Characterization of G-protein-binding region within TM helices of GPCRs. a, Comparison of the TM movement between the active (cyan) and inactive (grey) state of β_2-AR (top). TM6 moves 14 Å after activation. TM residues that contact the G_s protein and G_t peptide are shown as a matrix for β_2-AR (cyan) and rhodopsin (orange), respectively (bottom). Black dots above Ballesteros–Weinstein numbers indicate residues in the TM region that contact cytoplasmic-binding partners (G_s protein and G_t peptide) in both β_2-AR (cyan) and rhodopsin (orange). The percentage of all G_s protein or G_t peptide contacting residues within the TM region is shown as a bar plot to the left of the matrix. Other contacts (not shown) are made with residues in the intracellular region; for instance, ICL2 makes contacts with the N-terminal region of the Gα subunit[9]. **b**, Cytoplasmic view of β_2-AR and rhodopsin showing the interface of the G-protein interaction. Cα positions of residues in the receptor are shown as spheres. **c**, Schematic representation of the interaction of TM helices with the G protein (contacts with intracellular region are not shown). G protein and TM helices are shown as circles and the interactions are shown as green lines. The thickness of the line is proportional to the number of consensus contacts that are made at the interface of β_2-AR–G_s protein and rhodopsin–G_t peptide.

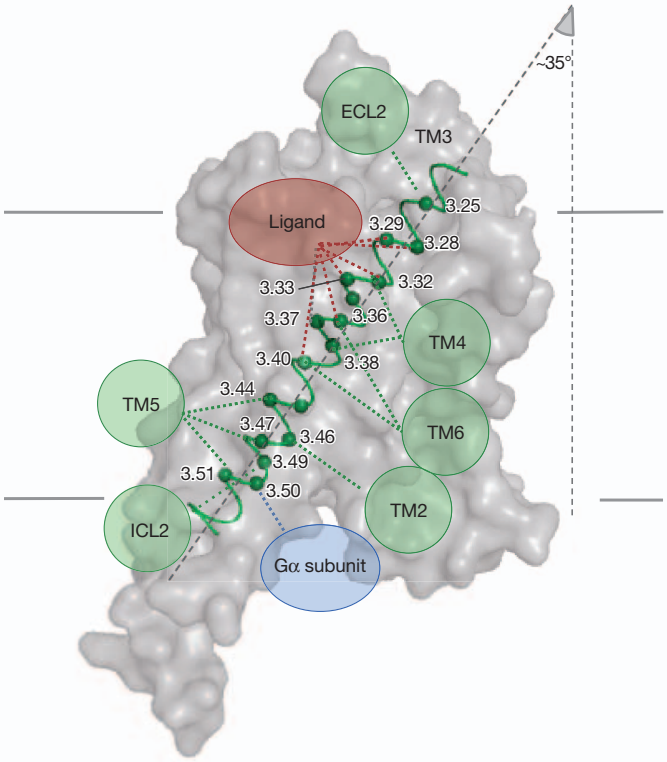

Figure 6 | Structural and functional hub role of TM3. The backbone of TM3 is shown as a ribbon helix in dark green. Ballesteros–Weinstein numbers of residues involved in making contacts with various parts of the receptor, ligand and the G protein are shown. Other TM helices and extracellular and intracellular regions interacting with TM3 are shown as green circles. Ligand and G protein are shown as a brown and blue ellipse, respectively. Note the unusual tilt-angle of TM3 with respect to the lipid bilayer.

Changes in the EC region during receptor activation

The EC region is unlikely to be a passive segment as NMR experiments have revealed conformational changes during receptor activation[70]. In rhodopsin, solid-state NMR data show that activation, which is initiated by the light-induced isomerization of 11-*cis*-retinal to all-*trans*-retinal, is accompanied by conformational changes in ECL2 (ref. 71). In β_2-AR, the existence of different conformations of a salt bridge connecting ECL2 and ECL3 lining the ligand-binding pocket, for agonists, neutral antagonists and inverse agonists was shown based on solution NMR spectroscopy using a modified lysine residue as a conformational probe[72]. These studies suggest that drugs targeting this surface could function as allosteric modulators with GPCR subtype-specific selectivity[72]. In this context, it has been reported that many of the autoantibodies that behave like agonists recognize the extracellular region of the receptor in diverse human diseases such as in Graves' disease. One possibility is that these antibodies stabilize distinct states of the extracellular loop, thereby allosterically modulating receptor activity, leading to activation of downstream signalling pathways in the absence of the ligand[40].

TM changes during receptor activation after agonist binding

The agonist-bound structures of rhodopsin[13,14,33], β_2-AR (refs 9, 34) and $A_{2A}R$ (refs 11, 37) show different patterns of ligand–receptor interactions. However, these interactions seem to result in a set of common structural rearrangements in the extracellular part of the TM bundle near the agonist-binding site. These changes can be summarized as follows: (1) small local structural changes in the Pro$^{5.50}$-induced distortion of TM5; (2) relocation of TM3 and TM7; and (3) translation/rotation of TM5 and TM6. In β_2-AR and $A_{2A}R$, agonist binding 'pulls' the extracellular sides of TM3, TM5 and TM7 together, whereas in rhodopsin retinal isomerization results in an increase in the volume of the binding pocket[14]. Furthermore, in $A_{2A}R$, agonist binding induces a 2 Å shift of TM3 along its axis and towards the extracellular side[11,73]. However, in all cases, these movements are accompanied by a rearrangement of a cluster of conserved hydrophobic and aromatic residues ('transmission switch', made of 3.40, 5.51, 6.44 and 6.48) deeper in the receptor core[58]. As a result, there is a rearrangement at the TM3–TM5 interface, and formation of new non-covalent contacts at the TM5–TM6 interface. Many of the residues in this transmission switch are highly conserved in class A GPCRs, suggesting that they are likely to constitute a common feature of GPCR activation.

These agonist-induced local structural changes near the binding site are translated into larger-scale helix movements through distinct activation pathways[58,59,73,74]. Specifically, the changes at the TM5 bulge involving Pro$^{5.50}$ are transmitted though the helix, resulting in rearrangements at its cytoplasmic side[75]. Also, rearrangement of the TM5–TM6 interface is linked to the rotation of TM6 near Phe$^{6.44}$, which is amplified owing to the strong kink of this helix, resulting in the large-scale relocation of the cytoplasmic side of TM6 (ref. 13). This opens the cleft required for binding of the G protein. This rearrangement was first demonstrated in rhodopsin using electron paramagnetic resonance spectroscopy[76], and further established by fluorescence spectroscopy, ultraviolet spectroscopy and chemical cross-linking of histidines, and by double electron–electron resonance spectroscopy experiments[77,78]. Infrared spectroscopy on rhodopsin labelled with genetically encoded probes suggests that smaller conformational changes in TM6 in early intermediate states precede its larger rigid-body movement[79]. The extent of the movement of TM6 varies among receptors[58], reaching 14 Å in the structure of the β_2-AR–G protein complex[9] (Fig. 5a). As discussed above, not all agonist-bound GPCR structures present these large-scale rearrangements in the cytoplasmic region, although many of them feature the rearrangement of the transmission switch[58]. These structures must therefore correspond either to an initial encounter complex between ligand and receptor or to 'intermediate' active states that have not manifested the full set of conformational changes to allow binding of the G protein. Thus, it seems that some receptors only adopt a fully active conformation when the G protein or other interaction partners (such as a peptide or antibody) stabilize the opening of the cytoplasmic domain. Water molecules have also been observed to facilitate conformation-specific contacts; for instance, in the inactive- and active-state rhodopsin structures, two distinct water-mediated hydrogen-bonding networks are present that involve many of the highly conserved residues of class A receptors[13,14].

TM–intracelluar region changes during receptor activation

Residues from the ICL2 and cytoplasmic end of TM3 (Arg$^{3.50}$ of the conserved DRY motif) interact with the G protein after activation[9]. In rhodopsin, Arg$^{3.50}$ forms a salt bridge with Glu$^{6.30}$ in the inactive state that is broken after activation. However, this 'ionic lock' is not a conserved feature of the inactive conformation of all GPCRs and has been observed to be conformationally plastic, thus implicating this region in basal activity of receptors[73]. The conformation of a native ICL3, which connects TM5 and TM6, is known only in rhodopsin[80] and $A_{2A}R$ (refs 7, 12). Comparison of the structures of $A_{2A}R$ T4–lysozyme chimaera[24] with that of the thermostabilized[12] or antibody-bound[7] $A_{2A}Rs$ shows that fusion of the lysozyme distorts the cytoplasmic sides of TM5 and TM6. Interestingly, some of the structures of β_1-AR with a shortened form of ICL3 are seen either with or without the ionic lock and suggests that this interaction may have a role in the regulation of receptor activation and also in basal activity[81].

Comparison of the active-state structures of the β_2-AR bound to the G protein (G_s)[9] and of rhodopsin (metarhodopsin II) bound to a peptide that resembles the C-terminal tail of G_t (ref. 33) allows the identification of a consensus interface in the TM helices for G-protein binding. This is formed by at least eight positions from TM3, TM5 and TM6 (Fig. 5). To create this interface, TM5 and TM6 move considerably compared to the inactive states (Fig. 5). It should be stressed, however, that the N terminus of the G protein interacts with the ICL2 and that the structure of

ICL2 is crucial to this interaction. Hydrogen-deuterium exchange mass spectrometry showed that the receptor perturbs the structure of the amino-terminal region of the α-subunit of G_s and consequently alters the 'P-loop' that binds the β-phosphate in GDP, thereby influencing the affinity to bind GTP[82]. It also seems that there is allosteric communication between the G-protein interface and the ligand-binding region as it has been shown that G proteins induce or stabilize a conformation in the receptor that binds agonists with a 100-fold higher affinity[83]. This suggests that the allosteric coupling between the ligand-binding site and the G-protein-binding site is bidirectional[7].

Biased signalling

Although GPCRs primarily operate by coupling to G proteins, interaction with other scaffold proteins, such as β-arrestins, can influence signalling events[84]. Certain ligands can preferentially trigger some of these signalling pathways, a phenomenon designated as biased agonism or functional selectivity[85]. Biased ligands that can selectively influence beneficial signalling pathways may have a high therapeutic potential as drug candidates. Nevertheless, it cannot be ignored that some biased ligands might affect undesirable signalling pathways leading to drug side effects. Despite its relevance to medical and biological fields, structural and mechanistic aspects underlying biased signalling are not yet well understood. However, recent studies are beginning to provide new insights in this direction. For instance, the first structures of β_1-AR bound to arrestin-biased ligands[36] show that they have weaker interactions with TM5 than full agonists, but contact additional residues in a 'minor' binding pocket near TM7 and involving ECL2, possibly involved in arrestin-biased signalling[86]. These thermostabilized structures do not show large conformational changes in the cytoplasmic side of the receptor. However, the additional ligand–receptor interactions with TM7 could affect the conformational states of this helix via a conserved activation pathway through TM2 or TM7 (ref. 74). In line with this, recent ^{19}F-NMR spectroscopy experiments show that binding of biased ligands alters the local environment of a chemically modified Cys at the TM7–H8 interface[87]. In addition, lanthanide-based resonance energy transfer spectroscopy studies on the arginine-vasopressin type 2 receptor showed that the G-protein-biased agonists stabilize a conformation of TM6 and TM7–H8 that is distinct from that stabilized by the arrestin-biased agonists[88]. Taken together, these studies suggest the existence of parallel and partially decoupled pathways of receptor activation that result in G-protein and β-arrestin signalling. However, we are far from a molecular understanding of how these conformational changes in the receptor are translated into cellular signalling events.

Structural features of class B, class C and other GPCRs

Although relatively little is known about the overall structure of non-class A GPCRs, important progress has been made to understand the structural and functional aspects of the extracellular ligand-binding domains of class B, class C and other receptors (covered extensively in ref. 39). In class B GPCRs, structures of N-terminal domains from different receptors have been solved by solution NMR and X-ray crystallography, revealing a common fold formed by an N-terminal α-helix and two β-sheets stabilized by three conserved disulphide bridges[89]. Ligands interact with these extracellular domains to induce receptor activation, but it is not clear whether the TM bundle contributes to a shared orthosteric-binding site[70]. Class C GPCRs possess a large N-terminal domain containing a bilobed structure that forms the ligand-binding site. The structure of this domain (the 'venus flytrap module') has been solved by X-ray crystallography for several members of this class. Receptor activation has been proposed to proceed through allosteric coupling between the ligand-bound extracellular domain and the transmembrane bundle[90]. Structures of the GPCR-autoproteolysis inducing (GAIN) domain from adhesion GPCRs are beginning to provide insights into the molecular basis of receptor function for the adhesion family of GPCRs[91]. The availability of structures of the TM domain region for these classes of GPCRs may provide the basis for understanding whether common structural features govern all GPCR classes. Given the limited variability in the G-protein repertoire, which is in sharp contrast to the enormous diversity in the shape and size of ligands across different GPCR classes, it is likely that the G-protein interaction interface of the receptor will be more similar than the ligand-binding site across the GPCR classes.

Outlook and future directions

The recent development of several technologies to facilitate GPCR structure determination will probably allow elucidation of structures of virtually any GPCR encoded in the human genome, although undoubtedly some will be more tractable than the others. Thus, structures of class B receptors (for example, peptide-hormone receptors) and class C receptors (for example, the metabotropic glutamate receptor) will probably be determined soon. These GPCRs share less sequence similarity with class A receptors, making them difficult to model on the basis of existing structures. Therefore, the structures of GPCRs from these additional classes may illuminate new modes of ligand binding and signal transmission, whereas comparison with class A structures will reveal universal principles underlying GPCR signalling.

Different ligands can affect whether a GPCR couples to one or several G proteins, or preferentially to arrestin and other intracellular effectors. This raises many key questions that are yet to be addressed: what is the molecular basis of the coupling of GPCRs with different intracellular effectors? How do different ligands selectively stabilize different active conformations or influence the conformational equilibrium? What is the role of water molecules, ions, cholesterol and native lipids in GPCR structure and function? An important step towards answering these questions is to obtain more high-resolution structures of GPCRs in the fully activated state in complex with different ligands and diverse intracellular effector proteins. With the determination of structures of increased resolution and application of complementary approaches, one can gain insights into how endogenous small molecules and the near native environment of the receptor can modulate function[92]. Another key structural problem is to understand how GPCRs associate to form homo- or hetero-oligomers. Some crystal structures suggest potential models for how GPCR dimers could form in the cell[25,27], but these need to be tested rigorously *in vivo*[93]. Furthermore, recent promising developments of free electron lasers will allow structure determination from small and easier to obtain nanocrystals. These advances will facilitate a better understanding of the structural determinants and functional implications of the activation mechanisms and oligomerization interfaces of receptors.

With approximately 30% of the known drugs targeting GPCRs, the pharmacological relevance of this family is firmly established. Structures of more GPCRs will facilitate structure-based *in silico* drug discovery and the development of therapeutic compounds with improved specificity and pharmacodynamics[48]. For example, the first preclinical compounds developed from structure-based drug design have recently been described for A_{2A}R as candidates for the treatment of Parkinson's disease[94]. Although GPCRs are particularly amenable to high-throughput *in silico* screening owing to deep binding cavities where the endogenous ligands bind, recent structural studies suggest other possible regions on the receptor for rational drug development. For example, one possibility would be to modulate receptor activity at the intracellular G-protein-binding site using short cell-penetrating peptides (pepducins) or single-domain antibodies. The structures of receptors highlight those regions that are unique to particular receptor subtypes, which may facilitate more rational developments of subtype-specific drugs, either using traditional small molecule chemistry or innovative approaches such as bicyclic or linear peptides[95]. Furthermore, these regions may be amenable to the design of allosteric modulators or bitopic ligands[96] such as small molecules, peptides or antibody domains[97]. Also, structure determination of GPCR mutants involved in human diseases will allow understanding of their molecular causes, and help design new therapeutic approaches for their treatment.

Although GPCR structures will be exceedingly informative, a systems-level understanding of the interactions between GPCRs and intracellular effectors in the cell will provide a holistic understanding of the cellular response under physiological and disease conditions. This will require a detailed characterization of which GPCRs and intracellular effectors are present in different cell types and, importantly, their abundance and the kinetic parameters of their interaction[98,99]. In addition to a systems biology approach and the discovery of new signalling functions, a genomic approach will be essential to understand the link between receptor polymorphisms in the population and how these may be linked to disease and drug efficacy. In this context, large international consortia that exploit next-generation sequencing technology such as the '1000 genome' and 'cancer genome' projects are identifying natural variants and discovering disease-causing mutations. Such receptor polymorphisms may affect activation kinetics and drug selectivity in subtle but important ways, perhaps explaining the heterogeneous response to drugs between individuals[33,35,36,100]. The growing number of solved structures provides a framework to now interpret such functional heterogeneity, paving the path towards personalized medicine and hence improving human health.

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