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## Structural insights into ligand recognition and selectivity for class A, B, and C GPCRs

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### Abstract

The G protein-coupled receptor (GPCR) superfamily constitutes the largest collection of cell surface signaling proteins with approximately 800 members in the human genome. GPCRs regulate virtually all aspects of physiology and they are an important class of drug targets with ~30% of drugs on the market targeting a GPCR. Breakthroughs in GPCR structural biology in recent years have significantly expanded our understanding of GPCR structure and function and ushered in a new era of structure-based drug design for GPCRs. Crystal structures for nearly thirty distinct GPCRs are now available including receptors from each of the major classes, A, B, C, and F. These structures provide a foundation for understanding the molecular basis of GPCR pharmacology. Here, we review structural mechanisms of ligand recognition and selectivity of GPCRs with a focus on selected examples from classes A, B, and C, and we highlight major unresolved questions for future structural studies.

### Keywords

GPCR structure; GPCR subtype; X-ray crystallography; Ligand recognition; Subtype selectivity

### 1. GPCR structural revolution

G protein-coupled receptors (GPCRs) are cell surface receptors that mediate biological responses to external stimuli by transducing signals across the plasma membrane to heterotrimeric G proteins and arrestins, which in turn activate cellular signaling cascades. GPCRs regulate many physiological processes and respond to a diverse array of endogenous ligands including neurotransmitters, peptide and protein hormones, amino acids, ions, lipids, and various other bioactive molecules (Lagerstrom and Schieth, 2008). In addition, chemosensory GPCRs are responsible for our senses of sight, smell, and taste by responding to photons, odorants, and tastants. GPCRs are important drug targets for many diseases because of their fundamental roles in critical processes and inherent druggability as cell

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surface proteins. It is estimated that ~ 30% of drugs on the market target a GPCR and yet a relatively small number of GPCRs are currently targeted by marketed drugs (Overington et al., 2006). There remains an immense opportunity for development of drugs targeting many other GPCRs. High-resolution protein structures, such as those obtained from X-ray crystallography, reveal the molecular basis for protein function and facilitate rational drug design. In this review we highlight recent progress on structural studies of GPCRs that have revolutionized the field by greatly expanding our understanding of the molecular basis of GPCR pharmacology and enabling structure-based drug design to be applied to this important protein family. Several excellent recent reviews cover GPCR structural topics including orthosteric and allosteric ligand binding (Katritch et al., 2012, 2013; Kruse et al., 2014), ligand efficacy (Warne and Tate, 2013), receptor activation (Audet and Bouvier, 2012; Tehan et al., 2014), and structure-based drug design (Congreve et al., 2014). Here, we focus on structural mechanisms of ligand recognition and selectivity with selected examples from three major GPCR classes.

The human genome encodes approximately 800 GPCRs, which are grouped as four classes, A, B, C, and F (Kolakowski, 1994) or as five families, Rhodopsin, Adhesion, Secretin, Glutamate, and Frizzled (Fredriksson et al., 2003). The class A/Rhodopsin family comprises the largest number of GPCRs and our structural understanding of these receptors is most advanced. Rhodopsin was the first GPCR to have its structure determined by X-ray crystallography in 2000 (Palczewski et al., 2000), followed by the  $\beta_2$ -adrenergic receptor structure in 2007 (Cherezov et al., 2007; Rosenbaum et al., 2007) and reports of other class A GPCR structures continually appearing since (reviewed in (Katritch et al., 2013)). We now have structures of over twenty distinct class A GPCRs including the landmark achievement of the ternary agonist-bound  $\beta_2$ -adrenergic receptor-Gs complex structure (Rasmussen et al., 2011b). The year 2013 saw reports of the first 7-transmembrane (7TM) domain structures for two class B/Secretin family GPCRs (Hollenstein et al., 2013; Siu et al., 2013) and of the class F/Frizzled family smoothed GPCR (Wang et al., 2013b). In 2014 the first 7TM domain structures for two class C/Glutamate family GPCRs were reported (Dore et al., 2014; Wu et al., 2014). We do not yet have a high-resolution crystal structure of a GPCR- $\beta$ -arrestin complex, but we are beginning to understand the architecture of this complex as revealed at low resolution by electron microscopy (Shukla et al., 2014).

The explosion of GPCR crystal structures was enabled by several technological breakthroughs that made possible the crystallization of these dynamic integral membrane proteins that were once thought “uncrystallizable”. Most of the structures utilized recombinant protein expressed in insect cells and codon optimization technology enabled increased expression levels. Development of novel detergents (Rasmussen et al., 2011b), lipid cubic phase crystallization (Cherezov, 2011), and X-ray microdiffraction beamlines (Riek et al., 2005) all contributed to making GPCR crystallography a reality. Perhaps the most important was the development of methods to stabilize the receptors for crystallization including the use of fusion protein approaches such as T4 lysozyme insertion into loops to facilitate crystal packing combined with lipid cubic phase crystallization (Cherezov et al., 2007; Rosenbaum et al., 2007) and the generation of thermostabilized receptors by mutagenesis (Tate, 2012). A recent twist on the thermostabilization approach utilizing

elegant directed evolution methods in *E. coli* (Scott and Pluckthun, 2013) resulted in the first crystal structure of a GPCR utilizing recombinant protein produced in *E. coli* (Egloff et al., 2014), a feat once thought impossible. Most GPCR structures are inactive antagonist- or inverse agonist-bound states. Crystallizing active state GPCRs is still a challenge, but this obstacle has been overcome by using nanobodies to stabilize the active state by mimicking G proteins (Kruse et al., 2013; Rasmussen et al., 2011a).

## 2. Class A GPCR structure and ligand binding

Class A/Rhodopsin family GPCRs make up the vast majority of the GPCR repertoire in the human genome with hundreds of receptors. Class A receptors consist of a 7-transmembrane (7TM) helical bundle in the membrane with three extracellular and three intracellular loops connecting the individual helices and an extracellular N-terminus and intracellular C-terminus (Fig. 1A) (Cherezov et al., 2007; Rosenbaum et al., 2007). A few class A GPCRs such as the glycoprotein hormone receptors have large N-terminal extracellular domains (ECDs) that bind ligands, but these will not be discussed here. The seven TM helices form a bundle; when viewed from the extracellular side helices I–VII are arranged in a counter-clockwise fashion (Fig. 1B). A short helix VIII that runs parallel to the membrane is often found near the C-terminus and includes sites of palmitoylation (Fig. 1A). TM helix III is a central hub critical to GPCR structure, ligand binding, and receptor activation (Venkatakrisnan et al., 2013). The orthosteric ligand binding pocket is positioned in the extracellular half of the bundle and is formed by residues from helices III, VI, and VII (Fig. 1A, B). In some cases residues from other helices also make specific ligand contacts such as with helix V for the  $\beta$ -adrenoceptors, which is an important determinant for ligand efficacy (Warne and Tate, 2013). In the  $\beta_2$ -adrenergic receptor access to the deep orthosteric pocket is available from the extracellular milieu because the extracellular loops do not completely cover the top of the receptor (Fig. 1B). An extracellular vestibule surrounded by the tops of helices V, VI, and VII and ECL2 and ECL3 is present above the orthosteric site. Molecular dynamics simulations detailed the pathway by which ligands first contact the extracellular vestibule and subsequently enter the deep orthosteric pocket (Dror et al., 2011).

The numerous class A GPCR structures available highlight the diversity in ligand binding sites and extracellular loops (Katritch et al., 2012). Although the orthosteric ligand binding sites are all in a similar position in the extracellular half of the helical bundle, the precise architectures and depth of the binding sites as well as access to the sites vary greatly. The  $\beta_2$ -adrenergic receptor ligand binding site is representative of the aminergic GPCRs (Fig. 1A, B). The lipid-binding sphingosine-1-phosphate (S1P) receptor has its orthosteric ligand binding pocket positioned very similar to the aminergic receptors, but access to the pocket from the extracellular milieu is blocked by an extended N-terminal  $\alpha$ -helix that covers the top of the receptor (Fig. 1C) (Hanson et al., 2012). A lateral opening between helices I and VII is presumed to allow the hydrophobic lipid ligand to access the orthosteric site from within the lipid bilayer (Fig. 1C). Peptide-binding class A GPCRs often have an ECL2 with a  $\beta$ -hairpin structural element and relatively open access at the top of the receptor (Fig. 1D). The neurotensin peptide binds the neurotensin type 1 receptor in an extended conformation and does not penetrate as deep into the receptor as observed for ligands of many other class

A GPCRs; as a consequence, the neurotensin peptide makes several contacts with the receptor extracellular loops (Fig. 1D) (Egloff et al., 2014; White et al., 2012).

### 3. Class A GPCR ligand selectivity

Crystal structures of several subtypes of class A GPCRs were recently revealed: serotonin 5-HT<sub>1B</sub> and 5-HT<sub>2B</sub> receptors, muscarinic acetylcholine M2 and M3 receptors, and four subtypes of opioid receptors (Fenalti et al., 2014; Granier et al., 2012; Haga et al., 2012; Kruse et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Wacker et al., 2013; Wang et al., 2013a; Wu et al., 2012). Although these receptors originate from several species (most from human, but some from rat and mouse) (Granier et al., 2012; Kruse et al., 2012; Manglik et al., 2012), structural comparisons provide insights into the mechanisms for receptor subtype selectivity. The opioid receptor crystal structures are useful to test the “message-address” hypothesis of opioid ligands that has been commonly assumed for opioid receptor subtype selectivity (Lipkowski et al., 1986; Portoghese, 1989).

#### 3.1 Serotonin receptors

Serotonin [5-hydroxytryptamine (5-HT)] is an endogenous neurotransmitter acting on multiple organs. Physiological effects of serotonin are mediated by a large family of serotonin receptors that are divided into seven subfamily groups (5-HT<sub>1-7</sub>) based on sequence homology and signaling pathways (Kroeze et al., 2002). The seven subfamily groups include several serotonin receptors, and total thirteen serotonin receptor subtypes have been reported in human (McCorvy and Roth, 2015). All the serotonin receptors are G protein-coupled receptors with one exception of the 5-HT<sub>3</sub> receptor, which is an ion channel. Recently, two subtype 5-HT<sub>1B</sub> and 5-HT<sub>2B</sub> receptors bound with an antimigraine drug ergotamine were reported (Wacker et al., 2013; Wang et al., 2013a). Although the main target of ergotamine is 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors (Tfelt-Hansen et al., 2000; Villalon et al., 1999), its usage is very limited due to off-target actions on the other 5-HT subtype receptors particularly on the 5-HT<sub>2B</sub> receptor that causes life-threatening valvulopathy on heart (Elangbam et al., 2008; Nebigil et al., 2001).

The orthosteric binding pockets of 5-HT<sub>1B</sub> and 5-HT<sub>2B</sub> receptors are located deeply in the transmembrane core, and they conserve key amino acids for ligand interactions (Wang et al., 2013a). One difference in the orthosteric binding pocket is that Y208<sup>5.38</sup> in the 5-HT<sub>1B</sub> receptor is unable to interact with ergotamine, whereas the corresponding F217<sup>5.38</sup> in the 5-HT<sub>2B</sub> receptor provides a hydrophobic cap (Fig. 2A, upper letters refer to Ballesteros-Weinstein nomenclature to indicate the amino acids in transmembrane domains (Ballesteros and Weinstein, 1995)). A 3.0 Å distance is observed between the α carbons of T209<sup>5.39</sup> in the 5-HT<sub>1B</sub> receptor and M218<sup>5.39</sup> in the 5-HT<sub>2B</sub> receptor (Fig. 2A). This shift provides a narrower opening of the extended binding pocket for the 5-HT<sub>2B</sub> receptor, compared to the 5-HT<sub>1B</sub> receptor. In addition, the side chain of M218<sup>5.39</sup> in the 5-HT<sub>2B</sub> receptor is bulkier than that of T209<sup>5.39</sup> in the 5-HT<sub>1B</sub> receptor. The narrower binding pocket in the 5-HT<sub>2B</sub> receptor appears to play a role in subtype selectivity. Docking simulations with a selective 5-HT<sub>1B</sub> agonist sumatriptan support this idea (Wang et al., 2013a). Although mutagenesis results with sumatriptan are unavailable, an M218<sup>5.39</sup>A mutation markedly increased the potency of other triptans (electriptan and donitriptan) by removing the methionine side chain

and expanding the binding pocket (Wang et al., 2013a). These mutagenesis results suggest that the opening of helix V in the serotonin receptor contributes to subtype selectivity.

Docking simulations of norfenfluramine with the serotonin receptors also suggest the mechanism for subtype selectivity (Wang et al., 2013a). Norfenfluramine is the active metabolite of fenfluramine that was once used as an antiobesity drug but now withdrawn due to severe side effects caused by the 5-HT<sub>2B</sub> receptor activation (e.g., valvulopathy) (Connolly et al., 1997). Although the antiobesity effects of norfenfluramine are mediated by the 5-HT<sub>2C</sub> receptor (Vickers et al., 2001), norfenfluramine also interacts with 5-HT<sub>1B</sub> and 5-HT<sub>2B</sub> receptors. Norfenfluramine activates the 5-HT<sub>2B</sub> receptor with higher potency and efficacy than those at the 5-HT<sub>1B</sub> receptor (Fitzgerald et al., 2000). Docking simulations with norfenfluramine suggested that F217<sup>5.38</sup> and M218<sup>5.39</sup> in the 5-HT<sub>2B</sub> receptor provide a hydrophobic cap and interact with norfenfluramine. F217<sup>5.38</sup>A and M218<sup>5.39</sup>V mutations that remove the hydrophobic components greatly reduced the potency of norfenfluramine (Setola et al., 2005; Wang et al., 2013a). In contrast, the corresponding Y208<sup>5.38</sup> and T209<sup>5.39</sup> in the 5-HT<sub>1B</sub> receptor are positioned away from norfenfluramine, and this appears to cause weak affinity of norfenfluramine for the 5-HT<sub>1B</sub> receptor. Together with results of sumatriptan, the simulations and mutagenesis results of norfenfluramine suggest that the extracellular end of helix V contributes to the opening of the binding pocket and that the consequential change of molecular interactions may mediate subtype selectivity of the 5-HT receptors.

### 3.2 Muscarinic acetylcholine receptors

Acetylcholine is a neurotransmitter that mediates multiple physiological actions by activating a family of GPCRs, muscarinic acetylcholine receptors (mAChRs). mAChRs are classified into five subtypes (M1-M5) that share a high degree of sequence homology. The high sequence conservation of mAChRs makes it challenging to design subtype selective ligands. Although there may be species-originated differences, the crystal structures of human M2 and rat M3 receptors provide clues to the mechanisms of Class A GPCR subtype selectivity.

The M2 receptor was crystallized with a non-subtype selective mAChR antagonist R-(−)-3-quinuclidinyl benzilate (QNB), and the M3 receptor was in complex with a muscarinic receptor inverse agonist tiotropium that is prescribed for chronic obstructive pulmonary disease (COPD) (Haga et al., 2012; Kruse et al., 2012). The orthosteric binding sites of the M2 and M3 receptors are well conserved, and the extracellular loops of M2 and M3 receptors also show high conformational similarity in the backbone fold (Haga et al., 2012; Kruse et al., 2012). Despite the high sequence similarity, the orthosteric and extended binding sites do have displacements that may be useful for subtype selectivity. One apparent difference is the positions of ECL2 F181 in the extended binding pocket at M2 and the corresponding L225 at M3 (Fig. 2B). A small side chain of L225 at M3 produces a more spacious binding pocket than that of M2. Another difference is a 2.8 Å upward shift of Y426<sup>7.39</sup> in the orthosteric binding pocket at M2 compared to its corresponding Y529<sup>7.39</sup> at M3. Slightly different conformations of the position 2.61 (Y80<sup>2.61</sup> in M2 and F124<sup>2.61</sup> in M3) are accompanied with the shift of Y<sup>7.39</sup> (Fig. 2B). These differences may help with the

design of subtype selective muscarinic receptor ligands targeting the orthosteric and extended binding sites.

The crystal structure of the M2 receptor was also reported with a positive allosteric modulator LY2119620 highlighting the allosteric binding site in the extracellular vestibule (Kruse et al., 2013). Positive allosteric modulators potentiate the orthosteric ligand interaction, and this can occur in a subtype selective manner with muscarinic acetylcholine receptors (Lazareno et al., 2004). Thus, allosteric interactions may be exploited for muscarinic receptor subtype selectivity. Several bitopic molecules that retain moieties that bind both orthosteric and allosteric sites also showed the selective interactions with muscarinic receptor subtypes (Disingrini et al., 2006; Steinfeld et al., 2007).

### 3.3 Opioid receptors

Opioid receptors interact with endogenous peptides such as endorphins, enkephalins, and dynorphins and opioid ligands including natural products and small molecules. Opioids were traditionally used for reducing pain as natural products, and the opioid receptors have been a drug target for alleviating severe pain. Crystal structures of all four opioid receptors ( $\delta$  receptor,  $\mu$  receptor,  $\kappa$  receptor, and nociceptin receptor) have been revealed (Fenalti et al., 2014; Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Wu et al., 2012). Comparison of these receptors provides structural information on receptor subtype selectivity and further confers evidence to validate the “message-address” hypothesis that has been used to explain the interactions between opioid receptors and ligands.

The ligand binding pockets of  $\delta$ ,  $\mu$ , and  $\kappa$  receptors are well conserved. Although chemically diverse opioid ligands can interact with these receptors, a phenolic hydroxyl group and a nearby positively charged amine are found in most ligands. This pharmacophore appears to mimic the tyrosine terminal of endogenous opioid peptides and is thereby a key interaction site with the opioid receptor (Bryant et al., 2003; Mosberg, 1999). Superposition of  $\delta$ ,  $\mu$ , and  $\kappa$  receptors suggests that the position 7.35 may be critical for opioid receptor subtype selectivity (Fig. 2C). L300<sup>7.35</sup> in the  $\delta$  receptor forms hydrophobic interactions with the indole group of a selective  $\delta$  receptor antagonist naltrindole (Granier et al., 2012). The corresponding residues in  $\mu$  and  $\kappa$  receptors are W318<sup>7.35</sup> and Y312<sup>7.35</sup> that contain a much larger aromatic side chain than L300<sup>7.35</sup> in the  $\delta$  receptor. Thus, the indole ring of naltrindole would produce steric hindrance with the bulky aromatic side chain at the position 7.35 in the  $\mu$  and  $\kappa$  receptors. Mutations of  $\mu$  receptor W318<sup>7.35</sup> to leucine and lysine greatly increased affinity of  $\delta$  receptor-selective ligands but considerably lowered affinity of  $\mu$  receptor-selective ligands (Bonner et al., 2000). These results support the critical role of the position 7.35 in opioid receptor subtype selectivity.

The nociceptin receptor has been classified into the opioid receptor family due to its high sequence homology with the other three opioid receptors. However, most opioid alkaloids show very low affinity to the nociceptin receptor (Mogil and Pasternak, 2001). Although the mechanism for the low affinity is unclear, mutations on V276, Q277, and V278 of TM VI helix, and A213 in the ECL2/TM V interface of the nociceptin receptor markedly increased the binding affinity of opioid ligands (Meng et al., 1998). This study introduced the bulky residues that are conserved among  $\delta$ ,  $\mu$ , and  $\kappa$  receptors to the nociceptin receptor. Thus,

the nociception receptor retain a relatively greater ligand binding pocket than those of  $\delta$ ,  $\mu$ , and  $\kappa$  receptors, and this may result in the low affinity of opioid ligands for the nociception receptor.

Opioid ligands have been proposed to contain two chemical components: one part for efficacy (message) and the other for subtype selectivity (address) (Dondio et al., 1997; Lipkowski et al., 1986; Portoghese, 1989). The message-address concept has been widely adopted to explain the pharmacology of opioid ligands. Crystal structures of the opioid receptors indicate that the lower part of the ligand binding pocket is highly conserved, receives the “message” of the ligand, and mediates receptor efficacy. In contrast, the upper part of the pocket shows sequence variability where the “address” part of the ligand elicits subtype selectivity. For instance, the morphinan group and tyrosine pharmacophore of naltrindole fit well in the lower binding pocket that accepts the “message” of the ligand (Granier et al., 2012). In addition, the upper binding pocket of the  $\delta$  receptor is markedly favorable for the interaction of the indole group in naltrindole versus  $\mu$  and  $\kappa$  receptors as exemplified with the position 7.35 (Fig. 2C). Although other factors may be involved in selective subtype interactions, the “message-address” concept is consistent with the crystal structures of the opioid receptors.

#### 4. Class B GPCR structure and ligand binding

The class B/Secretin family GPCRs comprise fifteen receptors in humans that are activated by peptide endocrine hormones, peptide paracrine factors, and neuropeptides (Hoare, 2005). The topology of class B receptors is similar to that of the class A receptors except that class B receptors have an N-terminal extracellular domain (ECD) of ~120 amino acids in addition to the 7TM domain. Peptide agonist binding to these receptors follows a two-domain model. The peptides are ~30–40 amino acids in length and their C-terminal region binds to the ECD to confer affinity and specificity whereas their N-terminal region binds to the 7TM helical bundle to activate the receptor. N-terminally truncated peptides typically act as antagonists. Several class B receptors are validated drug targets with drugs either on the market or in clinical trials for diseases including diabetes, osteoporosis, and migraine headache (Bortolato et al., 2014). In most cases the drugs are peptide agonists. For some class B receptors the desired drugs are antagonists, such as for the corticotropin releasing factor (CRF) 1 receptor for depression or the calcitonin gene-related peptide (CGRP) receptor for migraine headache, and small-molecule antagonists have been developed that target the 7TM of the CRF1 receptor or ECD of the CGRP receptor, respectively. Our structural understanding of ligand binding to class B GPCRs has increased with recent reports of structures of the isolated domains, but no structure of a full-length class B GPCR is available. The orientation of the ECD relative to the 7TM domain in the intact receptor is unclear and how peptides bind the 7TM domain remains poorly understood.

Crystal and NMR structures of several class B GPCR ECDs determined in complex with bound C-terminal peptide fragments confirmed a conserved ECD fold and mode of peptide binding (Grace et al., 2007; Grace et al., 2010; Pal et al., 2010; Parthier et al., 2007; Pioszak et al., 2009; Pioszak et al., 2008; Pioszak and Xu, 2008; Runge et al., 2008; Underwood et al., 2010). The ECD has an N-terminal  $\alpha$ -helix and a set of four  $\beta$ -strands that form a short

consensus repeat or sushi fold; these secondary structure elements are held together by three conserved disulfide bonds (Fig. 3A). Most of the class B receptor ECDs have a long N-terminal  $\alpha$ -helix, but this helix is significantly shortened in the CRF1 receptor. The peptide ligands form continuous  $\alpha$ -helices that occupy a shallow binding groove situated between the N- and C-termini of the ECD. This groove is generally hydrophobic, but specific polar contacts are also observed in the various structures. Most of the peptide-bound ECD structures show peptides in a position similar to that of parathyroid hormone (PTH), but CRF and the related urocortin (Ucn) peptides occupy a binding site that is slightly shifted compared to the other receptors (Fig. 3A). The position of the peptides in the ECD structures is such that their N-terminal regions would be directed towards the 7TM bundle.

Crystal structures of the isolated 7TM domain of the CRF1 receptor with a bound small molecule antagonist and of the ligand-free glucagon receptor 7TM domain were reported in 2013 (Hollenstein et al., 2013; Siu et al., 2013). These structures provided the first views of the class B 7TM bundle and allowed comparison to the class A 7TM bundle. The intracellular half of the class B 7TM bundle is similar to that of the class A receptors as expected for proteins that couple to the same G proteins. The class B and class A receptors are most different in their extracellular halves. The CRF1 receptor 7TM bundle is V-shaped with a prominent open cleft between ECL1/2 and ECL3 (Fig. 3B) (Hollenstein et al., 2013). The cleft presumably enables occupancy by the large N-terminal portion of the peptide ligand. There is considerable evidence for the N-terminal portion of the peptide forming  $\alpha$ -helical structure and contacting all three extracellular loops (Dong et al., 2014). Comparison of the CRF1 receptor structure to that of the antagonist-bound class A dopamine D3 receptor (Chien et al., 2010) reveals similar positioning of helices I, II, III, IV, and V, but a very different position of helices VI and VII and ECL3 that gives rise to the open cleft (Fig. 3C). The CRF1 receptor structure also revealed a dramatically different position of the antagonist-binding pocket as compared to class A receptors (Fig. 3B, C). The small-molecule CRF1 receptor antagonist occupies a pocket in the intracellular half of the 7TM bundle comprised of residues from helices III, V, and VI (Fig. 3B). The pocket is largely hydrophobic in nature, but there is a single hydrogen bond formed between the receptor and the ligand.

## 5. Class B GPCR peptide ligand selectivity

The structural basis for ligand selectivity is still poorly understood for class B receptors because of the lack of peptide-bound 7TM or full-length structures. Nonetheless, the ECD is sufficient to confer peptide selectivity for several class B receptors including the CRF1 and CRF2 receptors and the CGRP and adrenomedullin receptors (Moad and Pioszak, 2013; Pal et al., 2010). ECD-mediated selectivity is understood structurally for the CRF receptors, for which we have crystal structures of both subtype ECDs with bound CRF and Ucn1, -2, and -3 peptides (Pal et al., 2010; Pioszak et al., 2008). The related CRF and Ucn peptides regulate central stress responses, the cardiovascular system, and metabolism via activation of CRF1 and CRF2 receptors (Bale and Vale, 2004). CRF and Ucn1 activate both receptors whereas Ucn2 and -3 are selective for the CRF2 receptor. Comparison of the CRF-bound CRF1 receptor ECD and Ucn3-bound CRF2 receptor ECD structures indicated that the two  $\alpha$ -helical peptides occupy identical positions on the receptors and that peptide binding site



architectures of the CRF1 and CRF2 receptors are very similar other than a single crucial amino acid difference, CRF1 receptor E104 and CRF2 receptor P100, which is near peptide residues that differ, CRF/Ucn1 R35 and Ucn2/3 A35 (Fig. 4A). The electrostatic surface potential of the CRF1 receptor is considerably more negative near E104 as compared to the equivalent region near the nonpolar P100 in the CRF2 receptor (Fig. 4B, C). The nonpolar Ucn2/3 A35 appears to be incompatible with the negatively charged surface of the CRF1 receptor ECD, whereas CRF/Ucn1 R35 is electrostatically compatible with the negatively charged region of the CRF1 receptor and also the nonpolar P100 of the CRF2 receptor via the aliphatic portion of the R35 side chain. The charge compatibility mechanism is supported by peptide binding experiments showing that Ucn1 R35A abolished binding to the CRF1 receptor ECD with relatively minor impact on CRF2 receptor ECD binding and that Ucn3 A35R conferred binding ability to CRF1 receptor ECD while having a relatively minor effect on CRF2 receptor ECD binding (Pal et al., 2010).

## 6. Class C GPCR structure, ligand binding and selectivity

The class C/Glutamate family GPCRs comprise fifteen receptors in humans that are activated by small molecules such as amino acids and ions (Fredriksson et al., 2003). This family includes eight metabotropic receptors for the neurotransmitter glutamate, the GABA receptors, the calcium sensing receptor, and some taste receptors. Similar to the class B receptors, the class C receptors have an N-terminal ECD in addition to the 7TM domain, but the class C ECD is very large and it contains the entire orthosteric ligand binding site. The class C “venus flytrap domain” (VFT) ECD forms a bi-lobed structure with the orthosteric ligand binding pocket situated in a central cleft (Fig. 5A) (Kunishima et al., 2000). The VFT domain fluctuates between open and closed conformations; agonists generally stabilize the closed conformation whereas antagonists maintain the open conformation (Geng et al., 2013; Muto et al., 2007; Tsuchiya et al., 2002). In all cases except the GABA<sub>B</sub> receptor, a small cysteine-rich domain links the VFT domain to the 7TM domain. Class C GPCRs exist as heteromers, either as homodimers or in the case of the GABA<sub>B</sub> receptor as a heterodimer of GABA<sub>B</sub> receptor 1 and 2 (Pin et al., 2004). The VFT domain mediates dimerization (Fig. 5A). The mechanism by which agonist binding to the VFT results in activation of the 7TM domain is complex and not yet fully understood (Pin et al., 2004).

Drugs targeting several class C receptors are available in the market or in clinical trials for diseases including hyperparathyroidism, anxiety, schizophrenia, Fragile X syndrome, and Parkinson's (Urwiler, 2011). The orthosteric binding sites of the VFTs are generally well conserved and thus create challenges for designing selective drugs. Most of the drugs in development are allosteric modulators that bind to the 7TM domain at a site similar to the orthosteric site of class A GPCRs; the allosteric 7TM sites are generally less conserved and therefore enable subtype selectivity. In 2014 crystal structures of the 7TM domains of the metabotropic glutamate receptors mGlu<sub>1</sub> and mGlu<sub>5</sub> were reported, both with bound negative allosteric modulators (NAMs) (Dore et al., 2014; Wu et al., 2014). The mGlu<sub>1</sub> 7TM domain intriguingly crystallized as a homodimer with the dimer interface mediated by TM I interactions and a cluster of cholesterol molecules (Wu et al., 2014), but the physiological relevance of this dimer remains to be determined (Fig. 5B). The mGlu<sub>5</sub> 7TM structure did not form a dimer (Dore et al., 2014). The NAM binding sites were similar in both structures

and positioned equivalent to the orthosteric binding site of class A GPCRs in the extracellular half of the receptors near helices III, VI, and VII (Fig. 5B, C). The helical bundle of the class C GPCRs is more similar to that of the class A GPCRs than the class B GPCRs. The extracellular half of the class C helical bundle is compact with TM V and VII situated more towards the core of the receptor than in the class A receptors making the NAM binding pocket relatively small (Fig. 5C). The mGlu<sub>1</sub> structure provided a clear explanation for the selectivity of the NAM FITM for mGlu<sub>1</sub> over mGlu<sub>5</sub>. Residue T815 of mGlu<sub>1</sub> on helix VII makes a hydrogen bond with FITM whereas the equivalent position in mGlu<sub>5</sub> is M802, which lacks hydrogen bonding ability and would sterically clash with FITM (Fig. 5D). Consistent with the structures, the mGlu<sub>1</sub> T815M mutant exhibited significantly reduced FITM binding highlighting that this position is an important selectivity determinant (Wu et al., 2014).

## 7. Conclusions and future perspectives

The large number of GPCR crystal structures accumulated over the last several years is striking when considering that in early 2007 only one GPCR structure, that of Rhodopsin, was available. Representative structures for each of the major GPCR classes (other than the adhesion family) are now available and these have been invaluable for understanding ligand binding and selectivity as reviewed here. The surge in structural information has dramatically changed the field. We now have an atomic resolution structural understanding of orthosteric and allosteric ligand binding, ligand selectivity, efficacy, receptor activation, and G protein binding, and we are beginning to understand biased agonism (Liu et al., 2012; Wacker et al., 2013). The wealth of structural data has significantly expanded our understanding of GPCR pharmacology and opened up new avenues for GPCR structure-based drug design, which has enormous potential to yield drugs targeting GPCRs with optimal selectivity, potency, and efficacy profiles. Despite all of these advances, several areas remain in need of a structural explanation. A wish list of structures, in no particular order, includes a peptide bound class B GPCR 7TM domain and/or full-length receptor, full-length class C and class F GPCRs, active state structures for class B, C, and F receptors, GPCRs in complex with various G proteins, a GPCR-arrestin complex, and a GPCR-G protein-coupled receptor kinase (GRK) complex. In addition, there are many accessory/regulatory proteins that interact with GPCRs and we still understand very little about these interactions at the structural level. Lastly, despite the technological breakthroughs GPCR crystallography is still very expensive and labor-intensive. There is a need for methodological advances that make obtaining these structures easier and cheaper. These tasks will keep structural biologists busy for some time in the GPCR field.

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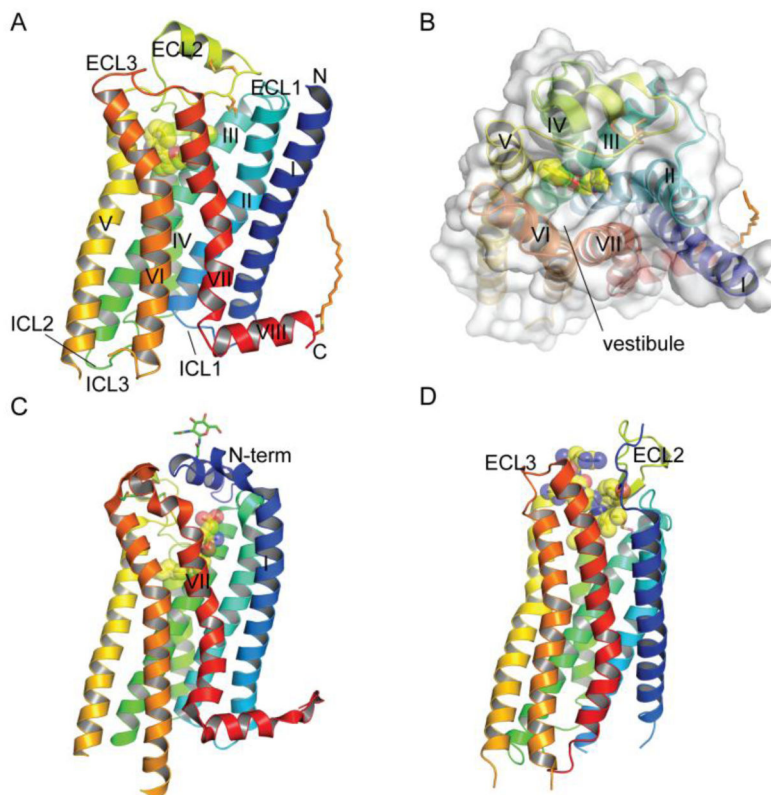
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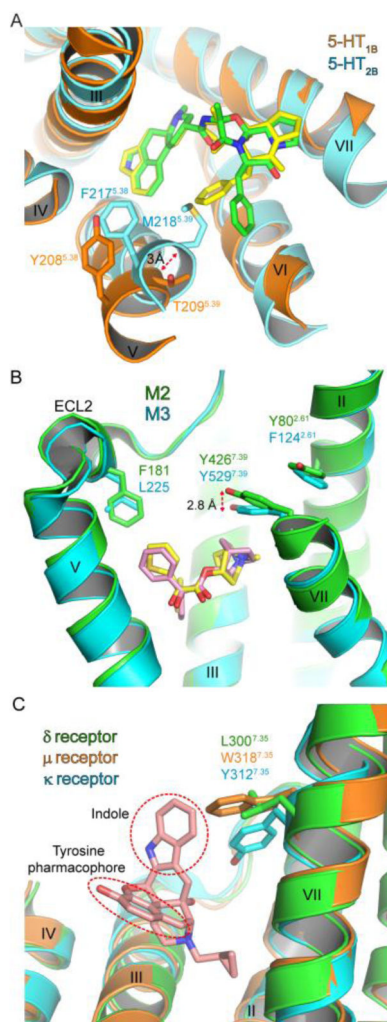
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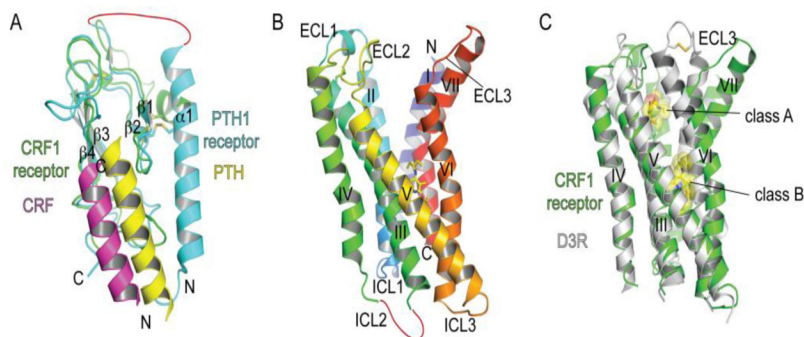


**Fig. 1.** Structures of representative class A GPCRs and positions of orthosteric ligand binding pockets. (A) Structure of the  $\beta_2$ -adrenergic receptor with a bound inverse agonist carazolol (PDB: 2RH1). (B) The  $\beta_2$ -adrenergic receptor with bound carazolol viewed from the extracellular side. (C) Structure of the sphingosine-1-phosphate receptor with a bound antagonist lipid-mimic ML056 (PDB: 3V2Y). (D) Structure of the NTSR1 neurotensin receptor with a bound agonist peptide neurotensin<sub>8-13</sub> (PDB: 4GRV). In all panels the receptor is color coded from N- to C-termini and the ligand is shown in stick representation and space-filling spheres. Extracellular (ECL) and intracellular (ICL) loops and the seven transmembrane helices are labeled. Disulfide bonds, palmitoylation at the C-terminus, and glycans at the N-terminus are also shown in stick representation. All figures were prepared with PyMol (Schrodinger).

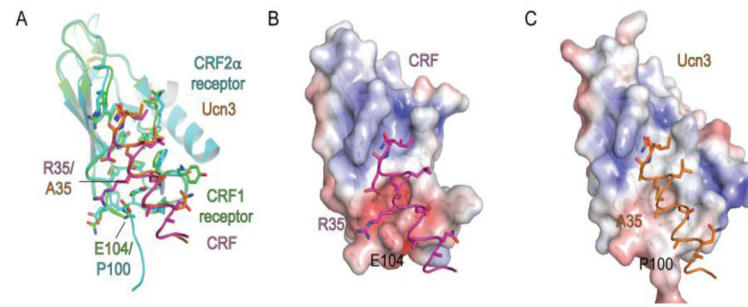




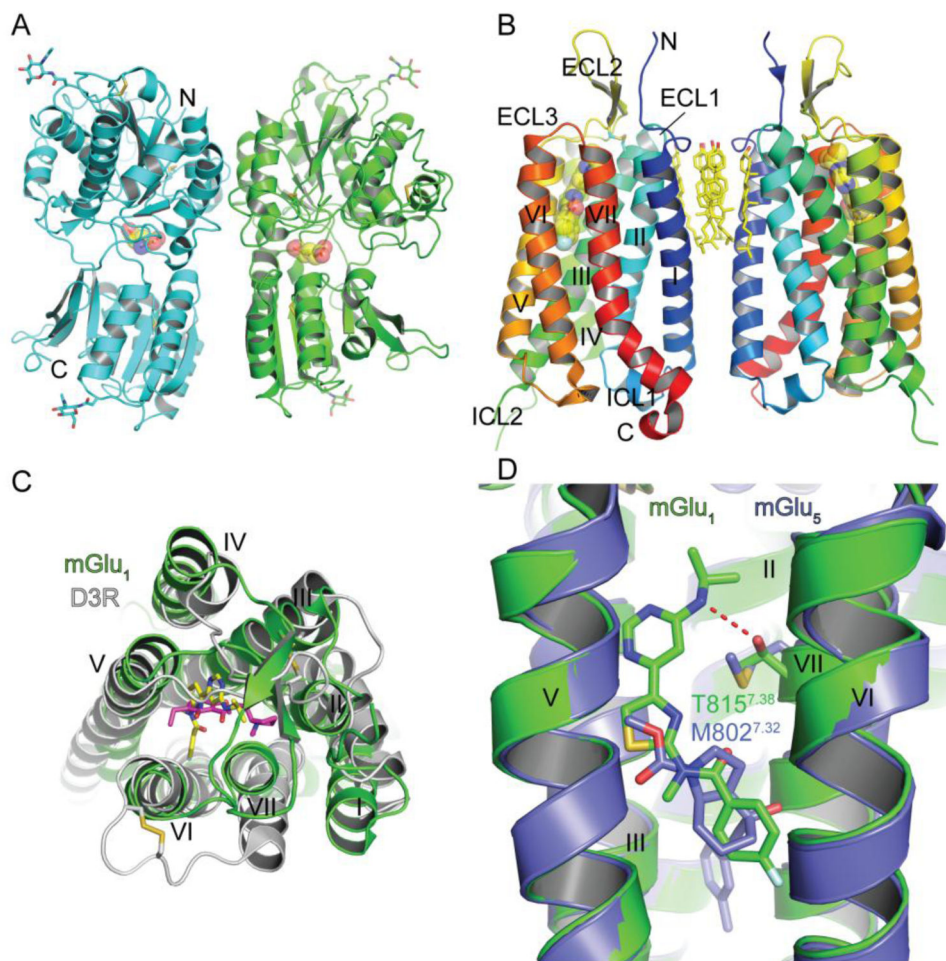
**Fig. 2.** Structural determinants for class A GPCR subtype selectivity. (A) Comparison of the serotonin 5-HT<sub>1B</sub> (PDB: 4IAR) and 5-HT<sub>2B</sub> (PDB: 4IB4) receptors viewed from the extracellular side. 5-HT<sub>1B</sub> and 5-HT<sub>2B</sub> receptors are colored orange and cyan, respectively. Ergotamine in 5-HT<sub>1B</sub> and 5-HT<sub>2B</sub> receptors is colored yellow and green, respectively. The top of helix V and the ECL3 are omitted for clarity. (B) Ligand binding pockets of the M2 (PDB: 3UON) and M3 (PDB: 4DAJ) muscarinic acetylcholine receptors. M2 and M3 receptors are colored green and cyan, respectively. QNB and tiotropium is colored pink and yellow, respectively. The top of helix VII and the entire helix VI are omitted for clarity. (C) Naltrindole selectivity for the δ receptor. The indole moiety and tyrosine pharmacophore of naltrindole are shown in red-dotted circles. The δ receptor (PDB: 4N6H) is colored green, the μ receptor (PDB: 4DKL) orange, the κ receptor (PDB: 4DJH) cyan, and naltrindole pink. The top of helix IV and the entire helices V and VI are eliminated for clarity.



**Fig. 3.** Structures of class B GPCR domains and positions of ligand binding surfaces and pockets. (A) N-terminal extracellular domain structures of the parathyroid hormone PTH1 receptor and corticotropin releasing factor CRF1 receptor with bound PTH (PDB: 3C4M) and CRF (PDB: 3EHU) peptide fragments, respectively. (B) The CRF1 receptor 7TM structure with a bound small molecule antagonist CP-376395 (PDB: 4K5Y) shown in stick representation. (C) Superposition of the class B CRF1 receptor 7TM and class A dopamine D3 receptor with a bound antagonist eticlopride (PDB: 3PBL) highlighting the difference in positions of the antagonist drug binding pockets and the open cleft in the CRF1 receptor. Ligands are shown in stick and space-filling representation.



**Fig. 4.** Structural determinants for ECD-mediated class B GPCR subtype selectivity. (A) Superposition of the CRF1 receptor and CRF2 $\alpha$  receptor ECDs with bound CRF (PDB: 3EHU) and Ucn3 (PDB: 3N93) peptide fragments, respectively. Receptor and peptide residues involved in selectivity are labeled. (B, C) Electrostatic surface potentials [from -5 (red) to +5 (blue) kT/e] of the CRF1 receptor ECD highlighting the negative area near CRF1 receptor E104 (B) and of the CRF2 $\alpha$  receptor ECD highlighting the neutral area near CRF2 $\alpha$  receptor P100 (C).



**Fig. 5.** Class C GPCR structure, ligand binding sites and ligand selectivity. (A) Structure of the homodimeric extracellular venus flytrap domain of mGlu<sub>1</sub> with a bound agonist glutamate shown in stick and space-filling representation in the orthosteric binding site (PDB: 1EWK). Glycans and disulfide bonds are shown in stick representation. (B) Structure of the mGlu<sub>1</sub> 7TM domain with a bound negative allosteric modulator FITM shown in stick and space-filling representation (PDB: 4OR2). Cholesterol molecules at the interface of the 7TM “homodimer” and disulfide bonds are shown in stick representation. (C) Superposition of the class C mGlu<sub>1</sub> 7TM domain and the class A dopamine D3 receptor with a bound antagonist eticlopride (PDB: 3PBL) viewed from the extracellular side. FITM is colored yellow and eticlopride magenta. FITM, eticlopride, and disulfide bonds are shown in stick representation. (D) Superposition of the mGlu<sub>1</sub> and mGlu<sub>5</sub> (PDB: 4OO9) 7TM structures highlighting a subtype selectivity determinant for negative allosteric modulator binding. FITM is colored green and mavoglurant is slate blue.