

## G protein-coupled receptors — recent advances

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The years 2000 and 2007 witnessed milestones in current understanding of G protein-coupled receptor (GPCR) structural biology. In 2000 the first GPCR, bovine rhodopsin, was crystallized and the structure was solved, while in 2007 the structure of  $\beta_2$ -adrenergic receptor, the first GPCR with diffusible ligands, was determined owing to advances in microcrystallization and an insertion of the fast-folding lysozyme into the receptor. In parallel with those crystallographic studies, the biological and biochemical characterization of GPCRs has advanced considerably because these receptors are molecular targets for many of currently used drugs. Therefore, the mechanisms of activation and signal transduction to the cell interior deduced from known GPCRs structures are of the highest importance for drug discovery. These proteins are the most diversified membrane receptors encoded by hundreds of genes in our genome. They participate in processes responsible for vision, smell, taste and neuronal transmission in response to photons or binding of ions, hormones, peptides, chemokines and other factors. Although the GPCRs share a common seven-transmembrane  $\alpha$ -helical bundle structure their binding sites can accommodate thousands of different ligands. The ligands, including agonists, antagonists or inverse agonists change the structure of the receptor. With bound agonists they can form a complex with a suitable G protein, be phosphorylated by kinases or bind arrestin. The discovered signaling cascades invoked by arrestin independently of G proteins makes the GPCR activating scheme more complex such that a ligand acting as an antagonist for G protein signaling can also act as an agonist in arrestin-dependent signaling. Additionally, the existence of multiple ligand-dependent partial activation states as well as dimerization of GPCRs result in a 'microprocessor-like' action of these receptors rather than an 'on-off' switch as was commonly believed only a decade ago.

**Key words:** G protein-coupled receptors, rhodopsin,  $\beta$ -adrenergic receptors, chemokine receptors, G protein, arrestin.

Received: 20 November, 2012; revised: 05 December, 2012; accepted: 10 December, 2012; available on-line: 18 December, 2012

### INTRODUCTION

In 2012 the Nobel Prize in chemistry was awarded jointly to two American scientists — Robert J. Lefkowitz of Duke University in Durham, North Carolina and Brian K. Kobilka of Stanford University School of Medicine in Palo Alto, California "for studies of G protein-coupled receptors (GPCRs)". The scientific achievements

of Lefkowitz and Kobilka have allowed the pharmaceutical industry to develop more selective drugs with the hope of fewer side effects through improved understanding of the signaling mechanisms initiated by endogenous substances such as adrenaline, serotonin, histamine, dopamine and many other hormones and neurotransmitters. Lefkowitz started his work on GPCRs by identifying adrenergic receptors ( $\alpha$  and  $\beta$ ) affected by adrenaline in the 1980s (Shorr *et al.*, 1981; Dixon *et al.*, 1986). With the help of his student, Kobilka, he noticed that the genes for  $\beta$ -adrenergic receptors shows remarkable similarities to the one for rhodopsin, known then as a light-sensing receptor of the eye (Filipek *et al.*, 2003; Palczewski, 2006), and concluded that there likely exists an entire protein family of such receptors with similar structure and function (Lefkowitz, 2000). Another key advance in Lefkowitz's research was identification of the  $\beta$ -adrenergic receptor kinase (Benovic *et al.*, 1986) followed by studies on  $\beta$ -arrestins (Luttrell & Lefkowitz, 2002; Lefkowitz & Shenoy, 2005). Continuing his work at Stanford, Kobilka together with Stevens from the Scripps Research Institute in California solved the crystal structure of  $\beta_2$ -adrenergic receptor stabilized by lysozyme (Cherezov *et al.*, 2007) and together with Schertler from MRC (Cambridge, UK) the structure of the same receptor stabilized by an antibody in the same year (Rasmussen *et al.*, 2007). The structure of the second GPCR (after vertebrate rhodopsin (Palczewski *et al.*, 2000)) confirmed the hypothesis about the common folding of all GPCRs comprising a seven-transmembrane helical bundle (Fig. 1). The structure of  $\beta_2$ -adrenergic receptor was followed by several crystal structures of other GPCRs solved by e.g. the Stevens' group (Jaakola *et al.*, 2008). Those investigators employed lysozyme, apocytochrome BRIL, and nanobody molecules (Cherezov, *et al.*, 2007) to stabilize not only the inactive state of these highly flexible receptors but also their extremely unstable active state in complex with trimeric G protein (Rasmussen *et al.*, 2011). Other investigators successfully employed

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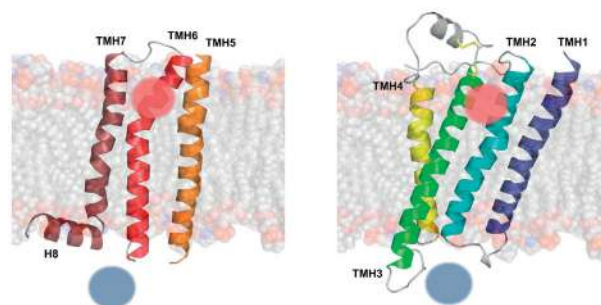
**Abbreviations:** A2AR, adenosine A2A receptor; BRIL, apocytochrome b(562)RIL; CRs, chemokine receptors; CXCR4, chemokine receptor CXCR4; D3R, dopamine D3 receptor; EC, extracellular; ECL, extracellular loop; GPCRs, G protein-coupled receptors; GRK, G protein-coupled receptor kinase; H1R, histamine H1 receptor; HBD, hormone-binding domain; IC, intracellular; ICL, intracellular loop; M2R, M3R, muscarinic M2 and M3 receptors; MCH, melanin-concentrating hormone; NOP, nociceptin opioid receptor; OR, opioid receptor; PDB, protein data bank; PDE, phosphodiesterase; PKA, protein kinase A; SOG, somatostatin, opioid and galanin receptors; TM, transmembrane; 7TMH, seven transmembrane helices

thermo-stabilizing mutations and truncations (Tate & Schertler, 2009; Lebon *et al.*, 2012).

Seven-transmembrane spanning GPCRs are critically involved in transmitting extracellular stimuli including light, hormones and neurotransmitters into specific cellular responses (Muller *et al.*, 2008). GPCRs are also pharmacologically important because they are the targets of about 30% of commercially available drugs (Salon *et al.*, 2011). The structure of vertebrate rhodopsin determined in 2000 marked the first three-dimensional atomic structure of any native GPCR. Significant advances in the field include the structural determination of truncated invertebrate rhodopsin, adrenergic, histamine H1, adenosine A2A, opioid, muscarinic acetylcholine, CXCR4, dopamine D3 and other receptors as well. In many cases GPCRs were modified with fusion proteins such as lysozyme. Whereas non-rhodopsin GPCRs require heterologous expression and extensive protein engineering to enable their stabilization and crystallization, bovine rhodopsin remains the only native, intact GPCR with a determined structure (Palczewski, 2012). GPCRs contain the seven-transmembrane helical bundle that provides a binding site for their ligands. The ligand binding triggers a slight change in GPCR conformation that is propagated through the whole protein, ultimately causing alterations at the receptor's cytoplasmic surface that permit binding to its cognate G protein. Today, crystal structures of all photoactivated intermediates of rhodopsin and several agonist- and antagonist-bound GPCRs have been determined (Palczewski, 2012). Moreover, the critically important GPCR-G protein complex structure also has become better characterized by low- and high-resolution experimental methods (Jastrzebska *et al.*, 2011b; Rasmussen, *et al.*, 2011; Orban *et al.*, 2012). Research on GPCRs still remains of a high priority. Hopefully, knowing better the connection between structure and function of GPCRs will improve our understanding of these molecular machines as well as will promote their potential pharmacological applications.

GPCRs comprise seven transmembrane  $\alpha$ -helices (7TMH) connected by three extracellular loops (ECL1-3) and three intracellular loops (ICL1-3) (Fig. 1). The extracellular (EC) region, which is responsible for ligand binding, also includes the N-terminus that can range from relatively short sequences in rhodopsin-like receptors to large extracellular domains in other classes of GPCRs, e.g. the hormone-binding domain (HBD) in adhesion receptors. The intracellular (IC) region interacts with G proteins, arrestins and other downstream effectors. It includes cytoplasmic helix H8 and a C-terminus that may provide sites for palmitoylation. The 7TM helical bundle contains a number of kinks (Fig. 1), mostly induced by Pro residues, that roughly divide the receptor into the EC and IC regions. The EC module responsible for ligand binding features a high structural diversity but small movement during activation. In contrast, the IC region, which is involved in binding downstream proteins including G proteins and arrestins, is more conserved in the GPCR family but is subjected to much larger conformational changes upon receptor activation than EC (Katrutch *et al.*, 2012).

The structures of GPCRs exhibit only limited differences despite the fact that the receptors were crystallized differently: in different crystal packing orientations, with different antagonists and inverse agonists bound and also in two different activation states, either activated (agonist bound) or inactivated (antagonist or inverse-agonist bound) (Rasmussen *et al.*, 2011). Even the conformations of extracellular loops (the most divergent part



**Figure 1.** A scheme of shapes and tilts of transmembrane helices of GPCRs based on the representative crystal structure of  $\beta_2$ -adrenergic receptor (PDB id: 2RH1).

Location of a ligand is marked by a red sphere whereas the location of protein G by a blue sphere.

of GPCR structures) can also be similar as is the case of crystal structures of the following receptors: dopamine receptor D3R (Chien *et al.*, 2010), muscarinic receptors M2R (Haga *et al.*, 2012) and M3R (Kruse *et al.*, 2012), CXCR4 (Wu *et al.*, 2010),  $\mu$ OR (Manglik *et al.*, 2012),  $\delta$ OR (Granier *et al.*, 2012),  $\kappa$ OR (Wu *et al.*, 2012), and nociceptin receptor (NOP) (Thompson *et al.*, 2012). In the more distant (by sequence homology) CXCR4 and opioid receptors, ECL2 forms a  $\beta$ -hairpin which is oriented nearly vertically to the membrane surface which is much different than that of rhodopsin, which is oriented horizontally and entirely covers the retinal. However, the ECL2 in rhodopsin is responsible for keeping the ligand in place, whereas in CXCR4 it is crucial for binding of either the small molecule IT1t, or the peptide antagonist CVX15 (they are both ligands in crystal structures of that receptor) that mimics the V3 loop of the HIV envelope glycoprotein gp120. In CXCR4 and opioid receptors, the ligand pocket is much larger than in other solved GPCR structures, and binding of peptide antagonists involves extensive interactions with ECL2. The N-terminus of GPCRs probably also participates in the binding of larger ligands, however, to date its structure has only been resolved in rhodopsin and partially in CXCR4 and also in crystallized peptide neurotensin receptor (White *et al.*, 2012) which is the first receptor structure in the  $\beta$  subfamily of rhodopsin-like GPCRs. Another important region in a GPCR structure, the cytoplasmic helix H8, is anchored to the membrane by palmitoylation and this feature is essential for receptor stabilization and its proper functioning (Maeda *et al.*, 2010). In a group of known GPCR structures, only in the case of CXCR4 and neurotensin receptor the helix H8 shows a disordered behavior. Nevertheless, it does not preclude the possibility of its formation in the cell membrane because this helix is present in all crystallized opioid receptor structures from the same g subfamily of rhodopsin-like GPCRs as CXCR4.

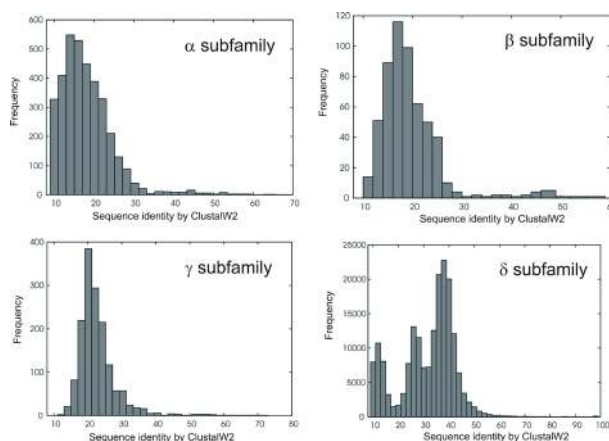
Our current understanding of the GPCR functioning has changed from a simple hypothesis of 'on-off' switches to the microprocessor-like action (Kenakin & Miller, 2010). Especially the phenomenon called 'functional selectivity', whereby certain ligands initiate only portions of the signaling mechanisms mediated by a given receptor, has opened new horizons for drug discovery. What should be discovered in the nearest future is a new receptor-ligand behavior with quantification of the drug effect in such complex systems. For example, some agonists selectively activate cellular pathways associated with a specific cell type and some antagonists actively induce receptor internalization without its activation. There are

also allosteric modulators which can be linked to the co-binding ligands. Agonists are now known to have multiple efficacies that are associated with selected signaling pathways coupled to the receptor. So called 'functional selectivity', defined as biased agonism and biased antagonism, is especially interesting in terms of its mechanism and potential therapeutic applications (see an extensive review by Park and colleagues (2008)).

## CLASSIFICATION OF GPCRS

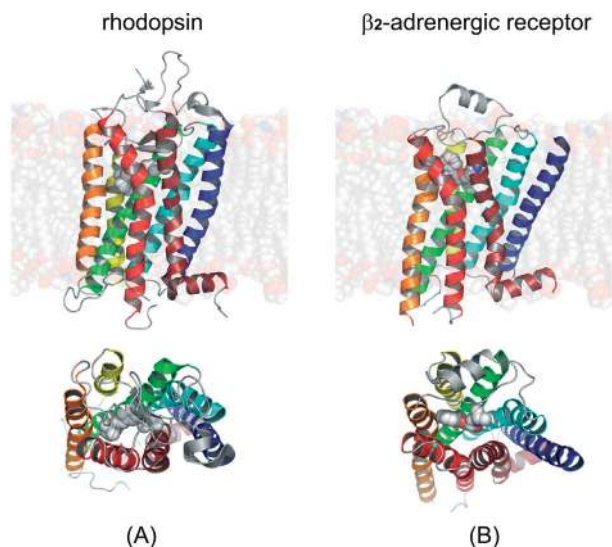
Human GPCRs form a large family of about 800 membrane receptors with sequence lengths between 289 (Mas-related GPCR — uniprot ref. no. Q86SM5) and 3312 (EGF-like protein 1 — uniprot ref. no. Q9NYQ7) residues (Nov. 2012, reviewed Uniprot entries only), with most GPCRs consisting of 300-500 amino acid residues (Mirzadegan *et al.*, 2003). The disparity of sequence lengths is mainly due to the extracellular portion of GPCRs involved in ligand recognition and/or cell signaling (Fig. 1) such as the N-terminus, extracellular loop 2 (ECL2) and ECL3 (Mirzadegan *et al.*, 2003). The intracellular region of GPCRs, less varied in length than the extracellular part, is involved in signal transduction by interactions with G protein and arrestin. Despite the high degree of sequence variability (Trzaskowski *et al.*, 2012) GPCRs share a common 7TMH core of a size exactly fitted to the cell membrane thickness. Interestingly, the transmembrane helices of GPCRs are frequently tilted with varied tilt and rotation angles that depend not only on the receptor type but also on its activation state. Unfortunately, precise prediction of kinks in the TMHs of GPCRs is still limited as these helical deformations cannot be explained only by the presence of the well-known helix-breakers such as Pro, Ser, Thr or Gly residues. Most likely these deformations are introduced by tertiary interactions which are difficult to capture without a 3D structure of the receptor (Yohannan *et al.*, 2004; Meruelo *et al.*, 2011). In the most commonly used GRAFS classification system (Schiöth & Fredriksson, 2005) the GPCR superfamily is divided into five main families: glutamate (former class C), rhodopsin (former class A), adhesion (part of former class B), frizzled/taste2 (former class F), and secretin (part of former class B). Other former classes D (fungal mating pheromone receptors) and E (cyclic AMP receptors) do not contain human receptors and they are not included in the GRAFS classification. So far, only members of the rhodopsin-like receptor family have been crystallized. As of November 2012 there are 15 unique GPCRs deposited through nearly 60 entries in the Protein Data Bank (PDB) involving a variety of ligands, activation states and point mutations. There also have been a few attempts to study GPCR structures by NMR in the 'membrane-mimicking' environment of phospholipid bilayers, e.g. CXCR1 by Park *et al.* (2012), but the resolution of such structures might be still too low to use them for drug discovery.

The rhodopsin family can be further divided into four subfamilies:  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  according to the classification of Fredriksson *et al.* (2003). The  $\alpha$  subfamily has five main branches: prostaglandin, amine, opsin, melatonin and adenosine receptors. Currently in the PDB there are crystal structures of amine receptors (histamine H1R, dopamine D3R, muscarinic M2R and M3R,  $\beta_1$ - and  $\beta_2$ -adrenergic receptors), opsins (rhodopsin), adenosine A2AR and lipid S1P1R receptors). The one-branch  $\beta$  subfamily includes hypocretin receptors, neuropeptide FF, tachykinin, cholecystokinin, neuropeptide Y,



**Figure 2. Histograms of sequence identity between members of four branches of rhodopsin-like family of GPCRs.**

endothelin-related, gastrin-releasing peptide, neuromedin B, uterinbombesin, neurotensin, growth hormone secretagogue, neuromedin, thyrotropin releasing hormone, ghrelin, arginine vasopressin, gonadotropin-releasing hormone, oxytocin and orphan receptors. In the  $\beta$  subfamily only neurotensin receptor has been crystallized so far. The  $\gamma$  subfamily consists of three main branches: SOG receptors (including crystallized  $\mu$ OR,  $\delta$ OR,  $\kappa$ OR and nociceptin opioid receptors), MCH receptors, and chemokine receptors (including crystallized CXCR4). The last  $\delta$  subfamily of rhodopsin-like GPCRs has four main branches: Mas-related (oncogene) receptors, glycoprotein receptors, nucleotide receptors and olfactory receptors. However, the  $\delta$  subfamily has no representative in the PDB so far and only the P2Y12 nucleotide receptor has been selected for crystallization in the near future by the Stevens' group (see [http://gpcr.scripps.edu/tracking\\_status.htm](http://gpcr.scripps.edu/tracking_status.htm)). The above classification of the rhodopsin family is still under discussion as other methods have provided different shapes for its phylogenetic tree (Surgand *et al.*, 2006; Deville *et al.*, 2009). For example, Pele *et al.* (2011) split the rhodopsin family into only four subfamilies: G0 — peptide receptors, opsin and melatonin receptors; G1 — somatostatin, opioid, chemokine and nucleotide receptors; G2 — amine and adenosine receptors; and G3 — including melanocortin, S1P and cannabinoid receptors, leucine-rich repeat (LRR)-containing receptors, prostaglandin and Mas-related receptors. The Pele's classification is not fully consistent with the previous one from Fredriksson *et al.* as members of G0 are included in both  $\alpha$  and  $\beta$  subfamilies, G1 is split between  $\delta$  and  $\gamma$ , G2 — only  $\alpha$ , and finally G3 corresponds to members of both  $\alpha$  and  $\delta$  subfamilies. Even if two GPCRs are classified as members of the same subfamily, they can significantly differ in their amino acid composition (see Fig. 2). A notable exception is the highly populated group of olfactory receptors belonging to the  $\delta$  subfamily in which most sequences are similar to each other (the two highest peaks in the  $\delta$  subfamily sequence identity histogram in Fig. 2). In general, sequence diversity is the highest within the extra- and intracellular loop regions, whereas the 7TMH core contains well conserved fragments (motifs) characteristic of GPCRs, for example: 'D/ERY' (TMH3), 'CwxP' (TMH6) and 'nPxxY' (TMH7). The high sequence diversity inside the rhodopsin family corresponds to the high diversity of kinks and bulges in the TM helices and distinct conformations of



**Figure 3. Crystal structures of rhodopsin (PDB id: 1F88) and  $\beta_2$ -adrenergic receptor (PDB id: 2RH1).** Top, view along the membrane plane, bottom, from the extracellular side.

loops. Even for members of the same subfamily (such as rhodopsin and  $\beta_2$ -adrenergic receptor (subfamily  $\alpha$ ) presented in Fig. 3), their structural diversity still makes homology modeling challenging.

#### CRYSTALLIZATION OF THE FIRST GPCRS — RHODOPSIN AND $\beta_2$ -ADRENERGIC RECEPTOR

The first X-ray crystal structure of any GPCR was that of ground-state rhodopsin (Palczewski, *et al.*, 2000). Refinement of crystallization conditions yielded higher resolution data, extending the model to 2.2 Å, the highest resolution of all rhodopsin structures determined to date (Okada *et al.*, 2004). The first structure of a diffusible ligand-responsive GPCR resulted from work of Kobilka and Stevens, who reported the crystal structure of a human  $\beta_2$ -adrenergic receptor-T4 lysozyme fusion protein bound to the partial inverse agonist carazolol at 2.4 Å resolution (Cherezov *et al.*, 2007; Rosenbaum *et al.*, 2007). Several structures of mutant and fusion GPCRs followed (Mustafi & Palczewski, 2009) culminating with a 1.8 Å resolution structure of an engineered human A2A adenosine receptor with its third intracellular loop replaced with apocytochrome b(562)RIL (Liu *et al.*, 2012). This is the highest resolution structure of any GPCR with well-defined Na<sup>+</sup> ions and water molecules. Water molecules play critical roles in the GPCR activation process by stabilizing intramolecular interactions (Wikstrom *et al.*, 2003; Osyczka *et al.*, 2005; Garczarek & Gerwert, 2006). Observed in key structurally sensitive areas of many GPCRs, water molecules along with amino acid side chains can form a signal transmission network extending from the ligand-binding site to the cytoplasmic surface (Angel *et al.*, 2009a; 2009b; Orban *et al.*, 2010). Despite their low sequence similarities, the overall folds of structurally determined GPCRs are remarkably similar. For all rhodopsin structures, RMSDs for transmembrane regions are within 1.8 Å, and when adrenergic receptors are compared with rhodopsin, these deviations are 3.3–3.5 Å for the  $\beta_2$ -adrenergic receptor and 4.3–4.7 Å for the

$\beta_1$ -adrenergic receptor (Jaakola, *et al.*, 2008; Lodowski *et al.*, 2009). Remarkably, preservation of only a few essential regions of a GPCR is required for activation of its cognate G protein (Mirzadegan *et al.*, 2003).

Rhodopsin in its inactive, ground state undergoes a series of photointermediate steps upon absorption of a photon and isomerization of its chromophore 11-*cis*-retinylidene. These photointermediate states exhibit unique absorption maxima and can be isolated by trapping alone or with a G protein-derived peptide analog. Ultimately, the photoisomerized chromophore is hydrolyzed and released from the binding pocket yielding opsin and free all-*trans*-retinal (Jastrzebska *et al.*, 2011a). Our laboratory expanded upon this work with the first photoactivated rhodopsin structure which exhibits spectral qualities of Meta II, the activated state (Salom *et al.*, 2006; Lodowski *et al.*, 2007). For rhodopsin, only the Meta II intermediate is capable of activating G<sub>t</sub> and it differs chemically from other photo-intermediates only by deprotonation of the Schiff base and uptake of a proton from bulk solvent (Salom *et al.*, 2006). By now, the structures of most photo-intermediates have been solved by X-ray and electron crystallographic methods (Breitman *et al.*, 1989; Park *et al.*, 2008; Lodowski *et al.*, 2009). Interestingly, the structures of those rhodopsin photo-intermediates did not exhibit large-scale movements of entire helices. Rather they showed that photoactivation was accomplished with just small-scale, local changes propagated to the cytoplasmic loops, especially the ends of helices V and VI. The 2–8 Å structural shift observed for GPCRs upon activation suggests that such subtle changes directly lead to different receptor activities or indirectly affect the key residues, e.g. the D(E)RY motif on the cytoplasmic surface responsible for the efficacy of G protein coupling along with further conformational changes. This observation is consistent with activation of other GPCRs (summarized by Sprang (2011)).

Structures of opsin revealed marked similarities to photoactivated rhodopsin (Topiol & Sabio, 2009). An additional structure of a photoactivated rhodopsin, obtained by regenerating opsin crystals with all-*trans*-retinal, superposed well with opsin structures and is spectrally indistinguishable from either our photoactivated rhodopsin structure or Meta II in solution (Choe *et al.*, 2011) (summarized in (Breitman *et al.*, 1989)). Moreover, structures of constitutively active mutants of rhodopsin have also been reported (Standfuss *et al.*, 2007; Standfuss *et al.*, 2011; Xie *et al.*, 2011; Deupi *et al.*, 2012), revealing changes around helices V and VI as compared with rhodopsin that are consistent with proposed models. When one considers the agonist-bound GPCR structures, it becomes readily apparent that the dynamics of the molecule (which make it both a difficult structural target and play a key role in its activation) are recapitulated. The inherent flexibility of GPCRs permits dynamic and conformational changes triggered by only a fraction of the energy derived from ligand binding. Because various agonists can bind to a GPCR leading to varying levels of activity, the small changes induced by agonist binding must account for the differences in the efficacy of such ligands in activating a given G protein (Rosenbaum *et al.*, 2009), suggesting that interaction with signaling proteins may induce further changes on the cytoplasmic surface of these receptors (Sprang 2011). NMR studies of GPCRs, particularly rhodopsin, further advanced and refined those activation models (Smith 2010; 2012; Struts *et al.*, 2011; Eilers *et al.*, 2012).

## INTERACTIONS WITH G PROTEIN AND ARRESTIN — PASSING ON THE SIGNAL

Activation of a GPCR triggers binding of the associated heterotrimeric G protein and nucleotide (GDP) release from its  $\alpha$ -subunit. This G protein activation is required for subsequent activation of the cascade of reactions, processes required to advance stepwise signal transduction. The G protein is released from the GPCR by GTP and both its  $\alpha$ - and  $\beta\gamma$ -subunits can activate the effector molecules as adenylyl cyclases and cation channels. Rather than to photon (as in rhodopsin), most GPCRs respond to molecules called ligands that upon binding to a particular GPCR cause a ligand-specific cellular response. The signal is attenuated by receptor phosphorylation and binding of a capping protein arrestin.

### G protein

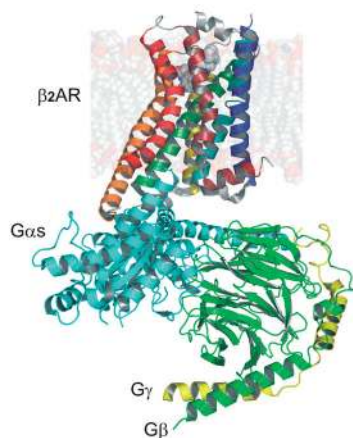
Heterotrimeric G proteins are composed of a nucleotide-binding  $\alpha$ -subunit ( $G\alpha$ ) and a dimer consisting of the  $\beta$ - and  $\gamma$ -subunits ( $G\beta\gamma$ ). In their inactive form,  $G\alpha$ -subunits are bound to GDP and tightly associated with  $G\beta\gamma$ . Interactions of  $\beta_2$ -adrenergic receptor and  $G_s$  (the stimulatory G protein that activates adenylyl cyclase) formed the foundation of the ternary complex model of GPCR activation (Ross *et al.*, 1977; De Lean *et al.*, 1980). In the ternary complex consisting of agonist, receptor and G protein, the affinity of the receptor for the agonist is enhanced and the specificity of the G protein for guanine nucleotides changes in favor of GTP over GDP. Agonist binding to the receptor promotes interactions with the GDP-bound  $G_s\alpha\beta\gamma$  heterotrimer, leading to the exchange of GDP for GTP and the functional dissociation of G protein into  $G\alpha$ -GTP and  $G\beta\gamma$  subunits. These separate subunits can modulate the activity of different cellular effectors (channels, kinases or other enzymes). The intrinsic GTPase activity of  $G\alpha$  leads to hydrolysis of GTP to GDP and the re-association of  $G\alpha$ -GDP and  $G\beta\gamma$  subunits with termination of signaling. The active state of a GPCR can be defined as that conformation that couples to and stabilizes a nucleotide-free G protein. In the agonist- $\beta_2$ -adrenergic receptor- $G_s$  ternary complex,  $G_s$  has a higher affinity for GTP than for GDP, and the  $\beta_2$ -adrenergic receptor has a roughly 100-fold higher affinity for agonists than does  $\beta_2$ -adrenergic receptor alone (Rasmussen *et al.*, 2011).

The  $\alpha$ -subunit ( $G\alpha$ ) of heterotrimeric G proteins mediates signal transduction in a variety of cell signaling pathways. These  $\alpha$ -subunits can be divided into four families:  $G\alpha_s$ ,  $G\alpha_i/G\alpha_o$ ,  $G\alpha_q/G\alpha_{11}$ , and  $G\alpha_{12}/G\alpha_{13}$ . Each family comprises various members that often show specific expression patterns. The  $\beta\gamma$ -complex of mammalian G proteins is assembled from a repertoire of five G protein  $\beta$ -subunits and twelve  $\gamma$ -subunits (Wettschurck & Offermanns, 2005). Most GPCRs are able to activate more than one G protein subtype. Therefore, the activation of a GPCR usually results in the activation of several signal transduction cascades *via* G protein  $\alpha$ -subunits as well as through the freed  $\beta\gamma$ -complex. G proteins of the  $G_i/G_o$  family are widely expressed and have been shown to mediate receptor-dependent inhibition of various types of adenylyl cyclases (Sunahara *et al.*, 1996). Because the expression levels of  $G_i$  and  $G_o$  are relatively high, their receptor-dependent activation results in the release of relatively high amounts of  $\beta\gamma$ -complexes. Activation of  $G_i/G_o$  is therefore believed to be the major coupling mechanism that results in the activation of  $\beta\gamma$ -mediated signaling (Clapham & Neer, 1997;

Robishaw 2004). The  $G_q/G_{11}$  family of G proteins couples receptors to  $\beta$ -isoforms of phospholipase C (Exton 1996; Rhee 2001). The G proteins  $G_{12}$  and  $G_{13}$  are often activated by receptors coupling to  $G_q/G_{11}$  (Strathmann & Simon, 1990; Dhanasekaran & Dermott, 1996). Analysis of cellular signaling processes regulated through  $G_{12}$  and  $G_{13}$  has been difficult because specific inhibitors of these G proteins are not available. In addition,  $G_{12}/G_{13}$ -coupled receptors usually activate other G proteins as well. The ubiquitously expressed G protein  $G_s$  couples many receptors to adenylyl cyclase and mediates activation increasing intracellular cAMP concentration (Beavo & Brunton, 2002; Chin *et al.*, 2002).  $\beta$ -adrenergic receptors couple primarily to  $G_s$ . The cAMP produced in response to  $G_s$  activation directly modulates the gating of hyperpolarization-activated, cyclic nucleotide-gated channels and activates protein kinase A (PKA). PKA in turn phosphorylates many proteins involved in excitation-contraction coupling including L-type  $Ca^{2+}$  channels, phospholamban, and/or troponin I (Bers 2002). Rhodopsin is coupled to rod-transducin ( $G_t$ , a homolog of  $G_o$ ), a member of the  $G\alpha_i/G\alpha_o$  family. Transducin couples the receptor in a stimulatory manner to cGMP-phosphodiesterase (PDE) by binding and sequestering the inhibitory  $\gamma$ -subunit of the retinal type 6 PDE (PDE6). Activation of PDE lowers cytosolic cGMP levels leading to a decreased probability of cGMP-regulated cation channels in the plasma membrane being open, which eventually causes hyperpolarization of photoreceptor cells (Arshavsky *et al.*, 2002).

The signal captured by photoactivated rhodopsin or an agonist-occupied GPCR propagates along a cell's plasma membrane to activate a G protein located 40 Å or more away to cause a cellular response. Perhaps our most advanced understanding of this activation process is derived from rhodopsin and the visual system. Absorption of a photon triggers a change in the conformation of rhodopsin's bound retinal chromophore which is propagated through this receptor, ultimately causing alterations at the cytoplasmic surface that permits binding of G protein transducin, its cognate G protein. This triggers nucleotide release from G protein and its subsequent activation, processes required to advance visual signal transduction. Most GPCRs respond to molecular signals in the form of ligands which upon binding elicit a ligand-specific cellular response. For most GPCRs, the ligand-binding site coincides with the retinylidene-binding pocket in rhodopsin. Thus, ligand binding causes similar conformational changes as those triggered by chromophore photoisomerization in rhodopsin, and the remaining molecular mechanisms for signal transduction are similar for all GPCRs. A model for the photoactivated rhodopsin-G protein complex was described as a 22 Å low-resolution structure from single particle analysis (Jastrzebska *et al.*, 2011b). Its molecular envelope is consistent with dimeric rhodopsin molecules together with one G protein heterotrimer, yielding a 2:1 molar ratio of photoactivated rhodopsin to G protein (Jastrzebska *et al.*, 2011b). The heteropentameric structure for this complex was obtained from native proteins, both rhodopsin and G protein (see also (Jastrzebska *et al.*, 2006)).

The crystal structure of an active state complex composed of agonist-occupied monomeric  $\beta_2$ -adrenergic receptor-T4-lysozyme fusion, a nucleotide-free  $G_s$  heterotrimer and a nanobody (Rasmussen *et al.*, 2011) (Fig. 4) may not represent a physiologically relevant complex. First, it was surprising that the receptor was in a monomeric state, as single particle analyses of a similar preparation indicated that the receptor exists to some degree



**Figure 4.** Crystal structure of  $\beta_2$ -adrenergic receptor in complex with heterotrimeric  $G\alpha\beta\gamma$  protein (PDB id: 3SN6). View along the membrane plane.

in a dimeric form in solution (Westfield *et al.*, 2011). Second, it was unexpectedly observed that the entire  $\alpha$ -helical domain (AH) of  $G\alpha$  was so largely displaced relative to the Ras-like GTPase domain (Rasmussen *et al.*, 2011). This displacement could result from the nanobody stabilization of the  $\beta_2$ -adrenergic receptor- $G_s$  complex in the crystal or from the crystallization conditions used. But these discrepancies cannot be explained by differences in the inherent structure of either this GPCR (as mentioned earlier) or its G protein. Both the visual G protein and  $G_s$  display a relatively small root-mean-square deviation ( $\sim 0.5$ – $1.0$  Å) between structures for the all three subunits. Future research should elucidate this discrepancy.

A wide range of different types of studies (Tesmer, 2010) have provided details of the conformational states of  $G\alpha$ , but the mechanism by which the GPCR interaction leads to release of the bound GDP from the  $G\alpha$  subunit and the structure of the resulting empty complex remain a major target for research in the field. The  $G\alpha$  subunit has two structural domains, namely a nucleotide binding or ras-like domain (Ras) and an  $\alpha$ -helical domain (AH) that partially occludes the bound nucleotide. Numerous studies indicate that the C-terminus of  $G\alpha$  is bound tightly to the receptor in the nucleotide-free complex (Oldham *et al.*, 2006). In addition, the N-terminal helix of  $G\alpha$  is associated with  $G\beta\gamma$  and with the membrane *via* N-terminal myristoylation (Resh, 1999). Together, these constraints fix the position of the nucleotide domain with respect to the membrane. The helical domain is connected to the ras-like domain through two flexible linkers. The receptor-catalyzed nucleotide exchange in G proteins suggested a large-scale reorientation of domains in the  $\alpha$ -subunit (Van Eps *et al.*, 2011; Westfield, *et al.*, 2011). As part of that, binding to a GPCR requires a movement of two helices in  $G\alpha$ , the so called  $\alpha N$  and  $\alpha 5$  helices at the  $G\alpha$  N- and C-terminus, respectively. A possible sequence of interactions during formation of the nucleotide-free complex has been proposed for the  $\beta_2$ -adrenergic receptor- $G_s$  structure (Rasmussen *et al.*, 2011). The first interaction of the  $\beta_2$ -adrenergic receptor with the  $G_s$  heterotrimer would require a movement of the  $\alpha 5$ -helix to permit interactions with the  $\beta_2$ -adrenergic receptor. The formation of more extensive interactions between the receptor and the amino terminus of  $G\alpha$  requires a rotation of  $G\alpha$  relative to the receptor. This is associated with further

conformational changes in both the  $\beta_2$ -adrenergic receptor and  $G\alpha$  Ras. One cannot say when GDP is released during the formation of the complex. However, it is suggested that the GDP release precedes the uncoupling of the two  $G\alpha$  domains.

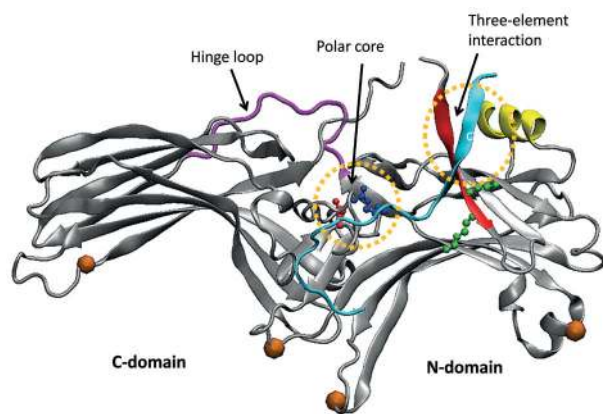
Although much progress has been made in understanding how  $G\alpha$  subunits interact with and regulate the activity of their downstream targets, it is less clear how activated GPCRs initiate this process by catalyzing nucleotide exchange on  $G\alpha\beta\gamma$ . The question is of great importance because it represents an essential, pharmacologically relevant interaction that can regulate nearly all aspects of eukaryotic cell physiology. Moreover, an atomic-resolution understanding will explain the GPCR functional selectivity, namely the ability of different agonists to elicit distinct downstream effects from a single GPCR.

### Arrestin

Additional knowledge about GPCRs and their interactions with desensitizing proteins has emanated from visual research (Kuhn & Dreyer, 1972; Kuhn, 1974; Weyand & Kuhn, 1990). Photoactivated rhodopsin is specifically phosphorylated by G protein-coupled receptor kinases (GRKs) (Maeda *et al.*, 2003; Singh *et al.*, 2008) and preferential binding of arrestin to activated rhodopsin blocks further G protein activation (Palczewski *et al.*, 1991; Freedman & Lefkowitz, 1996). Lefkowitz, as many other investigators, was interested in the mechanism of signal termination by GPCRs. He demonstrated that other GPCRs are phosphorylated in an agonist-specific manner and that the phosphorylated receptors bind  $\beta$ -arrestin (Lohse *et al.*, 1990), revealing a mechanism of receptor desensitization shared among rhodopsin and most GPCRs.

Mammals have four arrestin subtypes that demonstrate over 50% amino acid conservation and similar structures in their basal state. Arrestin-1 (also known as visual or rod arrestin) and arrestin-4 (cone arrestin) are predominantly expressed in photoreceptors, whereas arrestin-2 and -3 (also known as  $\beta$ -arrestin-1 and -2) are present in virtually every cell in the body with the greatest expression in mature neurons (Palczewski, 1994). Non-visual arrestins bind the great majority of GPCRs found in different mammalian species (Xiao *et al.*, 2007). Visual arrestin shows high selectivity for its cognate receptor rhodopsin, even though several other proteins have been identified to be bound by this arrestin subtype (Gurevich *et al.*, 2011).

The structures of arrestin (Fig. 5) determined by Granzin *et al.* (1998) followed by other structures of arrestin and homologs (Hirsch *et al.*, 1999; Han *et al.*, 2001; Sutton *et al.*, 2005; Zhan *et al.*, 2011) consist of two concave lobed regions, termed the C-domain and N-domain. The receptor-binding surface is mainly localized to the side of arrestin containing these two cavities. Several exposed residues in the C- and N-domains of arrestin have been identified as being responsible for GPCR recognition (Hanson, 2006; Vishnivetskii *et al.*, 2010; 2011). The two domains are linked together by a polar core of charged residues that form a network of salt bridges which stabilize their relative orientation. It seems that non-visual arrestins can form functionally different complexes with the same receptor depending on the number of receptor-attached phosphates and their positions. These receptor-attached phosphates play a major role in arrestin recruitment, whereas their positions apparently determine the functional consequences of arrestin bind-



**Figure 5. Crystal structure of arrestin (PDB id: 1CF1) with characteristic elements indicated.**

Orange balls indicate regions that change upon GPCR binding but are not directly involved in the interaction with receptor. Colored residues are important for arrestin stability (a salt bridge in blue and red in polar core region) or initial recognition of receptor (two Lys residues in green).

ing to the phosphoreceptor (Tobin *et al.*, 2008; Gimenez *et al.*, 2012). Phosphorylation sites on rhodopsin were well defined a decade ago *in vitro* and *in vivo* (Ohguro *et al.*, 1993; 1994; Ohguro & Palczewski, 1995; Ohguro *et al.*, 1995; Kennedy *et al.*, 2001). Lefkowitz's group has correlated the phosphorylation sites of  $\beta_2$ -adrenergic receptor with  $\beta$ -arrestin functions (Nobles *et al.*, 2011).

Arrestin-1 preferentially binds to activated and phosphorylated rhodopsin. It also specifically binds inactive phosphorylated and active non-phosphorylated forms, but with much lower affinity. This observation was the first indication that arrestin-1 can recognize activation and phosphorylation of rhodopsin independently of each other. A sequential multi-site binding model (Gurevich & Gurevich, 2004) posits that arrestin-1 first binds rhodopsin either *via* its structural elements that specifically interact with the light-activated rhodopsin conformation, or *via* residues that directly bind to the rhodopsin-attached phosphates. If the 'activated state' or the phosphates represent the only "attraction factor", then arrestin-1 binds with low affinity. However, when arrestin-1 encounters phosphorylated photoactivated rhodopsin, the engagement of both primary sites allows arrestin to switch into the high-affinity rhodopsin-binding state, bringing additional arrestin elements into contact with rhodopsin. The new contact surface provides extra energy to encourage the interaction, which accounts for arrestin-1's much greater affinity for phosphorylated photoactivated rhodopsin.

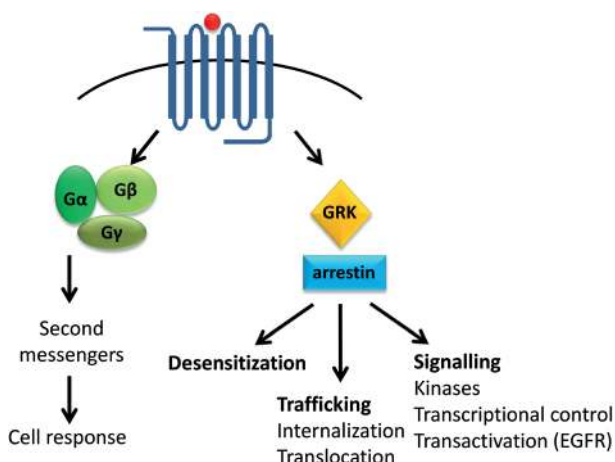
The "hinge" region of all arrestins (residues 179–191) is flexible, allowing movement of the N- and C-domains relative to each other. However, there is no large relative motion of arrestin domains during complex formation, but a concerted movement of multiple flexible loops most probably helps arrestin mold itself onto the receptor (Kim *et al.*, 2012) (Fig. 5). Lefkowitz's studies on the conformation of non-visual arrestins in their active state revealed that although the overall activation mechanism is the same for all arrestin types, the final conformations might differ (Nobles *et al.*, 2007). Moreover,  $\beta$ -arrestins might adopt multiple "active" conformations and, depending on their conformation, accomplish different functions (Shukla *et al.*, 2008).

The stoichiometry of rhodopsin complexes with its cognate proteins is a matter of debate. Historically, one-

to-one binding was usually assumed (Hanson *et al.*, 2007; Gurevich & Gurevich, 2008). However, it has also been proposed by Palczewski that a single arrestin molecule could accommodate two receptors (Liang *et al.*, 2003; Modzelewska *et al.*, 2006). Monomeric activated and phosphorylated rhodopsin in nanodiscs can bind arrestin (Tsukamoto *et al.*, 2010; Bayburt *et al.*, 2011). At the same time, it has been reported that although arrestin requires at least a single phosphorylated photoactivated rhodopsin to bind to the membrane, a single arrestin can actually interact with a pair of receptors composed of two different photo-intermediate states. The binding stoichiometry depends on the percentage of active receptors (Sommer *et al.*, 2011). From a physiological standpoint, the different binding modes of arrestin correspond well to the functional needs of the cell at different light intensities. In the single-photon range, arrestin binds monomeric phosphorylated photoactivated rhodopsin to quench signaling. But as the lighting level increases and photoactivates more rhodopsin, arrestin also binds dimeric photoactivated rhodopsin with only one phosphorylated protomer. Further studies have revealed that differentiated binding preferences of the two domains of arrestin allow it to accommodate the different functional forms of phosphorylated rhodopsin (Sommer *et al.*, 2012). A general hypothesis is formed that the N-domain of arrestin mediates binding to agonist-activated receptor, whereas the less specific C-domain may serve various functions depending on the requirements of the biological system. The asymmetric ability of arrestin to stimulate ligand binding within receptor dimers is in line with studies on  $\beta_2$ -adrenergic receptors (Gurevich *et al.*, 1997) and *N*-formyl-peptide receptor (Key *et al.*, 2001).

In addition to their role in GPCR desensitization,  $\beta$ -arrestins also participate in receptor internalization (Fig. 6). Lefkowitz's group identified internalization as a critical initial step in recycling of desensitized receptors (Sibley *et al.*, 1986) that also activates key mitogenic pathways within the cell (Daaka *et al.*, 1998).  $\beta$ -arrestin binds to clathrin *via* the adaptor protein AP2 (Goodman *et al.*, 1996) that causes arrestin-bound receptors to cluster in clathrin-coated pits. The clathrin-coated pit is pinched off from the plasma membrane by the motor protein dynamin, causing the desensitized receptor to enter an endosomal pool. After internalization,  $\beta$ -arrestin-mediated GPCR trafficking is regulated by both the location and variable binding affinities of different arrestin isoforms to GPCRs (Oakley *et al.*, 2000). Arrestin-2 is found in both the cytoplasm and the nucleus, whereas arrestin-3 is localized only in the cytoplasm.  $\beta$ -arrestins interact with GPCRs with differing affinities. Class A GPCRs, such as  $\beta_1$ -adrenergic receptors,  $\mu$  opioid receptors, and D1 dopamine receptors bind arrestin-3 with a greater affinity than arrestin-2 and their interactions are lost during internalization. Class B receptors, such as angiotensin AT1a receptor, neurotensin receptor 1 and vasopressin V2 receptor, bind arrestin-2 and -3 with equal affinity and their interaction remains intact during internalization. Internalized receptors are then sorted for degradation or recycling; trafficking is regulated by ubiquitination of  $\beta$ -arrestin as revealed by Shenoy *et al.* (2001) in the case of the  $\beta_2$ -adrenergic receptor. Receptors targeted for degradation traffic to lysosomes and are enzymatically degraded, whereas receptors for recycling traffic to acidified vesicles where they are de-phosphorylated and recycled back to the plasma membrane (Tan *et al.*, 2004).

$\beta$ -arrestins can also initiate a second wave of signaling which is independent of G-protein coupling and activa-



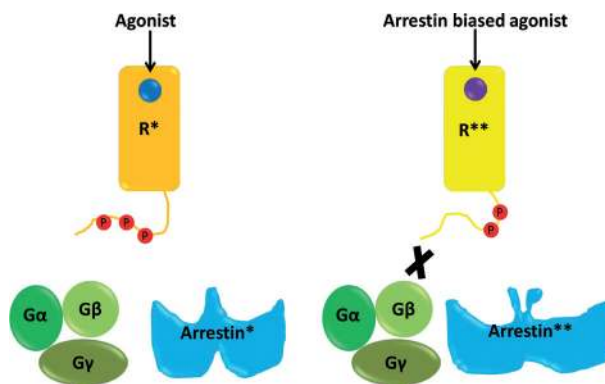
**Figure 6. GPCR signaling.**

In response to ligand binding a stimulation signaling can occur via G-protein-mediated pathway terminated by subsequent GRK/arrestin binding, or/and via  $\beta$ -arrestin-mediated pathway.

tion (Fig. 6). Here they serve as adaptor or scaffold molecules that bring crucial molecular components of specific signaling pathways in close proximity to an activated GPCR. Interestingly, depending on the type of activated GPCR, either both  $\beta$ -arrestin isoforms are required to activate the second wave of signaling (termed co-dependent), or only one isoform is required, whereas the other serves to inhibit the pathway (termed reciprocal regulation) (DeWire *et al.*, 2007). The group of Lefkowitz first found that arrestin-2 is complexed with the tyrosine kinase c-Src. This associates c-Src with the  $\beta_2$ -adrenergic receptor, resulting in activation of c-Src which initiates a tyrosine phosphorylation signaling cascade leading to stimulation of the Ras-ERK1/2 pathway (Luttrell *et al.*, 1999). Contributions of the Lefkowitz's group to understanding  $\beta$ -arrestin-mediated signaling have remained strong since their initiation.

In many cases, signals transmitted by arrestin binding are demonstrably independent of heterotrimeric G protein activation. This observation has triggered the concept of biased agonists, pathway-selective ligands that activate only a subset of the GPCR signaling repertoire (Kenakin, 2005). Biased agonism is best understood by a model in which different GPCR's active conformations are either competent for the full range of receptor activities or only for a subset of them. Thus, balanced ligands stabilize the conformations that are competent for signaling to all downstream pathways, whereas biased ligands stabilize only those conformations that are capable of promoting a subset of signaling effects (Rajagopal *et al.*, 2010) (Fig. 7). The latter ligands could more selectively target beneficial signaling and even block or negate detrimental or unwanted actions of full receptor activation (e.g. side effects, toxicity or tolerance). Over the last decade a diversity of biased ligands for GPCRs have been identified that selectively activate G proteins or  $\beta$ -arrestins (Whalen *et al.*, 2011), and several of these seem to have distinct functions when compared with traditional ligands with broad range efficacy.

Treatment with GPCR agonists can be limited by the development of tachyphylaxis, a decrease in responsiveness to the same dose of a drug, together with tolerance, whereby higher drug doses are required to obtain the same effect. Both processes, which limit the utility of therapeutics, are largely thought to be regulated by  $\beta$ -arrestin-dependent receptor desensitization and down-



**Figure 7. A schematic representation of arrestin-biased signaling.**

Binding of standard agonist to receptor induces an active conformation ( $R^*$ ) whereas binding of arrestin-biased agonist induces a different active conformation ( $R^{**}$ ). Distinct active conformations of receptor are coupled to different active conformation of arrestin which govern different functional outcomes.

regulation. For example, arrestin-2 is involved in cardiac  $\beta_2$ -adrenergic receptor desensitization (Conner *et al.*, 1997). Clinically the  $\beta$ -agonist, dobutamine is often used to provide inotropic support for patients with severe heart failure, but is associated with the development of tachyphylaxis. Thus a G-protein-biased ligand that does not promote  $\beta$ -arrestin recruitment would cause less tachyphylaxis and would be a more effective therapeutic agent.

Several disease states associated with alterations in receptor trafficking could benefit from therapies that modulate  $\beta$ -arrestin-mediated functions. HIV requires cell-surface co-receptors, either the CCR5 or CXCR4 chemokine receptor, to attach and gain entry into target cells. CCR5-tropic viruses are the predominant species in the early stages of infection and there has been a significant interest in targeting this receptor for the treatment of HIV infection. Indeed, modified CCR5 ligands promote receptor internalization. As  $\beta$ -arrestins regulate CCR5 trafficking, recycling and degradation (Oppermann, 2004), the modified ligands probably regulate CCR5 trafficking *via* changes in  $\beta$ -arrestin activity. Thus, use of a ligand that modifies  $\beta$ -arrestin-regulated CCR5 trafficking could represent an attractive therapy for the treatment of HIV.

Cardiac  $\beta_1$ -adrenergic receptors can stimulate arrestin-2- and -3-dependent signaling in the heart that results in transactivation of the cardioprotective epidermal growth factor receptor (EGFR) (Noma *et al.*, 2007). It is also thought that chronic  $\beta_2$ -adrenergic receptor activation is cardiotoxic and that this primarily involves  $G_s$  signaling (Xiao, 2001). These combined observations suggest that a  $\beta$ -arrestin-biased ligand acting as a classical antagonist of cardiotoxic G protein signaling while stimulating cardioprotective  $\beta$ -arrestin signaling could be therapeutically beneficial.

Arrestins regulate GPCR signaling by controlling desensitization, endocytosis and recycling/degradation of most GPCRs. They also function as ligand-regulated scaffolds that recruit functionally diverse proteins to GPCRs to confer novel signaling properties. Arrestin-dependent signals are involved in different processes *in vivo*, such as cell migration, neurotransmission, cardiac muscle contraction, and apoptosis. These signals can be initiated or antagonized independently of G protein acti-



**Table 1. Major diseases linked to chemokine receptors (adopted from Allen *et al.*, 2007).**

Disease	Chemokine	Chemokine receptor
Allograft	CCL3, CCL4, CCL5	CCR5
Asthma	CCL1, CCL17, CCL22	CCR3, CCR4, CCR8
Atherosclerosis	CCL2, CCL5	CCR2, CCR5, CXCR1, CXCR2, CX3CR1
Atopic dermatitis	CCL1, CCL13, CCL17-18, CCL27	CCR4, CCR8, CCR10
Crohn's	CCL28	CCR9
Chronic hepatitis	CCL3, CCL4	CCR5
Gut cancer	CCL25	CCR9
HIV	CCL3, CCL4, CCL5, CXCL12	CCR5, CXCR4
Ischemia-reperfusion	CCL2	CCR2
Lymph node cancer	CCL19, CCL21	CCR7
Multiple sclerosis	CCL2, CCL4, CCL5, CXCL9	CCR2, CCR5, CXCR3
Psoriasis	CCL4, CCL20, CCL27	CCR5, CCR6, CXCR3
Rheumatoid arthritis	CCL2, CCL3, CCL5, CXCL9, CXCL10	CCR1, CCR2, CCR5, CXCR3

vation. Although arrestins have been extensively studied by different groups, the molecular mechanisms of receptor-arrestin complex formation/function remain unclear and much additional work will be required for advances in this field.

#### IMPORTANCE OF OTHER GPCRS — CASE OF CHEMOKINE RECEPTORS

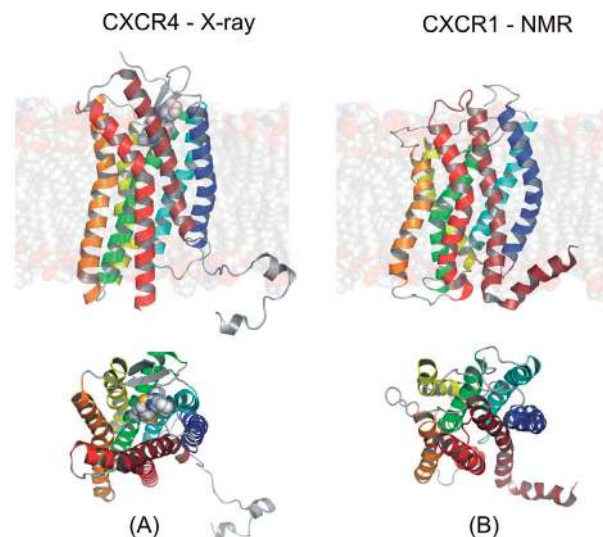
Chemokine receptors (CRs) are one of the most interesting families of GPCRs due to their key role in a number of diseases that affect millions of people worldwide. Chemokines are small chemotactic cytokines that regulate the trafficking of immune cells by binding to cell surface chemokine receptors (CRs). Chemokines coordinate the homeostatic circulation of leukocytes as well as their movement to sites of inflammation or injury (Murdoch & Finn, 2000). Structurally similar, they are small (8–10 kDa) proteins that share a relatively high sequence identity (20–50%). About 50 human chemokines that interact with 22 different receptors have been identified to date. Disregulated expression of chemokines and their receptors has been implicated in the development of many human diseases (see Table 1). As a result, considerable effort has been made to solve the three-dimensional structure of chemokine receptors and to develop drugs to modulate their activities. By the end of 2012 we have learned the connection between a disease and target protein for at least 15 CRs, developed agonists and antagonists for at least 10 different CRs and solved the 3D structures of two chemokine receptors, CXCR4 and CXCR1 (see Fig. 8). Here we review the status of our knowledge about CRs — their structures, involvement in human diseases and known agonists/antagonists. We also present computational approaches to model CR structures and perform rational drug design.

The largest subfamily of CRs is named after their ability to bind CC chemokines — a subfamily of chemokines with four or six Cys residues forming two or three disulfide bonds, with two conserved Cys residues always forming a CC motif. Members of this subfamily share substantial homology with the exception of CCR10. CCR1 was the first CC chemokine receptor identified. It shares a 62.3% sequence identity with CCR3 and binds similar chemokines, but is involved in different diseases than its close relative. Whereas CCR1 has been impli-

cated in multiple sclerosis, rheumatoid arthritis, psoriasis, transplant rejection, cancer and kidney disease, CCR3 has been connected only to asthma and allergic rhinitis. The first antagonists of CCR1 were reported by Hesselgesser *et al.* (1998) and Brown *et al.* (1998). Over 100 different small molecule CCR1 antagonists/agonists derived from at least 15 different scaffolds have been described (Pease & Horuk, 2009a; 2009b). Some reported drug candidates are potent antagonists with reported  $K_i$  values of around 1 nM, e.g. BX 471 (Liang *et al.*, 2000) and MLN3897 (Carson

& Harriman, 2004), and highly selective against CCR1. A few of the most potent and selective CCR1 inhibitors have progressed to clinical development, but despite their high therapeutic potential none have yet passed clinical trials (Gladue *et al.*, 2010).

The story of CCR3 antagonists is similar, although the first chemical compound showing high activity against it (SB-328437) was reported later (White *et al.*, 2000). As of the end of 2012 more than thirty different compounds interacting with CCR3 derived from at least ten different scaffolds have been identified, some with high CCR3 affinity ( $EC_{50}$  values of 1–5 nM (Naya *et al.*, 2003; Ting *et al.*, 2005; Morokata *et al.*, 2006)). Interestingly, both SB-328437 and another early CCR3 inhibitor, UCB 35625, are bi-specific, with nanomolar inhibitory activity towards both CCR3 and CCR1 (Naya *et al.*, 2001). The high homology and similarity in the transmembrane region allowed the design in this case of 'dual' antagonists

**Figure 8. Crystal structures of chemokine receptors.**

(A) Crystal structure of CXCR4 (PDB id: 3ODU) with small molecule antagonist IT1t. (B) NMR structure of CXCR1 (PDB id: 2LNL). Top, view along the membrane plane, bottom, from the extracellular side.

affecting both receptors. More dual inhibitors have been reported (Dhanak *et al.*, 2001a), some with subnanomolar activities against both CCR1 and CCR3 (Dhanak *et al.*, 2001b). Though some of those inhibitors were tested in patients with various diseases, none have yet succeeded.

CCR2 and CCR5 constitute another interesting pair of similar chemokine receptors, sharing 63.4% sequence identity. They also interact with some of the same chemokines and both have been linked to the same immunologic and cardiovascular diseases (Zhao, 2010). Of this pair, however, only CCR5 has achieved fame for serving as an entry factor for macrophage-tropic strains of HIV-1 (Moore *et al.*, 1997), a role that has instigated a search for small molecular antagonists of this receptor that could block viral entry.

The first CCR2 antagonists were described in the literature in 2000, six years after the successful cloning of this receptor (Forbes *et al.*, 2000; Mirzadegan *et al.*, 2000). By the end of 2012 several pharmaceutical companies have disclosed more than fifty CCR2 antagonists with different molecular scaffolds including piperidine, spiro-piperidine, aminopyrrolidine, compounds with bisubstituted cyclohexane groups, and others. Some of the optimized ligands evidenced CCR2 inhibition in the nanomolar range, with the lowest  $IC_{50}$  (subnanomolar) value reported by Teijin company for one of their optimized homopiperazine derivatives (Moree *et al.*, 2008). But as in the CCR1/CCR3 case, no drug has yet been approved for use against this receptor despite considerable effort.

Most CCR5 inhibitors were developed to prevent the cellular invasion of HIV. The first CCR5 inhibitor reported was TAK-779 synthesized by Takeda Chemical Industries in 1999 (Baba *et al.*, 1999). This compound bound to the receptor at nanomolar concentrations and inhibited HIV cellular entry *in vitro*. Unfortunately, it also exhibited a poor oral bioavailability, but still was used as a CCR5 inhibitor model by other pharmaceutical companies. In the last ten years at least forty different CCR5 antagonists have been identified and, due to the importance of AIDS, many were optimized to display inhibitory efficacy at nanomolar or subnanomolar concentrations. Based on favorable HIV inhibition data, a relatively large number of these compounds entered clinical trials for treatment of AIDS. Unfortunately, most CCR5 inhibitors experienced multiple problems in stage II or III clinical trials (Wilkin & Gulick, 2012) and only one (maraviroc) was cleared and approved as an anti-HIV drug targeting CCR5 (Hitchcock, 2005).

The second largest subfamily of CRs was named for their ability to bind CXC chemokines that possess the conserved CXC motif. CXCR4 is one of the two chemokine receptors (together with CCR5) used by HIV to enter human cells, a finding that has greatly accelerated structural research aimed at this protein (Oberlin *et al.*, 1996). The first small molecular antagonist of CXCR4 was described in 1992 (De Clercq *et al.*, 1992) and since then a number of potent antagonists (with binding in the nanomolar or even subnanomolar range) have been described (Ichiyama *et al.*, 2003; Zhan *et al.*, 2007). Moreover, the relatively large amount of data for this receptor provided insights into the binding modes of a number of antagonists (Vabeno *et al.*, 2006).

A breakthrough in CXCR4-based anti-HIV research occurred with the experimental solving of crystal structures of CXCR4 bound to either a small-molecular ligand or a cyclic peptide antagonist (Wu *et al.*, 2010) (Fig. 8A). Earlier it was suggested that both those ligands block the interaction between CXCR4 and its natural ligand CXC4,

and also inhibit CXCL12 interactions with the HIV-1 glycoprotein gp120. Indeed, both ligands found in those crystal structures interacted with the receptor's extracellular loops and N-terminus and most likely altered their conformation, making the interaction with CXCL12 and gp120 energetically unfavorable.

The structure of CXCR4 was rather surprising, especially because it revealed a number of relatively large differences from the crystal structures of the  $\beta_2$ -adrenergic receptor and other GPCRs obtained earlier. The most striking differences were the positions and rotations of helices 1, 2 and 6 that resulted in a much looser packing of all helices, and also the lengths of helices 5 and 7. Surprisingly, the binding cavity of CXCR4 also was larger, more open and located much closer to the extracellular surface than that of other known GPCRs. This investigation not only provides invaluable information about the structure of CXCR4 and possibly other CR-family members, but it also serves as a platform for rational drug design, contributing to understanding of the HIV-1 entry process along with the elucidated structures of native HIV-1 gp120 trimers (Liu *et al.*, 2008). This progress should lead to a series of new scaffolds and compounds targeting CXCR4 as well as dual inhibitors able to bind to both CXCR4 and CCR5 (Murray *et al.*, 2010). Interestingly, despite the known structure of CXCR4, no anti-HIV drug targeting this receptor has yet been approved for use. However, one of the first CXCR4 inhibitors found has been approved to be used to mobilize hematopoietic stem cells in cancer patients (AnorMED, 2007).

CXCR1 and CXCR2 form a pair of chemokine receptors which are most closely related to each other, with 76.6% sequence identity. The number of publications on the role of both receptors has increased exponentially within the last 10 years, in part due to the discovery of potent and selective CXCR1/CXCR2 dual antagonists. The first dual antagonist for this pair of proteins was shown to inhibit acute and chronic models of arthritis in the rabbit (Podolin *et al.*, 2002). There is also an interest in developing new CXCR1/CXCR2 antagonists as therapeutic agents for chronic obstructive pulmonary disease (COPD), asthma and various forms of cancer. Two such dual antagonists have passed all clinical trials and are now marketed — reparixin which attenuates inflammatory responses and promotes recovery of function after spinal cord injury (Gorio *et al.*, 2007) and navarixin, an anti-COPD drug.

CXCR1 is also the second chemokine receptor with a known three-dimensional structure. In 2012 its structure was obtained by using rotationally aligned solid-state NMR (Park *et al.*, 2012) (Fig. 8B). That novel method involved the use of NMR for the first time in GPCR structural studies and provided important information about this receptor in its natural phospholipid bilayer environment. A comparison of its structure with that of CXCR4 (32.9% sequence identity) shows a high homology and overall similarity. Four charged residues in the helical region of CXCR1 form a polar cluster in the ligand-binding site of this receptor. They most likely are important for ligand binding, similar to the three polar residues found in CXCR4. There also are some potentially important differences in positions and rotations of helices 1, 2 and 6, which alter the overall structure of the receptor's core and could contribute to the different biological activities exhibited by CXCR1 and CXCR4. As with CXCR4, solution of the CXCR1 structure hopefully will lead to the rational design of new CXCR1 and CXCR2 antagonists with improved properties.

Chemokine receptors are a family of GPCRs that have always attracted much attention from researchers and health professionals. Immediately after their recognition as potential targets in various diseases, these proteins became the focus of numerous programs run by pharmaceutical companies. Development of novel experimental and theoretical techniques has been crucial in finding and/or designing new CR antagonists. Of these recent innovations, the possibility of obtaining three-dimensional structures by using crystallography or NMR was one of the most important. This has led to the crystal structure of CXCR4 and the NMR structure of CXCR1 described above (Wu *et al.*, 2010; Park *et al.*, 2012). There are still many questions about the structure of other CRs, their probable oligomerization and dynamics in human cells, but our knowledge of this family of proteins is now increasing exponentially.

## FUTURE DIRECTIONS

The GPCR research will further shape the field of pharmacology and medicine in the decades ahead. Thus it will be challenging but necessary to determine high resolution structures of native GPCRs alone and in complexes with their G protein, receptor kinase and arrestin (Jastrzebska *et al.*, 2010). Moreover, GPCRs and G proteins are critically altered by several post-translational modifications which should be taken into account in structural studies. In addition to these modifications, water molecules have been shown to play a key role in all proteins, including transmembrane ones. It will be essential to determine the exact location of water molecules in these receptors and their re-arrangements during GPCR activation. Understanding the energy landscape of GPCRs corresponding to their folding pathway and activation is highly anticipated. Identification of key residues responsible for folding and membrane insertion is needed to explain the etiology of many human diseases associated with receptor mutations. Moreover, GPCRs are not monomeric — they have a propensity to interact not only with each other but also with other transmembrane proteins. In recent years, more than 50% of published papers in the field have explored the oligomerization of GPCRs. But we are still far from understanding “the logic” of oligomerization at both structural and functional levels. Understanding the structural complementarities of GPCR homo- and hetero-oligomerization offers several novel pharmacological opportunities to explore the high specificity of these interactions. A large number of GPCRs communicate with only a limited number of G proteins and even a smaller number of effectors such as enzymes and channels. Although the subcellular localization of specific sets of receptors and interacting proteins is clear, diffusible ligands such as cAMP or Ca<sup>2+</sup> allow a cross-talk between many specialized pathways. It will be important to determine the intersections of different relevant GPCR signaling pathways in native tissues of interest. Modern 3D structural electron microscopy (cryo-electron microscopy and tomography) and hybrid microscopic techniques will facilitate obtaining high resolution structures of signaling complexes between GPCRs, their cognate G proteins and effector molecules in native tissues. Such studies can determine how the GPCR signaling complexes are compartmentalized within cells to evoke their local effects. We are confident that the discovery of mutations responsible for genetic diseases will be dramatically accelerated due to the DNA and RNA sequencing of whole personal genomes as well as tissue

transcriptomes. This new methodology will enable evaluation of the impact of mutations and polymorphisms affecting GPCR expression levels on human conditions. Hence, GPCRs will certainly play a central role in drug research in the foreseeable future.

## Acknowledgements

This work was supported by funding from the National Institutes of Health, USA (EY008061) and the National Center of Science, Poland (DEC2011/03/B/NZ1/03204). K.P. is John H. Hord Professor of Pharmacology.

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