Review

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Emerging structural insights into biased GPCR signaling

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The discovery of biased signaling at G protein-coupled receptors (GPCRs), the largest class of cell surface receptors and primary drug targets for numerous human diseases, has redefined the classical concepts of receptor pharmacology. It not only highlights the depth of signaling diversity within the GPCR system, but also offers possibilities for novel and more-effective therapeutics. Here, we highlight the recent biophysical and structural advances in our understanding of ligand–receptor interactions and conformational changes in the receptors, which provide novel mechanistic insights into biased GPCR signaling. We also underline key aspects of GPCR-biased signaling that remain to be investigated in greater detail to develop a complete molecular understanding of this process and overall GPCR signaling.

G protein-coupled receptors: activation, signaling, and regulation

GPCRs are the largest class of cell surface receptors in the human genome and are the primary target of approximately 30–40% of currently marketed drugs [1–5]. GPCRs recognize a diverse array of ligands and transmit signals across the plasma membrane to induce a range of cellular and physiological responses. However, their activation, signaling, and regulatory mechanisms appear to be remarkably conserved. In the classical signaling paradigm of GPCRs, binding of an agonist leads to a conformational change in the receptor that is compatible with its coupling to heterotrimeric G proteins. Subsequent activation and dissociation of heterotrimeric G proteins leads to the generation of second messengers and cellular responses. Agonist-activated GPCRs undergo a conformational change that is suitable for phosphorylation, primarily by the GPCR kinases (GRKs) [5–7]. Phosphorylated GPCRs recruit β-arrestins, which compete with G protein coupling (through a steric mechanism), and desensitize the G protein signaling response [8–10] (Figure 1). Binding of β-arrestins to agonist-bound GPCRs also promotes their clathrin-dependent endocytosis, thereby providing an additional mechanism for dampening the signaling response [8,11].

Interestingly, it was discovered just over a decade ago that β-arrestins recruited to agonist-activated and phosphorylated receptors can also mediate a wave of G protein-independent signaling pathways [12,13]. This finding revealed the bimodal nature of GPCR signaling, a framework that is now firmly established for several different GPCRs and appears to be a conserved phenomenon in the GPCR superfamily. The repertoire of β-arrestin-mediated signaling includes scaffolding of mitogen-activated proteins kinases (MAPKs) (e.g., extracellular signal regulated kinase, ERK), nonreceptor tyrosine kinases (e.g., c-Src), glycogen synthase kinase 3 (GSK3), protein phosphatase 2A, and transcription factors (e.g., nuclear factor-κB) [13–18]. The cellular outcomes of β-arrestin-dependent signaling range from protein synthesis, cell migration, cytoskeletal rearrangement, and cell proliferation to apoptosis [14,19–25]. Furthermore, several global proteomics studies have started to reveal the diverse nature of signaling networks that β-arrestins can influence in response to activation of GPCRs [26–28], which often have distinct cellular and physiological outcomes compared with G protein-mediated signaling. Therefore, discovery

Glossary

Active-like conformation: used here in the context of agonist-bound receptor structures that display less pronounced features of a fully active conformation. In the spectrum of inactive to active receptor states, an active-like conformation can be conceptualized as an intermediate approaching the active state.

Biased agonism: the phenomenon of a ligand being an agonist for one pathway downstream of a receptor while being either a neutral antagonist or an inverse agonist for the other downstream signaling pathway(s).

Biased ligand: a ligand capable of selectively engaging one signaling pathway downstream of a receptor over the other downstream of the same receptor. A complete β-arrestin-biased ligand does not promote G protein coupling but induces robust β-arrestin recruitment. Similarly, a G protein-biased ligand leads to robust coupling and activation of G proteins but not of β-arrestins.

Bimane fluorescence assay: bromobimanes are small molecule chemical compounds that can be specifically attached to thiol groups (e.g., cysteines in proteins) and yield a particular fluorescence signature. The fluorescence intensity and profile of bimane dyes are sensitive to its surrounding and can be used as a read out of conformational changes in specific domains of proteins.

Chemical labeling: an approach where selected residues of a protein are labeled using small chemical probes that react to the side chains of specific amino acids. The accessibility (i.e., degree of labeling) of a particular amino acid indirectly reflects its conformational surrounding and surface exposure.

Inverse agonist: ligands that suppress the basal activity of the receptors (i.e., constitutive activity in the absence of activating ligands) are referred to as inverse agonists. Inverse agonists stabilize inactive conformation of the receptors and, in most cases, occupy the orthosteric ligand-binding pocket of the receptor.

Resonance energy transfer (RET) assays: a proximity assay that reports a qualitative assessment of distance and orientation of energy donor and acceptor moieties. Intramolecular RET assays involve donor and acceptor moieties on different domains of the same molecule (e.g., receptor or β-arrestin) and, therefore, indicate the relative domain movement as a readout of conformational change.

Unbiased ligand: also referred to as a 'full agonist' or 'balanced agonist,' it displays efficacies for all downstream effectors. Often, the endogenous agonist of GPCRs is considered an unbiased ligand and used as a reference ligand for comparing the bias of synthetic ligands.

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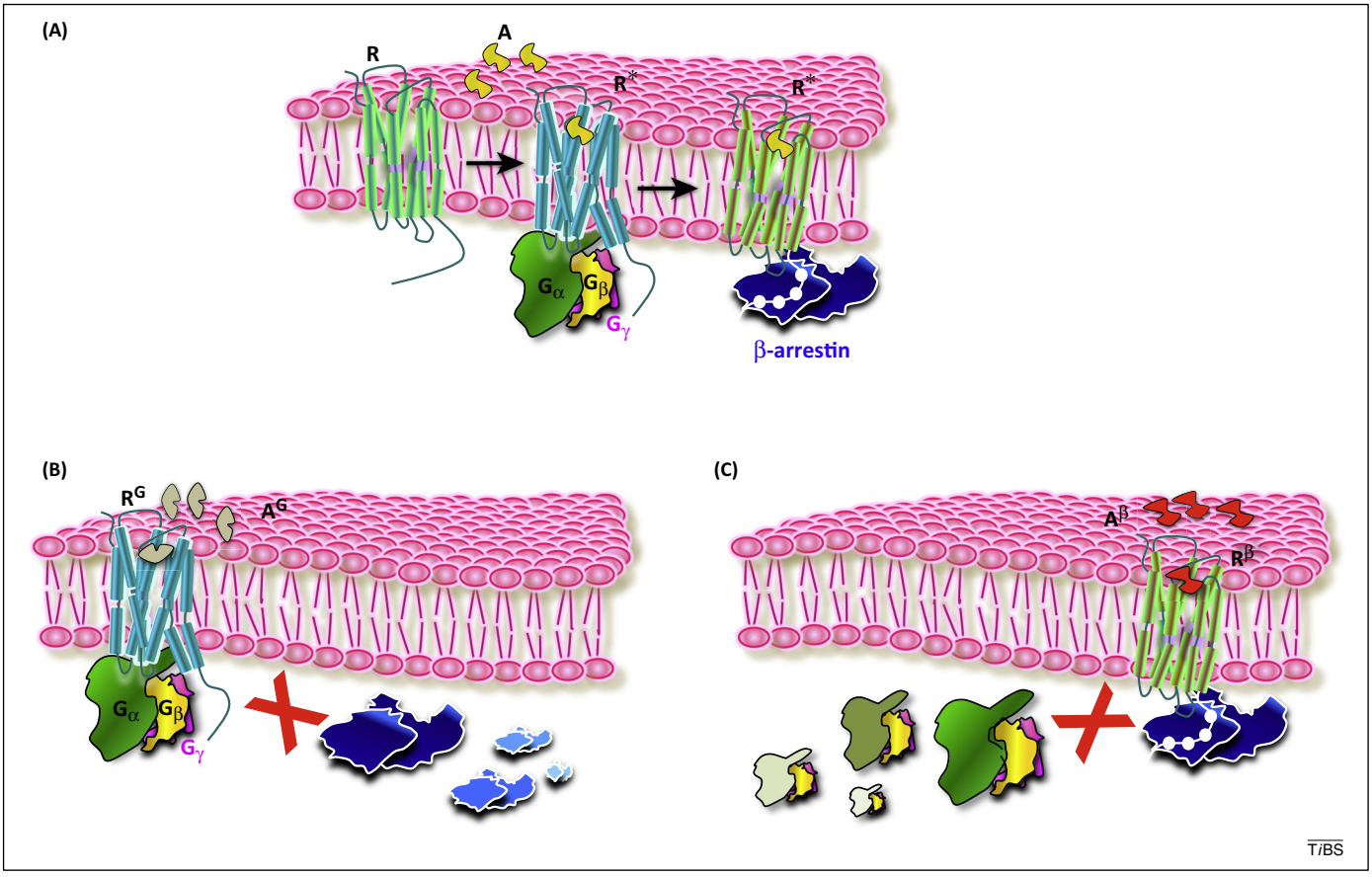

Figure 1. Schematic representation of unbiased and biased signaling at G protein-coupled receptors (GPCRs). **(A)** An unbiased ligand (A), also referred to as a 'full agonist' or 'balanced agonist' binds to the receptor (R), leading to activation of the receptor (R*) and coupling and activation of first the heterotrimeric G proteins (Gα, Gβ, and Gγ) and then β-arrestins. White circles represent phosphorylation in the C terminus of the activated receptor. **(B)** A G protein-biased ligand (A^G) stabilizes a receptor conformation (R^G) that selectively engages heterotrimeric G proteins without any detectable recruitment of β-arrestins. **(C)** A β-arrestin-biased ligand (A^β) stabilizes a receptor conformation (R^β) that leads to selective coupling and activation of β-arrestins without coupling and activation of heterotrimeric G proteins. Although biased signaling conformations of GPCRs (i.e., R^G versus R^β) are likely to display significant differences, the molecular details of such differences are currently not fully resolved.

of β-arrestin-dependent signaling creates a unique opportunity for the selective modulation of the downstream effects of GPCRs [29,30].

Interestingly, the G protein-dependent and β-arrestin-dependent signaling pathways are pharmacologically separable [31–34]. Ligands that selectively engage either G protein- or β-arrestin-dependent pathways are referred to as biased ligands (see Glossary; Figure 1) [34–42]. However, most of the endogenous ligands of GPCRs are capable of activating both the G protein and β-arrestin signaling pathways. Bias of pharmacological ligands is measured and described with respect to efficacies of endogenous ligands. Moreover, it has been possible to design mutants for several GPCRs that exhibit signaling bias for one or the other effector compared with the wild type receptors [31,43,44]. These mutants further confirm the independent nature and clear separation of the two signaling pathways.

The discovery, characterization, and physiological outcomes of biased ligands and biased signaling for GPCRs have been extensively reviewed and discussed in recent years [45–51]. However, the molecular basis of biased signaling is just emerging, and remains less well described. Here, we review recent advances in our understanding of ligand–receptor interactions, biased ligand-induced rearrangement of the transmembrane core, and conformational changes in the loop regions of the receptors. Together, these shed light on the mechanistic basis of biased GPCR signaling. We specifically focus on studies that utilize well-defined biased ligands to probe the conformational and structural changes in their cognate receptor to obtain novel insights into the process of biased signaling.

Shaping the functional outcome: conformation drives function

A central question that lies at the heart of biased signaling is how the same receptor is compatible with coupling to two entirely different effectors leading to distinct signaling outcomes. For unbiased ligands, coupling of the two different effectors (namely the heterotrimeric G protein and β-arrestins) is likely to be sequential, because their activation appears to be driven by activation and subsequent phosphorylation of the receptor, respectively. Interestingly, however, biased ligands selectively allow coupling of one but not the other effector. This implies an intrinsic difference in the receptor core that enables it to choose between the two effectors and prohibit coupling to the other effector. Accordingly, the concept of conformational coupling in the ligand–receptor–effector cascades has been put forward and has been tested for multiple receptor systems using several biophysical and structural approaches [1,12,34,51–55] (Table 1). An ideal scenario to fully decipher the structural basis of biased signaling would be to crystallize

Table 1. Summary of different methodologies used to study the structural basis of biased signaling, and their key conclusions

GPCR	Methodology	Major conclusion
β_2AR	Chemical labeling and ^{19F}-NMR	Unique conformational signature of β_2AR in response to β-arrestin-biased ligand carvedilol in third intracellular loop and TM7
β_1AR	X-ray crystallography	β-arrestin-biased ligand carvedilol makes additional ligand–receptor contacts in the ligand-binding pocket compared with an inverse agonist, cyanopindolol
Ghrelin receptor	Bimane fluorescence	Distinct receptor conformation for β-arrestin-biased ligand and a direct allosteric effect of effector proteins on receptor conformation
V_2R	Tryptophan fluorescence and RET	Distinct conformational change in the V_2R for β-arrestin-biased and G protein-biased ligands
5-HT_{1B} and 5-HT_{2B}	X-ray crystallography	ERG, a β-arrestin-biased ligand at 5-HT_{2B}, induces significantly different changes in the ligand-binding pocket, TM7 and the DRY motif compared with 5-HT_{1B}

a given receptor not only in inactive, active, and biased signaling conformations, but also bound to both G proteins and β-arrestins. Moreover, crystallographic observations must be coupled and interpreted in close association with dynamic studies of receptor–effector components. However, we are still far from having complete snapshots of structures and complementary dynamic insights for any given receptor. Therefore, the fine structural details of the mechanism of biased signaling are derived from the bits and pieces of information gathered on multiple different GPCRs studied by using several thematically converging biophysical approaches.

Unique conformational signature for a biased ligand: β2 adrenergic receptor

The β2 adrenergic receptor (β_2AR) has been a model system to conceptualize and delineate several different paradigms of GPCR activation and signaling. Not surprisingly, it has also been one of the GPCRs used most widely to study biased signaling and has contributed much to our understanding of these mechanisms [43,56–59]. Although a strongly biased ligand of the β_2AR is not available, carvedilol, originally thought to be an inverse agonist, has been characterized as a weak β-arrestin-biased ligand and has helped deduce several significant features of biased signaling at the β_2AR [56,60,61].

Although fully active structures of β_2AR bound to unbiased ligands are available, the extent of overlap between a biased signaling conformation and a fully active conformation remains to be established [62,63]. Still, some studies have begun to address this issue. Kahsai *et al.* used a chemical-labeling approach coupled with mass spectrometry (MS) analysis to investigate distinct conformational changes in the purified β_2AR in response to ligands of varying efficacy, including carvedilol [56]. Their experiments revealed that, although most residues move in a pattern as expected by the relative efficacy of the ligands used, Cys^{265}, in the distal region of the third intracellular loop of β_2AR, becomes more buried within the core of the protein in response to carvedilol compared with a reference ligand, isoproterenol. Interestingly, Lys^{263} in the distal end of the third intracellular loop, becomes more exposed with carvedilol compared with isoproterenol. These striking observations suggest a significantly different local conformation in this region in response to unbiased versus biased ligand. Unfortunately, a crystal structure of β_2AR in complex with carvedilol is lacking; therefore, the precise nature of conformational differences in this region for biased and unbiased ligands cannot be directly visualized at high resolution. Still, a comparison of the inactive and nanobody-stabilized active states of β_2AR suggests significant rearrangement of transmembrane (TM) 5 and 6 and, thereby, the third intracellular loop (Figure 2A). Given the highly dynamic nature of third intracellular loop in β_2AR, it is tempting to speculate that the significantly different accessibility of these two residues in response to carvedilol likely reflects a unique conformational signature of the β_2AR in a biased signaling conformation.

Additional support for the idea that GPCRs have unique conformational signatures in response to biased ligands comes from conformational probing of the β_2AR using ^{19}F-nuclear magnetic resonance (NMR) spectroscopy [60]. By using a covalently attached ^{19}F-labeled NMR probe, the authors followed the motions of three cytoplasmically facing cysteines (Cys^{265} in the third intracellular loop, Cys^{327} in the TM7 and Cys^{341} in the helix 8) of β_2AR during exposure to saturating concentrations of different ligands. Interestingly, the β-arrestin-biased ligand carvedilol appeared to result in a major receptor population that had a significant shift in the conformational equilibrium of TM6 and 7 compared with untreated receptor, as reported for Cys^{327} and Cys^{265}. By contrast, isoproterenol, an unbiased ligand, resulted in two distinct conformational populations of the receptor. This finding suggests the crucial involvement of TM6 and 7 in a β-arrestin-biased signaling conformation of the receptor. A comparison of the inactive and active β_2AR structures reveals significant rearrangement of TM6 and 7, which is in agreement with the ^{19}F NMR analysis (Figure 2B). Somewhat surprisingly, the chemical shift profile of Cys^{341}, which is located in helix 8 of the receptor, indicates that the overall position of helix 8 in β_2AR, including Cys^{341}, does not change significantly upon receptor activation (Figure 2C). However, previous studies implicated a critical role for helix 8 in receptor–arrestin interactions and predicted that it would have significant conformational reorientation upon interaction with β-arrestins [64–66]. It is plausible that conformational alterations in helix 8 are a part of an allosteric effect, and they can be detected robustly only in the context of a receptor–arrestin complex. Another possibility is that the lack of a conformational change in helix 8 reflects the weak efficacy of carvedilol on the β-arrestin pathway; therefore, the detected conformation is only representative of a partial β-arrestin biased conformation. In addition,

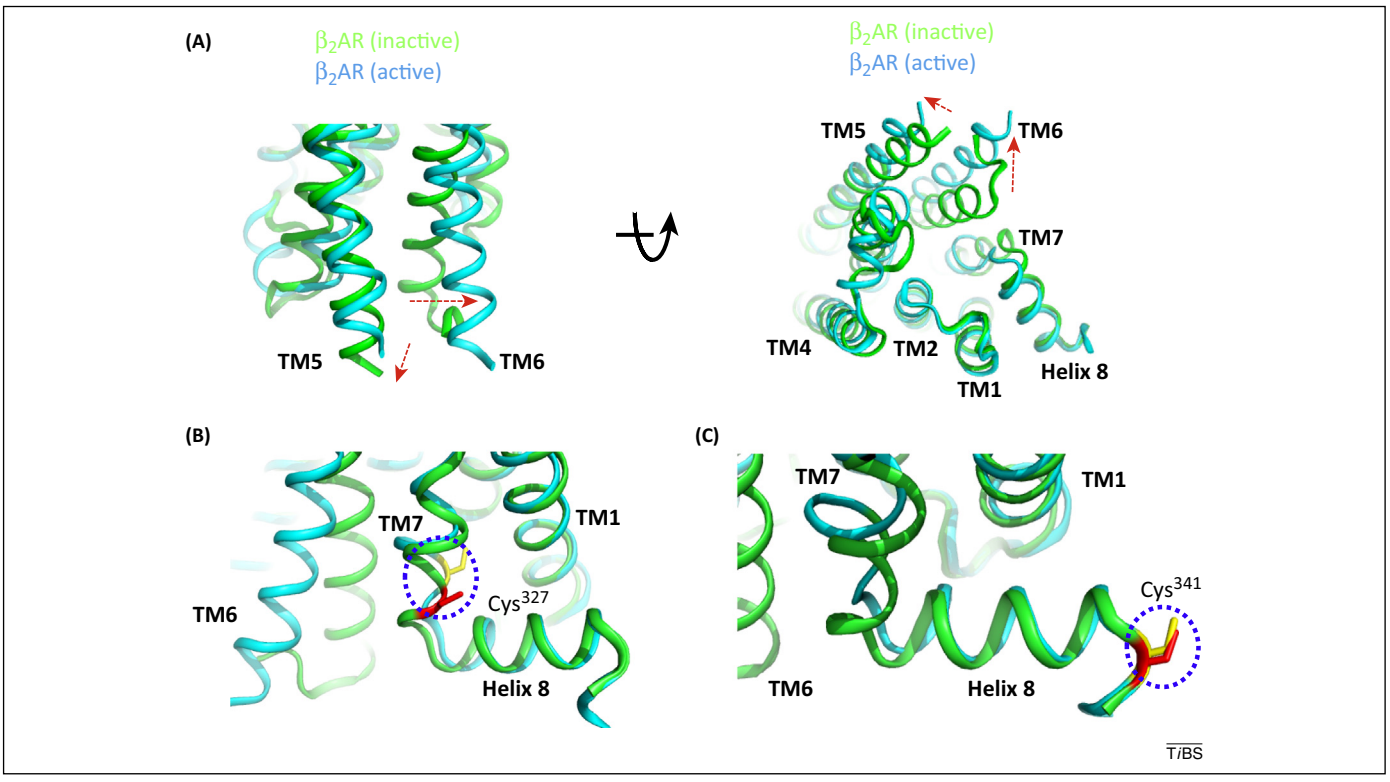

Figure 2. Structural transition of selected residues in β2-adrenergic receptors (β_2AR) potentially implicated in biased signaling conformation. **(A)** Structural changes in the transmembrane (TM) helices of the β_2AR upon agonist activation, focusing on TM5 and TM6 helices. The relative rearrangement of the TM5 and TM6 is likely to result in a conformational change in the third intracellular loop of the receptor. Broken red arrows indicate the outward movement of TM5 and TM6 upon receptor activation compared to inactive state. The right panel depicts cytoplasmic view of the receptors obtained through approximately 90° forward rotation of the structure overlays shown in left panel. **(B)** Rearrangement of TM7 upon receptor activation leads to a significant reorientation of Cys^{327} at the cytoplasmic end of TM7. **(C)** Relative orientation of Cys^{341} in helix 8 does not significantly alter between the inactive and active β_2AR conformations. The broken blue circle highlights the side chains of Cys^{327} in TM7 and Cys^{341} in TM8.

often ligands alone, even if they have extremely high affinity, cannot fully stabilize all components into active conformations [67,68]. Therefore, it is possible that the studies presented here that utilize receptor-biased ligand combinations represent only a small subset of conformational changes associated with biased signaling. Complete visualization of the conformational palette in biased signaling would require similar studies in the presence of additional conformation stabilizing agents or, better yet, on receptor–effector complexes.

The second intracellular loop and third extracellular loop as reporters of biased conformations: the ghrelin receptor

The highly conserved D(E)RY motif in the second intracellular loop of GPCRs is well documented to have a critical role not only in activation of GPCRs, but also in β-arrestin recruitment [69,70]. In addition, the third extracellular loop also appears to undergo a significant conformational alteration upon ligand binding [71,72]. Therefore, these two domains of GPCRs represent potential hot spots for detecting specific differences between biased and unbiased ligand-induced conformations. A recent study used two different fluorescence-based biosensors targeting these two regions in the ghrelin receptor to study ligand-induced conformational changes [73,74]. These sensors were singly labeled either at Cys^{146} in the second intracellular loop, in the vicinity of the DRY motif, or at Cys^{304} in the third extracellular loop, towards the proximal end of TM7. Subsequently, the effects of a series of ligands of differing efficacy on these two sensors were measured. Interestingly, the ligands that were biased for G protein signaling resulted in a smaller change in bimane fluorescence compared with the unbiased ligands. This differential change in bimane fluorescence suggests distinct conformational states of the receptor. Moreover, fluorescence decay experiments revealed two distinct subpopulations of conformations stabilized by functionally distinct ligands. These findings are in line with previous studies that implicate the DRY motif in the second intracellular loop in receptor activation and additional ligand contacts with TM7 residues in the ligand-binding pocket for biased ligands.

Monitoring the movements of TM6 versus TM7: the vasopressin receptor

Another interesting insight into GPCR-biased signaling comes from a study on ligand-induced structural changes in the human arginine vasopressin type 2 receptor (V_2R) [75]. While the endogenous peptide agonist arginine vasopressin (AVP) is used as a reference ligand for V_2R, a synthetic nonpeptide ligand SR121463 has been described as a β-arrestin-biased ligand and another nonpeptide ligand, MCF14, has been characterized as a G protein-biased ligand. In a recent study using purified V_2R reconstituted in nanodiscs, the addition of β-arrestin biased ligand SR121463 led to a decrease in overall intrinsic tryptophan fluorescence, indicating a conformational transition in response to ligand binding [75]. This is in stark

contrast with the unbiased ligand, AVP, and the G protein-biased ligand, MCF14, which both led to an increase in the overall intrinsic tryptophan fluorescence. Although these findings represent only a qualitative assessment, they clearly reveal the existence of two different receptor populations in response to biased and unbiased ligands. Furthermore, lanthanide resonance energy transfer-based intramolecular sensors of TM6 and TM7 movement with respect to helix 8, revealed two distinct conformational subpopulations of the receptor. Although fractional distribution of these two subpopulations was modulated only very minutely by addition of ligands, β-arrestin- and G protein-biased ligands differently affected the average lifetime constant of the major subpopulation of the receptor, indicating different conformational distribution. Given the biased nature of the ligands used here, it is tempting to speculate that TM6 undergoes major conformational rearrangement during G protein coupling, while the TM7–TM8 interface is a prominent player in arrestin engagement. These data are also in agreement with recent studies on the β_2AR–G_s complex [62] and the β_2AR–β-arrestin complex [66].

These studies present qualitative but direct evidence for unique conformational signatures of receptors in response to biased ligands. High-resolution details of such conformational signatures can be elucidated only by X-ray crystallography of the receptors, which is beginning to emerge.

Visualizing bias at high resolution: hints from crystallography

A recent revolution in crystallography of GPCRs provided the long-awaited breakthrough and opened the door for visualizing the atomic details of biased signaling. Structures of GPCRs bound to biased ligand have started to appear and they reveal not only the atomic details of ligand-receptor interaction, but also the specifics of overall global and local conformational changes in the receptor core and loop regions.

Coordinating biased ligands in an orthosteric pocket: the β1 adrenergic receptor

For several different GPCRs, biased ligands appear to be competitive with classical agonists and antagonists. This directly reflects the fact that these biased ligands occupy the same orthosteric ligand-binding pocket as the classical ligands, at least for most receptor–ligand contacts. However, it is also likely that biased ligands lack certain receptor–ligand contacts and make some distinct receptor–ligand interactions relative to unbiased ligands. Obviously, this unique set of receptor–ligand interactions is primarily responsible for dictating and inducing bias at the most proximal level. Interesting insights into this come from the crystal structures of a thermo-stabilized turkey β_1 adrenergic receptor bound to the β-arrestin-biased ligands bucindolol or carvedilol, and their direct comparison with the crystal structure of β_1AR bound to cyanopindolol, an inverse agonist (Figure 3A) [76,77]. While the global conformation of the receptor remains unaltered among these ligands, there are some interesting differences between the ligand-binding pocket of the inverse agonist-bound and biased ligand-bound structures of the receptor. Carvedilol, which has a bulky aromatic substituent at the amine end, makes additional van der Waals contact with residues Leu^{101} in TM2, Asp^{200} in extracellular loop 2 (ECL2), Tyr^{207} in ECL2, Trp^{330} in TM7, and a water-mediated hydrogen bond with Phe^{201} in ECL2 (Figure 3B). Interestingly, bucindolol, which is also a β-arrestin-biased ligand for β_1AR, appears to follow similar trends and form additional interactions with residues in

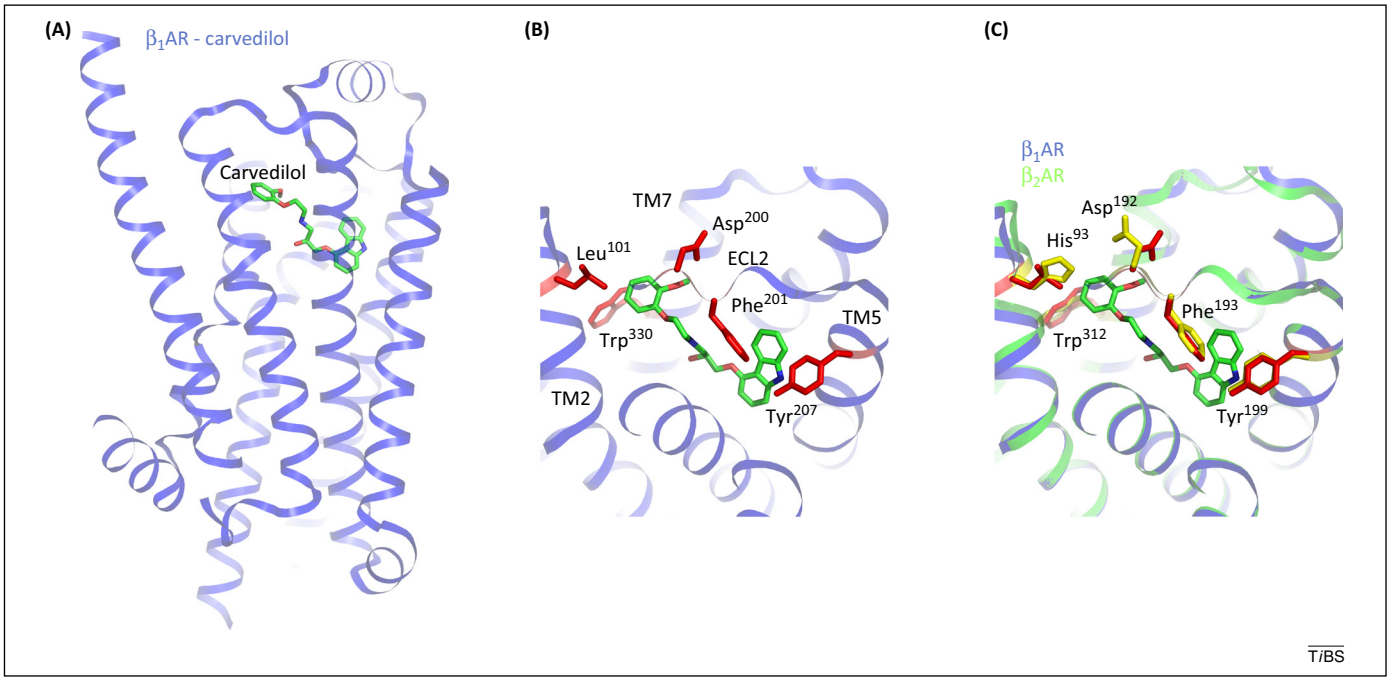

Figure 3. Binding of carvedilol, a β-arrestin-biased ligand, to the β-adrenoceptors (βARs). **(A)** Crystal structure of carvedilol-bound thermostabilized β_1 adrenergic receptor (β_1AR). Chemical structure of carvedilol is shown in green. **(B)** Additional ligand–receptor contacts in the ligand-binding pocket of β_1AR with carvedilol bound compared with the full agonist cyanopindolol (CYP) binding. Side chains of the residues that are involved in these additional contacts with carvedilol are highlighted in red. **(C)** Overlay of the carvedilol-bound β_1AR structure and an inactive conformation of β_2AR (PDB code: 2RH1). Residues that make additional contacts with carvedilol in β_1AR are also conserved in β_2AR, indicating a potentially conserved docking mechanism for biased-ligand carvedilol. Side chains of amino acids in the β_2AR are shown in yellow.

TM2, 3, 7, and ECL2. Superimposing the carvedilol-bound β_1AR structure over an inactive conformation of the β_2AR reveals that the residues that make additional contacts with carvedilol are conserved in β_2AR (Figure 3C). Therefore, it is tempting to speculate that these additional ligand–receptor contacts are responsible for inducing ligand bias and, in turn, might represent a conserved mechanism between these two βAR subtypes. However, a concrete validation of this hypothesis requires a crystal structure of the β_2AR bound to carvedilol and preferably stabilized with a biased conformation-stabilizing tool, such as a nanobody or an antibody fragment.

First steps towards a crystal-clear view of bias: serotonin receptors

One of the Holy Grails to understand the molecular basis of ligand bias is the determination and comparison of high-resolution crystal structures of a given GPCR bound with an unbiased and a biased ligand. Such a comparison is likely to reveal key features with respect to which conformational changes in the receptor are crucial for biased signaling. A step towards this goal was the recent determination of the structure of two subtypes of the 5-hydroxytryptamine serotonin receptor, namely 5-HT_{1B} and 5-HT_{2B}, bound to an ergotamine ligand (ERG) [78,79]. Pharmacological and signaling assays revealed that ERG exhibits strong β-arrestin bias for the 5-HT_{2B} receptor, but only a weak bias for the 5-HT_{1B} receptor. Although the overall arrangement of the receptor core between the two crystal structures looks similar, closer inspection reveals some interesting structural features. First, the second extracellular loop of ERG-bound 5-HT_{2B} forms an extra helical turn, which in turn leads to a small shrinkage of the ligand binding pocket (Figure 4A–E). As a result, ERG forms additional hydrophobic contacts with several residues in TM5, TM6, and TM7 when compared with the 5-HT_{1B} structure (Figure 4C). Interestingly, these additional contacts mirror the pattern observed for the β-arrestin-biased ligand carvedilol in the β_1AR [76]. Second, the so-called 'P-I-F' motif, which forms an interface between the TM 3, 5, and 6, has an intermediate conformation in the ERG-bound 5-HT_{2B} structure compared with the ERG-bound 5-HT_{1B} structure and fully activated β_2AR. Along the same lines, TM6 in 5-HT_{2B} closely resembles an inactive conformation, whereas TM7 is closer to an active conformation compared with the fully activated β_2AR structure. Another prominent difference is in the highly conserved DRY motif. While the 5-HT_{1B} structure shows an active-like conformation of this motif, as reflected by the broken salt bridge between the Arg^{153} and Asp^{152}, the 5-HT_{2B} structure shows an intact salt bridge (Figure 4E,F). Taken together, these observations suggest that the ERG-bound 5-HT_{2B} structure represents an intermediate between an

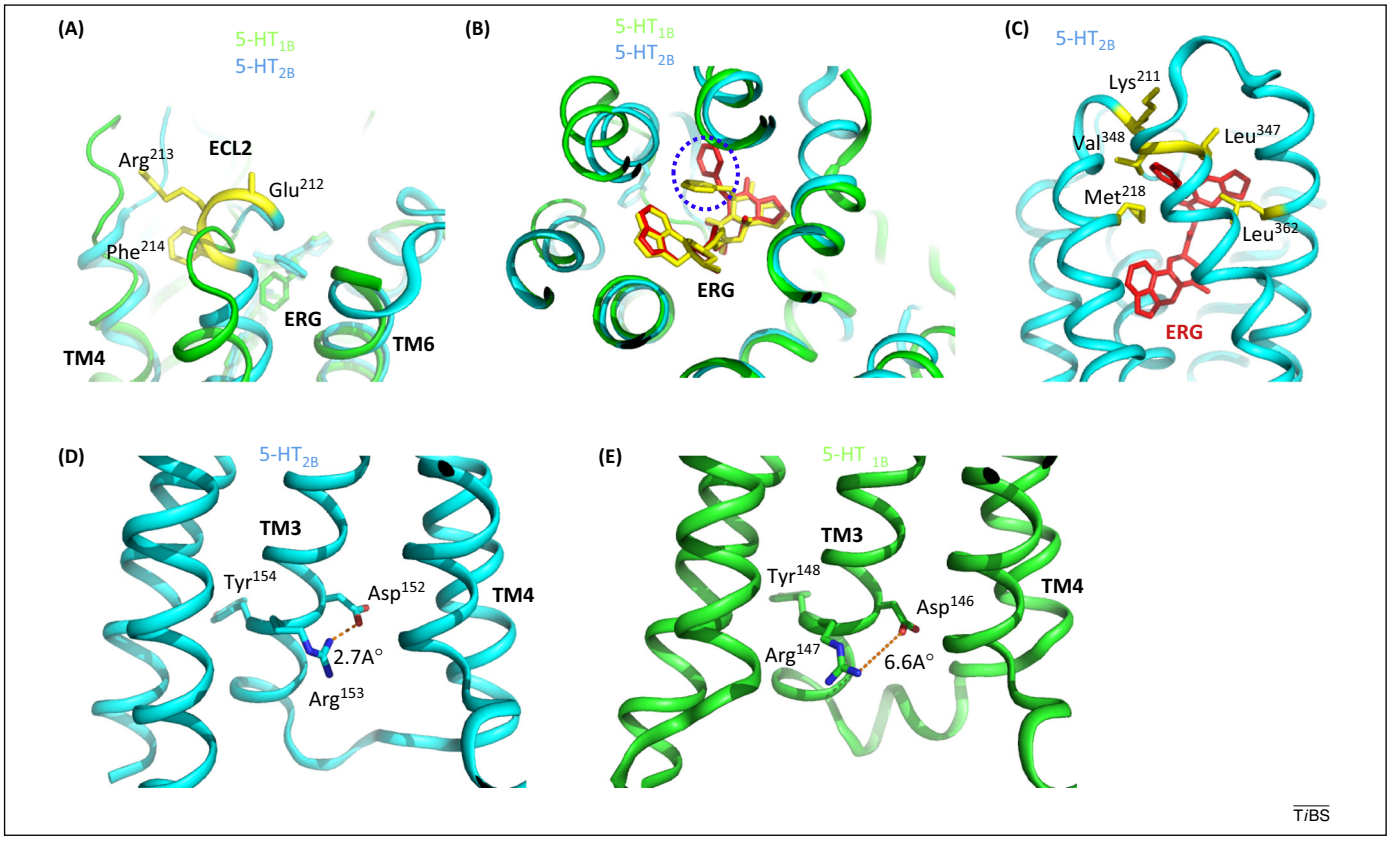

Figure 4. Prominent structural differences between ergotamine (ERG) ligand-bound 5-hydroxytryptamine type 1B receptors (5-HT_{1B}) and 5-HT 2B receptors (5-HT_{2B}) in the context of a biased signaling conformation. ERG is strong β-arrestin bias ligand at the 5-HT_{2B} receptor, while it displays relatively very weak bias at the 5-HT_{1B} receptor. Comparison of ERG-bound 5-HT_{1B} and 5-HT_{2B} receptor structures reveal interesting conformational differences relevant to bias. **(A)** Presence of an extra helical turn in the second extracellular loop (highlighted in yellow) of ERG-bound 5-HT_{2B} that is not seen in the 5HT_{1B} structure. **(B)** A conformational difference in the docking mode of ERG between 5-HT_{1B} and 5-HT_{2B} structures is highlighted by the broken blue circle. ERG is shown in yellow for 5-HT_{1B} and red for 5-HT_{2B}. **(C)** Additional ligand-receptor contacts observed in the ERG-bound 5-HT_{2B} structure compared with the ERG-bound 5-HT_{1B} receptor. Side chains of amino acids involved in additional contacts are shown in yellow and ERG is in red. **(D)** Intact salt bridge between Asp^{152} and Arg^{153} in the DRY motif of 5-HT_{2B} bound to ERG. **(E)** Lack of a corresponding salt bridge between Asp^{146} and Arg^{147} in the ERG-bound 5-HT_{1B} receptor structure.

inactive and fully active state compared with the ERG bound 5-HT_{1B} structure, which resembles an inactive conformation. Perhaps this intermediate conformation of the 5-HT_{2B} receptor represents a β-arrestin-biased signaling conformation, and an incomplete transition to the fully activated state dictates inefficient coupling to G proteins. However, ERG is not a completely biased ligand for 5-HT_{2B}, but rather it is more efficient in inducing β-arrestin signaling compared with G protein signaling. Furthermore, although the ligand structure (ERG) is the same, 5-HT_{1B} and 5-HT_{2B} represent two distinct subtypes of the 5-HT receptor. Therefore, the quest to obtaining a crystal-clear view of a β-arrestin signaling conformation remains open and requires further complementary studies to corroborate the intriguing structural features observed in the 5-HT_{2B} structure.

It is important to underline that the crystal structures represent only a static snapshot and ligand bias may arise from an ensemble of conformations. Therefore, structural insights derived from crystal structures should be interpreted in conjunction with functional data and complementary dynamic studies. In addition to visualizing the biased receptor conformations at high resolution, understanding the structural basis of signal transfer from receptor to effectors and corresponding structural alteration in the effector systems is also a key step in fully elucidating the mechanism of ligand bias. Several recent studies have started to provide key insights into how conformational changes in the receptor impact effector conformations.

Relaying the signal: conformational variability in the effector systems

For the signaling cascade to reach completion, a ligand-induced conformational change in a receptor must be transferred to the downstream effector, a concept that can be understood as conformational coupling in ligand–receptor–effector cascades. Given the challenges associated with the assembly and reconstitution of receptor–effector complexes *in vitro*, it is not surprising that direct structural and biophysical studies of receptor–effector complexes are only now beginning to surface. Striking evidence for conformational coupling between the receptor and its effector comes from the crystal structure of the β_2AR–Gα_s complex [62]. Upon interaction with an agonist occupied receptor, the Gα_s subunit displays a dramatic conformational rearrangement where the α-helical domain undergoes a major displacement relative to the Ras-like GTPase domain [62]. Further support for conformational coupling in the receptor–effector interaction comes from the crystal structure of β-arrestin 1 bound to the phosphorylated C terminus of a GPCR [80]. This interaction with the phosphorylated C terminus and subsequent activation leads to robust structural changes in β-arrestin 1, including a major rotation of the N and the C domains relative to each other. An intriguing emerging hypothesis is that binding of a biased ligand to a GPCR might also result in an effector conformation that is distinct from that induced by an unbiased ligand. In other words, the effector system is also capable of displaying multiple conformations tailored to match receptor conformations. Strong evidence to support this hypothesis stems from a bioluminescence resonance energy transfer (BRET)-based biosensor assay of β-arrestin 2 and a combination of biased ligand–receptor pairs [57]. Binding of β-arrestin to either an unbiased or biased ligand-occupied receptor results in strikingly different conformational outcomes for β-arrestin 2. Although this study reports only qualitative changes in β-arrestin conformation and additional investigations are necessary to elaborate the fine structural details of distinct β-arrestin conformations at high resolution, it does argue in favor of direct conformational matching of effectors to different receptor conformations.

Given that receptor–effector coupling is dynamic multistep process, it is plausible that there is a significant crosstalk between the receptor and effector with respect to their mutual conformational tuning. Several lines of evidence to support this notion have recently been documented for different receptor–effector complexes.

Receptor–effector interplay in conformational landscape

Given the large interface of receptor–effector interactions, it is likely that the coupling of effectors will further allosterically tune the receptor conformation. This might be a key factor in determining the range and span of downstream signaling. As mentioned above, structural visualization of distinct conformations of GPCRs and their signaling complexes has only now started to emerge. However, it is clear that interaction with an effector leads to a significant rearrangement of the receptor core and the intracellular surface. First, in the β_2AR–G protein complex, TM6 is positioned further outward compared with the agonist bound, nanobody-stabilized active state structure of the β_2AR alone [62,63]. Similarly, for the β_2AR–β-arrestin 1 complex, significant rearrangement of the phosphorylated C terminus of the β_2AR was required to accommodate the individual components of the complex (i.e., the β_2AR and β-arrestin 1) in an electron microscopy (EM)-based architecture of this complex. Furthermore, the presence of different effectors (such as Gα_q and arrestin) has distinct effects on the conformation of the ghrelin receptor, as shown by a qualitative assay [73–76]. These observations support the notion of effector coupling-induced conformational tuning in the receptor. A major knowledge gap remains at the level of these structural changes in the receptor upon interaction with different effectors and necessitates in-depth characterization of additional receptor–effector complexes.

Concluding remarks

The concept of biased agonism has come a long way over the past decade. Since the original discovery of the phenomenon, biased ligands for multiple GPCRs have been described and novel signaling pathways have been elucidated. Moreover, we have developed a crude conceptual framework to understand the ligand bias as an outcome of distinct receptor conformation and corresponding structural tuning of the effectors. In-depth details of structural basis of biased signaling are just starting to emerge and, with increasing advances in the area of GPCR structural biology and dynamic approaches, it is feasible that we

Box 1. Outstanding questions

- Does an unbiased ligand stabilize receptor in one conformation that is capable of coupling to both effectors or does it stabilize two distinct conformations, one for each effector? What atomic details will emerge from high-resolution crystal structures of biased agonist-bound GPCRs?
- Does a receptor undergo a conformational transition during effector coupling? What would a crystal structure of agonist–GPCR–β-arrestin complex show us about conformational coupling between the receptor and effector?
- What new insights would a crystal structure of a biased ligand-GPCR-effector complex provide?
- What is the extent of the diversity of GPCR bias (e.g., distinct G protein subtype binding, distinct β-arrestin isoform binding and additional signaling effectors)?
- What are the conformational requirements at the receptor level to accommodate different modes of GPCR-biased signaling?
- How can we learn more about biased signaling conformations of GPCRs through the development of novel, sophisticated tools, such as stabilizing antibody fragments, dynamic biophysical techniques, and natural bilayer like environments?

might have a crystal-clear view of ligand bias at high resolution in the not too distant future. An intriguing parallel of biased signaling is observed with some nuclear steroid hormone receptors, such as estrogen receptor-α (ERα), and their structural snapshots suggest specific helix rearrangement as the underlying mechanism of ligand-specific functional outcomes [81–84]. These observations further support the likelihood of distinct conformational signatures as fundamental mechanism of inducing ligand bias in GPCRs and highlight the generality of the ligand bias phenomenon in receptor families. One of the key long-term goals remains the structural characterization of receptor–effector signaling complexes at high resolution. It is fascinating to anticipate how the structural features of β-arrestins might differ when they are bound to a balanced agonist versus a β-arrestin-biased ligand occupied receptors. An analogous comparison for a G protein-biased ligand is equally intriguing. Another area that remains relatively less explored is a distinct set of receptors in the chemokine family of GPCRs that are intrinsically uncoupled from G proteins but robustly couple to β-arrestins. These receptors, referred to as 'noncanonical' or 'atypical' GPCRs, present a fascinating example of evolutionary divergence in the highly conserved superfamily of GPCRs. Their biophysical and structural analysis should reveal features that might have direct consequences for deciphering the mechanism of biased signaling for the entire GPCR family. Moreover, the extent of converging mechanisms of ligand bias among multiple receptors also remains to be explored in greater detail. Undoubtedly, the coming years will not only provide novel structural and biophysical insights into the mechanism of ligand bias, but also yield the missing links in a complete understanding of the depths of GPCR signaling (Box 1).

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