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Allosteric coupling and biased agonism in G protein-coupled receptors

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G protein-coupled receptors (GPCRs) are essential cell membrane signaling molecules and represent the most important class of drug targets. Some signaling pathways downstream of a GPCR may be responsible for drug adverse effects, while others mediate therapeutic efficacy. *Biased ligands* preferentially activate only a subset of all GPCR signaling pathways. They hold great potential to become next-generation GPCR drugs with less side effects due to their potential to exclusively activate desired signaling pathways. However, the molecular basis of biased agonism is poorly understood. GPCR activation occurs through *allosteric coupling*, the propagation of conformational changes from the extracellular ligand-binding pocket to the intracellular G protein-binding interface. Comparison of GPCR structures in complex with G proteins or β-arrestin reveals that intracellular transducer coupling results in closure of the ligand-binding pocket trapping the agonist inside its binding site. Allosteric coupling appears to be transducer-specific offering the possibility of harnessing this mechanism for the design of biased ligands. Here, we review the biochemical, pharmacological, structural, and biophysical evidence for allosteric coupling and delineate that biased agonism should be a consequence of preferential allosteric coupling from the ligand-binding pocket to one transducer-binding site. As transducer binding leads to large structural rearrangements in the extracellular ligand-binding pocket, we survey biased ligands with an extended binding mode that interact with extracellular receptor domains. We propose that biased ligands use ligand-specific triggers inside the binding pocket that are relayed through preferential allosteric coupling to a specific transducer, eventually leading to biased signaling.

Introduction

G protein-coupled receptors (GPCRs), with more than 800 members, form the largest class of cell membrane receptors and mediate the vast majority of physiological functions in humans [1,2]. GPCRs can sense a myriad of extracellular stimuli such as neurotransmitters, hormones, lipids, peptides, proteins, nucleotides, ions, photons, and odorants and relay this information into cellular responses by activating intracellular heterotrimeric G proteins. As the binding sites for extracellular ligands and G proteins are on opposite sides of the cell

Abbreviations
Ca^{2+}, calcium ions; cAMP, 3′,5′-cyclic adenosine monophosphate; DAG, diacylglycerol; DEER, double electron–electron resonance spectroscopy; GPCRs, G protein-coupled receptors; GRKs, G protein-coupled receptor kinases; IP3, inositol 1,4,5-trisphosphate; NMR, nuclear magnetic resonance spectroscopy; smFRET, single-molecule Foerster resonance energy transfer.

membrane, GPCR activation follows a process called *allosteric coupling* to relay information through the receptor core. Extracellular ligand binding leads to conformational changes in the receptor protein that allosterically favors binding of G proteins at the intracellular site of the receptor [3–5].

G protein activation subsequently triggers changes in the intracellular concentration of *second messengers* such as 3',5'-cyclic adenosine monophosphate (cAMP), calcium ions (Ca^{2+}), diacylglycerol (DAG), and inositol 1,4,5-trisphosphate (IP_3), which activate downstream target proteins, eventually leading to specific cellular responses. To exert spatiotemporal control over cell function, GPCR signaling is not limited to the cell membrane but can also emerge from intracellular compartments such as early endosomes, the trans-Golgi network, or the nucleus [6]. In addition, the concentration of second messengers at different locations in the cell is not uniform and it has been demonstrated that second messengers, especially Ca^{2+} and cAMP, are compartmentalized in cells [7–10], allowing GPCRs to fine-tune cell signaling with high precision in space and time. Thus, by controlling innumerable cellular processes in physiology and disease, GPCRs hold a pivotal role in cell signaling. Based on this key function, GPCRs have emerged as one of the most popular and most important drug targets accounting for more than a third of currently marketed drugs [11,12].

G proteins are organized in 4 different families (G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$) and comprise a total of 16 distinct subfamilies based on their Gα subunits [13]. Although it was commonly believed that every GPCR couples to a specific G protein subfamily, an overwhelming amount of evidence has now unequivocally shown that GPCRs are promiscuous signaling proteins in such that they can activate G proteins from multiple families. Upon activation, GPCRs are desensitized through phosphorylation by G protein-coupled receptor kinases (GRKs) and subsequent binding of β-arrestin, ultimately leading to receptor internalization [14]. Besides its role in GPCR internalization, β-arrestin can act as a scaffold for further signaling proteins such as kinases and phosphodiesterases among others [15]. Moreover, in recent years it has been appreciated that β-arrestin can bind to GPCRs in at least two distinct conformations, a 'tail' conformation favoring interactions with the phosphorylated C terminus of the receptor and a 'core' conformation comprising strong interactions with the receptor's transmembrane core [16–18]. To add further complexity, the distinct GPCR–arrestin complexes appear to exert distinct cellular functions [19–22].

Importantly, numerous GPCR ligands have been described that are able to preferentially activate one signaling pathway over others, for example, G protein signaling over β-arrestin recruitment or, more subtly nuanced, one G protein subfamily (e.g., $G\alpha_s$) over another (e.g., $G\alpha_{i/o}$) [14,23–25]. These ligands are commonly referred to as *biased ligands*. From a therapeutic point of view, this may be highly valuable as it has been suggested that some signaling pathways downstream of a particular GPCR may mediate therapeutically desired effects while others may be responsible for drug adverse effects. Due to their potential of presenting drugs with no or fewer side effects, biased ligands have emerged as a highly sought-after class of future GPCR drugs [12,26]. However, the structural basis of how biased ligand-mediated GPCR activation results in preferential coupling to a subset of signaling proteins at the expense of others is poorly understood. Along this line, for most diseases the signaling pathways responsible for drug adverse effects are yet not known. Therefore, it is currently extremely challenging to design biased ligands for GPCRs [27], a fact that is also mirrored by the very small number of marketed biased ligands.

Here, we review the biochemical, structural, biophysical, and computational evidence of allosteric coupling as the fundamental mechanism of GPCR activation. Special attention will be given to how biased ligands may selectively impact the allosteric coupling mechanism and how this may eventually lead to biased signaling.

Structural basis of GPCR activation

The last decade has witnessed a revolution in structural biology of GPCRs. To date, almost 500 structures have been solved including receptors in the apo state, receptors bound to ligands of diverse pharmacology (full agonists, partial agonists, antagonists, inverse agonists, and allosteric modulators), and, most importantly, dozens of ternary complexes of receptor, agonist, and G protein, the latter visualizing the essential GPCR signaling unit at atomic resolution. From this wealth of structural data, a common activation mechanism of the GPCR superfamily has been deduced and is covered in great detail by excellent recent reviews [3–5]. In brief, agonist binding to the extracellular part of the receptor stabilizes large-scale conformational changes through the receptor's transmembrane core that ultimately lead to binding of a G protein at the intracellular surface of the receptor. Importantly, the binding sites for the agonist and the G protein do not overlap, but are structurally linked via conformational

coupling, which is defined as *allosteric coupling*. Agonist binding leads to contraction of the ligand binding pocket and to conformational changes of the (mostly) conserved $P^{5.50}$ – $I^{3.40}$ – $F^{6.44}$ motif at the bottom of the binding pocket (numbering according to the Ballesteros-Weinstein nomenclature). This results in conformational changes in the conserved *toggle-switch* epitope $W^{6.48}$ causing a large outward swing of the inner half of transmembrane domain 6 (TM6) (Fig. 1). The intracellular outward movement of TM6 allows binding of the C-terminal part of the Gα subunit. Breaking of a salt-bride between $D(E)^{3.49}$ and $R^{3.50}$ upon receptor activation leads to rearrangement of the conserved $D(E)^{3.49}$ – $R^{3.50}$ – $Y^{3.51}$ motif stabilizing G protein binding particularly through a direct interaction of $R^{3.50}$ with the G protein. Moreover, the active G protein-coupled receptor state is stabilized by a rearrangement of the conserved $N^{7.49}$ – $P^{7.50}$ – x – x– $Y^{7.53}$ motif (Fig. 1). In contrast to these *conserved activation hot spots*, much less is known about how biased ligands may influence this process. This is mainly due to the paucity of biased ligand-bound GPCR structures in complex with two different signal transducers. Moreover, rather little is known about the dynamics of how conformational changes are relayed from the ligand-binding pocket to the intracellular G protein-binding site in general.

Allosteric coupling

Activation of GPCRs upon agonist binding is a classical allosteric process in which conformational changes in the extracellular ligand-binding pocket are allosterically linked to conformational changes in the intracellular transducer (G protein or β-arrestin)-binding interface. Already in 1976, Alfred G. Gilman and Robert J. Lefkowitz independently published two seminal papers in which they demonstrated that the affinity of agonists to β-adrenergic receptors is highly sensitive to the presence of guanine nucleotides [28,29]. Specifically, using equilibrium binding experiments where β-adrenergic receptors were labeled with the radioactive antagonists [^{125}I]iodohydroxybenzylpindolol [28,30] or [^{3}H]dihydroalprenolol [29], it was shown that agonist-binding curves were shifted significantly to the right in the presence of high concentrations of GTP, GDP, or guanyl-5'-yl imidodiphosphate, revealing a significant decrease in agonist affinity. Most importantly, this effect was exclusive to agonists as binding curves of antagonists were unaffected by the presence of guanine nucleotides. It was later shown that agonist competition binding curves are biphasic and characterized by two fractions representing high and low affinities of the agonist [31]. Addition of guanine nucleotides results in monophasic agonist competition curves due to elimination of the high-affinity fraction. These data have demonstrated that the high-affinity fraction of agonist binding corresponds to a G protein-bound state of receptors and, in fact, both the ratio of high to low agonist affinity and the size of the high-affinity fraction correlate well with agonist efficacy [31]. Most importantly, these biochemical studies have been formalized in quantitative terms and led to the development of the *ternary complex model* [32]. It describes conceptually

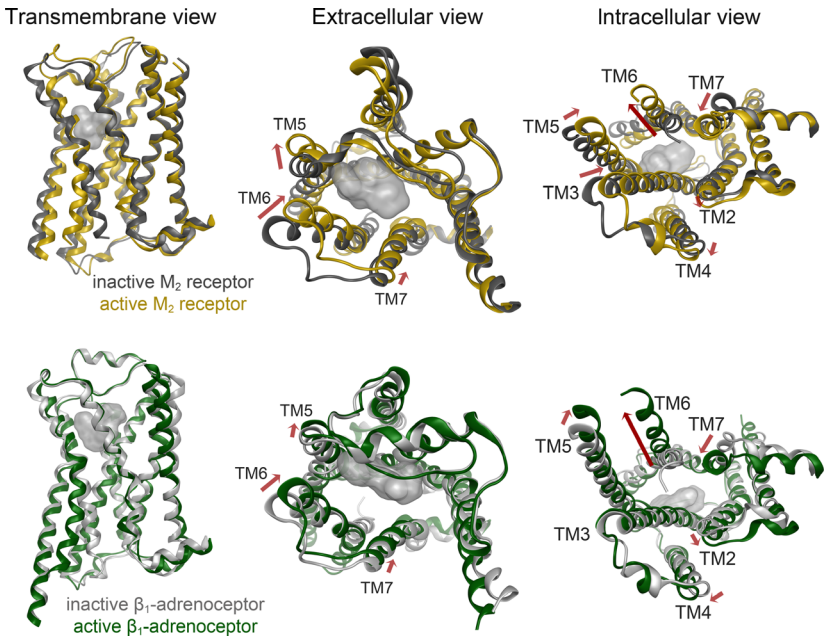

Fig. 1. Inactive and active GPCR crystal structures unveil common conformational changes upon receptor activation. The upper row shows a superimposition of the active (gold) and the inactive (dark gray) muscarinic M_2 receptor co-crystallized with the agonist iperoxo (PDB entry: 4MQS) [48] and QNB (PDB entry: 3UON) [47], respectively. The row below shows a superimposition of the active (green) and the inactive (light gray) β_1-adrenergic receptor (PDB entries: 2Y03 and 2YCW, respectively)[115,116]. While the largest conformational change at the extracellular side is depicted as an inward movement of TM6, the reciprocal effect (large outward movement of TM6) can be observed at the intracellular side.

and also quantitatively the allosteric coupling mechanism of GPCR activation in biochemical terms: Agonist binding favors G protein coupling and G protein coupling to the receptor increases agonist, but not antagonist, affinity. Based on the discovery of spontaneous activity of GPCRs, this model was later extended [33]. After the initial biochemical description of allosteric coupling at β-adrenergic receptors, increased agonist affinity in the presence of G proteins has been demonstrated at muscarinic [34], opioid [35], adenosine [36], and α-adrenergic receptors [37] and is now believed to be a common hallmark of all GPCRs.

Of note, ternary complex formation is not limited to G proteins. After the discovery of β-arrestin [38], it was shown that β-arrestin binding to GPCRs does also increase agonist affinity indicative of formation of an agonist/receptor/β-arrestin ternary complex analogous to the one observed with G proteins [39–42]. In quantitative terms, β-arrestin coupling to GPCRs has been formalized in the so-called *alternative ternary complex model* [39]. More recently, by comparing wild-type angiotensin receptors with angiotensin receptors fused to either $G\alpha_q$ or β-arrestin2, it has been demonstrated that competition binding curves of biased ligands (either $G\alpha_q$ over β-arrestin2 or *vice versa*) at either fusion proteins were left-shifted to a different extent in comparison with wild-type receptors [43]. For instance, competition binding curves of G protein-biased ligands (e.g., TRV055 and TRV056) at AT_1R-G_q receptors were much greater shifted to the left in comparison with AT_1R than competition curves at AT_1R-βarr2 receptors. In contrast, competition binding curves of βarr2-biased ligands (e.g., TRV023 and TRV026) were more prominently left-shifted at AT_1R-βarr2 receptors [43]. These data led to the hypothesis that the molecular nature of biased ligands may lie in preferential stabilization of G protein-stabilized ternary complexes over βarr2-stabilized ternary complexes or *vice versa*, thereby establishing the first biochemical link between divergent (i.e., transducer-specific) allosteric coupling and biased agonism.

Collectively, the biochemical evidence of more than 40 years has unequivocally shown that GPCR activation is an allosteric process. Of note, there is initial evidence that allosteric coupling may be dependent on the type of ligand and the type of signaling protein, suggesting that it may be possible to harness this process with designed ligands to ultimately control preferential transducer coupling and biased signaling.

Structural basis of allosteric coupling

Despite the detailed biochemical understanding of allosteric coupling, the structural basis of this fundamental process has remained enigmatic until recently. Using purified receptors (specifically β_2-adrenergic, muscarinic M_2, and μ-opioid receptors) and G proteins (or G protein mimetic nanobodies) reconstituted in high-density lipoprotein particles, it was revealed that binding of nucleotide-free G proteins to receptors severely impairs both the association and the dissociation of ligands to the extracellular binding pocket in a concentration-dependent manner [44]. Mapping these pharmacological data on the available crystal structures of the three receptors elucidated the molecular mechanism of allosteric coupling: Binding of a G protein to the intracellular receptor surface leads to closure of the extracellular ligand-binding pocket, virtually trapping the ligand in its binding site and preventing further ligand access to the binding site from the extracellular space [44]. In general, conformational changes in the extracellular loops 2 and 3 and the extracellular parts of TM5, TM6, and TM7 of the receptors appear to be responsible for forming a lid-like structure over the ligand-binding pocket [3] (Fig. 1). More specifically, in β-adrenergic [45,46] and muscarinic receptors [47,48] two aromatic residues in the ECL2 and upper part of TM7 move closer to one another. It should be noted that in muscarinic receptors, these aromatic residues form parts of the common allosteric-binding site and that their rearrangement during receptor activation allows designing allosteric modulators that favor binding to either the inactive or active states of the receptor [49,50]. Closure of the ligand-binding pocket hence provides the structural basis of the observed increase in agonist affinity in the presence of G proteins and is likely to be a conceptually common mechanism for allosteric coupling during GPCR activation [44,51].

Although there is detailed knowledge about the specific structural changes within both the ligand and the transducer-binding sites, much less is known about the structural dynamics of the conformational coupling of these regions. In particular, it is largely unknown how structural changes in the ligand-binding pocket are precisely relayed through the receptor core to the transducer-binding site and how this may be influenced by different ligands and different transducers.

As crystallography only provides extreme snapshots of GPCR activation, cryo-EM and other biophysical techniques such as nuclear magnetic resonance (NMR) spectroscopy, double electron–electron resonance (DEER) spectroscopy, and single-molecule Foerster resonance energy transfer (smFRET) have become invaluable to shed light on structural dynamics of allosteric coupling. As a representative example of the power of such biophysical methods, NMR

spectroscopy of multiple GPCRs equipped with different labels (^{13}C, ^{15}N, or ^{19}F) has uniformly demonstrated that allosteric coupling between the ligand-binding pocket and the G protein-binding site is loose [52–54]. Two key aspects can be extracted from these studies: First, agonist binding stabilizes an ensemble of different receptor conformations which are in equilibrium with each other and are able to interconvert, and second, the fully active state of the receptor is only reached upon addition of the G protein or a G protein mimicking nanobody [52–61]. The latter notion has also been confirmed by using DEER [52] or single-molecule FRET techniques [62]. Overall, this indicates that agonist binding alone sort of *primes* the receptor for subsequent engagement with a variety of intracellular transducers. However, the major structural changes observed at both the intracellular G protein-binding site and the extracellular closure of the ligand-binding pocket inevitably require the engagement of the G protein.

The residues that get labeled for NMR spectroscopy experiments are often predetermined by the amino acid sequence of the receptor under study (e.g., with ^{13}C-methionine, all methionine residues will be labeled) and it is possible to assign specific conformational changes to specific residues that are scattered all over the receptor protein. Therefore, NMR spectroscopy has the advantage to simultaneously sample structural changes at multiple locations all over the receptor protein due to labeling at multiple sites. This allows extracting much more dynamic information of the activation process of receptors. Most of the labeled residues in NMR spectroscopy are located in the vicinity of some conserved activation hot spots (e.g., the PIF, D(E)RY, or NPxxY motifs) so that NMR spectroscopy can provide direct evidence of the structural dynamics of the allosteric networks connecting the ligand-binding pocket and the intracellular transducer-binding interface.

Moreover, NMR spectroscopy allows to straightforwardly assess the impact of different ligands on allosteric coupling between the two opposite binding sites. By comparing conformational dynamics of specific residues in response to different ligands, it has been demonstrated that different ligands stabilize distinct receptor conformations [52–60]. Based on this, it is also likely that the allosteric pathways that link conformational changes in the ligand-binding pocket to the intracellular receptor site may be distinct and ligand-dependent [57,59,63].

To better elucidate the nature of those allosteric pathways, computational approaches have proven powerful tools to study allosteric communication pipelines upon receptor activation on a much larger scale. In general, a whole set of different ligand–receptor complexes in inactive and active states form the basis of structural data for computational analyses [64–67]. Diverse methods such as evolutionary trace analysis [68–71], phylogenetic analysis [65,66], or molecular dynamic simulations and subsequent analysis of residues contacts or torsion angles [72–75] have been applied to help elucidate the structural dynamics of allosteric coupling. Overarchingly, computational studies have discovered allosteric communication pipelines or allosteric networks, that is, residue contacts connecting the extracellular domains with the intracellular domains of the receptor (Fig. 2). Through concerted or subsequent motions of several connected residues, conformational changes triggered by the ligand can be relayed from the extracellular binding pocket throughout the receptor core to the intracellular G protein-binding interface. It is crucial to note that upon analysis of hundreds of receptor structures, it has become evident that presumably all class A GPCRs share a so-called common activation pathway that is characterized by conformational changes in conserved residues, motifs, and switches that ultimately converge at the G protein-binding site [64,65,67] (Fig. 2). However, the precise allosteric pathway is different at different receptors which provides the molecular basis that allows the diverse superfamily of GPCRs to become activated by ligands of exceptionally diverse chemical scaffolds while maintaining the common ability to activate the much smaller class of G proteins. Moreover, recent computational analysis of receptor structures in complex with biased ligands has suggested that allosteric communication pipelines toward the intracellular G protein or β-arrestin interfaces may be distinct highlighting the potential of harnessing specific allosteric communication pipelines for biased ligand design [75].

In summary, structural, biophysical, and computational studies have revealed key aspects of the structural dynamics of allosteric coupling during GPCR activation and have illustrated that the exact allosteric communication pipelines toward transducer-binding interfaces will likely be ligand-dependent.

How does allosteric coupling relate to biased agonism?

Biased agonists are endowed with the ability to preferentially couple to one transducer protein over another, thereby promoting preferential signaling through a limited subset of all possible pathways downstream of a specific GPCR. On the structural level, this

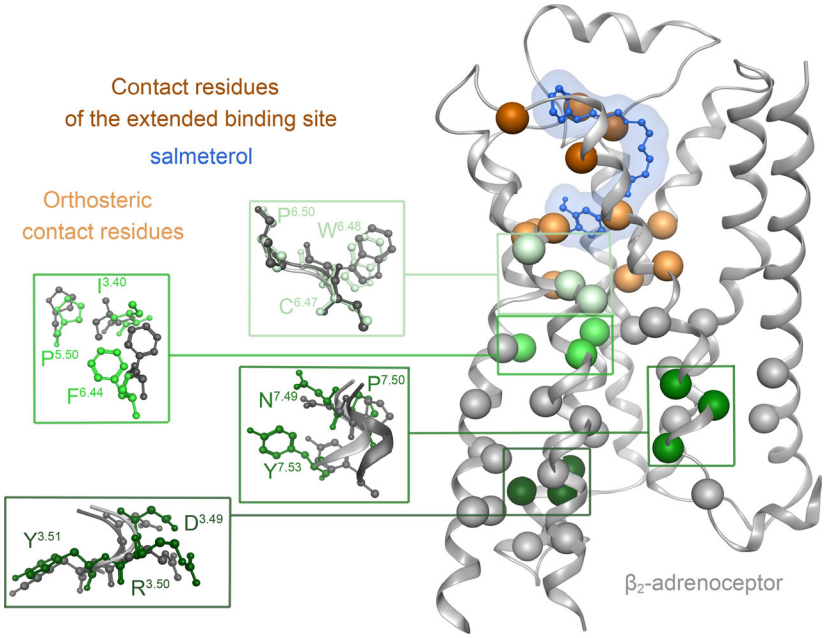

Fig. 2. A common allosteric network connects the extracellular ligand-binding pocket with the intracellular transducer-binding site. As an example, the salmeterol-bound crystal structure of the β_2 adrenergic receptor (PDB entry: 6MXT)[103] was used to map contact residues of the extracellular domains of the binding pocket (dark orange) and the classical orthosteric-binding site (light orange). The chemically encoded ligand information can be transferred to the intracellular side via a broad allosteric network consisting of some highly conserved structural motives (different shades of green) and more receptor-specific triggers (shown as gray spheres) [67].

phenomenon is hardly understood, mostly due to the lack of receptor structures of a biased ligand in complex with two different transducer proteins (e.g., G protein and β-arrestin, using the exact same receptor construct for all structure determinations). Nevertheless, a plausible explanation for preferential signaling pathway activation is that the ligand–receptor structure in complex with one transducer will be different from the structure of a complex with another transducer. Differential transducer coupling should be reasonable to assume because GPCR activation, as outlined above, is a prime allosteric process that should follow the hallmarks of allostery, that is, reciprocity and probe dependence. The reciprocity of allosteric coupling during GPCR activation has been clearly demonstrated on multiple levels. In biochemical terms, agonist binding stimulates G protein coupling, and reciprocally, G protein binding increases agonist affinity. In structural terms, agonist binding promotes an intracellular outward movement of TM6 facilitating G protein binding, and reciprocally, G protein coupling leads to closure of the extracellular ligand binding pocket. However, much less is known about the structural dynamics of probe dependency. Theoretically, it is legitimate to hypothesize that biased agonists promote preferential transducer coupling by preferential (i.e., more energetically favorable) formation or stabilization of one transducer-specific receptor complex over another. Based on the allosteric ternary complex model and by defining the transducer proteins as allosteric probes, it should directly follow that binding of one transducer (e.g., G protein) will exert a different effect on ligand–receptor interactions than binding of another transducer (e.g., β-arrestin) to the same receptor. In other words, the cooperativity between agonist and transducer in the receptor G protein complex should be different from their cooperativity in the β-arrestin complex.

An important question is how this diverging cooperativity may be reflected in structural terms, that is, how binding of different intracellular transducers would affect the conformation of the ligand-binding pocket. As outlined above, it has been elegantly shown that G protein binding closes off the ligand-binding pocket from the extracellular space. However, it has remained enigmatic whether binding of one receptor to G proteins from different families results in divergent conformational changes (e.g., different degrees of closure) of the ligand-binding pocket. Further, it has been unknown whether receptor coupling to β-arrestin leads to distinct conformations of the ligand-binding pocket different from the ones induced by G protein binding to the same receptor.

Recently, initial structural evidence has been provided that sheds light on these questions. High-resolution structures of three different receptors have been obtained each in complex with two different transducers: The neurotensin receptor 1 has been solved in complex with a G_i protein and β-arrestin1 [76,77], the β-adrenergic receptor has been solved in complex with the G_s-protein mimetic nanobody Nb80 [78], a G_s protein [79], and β-arrestin1 [78], and structures of the

muscarinic M_2 receptor have been obtained in complex with G_o and β-arrestin1 [80,81]. However, only for the muscarinic M_2 receptor and the β_1-adrenergic receptor structures in complex with two distinct transducers have been solved bound to the same agonist: The iperoxo-bound M_2 receptor has been solved in complex with G_o [80] and β-arrestin1 [81], and the structures of formoterol-bound β_1-adrenergic receptor in complex with the G_s-protein mimetic nanobody Nb80 and β-arrestin1 have been obtained [78]. Although, in both cases, the receptor constructs and the overall conditions for structure determination (detergent micelles *vs* phospholipid bilayers) are slightly different and nanobodies have some limitations [82], the structures provide invaluable insight into how the type of intracellular signaling protein changes the conformation of the extracellular ligand binding pocket. Interestingly, comparing the G protein-bound and β-arrestin-bound structures of the two receptors clearly reveals major differences in the extracellular parts of the ligand-binding pockets (Fig. 3). While G-protein coupling to both receptors already leads to a closure of the ligand-binding pocket, binding of β-arrestin appears to result in further conformational changes in the extracellular receptor domains. Specifically, in both receptors, there is an even more pronounced closure of the ligand-binding pocket mediated by further inward movements of the extracellular parts of TM6 and TM7 concomitantly leading to conformational changes in ECL3. Moreover, conformational changes in the upper parts of TM5, ECL1, and ECL2 contribute to the distinct overall structure of the binding pocket in comparison with the G protein (mimetic)-bound structures.

These studies provide direct evidence that allosteric coupling between the ligand and transducer-binding sites indeed depends on the type of transducer. However, it should be noted that the biochemical impact of these structural differences in the ligand-binding pocket has not yet been studied and it is not clear whether coupling to β-arrestin results in more severely restricted ligand access to the binding site. Nevertheless, the overall divergent conformations of the binding pockets of the receptors when in complex with G protein or β-arrestin provide an opportunity to design ligands that may take advantage of these divergent structural changes in such that it leads to preferential downstream signaling.

Biased agonists and allosteric coupling

Comparison of the G protein and β-arrestin complexes of M_2R and β_1-AR demonstrates structural changes in the overall binding pocket, in particular major conformational differences in the most extracellular parts of the binding pocket including the extracellular loops. Based on these structural data, we have surveyed the literature for recently reported biased ligands that have a so-called *extended binding mode*, that is, ligands with a molecular structure that spans the entire ligand-binding pocket including the upper parts of transmembrane helices and extracellular loops. Interestingly, there are a number of such biased ligands for several GPCRs for which it was reported that the molecular basis for their observed bias i the ligands' extensive contacts with the extracellular parts of the binding pocket.

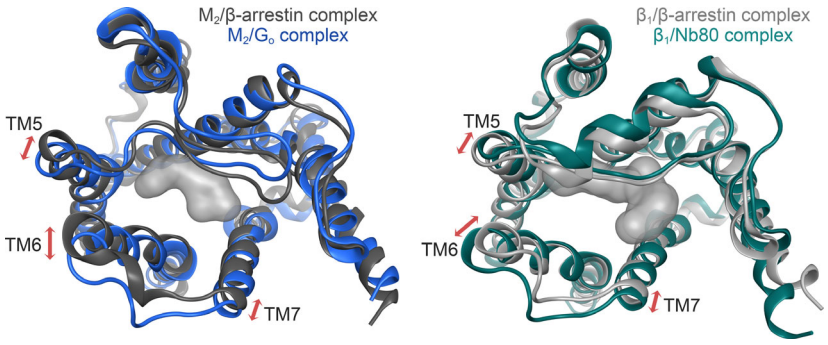

Fig. 3. Transducer-specific conformations of the extracellular ligandbinding pocket. Structures of two given ligand/receptor complexes coupled to two different intracellular transducers allow extracting and comparing transducer-specific allosteric coupling. For both receptor pairs, similar conformational changes occur when comparing differences between M_2 receptors (PDB entries: 6U1N and 6OIK) [80,81] and β_1-adrenoceptors (PDB entries 6TKO and 6IBL) [78] both bound to a G protein (or nanobody) and β-arrestin. Interestingly, the major conformational differences between the two structural overlays occur in the most extracellular parts of the ligand binding pocket.

Dopamine receptors

The dopamine D2 receptor is a major mediator of dopamine signaling in the central nervous system and serves as an important drug target for antipsychotics, drugs for Parkinson's disease, and antiemetics. D2 receptors couple to $G\alpha_{i/o}$ proteins but can also recruit β-arrestin. The structure of an active D2 receptor/G_i complex bound to bromocriptine [83], a drug for Parkinson's disease, has been solved and reveals a bitopic binding mode of bromocriptine where parts of the ligand form extensive contacts with residues of the extracellular loops of the D2 receptor including I184 in ECL2 (Fig. 4). Interestingly, bromocriptine has been shown to be a β-arrestin-biased agonist at D2 receptors [84]. In line with this, compound 2 [85], a congener of the drug aripiprazole [86,87] and a β-arrestin-biased agonist, interacts extensively with the extracellular domain including I184 as well (Fig. 4). The idea is that by strong ligand interactions with ECL2, compound 2 preferentially couples to β-arrestin [85]. Moreover, compound 2 shows less interactions with $S193^{5.42}$ at the bottom of the binding pocket which is an important trigger of $G\alpha_i$-protein coupling. MLS1547 is a $G_{i/o}$-biased ligand [88,89] and shows also strong interactions with a hydrophobic pocket in the extracellular domains comprising I184 and $F189^{5.38}$ at the extracellular part of TM5. In contrast to the two β-arrestin-biased ligands, the interaction of MLS1547 with $F189^{5.38}$ has been suggested to be responsible for impaired β-arrestin recruitment. Taken together, three biased ligands of diverse chemical scaffolds share a uniform mechanism of *preferential allosteric coupling*. Through strong interactions with

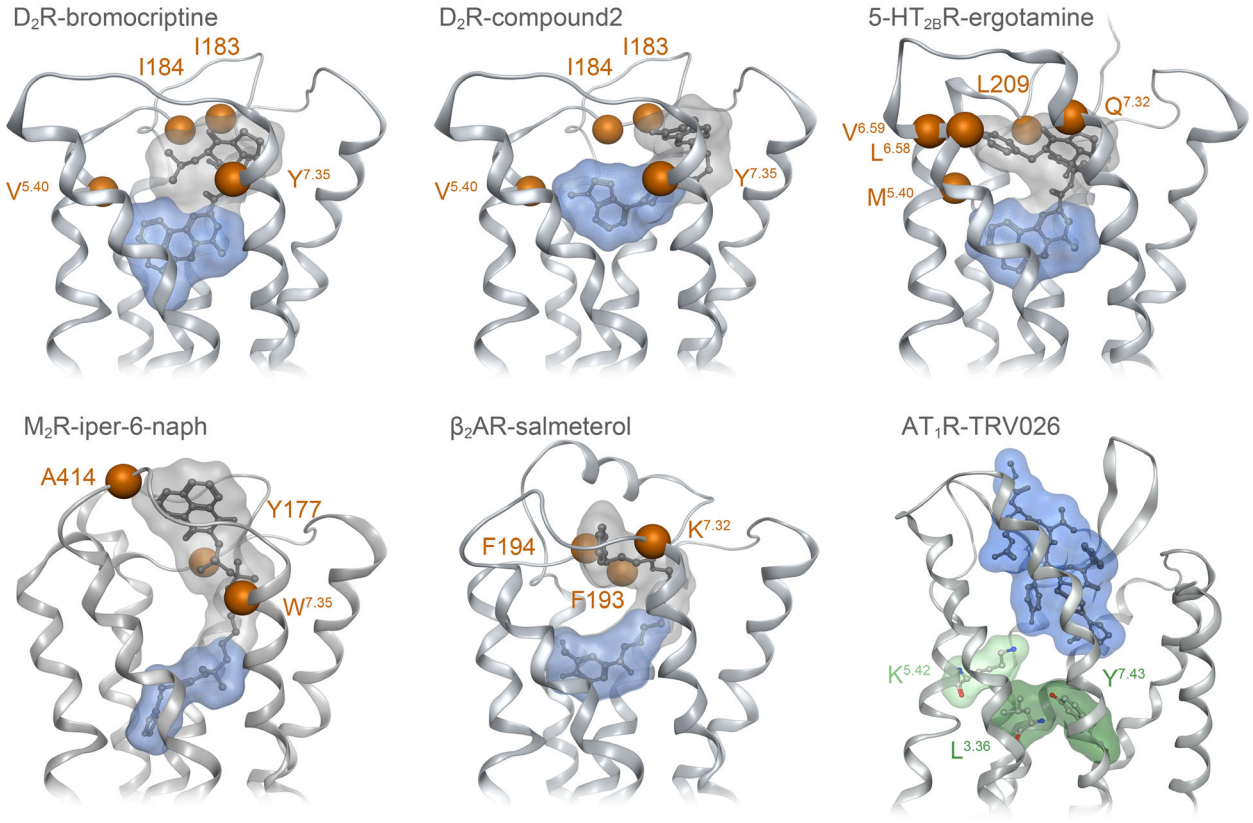

Fig. 4. Extended binding modes of biased ligands as structural hallmark for biased signaling. The examples for biogenic amine receptors include the dopamine D*2* receptor co-crystallized with the β-arrestin-biased drug bromocriptine (PDB entry: 6VMS)[83] or with a docked β-arrestin-biased compound 2 (according to [85]), the 5-HT_{2B} receptor co-crystallized with the β-arrestin-biased ergotamine (PDB entry: 4IB4) [90], the muscarinic M_2 receptor with the docked $G\alpha_i$-biased bitopic agonist iper-6-naph [51,113], and the β_2 adrenergic receptor co-crystallized with the $G\alpha_s$-protein-biased asthma therapeutic salmeterol (PDB entry: 6VMS)[103]. The blue and the gray surfaces indicate the classical orthosteric and extended molecule parts, respectively. Receptor-ligand contacts with extracellular domains of the binding pocket are highlighted in orange. A complementary way to interfere with allosteric coupling can be observed in the AT_1 receptor co-crystallized with the β-arrestin-biased peptide TRV026 (PDB entry: 6OS2)[98].

residues in the extracellular parts of the ligand-binding pocket, all three ligands trigger a distinct allosteric pathway from the extracellular domain preferentially leading to binding of only one transducer. In fact, for MLS1547 it has been shown that the interaction with F189$^{5.38}$ favors a distinct conformation of ICL2 that impairs β-arrestin coupling [89]. Thus, interaction with extracellular residues appears as trigger for biased signaling.

Serotonin receptors

Serotonin modulates a variety of physiological functions in humans, for example, in the cardiovascular system, the gastrointestinal tract, and the central nervous system. Serotonin receptor antagonists are important drugs in the treatment of nausea, migraine and other diseases. Moreover, serotonin receptors in the brain mediate hallucinogenic effects of psychedelic drugs such as LSD. LSD and ergotamine are two serotonin receptor agonists that have been crystallized in complex with the 5-HT$_{2B}$ receptor [90–92]. The 5-HT$_{2B}$ receptor couples preferentially to G$\alpha_{q/11}$ proteins bun can also recruit β-arrestin. Interestingly, ergotamine and LSD are both β-arrestin-biased ligands. The structures reveal an extended binding mode for both ligands and, in particular ergotamine, show direct interactions with extracellular parts of TM6 (L347$^{6.58}$, V348$^{6.59}$), TM7 (Q359$^{7.32}$), and ECL2 (L209) (Fig. 4). It has been suggested that the enhanced interactions of the ligands with the extracellular parts of the binding pocket are responsible for the observed β-arrestin bias.

Angiotensin receptors

AT$_1$ receptors mediate the majority of physiological functions of angiotensin in humans such as vasoconstriction. AT$_1$ receptor antagonists (e.g., losartan) serve as well-established drugs for the treatment of hypertension and heart failure. In recent years, there has been a lot of interest in β-arrestin-biased AT$_1$ receptor agonists as potential drugs for heart failure because studies have shown their antihypertensive effects to be comparable with angiotensin antagonists while β-arrestin recruitment appears to improve cardiac function [93,94]. Very recently, β-arrestin-biased AT$_1$ agonists have been discussed as potential drugs for COVID-19 [95]. Besides their therapeutic importance, AT$_1$ receptors are paradigmatic for studying the molecular nature of biased agonism. Numerous G$\alpha_{q/11}$ (e.g., TRV055 and TRV056) and β-arrestin-biased agonists (e.g., TRV023 TRV026) have been reported [43] and the structural basis and dynamics of their biased agonism have been elucidated recently [96–99]. A key structural element of all biased angiotensin analogues is the nature of the amino acid at position 8 which binds deep in the orthosteric pocket (a phenylalanine in angiotensin II, the endogenous agonist). G$\alpha_{q/11}$-biased agonists have a bulky amino acid at position 8 (e.g., TRV055 and TRV056) whereas β-arrestin-biased agonist contain a small amino acid (e.g., alanine in TRV026) or lack the eighth amino acid entirely (e.g., TRV023). Crystal structures and biophysical experiments have revealed that a bulky amino acid in position 8 of the angiotensin peptide is necessary to promote a rotamer shift of L112$^{3.36}$ at the bottom of the binding pocket that triggers a stronger outward swing of the lower parts of TM6 resulting in strong G$_{q/11}$ coupling (Fig. 4). In contrast, the lack of a bulky amino acid in position 8 impairs the outward movement of TM6 which forms the structural basis for β-arrestin-biased agonism at AT$_1$ receptors (Fig. 4). Of note, although angiotensin peptides are large molecules that span the entire ligand-binding pocket including extracellular receptor domains, the observed biased agonism in either direction (G$_{q/11}$ or β-arrestin) appears to stem entirely from different ligand interactions with residues at the bottom of the ligand-binding pocket such as L112$^{3.36}$. This interaction influences the conformation of W253$^{6.48}$ which is an important residue within the allosteric network coupling the ligand-binding pocket to the intracellular G$\alpha_{q/11}$ protein-binding site (Fig. 4). Thus, bulky amino acids at position 8 in angiotensin peptides promote *preferential allosteric coupling* to the G$\alpha_{q/11}$ protein-binding interface.

β-adrenergic receptors

β_1- and β_2-adrenergic receptors mediate essential physiological functions of the autonomic nervous system such as controlling heart rate and respiration. β_1-AR blockers form a fundamental class of drugs in the treatment of a variety of cardiovascular diseases, and β_2-AR agonists are pivotal in the treatment of bronchial asthma and chronic obstructive pulmonary disease. Both receptors have recently been crystallized with biased agonists which provides insights into the structural basis of biased signaling. The structures of the β_1-adrenergic receptor in complex with Nb80 (a Gα_s-protein mimetic) and β-arrestin both bound to formoterol, a β-arrestin-biased agonist [100], have been solved [78]. Formoterol displays an extended binding mode showing interactions with receptor residues of the upper parts of TM6 and ECL2 (Fig. 3). Comparison of the two structures has revealed that the β-

arrestin bias of formoterol may be due to differences at two interaction hot spots. There are less interactions of formoterol with the receptor in the deep orthosteric binding pocket which may impair a strong outward swing of the lower parts of TM6 and, thus, impair $G\alpha_s$-protein coupling. In stark contrast, formoterol displays much more interactions with residues in the extracellular parts of the binding pocket such as the upper parts of TM6 (Fig. 3). Of note, ligand interactions with extracellular residues have also been observed in the β_1-AR crystal structures bound to the β-arrestin-biased agonists bucindolol and carvedilol [101].

The β_2-adrenergic receptor has been crystallized with the $G\alpha_s$-biased partial agonist salmeterol [102,103], a well-known drug for the treatment of bronchial asthma. Salmeterol shows an extended binding mode comprising strong interactions with extracellular domains of the receptor (Fig. 4). Although salmeterol's bias relative to epinephrine has been suggested to result from a different hydrogen-bond network involving Ser204$^{5.43}$ and Asn293$^{6.55}$ deep in the orthosteric binding pocket, it shall be noted that salmeterol appears more β-arrestin biased than salbutamol (which is much smaller in size) [103], a pharmacological observation that may be due to salmeterol's interaction with extracellular receptor domains. Along this line, it has been shown that (R,R′)-fenoterol preferentially activates $G\alpha_s$-protein over $G\alpha_{i/o}$-protein signaling at β_2-adrenergic receptors. Interestingly, this biased agonism has been attributed to (R,R′)-fenoterol's interaction with Tyr308$^{7.35}$, a conserved residue in the extracellular part of the ligand-binding pocket [104].

Muscarinic acetylcholine receptors

Muscarinic receptors play a crucial function in the autonomous nervous system by mediating all parasympathetic effects such as controlling cardiac function, secretion of glands, and several processes in the central nervous system involved in memory and learning [49]. In line with this, muscarinic receptors serve as important drug targets for the treatment of a variety of diseases including Alzheimer's disease and chronic obstructive pulmonary disease. Besides their pivotal role in physiology and disease, muscarinic receptors are the paradigmatic receptors for allosteric modulation [105]. All 5 receptor subtypes possess a so-called common allosteric-binding site located on top of the orthosteric-binding pocket for acetylcholine. This site can be targeted with a huge variety of small molecules and peptides [48,106]. Bitopic ligands, that is, ligands that bind to the orthosteric and allosteric-binding site simultaneously, have first been described for muscarinic receptors and have been shown to have an extended binding mode [107–112]. This is similar to the binding mode of biased ligands at dopamine, serotonin, and β-adrenergic receptors (Fig. 4). In fact, bitopic ligands at muscarinic M_2 receptors have been shown to be $G\alpha_{i/o}$-biased agonists and fail to recruit β-arrestin [109,113]. Iper-6-naph, a well-characterized bitopic ligand, spans the entire ligand-binding pocket including residue contacts with extracellular receptor domains (Fig. 4). We have suggested that, due to its extended binding mode, iper-6-naph hampers the closure of the ligand-binding pocket [51,109,113]. While this is a structural hallmark during allosteric coupling, iper-6-naph binding to M_2 receptors appears to affect receptor coupling to β-arrestin more severely than $G\alpha_{i/o}$-protein coupling, providing a structural explanation for the observed biased agonism. Indeed, the recent cryo-EM structure of the M_2R/β-arrestin complex [81] shows an even more contracted binding pocket (Fig. 3) rationalizing why bitopic ligands fail to recruit β-arrestin at this receptor [109].

Recently, we have applied a similar strategy to muscarinic M_1 receptors [114]. Using a set of bitopic agonists, it was shown that progressive interference with binding pocket closure reduces the number of G protein subfamilies that can couple to the receptor. This is in line with the notion that ligand interactions with extracellular receptor domains may promote *preferential allosteric coupling* to selected transducers.

Conclusion

Allosteric coupling is the fundamental mechanism of GPCR activation and describes how these membrane receptors relay structural changes upon extracellular agonist binding through the transmembrane core to intracellular coupling of a variety of transducers such as G proteins and β-arrestin. Although this mechanism has been studied with biochemical and pharmacological methods for decades, only recently the structural basis for allosteric coupling has been elucidated. Binding of intracellular transducer proteins to GPCRs is conformationally coupled to pronounced structural changes in the extracellular domains of the ligand-binding pocket, resulting in closure of the binding pocket toward the extracellular space. The reciprocal structural changes in the binding pocket appear to be transducer-specific, suggesting that it may be possible to design biased ligand with desired signaling profiles. It should be noted that the most dramatic structural changes occur in the extracellular parts of the binding pocket such as the upper parts of transmembrane helices and extracellular loops. Therefore,

ligands with extended binding modes that span the entire binding pocket are likely to be promising candidates for biased signaling. In fact, several biased ligands with extended binding modes have been described for a variety of receptors. For many of these ligands, it has been demonstrated that their bias results from ligand interactions with the extracellular domains of the receptor. For others, distinct ligand–receptor interactions deep inside the orthosteric binding site are more likely to be responsible for the observed ligand bias. A mixture of both mechanisms is also likely to occur. However, despite the varying molecular flavors of ligand bias, we propose that it is likely that all biased ligands described above share a conceptually common mechanism of biased agonism. Based on the available data, we hypothesize that biased ligands use ligand-specific triggers within the binding pocket that initiate a *preferential allosteric pathway* to only one intracellular transducer-binding site. For some receptors, these molecular triggers may be located in the extracellular parts of the ligand-binding pocket (e.g., for biogenic amine receptors), whereas for other receptors, these triggers reside at the bottom of the ligand-binding pocket (e.g., for angiotensin receptors). Nevertheless, the initiation of *preferential allosteric coupling* with one transducer-binding site appears to be a common hallmark of all biased ligands at class A GPCRs. A deeper structural and pharmacological understanding of allosteric networks will likely boost the rational design of biased ligands as a new generation of GPCR drugs.

Author contributions

AB developed the scope of this review with input from MB. AB and MB designed figures. MB prepared figures. AB wrote the manuscript with edits from MB.

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Conflict of interest

The authors declare no conflict of interest.

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