Functional Selectivity in GPCR Signaling: Understanding the Full Spectrum of Receptor Conformations

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Abstract: The great versatility of G protein-coupled receptors (GPCRs), in terms of both their ability to bind different types of ligands and initiate a large number of distinct cellular signaling events, remains incompletely understood. In recent years, the classical view of the nature and consequences of ligand binding to GPCRs has dramatically changed. The notion of functional selectivity, achieved through both biased ligands and allosteric modulators, has brought substantial new insight into our comprehension of the pluridimensionality of signaling achieved by GPCRs. Moreover, receptor heterodimerization adds another important dimension to the diversity of cellular responses controlled by GPCRs. Here, we review these considerations and discuss how they will impact the design of improved therapeutics.

Keywords: GPCR, ligand-directed signaling, functional selectivity, biased ligand, allosterism, dimer.

1. INTRODUCTION

G protein-coupled receptors (GPCRs) are the largest and the most diverse family of cell surface receptors, representing approximately 2% of the human genome [1]. Thus, not surprisingly, GPCRs are involved in regulating, in one way or another, almost all physiological events [2]. The GPCR superfamily comprises over 800 different members, classified in five major classes (the glutamate receptor family or class C, the rhodopsin receptor-like family or class A, and the adhesin receptors, Frizzled/Taste receptors, peptide-regulated secretin receptor family or class B) according to their structural homology. At present, 50% or so of the currently marketed drugs directly target GPCRs or their downstream effectors [3]. However, despite their capacity to alter receptor signaling, most drugs exhibit undesirable side effects, poor subtype selectivity and often low efficacies. Historically, GPCRs were considered to act as molecular switches; however, a more complex and realistic model now describes and accounts for multiple conformations assumed by the receptor once bound by a given ligand, or through interactions with other signaling partners. During the last decade, a number of new GPCR modulators have been developed which possess the capacity to "select" among the distinct receptor states, which include, as we discuss below, both biased and allosteric ligands. In this review, we will discuss the different aspects of GPCR functional selectivity achieved with these modulators, the potential impact of receptor dimerization, and the implications for future drug development. As our main focus will be the notion that combining biased and allosteric properties may define a novel class of GPCR ligands, we will cite more detailed, recent reviews of individual subjects which readers can consult where necessary.

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2. FUNCTIONAL SELECTIVITY OF GPCR SIGNALING WITH BIASED-LIGANDS

2.1. Historical Aspects and Conceptual Innovations

Beginning with the development of the ternary complex model, ligand-bound GPCRs were assumed to couple with a single G protein, the latter being responsible for activating a downstream effector pathway ([4], see [5] for review). In this paradigm, GPCRs could exist in two different "states" or conformations: the inactive state, functionally and physically uncoupled from the G protein and the active state which was associated with a G protein, resulting in its activation. When it was recognized that agonist is not necessarily required to toggle receptors between the inactive and active forms, the "two-state model", was developed where the inactive receptor fluctuates from an inactivated (R), to an activated (R*) state [6]. Later, this simple, linear view was challenged, with studies showing the possibility of single receptors coupling to more than one G protein, leading to the activation of multiple downstream pathways [7].

There are currently a wide variety of orthosteric ligands (*i.e.* molecules binding to the endogenous ligand binding site of a given receptor) which bind GPCRs. These include classical agonists, antagonists (both reversible or irreversible), and inverse agonists (see Table **1** for definitions). Orthosteric agonists activate the receptor and engage heterotrimeric G protein coupling, leading to specific efficacies of activation on diverse signaling cascades by specific effectors [8]. Indeed, many classically defined agonists partially or fully activate and some antagonists (and inverse agonists) partially or fully block *all* the signaling pathways downstream of a given GPCR. Interestingly, some orthosteric ligands either recognize or induce specific conformations in the receptor, which "direct" signals toward a subset of these pathways. This property of engaging the

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Table 1. Definitions of Different Ligands Modulating Potency and Efficacy of GPCRs and the Functional Selectivity of Signaling.

Ligand	Definition
Ligands binding to the orthosteric binding site	
Agonist	A ligand that activates one or more responses downstream of receptor binding. These can be full (maximal response) or partial (less than maximal response). Most of the time, endogenous ligands are defined as agonists.
Neutral Antagonist	A ligand that has no effect on its own, but inhibits the agonist or inverse agonist effects. These an be competitive or non-competitive with the orthosteric binding site.
Inverse agonist	A ligand that reverses the constitutive activity of a given receptor. Usually, inverse agonists exert the opposite pharmacological effect as receptor agonists.
Biased ligand	A ligand that yields differential responses downstream of receptor activation, also called functionally selective.
Ligands binding to allosteric binding sites	
Allosteric modulator	A ligand which acts only in the presence of the orthosteric ligand. Can be positive (enhances) or negative (reduces) for modulation of a given signaling pathway (i.e. can also be biased).
Ago-allosteric modulator	An allosteric ligand that has an agonist effects on a given pathway in the absence of the orthosteric ligand.
Ligands binding to both orthosteric and allosteric binding sites	
Bitopic ligand	A synthetic ligand that possesses a combination of binding sites can be combinations of pharmacophores in the orthosteric ligand binding site or combine orthosteric and allosteric moieties.

receptor toward specific signaling outcomes has been termed biased-signaling, ligand-directed signaling or stimulus trafficking and leads to the "functional selectivity" of GPCR responses [9-11]. Therefore, orthosteric ligands with such properties have collectively been referred to as "biased ligands".

Kenakin first proposed the idea of "agonist-directed receptor trafficking" to explain inconsistencies between the two-state model and pharmacological data obtained experimentally [9]. One of the earliest studies reporting biased signaling demonstrated the capacities of agonist-bound α_2-adrenergic (α_2AR) [12] and cannabinoid receptors [13] to preferentially couple to either Gαs or Gαi, leading to biased efficacies for cyclic AMP (cAMP) production, depending on the ligand involved. Subsequent reports demonstrated that different pathways were triggered following activation of single serotonin [14, 15] and α_2AR [16] subtypes with the same type of ligand (*e.g.* different agonists). This led to the notion that there were multiple, discrete "active" states for one receptor, as seen for a change in the potency of the Gαs-mediated responses with different agonists of the calcitonin receptor type 2 [17]. G proteins also play a role in biased signaling, possibly by inducing specific conformations of GPCRs to which they are coupled. Different purine nucleotides, able to bind to the active site of Gαs, were able to induce different receptor conformations, leading to biased signaling depending on the ligand used to stimulate the β_2-adrenergic receptor (β_2AR) [18]. This reiterated the importance of the mutual interdependence in receptor/G protein coupling. Thus, GPCRs can be considered as "modular" entities, responding to distinct ligands with specific signaling outcomes depending on their specific sets of partners (see [19] and [20]).

2.2. The True Nature of GPCR Ligands Revealed

One intriguing question raised by these initial studies related to whether or not our definition of the nature of an individual ligand remained accurate with respect to the entire signaling phenome of a receptor. With the understanding of biased signaling came the idea of re-evaluating multiple pathways potentially triggered by known ligands. Many possibilities suggested themselves, the simplest case being that a classical full agonist for one G protein-mediated response, might be an antagonist or inverse agonist for another event via the same receptor [21]. The reason why canonical antagonists had not been studied in standard agonist paradigms comes from the fact that they were used in pre-treatment paradigms or simultaneously with agonist treatment, and in generally higher doses, probably high enough to desensitize the system beforehand in many cases, thus masking any latent agonist activity.

Not surprisingly, more recent studies have demonstrated agonist effects mediated by so-called neutral antagonists. For instance, antagonist-mediated endocytosis with or without β-arrestins, has been observed for the cholecystokinin receptor [22], serotonin receptors [23], the neuropeptide Y receptor [24] and the endothelin receptor [25]. Atosiban, an oxytocin receptor antagonist, was shown to stimulate Gαi-mediated inhibition of cell proliferation in both Madin-Darby canine kidney cells and in prostate cancer cells via persistent ERK1/2 mitogen-activated protein kinase (MAPK) activation while inhibiting the canonical Gαq-coupled pathway [26]. Classically defined antagonists can also act as inverse agonists, stabilizing receptors in inactive conformations for specific pathways, as was shown for the platelet-activating factor receptor (PAFR). Various

antagonists of PAFR showed distinct efficacies as inverse agonists for the inositol phosphate response with constitutively active receptor mutants (bearing the lysine-to-arginine L231R or the aspartate-to-asparagine D363N mutations) or wild type PAFR [27]. Not all antagonists exhibited inverse agonist effects on both mutants, suggesting that the conformational "state" of a receptor was critical for specifying the precise range of signaling outcomes. Another study showed the inverse agonist effects of SR144528, an antagonist for the cannabinoid receptor CB2, on constitutive adenylyl cyclase activity of this receptor expressed in COS cells [28].

In addition to their intrinsic interest to the signaling community, these findings suggest that inverse agonists, being a more recently defined class, could be used to reverse effects of constitutively activated mutant GPCRs seen in different pathologies [29-31]. However, inverse agonists, again redefined on a pathway-specific basis, can also act as agonists. A good example here is the β_2AR, for which inverse agonists for cAMP production, propranolol and ICI118551, have been shown to induce β-arrestin-dependent ERK1/2 activation [32]. Also, the patterns of ligand effects, when examined for two closely related GPCRs, the β_1AR and β_2AR, were distinct depending on the signaling output measured. Both receptors bind a variety of catecholamine derivatives with varying affinities. However, their use on both receptors lead to different efficacy profiles depending on whether cAMP production or MAPK activation was measured [33]. Signal transduction downstream of the angiotensin II (AngII) type I receptor (AT_1R) is another example of pluridimensional efficacy. Several derivatives of AngII, an octapeptide, have been synthesized, and when used to stimulate AT_1R, trigger unique patterns of activation of downstream signaling pathways [34-36]. A variation in efficacy has also been seen with SST_2R somatostatin receptors, where specific ligands present different efficacy profiles for adenylyl cyclase activation and ligand-mediated endocytosis [37]. Another report comparing the effects of SST peptides and synthetic agonists showed that peptide ligands where able to induce endocytosis of SST_2R, SST_3R and SST_5R, while synthetic agonists were only able to induce internalization of SST_2 and SST_3 [38]. Similar protean effects of ligands were observed with the vasopressin receptor antagonist SB121463B [32], and an endogenous inverse agonist for the melanocortin receptor, Agouti [39], suggesting that this type of bias may be generalizable to all GPCRs and their various signaling outcomes. Quantification of signaling efficacy in a pathway-specific fashion can be used as an indicator to establish the limits of functional selectivity of a group of ligands targeting the same receptor. This type of analysis, when first presented in Cartesian terms [33], indicated that most ligands would need to be re-evaluated and re-classified in terms of pathway-specific efficacy.

2.3. Time Dependence of Ligand-induced Functional Selectivity

The signal duration can also be a determinant in the functional selectivity of signaling. For instance, in the case of the ERK1/2 pathway, two waves of activation have been described: short-term activation being G protein-dependent, whereas longer stimulation is triggered by signaling mediated by endocytosed receptor-β-arrestin complexes [40, 41]. Neurokinin (NKA), a neuropeptide, can also activate two distinct waves of signaling, via its receptor NK2. A shorter form of neurokinin (NKA-4-10) triggers rapid and short-term calcium mobilization, whereas the full length peptide favours a distinct conformational state of the receptor, leading, in addition to calcium mobilization, to a delayed and prolonged cAMP production [42]. This differential kinetics of signaling may imply a different set of signaling partners leading to different biological outcomes. Moreover, some ligands drive ERK1/2 activation via transactivation of receptor tyrosine kinases (RTK) [43], through shedding of EGFR ligands following activation of matrix metalloproteinases [44] or Src [45]. This reinforces the idea that GPCRs are able to signal through multiple active conformations, and that, in principle, specific drugs could be designed to activate or inhibit subsets of these conformations. Interestingly, the time of treatment can also modulate the effects a ligand has on signaling. For example, it was shown using a constitutively activated cannabinoid CB_2 receptor that SR144528, defined as an antagonist, acted as an inverse agonist following short term treatment, but as a neutral antagonist following longer treatments [28]. Similar changes in ligand effects over time were seen with serotonin 5-HT receptors [46]. To date, little consideration to these types of effects has been given when such drugs are, or might, be used in clinical applications.

This sea change in our understanding of efficacy implies that individual ligands should be characterized pathway-by-pathway, rather than treating all receptors as identical *a priori*. Inverse agonists, agonists or antagonists induce or select their own conformations based on whether or not they recognize receptors alone or in pre-assembled signaling complexes, and it is now clear that there are multiple sub-conformations of the R and R* states required to explain the functional selectivity achieved by biased ligands. Moreover, many classical inverse agonists, agonists and antagonists may yet be revealed to be biased ligands, requiring an extensive re-evaluation of their effects on a multitude of GPCR effector pathways. Interested readers are directed to more detailed reviews of biased agonism [10, 19, 20, 47, 48]

3. GPCR FUNCTIONAL SELECTIVITY WITH ALLOSTERIC MODULATORS- MOVING BEYOND THE ORTHOSTERIC LIGAND BINDING SITE

3.1. Modulation of GPCRs By Allosteric Ligands

To date, the G protein is the best-characterized allosteric modulator of the receptor, through its capacity to modulate ligand-binding affinity. Some of the first models described the effect of G protein coupling to an activated or ligand-bound receptor as the "ternary complex model" [4, 49]. This model can also be used if the G protein is substituted for an allosteric ligand, which modulates differentially the activated receptor bound by orthosteric agonist [50]. By binding to a topographically distinct binding site with respect to the orthosteric binding site, allosteric ligands have rapidly become an interesting alternative to modulate the selectivity and functionality of receptors and more recently ligand-directed signaling [51-54]. Allosteric modulators, as such,

existed for a long time before their full potential was recognized. They were initially described as non-competitive antagonists [55, 56]. This non-competitive antagonism was often referred as insurmountable, because the receptor-antagonist complex was a new entity in its own right [57]. However, the ternary complex model of allosteric modulation indicated that this could be viewed, in principle, as an adaptation of the system rather than as insurmountable antagonism. Allosteric modulators can affect GPCRs at three different levels: 1) the binding of orthosteric ligand to the receptor, 2) the transmission of ligand binding information to other parts of the receptor or 3) the signaling downstream receptor activation. First, allosteric ligands have been shown to modulate the affinity of orthosteric drugs for their binding sites. In this type of modulation, cooperativity between the two binding sites can be neutral (no effect), positive (leftward shift of the binding curve) or negative (rightward shift of the binding curve). Interestingly, this allosteric effect is saturable, that is, when all the allosteric sites are occupied, no further allosteric modulation is observed. Moreover, the regulation of the different signaling modes downstream a given receptor can be achieved by modulating either the efficacy or potency of the response, with or without modulation of the binding affinity of the orthosteric ligand, or the coupling efficacy between the G protein and the receptor. For all the types of modulation described above, the allosteric ligand in question can therefore be a positive allosteric modulator (PAM) or a negative allosteric modulator (NAM, see Table **1** for definitions). One of the major characteristics of PAMs and NAMs is their inability to trigger GPCR-induced responses in the absence of the orthosteric ligand. There are, however, ago-allosteric ligands or allosteric agonists, which behave like agonists, but by binding to an allosteric binding site. Ago-allosteric modulators, which do not require the presence of the orthosteric ligand to induce agonist effects, are thought to be super-agonists when conjugated with orthosteric ligands. As a consequence, some ago-allosteric modulators have been shown to partially occupy the orthosteric binding site when acting alone [58]. Finally, there are synthetic ligands that can simultaneously bind to both allosteric and orthosteric binding sites, a subclass of the so-called bitopic ligands [59]. Bitopic ligands, in and of themselves are a very interesting class of molecules, which may combine a number of different pharmacophores, comprising allosteric and orthosteric ligands on the same receptor, or between receptor equivalents in homo- and hetero-oligomeric GPCRs. Interested readers are directed to comprehensive recent reviews ([60] and [61]).

3.2. Allosteric Modulation of Class C GPCRs

Although many examples have now been described for allosteric modulators of GPCR function (see [62] and [50] for review) and some are already used clinically (Table **2**), we will focus our discussion on the intersection between allosterism and biased signaling. Allosteric ligands, with all their advantages, can also "tune" agonist-bound receptor responses downstream, as a biased-ligand can, acting through the orthosteric binding site. Only a few examples of such modulation have been observed to date, in both class A and class C GPCRs. For the class C GPCRs, allosteric modulation involves not just the heptahelical core of the receptor, but the large extracellular N-terminal, or "Venus flytrap" (VFT) domain. The VFT domain is composed of two lobes, where conformational changes in this domain dictate the activation level of the receptor [63, 64]. For the metabotropic glutamate receptor 1α (mGluR1α), Gd^{3+} (gadolinium), a known modulator of glutamate-induced signaling, was revealed to be an allosteric ligand with biased properties. Indeed, by stabilization of the interface between both lobes of the VFT domain, Gd^{3+} facilitates changes from Gαs- to Gαq-coupled signaling following receptor stimulation [65]. Another example from this class of GPCR is the calcium-sensing receptor (CaSR), which regulates parathyroid hormone (PTH) production to control calcium homeostasis. This receptor is coupled to both Gαq-induced release of intracellular calcium and Gαi-induced inhibition of cAMP production, leading to MAPK ERK1/2 activation [66]. However, the balance between these two pathways was reversed when an autoantibody from an acquired hypocalciuric hypercalcemia patient was used to target the receptor. This autoantibody in fact acted as an allosteric modulator, increasing the relative contribution from the Gαq pathway compared to the Gαi pathway, decreasing ERK1/2 phosphorylation [67]. Interestingly, the same autoantibody used in presence of a calcimimetic had no effect on ERK1/2, making it insensitive to pertussis toxin, switching the MAP kinase signal dependence from Gαi to Gαq. Intriguingly, a recent study suggested that *N. meningitidis*, the Gram negative bacteria responsible for some forms of bacterial meningitis, also acts as an ago-allosteric ligand in that in binds to the N-terminal extracellular domain of the β_2AR and specifically stimulates β-arrestin recruitment to sites of bacterial infection in endothelial cells with activating the canonical Gαs signaling pathway [68].

Another rich source of allosteric binding sites, more common for modulators of class C GPCRs, is the heptahelical domain itself. One of this class of regulators, *N*-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*isoindol-2-yl)met-

Table 2. Reported allosteric Modulators of GPCRs Already Marketed or in Clinical Trials.

Receptor	Compound Name	Function	Reference
mGluR5	ADX10059	NAM for gastro-oesophageal reflux.	[151]
CaSR	Cinacalcet	PAM for diminution of parathyroid hormone levels in secondary hyperparathyroidism.	[152]
CCR5	Maraviroc	NAM for HIV entry to $CD4^+$ cells.	[153]

hyl]-phenyl}-2-hydroxybenzamide (CPPHA) potentiates mGluR5-induced calcium release [69] and was later shown to have biased properties, as it also resulted in inhibition of ERK1/2 MAPK activity [70]. Interestingly, 3,3'-difluororbenzaldazine (DFB), another allosteric modulator of mGluR5 binding to a site which overlaps with the CPPHA allosteric site [69], was able to potentiate both the calcium response and ERK1/2 activation. These results demonstrate that two different allosteric modulators, partially sharing a single allosteric binding site, can yield two specific signaling responses. These two examples highlight allosteric modulators of class C GPCRs which bind near the site of the orthosteric ligand even though it has often been suggested that the allosteric binding site of other class C GPCRs resides in the heptahelical domain (*e.g.* $GABA_BR$, [71] see below).

3.3. Allosteric Modulation of Class A GPCRs

In contrast to class C GPCRs, allosteric modulation of class A receptors is thought to occur via regions outside the heptahelical transmembrane domain, on the extracellular surface of the receptor, which are less conserved and thus more receptor-specific [72]. This modulation often stabilizes specific receptor conformations, altering coupling to distinct heterotrimeric G proteins. The first example of such a regulator was identified for the neurokinin NK2 receptor. When bound to its endogenous ligand, neurokinin A (NKA), NK2 can adopt distinct and sequential conformations, stabilized by two high affinity binding sites [42]. The first conformation, A1L, is thought to facilitate rapid dissociation kinetics of NKA from the receptor followed by Gαq-induced calcium mobilization, whereas the second conformation, A2L, results in slower ligand dissociation kinetics and leads to Gαs-induced cAMP production. An allosteric ligand for NK2R, LPI805, preferentially stabilized the A1L conformation, diminishing the intensity of Gαs-mediated cAMP accumulation, as the shorter version of the natural ligand NKA(4-10) would do (as discussed above) [73]. In a follow-up study, the authors were able to generate derivatives of the original allosteric ligand, which generated distinct selectivity profiles, acting as a PAM for calcium signaling and a NAM for cAMP production [74]. Another example of an allosteric modulator directly regulating coupling between the receptor and the G protein in a biased manner was shown for the prostaglandin F2α (PGF2α) receptor (FP). In this study, the authors developed a small molecule peptide mimic, PDC113.824, derived originally from the sequence of the second extracellular loop of FP [75]. The peptide itself was initially characterized as a tocolytic in a mouse model of pre-term labour [76]. As discussed below, this region of GPCRs may actually represent a hot-spot for allosteric regulation. PDC113.824 was demonstrated to stabilize a specific conformation of FP receptor, where a Gαq-induced PKC-ERK1/2 pathway was potentiated and a Gα12-induced, Rho-mediated cytoskeletal rearrangement was inhibited following PGF2αstimulation [75]. Interestingly, there were no direct signaling consequences in that neither effector pathway was modulated by PDC113.824 alone. However, basal levels of GTPγS incorporation were altered by PDC113.824 for both Gαq and Gα12, suggesting that the allosteric ligand recognized two distinct preformed, receptor/G protein complexes. As we will discuss below, this observation has implications regarding the actual molecular targets of biased and/or allosteric ligands. Moreover, the repercussions of such biased signaling were manifested *in vivo*, by inhibition of lipopolysaccharide- or PGF2α-induced preterm labour in mice, in the presence of PDC113.824. Interestingly, as for LPI805, the dissociation kinetics of [^{3}H]-PGF2α from FP were more rapid in the presence of PDC113.824, and Gαq coupling was enhanced. These studies suggest that allosteric modulators, which enhance GTP binding to G proteins in the absence of orthosteric ligand, may lead to alterations in orthosteric ligand affinity for the receptor, leading to specific G protein-dependent signaling patterns.

The studies cited above demonstrate that allosteric ligands can also be signaling- biased, further increasing their potential clinical utility. Another example of such biased-signaling was shown with the small molecule ADX61623, an allosteric modulator of the FSH receptor (FSHR) identified by high-throughput screening [77]. This ligand increases the affinity of the FSH for FSHR, acting as a PAM for orthosteric ligand binding. On the other hand, it was found to inhibit cAMP-induced progesterone production in ovarian primary cultures, acting as a NAM in this case. Interestingly, ADX61623 had no effect on estrogen production, indicating that the latter is Gαs-independent. However, using ADX61623 *in vivo*, concomitant with FSH treatment, showed no particular effect on follicular development. These discrepancies between the effects of an allosteric modulator in endogenous cell systems and in animal models suggest that the stability or delivery of such modulators may be affected *in vivo*. Also, this demonstrates that the complex and subtle regulatory controls in physiological systems may add an additionally complexity in controlling these systems with allosteric modulators.

Some allosteric modulators have been identified that have more complex effects; in that they have distinct sites and mechanisms of actions, which all impinge on GPCR signaling. For example, a recent study demonstrated that small molecular potentiators of A_1 adenosine receptor signaling, the so-called 2A3BT compounds, also have direct effects on G proteins distinct from their binding to the extracellular surface of the receptor [78]. A similar note of complexity and caution was raised in another study which showed that an allosteric ligands for the CXCR4 chemokine receptor also interacted with CXCR7 but produced precisely the opposite signaling phenotype [79].

3.4. The Use of Bitopic Ligands to Achieve Functional Selectivity

Functional selectivity can also be demonstrated using bitopic ligands. These ligands, composed of two distinct pharmacophore moieties, either combinations of orthosteric ligands or orthosteric and allosteric ligands, are also able to favour specific conformations of GPCRs [59]. Such ligands have been used to understand biased signaling via muscarinic acetylcholine receptors (mAChR), one of the most extensively studied GPCR subfamilies with respect to allosteric regulation. It was shown that the partial agonist McN-A-343 had differential efficacy for distinct mAChR

subtypes. McN-A-343 was unable to completely displace antagonist (N-methylscopolamine, NMS) binding to the M_2 mAChR [80] and was shown to have a different mode of binding to the receptor, compared to common allosteric modulators or antagonists, suggesting a unique mode of action [81]. McN-A-343 was also shown to exhibit functional selectivity for the Gα15 compared to Gαi-induced responses [82]. Later, using derivatives of the original molecule, it was shown that McN-A-343 was in fact a bitopic ligand, simultaneously binding to both orthosteric and allosteric sites of the M_2 mAChR [83]. Indeed, some of the derivatives were pure allosteric modulators with biased signaling properties. The allosteric moiety of the McN-A-343 was unable to displace [^{3}H]-NMS binding to M_2 mAChR, rather acting as a PAM for orthosteric antagonist binding (as shown by measuring dissociation kinetics of ligand binding to receptor) and as a NAM for allosteric agonist-mediated G protein/ERK1/2 activation.

As we have seen in this section, allosteric modulators show promise for use in biased signaling applications. Acting primarily in the presence of the orthosteric ligand, one can imagine many possibilities for clinical use from the perspective of more selective therapeutic and reduced off-target effects.

4. STRUCTURAL CORRELATES OF ALLOSTERIC AND BIASED SIGNALING

4.1. What the Crystal Structures Tells Us About GPCR Conformations

The recent crystal structures of antagonist-, inverse agonist- and more recently agonist-occupied GPCRs has highlighted the structural flexibility of most class A receptors as compared with rhodopsin [84-88]. Several features, important for ligand binding as well as receptor activation, have been revealed in recent years, with the crystallization of a number of class A GPCRs (see [89] for review). What are the implications of the recent structural work for biased and allosteric signaling? As suggested by *in silico* modeling of different agonists, antagonists and inverse agonists of β_2AR [90], there are different conformations associated with signaling phenotypes observed in biochemical studies. Each conformation would allow different residues of TM3, 6 and 7, as well as the second extracellular loop (ECL2) to make contact with the different ligands used. The recent ligand-bound β_1AR and β_2AR structures show, not surprisingly, differences between the activated and inactivated states of the receptors, in that agonist leads to an opening of the cytoplasmic face of the heptahelical [86, 88, 91-94]. Studies with the β_2AR demonstrated that conformations linked to specific signaling outcomes could be detected using FRET between two labelled residues within the receptor, C-terminal tail and the end of TM6 [95]. Another report demonstrated the loss of functional selectivity in the dopamine receptor D_2R when histidine 393 (in TM6) is mutated for an alanine [96]. Further, micro-switches, conserved residues inside the "barrel" formed by the seven transmembrane domains (TM1-7), are involved in receptor activation and in controlling the conformational state of the receptor, by inducing a rotamer change in the transmembrane helices (TMs). The main event following ligand binding, responsible for activation of the receptor, is the movement of the so-called "toggle switch". This switch involves a global repositioning of the TMs, as a vertical "see-saw" movement, which results in the tightening of the orthosteric pocket and the opening of the intracellular side to allow G protein coupling. Importantly, the contribution of the G protein is to be included in the allosteric communication model of receptor activation, as TM5-6 in the toggle-switch model adapts to the presence of the G protein [97] and as suggested by the incapacity of the agonist alone to generate a full R* state [93]. Therefore, as predicted, GPCRs can adopt multiple conformations, within the theoretical energy landscape generated to understand the effect of different ligands on β_2AR functional selectivity (reviewed in [98]).

4.2. ECL2 as a Key Player in Allosteric Modulation of GPCRs

In conjunction with X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy has also been used to understand ligand-induced changes in conformations in GPCRs [99]. A recent NMR study suggested the extracellular surface of the heptahelical domain, which is highly divergent in sequence between even homologous receptors, is an important target for both orthosteric and allosteric modulation, and therefore, functional selectivity [100]. The second extracellular loop or ECL2 normally has a cysteine which forms a disulfide bond with another cysteine residue in TM3, constraining this loop and forcing it to "shape" the entrance to the orthosteric binding site, regulating different states of the receptor [101]. Furthermore, to confirm the plasticity of binding sites in GPCRs, all the antagonists or inverse agonists used in the crystal structures generated to date have different contact positions inside the binding site and to ECL2 and ECL3, while maintaining key common residues for the general toggle-switch mechanism, reflecting the versatility of these receptors in accommodating ligands [89]. Interestingly, some GPCRs possess a second disulfide bond linking the N-terminal domain to ECL2, which may also have repercussions for receptor conformation and functional selectivity [102]. Several reports, using site-directed mutagenesis, have demonstrated that a putative allosteric binding site may be located in ECL2. Mutations of a cluster of acidic residues in ECL2 was enough to affect the functional selectivity of gallamine, an allosteric modulator of M_2 mAChR [103]. Mutation of tyrosine 177 was also sufficient to abrogate the allosteric effects on M_2 [83]. Interestingly, the addition of a second mutation on M_2 orthosteric site, at tyrosine 104, was enough to abrogate functional selectivity induced by 77-LH-28-1, an allosteric modulator [104]. Moreover, a recent report suggested that a molecule-mimic of ECL2 itself [76] could be used to allosterically modulate FP into two different G protein-linked conformations [75]. Another example of the functional selectivity achieved by the ECL2 was shown with the somatostatin receptor (SSTR), for which ECL2-specific antibodies were produced. These antibodies were unable to displace somatostatin binding to SSTR, but acted as agonists

[105]. Finally, these results show that residues in ECL2 are important regulators of ligand-induced functional selectivity of GPCRs. Orthosteric ligands, once inside the barrel formed by the heptahelical domain, can adopt receptor-specific interactions with ECL2 [89]. It is therefore not surprising that this region of the receptor may have evolved to control subtype selectivity, allowing it to transduce biased-signaling through the main binding pocket of GPCRs.

Class C GPCRs are quite different in their structure compared to class A or B receptors. Interestingly, for these receptors, with the exception of ions, the allosteric sites are not located in extracellular ligand-binding domains, but rather in the heptahelical domain, where the orthosteric ligand binds class A and B GPCRs. Indeed, in class A GPCRs, the situation is reversed in that the extracellular loops provide sites for allosteric regulation. This also suggests that ligands analogous to orthosteric ligands for class A GPCRs could be identified as allosteric regulators of class C GPCR signaling [106].

5. GPCR OLIGOMERS: ASYMMETRY IN STRUCTURE, ASYMMETRY IN SIGNALING

5.1. Class C GPCRs: the Effects of Constitutive Dimerization on Signaling Modulation

It has become clear in recent years that most if not all GPCRs can form dimers and possibly higher order structures (see [107-111] for review). Although allosteric and biased ligands have become interesting targets to control GPCR signaling more selectively, the potential of this approach is both increased *and* complicated by the existence of receptor homo- and hetero-oligomers. The notion that GPCRs can modulate one another allosterically, as the G protein does with respect to receptor ligand binding affinity, has in fact been demonstrated. Almost all class C GPCRs exist as dimers and the consequences of dimerization have been extensively studied for metabotropic glutamate receptors [112], calcium-sensing receptors [113], taste receptors [114], and $GABA_B$ receptors [115]. In fact, the most convincing example of dimerization in GPCRs remains the $GABA_B$ receptor. The $GABA_BR1$ harbors an ER retention signal and therefore must form a constitutive dimer with $GABA_BR2$ subunit to be targeted to the cell surface [115]. Each protomer of this dimer has a distinct function. $GABA_BR1$ is responsible for ligand binding via its large extracellular N-terminal VFT domain, characteristic of class C GPCRs. Once bound, $GABA_BR1$ transmits a conformational signal to $GABA_BR2$, which in return is responsible for activation of the G protein downstream [116, 117]. Until recently, the exact mechanism of the allosteric communication between both protomers of this dimer was unknown. Previous data suggested that transactivation was the only means by which $GABA_BR$ was able to initiate signaling, eliminating activation of the other protomer in *cis* [71]. Indeed, it was demonstrated that 1) the VFT domain of $GABA_BR2$ was not necessary for a functional heterodimer [118], and that 2) allosteric ligands can bind the heptahelical domain of $GABA_BR2$ [119], making a strong argument in favour of it serving a key regulatory role. However, it is now understood that allosteric modulation within the dimer occurs, through direct transactivation of the heptahelical domains of $GABA_BR1$ to $GABA_BR2$, after the VFT domain is primarily modulated by its ligand [118].

Some class C metabotropic glutamate receptors (mGluR, composed of mGluR1 to 8) have been shown to form constitutive dimers (see [120] for review). Chimeras of the metabotropic glutamate receptor 1 (mGluR1) with the C-terminal tails of either $GABA_BR1$ or $GABA_BR2$ were used to create constitutive dimers whose trafficking and assembly could be controlled. This was used to demonstrate the crosstalk between the two protomers [121]. These authors then created mutations within the heptahelical domain rendering one or both protomers able to bind MPEP, a non-competitive inverse agonist of mGluR5 (redefined in our discussion above as an allosteric modulator). When only one protomer of the dimer was bound by MPEP, the dimer was insensitive to its inverse agonist effect. However, when the first protomer was kept as the original chimera (mGluR1 with $GABA_BR1$ C-tail) and the second protomer was able to bind MPEP but unable to activate G proteins, the agonist response was enhanced, leading to a strong signaling response downstream. Finally, MPEP exhibited its full inhibitory effects when used with one protomer co-expressed with a second unable to bind MPEP or activate G proteins [121]. These results again demonstrate an allosteric interaction between both protomers of a receptor dimer, caused by conformational changes induced by ligand binding to one protomer which is transmitted to the other. This raised the question, however, of how the large N-terminal domains of such dimers, responsible for orthosteric ligand binding, might be implicated in the intensity and specificity of the signal transmitted to the heptahelical domain.

Conformational changes in the extracellular VFT have been characterized by X-ray crystallography for the mGluR1, in the presence or absence of glutamate [122]. It was revealed that two main conformations, an open or resting state and the closed or active state, were adopted by dimers of the VFT domains. Further, it was shown that in mGluR8, another metabotropic glutamate receptor, a single residue in the VFT domain is responsible for antagonistic effects on the whole receptor, by maintaining this domain in an open “state”. Mutating this residue to an alanine was sufficient to restore the closed or activated “state”, again showing the versatility and complexity of conformations that could be adopted by class C GPCRs [123]. The importance of the VFT domain as the first conformational step in receptor activation of the heptahelical domain was also reported. A recent study showed that the VFT of $GABA_BR2$ is necessary to control the intensity of the effect of an allosteric modulator, since the response to GABA was stronger with a dimer composed of $GABA_BR1$ and a chimera of $GABA_BR2$ lacking the VFT domain [118]. Thus, multiple conformational changes or “states” within the receptor extracellular and heptahelical domains of class C GPCRs are necessary to achieve signaling. These findings suggest that GABA and glutamate, the two major inhibitory and stimulatory transmitters in the human brain, evolved separately from the other classes of GPCRs, using both the VFT and the heptahelical domains to activate differential responses through unique conformational interactions.

5.2. Class A GPCRs: Different Partners Add Texture to Receptor Signaling

One of the most interesting features of GPCR heterodimers is the potential of each receptor equivalent to bias the signaling of the other and vice versa. For instance, heterodimerization of the β_2AR with the δ-opioid receptor leads to changes in trafficking and ERK1/2 MAPK activation [124]. Internalization of both receptors was observed when the dimer was treated with ligands specific for either receptor. However, β_2AR dimerization with the κ-opioid receptor (which does not internalize when expressed alone) did not result in internalization of the latter. This suggests that dimerization (especially heterodimerization) is a regulated event. The relationship between heterodimerization, trafficking and signaling has also been seen with members of the adrenergic receptor family. For example, the β_1- and β_2AR were shown to form heterodimers [125] with internalization and ERK1/2 activation profiles that were similar to when the β_1AR was expressed alone - *i.e.* it exhibited a dominant effect over the β_2AR. These findings may be relevant clinically, since both receptors are expressed in the heart and have been shown to dimerize in cardiomyocytes [126]. Another study detected heterodimers of the β_2- and β_3AR, showing a similar dominant phenotype of the β_3AR over the β_2AR with respect to receptor internalization [127]. This dominant dimer phenotype was also seen with adenosine A_{2A} and dopamine D_2R receptors. Here, production of cAMP via the Gαs-coupled A_{2A} receptor was blunted by the presence of the Gαi-coupled D_2R receptor [128]. With respect to the distinct signaling phenotypes in a receptor heterodimer, some recent studies of allosteric interactions between the receptors protomers are highly instructive. Of course, allosteric interactions between GPCRs in oligomers had been anticipated from studies of ligand binding cooperativity [129, 130].

Recently, the structure of the chemokine receptor, CXCR4, was elucidated. Five different dimer structures, bound to antagonists, were present as homodimers, containing different mutations allowing a better characterization of the allosteric changes within the dimer [131]. Of particular note, one of the structures (CXCR4-3) bearing a tyrosine-to-proline mutation at position 240 (T240P), causes disruption of transmembrane helix VI and was signaling impaired. When comparing the agonist-bound CXCR4 dimer structure not mutated for G protein coupling (CXCR4-1 or -2) versus CXCR4-3 dimer, the crystal packing arrangements were quite different. Despite the fact that both CXCR4-3 structures were bound by two different antagonists (one small molecule, one cyclic peptide), the results showed that the T240P mutation left the dimer in a specific conformation. The structure of the dimer with these antagonists revealed a change in the common interface between the two protomers that could eventually result in either negative or positive cooperativity.

Allosteric modulation within a receptor homodimer was elegantly demonstrated for the Class A dopamine D_2 receptor (D_2R) [132]. In this study, the authors used different combinations of free receptor and receptor/G protein fusions to demonstrate these allosteric interactions. Importantly, it was shown that one protomer actually provided a transactivating signal to the fused G protein of the other protomer when the former was occupied by agonist. Agonist occupation of the second protomer actually dampened signaling, likely through a mechanism involving negative cooperativity which had previously been demonstrated using hormone desorption experiments in other GPCRs [133-135]. Interestingly, as for the class C mGluR [121], binding of an agonist to the first protomer of the dimer in conjunction with inverse agonist binding to the second protomer lead to the highest efficacy [132]. Perhaps most intriguingly, this study showed that, as in Class C GPCRs, Class A homodimers may be arranged in an asymmetrical fashion with respect to the G protein. These findings need to be recapitulated in the context of Class A heterodimers as they have potentially important implications. The assembly of asymmetric heterodimers or hetero-oligomers implies that allosteric machines may be constructed in a cell that respond to a single ligand in terms of signaling output but could be allosterically regulated by ligands binding to different heterodimer partners. If receptor/G protein complexes are in fact pre-assembled, prior to reaching the plasma membrane (reviewed in [136, 137]), then different orientations of these machines might be constructed by reversing the specific asymmetric arrangement described above. Thus, two distinct, allosterically regulated receptors that respond as a single signaling unit, despite being a receptor heterodimer, may be regulated in distinct and cell-specific ways depending on how they are arranged with respect to each other. Thus, the formation of heterodimers could also lead to the formation of new signaling pathways, as demonstrated with D_1R/D_2R heterodimeric receptor complex. When expressed individually, these receptors do not couple to Gαq. However, when co-expressed, they are able to stimulate this pathway [138, 139]. Taken together, these findings reveal the capacity of individual protomers to interpret and bias signals delivered to GPCRs and transmit it into the cell in a myriad of new ways. These notions will need to be accommodated in screens for biased and allosteric ligands in future.

It is likely that the dimer is the minimal unit of GPCR organization and that oligomers exist for most receptors. As shown in Fig. (**1**), each GPCR protomer of a dimer is able to modulate its own conformation, when bound and when interacting with the other protomer of the complex. Each ligand might induce a specific conformation responsible for functional selectivity of signaling observed downstream. Several reports now discuss the notion of "receptor mosaics" that would each have specific functions and could be allosterically regulated by a number of unique signaling partners resident in any particular mosaic. Emerging imaging techniques such as resonance energy transfer (RET) or bimolecular fluorescence complementation (BiFC) are helping us understand the stoichiometry of these complexes [140-142]. The diversity of responses induced by GPCRs is not only dependent on different types of ligands, but on the arrangement of GPCR protomers within larger oligomeric complexes. More importantly, the formation of oligomers can explain the signaling diversity and the results obtained from ligand binding studies on native receptors expressed in tissue. Such hetero-oligomers, once fully characterized, may

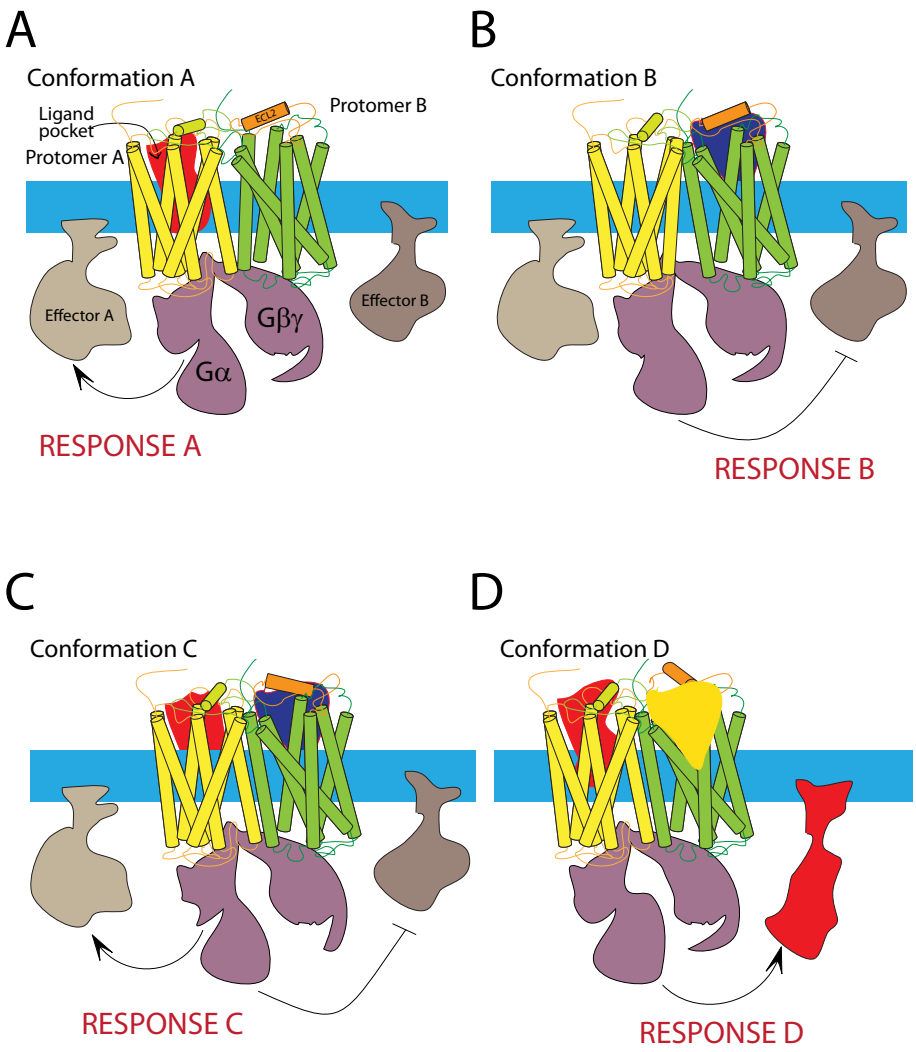

Fig. (1). Functional selectivity in GPCR heterodimers. Different conformations adopted by GPCR heterodimers when: *A*) protomer A is occupied, *B*) protomer B is occupied, *C*) protomer A and B are occupied by the same ligands as *A* and *B* and *D*) protomer A is occupied with the same ligand as in *A*, but protomer B is occupied by a different ligand. Each combination of ligands reveal aspects of the "texture" of the complex, modifying receptor conformation in the heptahelical domains and in the second extracellular loop (ECL2), leading to a change of conformation in the G protein, modulating a specific subset of effectors responsible for a unique physiological response. Ligand binding to the receptor is represented as a shaded shape in the orthosteric binding pocket. These ligands lead to functional selectivity of the response, using communication between and within GPCR protomers. Adding allosteric modulators beyond the orthosteric site for each protomer will also lead to distinct signaling outcomes. Lines ending in arrows indicate a stimulatory effector pathway, while lines ending in bars indicating a negative impact of a given ligand on effector activation

lead to the development of drugs selective for a given pathway in a given cell type, with fewer undesirable effects.

6. IMPLICATIONS FOR DRUG DEVELOPMENT

Our technical capability for discovery of new drugs has improved dramatically in the last two decades with continuing advances in high-throughput and high-content screening (HTS and HCS). However, these approaches will certainly have to be modified such that a great deal more of the complete receptor signaling phenotype will need to be evaluated and new assays will have to be developed in order to screen for both biased and allosteric ligands. This means going beyond the classic antagonist- and agonist-based screens. Several aspects of the GPCR lifecycle can now be evaluated in such screens, which will aid the characterization of improved biased ligands and new allosteric ligands. Biosensors generated from chimeric proteins designed to measure the activation of specific aspects of each or multiple signaling pathways are now commonly used to screen for biased ligands [143, 144]. Also, "label-free" sensors to detect subtle changes in the cell following GPCR stimulation with different ligands have been used to screen for new drugs in heterologous and more importantly, in endogenous systems [145, 146]. Another HTS system uses yeast to express GPCRs and detects functional selectivity via a GPCR/G protein/ERK1/2 pathway readout [147]. Moreover, sensors are now used in a dimerization paradigm, to find new receptor partners, using β-arrestin recruitment to GPCR

dimers using BRET [148]. *In silico* approaches such as molecular docking with large virtual ligand sets, using known GPCR structures or homology models GPCRs may help limit the extent of later screens [149]. In addition, NMR and spectroscopy techniques now allow for the complete characterization of energy landscapes, to develop ligand "maps" of possible intermediate conformations taken by ligand-receptor complexes [98, 150]. It will be eventually possible to design new drugs that stabilize particular conformational states, to control functional selectivity and reduce unwanted side effects. This will also involve the generation of very large data sets that will need to be analyzed as the outcomes from these more complicated screens.

7. CONCLUSION

GPCRs are versatile allosteric machines, able to either bias their shape to accommodate specific ligands, or to be specifically recognized as pre-assembled complexes by biased or allosteric ligands. This spectrum of responses to different ligands reflects the spectrum of possible GPCR signaling architectures and is responsible for the functional selectivity observed in the downstream events occurring in the cell. Modulators that exhibit both allosteric and biased effects would be ideal drugs, since they act only in the presence of the endogenous ligand (*in vivo*), are saturable and offer unparalleled specificity of signaling. Our definitions of the term "ligand" need to include qualifiers for the pathway being measured, and the global signaling repertoire of a given receptor needs to be considered when validating new drugs. The extracellular regions of GPCRs, with their receptor-specific sequences and unique conformational arrangements, will likely represent an important target for the design of new biased and allosteric therapeutics. Receptor protomers in GPCR oligomers can be viewed as allosteric modulators for the other protomers. As our understanding of receptor asymmetries increases, so will our ability to exploit distinct receptor-receptor interactions therapeutically. Finally, as our knowledge of the signalosomes of individual receptors increases, so to does the possibility of designing allosteric modulators of intracellular targets as well. The priority now is also to consider tissue- and ligand-specific "context" or "texture" in future drug screening campaigns.

CONFLICT OF INTREST

None.

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