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Teaching old receptors new tricks: biasing seven-transmembrane receptors

Sudarshan Rajagopal[*], **Keshava Rajagopal**[‡], and **Robert J. Lefkowitz**[*,§,‖]

[*] Department of Medicine, Duke University Medical Center, 2301 Erwin Road, Durham, North Carolina 27710, USA

[‡] Department of Surgery, Duke University Medical Center, 2301 Erwin Road, Durham, North Carolina 27710, USA

[§] Department of Biochemistry, Duke University Medical Center, 2301 Erwin Road, Durham, North Carolina 27710, USA

[‖] Howard Hughes Medical Institute, Duke University Medical Center, 2301 Erwin Road, Durham, North Carolina 27710, USA

Abstract

Seven-transmembrane receptors (7TMRs; also known as G protein-coupled receptors) are the largest class of receptors in the human genome and are common targets for therapeutics. Originally identified as mediators of 7TMR desensitization, β-arrestins (arrestin 2 and arrestin 3) are now recognized as true adaptor proteins that transduce signals to multiple effector pathways. Signalling that is mediated by β-arrestins has distinct biochemical and functional consequences from those mediated by G proteins, and several biased ligands and receptors have been identified that preferentially signal through either G protein- or β-arrestin-mediated pathways. These ligands are not only useful tools for investigating the biochemistry of 7TMR signalling, they also have the potential to be developed into new classes of therapeutics.

Seven-transmembrane receptors (7TMRs), also called G protein-coupled receptors (GPCRs), are the most common class of receptors, with more than 800 members identified in the human genome[1]. They are also the most commonly targeted receptor class for medicinal therapeutics[2]. Drugs that activate 7TMRs are thought to modulate the proportion of receptors that are in an active signalling conformation relative to those in an inactive, non-signalling conformation. Based on the classical model for 7TMR activity, agonist binding to the 7TMR causes the receptor to adopt a conformation that results in the activation of associated heterotrimeric G proteins. This activation involves the exchange of bound GDP for GTP by the Gα subunit of the G protein, leading to dissociation of the heterotrimeric protein complex into Gα and Gβγ subunits. This dissociation then promotes the production of and consequent

Correspondence to R.J.L. lefko001@receptor-biol.duke.edu.

Competing interests statement
The authors declare competing financial interests: see web version for details.

DATABASES
IUPHAR Databse of Receptors and Ion Channels: http://www.iuphar-db.org/index.jsp
5-HT$_{2A}$ | 5HT-$_{2C}$ | AT$_{1A}$ | β$_1$-adrenergic | β$_2$-adrenergic | CXCR4 | D$_2$ | δ-opioid | EP$_4$ | GPR109A | κ-opioid | NK$_1$ | PAR2 | PTH1
UniProtKB: http://ca.expasy.org/sprot
β-arrestin 1 | β-arrestin 2
FURTHER INFORMATION
Laboaratory homepage of Robert J. Lefkowitz: http://www.lefkolab.org
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signalling by second messenger systems, such as those involving cyclic AMP, diacylglycerol and calcium[3]. Signalling by the activated conformation of the 7TMR is terminated by phosphorylation of the cytoplasmic loops and tail of the 7TMR, which is catalysed predominantly by GPCR kinases (GRKs). This results in the binding of arrestins (most commonly β-arrestin 1 and β-arrestin 2) and consequent desensitization followed by internalization into clathrin-coated pits[4]. Thus, in the classical model, heterotrimeric G proteins mediate signal transduction via the receptor, and β-arrestins mediate receptor desensitization and internalization (FIG. 1a).

This classical model, however, is both over-simplified and incomplete. Over the past decade there has been a new appreciation regarding the capacity of β-arrestins to act not only as regulators of 7TMR desensitization, but also as multifunctional adaptor proteins that have the ability to signal through multiple mediators such as mitogen-activated protein kinases (MAPKs), SRC, nuclear factor-κB (NF-κB) and phosphoinositide 3-kinase (PI3K). This new perspective on 7TMR signalling represented a paradigm shift[5] (FIG. 1b). In this model, both heterotrimeric G proteins and β-arrestins are capable of interacting with and recruiting intracellular signalling molecules, with desensitization of the ligand-bound receptor mediated by β-arrestins. It is still unclear whether the same receptor conformations that result in β-arrestin-mediated signal transduction also lead to receptor desensitization. Biochemical data suggest that the signalling mediated by β-arrestins has distinct functional and physiological consequences from that mediated by G proteins[5]. However, much of the work comparing β-arrestin- and G protein-mediated signalling has been performed in transiently transfected cell lines and not in primary cells or animal models, which limits our current understanding of the physiology of β-arrestin-mediated signalling.

It was originally thought that most ligands that bind to 7TMRs have balanced or unbiased activity for signalling through β-arrestins and G protein pathways; that is, they signal equally through both[6]. However, some receptor–ligand systems display bias towards one pathway over the other; that is, they preferentially signal through either the G protein- or β-arrestin-mediated pathway[7]. This behaviour is an example of biased agonism (FIG. 1c), which is also referred to as collateral efficacy, functional selectivity or stimulus trafficking[8]. Biased agonism has important implications for the design of therapeutics that target 7TMRs, as signalling through these parallel pathways is thought to have distinct functional consequences. For example, two drugs could both act as agonists of G protein-mediated functions, but may have differing effects on β-arrestin-mediated signalling. This could result in markedly different signalling profiles *in vivo*, examples of which will be discussed in this Review. Frequently, drug screening assays are designed to detect signalling downstream of G proteins, such as an increase in intracellular calcium levels typical of G_q-coupled receptors or increased cAMP levels typical of G_s-coupled receptors. Such assays, however, may not be sensitive to β-arrestin-mediated signalling, the target pathways of which have yet to be fully characterized. In this Review, we discuss recent advances in the characterization of β-arrestin-mediated signalling and biased agonism at 7TMRs, and address the implications of these for drug discovery and design involving this ubiquitous superfamily of receptors.

The range of β-arrestin-mediated signalling

There are four arrestin isoforms[9]. Arrestin 1 and arrestin 4 are expressed only in the retina where they regulate signalling of the photosensors rhodopsin and the colour opsins. By contrast, arrestin 2 and arrestin 3 (commonly referred to as β-arrestin 1 and β-arrestin 2, respectively) are expressed throughout the body. The amino-acid sequences of β-arrestin 1 and β-arrestin 2 are nearly 80% identical, with most of the differences in the carboxyl termini of the proteins.

The initial role for which arrestin activity was noted was in the desensitization of rhodopsin, a 7TMR that is the visual pigment responsible for monochromatic vision in the dark. Rhodopsin is activated into its signalling conformation by photon absorption, which allows it to bind the signalling protein transducin. This is followed by phosphorylation of the receptor[10] by rhodopsin kinase[11], a member of the GRK family of serine/threonine kinases[12]. This phosphorylation results in the binding of arrestin 1 (visual arrestin) to rhodopsin[13], which sterically prevents further interaction of transducin with rhodopsin, thereby desensitizing the receptor.

A similar phosphorylation sequence of events was also observed in the β-adrenergic receptors[14]. In a reconstituted system with the β_2-adrenergic receptor (also known as β_2-adrenoceptor), it was subsequently shown that desensitization was dependent on GRKs and β-arrestins[15]. This mechanism was then demonstrated for several other 7TMRs, leading to the proposal of a classical paradigm for GPCR activity. In this scenario, signalling is performed by heterotrimeric G proteins followed by phosphorylation by GRKs, and desensitization is mediated by arrestins. However, it is now appreciated that β-arrestins act as multifunctional adaptor proteins that scaffold a diverse group of signalling proteins at the 7TMR and modulate the downstream activity of a number of signalling networks (reviewed in REF. [5]). Selected examples are discussed below and highlighted in BOX 1.

Box 1

Selected examples of cellular functions that are mediated by β-arrestins

Desensitization

- Mediate seven-transmembrane receptor (7TMR) desensitization[15,101], and recruit enzymes for second messenger degradation[102]

Trafficking

- Internalization: mediate7TMR internalization[103], and act as clathrin adaptors[104]
- Translocation: mediate translocation of Smoothened to the primary cilium[44]
- Exocytosis: mediate chemokine (C-X-C motif) receptor 1-induced exocytosis in granulocytes[105], and endothelin-1-stimulated translocation of glucose transporter 4 (GLUT4; also known as SLC2A4) to the plasma membrane[106]

Signalling

- Kinase regulation: regulate mitogen-activated protein kinases (MAPKs)[18–20], SRC[16,17], phosphoinositide 3-kinase[25] and AKT[26,29]
- Transcriptional regulation: are binding partners for inhibitor of nuclear factor-κBα (IκBα)[34,107]; control histone deacetylation by p300[38]; and control β-catenin transcriptional regulation mediated by Wnts[36]
- Chemotaxis: control protease-activated receptor 2-mediated chemotaxis[23]; angiotensin II type 1A receptor-mediated chemotaxis[24]; and CXCR4-mediated chemotaxis[108].
- Apoptotic/anti-apoptotic signalling: control substance P anti-apoptotic effects mediated by neurokinin 1 receptor[17]; retinal degeneration mediated by rhodopsin arrestin complexes[109,110]; inhibition of apoptotic signalling by *N*-formyl peptide receptor[111]; and anti-apoptotic signalling via BCL2-associated agonist of cell death (BAD) phosphorylation[27]

- Epidermal growth factor receptor (EGFR) transactivation: mediate transactivation of EGFR by prostaglandin E4 receptor[41], β_1-adrenergic receptor[42] and angiotensin II type 1A receptor[43]
- Protein synthesis: regulate mRNA translation and protein synthesis by MAPK interacting serine/threonine kinase 1 (MNK1; also known as MKNK1)[112]

Regulation of MAPKs

The first example of β-arrestin-mediated signalling reported was β-arrestin 1 recruitment of activated SRC, a non-receptor tyrosine kinase, leading to the downstream activation of extracellular signal-regulated kinase (ERK)[16,17]. In the case of the neurokinin 1 (NK_1) receptor, SRC recruitment by β-arrestin 1 is necessary for the prevention of apoptosis and the propagation of mitogenic signals[17]. For protease-activated receptor 2 (PAR2), β-arrestins recruit MAPK superfamily members upon agonist stimulation, resulting in the formation of a complex comprising activated receptor, β-arrestin 1, RAF1 and phosphorylated ERK[18]. Agonist binding to the angiotensin II type 1A (AT_{1A}) receptor results in the formation of a β-arrestin 2, RAF1, MAPK/ERK kinase (MEK1) and ERK1/2 signalling complex[19]. Additionally, overexpression of β-arrestin 1 or β-arrestin 2 decreases phosphoinositide hydrolysis and increases ERK activation upon stimulation of the AT_{1A} receptor with angiotensin II. However, this sequence of events is not accompanied by increased activity of the ELK1 transcription factor, which is typically associated with increased phosphorylated ERK generation mediated by G proteins[20], which then leads to the transcription of immediate-early response genes.

Formation of these signalling complexes is likely to be of physiological relevance as ERK activation that is mediated by β-arrestins seems to have different consequences at the biochemical level from those activated by G proteins. β-arrestin-mediated phosphorylated ERK is retained in endocytic vesicles[21] in a pathway that is spatially and temporally distinct from activation of phosphorylated ERK by G proteins. In HEK293 cells transiently transfected with the AT_{1A} receptor, G protein activation of phosphorylated ERK is maximal at early time points (after ~2 minutes), whereas β-arrestin-mediated activity peaks later and is more protracted, accounting for 100% of phosphorylated ERK activity at 30 minutes[22]. β-arrestin-dependent ERK activation does not show the typical nuclear localization that is characteristic of G protein-activated ERK, and therefore does not result in activation of transcription factors such as ELK1 (REF. [20]). Moreover, β-arrestin-dependent ERK activity seems to have predominant effects on chemotaxis and cytoskeletal rearrangements[23,24].

Regulation of other kinase families

The β-arrestins can also regulate signalling through several other kinase families. For example, in mouse embryonic fibroblasts lacking both β-arrestin isoforms, the receptor tyrosine kinase insulin-like growth factor 1 receptor (IGF1R) is unable to stimulate PI3K activity, an effect that is rescued by expression of exogenous β-arrestin 1 (REF. [25]).

AKT, a downstream target of PI3K, also has its activity upregulated and downregulated by β-arrestin-dependent mechanisms. In the case of protease-activated receptors, separate G protein and β-arrestin-1-dependent mechanisms for AKT activation have been identified, although the functional consequences of these differences are unclear[26]. β-arrestin 2 also regulates anti-apoptotic signalling through phosphorylation of BCL2-associated agonist of cell death (BAD) by AKT[27]. In diabetic mice, β-arrestin 2 is significantly downregulated, which results in decreased activity of SRC and AKT, leading to decreased insulin signalling[28]. However, for signalling mediated by the D_2 dopamine receptor in the corpus striatum, a complex comprising β-arrestin 2, AKT and protein phosphatase 2A (PP2A; also known as PPP2R4) leads to

dephosphorylation and inactivation of AKT and reciprocal activation of glycogen synthase kinase 3β (GSK3β), which is responsible for a subset of dopamine-dependent behaviours[29].

β-arrestins probably regulate the activity of several other kinase families. For instance, a proteomic analysis of β-arrestins identified a wide range of kinase families that interact with either β-arrestin 1 or β-arrestin 2 (REF. [30]).

Regulation of transcription

There are numerous examples of β-arrestins controlling transcription (reviewed in REF. [31]). Transcription is modulated either indirectly through the regulation of signalling pathways that control transcription factors or directly through its activities in the nucleus. Both β-arrestin isoforms have amino-terminal nuclear localization signals, although it seems that β-arrestin 1 plays a more significant role in nuclear processes as it is present in both the nucleus and the cytosol. By contrast, β-arrestin 2 contains a nuclear export signal and is constitutively exported to the cytosol. However, β-arrestin 2 can be shuttled to the nucleus, as it can regulate transcription upon stimulation of the odorant receptor OR17-4 (also known as OR1D2) in spermatozoa[32]. β-arrestins are capable of binding and stabilizing IκB (an inhibitor of NF-κB nuclear translocation and transcriptional activation), and both β-arrestin isoforms can stabilize the IκB–NF-κB complex[33,34]. Overexpression of either β-arrestin results in inhibition of NF-κB-mediated transcription[34], an effect that can be reversed by β-arrestin phosphorylation[33].

β-arrestin 2 can also increase the activity of nuclear receptors, such as the retinoic acid receptors, by regulating their phosphorylation by ERK[35]. Through its regulation of Wnt signalling via the frizzled family of 7TMRs, β-arrestins regulate the activity of β-catenin, thereby modulating the activity of the TCF/LEF family of transcription factors[36,37]. A more direct role of transcription regulation has been demonstrated for β-arrestin 1, which binds to the promoter regions of several genes, including *FOS* and *p27* (also known as *PSMD9*), upon stimulation of δ-opioid and κ-opioid receptors. This results in the recruitment of the histone acetyltransferase p300, through an interaction with the p300 binding partner cAMP responsive element binding protein (CREB), and leads to increased transcription of those genes[38]. Activated nuclear β-arrestin 1 can limit interferon-γ-stimulated transcription by recruiting T-cell tyrosine phosphatase, which leads to an increase in signal transducer and activator of transcription 1 (STAT1) tyrosine dephosphorylation[39].

It is likely that further examples of β-arrestin scaffolding complexes that regulate transcription in the nucleus will be discovered, as it represents a common regulatory mechanism for a wide range of receptors and their downstream gene targets.

Other β-arrestin-regulated processes

The β-arrestins regulate a wide range of other cellular processes such as 7TMR transactivation of the epidermal growth factor receptor (EGFR), receptor trafficking (in addition to internalization), and the activity of small GTPases.

EGFR transactivation by 7TMRs is a well-characterized process that occurs via ligand-dependent and ligand-independent mechanisms[40]. The β-arrestin-mediated transactivation of EGFR occurs in a SRC-dependent manner upon agonist binding to the prostaglandin E4 (EP_4) receptor[41], which has been shown to lead to an increase in the metastatic progression of colorectal cancer. Similar β-arrestin-mediated activity occurs at the β_1-adrenergic receptor[42], in which activation of EGFR results in the activation of cardioprotective pathways as well as of the AT_{1A} receptor[43].

A key role for β-arrestins in the control of 7TMR trafficking has been demonstrated in the translocation of the Smoothened signalling complex to the primary cilium through the activity of kinesin motors[44], a process that is distinct from receptor internalization or recycling.

Another pathway that is regulated by β-arrestins is the AT_{1A} receptor-dependent activation of the small GTPase RhoA, which leads to the formation of stress fibres in a β-arrestin-2-dependent manner[45].

Thus, the range of β-arrestin-mediated activity is wide, ranging from its classical function in desensitization, internalization and trafficking of receptors, to signalling via control of kinase signalling pathways, receptor transactivation and transcriptional regulation (BOX 1). β-arrestin-mediated signalling significantly expands the repertoire of 7TMR effectors from the well-characterized canonical pathways mediated by heterotrimeric G proteins to a wide array of other pathways mediated by β-arrestins that have yet to be fully explored.

The concept of biased agonism

Agonist binding to 7TMRs frequently results in the activation of multiple downstream effector pathways, a phenomenon that has been referred to as pluridimensional efficacy[46]. Initially, this behaviour was thought to be due to cell-specific effects and receptor heterogeneity[47]. However, it was then discovered that 7TMRs can couple to multiple G proteins and activate multiple signalling pathways[47]; for example, α_2-adrenergic receptor subtypes can couple to both $G\alpha_s$ and $G\alpha_i$ (REF. [48]). Moreover, signalling through these parallel pathways can differ depending on the ligand used to stimulate the receptor, thereby resulting in a biased response (reviewed in REF. [47]).

An early example of biased agonism was demonstrated with two agonists of the muscarinic acetylcholine receptor: carbachol and pilocarpine. Binding of carbachol results in a balanced response that is mediated by both $G\alpha_s$ and $G\alpha_q$. By contrast, other ligands such as pilocarpine do not lead to $G\alpha_s$-mediated adenylyl cyclase stimulation, but do lead to phospholipase C (PLC) activity that is mediated by $G\alpha_q$ (REFs [49,50]). Such ligands are said to exhibit $G\alpha_s$ bias over $G\alpha_q$. These types of observations in a number of systems led to the proposal of a theoretical framework for this behaviour, termed agonist–receptor trafficking[51] and which we refer to as biased agonism in this Review.

Biased agonism is a property of the ligand–receptor complex, and so a ligand or a receptor may be biased. A biased ligand (FIG. 2) favours one response over another (either G protein or β-arrestin) compared with the endogenous ligand, which is considered to be neutral. A biased receptor (FIG. 2) is only capable of signalling through a restricted subset of pathways that are typically available to that class of receptor. So far, most examples of β-arrestin-biased ligands have been generated by chemical modification of native ligands. These include SII angiotensin (Sar^1, Ile^4, Ile^8)[52,53], which signals through the AT_{1A} receptor, and PTH-βarr ((D-Trp^{12},Tyr^{34})-PTH(7–34))[54], which signals through the parathyroid hormone 1 (PTH1) receptor. Biased receptors that have been generated by mutating key residues involved in G protein coupling include the AT_{1A} receptor mutant AT_{1A}R(DRY/AAY), in which residues of the highly conserved DRY motif have been mutated to AAY[52,55], and the β_2-adrenergic receptor mutant β2AR(TYY), which contains mutations of three residues crucial for G protein coupling[56]. The existence of bias necessitates a more complex model of 7TMR activation to be developed (BOX 2).

Box 2

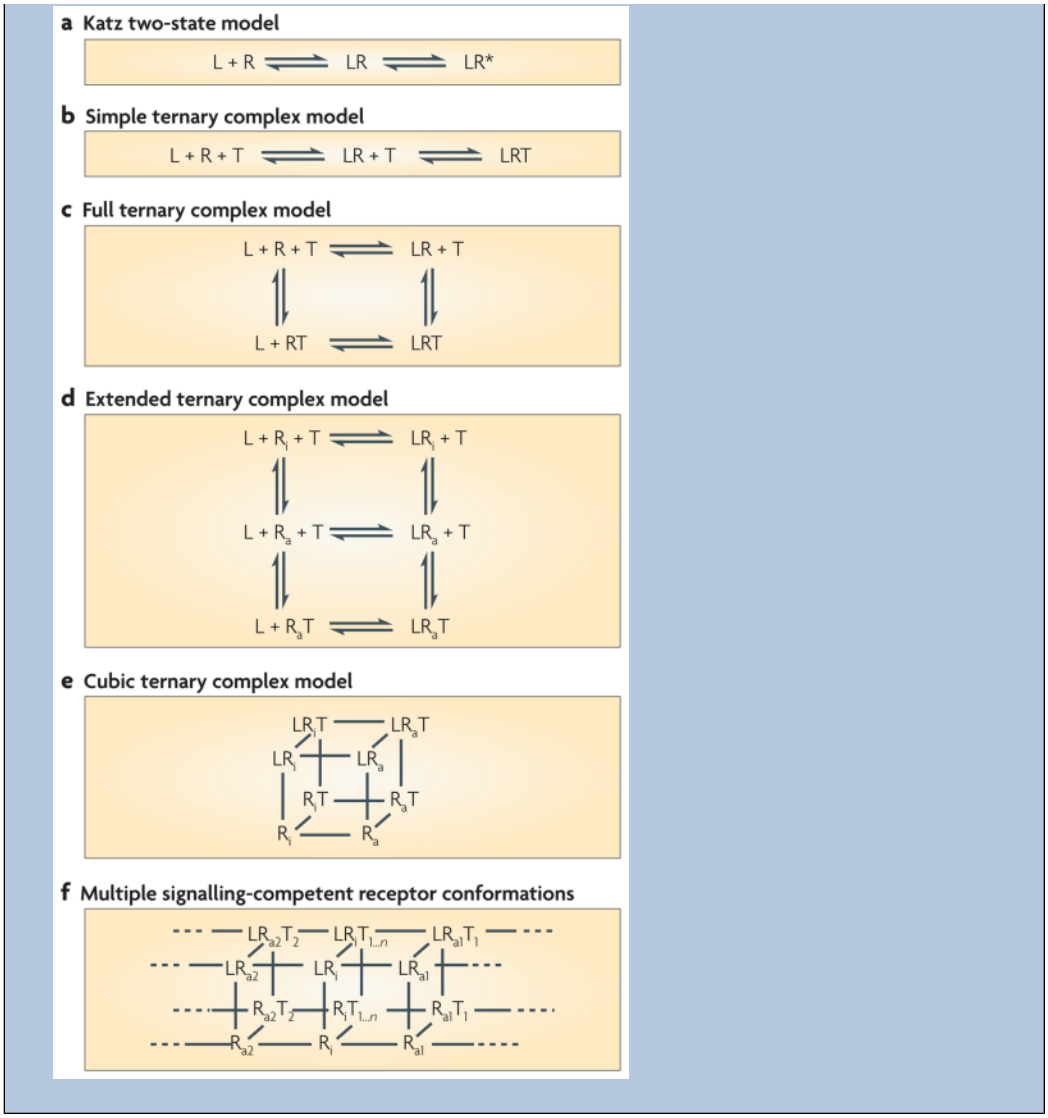
Models of seven-transmembrane receptor signalling

One of the oldest models for seven-transmembrane receptor (7TMR; R in the figure) activation is the Katz two-state model in which ligand (L) binding to the receptor results in formation of a ligand–receptor (LR) complex that generates an active receptor conformation (R*) that signals to downstream effectors (**a**). This model was subsequently modified to a simple ternary complex model, which included a ligand–receptor–G protein (or transducer; T) (LRT) complex (**b**). A more detailed ternary complex model[113] accounted for the high and low affinity states of the receptor and receptor transducer complex that the ligand could bind to (**c**). The discovery of constitutively active 7TMR mutants resulted in the development of an extended ternary complex model[114] (**d**), in which the receptor can adopt an active conformation (R_a) in the absence of ligand or transducer binding. A cubic model (**e**) of receptor activation includes distinct active and inactive receptor (R_i) conformations for transducer-bound receptor[115], which increases the number of states compared with the extended ternary complex.

In all of these models, it is assumed that the receptor has a single signalling-competent conformation that results in activation of all signalling pathways. So, ligands can be classified as partial or full agonists, which increases the percentage of receptors in the active state; as neutral antagonists, which do not change the percentage of receptors in an active state compared with the inactive state; and as inverse agonists, which decrease the number of receptors in the active state relative to the inactive state.

To account for biased agonism, a model of 7TMR signalling must take into account the fact that receptors can signal through different pathways with different efficacies. The idea that this occurs through distinct receptor conformations is supported by studies demonstrating such states are associated with differential signalling capacity[116,117]. An additional state(s) for receptor activation is therefore required, with distinct conformations for signalling through G proteins or β-arrestins (R_{a1}, R_{a2}, and so on), as well as additional ternary complexes with the receptor bound to other signal transducers (T_1, T_2, and so on) such as β-arrestin (**f**). The concept of such additional states is supported by the demonstration of low and high ligand-affinity receptor states associated with β-arrestin binding similar to those observed for receptor complexes with heterotrimeric G proteins[118].

A consequence of this model is the presence of biased agonists, which preferentially generate either a G protein or a β-arrestin ternary complex. Adding further to the complexity of multi-state models for receptor activation is the observation that some ligands bind at allosteric sites, which are structurally distinct from the orthosteric site, leading to effects on receptor activity. These allosteric modulators can significantly affect receptor activity and bias[119].



Quantifying ligand bias

Quantifying ligand bias — that is, how much a certain ligand produces bias in a given cellular context — is an active area of research. Ligands that have full efficacy for G protein pathways and half such efficacy for β-arrestin pathways, as well as other ligands that have full efficacy for G proteins and no efficacy for β-arrestins, would all be considered to be G protein-biased ligands. How can we differentiate between these levels of bias? One of the simplest ways to quantify agonist bias is to plot β-arrestin activity against G protein activity[57] (FIG. 3a). This activity could be related to efficacy, potency or another measure of quantifying the effective coupling of the ligand–receptor complex to a specific signalling pathway[8,58]. β-arrestin and G protein activity can also be represented as a matrix that incorporates data from multiple assays, or as ligand bias factors that compare β-arrestin activity against G protein activity in different assays (FIG. 3b). The quantification of the relative levels of bias is important in the identification of lead compounds and in the optimization of drug screening for biased agonists[8].

There are still considerable gaps in our understanding of bias and in the different receptor conformations that are responsible for signalling to G proteins and β-arrestins. Some

hypotheses explore regulated bias, in which G protein versus β-arrestin signalling can be controlled by GRKs or other cofactors. Although no clear examples of regulated bias have yet been identified, there is evidence that GRKs can be dynamically regulated, leading to effects on 7TMR signalling. For example, S-nitrosylation of GRK2 reduces its phosphorylation of β-adrenergic receptors and reduces the subsequent recruitment of β-arrestins to the receptor, resulting in the attenuation of receptor desensitization and internalization[59]. There is also evidence to suggest that the selective activity of GRKs mediates distinct responses by different ligands at the same receptor. The chemokine receptor CCR7 has two ligands, CCL19 and CCL21, which have different efficacies for calcium mobilization and receptor desensitization[60]. Binding of either CCL19 or CCL21 results in G protein coupling and β-arrestin recruitment in a GRK6-dependent manner. However, only CCL19 binding results in GRK3-dependent β-arrestin internalization and receptor desensitization[61]. These findings and others that follow will undoubtedly change our understanding of biased signalling by 7TMRs, and therefore will necessitate new models of their activity to be developed.

Biasing 7TMR physiology

The existence of 7TMR-mediated signal transduction that is G protein-independent requires a reassessment of the roles of specific biochemical inputs into all 7TMR-regulated physiological processes. Although the physiological consequences of β-arrestin-dependent signalling are only starting to be elucidated, several biased ligands and 7TMRs signal predominantly through either G protein- or β-arrestin-mediated pathways. These may serve to enhance our understanding of the different contributions of specific pathways to the regulation of physiological functions, and also as targeted agents for clinical therapies. In this section, we highlight the physiological consequences of this signalling in some of the better characterized systems.

β_1- and β_2-adrenergic receptors

Ligands for the $G\alpha_s$-coupled β_1- and β_2-adrenergic receptors — which are involved in the pathogenesis of diseases such as heart failure and asthma — are essential therapeutic agents. Specifically, agonists of β-adrenergic receptors function as positive inotropes, and are frequently used for the acute treatment of systolic ventricular dysfunction in decompensated heart failure. By contrast, antagonists of these receptors, commonly referred to as beta-blockers, are used for the chronic treatment of heart failure, in which they act via poorly understood mechanisms to mediate cardioprotection. In addition, β_2-adrenergic receptor agonists are used in the acute and chronic setting as bronchodilators in the treatment of obstructive lung diseases such as asthma and chronic obstructive pulmonary disease.

Recent studies have characterized the properties of medically relevant beta-blockers at the β_1- and β_2-adrenergic receptor level. In a study of 16 clinically relevant β_2-adrenergic receptor antagonists[62], the agents were roughly divided equally between weak partial agonists and inverse agonists with respect to $G\alpha_s$ activation; that is, no agent was a neutral antagonist. Of the inverse agonists, carvedilol was found to stimulate $G\alpha_s$-independent, β-arrestin-2-dependent activation of ERK. In a similar study that specifically examined β_1-adrenergic receptor-mediated EGFR transactivation — a process that was previously shown in the heart to be dependent upon β-arrestins[42] — only alprenolol and carvedilol induced EGFR internalization[63]. Both drugs stimulated EGFR-dependent ERK activation, and these effects were dependent upon phosphorylation of the β_1-adrenergic receptor and the presence of both β-arrestin 1 and β-arrestin 2. Thus, β-adrenergic receptor antagonists that are used in the clinical setting have divergent effects on $G\alpha_s$- and β-arrestin-mediated signalling. However, the physiological ramifications of these findings, and the implications of β-arrestin-mediated signalling via the β-adrenergic receptors in general, are currently unknown.

AT_{1A} receptor

One of the first examples of a β-arrestin-biased ligand was SII angiotensin, a synthetically modified form of angiotensin II that binds the AT_{1A} receptor[52,53]. It is unable to activate $G\alpha_q$ signalling (as evidenced by the lack of phosphoinositide hydrolysis, calcium mobilization or diacylglycerol activity), but retains the ability to recruit β-arrestin 2, resulting in receptor internalization and activation of ERK in an entirely β-arrestin-2-dependent manner[52]. In a live *ex vivo* cardiomyocyte system, SII angiotensin exhibits positive inotropic and lusitropic properties[64]. Furthermore, while inhibition of the G protein-mediated pathway via protein kinase C (PKC) failed to affect the positive inotropic and lusitropic effects of SII angiotensin, deficiency of β-arrestin 2 or of the proximal kinase GRK6 abolished these effects. By contrast, the positive inotropic and lusitropic effects of angiotensin were markedly reduced by PKC inhibition, but were unaffected by deficiency of any of the β-arrestins or GRKs assessed[64]. Thus, angiotensin and SII angiotensin seem to be leading to the same positive effects in cardiomyocytes through pathways that are mediated by G proteins and β-arrestin 2, respectively. These data identify a role for G protein-independent, β-arrestin-dependent signalling (independent of effects on desensitization of G protein-mediated signalling), in the regulation of cardiovascular functions.

In a study designed to understand the role of the intracellular loops in signalling by the AT_{1A} receptor to G proteins[65], a mouse strain with cardiac-specific over-expression of a modified receptor with mutations in the second intracellular loop (called i2m) was shown to be uncoupled from $G\alpha_q$. In comparison with mice over-expressing a wild-type AT_1 receptor, overexpression of the i2m mutant resulted in marked ventricular dilation and eccentric hypertrophy, which was accompanied by diminished cardiomyocyte apoptosis. The effects on ventricular function were less clear, with only minor functional differences under resting conditions between the hearts from wild-type mice and i2m AT_1 receptor-overexpressing mice. Although we can conclude that these differences were due to G protein-independent signalling, the authors did not test whether such signalling was β-arrestin-dependent. Interestingly, the hearts from the i2m mice revealed activation of SRC and cytoplasmic sequestration of activated ERK, which is consistent with selective β-arrestin-mediated signalling. From these studies it seems that AT_1 receptor β-arrestin pathway-biased ligands may have beneficial effects on ventricular function while enhancing cardiomyocyte survival.

μ-opioid receptor

The μ-opioid receptor is the target for endogenous enkephalin peptides, as well as for exogenous opioid analgesics (that are agonists) such as morphine. In addition, antagonists of the μ-opioid receptor, such as naloxone and its derivatives, are used in the treatment of substance abuse. Enkephalins are balanced agonists for G protein- and β-arrestin-mediated activities, whereas morphine provokes considerably less receptor phosphorylation and internalization, which is consistent with bias towards G protein-mediated signalling[66]. However, in β-arrestin 2 knockout mice, morphine-induced analgesia is amplified and prolonged relative to wild-type mice, which is consistent with the presence of some morphine-induced β-arrestin-mediated desensitization[67]. Surprisingly, loss of β-arrestin 2 has no effect on tolerance induced by balanced agonists that strongly recruit β-arrestin 2, such as fentanyl and methadone[68]. Although, the role of β-arrestin 2 recruitment and signalling in the development of opioid tolerance is still unclear, β-arrestin 2 knockout mice are protected from the side effects of morphine, including respiratory depression and constipation, suggesting that β-arrestin-mediated pathways control these peripheral side effects[69]. Therefore, a purely G protein-biased agonist may be expected to have the antinociceptive effects of the opioid analgesics without some of their problematic side effects.

D_2 dopamine receptor

D_2 dopamine receptors are involved in mental illness, an effect that was originally thought to occur through $G\alpha_i/G\alpha_o$-mediated inhibition of adenylyl cyclase[70]. However, more recent behavioural and biochemical evidence has demonstrated that β-arrestin 2 plays a crucial role in signal transduction by D_2 dopamine receptors through regulation of the AKT–GSK3 pathway[71]. Stimulation of D_2 dopamine receptors results in the formation of a protein complex comprising β-arrestin 2, AKT and PP2A, which facilitates the dephosphorylation of AKT in response to dopamine[29]. This complex is a target of lithium — a drug used for the treatment of bipolar disorder and other psychiatric illnesses — the behavioural effect of which is lost in β-arrestin 2 knockout mice[72]. β-arrestin 2 knockout mice also display a number of defects in behaviours regulated by dopamine, including reduced apomorphine-induced climbing and reduced responsiveness to dopamine-dependent actions of amphetamine and morphine[29,73]. In addition, these knockout mice have a reduction in the typical novelty-induced locomotor hyperactivity phenotype of dopamine transporter knockout mice[74].

5-HT_{2A} and 5-HT_{2C} receptors

The 5-hydroxytryptamine 2A (5-HT_{2A}) and 5-HT_{2C} members of the 5-HT (also known as serotonin) receptor family exhibit bias towards the activation of different G proteins (reviewed in REF. [75]). Activation of these receptors by different agonists can lead to different levels of phospholipase A2 (PLA2)-mediated arachidonic acid release (mediated by $G\alpha_i$) and PLC-mediated phosphoinositide hydrolysis (mediated by $G\alpha_q$)[76,77]. β-arrestin 2 colocalizes with 5-HT_{2A} receptors in rat prefrontal cortical neurons[78], and β-arrestins are essential for mediating 5-HT_{2A} receptor internalization by serotonin but not by the selective 5-HT_2 receptor agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI)[79]. Notably, generation of phosphorylated ERK by DOI is completely blocked by inhibition of PLC, whereas serotonin-mediated activation of phosphorylated ERK seems to be regulated by independent β-arrestin- and PLC-mediated pathways. *In vivo*, β-arrestin 2 knockout mice do not display a typical head-twitch response to high doses of serotonin, whereas treatment of these mice with DOI results in an equivalent head-twitch response to wild-type mice[79]. Therefore, it seems that DOI is a G protein-biased agonist for 5-HT_{2A} receptors, although the full physiological consequences of this are unclear.

CXCR4

The chemokine (C-X-C motif) receptor CXCR4 is a $G\alpha_i$-coupled 7TMR that binds the endogenous agonist ligand CXCL12 (also known as SDF-1a). A truncation mutation in the cytoplasmic tail of CXCR4 is found in WHIM (warts, hypogammaglobulinaemia, infections, myelokathexis) syndrome[80], and leukocytes from patients with this syndrome display defective CXCR4 desensitization and enhanced chemotaxis. In WHIM syndrome, agonist-induced receptor internalization of CXCR4 — which is regulated by GRK3 and β-arrestin 2 (REF. [80]) — is defective, although this internalization does not underlie the enhanced chemotaxis. Chemotaxis of CXCR4-bearing leukocytes from patients with WHIM syndrome requires β-arrestin 2 signalling, as additional mutation of residues that are necessary for β-arrestin 2 recruitment (without affecting G protein coupling) abolishes the augmented chemotactic responses of leukocytes derived from patients with WHIM syndrome. This suggests that the truncated receptor is capable of regulating β-arrestin-2-mediated signalling but not β-arrestin-2-mediated internalization or endocytosis.

PTH1 receptor

PTH is an 84 residue peptide and is the principal regulator of calcium and phosphate homeostasis. Binding of the N-terminal 34 residues of PTH, PTH(1–34), to the $G\alpha_s$- and $G\alpha_{q/11}$-coupled PTH1 receptor, results in full agonist activity. PTH-βarr, an inverse agonist of PTH1 receptor-mediated G protein-mediated signalling, results in β-arrestin-mediated ERK

activation[54]. PTH-βarr also induces anabolic bone formation in mice in a similar manner to PTH(1–34), an effect that is attenuated in β-arrestin 2 knockout mice[81]. Thus, it seems that both G protein and β-arrestin-mediated pathways are involved in the anabolic response to PTH1 receptor activation. However, the biochemical differences between these two pathways have yet to be fully elucidated, and the full physiological impact of biased signalling at this receptor remains to be understood.

GPR109A

Niacin (also known as nicotinic acid or vitamin B3) — which stimulates the 7TMR GPR109A and acts through $G\alpha_i/G\alpha_o$ proteins — lowers triglycerides and raises high-density lipoprotein, but its clinical use is limited by cutaneous flushing[82]. Niacin stimulation of GPR109A lowers cAMP levels and recruits β-arrestins to the receptor[83]. This leads to β-arrestin-mediated signalling to ERK/MAPKs and binding of β-arrestins to activated cytosolic PLA2. Although the interaction of β-arrestin 1 with PLA2 generates arachidonate, which ultimately leads to the flushing response, this signalling does not result in the lowering of serum free fatty-acid levels. As β-arrestin 1 mediates the cutaneous flushing and G proteins mediate the lowering of free fatty-acid levels in serum, it might be expected that a G protein-biased ligand would provide the beneficial effects of lowering lipid levels without the side effect of flushing. Indeed, a recently synthesized partial agonist of the receptor that retains its antilipolytic activity without vasodilatory side effects seems to work via a G protein-biased signalling mechanism[84].

Discovery of biased agonists as drugs

G protein- and β-arrestin-biased agonism have important implications for the design of therapeutics that target 7TMRs, as signalling through these parallel pathways has distinct consequences at molecular and functional levels. As our understanding of these differences grows, there will be better appreciation of the importance and relevance of different aspects of biased signalling at 7TMRs. Although experiments have demonstrated significant functional differences between G protein- and β-arrestin-mediated signalling using biased agonists (for example, for AT_{1A} receptor, PTH1 receptor and β_2-adrenergic receptor), most have utilized genetically modified systems (for example, transgenic animals or small interfering RNA knockdown). Moreover, understanding of the physiology of β-arrestin-mediated signalling is, so far, largely limited to studies in cell culture or in small animals, with little or no data from large animal or human studies. Thus, the true therapeutic impact of biased agonists will not be known until they are tested in clinical trials. As described above, there are already several well-documented situations in which development and testing of such agents seems clearly justified. It seems likely that many more remain to be discovered.

The discovery of β-arrestin-mediated signalling necessitates a reassessment of the methods used for 7TMR drug discovery, otherwise potentially useful drugs that act primarily through non-canonical pathways, such as those mediated by β-arrestins, will continue to go unrecognized. Assays for drug discovery at 7TMRs have typically focused on identifying proximal responses that are typical of signalling by Gα and Gβγ subunits. These include the use of cell-based assays[85] that measure reporter-gene expression or levels of second messengers that can be easily read with multi-well plate readers, for example, increases or decreases in second messengers such as cAMP or calcium[86]. As these assays of canonical G protein activity alone give an incomplete picture of a compound's effects on cell signalling, several approaches directed towards addressing β-arrestin recruitment and signalling have been developed over the past few years[87].

Redistribution assays

As β-arrestins are recruited to 7TMRs to facilitate their internalization, a number of assays (such as the Thermo Redistribution assay and the TransFluor assay[88] from Molecular Devices) have been designed on the basis of visualizing agonist-stimulated changes in fluorescently-labelled 7TMR or β-arrestin distribution[89] (TABLE 1).

In β-arrestin redistribution assays, treatment with ligand results in one of two patterns for recruitment of fluorescently labelled β-arrestin (FIG. 4a). In the class A pattern, which is observed for receptors such as the β_2-adrenergic receptor, β-arrestin is recruited to the plasma membrane and the 7TMR typically undergoes rapid recycling after internalization. In the class B pattern, which is observed for receptors such as the AT_{1A} receptor, there is stronger and more prolonged binding of β-arrestin to the 7TMR such that following recruitment to clathrin-coated pits the receptor and β-arrestin remain bound together on the surface of endocytic vesicles and demonstrate a slow recycling pattern. The functional significance of this difference between class A and B patterns in β-arrestin recruitment is not completely understood. The prolonged association of β-arrestins with the receptor is thought to inhibit 7TMR resensitization, although other evidence argues for different mechanisms regulating receptor recycling (reviewed in REF. [90]).

If the receptor is labelled in a redistribution assay, receptor internalization in response to agonist stimulation can be monitored (although no difference in β-arrestin recruitment pattern can be observed). In either labelling scheme, of β-arrestin or receptor, the formation of endocytic vesicles can be visualized by fluorescence microscopy or quantified by automated image acquisition and analysis.

Proximity assays

Proximity assays monitor changes in the proximity of β-arrestins to a 7TMR C terminus upon agonist binding (TABLE 1). Such assays are usually straightforward to scale up for high-throughput screening and, depending on the technology used and the system being studied, can be extremely sensitive to the β-arrestin–receptor interaction. Usually, proximity assays monitor the distance between the 7TMR and the β-arrestin by using reporter molecules attached to both the 7TMR and β-arrestin. Frequently, these reporters are spectroscopically active, allowing changes in intermolecular resonance energy transfer between fluorescent[91] or bioluminescent[92] probes — known as fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET), respectively — to be detected between suitably labelled 7TMRs and β-arrestins (FIG. 4b).

Other proximity assays are based on enzyme complementation or other strategies. For example, when β-galactosidase is fused to the C-terminal tail of the 7TMR and its complementarity deletion mutant is fused to β-arrestin[93,94], β-arrestin recruitment results in complementation and hydrolysis of a chemiluminescent substrate (FIG. 4b). Alternatively, the C terminus of the 7TMR can be extended with a protease cleavage site and transcription factor with the corresponding protease linked to β-arrestin. β-arrestin recruitment results in cleavage of the C terminus of the 7TMR, release of the transcription factor and subsequent induction of reporter-gene expression[95] (FIG. 4b). Proximity assays based on enzyme activity tend to be highly sensitive because of the significant signal amplification associated with enzyme activity or reporter-gene expression, thereby allowing the identification of weak partial agonists that are not identified by other assays.

Conformation assays

New methods for monitoring receptor and β-arrestin conformations associated with signalling are currently being developed[87]. Receptor conformation has been monitored through

fluorescent labelling of loops or segments that are involved in receptor activation[96]. This has been performed for polymorphic variants of the β_1-adrenergic receptor, which demonstrated discrete conformations depending on the type of ligand bound to the receptor[97] (FIG. 4c). Therefore, monitoring β-arrestin conformation is likely to be a fruitful approach to screen both for 7TMR binding and for conformations that may be associated with specific signalling pathways, thus acting as a primary screen for ligand binding as well as a screen for downstream activity.

An intramolecular β-arrestin 2 BRET biosensor has been designed in which β-arrestin 2 is sandwiched between luciferase and yellow fluorescent protein (YFP). Upon 7TMR activation, β-arrestin 2 undergoes a conformational change that results in an alteration in distance or orientation between the luciferase and YFP, thereby changing the BRET signal[98]. This biosensor displays different BRET signatures associated with receptor binding to β-arrestin-biased agonists compared with balanced or unbiased agonists at the AT_{1A} receptor, the β_2-adrenergic receptor and the PTH1 receptor[99] (FIG. 4c). These results suggest that distinct β-arrestin conformations are responsible for functionally selective responses. If these β-arrestin conformations could be correlated with distinct responses in a relevant system — for example, MAPK activation — such assays could yield insights into ligand binding and its downstream consequences, an area that is currently experiencing active investigation.

Signalling assays

As the full range of β-arrestin-mediated cell signalling has yet to be completely characterized, assays of cell signalling for drug screening are currently limited, but such assays may be fruitful in the future. Monitoring ERK1/2 phosphorylation, a well-characterized pathway regulated by β-arrestins, has been proposed as one method for assessing β-arrestin-mediated signalling[7]. Based on the presence of β-arrestin-mediated transcriptional activity, reporter assays could also be designed based on activation of selected signalling pathways. Other plausible strategies include assays of other characterized responses that are mediated by β-arrestins (such as chemotaxis[24] or stress-fibre formation[45]), and performing high-throughput assays in the presence of inhibitors of G protein signalling (such as pertussis toxin for $G\alpha_i$). Unfortunately, the applicability of such assays is limited by the current incomplete knowledge regarding β-arrestin-mediated signalling in many 7TMR systems.

Perspective on assays to measure β-arrestin activity

Currently, redistribution and proximity assays are the most well-developed and the easiest to apply to most 7TMR systems; however, they do have limitations (TABLE 1). One drawback of proximity assays is the requirement for labelling of the receptor with a probe, usually at the C terminus. Although such modifications usually do not affect ligand binding, theoretically they may have an effect on signalling and thus modify the recruitment of β-arrestins. This drawback of proximity assays is balanced by their easy scalability to high-throughput formats and their high sensitivity, which results from the amplification that is inherent to enzymatic or reporter systems. Redistribution assays may not require labelling of the receptor, but are not as straightforward to scale up in a high-throughput manner.

Conformational assays hold the promise of being surrogates for functional responses by the identification of active conformations of a receptor or a β-arrestin. Unfortunately, conformational assays lack sensitivity, thus limiting their utility for primary screening.

β-arrestin-mediated signalling assays need to be validated for the 7TMR and cell type being studied, but hold the promise of being as high throughput as the cell-based assays currently used for assaying G protein-mediated signalling. This field is still young and as we gain a better

understanding of β-arrestin-mediated signalling pathways over the coming years, the role of these different assays in the drug development process will become clearer.

Concluding thoughts

Most classes of cell-surface receptors are appreciated to signal via various proximal effector molecules. Until recently, an exception to this rule was 7TMRs, which had been viewed as a simple on-off switch that used a single class of effector molecule: the heterotrimeric G proteins. However, converging lines of evidence demonstrate the existence of G protein-independent signal transduction and its unique biochemical and physiological effects.

Most prominent among these pathways is signal transduction mediated through the GRK–β-arrestin system, which not only desensitizes G protein-mediated signalling, but also initiates distinct signalling cascades. Recent studies highlight the complexity of signal transduction via the GRK–β-arrestin system, and the development of biased ligands suggests the ability to modulate 7TMR signal transduction efficaciously and specifically, selectively targeting a wide range of possible biochemical and physiological responses. Thus, a diverse array of ligands can potentially be developed for a given 7TMR, each of which may have unique signalling properties.

These biased ligands may be exploited for use as tools for understanding the basic biology of 7TMRs, and, potentially and most importantly, as fine-tuned therapeutics that maximize beneficial effects and minimize adverse effects[100]. For example, for conditions such as asthma or catecholamine-resistant shock, β-adrenergic receptor agonists that have the capacity to signal through G proteins without desensitization mediated by β-arrestins would be predicted to be more effective than current β-adrenergic receptor agonists that display significant tachyphylaxis. The potential therapeutic superiority of biased over unbiased ligands in these and other circumstances remains to be demonstrated in clinically relevant systems. As more work is done to better understand the full range of β-arrestin-mediated signalling from both biochemical and physiological perspectives, it seems likely that numerous other targets will emerge. A systems biology approach using mass spectrometry has yielded a number of possible roles for β-arrestins by identifying their binding partners[30]. Such experiments, combined with physiological studies of biased agonists in animal models, will yield much greater insights into these areas.

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Glossary

G protein	A heterotrimeric protein that exchanges GDP for GTP in its α-subunit on agonist binding to a seven-transmembrane receptor, resulting in dissociation of the complex into Gα and Gβγ subunits. These subunits lead to the activation of second messenger systems through the regulation of enzymes such as adenylyl cyclase or phospholipase C
Arrestins	Multifunctional adaptor proteins that are important in regulating desensitization and signalling by seven- transmembrane receptors and other transmembrane receptors

β-catenin	A multifunctional adaptor protein. One of its roles includes the regulation of TCF/LeF transcription factors in response to signalling by Wnts through the frizzled seven-transmembrane receptors
Transactivation	The activation of a transmembrane receptor that results from signalling caused by activation of another receptor. In the case of epidermal growth factor receptor (eGFR) transactivation, agonist binding at a number of seven-transmembrane receptors activates a pathway that releases a membrane- bound eGF ligand by proteolytic cleavage, which then activates the eGFR
Biased agonist	A ligand that results in the activation of select, but not all, available signalling pathways that are known to be activated by the receptor
Positive inotropes	An agent that increases the force of the heart's contraction. Negative inotropes decrease the force of contraction
Antagonist	A term that is commonly used to refer broadly to neutral antagonists, weak partial agonists and inverse agonists
Partial agonist	A ligand that when bound to a receptor results in a submaximal response. Partial agonists can antagonize full agonists
Inverse agonist	A ligand that decreases the signalling activity of the receptor on binding compared with the ligand-unbound state
Neutral antagonist	A ligand that results in no change in activity of the receptor on binding compared with the ligand-unbound state
Allosteric site	A binding site on a seven- transmembrane receptor that is different than the orthosteric site
Orthosteric site	The binding site on a seven-transmembrane receptor to which the endogenous agonist binds
Allosteric modulator	A ligand that binds to the allosteric site of the receptor and affects receptor responses to orthosteric ligands. Some allosteric modulators are capable of generating biased responses
Lusitropic	Relates to the relaxation and filling of the heart. Positive lusitropic agents improve the heart's relaxation and filling
Full agonist	A ligand that completely activates the receptor on binding

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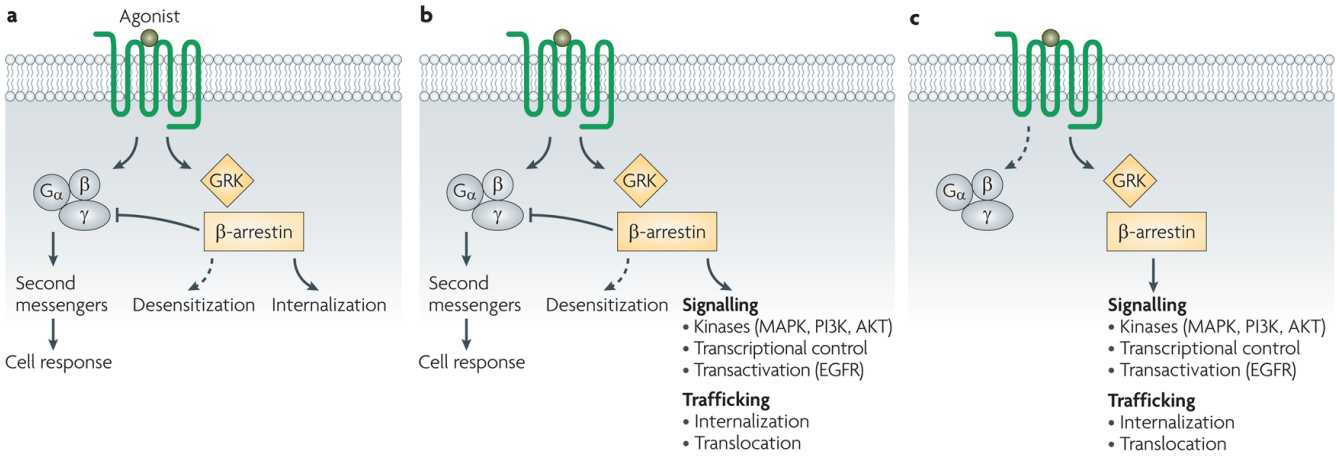

Figure 1. G protein and β-arrestin-mediated signalling

a | In the classical model for seven-transmembrane receptor (7TMR) activation, signalling is mediated by G proteins and desensitization is mediated by β-arrestins. **b** | In the current model for 7TMR activation, binding of a ligand results in activation of signalling by G proteins and β-arrestins, as well as desensitization and internalization by β-arrestins. **c** | In a system with biased agonism (β-arrestin-biased in this example), signalling only proceeds through one pathway. EGFR, epidermal growth factor receptor; GRK, G protein-coupled receptor kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase.

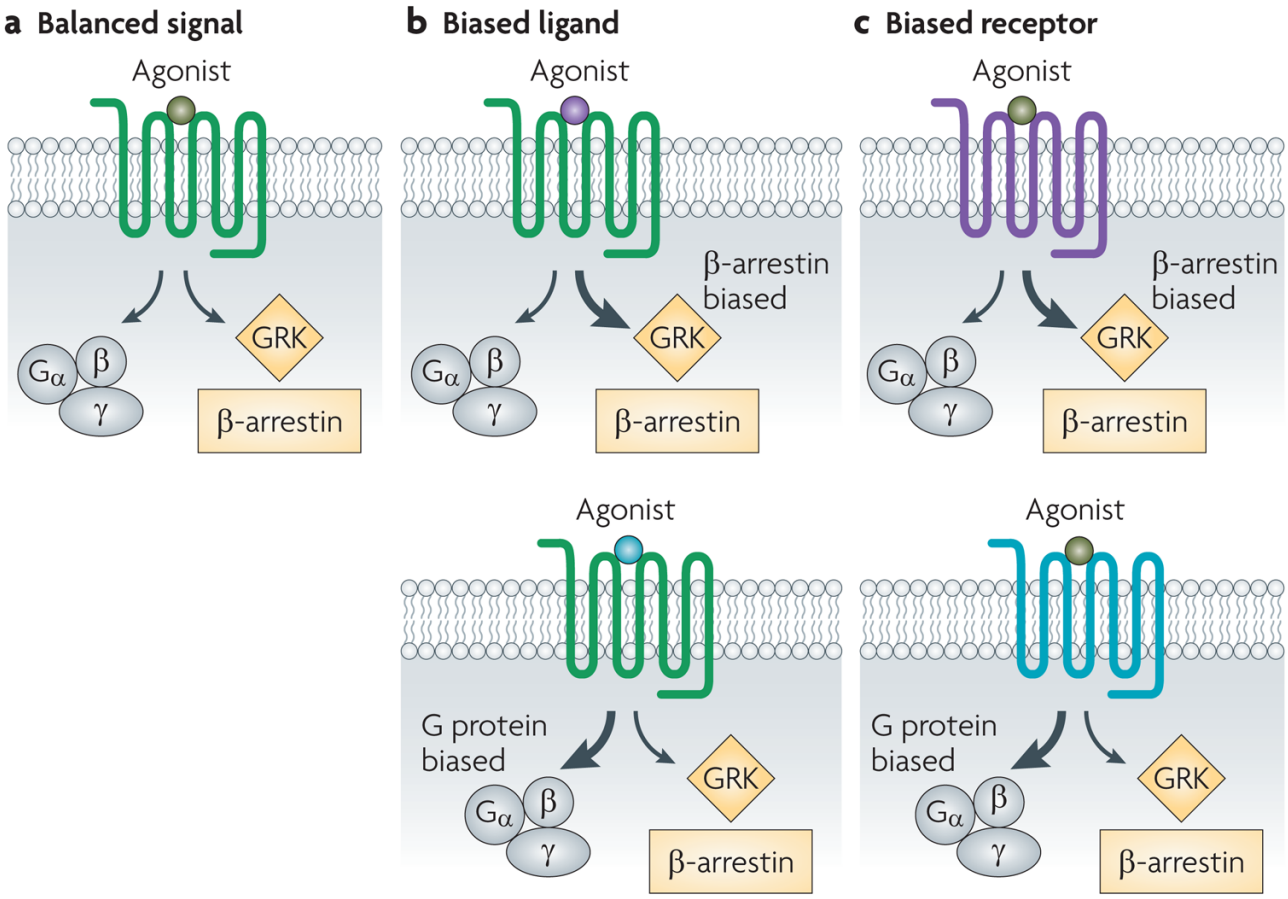

Figure 2. Biased ligands and biased receptors

In balanced signalling (**a**; green), binding of a ligand results in activation of signalling by G proteins and β-arrestins, as well as desensitization and internalization by β-arrestin alone. Two cases of biased agonism exist. In the case of a biased ligand (**b**), binding of a ligand (β-arrestin-biased, purple; G protein-biased, blue) to an unbiased receptor results in a biased response. In the case of a biased receptor (**c**), binding of an unbiased ligand to the biased receptor (β-arrestin-biased, purple; G protein-biased, blue) also results in a biased response. GRK, G protein-coupled receptor kinase.

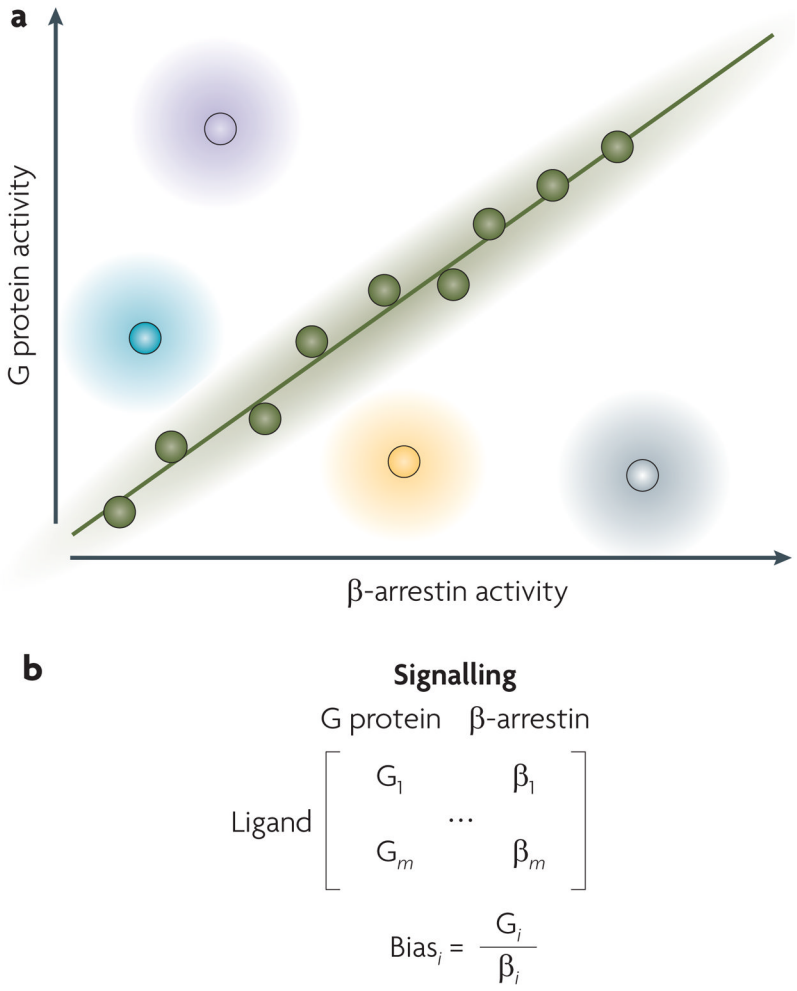

Figure 3. Quantifying the pluridimensional efficacies of seven-transmembrane receptors

a | One way to compare signalling between G protein- and β-arrestin-mediated pathways is to plot β-arrestin activity on the *x* axis and G protein activity on the y axis. Unbiased ligands would be expected to have equal levels of efficacy for β-arrestin- and G protein-mediated pathways, as shown by the green circles and the line. For biased ligands, there would be differing levels of β-arrestin- and G protein-mediated efficacies, as illustrated for β-arrestin-biased full agonists (grey), β-arrestin-biased partial agonists (yellow), G protein-biased partial agonists (blue) and G protein- biased full agonists (purple). **b** | Such data can also be represented in matrix form for *m* ligands (upper) or in terms of a bias factor for each ligand *i* (bottom).

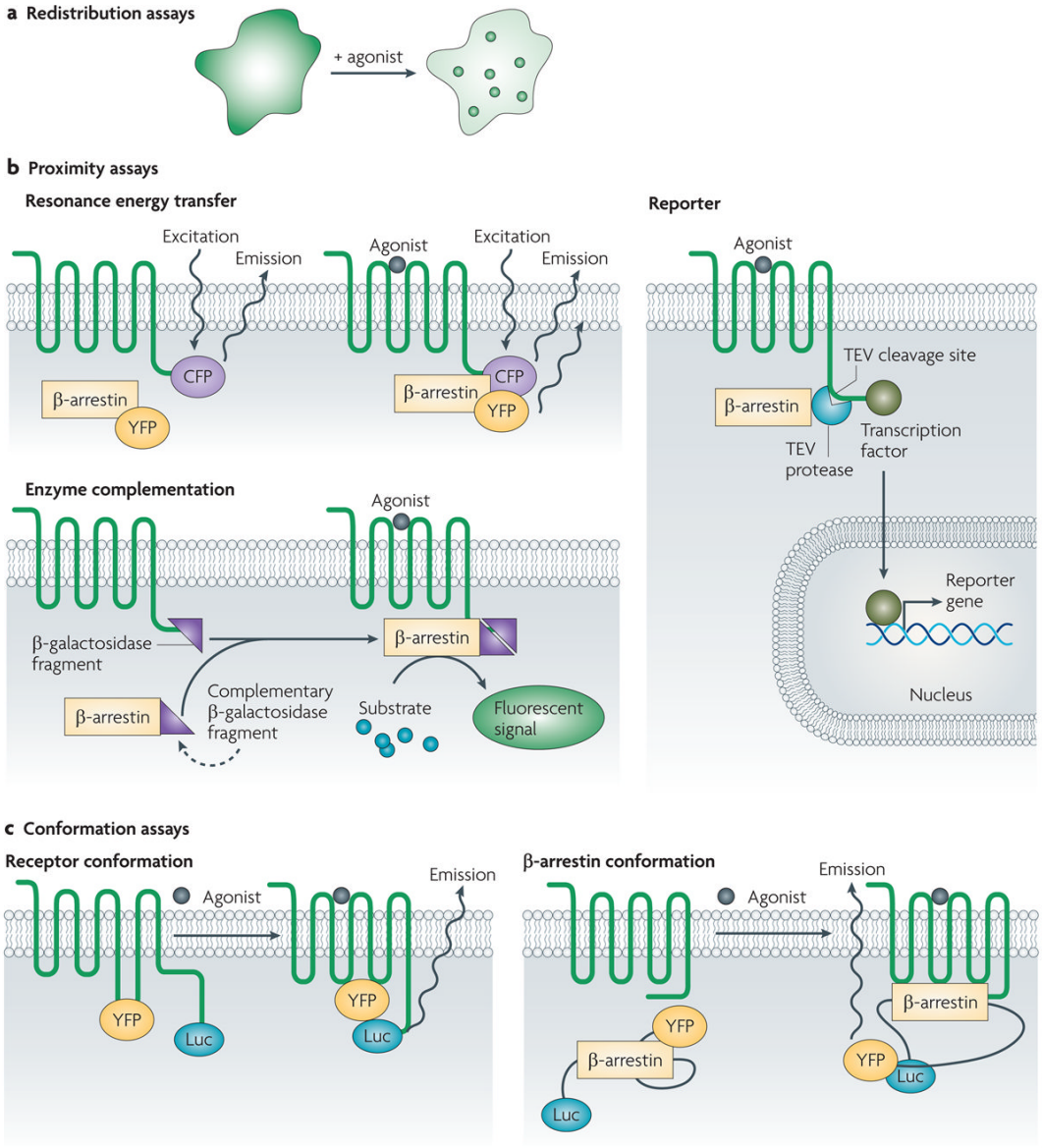

Figure 4. Assays for β-arrestin recruitment and activation

a | In redistribution assays, changes in distribution of β-arrestins (green) or receptor (not shown) can be used as a response. Before stimulation, the receptor is usually localized to the membrane, whereas β-arrestins are found largely diffusely in the cytosol. In class A receptors, β-arrestins translocate to the membrane while the receptor is internalized into rapid recycling endosomes. In class B receptors, β-arrestins are internalized with the receptors into slow recycling endosomes. **b** | In proximity assays, the proximity between the β-arrestin and the receptor is monitored using a number of strategies. In resonance energy transfer (RET) assays, a donor probe and an acceptor probe can be attached to the receptor and β-arrestin, respectively. Upon recruitment of the β-arrestin, emission of the donor probe, by excitation with light in fluorescence RET (FRET) or with chemiluminescent substrate in bioluminescence RET (BRET), results in energy transfer to the acceptor probe. Proximity can also be assessed using reporter-based assays, such as the Tango assay. Upon recruitment of the β-arrestin, a protease — tobacco etch virus (TEV) in this example — covalently linked to the β-arrestin cleaves a site and releases a transcription factor that was attached to a modified receptor. This

transcription factor then translocates to the nucleus resulting in reporter-gene expression. In assays based on enzyme complementation, the β-arrestin and the receptor are modified with fragments of an enzyme, which, upon recruitment of the β-arrestin, results in the formation of a functional enzyme. **c** | In conformation assays, changes in receptor or β-arrestin conformation are used to assess ligand activity and binding. In assays of receptor conformation, site-specific probes are incorporated in areas of the receptor that are thought to undergo significant structural change after ligand binding. Monitoring of FRET between these two fluorescent probes shows distinct changes associated with binding of the ligand. Similar strategies have been used to study the conformation of β-arrestin. Here, intramolecular BRET between luciferase (Luc) and yellow fluorescent protein (YFP) attached to the amino and carboxyl termini of the β-arrestin is used to monitor changes in β-arrestin conformation.

Table 1

Assays used in the discovery of β-arrestin-mediated signalling

Assay type	Technology	strengths	Weaknesses
Redistribution	• Fluorescent labelling of β-arrestin or seven-transmembrane receptor	• Can work with native receptor (that is, free of tags) when labelling β-arrestin • Easy to apply to receptor of interest in transiently transfected cells	• Difficult to quantify level of β-arrestin recruitment • May miss ligands that lead to weak β-arrestin recruitment that is still functional
Proximity	• FRET or BRET between seven-transmembrane receptor and β-arrestin • Enzyme complementation • Reporter-based assays	• Depending on the technology, can be highly sensitive	• Receptor must be modified, which could change its properties
Conformation	• Intramolecular BRET or FRET of β-arrestin or seven-transmembrane receptor	• Gives insight into β-arrestin recruitment and activation	• Can be difficult to interpret signals • May be necessary to perform in tandem with functional studies
Signalling	• MAPK activation • Stress fibre formation • Chemotaxis	• Directly assays a functional response that may be physiologically relevant	• May be difficult to scale up for HTS • Requires negative controls to rule out signalling through other endogenous receptors

BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; HTS, high-throughput screening; MAPK, mitogen-activated protein kinase.