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Biased Receptor Signaling in Drug Discovery

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Abstract	268
I. Introduction	268
II. Pleiotropic Receptor Systems.....	271
A. G Proteins	271
B. β -Arrestins	272
III. Bias and Pluridimensional Efficacy and Affinity	272
A. Receptor/G Protein Interactions	272
B. Receptor– β -Arrestin Interactions	273
C. Other Receptor Signaling Partners and Receptor Behaviors	274
D. Internal Signaling	275
IV. Naturally Biased Signaling in Physiology.....	275
V. Therapeutic Application of Biased Signaling.....	277
A. Assessing the Impact of Biased Signaling on Natural Physiology.....	277
B. Assessing the Impact of Biased Signaling from Known Ligands	279
VI. Molecular Mechanism(s) of Ligand Bias	280
VII. Detection and Quantification of Biased Signaling.....	283
A. Deviations from Monotonic Signaling	283
B. Biased Agonism, Antagonism, and Strength of Signal.....	285
C. Biased Antagonism	286
D. Assay Effects in Biased Signaling Measurement	288
E. Methods to Quantify Biased Signaling	289
1. Transducer Coefficients: Log(Ratio of Agonist Efficacy/Functional Affinity) Values	289
2. Log(Maximal Response/Concentration of Agonist Producing 50% of the Agonist Maximal Response)	290
3. Relative Efficacy: Log(Agonist Efficacy) Values	290
4. Relative Activity Ratios.....	291
5. Double-Reciprocal Null Comparison of Agonism	291
6. Reference Intrinsic Activity Trajectory and Rank Order Method	291
F. Temporal Effects on Measured Biased Signaling.....	292
G. Representations of Biased Signaling Profiles.....	293
H. Biased Signaling in Screening and Lead Optimization	295
I. Applications of Biased Scales in Pharmacology	296
J. Allosterically Induced Bias	297
VIII. Translation of In Vitro Bias to In Vivo Physiology	299
A. Translation from Pathways to Whole Cells	299
B. Translation to In Vivo Therapeutic Systems	302
1. Translational Gaps in the Conversion of In Vitro to In Vivo Bias	302
2. Affinity-Dominant versus Efficacy-Dominant Biased Signaling	302
IX. Future Considerations and Possible Ways Forward	304
References.....	306

Abstract—A great deal of experimental evidence suggests that ligands can stabilize different receptor active states that go on to interact with cellular signaling proteins to form a range of different complexes in varying quantities. In pleiotropically linked receptor systems, this leads to selective activation of some signaling pathways at the expense of others (biased signaling). This article summarizes the current knowledge about the complex components of receptor systems, the evidence that biased signaling is used in natural physiology to fine-tune signaling, and the current thoughts on how this mechanism may be applied to the design of better drugs. Although this is a fairly newly discovered phenomenon, theoretical and experimental data suggest that it is a ubiquitous

behavior of ligands and receptors and to be expected. Biased signaling is simple to detect in vitro and there are numerous methods to quantify the effect with scales that can be used to optimize this activity in structure-activity medicinal chemistry studies. At present, the major hurdle in the application of this mechanism to therapeutics is the translation of in vitro bias to in vivo effect; this is because of the numerous factors that can modify measures of bias in natural physiologic systems. In spite of this, biased signaling still has the potential to justify revisiting of receptor targets previously thought to be intractable and also furnishes the means to pursue targets previously thought to be forbidden due to deleterious physiology (as these may be eliminated through biased signaling).

I. Introduction

...[The] possibility is raised that selective agonists and antagonists might be developed which have specific effects on a particular receptor-linked effector system...

Roth and Chuang (1987)

What is bias? As the word implies, *bias* suggests an inequality. When this term is applied to cellular signaling mediated by seven-transmembrane receptors (7TMRs), it refers to a pleiotropically linked receptor (one that is coupled to more than one signaling pathway) producing more of some of the signals at the expense of others. Bias can be observed at different levels in the receptor pathway, from the elemental interactions of the receptor with signaling proteins at the beginning of

cellular cascades to the end-product whole cell signals themselves. This article will describe biased signaling from the point of view of considering it as a viable pharmacological mechanism that modifies 7TMR signaling to confer possibly better therapeutic profiles on receptor drugs, namely agonists, allosteric modulators, and antagonists.

It is important to differentiate system and measurement bias from true ligand-dependent bias, since it is only the latter effect that can result in an effective therapeutic advantage. System bias concerns the relative sensitivity of pathways connected to the receptor and this is hard-wired by the physiology of the system. Physiologic processes are linked to receptors by cells presumably for optimal signaling efficiency for the cells' needs; that is, there is no a priori reason that pleiotropic signals should be linked to a 7TMR with equal efficiency.

ABBREVIATIONS: 5-HT, serotonin; 7TMR, seven-transmembrane receptor; [³⁵S]GTPγS, 5'-O-(3-[³⁵S]thio)triphosphate; A-77636, (1*R*,3*S*)-3-adamantyl-1-(aminomethyl)-3,4-dihydro-5,6-dihydroxy-1*H*-2-benzopyran hydrochloride hydrate; AZ1729, *N*-[3-(2-carbamimidamido-4-methyl-1,3-thiazol-5-yl)phenyl]-4-fluoro benzamide; BAY 60-6583, 2-[(6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide; BQCA, 1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; BRET, bioluminescence resonance energy transfer; CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C motif) receptor; CGP-12177, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one hydrochloride; CHO, Chinese hamster ovary; CP55,940, (–)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; CXCR, C-X-C chemokine receptor; DAMGO, [D-Ala²,Nme-Phe⁴,Gly-ol⁵]-enkephalin; DPFE, 1-(4-(2,4-difluorophenyl) piperazin-1-yl)-2-((4-fluorobenzyl)oxy)ethanone; DREAD, designer receptor exclusively activated by designer drugs; Dyn1-11, dynorphin 1-11; ERK, extracellular signal-regulated kinase; GLP, glucagon-like peptide; GPCR, G protein-coupled receptor; GR89696, 4-[(3,4-dichlorophenyl)acetyl]-3-(1-pyrrolidinylmethyl)-1-piperazinecarboxylic acid methyl ester fumarate salt; GRK, G protein-coupled receptor kinase; GUE1654, 7-(methylthio)-2-[(2,2-diphenylacetyl)amino]benzo[1,2-*d*:4,3-*d'*]bisthiazole; HEK293, human embryonic kidney 293; HIV, human immunodeficiency virus; hNPS, human neuropeptide S; ICI204448, (*RS*)[3-[1-[[3,4-dichlorophenyl)-acetyl]methylamino]-2-(1-pyrrolidinyl)ethyl]phenoxy]acetic acid hydrochloride; (ICL)1-9, TAIKFERLLQTVTNFYFIT; IP1, inositol phosphate; IP3, inositol triphosphate; J113863, 1,4-*cis*-1-(1-cycloocten-1-ylmethyl)-4-[[2,7-dichloro-9*H*-xanthen-9-yl)carbonyl]amino]-1-ethylpiperidinium iodide; JNK, c-Jun N-terminal kinase; MLS1547, 5-chloro-7-[[4-(2-pyridinyl)-1-piperazinyl]methyl]-8-quinolinol; NAM, negative allosteric modulator; NECA, 1-(6-amino-9*H*-purin-9-yl)-1-deoxy-*N*-ethyl-β-D-ribofuranuronamide; NHERF, Na⁺/H⁺ exchange regulatory factor; PACAP, pituitary adenylate cyclase-activating polypeptide; PAM, positive allosteric modulator; PAR, protease-activated receptor; PLC, phospholipase C; PR, potency ratio; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; PZM21, 1-(2*S*)-2-(dimethylamino)-3-(4-hydroxyphenyl)propyl]-3-(2*S*)-1-(thiophen-3-yl)propan-2-yl]urea; RA, relative activity; RAMP, receptor activity-modifying protein; RANTES, regulated on activation normal T cell expressed and secreted; RASSL, receptor activated solely by a synthetic ligand; SAR, structure-activity relationship; SII, [Sar,Ile4,Ile8]-angiotensin II; SNC80, [(+)-4-(α*R*)-α-(2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-*N,N*-diethylbenzamide; SR592230A, 3-(2-ethylphenoxy)-1-[(1*S*)-1,2,3,4-tetrahydronaph-1-ylamino]-2*S*-2-propanol oxalate; TRV120027 (also TRV120), (2*R*)-2-[[2*S*)-1-[(2*S*)-2-[(2*S*)-2-[(2*S*)-2-[(2*S*)-5-carbamimidamido-2-[2-(methylamino)acetamido]pentanamido]-3-methylbutanamido]-3-(4-hydroxyphenyl)propanamido]-3-methylpentanamido]-3-(1*H*-imidazol-5-yl)propanoyl]pyrrolidin-2-yl]formamido]propanoic acid; TRV130, *N*-[3-(methoxythiophen-2-yl)methyl]-2-(9-pyridin-2-yl)-6-oxaspiro[4,5]decan-9-yl)ethanamine; UCB35625, 1,4-*trans*-1-(1-cycloocten-1-ylmethyl)-4-[[2,7-dichloro-9*H*-xanthen-9-yl)carbonyl]amino]-1-ethylpiperidinium iodide; UNC9975, 7-[4-[4-(2,3-dichlorophenyl)-1,4-diazepan-1-yl]butoxy]-1,2,3,4-tetrahydro-1,8-naphthyridin-2-one; VU0360172, *N*-cyclobutyl-6-[(3-fluorophenyl)ethynyl] picolinamide.

For example, cardiac cells react to elevation of cAMP by increasing calcium for inotropy (increased force of contraction) and also by increasing the rate of calcium uptake back into the sarcoplasmic reticulum for increased cardiac muscle relaxation (lusitropy). The cardiac cell is naturally biased toward lusitropy as a mechanism and lower concentrations of cAMP are required to elevate lusitropy than those required to elevate inotropy (Kenakin et al., 1991). As will be seen, these differential effects are readily seen in bias plots.

To further discuss system (and measurement) versus ligand bias, the most important tool to use is the bias plot; these simply express the response produced in one signaling pathway as a function of the response produced by the same concentrations of the activator in another pathway. Bias plots are a direct measure of the relative effectiveness of a receptor stimulator on two signaling pathways that do not involve mathematical modeling or assumptions about the origins of the signal, and they can be used to assess the uniformity of agonists in producing two signals emanating from the same receptor. Bias plots compare signaling pathways, most often driven by receptor activation. An example of a bias plot for the cAMP pathway is shown in Fig. 1B, which

expresses the lusitropic response to forskolin and dibutyryl cAMP as a function of the inotropic response (Fig. 1A). The hyperbolic shape of this function indicates the direction of the system bias (in this case, toward lusitropy). The fact that the trajectories of the two bias plots for forskolin and dibutyryl cAMP are similar suggests that both agents are subject to a uniform system bias; that is, there is no evidence to suggest that forskolin and dibutyryl cAMP affect the two pathways in different ways. Note that a bias plot showing uniform system bias need not be linear but rather will reflect the relative sensitivity of the two signaling pathways as they are used in physiology. Elevations of cAMP through activation of β -adrenoceptors by agonists in rat atria leads to the same lusitropic bias as seen with forskolin and dibutyryl cAMP (see Fig. 1D). As with the previous example, the uniformity of the bias plot trajectories suggests no signaling bias with respect to these two signals produced by these two agonists. Agonists such as those shown in Fig. 1C have been termed “unbiased” or “balanced,” since their effects do not deviate from the normal physiology of the system. However, these are misnomers since they imply that these molecules will produce similar levels

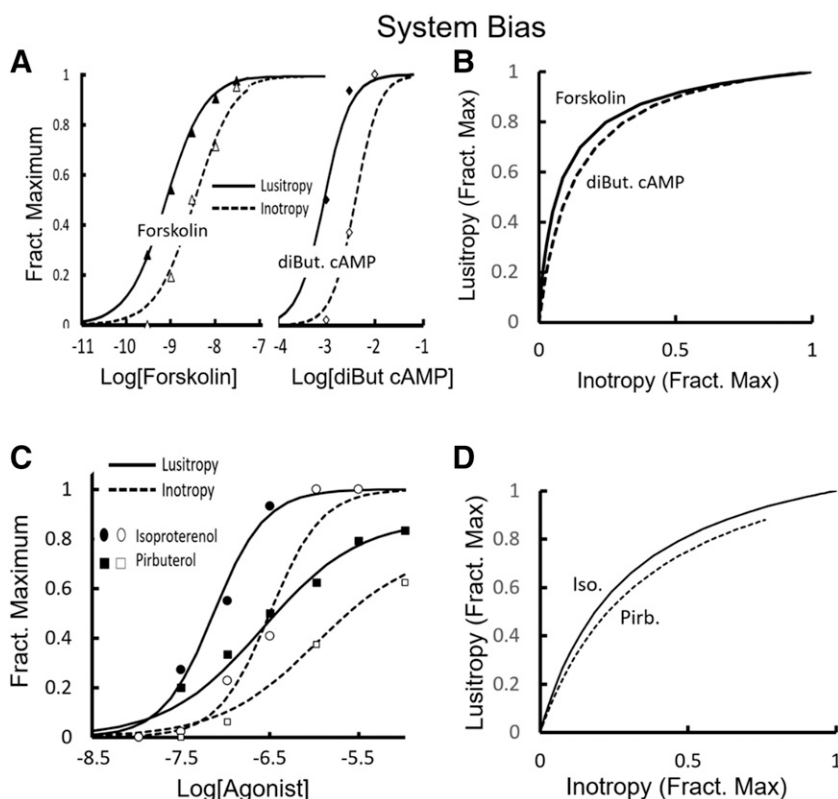


Fig. 1. System bias reflected in a comparison of the ability of elevated cAMP levels in the rat atrial cell, through activation of adenylate cyclase by forskolin and direct addition of cell-permeable cAMP (dibutyryl cAMP) to produce lusitropic (relaxation) and inotropic (force of contraction) responses. (A) Concentration-response curves for lusitropy (solid DR curve lines) and inotropy (dotted lines) for forskolin and dibutyryl cAMP. (B) Bias plot showing the lusitropic response (ordinates) expressed as a function of the inotropic response (abscissae) produced by common concentrations of forskolin and dibutyryl cAMP. Rat atrial cell system bias imposed on elevation of cAMP due to activation of β -adrenoceptors by the agonists isoproterenol (filled and open circles) and pirbuterol (filled and open squares). (C) Concentration-response curves to isoproterenol and pirbuterol for lusitropy (solid lines) and inotropy (dotted lines). (D) Bias plot showing lusitropic responses (ordinates) produced by the agonists as a function of inotropic responses (abscissae) produced by the same concentrations of agonist. DR, dose response. Data are from Kenakin et al. (1991).

of signaling from the dual pathways, a fact that may or may not be true depending on the natural system bias of the tissue. True biased agonism (or antagonism) is relevant only when the agonists are compared with a defined agonist and is usually measured in contrast to a natural endogenous agonist effect (physiologic system bias). That being the case, natural agonists that are unbiased in comparison with true biased synthetic ligands should be referred to as having “natural” bias or at least qualified as naturally unbiased compared with a truly biased ligand.

Since bias plots compare sensitivities of responses, the efficiency of the assay system necessarily is involved in the measurement; that is, system bias as measured in vitro with two functional assays is also a measure of the physiologic relative sensitivity of the receptor coupling to the pathways and also the relative sensitivity of the functional assays used to make the measurements. System bias reflects any difference from the production and transduction of the receptor stimulus to the point of measurement. For example, if two functional assays have differential sensitivity, then this will be reflected in a curved bias plot. Second messenger assays such as the measurement of cAMP are often highly amplified and agonists have correspondingly high potency. In contrast, β -arrestin complementation assays are usually not highly amplified and agonists have a correspondingly low potency. Comparison of cAMP and β -arrestin assays through a bias plot therefore often shows a high bias toward cAMP simply because of the mechanics of the assay transduction of signals. It is the *relative* nonlinearity between ligands in a bias plot, not the magnitude of the nonlinearity, that is important in the detection and quantification of ligand-based signaling bias of possible value in therapy. In assessing biased signaling associated with particular ligands, it is essential that system (and measurement) bias be canceled and this is done by comparison with a common reference agonist in the two pathways (*vide infra*).

Selective ligand bias is manifest as a further bias of the signaling superimposed upon the system bias in the functional assays. An example of this for κ -opioid agonists is shown in Fig. 2. In this case, it is clear that dynorphin 1-11 (Dyn1-11) (solid line curve) is biased toward G protein signaling, whereas GR89696 [4-([3,4-dichlorophenyl]acetyl)-3-(1-pyrrolidinylmethyl)-1-piperazinecarboxylic acid methyl ester fumarate salt] (broken line curve) is biased toward β -arrestin; the other agonists follow a uniform system bias (White et al., 2014). The fact that the other agonists appear to have near linear relationships for the two signaling pathways in this case has no significance, since the relationship defined by system bias is a complex function of assay sensitivity and efficiency of receptor coupling to cellular signaling. As seen in Fig. 2, the ligand bias for Dyn1-11 and GR89696 can be detected through a bias plot and it is this specific property of these ligands that may be exploited for therapeutic advantage.

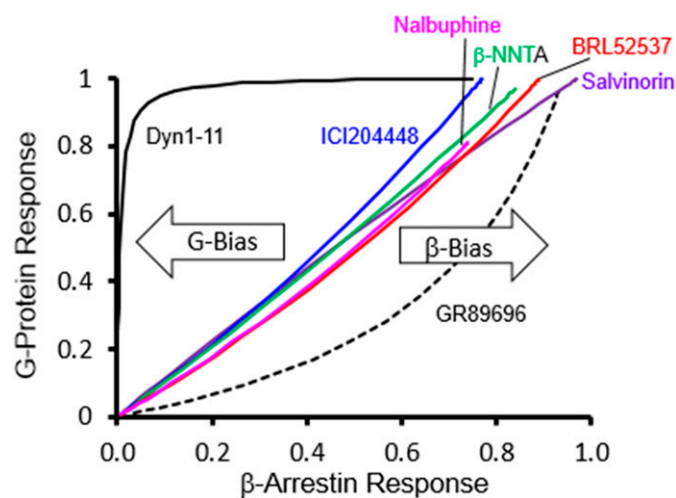


Fig. 2. Bias plot for κ -opioid agonists activating G proteins and β -arrestin (in HEK cells). Ordinates show the fraction of maximal activation of G protein signaling. Abscissae show the fraction of maximal β -arrestin activation. Nalbuphine, β -NNTA, BRL52537, and salvinorin produce similar bias profiles, whereas Dyn1-11 is biased toward G protein signaling and GR89696 is biased toward β -arrestin signaling. β -NNTA, N-naphthoyl- β -naltrexamine; BRL52537, (6)-1-(3,4-dichlorophenyl)acetyl-2-(1-pyrrolidinyl) methylpiperidine hydrochloride. Data are redrawn from White et al. (2014).

When assessing bias with two separate functional assays in vitro, the system and measurement sensitivity can give an erroneous impression of nonconcomitant and sequential signaling from the same receptor. For example, Fig. 3A shows the activation of chemokine (C-C motif) receptor CCR5 by chemokine (C-C motif) ligand CCL3 (Kenakin et al., 2012); the shaded area appears to show a concentration range where CCL3 produces increased inositol phosphate (IP1) with no receptor internalization; moreover, these curves make it appear that internalization commences at concentrations >300 times those needed to elevate IP1; this type of nonsynchronous activation of signaling from the same receptor is not compatible with mass action kinetics. Expression of the same curves as bias plots in Fig. 3B resolves this apparent dichotomy, as it shows that both processes (IP1 and internalization) occur concomitantly with CCL3 receptor occupation but that the signals are of different strength; that is, the receptor reserve for IP1 and the sensitivity of the IP1 assay is greater than that for internalization. Therefore, a direct correspondence between in vitro biased estimates in terms of what will occur in vivo may not be expected.

It is useful to consider expectations of biased ligands in physiology. The implication associated with biased signaling is that a reduction in the pleiotropy of natural signaling will occur; that is, the biased agonist will reduce the variety of signals that are not beneficial for therapy (i.e., reduce side effects) but this need not be the case. Natural physiology uses biased signaling to achieve fine control in normal healthy organ systems, and biased ligands basically interfere with this natural balance to yield an unnatural (or at least “unbalanced”)

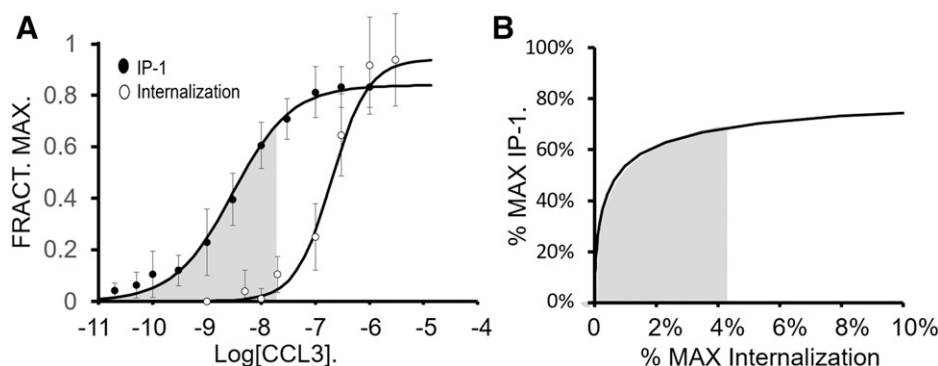


Fig. 3. Activation of the CCR5 receptor with the chemokine agonist CCL3. (A) Responses shown for IP1 production and receptor internalization. The shaded area represents concentrations of CCL3 with apparently no receptor internalizing activity. Data are redrawn from Kenakin et al. (2012). (B) Bias plot of the data shown in (A). It can be seen that both responses occur concomitantly with CCL3 binding but that the intensity of the IP1 response is much greater than the internalization response.

outcome (Luttrell and Gesty-Palmer, 2010; Luttrell et al., 2018). In fact, biased ligands have been seen to recruit signaling pathways that normally are not activated by a given receptor and natural agonist pair (Saulière et al., 2012; Santos et al., 2015). Finally, as with all agonists, the sensitivity of the system controls whether agonism is observed at all. That is, the receptor density and/or stoichiometry of the receptor/signaling elements of the cell may be inadequate to allow a low-efficacy agonist to manifest agonism; under these circumstances, such a molecule will be an antagonist. For orthosteric biased ligands, these occupy the receptor required by the endogenous agonist and this means that in vivo, an important component of biased agonism is antagonism of the natural signaling system (vide infra). In fact, as in the case of the biased angiotensin ligand TRV120027 (also TRV120; (2R)-2-[(2S)-1-[(2S)-2-[(2S,3S)-2-[(2S)-2-[(2S)-5-carbamimidamido-2-[2-(methylamino)acetamido]pentanamido]-3-methylbutanamido]-3-(4-hydroxyphenyl)propanamido]-3-methylpentanamido]-3-(1H-imidazol-5-yl)propanoyl]pyrrolidin-2-yl]formamido]propanoic acid) for heart failure (Violin et al., 2010), the antagonist effect of the molecule may be the predominant therapeutic effect, not the agonism. In general, current studies in vivo indicate that biased ligands, through mixed selective agonism and antagonism, produce effects that are different from those seen with standard agonists and antagonists (Luttrell et al., 2018). As a preface to considering how this drug property may be applied in therapy, a discussion of cellular pleiotropic receptor-based signaling is useful to define the pharmacological systems interacting with drug molecules.

II. Pleiotropic Receptor Systems

7TMRs, also known as G protein-coupled receptors (GPCRs), are nature's prototypical allosteric proteins and they are designed to bind multiple ligands and change their conformation accordingly to bind multiple intracellular bodies (e.g., signaling proteins) to transmit signals from the extracellular to the intracellular space.

Early depictions of 7TMRs describe them as switches binding extracellular ligands (e.g., hormones, neurotransmitters) and subsequently activating signaling proteins in the cell cytosol; in this type of system, receptor signaling is uniform with the only variation with system parameters (e.g., ligand concentration, type) being strength of signal. A great deal of research over the past years has necessitated a revision of this model to an alternative model of receptors as microprocessors able to receive a range of incoming signals and produce a modified range of outgoing signals (Kenakin, 2015a). Receptors demonstrate a wealth of behaviors in the process of signal transduction, beginning with the range of forms they present to the cell as ensembles of microstates of different conformations that can differentially engage a wide array of signaling partners. As a preface to the discussion of how these receptor systems function in the cell, it is useful to consider the various components involved.

A. G Proteins

Canonical 7TMR signaling was first described as an interaction with heterotrimeric G proteins ($G[\alpha]/G[\beta\gamma]$) leading to a ligand/GPCR-catalyzed GDP/GTP exchange on the $G[\alpha]$ subunit to induce structural rearrangement or dissociation of $G[\alpha]$ -GTP and $G[\beta\gamma]$ (Denis et al., 2012). The numerous $G[\alpha]$ subunits are transducers of adenylyl cyclase ($G[\alpha]_s$ stimulation, $G[\alpha]_i$ inhibition), phospholipase C (PLC) ($G[\alpha]_q$), and RhoGEFs ($G[\alpha]_{12/13}$) (Neer, 1995; Luttrell, 2008; Walther and Ferguson, 2015) to produce a range of intracellular second messengers such as cAMP, inositol triphosphate (IP3), and diacylglycerol (Neves et al., 2002). Receptor G protein-induced production of second messengers such as cAMP also can be temporally and spatially separated from membrane events (Gidon et al., 2014; Luttrell, 2014). First reported in yeast (Slessareva et al., 2006), nonmembrane-based cAMP production has since been reported in mammalian cell systems for dopamine D_1 receptors (Kotowski et al., 2011), β_2 -adrenergic receptors (Irannejad et al., 2013), glucagon-like peptide (GLP)-1 receptors (Kuna et al., 2013),

pituitary adenylate cyclase-activating polypeptide (PACAP) type 1 receptors (Merriam et al., 2013), thyroid-stimulating hormone receptors (Calebiro et al., 2015), parathyroid hormone (PTH) receptors (Castro et al., 2005; Ferrandon et al., 2009), sphingosine-1-phosphate receptors (Mullershausen et al., 2009), melanocortin-4 receptors (Molden et al., 2015), and vasopressin type 2 receptors (Feinstein et al., 2013). In addition, G[$\beta\gamma$] variants (dissociated from G α subunits) can function as independent sources of second messengers (Clapham and Neer, 1997; Dupré et al., 2009). It will be seen that this wide array of G protein subunits forms the basis for a diverse potential for biased signaling.

B. β -Arrestins

GPCR activation sequelae lead to phosphorylation of receptor cytoplasmic domains by second messenger-dependent protein kinases or the G protein-coupled receptor kinase (GRK) family (GRK 1–7), a class of seryl-threonyl kinases that phosphorylate the cytoplasmic tail of agonist-occupied receptors (Pitcher et al., 1998; Lefkowitz and Shenoy, 2005; Drake et al., 2006). The phosphorylated receptors then have a high affinity for a family of proteins called arrestins (four isoforms consisting of two “visual” arrestins, arrestin1 and arrestin4, and two ubiquitous cellular arrestins involved in 7TMR signaling, β -arrestin1 and β -arrestin2). The binding of arrestins to receptors, first reported for rhodopsin by Kühn et al. (1984), results in suppression of G protein activation through competition (Wilden et al., 1986; Wilden, 1995). First characterized for their role in receptor desensitization in mammalian systems (Ferguson, 2001), a variety of subsequent studies confirmed the role of β -arrestin in receptor desensitization; Kohout and Lefkowitz, 2003; Lefkowitz and Whalen, 2004; Shenoy and Lefkowitz, 2005; Moore et al., 2007). These receptor interactions with β -arrestins also cause receptor internalization and vesicular trafficking, routing, and desensitization (Luttrell, 2008; Walther and Ferguson, 2013, 2015). As early as 1999, it was reported that β -arrestin2 bound nonreceptor tyrosine kinase and c-Src recruited β_2 -adrenoceptors (Luttrell et al., 1999); subsequent studies revealed that receptor- β -arrestin interactions can lead to the formation of signalsomes (receptorsomes) by functioning as scaffolding proteins for recruitment and further activation of cytoplasmic proteins (Shenoy et al., 2006; Noma et al., 2007; Irannejad et al., 2013). These receptor-bound β -arrestins can interact with extracellular signal-regulated kinase (ERK1/2), protein kinase B, mitogen-activated protein kinase kinase, Raf-1, ubiquitin kinase, and other cytoplasmic proteins (Luttrell et al., 2001; Shenoy et al., 2001; Beaulieu et al., 2005; Del’guidice et al., 2011; Urs et al., 2011; Kuhar et al., 2015), the Src family of kinases (Barlic et al., 2000; DeFea et al., 2000a), E3 ubiquitin ligase Mdm2 (Shenoy

et al., 2001), c-Jun N-terminal kinase (JNK) 3 mitogen-activated protein kinase cascades (DeFea et al., 2000b; McDonald et al., 2000; Luttrell et al., 2001), cAMP phosphodiesterases 4D3/5 (Perry et al., 2002), the inhibitor of nuclear factor- κ B I κ B α (Gao et al., 2004; Witherow et al., 2004), the Ral-GDP dissociation stimulator (Bhattacharya et al., 2002), the actin filament-severing protein cofilin (Zoudilova et al., 2007), diacylglycerol kinase (Nelson et al., 2007), and serine/threonine protein phosphatase 2A (Beaulieu et al., 2004, 2005). In addition, β -arrestins have been shown to potentiate G α_s activity through a β -arrestin–G[$\beta\gamma$] complex that allows multiple rounds of G α_s association and dissociation (Wehbi et al., 2013). Receptors can interact with other signaling partners in addition to G protein and β -arrestins to produce cytoplasmic signaling (Ritter and Hall, 2009), including PDZ domains [multi-PDZ domain protein 1, synapse-associated protein 97, postsynaptic density protein 95, sorting nexin family member 27, Na⁺/H⁺ exchange regulatory factors (NHERFs), membrane-associated guanylate kinase] and non-PDZ domain [A-kinase-anchoring proteins, Jak2, 14-3-3]–containing scaffolds (Walther and Ferguson, 2015). Thus, structurally diverse receptorsomes created in different cells can influence pluridimensional efficacy profiles for 7TMR ligands (Maudsley et al., 2011, 2013).

III. Bias and Pluridimensional Efficacy and Affinity

Biased signaling results in a textured cellular response made up of individual elements of biochemical cascade reactions, and this ensures that the efficacy of a ligand has a quality composed of the summation of these various elements. The complexity of this overall efficacy depends on the vantage point taken to observe ligand response; the further toward the endpoint (cellular response), the more complex and textured will be the result. Ligand efficacy can be defined as the imposition of altered behavior of 7TMRs (toward the cell) induced by receptor-ligand interaction. These behaviors are initiated by biochemical reactions resulting from the initial ligand-receptor complex interaction with cellular signaling proteins. It is useful to consider the various starting points (i.e., immediate receptor behaviors) known for 7TMRs—in effect, the individual components of what makes up cellular efficacy.

A. Receptor/G Protein Interactions

As discussed, the first and most prominent interaction noted for 7TMRs is their interaction with G proteins. Mammalian genomes code for 16 different G protein G α subunits capable of interacting with 7TMRs, and it has been suggested that choices between these constitute the largest source of functional selectivity (Hermans, 2003). These 16 types of G α subunits associate with an equally diverse set of G $\beta\gamma$ subunits

(Hermans, 2003; Oldham and Hamm, 2008) made up of six different β -subunits and 12 γ -subunits (Gautam et al., 1998; Vanderbeld and Kelly, 2000) that form a large network of possible signaling units. Whereas $G\beta\gamma$ subunits are thought to be functionally interchangeable (Smrcka, 2008), activated $G\alpha$ subunits regulate distinct and nonredundant cellular effectors (Wettschureck and Offermanns, 2005; Hubbard and Hepler, 2006). In addition, restricted tissue expression of some of these elements prevents some combinations. Within this context, the potential compartmentalization and concentrations of some of these elements in cells may contribute to cell-based modifications of biased signaling. This diverse system of G proteins is generally classified into four main functional groupings according to the family of $G\alpha$ subunits that are predominantly activated: namely $G_{i/o}$, $G_{q/11}$, G_s , and $G_{12/13}$. 7TMRs generally can interact with members spanning across these general groupings despite the fact that the G protein functional outcomes can be very different (Michal et al., 2007; Inoue et al., 2012; Saulière et al., 2012). For instance, cannabinoid receptors interact with $G\alpha_z$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (Diez-Alarcia et al., 2016; Laprairie et al., 2017). Ligand-stabilized receptor conformations drive the selection of G protein interactions and these selections can be discerned within G protein subunit families; for instance, studies with bioluminescence resonance energy transfer (BRET) biosensors have shown that oxytocin analogs differentiate between individual $G_{i/o}$ family members ($G\alpha_q$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oA}$, $G\alpha_{oB}$; Busnelli et al., 2012). Ligand bias between subunit members of G protein families is increasingly observed with agonists for μ -opioid receptors ($G\alpha_{i1}$, $G\alpha_{oA}$; Saidak et al., 2006), dopamine receptors ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oA}$, $G\alpha_{oB}$; Möller et al., 2017), and PTH receptors ($G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{i/o}$; Appleton et al., 2013). Signaling also results from activation of $G\beta$ subunits (activation or deactivation of adenylate cyclase, PLCs, phosphatidylinositol 3-kinase) and $G\gamma$ subunits (protein kinase D) (Morris and Malbon, 1999; Vanderbeld and Kelly, 2000). Bias between $G\alpha$ and $G\beta\gamma$ subunit activation also has been observed. For example, the small molecule agonist GUE1654 [7-(methylthio)-2-[(2,2-diphenylacetyl)amino]benzo[1,2-*d*:4,3-*d'*]bisthiazole] for $G_{i/o}$ -coupled oxoeicosanoid receptors produces inhibition of $G\beta\gamma$ otherwise allowing activation of $G\alpha$ -dependent pathways (Blättermann et al., 2012). This type of bias may be important to drug therapy, as differential activation of specific combinations of $G\alpha$ and $G\beta\gamma$ subunits is beginning to be recognized in terms of clinical benefits (Piñeyro, 2009; Lin and Smrcka, 2011).

B. Receptor- β -Arrestin Interactions

Another major set of 7TMR-signaling protein interactions occurs with arrestins. In fact, of 350 nonolfactory human 7TMRs, nearly all couple to β -arrestin (Roth and

Marshall, 2012; Kroeze et al., 2015). The interaction of receptors with β -arrestins is promoted by receptor activation, phosphorylation of receptors by GRKs (Benovic et al., 1986), and, in certain instances, special interactions and post-translational processes such as palmitoylation (Charest and Bouvier, 2003). Receptor phosphorylation is particularly important to receptor-arrestin interactions (Tobin, 2008; Tobin et al., 2008; Reiter et al., 2012). This phosphorylation of serine/threonine residues produces a distinct pattern at the C terminus or intracellular loops of the receptor referred to as a “barcode” (Tobin et al., 2008; Nobles et al., 2011); different patterns for these have been shown through mass spectrometry proteomics for ligand interactions with β -adrenoceptors (Nobles et al., 2011). Mass spectrometry and receptor phosphorylation-specific antibodies also can be employed to elucidate these unique barcodes (Prihandoko et al., 2015).

In terms of signaling bias, the nature of the ligand-receptor complex has been shown to influence the phosphorylation barcode of the receptor to further influence the signaling outcome of the complex (i.e., signaling bias) (Kim et al., 2005; Tobin et al., 2008; Zidar et al., 2009; Butcher et al., 2011; Nobles et al., 2011; Zhou et al., 2017a). Agonist-specific phosphorylation barcodes have been reported for angiotensin II type I receptors (Xiao et al., 2007; Christensen et al., 2010), opioid receptors (Just et al., 2013), and serotonin 5-HT_{2A} receptors (González-Maeso et al., 2007). For the β_1 -adrenoceptor, structural evidence that this occurs through unique binding modes for carvedilol and bucindolol has been given (Warne et al., 2012) to describe special conformations that go on to provide unique signaling effects (Reiter et al., 2012). Phosphorylation also has been shown to modify G protein interactions for 5-HT₆ receptor changes from ligand-dependent $G\alpha_s$ coupling to a ligand-independent coupling to Cdc42 (Duhr et al., 2014).

There are at least three major possible outcomes of activated receptor- β -arrestin interaction: cessation of G protein signaling (Lohse et al., 1990), internalization of receptors (Lefkowitz, 1998; Ferguson, 2001; Luttrell, 2008; Ahn et al., 2009; Walther and Ferguson, 2013, 2015), and formation of an intracellular scaffold for internal cellular signaling (Luttrell et al., 1999; DeFea et al., 2000a; Lefkowitz and Shenoy, 2005). These mechanisms have been associated with a wealth of physiologic responses to pharmacological activity, including receptor desensitization (Deshpande et al., 2008; Wang et al., 2009; Whalen et al., 2011), receptor internalization (Ferguson et al., 1996; Goodman et al., 1996; Laporte et al., 1999; Hanyaloglu and von Zastrow, 2008), cell apoptosis (Chen et al., 2009), protein cell synthesis (DeWire et al., 2008; Ahn et al., 2009), central nervous system reward (Bohn et al., 2003), and learning and memory (Poulin et al., 2010).

The dependence of receptor internalization on β -arrestin has been demonstrated in numerous studies but there are a diverse number of mechanisms involved

in this process. The most extensively characterized involves the receptor/ β -arrestin complex binding to clathrin and its adapter protein AP2 (Wilden et al., 1986; Lohse et al., 1990; Ferguson et al., 1996; Goodman et al., 1996; Laporte et al., 1999), the clathrin heavy chain (Goodman et al., 1996), and E3 ligase Mdm2 (Shenoy et al., 2007) to form clathrin-coated vesicles that traffic to the endosome with subsequent possible transfer to lysosomes for degradation or recycling back to the plasma membrane (Cao et al., 1998). The introduction of green fluorescent protein–tagged receptors has moved these types of experiments forward (Barak et al., 1997). A classification system, based on differential affinity of the receptor for arrestin, has evolved from these studies, with class A 7TMRs (e.g., β_2 -adrenoceptors, μ -opioid receptors, dopamine D_1) preferentially binding β -arrestin1 (also known as arrestin2) to be rapidly dephosphorylated and recycled. In contrast, class B 7TMRs (e.g., angiotensin II type A, vasopressin V2, substance P) bind β -arrestin1 and β -arrestin2 (also known as arrestin3) and have equal affinity to form stable complexes that can be retained in the cytosol, recycled, or degraded (Walther and Ferguson, 2013).

Biased signaling involving β -arrestins can be extremely complex due to the fact that arrestins have so many consequential actions in the cell with respect to receptor disposition and signaling and also because there are a number of alternative activities that can be exploited therapeutically. For instance, biased signaling toward β -arrestin–receptor interaction has alternately been suggested to be beneficial for some receptors, such as promotion of antipsychotic dopamine D_2 receptor activity (Lawler et al., 1999; Mailman and Murthy, 2010; Allen et al., 2011) and angiotensin-mediated cardioprotection (Violin et al., 2010), or detrimental in conditions such as κ -opioid receptor–mediated dysphoria (White et al., 2014). In terms of internal signaling, receptor-arrestin complexes have been reported to interact with the Src family of tyrosine kinases (Luttrell et al., 1999), a number of mitogen-activated protein kinases (Chavkin et al., 2014), ERK1/2 and JNK3, and p38 (Seo et al., 2011). These effectors can use β -arrestin/receptor complexes as scaffolds to form different signalsomes (Peterson and Luttrell, 2017) to produce long-lasting signals in the cytosol (Luttrell et al., 1999; McDonald et al., 2000; Gong et al., 2008; Song et al., 2009). For some specific receptors, β -arrestin has been shown to scaffold AKT (Schmid and Bohn, 2010; Schmid et al., 2013), PI3K, and phosphodiesterase 4 (DeWire et al., 2007) and also to produce mediation of nuclear signaling such as microRNA processing after β_1 -adrenoceptor activation (Kim et al., 2014). In addition, β -arrestin complexes have been implicated in ubiquitination (Shenoy et al., 2001) of receptors. Finally, although β -arrestin2 has received the most attention in the literature for these activities, increasing studies with β -arrestin1 have revealed a further variety of cellular effects (Srivastava et al.,

2015). For example, GLP-1 receptors interact with β -arrestin1 to promote insulin release in the pancreatic β cell (with application to the treatment of diabetes; Sonoda et al., 2008). Biased signaling through selective arrestin coupling has also been observed for δ -opioid receptors. Specifically, whereas the highly internalizing δ -opioid agonist SNC80 [(+)-4-[(αR)- α -((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N,N*-diethylbenzamide] initiates receptor interaction with β -arrestin1, the lower internalizing agonists ARM930 and JNJ2078860 preferentially recruit β -arrestin2 (Pradhan et al., 2016).

In terms of receptor internalization, the ligand-receptor conformational complex may or may not code for internalization; for instance, although agonists such as quinpirole promote dopamine D_2 receptor internalization, the biased ligand aripiprazole does not (Allen et al., 2011). Receptor internalization can occur with subsequent rapid recycling or with receptor degradation (Tsao et al., 2001; Whistler et al., 2002). For instance, for GLP-1 receptors, the agonist GLP-1 mediates a faster recycling rate than do the synthetic agonists exendin-4 and liraglutide, leading to temporal differences in levels of activation (Roed et al., 2014). There can be even more diverse trafficking ligand effects as in the case of CCR5. This receptor mediates human immunodeficiency virus (HIV)-1 infection, and one therapeutic approach to limiting HIV-1 infection is to internalize the receptor so that the gp120 protein on the HIV viral coat cannot bind. Two chemokines have very different effects: RANTES (regulated on activation normal T cell expressed and secreted; CCL5) promotes rapid internalization of receptor with rapid recycling, whereas the analog AOP-RANTES promotes rapid internalization but with much less and slower recycling of the receptor to the cell surface (Mack et al., 1998).

C. Other Receptor Signaling Partners and Receptor Behaviors

Some 7TMRs express an endogenous PDZ domain at their distal carboxyl termini (Bockaert et al., 2003), allowing them to interact with PDZ domain proteins (Bockaert et al., 2004). These domains generally consist of 80 to 100 residues forming six β -strands and two α -helices. The carboxyl-terminal tail of the receptor can then interact with an elongated surface groove that is situated between the second β -strand and the second α -helix. Three classes of PDZ ligands have been described: class I (–E–S/T–xV/I), class II (– φ – φ –), and class III (ψ –x– φ –), in which ψ represents an acidic residue and φ represents a hydrophobic residue (Sheng and Sala, 2001). Many such partners have been proposed (C kinase 1 protein, Golgi reassembly stacking protein, glutamate receptor-interacting protein, and nebulin molecules) but the most extensively characterized interaction is with NHERF1 and NHERF2. For instance, PTH receptor 1 facilitates PLC signaling while inhibiting

$G\alpha/cAMP$ signaling (Mahon et al., 2002; Mahon and Segre, 2004) through interactions with NHERF1 and NHERF2. In general, these interactions result in effects on transcriptional regulation, intracellular trafficking, and cell growth.

With increasing assay technology has come an appreciation of the wealth of receptor behaviors practiced by 7TMRs during cell signaling and function. In addition to receptor phosphorylation, coupling of receptors to G proteins, β -arrestins and other cytosolic proteins, and ligands may change other behaviors as well. For instance, the μ -opioid receptor agonists DAMGO ([D-Ala², Nme-Phe⁴, Gly-ol⁵]-enkephalin) and morphine increase (whereas endomorphin-2 decreases) lateral mobility of μ -opioid receptors in the cell membrane; the interaction of these effects with membrane cholesterol content produces variable signaling and this suggests a possible mechanism of bias variation with cell type (Melkes et al., 2016).

In addition, 7TMRs are known to oligomerize with other receptors to form homodimers and heterodimers (Gomes et al., 2016). Through such activity, receptor signaling can be increased (dopamine D₁-D₃ heteromer; Fiorentini et al., 2008), diminished (adenosine A_{2A}-dopamine D₂ heteromer; Strömberg et al., 2000), or completely changed (dopamine D₁-D₂ heteromer; Rashid et al., 2007). In some systems (notably chemokine receptors), receptor dimerization has been proposed as a natural mechanism required for activation (Rodríguez-Frade et al., 1999; Vila-Coro et al., 1999; Trettel et al., 2003; Hernanz-Falcón et al., 2004). In fact, some receptors such as C-X-C chemokine receptor CXCR4 are proposed to function constitutively as dimers (Babcock et al., 2003). Given that oligomerization theoretically offers a new ligand target and/or choices for ligand efficacy, the possibility of bias in such systems would be predicted (Zhou and Giraldo, 2018). Although there are currently little data to definitively suggest that dimerization is involved in biased signaling, a recent study with melanocortin receptors suggests that this may be a fruitful line of research for selective receptor activation (Lensing et al., 2019).

D. Internal Signaling

Finally, a relatively new receptor behavior has been described, whereby receptor-G protein signaling complexes translocate to the endoplasmic reticulum, Golgi apparatus, and nucleus (Revankar et al., 2005; Re et al., 2010) and continue to signal (Castro et al., 2005; Hein et al., 2006; Boivin et al., 2008). In fact, such intracellular signaling has been described in unique terms therapeutically in treatments for multiple sclerosis (Mullershausen et al., 2009), pain (Geppetti et al., 2015; Cahill et al., 2017), and nociception (Jensen et al., 2017). The vast array of interactions possible within receptor systems offers the opportunity for natural fine-tuning of cell signaling.

IV. Naturally Biased Signaling in Physiology

The stabilization of select multiple conformations of the receptor with a wide range of signaling effector molecules is theoretically optimal for fine-tuning cellular response, and this opens the question of whether these mechanisms are used by natural physiology. There are three lines of thought that would suggest this to be the case; the first two are theoretical. If it is accepted that receptors form ensembles of multiple conformations (in varying quantities) and that ligands form unique ensembles according to the differential affinity they have for each of the ensemble members (Burgen, 1981; Bosshard, 2001; Vogt and Di Cera, 2013), then for two agonists to have an identical pattern of bias they would need to have identical affinities for each of the natural ensemble members (and thus produce identical ligand-bound ensembles), an unlikely scenario. Conformational selection by a ligand binding to an ensemble of n members differing in relative quantity by an allosteric constant L_i ($L_i = [R_i]/[R_{ref}]$, $[R_{ref}]$ being a common reference conformation) and where the affinity of ligand for each member differs by a value α_i is given by (Kenakin, 2013) as shown in eq. 1:

$$\frac{\rho_{\infty}}{\rho_0} = \frac{\sum_{i=1}^n \alpha_{i+1} L_{i+1} \left(1 + \sum_{i=1}^n L_{i+1} \right)}{\left(1 + \sum_{i=1}^n \alpha_{i+1} L_{i+1} \right) \sum_{i=1}^n L_{i+1}} \quad (1)$$

where ρ_0 represents the ensemble configuration in the absence of the ligand and ρ_{∞} is the configuration in the presence of a saturating concentration of ligand. It can be seen from this equation that ligand binding will not change the configuration of the ensemble only if $\alpha = 1$ for each and every conformation (i.e., the ligand has identical affinity for every conformation). If the ligand has a different affinity for any conformation, the relative amounts of the conformations will change upon ligand binding. This would suggest that different ligands would naturally be at least slightly biased, in terms of signaling, with respect to each other. This leads to the notion that natural multiple ligands for given receptors would be internally biased and thus produce different qualities of efficacy in natural physiologic systems. In fact, it has been seen that with complex downstream signaling patterns through analysis of gene arrays, synthetic ligands invariably produce biased signaling fingerprints compared with natural endogenous agonists (Luttrell and Kenakin, 2011; Maudsley et al., 2012).

The second theoretical idea comes from the known behavior of allosteric proteins. Specifically, the effect of a ligand on an allosteric protein is probe specific, such that the influence of a ligand on the subsequent interaction of the ligand-protein complex with proteins and

ligands will be unique to that particular ternary complex of ligand/receptor/signaling protein (Edelstein and Changeux, 2016); this is the essence of agonist efficacy. This being the case, each ligand-bound receptor would have a different propensity to interact with the array of signaling proteins available for subsequent binding. To infer that two ligands will have identical bias would further imply that they would have identical probe dependence.

A third line of thought supporting a general acceptance of natural signaling bias comes from emerging experimental data showing that multiple natural ligands for common receptors, when subjected to scrutiny, actually do demonstrate signaling bias. The first documented case of natural signaling bias was for the PACAP receptor expressed in Lilly Laboratories cell-porcine kidney 1 cells, where it was shown that two natural peptides for this receptor (PACAP₁₋₂₇ and PACAP₁₋₃₈) produce differential activation of cAMP and IP₃ signaling. Specifically, PACAP₁₋₃₈ produces a rank order of activity of cAMP > IP₃, whereas PACAP₁₋₂₇ produces a reverse rank order of IP₃ > cAMP through the same receptor (Spengler et al., 1993). In fact, systems with multiple natural endogenous agonists and/or antagonists are clear targets for investigating the notion that natural physiology employs biased signaling to fine-tune signaling. For example, the melanocortin receptor system, which has the natural peptide agonist α -melanocyte-stimulating hormone and the natural antagonist agouti-related peptide, has been reported to show signaling bias within these molecules (Yang and Tao, 2016). Similarly, the protease-activated receptor 2 (PAR₂) has multiple natural agonists associated with the variety of proteases that cleave the receptor at different sites; these different resultant agonists have been shown to differentially signal through the multiple pathways linked to PAR₂ (Suen et al., 2014; Jiang et al., 2017). Similarly, whereas trypsin and tryptase neutrophil elastase cleave the receptor to generate an agonist that activates all known PAR₂ receptor signaling, neutrophil elastase cleaves the receptor to form an agonist that activates ERK but not calcium signaling (Zhao et al., 2014b).

A prominent system for possible biased signaling is the chemokine receptor system for the control of leukocyte migration in homeostatic and inflammatory physiologic processes. In this system, 19 receptors are activated by 47 chemokines and redundancy apparently abounds. For example, the CCR5 receptor interacts with seven natural chemokines, two of which also interact with CCR2 and three of which also interact with CCR1 (Wells et al., 2006). Reported biased signaling within this natural system has been found for the CCR7 chemokine receptor. Specifically, the endogenous agonists CCL19 and CCL21 are biased, in terms of signaling, with respect to each other. Although both produce G protein activation, only CCL19 (not CCL21) causes receptor agonist-dependent phosphorylation and

recruitment of β -arrestin to terminate the G protein stimulus (Kohout et al., 2004). Later studies on this receptor confirmed and extended this finding (Byers et al., 2008; Hauser and Legler, 2016). Another example of natural biased signaling is found in the expression of splice variants of CXCR3 (the CXCR3 primary transcript has three natural alternative splice variants). Specifically, four natural agonists for this receptor (CXCL4, CXCL9, CXCL10, and CXCL11) demonstrate very different biased signaling (with respect to G protein vs. β -arrestin) on these variants to affect cell-based signaling selectivity (Berchiche and Sakmar, 2016). The chemokine system is currently an active target for drug discovery and strategies employing biased signaling are under investigation (Amarandi et al., 2016; Anderson et al., 2016; Roy et al., 2017). Another multiple natural agonist system involves the Class Frizzled (FZD1–10) receptors activated by the WNT family of lipoglycoproteins; these endogenous ligands are shown to have natural bias toward different downstream signaling pathways producing functional selectivity within a complex network of signaling pathways (Dijksterhuis et al., 2015). Finally, although there is evidence for biased signaling within collections of natural multiple endogenous agonists, this mechanism also is operable for metabolites of natural agonists to produce modified signaling after agonist metabolism. For example, the catabolism of adenosine to inosine produces a biased new agonist with altered signaling properties (Welihinda et al., 2016). As will be seen in later sections of this article, the production of unique active-state receptor conformations to produce biased signaling is a mechanism that can produce cell-based biased effects due to the relative stoichiometry of receptors and signaling proteins. Thus, natural signaling can further be diversified at the level of the cell. Table 1 shows other natural pleiotropic receptor systems demonstrating natural signaling bias within the array of endogenous agonists known for those receptors.

The acceptance of natural signaling bias also opens the question of alteration in natural signaling with changes in physiology (i.e., through protein mutation). It might be expected that changes in receptors and/or signaling proteins would lead to changes in natural biased signaling and, in fact, this has been observed. Thus, mutations of critical amino acid residues have been seen to produce alterations in bias between G proteins and β -arrestin for the muscarinic M₂ receptor (Gregory et al., 2010) and between G_q and G_s proteins for the NK₁ receptor (Valentin-Hansen et al., 2015). Differences in bias also have been documented for receptor isoforms such as the histamine H₂ receptor (Riddy et al., 2017). These effects extend to mutations found in disease such as the alterations in signaling bias seen for the prokineticin receptor 2 in Kallman syndrome (Sbai et al., 2014).

TABLE 1
Naturally biased signaling

Receptor	Agonist 1	Signaling	Agonist 2	Signaling	Reference
GLP-1 PAC1	Oxyntomodulin PACAP ₁₋₂₇	Preferred cAMP cAMP > IP3	GLP-1 PACAP ₁₋₃₈	cAMP/ β -Arr1/ β -Arr2 IP3 > cAMP	Jorgensen et al. (2007) Spengler et al. (1993), Blechman and Levkowitz (2013)
CCR7 P2Y2 μ -Opioid CXCR4	CCL19 ATP β -Endorphin Ubiquitin	β -Arr2 > β -Arr1 β -Arr1 > β -Arr2 β -Arr2 > cAMP IP3 > β -Arr2	CCL21 UTP Endomorphin-2 CXCL12	$\neq \beta$ -Arr2 $\neq \beta$ -Arr1 β -Arr1 = β -Arr2 cAMP > β -Arr2 IP3/ β -Arr2	Byers et al. (2008), Hauser and Legler (2016) Hoffmann et al. (2008) Thompson et al. (2015) Eby et al. (2017)
Ang II type 1 PAR1 Adenosine _{A2A} V ₂ R	AT-(1-7) APC Inosine Vasopressin	β -Arr2 > G _q β -Arr2 > G protein ERK1/2 > cAMP Prolonged cAMP	Ang II Thrombin Adenosine Oxytocin	G _q / β -Arr2 G protein/ β -Arr2 cAMP/ERK1/2 Transient cAMP	Galandrin et al. (2016a), Teixeira et al. (2017) Roy et al. (2016) Welihinda et al. (2016) Feinstein et al. (2013)

Ang, angiotensin; APC, activated protein C; Arr, arrestin; AT, angiotensin; P2Y2, purinergic receptor Y2; PAC, pituitary adenylate cyclase-activating polypeptide type I; V₂R, vasopressin receptor 2.

V. Therapeutic Application of Biased Signaling

From the very first discussions of receptor signaling bias, the concept has been proposed as a means to make more selective and effective drugs. The first application of this idea was toward the design of better antipsychotic drugs by Mailman and colleagues (Lawler et al., 1994, 1999), studies which led to the identification of the atypical antipsychotic drug aripiprazole (Urban et al., 2007). Based on early studies showing that the angiotensin analog SII ([Sar,Ile4,Ile8]-angiotensin II) does not activate G_q protein but does trigger β -arrestin recruitment to the receptor (with subsequent ERK1/2 activation) (Azzi et al., 2003; Wei et al., 2003), an early biased ligand to be taken into the clinic was the analog TRV027 for heart failure (Violin et al., 2010; Felker et al., 2015); this molecule was developed as an improvement on existing angiotensin receptor blockers such as losartan. Specifically, although blockade of angiotensin receptor-mediated pressor effects is beneficial in heart failure (reduction in cardiac afterload), the added β -arrestin signaling properties of TRV027 are proposed to offer an advantage through cardioprotective effects (Violin et al., 2010; Monasky et al., 2013). Thus, TRV027 enhances cardiac contractility and output (Violin et al., 2010) and is distinguishable from angiotensin-converting enzyme inhibitors and angiotensin receptor blockers by controlling vascular effects to prevent prolonged hypotension (Violin et al., 2014). This line of research for heart failure is being continued with another biased angiotensin ligand TRV067, which blocks G_q protein signaling while producing sensitization of myofilament calcium-responsiveness in a genetic mouse model of dilated cardiomyopathy (Ryba et al., 2017).

A second biased drug, TRV130 (*N*-[(3-methoxythiophen-2-yl)methyl]-2-(9-pyridin-2-yl)-6-oxaspiro[4,5]decan-9-yl)ethanamine), a μ -opioid receptor agonist for postoperative pain, is also currently in clinical trials (Chen et al., 2013a; Violin et al., 2014). The rationale for advancing this molecule is the preclinical data showing that analgesia is associated with G α_i activation, whereas gastrointestinal dysfunction, respiratory depression, and tolerance may be linked to β -arrestin2 recruitment

(Bohn et al., 1999, 2000; Ikeda et al., 2002; Raehal et al., 2005; Li et al., 2009; Yang et al., 2011; DeWire et al., 2013). Although the treatment of moderate to severe acute pain has been confirmed for TRV130 (Soergel et al., 2014; Viscusi et al., 2016), typical opioid agonist side effects were still found to occur (Viscusi et al., 2016). This is consistent with effects seen in mice, in which TRV130 produced antinociception but concomitant inhibition of gastrointestinal function and weak abuse-related effects. However, repeated treatment failed to produce tolerance seen with morphine (Altarifi et al., 2017). Subsequent studies in this area have advanced a similar analgesic in TRV734 (White et al., 2015). In addition, medicinal chemical strategies to direct μ -opioid receptor stimulus toward G protein activation versus β -arrestin2 in scaffolds such as PZM21 (1-[(2*S*)-2-(dimethylamino)-3-(4-hydroxyphenyl)propyl]-3-[(2*S*)-1-(thiophen-3-yl)propan-2-yl]urea) (Manglik et al., 2016; Hill et al., 2018) have resulted in a profile of analgesia with minimal constipation and respiratory depression (Soergel et al., 2014; Viscusi et al., 2016).

The translation of biased signaling to in vivo systems involves assessments of the impact of the unique signaling profiles of molecules. An important aspect of this question is the determination of the effects of biased signaling on natural physiology; this furnishes data to guide the rational design of new biased molecules for therapeutic advantage. Genetically modified systems have been instrumental in this process.

A. Assessing the Impact of Biased Signaling on Natural Physiology

One of the main tools available to pharmacologists in the interpretation of in vitro bias in terms of what it may mean in vivo is the genetic knockout system (i.e., a frequent approach is to produce genetic knockouts for β -arrestin to assess the importance of this signaling system). Although deletion of both nonvisual arrestin isoforms is lethal, individual deletion of β -arrestin1 or β -arrestin2 can be studied (Kohout et al., 2001). Thus, it can be seen that opioid analgesics such as morphine produce less respiratory depression in β -arrestin knockout

mice (compared with wild-type mice) (Raehal et al., 2005), leading to the hypothesis that μ -opioid agonists with less propensity to induce receptor- β -arrestin interaction might offer a better margin of analgesia (over respiratory depression; e.g., see data with TRV027; Violin et al., 2014). Mice devoid of β -arrestin2 (but not β -arrestin1) have demonstrated altered behavioral responses to addicting drugs such as morphine (Bohn et al., 2003; Urs and Caron, 2014), amphetamine (Urs and Caron, 2014), and alcohol (Li et al., 2013). In general, the use of transgenic mice has identified β -arrestin2 as a clear mediator of unwanted μ -opioid receptor agonism (Raehal et al., 2005). Similarly, the fact that PTH analogs do not effectively produce bone in β -arrestin knockout mice leads to the hypothesis that G protein-biased PTH agonists could offer better profiles for therapy in osteoporosis (Ferrari et al., 2005). There are cases in which genetic modification of systems can be linked to actual therapeutically relevant drug profiles. For instance, the biased ligand UNC9975 (7-[4-[4-(2,3-dichlorophenyl)-1,4-diazepan-1-yl]butoxy]-1,2,3,4-tetrahydro-1,8-naphthyridin-2-one) displays potent antipsychotic-like activity without induction of motoric side effects in inbred C57BBL/6 mice. Furthermore, genetic deletion of β -arrestin2 attenuates antipsychotic activity, thus transforming UNC9975 from an atypical to a typical antipsychotic (Allen et al., 2011).

Technological advances have enabled genetic knock-out systems to be made (e.g., Rohrer and Kobilka, 1998) and these have enabled the study of physiologic systems without selected components in studies to determine the importance of those components to the physiology. In general, knockout animals have been instrumental in identifying physiologically relevant pathways for drug candidates for dopamine D₁ receptors (Xu et al., 1994a,b), metabotropic glutamate 1 receptors (Aiba et al., 1994), 5-HT_{2B} receptors (Saudou et al., 1994), angiotensin 1A receptors (Ito et al., 1995; Coffman, 1997), μ -opioid receptors (Sora et al., 1997), α_{2b} -adrenoceptors, (Link et al., 1996; MacMillan et al., 1996), α_{1b} -adrenoceptors (Cavalli et al., 1997), β_1/β_2 -adrenoceptors (Rohrer et al., 1999), β_3 -adrenoceptors (Susulic et al., 1995), and muscarinic M₃ receptors (Duttaroy et al., 2004). Similar outcomes have been observed through ablation of receptor effects through application of RNA-guided CRISPR/Cas9 endonucleases (Naylor et al., 2016).

Complimentary data are obtained from knock-in studies whereby the endogenous GPCR gene is replaced with a gene for a mutant receptor and the expression of that mutant is driven by the wild-type promoter; the aim of this approach is to express the mutant receptor in the same tissue types and at the same receptor levels as the wild-type receptor. An example of the application of this technology is found in the elucidation of the relative contributions of δ - and μ -opioid receptors in the sensation of mechanical and heat pain with enhanced green fluorescent protein δ -opioid receptors (Scherrer et al., 2006,

2009; Pradhan et al., 2009; Shenoy and Lefkowitz, 2011; Faget et al., 2012). Similarly, the impact of muscarinic M₃ receptor phosphorylation on learning, glucose tolerance, and insulin release has been studied with phosphorylation-deficient M₃ receptors (Kong et al., 2010; Poulin et al., 2010). These phosphorylation-deficient muscarinic M₃ receptors do not internalize but couple normally to G protein-dependent signaling such as PLC/calcium mobilization mechanisms (Budd et al., 2001; Urban and Roth, 2015).

Inserting a coding sequence of a mutant receptor that is only activated by a synthetic ligand [to code for a designer receptor exclusively activated by designer drugs (DREAD); Conklin et al., 2008; Urban and Roth, 2015] is a powerful technology whereby the relevance of certain signaling to cognate physiology can be assessed (Peng et al., 2008). The first application of this approach was made with the κ -opioid receptor modified to contain the second extracellular loop of the δ -opioid receptor (Coward et al., 1998) to yield a receptor with a 200-fold reduction in the binding of the endogenous opioid agonist dynorphin (and reductions in the binding of 21 other opioid peptides) but maintained binding and activation for the synthetic agonist spiradoline. This receptor was given the name RASSL for "receptor activated solely by a synthetic ligand." A problem with early studies with RASSLs was that the retention of activity of the synthetic ligand for native receptors caused concomitant activation of native receptors in the transgenically modified animals in addition to the RASSLs (Redfern et al., 1999). In addition, RASSLs often have a high level of constitutive activity, further complicating interpretation of experimental data (Hsiao et al., 2008). These drawbacks led to the development of second-generation RASSLs named DREADs, in which the agonist (clozapine-*N*-oxide) has no other activating properties for native receptors (Armbruster et al., 2007; Conklin et al., 2008; Giguere et al., 2014; Urban and Roth, 2015). Using this technology, the role of M₃ receptor signaling (Armbruster et al., 2007; Dong et al., 2010) and free fatty acid receptor-2 signaling (Hudson et al., 2012) has been explored. DREADs have been used to evaluate the importance of biased signaling in different cell types as in studies on the cell type-specific expression of a muscarinic M₃ receptor DREAD mutationally modified to not interact with β -arrestin but rather only G_{q/11} proteins (Hu et al., 2016). A related approach allows the activation of the mutant receptor optically through light (Levitz et al., 2013), a new technology as yet to be applied to the study of signaling bias.

Finally, there are obvious caveats to the interpretation of these studies to human therapeutics. Differences in animal versus human signaling can confuse conclusions from the data. For instance, it has been suggested that cannabinoid CB₁ receptor signaling differs between humans and rodents (Straiker et al., 2012). In

addition, the known changes in signaling preferences of receptors with receptor mutation (*vide infra*), as well as the expectation of signaling signatures different from natural ones with synthetic agonists such as clozapine-*N*-oxide, raises the specter that DREADDs will give misleading signaling profiles in natural physiology. At present, studies to assess this are consistent with this not being a tangible problem (Alvarez-Curto et al., 2011).

B. Assessing the Impact of Biased Signaling from Known Ligands

Retrospective analyses have provided insights into how some uniquely beneficial currently used therapeutic drugs achieve their favorable profiles through biased signaling. Thus, the beneficial effects of carvedilol, a nonselective β -adrenoceptor inverse agonist for G_{α_s} -mediated cAMP production in congestive heart failure, have been attributed to its β -arrestin-mediated partial agonist activity for activation of ERK1/2 (Wisler et al., 2007; Kim et al., 2008). Similar signaling profiles have been associated with nebivolol (Erickson et al., 2013), alprenolol (Kim et al., 2008), and propranolol (Azzi et al., 2003; Baker et al., 2003). The diminished respiratory depression potential of the opioid analgesic levorphanol (over morphine) has been attributed to its biased signaling profile (lack of β -arrestin2 recruitment) (Le Rouzic et al., 2019). In fact, the dependence liability of oxycodone, hydrocodone/paracetamol, and hydromorphone has been attributed to biased signaling (toward G protein vs. β -arrestin) (Johnson et al., 2017). The cardioprotective and cardiac fibrosis-modulating properties of the adenosine agonist capadenoson (currently in clinical trials) have been attributed to its biased cAMP activity through adenosine 2b receptor activity (Baltos et al., 2017). The biased activation of G_s protein (over nonspecific dual G_s and G_i activation) for fenoterol has been proposed as a favorable property for this bronchodilator (Jozwiak et al., 2010). Similarly, the tolerance seen with morphine, as opposed to other μ -opioid receptor agonists, has been attributed to this agonist's selective signaling through β -arrestin2 as opposed to β -arrestin1 (Raehal and Bohn, 2011).

Irrespective of novel therapeutics, biased ligands can be valuable probes of physiologic processes and disease states. For example, PAR₂, which is highly expressed in HT-29 colorectal carcinoma cells, is implicated in cancer (Elste and Petersen, 2010). Through observation of the effects of newly developed PAR₂ biased agonists, the relative importance of ERK1/2 versus calcium signaling in human cancer through this receptor has been studied (Jiang et al., 2017). Similarly, comparison of nonpeptide biased agonists of the nociception/orphanin FQ receptor has been applied to study the pharmacology of nociceptin orphanin activation in disease states (Ferrari et al., 2017). Elegant studies with a range of biased PTH analogs have been valuable in elucidating the complicating bone-building and bone resorption effects of PTH

for therapy of osteoporosis (Luttrell et al., 2018). The study of the biased κ -opioid receptor antagonist norbinaltorphimine has enabled linkage of JNK signaling to long-term blockade of antinociception (with no ERK activity) and selective long-term effects on regulation of κ -opioid receptors (Jamshidi et al., 2016). A novel application of bias in the delineation of the role of β -arrestin signaling in cardiac β -adrenoceptor function has been suggested in the use of a biased pepducin, (ICL)-1-9 [TAIAKFERLLQTVTNFYFIT], to decouple β -arrestin signaling from occupation of the receptor (Carr et al., 2016). Biased ligands have been especially valuable in the study of systems where there appears to be duplication and crossover between ligands and receptors such as the chemokine receptor system (Amarandi et al., 2016; Milanos et al., 2016a).

For peptide receptors, truncation of the natural peptide can lead to biased analogs of value in the delineation of physiologic pathways. For example, a biased analog of human neuropeptide S, hNPS-(1-10) lacks 10 residues from the C terminus of the natural peptide and preferentially activates G_{α_q} -mediated calcium mobilization with less activity at G_{α_s} (compared with the natural peptide); this analog produces no physiologic effect *in vivo*, providing a unique probe of the physiology and therapeutic potential of hNPS-directed signaling (Liao et al., 2016). Biased agonists can dissect complex signaling patterns of endogenous agonists to determine dominant signaling. For instance, the dependence on various physiologic endpoints of free fatty acid receptor-2 stimulation on different signaling was determined through studies with the biased agonist AZ1729 (*N*-[3-(2-carbamimidamido-4-methyl-1,3-thiazol-5-yl)phenyl]-4-fluoro benzamide), which predominantly activates only G_i (not G_q/G_{11}) signaling (Bolognini et al., 2016).

Beyond using biased ligands to probe natural physiology, this idea has been advanced as a strategy for the design of better (i.e., more selective) drug therapy with fewer side effects. There are basically four rationales for this approach: 1) emphasis of a therapeutically favorable signal (i.e., PTH in osteoporosis; Gesty-Palmer and Luttrell, 2011; Gesty-Palmer et al., 2013), 2) de-emphasis of an unfavorable signal (respiratory depression for opioid agonists; Raehal et al., 2005; Kelly, 2013; Koblish et al., 2017), 3) production of limited signaling to allow prosecution of otherwise forbidden drug targets (i.e., κ -opioid receptors; White et al., 2014; Brust et al., 2016), and 4) emphasis of a favorable signal and prevention of the natural system production of an unfavorable signal (i.e., angiotensin in heart failure; Violin et al., 2006, 2010). Based on these general ideas, Table 2 shows a sampling of receptors and therapeutic applications of biased signaling that have been proposed in the literature as possible avenues toward better drug therapy.

One important consideration in the evaluation of biased signaling in therapeutics is the difference between natural nondiseased systems that are used for ligand characterization and pathologically modified systems in therapy (Insel et al., 2015). In disease states, the relative stoichiometry or components and sensitivities of cells are known to vary. For example, GRK2 is upregulated in heart failure, leading to an increased phosphorylation of β -adrenoceptors and downregulation of receptors (Casey et al., 2010). In hypertrophic myocytes from mice with heart failure, levels of G protein were found to be upregulated (i.e., $G\alpha_o$, 7.5-fold; and $G\alpha_{11}$, 12.5-fold) leading to differences in β -adrenoceptor agonist biased signaling (Onfroy et al., 2017). Similarly, the apelin pathway is known to be downregulated in heart failure (Yang et al., 2015). The natural signaling bias of the calcium-sensing receptor is also altered in disease states (for review, see Leach et al., 2015). Models of dystonia (a common movement disorder) involving mutation of the protein torsinA demonstrate a pathologic increase in cholinergic tone to affect dopamine interneurons, and there is a change in dopamine

signaling polarity and a bias introduced into dopamine signaling from primarily $G_{i/o}$ to noncanonical β -arrestin signaling (Scarduzio et al., 2017). In general, biased signaling is an obvious mechanism to exploit for drug therapy and the existing data with characterized biased ligands certainly show different phenotypical signaling profiles in vivo. What is lacking at this time is a systematic linkage between in vitro profiles of biased signaling and the translation to in vivo systems.

VI. Molecular Mechanism(s) of Ligand Bias

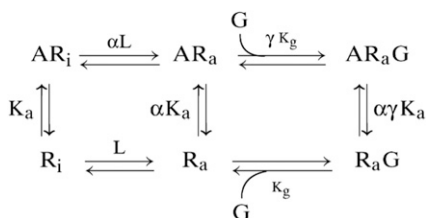
The first proposed and still most commonly cited mechanism of agonist-induced biased signaling is the selective stabilization of unique receptor conformational “active” states (from the point of view of interacting in a fruitful way with a signaling protein to induce a cellular signal) (Kenakin and Morgan, 1989; Kenakin, 1995); subsequent literature supports this hypothesis (Nickolls et al., 2005). It is worth examining this idea in light of our present understanding of receptor systems. 7TMRs are pleiotropic with respect to the proteins with which they

TABLE 2
Preconceived strategies for applying biased signaling to therapeutic advantage

Receptor	Therapeutic Application	References
Dopamine	Neuropsychiatric disorders	Lawler et al. (1994, 1999), Roth et al. (2004), Beaulieu et al. (2007), Allen et al. (2011), Möller et al. (2017)
Muscarinic M_1	Alzheimer disease	Galandrin et al. (2007)
Muscarinic M_3	Alzheimer disease	Poulin et al. (2010)
Calcium	Kidney disease, hyperparathyroidism	Thomsen et al. (2012), Leach et al. (2015)
Ghrelin-R1a	Obesity, growth hormone secretion	Evron et al. (2014), M'Kadmi et al. (2015)
Cannabinoid-1	Pain management, addiction, energy metabolism, diabetes, Huntington disease, Parkinson disease, multiple sclerosis	Laprairie et al. (2017)
Apelin	Heart failure, pulmonary artery hypertension, myocardial infarction	Japp et al. (2010), Yang et al. (2015), Read et al. (2016)
CXCR4, CCR6, CCR7	Cancer metastasis	Roy et al. (2014, 2017)
μ -Opioid	Analgesia	Bohn et al. (2004), DeWire et al. (2013), Manglik et al. (2016), Viscusi et al. (2016), Altarifi et al. (2017)
Adenosine A_{2B}	Cardioprotection, diabetes, cancer	Wei et al. (2013), Merighi et al. (2015), Vecchio et al. (2016)
Adenosine A_1	Ischemia reperfusion injury, paroxysmal supraventricular tachycardia	Valant et al. (2014)
κ -Opioid	Analgesia	White et al. (2015)
Sphingosine 1 phosphate 1	Immunomodulation, multiple sclerosis, allograft rejection	Oo et al. (2007)
Hydroxyl carboxylic acid receptor	Lipid lowering	Walters et al. (2009)
Urotensin receptor	Hypertension, heart failure, cardiac fibrosis	Brulé et al. (2014)
PAR-1	Thrombin with decreased bleeding	McLaughlin et al. (2005)
δ -opioid	Migraine, Parkinson disease, neuropathic pain	Pradhan et al. (2011), White et al. (2015)
PTH receptor	Osteoporosis	Gesty-Palmer et al. (2009)
PACAP	Chronic pain, stress-related disorders	May and Parsons (2017)
PAR-1/2	Cancer, gastrointestinal disorders, inflammation	Zhao et al. (2014), Bar-Shavit et al. (2016), Jiang et al. (2017), Sebastiano et al. (2017)
β -adrenergic	Heart failure	Haney and Hancox (2006), Jozwiak et al. (2010)
Endothelin	Cancer	Maguire (2016), Bologna et al. (2017)
Formyl peptide receptor	Myocardial infarction	Qin et al. (2017)
GLP-1	Diabetes	Koole et al. (2013)
Angiotensin-1R	Renal fibrosis, cardiomyopathy	Maning et al. (2017), Ryba et al. (2017); Wang et al. (2017)
Neurotensin-1	Addiction	Barak et al. (2016)
MC4R	Obesity	Yang and Tao (2017)
Oxytocin	Dysfunctional labor, hemorrhage	Grotegut et al. (2011)
mGluR7	Learning, memory disorders	Wang et al. (2016)
Histamine H_1/H_2	Cancer	Monczor and Fernandez (2016)

MC4R, melanocortin receptor 4; mGluR, metabotropic glutamate receptor.

interact. The coding for these interactions is embodied in the tertiary conformation of the receptor either through a spontaneous isomerization (i.e., constitutive activity) or due to the interaction with another body such as a ligand or accessory protein. The simplest model for such activation is the formation of a single uniform receptor active state that triggers activation of all signaling bodies interacting with the receptor. Ostensibly, this idea appears to be contained in the simple extended ternary complex model for 7TMRs published in 1993 (Samama et al., 1993):



where an equilibrium exists between the inactive ($[R_i]$) and active ($[R_a]$) state of the receptor and is controlled by an allosteric constant L . The equilibrium association constants for ligand $[A]$ and G protein $[G]$ for the receptor are K_a and K_g , respectively. α represents the difference in the affinity of the ligand for the active state over the inactive state, and γ is the difference in the affinity of the agonist-bound receptor when the active-state receptor ($[R_a]$) is and is not bound by agonist. However, such a simplistic interpretation of a single receptor active state within this model is an illusion, since it can be seen that variation in the γ term describing the affinity of the agonist-bound receptor and G protein is a variable that can change with agonist type (i.e., this model basically describes an infinite number of receptor active states contained in the value of γ with each agonist binding to the receptor). This is in accordance with standard allosteric theory, which dictates that allosterically interacting bodies (in this case, the ligand and G protein both interacting with the receptor) practice probe dependence; that is, the effect of different probes on the receptor conformation with respect to the interaction with other probes will differ with the nature of that probe. Since allosteric energy is reciprocal, there is another probe dependence that becomes operative as the ligand-bound receptor interacts with a signaling protein (namely a dependence relating to the type of signaling protein). Thus, as the agonist-bound receptor binds to different G proteins, there will be a unique γ value for every G protein (or indeed any other signaling protein in the membrane). It can be seen that this can theoretically lead to a very large number of unique possibilities. As a preface to the discussion of these types of systems, consideration of the nature of agonist efficacy is useful (i.e., how does a ligand participate in the transformation from R_i to R_a ?).

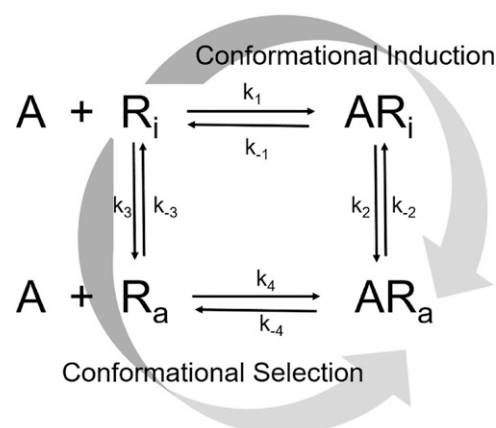


Fig. 4. Receptor system whereby the receptor can exist in an active (R_a) and inactive (R_i) state. A ligand (A) binds to both with varying affinity. Conformational selection is where the ligand preferentially binds to a preexisting R_a state, and conformational induction is where the ligand binds to the R_i state to convert it to the R_a state.

Thermodynamic considerations for the scheme shown in Fig. 4 strongly suggest that conformational selection would be the only feasible mechanism to yield production of AR_a by a ligand within the timeframe required to sustain life in cells (Burgen, 1981; Bosshard, 2001; Vaidehi and Kenakin, 2010; Vogt and Di Cera, 2013). This being the case, the first consideration for agonist-induced biased signaling is the number of choices the ligand has to select from. The scheme shown in above suggests only two, but the allosteric nature of functional receptor systems (Tucek, 1997), as well as the natural flexible nature of 7TMRs (Liapakis et al., 2012), argues against a simple two-state selection. In fact, the inherent flexibility of proteins possessing marginal conformational stability under physiologic conditions ($\Delta G_{\text{folding}} = -5$ to -10 kcal/mol; Privalov and Khechinashvili, 1974; Williams et al., 2007) ensures a high degree of function-related conformational flexibility (Frauenfelder et al., 1979; Tang and Dill, 1998; Williams et al., 2007). In addition, a great deal of experimental evidence since the proposal of the extended ternary complex model and the introduction of molecular dynamics into pharmacology has provided an alternative view, namely the selection of receptor conformations from a preexisting ensemble of similar but different conformations (Boehr et al., 2009; Dror et al., 2010, 2011; Park, 2012; Nygaard et al., 2013; Motlagh et al., 2014). This ensemble of receptor conformations forms a dynamic system (Vardy and Roth, 2013; Manglik and Kobilka, 2014; Manglik et al., 2015), which then interacts with signaling systems through a full range of allosteric linkages (Monod et al., 1965; Changeux and Edelstein, 2005); these ideas have been discussed in terms of oscillating dynamic systems of multiple conformations (Cui and Karplus, 2008; Changeux and Edelstein, 2011) that produce “fluctuating networks” operating on a real-time scale of micro-seconds (Ichikawa et al., 2016).

Many techniques have demonstrated that receptors can be stabilized by ligands into a range of different conformations (Luttrell and Kenakin, 2011). For instance, in studies with β -adrenoceptors, multiple conformations have been demonstrated through the use of a monobromobimane-labeled receptor (Yao et al., 2006), whereas hydrogen/deuterium exchange coupled with mass spectrometry reveals a range of changes in the kinetic behavior of the β -adrenoceptor in different regions (West et al., 2011). Fluorescence spectroscopy has also been used to study conformational heterogeneity for vasopressin receptors (Rahmeh et al., 2012), whereas differential ligand modulation of the β_2 -adrenoceptor energy landscape has been shown through dynamic single-molecule force spectroscopy (Zocher et al., 2012). More recently, NMR studies have shown that different ligands stabilize different conformations within these ensembles (Kofuku et al., 2012; Liu et al., 2012; Nygaard et al., 2013). This idea has been extended to the stabilization of unique receptor conformations by nanobodies for β -adrenoceptors (Rasmussen et al., 2011), which show a variety of effects on cAMP signaling and β -arrestin recruitment (Staus et al., 2016).

Theoretical computational methods can be used to rationally design ligand-receptor active-state complexes. These active-state conformations have higher energy than inactive states and are thus more unstable. For this reason, computational methods are biased toward lower energy structures. However, recent advances in computational techniques have made inroads into the prediction of ligand-receptor active-state conformations of higher energy (Milanos et al., 2016b; Dong et al., 2017). In allosteric systems, it is important to consider all of the interactants, as each will have an influence on the behavior of the others. Therefore, it is important to consider the variety of signaling proteins and their conformations (specifically, the fact that these too form ensembles). Arrestins are known to exist in at least three distinct conformations (free, receptor bound, and microtubule bound; Gurevich et al., 2018) and within these categories, further heterogeneity exists. For instance, fluorescent arsenical hairpin BRET probes reveal that β -arrestin2 exists as a dynamic conformational ensemble (Lee et al., 2016). In fact, there is considerable evidence that structural disorder of arrestin elements appears to be important to their functionality (Gurevich et al., 2018). In terms of arrestin ensembles, the complexation of arrestins with receptors initiates signaling that free arrestins do not (Peterson and Luttrell, 2017), as shown by the enhancement of the affinity of ERK1/2 to arrestin by receptor recruitment (Luttrell et al., 2001) with activation occurring only after receptor stimulation (Luttrell et al., 2001; Coffa et al., 2011). Active-state β -arrestin2 conformations that lead to cellular signaling, promoted by the angiotensin ligands SII and angiotensin II and distinct from other conformations in the ensemble, have been described

(Shukla et al., 2008). In fact, it has been shown through biophysical (Nobles et al., 2007), mutation (Gurevich and Gurevich, 2006), and crystallographic (Shukla et al., 2013) experiments that β -arrestins undergo extensive conformational changes upon binding to phosphorylated receptors. Furthermore, it has been shown that different β -arrestin2 active-state conformations lead to different downstream signaling outcomes in the cell (Shukla et al., 2008; Zimmerman et al., 2012). In fact, it has been shown that different ligands binding to the same receptor can change the “population average conformational signature of arrestins” (Luttrell et al., 2018) to produce variable signaling outcomes (Lee et al., 2016; Nuber et al., 2016). For example, the application of fluorescence resonance energy transfer–based β -arrestin2 biosensors in real time in living human cells indicates that β -arrestins remain active after dissociation from receptors to signal independently at the cell surface (Nuber et al., 2016).

Evidence of heterogeneous agonist-receptor complexes with different signaling proteins has been generated with biosensors on receptor proteins that measure the probe environment with conformational change; an early study on β -adrenoceptors with this technique was published by Ghanouni et al. (2001). More recent studies with biosensors can discriminate between different ternary complexes (agonist/receptor/signaling protein). An example of this technique is seen with the angiotensin II type 1 receptor (Devost et al., 2017). Specifically, bioluminescence signals reporting energy transfer from the interaction of a Renilla luciferase as an energy donor placed at the distal end of the receptor C-tail and a small fluorescent arsenical hairpin molecule as an energy acceptor placed at various positions in the intracellular loops of the receptor were used to measure conformational changes (see Fig. 5A; Devost et al., 2017). With this system, it was observed that a range of angiotensin II type 1 receptor agonists produce unique receptor–G protein complexes from the point of view of conformation. Specifically, probes placed in three different regions of the receptor reflect differing BRET signals indicating varying distances and thus differing conformations (see Fig. 5B). BRET experiments have been used to identify δ -opioid receptor agonist-selective receptor conformations as well (Audet et al., 2008).

Finally, it is not yet clear how some subtle trafficking of stimulus is achieved further down into the cell cytosol. Two possible mechanisms for this are 1) a persistent binding of the agonist to the receptor to code for cytosolic control of effector interaction and 2) agonist-dependent stabilization of conformations that then are phosphorylated with different barcodes to determine subsequent interactions in the cytosol (Yang et al., 2015). A variant of the first mechanism is the idea that the receptor may have a “memory” of the conformational stabilization produced at the cell surface, which then lasts as the receptor continues interacting with effectors in the

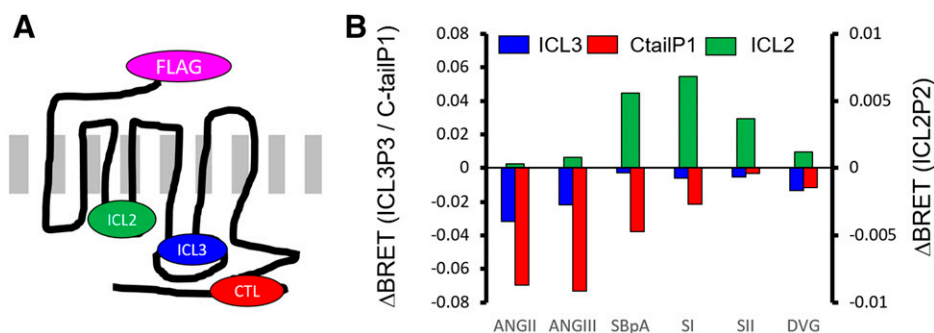


Fig. 5. (A) Ligand-selective conformational states of the angiotensin II type 1 receptor revealed with biosensors. A Renilla luciferase energy donor interacts with a small fluorescent molecule FLAsH as an energy acceptor placed at different positions on the intracellular loops of the receptor. (B) Unique ligand-selective complexes are indicated by the varying BRET signals (indicative of varying distances between the elements) produced by the sensors. ANG, angiotensin; CTL, C tail; DVG, (Asp¹,Val⁵,Gly⁸)-AngII; FLAG, FLAsHwalk-tagged epitope; FLAsH, fluorescent arsenical hairpin; ICL, intracellular loop; SBpA, (Sar¹,Bpa⁸)-AngII; SI, (Sar¹,Ile⁸)-AngII; SII, (Sar¹,Ile⁴,Ile⁸)-AngII. Redrawn from Devost et al. (2017).

cytosol after the ligand has dissociated from the receptor. Some evidence for this has been reported for receptor-arrestin3 complexes where specific conformations of the receptor are maintained after dissociation of arrestin3 (Nuber et al., 2016). Recently, a striking variation on the theme of subcellular compartmentalization of signaling was reported for muscarinic M₃ receptors and β_2 -adrenoceptors in the form of preassembled GPCR signaling complexes at the cell membrane that mediate responses to extremely low (as low as attomolar) concentrations of agonist (Civciristov et al., 2018).

More detailed insights into bias mechanisms have been gained by linking structure-activity relationship (SAR) experiments to receptor structural data (Shukla et al., 2014; Ranjan et al., 2017; Wacker et al., 2017; Zhou et al., 2017; Lee et al., 2018; McCorvy et al., 2018). Such structural studies have been targeted toward β_2 -adrenoceptors (Reiter et al., 2012), κ -opioid receptors (Zheng et al., 2017; Che et al., 2018), 5-HT_{1B} and 5-HT_{2B} receptors (Wacker et al., 2013; Wang et al., 2013), muscarinic M₁ acetylcholine receptors (Kruse et al., 2013; Abdul-Ridha et al., 2014), and muscarinic M₂ receptors (Dror et al., 2013). Receptor structures of ligand complexes with the β_1 -adrenoceptor have been particularly useful in the study of biased signaling. Specifically, crystal structures of the receptor complexed with the differentially biased ligands bucindolol, dobutamine, and carvedilol suggest that varying interactions of the ligands at extracellular loop 2 of the receptor through direct or water-mediated effects are critical to biased signaling at this receptor (Warne et al., 2012). Similarly, for the β_2 -adrenoceptor, NMR studies have shown that “balanced” agonists for β_2 -adrenoceptors shift the ensemble equilibrium toward the G protein-specific active state of helix 6 of the receptor, whereas β -arrestin-biased ligands regulate the conformational states in helix 7 (Liu et al., 2012). Similarly, bias SAR information regarding regions of receptors that are involved in biased signaling has also been obtained through site-directed mutagenesis as seen, for example,

with G protein receptor 183 (Daugvilaite et al., 2017) and μ -opioid receptors (Hothersall et al., 2017).

VII. Detection and Quantification of Biased Signaling

A. Deviations from Monotonic Signaling

Biased signaling is detected by measuring deviations from a pattern and they are quantified by measuring the degree of that deviation. As discussed in section I, all systems are biased by physiology in terms of the needs of the organ system. Thus, receptor levels, relative stoichiometry of receptors to signaling proteins, and efficiency of stimulus-response coupling are unique in every organ system and dictate the sensitivity of those organ systems to endogenous agonism. Much of the challenge of translating in vitro effects to in vivo systems is due to this customization of organ sensitivity in the body.

The molecular mechanisms involved in the production of biased receptor signals were suggested from the early work describing deviations from predictions of monotonic (unbiased) receptor signaling. As first presented, agonist efficacy was a monotonic signal emanating from the receptor upon binding of the agonist to the receptor (Ariens, 1954; Stephenson, 1956; Furchgott, 1972; Mackay and Van Rossum, 1977). This signal was assumed to be homogeneous in nature, varying only in signal strength; the assumption for this was rooted in the lack of knowledge of the nature of cellular receptor signals and the fact that only a single readout of drug response was normally available in the assays used for characterizing agonism. For example, Stephenson (1956) characterized ligands with efficacy as those producing contraction of guinea pig ileum in functional in vitro experiments. This concept led to the derivation of one of the most useful tools in quantitative pharmacology for the classification of therapeutic agonists, namely the agonist potency ratio (PR). Through application of null experiments and the assumption that agonists produce a homogeneous signal of activation

from the receptor termed “stimulus” (Stephenson, 1956), the idea emerged that equiactive concentrations of agonists could be used to derive a ratio of potency that would be receptor agonist specific and thus transcend the test system in which it was measured. This would be the case since the cellular system translating the receptor stimulus would serve only as an amplifier that does not change the nature of the signal. Under these circumstances, PRs of agonists could be derived in test systems and used to predict relative agonist potency in all systems including the therapeutic one; this is an enormously useful idea since agonists are usually developed in test systems, not the therapeutic one. The system independence of PR values can be illustrated through examination of agonist response through the Black/Leff operational model (Black and Leff, 1983) (eq. 2):

$$\text{Response} = [A]/K_A\tau/([A]/K_A(1 + \tau) + 1) \quad (2)$$

where τ is efficacy and K_A is the equilibrium dissociation constant of the agonist-receptor complex. Equation 2 predicts that potency as quantified by the EC_{50} of the agonist (concentration producing the half-maximal effect), which is given by eq. 3:

$$\text{Agonist Potency} = EC_{50} = K_A/(1 + \tau) \quad (3)$$

For full agonists where $\tau \gg 1$, the EC_{50} becomes K_A/τ , an expression derived from the ratio of the affinity and efficacy of the agonist. It follows that the PR of two agonists (A_1 and A_2) measured in the same functional system is calculated as shown in eq. 4:

$$PR = K_{A-1}\tau_2/K_{A-2}\tau_1 \quad (4)$$

As defined by Black and Leff (1983), τ is a term formally identical to Stephenson's efficacy term, in that it contains elements related strictly to the agonist and also the test system in which it measured. However, ratios of τ cancel the tissue-related elements and leave a ratio of strictly agonist-related efficacy. Therefore, ratios of τ/K_A values for two agonists become tissue-independent measures of the relative power of the two agonists to induce response in any system.

Although PR values were and are important quantitative parameters for the measurement of agonist in pharmacology, their intrinsic value is predicated on the assumption that the nature of the signal produced by the two agonists at the level of the receptor is identical and that the processing of that signal is a monotonic function (one y for every x in a Cartesian system of stimulus to response) by the cell. It follows that deviations of PR values seen experimentally, presuming they are not due to experimental error, furnish evidence negating the monotonic assumption of signal processing. The key to determining such heterogeneity in signaling is the availability of multiple measures of agonist response (i.e., the

delineation of the nature of agonist response into its elements at the level of the receptor-signaling protein interface). If independent observation of two signals from the same agonist-receptor interaction can be obtained, then a direct test of the monotonic nature of stimulus-response coupling can be made. This was predicted in theoretical terms for a system of receptor interaction with two G proteins (Kenakin and Morgan, 1989). In this study, the effect of the production of different active states of the receptor with two agonists on the resulting affinity of interaction of that receptor with two G proteins on the observed potency of the agonists was simulated. Assuming that different active states (i.e., different tertiary conformations of the receptor) will have correspondingly different affinities for the two G proteins in the system, it is predicted that differences in the relative potency of the agonists will be observed. Thus, variation in PR values would constitute evidence of the production of different receptor active states by the agonists (i.e., biased agonism would be predicted) (Kenakin and Morgan, 1989).

As agonists were tested in more and different functional systems, reports increasingly cited instances where the simple relationship between occupancy and response predicted by monotonic efficacy systems were not verified (for example, see Roth and Chuang, 1987; Mottola et al., 1991; Roerig et al., 1992; Fisher et al., 1993; Gurwitz et al., 1994; Lawler et al., 1994, 1999; Ward et al., 1995; Heldman et al., 1996; Mailman et al., 1998). Incontrovertible evidence of deviation from monotonic signaling was furnished for the PACAP receptor in a system whereby cAMP and IP signaling could be monitored from the same receptor as a function of activation by two agonists, PACAP₁₋₂₇ and PACAP₁₋₃₈ (Spengler et al., 1993). These experiments showed a reversal of relative potency of PACAP₁₋₂₇ and PACAP₁₋₃₈ for these two pathways, indicating undisputable variation in affinity and/or efficacy of the agonists as the receptor interacted with different signaling pathways. Specifically, as shown by eq. 4, reversed PR values can only occur through different values of K_A and τ and this most likely is the result of different receptor conformations stabilized by PACAP₁₋₂₇ and PACAP₁₋₃₈. These and other data led to a formal declaration of the stabilization of different receptor active states by different agonists as the source of observed biased agonism (Kenakin, 1995).

As a preface to discussion of the various methods available to detect and quantify biased signaling, it is important to consider an important mechanism of apparent deviation from signaling pattern that is not due to bias. Specifically, where the strength of agonist signal is not adequate to produce response but rather converts the ligand from an agonist to partial agonist or antagonist; this is due to the interaction of the strength of the magnitude of efficacy and the sensitivity of the system.

B. Biased Agonism, Antagonism, and Strength of Signal

Historically, ligands that block responses to agonists and produce no further direct effect have been termed antagonists with the assumption that these molecules simply occupy the agonist binding site to prevent activation and thus block functional response. A well known phenomenon in pharmacology is the observed range of behaviors of low-efficacy ligands from showing agonism in sensitive functional systems to antagonism in systems of low sensitivity. For example, the low-efficacy β -adrenoceptor ligand prenalterol is nearly a full agonist in the thyroxine-treated guinea pig right atria to a complete antagonist in the guinea pig extensor digitorum longus muscle (Kenakin, 1985). This indicates that prenalterol produces a change in receptor conformation even though low-sensitivity systems cannot reveal positive efficacy. Low-efficacy ligands can be classified as partial agonists or antagonists depending on the sensitivity of the functional assay used to measure effects. This has been shown in studies with some classic antipsychotic drugs for dopamine D_2/β receptors that are reported to be partial agonists in some functional assays (Allen et al., 2011) and silent antagonists in other cell systems (Masri et al., 2008). Similarly, the chemokine ligands (CCR2 and CCR5) J113863 [1,4-*cis*-1-(1-cycloocten-1-ylmethyl)-4-[(2,7-dichloro-9*H*-xanthen-9-yl)carbonyl]amino]-1-ethylpiperidinium iodide] and UCB35625 [1,4-*trans*-1-(1-cycloocten-1-ylmethyl)-4-[(2,7-dichloro-9*H*-xanthen-9-yl)carbonyl]amino]-1-ethylpiperidinium iodide] can function as antagonists, partial agonists, or full agonists depending on the receptor and signaling pathway being observed (Corbisier et al., 2017). When such changes in system sensitivity occur with different signaling from the same receptor, it can take on the profile of biased signaling.

With the ability to measure multiple signaling from receptors has come the realization that ligands can have multiple efficacies (i.e., “pluridimensional efficacy”; Galandrin and Bouvier, 2006). In addition, the stabilization of unique receptor conformations by antagonists opens possibilities that these conformations may confer signaling under some conditions. For example, the PTH receptor ligand [D-Trp¹², Tyr³⁴]-bPTH(7-34) is a neutral antagonist of calcium signaling and an inverse agonist for cAMP production but a positive partial agonist for ERK1/2 (Luttrell et al., 2018); the simple labels of “antagonist” and “agonist” fail to capture the pharmacology of ligands in a biased conformational world (Kenakin, 2008).

The sensitivity of the functional assay used to identify signaling can have profound effects on the determination of bias and/or efficacy. A great deal of research on signaling bias involves G protein versus β -arrestin signaling. However, it is very important to consider that well coupled sensitive assays such as second messenger assays are generally more sensitive than

β -arrestin assays (which are not normally amplified), leading to apparent bias toward weak agonists producing second messenger responses without concomitant β -arrestin responses. This gives the illusion of “perfect bias” in that no response in one of the pathways is observed, further implying no interaction of the receptor with that signaling protein. However, experiments in which the “perfectly biased” ligand is used as an antagonist indicate that an interaction between the receptor and β -arrestin actually is taking place but no overt agonism can be displayed (Kenakin, 2015b; Stahl et al., 2015). A classic example of this is shown with the angiotensin biased ligand TRV120027, where the lack of G_q protein response is further shown to be a competitive antagonism of angiotensin G_q -mediated responses through Schild analysis (Violin et al., 2010). Similarly, the dopamine D_2 biased ligand MLS1547 [5-chloro-7-[[4-(2-pyridinyl)-1-piperazinyl]methyl]-8-quinolinol] activates cAMP but does not promote receptor β -arrestin BRET signaling at any concentrations ranging from 1 pM to 100 μ M, thereby giving the illusion of perfect bias. However, further experimentation reveals that MLS1547 is an antagonist of dopamine/ β -arrestin effects, with an IC_{50} of 1 μ M (Free et al., 2014). This dependence on assay sensitivity can be demonstrated within G proteins as well. For example, a lack of G_q protein activation and β -arrestin activation for the biased angiotensin agonist SII was reported in early studies (Wei et al., 2003), but subsequent work with more sensitive G protein assays in fact did demonstrate the G protein activation by SII (Saulière et al., 2012). Thus, the observation of “perfect bias” (i.e., where no signaling is observed in one signaling pathway) is not necessarily evidence of bias until the second pathway response can be observed and quantified in another assay.

A major way in which cells control their signaling is through variation of the relative stoichiometry of signaling elements, both receptors and signaling proteins. Experimentally, it can be shown that changes in cell surface receptor levels result in concomitant changes in sensitivity to agonists. For example, the β_3 -adrenoceptor ligand SR592230A [3-(2-ethylphenoxy)-1-[(1*S*)-1,2,3,4-tetrahydronaph-1-ylamino]-2*S*-2-propanol oxalate] is normally an antagonist but it produces agonism for cAMP formation in high-density β_3 -adrenoceptor cells (Sato et al., 2007). Moreover, for pleiotropically coupled receptors, receptor levels are linked to the actual quality of signal through recruitment of signaling pathways with increasing receptor levels. Thus, it has been shown that increased expression of α_2 -C10-adrenoceptor levels in transfected Chinese hamster ovary (CHO) cells show a pattern of initial coupling to G_i protein (the most sensitively linked pathway), followed by a recruitment of G_s signaling at higher receptor levels (Eason et al., 1992). Similar effects have been shown with the human calcitonin receptor, with initial G_s coupling evolving into mixed G_s and G_q

coupling with increasing receptor expression (Kenakin, 1997). These types of effects can produce fine-tuning of response to low-efficacy agonists in vivo and can also lead to discontinuities in the translation of biased effects from simple in vitro assays to in vivo systems. These effects also can confound predictions of in vivo effects from in vitro profiles in pathologic systems, as changes in sensitivity in these latter tissues can occur due to altered stoichiometry between receptors and signaling elements. For example, downregulation of β -adrenoceptors and $G\alpha_i$ proteins (Eschenhagen et al., 1992), $G\alpha_s$ proteins (Longabaugh et al., 1988), and even adenylyl cyclases V/VI (Ishikawa et al., 1994) has been seen in congestive heart failure and these effects are known to change receptor signaling (Bristow et al., 1982). Altered G protein levels also have been noted in Parkinson disease (Corvol et al., 2004). In cancer, huge overexpression of receptors (notably of vasoactive intestinal peptide receptors) has been noted (for review, see Kenakin, 2001). The fact that very low-efficacy ligands can primarily function as antagonists raises the possibility that, like biased agonists, the same mechanisms may lead to biased antagonism. At this point, it is worth considering the evidence available to suggest that receptor conformational states can contribute to differential affinity for different ligands and signaling pathways.

C. Biased Antagonism

Since affinity and efficacy are both components of biased signaling, it is logical to consider that the same mechanisms may cause biased antagonism as well. There are a number of biased agonists that are of very low efficacy and thus function as antagonists of some signaling pathways. The mechanism of stabilization of unique receptor conformations can lead to differences in receptor affinity as well as efficacy, an effect shown in the observed affinity of weak partial agonists. Specifically, the EC_{50} of a partial agonist is a good approximation of the affinity (K_A , equilibrium dissociation constant of the partial agonist-receptor complex) through the relationship $EC_{50} = K_A/(1 + \tau)$ (Black et al., 1985), where τ is efficacy. When efficacy is low ($\tau \rightarrow 0$), the $EC_{50} \rightarrow K_A$ (Bdioui et al., 2018). There are systems in which a weak partial agonist produces submaximal concentration-response curves for both pathways and where it is clear that a single estimate of affinity of the partial agonist for the receptor cannot be used to fit the curves (Kenakin, 2014). For example, extremely divergent EC_{50} values indicate variation in ligand affinity for different signaling pathways for adenosine A_{2B} receptors where it is impossible to fit the curves for the partial agonist BAY 60-6583 (2-[(6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]acetamide) with a single value for receptor affinity (see Fig. 6). In fact, a 19-fold difference in the affinity of BAY 60-6583 is required to fit BAY 60-6583 curves in IP1 versus ERK assays (Baltos

et al., 2017). A similar effect is seen with dopamine D_2 receptors. Specifically, the partial agonist 16c is 700-fold biased toward $G\alpha_{oA}$ over $G\alpha_{i2}$ (compared with the agonist quinpirole) but this effect cannot be accounted for completely by selective efficacy. This is because the concentration-response curves to 16c cannot be fit to the operational model assuming the same affinity differing only in efficacy. The fact that there are divergent EC_{50} values for 16c for these two G protein pathways indicates a 158-fold difference in the affinity of an agonist for the dopamine D_2 receptor when the receptor binds to $G\alpha_{oA}$ versus $G\alpha_{i2}$ protein (Möller et al., 2017).

Divergent pathway-selective affinity is consistent with the allosteric nature of receptor systems. Specifically, receptors alter their properties when a second body is bound to them and among those properties is the affinity for ligands through a cooperativity factor imposed on the receptor by the cobinding protein (denoted with the parameter α) (Ehlert, 2005; Kenakin, 2005; Price et al., 2005). Allosteric modulators can have varying effects on different molecules interacting with the receptor proteins to either increase or decrease the affinity of the receptor for ligands. As shown by Staus et al. (2016), the nanobody Nb80 enhances affinity 1000-fold, whereas the nanobody Nb60 reduces the affinity of noradrenaline for β_2 -adrenoceptors by a factor of 100.

The power of allosteric mechanisms to alter receptor antagonist activity is demonstrated in natural physiology. Thus, cellular coexpression of receptor activity-modifying proteins (RAMPs) produces a wealth of different phenotypes for the calcitonin/calcitonin gene-related peptide (CGRP) family of peptides that include calcitonin, α - and β -CGRP, amylin, adrenomedullin, and adrenomedullin 2/intermedin (Hay et al., 2018). For example, coexpression of calcitonin receptors and RAMP3 yields a receptor with a selectively high affinity for amylin. In cells containing RAMP3, the calcitonin receptor forms a complex that has completely different pharmacology compared with cells not containing RAMP3 and this leads to a selective change in antagonist potency of peptide antagonists such as AC66 (Armour et al., 1999). Without RAMP3, AC66 has a K_B of 0.25 nM for blockade of responses to amylin and calcitonin; with coexpressed RAMP3, there is a selective 7-fold decrease in potency of AC66 for blockade of amylin responses (potency = 1.8 nM) and no concomitant change in potency for calcitonin responses. In this regard, the presence or absence of RAMPs in various cell types produces induced bias for ligands (i.e., amylin and human calcitonin) much like what is seen with synthetic positive allosteric modulators (PAMs) (vide infra).

Biased agonists stabilize different receptor active-state conformations and telegraph their selective conformations through distinct signaling patterns. Stabilization of unique receptor conformations by biased antagonists may not be as evident if an assay is not available to detect the

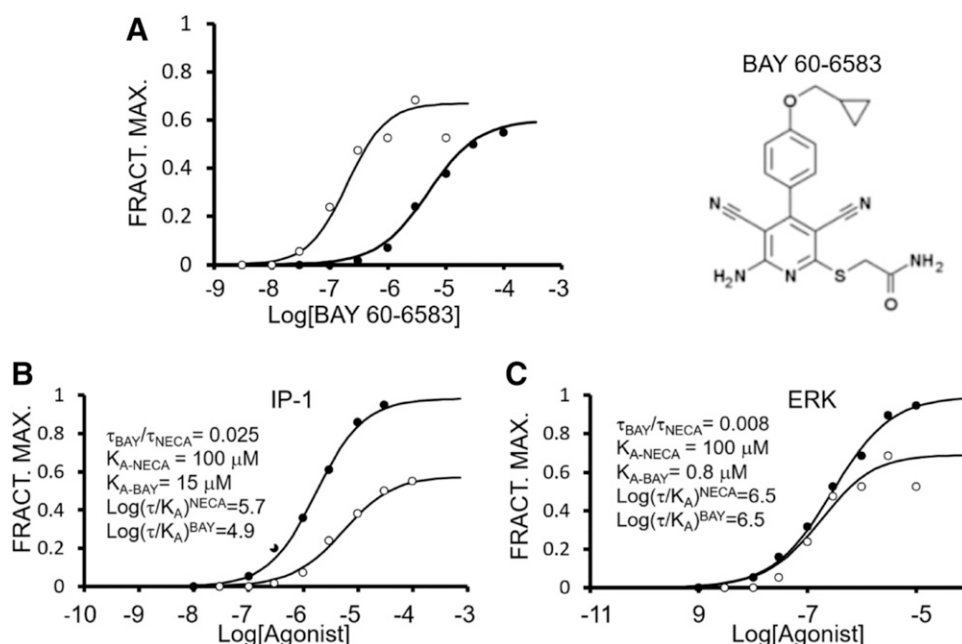


Fig. 6. Concentration-response curves for adenosine A_{2B} receptor agonists NECA and BAY 60-6583 for IP1 metabolism and ERK1/2 activation. (A) BAY 60-6583 is a partial agonist for IP1 metabolism (filled circles) and for ERK1/2 activation (open circles) with significantly different EC_{50} values for each pathway. (B) DR curves for IP1 metabolism for NECA (filled circles) and BAY 60-6583 (open circles). Fitting the Black/Leff operational model yields values for affinity (K_A) and relative efficacy ($\tau_{\text{BAY}}/\tau_{\text{NECA}}$) for these agonists activating this pathway. (C) DR curves for NECA (filled circles) and BAY 60-6583 (open circles) with values for relative affinity and efficacy as in (B) but for the ERK1/2 pathway. Fitting the Black/Leff operational model yields estimates of the affinity of BAY 60-6583 for the adenosine A_{2B} receptor differing by a factor of 19 ($K_{A\text{-IP1}} = 15 \mu\text{M}$, $K_{A\text{-ERK}} = 0.8 \mu\text{M}$). DR, dose response. Data are redrawn from Baltos et al. (2017).

conformational change. For example, the G_q protein assay denoting the competitive angiotensin antagonism by TRV120027 belies any production of a new receptor conformation until it is revealed through the β -arrestin assay (Violin et al., 2010). This type of dissimulation has been demonstrated for inverse agonists as well; for a selection of 380 apparently silent simple competitive antagonists for 73 receptors, 322 (85%) were found to actually be inverse agonists when tested in a constitutively active system (Kenakin, 2004). Thus, the stabilization of an inactive state receptor was not evident until the appropriate assay (constitutive receptor activity) was in place to detect it.

The profile of G_q protein blockade and β -arrestin agonism seen with TRV120027 highlights the essentially semantic issues with the nomenclature. From the point of view of β -arrestin effect, TRV120027 is a biased agonist; in terms of G_q protein signaling, it is an antagonist. It should be stressed that this is not because of differences in signal strength, as the bias is calculated with respect to a reference agonist and strength of signal is canceled. In the case of TRV120027, the antagonism is not selective (i.e., the EC_{50} for β -arrestin effect is equal to the pK_B for G_q protein blockade). However, there are cases of ligand-directed selective antagonism whereby stabilization of unique receptor conformations results in differences of affinity. For example, the PACAP receptor antagonist PACAP₁₋₆ has varying potency when blocking different PACAP agonists. Specifically, PACAP₁₋₂₇ and PACAP₁₋₃₈ produce

elevation of cAMP but PACAP₁₋₆ is significantly more potent in blocking the effects of PACAP₁₋₂₇ than the effects of PACAP₁₋₃₈ (Walker et al., 2014). A similar effect is seen with the β -blocking drug propranolol, which produces blockade of conventional agonists such as isoproterenol but less antagonism of the β -adrenoceptor agonist CGP-12177 (4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one hydrochloride) (Konkar et al., 2000; Baker and Hill, 2007).

Similar arguments to those proposing therapeutic advantage for biased agonism can be put forward for biased antagonism as well. In terms of therapeutics, biased antagonism would differ from biased agonism, in that selective signaling would emerge through blockade of endogenous agonists and the quality of endogenous efficacy would change with the biased antagonist. In this case, the nature of the agonist would dictate the degree of blockade by the antagonist (true agonist-mediated biased antagonism), and this would allow for preferential blockade of some endogenous agonists and not others or selective blockade of some signaling pathways but not others. Historical data suggest that biased antagonism can be of therapeutic benefit. Thus, the β_1 -adrenoceptor-mediated β -arrestin signaling effects leading to cardioprotective transactivation of epidermal growth factor receptors have been proposed as the reason carvedilol and alprenolol provide a survival advantage over 18 other drugs in heart failure (Wisler et al., 2007). Specific cases can be made for biased antagonism as well for future work. For example,

the natural agonist endothelin 1 activates endothelin A receptors to cause oncogenic (G_{α_q} -coupled and/or β -arrestin signaling; Spinella et al., 2004; Rosanò et al., 2013; Teoh et al., 2014) and tumor-suppressive (G_{α_s} -signaling; Takahashi et al., 2009; Follin-Arbelet et al., 2013; Teoh et al., 2014) effects. On balance, patients with ovarian cancer and high levels of endothelin A receptors were shown to have a low survival rate (Follin-Arbelet et al., 2013), yet endothelin receptor antagonists appeared to be ineffective as a cancer treatment (Cognetti et al., 2013). This has led researchers to postulate that nonspecific endothelin A receptor blockade appears to inhibit both the oncogenic and tumor-suppressive effects to negate a beneficial effect. This further suggests that a biased antagonist of the G_{α_q} and/or β -arrestin effects of endothelin 1 would be beneficial (Bologna et al., 2017).

D. Assay Effects in Biased Signaling Measurement

Pharmacological assays are the “eyes to see” the physiologic effects of ligands and having optimal assays is crucial to the successful detection and quantification of signaling bias. Historically, the paucity of pharmacological assays made drug response a simple dependent variable, usually the physiologic effect from an isolated tissue. In some cases, texture for these responses could be obtained as in the measurement of cardiac inotropy (force of contraction) and lusitropy (myocardial relaxation) from a single cardiac preparation (Kenakin et al., 1991) but in general, a single output for response (and therefore efficacy) was used to quantify and classify drug effect. In this type of environment, signaling bias was moot since there were few choices for signal measurement. Beginning in the 1980s, increasing technology made evident through different functional assays showed that agonists have many efficacies. More than any single factor, the availability of multiple measures of agonist response from a single receptor led to the discovery of signaling bias. It is worth considering the characteristics of these assays and how these characteristics affect the measurement of signaling.

One well known factor contributing to heterogeneity in observed agonist effect is variation in levels of receptor expression (Zhu et al., 1994; Nasman et al., 2001). Thus, low sensitivity resulting from low receptor expression leads to an absence of measured response for weak agonists and differential sensitivity of different assays (i.e., second messenger assays vs. β -arrestin complementation; Rajagopal et al., 2010) contributes to system bias. The effects of assay sensitivity are illustrated with the determination of receptor G protein signaling. The most widely applied method to determine G protein activation has been stimulation of 5'-O-(3-[35 S]thio)triphosphate ([35 S]GTP γ S) binding, a method that is relatively insensitive to weak stimulation and is restricted mainly to the $G_{\alpha_{i/o}}$ family (cannot readily distinguish $G_{\alpha_{i/o}}$ isoforms) (Denis et al., 2012). Vast

improvements in G protein signaling detection have been made with new BRET-based biosensors, which can directly measure activation of all G proteins in living cells (Lohse et al., 2012). There are indirect methods available as well (e.g., for G_q protein activation, via IP1 metabolism or calcium release) (Trinquet et al., 2006, 2011). In fact, changes in assay sensitivity have led to the observation of more subtle variation in signaling for the angiotensin ligand SII. As noted earlier, the lack of G protein signaling effect for this molecule with GTP γ S or IP3 radioactive assays led to its classification as a β -arrestin agonist and G_q protein antagonist (Wei et al., 2003). Application of a more sensitive IP1-homogeneous time-resolved fluorescence-based assay by the same group (Strachan et al., 2014) confirmed a different classification for SII (Saulière et al., 2012), namely one suggesting that SII does couple to some G proteins. BRET- and fluorescence resonance energy transfer-based biosensors have been used to monitor rearrangement of $G\alpha\beta\gamma$ subunits (Galés et al., 2005; Nikolaev et al., 2006; Audet et al., 2008; Masuho et al., 2015), receptors and G protein (Galés et al., 2005; Audet et al., 2008), receptors and β -arrestin (Zimmerman et al., 2012), and G proteins and downstream effectors (Riven et al., 2006; Richard-Lalonde et al., 2013). Single platform optical formats utilizing BRET signals generated by dissociated $\beta\gamma$ G protein subunits and GRK further increase the power of these assays to differentiate G protein receptor interactions (Masuho et al., 2015). In fact, the development of genetically encoded fluorescence-based biosensors has led to an explosion in terms of the observation of signaling events in living cells (Oldach and Zhang, 2014; Miyawaki and Niino, 2015; Galandrin et al., 2016b). This strategy has been extended to probe ligand-induced changes in β -arrestin conformation through intramolecular BRET-based biosensors (Charest et al., 2005). BRET-based biosensors have been used to study complex signaling for neurotensin type 1 receptors (Besserer-Offroy et al., 2017), angiotensin AT1 receptors (Saulière et al., 2012; Devost et al., 2017), κ -opioid receptors (Rives et al., 2012), a mutant somatostatin 5 receptor (Peverelli et al., 2013), and oxytocin receptors (Busnelli et al., 2012).

Assay sensitivity can affect observed system bias as seen in the highly amplified second messenger versus nonamplified β -arrestin signals, but more subtle effects can also change the magnitude of in vitro bias. As shown in Fig. 7, in some cases reversals in the relative potency of agonists are observed with varying assay format assessment of a single pathway, namely β -arrestin activity. Thus, a BRET method versus an enzyme complementation assay for dopamine D_2 receptor/ β -arrestin2 interactions (Path-Hunter) indicates differences in the bias of some agonists when comparing G_i protein versus β -arrestin interactions (relative to the reference quinpirole) due to the nature of the assay format (Möller et al., 2017). Finally, one experimental strategy that can be employed to expand detection capability is to manipulate assay

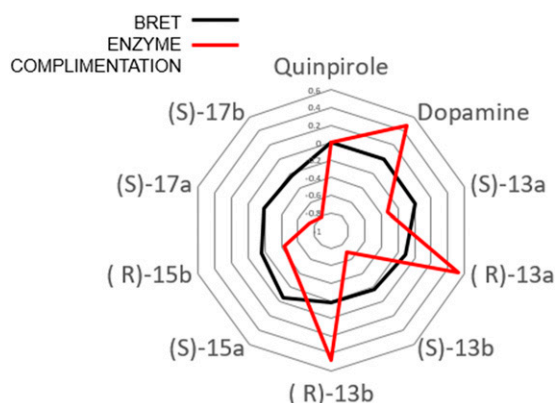


Fig. 7. Bias activity for dopamine D_2 receptor $G\alpha_{i1}$ activation/ β -arrestin interaction for 10 agonists measured either by BRET or enzyme complementation. Data are redrawn from Möller et al. (2017).

sensitivity. For example, the usually low sensitivity of β -arrestin assays can be increased through coexpression of GRKs (phosphorylation of receptors increases their sensitivity to β -arrestin) (Urs et al., 2016).

It is important to note that the assay used to quantify biased effects must accurately reflect the agonist activity; this is illustrated by the dissimulation of G_q protein-activating effects of the muscarinic PAM-agonist BQCA [1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid]. Specifically, agonism determined with calcium transient responses (a hemi-equilibrium assay) indicates an apparent 253-fold bias toward IP1 metabolism over calcium (compared with acetylcholine). However, the known inability of calcium assays to accurately reflect the response to slow-acting agonists (Unett et al., 2013), coupled with the fact that the agonism for BQCA cannot be reconciled with its allosteric receptor occupancy with the calcium assay (Bdioui et al., 2018), indicates that the agonism observed with the hemi-equilibrium calcium assay yields an erroneous location parameter for the agonist concentration-response curve and a concomitant erroneous estimate of bias. These effects illustrate the importance of the assay in bias measurements.

A valuable addition to the study of biased signaling has been made with label-free assays. In these experiments, the cellular responses to agonists are measured either as changes in dynamic mass redistribution (Fang and Ferrie, 2008; Kebig et al., 2009; Deng et al., 2013) or cellular impedance (Peters and Scott, 2009). These assays are highly sensitive and yield textured nuances in drug effect due to the fact that the complex signaling components of the cell (i.e., $G\alpha_s$ -, $G\alpha_i$ - and $G\beta\gamma$ -dependent signaling events, including activation canonical cAMP and ERK1/2 pathways) contribute to the final magnitude of response. In addition, virtually any cell type may be used, thereby allowing the testing of biased signaling in a variety of cell backgrounds and contexts (Ferrie et al., 2011; Stallaert et al., 2012; Deng et al., 2013; Morse et al., 2013).

E. Methods to Quantify Biased Signaling

If biased signaling is considered to be a valuable therapeutic property of drug candidate molecules, then a continuous scale of bias is required to allow medicinal chemists to optimize it. In general, a quantitative method for quantifying biased signaling should have the following properties:

- *Sensitive/system independent*: The method must provide an index of activity that is scaled to a reference within the series of measurements made and becomes an independent inner scale that can then be compared across assays to gauge differential signaling (bias).
- *Quantifiable* (have a scale): The method must provide a continuous numerical scale reflecting the degree of differential signaling (i.e., rank order is insufficient).
- *Theoretically sound* (relevant pharmacological parameters): The method must be grounded in mass action receptor models to relate the biased measurements to a molecular interaction between the ligand and the receptor.
- *High throughput* (or at least suitable for multiple molecules): For active SAR medicinal chemistry programs aimed at optimizing biased effects, the method should be amenable to rapid comparison of multiple molecules (null methods comparing two agonists in a single functional experiment are rigorous but not amenable to multiple-molecule SARs).
- *Statistically verifiable/bounded*: The method should ideally furnish statistical estimates of variability leading to assessment of significant difference (i.e., 95% confidence limits).

There are a number of methods proposed for the quantification of bias in the literature; an excellent discussion of these is given by Onaran et al. (2017).

1. *Transducer Coefficients: Log(Ratio of Agonist Efficacy/Functional Affinity) Values.* A method based on the Black/Leff operational model of agonism (Black and Leff, 1983) characterizes agonism as a single number, specifically the ratio of the agonist efficacy (τ) and functional affinity (K_A) (Kenakin et al., 2012). In this process, agonist concentration-response curves are fit to the Black/Leff operational model to furnish these estimates in the form of $\text{Log}(\tau/K_A)$ values for responses in a signaling pathway and then scaled to a common reference agonist (usually the natural endogenous agonist) to yield relative values in the form of $\Delta\text{Log}(\tau/K_A)$ values. In the case of multiple endogenous agonists, a choice must be made to one reference agonist and all calculations referenced to that agonist. It should be noted that the actual choice of agonist does not make a difference in the relative bias of a series of agonists, only to the absolute magnitude of the values. This scales the

relative power of the agonists to activate a given signaling system relative to the reference agonist. This is then done for another signaling pathway (the same reference agonist must be used) and then these relative values for each pathway are further compared with yield $\Delta\Delta\text{Log}(\tau/K_A)$ values between pathways. The fact that this method reduces agonism to a single value allows for statistical analysis of significance. Statistical formulae allow calculation of 95% confidence limits on all estimates of bias without regard to number of replications (Kenakin et al., 2012; see Table 3). In general, transducer coefficients, specifically $\text{Log}(\tau/K_A)$ values, provide a quantifiable and scalable method to characterize ligand bias due to selective efficacy and/or affinity that can be used to analyze multiple agonists.

2. *Log(Maximal Response/Concentration of Agonist Producing 50% of the Agonist Maximal Response)*. A method related to the transducer coefficient method that is simpler utilizes a ratio of the maximal response and EC_{50} (concentration of agonist producing 50% of the agonist maximal response); this method avoids some of the difficulties encountered with fitting the Black/Leff operational equation (Kenakin, 2017). Developed through allosteric equations depicting agonists as PAMs of receptor-signaling protein interaction (Kenakin, 2017), this yields a theoretically sound scale comparable to transducer coefficients for all agonists except those with very low efficacy (i.e., producing <30% maximal response) and/or having shallow concentration-response curves with Hill coefficients <0.5. This scale can be used for multiple agonists, is amenable to statistical estimation of variability and significance, and also considers both efficacy and affinity.

3. *Relative Efficacy: Log(Agonist Efficacy) Values*. A method that relates the relative efficacy of agonists for production of signaling pathway activation has been proposed, which assumes that biased interactions produce no change in the affinity of the receptor for the ligand but only changes in the efficacy of the agonist (Rajagopal et al., 2011; Onaran et al., 2014). If this assumption is accepted, then this method is theoretically sound but any changes in the affinity of the receptor due to differential signal protein interaction will not be considered and the bias estimated on the basis of only efficacy may differ from estimates with transduction coefficients and/or $\text{Log}(\text{max}/\text{EC}_{50})$ values. There are systems where affinity is not altered and only efficacy accounts for biased signaling. For example, TRV120027 shows an EC_{50} for β -arrestin activation of 12 μM (for partial agonism, this is an acceptable estimate of affinity) and the affinity for blockade of G_q protein effects through Schild analysis is 15 μM (Violin et al., 2010). Therefore, in this case, there is no difference in affinity associated with the two signaling pathways and the bias is due totally to differences in efficacy. However, affinity differences for agonists with a signaling pathway have been noted in other studies (Kenakin, 2014), leading to clear examples of systems in which a single estimate of affinity is not able to fit concentration-response curves to a biased ligand for two signaling pathways. For example, data for the adenosine A_{2B} receptor partial agonist BAY-6583 shown in Fig. 6 illustrate how pathway differences in affinity cannot be ignored (Baltos et al., 2017). In general, the lack of consideration of possible differences in functional affinity can be a major problem with this method. On the other hand, there are examples in which affinity does not

TABLE 3
Statistical formulae to assess significance of bias through $\text{Log}(\tau/K_A)$

No.	Parameter	Formula	Description
1	Error for mean $\text{Log}(\tau/K_A)$ values	$S_{ij}^2 = \frac{1}{n_{ij}-1} \sum_{k=1}^{n_i} (y_{ijk} - y_{\text{mean}})^2$	For a collection of n $\text{Log}(\tau/K_A)$ values for an agonist, squares of the deviations yield an estimate of variability of the estimate for that agonist
2	Pooled variance for $\text{Log}(\tau/K_A)$ values	$S_{\text{pooled}} = \sqrt{\frac{\sum_{i=1}^K \sum_{j=1}^2 s_{ij}^2}{df_{\text{error}}}}$	The error values from each estimate for all compounds for both pathways are used to produce a general estimate of the variation of the assays; this estimate is applied to all estimates in the analysis (including if there are singlet $n = 1$ values)
3	Degrees of freedom for pooled variance	$df_{\text{error}} = \sum_{i=1}^K \sum_{j=1}^2 (n_{ij} - 1)$	For k pathways and j groups of agonists with i values in each group, the degrees of freedom of the pooled variance is obtained; this allows calculation of significance and confidence limits
4	95% CL of mean $\text{Log}(\tau/K_A)$ values	(estimate) $\pm T(df_{\text{error}}, 0.975)(S.E.)$	This allows calculation of 95% confidence limits on the individual mean values of $\text{Log}(\tau/K_A)$
5	S.E. for calculation of 95% CLs shown in eq. 4 for mean values of $\text{Log}(\tau/K_A)$	$S.E. = S_{\text{pooled}} \sqrt{\frac{1}{n_{ij}}}$	For $\text{Log}(\tau/K_A)$ values, eq. 4 is used to calculate 95% CL with this formula for S.E.
6	S.E. for 95% CL on estimates of $\Delta\text{Log}(\tau/K_A)$ within each pathway	$S.E. = S_{\text{pooled}} \sqrt{\frac{1}{n_{ij}} + \frac{1}{n_{i'j}}}$	For $\Delta\text{Log}(\tau/K_A)$ values within each pathway, eq. 4 is used to calculate 95% CL with this formula for S.E.
7	S.E. for calculation of 95% CL on estimates of $\Delta\Delta\text{Log}(\tau/K_A)$ between pathways	$S.E. = S_{\text{pooled}} \sqrt{\frac{1}{n_{i1}} + \frac{1}{n_{i'1}} + \frac{1}{n_{i2}} + \frac{1}{n_{i'2}}}$	For $\Delta\Delta\text{Log}(\tau/K_A)$ values between pathway, eq. 4 is used to calculate 95% CL with this formula for S.E.

CL, confidence limit.

change with the signaling pathway and bias mechanisms based on efficacy (as opposed to affinity) are more robust and predicted to better translate in vivo (vide infra).

4. Relative Activity Ratios. A method described by Ehlert (2008) compares responses to two agonists in the same functional assay to determine a “relative activity” (RA) value gauging the relative power of those agonists to induce the response being measured. This method is theoretically sound and does not require prior knowledge of the affinities or efficacies of the agonists involved in the analysis. This is because the approach obtains RA values through null comparison of the agonist concentration-response curves. Formally, for concentration-response curves of unit Hill coefficients, the ratio of RA values can also be a ratio of (max/EC₅₀) values, although RA should not be equated with max/EC₅₀ because it is derived from a null comparison of two curves and not a single estimate of curve parameters. This is a powerfully rigorous and complete method that is excellent for detailed analysis of agonists of interest but lacks the high-throughput characteristics needed for simultaneous analyses of multiple candidate ligands.

5. Double-Reciprocal Null Comparison of Agonism. Barlow et al. (1967) published a method to compare agonism of full and partial agonists, which (like the RA method) does not require prior knowledge of the affinity and/or efficacy of the agonists. Rather, reciprocals of equiactive concentrations of the agonists are used to construct a double-reciprocal plot, the slope of which is an estimate of the logarithm of the efficacy/affinity ratio [i.e., $\text{Log}(\tau/K_A)$] according to eq. 5:

$$\frac{1}{[A_1]} = \frac{1}{[A_2]} \cdot \frac{\tau_{A1}K_{A-2}}{\tau_{A2}K_{A-1}} + \frac{\tau_{A2}K_{A2}}{\tau_{A2}K_{A-1}} \quad (5)$$

where agonists A₁ and A₂ have respective efficacies of τ_{A1} and τ_{A2} and respective affinities K_{A-1} and K_{A-2} . An

example of this procedure is shown in Fig. 8, where the relative power of the chemokines CCL3-like 1 and CCL5 are compared in an assay measuring CCR5 receptor internalization (data redrawn from Kenakin et al., 2012). Although this method is theoretically sound, there are some practical issues with applying it—namely that the concentration-response curves must diverge (see Fig. 8A) to allow convergence of the plot. There are also serious statistical limitations with the use of double-reciprocal plots, which can skew errors and emphasize certain regions of the data set over others (see Fig. 8B). In addition, like RA, this is a null method requiring the comparison of two agonists in the same assays, thereby limiting analyses of large data sets for SARs.

6. Reference Intrinsic Activity Trajectory and Rank Order Method. A model-free method of assessing possible signaling bias with two variations was presented by Onaran et al. (2017). This method is based on the fact that system bias tightly links the maximal responses of agonists of different efficacy into a regular pattern, usually a hyperbolic relationship. It should be noted that there are no assumptions about a model determining these effects; rather, the method is based on what is found in experimental pharmacology. A plot of the maximal responses to a range of agonists obtained in one pathway as a function of the maximal response found in another will be a singular continuous function if the activation of the two pathways by the agonists has the same mechanism; in essence, a trajectory of intrinsic activities (relative maximal responses) will be defined by a ratio of the maximal responses to the agonists (see Fig. 9). This defines the system bias for the two pathways and will be referred to as a “reference I.A. trajectory.” In addition, another method of depicting such a relationship is to plot the rank order of the magnitude of the maximal effects for both pathways. If the rank order in each pathway is the same (this will yield a straight-line correlation of unit

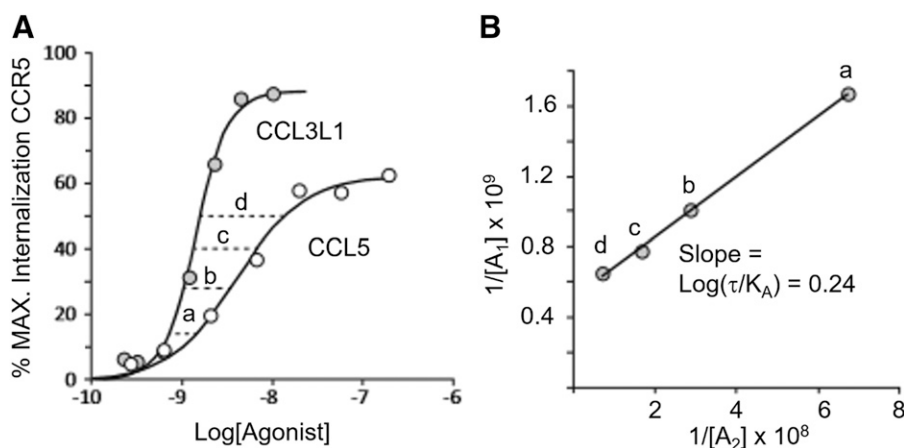


Fig. 8. Determination of the relative $\text{Log}(\tau/K_A)$ values for CCL3-like 1 (CCL3L1) and CCL5 mediation of internalization of the CCR5 receptor. (A and B) Reciprocals of equiactive concentrations of agonist (a–d) (A) are used to construct a linear regression (B) according to eq. 4. Data are redrawn from Kenakin et al. (2012).

slope), this would reflect system bias but suggest no ligand bias (see inset in Fig. 9). If a given agonist departs from this trajectory (i.e., the ratio of maximal responses deviates from the pattern), this would suggest ligand bias for that agonist. A classic example and one that furnished among the first pieces of evidence to support ligand bias was published by Berg et al. (1998) for 5-HT_{2A} receptor agonists. Specifically, these studies revealed agonists that actually reverse their efficacy for different signaling pathways (IP1 metabolism vs. arachidonate release) upon receptor activation. This is clear evidence of a reversal of efficacy for these pathways by these agonists that is shown in departures from the reference I.A. trajectory for these two signaling pathways (Fig. 10) and also rank order of intrinsic activities (inset in Fig. 10). The I.A. trajectory and rank order methods are essentially identical to the Log(τ) method; as such, they are solely based on differences in efficacy and ignore any effects on affinity. One of the main disadvantages of the I.A. trajectory (and rank order) method(s) is that a series of agonists with a wide range of intrinsic efficacies is needed to define the trajectory; if this is not available in a synthetic program, then the control (system) bias cannot be determined.

In general, it can be seen that there are numerous methods proposed to detect and, in some cases, quantify signaling bias. These methods, along with their advantages and disadvantages, are summarized in Table 4. Analysis of bias estimates with these different methods unveils systematic and nonsystematic deviation of actual bias values (Onaran et al., 2017), which is a problem for exact application of the bias scale numbers. However, it will be seen that these *in vitro* assays essentially identify bias and rank compounds in terms of magnitude of bias, with less emphasis on the expectation that these actual numbers will accurately translate to *in vivo* therapy. This is due to the fact that

there are a number of factors that can change these *in vitro* bias numbers as the ligands activate receptors *in vivo* (*vide infra*). This being the case, the *in vitro* assays should be considered only in terms of how serviceable they are to yield scales for the sorting of compounds (and optimize SARs) for further testing and not sources of immutable numbers. Thus, features such as high-throughput capability, continuity of the scale, and amenability to statistical analysis become the most important features of an *in vitro* method to quantify signaling bias.

F. Temporal Effects on Measured Biased Signaling

The real-time kinetics of the development and maintenance of cellular signaling can affect the estimation of biased signaling of ligands when snapshots in time are taken to make the measurements. As shown in Fig. 11, the relative potency of three agonists that have different real-time kinetics of response production varies with the actual time that the response is measured ("snapshot" format of response measurement). An obvious dichotomy exists between the kinetics of G protein activation (rapid and transient) and β -arrestin signaling (slow and sustained). Kinetic differences can occur due to differences in on and off rates of agonists with the receptor (differences in drug-target residence time; Strasser et al., 2017). For instance, while PTH(1-34) and parathyroid hormone-related peptide PTHrP(1-36) both elevate cAMP, PTH(1-34) has a rapid onset and slow offset compared with PTHrP(1-36), leading to a selectively prolonged activation for the latter. Similarly, the kinetics of cAMP production differs depending on whether the bulk of the signal comes from membrane or endosomal (intracellular) sources. Although PTH and PTHrP both elevate cAMP, only PTH produces sustained cAMP elevation due to production from early

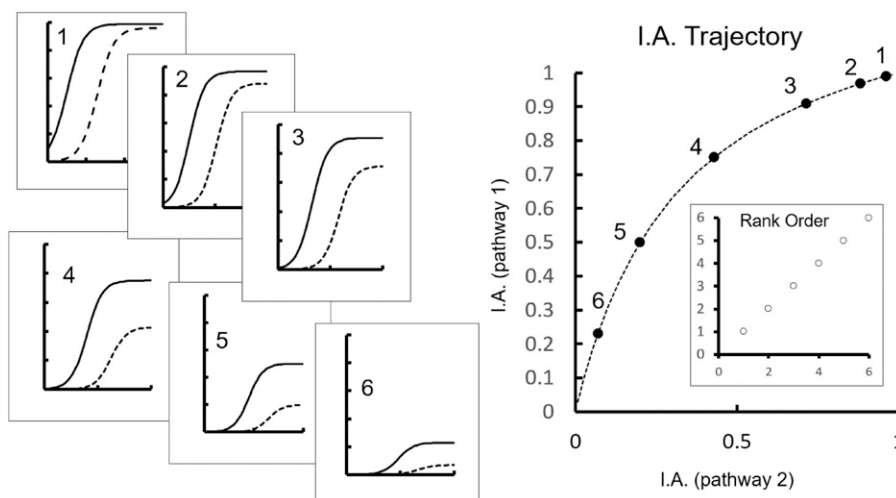


Fig. 9. Concentration-response curves for agonists with a constant ratio of efficacies for two signaling pathways (denoted by solid and dotted curves in 1–6). The panels are shown for agonists of descending efficacies from highest (1) to lowest (6). The ratios of the intrinsic activities (maximal responses) for each pathway are plotted to produce a trajectory depicting the system bias for the two assays with no ligand-based bias operative. Ratios of intrinsic activities that lie on this trajectory would simply indicate system bias and not ligand bias. The inset ranks the intrinsic activities of the agonists from highest to lowest for each pathway. A linear regression with no deviations indicates no ligand bias.

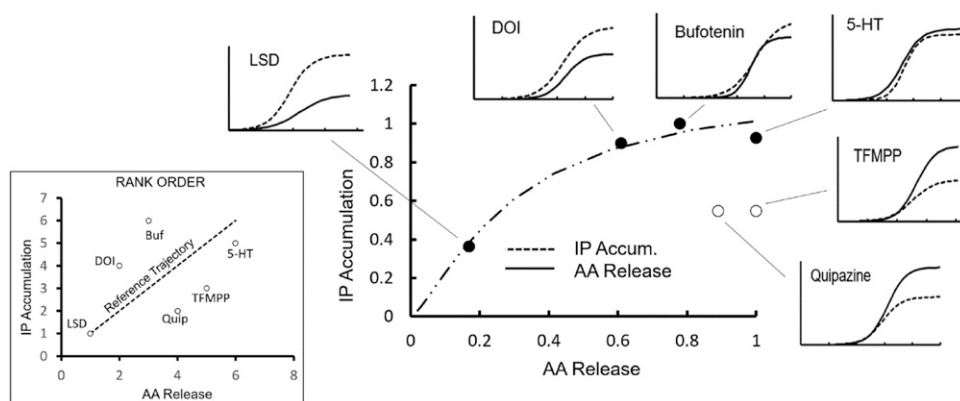


Fig. 10. Relative intrinsic activities for 5-HT_{2A} receptor agonists for two responses linked to the 5-HT_{2A} receptor: arachidonate release and IP1 accumulation. Whereas LSD, DOI, bufotenin, and 5-HT_{2A} lie on a single I.A. trajectory (system bias), TFMPP and quipazine clearly deviate, indicating biased signaling. This is due to a difference in efficacy for these pathways as the intrinsic activities actually reverse. This bias is also reflected in the rank order inset plot. AA, arachidonic acid; DOI, (6)-1-(2,5-dimethoxy-4iodophenyl)-2-aminopropane; LSD, lysergic acid diethylamide; TFMPP, 3-trifluoromethylphenyl-piperazine. Data redrawn from Berg et al. (1998).

endosomes (Ferrandon et al., 2009; Feinstein et al., 2011). In general, agonist-dependent sensitivity of disruption of the G protein complex by GTP (resulting in divergent agonist-dependent receptor-residency times for the G protein heterotrimer complex and effectors) has been correlated with agonist bias (Furness et al., 2016).

Since biased signaling is basically a comparison of relative potency for different signaling pathways, temporal differences can translate to differences in bias estimates (Klein Herenbrink et al., 2016). In many cases, this results in modification of actual indices of bias but usually not in general gross direction of bias. However, there are cases in which temporal effects can be critical to the determination of signaling bias. For instance, norepinephrine and oxymetazoline both induce α_{1A} -adrenoceptor phosphorylation and internalization. Whereas the effects of oxymetazoline are rapid, the effect of norepinephrine requires long time periods; therefore, when measurements of internalization are made at 30 minutes, oxymetazoline produces full agonism, whereas norepinephrine is inactive (apparently perfect bias) (Akinaga et al., 2013).

G. Representations of Biased Signaling Profiles

Depending on the number of pathways, ligands, and systems involved in biased signaling measurement, there are various methods employed to display the results. The main aim of these representations is to view a totality of data to draw general conclusions regarding the structure of ligands and physiologic selectivity. As a general rule, the farther the vantage point for measurement from the ligand-receptor interaction, the more complex (and textured) the pattern of biased signaling will be. There are a number of systems that have been employed to depict these patterns. As discussed earlier, the most straightforward method is to construct a bias plot where the responses to an agonist for one pathway are plotted as a function of the responses to the same agonist concentration in another

pathway (see Fig. 1). While bias plots make evident signaling bias in agonists, they do not furnish a quantitative scale to judge the degree to which ligand bias exceeds system bias.

If a quantitative scale of bias is employed then a simple correlation of $\Delta\Delta\text{Log}(\tau/K_A)$ values can be useful. Figure 12 shows $\Delta\Delta\text{Log}(\tau/K_A)$ values for a series of bitopic adenosine A_1 receptor agonists (referenced to NECA [1-(6-amino-9H-purin-9-yl)-1-deoxy-N-ethyl- β -D-ribofuranuronamide]) for calcium and cAMP responses; it can be seen that while eight compounds correlate fairly well [uniform $\Delta\Delta\text{Log}(\tau/K_A)$ values], the eight compounds in red are biased toward cAMP over calcium (Aurelio et al., 2018). Other methods are used if more than one signaling pathway is involved. A common method of representing multiple activities (i.e., efficacies) in molecules is with radar plots; for example, the efficacy of β -adrenoceptor ligands is displayed on multiple intersecting axes to yield a “web of efficacy” (Evans et al., 2010). Radar plots can be used to depict actual differences in agonist potency [i.e., $\text{Log}(\tau/K_A)$ or $\text{Log}(\text{max}/\text{EC}_{50})$ values; see Figs. 17, A and C, 18, and 19], or relative agonist activity compared with a reference agonist [$\Delta\text{Log}(\tau/K_A)$ or $\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values; e.g., see Figs. 13, 15, 16, 17, B and D, and 20–22]. Finally, actual differences in bias can be depicted with radar plots of $\Delta\Delta\text{Log}(\tau/K_A)$ or $\Delta\Delta\text{Log}(\text{max}/\text{EC}_{50})$ (i.e., “webs of bias”; Baltos et al., 2016a,b). In general, radar plots are used as representations of departures from system and measurement bias.

Although a great deal of literature is available to report receptor biased signaling between the G protein and β -arrestin pathways, the largest source of functional selectivity, in light of the G protein diversity available in the genome, may well be selective activation of G protein subunits (Hermans, 2003). Despite the technical challenge of measuring low-level G protein activation through $\text{GTP}\gamma\text{S}$ measurements, G protein selectivity has been determined with this method for

TABLE 4
Methods to quantify biased signaling

No.	Method	Procedure	Advantages	Disadvantages	Reference
1	Transducer coefficients [$\Delta\text{Log}(\tau/K_A)$]	Fit DR curves to operational model/calculate ratios of $\text{Log}(\tau/K_A)$ within each pathway ($\Delta\text{Log}(\tau/K_A)$); calculate Log bias as $\Delta\Delta\text{Log}(\tau/K_A)$ across pathways	Sensitive/system independent Quantifiable to yield a scale High throughput Statistically verifiable/bounded Accommodates full agonists Theoretically sound	Possible difficulties in fitting Black/Leff operational model to DR curves	Kenakin et al. (2012)
2	$\Delta\text{Log}(\text{max/EC}_{50})$	Calculate $\text{Log}(\text{max/EC}_{50})$ of DR curves; calculate $\Delta\text{Log}(\text{max/EC}_{50})$ within each pathway; calculate $\Delta\Delta\text{Log}(\text{max/EC}_{50})$ across pathways for Log bias	Sensitive/system independent Simple/quantifiable yielding a scale High throughput Statistically verifiable/bounded Accommodates full agonists No difficulties fitting curves to operational model Theoretically sound	Cannot be used if $n < 1$ and/or intrinsic activity $< 30\%$	Kenakin (2017)
3	Relative efficacy [$\Delta\text{Log}(\tau)$]	Fit DR curves for both functional pathways with a single measurement of affinity (binding) to obtain efficacy (τ values); express bias as relative $\text{Log}(\tau)$ values	Sensitive/system independent Quantifiable to yield scale High throughput Statistically verifiable/bounded	Not theoretically sound (ignores possible Δ affinity) Binding affinity must be used for full agonists Possible difficulties in fitting Black/Leff operational model to DR curves	Rajagopal et al. (2011)
4	RA	Equiactive concentrations from DR curves for two agonists are compared with null methods in each pathway to yield RA values. Bias is then evaluated through $\Delta\text{log(RA)}$ values	Sensitive/system independent Quantifiable to yield a scale Statistically variable/bounded No difficulties fitting curves to operational model Theoretically sound	Not high throughput (need dual agonist simultaneous comparison) Works best with divergent curves (i.e., full vs. partial agonist)	Ehlert (2008)
5	Method of Barlow, Scott, and Stephenson	Double-reciprocal plot of equiactive concentrations of two agonists in a functional system are used to yield a ratio of efficacy and affinity in each pathway. These ratios are then compared across pathways to yield estimate of bias	Sensitive/system independent Quantifiable to yield scale Theoretically sound No difficulties fitting curves to operational model	Not high throughput (need dual agonist simultaneous comparison) Needs divergent curves (i.e., full vs. partial agonist) Double-reciprocal plots often skewed and yield erroneous parameters	Barlow et al. (1967)
6	Trajectory and rank order	Maximal responses to a range of agonists plotted for each pathway to determine trajectory relationship defining system bias. Outliers (either from plot itself or rank order) identified as biased	Model independent Sensitive/system independent No difficulties fitting curves to operational model Theoretically sound	No quantifiable scale Requires a range of agonist intrinsic activities to define trajectory Ignores affinity (may miss bias in some compounds)	Onaran et al. (2017)

DR, dose response.

some receptors. For example, dopamine receptor selectivity between $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, plus $G\beta_1$ and $G\gamma_2$ has been reported using GTP γ S assays (Gazi et al., 2003). Similarly, selectivity within $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_z$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ has been reported with cannabinoid receptor agonism (Diez-Alarcia et al., 2016). Recently, the application of sensitive BRET probes to directly measure G protein activation in living cells has been applied to the measurement of G protein subunit selectivity (Lohse et al., 2012; Saulière et al., 2012). These new methods allow delineation of particularly difficult to distinguish G protein subunit isoforms within the $G_{i/o}$ family (Saulière et al., 2012; Bellot et al., 2015). BRET technology has been applied to the study of multiple $G\alpha$ subunit interactions (Soto et al., 2015) with receptors. Specifically, multiple G protein subunit interactions with oxytocin receptors (Busnelli et al., 2012), κ -opioid receptors (Rives et al., 2012), and ghrelin receptors (M'Kadmi et al., 2015) have been described. This latter study shows how a simple profile for ghrelin receptor agonists indicating G protein bias (over β -arrestin 2) can be further expanded to show a diverse texture of bias among G protein subunits (see Fig. 13). Recently, an all-inclusive single platform optical method to directly monitor G protein activation in live cells, to yield signal magnitude, activation rates, and varying efficacy and kinetics of ligand activator in real time to provide efficacy fingerprints, has been reported (Masuho et al., 2015).

More complex texture in signaling data can be presented as heat maps in which multiple responses are compared (i.e., Soethoudt et al., 2017). Added value to such heatmaps can be gained if the resulting arrays are statistically clustered; this can yield useful similarity data for SARs (Huang et al., 2009; Stallaert et al., 2012; Kenakin, 2015c). An example of this technique is shown in Fig. 14, where $\text{Log}(\text{max}/\text{EC}_{50})$ values for 15 opioid agonists in six signaling assays are clustered for similarity to produce nine groups linked by their unique signaling pattern in these assays (Kenakin, 2015c). The clusters obtained from signaling profiles often link structurally diverse molecules (i.e., see dynorphin A and morphine in Fig. 14).

The idea that receptor signaling becomes more complex and textured as measurements are made further from the ligand-receptor interaction leads to the demonstration that ligands produce differences in these patterns not evident at the cell membrane. One window into such patterns is receptor-mediated global changes in protein phosphorylation. For example, it has been shown that a 5-minute exposure of osteoblastic cells in vitro to PTH(1-34) leads to changes in the phosphorylation of 224 distinct proteins in the cell (Williams et al., 2016). One of the most complex "fingerprints" of biased signaling involving both ligand bias and cell background is obtained through transcriptome analysis, namely the comparison of differential mRNA

expression from tissue cDNA (Maudsley et al., 2011, 2013; Chen et al., 2013b). In vitro studies from mouse calvarial bone cells indicate that bPTH(7-34) regulates 192 genes (47 upregulated and 145 downregulated) to yield a unique fingerprint for PTH receptor activation (Gesty-Palmer et al., 2013). Similarly, in bone tissue from wild-type and congenic β -arrestin2^{-/-} mice, dramatic differences in the fingerprints are seen with hPTH(1-34) and [D-Trp¹², Tyr³⁴]-bPTH(7-34) (Maudsley et al., 2015).

H. Biased Signaling in Screening and Lead Optimization

Since pharmacological assays can be used to detect, measure, and quantify ligand-directed biased signaling, it follows that medicinal chemistry can be used to optimize desirable biased profiles. In fact, the detection of chemical scaffolds that demonstrate biased signaling can be achieved at the screening step in the drug discovery process. Thus, once molecules have been screened in one signaling format, a broad selection of the "hits" can then be cross-screened in another signaling format to produce a range of activity; that is, it is nearly guaranteed that a lack of uniformity in activity in the two assays will be seen. At this stage, an informed decision can be made to progress molecules known to be different (i.e., known to stabilize different receptor active states) to a secondary assay rather than only the most potent hits from the initial screening assay. This should, in turn, ensure the optimal opportunity for the discovery of unique therapeutic phenotypes in secondary assays (Kenakin, 2011). Application of such parallel primary screening has been applied to the detection of apelin receptor hits for cardioprotection (McAnally et al., 2017). The utilization of whole cell response assays for such screening optimizes for detection of activity (most sensitive) and biased signaling diversity (differences in pathway activation) (Kenakin, 2012). In this regard, label-free assays can be used for effective screening of biased molecules (Peters and Scott, 2009; Hou et al., 2016). Newer technologies such as those employing sensor surface-immobilized receptors also have been described as systems for the detection of biased ligands (Kumari et al., 2015). In addition, virtual screening techniques have been applied to the detection of biased molecules (Li et al., 2015; Manglik et al., 2016).

The ability to quantify biased signaling to a scale also powers SARs and enables a rational alteration of the effect through medicinal chemistry. Modifications of ligand-receptor function lead to changes in allosteric interaction and these can lead to precipitous changes in activity with small changes in ligand structure; this has been noted in the SAR for negative allosteric modulators (NAMs) and PAMs. For example, very subtle changes in chemical structures are known to radically convert a PAM to a NAM in a 5-(phenylethynyl)pyrimidine scaffold (Sharma et al., 2008); this phenomenon has been

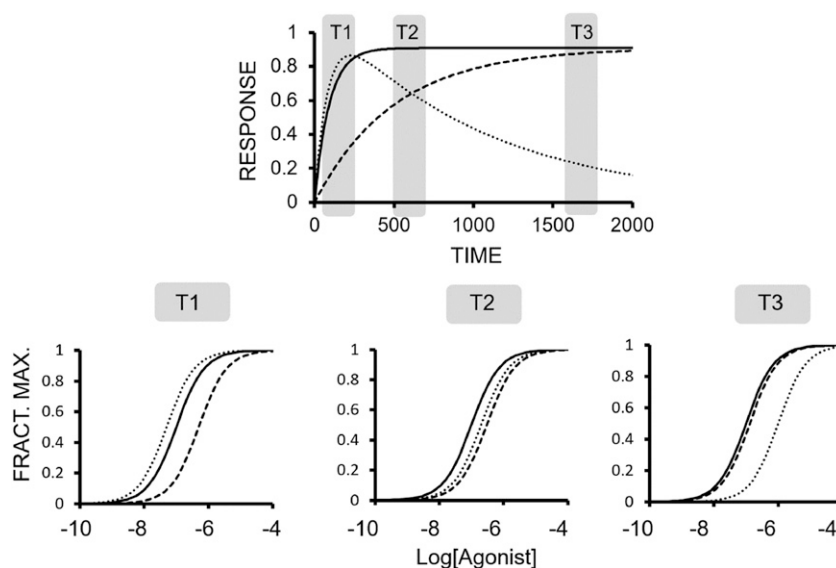


Fig. 11. Time course for production of response by three agonists (denoted by different solid and broken lines); measurement of response at three time points (T1, T2, T3) yields three concentration-response curves for the agonists with varying relative potencies.

given the term “activity switching.” Since biased signaling is no more than the imposition of selective PAM effects for different signaling proteins, this suggests that changes in bias could be equally sensitive to changes in ligand structure. Alteration of biased signaling has been aided in many cases by linking effects with the structure of the receptor. For instance, the 5-HT_{2A} and β -adrenoceptor crystal structures have identified transmembrane domains specifically linked to biased ligand interaction (Warne et al., 2012; Wacker et al., 2013). Similarly, protein mutation has been employed to generate SAR data for biased signaling (G protein receptor 183, Daugvilaite et al., 2017; GLP-1, Wootten et al., 2016). Chemical scaffolds yielding biased molecules range from modification of natural endogenous agonists to natural products (Gupta et al., 2016). In general, there has been a steady increase in the number of studies on the structural features of molecules required for biased signaling (Tan et al., 2018; and see Table 5).

I. Applications of Biased Scales in Pharmacology

Quantitative scales for biased signaling, such as $\Delta\Delta\text{Log}(\tau/K_A)$ and $\Delta\Delta\text{Log}(\text{max}/\text{EC}_{50})$, can be used for a variety of purposes in pharmacology to gauge ligand selectivity and their impact on physiologic changes in systems. Just as these scales quantify agonist intracellular selectivity (biased signaling), they also can be used to quantify extracellular (receptor) selectivity. The inherent advantage of this is the ability to compare full and partial agonism, something that is not possible with simple potency ratios (Kenakin, 2017). By comparing $\Delta\text{Log}(\tau/K_A)$ or $\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values, the sensitivity of each test system for the receptor type can be accounted for (by comparison with a reference agonist) to reveal the selectivity of test agonists. Figure 15A shows $\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values for eight agonists for

three 5-HT receptor subtypes illustrating the application of this method (lorcaserin is 5-HT_{2C} selective) (Zhang et al., 2017).

Basically, any type of selectivity can be quantified with the same techniques used to quantify biased signaling. For example, cell type can confer selectivity on agonists (actually it can modify pathway-selective effects seen in in vitro assays; vide infra). This can readily be seen with label-free assays in which the agonist activity can be measured and compared as $\Delta\text{Log}(\tau/K_A)$ or $\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values. Figure 15B shows $\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values for five agonists on dopamine D₂ receptors measured in two cell types with a label-free assay (Peters and Scott, 2009). It can be seen that A-77636 [(1)-(1R,3S)-3-adamantyl-1-(amino-methyl)-3,4-dihydro-5,6-dihydroxy-1H-2-benzopyran hydrochloride hydrate] is selectively 11-fold more potent in U-2 cells (over SK-N-MC cells), thereby identifying this agonist as cell type specific. This type of analysis

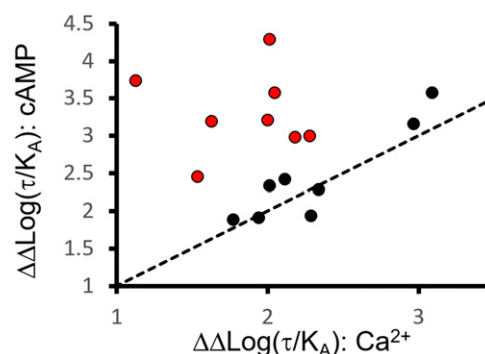


Fig. 12. Measurements of bias [as $\Delta\Delta\text{Log}(\tau/K_A)$ values] for biotopic adenosine A₁ receptor agonists (referenced to NECA) for calcium (abscissae) and cAMP (ordinates) responses. Agonists denoted with black solid circles show uniform activation of both pathways (no bias), whereas circles in red indicate bias toward cAMP signaling over calcium. Data redrawn from Aurelio et al. (2018).

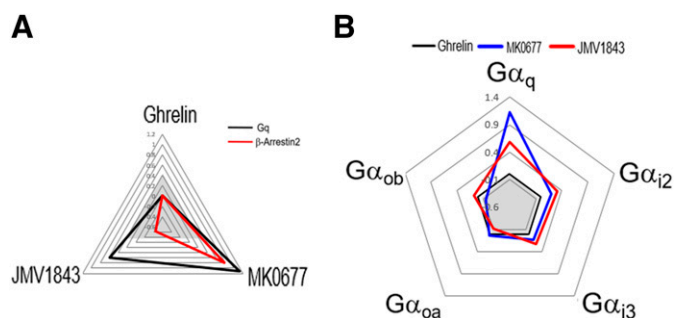


Fig. 13. Relative agonist activity of three ghrelin agonists for the ghrelin receptor mediating various signaling pathways in HEK293T cells. (A) Radar plot based on agonist type for G protein and β -arrestin signaling [$\Delta\text{Log}(\tau/K_A)$ values] shown relative to ghrelin as the reference agonist. (B) More textured depiction of G protein selectivity. Radar plot based on G protein type showing $\Delta\text{Log}(\tau/K_A)$ values (with ghrelin as the reference agonist); the filled pentagon shows activity equal to ghrelin. Data are shown for agonists MK0677 and JMV1843. Redrawn from M'Kadmi et al. (2015). JMV1843, 2-methylalanyl-N-[(1R)-1-(formylamino)-2-(1H-indol-3-yl)ethyl]-D-tryptophanamide; MK0677, 2-amino-2-methyl-N-[(2R)-1-(1-methylsulfonylspiro[2H-indole-3,4'-piperidine]-1'-yl)-1-oxo-3-phenylmethoxypropan-2-yl]propanamide.

could be applied to identifying selectivity for pathologic systems over normal physiologic systems (i.e., cancer vs. healthy host, normal cardiac vs. cells from heart failure models, etc.) to identify disease-selective therapeutic molecules (Kenakin, 2015b).

Finally, biased signaling scales can be used to characterize and quantify physiology in a system-dependent manner as in the characterization of the effect of mutation on receptor function. Figure 16 shows $\Delta\text{Log}(\text{max}/\text{EC}_{50})$

values for ghrelin activity on wild-type and mutated analogs of the ghrelin receptor (Mokrosiński et al., 2012). Ideally, $\Delta\text{Log}(\tau/K_A)$ or $\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values should be calculated for two agonists to account for differences in receptor expression (Tschammer et al., 2011; Kenakin, 2017). While conventional analyses of biased signaling for new agonists compares the synthetic agonist profile to the endogenous agonist (to gain an insight into the new signaling that might be expected with the synthetic agonist), in mutational studies the question more often relates to what effect a receptor mutation will have on the endogenous signaling in a pathologic condition. Therefore, the analysis usually uses a synthetic agonist as the reference to control for differences in receptor expression and centers on the impact of the mutation on the endogenous agonist. Ideally, specific residues may be identified in such studies to guide medicinal chemists to regions of the receptor that can change ligand selectivity (e.g., see Daugvilaite et al., 2017).

J. Allosterically Induced Bias

In view of the fact that allosteric modulators can completely change the receptor through stabilization of different global conformations, there is no a priori reason to believe that the allosterically modified receptor should retain its preallosteric modified signaling bias after binding the allosteric ligand. This being the case, the modulator can induce a new bias to the agonist-receptor system; this will be referred to as

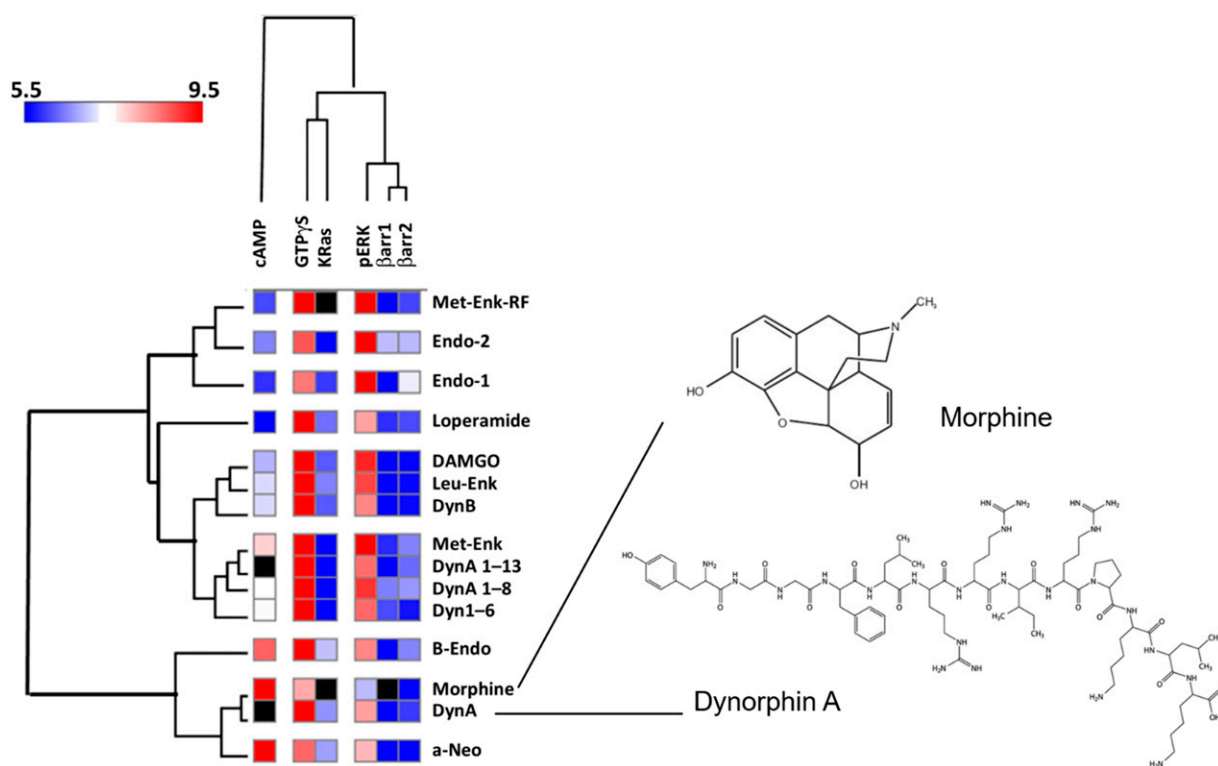


Fig. 14. Cluster analysis of 15 μ -opioid agonists in six different functional assays (data from Thompson et al., 2015). The gene cluster program GENE-E groups the agonists according to their $\text{Log}(\text{max}/\text{EC}_{50})$ values in each assay. Shown is a grouping of two agonists (morphine and dynorphin A) of very different chemical structure. Analysis and data are redrawn from Kenakin (2015c).

TABLE 5	
Structure-activity studies for alteration of biased signaling	
Receptor	Reference
Adenosine A ₃	Baltos et al. (2016b)
PAR ₂	Jiang et al. (2017)
Adenosine A ₁	Glukhova et al. (2017), Aurelio et al. (2018), Vecchio et al. (2018)
β ₂ -adrenoceptor	Reiner et al. (2010)
Adenosine A _{2b}	Vecchio et al. (2016)
EP ₂	Ogawa et al. (2016a,b)
Dopamine D ₃	Xu et al. (2017)
PTH	van der Lee et al. (2013)
Dopamine D ₂	Allen et al. (2011), Chen et al. (2012), Free et al. (2014), Bonifazi et al. (2017), Möller et al. (2017), Chun et al. (2018), Weiwer et al. (2018)
GLP-1	Hager et al. (2016), Wootten et al. (2016)
α ₁ -adrenoceptor	Evans et al. (2011)
CRF-1	Beyermann et al. (2007)
Angiotensin II type 1	Violin et al. (2010)
α _{2C} -adrenoceptor	Kurko et al. (2014)

CRF, corticotropin-releasing hormone receptor 1; EP, prostanoid receptor.

allosterically induced bias. With the activation of multiple signaling pathways by an agonist will come a change in the efficacy fingerprint of that agonist. Induced bias in normal physiology has been documented. For instance, the accessory protein RAMP2 binds to the vasoactive intestinal polypeptide-pituitary adenylyl cyclase-activating peptide 1 receptor to selectively enhance G_{q/11} signaling (receptor-mediated phosphoinositide turnover) without altering G_s-coupled cAMP production (Christopoulos et al., 2003). Similarly, neurochondrin interaction with the melanin-concentrating hormone receptor 1 leads to inhibition of agonist-induced G_{i/o} and G_{q/11} signaling with no concomitant internalization (Francke et al., 2006).

Induced bias is increasingly seen with synthetic drug-like allosteric modulators. For example, the agonist for the metabotropic glutamate receptor 5(*S*)-3,5-dihydroxyphenylglycine produces activation of calcium, IP1, and

phosphor-ERK1/2 signaling in mouse cortical neurons (Sengmany et al., 2017). Values of Log(τ/K_A) for activation of these pathways in the absence and presence of a high concentration of PAM agonists for this receptor indicate that the pattern of activation of these three pathways changes significantly after allosteric modification (see Fig. 17). Thus, the PAM-agonist VU0360172 [*N*-cyclobutyl-6-((3-fluorophenyl)ethynyl) picolinamide] produces a 12-fold bias toward calcium, while the PAM-agonist DPFE [1-(4-(2,4-difluorophenyl) piperazin-1-yl)-2-((4-fluorobenzyl)oxy)ethanone] produces a 10-fold bias toward calcium. Induced bias has been observed for NAMs of NK2 receptors (Maillet et al., 2007), prostaglandin D₂ receptors (Mathiesen et al., 2005), lysophosphatidic acid receptors (Shimizu and Nakayama, 2017), and calcium-sensing receptors (Davey et al., 2012; Cook et al., 2015). The effect has also been reported for PAMs for GLP-1 receptors (Koole et al., 2010), adenosine A₁ receptors (Aurelio et al., 2009), muscarinic M₁ receptors (Davoren et al., 2016), and metabotropic glutamic acid 5 receptors (Bradley et al., 2011). In addition, induced bias has also been seen with other types of allosteric modulators such as PAM antagonists (Kenakin and Strachan, 2018); for example, this has been documented for the cannabinoid CB₁ molecule Org27569 (5-Chloro-3-ethyl-N-[2-[4-(1-piperidiny)phenyl]ethyl-1H-indole-2-carboxamide], which blocks cAMP production but not ERK1/2 phosphorylation for some cannabinoid orthosteric agonists (Khajehali et al., 2015). In keeping with the cell-dependent modification of measured biased signaling, cell-dependent modulator-induced bias also has been reported for the follicle-stimulating hormone receptor, where differential effects on follicle-stimulating hormone receptor-stimulated steroidogenesis and ovulation in murine Leydig tumor cell line-1 and rat primary Leydig cells are observed (Ayoub et al., 2016).

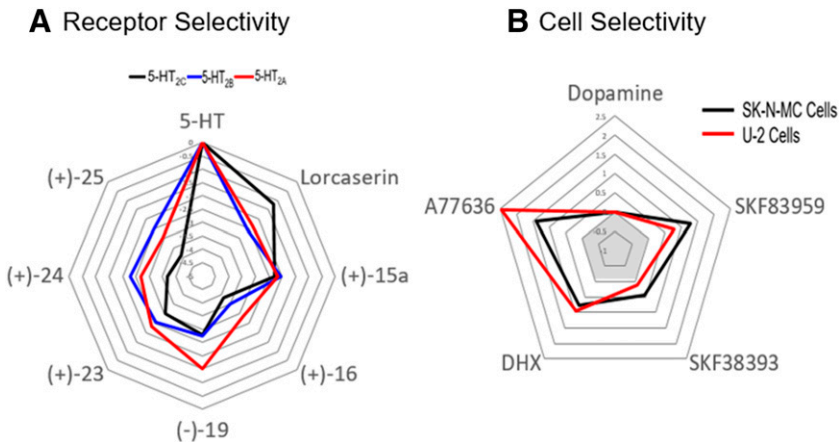


Fig. 15. Agonist selectivities depicted with radar plots. (A) Receptor selectivity [as $\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values] compared with 5-HT for agonists activating three 5-HT receptor subtypes. Data are redrawn from Zhang et al. (2017). (B) Selectivity of dopamine agonists [as $\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values] from label-free activation of dopamine D₂ receptor in two different cell lines. Data indicate a cell-type specificity for A-77636. DHX, dihydroxydine; SKF38393, (±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrobromide. Data are redrawn from Peters and Scott (2009).

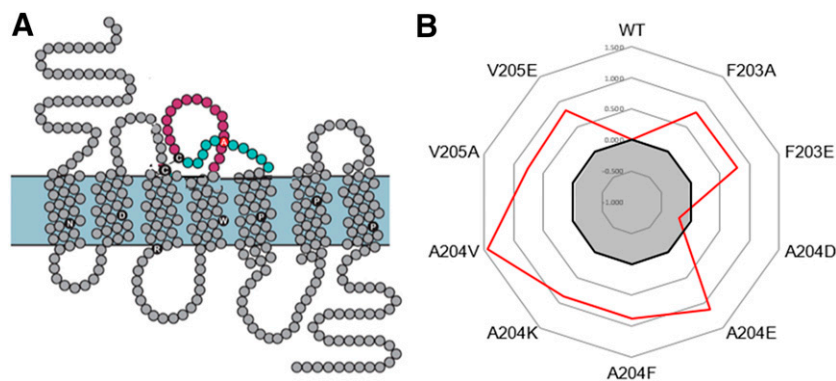


Fig. 16. Effect of ghrelin receptor mutation on $\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values for ghrelin. (A) Mutations made in the second extracellular loop of the receptor. (B) The shaded area represents normal wild-type receptor activity for ghrelin, and the red line indicates departure of activity made by mutation. No large differences in receptor expression were seen in these experiments. WT, wild type. Data are redrawn from Mokrosiński et al. (2012).

VIII. Translation of In Vitro Bias to In Vivo Physiology

A. Translation from Pathways to Whole Cells

Although measurements of signaling bias can be made with simplified in vitro assay systems (e.g., biosensor interactions of receptors and effectors), ultimately the relevant outcome is the in vivo overall cellular effect of the biased agonist. There are a number of factors that contribute to the modification of simple dual pathway estimates of bias by the cell. Convergence of signaling pathways occurring in cells can change the overall outcome of signals initiated with different relative strengths of signal (Alvarez et al., 2002; Ostrom et al., 2012; Charfi et al., 2014). Initial events at the cell membrane may also have differential consequences for the whole cell. For example, while the cannabinoid CB₂ agonists CP55,940 [(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol] and WIN55,212-2 both promote β -arrestin2 recruitment at the cell membrane, only CP55,940 induces receptor internalization (Atwood et al., 2012). Similarly, seemingly identical cellular outcomes may result from different intracellular signaling pathway activation. For example, in microphysiometry studies (measurement of extracellular acidification, a whole cell response), it was observed that two β_3 -adrenoceptor agonists, CL316,243 and SR59,230A produce full agonism. However, when studied further, it was seen that whereas CL316,243 produces response through dual activation of cAMP and p38, SR59,230A produces the same end organ response through sole activation of p38 (Sato et al., 2007). Similarly, a range of PTH receptor agonists activate cAMP and ERK1/2 signaling and also produce anabolic bone response in vivo; paradoxically, the ligand (D-Trp¹², Tyr³⁴)-bPTH(7-34) has a dramatically different in vitro signaling profile (inverse agonism for cAMP and ERK1/2) but still produces positive anabolic bone responses (Appleton et al., 2013).

Possible further complication may result from different agonist-stabilized receptor active conformations interacting with different conformations of effector; for

example, two angiotensin β -arrestin-biased agonists produce two different and distinct patterns of downstream signaling in cells by stabilization of different conformations of β -arrestin (Santos et al., 2015). As conformationally coded receptors interact with various components of the cell cytosol, the relative stoichiometry of those components becomes a factor in the overall impact of any given pathway. Data from intramolecular-bioluminescence resonance energy experiments indicate that different ligands binding to the same receptor can produce unique population average conformational signatures for arrestins (Lee et al., 2016; Nuber et al., 2016). These signatures become instrumental in the assembly of distinct signaling β -arrestin “signalsomes” (Peterson and Luttrell, 2017).

The stoichiometry of interacting components, namely receptors and signaling proteins, is known to vary between different cell types (Kenakin, 1997; Newman-Tancredi et al., 1997, 2000). Proteomic techniques using mass spectrometry show marked differences in the expression level of proteins in 11 different human cell lines (Geiger et al., 2012) and quantification of mRNA showed large differences in receptors and signaling proteins in cell lines often used in pharmacologic experiments, specifically human embryonic kidney 293 (HEK293), AtT20, BV2, and N18 cells (Atwood et al., 2011). Differences in receptor/G protein stoichiometry have been shown to directly influence estimates of signaling bias (for review, see Gurevich and Gurevich, 2018). Figure 18A shows the variation in bias measured for β -adrenoceptor agonists under conditions of varying $G\alpha$ protein subunit expression (Onfroy et al., 2017). Similarly, the relative stoichiometry of $G\alpha_s$ protein and calcitonin receptors has been shown to reverse the relative order of potency for calcitonin agonists (see Fig. 18B) (Watson et al., 2000). Theoretical modeling confirms that variation in the relative quantity of different signaling proteins in cells can lead to variation in the whole cell estimate of biased signaling for biased agonists (Kenakin, 2016). In fact, experimentally demonstrated effects have been shown to translate to whole

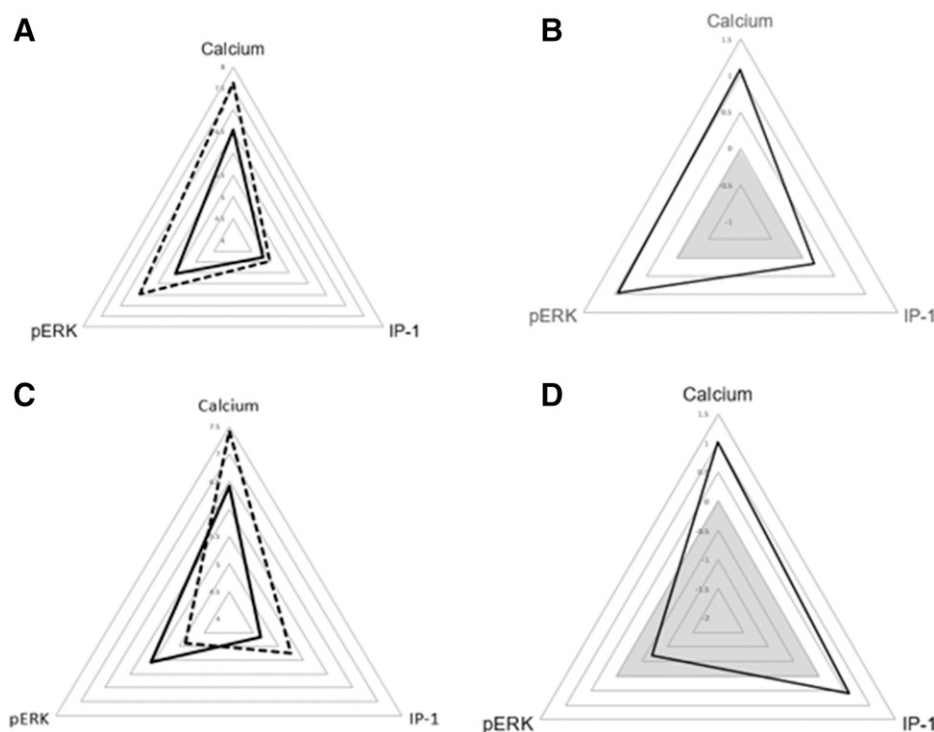


Fig. 17. Allosterically induced bias. (A) $\text{Log}(\tau/K_A)$ values for the metabotropic glutamate receptor 5 agonist DHPG for the signaling pathways in mouse cortical neurons. (B) The solid line represents control values; dotted line values in presence of the PAM-agonist VU0360172. Data in (A) are presented as ratios of control $\text{Log}(\tau/K_A)$ values [$\Delta\text{Log}(\tau/K_A)$ values] and values after allosteric modulation. The shaded triangle indicates the native receptor signaling before allosteric modification, and the solid line indicates the change in this signaling in the presence of VU0360172. Values extending beyond the shaded triangle indicate increased signaling in the presence of the allosteric modulator and inside the triangle a reduced signaling. (C) $\text{Log}(\tau/K_A)$ control values are indicated as a solid line; values in the presence of the PAM-agonist DPFE are shown as a dotted line. (D) Data in (C) are presented as ratios of control $\text{Log}(\tau/K_A)$ values [$\Delta\text{Log}(\tau/K_A)$ values]. The shaded triangle indicates the native receptor signaling before allosteric modification, and the solid line indicates the change in this signaling in the presence of DPFE. Values extending beyond the shaded triangle indicate increased signaling in the presence of the allosteric modulator and inside the triangle a reduced signaling. DHPG, 5-(S)-3,5-dihydroxyphenylglycine; VU0360172, *N*-cyclobutyl-6-((3-fluorophenyl)ethynyl) picolinamide. Figure drawn from data recalculated from Sengmany et al. (2017).

cell differences in experimental cell systems (Masuho et al., 2015). In addition, cell background context (HEK293 vs. rat vascular smooth muscle cells) has been seen to affect basal angiotensin AT1 receptor conformation as detected by receptor conformationally sensitive biosensors (Devost et al., 2017), consistent with the reciprocal allosteric effects of receptors and G proteins. These data show that signaling partners such as G proteins influence the agonist-induced conformational changes and modify them when they are present in the membrane (or deleted with CRISPR). For instance, the absence of $G_{q/11}$ confers sensitivity onto the biosensor at intracellular loop 2P2, which then becomes sensitive to angiotensin II and III. Additionally, the nature of the host cell can modify both the basal conformation of the receptor and the changes induced by agonists. This latter effect is demonstrated by the different conformations (as sensed through ΔBRET changes in the second and third intracellular loops of the receptor) produced by two agonists, namely angiotensin II and the biased agonist SI. The effects of these agonists are of different magnitude and, notably, vary in the direction of conformation change when measured in HEK cells versus vascular smooth muscle cells (Devost et al., 2017). These data indicate that the cell type can change the

conformational ensemble of the receptor and the nature and magnitude of agonist-induced signaling bias. Similarly, the lipid environment of the receptor has long been postulated to affect receptor function; a recent report utilizing mass spectrometry on preferential binding of phosphatidylinositol-4,5-bisphosphate to adenosine A_{2A} receptor- and β_1 -adrenoceptor-G protein complexes shows variation in receptor-G protein coupling efficiency, thereby illustrating the influence of lipids (and therefore cell type) on receptor function (Yen et al., 2018). Variation in the stoichiometry of intracellular signaling modulators also can have profound effects on responses to agonists in terms of G protein selectivity. For example, Fig. 19 shows measurements of the maximal muscarinic M_3 receptor and selective G protein response produced by acetylcholine in control HEK293T/17 cells and in the presence of a cotransfected regulator of G protein signaling (RGS8) and presence of a cotransfected activator of G protein signaling (AGS1); it can be seen that the interaction of the receptor with individual G protein subtypes changes considerably (Masuho et al., 2015).

Yet another whole cell variable may involve the phosphorylation of receptors (Tobin, 2008; Tobin et al., 2008) referred to as receptor “barcoding” (Tobin et al.,

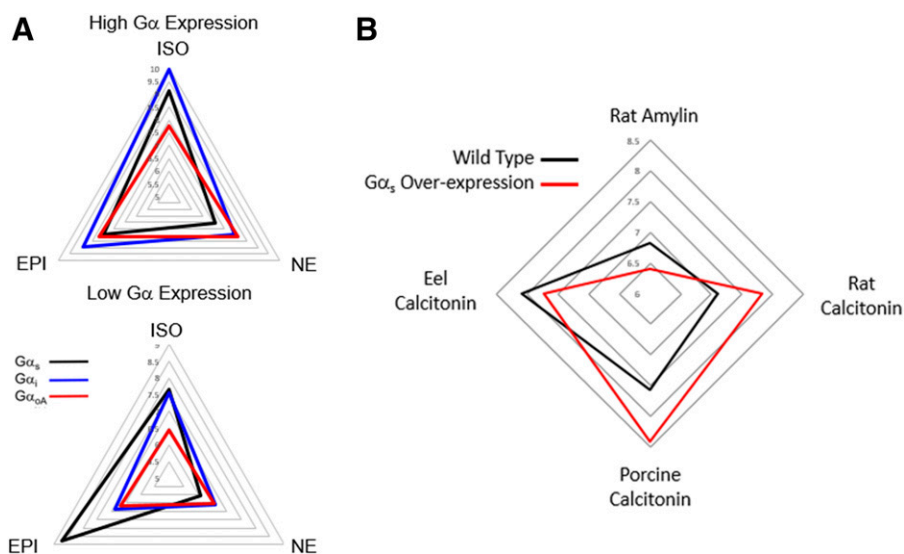


Fig. 18. Variation in receptor/G protein stoichiometry can change bias estimates. (A) Log(max/EC₅₀) values for three β -adrenoceptor agonists in HEK293 cells varying in levels of G protein expression, drawn from Onfroy et al. (2017). (B) Effect of coexpression of G α_s protein into HEK cells on calcitonin receptor agonist Log(max/EC₅₀) values, drawn from Watson et al. (2000). EPI, epinephrine; ISO, isoproterenol; NE, norepinephrine.

2008; Zidar et al., 2009; Nobles et al., 2011). It has been established that different receptor ligands can induce different phosphorylation barcodes (Butcher et al., 2011; Just et al., 2013) to induce different signaling outcomes (Tobin et al., 2008). This may be the result of specific phosphorylation-dependent interactions of receptors with β -arrestins (DeWire et al., 2007) that change from an inactive to active form upon interaction with the phosphorylated receptor (Xiao et al., 2004; Nobles et al., 2007; Shukla et al., 2013). Whole cell modification of signaling bias can be affected, as phosphorylation has been shown to be tissue specific (Torrecilla et al., 2007).

Finally, other points of cellular control that can influence agonist whole cell response have been described. For example, the β_2 -adrenoceptor agonists clenbuterol, cimaterol, procaterol, and terbutaline were shown to be biased toward cAMP production (with weak activity on ERK phosphorylation) in HEK293 cells only in adherent cells with 100% confluence; reduction in cell-cell contact by either decreasing the cell density or bringing the cells into suspension abolished this bias (Kaya et al., 2012). Temporal encoding, in which each of these components brings with it a real-time kinetic element, can change the emphasis of various pathways in whole cell response in cell-dependent ways (Grundmann and Kostenis, 2017). In addition, cell-dependent expression of receptor splice variants (as in the case of CXCR3 receptors) also has been shown to produce cell-dependent differences in biased signaling (Berchiche and Sakmar, 2016).

In view of the various points of possible variation in biased signaling measurements to be found in different cell types, it is not surprising that bias measurements have been seen to vary with cell host. For example, variation in cAMP production and receptor

internalization has been seen for opioid receptor ligands when measured in CHO versus AtT20 cells (Thompson et al., 2015, 2016). Similarly, the array of signaling pathways activated by relaxin has been shown to markedly differ in seven different cell types, an effect possibly related to varying levels of G α_{i3} and G α_o (Halls et al., 2009). The fact that label-free assays use total cellular response as the output for agonism makes this format ideal to detect cell type variation (Peters and Scott, 2009). For example, seven muscarinic M₃ receptor agonists have been shown to produce diverse signaling profiles in six different cell lines through label-free assay technology (Deng et al., 2013). In keeping with the idea that more complex outputs of agonist response increase the capability to detect diversity in signaling, mRNA microarrays also offer a useful technology to detect cell type variation in agonist signaling (Atwood et al., 2011).

Cell type differences also extend to comparisons of recombinant versus natural cell systems and even between different recombinant systems utilizing different host cells (Christmanson et al., 1994). In this case, the expression of human calcitonin receptors into COS and CHO cells yields strikingly different relative potency ratios, indicating a clear cellular control of biased signaling for these agonists. When comparing recombinant versus natural cell lines, it has been shown that opioid and cannabinoid receptors access the same pool of G proteins in recombinant cell systems but mediate signals through distinctly different pools in endogenous natural cell systems (Shapira et al., 2000). Similarly, while protein kinase C and GRK2 are important for δ -opioid receptor internalization in cortical neurons, they are less so in HEK293 cells (Charfi et al., 2014). Different pharmacology of PACAP agonists PACAP-27 and PACAP-38 has been reported between transfected

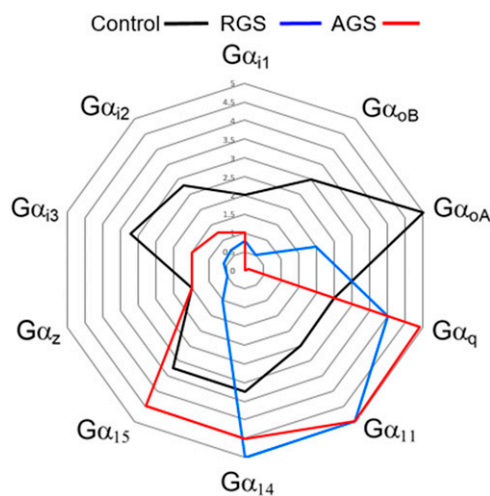


Fig. 19. Maximal effect of muscarinic M₃ receptor activation with different G α subunits in HEK293T/17 cells (control) and cells cotransfected with regulator of G protein signaling RGS8 protein (blue) and activator of G protein signaling AGS1 (red). Data redrawn from Masuho et al. (2015).

PAC_{in} cells and natural trigeminal neurons and glia (Walker et al., 2014). In general, beyond variation in the relative stoichiometry of signaling components in recombinant systems, variation in the integration of the transgene in stable transfection systems can permanently alter the cell genotype/phenotype (Lin et al., 2014). However, it should not be assumed that all recombinant versus natural cell systems show divergence. For instance, the G protein bias of the κ -opioid receptor agonist 6'-guanidinonaltrindole originally detected in HEK cells (Rives et al., 2012) retains this profile in primary neuronal cultures where κ -opioid receptors are expressed endogenously (Schmid et al., 2013).

In view of the fact that bias measurements made in recombinant systems often differ from those seen in natural systems and also that cell type can change estimates of bias, it is useful to quantitatively compare different functional systems and measurements of bias. Figure 20 shows data for allosteric agonists of the metabotropic glutamate receptor 5 for functional responses (calcium, IP₁, ERK) when the receptor is transfected into HEK cells versus that endogenously found in cultured cortical neurons (Sengmany et al., 2017). These data show a trend of greater agonist activity and increased bias [$\Delta\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values] in HEK cells versus cortical neurons. This shows that these agonists appear to be more biased in the recombinant system over the natural system.

It is interesting to see how the vantage point for observing response can change the magnitude of measured bias. Figure 21 shows how increases in cAMP mediated by dopamine D₂ receptor agonism translate to label-free whole cell response with the resulting changes in quantitative biased signaling (Peters et al., 2007). A more extensive analysis of the changes occurring throughout the stimulus-response cascades controlling whole cell response are shown in Fig. 22 (Klein

Herenbrink et al., 2016). Specifically, it can be seen that the part of the overall pathway chosen to make the bias measurements produces different estimates of the relative bias of the agonists.

B. Translation to In Vivo Therapeutic Systems

1. Translational Gaps in the Conversion of In Vitro to In Vivo Bias. When a biased ligand is introduced to an in vivo system, it will interact with a vast array of tissues with varying sensitivities, relative stoichiometries of receptor to signaling proteins, variable coexpression of receptors and interconnections between codependent systems (especially in the brain), and varying endogenous physiologic chemical contexts. These variations have created a serious translational gap between in vitro and in vivo coordination of bias ligand properties (Appleton et al., 2013; Kenakin, 2018). Much of this unpredictability results from the complex nature of in vivo systems and the fact that biased ligands do not uniformly produce “edited” versions of natural receptors but rather will produce completely different receptors with the concomitant ability to increase, reduce, or not change a broad range of physiologic processes (Luttrell et al., 2018). For example, in bone remodeling studies in mice, the biased PTH receptor agonist [D-Trp¹², Tyr³⁴]-bPTH(7-34) produces a transcriptome with remarkably little similarity to the one produced by the natural agonist PTH, leading to the conclusion that in vivo bone mass effects of the biased agonist could not have been predicted by its in vitro bias profile (Appleton et al., 2013; Gesty-Palmer et al., 2013; Luttrell et al., 2018).

2. Affinity-Dominant versus Efficacy-Dominant Biased Signaling. In cases where in vivo agonism may be relevant to therapeutic effect, it is important to note that ligand efficacy and not bias is the important factor. While bias determines the relative concentrations at which two responses will appear (in relation to each other) when the responses actually do appear, whether any response occurs at all remains in the realm of the ligand efficacies for that pathway and the sensitivity of the tissue involved. As illustrated by eq. 3, agonist potency depends on both affinity and efficacy; thus, high potency can be achieved through high affinity or high efficacy. However, the dependence of agonism on tissue sensitivity differs depending on the dominating factor for agonist potency. If agonist potency depends largely on affinity (“affinity-dominant” agonists), then decreases in tissue sensitivity will have much more effect on whether agonism is produced than if the potency depends on efficacy (“efficacy-dominant” agonists) (Kenakin, 1984). In general, affinity-dominant agonists are much more prone to show no agonism in less sensitive tissues than efficacy-dominant agonists that produce agonism in all tissues. For example, decreases in α -adrenoceptor density through chemical alkylation depress the response to the more potent affinity-dominant agonist oxymetazoline to a much greater extent than the responses to the less potent but

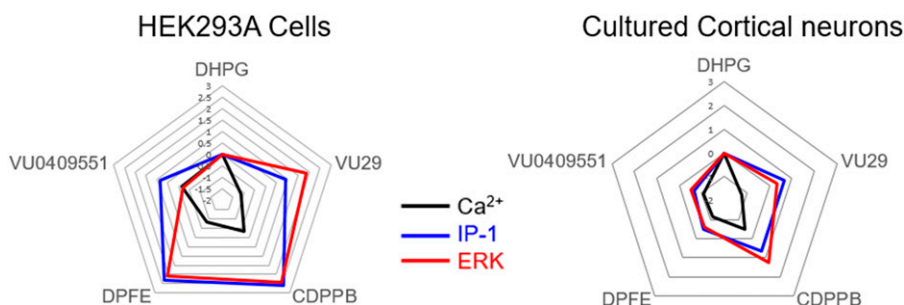


Fig. 20. Bias measured in HEK cells and cultured cortical neurons. The left panel shows $\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values for allosteric agonist activation of metabotropic glutamate receptor 5 (DHPG as the reference agonist) for VU0409551, DFPE, and CDPPB for calcium, IP1, and ERK responses in HEK293A cells. The right panel shows the same responses in cultured cortical neurons. These radar plots indicate that bias is more pronounced in HEK cells than natural cultured cortical neurons. CDPPB, 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide; DFPE, 1-(4-(2,4-difluorophenyl)piperazin-1-yl)-2-((4-fluorobenzyl)oxy)ethanone; DHPG, 5-(*S*)-3,5-dihydroxyphenylglycine; VU0409551, ((4-fluorophenyl) (2-(phenoxy)methyl)-6,7-dihydrooxazolo[5,4-*c*]pyridin-5(4*H*)-yl) methanone; VU29, *N*-(1,3-diphenyl-1*H*-pyrazolo-5-yl)-4-nitrobenzamide. Figure drawn from data recalculated from Sengmany et al. (2017).

efficacy-dominant agonist norepinephrine (Kenakin, 1984). Since bias is simply a ratio of efficacy to affinity for two pathways, bias can also be affinity dominant or efficacy dominant. As seen in Fig. 23, two κ -opioid agonists, ICI204448 [(*RS*)[3-[1-[[3,4-dichlorophenyl]-acetyl]methylamino]-2-(1-pyrrolidinyl)ethyl]phenoxy]acetic acid hydrochloride and RB-59, have the same intrinsic activities for cAMP and β -arrestin responses but very different relative potencies (White et al., 2014). The fact that both agonists lie on the same intrinsic activity reference trajectory indicates that efficacy alone cannot account for the bias of these agonists (RB-59 is 147-fold biased toward G protein compared with ICI204448 using the reference agonist salvanorin); rather, differences in affinity play a key role in determining the bias. Parenthetically, this illustrates a major shortcoming of utilizing methods that only involve efficacy for the quantification of bias, such as $\text{Log}(\tau)$, I.A. reference trajectory, and rank order (see section VII.E). As with agonism, efficacy-dominant bias will be more robust in terms of activating biased signaling pathways in a

wider range of tissues in vivo than will affinity-dominant bias. This is demonstrated in Fig. 24, which shows the response to two identically biased agonists in a range of tissues with varying levels of receptors. It can be seen from this figure that there is a greater range of tissues (as seen by a greater range of receptor densities) that will demonstrate biased agonism (as opposed to antagonism) in vivo (in the presence of an EC_{50} of endogenous agonist response) for the efficacy-dominant biased agonist than for the affinity-dominant biased agonist. These simulations underscore the importance of efficacy, as opposed to only bias, in the prediction of agonist response in vivo.

The complex interplay of bias and efficacy in the production of therapeutically relevant responses is illustrated by the effects of dopaminergic ligands for the treatment of schizophrenia, a disease postulated to be caused by striatal hyperdopaminergic and cortical hypodopaminergic activity. Partial agonists such as aripiprazole, developed to treat this disease, still are insufficient to correct cortical function; this profile is

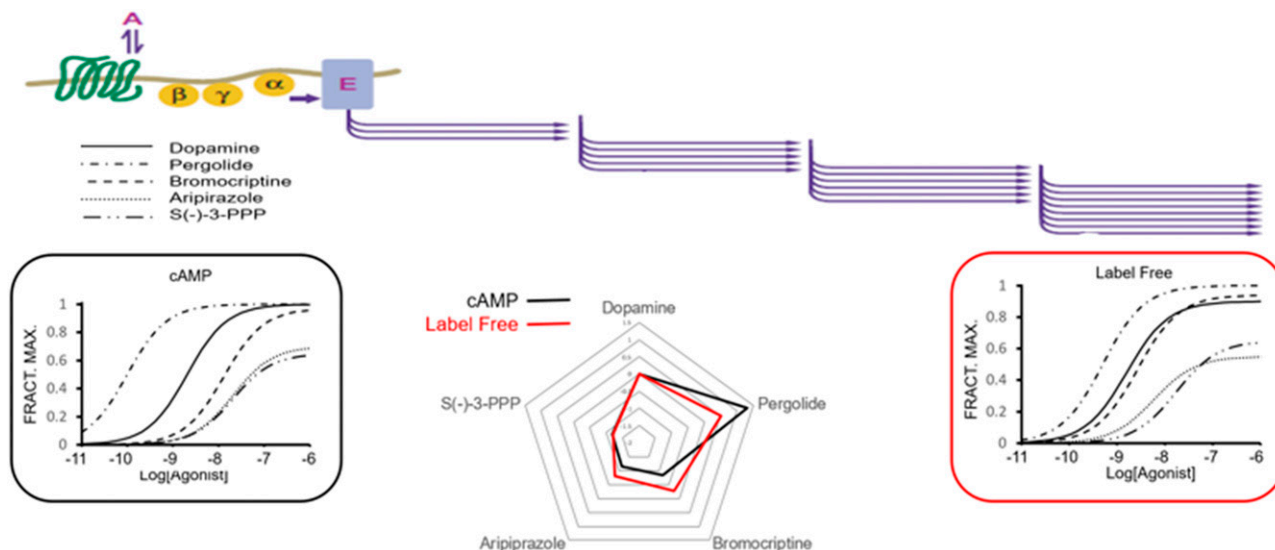


Fig. 21. Concentration-response curves from dopamine D_2 receptor agonists showing relative potency at the point of receptor/G protein interaction (cAMP) and whole cell end-organ response (cellular impedance in label-free format). The center radar plot shows $\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values from both vantage points, indicating differences in bias estimates. Data calculated from Peters et al. (2007).

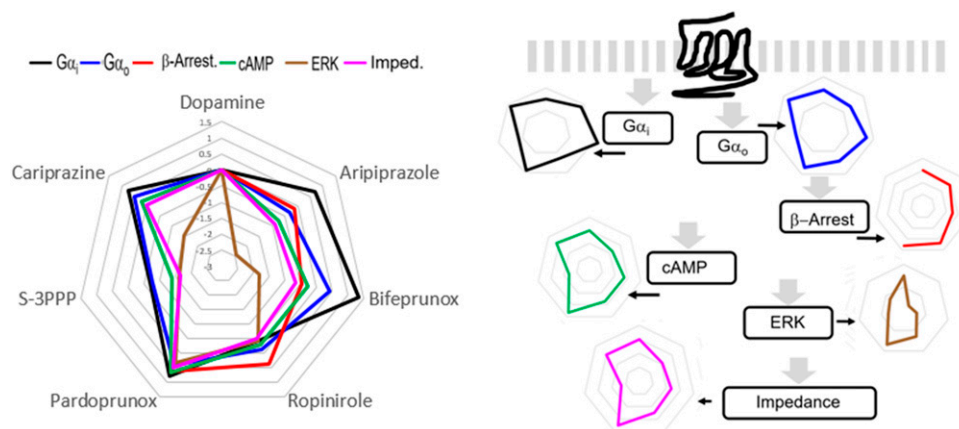


Fig. 22. Different relative agonist activity measurements [$\Delta\text{Log}(\text{max}/\text{EC}_{50})$ vs. dopamine] for seven dopamine D₂ receptor agonists with estimates made at various points along the stimulus-response pathway in Flp-In-CHO cells. The left panel shows $\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values for the agonists measured at six points along the stimulus-response cascade (beginning with receptor/G protein and β -arrestin interaction to whole cell impedance response). Of note are the different patterns of agonism, indicating that bias estimates at each of these points are different, as shown in the right panel. S-3PPP, [3-(3-hydroxyphenyl)-N-propylpiperidine]. Data calculated from Klein Herenbrink et al. (2016).

corrected with β -arrestin2 biased partial agonists that enhance firing of cortical fast-firing neurons (Urs et al., 2016). In general, the profile of striatal antagonism and cortical agonism leads to an improved antipsychotic profile, in that the lack of G protein signaling (bias) of these compounds coupled to the elevated expression of β -arrestin2 and GRK2 in the cortex (vs. striatum) produces the optimal conditions for the biased effect. It is proposed that the interplay of agonism and antagonism present in β -arrestin2 biased partial agonists such as the aripiprazole-derived analog compound 94A is critical to the favorable therapeutic potential for the treatment of schizophrenia (Allen et al., 2011; Urs et al., 2014, 2016).

IX. Future Considerations and Possible Ways Forward

Although the pharmacologic phenomenon of biased signaling is established and easily demonstrated in vitro, the value of this effect is still not really demonstrated in therapy. As pointed out, at the time of this writing, few biased molecules have been tested in humans and the first, TRV120027, failed to meet either the primary or secondary endpoints in the phase IIb Biased Ligand of the Angiotensin Receptor Study in Acute Heart Failure study (Pang et al., 2017). Two other molecules, TRV067 (a blocker of G_q protein-mediated vasoconstriction while sensitizing myofilament calcium-responsiveness in a genetic mouse model of dilated cardiomyopathy; Ryba et al., 2017) and TRV130 (the μ -opioid receptor agonist for postoperative pain; Chen et al., 2013a; Violin et al., 2014), are currently in clinical trials. These are examples of applying the concept of biased signaling through prospective means (the theoretical rationale precedes the creation of the molecule). Interestingly, there are retrospective examples of biased ligands that demonstrate a divergent clinical profile in treatment

outcome before they were identified as being biased as in the case of carvedilol (Wisler et al., 2007; Kim et al., 2008). Similar examples of retrospective attribution of selective activity to biased signaling were discussed earlier in section VII.B. Although to date no designed biased molecule has been successful in the clinic, data with these retrospectively identified biased molecules furnish a roundabout proof of concept of the value of biased signaling.

The translational gaps involved in the progression of biased molecules from in vitro characterization studies to in vivo therapeutic conditions are expected and indeed predicted by the tenets of cellular pharmacology. Moreover, these gaps have been realized in the few attempts made to make the transfer into the therapeutic realm, raising the obvious question: What are ways forward to minimize attrition at this crucial step in the drug discovery and development process? One obvious but not very helpful approach would be to identify exemplar molecules in the initial stages of screening and lead optimization and then put them into the most therapeutically relevant systems as quickly as possible. However, the general theme of increasing texture in the pharmacological outcome of assays of greater complexity (i.e., from simple in vitro to highly complex in vivo) usually means that a set of “exemplars” will further differentiate into many subsets, leading to too many possible molecules to progress to the costly final steps of drug testing in humans.

There may be further less radical steps in the development process that can refine choices of biased molecules and result in more informed decisions for therapeutic progression. The use of primary cell systems is one approach that has been cited as a valuable intermediate step in the development process. For instance, cultured trigeminal neurons and glia have been reported to furnish useful data to characterize PACAP receptor biased molecules of possible value in regulation of circadian rhythms, reproduction and

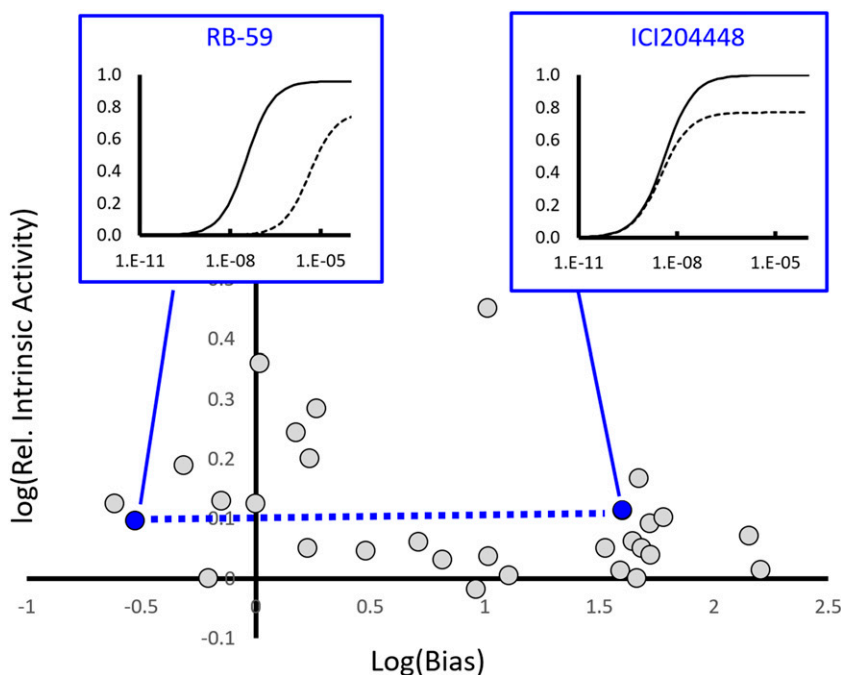


Fig. 23. Regression of the relative intrinsic activities of κ -opioid receptor agonists for G protein/ β -arrestin responses on the bias of these agonists for each pathway. It can be seen that there are agonists with identical bias values but differing relative efficacies (reflected as intrinsic activity values) for each pathway and vice versa (identical maximal intrinsic activities but varying bias). This latter effect is demonstrated by RB-59 and ICI204448, which have very different bias values but essentially identical efficacies for each pathway (would lie on the same I.A. trajectory). This illustrates the importance of variable affinity with signaling pathways. Data redrawn from White et al (2014).

development, cognitive behavior, pain transmission, neuroprotection, and neuromodulation (Walker et al., 2014). Another approach is the more effective use of whole animals and complex readouts of biased response such as transcriptome analysis in control and genetically modified animals (Luttrell et al., 2018). Specifically, analyses from such studies often identify many

differences, but it may be that concentrating on conserved elements across tissues with biased molecules may be more fruitful. For example, when the transcriptome in studies on bone metabolism with [D-Trp¹², Tyr³⁴]-bPTH(7-34) and hPTH(1-34) are compared in six different murine tissues, some interesting general phenomena are noted. Thus, the arrestin-biased molecule

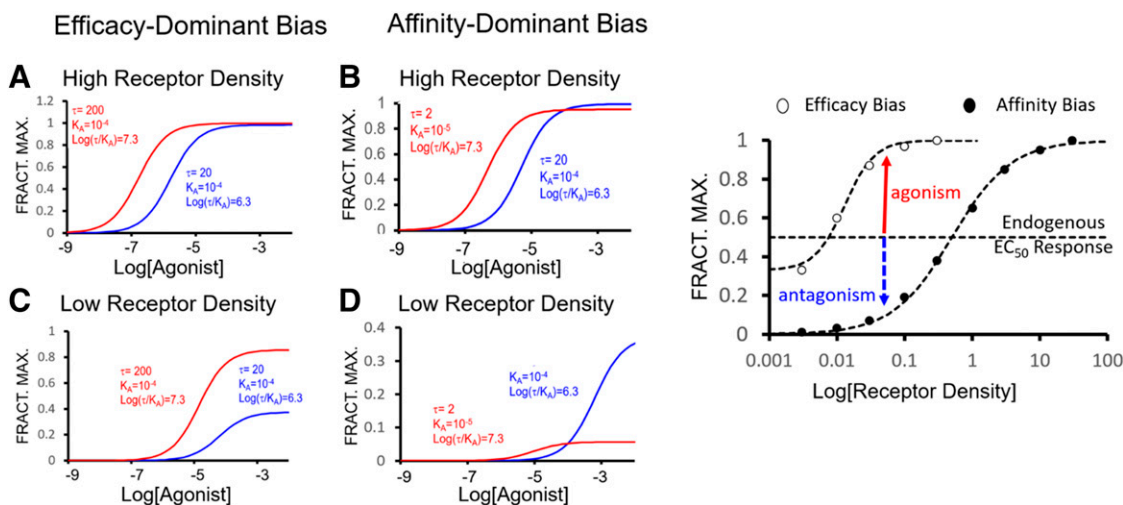


Fig. 24. Agonist and antagonist effects of two identically biased agonists in a range of tissues with varying sensitivities (receptor densities). (A–D) The agonist in (A) and (C) achieves bias through selective efficacy, whereas the agonist in (B) and (D) is biased because of selective affinity. It can be seen that in high-sensitivity tissues (A and B), both agonists produce identical profiles of biased effect. However, in low-sensitivity tissues (low receptor density), the response in the preferred pathway (red curve) is selectively depressed for affinity-dominant bias (D) when compared with efficacy-dominant bias (C). In such tissues, this agonist will function as an antagonist. The right panel depicts the observed response to these agonists over a range of tissue sensitivities. It can be seen that with low receptor density, no agonist response will be produced and the ligands will function as antagonists. With increasing tissue sensitivity, the direct agonist effect of the agonists emerges. However, the range of tissues (as indicated by the range in receptor densities) demonstrating agonism is far greater for the efficacy-dominant agonist than it is for the affinity-dominant agonist (i.e., biased agonism is more robust in vivo for efficacy-dominant bias).

generates a more conserved response over the conventional ligand with regard to cell growth, development, and survival (Maudsley et al., 2015, 2016). In this regard, the application of more complex models may furnish more textured data capable of yielding therapeutically relevant concepts for biased molecules (Bradley and Tobin, 2016). Another factor in further defining useful in vivo phenotypes is the creation of tissue-specific gene knockouts. For example, the β -arrestin-biased angiotensin ligand TRV067 demonstrates cardioprotective effects in cardiomyocytes (Ryba et al., 2017). However, β -arrestin overall knockouts in vivo affect other tissues such as the vasculature and the kidney; therefore, it is difficult to ascribe in vivo effects directly to cardiac function (Woo et al., 2017). Often, a limitation to adequate determination of in vivo phenotypes is the difficulty in obtaining responses from the therapeutically relevant tissue; in this regard, the application of label-free technology may offer a way forward, as cell-specific responses can be measured with this format. Finally, the application of new technology and assay techniques such as whole animal functional resonance imaging may assist in the determination of therapeutically phenotypes in vivo; the tracking of brain region 5-HT_{1A} function has been used to correlate in vitro bias with in vivo effect for biased 5-HT agonists (Becker et al., 2016).

In general, two major ideas have revolutionized receptor pharmacology over the past 15 years and spawned the concepts now discussed as biased receptor signaling. The first relates to the allosteric nature of 7TMRs and the view of multiconformational receptor states envisioned with molecular dynamics. The second idea involves the application of the plethora of new functional receptor assays now available to separately observe the wealth of behaviors practiced by 7TMRs. The observation of separate receptor signaling pathways indicates that classic receptor theory describing a simplistic active versus inactive receptor state is incapable of accommodating what is observed in experimental pharmacology. This article discusses the elements involved in biased receptor signaling (defined in section I) beginning with the pleiotropic components of receptor systems (section II) and the observed interactions between them (section III) that lead to the diversity of signaling produced in natural systems to fine-tune physiologic response (section IV). The discussion then progresses to the ideas around harnessing this mechanism for therapeutic advantage (section V); the molecular nature of the mechanism is then discussed in section VI. Section VII discusses the tools that have been proposed to quantify biased signaling so that it can be modified through medicinal chemistry to produce therapeutic molecules. Finally, the important caveats and hurdles in the translation of easily measured and quantified biased signaling effects observed when translating in vitro to in vivo systems for therapeutic

advantage are discussed in section VIII. At the time of writing, this is an untested idea in terms of being a fruitful strategy to make better drugs. However, there is a strong rationale for believing that biased signaling could lead to valuable drug therapeutics; in terms of the minimal resources required to sustain efforts to detect and measure possible biased effects in new ligands (Kenakin, 2017), the value proposition for continuing exploration of this effect is favorable.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Kenakin.

References

- Abdul-Ridha A, López L, Keov P, Thal DM, Mistry SN, Sexton PM, Lane JR, Canals M, and Christopoulos A (2014) Molecular determinants of allosteric modulation at the M1 muscarinic acetylcholine receptor. *J Biol Chem* **289**:6067–6079.
- Ahn S, Kim J, Hara MR, Ren XR, and Lefkowitz RJ (2009) (beta)-Arrestin-2 mediates anti-apoptotic signaling through regulation of BAD phosphorylation. *J Biol Chem* **284**:8855–8865.
- Aiba A, Chen C, Herrup K, Rosenmund C, Stevens CF, and Tonegawa S (1994) Reduced hippocampal long-term potentiation and context-specific deficit in associative learning in mGluR1 mutant mice. *Cell* **79**:365–375.
- Akinaga J, Lima V, Kiguti LR, Hebeler-Barbosa F, Alcántara-Hernández R, García-Sáinz JA, and Pupo AS (2013) Differential phosphorylation, desensitization, and internalization of α 1A-adrenoceptors activated by norepinephrine and oxymetazoline. *Mol Pharmacol* **83**:870–881.
- Allen JA, Yost JM, Setola V, Chen X, Sassano MF, Chen M, Peterson S, Yadav PN, Huang XP, Feng B, et al. (2011) Discovery of β -arrestin-biased dopamine D2 ligands for probing signal transduction pathways essential for antipsychotic efficacy. *Proc Natl Acad Sci USA* **108**:18488–18493.
- Altarifi AA, David B, Muchhala KH, Blough BE, Akbarali H, and Negus SS (2017) Effects of acute and repeated treatment with the biased mu opioid receptor agonist TRV130 (oliceptine) on measures of antinociception, gastrointestinal function, and abuse liability in rodents. *J Psychopharmacol* **31**:730–739.
- Alvarez VA, Arttamangkul S, Dang V, Salem A, Whistler JL, Von Zastrow M, Grandy DK, and Williams JT (2002) mu-Opioid receptors: ligand-dependent activation of potassium conductance, desensitization, and internalization. *J Neurosci* **22**:5769–5776.
- Alvarez-Curto E, Prihandoko R, Tautermann CS, Zwier JM, Pediani JD, Lohse MJ, Hoffmann C, Tobin AB, and Milligan G (2011) Developing chemical genetic approaches to explore G protein-coupled receptor function: validation of the use of a receptor activated solely by synthetic ligand (RASSL). *Mol Pharmacol* **80**:1033–1046.
- Amarandi RM, Hjortø GM, Rosenkilde MM, and Karlshøj S (2016) Probing biased signaling in chemokine receptors. *Methods Enzymol* **570**:155–186.
- Anderson CA, Solari R, and Pease JE (2016) Biased agonism at chemokine receptors: obstacles or opportunities for drug discovery? *J Leukoc Biol* **99**:901–909.
- Appleton KM, Lee M-H, Alele C, Alele C, Luttrell DK, Peterson YK, Morinelli TA, and Luttrell LM (2013) Biasing the parathyroid hormone receptor: relating in vitro ligand efficacy to in vivo biological activity. *Methods Enzymol* **522**:229–262.
- Ariens EJ (1954) Affinity and intrinsic activity in the theory of competitive inhibition. I. Problems and theory. *Arch Int Pharmacodyn Ther* **99**:32–49.
- Armbruster BN, Li X, Pausch MH, Herlitze S, and Roth BL (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potentially activated by an inert ligand. *Proc Natl Acad Sci USA* **104**:5163–5168.
- Armour SL, Foord S, Kenakin T, and Chen W-J (1999) Pharmacological characterization of receptor-activity-modifying proteins (RAMPs) and the human calcitonin receptor. *J Pharmacol Toxicol Methods* **42**:217–224.
- Atwood BK, Lopez J, Wager-Miller J, Mackie K, and Straiker A (2011) Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics* **12**:14.
- Atwood BK, Wager-Miller J, Haskins C, Straiker A, and Mackie K (2012) Functional selectivity in CB(2) cannabinoid receptor signaling and regulation: implications for the therapeutic potential of CB(2) ligands. *Mol Pharmacol* **81**:250–263.
- Audet N, Galés C, Archer-Lahlou E, Vallières M, Schiller PW, Bouvier M, and Pineyro G (2008) Bioluminescence resonance energy transfer assays reveal ligand-specific conformational changes within preformed signaling complexes containing delta-opioid receptors and heterotrimeric G proteins. *J Biol Chem* **283**:15078–15088.
- Aurelio L, Baltos J-A, Ford L, Nguyen ATN, Jörg M, Devine SM, Valant C, White PJ, Christopoulos A, May LT, et al. (2018) A structure-activity relationship study of bitopic N6-substituted adenosine derivatives as biased adenosine A1 receptor agonists. *J Med Chem* **61**:2087–2103.
- Aurelio L, Valant C, Flynn BL, Sexton PM, Christopoulos A, and Scammells PJ (2009) Allosteric modulators of the adenosine A1 receptor: synthesis and pharmacological evaluation of 4-substituted 2-amino-3-benzoylthiophenes. *J Med Chem* **52**:4543–4547.
- Ayoub MA, Yvinec R, Jégot G, Dias JA, Poli S-M, Poupon A, Crépieux P, and Reiter E (2016) Profiling of FSHR negative allosteric modulators on LH/CGR reveals biased antagonism with implications in steroidogenesis. *Mol Cell Endocrinol* **436**:10–22.
- Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M, and Pineyro G (2003) Beta-arrestin-mediated activation of MAPK by inverse agonists reveals

- distinct active conformations for G protein-coupled receptors. *Proc Natl Acad Sci USA* **100**:11406–11411.
- Babcock GJ, Farzan M, and Soderstrom J (2003) Ligand-independent dimerization of CXCR4, a principal HIV-1 coreceptor. *J Biol Chem* **278**:3378–3385.
- Baker JG, Hall IP, and Hill SJ (2003) Agonist and inverse agonist actions of beta-blockers at the human beta 2-adrenoceptor provide evidence for agonist-directed signaling. *Mol Pharmacol* **64**:1357–1369.
- Baker JG and Hill SJ (2007) Multiple GPCR conformations and signalling pathways: implications for antagonist affinity estimates. *Trends Pharmacol Sci* **28**:374–381.
- Baltos J-A, Gregory KJ, White PJ, Sexton PM, Christopoulos A, and May LT (2016a) Quantification of adenosine A(1) receptor biased agonism: implications for drug discovery. *Biochem Pharmacol* **99**:101–112.
- Baltos JA, Paoletta S, Nguyen AT, Gregory KJ, Tosh DK, Christopoulos A, Jacobson KA, and May LT (2016b) Structure-activity analysis of biased agonism at the human adenosine A3 receptor. *Mol Pharmacol* **90**:12–22.
- Baltos JA, Vecchio EA, Harris MA, Qin CX, Ritchie RH, Christopoulos A, White PJ, and May LT (2017) Capadenoson, a clinically trialed partial adenosine A₁ receptor agonist, can stimulate adenosine A_{2B} receptor biased agonism. *Biochem Pharmacol* **135**:79–89.
- Bar-Shavit R, Maoz M, Kancharla A, Jaber M, Agranovich D, Grisaru-Granovsky S, and Uzieli B (2016) Protease-activated receptors (PARs) in cancer: novel biased signaling and targets for therapy. *Methods Cell Biol* **132**:341–358.
- Barak LS, Bai Y, Peterson S, Evron T, Urs NM, Peddibhotla S, Hedrick MP, Hershberger P, Maloney PR, Chung TD, et al. (2016) ML314: a biased neurotensin receptor ligand for methamphetamine abuse. *ACS Chem Biol* **11**:1880–1890.
- Barak LS, Ferguson SSG, Zhang J, and Caron MG (1997) A β -arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J Biol Chem* **272**:27497–27500.
- Barlic J, Andrews JD, Kelvin AA, Bosinger SE, DeVries ME, Xu L, Dobransky T, Feldman RD, Ferguson SS, and Kelvin DJ (2000) Regulation of tyrosine kinase activation and granule release through beta-arrestin by CXCR1. *Nat Immunol* **1**:227–233.
- Barlow RB, Scott NC, and Stephenson RP (1967) The affinity and efficacy of onium salts on the frog rectus abdominis. *Br J Pharmacol Chemother* **31**:188–196.
- Bdioui S, Verdi J, Pierre N, Trinquet E, Roux T, and Kenakin T (2018) Equilibrium assays are required to accurately characterize the activity profiles of drugs modulating Gq-protein-coupled receptors. *Mol Pharmacol* **94**:992–1006.
- Beaulieu JM, Gainetdinov RR, and Caron MG (2007) The Akt-GSK-3 signaling cascade in the actions of dopamine. *Trends Pharmacol Sci* **28**:166–172.
- Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR, and Caron MG (2005) An Akt/beta-arrestin/2PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell* **122**:261–273.
- Beaulieu JM, Sotnikova TD, Yao WD, Kockeritz L, Woodgett JR, Gainetdinov RR, and Caron MG (2004) Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade. *Proc Natl Acad Sci USA* **101**:5099–5104.
- Becker G, Bolbos R, Costes N, Redouté J, Newman-Tancredi A, and Zimmer L (2016) Selective serotonin 5-HT_{1A} receptor biased agonists elicit distinct brain activation patterns: a pharmacMRI study. *Sci Rep* **6**:26633.
- Bellot M, Galandrin S, Boularan C, Matthies HJ, Despas F, Denis C, Javitch J, Mazères S, Sanni SJ, Pons V, et al. (2015) Dual agonist occupancy of AT₁-R- α 2C-AR heterodimers results in atypical Gs-PKA signaling. *Nat Chem Biol* **11**:271–279.
- Benovic JL, Strasser RH, Caron MG, and Lefkowitz RJ (1986) Beta-adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc Natl Acad Sci USA* **83**:2797–2801.
- Berchiche YA and Sakmar TP (2016) CXC chemokine receptor 3 alternative splice variants selectively activate different signaling pathways. *Mol Pharmacol* **90**:483–495.
- Berg KA, Maayani S, Goldfarb J, Scaramellini C, Leff P, and Clarke WP (1998) Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. *Mol Pharmacol* **54**:94–104.
- Besserer-Offroy É, Brouillette RL, Lavenus S, Froehlich U, Brumwell A, Murza A, Longpré JM, Marsault É, Grandbois M, Sarret P, et al. (2017) The signaling signature of the neurotensin type 1 receptor with endogenous ligands. *Eur J Pharmacol* **805**:1–13.
- Beyermann M, Heinrich N, Fechner K, Furkert J, Zhang W, Kraetke O, Bienert M, and Berger H (2007) Achieving signalling selectivity of ligands for the corticotropin-releasing factor type 1 receptor by modifying the agonist's signalling domain. *Br J Pharmacol* **151**:851–859.
- Bhattacharya M, Anborgh PH, Babwah AV, Dale LB, Dobransky T, Benovic JL, Feldman RD, Verdi JM, Rylett RJ, and Ferguson SS (2002) Beta-arrestins regulate a Ral-GDS Ral effector pathway that mediates cytoskeletal reorganization. *Nat Cell Biol* **4**:547–555.
- Black JW and Leff P (1983) Operational models of pharmacological agonism. *Proc R Soc Lond B Biol Sci* **220**:141–162.
- Black JW, Leff P, Shankley NP, and Wood J (1985) An operational model of pharmacological agonism: the effect of E/[A] curve shape on agonist dissociation constant estimation. *Br J Pharmacol* **84**:561–571.
- Blättermann S, Peters L, Ottersbach PA, Bock A, Konya V, Weaver CD, Gonzalez A, Schröder R, Tyagi R, Luschni P, et al. (2012) A biased ligand for OXER-uncouples $G\alpha$ and $G\beta\gamma$ signaling within a heterotrimer. *Nat Chem Biol* **8**:631–638.
- Blechnan J and Levkowitz G (2013) Alternative splicing of the pituitary adenylate cyclase-activating polypeptide receptor PAC1: mechanisms of fine tuning of brain activity. *Front Endocrinol (Lausanne)* **4**:55.
- Bockaert J, Fagni L, Dumuis A, and Marin P (2004) GPCR interacting proteins (GIP). *Pharmacol Ther* **103**:203–221.
- Bockaert J, Marin P, Dumuis A, and Fagni L (2003) The 'magic tail' of G protein-coupled receptors: an anchorage for functional protein networks. *FEBS Lett* **546**:65–72.
- Boehr DD, Nussinov R, and Wright PE (2009) The role of dynamic conformational ensembles in biomolecular recognition. *Nat Chem Biol* **5**:789–796.
- Bohn LM, Dykstra LA, Lefkowitz RJ, Caron MG, and Barak LS (2004) Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. *Mol Pharmacol* **66**:106–112.
- Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, and Caron MG (2000) Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* **408**:720–723.
- Bohn LM, Gainetdinov RR, Sotnikova TD, Medvedev IO, Lefkowitz RJ, Dykstra LA, and Caron MG (2003) Enhanced rewarding properties of morphine, but not cocaine, in beta(arrestin)-2 knock-out mice. *J Neurosci* **23**:10265–10273.
- Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, and Lin FT (1999) Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* **286**:2495–2498.
- Boivin B, Vaniotis G, Allen BG, and Hébert TE (2008) G protein-coupled receptors in and on the cell nucleus: a new signaling paradigm? *J Recept Signal Transduct Res* **28**:15–28.
- Bologna Z, Teoh J-P, Bayoumi AS, Tang Y, and Kim I-M (2017) Biased G protein-coupled receptor signaling: new player in modulating physiology and pathology. *Biomol Ther (Seoul)* **25**:12–25.
- Bolognini D, Moss CE, Nilsson K, Petersson AU, Donnelly I, Sergeev E, König GM, Kostenis E, Kurowska-Stolarska M, Miller A, et al. (2016) A novel allosteric activator of free fatty acid 2 receptor displays unique Gi-functional bias. *J Biol Chem* **291**:18915–18931.
- Bonifazi A, Yano H, Ellenberger MP, Muller L, Kumar V, Zou MF, Cai NS, Guerrero AM, Woods AS, Shi L, et al. (2017) Novel bivalent ligands based on the sumanirepharmacophore reveal dopamine D₂ receptor (D₂R) biased agonism. *J Med Chem* **60**:2890–2907.
- Bosshard HR (2001) Molecular recognition by induced fit: how fit is the concept? *Neuro Physiol Sci* **16**:171–173.
- Bradley SJ, Langmead CJ, Watson JM, and Challiss RA (2011) Quantitative analysis reveals multiple mechanisms of allosteric modulation of the mGlu5 receptor in rat astroglia. *Mol Pharmacol* **79**:874–885.
- Bradley SJ and Tobin AB (2016) Design of next-generation G protein-coupled receptor drugs: linking novel pharmacology and in vivo animal models. *Annu Rev Pharmacol Toxicol* **56**:535–559.
- Bristow MR, Ginsburg R, Minobe W, Cubicciotti RS, Sageman WS, Lurie K, Billingham ME, Harrison DC, and Stinson EB (1982) Decreased catecholamine sensitivity and beta-adrenergic-receptor density in failing human hearts. *N Engl J Med* **307**:205–211.
- Brulé C, Perzo N, Joubert JE, Sainsily X, Leduc R, Castel H, and Prézeau L (2014) Biased signaling regulates the pleiotropic effects of the urotensin II receptor to modulate its cellular behaviors. *FASEB J* **28**:5148–5162.
- Brust TF, Morgenweck J, Kim SA, Rose JH, Locke JL, Schmid CL, Zhou L, Stahl EL, Cameron MD, Scarry SM, et al. (2016) Biased agonists of the kappa opioid receptor suppress pain and itch without causing sedation or dysphoria. *Sci Signal* **9**:ra117.
- Budd DC, Willars GB, McDonald JE, and Tobin AB (2001) Phosphorylation of the Gq/11-coupled m3-muscarinic receptor is involved in receptor activation of the ERK1/2 mitogen-activated protein kinase pathway. *J Biol Chem* **276**:4581–4587.
- Burgen ASV (1981) Conformational changes and drug action. *Fed Proc* **40**:2723–2728.
- Busnelli M, Saulière A, Manning M, Bouvier M, Galés C, and Chini B (2012) Functional selective oxytocin-derived agonists discriminate between individual G protein family subtypes. *J Biol Chem* **287**:3617–3629.
- Butcher AJ, Prihandoko R, Kong KC, McWilliams P, Edwards JM, Bottrill A, Mistry S, and Tobin AB (2011) Differential G-protein-coupled receptor phosphorylation provides evidence for a signaling bar code. *J Biol Chem* **286**:11506–11518.
- Byers MA, Calloway PA, Shannon L, Cunningham HD, Smith S, Li F, Fassold BC, and Vines CM (2008) Arrestin 3 mediates endocytosis of CCR7 following ligation of CCL19 but not CCL21. *J Immunol* **181**:4723–4732.
- Cahill TJ 3rd, Thomsen AR, Tarrasch JT, Plouffe B, Nguyen AH, Yang F, Huang LY, Kahsai AW, Bassoni DL, Gavino BJ, et al. (2017) Distinct conformations of GPCR- β -arrestin complexes mediate desensitization, signaling, and endocytosis. *Proc Natl Acad Sci USA* **114**:2562–2567.
- Calebiro D, Godbole A, Lyga S, and Lohse MJ (2015) Trafficking and function of GPCRs in the endosomal compartment. *Methods Mol Biol* **1234**:197–211.
- Cao TT, Mays RW, and von Zastrow M (1998) Regulated endocytosis of G-protein-coupled receptors by a biochemically and functionally distinct subpopulation of clathrin-coated pits. *J Biol Chem* **273**:24592–24602.
- Carr R 3rd, Schilling J, Song J, Carter RL, Du Y, Yoo SM, Traynham CJ, Koch WJ, Cheung JY, Tilley DG, et al. (2016) β -arrestin-biased signaling through the β 2-adrenergic receptor promotes cardiomyocyte contraction. *Proc Natl Acad Sci USA* **113**:E4107–E4116.
- Casey LM, Pistner AR, Belmonte SL, Migdalovich D, Stolpnik O, Nwankma FE, Vorobiof G, Dunaevsky O, Matavel A, Lopes CM, et al. (2010) Small molecule disruption of G beta gamma signaling inhibits the progression of heart failure. *Circ Res* **107**:532–539.
- Castro M, Nikolaev VO, Palm D, Lohse MJ, and Vilardaga JP (2005) Turn-on switch in parathyroid hormone receptor by a two-step parathyroid hormone binding mechanism. *Proc Natl Acad Sci USA* **102**:16084–16089.
- Cavalli A, Lattion AL, Hummler E, Nenniger M, Pedrazzini T, Aubert JF, Michel MC, Yang M, Lembo G, Vecchiene C, et al. (1997) Decreased blood pressure response in mice deficient of the α 1b-adrenergic receptor. *Proc Natl Acad Sci USA* **94**:11589–11594.
- Changeux JP and Edelstein SJ (2005) Allosteric mechanisms of signal transduction. *Science* **308**:1424–1428.
- Changeux JP and Edelstein S (2011) Conformational selection or induced fit? 50 years of debate resolved. *F1000 Biol Rep* **3**:19.
- Charest PG and Bouvier M (2003) Palmitoylation of the V2 vasopressin receptor carboxyl tail enhances beta-arrestin recruitment leading to efficient receptor endocytosis and ERK1/2 activation. *J Biol Chem* **278**:41541–41551.

- Charest PG, Terrillon S, and Bouvier M (2005) Monitoring agonist-promoted conformational changes of beta-arrestin in living cells by intramolecular BRET. *EMBO Rep* **6**:334–340.
- Charfi I, Nagi K, Mnie-Filali O, Thibault D, Balboni G, Schiller PW, Trudeau LE, and Pineyro G (2014) Ligand- and cell-dependent determinants of internalization and cAMP modulation by delta opioid receptor (DOR) agonists. *Cell Mol Life Sci* **71**: 1529–1546.
- Chavkin C, Schattauer SS, and Levin JR (2014) Arrestin-mediated activation of p38 MAPK: molecular mechanisms and behavioral consequences. *Handb Exp Pharmacol* **219**:281–292.
- Che T, Majumdar S, Zaidi SA, Ondachi P, McCorvy JD, Wang S, Mosier PD, Uprety R, Vardy E, Krumm BE, et al. (2018) Structure of the nanobody-stabilized active state of the kappa opioid receptor. *Cell* **172**:55–67.e15.
- Chen H, Martin B, Daimon CM, Siddiqui S, Luttrell LM, and Maudsley S (2013b) Textrools: extracting semantic textual meaning from gene sets. *PLoS One* **8**: e62665.
- Chen J, Rusnak M, Lombroso PJ, and Sidhu A (2009) Dopamine promotes striatal neuronal apoptotic death via ERK signaling cascades. *Eur J Neurosci* **29**:287–306.
- Chen X, Sassano MF, Zheng L, Setola V, Chen M, Bai X, Frye SV, Wetsel WC, Roth BL, and Jin J (2012) Structure-functional selectivity relationship studies of beta-arrestin-biased dopamine D₂ receptor agonists. *J Med Chem* **55**:7141–7153.
- Chen XT, Pitis P, Liu G, Yuan C, Gotchev D, Cowan CL, Rominger DH, Koblisch M, Dewire SM, Crombie AL, et al. (2013a) Structure-activity relationships and discovery of a G protein biased μ opioid receptor ligand, [(3-methoxythiophen-2-yl)methyl]-(2-[(9R)-9-(pyridin-2-yl)-6-oxaspiro-[4.5]decan-9-yl)ethyl)amine (TRV130), for the treatment of acute severe pain. *J Med Chem* **56**:8019–8031.
- Christensen GL, Kelstrup CD, Lyngsø C, Sarwar U, Bøgebo R, Sheikh SP, Gammeltoft S, Olsen JV, and Hansen JL (2010) Quantitative phosphoproteomics dissection of seven-transmembrane receptor signaling using full and biased agonists. *Mol Cell Proteomics* **9**:1540–1553.
- Christmansson L, Westermarck P, and Betsholtz C (1994) Islet amyloid polypeptide stimulates cyclic AMP accumulation via the porcine calcitonin receptor. *Biochem Biophys Res Commun* **205**:1226–1235.
- Christopoulos A, Christopoulos G, Morfis M, Udawela M, Laburthe M, Couvineau A, Kuwasako K, Tilakaratne N, and Sexton PM (2003) Novel receptor partners and function of receptor activity-modifying proteins. *J Biol Chem* **278**:3293–3297.
- Chun LS, Vekariya RH, Free RB, Li Y, Lin DT, Su P, Liu F, Namkung Y, Laporte SA, Moritz AE, et al. (2018) Structure-activity investigation of a G protein-biased agonist reveals molecular determinants for biased signaling of the D₂ dopamine receptor. *Front Synaptic Neurosci* **10**:2.
- Civciristov S, Ellisdson AM, Suderman R, Pon CK, Evans BA, Kleinfeld O, Charlton SJ, Hlavacek WS, Canals M, and Halls ML (2018) Preassembled GPCR signaling complexes mediate distinct cellular responses to ultralow ligand concentrations. *Sci Signal* **11**:eaan1188.
- Clapham DE and Neer EJ (1997) G protein beta gamma subunits. *Annu Rev Pharmacol Toxicol* **37**:167–203.
- Coffa S, Breiman M, Spiller BW, and Gurevich VV (2011) A single mutation in arrestin-2 prevents ERK1/2 activation by reducing c-Raf1 binding. *Biochemistry* **50**:6951–6958.
- Coffman TM (1997) A genetic approach for studying the physiology of the type 1A (AT1A) angiotensin receptor. *Semin Nephrol* **17**:404–411.
- Cognetti F, Bagnato A, Colombo N, Savarese A, Scambia G, Sehoul J, Wimberger P, Sorio R, Harter P, Mari E, et al. (2013) A phase II, randomized, double-blind study of zibotentan (ZD4054) in combination with carboplatin/paclitaxel versus placebo in combination with carboplatin/paclitaxel in patients with advanced ovarian cancer sensitive to platinum-based chemotherapy (AGO-OVAR 2.14). *Gynecol Oncol* **130**:31–37.
- Conklin BR, Hsiao EC, Claeysen S, Dumuis A, Srinivasan S, Forsayeth JR, Guettier JM, Chang WC, Pei Y, McCarthy KD, et al. (2008) Engineering GPCR signaling pathways with RASSLs. *Nat Methods* **5**:673–678.
- Cook AE, Mistry SN, Gregory KJ, Furness SG, Sexton PM, Scammells PJ, Conigrave AD, Christopoulos A, and Leach K (2015) Biased allosteric modulation at the CaS receptor engendered by structurally diverse calcimimetics. *Br J Pharmacol* **172**:185–200.
- Corbisier J, Huszagh A, Galés C, Parmentier M, and Springael JY (2017) Partial agonist and biased signaling properties of the synthetic enantiomers J113863/UCB35625 at chemokine receptors CCR2 and CCR5. *J Biol Chem* **292**:575–584.
- Corvol JC, Muriel MP, Valjent E, Féger J, Hanoun N, Girault JA, Hirsch EC, and Hervé D (2004) Persistent increase in olfactory type G-protein alpha subunit levels may underlie D1 receptor functional hypersensitivity in Parkinson disease. *J Neurosci* **24**:7007–7014.
- Coward P, Wada HG, Falk MS, Chan SD, Meng F, Akil H, and Conklin BR (1998) Controlling signaling with a specifically designed Gi-coupled receptor in transgenic mice. *Nat Biotechnol* **17**:165–169.
- Cui G and Karplus M (2008) Allosteric and cooperativity revisited. *Protein Sci* **17**: 1295–1307.
- Daugvilaite V, Madsen CM, Lückmann M, Echeverria CC, Sailer AW, Frimurer TM, Rosenkilde MM, and Bølleby-Jensen T (2017) Biased agonism and allosteric modulation of G protein-coupled receptor 183 - a 7TM receptor also known as Epstein-Barr virus-induced gene 2. *Br J Pharmacol* **174**:2031–2042.
- Davey AE, Leach K, Valant C, Conigrave AD, Sexton PM, and Christopoulos A (2012) Positive and negative allosteric modulators promote biased signaling at the calcium-sensing receptor. *Endocrinology* **153**:1232–1241.
- Davoren JE, Lee CW, Garnsey M, Brodney MA, Cordes J, Dlugolenski K, Edgerton JR, Harris AR, Helal CJ, Jenkinson S, et al. (2016) Discovery of the potent and selective M1 PAM-agonist N-[(3R,4S)-3-hydroxytetrahydro-2H-pyran-4-yl]-5-methyl-4-[(4-1,3-thiazol-4-yl)benzyl]pyridine-2-carboxamide (PF-06767832): evaluation of efficacy and cholinergic side effects. *J Med Chem* **59**:6313–6328.
- DeFea KA, Vaughn ZD, O'Bryan EM, Nishijima D, Déry O, and Bunnett NW (2000a) The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta-arrestin-dependent scaffolding complex. *Proc Natl Acad Sci USA* **97**:11086–11091.
- DeFea KA, Zalevsky J, Thoma MS, Déry O, Mullins RD, and Bunnett NW (2000b) Beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol* **148**:1267–1281.
- Delguidice T, Lemasson M, and Beaulieu JM (2011) Role of beta-arrestin 2 downstream of dopamine receptors in the basal ganglia. *Front Neuroanat* **5**:58.
- Deng H, Sun H, and Fang Y (2013) Label-free cell phenotypic assessment of the biased agonism and efficacy of agonists at the endogenous muscarinic M3 receptors. *J Pharmacol Toxicol Methods* **68**:323–333.
- Denis C, Saulière A, Galandrin S, Sénard JM, and Galés C (2012) Probing heterotrimeric G protein activation: applications to biased ligands. *Curr Pharm Des* **18**:123–144.
- Deshpande DA, Theriot BS, Penn RB, and Walker JK (2008) Beta-arrestins specifically constrain beta2-adrenergic receptor signaling and function in airway smooth muscle. *FASEB J* **22**:2134–2141.
- Devost D, Sleno R, Pétrin D, Zhang A, Shinjo Y, Okde R, Aoki J, Inoue A, and Hébert TE (2017) Conformational profiling of the AT1 angiotensin II receptor reflects biased agonism, G protein coupling, and cellular context. *J Biol Chem* **292**: 5443–5456.
- DeWire SM, Ahn S, Lefkowitz RJ, and Shenoy SK (2007) β -arrestins and cell signaling. *Annu Rev Physiol* **69**:483–510.
- DeWire SM, Kim J, Whalen EJ, Ahn S, Chen M, and Lefkowitz RJ (2008) Beta-arrestin-mediated signaling regulates protein synthesis. *J Biol Chem* **283**: 10611–10620.
- DeWire SM, Yamashita DS, Rominger DH, Liu G, Cowan CL, Graczyk TM, Chen XT, Pitis PM, Gotchev D, Yuan C, et al. (2013) A G protein-biased ligand at the μ -opioid receptor is potentially analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *J Pharmacol Exp Ther* **344**:708–717.
- Diez-Alarcia R, Ibarra-Lecue I, Lopez-Cardona AP, Meana J, Gutierrez-Adán A, Callado LF, Agirregoitia E, and Uruguén L (2016) Biased agonism of three different cannabinoid receptor agonists in mouse brain cortex. *Front Pharmacol* **7**:415–428.
- Dijksterhuis JP, Baljinnyam B, Stanger K, Sercan HO, Ji Y, Andres O, Rubin JS, Hannoush RN, and Schulte G (2015) Systematic mapping of WNT-FZD protein interactions reveals functional selectivity by distinct WNT-FZD pairs. *J Biol Chem* **290**:6789–6798.
- Dong S, Rogan SC, and Roth BL (2010) Directed molecular evolution of DREADDs: a generic approach to creating next-generation RASSLs. *Nat Protoc* **5**:561–573.
- Dong SS, Goddard WA 3rd, and Abrol R (2017) Identifying multiple active conformations in the G protein-coupled receptor activation landscape using computational methods. *Methods Cell Biol* **142**:173–186.
- Drake MT, Shenoy SK, and Lefkowitz RJ (2006) Trafficking of G protein-coupled receptors. *Circ Res* **99**:570–582.
- Dror RO, Arlow DH, Maragakis P, Mildorf TJ, Pan AC, Xu H, Borhani DW, and Shaw DE (2011) Activation mechanism of the β 2-adrenergic receptor. *Proc Natl Acad Sci USA* **108**:18684–18689.
- Dror RO, Green HF, Valant C, Borhani DW, Valcourt JR, Pan AC, Arlow DH, Canals M, Lane JR, Rahmani R, et al. (2013) Structural basis for modulation of a G-protein-coupled receptor by allosteric drugs. *Nature* **503**:295–299.
- Dror RO, Jensen MO, Borhani DW, and Shaw DE (2010) Exploring atomic resolution physiology on a femtosecond to millisecond timescale using molecular dynamics simulations. *J Gen Physiol* **135**:555–562.
- Duhr F, Deléris P, Raynaud F, Séveno M, Morisset-Lopez S, Mannoury la Cour C, Millan MJ, Bockaert J, Marin P, and Chaudmont-Dubel S (2014) Cdk5 induces constitutive activation of 5-HT6 receptors to promote neurite growth. *Nat Chem Biol* **10**:590–597.
- Dupré DJ, Robitaille M, Rebois RV, and Hébert TE (2009) The role of Gbetagamma subunits in the organization, assembly, and function of GPCR signaling complexes. *Annu Rev Pharmacol Toxicol* **49**:31–56.
- Duttaroy A, Zimlik CL, Gautam D, Cui Y, Mears D, and Wess J (2004) Muscarinic stimulation of pancreatic insulin and glucagon release is abolished in m3 muscarinic acetylcholine receptor-deficient mice. *Diabetes* **53**:1714–1720.
- Eason MG, Kurose H, Holt BD, Raymond JR, and Liggett SB (1992) Simultaneous coupling of alpha 2-adrenergic receptors to two G-proteins with opposing effects. Subtype-selective coupling of alpha 2C10, alpha 2C4, and alpha 2C2 adrenergic receptors to Gi and Gs. *J Biol Chem* **267**:15795–15801.
- Eby JM, Abdelkarim H, Albee LJ, Tripathi A, Gao X, Volkman BF, Gaponenko V, and Majetschak M (2017) Functional and structural consequences of chemokine (C-X-C motif) receptor 4 activation with cognate and non-cognate agonists. *Mol Cell Biochem* **434**:143–151.
- Edelstein SJ and Changeux JP (2016) Biased allosteric. *Biophys J* **111**:902–908.
- Ehlert FJ (2008) On the analysis of ligand-directed signaling at G protein-coupled receptors. *Naunyn Schmiedeberg Arch Pharmacol* **377**:549–577.
- Ehlert FJ (2005) Analysis of allosterism in functional assays. *J Pharmacol Exp Ther* **315**:740–754.
- Elste AP and Petersen I (2010) Expression of proteinase-activated receptor 1-4 (PAR 1-4) in human cancer. *J Mol Histol* **41**:89–99.
- Erickson CE, Gul R, Blessing CP, Nguyen J, Liu T, Pulakat L, Bastepe M, Jackson EK, and Andresen BT (2013) The β -blocker nebivolol is a GRK/ β -arrestin biased agonist. *PLoS One* **8**:e71980.
- Eschenhagen T, Mende U, Nose M, Schmitz W, Scholz H, Haverich A, Hirt S, Döring V, Kalmár P, Höppner W, et al. (1992) Increased messenger RNA level of the inhibitory G protein alpha subunit Gi alpha-2 in human end-stage heart failure. *Circ Res* **70**:688–696.
- Evans BA, Broxton N, Merlin J, Sato M, Hutchinson DS, Christopoulos A, and Summers RJ (2011) Quantification of functional selectivity at the human α 1A-adrenoceptor. *Mol Pharmacol* **79**:298–307.
- Evans BA, Sato M, Sarwar M, Hutchinson DS, and Summers RJ (2010) Ligand-directed signalling at β -adrenoceptors. *Br J Pharmacol* **159**:1022–1038.
- Evron T, Peterson SM, Urs NM, Bai Y, Rochelle LK, Caron MG, and Barak LS (2014) G Protein and β -arrestin signaling bias at the ghrelin receptor. *J Biol Chem* **289**: 33442–33455.

- Faget L, Erbs E, Le Merrer J, Scherrer G, Matifas A, Benturquia N, Noble F, Decossas M, Koch M, Kessler P, et al. (2012) In vivo visualization of delta opioid receptors upon physiological activation uncovers a distinct internalization profile. *J Neurosci* **32**:7301–7310.
- Fang Y and Ferrie AM (2008) Label-free optical biosensor for ligand-directed functional selectivity acting on beta(2) adrenoceptor in living cells. *FEBS Lett* **582**: 558–564.
- Feinstein TN, Wehbi VL, Ardura JA, Wheeler DS, Ferrandon S, Gardella TJ, and Vilardaga JP (2011) Retromer terminates the generation of cAMP by internalized PTH receptors. *Nat Chem Biol* **7**:278–284.
- Feinstein TN, Yui N, Webber MJ, Wehbi VL, Stevenson HP, King JD Jr, Hallows KR, Brown D, Bouley R, and Vilardaga JP (2013) Noncanonical control of vasopressin receptor type 2 signaling by retromer and arrestin. *J Biol Chem* **288**:27849–27860.
- Felker GM, Butler J, Collins SP, Cotter G, Davison BA, Ezekowitz JA, Filippatos G, Levy PD, Metra M, Ponikowski P, et al. (2015) Heart failure therapeutics on the basis of a biased ligand of the angiotensin-2 type 1 receptor. Rationale and design of the BLAST-AHF study (Biased Ligand of the Angiotensin Receptor Study in Acute Heart Failure). *JACC Heart Fail* **3**:193–201.
- Ferguson SS (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* **53**:1–24.
- Ferguson SS, Downey WE 3rd, Colapietro AM, Barak LS, Ménard L, and Caron MG (1996) Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science* **271**:363–366.
- Ferrandon S, Feinstein TN, Castro M, Wang B, Bouley R, Potts JT, Gardella TJ, and Vilardaga JP (2009) Sustained cyclic AMP production by parathyroid hormone receptor endocytosis. *Nat Chem Biol* **5**:734–742.
- Ferrari F, Malfacini D, Journigan BV, Bird MF, Trapella C, Guerrini R, Lambert DG, Calo G, and Zaveri NT (2017) In vitro pharmacological characterization of a novel unbiased NOP receptor-selective nonpeptide agonist AT-403. *Pharmacol Res Perspect* **5**:e00333.
- Ferrari SL, Pierroz DD, Glatt V, Goddard DS, Bianchi EN, Lin FT, Manen D, and Boussein ML (2005) Bone response to intermittent parathyroid hormone is altered in mice null for β -arrestin2. *Endocrinology* **146**:1854–1862.
- Ferrie AM, Sun H, and Fang Y (2011) Label-free integrative pharmacology on-target of drugs at the $\beta(2)$ -adrenergic receptor. *Sci Rep* **1**:33.
- Fiorentini C, Busi C, Gorruso E, Gotti C, Spano P, and Missale C (2008) Receptor regulation of dopamine D1 and D3 receptor function and trafficking by heterodimerization. *Mol Pharmacol* **74**:59–69.
- Fisher A, Heldman E, Gurwitz D, Haring R, Barak D, Meshulam H, Marciano D, Brandeis R, Pittel Z, Segal M, et al. (1993) Selective signaling via unique M1 muscarinic agonists. *Ann N Y Acad Sci* **695**:300–303.
- Follin-Arbelet V, Torgersen ML, Naderi EH, Misud K, Sundan A, and Blomhoff HK (2013) Death of multiple myeloma cells induced by cAMP-signaling involves downregulation of Mcl-1 via the JAK/STAT pathway. *Cancer Lett* **335**:323–331.
- Francke F, Ward RJ, Jenkins L, Kellett E, Richter D, Milligan G, and Bächner D (2006) Interaction of neurochondrin with the melanin-concentrating hormone receptor 1 interferes with G protein-coupled signal transduction but not agonist-mediated internalization. *J Biol Chem* **281**:32496–32507.
- Frauenfelder H, Petsko GA, and Tsernoglou D (1979) Temperature-dependent X-ray diffraction as a probe of protein structural dynamics. *Nature* **280**:558–563.
- Free RB, Chun LS, Moritz AE, Miller BN, Doyle TB, Conroy JL, Padron A, Meade JA, Xiao J, Hu X, et al. (2014) Discovery and characterization of a G protein-biased agonist that inhibits β -arrestin recruitment to the D2 dopamine receptor. *Mol Pharmacol* **86**:96–105.
- Furchgott RF (1972) The classification of adrenoceptors (adrenergic receptors): an evaluation from the standpoint of receptor theory, in *Catecholamines* (Blaschko H and Muscholl E eds) vol 33, pp 283–335, Springer-Verlag, Berlin.
- Furness SGB, Liang YL, Nowell CJ, Halls ML, Wookley PJ, Dal Maso E, Inoue A, Christopoulos A, Wootton D, and Sexton PM (2016) Ligand-dependent modulation of G protein conformation alters drug efficacy. *Cell* **167**:739–749.
- Galandrin S and Bouvier M (2006) Distinct signaling profiles of beta1 and beta2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol* **70**:1575–1584.
- Galandrin S, Denis C, Boularan C, Marie J, MKadmi C, Pilette C, Dubroca C, Nicaise Y, Seguelas MH, N'Guyen D, et al. (2016a) Cardioprotective angiotensin-(1-7) peptide acts as a natural-biased ligand at the angiotensin II type 1 receptor. *Hypertension* **68**:1365–1374.
- Galandrin S, Oligny-Longpré G, and Bouvier M (2007) The evasive nature of drug efficacy: implications for drug discovery. *Trends Pharmacol Sci* **28**:423–430.
- Galandrin S, Onfroy L, Poirat MC, Sénard J-M, and Galés C (2016b) Delineating biased ligand efficacy at 7TM receptors from an experimental perspective. *Int J Biochem Cell Biol* **77**:251–263.
- Galés C, Rebois RV, Hogue M, Trieu P, Breit A, Hébert TE, and Bouvier M (2005) Real-time monitoring of receptor and G-protein interactions in living cells. *Nat Methods* **2**:177–184.
- Gao H, Sun Y, Wu Y, Luan B, Wang Y, Qu B, and Pei G (2004) Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways. *Mol Cell* **14**:303–317.
- Gautam N, Downes GB, Yan K, and Kisselev O (1998) The G-protein betagamma complex. *Cell Signal* **10**:447–455.
- Gazi L, Nickolls SA, and Strange PG (2003) Functional coupling of the human dopamine D2 receptor with G alpha i1, G alpha i2, G alpha i3 and G alpha o G proteins: evidence for agonist regulation of G protein selectivity. *Br J Pharmacol* **138**:775–786.
- Geiger T, Wehner A, Schaab C, Cox J, and Mann M (2012) Comparative proteomic analysis of eleven common cell lines reveals ubiquitous but varying expression of most proteins. *Mol Cell Proteomics* **11**:M111.014050.
- Geppetti P, Veldhuis NA, Lieu T, and Bunnett NW (2015) G protein-coupled receptors: dynamic machines for signaling pain and itch. *Neuron* **88**:635–649.
- Gesty-Palmer D, Flannery P, Yuan L, Corsino L, Spurney R, Lefkowitz RJ, and Luttrell LM (2009) A beta-arrestin-biased agonist of the parathyroid hormone receptor (PTH1R) promotes bone formation independent of G protein activation. *Sci Transl Med* **1**:1ra1.
- Gesty-Palmer D and Luttrell LM (2011) 'Biasing' the parathyroid hormone receptor: a novel anabolic approach to increasing bone mass? *Br J Pharmacol* **164**:59–67.
- Gesty-Palmer D, Yuan L, Martin B, Wood WH 3rd, Lee MH, Janech MG, Tsoi LC, Zheng WJ, Luttrell LM, and Maudsley S (2013) β -arrestin-selective G protein-coupled receptor agonists engender unique biological efficacy in vivo. *Mol Endocrinol* **27**:296–314.
- Ghanouni P, Gryczynski Z, Steenhuis JJ, Lee TW, Farrens DL, Lakowicz JR, and Kobilka BK (2001) Functionally different agonists induce distinct conformations in the G protein coupling domain of the beta 2 adrenergic receptor. *J Biol Chem* **276**:24433–24436.
- Gidon A, Al-Bataineh MM, Jean-Alphonse FG, Stevenson HP, Watanabe T, Louet C, Khatri A, Calero G, Pastor-Soler NM, Gardella TJ, et al. (2014) Endosomal GPCR signaling turned off by negative feedback actions of PKA and v-ATPase. *Nat Chem Biol* **10**:707–709.
- Giguere PM, Kroeze WK, and Roth BL (2014) Tuning up the right signal: chemical and genetic approaches to study GPCR functions. *Curr Opin Cell Biol* **27**:51–55.
- Glukhova A, Thal DM, Nguyen AT, Vecchio EA, Jörg M, Scammells PJ, May LT, Sexton PM, and Christopoulos A (2017) Structure of the adenosine A1 receptor reveals the basis for subtype selectivity. *Cell* **168**:867–877.e13.
- Gomes I, Ayoub MA, Fujita W, Jaeger WC, Pfleger KDG, and Devi LA (2016) G protein-coupled receptor heteromers. *Annu Rev Pharmacol Toxicol* **56**:403–425.
- Gong K, Li Z, Xu M, Du J, Lv Z, and Zhang Y (2008) A novel protein kinase A-independent, β -arrestin-1-dependent signaling pathway for p38 mitogen-activated protein kinase activation by β 2-adrenergic receptors. *J Biol Chem* **283**: 29028–29036.
- González-Maeso J, Weisstaub NV, Zhou M, Chan P, Ivic L, Ang R, Lira A, Bradley-Moore M, Ge Y, Zhou Q, et al. (2007) Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling pathways to affect behavior. *Neuron* **53**: 439–452.
- Goodman OB Jr, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, and Benovic JL (1996) Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature* **383**:447–450.
- Gregory KJ, Hall NE, Tobin AB, Sexton PM, and Christopoulos A (2010) Identification of orthosteric and allosteric site mutations in M2 muscarinic acetylcholine receptors that contribute to ligand-selective signaling bias. *J Biol Chem* **285**:7459–7474.
- Grotegut CA, Feng L, Mao L, Heine RP, Murtha AP, and Rockman HA (2011) β -arrestin mediates oxytocin receptor signaling, which regulates uterine contractility and cellular migration. *Am J Physiol Endocrinol Metab* **300**:E468–E477.
- Grundmann M and Kostenis E (2017) Temporal bias: time-encoded dynamic GPCR signaling. *Trends Pharmacol Sci* **38**:1110–1124.
- Gupta A, Gomes I, Bobeck EN, Fakira AK, Massaro NP, Sharma I, Cavé A, Hamm HE, Parelo J, and Devi LA (2016) Collybolide is a novel biased agonist of κ -opioid receptors with potent antipruritic activity. *Proc Natl Acad Sci USA* **113**:6041–6046.
- Gurevich VV and Gurevich EV (2006) The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. *Pharmacol Ther* **110**:465–502.
- Gurevich VV and Gurevich EV (2018) GPCRs and signal transducers: interaction stoichiometry. *Trends Pharmacol Sci* **39**:672–684.
- Gurevich VV, Gurevich EV, and Uversky VN (2018) Arrestins: structural disorder creates rich functionality. *Protein Cell* **9**:986–1003.
- Gurwitz D, Haring R, Heldman E, Fraser CM, Manor D, and Fisher A (1994) Discrete activation of transduction pathways associated with acetylcholine m1 receptor by several muscarinic ligands. *Eur J Pharmacol* **267**:21–31.
- Hager MV, Johnson LM, Wootton D, Sexton PM, and Gellman SH (2016) β -arrestin-biased agonists of the GLP-1 receptor from β -amino acid residue incorporation into GLP-1 analogues. *J Am Chem Soc* **138**:14970–14979.
- Halls ML, Hewitson TD, Moore X-L, Du X-J, Bathgate RAD, and Summers RJ (2009) Relaxin activates multiple cAMP signaling pathway profiles in different target cells. *Ann N Y Acad Sci* **1160**:108–111.
- Haney S and Hancox RJ (2006) Recovery from bronchoconstriction and bronchodilator tolerance. *Clin Rev Allergy Immunol* **31**:181–196.
- Hanyaloglu AC and von Zastrow M (2008) Regulation of GPCRs by endocytic membrane trafficking and its potential implications. *Annu Rev Pharmacol Toxicol* **48**: 537–568.
- Hauser MA and Legler DF (2016) Common and biased signaling pathways of the chemokine receptor CCR7 elicited by its ligands CCL19 and CCL21 in leukocytes. *J Leukoc Biol* **99**:869–882.
- Hay DL, Gareja ML, Poyner DR, and Walker CS (2018) Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review 25. *Br J Pharmacol* **175**: 3–17.
- Hein P, Rochais F, Hoffmann C, Dorsch S, Nikolaev VO, Engelhardt S, Berlot CH, Lohse MJ, and Bütemann M (2006) Gs activation is time-limiting in initiating receptor-mediated signaling. *J Biol Chem* **281**:33345–33351.
- Heldman E, Barg J, Fisher A, Levy R, Pittel Z, Zimlichman R, Kushnir M, and Vogel Z (1996) Pharmacological basis for functional selectivity of partial muscarinic receptor agonists. *Eur J Pharmacol* **297**:283–291.
- Hermans E (2003) Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacol Ther* **99**:25–44.
- Hernanz-Falcón P, Rodríguez-Frade JM, Serrano A, Juan D, del Sol A, Soriano SF, Roncal F, Gómez L, Valencia A, Martínez-A C, et al. (2004) Identification of amino acid residues crucial for chemokine receptor dimerization. *Nat Immunol* **5**: 216–223.
- Hill R, Disney A, Conibear A, Sutcliffe K, Dewey W, Husbands S, Bailey C, Kelly E, and Henderson G (2018) The novel μ -opioid receptor agonist PZM21 depresses respiration and induces tolerance to antinociception. *Br J Pharmacol* **175**:2653–2661.
- Hoffmann C, Ziegler N, Reiner S, Krasel C, and Lohse MJ (2008) Agonist-selective, receptor-specific interaction of human P2Y receptors with β -arrestin-1 and -2. *J Biol Chem* **283**:30933–30941.
- Hothersall JD, Torella R, Humphreys S, Hooley M, Brown A, McMurray G, and Nickolls SA (2017) Residues W320 and Y328 within the binding site of the μ -opioid receptor influence opiate ligand bias. *Neuropharmacology* **118**:46–58.

- Hou T, Shi L, Wang J, Wei L, Qu L, Zhang X, and Liang X (2016) Label-free cell phenotypic profiling and pathway deconvolution of neurotensin receptor-1. *Pharmacol Res* **108**:39–45.
- Hsiao EC, Boudignon BM, Chang WC, Bencsik M, Peng J, Nguyen TD, Manalac C, Halloran BP, Conklin BR, and Nissenson RA (2008) Osteoblast expression of an engineered Gs-coupled receptor dramatically increases bone mass. *Proc Natl Acad Sci USA* **105**:1209–1214.
- Hu J, Stern M, Gimenez LE, Wanka L, Zhu L, Rossi M, Meister J, Inoue A, Beck-Sickingner AG, Gurevich VV, et al. (2016) A G protein-biased designer G protein-coupled receptor useful for studying the physiological relevance of Gq₁₁-dependent signaling pathways. *J Biol Chem* **291**:7809–7820.
- Huang XP, Setola V, Yadav PN, Allen JA, Rogan SC, Hanson BJ, Revankar C, Robers M, Doucette C, and Roth BL (2009) Parallel functional activity profiling reveals valvulopathogens are potent 5-hydroxytryptamine_{2B} receptor agonists: implications for drug safety assessment. *Mol Pharmacol* **76**:710–722.
- Hubbard KB and Hepler JR (2006) Cell signalling diversity of the Gq α family of heterotrimeric G proteins. *Cell Signal* **18**:135–150.
- Hudson BD, Christiansen E, Tikhonova IG, Grundmann M, Kostenis E, Adams DR, Ulven T, and Milligan G (2012) Chemically engineering ligand selectivity at the free fatty acid receptor 2 based on pharmacological variation between species orthologs. *FASEB J* **26**:4951–4965.
- Ichikawa O, Fujimoto K, Yamada A, Okazaki S, and Yamazaki K (2016) G-protein/ β -arrestin-linked fluctuating network of G-protein-coupled receptors for predicting drug efficacy and bias using short-term molecular dynamics simulation. *PLoS One* **11**:e0155816.
- Ikeda K, Kobayashi T, Kumanishi T, Yano R, Sora I, and Niki H (2002) Molecular mechanisms of analgesia induced by opioids and ethanol: is the GIRK channel one of the keys? *Neurosci Res* **44**:121–131.
- Inoue A, Ishiguro J, Kitamura H, Arima N, Okutani M, Shuto A, Higashiyama S, Ohwada T, Arai H, Makide K, et al. (2012) TGF α shedding assay: an accurate and versatile method for detecting GPCR activation. *Nat Methods* **9**:1021–1029.
- Insel PA, Wilderman A, Zambon AC, Snead AN, Murray F, Aroonsakool N, McDonald DS, Zhou S, McCann T, Zhang L, et al. (2015) G protein-coupled receptor (GPCR) expression in native cells: “novel” endoGPCRs as physiologic regulators and therapeutic targets. *Mol Pharmacol* **88**:181–187.
- Irannejad R, Tomshine JC, Tomshine JR, Chevalier M, Mahoney JP, Steyaert J, Rasmussen SG, Sunahara RK, El-Samad H, Huang B, et al. (2013) Conformational biosensors reveal GPCR signalling from endosomes. *Nature* **495**:534–538.
- Ishikawa Y, Sorota S, Kiuchi K, Shannon RP, Komamura K, Katsushika S, Vatner DE, Vatner SF, and Homcy CJ (1994) Downregulation of adenylyl cyclase types V and VI mRNA levels in pacing-induced heart failure in dogs. *J Clin Invest* **93**:2224–2229.
- Ito M, Oliverio MI, Mannon PJ, Best CF, Maeda N, Smithies O, and Coffman TM (1995) Regulation of blood pressure by the type 1A angiotensin II receptor gene. *Proc Natl Acad Sci USA* **92**:3521–3525.
- Jamshidi RJ, Sullivan LC, Jacobs BA, Chavera TA, Berg KA, and Clarke WP (2016) Long-term reduction of kappa opioid receptor function by the biased ligand, norbinaltorphimine, requires c-Jun N-terminal kinase activity and new protein synthesis in peripheral sensory neurons. *J Pharmacol Exp Ther* **359**:319–328.
- Japp AG, Cruden NL, Barnes G, van Gemenen N, Mathews J, Adamson J, Johnston NR, Denvir MA, Megson IL, Flapan AD, et al. (2010) Acute cardiovascular effects of apelin in humans: potential role in patients with chronic heart failure. *Circulation* **121**:1818–1827.
- Jensen DD, Lieu T, Halls ML, Veldhuis NA, Imlach WL, Mai QN, Poole DP, Quach T, Aurelio L, Conner J, et al. (2017) Neurokinin 1 receptor signaling in endosomes mediates sustained nociception and is a viable therapeutic target for prolonged pain relief. *Sci Transl Med* **9**:eaal3447.
- Jiang Y, Yau MK, Kok WM, Lim J, Wu KC, Liu L, Hill TA, Suen JY, and Fairlie DP (2017) Biased signaling by agonists of protease activated receptor 2. *ACS Chem Biol* **12**:1217–1226.
- Johnson TA, Milan-Lobo L, Che T, Ferwerda M, Lambu E, McIntosh NL, Li F, He L, Lorig-Roach N, Crews P, et al. (2017) Identification of the first marine-derived opioid receptor “balanced” agonist with a signaling profile that resembles the endorphins. *ACS Chem Neurosci* **8**:473–485.
- Jorgensen R, Kubale V, Vrecl M, Schwartz TW, and Elling CE (2007) Oxyntomodulin differentially affects glucagon-like peptide-1 receptor β -arrestin recruitment and signaling through Galphas. *J Pharmacol Exp Ther* **322**:148–154.
- Jozwiak K, Toll L, Jimenez L, Woo AY-H, Xiao R-P, and Wainer IW (2010) The effect of stereochemistry on the thermodynamic characteristics of the binding of fenoterol stereoisomers to the beta(2)-adrenoceptor. *Biochem Pharmacol* **79**:1610–1615.
- Just S, Illing S, Trester-Zedlitz M, Lau EK, Kotowski SJ, Miess E, Mann A, Doll C, Trinidad JC, Burlingame AL, et al. (2013) Differentiation of opioid drug effects by hierarchical multi-site phosphorylation. *Mol Pharmacol* **83**:633–639.
- Kaya AI, Onaran HO, Özcan G, Ambrosio C, Costa T, Balli S, and Ugur Ö (2012) Cell contact-dependent functional selectivity of β 2-adrenergic receptor ligands in stimulating cAMP accumulation and extracellular signal-regulated kinase phosphorylation. *J Biol Chem* **287**:6362–6374.
- Kebig A, Kostenis E, Mohr K, and Mohr-Andrä M (2009) An optical dynamic mass redistribution assay reveals biased signaling of dualistic GPCR activators. *J Recept Signal Transduct Res* **29**:140–145.
- Kelly E (2013) Efficacy and ligand bias at the μ -opioid receptor. *Br J Pharmacol* **169**:1430–1446.
- Kenakin T (1995) Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends Pharmacol Sci* **16**:232–238.
- Kenakin T (1997) Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol Sci* **18**:456–464.
- Kenakin T (2001) Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J* **15**:598–611.
- Kenakin T (2004) Efficacy as a vector: the relative prevalence and paucity of inverse agonism. *Mol Pharmacol* **65**:2–11.
- Kenakin T (2005) New concepts in drug discovery: collateral efficacy and permissive antagonism. *Nat Rev Drug Discov* **4**:919–927.
- Kenakin T (2011) Functional selectivity and biased receptor signaling. *J Pharmacol Exp Ther* **336**:296–302.
- Kenakin T (2012) Casting a wider net: whole-cell assays to capture varied and biased signaling. *Mol Pharmacol* **82**:571–574.
- Kenakin T (2013) New concepts in pharmacological efficacy at 7TM receptors: IUPHAR Review 2. *Br J Pharmacol* **168**:554–575.
- Kenakin T (2014) What is pharmacological “affinity”? Relevance to biased agonism and antagonism. *Trends Pharmacol Sci* **35**:434–441.
- Kenakin T (2015a) Gaddum Memorial Lecture 2014: receptors as an evolving concept: from switches to biased microprocessors. *Br J Pharmacol* **172**:4238–4253.
- Kenakin T (2015b) The effective application of biased signaling to new drug discovery. *Mol Pharmacol* **88**:1055–1061.
- Kenakin T (2015c) New lives for seven transmembrane receptors as drug targets. *Trends Pharmacol Sci* **36**:705–706.
- Kenakin T (2017) A scale of agonism and allosteric modulation for assessment of selectivity, bias, and receptor mutation [published correction appears in *Mol Pharmacol* (2017) 92:501]. *Mol Pharmacol* **92**:414–424.
- Kenakin T (2018) Is the quest for signaling bias worth the effort? *Mol Pharmacol* **93**:266–269.
- Kenakin T and Strachan RT (2018) PAM-antagonists: a better way to block pathological receptor signaling? *Trends Pharmacol Sci* **39**:748–765.
- Kenakin T, Watson C, Muniz-Medina V, Christopoulos A, and Novick S (2012) A simple method for quantifying functional selectivity and agonist bias. *ACS Chem Neurosci* **3**:193–203.
- Kenakin TP (1984) The relative contribution of affinity and efficacy to agonist activity: organ selectivity of noradrenaline and oxymetazoline with reference to the classification of drug receptors. *Br J Pharmacol* **81**:131–141.
- Kenakin TP (1985) Prenalterol as a selective cardiostimulant: differences between organ and receptor selectivity. *J Cardiovasc Pharmacol* **7**:208–210.
- Kenakin TP (2008) Pharmacological onomastics: what’s in a name? *Br J Pharmacol* **153**:432–438.
- Kenakin TP (2016) Synoptic pharmacology: detecting and assessing the pharmacological significance of ligands for orphan receptors. *Pharmacol Res* **114**:284–290.
- Kenakin TP, Ambrose JR, and Irving PE (1991) The relative efficiency of beta adrenoceptor coupling to myocardial inotropy and diastolic relaxation: organ-selective treatment for diastolic dysfunction. *J Pharmacol Exp Ther* **257**:1189–1197.
- Kenakin TP and Morgan PH (1989) Theoretical effects of single and multiple transducer receptor coupling proteins on estimates of the relative potency of agonists. *Mol Pharmacol* **35**:214–222.
- Khajehali E, Malone DT, Glass M, Sexton PM, Christopoulos A, and Leach K (2015) Biased agonism and biased allosteric modulation at the CB1 cannabinoid receptor. *Mol Pharmacol* **88**:368–379.
- Kim IM, Tilley DG, Chen J, Salazar NC, Whalen EJ, Violin JD, and Rockman HA (2008) β -blockers alprenolol and carvedilol stimulate β -arrestin-mediated EGFR transactivation. *Proc Natl Acad Sci USA* **105**:14555–14560.
- Kim IM, Wang Y, Park KM, Tang Y, Teoh JP, Vinson J, Traynham CJ, Pironti G, Mao L, Su H, et al. (2014) β -arrestin1-biased β 1-adrenergic receptor signaling regulates microRNA processing. *Circ Res* **114**:833–844.
- Kim J, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei H, and Lefkowitz RJ (2005) Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling. *Proc Natl Acad Sci USA* **102**:1442–1447.
- Klein Herenbrink C, Sykes DA, Donthamsetti P, Canals M, Coudrat T, Shonberg J, Scammells PJ, Capuano B, Sexton PM, Charlton SJ, et al. (2016) The role of kinetic context in apparent biased agonism at GPCRs. *Nat Commun* **7**:10842.
- Koblish M, Carr R 3rd, Siuda ER, Rominger DH, Gowen-MacDonald W, Cowan CL, Crombie AL, Violin JD, and Lark MW (2017) TRV0109101, a G protein-biased agonist of the μ -opioid receptor, does not promote opioid-induced mechanical allodynia following chronic administration. *J Pharmacol Exp Ther* **362**:254–262.
- Kofuku Y, Ueda T, Okude J, Shiraiishi Y, Kondo K, Maeda M, Tsujishita H, and Shimada I (2012) Efficacy of the β 2-adrenergic receptor is determined by conformational equilibrium in the transmembrane region. *Nat Commun* **3**:1045.
- Kohout TA and Lefkowitz RJ (2003) Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Mol Pharmacol* **63**:9–18.
- Kohout TA, Lin FS, Perry SJ, Conner DA, and Lefkowitz RJ (2001) Beta-arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. *Proc Natl Acad Sci USA* **98**:1601–1606.
- Kohout TA, Nicholas SL, Perry SJ, Reinhart G, Junger S, and Struthers RS (2004) Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. *J Biol Chem* **279**:23214–23222.
- Kong KC, Butcher AJ, McWilliams P, Jones D, Wess J, Hamdan FF, Werry T, Rosethorne EM, Charlton SJ, Munson SE, et al. (2010) M3-muscarinic receptor promotes insulin release via receptor phosphorylation/arrestin-dependent activation of protein kinase D1. *Proc Natl Acad Sci USA* **107**:21181–21186.
- Konkar AA, Zhu Z, and Granneman JG (2000) Aryloxypropanolamine and catecholamine ligand interactions with the β (1)-adrenergic receptor: evidence for interaction with distinct conformations of β (1)-adrenergic receptors. *J Pharmacol Exp Ther* **294**:923–932.
- Koole C, Savage EE, Christopoulos A, Miller LJ, Sexton PM, and Wootten D (2013) Minireview: signal bias, allostereism, and polymorphic variation at the GLP-1R: implications for drug discovery. *Mol Endocrinol* **27**:1234–1244.
- Koole C, Wootten D, Simms J, Valant C, Sridhar R, Woodman OL, Miller LJ, Summers RJ, Christopoulos A, and Sexton PM (2010) Allosteric ligands of the glucagon-like peptide 1 receptor (GLP-1R) differentially modulate endogenous and exogenous peptide responses in a pathway-selective manner: implications for drug screening. *Mol Pharmacol* **78**:456–465.
- Kotowski SJ, Hopf FW, Seif T, Bonci A, and von Zastrow M (2011) Endocytosis promotes rapid dopaminergic signaling. *Neuron* **71**:278–290.

- Kroeze WK, Sassano MF, Huang XP, Lansu K, McCorvy JD, Giguère PM, Sciaky N, and Roth BL (2015) PRESTO-Tango as an open-source resource for interrogation of the druggable human GPCRome. *Nat Struct Mol Biol* **22**:362–369.
- Kruse AC, Ring AM, Manglik A, Hu J, Hu K, Eitel K, Hübner H, Pardon E, Valant C, Sexton PM, et al. (2013) Activation and allosteric modulation of a muscarinic acetylcholine receptor. *Nature* **504**:101–106.
- Kuhar JR, Bedini A, Melief EJ, Chiu YC, Striegel HN, and Chavkin C (2015) Mu opioid receptor stimulation activates c-Jun N-terminal kinase 2 by distinct arrestin-dependent and independent mechanisms. *Cell Signal* **27**:1799–1806.
- Kühn H, Hall SW, and Wilden U (1984) Light-induced binding of 48-kDa protein to photoreceptor membranes is highly enhanced by phosphorylation of rhodopsin. *FEBS Lett* **176**:473–478.
- Kumari P, Ghosh E, and Shukla AK (2015) Emerging approaches to GPCR ligand screening for drug discovery. *Trends Mol Med* **21**:687–701.
- Kuna RS, Girada SB, Asalla S, Vallentyne J, Maddika S, Patterson JT, Smiley DL, DiMarchi RD, and Mitra P (2013) Glucagon-like peptide-1 receptor-mediated endosomal cAMP generation promotes glucose-stimulated insulin secretion in pancreatic β -cells. *Am J Physiol Endocrinol Metab* **305**:E161–E170.
- Kurko D, Kapui Z, Nagy J, Lendvai B, and Kolok S (2014) Analysis of functional selectivity through G protein-dependent and -independent signaling pathways at the adrenergic $\alpha(2C)$ receptor. *Brain Res Bull* **107**:89–101.
- Laporte SA, Oakley RH, Zhang J, Holt JA, Ferguson SS, Caron MG, and Barak LS (1999) The beta2-adrenergic receptor/beta-arrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc Natl Acad Sci USA* **96**:3712–3717.
- Laprairie RB, Bagher AM, and Denovan-Wright EM (2017) Cannabinoid receptor ligand bias: implications in the central nervous system. *Curr Opin Pharmacol* **32**:32–43.
- Lawler CO, Watts VJ, Booth RG, Southerland SB, and Mailman RB (1994) Discrete functional selectivity of drugs: OPC-14597, a selective antagonist for post-synaptic dopamine D2 receptors (Abstract). *Soc Neurosci Abstr* **20**:525.
- Lawler CP, Prioleau C, Lewis MM, Mak C, Jiang D, Schetz JA, Gonzalez AM, Sibley DR, and Mailman RB (1999) Interactions of the novel antipsychotic aripiprazole (OPC-14597) with dopamine and serotonin receptor subtypes. *Neuropsychopharmacology* **20**:612–627.
- Le Rouzic V, Narayan A, Hunkle A, Marrone GF, Lu Z, Majumdar S, Xu J, Pan YX, and Pasternak GW (2019) Pharmacological characterization of levorphanol, a G-protein biased opioid analgesic. *Anesth Analg* **128**:365–373.
- Leach K, Conigrave AD, Sexton PM, and Christopoulos A (2015) Towards tissue-specific pharmacology: insights from the calcium-sensing receptor as a paradigm for GPCR (patho)physiological bias. *Trends Pharmacol Sci* **36**:215–225.
- Lee MH, Appleton KM, Strungs EG, Kwon JY, Morinelli TA, Peterson YK, Laporte SA, and Luttrell LM (2016) The conformational signature of β -arrestin2 predicts its trafficking and signalling functions. *Nature* **531**:665–668.
- Lee Y, Basith S, and Choi S (2018) Recent advances in structure-based drug design targeting class A G protein-coupled receptors utilizing crystal structures and computational simulations. *J Med Chem* **61**:1–46.
- Lefkowitz RJ (1998) G protein-coupled receptors. III. New roles for receptor kinases and β -arrestins in receptor signaling and desensitization. *J Biol Chem* **273**:18677–18680.
- Lefkowitz RJ and Shenoy SK (2005) Transduction of receptor signals by β -arrestins. *Science* **308**:512–517.
- Lefkowitz RJ and Whalen EJ (2004) β -arrestins: traffic cops of cell signaling. *Curr Opin Cell Biol* **16**:162–168.
- Lensing CJ, Freeman KT, Schnell SM, Speth RC, Zarth AT, and Haskell-Luevano C (2019) Developing a biased unmatched bivalent ligand (BUMBUL) design strategy to target the GPCR homodimer allosteric signaling (cAMP over β -arrestin 2 recruitment) within the melanocortin receptors. *J Med Chem* **62**:144–158.
- Levitz J, Pantoja C, Gaub B, Janovjak H, Reiner A, Hoagland A, Schoppik D, Kane B, Stawski P, Schier AF, et al. (2013) Optical control of metabotropic glutamate receptors. *Nat Neurosci* **16**:507–516.
- Li A, Yang D, Zhu M, Tsai KC, Xiao KH, Yu X, Sun J, and Du L (2015) Discovery of novel FFA4 (GPR120) receptor agonists with β -arrestin2-biased characteristics. *Future Med Chem* **7**:2429–2437.
- Li H, Tao Y, Ma L, Liu X, and Ma L (2013) β -arrestin-2 inhibits preference for alcohol in mice and suppresses Akt signaling in the dorsal striatum. *Neurosci Bull* **29**:531–540.
- Li Y, Liu X, Liu C, Kang J, Yang J, Pei G, and Wu C (2009) Improvement of morphine-mediated analgesia by inhibition of β -arrestin2 expression in mice periaqueductal gray matter. *Int J Mol Sci* **10**:954–963.
- Liapakis G, Cordomi A, and Pardo L (2012) The G-protein coupled receptor family: actors with many faces [published correction appears in *Curr Pharm Des* (2012) 18:4583]. *Curr Pharm Des* **18**:175–185.
- Liao Y, Lu B, Ma Q, Wu G, Lai X, Zang J, Shi Y, Liu D, Han F, and Zhou N (2016) Human neuropeptide S receptor is activated via a G_{aq} protein-biased signaling cascade by a human neuropeptide S analog lacking the C-terminal 10 residues. *J Biol Chem* **291**:7505–7516.
- Lin Y and Smrcka AV (2011) Understanding molecular recognition by G protein $\beta\gamma$ subunits on the path to pharmacological targeting. *Mol Pharmacol* **80**:551–557.
- Lin YC, Boone M, Meuris L, Lemmens I, Van Roy N, Soete A, Reumers J, Moisse M, Plaisance S, Drmanac R, et al. (2014) Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. *Nat Commun* **5**:4767.
- Link RE, Desai K, Hein L, Stevens ME, Chruscinski A, Bernstein D, Barsh GS, and Kobilka BK (1996) Cardiovascular regulation in mice lacking $\alpha(2A)$ -adrenergic receptor subtypes b and c. *Science* **273**:803–805.
- Liu JJ, Horst R, Katritch V, Stevens RC, and Wüthrich K (2012) Biased signaling pathways in $\beta(2)$ -adrenergic receptor characterized by 19F-NMR. *Science* **335**:1106–1110.
- Lohse MJ, Benovic JL, Codina J, Caron MG, and Lefkowitz RJ (1990) Beta-arrestin: a protein that regulates beta-adrenergic receptor function. *Science* **248**:1547–1550.
- Lohse MJ, Nuber S, and Hoffmann C (2012) Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein-coupled receptor activation and signaling. *Pharmacol Rev* **64**:299–336.
- Longabaugh JP, Vatner DE, Vatner SF, and Homcy CJ (1988) Decreased stimulatory guanosine triphosphate binding protein in dogs with pressure-overload left ventricular failure. *J Clin Invest* **81**:420–424.
- Luttrell LM (2008) Reviews in molecular biology and biotechnology: transmembrane signaling by G protein-coupled receptors. *Mol Biotechnol* **39**:239–264.
- Luttrell LM (2014) Minireview: more than just a hammer: ligand “bias” and pharmaceutical discovery. *Mol Endocrinol* **28**:281–294.
- Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, et al. (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* **283**:655–661.
- Luttrell LM and Gesty-Palmer D (2010) Beyond desensitization: physiological relevance of arrestin-dependent signaling. *Pharmacol Rev* **62**:305–330.
- Luttrell LM and Kenakin TP (2011) Refining efficacy: allosterism and bias in G protein-coupled receptor signaling. *Methods Mol Biol* **756**:3–35.
- Luttrell LM, Maudsley S, and Gesty-Palmer D (2018) Translating in vitro ligand bias into in vivo efficacy. *Cell Signal* **41**:46–55.
- Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL, and Lefkowitz RJ (2001) Activation and targeting of extracellular signal-regulated kinases by β -arrestin scaffolds. *Proc Natl Acad Sci USA* **98**:2449–2454.
- MKadmi C, Leyris JP, Onfroy L, Galès C, Saulière A, Gagne D, Damian M, Mary S, Maingot M, Denoyelle S, et al. (2015) Agonism, antagonism, and inverse agonism bias at the ghrelin receptor signaling. *J Biol Chem* **290**:27021–27039.
- Mack M, Luckow B, Nelson PJ, Cihak J, Simmons G, Clapham PR, Signoret N, Marsh M, Stangassinger M, Borlat F, et al. (1998) Aminoxy-pentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. *J Exp Med* **187**:1215–1224.
- Mackay D and Van Rossum JM (1977) A critical survey of receptor theories of drug action, in *Kinetics of Drug Action* (Van Rossum JM ed) pp 255–322, Springer-Verlag, Berlin.
- MacMillan LB, Hein L, Smith MS, Piascik MT, and Limbird LE (1996) Central hypotensive effects of the $\alpha(2A)$ -adrenergic receptor subtype. *Science* **273**:801–803.
- Maguire JJ (2016) Evidence for biased agonists and antagonists at the endothelin receptors. *Life Sci* **159**:30–33.
- Mahon MJ, Donowitz M, Yun CC, and Segre GV (2002) Na⁺/H⁺ exchanger regulatory factor 2 directs parathyroid hormone 1 receptor signalling. *Nature* **417**:858–861.
- Mahon MJ and Segre GV (2004) Stimulation by parathyroid hormone of a NHERF-1-assembled complex consisting of the parathyroid hormone 1 receptor, phospholipase C β eta, and actin increases intracellular calcium in opossum kidney cells. *J Biol Chem* **279**:23550–23558.
- Maillet EL, Pellegrini N, Valant C, Bucher B, Hibert M, Bourguignon JJ, and Galzi JL (2007) A novel, conformation-specific allosteric inhibitor of the tachykinin NK2 receptor (NK2R) with functionally selective properties. *FASEB J* **21**:2124–2134.
- Mailman RB, Lawler CP, Lewis MM, and Blake B (1998) Functional effects of novel dopamine ligands: dihydrexidine and Parkinson's disease as a first step, in *Dopamine Receptor Subtypes: From Basic Science to Clinical Application* (Jenner P and Demirdamar R eds) pp 112–119, IOS Press, Amsterdam.
- Mailman RB and Murthy V (2010) Third generation antipsychotic drugs: partial agonism or receptor functional selectivity? *Curr Pharm Des* **16**:488–501.
- Manglik A, Kim TH, Masurel M, Altenbach C, Yang Z, Hilger D, Lerch MT, Kobilka TS, Thian FS, Hubbell WL, et al. (2015) Structural insights into the dynamic process of $\beta(2)$ -adrenergic receptor signaling [published correction appears in *Cell* (2015) 162:1431]. *Cell* **161**:1101–1111.
- Manglik A and Kobilka B (2014) The role of protein dynamics in GPCR function: insights from the $\beta(2)$ AR and rhodopsin. *Curr Opin Cell Biol* **27**:136–143.
- Manglik A, Lin H, Aryal DK, McCorvy JD, Dengler D, Corder G, Levit A, Kling RC, Bernat V, Hübner H, et al. (2016) Structure-based discovery of opioid analgesics with reduced side effects. *Nature* **537**:185–190.
- Maning J, Negussie S, Clark MA, and Lymperopoulos A (2017) Biased agonism/antagonism at the AngII-AT1 receptor: implications for adrenal aldosterone production and cardiovascular therapy. *Pharmacol Rev* **125**:14–20.
- Masri B, Salahpour A, Didriksen M, Ghisi V, Beaulieu J-M, Gainetdinov RR, and Caron MG (2008) Antagonism of dopamine D2 receptor/beta-arrestin 2 interaction is a common property of clinically effective antipsychotics. *Proc Natl Acad Sci USA* **105**:13656–13661.
- Masuho I, Ostrovskaya O, Kramer GM, Jones CD, Xie K, and Martemyanov KA (2015) Distinct profiles of functional discrimination among G proteins determine the actions of G protein-coupled receptors. *Sci Signal* **8**:ra123.
- Mathiesen JM, Ulven T, Martini L, Gerlach LO, Heinemann A, and Kostenis E (2005) Identification of indole derivatives exclusively interfering with a G protein-independent signaling pathway of the prostaglandin D2 receptor CRTH2. *Mol Pharmacol* **68**:393–402.
- Maudsley S, Chadwick W, Wang L, Zhou Y, Martin B, and Park SS (2011) Bioinformatic approaches to metabolic pathways analysis. *Methods Mol Biol* **756**:99–130.
- Maudsley S, Martin B, Gesty-Palmer D, Cheung H, Johnson C, Patel S, Becker KG, Wood WH 3rd, Zhang Y, Lehmann E, et al. (2015) Delineation of a conserved arrestin-biased signaling repertoire in vivo. *Mol Pharmacol* **87**:706–717.
- Maudsley S, Martin B, Janssens J, Etienne H, Jushaj A, van Gastel J, Willemsen A, Chen H, Gesty-Palmer D, and Luttrell LM (2016) Informatic deconvolution of biased GPCR signaling mechanisms from in vivo pharmacological experimentation. *Methods* **92**:51–63.
- Maudsley S, Patel SA, Park SS, Luttrell LM, and Martin B (2012) Functional signaling biases in G protein-coupled receptors: game theory and receptor dynamics. *Mini Rev Med Chem* **12**:831–840.
- Maudsley S, Siddiqui S, and Martin B (2013) Systems analysis of arrestin pathway functions. *Prog Mol Biol Transl Sci* **118**:431–467.
- May V and Parsons RL (2017) G protein-coupled receptor endosomal signaling and regulation of neuronal excitability and stress responses: signaling options and lessons from the PAC1 receptor. *J Cell Physiol* **232**:698–706.

- McAnally D, Siddiquee K, Sharir H, Qi F, Phatak S, Li JL, Berg E, Fishman J, and Smith L (2017) A systematic approach to identify biased agonists of the apelin receptor through high-throughput screening. *SLAS Discov* **22**:867–878.
- McCorvey JD, Butler KV, Kelly B, Rechsteiner K, Karpiak J, Betz RM, Kormos BL, Shoichet BK, Dror RO, Jin J, et al. (2018) Structure-inspired design of β -arrestin-biased ligands for aminergic GPCRs. *Nat Chem Biol* **14**:126–134.
- McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin F-T, Davis RJ, and Lefkowitz RJ (2000) Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* **290**:1574–1577.
- McLaughlin JN, Shen L, Holinstat M, Brooks JD, Dibenedetto E, and Hamm HE (2005) Functional selectivity of G protein signaling by agonist peptides and thrombin for the protease-activated receptor-1. *J Biol Chem* **280**:25048–25059.
- Melkes B, Hejnova L, and Novotny J (2016) Biased μ -opioid receptor agonists diversely regulate lateral mobility and functional coupling of the receptor to its cognate G proteins. *Naunyn Schmiedebergs Arch Pharmacol* **389**:1289–1300.
- Merighi S, Borea PA, and Gessi S (2015) Adenosine receptors and diabetes: focus on the A(2B) adenosine receptor subtype. *Pharmacol Res* **99**:229–236.
- Merriam LA, Baran CN, Girard BM, Hardwick JC, May V, and Parsons RL (2013) Pituitary adenylate cyclase 1 receptor internalization and endosomal signaling mediate the pituitary adenylate cyclase activating polypeptide-induced increase in guinea pig cardiac neuron excitability. *J Neurosci* **33**:4614–4622.
- Michal P, El-Fakahany EE, and Dolezal V (2007) Muscarinic M2 receptors directly activate Gq/11 and Gs G-proteins. *J Pharmacol Exp Ther* **320**:607–614.
- Milanos L, Brox R, Frank T, Poklukur G, Palmisano R, Waibel R, Einsiedel J, Dürr M, Ivanović-Burmazović I, Larsen O, et al. (2016a) Discovery and characterization of biased allosteric agonists of the chemokine receptor CXCR3. *J Med Chem* **59**:2222–2243.
- Milanos L, Saleh N, Kling RC, Kaindl J, Tschammer N, and Clark T (2016b) Identification of two distinct sites for antagonist and biased agonist binding to the human chemokine receptor CXCR3. *Angew Chem Int Ed Engl* **55**:15277–15281.
- Miyawaki A and Niino Y (2015) Molecular spies for bioimaging—fluorescent protein-based probes. *Mol Cell* **58**:632–643.
- Mokrosiński J, Frimurer TM, Sivertsen B, Schwartz TW, and Holst B (2012) Modulation of constitutive activity and signaling bias of the ghrelin receptor by conformational constraint in the second extracellular loop. *J Biol Chem* **287**:33488–33502.
- Molden BM, Cooney KA, West K, Van Der Ploeg LHT, and Baldini G (2015) Temporal cAMP signaling selectivity by natural and synthetic MC4R agonists. *Mol Endocrinol* **29**:1619–1633.
- Möller D, Banerjee A, Uzuneser TC, Skultety M, Huth T, Plouffe B, Hübner H, Alzheimer C, Friedland K, Müller CP, et al. (2017) Discovery of G protein-biased dopaminergics with a pyrazolo[1,5-*a*]pyridine substructure. *J Med Chem* **60**:2908–2929.
- Monasky MM, Taglieri DM, Henze M, Warren CM, Utter MS, Soergel DG, Violin JD, and Solaro RJ (2013) The β -arrestin-biased ligand TRV120023 inhibits angiotensin II-induced cardiac hypertrophy while preserving enhanced myofilament response to calcium. *Am J Physiol Heart Circ Physiol* **305**:H856–H866.
- Monczor F and Fernandez N (2016) Current knowledge and perspectives on histamine H1 and H2 receptor pharmacology: functional selectivity, receptor cross-talk, and repositioning of classic histaminergic ligands. *Mol Pharmacol* **90**:640–648.
- Monod J, Wyman J, and Changeux JP (1965) On the nature of allosteric transitions: a plausible model. *J Mol Biol* **12**:88–118.
- Moore CA, Milano SK, and Benovic JL (2007) Regulation of receptor trafficking by GRKs and arrestins. *Annu Rev Physiol* **69**:451–482.
- Morris AJ and Malbon CC (1999) Physiological regulation of G protein-linked signaling. *Physiol Rev* **79**:1373–1430.
- Morse M, Sun H, Tran E, Levenson R, and Fang Y (2013) Label-free integrative pharmacology on-target of opioid ligands at the opioid receptor family. *BMC Pharmacol Toxicol* **14**:17–35.
- Motlagh HN, Wrabl JO, Li J, and Hilser VJ (2014) The ensemble nature of allostery. *Nature* **508**:331–339.
- Mottola DM, Cook LL, Jones SR, Booth RG, Nichols DE, and Mailman RB (1991) Dihydroxidine, a selective dopamine receptor agonist that may discriminate postsynaptic D2 receptors. *Abstr Soc Neurosci* **17**:818.
- Müllershausen F, Zecri F, Cetin C, Billich A, Guerini D, and Seuwen K (2009) Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors. *Nat Chem Biol* **5**:428–434.
- Nasman J, Kukkonen JP, Ammoun S, and Akerman KE (2001) Role of G-protein availability in differential signaling by alpha 2-adrenoceptors. *Biochem Pharmacol* **62**:913–922.
- Naylor J, Suckow AT, Seth A, Baker DJ, Sermadiras I, Ravn P, Howes R, Li J, Snaith MR, Coghlan MP, et al. (2016) Use of CRISPR/Cas9-engineered INS-1 pancreatic β cells to define the pharmacology of dual GIPR/GLP-1R agonists. *Biochem J* **473**:2881–2891.
- Neer EJ (1995) Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* **80**:249–257.
- Nelson CD, Perry SJ, Regier DS, Prescott SM, Topham MK, and Lefkowitz RJ (2007) Targeting of diacylglycerol degradation to M1 muscarinic receptors by beta-arrestins. *Science* **315**:663–666.
- Neves SR, Ram PT, and Iyengar R (2002) G protein pathways. *Science* **296**:1636–1639.
- Newman-Tancredi A, Audinot V, Moreira C, Verrièle L, and Millan MJ (2000) Inverse agonism and constitutive activity as functional correlates of serotonin 5-HT_{1B} (1B) receptor/G-protein stoichiometry. *Mol Pharmacol* **58**:1042–1049.
- Newman-Tancredi A, Conte C, Chaput C, Verrièle L, and Millan MJ (1997) Agonist and inverse agonist efficacy at human recombinant serotonin 5-HT_{1A} receptors as a function of receptor:G-protein stoichiometry. *Neuropharmacology* **36**:451–459.
- Nickolls SA, Fleck B, Hoare SR, and Maki RA (2005) Functional selectivity of melanocortin 4 receptor peptide and nonpeptide agonists: evidence for ligand-specific conformational states. *J Pharmacol Exp Ther* **313**:1281–1288.
- Nikolaev VO, Hoffmann C, Bünemann M, Lohse MJ, and Vilardaga JP (2006) Molecular basis of partial agonism at the neurotransmitter alpha2A-adrenergic receptor and Gi-protein heterotrimer. *J Biol Chem* **281**:24506–24511.
- Nobles KN, Guan Z, Xiao K, Oas TG, and Lefkowitz RJ (2007) The active conformation of β -arrestin1: direct evidence for the phosphate sensor in the N-domain and conformational differences in the active states of β -arrestins1 and -2. *J Biol Chem* **282**:21370–21381.
- Nobles KN, Xiao K, Ahn S, Shukla AK, Lam CM, Rajagopal S, Strachan RT, Huang TY, Bressler EA, Hara MR, et al. (2011) Distinct phosphorylation sites on the $\beta(2)$ -adrenergic receptor establish a barcode that encodes differential functions of β -arrestin. *Sci Signal* **4**:ra51.
- Noma T, Lemaire A, Naga Prasad SV, Barki-Harrington L, Tilley DG, Chen J, Le Corvoisier P, Violin JD, Wei H, Lefkowitz RJ, et al. (2007) Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. *J Clin Invest* **117**:2445–2458.
- Nuber S, Zabel U, Lorenz K, Nuber A, Milligan G, Tobin AB, Lohse MJ, and Hoffmann C (2016) β -arrestin biosensors reveal a rapid, receptor-dependent activation/deactivation cycle. *Nature* **531**:661–664.
- Nygaard R, Zou Y, Dror RO, Mildorf TJ, Arlow DH, Manglik A, Pan AC, Liu CW, Fung JJ, Bokoch MP, et al. (2013) The dynamic process of $\beta(2)$ -adrenergic receptor activation. *Cell* **152**:532–542.
- Ogawa S, Watanabe T, Moriyuki K, Goto Y, Yamane S, Watanabe A, Tsuboi K, Kinoshita A, Okada T, Takeda H, et al. (2016a) Structural optimization and structure-functional selectivity relationship studies of G protein-biased EP2 receptor agonists. *Bioorg Med Chem Lett* **26**:2446–2449.
- Ogawa S, Watanabe T, Sugimoto I, Moriyuki K, Goto Y, Yamane S, Watanabe A, Tsuboi K, Kinoshita A, Kigoshi H, et al. (2016b) Discovery of G protein-biased EP2 receptor agonists. *ACS Med Chem Lett* **7**:306–311.
- Oldach L and Zhang J (2014) Genetically encoded fluorescent biosensors for live-cell visualization of protein phosphorylation. *Chem Biol* **21**:186–197.
- Oldham WM and Hamm HE (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol* **9**:60–71.
- Onaran HO, Ambrosio C, Uğur Ö, Madaras Konz E, Grö MC, Vezzi V, Rajagopal S, and Costa T (2017) Systematic errors in detecting biased agonism: analysis of current methods and development of a new model-free approach. *Sci Rep* **7**:44247.
- Onaran HO, Rajagopal S, and Costa T (2014) What is biased efficacy? Defining the relationship between intrinsic efficacy and free energy coupling. *Trends Pharmacol Sci* **35**:639–647.
- Onfroy L, Galandrin S, Pontier SM, Seguelas MH, N'Guyen D, Sénard JM, and Galés C (2017) G protein stoichiometry dictates biased agonism through distinct receptor-G protein partitioning. *Sci Rep* **7**:7885.
- Oo ML, Thangada S, Wu MT, Liu CH, Macdonald TL, Lynch KR, Lin C-Y, and Hla T (2007) Immunosuppressive and anti-angiogenic sphingosine 1-phosphate receptor-1 agonists induce ubiquitinylation and proteasomal degradation of the receptor. *J Biol Chem* **282**:9082–9089.
- Ostrom RS, Bogard AS, Gros R, and Feldman RD (2012) Choreographing the adenylyl cyclase signalosome: sorting out the partners and the steps. *Naunyn Schmiedebergs Arch Pharmacol* **385**:5–12.
- Pang PS, Butler J, Collins SP, Cotter G, Davison BA, Ezekowitz JA, Filippatos G, Levy PD, Metra M, Ponikowski P, et al. (2017) Biased ligand of the angiotensin II type 1 receptor in patients with acute heart failure: a randomized, double-blind, placebo-controlled, phase IIB, dose ranging trial (BLAST-AHF). *Eur Heart J* **38**:2364–2373.
- Park PS (2012) Ensemble of G protein-coupled receptor active states. *Curr Med Chem* **19**:1146–1154.
- Peng J, Bencsik M, Louie A, Lu W, Millard S, Nguyen P, Burghardt A, Majumdar S, Wronski TJ, Halloran B, et al. (2008) Conditional expression of a Gi-coupled receptor in osteoblasts results in trabecular osteopenia. *Endocrinology* **149**:1329–1337.
- Perry SJ, Baillie GS, Kohout TA, McPhee I, Magiera MM, Ang KL, Miller WE, McLean AJ, Conti M, Houslay MD, et al. (2002) Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. *Science* **298**:834–836.
- Peters MF, Knappenberger KS, Wilkins D, Sygowski LA, Lazor LA, Liu J, and Scott CW (2007) Evaluation of cellular dielectric spectroscopy, a whole-cell, label-free technology for drug discovery on Gi-coupled GPCRs. *J Biomol Screen* **12**:312–319.
- Peters MF and Scott CW (2009) Evaluating cellular impedance assays for detection of GPCR pleiotropic signaling and functional selectivity. *J Biomol Screen* **14**:246–255.
- Peterson YK and Luttrell LM (2017) The diverse roles of arrestin scaffolds in G protein-coupled receptor signaling. *Pharmacol Rev* **69**:256–297.
- Peverelli E, Busnelli M, Vitali E, Giardino E, Galés C, Lania AG, Beck-Peccoz P, Chini B, Mantovani G, and Spada A (2013) Specific roles of G(i) protein family members revealed by dissecting SST5 coupling in human pituitary cells. *J Cell Sci* **126**:638–644.
- Piñeyro G (2009) Membrane signalling complexes: implications for development of functionally selective ligands modulating heptahelical receptor signalling. *Cell Signal* **21**:179–185.
- Pitcher JA, Freedman NJ, and Lefkowitz RJ (1998) G protein-coupled receptor kinases. *Annu Rev Biochem* **67**:653–692.
- Poulin B, Butcher A, McWilliams P, Bourgognon JM, Pawlak R, Kong KC, Bottrill A, Mistry S, Wess J, Rosethorne EM, et al. (2010) The M3-muscarinic receptor regulates learning and memory in a receptor phosphorylation/arrestin-dependent manner. *Proc Natl Acad Sci USA* **107**:9440–9445.
- Pradhan AA, Becker JA, Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, Massotte D, Gaviériaux-Ruff C, and Kieffer BL (2009) In vivo delta opioid receptor internalization controls behavioral effects of agonists. *PLoS One* **4**:e5425.
- Pradhan AA, Belfort K, Nozaki C, Gaviériaux-Ruff C, and Kieffer BL (2011) The delta opioid receptor: an evolving target for the treatment of brain disorders. *Trends Pharmacol Sci* **32**:581–590.
- Pradhan AA, Perroy J, Walwyn WM, Smith ML, Vicente-Sanchez A, Segura L, Bana A, Kieffer BL, and Evans CJ (2016) Agonist-specific recruitment of arrestin isoforms differentially modify delta opioid receptor function. *J Neurosci* **36**:3541–3551.

- Price MR, Baillie GL, Thomas A, Stevenson LA, Easson M, Goodwin R, McLean A, McIntosh L, Goodwin G, Walker G, et al. (2005) Allosteric modulation of the cannabinoid CB1 receptor. *Mol Pharmacol* **68**:1484–1495.
- Prihondok R, Bradley SJ, Tobin AB, and Butcher AJ (2015) Determination of GPCR phosphorylation status: establishing a phosphorylation barcode. *Curr Protoc Pharmacol* **69**:2.13.1–2.13.26.
- Privalov PL and Khechinashvili NN (1974) A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. *J Mol Biol* **86**:665–684.
- Qin CX, May LT, Li R, Cao N, Rosli S, Deo M, Alexander AE, Horlock D, Bourke JE, Yang YH, et al. (2017) Small-molecule-biased formyl peptide receptor agonist compound 17b protects against myocardial ischemia-reperfusion injury in mice. *Nat Commun* **8**:14232.
- Raeal KM and Bohn LM (2011) The role of beta-arrestin2 in the severity of antinociceptive tolerance and physical dependence induced by different opioid pain therapeutics. *Neuropharmacology* **60**:58–65.
- Raeal KM, Walker JK, and Bohn LM (2005) Morphine side effects in β -arrestin 2 knockout mice. *J Pharmacol Exp Ther* **314**:1195–1201.
- Rahmeh R, Damian B, Cottet M, Orcl H, Mendre C, Durroux T, Sharma KS, Durand G, Pucci B, Trinquet E, et al. (2012) Structural insights into biased G protein-coupled receptor signaling revealed by fluorescence spectroscopy. *Proc Natl Acad Sci USA* **109**:6733–6738.
- Rajagopal S, Ahn S, Rominger DH, Gowen-MacDonald W, Lam CM, Dewire SM, Violin JD, and Lefkowitz RJ (2011) Quantifying ligand bias at seven-transmembrane receptors. *Mol Pharmacol* **80**:367–377.
- Rajagopal S, Rajagopal K, and Lefkowitz RJ (2010) Teaching old receptors new tricks: biasing seven-transmembrane receptors. *Nat Rev Drug Discov* **9**:373–386.
- Ranjan R, Dwivedi H, Baidya M, Kumar M, and Shukla AK (2017) Novel structural insights into GPCR- β -arrestin interaction and signaling. *Trends Cell Biol* **27**:851–862.
- Rashid AJ, So CH, Kong MM, Furtak T, El-Ghundi M, Cheng R, O'Dowd BF, and George SR (2007) D1-D2 dopamine receptor heterooligomers with unique pharmacology are coupled to rapid activation of Gq/11 in the striatum. *Proc Natl Acad Sci USA* **104**:654–659.
- Rasmussen SG, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, Devree BT, Rosenbaum DM, Thian FS, Kobilka TS, et al. (2011) Structure of a nanobody-stabilized active state of the $\beta(2)$ adrenoceptor. *Nature* **469**:175–180.
- Re M, Pampillo M, Savard M, Dubuc C, McArdle CA, Millar RP, Conn PM, Gobeil F Jr, Bhattacharya M, and Babwah AV (2010) The human gonadotropin releasing hormone type I receptor is a functional intracellular GPCR expressed on the nuclear membrane. *PLoS One* **5**:e11489.
- Read C, Fitzpatrick CM, Yang P, Kuc RE, Maguire JJ, Glen RC, Foster RE, and Davenport AP (2016) Cardiac action of the first G protein biased small molecule apelin agonist. *Biochem Pharmacol* **116**:63–72.
- Redfern CH, Coward P, Degtyarev MY, Lee EK, Kwa AT, Hennighausen L, Bujard H, Fishman GI, and Conklin BR (1999) Conditional expression and signaling of a specifically designed Gi-coupled receptor in transgenic mice. *Nat Biotechnol* **17**:165–169.
- Reiner S, Ambrosio M, Hoffmann C, and Lohse MJ (2010) Differential signaling of the endogenous agonists at the beta2-adrenergic receptor. *J Biol Chem* **285**:36188–36198.
- Reiter E, Ahn S, Shukla AK, and Lefkowitz RJ (2012) Molecular mechanism of β -arrestin-biased agonism at seven-transmembrane receptors. *Annu Rev Pharmacol Toxicol* **52**:179–197.
- Revankar CM, Cimino DF, Sklar LA, Arterburn JB, and Prossnitz ER (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* **307**:1625–1630.
- Richard-Lalonde M, Nagi K, Audet N, Sleno R, Amraei M, Hogue M, Balboni G, Schiller PW, Bouvier M, Hébert TE, et al. (2013) Conformational dynamics of Kir3.1/Kir3.2 channel activation via δ -opioid receptors. *Mol Pharmacol* **83**:416–428.
- Riddy DM, Cook AE, Diepenhorst NA, Bosnyak S, Brady R, Mannoury la Cour C, Mocaer E, Summers RJ, Charman WN, Sexton PM, et al. (2017) Isoform-specific biased agonism of histamine H3 receptor agonists [published correction appears in *Mol Pharmacol* (2017) 91:263]. *Mol Pharmacol* **91**:87–99.
- Ritter SL and Hall RA (2009) Fine-tuning of GPCR activity by receptor-interacting proteins. *Nat Rev Mol Cell Biol* **10**:819–830.
- Riven I, Iwanir S, and Reuveny E (2006) GIRK channel activation involves a local rearrangement of a preformed G protein channel complex. *Neuron* **51**:561–573.
- Rives ML, Rossillo M, Liu-Chen LY, and Javitch JA (2012) 6'-Guanidinonaltrindole (6'-GNTI) is a G protein-biased κ -opioid receptor agonist that inhibits arrestin recruitment. *J Biol Chem* **287**:27050–27054.
- Rodriguez-Frade JM, Vila-Coro AJ, de Ana AM, Albar JP, Martínez-A C, and Mellado M (1999) The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2. *Proc Natl Acad Sci USA* **96**:3628–3633.
- Road SN, Wismann P, Underwood CR, Kulahin N, Iversen H, Cappelen KA, Schäffer L, Lehtonen J, Hecksher-Soerensen J, Secher A, et al. (2014) Real-time trafficking and signaling of the glucagon-like peptide-1 receptor. *Mol Cell Endocrinol* **382**:938–949.
- Roerig SC, Loh HH, and Law PY (1992) Identification of three separate guanine nucleotide-binding proteins that interact with the delta-opioid receptor in NG108-15 neuroblastoma x glioma hybrid cells. *Mol Pharmacol* **41**:822–831.
- Rohrer DK, Chruscinski A, Schauble EH, Bernstein D, and Kobilka BK (1999) Cardiovascular and metabolic alterations in mice lacking both β 1- and β 2-adrenergic receptors. *J Biol Chem* **274**:16701–16708.
- Rohrer DK and Kobilka BK (1998) G protein-coupled receptors: functional and mechanistic insights through altered gene expression. *Physiol Rev* **78**:35–52.
- Rosanò L, Cianfrocca R, Tocci P, Spinella F, Di Castro V, Spadaro F, Salvati E, Birocchio AM, Natali PG, and Bagnato A (2013) β -arrestin-1 is a nuclear transcriptional regulator of endothelin-1-induced β -catenin signaling. *Oncogene* **32**:5066–5077.
- Roth BL and Chuang DM (1987) Multiple mechanisms of serotonergic signal transduction. *Life Sci* **41**:1051–1064.
- Roth BL and Marshall FH (2012) NOBEL 2012 chemistry: studies of a ubiquitous receptor family. *Nature* **492**:57.
- Roth BL, Sheffler DJ, and Kroeze WK (2004) Magic shotguns versus magic bullets: selectively non-selective drugs for mood disorders and schizophrenia. *Nat Rev Drug Discov* **3**:353–359.
- Roy I, Evans DB, and Dwinell MB (2014) Chemokines and chemokine receptors: update on utility and challenges for the clinician. *Surgery* **155**:961–973.
- Roy I, Getschman AE, Volkman BF, and Dwinell MB (2017) Exploiting agonist biased signaling of chemokines to target cancer. *Mol Carcinog* **56**:804–813.
- Roy RV, Ardeshtyrlajimi A, Dinarvand P, Yang L, and Rezaie AR (2016) Occupancy of human EPCR by protein C induces β -arrestin-2 biased PAR1 signaling by both APC and thrombin. *Blood* **128**:1884–1893.
- Ryba DM, Li J, Cowan CL, Russell B, Wolska BM, and Solaro RJ (2017) Long-term biased β -arrestin signaling improves cardiac structure and function in dilated cardiomyopathy. *Circulation* **135**:1056–1070.
- Saidak Z, Blake-Palmer K, Hay DL, Northup JK, and Glass M (2006) Differential activation of G-proteins by mu-opioid receptor agonists. *Br J Pharmacol* **147**:671–680.
- Samama P, Cotecchia S, Costa T, and Lefkowitz RJ (1993) A mutation-induced activated state of the β 2-adrenergic receptor. Extending the ternary complex model. *J Biol Chem* **268**:4625–4636.
- Santos GA, Duarte DA, Parreiras-E-Silva LT, Teixeira FR, Silva-Rocha R, Oliveira EB, Bouvier M, and Costa-Neto CM (2015) Comparative analyses of downstream signal transduction targets modulated after activation of the AT1 receptor by two β -arrestin-biased agonists. *Front Pharmacol* **6**:131–140.
- Sato M, Horinouchi T, Hutchinson DS, Evans BA, and Summers RJ (2007) Ligand-directed signaling at the beta3-adrenoceptor produced by 3-(2-ethylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronaph-1-ylaminol]-2S-2-propanol oxalate (SR59230A) relative to receptor agonists. *Mol Pharmacol* **72**:1359–1368.
- Saudou F, Amara DA, Dierich A, LeMeur M, Ramboz S, Segu L, Buhot MC, and Hen R (1994) Enhanced aggressive behavior in mice lacking 5-HT1B receptor. *Science* **265**:1875–1878.
- Saulière A, Bellot M, Paris H, Denis C, Finana F, Hansen JT, Altié MF, Seguelas MH, Pathak A, Hansen JL, et al. (2012) Deciphering biased-agonism complexity reveals a new active AT1 receptor entity. *Nat Chem Biol* **8**:622–630.
- Sbai O, Monnier C, Dodé C, Pin J-P, Hardelin J-P, and Rondard P (2014) Biased signaling through G-protein-coupled PROKR2 receptors harboring missense mutations. *FASEB J* **28**:3734–3744.
- Scarduzio M, Zimmerman CN, Jaunarajs KL, Wang Q, Standaert DG, and McMahon LL (2017) Strength of cholinergic tone dictates the polarity of dopamine D2 receptor modulation of striatal cholinergic interneuron excitability in DYT1 dystonia. *Exp Neurol* **295**:162–175.
- Scherrer G, Tryoen-Tóth P, Filliol D, Matifas A, Laustriat D, Cao YQ, Basbaum AI, Dierich A, Vonesh JL, Gavériaux-Ruff C, et al. (2006) Knockin mice expressing fluorescent delta-opioid receptors uncover G protein-coupled receptor dynamics in vivo. *Proc Natl Acad Sci USA* **103**:9691–9696.
- Scherrer G, Inamachi N, Cao YQ, Contet C, Mennicken F, O'Donnell D, Kieffer BL, and Basbaum AI (2009) Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. *Cell* **137**:1148–1159.
- Schmid CL and Bohn LM (2010) Serotonin, but not N-methyltryptamines, activates the serotonin 2A receptor via a β -arrestin2/Src/Akt signaling complex in vivo. *J Neurosci* **30**:13513–13524.
- Schmid CL, Streicher JM, Groer CE, Munro TA, Zhou L, and Bohn LM (2013) Functional selectivity of 6'-guanidinonaltrindole (6'-GNTI) at κ -opioid receptors in striatal neurons. *J Biol Chem* **288**:22387–22398.
- Sebastiano M, Momi S, Falcinelli E, Bury L, Hoylaerts MF, and Gresele P (2017) A novel mechanism regulating human platelet activation by MMP-2-mediated PAR1 biased signaling. *Blood* **129**:883–895.
- Sengmany K, Singh J, Stewart GD, Conn PJ, Christopoulos A, and Gregory KJ (2017) Biased allosteric agonism and modulation of metabotropic glutamate receptor 5: implications for optimizing preclinical neuroscience drug discovery. *Neuropharmacology* **115**:60–72.
- Seo J, Tsakem EL, Breitman M, and Gurevich VV (2011) Identification of arrestin-3-specific residues necessary for JNK3 kinase activation. *J Biol Chem* **286**:27894–27901.
- Shapira M, Vogel Z, and Sarne Y (2000) Opioid and cannabinoid receptors share a common pool of GTP-binding proteins in cotransfected cells, but not in cells which endogenously coexpress the receptors. *Cell Mol Neurobiol* **20**:291–304.
- Sharma S, Rodriguez AL, Conn PJ, and Lindsley CW (2008) Synthesis and SAR of a mGluR5 allosteric partial antagonist lead: unexpected modulation of pharmacology with slight structural modifications to a 5-(phenylethynyl)pyrimidine scaffold. *Bioorg Med Chem Lett* **18**:4098–4101.
- Sheng M and Sala C (2001) PDZ domains and the organization of supramolecular complexes. *Annu Rev Neurosci* **24**:1–29.
- Shenoy SK, Barak LS, Xiao K, Ahn S, Berthouze M, Shukla AK, Luttrell LM, and Lefkowitz RJ (2007) Ubiquitination of β -arrestin links seven-transmembrane receptor endocytosis and ERK activation. *J Biol Chem* **282**:29549–29562.
- Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Pre-mont RT, Lichtarge O, and Lefkowitz RJ (2006) Beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J Biol Chem* **281**:1261–1273.
- Shenoy SK and Lefkowitz RJ (2005) Seven-transmembrane receptor signaling through beta-arrestin. *Sci STKE* **2005**:cm10.
- Shenoy SK and Lefkowitz RJ (2011) β -arrestin-mediated receptor trafficking and signal transduction. *Trends Pharmacol Sci* **32**:521–533.
- Shenoy SK, McDonald PH, Kohout TA, and Lefkowitz RJ (2001) Regulation of receptor fate by ubiquitination of activated β 2-adrenergic receptor and β -arrestin. *Science* **294**:1307–1313.
- Shimizu Y and Nakayama M (2017) Discovery of novel Gq-biased LPA1 negative allosteric modulators. *SLAS Discov* **22**:859–866.

- Shukla AK, Manglik A, Kruse AC, Xiao K, Reis RI, Tseng WC, Staus DP, Hilger D, Uysal S, Huang LY, et al. (2013) Structure of active β -arrestin-1 bound to a G-protein-coupled receptor phosphopeptide. *Nature* **497**:137–141.
- Shukla AK, Singh G, and Ghosh E (2014) Emerging structural insights into biased GPCR signaling. *Trends Biochem Sci* **39**:594–602.
- Shukla AK, Violin JD, Whalen EJ, Gesty-Palmer D, Shenoy SK, and Lefkowitz RJ (2008) Distinct conformational changes in β -arrestin report biased agonism at seven-transmembrane receptors. *Proc Natl Acad Sci USA* **105**:9988–9993.
- Slessareva JE, Rott SM, Temple B, Bankaitis VA, and Dohlman HG (2006) Activation of the phosphatidylinositol 3-kinase Vps34 by a G protein alpha subunit at the endosome. *Cell* **126**:191–203.
- Smrcka AV (2008) G protein $\beta\gamma$ subunits: central mediators of G protein-coupled receptor signaling. *Cell Mol Life Sci* **65**:2191–2214.
- Soergel DG, Subach RA, Burnham N, Lark MW, James IE, Sadler BMF, Skobieranda F, Violin JD, and Webster LR (2014) Biased agonism of the μ -opioid receptor by TRV130 increases analgesia and reduces on-target adverse effects versus morphine: a randomized, double-blind, placebo-controlled, crossover study in healthy volunteers. *Pain* **155**:1829–1835.
- Soethoudt M, Grether U, Fingerle J, Grim TW, Fezza F, de Petrocellis L, Ullmer C, Rothenhäusler B, Perret C, van Gils N, et al. (2017) Cannabinoid CB₂ receptor ligand profiling reveals biased signalling and off-target activity. *Nat Commun* **8**:13958.
- Song X, Coffa S, Fu H, and Gurevich VV (2009) How does arrestin assemble MAPKs into a signaling complex? *J Biol Chem* **284**:685–695.
- Sonoda N, Imamura T, Yoshizaki T, Babendure JL, Lu JC, and Olefsky JM (2008) Beta-arrestin-1 mediates glucagon-like peptide-1 signaling to insulin secretion in cultured pancreatic beta cells. *Proc Natl Acad Sci USA* **105**:6614–6619.
- Sora I, Takahashi N, Funada M, Ujike H, Revay RS, Donovan DM, Miner LL, and Uhl GR (1997) Opiate receptor knockout mice define μ receptor roles in endogenous nociceptive responses and morphine-induced analgesia. *Proc Natl Acad Sci USA* **94**:1544–1549.
- Soto AG, Smith TH, Chen B, Bhattacharya S, Cordova IC, Kenakin T, Vaidehi N, and Trejo J (2015) N-linked glycosylation of protease-activated receptor-1 at extracellular loop 2 regulates G-protein signaling bias. *Proc Natl Acad Sci USA* **112**:E3600–E3608.
- Spengler D, Waeber C, Pantaloni C, Holsboer F, Bockaert J, Seeburg PH, and Journot L (1993) Differential signal transduction by five splice variants of the PACAP receptor. *Nature* **365**:170–175.
- Spinella F, Rosanò L, Di Castro V, Nicotra MR, Natali PG, and Bagnato A (2004) Inhibition of cyclooxygenase-1 and -2 expression by targeting the endothelin A receptor in human ovarian carcinoma cells. *Clin Cancer Res* **10**:4670–4679.
- Srivastava A, Gupta B, Gupta C, and Shukla AK (2015) Emerging functional divergence of β -arrestin isoforms in GPCR function. *Trends Endocrinol Metab* **26**:628–642.
- Stahl EL, Zhou L, Ehler FJ, and Bohn LM (2015) A novel method for analyzing extremely biased agonism at G protein-coupled receptors. *Mol Pharmacol* **87**:866–877.
- Stallaert W, Dorn JF, van der Westhuizen E, Audet M, and Bouvier M (2012) Impedance responses reveal β_2 -adrenergic receptor signaling pluridimensionality and allow classification of ligands with distinct signaling profiles. *PLoS One* **7**:e29420.
- Staus DP, Strachan RT, Manglik A, Pani B, Kahsai AW, Kim TH, Winkler LM, Ahn S, Chatterjee A, Masoudi A, et al. (2016) Allosteric nanobodies reveal the dynamic range and diverse mechanisms of G-protein-coupled receptor activation. *Nature* **535**:448–452.
- Stephenson RP (1956) A modification of receptor theory. *Br J Pharmacol Chemother* **11**:379–393.
- Strachan RT, Sun JP, Rominger DH, Violin JD, Ahn S, Rojas Bie Thomsen A, Zhu X, Kleist A, Costa T, and Lefkowitz RJ (2014) Divergent transducer-specific molecular efficacies generate biased agonism at a G protein-coupled receptor (GPCR). *J Biol Chem* **289**:14211–14224.
- Straiker A, Wager-Miller J, Hutchens J, and Mackie K (2012) Differential signalling in human cannabinoid CB1 receptors and their splice variants in autaptic hippocampal neurons. *Br J Pharmacol* **165**:2660–2671.
- Strasser A, Wittmann H-J, and Seifert R (2017) Binding kinetics and pathways of ligands to GPCRs. *Trends Pharmacol Sci* **38**:717–732.
- Strömberg I, Popoli P, Müller CE, Ferré S, and Fuxe K (2000) Electrophysiological and behavioural evidence for an antagonistic modulatory role of adenosine A2A receptors in dopamine D2 receptor regulation in the rat dopamine-denervated striatum. *Eur J Neurosci* **12**:4033–4037.
- Suen JY, Cotterell A, Lohman RJ, Lim J, Han A, Yau MK, Liu L, Cooper MA, Vesey DA, and Fairlie DP (2014) Pathway-selective antagonism of proteinase activated receptor 2. *Br J Pharmacol* **171**:4112–4124.
- Susulic VS, Frederich RC, Lawitts J, Tozzo E, Kahn BB, Harper M-E, Himms-Hagen J, Flier JS, and Lowell BB (1995) Targeted disruption of the β 3-adrenergic receptor gene. *J Biol Chem* **270**:29483–29492.
- Takahashi A, Kato K, Kuboyama A, Inoue T, Tanaka Y, Kuhara A, Kinoshita K, Takeda S, and Wake N (2009) Induction of senescence by progesterone receptor-B activation in response to cAMP in ovarian cancer cells. *Gynecol Oncol* **113**:270–276.
- Tan L, Yan W, McCorvy JD, and Cheng J (2018) Biased ligands of G protein-coupled receptors (GPCRs): structure-functional selectivity relationships (SFSRs) and therapeutic potential. *J Med Chem* **61**:9841–9878.
- Tang KES and Dill KA (1998) Native protein fluctuations: the conformational-motion temperature and the inverse correlation of protein flexibility with protein stability. *J Biomol Struct Dyn* **16**:397–411.
- Teixeira LB, Parreiras-E-Silva LT, Bruder-Nascimento T, Duarte DA, Simões SC, Costa RM, Rodriguez DY, Ferreira PAB, Silva CAA, Abreu EP, et al. (2017) Ang-(1-7) is an endogenous β -arrestin-biased agonist of the AT₁ receptor with protective action in cardiac hypertrophy. *Sci Rep* **7**:11903.
- Teoh JP, Park KM, Wang Y, Hu Q, Kim S, Wu G, Huang S, Maihle N, and Kim IM (2014) Endothelin-1/endothelin A receptor-mediated biased signaling is a new player in modulating human ovarian cancer cell tumorigenesis. *Cell Signal* **26**:2885–2895.
- Thompson GL, Lane JR, Coudrat T, Sexton PM, Christopoulos A, and Canals M (2015) Biased agonism of endogenous opioid peptides at the μ -opioid receptor. *Mol Pharmacol* **88**:335–346.
- Thompson GL, Lane JR, Coudrat T, Sexton PM, Christopoulos A, and Canals M (2016) Systematic analysis of factors influencing observations of biased agonism at the μ -opioid receptor. *Biochem Pharmacol* **113**:70–87.
- Thomsen AR, Worm J, Jacobsen SE, Stahlhut M, Latta M, and Bräuner-Osborne H (2012) Strontium is a biased agonist of the calcium-sensing receptor in rat medullary thyroid carcinoma 6-23 cells. *J Pharmacol Exp Ther* **343**:638–649.
- Tobin AB (2008) G-protein-coupled receptor phosphorylation: where, when and by whom. *Br J Pharmacol* **153** (Suppl 1):S167–S176.
- Tobin AB, Butcher AJ, and Kong KC (2008) Location, location, location...site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. *Trends Pharmacol Sci* **29**:413–420.
- Torreclilla I, Spragg EJ, Poulin B, McWilliams PJ, Mistry SC, Blaukat A, and Tobin AB (2007) Phosphorylation and regulation of a G protein-coupled receptor by protein kinase CK2. *J Cell Biol* **177**:127–137.
- Trettel F, Di Bartolomeo S, Lauro C, Catalano M, Ciotti MT, and Limatola C (2003) Ligand-independent CXCR2 dimerization. *J Biol Chem* **278**:40980–40988.
- Trinquet E, Bouhelal R, and Dietz M (2011) Monitoring Gq-coupled receptor response through inositol phosphate quantification with the IP-One assay. *Expert Opin Drug Discov* **6**:981–994.
- Trinquet E, Fink M, Bazin H, Grillet F, Maurin F, Bourrier E, Ansanay H, Leroy C, Michaud A, Durroux T, et al. (2006) D-myo-inositol 1-phosphate as a surrogate of D-myo-inositol 1,4,5-tris phosphate to monitor G protein-coupled receptor activation. *Anal Biochem* **358**:126–135.
- Tsao P, Cao T, and von Zastrow M (2001) Role of endocytosis in mediating downregulation of G-protein-coupled receptors. *Trends Pharmacol Sci* **22**:91–96.
- Tschammer N, Bollinger S, Kenakin T, and Gmeiner P (2011) Histidine 6.55 is a major determinant of ligand-biased signaling in dopamine D2L receptor. *Mol Pharmacol* **79**:575–585.
- Tucek S (1997) Is the R and R dichotomy real? *Trends Pharmacol Sci* **18**:414–416.
- Unett DJ, Gatlin J, Anthony TL, Buzard DJ, Chang S, Chen C, Chen X, Dang HT, Frazer J, Le MK, et al. (2013) Kinetics of 5-HT_{2B} receptor signaling: profound agonist-dependent effects on signaling onset and duration. *J Pharmacol Exp Ther* **347**:645–659.
- Urban DJ and Roth BL (2015) DREADDs (designer receptors exclusively activated by designer drugs): chemogenetic tools with therapeutic utility. *Annu Rev Pharmacol Toxicol* **55**:399–417.
- Urban JD, Vargas GA, von Zastrow M, and Mailman RB (2007) Aripiprazole has functionally selective actions at dopamine D2 receptor-mediated signaling pathways. *Neuropsychopharmacology* **32**:67–77.
- Urs NM and Caron MG (2014) The physiological relevance of functional selectivity in dopamine signalling. *Int J Obes Suppl* **4** (Suppl 1):S5–S8.
- Urs NM, Daigle TL, and Caron MG (2011) A dopamine D1 receptor-dependent β -arrestin signaling complex potentially regulates morphine-induced psychomotor activation but not reward in mice. *Neuropsychopharmacology* **36**:551–558.
- Urs NM, Gee SM, Pack TF, McCorvy JD, Evron T, Snyder JC, Yang X, Rodriguez RM, Borrelli E, Wetzel WC, et al. (2016) Distinct cortical and striatal actions of a β -arrestin-biased dopamine D2 receptor ligand reveal unique antipsychotic-like properties. *Proc Natl Acad Sci USA* **113**:E8178–E8186.
- Urs NM, Nicholls PJ, and Caron MG (2014) Integrated approaches to understanding antipsychotic drug action at GPCRs. *Curr Opin Cell Biol* **27**:56–62.
- Vaidehi N and Kenakin T (2010) The role of conformational ensembles of seven transmembrane receptors in functional selectivity. *Curr Opin Pharmacol* **10**:775–781.
- Valant C, May LT, Aurelio L, Chuo CH, White PJ, Baltos JA, Sexton PM, Scammells PJ, and Christopoulos A (2014) Separation of on-target efficacy from adverse effects through rational design of a bitopic adenosine receptor agonist. *Proc Natl Acad Sci USA* **111**:4614–4619.
- Valentin-Hansen L, Frimurer TM, Mokrosinski J, Holliday ND, and Schwartz TW (2015) Biased Gs versus Gq proteins and β -arrestin signaling in the NK1 receptor determined by interactions in the water hydrogen bond network. *J Biol Chem* **290**:24495–24508.
- van der Lee MM, Verkaar F, Wat JWY, van Offenbeek J, Timmerman M, Voorneveld L, van Lith LHJC, and Zaman GJR (2013) β -arrestin-biased signaling of PTH analogs of the type 1 parathyroid hormone receptor. *Cell Signal* **25**:527–538.
- Vanderbeld B and Kelly GM (2000) New thoughts on the role of the β -gamma subunit in G-protein signal transduction. *Biochem Cell Biol* **78**:537–550.
- Vardy E and Roth BL (2013) Conformational ensembles in GPCR activation. *Cell* **152**:385–386.
- Vecchio EA, Baltos JA, Nguyen ATN, Christopoulos A, White PJ, and May LT (2018) New paradigms in adenosine receptor pharmacology: allostery, oligomerization and biased agonism. *Br J Pharmacol* **175**:4036–4046.
- Vecchio EA, Chuo CH, Baltos J-A, Ford L, Scammells PJ, Wang BH, Christopoulos A, White PJ, and May LT (2016) The hybrid molecule, VCP746, is a potent adenosine A2B receptor agonist that stimulates anti-fibrotic signalling. *Biochem Pharmacol* **117**:46–56.
- Vila-Coro AJ, Rodríguez-Frade JM, Martín De Ana A, Moreno-Ortiz MC, Martínez-A C, and Mellado M (1999) The chemokine SDF-1 α triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *FASEB J* **13**:1699–1710.
- Violin JD, Crombie AL, Soergel DG, and Lark MW (2014) Biased ligands at G-protein-coupled receptors: promise and progress. *Trends Pharmacol Sci* **35**:308–316.
- Violin JD, Dewire SM, Barnes WG, and Lefkowitz RJ (2006) G protein-coupled receptor kinase and beta-arrestin-mediated desensitization of the angiotensin II type 1A receptor elucidated by diacylglycerol dynamics. *J Biol Chem* **281**:36411–36419.
- Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, Whalen EJ, Gowen M, and Lark MW (2010) Selectively engaging β -arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. *J Pharmacol Exp Ther* **335**:572–579.

- Viscusi ER, Webster L, Kuss M, Daniels S, Bolognese JA, Zuckerman S, Soergel DG, Subach RA, Cook E, and Skobieranda F (2016) A randomized, phase 2 study investigating TRV130, a biased ligand of the μ -opioid receptor, for the intravenous treatment of acute pain. *Pain* **157**:264–272.
- Vogt AD and Di Cera E (2013) Conformational selection is a dominant mechanism of ligand binding. *Biochemistry* **52**:5723–5729.
- Wacker D, Stevens RC, and Roth BL (2017) How ligands illuminate GPCR molecular pharmacology. *Cell* **170**:414–427.
- Wacker D, Wang C, Katritch V, Han GW, Huang X-P, Vardy E, McCorvy JD, Jiang Y, Chu M, Siu FY, et al. (2013) Structural features for functional selectivity at serotonin receptors. *Science* **340**:615–619.
- Walker CS, Sundrum T, and Hay DL (2014) PACAP receptor pharmacology and agonist bias: analysis in primary neurons and glia from the trigeminal ganglia and transfected cells. *Br J Pharmacol* **171**:1521–1533.
- Walters RW, Shukla AK, Kovacs JJ, Violin JD, DeWire SM, Lam CM, Chen JR, Muehlbauer MJ, Whalen EJ, and Lefkowitz RJ (2009) Beta-arrestin1 mediates nicotinic acid-induced flushing, but not its antipolytic effect, in mice. *J Clin Invest* **119**:1312–1321.
- Walther C and Ferguson SS (2013) Arrestins: role in the desensitization, sequestration, and vesicular trafficking of G protein-coupled receptors. *Prog Mol Biol Transl Sci* **118**:93–113.
- Walther C and Ferguson SS (2015) Minireview: role of intracellular scaffolding proteins in the regulation of endocrine G protein-coupled receptor signaling. *Mol Endocrinol* **29**:814–830.
- Wang C, Jiang Y, Ma J, Wu H, Wacker D, Katritch V, Han GW, Liu W, Huang X-P, Vardy E, et al. (2013) Structural basis for molecular recognition at serotonin receptors. *Science* **340**:610–614.
- Wang WC, Muhlbacher KA, Brunnett AC, and Liggett SB (2009) Targeted transgenesis reveals discrete attenuator functions of GRK and PKA in airway beta2-adrenergic receptor physiologic signaling. *Proc Natl Acad Sci USA* **106**:15007–15012.
- Wang WY, Wu XM, Jia LJ, Zhang HH, Cai F, Mao H, Xu WC, Chen L, Zhang J, and Hu SF (2016) Beta-arrestin1 and 2 differentially modulate metabotropic glutamate receptor 7 signaling in rat developmental sevoflurane-induced neuronal apoptosis. *Neuroscience* **313**:199–212.
- Wang Y, Huang J, Liu X, Niu Y, Zhao L, Yu Y, Zhou L, Lu L, and Yu C (2017) β -arrestin-biased AT1R stimulation promotes extracellular matrix synthesis in renal fibrosis. *Am J Physiol Renal Physiol* **313**:F1–F8.
- Ward JS, Merritt L, Calligaro DO, Bymaster FP, Shannon HE, Sawyer BD, Mitch CH, Deeter JB, Peters SC, Sheardown MJ, et al. (1995) Functionally selective M1 muscarinic agonists. 3. Side chains and azacycles contributing to functional muscarinic selectivity among pyrazinylazacycles. *J Med Chem* **38**:3469–3481.
- Warne T, Edwards PC, Leslie AG, and Tate CG (2012) Crystal structures of a stabilized β_1 -adrenoceptor bound to the biased agonists bucindolol and carvedilol. *Structure* **20**:841–849.
- Watson C, Chen G, Irving P, Way J, Chen WJ, and Kenakin T (2000) The use of stimulus-biased assay systems to detect agonist-specific receptor active states: implications for the trafficking of receptor stimulus by agonists. *Mol Pharmacol* **58**:1230–1238.
- Wehbi VL, Stevenson HP, Feinstein TN, Calero G, Romero G, and Vilardaga JP (2013) Noncanonical GPCR signaling arising from a PTH receptor-arrestin-G β complex. *Proc Natl Acad Sci USA* **110**:1530–1535.
- Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, and Lefkowitz RJ (2003) Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci USA* **100**:10782–10787.
- Wei Q, Costanzi S, Balasubramanian R, Gao Z-G, and Jacobson KA (2013) A2B adenosine receptor blockade inhibits growth of prostate cancer cells. *Purinergic Signal* **9**:271–280.
- Weißer M, Xu Q, Gale JP, Lewis M, Campbell AJ, Schroeder FA, Van de Bittner GC, Walk M, Amaya A, Su P, et al. (2018) Functionally biased D2R antagonists: targeting the β -arrestin pathway to improve antipsychotic treatment. *ACS Chem Biol* **13**:1038–1047.
- Welihinda AA, Kaur M, Greene K, Zhai Y, and Amento EP (2016) The adenosine metabolite inosine is a functional agonist of the adenosine A2A receptor with a unique signaling bias. *Cell Signal* **28**:552–560.
- Wells TNC, Power CA, Shaw JP, and Proudfoot AEI (2006) Chemokine blockers—therapeutics in the making? *Trends Pharmacol Sci* **27**:41–47.
- West GM, Chien EY, Katritch V, Gatchalian J, Chalmers MJ, Stevens RC, and Griffin PR (2011) Ligand-dependent perturbation of the conformational ensemble for the GPCR β_2 adrenergic receptor revealed by HDX. *Structure* **19**:1424–1432.
- Wettschreck N and Offermanns S (2005) Mammalian G proteins and their cell type specific functions. *Physiol Rev* **85**:1159–1204.
- Whalen EJ, Rajagopal S, and Lefkowitz RJ (2011) Therapeutic potential of β -arrestin- and G protein-biased agonists. *Trends Mol Med* **17**:126–139.
- Whistler JL, Enquist J, Marley A, Fong J, Gladher F, Tsuruda P, Murray SR, and Von Zastrow M (2002) Modulation of postendocytic sorting of G protein-coupled receptors. *Science* **297**:615–620.
- White KL, Robinson JE, Zhu H, DiBerto JF, Polepally PR, Zjawiony JK, Nichols DE, Malanga CJ, and Roth BL (2015) The G protein-biased κ -opioid receptor agonist RB-64 is analgesic with a unique spectrum of activities in vivo. *J Pharmacol Exp Ther* **352**:98–109.
- White KL, Scopton AP, Rives ML, Bikkulavov RV, Polepally PR, Brown PJ, Kenakin T, Javitch JA, Zjawiony JK, and Roth BL (2014) Identification of novel functionally selective κ -opioid receptor scaffolds. *Mol Pharmacol* **85**:83–90.
- Wilden U (1995) Duration and amplitude of the light-induced cGMP hydrolysis in vertebrate photoreceptors are regulated by multiple phosphorylation of rhodopsin and by arrestin binding. *Biochemistry* **34**:1446–1454.
- Wilden U, Hall SW, and Kühn H (1986) Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proc Natl Acad Sci USA* **83**:1174–1178.
- Williams GR, Bethard JR, Berkaw MN, Nagel AK, Luttrell LM, and Ball LE (2016) Exploring G protein-coupled receptor signaling networks using SILAC-based phosphoproteomics. *Methods* **92**:36–50.
- Williams PD, Pollock DD, and Goldstein RA (2007) Functionality and the evolution of marginal stability in proteins: inferences from lattice simulations. *Evol Bioinform Online* **2**:91–101.
- Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, Shenoy SK, and Lefkowitz RJ (2007) A unique mechanism of β -blocker action: carvedilol stimulates β -arrestin signaling. *Proc Natl Acad Sci USA* **104**:16657–16662.
- Witherow DS, Garrison TR, Miller WE, and Lefkowitz RJ (2004) Beta-arrestin inhibits NF- κ B activity by means of its interaction with the NF- κ B inhibitor I κ B α . *Proc Natl Acad Sci USA* **101**:8603–8607.
- Woo AY, Komuro I, and Xiao RP (2017) Biased agonism at the angiotensin receptor: blocker and calcium sensitizer at the same time. *Circulation* **135**:1071–1074.
- Wooten D, Reynolds CA, Smith KJ, Mobarec JC, Furness SG, Miller LJ, Christopoulos A, and Sexton PM (2016) Key interactions by conserved polar amino acids located at the transmembrane helical boundaries in class B GPCRs modulate activation, effector specificity and biased signalling in the glucagon-like peptide-1 receptor. *Biochem Pharmacol* **118**:68–87.
- Xiao K, McClatchy DB, Shukla AK, Zhao Y, Chen M, Shenoy SK, Yates JR 3rd, and Lefkowitz RJ (2007) Functional specialization of beta-arrestin interactions revealed by proteomic analysis. *Proc Natl Acad Sci USA* **104**:12011–12016.
- Xiao K, Shenoy SK, Nobles K, and Lefkowitz RJ (2004) Activation-dependent conformational changes in β -arrestin 2. *J Biol Chem* **279**:55744–55753.
- Xu M, Hu XT, Cooper DC, Moratalla R, Graybiel AM, White FJ, and Tonegawa S (1994a) Elimination of cocaine-induced hyperactivity and dopamine-mediated neurophysiological effects in dopamine D1 receptor mutant mice. *Cell* **79**:945–955.
- Xu M, Moratalla R, Gold LH, Hiroi N, Koob GF, Graybiel AM, and Tonegawa S (1994b) Dopamine D1 receptor mutant mice are deficient in striatal expression of dynorphin and in dopamine-mediated behavioral responses. *Cell* **79**:729–742.
- Xu W, Wang X, Tocker AM, Huang P, Reith ME, Liu-Chen LY, Smith AB 3rd, and Kortagere S (2017) Functional characterization of a novel series of biased signaling dopamine D3 receptor agonists. *ACS Chem Neurosci* **8**:486–500.
- Yang CH, Huang HW, Chen KH, Chen YS, Sheen-Chen SM, and Lin CR (2011) Antinociceptive potentiation and attenuation of tolerance by intrathecal β -arrestin 2 small interfering RNA in rats. *Br J Anaesth* **107**:774–781.
- Yang F, Yu X, Liu C, Qu CX, Gong Z, Liu HD, Li FH, Wang HM, He DF, Yi F, et al. (2015) Phospho-selective mechanisms of arrestin conformations and functions revealed by unnatural amino acid incorporation and (19)F-NMR. *Nat Commun* **6**:8202.
- Yang LK and Tao YX (2017) Biased signaling at neural melanocortin receptors in regulation of energy homeostasis. *Biochim Biophys Acta Mol Basis Dis* **1863**:2486–2495.
- Yang P, Maguire JJ, and Davenport AP (2015) Apelin, Elabela/Toddler, and biased agonists as novel therapeutic agents in the cardiovascular system. *Trends Pharmacol Sci* **36**:560–567.
- Yang Z and Tao YX (2016) Biased signaling initiated by agouti-related peptide through human melanocortin-3 and -4 receptors. *Biochim Biophys Acta* **1862**:1485–1494.
- Yao X, Parnot C, Deupi X, Ratnala VR, Swaminath G, Farrens D, and Kobilka B (2006) Coupling ligand structure to specific conformational switches in the beta2-adrenoceptor. *Nat Chem Biol* **2**:417–422.
- Yen H-Y, Hoi KK, Liko I, Hedger G, Horrell MR, Song W, Wu D, Heine P, Warne T, Lee Y, et al. (2018) PtdIns(4,5)P₂ stabilizes active states of GPCRs and enhances selectivity of G-protein coupling. *Nature* **559**:423–427.
- Zhang G, Cheng J, McCorvy JD, Lorello PJ, Caldaroni BJ, Roth BL, and Kozikowski AP (2017) Discovery of N-substituted (2-phenylcyclopropyl)methylamines as functionally selective serotonin 2C receptor agonists for potential use as antipsychotic medications. *J Med Chem* **60**:6273–6288.
- Zhao P, Lieu T, Barlow N, Metcalf M, Veldhuis NA, Jensen DD, Kocan M, Sostegni S, Haerteis S, Baraznenok V, et al. (2014a) Cathepsin S causes inflammatory pain via biased agonism of PAR2 and TRPV4. *J Biol Chem* **289**:27215–27234.
- Zhao P, Metcalf M, and Bunnett NW (2014b) Biased signaling of protease-activated receptors. *Front Endocrinol (Lausanne)* **5**:67–78.
- Zheng Z, Huang XP, Mangano TJ, Zou R, Chen X, Zaidi SA, Roth BL, Stevens RC, and Katritch V (2017) Structure-based discovery of new antagonist and biased agonist chemotypes for the kappa opioid receptor. *J Med Chem* **60**:3070–3081.
- Zhou B and Giraldo J (2018) An operational model for GPCR homodimers and its application in the analysis of biased signaling. *Drug Discov Today* **23**:1591–1595.
- Zhou XE, He Y, de Waal PW, Gao X, Kang Y, Van Eps N, Yin Y, Pal K, Goswami D, White TA, et al. (2017a) Identification of phosphorylation codes for arrestin recruitment by G protein-coupled receptors. *Cell* **170**:457–469.e13.
- Zhou XE, Melcher K, and Xu HE (2017b) Understanding the GPCR biased signaling through G protein and arrestin complex structures. *Curr Opin Struct Biol* **45**:150–159.
- Zhu X, Gilbert S, Birnbaumer M, and Birnbaumer L (1994) Dual signaling potential is common among Gs-coupled receptors and dependent on receptor density. *Mol Pharmacol* **46**:460–469.
- Zidar DA, Violin JD, Whalen EJ, and Lefkowitz RJ (2009) Selective engagement of G protein coupled receptor kinases (GRKs) encodes distinct functions of biased ligands. *Proc Natl Acad Sci USA* **106**:9649–9654.
- Zimmerman B, Beaudrait A, Aguila B, Charles R, Escher E, Claing A, Bouvier M, and Laporte SA (2012) Differential β -arrestin-dependent conformational signaling and cellular responses revealed by angiotensin analogs. *Sci Signal* **5**:ra33.
- Zocher M, Fung JJ, Kobilka BK, and Müller DJ (2012) Ligand-specific interactions modulate kinetic, energetic, and mechanical properties of the human β_2 adrenergic receptor. *Structure* **20**:1391–1402.
- Zoudilova M, Kumar P, Ge L, Wang P, Bokoch GM, and DeFea KA (2007) Beta-arrestin-dependent regulation of the cofilin pathway downstream of protease-activated receptor-2. *J Biol Chem* **282**:20634–20646.