Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


# A pluridimensional view of biased agonism

Claudio M. Costa-Neto, Lucas T. Parreiras-e-Silva, & Michel Bouvier

Department of Biochemistry and Immunology, Ribeirao Preto Medical School, University of São Paulo, Ribeirao Preto, SP, 14049-900, Brazil; CMCN, LTPS.
Department of Biochemistry and Molecular Medicine and Institute for Research in Immunology and Cancer, University of Montréal, Montréal, QC, H3C-3J7, Canada; LTPS, MB.

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.



**Running title:** GPCR biased signaling

**Corresponding author information:**
Claudio M. Costa-Neto, Ph.D.
Department of Biochemistry and Immunology, Ribeirao Preto Medical School, University of São Paulo, Ribeirao Preto, SP, 14049-900, Brazil.
E-mail: claudio@fmrp.usp.br
Phone: +55 16 3315 3261

&

Michel Bouvier, Ph.D.
Department of Biochemistry and Molecular Medicine and Institute for Research in Immunology and Cancer, University of Montréal, Montréal, QC, H3C-3J7, Canada.
E-mail: michel.bouvier@umontreal.ca
Phone: 1-(514) 343-6319

**Document statistics:**
Total number of pages: 38
Number of text pages: 17
Total number of words: 7,987
Number of words in abstract: 223
Number of words in Introduction: 1,143
Number of words in Discussion: 492
Number of tables: 0
Number of Figures: 4
Number of references: 89

**List of non-standard abbreviations:** GPCR, G protein-coupled receptor; G protein, guanine nucleotide-binding protein; GRK, G protein-coupled receptor kinase; 5HT, 5-hydroxytryptamine; PI, phosphoinositide; PACAP Pituitary adenylate cyclase-activating polypeptide; cAMP, cyclic adenosine monophosphate; IP3, inositol trisphosphate; PKA, protein kinase A; PKC, protein kinase C; NMR, nuclear magnetic resonance; ERK1/2, extracellular signal–regulated kinases; V2R, V2 vasopressin receptor; PTHR, parathyroid hormone receptor; CCR7, C-C chemokine receptor type 7; CCL19, chemokine (C-C motif) ligand 19; CCL21, chemokine (C-C motif) ligand 21; AT1R, angiotensin II type 1 receptor; $K_{on}$, association rate; $K_{off}$, dissociation rate; RGS, regulators of G protein signaling; PLC, phospholipase C; iPS, induced pluripotent stem cells; BRET, bioluminescence resonance energy transfer.

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


**Abstract:**

When studying GPCR signaling and ligand-biased agonism, at least three “dimensional spaces” must be considered: 1) the distinct conformations that can be stabilized by different ligands promoting the engagement of different signaling effectors and accessory regulators; 2) the distinct subcellular trafficking that can be conferred by different ligands, which results in spatially distinct signals; and, 3) the differential binding kinetics that maintain the receptor in specific conformation and or subcellular localization for different periods of time, allowing for the engagement of distinct signaling effector subsets. These three pluridimensional aspects of signaling contribute to different faces of functional selectivity and provide a complex, interconnected way to define the signaling profile of each individual ligand acting at GPCRs. In this review we discuss how each of these aspects may contribute to the diversity of signaling, but also how they shed light on the complexity of data analyses and interpretation. The impact of phenotype variability as a source of signaling diversity, and the influence of novel and more sensitive assays in the detection and analysis of signaling pluridimensionality, is also discussed. Finally, we discuss perspectives for the use of the concept of pluridimensional signaling in drug-discovery, where we highlight future predictive tools that may facilitate the identification of compounds with optimal therapeutic and safety properties based on the “signaling signatures” of drug candidates.

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


**Introduction:**

For many decades, GPCRs have been described as plasma membrane proteins transducing signals from a variety of extracellular molecules to the intracellular milieu by coupling to and activating G proteins (e.g. Wess, 1997; Dohlman et al., 1991). The original model proposed that binding of agonists would promote an active receptor state that could engage and activate its cognate G protein, whereas binding of competitive antagonists would block activation by impeding agonist interaction. Therefore, GPCR functionality was measured and interpreted in a linear way as a switch turning a given signaling pathway “ON” or “OFF”. Nowadays it is known that a plethora of GPCRs signals can occur and are modulated by multiple mechanisms, including interaction with different G proteins, β-arrestins, GRKs and other effectors.

The first evidence that select GPCRs could interact with different G proteins isoforms was published in the 1980’s (Burch et al., 1986; Brown and Goldstein, 1986; Murayama and Ui, 1985; Kelly et al., 1985; for a review see Garcia-Sainz, 1987). Further insight regarding diversity of signaling and biased agonism, its possible therapeutic applications, and potential underlying mechanisms were introduced by Roth and Chuang in 1987. “The 5HT2 receptors may be coupled, directly or indirectly, to: (1) PI metabolism; (2) voltage-gated calcium channels and (3) prostaglandin metabolism. If these responses represent distinct coupling mechanism, it might be possible to design selective 5HT agonists and antagonists that alter only one or another of these systems” (Roth & Chuang, 1987). To explain such a phenomenon, it was then hypothesized that receptors could be stabilized in different active conformations, and that ligands with different structures would be able to

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


stabilize such different conformations of the receptor (Kenakin & Morgan, 1989). Federman and colleagues in 1992 showed that the α2-adrenoceptor could couple to both Gi and Gs isoforms, a profile that could be detected depending on the used cell type due to the distinct repertoire of co-expressed receptors and intracellular effectors (Federman et al., 1992). In 1994, using the same receptor, Eason and coworkers reported that Gs or Gi selection could be defined by the agonist in use (Eason et al., 1994). In this article, Stephen Liggett's group showed that six different agonists presented very similar efficacies towards Gi coupling, whereas significant differences were observed in Gs signaling. One year before, in 1993, Spengler and coworkers showed that splicing variants of the PACAP receptor triggered different kinds of response after stimulation with the same ligands. These authors provided clear evidence of the reversal of potency for the ligands PACAP-27 and PACAP-38 when triggering cAMP and IP3 responses (Spengler et al., 1993). The term agonist-receptor trafficking was introduced in 1995 by Terry Kenakin (Kenakin, 1995), and few years later, the term "biased agonism" was used for the first time (Jarpe et al., 1998).

The first evidence was also obtained during the 1980s showing that agonist stimulation leads to GPCR phosphorylation catalyzed either by second messenger-dependent kinases (e.g. PKA and PKC) or by G protein coupled receptor kinases (GRKs), leading to desensitization of the response (e.g. Nambi et al., 1985; Bouvier et al., 1987; Bouvier et al., 1988; for a review see Lefkowitz et al., 1990). GRK-promoted phosphorylation was then shown to promote the recruitment of the regulatory protein β-arrestin to the receptor, leading to internalization and termination of G protein signaling (e.g. Benovic et al., 1987;

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


Lohse et al., 1990; Ferguson et al., 1996; for reviews see Lefkowitz, 1998; Zhang et al., 1997). Although such findings revealed the roles of new important partners, the overall view of GPCR functionality remained linked to G protein signaling. However, these findings introduced the notion that modifications in the receptor structure could regulate its ability to interact with different intracellular partners, and thus modulate its signaling properties.

An understanding of the dynamic nature of the equilibrium between active and inactive conformations of the receptor was significantly enriched by the first reports of ligands that were able to decrease the basal level of receptor activity (e.g. Costa & Herz, 1989; Costa et al., 1990; Chidiac et al., 1994; Barker et al., 1994; Samama et al., 1994; Leeb-Lundberg et al., 1994). Such reports underpinned the concept that receptors could spontaneously isomerize between active and inactive conformations, and that the equilibrium state in the absence of ligand determined the level of constitutive activity (Bond et al., 2001). These observations led to the discovery of inverse agonists, which shift the equilibrium from the active to the inactive conformation of the receptor and silence constitutive activity. This is in contrast to agonists, which promote the equilibrium shift towards the active state. Although the concept of inverse agonism added the idea of dynamic transition between different receptor conformations with important mechanistic and possibly therapeutic implications, the concept still fits into the perspective of receptor activation by engagement of G proteins.

The paradigm of G protein coupling as the unique effector of GPCRs' activation was challenged by the first reports describing that some compounds could either promote cellular responses even when unable to trigger G protein

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


activation or even act as inverse agonists on G protein-dependent signaling (Azzi et al., 2003; Baker et al., 2003; Wei et al., 2003, Gbahou et al., 2003). These studies indicated that the G protein-independent component of GPCR activation could occur via β-arrestins, consistent with previous reports demonstrating that after interacting with receptors, β-arrestin serves as a scaffolding protein coordinating the actions of other effectors, including kinases, involved in downstream signaling (Milano et al., 2002; McDonald et al., 2000; Luttrell et al., 1999; Luttrell et al., 2001). The discovery of molecules bearing the capacity of activating distinct pathways after interacting with the same receptor resulted in the concept of functional selectivity and biased agonism. Therefore, agonists able to preferentially trigger β-arrestin-dependent signaling pathways became known as β-arrestin-biased agonists, and those able to preferentially trigger G protein-dependent pathways as G protein-biased agonists. More recently, compounds biased toward distinct G protein subtypes were described (Busnelli et al., 2012; Saulière et al., 2012).

The concept of biased agonism therefore shifted our view of GPCR from unidimensional regulators of linear signaling cascades to multidimensional transducers that can engage diverse signaling pathways and differentially regulate them. This has clear implications for pharmacological nomenclature, and indeed it has become insufficient to qualify a ligand as “agonist” or “inverse agonist” and it is now necessary to specify toward which pathway a given ligand is an agonist or an inverse agonist. This new vision of GPCR signaling also raises the issue of how to quantify the relative efficacy toward the different pathways. There are currently rich and active discussions about the best way of calculating bias. This topic is out of the scope of this mini-review, but articles

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


addressing it can be found elsewhere (e.g. Stallaert et al., 2011; Kenakin 2014; Van der Westhuizen et al., 2014; Kenakin et al., 2012; Kenakin and Christopoulos, 2013).

**The pluridimensionality of signaling and of biased agonism:**

Different structures/conformations, different active states:

The main hypothesis proposed to explain biased signaling tenders that differences from the ligand structure are reflected in differences of the final structures/conformations of the receptor, which in turn underlie the selective engagement of different subsets of effectors and/or the stabilization of different conformational states of the effectors. As will be discussed further below, in addition to this conformational hypothesis, different kinetics of ligand binding, differential post-translational modification, as well as distinct subcellular localization of the signaling complexes most-likely also contribute to the pluridimensionality of GPCR signaling and biased signaling.

The conformational hypothesis of ligand-biased signaling is supported by an increasing number of studies directly demonstrating that chemically diverse ligands can stabilize different conformations of receptors as assessed by NMR studies (Nygaard et al., 2013). Energy landscape can be used as an approach to visualize the various conformational states of proteins (Henzler-Wildman & Kern, 2007) and also of GPCR through their activation steps (Deupi & Kobilka, 2010). In Figure 1 we represent part of an activation coordinate path for a hypothetical GPCR and the corresponding energy landscapes until reaching the G protein active state(s). In panel A, we represent one possible active conformation, which is correlated to the G protein signaling pathway. Our

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


representation on panel B highlights the existence of different intermediate active conformations of the ligand-receptor complex, which besides being able to interact with G proteins, are also able to interact with additional effectors. Distinct ligands can lead to stabilization of different conformations, hence generating signaling diversity.

Cellular compartments and microdomains unveils new dimensions of signaling:

The role of distinct membrane microdomains, receptor trafficking, and endosomal signaling are among the most actively debated topics in the field of GPCR signaling (e.g. Eichel et al., 2016; Irannejad et al., 2013; for a review see Calebiro et al., 2010). The concept of endosomal signaling has first been proposed for receptors of the tyrosine kinase family (for review see Wiley & Burke, 2001). Evidence has also accumulated indicating that endocytosed GPCRs can activate the mitogen-activated protein kinases ERK1/2 in a β-arrestin-dependent manner (Luttrell et al., 2001; Luttrell & Lefkowitz, 2002). More recently, sustained cAMP signaling has been proposed to result from a second wave of activation of Gs in the endosomes (for a review see Vilardaga et al., 2014). For instance, such non-canonical activation of Gs has been shown for the vasopressin type 2 receptor (V2R) and the parathyroid hormone receptor (PTHR), and that long lasting receptor-arrestin complexes correlate with different endocytosis patterns and sustained Gs signaling from endosomes (Feinstein et al., 2011; Vilardaga et al., 2012; Wehbi et al., 2013; Feinstein et al., 2013).

Such distinct signaling location, that was neglected until recently for GPCR drug discovery, may have a significant impact as it may be a key factor

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


controlling response to drugs. Supporting this notion, it was recently reported that the cAMP produced in endosomes promotes a different gene regulation profile than that resulting from cAMP generated at the plasma membrane (Tsvetanova & von Zastrow, 2014).

The role of distinct subcellular compartment signaling takes particular importance when considering that different ligands can promote distinct endocytosis and differential subcellular trafficking and recycling paths. For instance, it has been described that activation of the CCR7 receptor by CCL19 or CCL21 resulted in different patterns of receptor internalization despite the ability of both ligands to similarly induce recruitment of β-arrestin 2. It was also shown that activation by CCL19 resulted in receptor phosphorylation by GRK3 and GRK6, while CCL21 only involved GRK6. Such differences probably yielded distinct phosphorylation patterns involved in differential engagement of the internalization and trafficking machinery (Zidar et al., 2009). The finding that different β2-adrenergic ligands can promote distinct receptor phosphorylation patterns leading to different conformations and function of the engaged β-arrestin (desensitization vs MAPK signaling) lead to the concept of “barcode”, related to different phosphorylation patterns in the receptor by GRKs (Nobles et al., 2011). Stabilization of distinct β-arrestin conformations by different ligands has been adding a new dimension to the concept of biased signaling (Zimmerman et al., 2012; Santos et al., 2015), and recently a β-arrestin “conformational signature” has been elegantly proposed (Lee et al., 2016). It has also been recently shown that different ligands for the AT1R were able to promote distinct intracellular trafficking (Szakadáti et al., 2015; Namkung et al., 2016)

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


The kinetic component of biased signaling:

When considering biased ligand signaling, it is also critical to bear in mind the influence of time on determined signaling profiles. Time and kinetics can indeed influence the interpretation of signaling data on different fronts. Among these, times of measurements and ligand binding kinetics represent the most obvious parameters that need to be considered.

Signal and assay time courses:

The different signals triggered by receptor activation as well as the assays used to monitor them can vary significantly. Therefore, two ligands with similar affinities for the same receptor and in the same given system may yield similar or completely contrasting signaling profiles depending on the time of data acquisition. Kinetics analyses of different pathways and the use of different readouts should reveal the best time for data acquisition for each pathway and also suggest which pathways can be compared at a given time-point. For instance, the same time point cannot be used for comparing transient signals, such as calcium or ERK1/2 activation with the more sustained cAMP accumulation. As illustrated in Figure 2, pathways **A** to **H** are activated by a given ligand to a similar extent at 5 and 10 minutes after stimulus, while only pathways **A**, **B**, and **F** maintain a similar profile of activation when comparing data acquired at 5 and 30 minutes. In this hypothetical example, if a kinetics analyses had not been carried out, one or the other time of data acquisition could have been chosen (e.g. 5 or 30 minutes), therefore leading to completely

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


different interpretation. It follows that time-course profiles of responses should be carried out for each pathway considered and the consequence of different kinetics taken into consideration when analyzing apparent bias. In a recent study, Thompson and coworkers (2016) revisited their own data on biased signalling of opioid ligands, taking into consideration various cellular background and assay format parameters. Although the ligands considered as biased in previous studies retained their uniqueness, different profiles were observed providing broader knowledge on the characteristics of each ligand (Thompson et al., 2016). Also, in an elegant work by Rob Lane and colleagues, the influence of experimental kinetics and the kinetics of ligand-receptor interaction in signaling results, along with the possible misinterpretations about biased agonism that may arise were reported. Among other interesting data, the authors state that the distinct choice of time acquisition can even lead to a reversal of bias direction for some agonists for the D2 receptor (Klein Herenbrink et al., 2016).

The contribution of ligand's interaction kinetics to conformation stabilization:

Another kinetic aspect to be considered in the context of biased signaling concerns ligand binding on-rates and off-rates. Although some ligands have similar affinities determined by equilibrium radio-ligand binding or competition assays, they may have distinct $K_{on}$ and $K_{off}$ rates. Such differences in the "time of residence" of ligands bound to a GPCR can ultimately play a pivotal role in stabilizing specific conformations of the receptor for longer periods of time. This can lead to significant differences in signaling profiles, notably those that

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


depend on lower affinity interaction with effectors (Zhang et al., 2015; Guo et al., 2014). Although we are aware that such approaches cannot be easily implemented in drug discovery high-throughput screenings, recent data highlights the relevance of the ligand's association and dissociation rates in addition to characterizing the properties of drug candidates and assessing the contribution of their $K_{on}$ and $K_{off}$ to the biased signaling observed (Guo et al., 2016; Klein Herenbrink et al., 2016). The observation that binding kinetics may influence the signaling profile of ligands adds the dimension of time to those of conformation and localization, all of which control GPCR functional selectivity.



The influence of phenotypic diversity to signal texture:

Subtle changes in ligands' structures can ultimately result in different receptor conformations, but it is also predicted that other factors such as membrane composition, presence of different effectors, and interacting proteins or other receptors will also impact the conformation of the receptor and influence its stabilization in distinct active states that may lead to distinct signaling profiles. Also, the relative abundance of the receptor's signaling partners influences the propensity of the receptor to engage some of the pathways simply by the law of mass action. For example, the low expression level of a given G protein in a specific cell type may allow a receptor that is normally highly coupled to this G protein to now engage other G protein subtypes for which it has moderate affinity. In a recent article, Broad and coworkers analyzed the functional profiles of κ-opioid receptors present in heterologous systems and in primary cell cultures. ICI204448 and Asimadoline are usually referred to as full agonists for the Gi coupled pathway, and Gi

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


activation assays performed in this study using HEK293 cells expressing the human κ-opioid receptor indeed showed that both drugs acted with similar potencies and seemed to be full agonists for this pathway. On the other hand, in human colon cells, ICI204448 acted at very low concentrations while Asimadoline was only effective in much higher concentrations (Broad et al., 2016), an effect that we believe probably includes differences in receptor expression levels when comparing heterologous and primary systems.

Even when comparing cells having similar G protein repertoires, differences in the expression levels of other signaling partners such as regulators of G protein signaling (RGS), G protein receptor kinases (GRKs), second messenger-dependent kinases, phosphatases, other proteins involved in downstream signaling cascades, as well as other GPCRs that may indirectly interact by functional crosstalk or directly by receptor dimerization undoubtedly impact the signaling profiles observed. For instance, μ- and δ- opioid receptor heterodimerization has been proposed to affect subcellular trafficking, which results in lower cell surface expression (Décaillot et al., 2008), and unveiled a pertussis toxin-resistant signaling pathway (George et al., 2000).

The role of GRK expression levels in defining the signaling profile of the receptor has also been exemplified for the μ-opioid receptor, which in many cell types has been shown not to recruit and activate β-arrestin upon morphine treatment. However, over-expression of GRK2 has been shown to allow the recruitment of β-arrestin to the morphine-activated receptor and to restore its endocytosis (Zhang et al., 1998). These data indicate that the functional selectivity of morphine may be affected by the relative expression levels of GRKs in different tissues.

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


The composition of biological membranes might also play a significant role in defining the activation profile of a given GPCR. This can be easily understood when considering Gq/11 coupled receptors, which are functionally linked to PLC action on membrane phospholipids. Cholesterol has also been proposed to influence GPCR signal transduction (e.g. Pontier et al., 2008; Levitt et al., 2009; for reviews see Head et al., 2014; Oates & Watts, 2011). Obviously, differential membrane composition of molecules involved in internalization and/or endosome formation processes, such as lipids, clathrin and caveolae, could also have critical impact on signal transduction (for review see Head et al., 2014). Because membrane compartmentalization, endosome formation and trafficking are also dependent on the repertoire of membrane and intracellular proteins, it becomes clear that different cell systems may result in distinct profiles, and therefore must be taken into account when designing, analyzing and interpreting signal transduction data and biased signaling.

Based on the above discussion, it is predictable that the same ligands could have distinct signaling profiles in different cellular system as a result of the specific components present in each cell (Devost et al., 2016). However, if as theoretically proposed, functional selectivity is an intrinsic property of a ligand-receptor complex, it would be reasonable to propose that compounds sharing similar signaling profiles in a given cell type will still share a similar signaling signature in a different cellular background, albeit different from the first cell type. If this prediction is borne out, it would allow the clustering of compounds in different signaling groups in generic cell lines and the testing of representatives of these groups for their biological and clinical outcomes (i.e., therapeutic efficacy, side effects, and safety). Although such classification of

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


compounds may not allow for the identification of the specific pathways involved in the desirable or undesirable effects, it may still be very useful to stratify different drug lead series identified in drug discovery programs. To validate this hypothesis, the signaling profile of the same ligands would need to be tested in different cell types, including primary cells and/or iPS cells differentiated in physiologically relevant cells to determine if the compounds cluster in the same subgroups independently of the cell background (Figure 3). If it is the case, it will then be important to determine how many signaling pathways need to be tested in order to establish “signaling signatures” of clusters that bear predictive biological and clinical values.

Discovering new signaling dimensions:

The discovery of ligand-biased signaling stimulated the development of assays with the required sensitivity to monitor GPCR functional selectivity, which allowed for the detection of multiple signaling pathways. Among these, bioluminescence resonance energy transfer (BRET)-based assays monitoring G protein activation (Galés et al., 2006), β-arrestin engagement (Angers et al., 2000) and activation (Charest et al., 2005), as well as second messenger generation (Jiang et al., 2007; Leduc et al., 2009) have attracted considerable attention and have been used to describe the signaling profile of an increasing number of ligands and receptors (Saulière et al., 2012; Quoyer et al., 2013; for reviews see Schann et al., 2013; Lohse et al., 2012). The granularity and sensitivity of the assays used is of primary importance to allow the full characterization of the signaling texture of ligands. As illustrated in Figure 4A, activation of pathways by different compounds may appear similar or even

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


equal when tested by poorly sensitive assays, whereas a more sensitive assay and/or the broadening of the evaluation to downstream targets could reveal differences (Figure 4B and 4C) that would be neglected otherwise. Examples of the importance of the assay format on the detection of biased signaling involve β-arrestin; using an assay with high sensitivity detected the conformational rearrangement of β-arrestin following its activation, and this allowed researchers to distinguish the β-arrestin activation profile of the angiotensin AT1 receptor (AT1R) ligands that otherwise recruited β-arrestin to a similar extent to the receptor (Zimmerman et al., 2012). More recently, we analyzed downstream signaling profiles of two β-arrestin biased ligands in the AT1R, and observed significant differences, unveiling that broader analyses of signaling pathways and downstream targets can generate a more complete activation profile for a given ligand (Santos et al., 2015).

**Discussion:**

How the pluridimensional aspects discussed above can help or confuse biased agonism measurement and interpretation.

Analyses of diverse and pluridimensional aspects of GPCR signaling certainly will bring significant knowledge to the field and broaden our concept of biased agonism. The frequently used dichotomy of biased agonism towards β-arrestin or G protein can substantially change with inclusion of other pathways, as well as inclusion of spatiotemporal perspectives. On the other hand, it is important to remember that the concept of biased agonism is intrinsically dependent on a comparison with a second ligand acting on the same receptor that should ideally be under identical conditions. Therefore, differences in

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


parameters such as the assay system or assay conditions (temperature, time of acquisition, etc) can lead to data misinterpretations or even contradictory data.

Perspectives for pluridimensional signaling in drug discovery:

A key point when considering analyses of large amounts of complex data is to extract those pathways that are indeed crucial for characterizing a given molecule in a predictive way in a drug discovery program. In other words, how much is it necessary to know? Is it mandatory to evaluate all possible pathways that are conceivably activated by a given ligand-receptor complex (including those of very low activation levels)? Or would it be possible to identify some pathways that when presenting a certain pattern of activation, would be enough to suggest the next directions? Perhaps those questions cannot be fully answered yet, but what we believe is that in the coming years, the increasing level of data granularity about GPCR pluridimensional signaling obtained in different systems, combined with pre-clinical and clinical data from successful molecules, will allow the creation of a predictive signaling/outcome framework. More importantly, meta-analyses extraction and predictive software should allow identification of the key pathways and corresponding patterns of activation, generating a “signaling signature” that is relevant for a given drug discovery pipeline or indication (see Figure 3). Therefore, whereas the methodological advances and sensitivities of the assays will allow us to discover the diversity of signaling pathways, the use of artificial intelligence tools should facilitate the early identification of promising molecules based on “learned data” from previous successful molecules. Such machine learning tools should allow us to predict physiological outcomes based on the signaling

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


patterns previously associated with specific physiological responses. Similarly to a cell phone predictive text typing application that “learns” from our regular insertion of the most used/required words, the predictive signaling pathway identification tool would “learn” from the continuous insertion of pluridimensional signaling data combined with physiological outcomes from pre-clinical and clinical studies.

In conclusion, although the phenotypic diversity, the influence of binding kinetics and/or time of data acquisition, and other factors can complicate the analysis of biased signaling, we believe that such information can be taken into consideration and factored in formal analyses in order to provide robust prediction of the biological outcomes of different ligands.

**Authorship contribution:**

Designed the review: Costa-Neto, and Bouvier.

Wrote the review: Costa-Neto, Parreiras-e-Silva, and Bouvier.

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


**References:**

Angers S, Salahpour A, Joly E, Hilairet S, Chelsky D, Dennis M, Bouvier M (2000) Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc Natl Acad Sci USA **97**: 3684-3689.

Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M, Piñeyro 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.

Baker JG, Hall IP, 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.

Barker EL, Westphal RS, Schmidt D, Sanders-Bush E (1994) Constitutively active 5-hydroxytryptamine2C receptors reveal novel inverse agonist activity of receptor ligands. J Biol Chem **269**: 11687-11690.

Benovic JL, Kühn H, Weyand I, Codina J, Caron MG, Lefkowitz RJ (1987) Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). Proc Natl Acad Sci USA. **84**: 8879-8882.

Benovic JL, Pike LJ, Cerione RA, Staniszewski C, Yoshimasa T, Codina J, Caron MG, Lefkowitz RJ (1985) Phosphorylation of the mammalian beta-adrenergic receptor by cyclic AMP-dependent protein kinase. Regulation of the rate of receptor phosphorylation and dephosphorylation by agonist occupancy and effects on coupling of the receptor to the stimulatory guanine nucleotide regulatory protein. J Biol Chem 260: 7094-7101.

Bond R, Milligan G, Bouvier M (2001) Inverse agonism, in Handbook of Experimental Pharmacology. The pharmacology of functional, biochemical, and recombinant receptor system (Kenakin T and Angus JA eds) vol 148, pp 167-182, Springer-Verlag publisher.

Bouvier M, Hausdorff WP, De Blasi A, O'Dowd BF, Kobilka BK, Caron MG, Lefkowitz RJ (1988) Removal of phosphorylation sites from the beta 2-adrenergic receptor delays onset of agonist-promoted desensitization. Nature **333**: 370-373.

Bouvier M, Leeb-Lundberg LM, Benovic JL, Caron MG, Lefkowitz RJ (1987) Regulation of adrenergic receptor function by phosphorylation. II. Effects of agonist occupancy on phosphorylation of alpha 1- and beta 2-adrenergic receptors by protein kinase C and the cyclic AMP-dependent protein kinase. J Biol Chem **262**: 3106-3113.

Broad J, Maurel D, Kung VW, Hicks GA, Schemann M, Barnes MR, Kenakin TP, Granier S and Sanger GJ (2016) Human native kappa opioid receptor

functions not predicted by recombinant receptors: Implications for drug design. Sci Rep **6**: 30797.

Brown JH and Goldstein D (1986) Differences in muscarinic receptor reserve for inhibition of adenylate cyclase and stimulation of phosphoinositide hydrolysis in chick heart cells. Mol Pharmacol **30**: 566-570.

Burch RM, Luini A and Axelrod J (1986) Phospholipase A2 and phospholipase C are activated by distinct GTP-binding proteins in response to alpha 1-adrenergic stimulation in FRTL5 thyroid cells. Proc Natl Acad Sci USA **83**: 7201-7205.

Busnelli, M, Saulière, A, Manning, M, Bouvier, M, Gales, C and Chini, B (2012) Functionally selective Oxytocin-derived agonists discriminate individual G protein family subtypes. J Biol Chem **287**: 3617-3629.

Calebiro D, Nikolaev VO, Lohse MJ (2010) Imaging of persistent cAMP signaling by internalized G protein-coupled receptors. J Mol Endocrinol **45** :1-8.

Costa T and Herz A (1989) Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. Proc Natl Acad Sci USA **86**: 7321-7325.

Charest PG, Terrillon S, Bouvier M (2005) Monitoring agonist-promoted conformational changes of beta-arrestin in living cells by intramolecular BRET. EMBO Rep **6**: 334-340.

Chidiac P, Hebert TE, Valiquette M, Dennis M, Bouvier M (1994) Inverse agonist activity of beta-adrenergic antagonists. Mol Pharmacol. **45**: 490-499.

Costa T, Lang J, Gless C, Herz A (1990) Spontaneous association between opioid receptors and GTP-binding regulatory proteins in native membranes: specific regulation by antagonists and sodium ions. Mol Pharmacol. **37**: 383-394.

Décaillot FM, Rozenfeld R, Gupta A, Devi LA (2008) Cell surface targeting of mu-delta opioid receptor heterodimers by RTP4. Proc Natl Acad Sci USA. **105**: 16045-16050.

Deupi X, Kobilka BK (2010) Energy landscapes as a tool to integrate GPCR structure, dynamics, and function. Physiology (Bethesda) **25**: 293-303.

Devost D, Audet N, Zhou C, Kobayashi H, Bonin H, Lukashova V, Le Gouill C, Bouvier M, Hébert TE (2016) Cellular and subcellular context determine outputs from signaling biosensors. Methods Cell Biol **132**: 319-337.

Dohlman HG, Thorner J, Caron MG, Lefkowitz RJ (1991) Model systems for the study of seven-transmembrane-segment receptors. Annu Rev Biochem **60**: 653-688.

Eason MG, Jacinto MT and Liggett SB (1994) Contribution of ligand structure to activation of alpha 2-adrenergic receptor subtype coupling to Gs. Mol Pharmacol **45**: 696-702.

Eichel K, Jullié D, von Zastrow M (2016) β-Arrestin drives MAP kinase signalling from clathrin-coated structures after GPCR dissociation. Nat Cell Biol **18**: 303-310.

Federman AD, Conklin BR, Schrader KA, Reed RR and Bourne HR (1992) Hormonal stimulation of adenylyl cyclase through Gi-protein beta gamma subunits. Nature **356**: 159-161.

Feinstein TN, Wehbi VL, Ardura JA, Wheeler DS, Ferrandon S, Gardella TJ, 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, Vilardaga JP (2013) Noncanonical control of vasopressin receptor type 2 signaling by retromer and arrestin. J Biol Chem **288**: 27849-27860.

Ferguson SS, Downey WE 3rd, Colapietro AM, Barak LS, Ménard L, Caron MG (1996). Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. Science **271**: 363-366.

Galés C, Van Durm JJ, Schaak S, Pontier S, Percherancier Y, Audet M, Paris H, Bouvier M (2006) Probing the activation-promoted structural rearrangements in preassembled receptor-G protein complexes. Nat Struct Mol Biol **13**: 778-786.

Garcia-Sainz JA (1987) Pathways of alpha 1-adrenergic action: comparison with V1-vasopressin and A1-angiotensin. Circ Res **61**(5 Pt 2): II1-5.

Gbahou F, Rouleau A, Morisset S, Parmentier R, Crochet S, Lin JS, Ligneau X, Tardivel-Lacombe J, Stark H, Schunack W, Ganellin CR, Schwartz JC, Arrang JM (2003) Protean agonism at histamine H3 receptors in vitro and in vivo. Proc Natl Acad Sci USA **100**: 11086-11091.

George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, O'Dowd BF (2000) Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. J Biol Chem **275**: 26128-26135.

Guo D, Dijksteel GS, van Duijl T, Heezen M, Heitman LH, IJzerman AP (2016) Equilibrium and kinetic selectivity profiling on the human adenosine receptors. Biochem Pharmacol **105**: 34-41.

Guo D, Hillger JM, IJzerman AP, Heitman LH (2014) Drug-target residence time - a case for G protein-coupled receptors. Med Res Rev **34**: 856-892.

Head BP, Patel HH, Insel PA (2014) Interaction of membrane/lipid rafts with the cytoskeleton: impact on signaling and function: membrane/lipid rafts, mediators of cytoskeletal arrangement and cell signaling. Biochim Biophys Acta **1838**: 532-545.

Henzler-Wildman K, Kern D (2007) Dynamic personalities of proteins. Nature **450**: 964-972.

Irannejad R, Tomshine JC, Tomshine JR, Chevalier M, Mahoney JP, Steyaert J, Rasmussen SG, Sunahara RK, El-Samad H, Huang B, von Zastrow M (2013) Conformational biosensors reveal GPCR signalling from endosomes. Nature **495**: 534-538.

Jarpe MB, Knall C, Mitchell FM, Buhl AM, Duzic E and Johnson GL (1998) [D-Arg1,D-Phe5,D-Trp7,9,Leu11]Substance P acts as a biased agonist toward neuropeptide and chemokine receptors. J Biol Chem **273**: 3097-3104.

Jiang LI, Collins J, Davis R, Lin KM, DeCamp D, Roach T, Hsueh R, Rebres RA, Ross EM, Taussig R, Fraser I, Sternweis PC (2007) Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. J Biol Chem **282**: 10576-10584.

Kelly E, Rooney TA and Nahorski SR (1985) Pertussis toxin separates two muscarinic receptor-effector mechanisms in the striatum. Eur J Pharmacol **119**: 129-130.

Kenakin T (1995) Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. Trends Pharmacol Sci **16**: 232-238.

Kenakin T (2014) What is pharmacological 'affinity'? Relevance to biased agonism and antagonism. Trends Pharmacol Sci **35**: 434-441.

Kenakin T, Christopoulos A (2013) Signalling bias in new drug discovery: detection, quantification and therapeutic impact. Nat Rev Drug Discov **12**: 205-216.

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.

Kenakin T, Watson C, Muniz-Medina V, Christopoulos A, Novick S (2012) A simple method for quantifying functional selectivity and agonist bias. ACS Chem Neurosci **3**: 193-203.

Klein Herenbrink C, Sykes DA, Donthamsetti P, Canals M, Coudrat T, Shonberg J, Scammells PJ, Capuano B, Sexton PM, Charlton SJ, Javitch JA,

Christopoulos A and Lane JR (2016) The role of kinetic context in apparent biased agonism at GPCRs. Nat Commun **7**: 10842.

Leduc M, Breton B, Galés C, Le Gouill C, Bouvier M, Chemtob S, Heveker N (2009) Functional selectivity of natural and synthetic prostaglandin EP4 receptor ligands. J Pharmacol Exp Ther **331**: 297-307.

Lee MH, Appleton KM, Strungs EG, Kwon JY, Morinelli TA, Peterson YK, Laporte SA and Luttrell LM (2016) The conformational signature of beta-arrestin2 predicts its trafficking and signalling functions. Nature **531**: 665-668.

Leeb-Lundberg LM, Mathis SA, Herzig MC (1994) Antagonists of bradykinin that stabilize a G-protein-uncoupled state of the B2 receptor act as inverse agonists in rat myometrial cells. J Biol Chem **269**: 25970-25973.

Lefkowitz RJ, Hausdorff WP, Caron MG (1990) Role of phosphorylation in desensitization of the beta-adrenoceptor. Trends Pharmacol Sci **11**: 190-194.

Lefkowitz RJ (1998) G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. J Biol Chem **273**: 18677-18680.

Levitt ES, Clark MJ, Jenkins PM, Martens JR, Traynor JR (2009) Differential effect of membrane cholesterol removal on mu- and delta-opioid receptors: a

parallel comparison of acute and chronic signaling to adenylyl cyclase. J Biol Chem **284**: 22108-22122.

Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ (1990) beta-Arrestin: a protein that regulates beta-adrenergic receptor function. Science **248**: 1547-1550.

Lohse MJ, Nuber S, Hoffmann C (2012) Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein-coupled receptor activation and signaling. Pharmacol Rev **64**: 299-336.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science **283**: 655-661.

Luttrell LM, Lefkowitz RJ (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci **115**: 455-465.

Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL, Lefkowitz RJ (2001) Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. Proc Natl Acad Sci USA **98**: 2449-2454.

McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, Davis RJ, Lefkowitz RJ (2000) Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. Science **290**: 1574-1577.

Milano SK, Pace HC, Kim YM, Brenner C, Benovic JL (2002) Scaffolding functions of arrestin-2 revealed by crystal structure and mutagenesis. Biochemistry **41**: 3321-3328.

Murayama T and Ui M (1985) Receptor-mediated inhibition of adenylate cyclase and stimulation of arachidonic acid release in 3T3 fibroblasts. Selective susceptibility to islet-activating protein, pertussis toxin. J Biol Chem **260**: 7226-7233.

Nambi P, Peters JR, Sibley DR, Lefkowitz RJ (1985) Desensitization of the turkey erythrocyte beta-adrenergic receptor in a cell-free system. Evidence that multiple protein kinases can phosphorylate and desensitize the receptor. J Biol Chem **260**: 2165-2171.

Namkung Y, Le Gouill C, Lukashova V, Kobayashi H, Hogue M, Khoury E, Song M, Bouvier M, Laporte SA (2016) Monitoring G protein-coupled receptor and β-arrestin trafficking in live cells using enhanced bystander BRET. Nat Comun - in Press.

Nobles KN, Xiao K, Ahn S, Shukla AK, Lam CM, Rajagopal S, Strachan RT, Huang TY, Bressler EA, Hara MR, Shenoy SK, Gygi SP, Lefkowitz RJ (2011)

Distinct phosphorylation sites on the β(2)-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. Sci Signal **4** (185): ra51.

Nygaard R, Zou Y, Dror RO, Mildorf TJ, Arlow DH, Manglik A, Pan AC, Liu CW, Fung JJ, Bokoch MP, Thian FS, Kobilka TS, Shaw DE, Mueller L, Prosser RS, Kobilka BK (2013) The dynamic process of β(2)-adrenergic receptor activation. Cell **152**: 532-542.

Oates J, Watts A (2011) Uncovering the intimate relationship between lipids, cholesterol and GPCR activation. Curr Opin Struct Biol **21**: 802-807.

Pontier SM, Percherancier Y, Galandrin S, Breit A, Galés C, Bouvier M (2008) Cholesterol-dependent separation of the beta2-adrenergic receptor from its partners determines signaling efficacy: insight into nanoscale organization of signal transduction. J Biol Chem **283**: 24659-24672.

Quoyer J, Janz JM, Luo J, Ren Y, Armando S, Lukashova V, Benovic JL, Carlson KE, Hunt SW 3rd, Bouvier M (2013) Pepducin targeting the C-X-C chemokine receptor type 4 acts as a biased agonist favoring activation of the inhibitory G protein. Proc Natl Acad Sci USA **110**: E5088-5097.

Roth BL and Chuang DM (1987) Multiple mechanisms of serotonergic signal transduction. Life Sci **41**: 1051-1064.

Samama P, Pei G, Costa T, Cotecchia S, Lefkowitz RJ (1994) Negative antagonists promote an inactive conformation of the β2-adrenergic receptor. Mol Pharmacol **45**: 390-394.

Santos GA, Duarte DA, Parreiras-E-Silva LT, Teixeira FR, Silva-Rocha R, Oliveira EB, Bouvier M, 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.

Saulière A, Bellot M, Paris H, Denis C, Finana F, Hansen JT, Altié MF, Seguelas MH, Pathak A, Hansen JL, Sénard JM, Galés C (2012) Deciphering biased-agonism complexity reveals a new active AT1 receptor entity. Nat Chem Biol **8**: 622-630.

Schann S, Bouvier M, Neuville P (2013) Technology combination to address GPCR allosteric modulator drug-discovery pitfalls. Drug Discov Today Technol **10**: e261-267.

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.

Stallaert W, Christopoulos A, Bouvier M (2011) Ligand functional selectivity and quantitative pharmacology at G protein-coupled receptors. Expert Opin Drug Discov **6**: 811-825.

Szakadáti G, Tóth AD, Oláh I, Erdélyi LS, Balla T, Várnai P, Hunyady L, Balla A (2015) Investigation of the fate of type I angiotensin receptor after biased activation. Mol Pharmacol **87**: 972-981.

Thompson GL, Robert Lane J, Coudrat T, Sexton PM, Christopoulos A, Canals M (2016) Systematic analysis of factors influencing observations of biased agonism at the mu-opioid receptor. Biochem Pharmacol - in Press.

Tsvetanova NG, von Zastrow M (2014) Spatial encoding of cyclic AMP signaling specificity by GPCR endocytosis. Nat Chem Biol 10: 1061-1065.

van der Westhuizen ET, Breton B, Christopoulos A, Bouvier M (2014) Quantification of ligand bias for clinically relevant β2-adrenergic receptor ligands: implications for drug taxonomy. Mol Pharmacol **85**: 492-509.

Vilardaga JP, Gardella TJ, Wehbi VL, Feinstein TN (2012) Non-canonical signaling of the PTH receptor. Trends Pharmacol Sci **33**: 423-431.

Vilardaga JP, Jean-Alphonse FG, Gardella TJ (2014) Endosomal generation of cAMP in GPCR signaling. Nat Chem Biol **10**: 700-706.

Wehbi VL, Stevenson HP, Feinstein TN, Calero G, Romero G, Vilardaga JP (2013) Noncanonical GPCR signaling arising from a PTH receptor-arrestin-Gβγ complex. Proc Natl Acad Sci USA **110**: 1530-1535.

Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, 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.

Wess J (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. FASEB J **11**: 346-354.

Wiley HS, Burke PM (2001) Regulation of receptor tyrosine kinase signaling by endocytic trafficking. Traffic **2**: 12-18.

Zhang J, Ferguson SS, Barak LS, Aber MJ, Giros B, Lefkowitz RJ, Caron MG (1997) Molecular mechanisms of G protein-coupled receptor signaling: role of G protein-coupled receptor kinases and arrestins in receptor desensitization and resensitization. Receptors Channels **5**: 193-199.

Zhang J, Ferguson SS, Barak LS, Bodduluri SR, Laporte SA, Law PY, Caron MG (1998) Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. Proc Natl Acad Sci USA. **95**: 7157-7162

Zhang X, Stevens RC, Xu F (2015) The importance of ligands for G protein-coupled receptor stability. Trends Biochem Sci **40**: 79-87.

Zidar DA, Violin JD, Whalen EJ, 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, Beautrait A, Aguila B, Charles R, Escher E, Claing A, Bouvier M, Laporte SA (2012) Differential β-arrestin-dependent conformational signaling and cellular responses revealed by angiotensin analogs. Sci Signal **5**: ra33.

**Footnotes:**

CMCN holds a Brazilian National Council for Scientific and Technological Development, CNPq, research fellowship and his research on the topic of this review is supported by the Sao Paulo State Research Foundation, FAPESP, [Grant 2012/20148-0] and by the Ribeirao Preto Medical School, University of São Paulo. LTPS holds a FAPESP post-doctoral fellowship and was a recipient of a FAPESP international fellowship for a training period of one year at MB's laboratory. MB holds the Canada Research Chair in Signal Transduction and Molecular Pharmacology and his Research on the topic of this review is supported by the Canadian Institute for Health Research. CMCN and MB have a joint international cooperation grant funded by FAPESP [Grant SPRINT 2015/50086-4] on the subject of this review.

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


**Legends to the Figures:**

**Figure 1. Representation of energy landscapes for GPCR activation.** The energy landscape of GPCR is represented as a function of the activation path of a receptor from the resting state to the "G protein active state" following stimulation with ligands. The represented minima in each landscape graph correspond to stable conformations. Panel **A** illustrates the classical view that the unbound receptor (R), upon ligand binding (RL), would reach a unique ternary complex ligand-receptor-G protein (RLG*) low energy state, corresponding to the active conformation that triggers G protein dependent signaling. Panel **B** illustrates our current view that upon ligand binding, the receptor can be stabilized in several intermediate active conformations. All such intermediate active conformations allow coupling to G proteins, but also bear structural differences that could be reflected in coupling/activation of other pathways. In panel **B**, the distinct intermediate active states are represented by LRG*$^{a}$, LRG*$^{b}$, LRG*$^{c}$, and LRG*$^{d}$. Panel **B** also illustrates that Ligands X and Y, despite being able to both trigger G protein activation, may yield distinct signaling profiles since they differentially stabilize different subsets of intermediate active conformations.

**Figure 2. Impact of different data acquisition times on signaling profiles.** The signaling profiles for each ligand is illustrated using a web representation of activity where each radius of the web represents the extent of activation of a specific pathway arbitrarily named **A** to **H** in the present hypothetical example. The webs represent the hypothetical activation of a given receptor by the same

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


ligand, where activation of signaling pathways **A** to **H** are measured at 5, 10, and 30 minutes after stimulus. As can be observed in the red and blue overlapping web-chart, pathways **A** to **H** reach similar extents of activation at 5 and 10 minutes after stimulus. On the other hand, the blue and green overlapping web-chart shows that only pathways **A**, **B** and **F** display a similar activation profile when comparing data acquired at 5 and 30 minutes. This figure highlights that conclusions about the distinct signaling profiles of different ligands can be greatly influenced by the choice of data acquisition times.

**Figure 3. Schematic representation of a hypothetical platform for "functional prediction" based on clustering of compounds with similar "signaling signatures" in different cell lines.** Although the signaling pathways triggered by a given compound are expected to be different in different cell lines, compounds sharing similar signaling profiles in a given cell line would share a common "signaling signature" in another cell line allowing a clustering of compounds in distinct groups. In the example, although compound A, D and F have different signaling profiles in the 3 cell types tested, they remained clustered within each of the cell types representing a "signaling signature". Such clustering would then be used to link signaling signatures to specific biological/clinical outcomes and make predictions about the efficacy and safety of drug candidates.

**Figure 4. Hypothetical heat-map representing signaling activation patterns triggered by different compounds.** Panel **A** shows the activation patterns of compounds A to F in pathways 1 to 5 using a low sensitivity assay. As

Molecular Pharmacology Fast Forward. Published on September 16, 2016 as DOI: 10.1124/mol.116.105940
This article has not been copyedited and formatted. The final version may differ from this version.


highlighted, compounds A and E seem to yield a similar activation profile in pathway 4. Panels **B** and **C** illustrate that when a more sensitive and/or amplified assay is used, such as evaluation of downstream targets or gene expression, important differences can be unveiled.

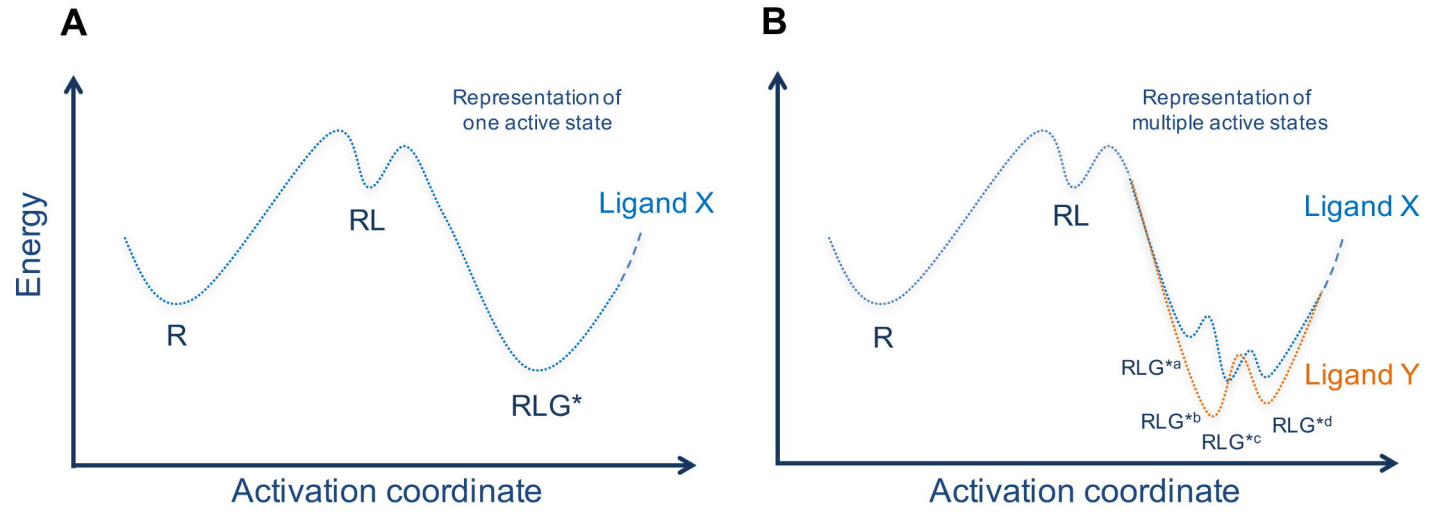
A
Representation of
one active state
Energy
RL
Ligand X
R
RLG*
Activation coordinate
B
Representation of
multiple active states
RL
Ligand X
R
RLG*a
Ligand Y
RLG*b
RLG*c
RLG*d
Activation coordinate

**Figure 1**

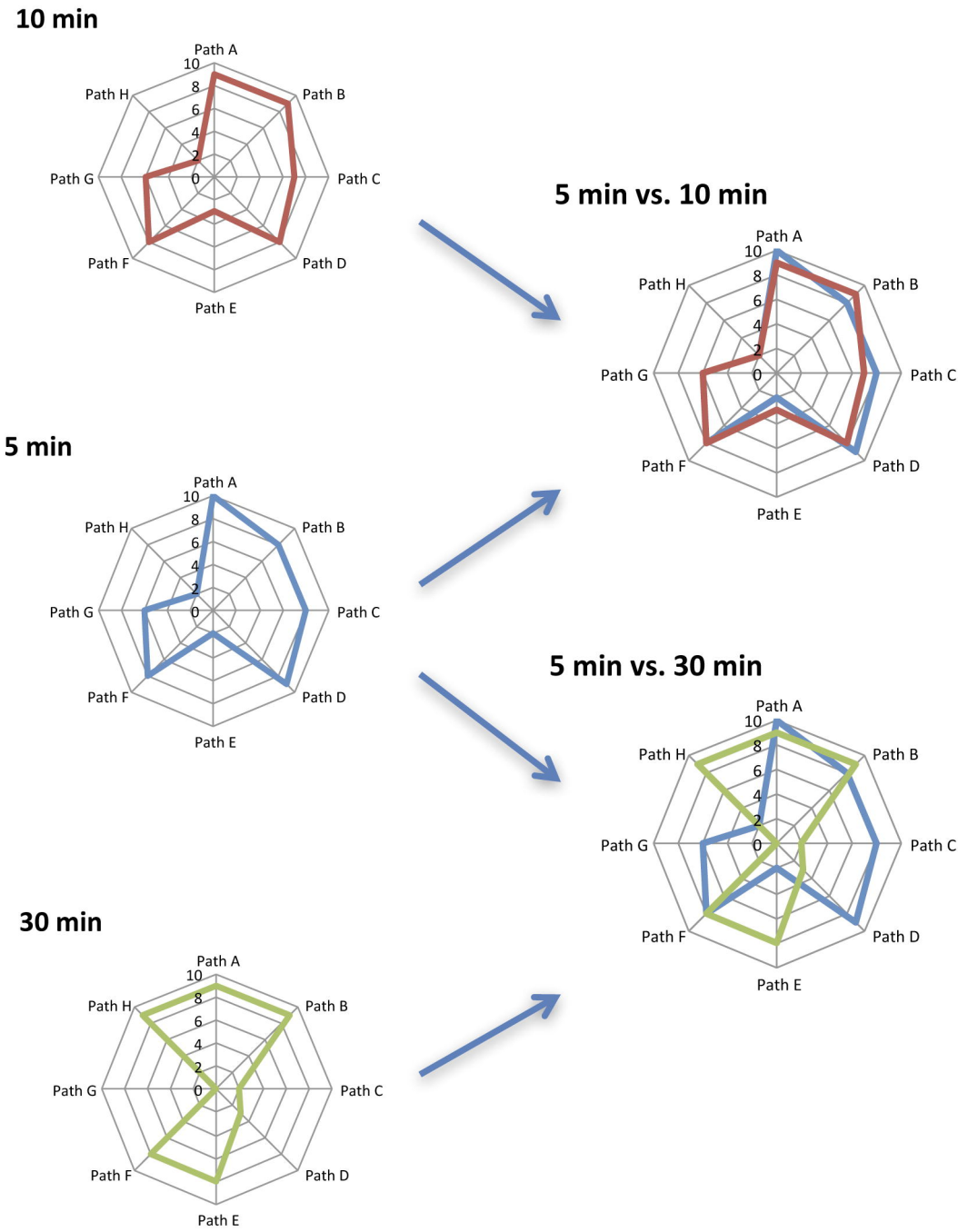
10 min
Path A
Path B
Path C
Path D
Path E
Path F
Path G
Path H
10
8
6
4
2
0
5 min vs. 10 min
5 min
5 min vs. 30 min
30 min

Figure 2

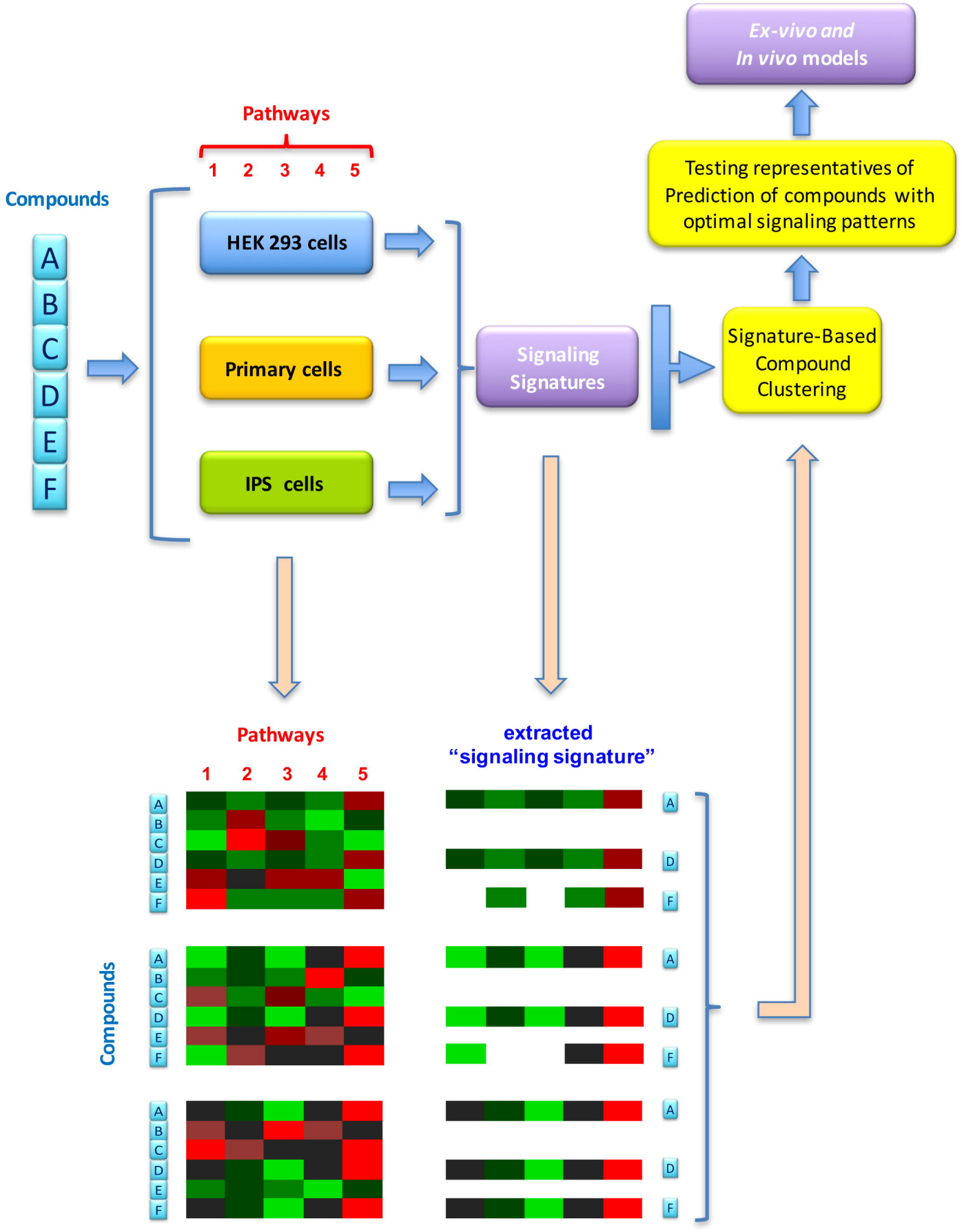
Ex-vivo and
In vivo models
Testing representatives of
Prediction of compounds with
optimal signaling patterns
Signature-Based
Compound
Clustering
Signaling
Signatures
Pathways
1 2 3 4 5
Compounds
A
B
C
D
E
F
HEK 293 cells
Primary cells
IPS cells
Pathways
1 2 3 4 5
extracted
"signaling signature"
Compounds

**Figure 3**

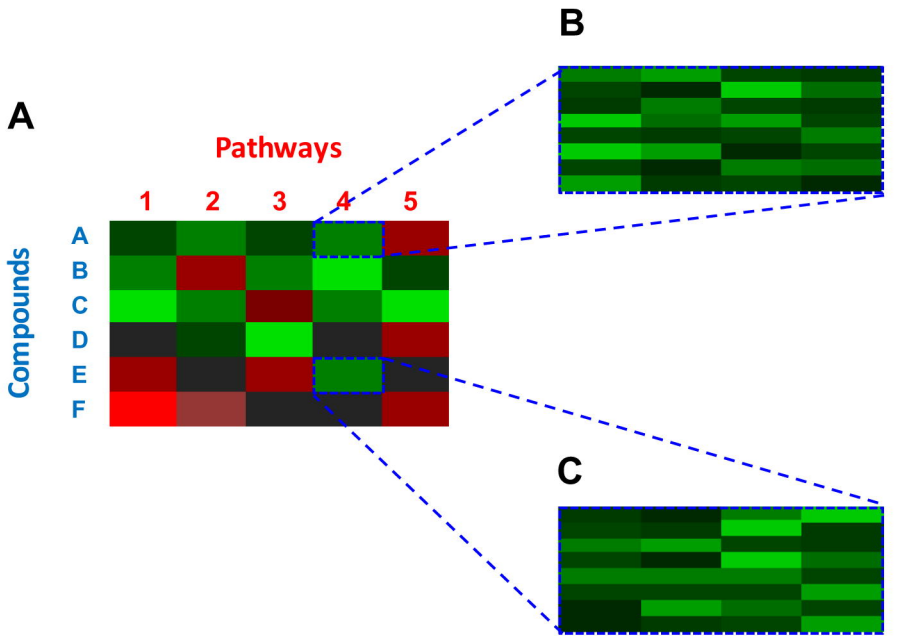


**Figure 4**