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Delineating biased ligand efficacy at 7TM receptors from an experimental perspective

Ségolène Galandrin[a,1], Lauriane Onfroy[a,1], Mathias Charles Poirot[a,1], Jean-Michel Sénard[a,b], Céline Galés[a,*]

[a] Institut des Maladies Métaboliques et Cardiovasculaires (I2MC), INSERM, UMR 1048, Université Toulouse, F-31432 Toulouse, France
[b] Service de Pharmacologie Clinique, Faculté de médecine, Centre Hospitalier Universitaire de Toulouse, Université de Toulouse, F-31000 Toulouse, France

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ABSTRACT

During the last 10 years, the concept of "biased agonism" also called "functional selectivity" swamped the pharmacology of 7 transmembrane receptors and paved the way for developing signaling pathway-selective drugs with increased efficacy and less adverse effects. Initially thought to select the activation of only a subset of the signaling pathways by the reference agonist, bias ligands revealed higher complexity as they have been shown to stabilize variable receptor conformations that associate with distinct signaling events from the reference. Today, one major challenge relies on the *in vitro* determination of the bias and classification of these ligands, as a prerequisite for future *in vivo* and clinical translation. In this review, current experimental considerations for the bias evaluation related to choice of the cellular model, of the signaling pathway as well as of the assays are presented and discussed.

1. Biased agonism at a glance

Seven transmembrane (7TM) receptors also commonly known as G protein coupled receptors (GPCRs) constitute the largest family of cell surface receptors. As general sensors/convertors of extracellular stimuli at the plasma membrane into intracellular signaling modules, they ensure cells to adapt to their environment, thus orchestrating a broad range of physiological processes. Obviously, they have for long time been considered attractive pharmaceutical targets with, today, ~35% of marketed drugs. However, despite providing the desirable effect, these drugs generally face the problem of concomitant adverse events that sometimes unbalance the clinical benefit/risk ratio and thus limit their use. They have been classically associated for long with an inherent lack of receptor selectivity of these drugs, *i.e.* off-target effects. However, these last 15 years, careful dissection of the molecular mechanisms underlying GPCRs ligand efficacy shed new light on this aspect.

According to the classical pharmacology, 7TM receptors were believed to work linearly as on/off switches, activating or shutting down the whole signaling array associated with a given receptor and with all agonists activating a similar set of signaling pathways (Fig. 1A). In line with this concept, ligand efficacy has been defined according to its ability to oscillate the receptor from its inactive (off) to active (on) state. Accordingly, full and partial agonists oscillate the receptor toward the active state, full and partial inverse agonists favor the transition of the receptor toward inactive state, while neutral antagonists do not modify the on-off equilibrium while blocking agonist/inverse agonist action. However, the revolutionary concept of "biased agonism" or ligand-functional selectivity" (Kenakin, 1995, 2011) postulated that some ligands can indeed stabilize different active receptor conformations and thus elicit activation of only a subset of signaling pathways associated with the receptor-reference ligand (Fig. 1B) or even new signaling (Fig. 1C) (Santos et al., 2015; Sauliere et al., 2012). This now well-established paradigm dramatically impacts 7TM receptors drug discovery, since it was clearly demonstrated that biased ligands can activate or block specific intracellular pathways linked to disease outcomes. In the end, biased agonism paves the way for the development of pathway-specific drugs with increased efficacy and less adverse effects for an ensuing better clinical benefit/risk ratio. For instance, TRV130, a G protein-biased ligand currently in Phase III clinical trials for the treatment of acute pain dissociates opiates-

* Corresponding author at: INSERM, UMR1048, Institut des Maladies Métaboliques et Cardiovasculaires - I2MC 1, Avenue Jean-Poulhès - BP84225, 31432 Toulouse, Cedex 4, France.
E-mail address: celine.gales@inserm.fr (C. Galés).
[1] These authors contributed equally to this work.

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related respiratory depression from desirable analgesia by favoring the G protein dependent signaling of the μ opioid receptor over the β-arrestin deleterious one (Viscusi et al., 2016). Conversely, TRV120027, a β-arrestin-biased AT1-R ligand, is currently in Phase IIb for the treatment of acute heart failure with promising specific targeting of the β-arrestin dependent myocardial protection (increased contractility and decreased apoptosis) while antagonizing the deleterious Gq-dependent vasoconstriction (Felker et al., 2015).

Biased agonism deeply modifies the pharmacological classification of ligand efficacy which does no longer exclusively relate on a ligand-receptor (L-R) pair as previously stated, but rather on a ligand-receptor-effector (L-R-E) tripartite, thus highlighting the pluridimensionality of efficacy (Galandrin et al., 2007). Accordingly, one ligand can elicit agonist efficacy on the R-E1 couple while acting at the same time as an antagonist on the R-E2 one, for the same receptor. The 6′-Guanidinonaltrindole (6′-GNTI) dual G protein-biased agonist and β-arrestin antagonist at the κ-oipoid receptor nicely illustrates this notion (Rives et al., 2012). Now, from drug discovery standpoint, identification of biased ligands in the context of the pleiotropic 7TM receptor signaling is not an easy task and raises several questions: which cellular model to select? Which signaling pathway to probe? Which assay to use? How to quantify/illustrate it? We will try to discuss these different aspects in the following sections.

2. Choice of the cellular model

2.1. *Primary cell lines* versus *heterologous systems*

One of the first questions arising when initiating the identification of a biased ligand relies on the choice of the cellular model to be used. Indeed, it is clear that the significance of the *in vitro* bias of a ligand with its *in vivo* activity remains totally elusive. Obviously, the *in vivo* response to a ligand does not arise from a unique cell response but most probably results from the overall actions of specific concentration of both target receptor and ligand in different cell types in the organism, all producing different levels of bias. Moreover, we are still far from understanding the physiological relevance of the intracellular signaling pathways, thus making difficult the assumption to screen for a specific signaling pathway over the other in view of an expected clinical outcome. Another complexity arises from the pathophysiological state when using such biased ligands since diseases can also impact on the intracellular pathways and thus will modify the bias activity. In this context, *in vitro* biased ligand characterization has to be viewed as a simple first step for the identification of compounds exhibiting selective signaling signature with no *a priori* on the cell line. The cell can thus be imaged as a test tube allowing screening for a maximum of signaling possibilities. Nevertheless, cell lines can really differ in terms of intracellular 7TM receptor effector content that will largely influence the output signaling (see Section 2.2). This will have major impact particularly when evaluating biased ligand activity using cell assays that play with all the endogenous signaling machinery. Thus, incorporating at least two quite different cell lines for the bias detection should increase the probability of positive hits.

Despite not necessarily essential for a first screening, the use of primary human cell types from multiple tissues offers the possibility to determine ligand biased activity in terms of phenotypic impact on relevant diseases with endogenous level of receptors, *i.e.* the use of immune cells in the context of inflammation, fibroblasts in fibrosis, but their scarcity impedes large scale screening. It will also comfort about drug pharmacological profile and its future *in vivo* translation when bias activity identified in a simplified culture system is preserved in a more physiologically relevant system. For instance, the original pharmacological profile of 6′-GNTI as a G protein-biased agonist but β-arrestin pathway antagonist at the κ-opioid receptor was originally described in HEK cells (Rives et al., 2012) and was further confirmed in CHO and also in primary neuronal cultures where κ-opioid receptor is expressed endogenously, paving the way for its *in vivo* use as a potential valuable nonaddictive analgesic with less β-arrestin dependent-dysphoric adverse effect (Schmid et al., 2013). Recombinant immortalized cell lines could be considered as the better alternative for the bias detection. To date, more than 4000 cell lines relevant for multiple physiological and pathological area are available (ATCC bank) but CHO and HEK293T are the most popular ones for 7TM receptors drug screening. They are generally easy to culture, reasonably amenable for transfection thus facilitating the creation of a functional assay and more importantly allowing an accurate setting of receptor/signaling effectors stoichiometry known to easily influence bias responses, and finally they are prone to high throughput screening (HTS) (Sato et al., 2007). Essentially dictated by functional assay restrictions, the bias characterization results in most cases from multiple comparisons between mixed functional tests obtained from both transient transfected heterologous cell lines (over-expressing the receptor of interest and some signaling probes) and from stable transfected ones (stably-expressing the receptor of interest). However, delineating the bias of a ligand relies on its capability to favor the activation of specific intracel-

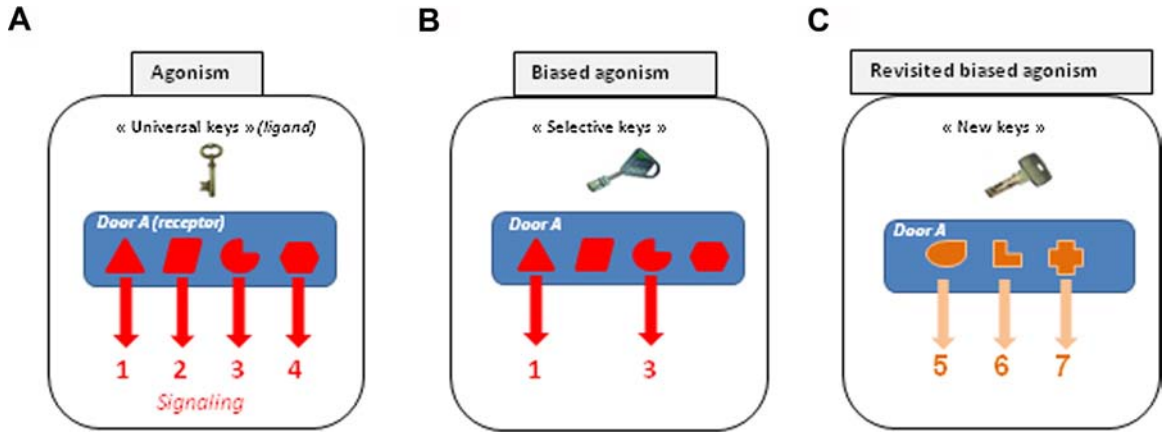

Fig. 1. Past and present of agonism. (A) Classical model of agonism (*Universal keys*): the agonists activate simultaneously all signaling pathways (1–4) associated with a receptor (*Door A*). (B) Recent concept of biased agonism: some agonists can select among receptor signaling pathways (1–3). (C) Revisited biased agonism: some agonists can promote activation of signaling pathways unrelated to those activated by the reference agonist.

lular signaling pathways (*e.g.* G protein over β-arrestin pathway), and thus to compare the pharmacological profile of the drug on the different pathways. Also, one would expect to be allowed to compare the signaling pathways, meaning to use at least a common cellular background with similar receptor densities for each assay. Up to now, the field largely fails on this criterion as some assays to explore receptor activity require receptor as well as effector overexpression (*i.e.* β-arrestin assays, see Section 4.2). Consequently, such experiments using different cell systems should be interpreted with caution. Beyond the questions of protein stoichiometry, another problem restricting the comparison between results obtained in transient *versus* stable cell lines relates to the integration of the transgene in stable transfection since it can permanently alter the cell genotype/phenotype (Lin et al., 2014) and thus potentially modify the standard signaling pathways of a given cell. By contrast, transiently transfected genes are only expressed for a limited period of time and are not integrated into the genome. Overall, transient transfection of receptors together with the different signaling probes, followed by an accurate calibration of the receptor/signaling probes in each experimental setting appears maybe as a model of choice to compare different signaling pathways in the context of biased behavior of 7TM receptors ligands.

More recently, the fruit fly has gained interest for drug screening despite not yet used in the 7TM receptor biased agonism field (Dar et al., 2012; Gasque et al., 2013). However, this model harbors the perfect characteristics expected for a rapid screening of 7TM receptor biased ligands: integration of the drug bias signaling on the whole organism with physiological outcomes. The fruit fly model indeed parallels to a certain level the human physiology/pathophysiology by involving similar molecular pathways that play a role in the main functions/dysfunctions as for instance in cancer or diabetes (Pandey and Nichols, 2011). The easy genetic manipulation of *Drosophila* also allows a rapid understanding of the interconnection between a specific signaling pathway and a physiological/pathophysiological outcome. This approach recently led to the elegant identification of different effectors from specific intracellular pathways whose pharmacological targeting led to an optimum therapeutic index for cancer (Dar et al., 2012).

2.2. Cellular content and protein expression levels

Taking a cellular model as bait for drug screening tackles the problem of its endogenous protein background that will differ in between cells and will ultimately dictate different signaling responses. Mass spectrometry-based proteomics of 11 human cell lines revealed marked disparities in the expression levels of the proteins despite high similarities in the proteome by itself (Geiger et al., 2012). Thanks to mRNA quantification, Atwood et al., also confirmed huge disparities in the pattern of expression of all class A, B and C 7TM receptors and related signaling proteins in four cell lines commonly used for the study of 7TM receptors signaling (HEK293, AtT20, BV2 and N18) (Atwood et al., 2011). The stoichiometry of 7TM receptors and their intracellular signaling effectors, *i.e.* G proteins, has long been shown to be crucial in conducting specific signaling pathways both in primary and heterologous cells (Kenakin, 1997; Newman-Tancredi et al., 2000; Newman-Tancredi et al., 1997) and it is therefore not surprising that a ligand-receptor pair can promote different output signaling in two different cell lines (Halls et al., 2009; Sauliere et al., 2012). Paradoxically, this concept has been often forgotten in the pharmacological characterization of biased molecules and studies, most of the time, tend to delineate biased activity of a compound based on the comparison of diverse signaling pathways each performed in a cell line exhibiting different expression levels of receptor and signaling effectors (see Section 2.1). Furthermore, cell-type variation in ligand efficacy has been observed using cellular impedance or dynamic mass redistribution assays (Deng et al., 2013; Peters and Scott, 2009) or conventional signaling measurements (Charfi et al., 2014; Sauliere et al., 2012). For example, in 2013, Deng et al., systematically measured efficacies of seven agonists of the endogenous M3 muscarinic receptor expressed to different levels in six different cell lines and demonstrated that the receptor can couple to diverse signaling pathways with distinct biased activities of muscarinic agonists depending on the cell line (Deng et al., 2013). Finally, one would generally give preference to biased activity screening on so-called "physiological expression levels of receptors" when possible. However, the ultimate purpose of biased ligands relies on their use in pathophysiological conditions when both 7TM receptors and signaling effectors are prone to radical modifications of their expression levels. In heart failure, cardiomyocytes undergo downregulation of β1-AR expression as well as Gαi2 proteins (Eschenhagen et al., 1992) or Gαs (Longabaugh et al., 1988) but also secondary effectors like adenylyl cyclases V/VI (Ishikawa et al., 1994) that contribute to alterations of the β-signaling (Bristow et al., 1982). Cancer (Yajima et al., 2012) or Parkinson (Corvol et al., 2004) diseases have also been associated with alteration of the expression levels of G protein subunits. Besides, it is clear that receptor expression levels can influence ligand efficacy as shown for high density of β3-AR that turned SR59230A from an antagonist to an agonist for cAMP formation when compared to low receptor densities (Sato et al., 2007). Hence, there is no perfect cellular expression receptor/signaling effectors level for biased research but considering two (or more) expression levels of the 7TM receptor of interest in a similar cellular background could nevertheless favor the appreciation of the different "textures" of a ligand at this receptor.

2.3. Physical properties of cellular plasma membranes

Given that 7TM receptors are transmembrane proteins, it is not surprising that the plasma membrane can markedly influence their activity by modulating their 3D conformation. Accordingly, biased activity of the ligands should be impacted as well. Plasma membrane is a nano-organized structure composed of a variety of lipids and proteins whose proportion differs among cells according to their origin, specialization and pathophysiological state. Those differences can impact on 7TM receptors conformational landscape through three mechanisms:

– First, lipid and protein composition modifies the physical constraints the plasma membrane exerts on 7TM receptors landscape. This has been well demonstrated for the rhodopsin receptor (Botelho et al., 2002). Recently, membrane stretch has also been shown to allosterically modulate β-arrestin-biased Angiotensin II type I receptor signaling (Tang et al., 2014).

– Second, the nanoscale organization of the plasma membrane promotes subcellular compartmentalization of 7TM receptors and related proteins in specific domains. For instance, spatiotemporal diffusion and bioactivity of 7TM receptors and membrane-anchoring proteins are partially controlled by the actin-based cytoskeleton (Kusumi et al., 2012). A large body of evidence, obtained from primary and heterologous cell lines, also clearly established that plasma membrane cholesterol- and sphingolipids-enriched domains, so-called lipid raft nanodomains, can influence many GPCR signaling cascades by partitioning 7TM receptors, G proteins and their various effectors. This phenomenon has been observed for many receptors (Insel et al., 2005) and was largely exemplified for β-adrenergic signals in cardiomyocytes (Xiang, 2011) or heterologous cells (Pontier et al., 2008). Agonist-directed trafficking is also determined by receptor location within the membrane domains as shown for the μ-opioid receptor (Zheng et al., 2008). In the same line of evidence, membrane lipids composition can modulate 7TM/G proteins interaction (Inagaki et al., 2012).

Moreover, lipid rafts have been assigned a crucial role in pathological contexts such as heart failure, Alzheimer's disease or spongiform encephalopathies, either by their disruption or their ability to concentrate ectopic proteins, thus disrupting the physiological signaling (Michel and Bakovic, 2007).

– Third, the influence of lipids on 7TM receptors signaling could arise from a direct mechanism, mediated by lipids interaction with specific domains of the 7TM receptor. Cholesterol binding motif has been shown to be present in several transmembrane proteins (Li et al., 2001; Scolari et al., 2010) and accordingly, cholesterol was reported to have a regulatory role on the protein functions (Derler et al., 2016). In 2007 and 2008, crystal structures of the β2-AR receptor allowed the observation of a direct cholesterol-receptor interaction (Cherezov et al., 2007; Hanson et al., 2008). However, the first identification of a cholesterol-binding motif in β2-AR, as well as in rhodopsin and serotonin-1A receptors, was established in 2011 by Chattopadhyay and colleagues (Jafurulla et al., 2011). This direct cholesterol-7TM receptors interaction could be important for the modulation of the conformational landscape of the activated 7TM receptor. Indeed, cholesterol has been shown to modulate biophysical properties of the β2-AR (Zocher et al., 2012). Apart from cholesterol, Govaerts and colleagues also demonstrated that phospholipids can act as positive (phosphatidylcholine) or negative (phophatidylethanolamine) allosteric modulators influencing agonist/antagonist binding and activation state of the β2-AR (Dawaliby et al., 2016).

Based on the preceding observations, an important technical aspect when comparing different signaling pathways in a cell line relies on the nature of the agent used for transfection among assays or when comparing assays using transfected and non-transfected cells for evaluation of biased efficacy of ligands (*i.g.* II messenger assay *versus* β-arrestin recruitment, see Section 4). Indeed, many transfection methods have been developed to introduce foreign nucleic acids into cells (biological, chemical, and physical) but the use of cationic lipids (lipofection) is the most popular. However, this later method is not completely neutral and can influence the physical properties of the cellular plasma membrane as liposome vesicle DNA carriers are made of phospholipid layer that can merge with phospholipids of the plasma membrane. This can thus influence the conformations of 7TM receptors as well as those of the signaling transducers anchored in the plasma membrane. In agreement, Lonez et al., reported that cationic lipids activate intracellular signaling pathways (Lonez et al., 2012). Thus, lipid-based transfection can change the basal signaling background of the cell and then could potentially interfere with the determination of ligand biased activity.

2.4. Microenvironment, extracellular matrix and cell density

The cell microenvironment includes all factors and elements surrounding the cell that influence its activity. Those factors include other cells, signaling molecules and the extracellular matrix (ECM). In multicellular organized tissues, the extracellular space surrounding cells is composed of a complex layout of tissue-specific polysaccharides and proteins, such as collagen, laminin, fibronectin, elastin constituting the ECM. It is now well established that the ECM is not just a structural scaffold for cells but more an instructive and communicant environment dictating the cell behavior as its morphology and function (migration, survival) (Gospodarowicz et al., 1980; Kim et al., 2011). The bi-directional communication between cells and ECM, mostly *via* integrins, allows a constant dynamic change of the ECM to facilitate cell plasticity as required in development. The cell response to ECM components is highly variable. It has been well shown that different ECM coating proteins in tissue culture modify cell properties such as adherence, morphology and proliferation (Ragetly et al., 2010; Vleggeert-Lankamp et al., 2004), a phenomenon that can rely on differential gene expression regulation (Heng et al., 2010). It follows that the comparison of the cellular signaling pathways for characterization of a biased ligand should pay attention to the use of similar ECM conditioning of the cell for each of these pathways. However, we are far from such assay calibration since some bias studies are mixing assays performed on adherent *versus* suspended cells (*i.e.* BRET studies in cell suspension *versus* II messengers studies on adherent cells) despite we know that the loss of cell adhesion can profoundly affect cellular intracellular signaling as it was shown for cAMP production (Norambuena and Schwartz, 2011). In a first biased screen, the importance is not to support a cell status *versus* another one but mainly to have a consensus on the cell status in between signaling assays so to be allowed to establish the biased signature of the ligand.

Finally, cell density/contact can also differently impact on biological processes. Hence, Heng et al., demonstrated that the proliferation rate of human umbilical vein endothelial cells is tightly under control of cell-seeding density (Heng et al., 2011). Thus, cell seeding below/above a specific density threshold can cause a non-optimal cell proliferation. Moreover, cell density has been shown to be an important modulatory factor in ligand agonism (Koizumi et al., 2003) as well as in biased efficacy of the ligands (Kaya et al., 2012; Satoet al., 2007). Cell density will also selectively influence the signaling output. For example, Koizumi et al., demonstrated that a high level of MC3T3-E1 density increase the $[Ca^{2+}]_i$ at basal state while decreasing the cell-response sensitivity to ligand stimulation (Koizumi et al., 2003). Kaya et al., studied cAMP and ERK responses in confluent or suspended HEK cells upon stimulation with β2-AR ligands (Kaya et al., 2012). They showed that the Gs-induced cAMP-mediated ERK activation through β2-AR was apparently inhibited by cell-contact whereas the Gi/o-mediated ERK activation was insensitive.

3. Evaluation of biased agonism: choice of the signaling pathways

3.1. 7TM receptors canonical versus non-canonical signaling pathways

3.1.1. G protein signaling pathways

Despite recent evidences suggesting the existence of some G protein-independent signaling, the activation of heterotrimeric G proteins represents the only hallmark common to all 7TM receptors and initiated immediately after agonist binding to the receptor (Denis et al., 2012). Heterotrimeric G proteins are composed of three αβγ associated subunits (α, each encompassing multiple isoforms with potential creation of a myriad of heterotrimers although each cell type displays selectivity in G protein expression. Basically, they have been classified into four families Gαs, Gαi/o, Gαq/11 and Gα12/13, relying on the Gα subunit isoform that dictates the specificity of the receptor intracellular signaling (Fig. 2).

Only few biased ligands that specifically target the G protein signaling have been identified so far with some of them presenting potential therapeutic values. As extreme example of G protein biased ligands, oxytocin-receptor ligand atosiban selectively activates Gαi1 and Gαi3 but completely fails to recruit β-arrestin (Busnelli et al., 2012) while 6′-GNTI mediates partial G protein activation at the κ-opioid receptor while antagonizing the β-arrestin recruitment to the receptor (Rives et al., 2012; Schmidet al., 2013) and so far, no-one of these drugs have been yet tested for their *in vivo* performances. TRV130 acts as a potent analgesic without the β-arrestin-related gastrointestinal and respiratory dysfunctions in mice, consistent with its selective G protein-biased agonism at the μ-opioid receptor with no activity on the β-arrestin pathway

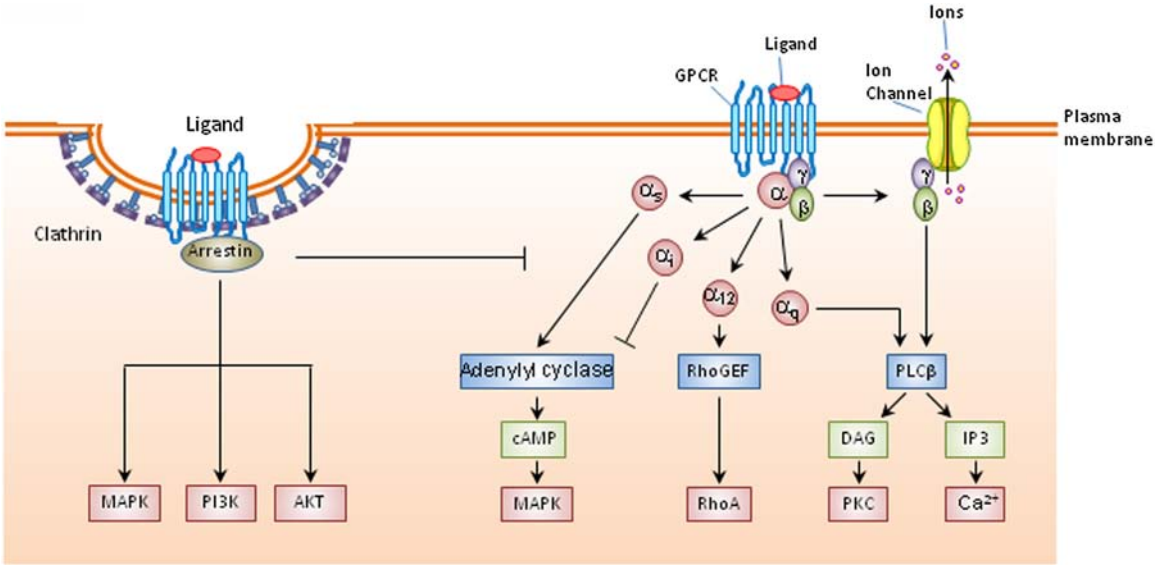

Fig. 2. 7TM receptors canonical signaling pathways. Illustration of the main G protein-dependent and independent signaling induced upon 7TM receptor activation.

(DeWire et al., 2013). More recently, preclinical studies testing a new potent and selective Glucagon-like peptide-1 receptor (GLP-1R) G protein-biased agonist in diabetic mice demonstrated its remarkable antidiabetic effects, suggesting GLP-1R-G protein-biased agonists as a novel therapeutic approach to type 2 diabetes mellitus (Zhang et al., 2015). Indeed, the *in vivo* effects of G protein pathway-selective biased ligands are unlikely predictable and up to now the physiological significance of G protein-signaling have been more likely inferred indirectly from the use of β-arrestin knockout mice (Allen et al., 2011; Bohn et al., 2000; Bohn et al., 1999; Conner et al., 1997). However, some of the G protein-mediated processes have been elucidated using genetic mouse models in physiology and pathophysiology (Wettschureck et al., 2004), but G protein redundancy and lethality of the knockout models provide a mean for generation of tissue-specific or inducible gene deletion of the G protein subunits so to delineate the specific *in vivo* functions of each individual G protein subunits in the different tissues. There is thus a need in the future to explore the wide-range biological functions of the heterotrimeric G proteins but also to get further insight into the *in vivo* relevance of G protein-biased ligands.

Although Gβγ complex was initially thought to only favor Gα anchorage to the plasma membrane and to stabilize its inactive state, it is now well established that the Gβγ complex is also able to control many different signaling pathways on their own following 7TM receptors activation (Dupre et al., 2009). The effectors of Gβγ-signaling include various ion channels, such as G-protein-regulated inwardly rectifying K^+ channels (GIRKS), P/Q and N-type voltage-gated Ca^{2+} channels, as well as some isoforms of adenylyl cyclases and PLC, along with some phosphoinositide-3-kinase (PI3 K) isoforms. The existence of dual independent Gα and Gβγ signaling was nicely illustrated by the recent discovery of the first biased ligand that discriminates between Gα and Gβγ subunits (Blattermann et al., 2012). In this study, the authors have identified Gue1654 as a specific oxoeicosanoid receptor (OXE-R) biased compound which selectively inhibits Gβγ but exclusively activates Gαi signaling.

3.1.2. G protein-independent signaling pathways

Despite 7TM receptors intracellular signals were long thought to arise from a unique proximal heterotrimeric G protein processor, it became clear over years that 7TM receptors can directly interact with several other intracellular proteins (Ritter and Hall, 2009) leading to G protein-independent signaling pathways with β-arrestin-dependent signaling as the most popular example. Paradoxically, β-arrestin was originally discovered as a negative regulator of the G protein pathway upon sustained stimulation of the receptor thus leading to G protein desensitization and receptor internalization (Luttrell and Lefkowitz, 2002) (Fig. 2). However, β-arrestins (β-arrestin 1 and 2) also act as scaffold proteins that promote multi-molecular complexes assembly and control intracellular signaling pathways including those of mitogen-activated protein kinases (MAPKDeWire et al., 2007; Lefkowitz and Shenoy, 2005) (Fig. 2). Generally, both G protein- and β-arrestin-dependent signaling naturally coexist following stimulation of 7TM receptors with endogenous ligand. Nevertheless CXCR7, a member of the decoys 7TM receptors originally thought to be a non-signaling receptor but an extracellular ligand scavenger, was finally identified as an endogenous biased 7TM receptor that signals through the β-arrestin in the absence of G protein activation (Rajagopal et al., 2010). The β-arrestin-dependent pathway became more largely popular with the discovery of biased-drugs favoring β-arrestin signals over those conducted by the G proteins (Whalen et al., 2011), early initiated by Lefkowitz's and Bouvier's groups (Azzi et al., 2003; Wei et al., 2003). Up to now, β-arrestin biased ligands were mostly identified for β-arrestin 2 isoform. However, given the existence of two isoforms, β-arrestin 1 and 2, both controlling distinct aspects of 7TM receptors signaling (Srivastava et al., 2015), it should be not surprising that β-arrestin 1 biased emerges as well in the future. Taking advantages of the β-arrestin1/2 KO mice, several β-arrestin biased agonists with putative therapeutic advantages have been identified. *In vivo*, UNC9975, an antagonist of the Dopamine D2-R/Gi axis and partial agonist at D2-R/β-arrestin, displayed potent β-arrestin-dependent antipsychotic activity without the Gi-associated motor side effects that are classically shared by antipsychotic drugs, thus highlighting the therapeutic benefit of blocking the G protein pathway (Allen et al., 2011). As an additional example, TRV120027 and TRV120023 engage the beneficial AT1-R/β-arrestin axis at AT1-R and these molecules elicit cardioprotective properties *in vivo*, reducing blood pressure (TRV120027) while acting as a mild inotropes (TRV120027 and TRV120023) (Violin et al., 2014). TRV120027 is currently in phase II clinical trials for the treatment of acute heart failure (Felker et al., 2015). Numerous other receptors support biased agonism at β-arrestin with promising clinical advantages (Violin et al., 2014).

3.2. Common effectors for diverse signaling pathways: how to select a pathway?

The massive evolution of the concept of biased agonism in the 7TM receptors field these last 10 years has introduced a somehow simplistic caricature of the 7TM receptors signaling with G protein-dependent signaling and β-arrestin-dependent/G protein-independent pathways and biased efficacy of the ligands has been screened and classified accordingly. However, G proteins and β-arrestins constitute common effectors of multiple intracellular pathways, each of them responsible for specific cellular outcomes. For instance, ERK1/2 activation, that regulates mitotic and post-mitotic cell function, can be elicited by G protein or β-arrestin or both (Sauliere et al., 2012). Mitotic state of the cell has been also recently shown to be tightly controlled by another G protein-dependent Hippo/YAP pathway (Yu et al., 2012). Tyrosine kinase receptor transactivation by 7TM receptors can also initiate β-arrestin recruitment or involve G protein interaction at these receptors (Cattaneo et al., 2014). It thus follows that *in vivo* effects of G protein- and β-arrestin biased ligands are finally unpredictable and care must be thus taken when trying to correlate the signaling responses observed *in vitro* in cell-based assays with the pharmacological profile obtained *in vivo*. In line with this concept, multiple ligands can cause a similar cellular response but using distinct transductions pathways. For instance, Sato et al., compared extracellular acidification rates (ECAR) promoted by CL316,243 and SR59,230A *versus* isoproterenol agonist reference at β3-AR and found that all behave as full agonists (Sato et al., 2007). However, when dissecting the molecular mechanisms leading to ECAR activation, they found that while CL316,243 involves a dual cAMP and p38 pathway, SR59,230A almost exclusively leads to ECAR activation through a p38 pathway. In the same line of evidence, while TRV120027 (in Phase IIb for acute heart failure; see Section 1) was originally developed as a high affinity [Sar^1, Ile^4, Ile^8]-AngII (SII)-like β-arrestin-biased agonist, comparison of TRV120027 and SII downstream signaling events (kinase phosphorylation/gene expression profiling) revealed unexpected specific and different activation profiling for each peptide also differing from that of the reference agonist Angiotensin II, demonstrating that β-arrestin can be part of distinct signaling cascades (Santos et al., 2015). This result is in agreement with a previous work from Saulière et al. that explored and dissected the β-arrestin- and G protein-dependence of the ERK1/2 pathway in different cell lines (Sauliere et al., 2012) and found that SII was not a mimicking peptide of the β-arrestin-dependent pathway activated by Angiotensin II but rather used similar G protein and β-arrestin effectors in different signaling. Conversely, a therapeutic advantage of a drug can arise from various pharmacological *in vitro* profiles. Thus, classical antipsychotic activity of therapeutic agents used in the clinic for schizophrenia treatment has been assigned a common antagonism of the D2-R/β-arrestin pathway (Masri et al., 2008). However, a more recent study identified new highly potent antipsychotic drugs (UNC9975/UNC9994) acting at D2-R but promoting partial activation of the β-arrestin while blocking the Gi pathway and without the deleterious motor adverse effects (Allen et al., 2011).

Overall these results clearly indicate that diversity of activation can be observed in both the “G protein-dependent” and the "β-arrestin-dependent” signaling, highlighting that bias profiling of a ligand has to be established on a maximum of effectors/signaling events. Moreover, while so far “canonical” effectors have been mainly sensed (i.e, G protein, β-arrestin, ERK1/2, cAMP, Ca^{2+}...), more specific effectors should be introduced in the screening stage of biased ligands so to appreciate the differential texture of the activated pathways. Thus, while cAMP production originates from common adenylyl cyclase proteins, its two downstream effectors (PKA and Epac existing in multiple isoforms) direct different cAMP-outcomes (Boularan and Gales, 2015; Bruzzone et al., 2014). In this context, the use of PKA or Epac full-length protein biosensors (*i.e.* FRET/BRET) could thus help dissecting the cAMP pathway (Bruzzone et al., 2014). Disruption of the interaction between 5-HT2C receptors and the tumor suppressor PTEN has been shown to selectively suppress behavioral responses to drug abuse (nicotine and Δ9-tetrahydrocannabinol) (Ji et al., 2006), suggesting that a biosensor directly measuring the interaction between 5-HT2C receptor and PTEN could help identifying specific ligands antagonizing the interaction. Beyond the activation state, evaluation of other specific parameters of the effectors could be predictive of differential signaling of the ligands. The different conformations of β-arrestin (Shukla et al., 2008) or of the receptor-β-arrestin/receptor-G protein complexes (Bellot et al., 2015) stabilized by different ligands have been shown to dictate different intracellular signaling. Since 7TM receptors signaling consists in tightly regulated spatio-temporal events, kinetics of G protein or β-arrestin activation could also be indicative of different output signals and was recently used in bias screening (Masuho et al., 2015; Sauliere et al., 2012). Another important consideration is the sensing of distal *versus* proximal signaling events from the activated receptor. While measurement of proximal events will most probably increase the efficacy of the screen (less false positives), *i.e* G protein and β-arrestin, downstream signaling will conversely increase its sensitivity due to the signal amplification. Therefore, multiplexing all these signaling events will probably favor the detection and the establishment of biased ligand signatures.

4. Choice of the assays

Measurement of biased ligand efficacy and thus ligand pharmacological classification directly results from the availability of the assays and from their sensitivity to sense the different signaling events. Hence, delineation of ligand efficacy has constantly evolved according to the pleiotropic signaling of 7TM receptors (see Section 3) and mainly to the technological breakthroughs in the field of bioassays. Biased ligands do not escape this rule since their generally weak efficacy constitutes an impediment to their detection, thus requiring highly sensitive and specific assays. Moreover, their precise pharmacological profiling constitutes a hard task to characterize since it depends on the simultaneous comparison of ligand efficacy and potency on several signaling pathways using different assays.

4.1. II messenger’s based assays

Most likely because intracellular II messengers were the first identified intracellular mediators of active 7TM receptors at the plasma membrane and easier to quantify than the G protein activity by itself, assays measuring their intracellular levels still represent the most frequently used ones to characterize ligand efficacy and biased agonism. II messengers levels are classically measured according to the nature of the G protein coupled to the receptor and thus constitute indirect sensors of G protein activity (see Section 3.1.1). Basically, cAMP levels are quantified for 7TM receptors coupled to Gs or Gi/o as a readout of adenylyl cyclase activity whereas for receptors coupled to Gq, IP_3/IP_1 and Ca^{2+} levels are evaluated as a readout of phospholipase C activity. Originally, these measurements were performed by direct quantification using radiometric assays (Palmer et al., 1989; Pineyro et al., 2005). In an effort to skip the use of radioactivity, II messengers assays largely rely now on the use fluorescence/bioluminescence-based technologies and a wide variety of assays are currently available (Zhang and Xie, 2012). Most of them have initially been developed in an HTS per-

spective aiming to screen a large number of molecules. Thus, the main characteristics of these assays are rapidity, robustness, reliability and cost-effectiveness but they are often not necessarily highly quantitative. For cAMP or IP_3/IP_1 detection, assays mostly rely on a immunoassay based on the competitive binding between a radioactive (*i.e.* RIA) or labeled (*i.e.* HTRF) antigen ("tracer") (cAMP, IP_1...) and the antigen/II messengers to be quantified. Cytosolic BRET-based biosensors using the II-messenger specific binding domain (*e.g.* cAMP domain of Epac or PKA) have been also recently used for the quantification of ligand bias (Masri et al., 2008; van der Westhuizen et al., 2014). Popular calcium assays use the cell-permeable Ca^{2+}-sensitive fluorescent dyes (Fluo-3/Fluo-4) but luminescent-Ca^{2+}-sensitive sensors (aequorine/obelin) have been used as well in the context of ligand bias (van der Westhuizen et al., 2014; Zhang and Xie, 2012). Despite high sensitivity, these calcium assays suffer from several limitations. For instance, they cannot detect inverse agonist efficacy. Moreover their quantification constitutes a complex task. Indeed, depending on the affinity of the dye and the system used, a dye saturation could be observed, thus resulting in an overestimation of agonist potency and partial agonist efficacy. Also, the fact that the signal is measured in non-equilibrium conditions could modify ligand efficacy parameters (Charlton and Vauquelin, 2010). Intracellular calcium signal is also highly textured since it results from highly compartmentalized signals that are time regulated, each governing specific functional outputs. This orchestration is conducted by the generation of specific multiple second messengers acting on specific Ca^{2+} channels. For example, IP_3 activates IP_3-Receptor channels but is not the only II messenger to regulate Ca^{2+} release since for example cADPR (cyclic-ADP-ribose) activates RyR-R (Ryanodin receptor) channels and NAADP (Nicotinic acid adenine dinucleotide phosphate) acts on NAADP-R channels. Hence, each GPCR/ligand will encode a combination of channels activators to generate a specific signal (Kiselyov et al., 2003). Hence, pharmacological translation of $Ca2^{+}$ signals is quite difficult using classical calcium assays. Finally, another alternative for II messenger quantification consists in the use of cell-based reporter assays relying on a II messenger responsive element upstream of a promoter controlling the transcription of various enzymes (luciferase, β-galactosidase...) that can be easily quantified and offers a large signal to background window.

The measurement of G protein-mediated II messengers downstream the active receptor offers advantages for the detection of biased ligand with weak activity since they are prone to signal amplification. Nevertheless, despite signal amplification, the assay threshold can prevent detection of low II messengers production. The recent development of broad range of II messenger cell-based assays has helped resolving this problem and provides now high resolution detection. For instance, the α2C-AT1-R dimer coupling to the Gs/cAMP pathway promoted by the co-stimulation with noradrenaline and angiotensin II was only detectable using the high sensitive cAMP femto kit from Cisbio allowing detection of as little as 28 pM cAMP (Bellot et al., 2015). Now besides the bright side, measurement of the II messengers can also lead to complex interpretations. Indeed, because their production results from a combination of several signaling outcomes downstream the active receptor, they could not discriminate between the stimulation of distinct effectors (*e.g.* G proteins, adenylyl cyclase or Phospholipase C isoforms). Thus, as some 7TM receptors dually couple to Gs and Gi (*e.g.* β2-AR), cAMP levels produced at one time point can result from both activation of the adenylyl cyclase *via* Gs proteins and its inhibition mediated by the various Gi isoforms, making signaling analysis hard to achieve. However, the use of pharmacological tools modulating the G protein activity can be helpful. In this way, CHO cells pretreatment with Pertussis Toxin (PTX)-Gi inhibitor and Cholera Toxin (CTX)-Gs activator allowed to demonstrate that prototypical Gi-coupled α2-adrenergic receptors (α2A/B/C-AR) can indeed couple also to Gs in a α2-subtype and ligand-structure specific manner (Eason et al., 1994). Similarly, elevation of cytosolic calcium concentrations can result from different sources through voltage dependent ion channels *via* the G protein Go or from the activation of PLC *via* Gq, G_{11} or Gi-derived βγ subunits. Furthermore, II messenger production is not always a linear causal process and for example, in sympathetic neurons, acetylcholine leads to DAG production without calcium release (Delmas et al., 2002). This observation is of interest for biased activity detection since the Gq pathway is generally sensed through a calcium or a $IP_1/_3$ assay but it follows that both messengers should be systematically screened so to better appreciate the bias. Likewise, the choice of the II messenger assay relies on the canonical coupling recognized for the receptor of interest. However, recent studies reported the capacity of some G proteins to activate unexpected secondary effectors. Hence, GXLαs has been shown to enhance IP_3 production through the parathyroid hormone receptor (He et al., 2015) while Gαi1 can directly activate the transient receptor potential canonical channel TRPC4 (Jeon et al., 2013). Thus, the prototypical Gi-coupled α2-AR atypically activates the Gq/PLC pathway in kidney distal convoluted tubule cells (Gesek, 1996). Overall, these results argue that ligand bias should be finally screened without any *a priori* on the G protein or other signaling effectors to increase their window detection. Amplification of the signal can also result in an overestimation of the relative ligand efficacy because of the presence of "receptor reserve" with a partial agonist leading to the same maximal response as a full agonist and acting thus as a confusing factor for ligand bias classification (Trzeciakowski, 1999a,b). Finally, II messenger-based assays, due to their high sensitivity, can allow the detection of endogenous receptor activity, thus making difficult bias analysis. As example, β-adrenergic receptors are often found as endogenous background in HEK293 cells and interfere with different ligand efficacies tested on overexpressed adrenergic receptors (Bellot et al., 2015; van der Westhuizen et al., 2014).

4.2. Assays evaluating initial signaling events

Given the pleiotropic 7TM receptors signaling, measuring the initial and common heterotrimeric G protein activation rather than second intracellular effectors (see Section 3.1.1; Adenylyl cyclase, Phospholipases, MAPK, GIRK channels, Ca^{2+} channels...), will certainly more accurately depict ligand efficacy. However, 7TM receptors usually couple to multiple G protein isoforms, but accurate evaluation of the activation of each individual isoform remains elusive despite they are prone to functional selectivity (Hermans, 2003). To date, evaluation of G protein activation essentially relies on two assays. The radioactive [^{35}S]GTPγS binding assay on cell membrane extracts is the most commonly used and largely proved its efficiency to determine ligand potency and efficacy toward G proteins (Harrison and Traynor, 2003). However, its greater specificity for the Gi family limits its use to decipher ligand biased efficacy. More recently, distance-proximity BRET/FRET-based assays directly measuring the interaction between receptors and G protein subunits or interactions between Gα and Gγ subunits as sensors of G protein activation have been developed in living cells (Denis et al., 2012; Lohse et al., 2012) These assays are now available to directly monitor all major Gα isoforms (Gi/o, Gs, Gq/11, G12/13) activation, offering the possibility to depict biased agonism among G proteins isoforms particularly difficult to discriminate (*i.e* the Gi/o family) (Sauliere et al., 2012; Bellot et al., 2015). Using such probes, a potent GαoA-biased ligand at κ-opioid receptor was identified in agreement with its efficacy on adenylyl cyclase inhibition (Rives et al., 2012). Screening at oxytocin receptor led to the identification of original G protein-biased ligands strongly discriminating between the Gi/o family members (Busnelli et al., 2012). The G protein BRET probes allowed the comprehension of antiprolifer-

ative and antisecretory effects of a natural mutant of SST5 receptor displaying a specific blunt on $G\alpha_{oA}$ activation in human pituitary cells (Peverelli et al., 2012). These probes also facilitate the detection of ligand activity on more atypical G proteins like Gα12 and Gα13 for which downstream signaling cannot be easily screened, thus extending the repertoire of bias detection (M'Kadmi et al., 2015; Sauliere et al., 2012). Finally, they high sensitivity allowed depicting the capability of some ligands, such as the Angiotensin II-related peptide SII, to activate G proteins (Sauliere et al., 2012). while initially classified as β-arrestin-biased agonists (Wei et al., 2003), was identified and further confirmed using specific G protein inhibitors on downstream signaling Interestingly, agonist efficacy determined for SII on the G protein pathway seems to rely just on assay sensitivity since this compound was initially described as unable to induce G protein activation in AT1-R-expressing HEK293T when using radioactive GTPγS or IP3 radioactive assays (Wei et al., 2003), while the same group recently confirmed the ability of SII to couple to G proteins in similar cell system but using a sensitive IP1HTRF-based assay (Strachan et al., 2014). BRET probes sensing G protein activity also provide powerful and sensitive tools to depict and quantify constitutive activity of 7TM receptors, thus allowing for instance the recent identification of Gαq and Gα13 inverse agonist bias at ghrelin receptor (M'Kadmi et al., 2015).

Another early signaling step subsequent to 7TM receptors activation is the β-arrestin recruitment, generally leading to G protein uncoupling and ensuing receptor internalization or formation of new intracellular signalosomes (DeWire et al., 2007). β-arrestin assays basically measure protein recruitment from the cytosol to cell surface receptor (Verkaar et al., 2008), and rely either on classical cell imaging studies, not really adapted to accurately quantify discrete recruitment modulations, or on more quantitative Resonance Energy Transfer (RET)-based assays. To date, BRET/FRET-based assays measuring the direct interaction between the receptor and β-arrestin provide sensitive tools to quantitatively evaluate the ligand efficacy, including in HTS applications (Hamdan et al., 2005). They allow real-time measurement of β-arrestin recruitment in living cells, offering the possibility not only to easily quantify the maximal protein amount recruited to the receptor but also to appreciate its recruitment rate by opposition to one time point recruitment assays (DiscoveRx PathHunter™ eXpress ß-Arrestin Assay or ThermoFisher Tango™ GPCR assay system). However, data from β-arrestin RET assays have to be interpreted cautiously since determination of ligand efficacy on the β-arrestin recruitment cannot easily be inferred from the RET-based assay. In fact, the maximal RET signal is generally taken as a readout of the maximum ligand-promoted β-arrestin recruitment. However, because RET signal depends on both dipole orientation and distance between RET partners, a part of this signal may also senses the conformational state of the receptor/β-arrestin complex. It follows that biased ligands recruiting similar levels of β-arrestin, but leading to different conformations of the receptor-β-arrestin complex with different distances between the energy donor and acceptor, will be sensed as different maximum BRET ratio (Fig. 3). Conversely, biased ligands recruiting different β-arrestin levels leading to different receptor-β-arrestin complex conformations but with similar distances between the energy donor and acceptor will be sensed as identical maximum BRET signals. This theoretical RET analysis has been previously validated when examining the interaction between receptors and G proteins (Gales et al., 2006) and has been recently confirmed experimentally by Bellot et al., who examined β-arrestin recruitment to the α2C-AT1-R dimer by BRET (Bellot et al., 2015). The authors showed that the use of a N-terminal Rluc-tagged β-arrestin2 probe *vs* a C-terminal one results in different ligand-induced maximum recruitment profiles more likely in agreement with a different conformational sensing of the receptor-β-arrestin complex. It follows that, when performed as a single readout of the β-arrestin pathway, the RET-based assays could lead to equivocal classification of ligand bias efficacy. This could also explain, in part, distortions observed between the results from β-arrestin translocation RET-based assays *vs* confocal microscopy experiments (Reiner et al., 2010). Thus, assays appreciating the conformation of β-arrestin (double brilliance β-arrestin, (Charest et al., 2005) or β-arrestin/receptor complexes (RET assays) will reinforce the signature of biased ligand efficacy since different β-arrestin conformations correlate with different output signaling (Shukla et al., 2008; Zimmerman et al., 2012). Besides, assays directly measuring β-arrestin binding to the receptor (Verkaar et al., 2008) that only quantify the total amount of β-arrestin associated with the receptor would complement the pharmacological characterization of the compound at the efficacy level.

4.3. Assays monitoring temporal and spatial events

The texture of ligand efficacy can also be appreciated through a spatio-temporal standpoint (Lohse and Hofmann, 2015) since signaling must be coordinated in both space and time to fine-tune a specific response of the cell. Cell signal compartmentalization leading to subtle local II messenger generation/effector activation, as largely highlighted in the cardiomyocytes (Xiang, 2011), could constitute a priority target for some biased ligands as it implies a fine degree of spatial and temporal control over the cellular signaling. The development of several fluorescence-based probes has allowed the investigation of several signaling outputs in both a spatial and temporal manner in living cells. Early sensors include small fluorescent dyes, among them the most widely used Fura-2 ratiometric dye that binds intracellular Ca^{2+} and permit the observation of a local change of calcium gradients in single living smooth muscle cells (Williams et al., 1985). More recently a plethora of genetically-encoded fluorescence-based biosensors has been developed, allowing to monitor the dynamics of a large array of signaling events in living cells (Miyawaki and Niino, 2015; Oldach and Zhang, 2014) in highly specialized cell structures, such as primary cilia (Su et al., 2013). These probes based on the appreciation of protein–protein interactions or protein conformational changes have demonstrated spatio-temporal regulation at different levels of the signaling cascade. Recently, Masuho et al. observed functional selectivity among G protein activation kinetics (Masuho et al., 2015). Also, a recent study, by combining real-time measurements of receptor internalization and cAMP signaling in living cells, identified functional selectivity on kinetics of GLP-1R internalization, recycling and signaling following stimulation with the natural ligand GLP-1 and the two stable analogues (Roed et al., 2014). Interestingly, Molden et al., by analyzing intracellular cAMP kinetics in a FRET-assay, have shown that distinct melanocortin-4 receptor agonists can exert temporal selectivity to modulate the duration of the cAMP response and potentially the cellular compartment from which the signal is generated in response to acute agonist stimulation (Molden et al., 2015).

4.4. Quantifying biased activity or depicting ligand texture?

Ligand biased efficacy can be grasped in both quantitative and qualitative ways. But whatever the case, their classification of biased ligands will always rely on the comparison with a reference ligand, often the physiological ligand. Basically, the choice of the signaling pathway for the characterization of the bias is generally dictated by the signaling commonly associated with the natural receptor agonist used as the reference. However, since it has been shown that some ligands can engage specific receptor signaling outputs not related to those of the "reference agonist" (Santos et al., 2015; Sauliere et al., 2012), a panel of signaling effectors should be systematically tested without *a priori*. Moreover, the reference by

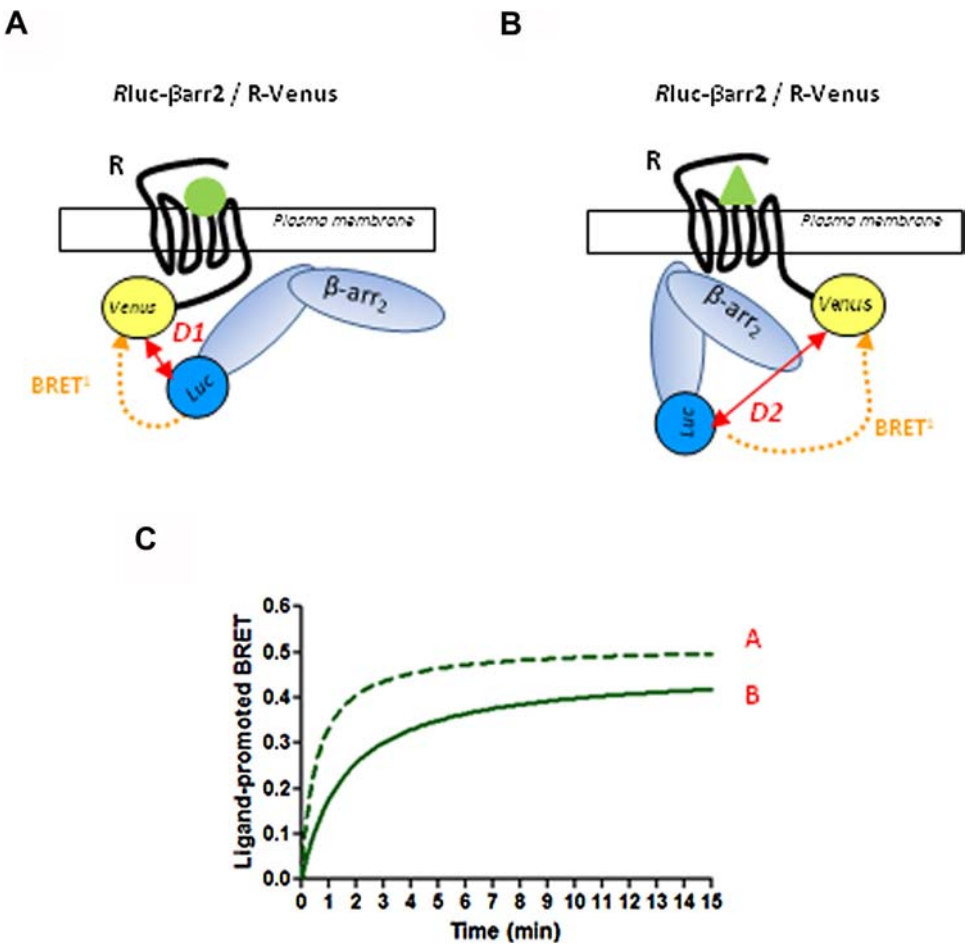

Fig. 3. Classical BRET-based β-arrestin assay: example of 2 biased ligands recruiting similar amount of β-arrestin to a 7TM receptor. (A) $BRET^1$ was measured between *R*luc-β-arrestin2 (*R*luc-β-arr_2) and the receptor-Venus (R-Venus) following agonist binding (green) to the receptor, and allowing close distance (D1) between Venus and *R*luc BRET partners. (B) Model of *R*luc-β-arrestin2 recruited to the same receptor in the presence of a biased ligand stabilizing different receptor and β-arrestin2 conformation. Distances between *R*luc and Venus are increased when compared to that in A (D2 > D1). (C) Real time kinetics $BRET^1$ measurement of β-arrestin2-*R*luc recruitment to the receptor-Venus allowing quantification of both the rate and the maximal ligand-promoted BRET signal. Maximal BRET signals will be different in A and B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

itself may be somehow difficult to define since many 7TM receptors bind multiple endogenous ligands, *i.e.* the chemokine receptor family, and these ligands sometimes exhibit biased activity (Corbisier et al., 2015; Haessler et al., 2011; Reiner et al., 2010).

These last 5 years, several groups tried to accurately quantify the biased activity of ligands so to establish a quantitative "fingerprint". Different calculation models have been proposed to establish a bias factor and there is currently controversy regarding the best way to calculate it (Kenakin and Christopoulos, 2013; Kenakin et al., 2012; Nagi and Pineyro, 2016; Rajagopal, 2013; Rajagopal et al., 2011; Stott et al., 2016). As already discussed, all these quantification models mostly rely on the use of a reference ligand and on classical parameters obtained from concentration-response curves (Emax and EC50) on two different signaling pathways. In addition, some approaches incorporate ligand affinity parameter derived from competition binding experiments, but assuming that the affinity remains constant for all receptor/effector complexes. However, Rasmussen et al. reported that conformational changes promoted by the effector on the receptor alter binding properties of ligands, suggesting that receptor/effector complex can dictate the functionnal selectivity (Rasmussen et al., 2011). Thus, taking into account this observation, other bias quantification tried to introduce in the model variable ligand affinities for the different agonist/receptor/effector complexes (Kenakin et al., 2012; van der Westhuizen et al., 2014).

The calculation of the bias factor provides a useful comparison between several compounds as exemplified by a study on clinically relevant β2-adrenergic receptor ligands on four signaling outputs (van der Westhuizen et al., 2014). However, because the calculation of the bias factor is restricted to the comparison of only two signaling pathways, it is quite difficult to easily appreciate the overall texture of each ligand for each signaling in a 3D representation (Fig. 4A). An alternative to bypass this problem consists in the establishment of a matrix of pairwise-determined bias factors for a given ligand in *n* dimensions (Reiter et al., 2012) (Fig. 4B). However, despite its interest, this representation does not allow the face to face comparison of ligands. A more didactic and visual representation of the pluri-dimensionality of the bias could be depicted in multiaxial illustration (Webs of bias) (Fig. 4C). In this representation, each axis relates to a pharmacological parameter of choice (Emax, EC50, kinetics parameter) plotted on a specific signaling output (Appleton et al., 2013; Koole et al., 2013; Sauliere et al., 2012). It can be conducted simultaneously on different ligands as well as on different signaling pathways, thus offering a brief but clear overview of the signature of a ligand *versus* another.

Nevertheless, before any "bias factor" calculation and representation, the possibility of an experimental bias that will potentially pollute data should be kept in mind. First of all, does calculation of a bias factor have any sense when the comparison of ligand effects on two signaling pathways arise from tests carried out using differ-

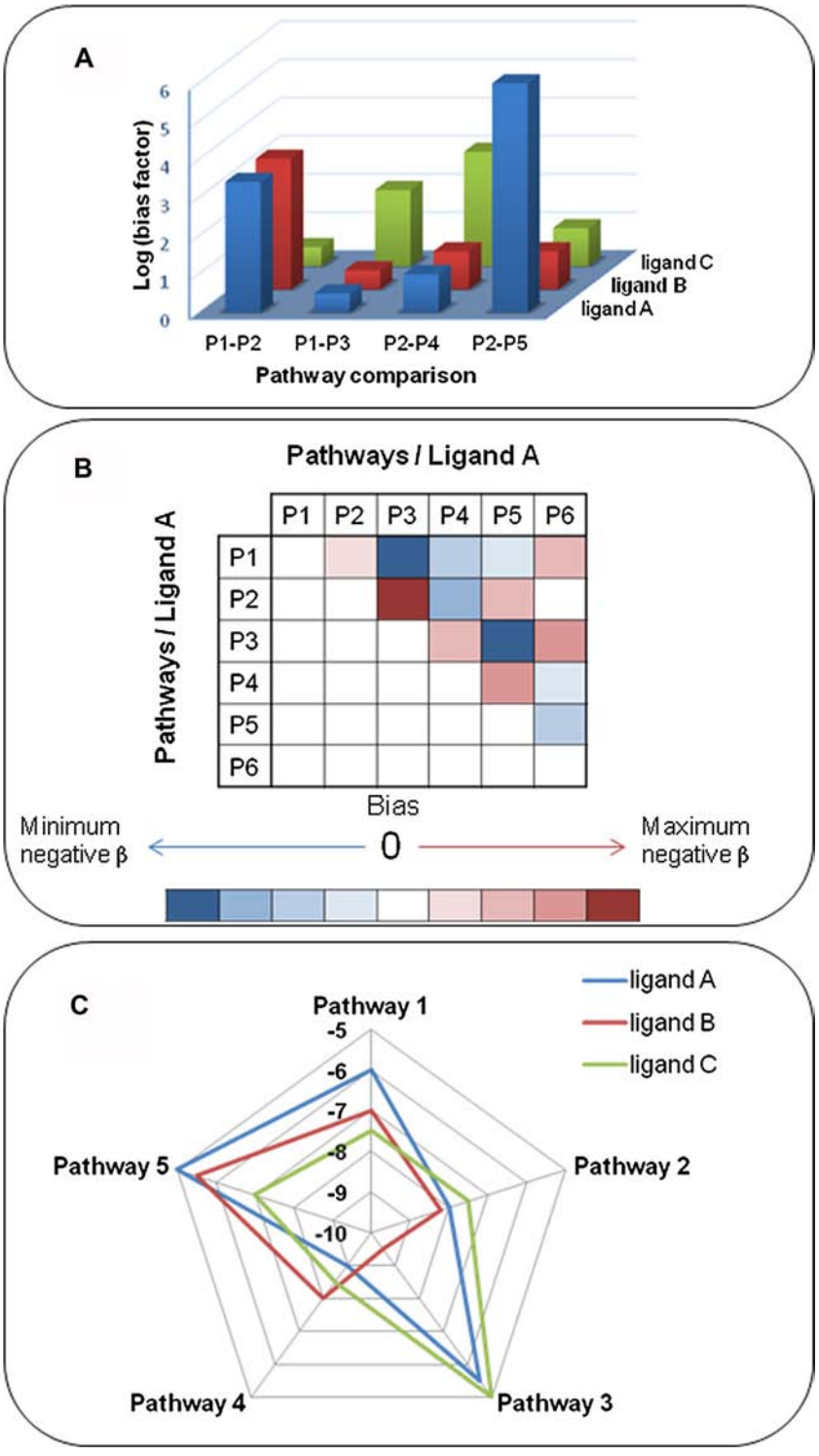

Fig. 4. Representations of the pluridimensional efficacies of biased ligands. (A) 3D representation of the bias factor where ligands are compared for their efficacy on one signaling pathway *versus* another (adapted from van der Westhuizen et al., 2014). (B) Matrix representation of pairwise bias factors β of one ligand for 6 pathways (adapted from Reiter et al., 2012). (C) The multiaxial representation "Web of bias". Efficacy parameters (here the log(EC_{50})) are plotted on each axis, that relates to a specific pathway.

ent receptor and effector stoichiometry (see Section 2.2)? Indeed, the assays available to evaluate multiple pathways are difficult to use exactly in identical conditions setting. As previously discussed, most assays require probe overexpression and are often performed in different buffers. They may require either adherent or suspended cells or even cell membranes. Moreover, comparing transient responses such as Ca^{2+} release or ERK1/2 activation with long-lasting ones (cAMP, IP_3 production) may lack of relevance. The kinetics of signaling also account for biased ligand efficacy texture and could be quantified, as well exemplified by Masuho et al., showing that G protein activation rate ($\tau/2$) was ligand dependent (Masuho et al., 2015).

5. Concluding remarks

Functional selectivity of ligands has recently emerged as a promising avenue for alleviating adverse effects of drugs targeting

7TM receptors. In this context, *in vitro* determination and comparison of efficacy on multiple signaling pathways to identify ligand bias became a mandatory option. However, despite the remarkable progress in the quantification of the ligand bias, the experimental angle has not yet been thoroughly examined. In this review, we have specifically highlighted the sensitivity of ligand efficacies to the cell system, the cell signaling, its kinetics, it emphasizes the importance to accurately calibrate the assays used to monitor the different signaling pathways to permit comparison of the ligand efficacies and generate bias fingerprint profiles. These methodological settings still remain unexplored for delineation of biased signaling and should come under urgent considerations. This could explain, at least in part, the discrepancies observed in between studies for the bias fingerprint delineating for a given ligand.

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