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Themed Section: Opioids: New Pathways to Functional Selectivity

REVIEW

Novel GPCR paradigms at the μ-opioid receptor

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Opioids, such as morphine, are the most clinically useful class of analgesic drugs for the treatment of acute and chronic pain. However, the use of opioids is greatly limited by the development of severe adverse side effects. Consequently, drug discovery efforts have been directed towards improving the therapeutic profile of opioid-based treatments. Opioid receptors are members of the family of GPCRs. As such, the recent GPCR paradigms of biased agonism and allosterism may provide novel avenues for more effective analgesics. Biased agonism (or functional selectivity) has been described for all the opioid receptor family members. Furthermore, the first allosteric modulators of opioid receptors have very recently been described. However, identification and quantification of biased agonism in a manner that is informative to medicinal chemists and drug discovery programmes still remains a challenge. In this review, we examine the progress, to date, towards identification and quantification of biased agonism and allosterism at the μ-opioid receptor in the context of its implications for the discovery of better and safer analgesics.

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Abbreviations
DAMGO, [D-Ala[2], NMe-Phe[4], Gly-ol[5]]-enkephalin; DOR, δ-opioid receptor; GRK2, GPCR kinase 2; KOR, κ-opioid receptor; MOR, μ-opioid receptor; PAM, positive allosteric modulator; SAR, structure–activity relationship

GPCRs can mediate a spectrum of acute signalling and longer-term regulatory behaviours that can be modulated in a ligand-specific manner. Such functional versatility cannot be explained by a simple 'on–off' switch model of receptor activation, and is more compatible with dynamic and flexible structures. Indeed, during the last decade, we have witnessed the experimental confirmation of previously theoretical concepts demonstrating that GPCRs exist in many, temporally related, micro-conformations (Deupi and Kobilka, 2010). Approaches such as NMR spectroscopy have provided evidence that GPCRs are highly dynamic proteins that exist in several functionally relevant conformational states (Hofmann *et al.*, 2009; Bokoch *et al.*, 2010). Two paradigms that are fundamentally linked to such inherent plasticity of GPCRs are biased agonism (or functional selectivity) and allosterism.

Biased agonism describes the phenomenon whereby the binding of different ligands to the same receptor in an identical cellular background results in differential activation of cell signalling pathways, and eventually, in different physiological outcomes (Kenakin, 2011). At a molecular level, this is a consequence of the fact that different agonists do not activate receptors through stabilization of the same active state; rather, they can stabilize different functionally active states that lead to the engagement of a limited subset of intracellular effectors, and in turn, the activation of specific signalling pathways. The ability of distinct GPCR-agonist complexes to differentially activate intracellular signals provides new avenues for the development of drugs that are not only receptor-specific, but also 'pathway-specific', and has opened the way to the discovery of ligands that selectively activate signalling pathways mediating the desired

physiological effects while minimizing 'on-target' side effects that are elicited by activation of other signalling pathways via the same receptor. However, although this concept is very attractive, there are significant challenges to its translation from the field of medicinal chemistry into effective therapies. On the one hand, the identification of the signalling pathways responsible for therapeutic effects and of those responsible for the deleterious side effects is not straightforward; on the other hand, analytical tools for the detection and quantification of biased agonism are becoming available for drug development efforts aimed in this direction (Kenakin and Christopoulos, 2013).

The phenomenon of allosterism is also a consequence of the conformational plasticity of GPCRs. Allosteric ligands influence receptor activity by binding to sites that are topographically distinct from the site where the endogenous (orthosteric) ligand binds. Classical models of allosterism already postulated the need for multiple conformational states in the absence of ligand as a fundamental characteristic of allosteric proteins (Monod *et al.*, 1965). These states exist in a dynamic equilibrium, and the binding of a ligand to an allosteric protein stabilizes some states at the expense of others (Canals *et al.*, 2011). As such, allosteric ligands mediate their effects by promoting conformational changes in the GPCR protein that are transmitted from the allosteric binding pocket to the orthosteric site, or directly to the effector sites. In terms of drug development, allosteric GPCR ligands offer significant advantages over targeting of the orthosteric site. First, because of the lack of evolutionary pressure on sites other than that where the endogenous ligand binds, allosteric sites have not necessarily been conserved and therefore offer greater potential for receptor subtype selectivity. Second, in the absence of intrinsic efficacy, allosteric modulators will only exert their effect when and where the endogenous ligand is present, therefore tuning cellular responses and maintaining the temporal and spatial characteristics of endogenous signals. Furthermore, as the effect of an allosteric ligand is limited by its cooperativity, such class of ligands may become safer therapies with fewer on-target overdosing risks.

Studies on opioid receptors have provided prototypical examples of ligand-dependent signalling and regulation (Raehal *et al.*, 2011), and recently, allosteric modulators of the μ-opioid receptor (MOR; receptor nomenclature follows Alexander *et al.*, 2013) have been described (Burford *et al.*, 2013). In this review, we examine the progress, to date, towards identification and quantification of biased agonism and allosterism at the MOR, in the context of its implications for the discovery of better and safer analgesics.

Ligand-dependent effects at the MOR

Opioids have been used for millennia for the treatment of moderate to severe pain, and remain the most effective and widely used analgesics to date. Of the four subtypes of opioid receptors, the MOR subtype is the therapeutic target for most currently used opioids as the analgesic effects of morphine were abolished in a MOR knockout mouse (Kieffer and Gavériaux-Ruff, 2002). However, it is well known that opioid analgesics, including morphine, oxycodone and fentanyl, suffer from very limiting side effects such as tolerance, dependence and addiction, respiratory depression, and constipation, which severely limit their clinical use. Therefore, there is a need for new compounds that provide effective analgesia, but without the serious side effects.

As mentioned previously, biased agonism offers such potential, and there are clear indications of the existence of ligand-specific effects on MOR signalling and regulation (see Raehal *et al.*, 2011; Williams *et al.*, 2013). Two key observations on the actions of morphine triggered most of the studies on functional selectivity at the MOR. First, morphine induces relatively less internalization of MORs internalization, in spite of its efficacy in mediating G-protein activation (Keith *et al.*, 1996; Sternini *et al.*, 1996). Second, morphine-induced respiratory depression and constipation are dramatically attenuated in a β-arrestin2 knockout mouse while analgesia is enhanced (Raehal *et al.*, 2005). This latter result also provided clear evidence of the tissue-specific mechanisms of receptor activity and regulation. Taken together, these reports have sparked the search for the potentially different signalling mechanisms that mediate the diverse physiological actions of MOR agonists as well as for ligands that exploit such mechanisms. However, most descriptions of biased agonism at the MOR have been based on qualitative comparisons between ligands. The number of studies quantifying bias is still very low (see later). Yet, in order to apply biased agonism therapeutically and effectively, it is necessary to incorporate parameters that describe the degree of bias in a manner that can inform future drug development.

Quantifying biased agonism

Although biased agonism is now widely accepted, the majority of studies to date on functional selectivity have used largely qualitative observations, such as reversals in agonist potency orders or maximal agonist effects between different pathways, as indicators of biased agonism. However, such approaches are not optimal. The observed response of an agonist at a given pathway is not only the result of unique ligand-induced receptor conformations, but is also affected by 'system bias', which reflects the differing coupling efficiencies of the receptor to a given signalling pathway, and by 'observation bias', which results from differing assay sensitivity and conditions (Kenakin and Christopoulos, 2013). It is the bias imposed by the ligand on the receptor that is the only source of bias that can be chemically optimized to improve the therapeutic profile of a drug. It is therefore important to quantify signalling bias in such a way that it excludes system and observation bias, in order to reveal the unique signalling profile that is induced by the ligand.

The potency of a ligand is determined by both its affinity for the receptor state coupled to that particular pathway, as well as its intrinsic efficacy for generating a response in that pathway. In contrast, the maximal effect of a ligand at saturating concentrations is only determined by intrinsic efficacy. In addition, contributors to system bias, signal amplification and receptor expression need to be taken into account as they have markedly different effects on potencies and efficacies of differently efficacious ligands. Therefore, a rigorous and useful analysis of bias should take into account both potency

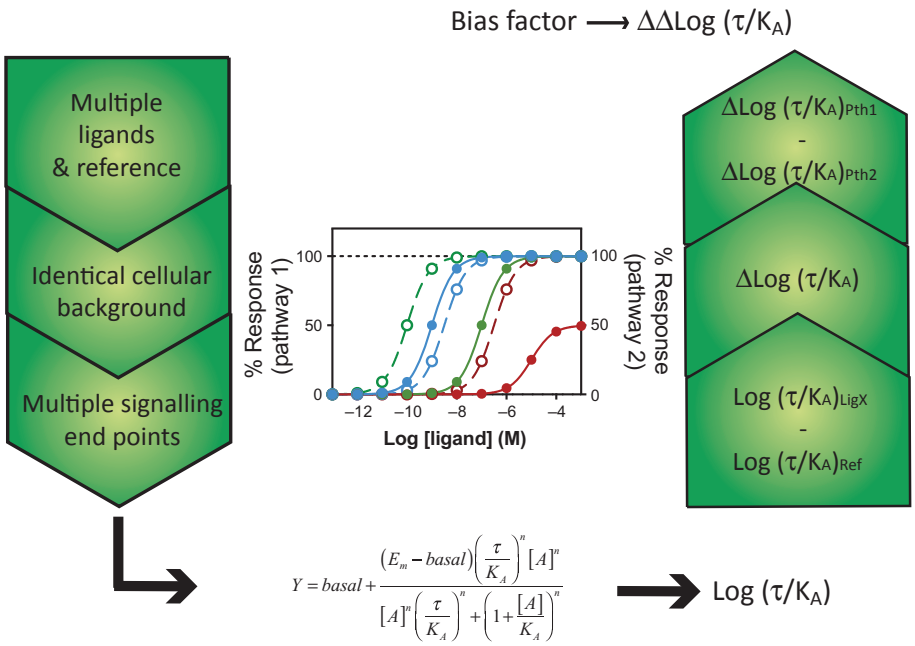

Figure 1

Quantification of biased agonism using relative transduction ratios. In order to quantify the signalling bias of a set of ligands, it is necessary to measure bias in an identical cellular background across several signalling end points. Full concentration–response curves for each pathway are fitted to the Black–Leff operational model to estimate a transduction coefficient (log[τ/K_A]) for each agonist (in the example, solid lines for pathway 1 and dotted lines for pathway 2). Next, the effects of system and observation bias are removed by adjusting these values to a reference ligand to yield the $\Delta\log[\tau/K_A]$. Finally, the relative transduction ratio ($\Delta\Delta\log[\tau/K_A]$) is obtained by subtraction of the $\Delta\log[\tau/K_A]$ value of two pathways one from another. The $\Delta\Delta\log[\tau/K_A]$ values represent ligand bias on a linear scale, which is amenable to statistical analysis. [A], agonist concentration; K_A, operational affinity; τ, efficacy; n, slope of the fitting parameter; E_m, maximal response of the system. For a step-by-step method to measure bias, see Appendix 1 or van der Westhuizen *et al.* (2014).

and maximal effect of a ligand, eliminate effects of system and observation bias, and should be broadly applicable to routinely derived concentration–response data. Such analysis would not only allow the signalling bias profiles of ligands in different cell types to be compared, but would also aid the efforts of medicinal chemists to discover new biased ligands.

Several analytical approaches have been described to quantify biased agonism (see Kenakin and Christopoulos, 2013). Of these, the relative transduction ratio [$\Delta\Delta\log(\tau/K_A)$] is one of the most robust and widely applicable method for bias quantification. This method applies the operational model of agonism first derived by Black and Leff (1983). Application of this model to concentration–response curves estimates a 'transduction coefficient' $\log(\tau/K_A)$ which is comprised of the functional equilibrium dissociation constant (operational affinity, K_A), a measure of the affinity for the receptor coupled to a particular effector protein or signalling pathway, which is different from the affinity of the ligand for the bare receptor determined in radioligand binding experiments (see later); and τ which encompasses both the intrinsic efficacy of the agonist in activating a particular signalling response and receptor density. The $\log(\tau/K_A)$ is thus an overall measure of the relative 'power' of an agonist to induce a response. In order to eliminate the effects of system and observation bias, the $\log(\tau/K_A)$ is normalized to a reference agonist, yielding values of $\Delta\log(\tau/K_A)$. Finally, these values can be compared across two signalling pathways for a given agonist to obtain the relative transduction ratio $\Delta\Delta\log(\tau/K_A)$ as measures of agonist bias (Figure 1 and Appendix 1 for a step-by-step method to measure bias).

Quantification of biased agonism at MOR

The effects of morphine in the β-arrestin2 knockout mice (Raehal *et al.*, 2005) together with the substantial evidence of the distinct effects of morphine in MOR desensitization and internalization (Johnson *et al.*, 2006; Dang *et al.*, 2011) suggest that ligands that display bias towards G-protein-mediated pathways and away from β-arrestin2 recruitment, may have improved therapeutic profiles as analgesics. Such ligands offer the potential to provide pain relief with less adverse effects normally associated with the opioid agonists, including tolerance, dependence and addiction, constipation, nausea, and respiratory depression. For this reason, most of the studies focused on detection and quantification of biased agonism have utilized these two pathways, G-protein activation and β-arrestin2 recruitment, albeit using different approaches for such determinations (Borgland, 2003; McPherson *et al.*, 2010; Molinari *et al.*, 2010; Frölich *et al.*, 2011; Rivero *et al.*, 2012).

Using a BRET approach, Molinari *et al.* (2010) investigated the ability of MOR and δ-opioid receptor (DOR) to activate

G-proteins and recruit β-arrestins. G-protein activation by a wide range of opioid ligands was measured as changes in the BRET signal between the receptor and the β1 subunit of the G-protein in cell membranes, while β-arrestin recruitment to the receptor was performed in whole cells. Plotting the relative intrinsic activities (i.e. the maximal response of a given ligand relative to the maximal response of a reference agonist) of all ligands, using the two signalling end points, revealed a hyperbolic relationship between the two pathways. This is in line with the fact that most of the tested ligands displayed full agonism for G-protein activation and that comparison of intrinsic activities fails to differentiate between full agonists (Stallaert *et al.*, 2011). A number of ligands, including morphine, oxymorphone and ethylketocyclazocine clustered as ligands with low intrinsic activities for β-arrestin2 recruitment and high intrinsic activities for G-protein activation. Indeed, this result is also expected when the coupling efficiency of different pathways is taken into consideration, as the response of partial agonists will be lower in less efficiently coupled signalling pathways such as β-arrestin2 recruitment. Estimation of the bias factor $\Delta\Delta\log(\tau/K_A)$ relative to etorphine of the corresponding concentration–response curves reveals that different intrinsic activities (such as oxymorphone vs. etorphine, endomorphin-2 or lofentanyl) do not necessarily translate into significant ligand bias (Figure 2A).

As bias is an intrinsic characteristic of a ligand, it follows that metabolites of a given ligand do not necessarily have to mimic the bias of the original compound. Interestingly, this has been evaluated *in vitro* for morphine, and its metabolites (Frölich *et al.*, 2011) using FRET approaches to detect $G\alpha_{i1}$ activation and β-arrestin2 recruitment. Comparison of relative efficacies of all the metabolites with morphine, suggested that only three metabolites (normorphine, 6-acetylmorphine, morphine-6-glucuronide) showed bias towards β-arrestin2 recruitment. However, when using morphine as the reference ligand to estimate $\Delta\Delta\log(\tau/K_A)$ values, nearly all the metabolites are significantly biased towards β-arrestin2 compared with morphine (Figure 2B). This illustrates additional information that can be obtained by application of the operational model of agonism to detect and quantify bias because, as mentioned previously, comparison of relative efficacies fails to distinguish between full agonists as their activity is limited by the system. In this case, the signalling bias of higher-efficacy agonists may be overlooked or may be mistakenly considered as biased when they are in fact not. Therefore, in the case of the higher-efficacy agonists, a scale that incorporates both the maximal response and potency, such as the transduction coefficient, is required. Such results also suggest that morphine metabolites possess divergent signalling bias, an aspect that will need to be taken into consideration when interpreting the effects of morphine signalling *in vivo*. Finally, it should be noted that in these experiments, GRK2 was only overexpressed when measuring β-arrestin2 recruitment, and as described later, the cellular content of relevant proteins or other agents can also play a role in the direction of bias. It would therefore be very informative to quantify bias of morphine metabolites under similar conditions across different signalling pathways.

In a systematic approach to evaluate biased agonism at the MOR, McPherson *et al.* examined the signalling bias of a wide range of ligands including endogenous opioid peptides and synthetic opioids (McPherson *et al.*, 2010; Rivero *et al.*, 2012). As in the previous reports, G-protein activation and β-arrestin2 recruitment were measured by [^{35}S]GTPγS binding, and an enzymatic complementation assay respectively. In these studies, endomorphin-2 was the only ligand that showed statistically significant bias towards β-arrestin2 recruitment. Agonist bias was determined by fitting concentration–response curves to the Black–Leff operational model to estimate the efficacy parameter τ. However, instead of estimating the functional affinity of the ligand-bound

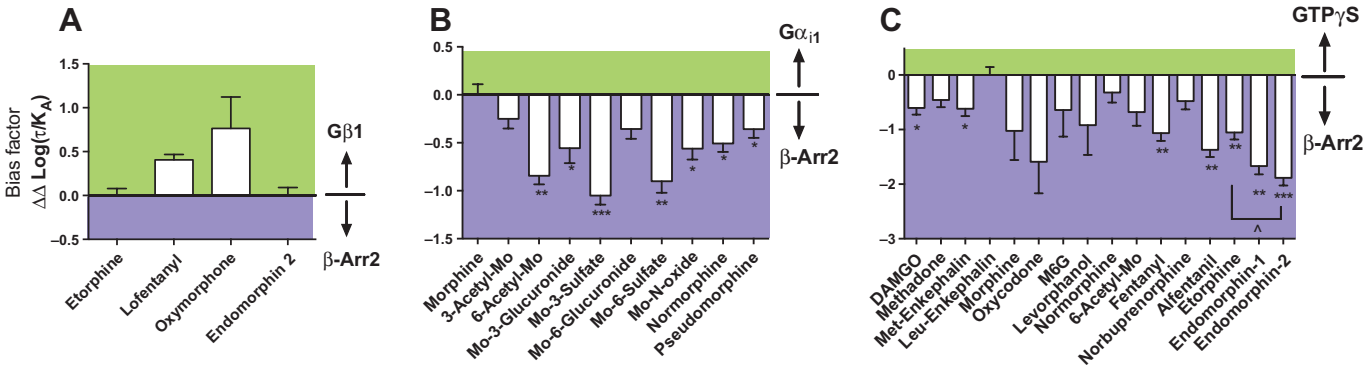

Figure 2

Quantification of signalling bias between G-protein and β-arrestin interactions at the MOR. (A) Relative transduction ratios estimated for data from Molinari *et al.* 2010 between assays for Gβ1 and β-arrestin2 interactions using etorphine as the reference ligand showed no significant bias between ligands (B) Relative transduction ratios between $G\alpha_i1$ and β-arrestin2 recruitment estimated using data from Frölich *et al.* (2011) using morphine as the reference ligand shows that most morphine metabolites are significantly biased towards recruitment of β-arrestin2 compared with morphine (C) Relative transduction ratios between GTPγS binding and β-arrestin2 recruitment from McPherson *et al.* (2010) estimated using Leu-enkephalin as the reference ligand show that in addition to endomorphin-2, endomorphin-1, etorphine and several other ligands are biased towards β-arrestin2 recruitment. The two-tailed *t*-test was used to determine whether transduction ratios were statistically different from the reference ligand * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ^ $P \leq 0.05$.

receptor in each particular pathway, the affinity parameter in these calculations remained constant across pathways and was determined from radioligand binding experiments (Rajagopal *et al*., 2011). Given that a ligand can have differing affinities for distinct receptor states (e.g., for the G-protein-bound and unbound states), such differing affinities have to be taken into account when measuring biased agonism. Although in some situations, the binding affinity and the functional affinity can be very similar, this will not always be the case (see Kenakin and Christopoulos, 2013; Shonberg *et al*., 2013). When the dissociation constant is obtained from concentration–response curves using the Black–Leff operational model and bias factors are estimated, it becomes apparent that other ligands, apart from endomorphin-2 also show significant bias towards β-arrestin2 recruitment when compared with Leu-enkephalin as a reference ligand (Figure 2C). Interestingly, some ligands used in this analysis had previously been used in other studies. However, the relative transduction ratio analysis suggests that morphine-6-glucuronide, 6-acetyl-morphine and normorphine did not show significant bias towards β-arrestin2 recruitment when compared with morphine in contrast to previous reports (Frölich *et al*. (2011). Similarly, this analysis also revealed a significant difference between etorphine and endomorphin2 that had not been detected by Molinari *et al*. (2010; Figure 2C).

Finally, the ability of different ligands to mediate G-protein activation, receptor desensitization and receptor internalization has been examined using inhibition of calcium channel currents (I_{Ca}) and immunocytochemistry, respectively, as functional readouts (Borgland, 2003). The relative efficacies of [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO), morphine, methadone and pentazocine were measured for acute inhibition of calcium currents, for homologous desensitization of these currents and for receptor endocytosis. Importantly, relative efficacies were calculated using functional affinity constants determined experimentally from receptor depletion experiments. These experiments revealed that the efficacy for I_{Ca} inhibition did not correlate with the efficacy of desensitization or receptor internalization, suggesting that morphine and, potentially, pentazocine are biased, relative to DAMGO, and that acute desensitization is not dependent on receptor internalization.

Overall, these studies have provided valuable insight into biased signalling of opioids at MOR although it still remains to be seen whether they are able to predict differential responses *in vivo*. These studies also illustrate the importance of a number of factors that influence the identification and quantification of biased agonism such as cellular content, the pluridimensionality of efficacy and the choice of a reference agonist. It is important to consider these key aspects when interpreting information obtained from studying biased signalling *in vitro*.

Effect of cellular content on biased agonism

The content of signalling effectors among different cells is not identical. As a consequence, biased agonism across different cellular systems is likely to change. This has important implications *in vivo* as, for instance, the effect of the same ligand in primary cells isolated from different tissues can show opposite bias. One relevant example in the case of the MOR-biased agonism is the effect of the levels of GRK2 expression. Overexpression of GRK2 has sometimes been used as a strategy to increase the sensitivity of the detection of β-arrestin2 recruitment to several GPCRs including the MOR (Hoffmann *et al*., 2008; Allen *et al*., 2011; Frölich *et al*., 2011). However, there is substantial evidence to demonstrate that receptor phosphorylation is also subject to bias (ligand-dependent) and that this phosphorylation can have downstream consequences such as differential engagement of signalling kinases or differential receptor regulation (Tobin *et al*., 2008; Butcher *et al*., 2011; Doll *et al*., 2011; Just *et al*., 2013). Thus, overexpressing a particular kinase may have differential effects on the efficacy of distinct ligands, thus changing the bias profile of the entire set of ligands. One approach to minimize such artificial introduction of bias is to evaluate all the signalling end points under exactly the same cellular conditions and content, for example overexpressing GRK2 when investigating all the pathways in addition to β-arrestin2 recruitment.

However, the issue of differential contents of effector and regulatory proteins in different tissues still remains. For example, high levels of GRK2 are found in brain, leukocytes, heart and spleen, followed by lung and kidney (Aragay *et al*., 1998). Thus, quantification of signalling bias in recombinant cells can be used to reveal ligands with unique signalling profiles that can then be used as pharmacological tools for studying the consequences of biased agonism *in vivo*. It is therefore important to adopt a global perspective on the concept of bias, that is as an indicator of differential behaviours, fingerprints or activity profiles across ligands at the same receptor that can ultimately translate to different physiological outcomes.

Efficacy is pluridimensional

Most of the descriptions of biased agonism to date have focused on the differential activation of G-protein-mediated events and β-arrestin2 recruitment. However, it is evident that most GPCRs pleiotropically couple to a myriad of signalling effectors. The ability of compounds to promote unique, ligand-selective conformations of GPCRs that are able to engage different transduction pathways or regulatory events underlies the mechanism for the pluridimensionality of efficacy (Galandrin *et al*., 2007). As such, the detection of bias should be extended beyond differences between G-protein activation and β-arrestin2 recruitment. Additionally, it is now clear that signalling efficacy through GPCRs is not linear, and that multiple mechanisms control the responsiveness of receptor systems such as desensitization and internalization, resulting from receptor phosphorylation (Kenakin, 2007). It is now established in several GPCR models, including the MOR, that not all ligands with similar apparent efficacy towards a given signalling pathway display a similar propensity to trigger these regulatory events (Sternini *et al*., 1996).

The MOR has been shown to couple to many signalling effectors via G-proteins and β-arrestins (see Law, 2011; Raehal *et al*., 2011; Georgoussi *et al*., 2012). Furthermore, the MOR has also been shown to directly interact with phospholipase D2 (Koch *et al*., 2003), and with proteins that control its localization in lipid raft microdomains (Ge *et al*., 2009). As such, limiting bias studies to these two proximal events directly limits the detection of functional selectivity. In addition, there is now evidence of biased activation of G-protein subunits by the MOR, which is not detected in proximal

G-protein activation assays (such as cAMP inhibition), but may differentially affect downstream signalling (Massotte *et al.*, 2002; Clark and Traynor, 2006; Saidak *et al.*, 2009). Similarly, biased effects on β-arrestin activity are not completely captured in β-arrestin recruitment assays. β-arrestins have a diverse range of functions that affect signalling and receptor regulation, which are dependent on both cellular content and the ligand itself. The strength of interaction between the receptor and β-arrestin, as measured in a β-arrestin recruitment assay, is not necessarily indicative of the subsequent effects on downstream signalling and receptor regulation.

The systematic analysis of many signalling end points will maximize the information obtained from biased signalling studies *in vitro* as such approaches will provide 'textures' of ligands in cellular models. Different 'textures' *in vitro* may be indicative of distinct physiological fingerprints when translating bias into physiologically relevant systems.

Relevance of the reference ligand

As mentioned earlier, signalling bias is a relative measure; it is always in comparison with another ligand. As such, choosing a reference ligand is a key aspect of the quantification. The reference ligand itself is not unbiased (there is no such thing), but ideally, the reference ligand should show activity in most pathways as well as possess a signalling profile similar to the endogenous ligand or to most ligands that target that particular GPCR. It can be misleading to use a reference agonist such as morphine, which is known to exhibit differential signalling when compared with most endogenous opioid peptides. Under most conditions, morphine will be biased towards G-protein-mediated signalling, so if it is used as a reference agonist, most other ligands will be clustered as β-arrestin biased. This is illustrated in Figure 3, where bias between G-protein activation and β-arrestin2 recruitment measured by Frölich *et al.* (2011), is quantified using two different reference ligands. As shown earlier, when morphine is used as the reference ligand to estimate values of $\Delta\Delta\log(\tau/K_A)$, nearly all the morphine metabolites are biased towards β-arrestin2 recruitment. However, when an agonist with higher efficacy in the β-arrestin2 recruitment assay, such as normorphine, is used as the reference agonist, the calculated bias for many of the metabolites appears to change. This shows that the majority of the metabolites are similar to normorphine, whereas morphine becomes significantly G-protein-biased and there are now only three metabolites that are biased towards β-arrestin2 recruitment. However, it is important to note that while the absolute bias factors ascribed to an agonist will change depending upon the ligand designated as reference, whether or not a significant difference exists between any ligand pair will not change regardless of which ligand is designated as reference.

In order to make direct comparisons of signalling bias among different studies, it is important that the same ligand is used as a reference for quantification. The reference ligand is also very important when attempting to predict bias *in vivo*. As mentioned earlier, ligand bias is cell-dependent, making predictions of *in vivo* bias difficult. However, extensive knowledge of the physiological activity and signalling by the reference ligand, will allow links between signalling profiles and physiological effects to be made. Hence, the reference should ideally be a ligand that has been very widely studied. The obvious choice in most cases would be to use the endogenous ligand; however, that is not straightforward with opioid receptors because of the existence of many endogenous opioid peptides. As DAMGO and Met-enkephalin have been the ligands most widely studied in MOR biology, these two ligands would potentially be ideal 'universal' candidates.

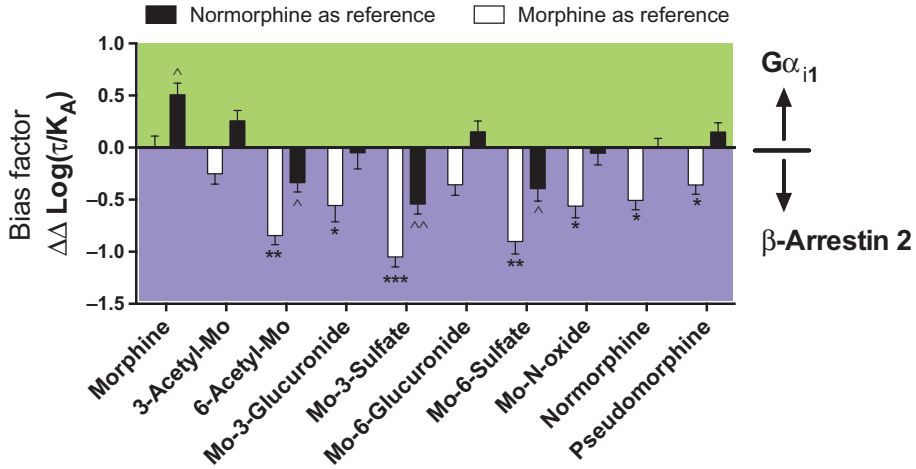

Figure 3

Quantification of bias using different reference ligands. Concentration–response curves from Frölich *et al.* 2011 for G-protein activation and β-arrestin2 recruitment were fitted to the operational model to estimate values of $\Delta\Delta\log(\tau/K_A)$ between the two pathways using either morphine or normorphine as the reference ligand. When morphine is used as the reference ligand most morphine metabolites are biased towards β-arrestin2 recruitment. When normorphine is used as the reference, morphine becomes G-protein-biased and its derivatives, 6-acetyl-morphine (6-acetyl-Mo), Mo-3-sulfate and Mo-6-sulfate are β-arrestin2-biased. The two-tailed t test was used to determine whether transduction ratios were statistically different from the reference ligand, morphine $^*P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$ or normorphine $^{\wedge}P \le 0.05$, $^{\wedge\wedge}P \le 0.01$.

However, the choice of reference ligands will always depend on the question that a particular study is trying to address.

Use of biased agonism in drug discovery to improve pharmacological profiles of analgesics

Quantification and determination of bias *in vitro* can guide medicinal chemists towards the design of biased GPCR ligands for those receptors where the signalling cascades responsible for therapeutic versus side effects are known. Structure–activity relationship (SAR) studies at the angiotensin AT_1 receptor have resulted in a β-arrestin2-biased ligand (TRV120027) that is currently in clinical trials for acute decompensated heart failure (Boerrigter *et al.*, 2011). Similarly, at the dopamine D_2 receptor, biased partial agonists have been identified by exploring the structure of the antipsychotic aripiprazole through a combinatorial chemistry approach (Allen *et al.*, 2011) and, more recently, by classical SAR studies supplemented with parameters of bias and functional affinity determined using the transduction coefficient method (Shonberg *et al.*, 2013).

Given the phenotype of the β-arrestin2 knockout mouse and the accumulated evidence of ligand-directed signalling at the MOR, SAR approaches for the biased activation of this receptor have also been developed and have yielded promising compounds with analgesic function and improved side effect profile (Varamini *et al.*, 2012; DeWire *et al.*, 2013). Recently, new MOR ligands (low MW compounds and endomorphin-1 derivatives) that produce analgesia with less gastrointestinal dysfunction and respiratory depression have been reported. One of these compounds, TRV130, is the result of SAR screening studies by Trevena, Inc. focused on the discovery of G-protein-biased (as opposed to β-arrestin2 biased) ligands at the MOR (Chen *et al.*, 2013). *In vitro*, and compared with morphine, TRV130 displayed markedly different responses when assessed by two different signalling end points – inhibition of forskolin-induced cAMP and β-arrestin2 recruitment. Additionally, TRV130 showed decreased phosphorylation of the receptor at Ser^{375} and failed to internalize receptors. The authors examined bias between adenylate cyclase inhibition and β-arrestin2 recruitment by comparison of relative intrinsic activities (Rajagopal *et al.*, 2011) of TRV130 and morphine, and showed that TRV130 was biased towards adenylate cyclase inhibition. However, statistical comparison of bias using this method was hampered by the low efficacy of TRV130 in β-arrestin2 recruitment. As an alternative approach, the authors constructed a 'bias plot', where the normalized responses as changes in cAMP were shown as a function of the corresponding response in β-arrestin recruitment (Gregory *et al.*, 2010a; Kenakin and Christopoulos, 2013). Bias plots are useful graphical tools for visualizing bias of ligands between two pathways, but they incorporate all three types of bias, observation, system bias and ligand bias. This means ligand bias can only be observed when there are extreme differences between ligands, and also makes bias plots unsuitable for quantifying bias. Estimation of the bias factor using the operational model of agonism described above showed that the relative transduction ratio of TRV130 was not statistically different from morphine. The reduced β-arrestin2 recruitment observed with TRV130 in HEK cells could be attributed to the fact that it is a low-efficacy agonist and hence, poorly stimulates signalling pathways with low coupling efficiency. However, this moderate difference in the bias factors is very likely to be more relevant *in vivo*, which accounts for the improved pharmacological profile of TRV130. A more comprehensive analysis of bias of TRV130 across many signalling pathways, and compared with other opioid ligands will reveal more information about its unique bias profile, and provide insight into how TRV130 exerts its effects *in vivo*.

Finally, it is worth noting that most of the SAR studies performed so far that were focused on the discovery of biased ligands, have utilized differences between G-protein and β-arrestin-mediated pathways. However, it is tempting to anticipate that, in the coming years, there will be an increase in the number of studies that investigate a more diverse array of signalling end points that will reveal differential 'textures' of GPCR ligands.

Allosterism at the MOR

Although topographically distinct, druggable, allosteric sites have been postulated to be present in all GPCRs (May *et al.*, 2007; Gregory *et al.*, 2010b), the discovery of allosteric ligands that bind to the opioid receptors has remained a challenge until recently. Allosteric ligands induce conformational changes that are transmitted from the allosteric binding pocket to the orthosteric binding site. They offer the potential of improved subtype selectivity, decreased risk of overdose and maintained spatial and temporal activity of the target receptor (Keov *et al.*, 2011). All these pharmacological characteristics are of particular relevance for opioid-based therapies, as they may offer the potential to overcome the tolerance and dependence developed upon chronic/prolonged receptor activation. However, several considerations need to be taken into account regarding the effects of allosteric modulators on GPCRs.

The classical view of GPCR allosterism has focused on the change elicited on the properties of the orthosteric ligand; however, the conformational changes induced by the binding of an allosteric modulator can have similar consequences when considering the cytosolic proteins that interact with the receptor and mediate signal transduction. More importantly, this effect can vary depending on the different intracellular effectors. Macroscopically, this translates in some pathways being modulated, in either a positive or negative direction, at the expense of others. Finally, allosteric ligands can also display efficacy in their own right, and as such, they can potentially activate signalling pathways that are distinct from those activated by the orthosteric ligands. It is therefore important that the characterization of allosteric ligands includes the assessment of many relevant signalling pathways as well as the intrinsic efficacy of allosteric ligands on their own.

Interestingly, compared with other family A GPCRs, there have been significantly fewer allosteric ligands discovered for the opioid receptors. This is in contrast with the several descriptions of 'allosteric interactions' across opioid receptor

dimers, whether homodimers or heterodimers (Jordan and Devi, 1999; Wang, 2005; Milligan, 2009; Yekkirala *et al.*, 2012). With regards to the MOR subtype, the crystal structure of the MOR already suggests an oligomeric arrangement of this receptor (Manglik *et al.*, 2012), and allosteric interactions have been described between MOR and mGluR5, CB_1, DOR and κ–opioid receptors (Yekkirala *et al.*, 2011; Metcalf *et al.*, 2012; Akgün *et al.*, 2013; Le Naour *et al.*, 2013).

In terms of – low MW allosteric modulators, Burford *et al.* (2013) recently discovered the first allosteric modulators of the MOR using high throughput screening with a complementation approach to measure β-arrestin2 recruitment. This screening resulted in the identification of two positive allosteric modulators (PAMs) and two silent allosteric modulators (which bind to the allosteric site of the receptor but have neutral cooperativity with the orthosteric ligand). BMS-986121 and BMS-986122 positively modulated the binding of DAMGO to the MOR and potentiated the effects of endomorphin-1, DAMGO and morphine in β-arrestin2 recruitment, G-protein activation and cAMP inhibition. This exciting discovery has provided the tools to investigate the effects of allosterism on ligand-dependent effects at the MOR. For example, how do PAMs affect MOR regulation by different agonist? Do PAMs differently affect synthetic versus endogenous opioid ligands? It will also be extremely interesting to investigate whether PAMs can potentiate the analgesic effects of current opioid drugs or endogenous opioids, without potentiation of the side effects.

Concluding comments

Although GPCRs are coupled to a plethora of signalling pathways, most descriptions of biased agonism have been based on the comparison of two signalling events across different ligands. It is therefore quite likely that the 'relevant' signalling event has been omitted by the initial selection of signalling end points. Additionally, it is unlikely that a response derives from the activation of a very distinct signaling pathway. Rather, physiological responses are likely to reflect complex outputs from a tightly controlled and selective activation of a particular group of intracellular signals. Such a holistic view represents a major challenge for pharmacologists and medicinal chemists. One potential approach to predict physiological outcomes is the thorough investigation of many signaling end points in simple cellular models to generate ligand activity profiles. Subsequently these specific fingerprints can be related to more complex physiological responses. This approach requires not only a robust and systematic quantification method, but also the validation of signaling profiles in more relevant systems. However, once obtained, these fingerprints will represent a framework that will offer the potential to predict the physiological outcome from a novel drug.

Parallel with the discovery of new biased MOR ligands with improved therapeutic windows, the discovery of opioid receptor allosteric modulators will also open new avenues to overcome the current limitations of opioid ligands as analgesics. As such, the evaluation of this new class of compounds *in vivo* will be extremely informative in terms of whether allosterism can be exploited to generate better and safer analgesics.

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Conflict of interest

The authors state no conflict of interest.

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Appendix 1

Step-by-step quantification of bias using the operational model of agonism.

1. Concentration–response data should be normalized to the response of a reference ligand.
2. The operational model is used to determine the transduction ratios (τ/K_A) for the different agonists using the following equation which derives from the standard form of the operational model shown in Figure 1 [for a complete derivation, see van der Westhuizen *et al*. (2014)]

$$E = Basal + \frac{(E_m - Basal)}{1+\left(\frac{\left(\frac{[A]}{10^{\log K_A}}+1\right)}{10^{\log R\times[A]}}\right)^n}$$

   where E is the response of the ligand, $[A]$ is the concentration of agonist, E_m is the maximal possible response of the system, *Basal* is the basal level of response in the absence of agonist, $\log K_A$ denotes the logarithm of the functional equilibrium dissociation constant of the agonist, n is the slope of the transducer function that links occupancy to response, and logR is the logarithm of the 'transduction coefficient' (or 'transduction ratio'), τ/K_A, where τ is an index of the coupling efficiency (or efficacy) of the agonist. For the analysis, all families of agonist curves at each pathway are globally fitted to the model with the parameters, *Basal*, E_m and n shared between all agonists. For full agonists, the $\log K_A$ should be constrained to a value of zero, whereas for partial agonists this is directly estimated by the curve fitting procedure (van der Westhuizen *et al*., 2014). The logR [i.e. $\log(\tau/K_A)$] parameter is estimated as a unique measure of activity for each agonist.
3. Elimination of system and observation bias is achieved by comparing ligand activity at a given signalling pathway with that of a reference compound. As such, the difference between the $\log\tau/K_A$ of an agonist and the reference ligand is calculated using the equation:

$$\Delta\log\left(\frac{\tau}{K_A}\right) = \log\left(\frac{\tau}{K_A}\right)_{Ligand} - \log\left(\frac{\tau}{K_A}\right)_{Reference}$$

4. Ligand bias is finally calculated as the difference between the $\Delta\log(\tau/K_A)$ values obtained from the previous equation at two different pathways

$$\Delta\Delta\log\left(\frac{\tau}{K_A}\right) = \Delta\log\left(\frac{\tau}{K_A}\right)_{Pathway\ 1} - \Delta\log\left(\frac{\tau}{K_A}\right)_{Pathway\ 2}$$

5. The bias factor is then the anti-logarithm of $\Delta\Delta\log(\tau/K_A)$.

$$\text{bias factor} = 10^{\Delta\Delta\log\left(\frac{\tau}{K_A}\right)}$$

6. For complete GraphPad Prism equations and fitting parameters, see van der Westhuizen *et al*. (2014).