ANNUAL REVIEWS

Annual Review of Pharmacology and Toxicology

Biased Agonism: Lessons from Studies of Opioid Receptor Agonists

Eamonn Kelly, Alexandra Conibear, and Graeme Henderson

School of Physiology, Pharmacology and Neuroscience, University of Bristol, Bristol, United Kingdom; email: E.Kelly@bristol.ac.uk

Annu. Rev. Pharmacol. Toxicol. 2023. 63:491–515

First published as a Review in Advance on September 28, 2022

The *Annual Review of Pharmacology and Toxicology* is online at pharmtox.annualreviews.org

https://doi.org/10.1146/annurev-pharmtox-052120-091058

Keywords

ligand bias, opioid receptor, G protein, β-arrestin

Abstract

In ligand bias different agonist drugs are thought to produce distinct signaling outputs when activating the same receptor. If these signaling outputs mediate therapeutic versus adverse drug effects, then agonists that selectively activate the therapeutic signaling pathway would be extremely beneficial. It has long been thought that μ-opioid receptor agonists that selectively activate G protein– over β-arrestin-dependent signaling pathways would produce effective analgesia without the adverse effects such as respiratory depression. However, more recent data indicate that most of the therapeutic and adverse effects of agonist-induced activation of the μ-opioid receptor are actually mediated by the G protein–dependent signaling pathway, and that a number of drugs described as G protein biased in fact may not be biased, but instead may be low-intrinsic-efficacy agonists. In this review we discuss the current state of the field of bias at the μ-opioid receptor and other opioid receptor subtypes.

INTRODUCTION: LIGAND BIAS AT G PROTEIN–COUPLED RECEPTORS

GPCR kinase (GRK): a class of protein kinases (GRK1–GRK5) that mediate agonist-induced phosphorylation of GPCRs

β-arrestins: a class of proteins that interact with activated, GRK-phosphorylated GPCRs; the subtypes β-arrestin 1 and β-arrestin 2 are found in most cells

On target: effects produced by a drug following its interaction with a defined receptor as opposed to actions via other sites

Desensitization: the tendency of the response to agonist activation of a receptor to decrease following prolonged exposure to the drug

The idea of ligand bias, also known as functional selectivity, is one that has transformed molecular pharmacology and has the potential to lead to the development of new, more effective medicines with fewer adverse effects (1–4). The basis of ligand bias is the idea that some agonists possess the ability to stabilize active conformations of a G protein–coupled receptor (GPCR) that are distinct from those that are stabilized by other agonists, and that these conformations can couple differently to downstream signaling proteins such as G proteins and GPCR kinases (GRKs)/β-arrestins (5) (**Figure 1**). This idea has led to descriptions of G protein–biased or β-arrestin-biased agonists, and many such agonists have now been described for different GPCRs (6). However, bias can also exist with respect to the activation of any receptor signaling mechanism, such as the ability to selectively activate different G protein subtypes (7). An important consequence of the idea of biased agonists is that if such agonists are to be useful, then the therapeutic and on-target adverse effects produced by an agonist must be mediated by different signaling mechanisms in the relevant cells of the body. If this is not the case, then the hunt for biased agonists as more effective medicines may well be largely a waste of time and money.

μ-Opioid Receptor Signaling

Before discussing bias, we define the signaling processes mediated through opioid receptors. The μ-opioid receptor is a $G_{i/o}$-coupled GPCR whose downstream functional signaling can be grouped into four general (and at times overlapping) areas: G protein–dependent signaling, G protein–independent signaling, desensitization, and internalization (**Figure 2*a***). The last two are included in signaling because desensitization produces reduced signaling (8) and receptor internalization can result in intracellular signaling (9) as well as downregulation (10). G protein–dependent signaling mediated by activation of μ-opioid receptors involves inhibition of adenylyl cyclase and the regulation of various plasma membrane ion channels, including activation of G protein–coupled inwardly rectifying potassium channels (GIRKs) and inhibition of presynaptic N- and P-/Q-type Ca^{2+} channels (11). Inhibition of such channels in primary afferent neurons

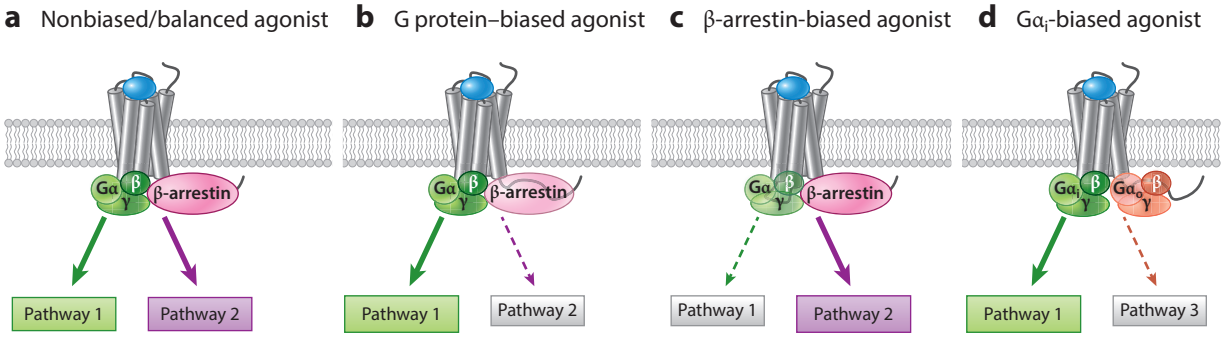

Figure 1

Signaling of biased agonists at G protein–coupled receptors. (*a*) It is generally accepted that most agonists regarded as nonbiased or balanced can efficiently activate both G protein– and β-arrestin-dependent signaling. (*b*,*c*) Relative to the nonbiased agonist, in what are considered the most common forms of bias, the biased agonists preferentially activate either G protein– or β-arrestin-dependent signaling, as shown by the solid arrows. (*d*) Bias can extend to any signaling pathway downstream of the receptor, and in this case the agonist displays bias between the signaling pathways mediated by two different G protein subtypes, $G\alpha_i$ and $G\alpha_o$. Figure adapted with permission from Reference 19; copyright 2019 the American Society for Pharmacology and Experimental Therapeutics.

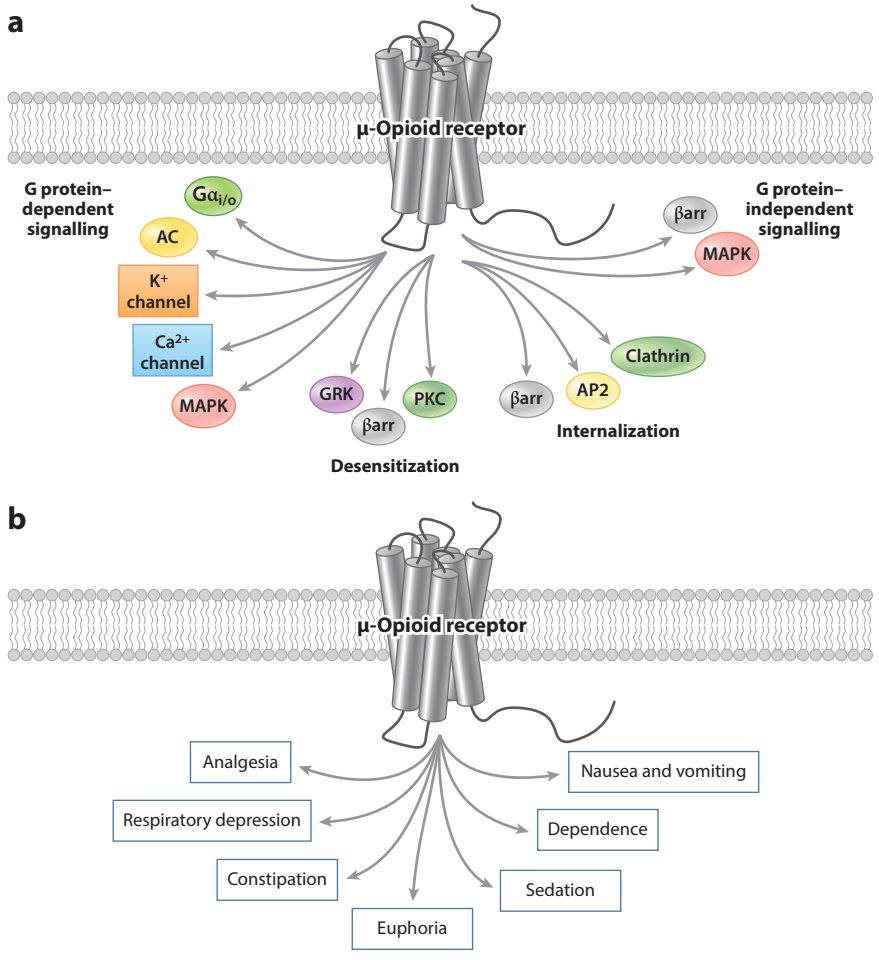

Figure 2

(*a*) Cell signaling pathways mediated by agonist activation of the μ-opioid receptor and (*b*) effects of μ-opioid receptor activation in vivo. In panel *a*, the outputs have been placed into four categories: G protein–dependent signaling, G protein–independent signaling, desensitization, and internalization. Abbreviations: βarr, β-arrestin; AC, adenylyl cyclase; AP2, adaptor protein 2; GRK, G protein–coupled receptor kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C. Panel *a* adapted with permission from Reference 10; copyright 2013 the American Society for Pharmacology and Experimental Therapeutics. Panel *b* adapted from Reference 61; copyright 2013 British Pharmacological Society.

represents a major site of antinociceptive activity mediated by μ-opioid receptor agonists (12, 13). G protein–independent signaling includes that mediated by the β-arrestins: β-arrestin 1 and β-arrestin 2 (14). The cell signaling that occurs downstream of β-arrestins following opioid receptor activation is largely unknown but is assumed to be similar to β-arrestin signaling by other GPCRs (14). The β-arrestin signaling is also considered to occur over a slower timescale than does G protein–dependent signaling, particularly if the response involves changes in gene expression (14). The association of the μ-opioid receptor with β-arrestins is dependent on both agonist occupation of the receptor and phosphorylation of the receptor's COOH terminus by GRKs (10). Association of β-arrestins with the GRK-phosphorylated receptor is then thought to

Table 1 Signaling bias and efficacy profile of μ-opioid receptor agonists

Drug	Biased at the μ-opioid receptor?	Efficacy in cellular signaling[a]	Use
Morphine	No (47, 59, 61)	Medium/low (partial agonist in most cases)	Analgesic
DAMGO	Commonly used as reference/ balanced agonist (e.g., 51)	High (full agonist in all systems)	Experimental
Fentanyl	No (57, but see 51)	Medium/high (full agonist in most cases)	Analgesic
Methadone	No (57)	High (full agonist in most cases)	Analgesic, opioid use disorder
Oxycodone	No (47)	Medium/low (partial agonist in most cases)	Analgesic
Endomorphin-1, endomorphin-2	Some β-arrestin bias (57, 59)	Medium/high (full agonist in most cases)	Experimental
TRV130 (oliceridine)	Possible G protein bias (71) but disputed (47)	Low (weak partial agonist in most cases)	Analgesic
PZM21	Possible G protein bias (82) but disputed (47, 84)	Low (weak partial agonist in most cases)	Experimental
SR-17018	Possible G protein bias (51) but disputed (47)	Low (weak partial agonist in most cases)	Experimental
Mitragynine	Possible G protein bias (152)	Medium/low (partial agonist in most cases)	Experimental

[a]Many μ-opioid receptor agonists that behave as partial agonists within in vitro cell signaling assays are full agonists with in vivo measures of agonist activity (e.g., antinociception).

lead to receptor desensitization as well as internalization of the receptor (**Figure 2*a***), although the exact relationship of these mechanisms to each other and to in vivo tolerance to opioid agonists remains unclear (10).

Tolerance: the tendency of agonist effects in a tissue or organism to decrease following prolonged exposure to the drug

Dependence: a state characterized by behavioral and other responses resulting in compulsions to take a drug

Opioid epidemic: the recent large-scale increase in the abuse of opioids, particularly in North America, leading to large numbers of overdose deaths

Opioid Receptors and Ligand Bias

Opioid receptors represent some of the most important pharmacological targets in the body (15); for example, μ-opioid receptor agonists such as morphine and oxycodone (see **Table 1** for summary details of these drugs and others mentioned in the review) provide relief from severe pain but also produce significant adverse effects, including respiratory depression and constipation, as well as euphoria, dependence, and addiction (16) (**Figure 2*b***). Clearly, given the severity and life-threatening nature of some of these adverse effects, novel μ-opioid receptor agonists that are able to induce strong and long-lasting analgesia but not, for example, dependence or respiratory depression would be desirable. The current opioid epidemic in North America (17), and the increasing misuse of prescribed and illicit opioids in other countries, adds impetus to this search. However, while the widespread search for biased agonists at the μ-opioid receptor has been ongoing for several years, it can be argued that such agonists, that is, μ-opioid receptor agonists that are effective analgesics yet are devoid of serious adverse effects, remain elusive.

To varying degrees, the possibility of ligand bias has been investigated at each of the opioid receptor subtypes, μ-, δ-, and κ-opioid receptors, as well as the related nociceptin (NOP) receptor (18–23). Not surprisingly, most studies so far have focused on the μ-opioid receptor due to its physiological, therapeutic, and societal importance. Ironically, however, in the end it may be the other opioid receptor subtypes that become more successful targets in terms of biased agonists leading to new therapeutics. In this review we first assess the evidence that the study of bias at

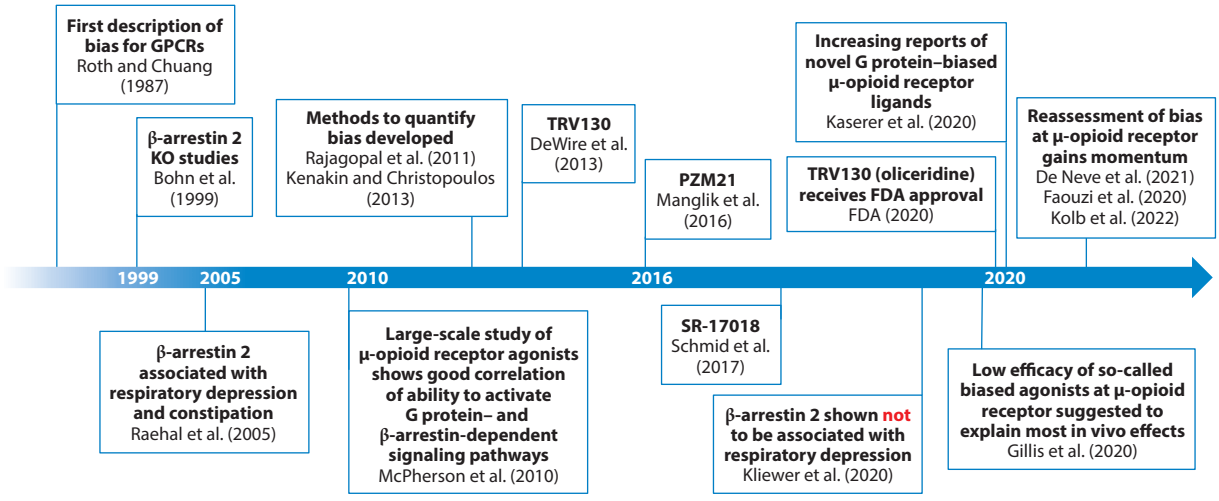

Figure 3

Timeline of important developments in ligand bias at the μ-opioid receptor. From left to right, References 156, 24, 26, 57, 55, 2, 71, 82, 51, 28, 47, 73, 94, 21, 22, and 118 are cited.

the μ-opioid receptor is a worthwhile venture that is likely to lead to new therapeutics, and then discuss whether the putative biased agonist ligands at the μ-opioid receptor so far reported are indeed what they claim to be, and whether current evidence indicates that they have real therapeutic potential. Additionally, we discuss the therapeutic potential of ligand bias at the other opioid receptors. In later sections we consider the lessons to be learned from examining over 20 years of published work in the field of bias at opioid receptors; a timeline of notable points in the story of biased agonism at the μ-opioid receptor is provided in **Figure 3**.

BIASED AGONISM AT μ-OPIOID RECEPTORS: LESSONS FROM GENETICALLY MODIFIED MICE

The rationale to develop biased agonists at the μ-opioid receptor as a therapeutic strategy had its beginnings over 20 years ago when it was reported that morphine produced more effective antinociception with less propensity to develop tolerance in mice lacking β-arrestin 2 (β-arrestin 2 knockout mice) than in wild-type mice (24, 25). In addition, [^{35}S]-GTPγS binding stimulated by DAMGO ([D-Ala^2, *N*-MePhe^4, Gly-ol^5]-enkephalin) was increased in brain membranes from mice lacking β-arrestin 2 (25), suggesting that in vivo regulation of μ-opioid receptor function was mediated in large part by β-arrestin 2. Thus, it was envisaged that μ agonists that selectively engage G protein signaling pathways could be more effective analgesics, particularly as such agonists would exhibit less tolerance. In a later study an additional observation transformed the opioid field: Morphine-induced respiratory depression was reported to be markedly reduced or even absent in mice lacking β-arrestin 2 (26). In addition, some markers of constipation, including morphine-induced inhibition of fecal boli production, were also reported to be reduced in mice lacking β-arrestin 2 (26). The conclusion was that G protein activation mediates the analgesic effects of μ-opioid receptor agonists, whereas tolerance and adverse effects such as respiratory depression and constipation are mediated largely by β-arrestin 2–dependent pathways (26). Thus, the idea that G protein–biased agonists at the μ-opioid receptor would be more effective and safer medicines because they would induce strong analgesia with limited tolerance and display fewer adverse effects, particularly the potentially lethal respiratory depression, gained a strong footing

DAMGO: [D-Ala^2, *N*-MePhe^4, Gly-ol^5]-enkephalin; a high-efficacy peptide agonist selective for the μ-opioid receptor; used as a standard in many experiments

(27). It is fair to say that this idea has to a large extent dominated the field of bias at μ-opioid receptors ever since (20), even following the appearance of contradictory evidence, as detailed below.

More Recent Studies with Genetically Modified Mice

For 20 years, no other research groups reported attempts to repeat the study with morphine in mice lacking β-arrestin 2. Eventually, in a collaborative effort, three laboratories—including our own—in different countries tested the effects of morphine on respiration in wild-type mice and in mice lacking β-arrestin 2 (28). In stark contrast to the original report (26), each laboratory found that the ability of morphine to depress respiration was the same with or without the presence of β-arrestin 2. Since then, this finding has been confirmed in no fewer than four further studies (29–32). Indeed, one of those studies (30) found that following high doses of fentanyl, mice lacking β-arrestin 2 were actually more likely to stop breathing and die than were wild-type mice, a result that certainly does not indicate that G protein–biased agonists at the μ-opioid receptor will be safer. An alternative approach (33) used mice with knock-in mutant μ-opioid receptors lacking COOH terminus phosphorylation sites. These mutant receptors are unable to recruit β-arrestins, but in the mice expressing them both morphine and fentanyl depressed respiration to the same extent or even slightly more than that in wild-type mice (33). Together these studies make a compelling case that morphine-induced respiratory depression is not mediated by β-arrestins; thus, the hypothesis that G protein–biased agonists at the μ-opioid receptor will be safer therapeutics because they are less likely to depress respiration has little foundation. It was in any case difficult to envisage just how β-arrestin-mediated signaling, which is slow relative to G protein signaling (34), could provide rapid second-to-second regulation of respiratory neurons (35); indeed, there is now good evidence to indicate that opioid-induced respiratory depression is mediated by G protein modulation of ion channels (36, 37) located on neurons within defined brain nuclei (35). Nevertheless, the idea that β-arrestin 2 engagement mediates many of the adverse effects of μ-opioid receptor activation has been extremely influential and continues to be cited as a basis for the development of novel G protein–biased agonists at the μ-opioid receptor (38, 39). The reason for the original observation of loss of morphine-induced respiratory depression (26) may lie in the genetic background of the mice lacking β-arrestin 2 used in these experiments (40). The initial studies from the Bohn laboratory (26) were performed on mice lacking β-arrestin 2 that were of a mixed strain background, having been bred from knockout 129/SvJ males and wild-type C57BL/6 females. These particular animals are in fact less sensitive to morphine for reasons not related to β-arrestin 2 engagement. Indeed, there is evidence of strain variation in opioid-induced antinociception, respiratory depression, and tolerance (40–43). The major lesson to be drawn from these studies using genetically modified mice is the importance of independent laboratories and/or other experimental approaches corroborating key findings before they become the basis for major investment of research effort.

Other Issues with Using Genetically Modified Mice

While a major focus of the studies with genetically modified mice was the apparent loss of morphine's ability to depress respiration in the absence of β-arrestin 2 (26), other findings from the earlier studies should have given serious pause for thought in terms of safer opioid therapeutics. The authors of the original report with mice lacking β-arrestin 2 (24) had made the important observation that measures of opioid dependence, assessed following withdrawal of morphine treatment, were unchanged in mice lacking β-arrestin 2 (25). This finding was recently confirmed in mice expressing mutant μ-opioid receptors that are unable to recruit β-arrestins (33). In addition,

reward-seeking behavior was actually increased in mice lacking β-arrestin 2 (44). This finding suggests that the whole spectrum of available data from different approaches should be considered before determining the suitability of a new drug class, such as biased agonists, for development as novel therapeutics.

Another issue regarding the role of β-arrestins in μ-opioid receptor function relates to agonist-dependent effects in mice lacking β-arrestin 2. Whereas as described above the analgesic response to morphine in mice lacking β-arrestin 2 was enhanced and prolonged (24), the response to agonists such as fentanyl, oxycodone, and methadone was not (45, 46). The reason for this difference remains unclear and is particularly intriguing for oxycodone, as the efficacy profile of G protein and β-arrestin engagement for this drug is similar to that for morphine (47). However, in mice with mutant μ-opioid receptors unable to recruit β-arrestins, tolerance to both morphine and fentanyl was blunted (33). Irrespective of whether these results reflect differences in the animal models used in these studies (33, 45, 46) or differences in the experimental protocols employed (48), these data again raise questions about adopting a simple model in which G protein–biased agonists at the μ-opioid receptor will inevitably increase responsiveness to the agonist by exhibiting reduced tolerance.

Furthermore, what these studies of mice lacking β-arrestin engagement with the μ-opioid receptor really highlight is the need to clearly identify cellular signaling pathways that mediate the different behavioral effects produced by agonist activation of the receptor (1). In the case of β-arrestins and the effects of μ-opioid receptor agonists, this had not been done properly; indeed, the evidence now points to the same pathway, that is, G protein–dependent signaling, mediating most of the therapeutic and adverse effects of μ-opioid receptor activation (28, 33). Apart from β-arrestin signaling contributing to receptor desensitization (49) and tolerance in some cases, its role in opioid function in vivo remains relatively obscure. Ironically a recent study (31) suggests that the improved adverse effect profile of μ-opioid receptor agonists actually correlates with increased interaction of the receptor with β-arrestins—the opposite of the original hypothesis: that reduced interaction of the μ-opioid receptor with β-arrestins is desirable (20, 26). Further studies are now needed to explore this latest idea (31, 50), which if correct would overturn many of the assumptions about μ-opioid receptor function from the past two decades.

BIASED AGONISTS AT μ-OPIOID RECEPTORS: A CASE OF SMOKE AND MIRRORS?

Another way to assess the in vivo potential of bias at the μ-opioid receptor is to develop strongly biased agonists that can then be compared to the effects of unbiased agonists in vivo. Of course, this requires the in vitro identification of μ agonists with clear bias, but again this has led opioid researchers along a somewhat rocky road as the extent of bias, and indeed whether bias exists at all for some of these new ligands (**Figure 4**), remains an area of contention (47). Furthermore, the issues of receptor specificity and pharmacokinetics complicate the interpretation of results from in vivo studies (47, 48).

How Is Ligand Bias Measured?

For potentially biased ligands, different in vitro studies using distinct experimental approaches and methods of data analysis have at times reached different conclusions regarding the presence or absence of bias (19, 21, 22, 40). Therefore, the accurate and reliable assessment of ligand bias at the receptor is of prime importance. Methods to do this are based on the analysis of agonist concentration-response curves from in vitro signaling assays of two pathways (see **Table 2**), typically ones mediated by G proteins and β-arrestins. In all cases, bias must be assessed relative

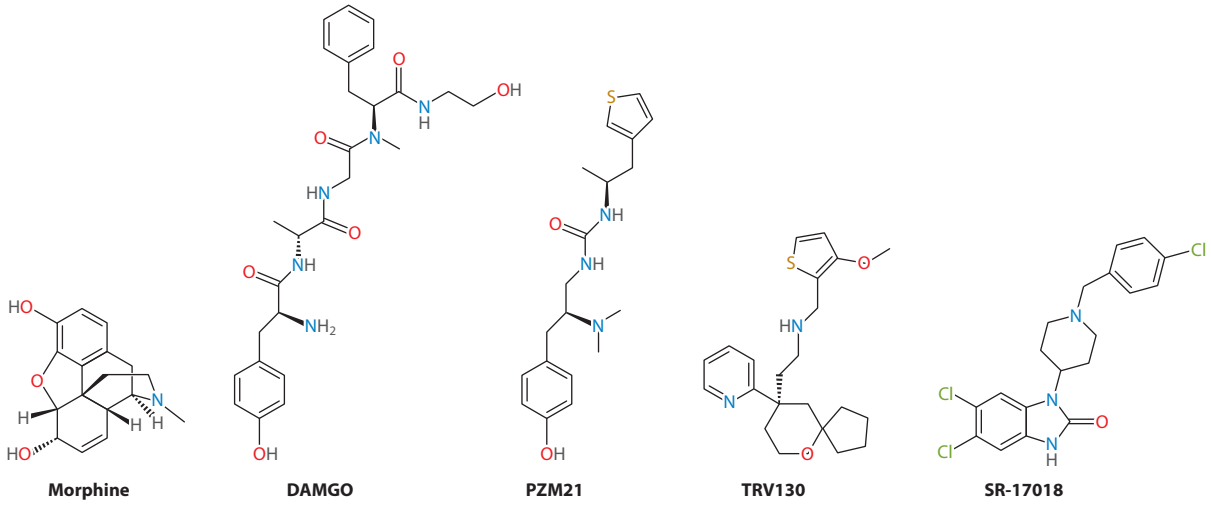

Figure 4

Structures of the main ligands discussed in the review. TRV130 (oliceridine), PZM21, and SR-17018 are proposed to be G protein–biased agonists at the μ-opioid receptor. Abbreviations: DAMGO, [D-Ala^2, *N*-MePhe^4, Gly-ol^5]-enkephalin; PZM21, 1-[(2*S*)-2-(dimethylamino)-3-(4-hydroxyphenyl)propyl]-3-[(2*S*)-1-(thiophen-3-yl)propan-2-yl]urea; TRV130, *N*-[(3-methoxythiophen-2-yl)methyl]-2-[(9*R*)-9-pyridin-2-yl-6-oxaspiro[4.5]decan-9-yl]ethanamine; SR-17018, 5,6-dichloro-1-(1-(4-chlorobenzyl)piperidin-4-yl)-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one.

to a "reference" agonist, usually a well characterized agonist that is a full agonist at both pathways being assessed: For the μ-opioid receptor, this is often the widely used high-efficacy agonist DAMGO (51) (**Figure 4**). The analysis usually involves fitting the curves to the operational model of pharmacological agonism (52) and calculating a measure of agonism, in this case the transduction ratio (referred to mathematically as τ/K_A) (53) (see **Table 2**). The difference in transduction ratio between the reference ligand and the ligand of interest for a particular signaling pathway is determined [$\Delta\log(\tau/K_A)$], and then this difference is compared for the two signaling pathways being studied [$\Delta\Delta\log(\tau/K_A)$]. For an agonist that is biased relative to the reference agonist, then the value of $\Delta\Delta\log(\tau/K_A)$ will be significantly different from zero. This and other approaches to determine bias, including relative activity (54) and relative efficacy methods (55), are compared in **Table 2**.

Studies of Bias with Established μ-Opioid Receptor Agonists

Some early studies had compared the relative strengths of downstream signaling outputs for small numbers of μ-opioid receptor agonists (45, 56). However, the possibility that a spectrum of μ-opioid receptor agonists can show different preferences for downstream signaling events (i.e., ligand bias) was first addressed by us (57) and Molinari et al. (58) in 2010. Although our study (57) did not undertake bias calculations, using μ-opioid receptors heterologously expressed in HEK293 cells the correlation of G protein activation with the ability to induce μ-opioid receptor phosphorylation, β-arrestin 2 recruitment, and receptor internalization was investigated. For most of the 22 μ-opioid receptor agonists we examined, there was a strong positive correlation between these measures, which looking back turns out to be perhaps the most significant finding from this study (57), and one that perhaps should have received more attention over the past decade, as this relationship may govern the signaling behavior of most μ-opioid receptor agonists,

Table 2 Details and comparison of methods used to estimate ligand bias

Method and curve fit	Equations	Comments	Issues
Transduction coefficients [$\Delta\log(\tau/K_A)$] Operational model fit (53)	$\Delta\log(\tau/K_A) = \log(\tau/K_A)_{lig} - \log(\tau/K_A)_{ref}$, $\Delta\Delta\log(\tau/K_A) = \Delta\log(\tau/K_A)_{path1} - \Delta\log(\tau/K_A)_{path2}$, and $\beta = 10^{\Delta\Delta\log(\tau/K_A)}$	This approach estimates agonism as a single calculated parameter: the log ratio of agonist efficacy (τ) and functional affinity (K_A). Ligand bias factors [$\Delta\log(\tau/K_A)$] are expressed after normalization against a reference ligand. These values can be compared across two signaling pathways for a given agonist to obtain the relative transduction ratio [$\Delta\Delta\log(\tau/K_A)$] as measures of agonist bias. For the calculation of bias factors, the $\Delta\Delta\log(\tau/K_A)$ values should be transformed to the corresponding antilog value.	Ambiguous fits to the operational model lead to difficulties, particularly for low-efficacy agonists. Ambiguous fits can result in large errors associated with the calculated bias factors. The maximum effect of the system must be clearly defined.
Relative activities [$\Delta\log(E_{max}/EC_{50})$] Logistic fit (54, 153)	$\Delta\log(E_{max}/EC_{50}) = \log(E_{max}/EC_{50})_{lig} - \log(E_{max}/EC_{50})_{ref}$, $\Delta\Delta\log(E_{max}/EC_{50}) = \Delta\log(E_{max}/EC_{50})_{path1} - \Delta\log(E_{max}/EC_{50})_{path2}$, and $\beta = 10^{\Delta\Delta\log(E_{max}/EC_{50})}$	This approach is relatively straightforward, only requiring E_{max} and EC_{50} values for each ligand. The log ratio of a test ligand is first compared to that of a reference ligand and subsequently across two signaling pathways. For ligands with concentration-response curves with a Hill coefficient of 1, the $\Delta\Delta\log(E_{max}/EC_{50})$ values are equivalent to $\log(\tau/K_A)$ values. For the calculation of bias factors, the $\Delta\Delta\log(E_{max}/EC_{50})$ values should be transformed to the corresponding antilog value.	The Hill coefficient of the concentration-response curves must not deviate from unity.
Relative efficacy (σ_{lig}) Operational model fit (55)	$\sigma_{lig} = \log(\tau_{lig}/\tau_{ref})$ and $\beta_{lig} = (\sigma_{lig}{}^{path1} - \sigma_{lig}{}^{path2})/\sqrt{2}$[a]	This approach yields both bias factors and estimates of efficacy. For each agonist within a pathway the effective signaling (σ_{lig}) is calculated, where τ_{lig} is the operational efficacy of a ligand for a particular pathway, and τ_{ref} is the operational efficacy for the reference agonist.	This approach relies on a single estimate of K_A that is determined experimentally and assumed to be constant across different signaling pathways. This method is also susceptible to difficulties associated with ambiguous fits to the operational model.

[a]In some iterations of this model, the $\sqrt{2}$ has been removed from the equation (see 108).

including some of those currently considered by some to be biased agonists (47). Two exceptions were the peptides endomorphin-1 and endomorphin-2, which appeared to be β-arrestin-2 biased (57, 59). However, the problem with peptide ligands is that they possess poor ability to cross the blood-brain barrier and so assessing the in vivo effects of such ligands is not straightforward.

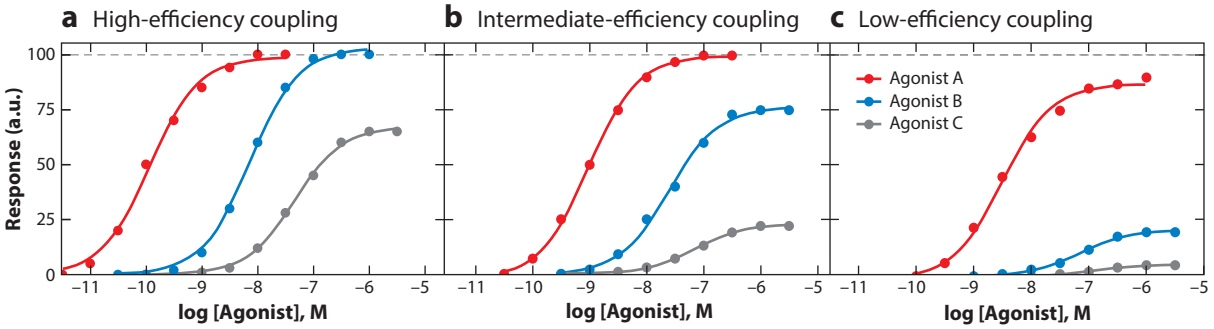

Figure 5

In terms of bias, concentration-response curves can be deceptive. Simulated responses to three agonists in systems with different levels of signal amplification are shown in panels *a–c*. If panel *a* was recording a G protein response and panel *c* recording a β-arrestin response, it might be concluded that agonists B (*blue*) and C (*gray*) are G protein biased relative to agonist A (*red*), but actually they may not be. Apart from changes in the maximum responses, note the rightward shift of the concentration-response curves as coupling efficiency decreases: This important factor, that decreases in efficacy can manifest as a shift of the concentration-response curve of a full agonist to the right and by a reduction in maximum response, is often overlooked in analyses of proposed biased agonists. Thus, while the maximum responses to agonists B and C have been greatly depressed in panel *c* compared with panel *a*, the potency of agonist A has also decreased by approximately 17-fold from panel *a* to panel *c*. The curves for agonists B and C in panel *c* also highlight the problem of accurately measuring E_{max} and EC_{50} for weak partial agonists in such analyses, which can confound bias calculations.

The prototypical μ-opioid receptor agonist morphine was the first opioid whose profile of downstream signaling was considered to be somewhat different from other well-characterized agonists such as DAMGO (60). Morphine activated G protein pathways with efficacy higher than that with which it promoted receptor phosphorylation, β-arrestin recruitment, or receptor internalization (45, 56). However, subsequent studies and further analyses have generally concluded that morphine is not a G protein–biased agonist; rather, it is an unbiased μ-opioid receptor agonist of moderate intrinsic efficacy (47, 49, 61). Indeed, a recurring theme in bias studies is that agonists are considered to be G protein biased simply because they have sufficient intrinsic efficacy to produce a maximum or near-maximum response in G protein signaling readouts, but their intrinsic efficacy is only sufficient to produce a weak response in β-arrestin readouts (19, 40, 62). To the naked eye this combination of relative response magnitudes gives the appearance of G protein bias, but this is actually often not the case (19) and can be explained by the normally large receptor reserve and efficient coupling for G protein–mediated signaling events and the small or nonexistent receptor reserve and low-amplification coupling for β-arrestin recruitment and related signaling events (**Figure 5**). Once the signaling of agonists such as morphine is carefully quantified with the use of reference agonists such as DAMGO, then the absence of bias becomes evident (47, 59). Nevertheless, some aspects of morphine's signaling are different from those of high-intrinsic-efficacy agonists, such as the involvement of protein kinase C rather than GRK and β-arrestins in morphine-induced μ-opioid receptor desensitization and tolerance (63, 64), but this difference may be related to the relative intrinsic efficacy of the agonists rather than to bias as such (63, 65).

Intrinsic efficacy: the ability of a drug to activate a receptor

Of importance, the established μ-opioid receptor agonist fentanyl is also reported to be a biased agonist, but this time to be β-arrestin biased (51). Indeed, the proposal that this bias could be responsible for the lethality of fentanyl (51) was based on the idea that β-arrestin signaling mediates respiratory depression induced by the μ-opioid receptor, now known to be incorrect. Fentanyl's lethality is instead likely to be due to factors such as high in vivo potency and the ability to induce

respiratory muscle stiffness, also known as wooden chest syndrome (66). Other studies have been based on this assumption that fentanyl is a β-arrestin-biased agonist (67, 68). However, in our own experiments we did not find fentanyl to be β-arrestin biased (57, 59), and this has been confirmed by others (47, 69, 70). In our opinion fentanyl should not be considered a β-arrestin-biased agonist (66).

Opioid use disorder (OUD): the chronic use of opioids that causes clinically significant distress or impairment

Are TRV130, PZM21, and SR-17018 Novel G Protein–Biased Agonists at the μ-Opioid Receptor?

The first synthetic ligand to be reported as a G protein–biased agonist was TRV130 (later named oliceridine; **Figures 3** and **4**; **Table 1**) (71). While the bias reported was moderate (estimated to be threefold relative to morphine but did not reach statistical significance), oliceridine had a favorable profile in that it appeared less likely than morphine to depress respiration or induce constipation (71) and also less likely to induce tolerance (72). Oliceridine has since received FDA approval for use as an analgesic administered by discrete intravenous doses (73). However, the overall clinical advantages of this drug over standard opioids such as morphine in terms of therapeutic window are generally moderate, although more recent studies did not find oliceridine to be G protein biased (47), and the balance of behavioral studies indicates that oliceridine retains at least some of the drug-seeking behavior of morphine and other established opioid drugs (72–75). Other studies have found oliceridine to be a weak partial agonist at the μ-opioid receptor (76) and that, under the appropriate conditions of reduced μ-opioid receptor expression, it can induce tolerance to its own analgesic effect (77). On the other hand, oliceridine is effective in decreasing relapse to oxycodone seeking in rats (78, 79), so new drugs such as oliceridine may have a place in the future treatment of opioid use disorder (OUD). It remains to be seen whether the clinical profile of oliceridine is due to a modicum of G protein bias, overall low intrinsic efficacy at the receptor, its pharmacokinetic properties [oliceridine has a short plasma half-life and has to be administered parenterally (80)], or a combination of these properties (81).

Another synthetic ligand, PZM21 (**Figures 3** and **4**; **Table 1**), was reported to be G protein biased (82), but this was not based on a formal calculation of bias, as the β-arrestin 2 response was too small to quantify accurately, although in vivo it was reported to induce effective antinociception but not respiratory depression nor drug-seeking behavior as assessed by a conditioned place preference protocol (82). However, we undertook a subsequent study in which we found PZM21 to produce respiratory depression and analgesic tolerance similar to that of morphine (83). In addition, in cell signaling experiments we did not find PZM21 to be G protein biased, a finding confirmed by others (47, 84). On the other hand, a subsequent study (47) reported that PZM21 induced relatively weak respiratory depression, although in that study the first reading of respiratory depression was taken 30 min after PZM21 administration, whereas in our study (83) the peak respiratory depression to this drug was observed within 15 min of drug administration. Overall, PZM21, like oliceridine, appears not to be biased but instead a weak partial agonist at the μ-opioid receptor (76, 84, 85). An interesting observation was that although PZM21 can induce tolerance and dependence in vivo, it was also able to antagonize the ability of morphine to induce conditioned place preference (85), which again suggests that such ligands as PZM21 could have a place in the treatment of OUD. As with oliceridine, however, it is not clear what properties of the drug might be responsible for its in vivo profile.

A further major development in the field was the description of a series of piperidine-benzimidazole compounds, with the ligand of main interest being SR-17018 (51) (**Figures 3** and **4**; **Table 1**). Some of these ligands expressed large degrees of bias, with SR-17018 exhibiting

a 100-fold bias for G protein activation over β-arrestin 2 recruitment. However, a later study reported that SR-17018 was not biased (47). The reason for this surprising disparity is not clear but may relate to some of the assumptions about bias calculations made in the original study due to the inability of the agonist to invoke a quantifiable maximum response in the β-arrestin 2 assay employed (51). Whether or not SR-17018 is actually a G protein–biased ligand at the μ-opioid receptor, it is an unusual ligand. Thus, in a cellular assay high concentrations of SR-17018 (>10 μM) were reported to not antagonize an approximately EC_{50} concentration (300 nM) of DAMGO for recruitment of β-arrestin 2 (51), whereas basic receptor theory predicts that this should not be the case for ligands reversibly binding to the same site and thus competing with each other. The kinetics of SR-17018 action in vivo are slow relative to other ligands (47, 51), and the kinetics of μ-opioid receptor phosphorylation and dephosphorylation following treatment with SR-17018 are remarkably slow (47, 86). Some of these effects could be explained by slow receptor dissociation kinetics, as seen for buprenorphine, but data on receptor binding kinetics for SR-17018 have not been reported; however, this is of interest because ligand binding kinetics represent one factor proposed to determine whether bias occurs for a particular agonist (87). In addition, the limited solubility of SR-17018 would mean that the assumed and actual free concentrations of drug present in an experiment are not the same, which could interfere with bias calculations (47). SR-17018 is, however, able to reverse morphine tolerance while preventing morphine withdrawal symptoms (88), but it is unclear what the mechanisms of these effects are. The effects of putative G protein–biased agonists (oliceridine, PZM21, and SR-17018) on abuse potential and addiction-related behaviors have been recently reviewed in detail, and this area represents an avenue of further interest and potential exploration for these ligands (74, 89).

Other Ligands Proposed to Be Biased at the μ-Opioid Receptor

Several other ligands have more recently been reported as G protein–biased ligands at the μ-opioid receptor, including mitragynine and its derivatives (90, 91), as well as novel compounds such as peptides (92), cyclic peptides (84, 93), plant-derived alkaloids (94), a series of piperidine-benzimidazolone derivatives (95), and most recently ligands based on the structure of either TRV130/oliceridine (96) or PZM21 (97). Further work is needed to establish the in vivo profiles and therapeutic potential of these novel ligands. Interestingly, the pharmacological properties of such compounds may not always be predictable on the basis of their in vitro signaling profile. Recently, a cyclic peptide (compound 1) was shown in cellular assays to be significantly G protein biased with little ability to recruit β-arrestins, but when tested on endogenous μ-opioid receptors in brain slices, the compound could induce rapid GRK-mediated desensitization of the receptor response similar to nonbiased μ agonists that efficiently recruit β-arrestins to the receptor (84). Thus, care must be taken when assuming the in vivo mode of action and regulatory properties of agonists on the basis of in vitro–biased signaling behavior, particularly where the latter is based on assays in which the receptor or other components of the reporter signaling system are overexpressed in nonnative cell systems such as HEK293 cells. It is preferable, although challenging, to obtain in vitro signaling data for bias analysis where possible from preparations (e.g., neuronal cultures or brain slices) with endogenous expression of μ-opioid receptor and signaling components (98), or at least cells in which heterologous expression of these components reflects endogenous levels of these proteins. It remains to be seen whether these newer G protein–biased agonists express an in vivo pharmacology that can be understood on the basis of their in vitro cell signaling profiles and have improved side effect profiles with, for example, less respiratory depression than established agonists.

LESSONS TO BE LEARNED FROM BIASED AGONISTS AT THE μ-OPIOID RECEPTOR

The consequences of classifying novel ligands as biased without either numerical justification of statistically significant bias or corroboration of bias data from different studies are now becoming evident in areas beyond the confines of in vitro cell signaling and in vivo animal studies. In the clinical literature there is continuing reference to these new μ-opioid receptor agonists as being advantageous to the patient on the basis of their G protein bias, thus implying to the medical community mechanisms of drug action, such as G protein bias, that may not be justified at this time (99–103). Therefore, a careful assessment is needed of where we are in terms of biased ligands at the μ-opioid receptor.

Bioluminescence resonance energy transfer (BRET): a cell signaling assay system based on energy transfer between heterologously expressed signaling components

Bias or Low Intrinsic Efficacy?

By far the most important current issue regarding potentially G protein–biased ligands is whether the in vivo profile of the drug really reflects biased signaling or some other property of the drug such as low intrinsic efficacy at the μ-opioid receptor (40, 62, 104, 105). It is worth considering that all three of the drugs described above as potentially G protein biased (oliceridine, PZM21, and SR-17018) are low-intrinsic-efficacy agonists even for G protein activation at the μ-opioid receptor, as determined in a range of signaling assays (47). They are certainly agonists with much lower intrinsic efficacy at the μ-opioid receptor than standard high-intrinsic-efficacy agonists such as DAMGO, methadone, and fentanyl and also with significantly lower intrinsic efficacy than morphine itself (47). In a recent commentary (62) a particular problem surrounding the nature of the signaling assays used in most studies of bias was highlighted, but it is one that has been raised previously (69, 106–108). The standard in vitro bias assay format usually involves a cellular assay of G protein activity with a degree of response amplification, which can be large (e.g., in assays of adenylyl cyclase activity or when there are high levels of receptor expression) or small (e.g., in an assay of β-arrestin recruitment involving little or no response amplification; this would be the case, for example, if **Figure 5*a*** represented G protein signaling and **Figure 5*c*** represented β-arrestin recruitment). In both types of assay there are issues in the quantification of bias due to the constraints of the operational model of pharmacological agonism (52), which as noted above is the usual vehicle of analysis for determining the presence of bias (53). Thus, when the amplification factors in the assays are so different, the estimations of relative intrinsic efficacy of ligands may be inaccurate, possibly leading to erroneous conclusions about the presence or otherwise of ligand bias (62). Specifically, the operational model analysis can lead on the one hand to overestimation of the intrinsic efficacy of agonists for G protein–dependent signaling pathways, particularly for low-intrinsic-efficacy agonists, or on the other hand to underestimation of differences in the relative intrinsic efficacies of the ligands for β-arrestin-dependent pathways (62). Accordingly, some of the novel ligands considered to be biased may be not biased at all, but instead, and more straightforwardly, might be low-intrinsic-efficacy agonists at the μ-opioid receptor (but see 109). A potential solution suggested for this problem (62, 108) is to make the assays on which the bias is determined more comparable in terms of signal amplification. Thus, it is possible to reduce the amplification of the G protein signal that is being measured by using, for example, G protein nanobodies in bioluminescence resonance energy transfer (BRET) assays (47), where the amplification factor for the output signal of G protein nanobody interaction with the receptor is much less than that for downstream signaling outputs such as changes in cyclic AMP generation. Alternatively, the G protein signaling output can be limited by reducing receptor number with an irreversible antagonist such as β-funaltrexamine (77). On the other hand, β-arrestin signal output can be amplified by coexpressing GRKs to enhance agonist-induced receptor phosphorylation and

hence β-arrestin recruitment to the receptor (47, 110). These methodological modifications are themselves not without issues; when GRKs are overexpressed to enhance β-arrestin recruitment, the effects of GRK overexpression on G protein signaling are usually not investigated but are assumed, with limited justification, to be insignificant (see 111). Moreover, the amplifying effects of GRK overexpression on β-arrestin recruitment may vary between agonists, as has been recently shown (110) for μ-opioid receptor internalization (which is dependent on β-arrestin recruitment). The bottom line is that future measurements of bias should at least avoid using assays with widely differing assay amplification factors.

Other Issues in the Experimental Determination of Ligand Bias at Opioid Receptors

The use of a single G protein subtype and a single subtype of β-arrestin, common for most bias studies for the μ-opioid receptor, may underestimate the complexity of signaling through this receptor (112). Two subtypes of β-arrestin are expressed in most cells of the body, β-arrestin 1 and β-arrestin 2, but few studies measure agonist-induced recruitment of both (113), let alone their signaling, and recent evidence suggests that these subtypes mediate at least some distinct functions at the μ-opioid receptor (114). More widely, it is now known that the interaction of β-arrestins with a GPCR can involve both receptor phosphorylation–dependent and receptor phosphorylation–independent interactions (115), that these sites of receptor–β-arrestin interaction can mediate distinct downstream cellular functions (116), and that, as shown for the angiotensin type I receptor, agonists can differentially promote these distinct receptor–β-arrestin interactions (117). Clearly, this is a much more complex and nuanced picture than is currently drawn by most bias studies, in which the simple recruitment of β-arrestin 2 to the μ-opioid receptor is taken as a proxy for overall β-arrestin function, including desensitization of G protein responsiveness, receptor trafficking, and any form of β-arrestin-dependent signaling. The work undertaken with other GPCRs suggests that more sophisticated analyses of β-arrestin interaction with the μ-opioid receptor are warranted for bias studies (112). Similar conclusions could be drawn about the potential complexity of G protein signaling with regard to bias studies (7).

Apart from the issue of assay amplification factors discussed above, it is also important for confirmatory studies to be undertaken before ligands are generally accepted as biased. This applies particularly to ligands for which the original claim for bias was not based on a statistically significant observation such as when bias has been inferred simply from visual inspection of concentration-response curves. Ideally, such confirmation would include studies with receptors expressed in different cell types, and with different levels of receptor expression (77), and also studies using more than one assay of G protein or β-arrestin signaling [e.g., BRET, complementation assay, GTPγS assay, receptor internalization (47, 105)]. Furthermore, different methods to calculate bias should be considered when determining the presence of bias or otherwise. For the μ-opioid receptor and many other GPCRs, the standard method is the transduction ratio method, as discussed above. Each of the methods (**Table 2**) has its nuances and it is becoming apparent that there are limitations in the accuracy by which biased agonism can be quantified experimentally (62, 111). Rather confusingly, the application of these different methods can lead to different conclusions about the presence or absence of bias for a ligand, particularly if the potential bias is moderate anyway (for a detailed discussion of each of the methods, see 6). A recent IUPHAR (International Union of Basic and Clinical Pharmacology) review (118) recommends the use of either the transduction ratio method [$\Delta\Delta\log(\tau/K_A)$] or the relative activity method [$\Delta\Delta\log(E_{max}/EC_{50})$] (**Table 2**).

Also, the choice of reference ligand can determine whether a novel ligand is concluded to be biased, as the degree or nature of the bias will depend on the signaling properties of the reference

agonist. Technically, a biased ligand should always be reported as being biased relative to the reference ligand. In most cases it is probably best to use as the reference ligand a well-characterized agonist that is a full agonist in both G protein and β-arrestin assays, such as DAMGO, and indeed, this is the reference agonist in most assays of bias at the μ-opioid receptor. Other reference ligands have been used, however, including the lower-intrinsic-efficacy ligands morphine (71) and hydromorphone (119). Reference ligands such as DAMGO are often described as signaling equally through G protein and β-arrestin pathways, giving rise to the term balanced agonist for agonists such as DAMGO. However, in most signaling assays, the potency with which DAMGO activates the G protein response is usually much higher than the potency with which it recruits β-arrestin to the receptor, 100-fold or more in some cases (120). As discussed above, this difference becomes important when trying to understand comparisons of concentration-response curves for potentially biased agonists. One important issue with DAMGO as a reference ligand is that it is a peptide agonist that will not penetrate the blood-brain barrier significantly, and so cannot be readily used as the reference agonist if the experimenter wants to progress to assessment of the in vivo actions of the potentially biased ligands.

Finally, in attempts to understand the structural basis of ligand bias at the μ-opioid receptor, several in silico studies have employed molecular dynamics simulations to explore the ability of ligands such as oliceridine and PZM21 to interact with and induce particular μ-opioid receptor conformations (82, 121). These are then compared with interactions and receptor conformations induced by reference μ-opioid receptor ligands, which by definition are not biased. However, given the above issues concerning the proposed bias of these ligands, the studies may be revealing as much about the mechanistic detail of partial agonism as about ligand bias at the μ-opioid receptor. Nevertheless, studies with recently developed ligands proposed to be G protein biased at the μ-opioid receptor (38, 97, 120) may begin to reveal important information about ligand–receptor interactions and structural changes associated with bias.

BIASED LIGANDS AT δ-OPIOID, κ-OPIOID, AND NOCICEPTIN RECEPTORS?

δ-Opioid Receptor

The δ-opioid receptor is a promising target for the treatment of chronic pain states, anxiety, and other mood disorders (122). Unlike the μ-opioid receptor, activation of the δ-opioid receptor does not induce respiratory depression, euphoria, or dependence (123, 124), but it has been associated with convulsive activity (125) and the development of tolerance to the analgesic effects (126), two confounding issues that have previously limited the development of novel δ-opioid receptor ligands. Recent studies suggest that the convulsive activity and tolerance are not properties common to all δ-opioid receptor ligands (123, 127) and research efforts have focused on the development of G protein–biased ligands as a potential mechanism to improve the therapeutic profile of δ-opioid receptor ligands and avoid unwanted adverse effects. Biased agonism at the δ-opioid receptor has been studied less extensively than at the μ-opioid receptor, but novel G protein–biased ligands such as PN6047 (127) and Trevena's TRV250 (128) are reported to have a desirable therapeutic profile in preclinical models of chronic pain; however, the in vitro signaling data and extent of G protein bias for TRV250 have not yet been published.

Various studies have reported ligand-specific differences in signaling and regulation of the δ-opioid receptor (129), supporting the idea that it may be possible to engage selectively signaling pathways that mediate therapeutic versus adverse effects. However, some of the ligand-specific differences appear to be due to differences in agonist intrinsic efficacy and receptor reserve, not biased agonism (130). The δ-opioid receptor is regulated by both β-arrestin 1 and β-arrestin 2, and

each β-arrestin subtype is thought to mediate distinct behaviors; high-internalizing agonists such as SNC80 are reported to preferentially recruit β-arrestin 1 and result in receptor internalization and degradation, whereas low-internalizing agonists such as ARM390 favor engagement between the δ-opioid receptor and β-arrestin 2 (126, 131, 132). The preferential recruitment of β-arrestin 2 over β-arrestin 1 for low-internalizing agonists is suggested to protect against acute analgesic tolerance (126, 131, 133), and several low-internalizing agonists have a reduced propensity to induce convulsions (123). Studies using genetically modified mice have provided further support for the separation of signaling pathways that mediate the differential behavioral outcomes (134). SNC80-induced convulsions were potentiated in mice lacking β-arrestin 1 but not in mice lacking β-arrestin 2, $G\alpha_o$ heterozygous knockout mice, or RGS-insensitive heterozygous knock-in mice (which display enhanced signaling from $G\alpha_o$) (134), whereas in $G\alpha_o$ heterozygous knockout mice SNC80-induced antihyperalgesia was abolished. Conversely, the potency of SNC80 to produce antihyperalgesia and antidepressant-like effects was enhanced in the RGS-insensitive heterozygous knock-in mice, suggesting a role for G protein–mediated signaling in the desired therapeutic effects arising from the δ-opioid receptor. Why knockout of β-arrestin 1 potentiates convulsive activity remains to be determined: If the convulsions arise due to agonist-induced recruitment of β-arrestin 1, then it would be expected that the convulsions would be absent in mice lacking β-arrestin 1.

RGS protein: regulator of G protein signaling proteins; inhibits G protein signaling by enhancing the GTPase activity of the Gα subunit

Hyperalgesia: an increase in pain perception above the normal response to the stimulus

In the development of novel biased ligands for the δ-opioid receptor, certain differences between the μ- and the δ-opioid receptors should be considered and some lessons can be learned from the development of μ-opioid receptor–biased ligands. The differences between the functions of the two β-arrestin subtypes in behaviors mediated by the δ-opioid receptor highlight the importance of characterizing the recruitment of both β-arrestin subtypes in vitro for bias calculations, as discussed above for the μ-opioid receptor. Additionally, the δ-opioid receptor undergoes agonist-induced downregulation (129), whereas after internalization the μ-opioid receptor typically recycles back to the plasma membrane (113), but how such δ-opioid receptor downregulation affects biased signaling remains to be determined. For several novel δ-opioid receptor ligands (purportedly biased or unbiased) the lack of quantitative in vitro signaling data impedes our interpretation of in vivo data. By comparison, one of the benefits of studying biased agonism at the μ-opioid receptor is the significant number of studies that have characterized the signaling of many different ligands within that same study (47, 57, 135, 136).

While the evidence to support the development of G protein–biased ligands for the δ-opioid receptor is accumulating, in a similar vein to the μ-opioid receptor (and other GPCRs), our understanding of the underlying physiology and molecular mechanisms may not yet be sufficient to justify this approach conclusively. It may be that for the δ-opioid receptor, low-intrinsic-efficacy agonism, i.e., partial agonism, offers an improved therapeutic profile, although the observation that a low-intrinsic-efficacy agonist (BU48) induces convulsions suggests otherwise (130, 137). It is important going forward that we do not attempt to oversimplify the signaling paradigms and repeat the errors made with the μ-opioid receptor (i.e., G protein signaling = good and β-arrestin signaling = bad).

κ-Opioid Receptor

For the κ-opioid receptor, a similar theme appears in the literature: G protein–biased ligands are suggested to provide beneficial effects (antinociception and antipruritic activity) without the dysphoria, sedation, and other side effects typically associated with κ-opioid receptor agonists (22, 138). Several purportedly G protein–biased compounds have been developed and one compound, nalfurafine (also known as TRK-820), has been approved in Japan for the treatment of

resistant pruritus in patients undergoing hemodialysis (139). Nalfurafine has both analgesic and antipruritic effects (140, 141) but does not produce dysphoria, and only a limited number of patients have reported hallucinations (142, 143). Despite the apparent success of nalfurafine, there are questions regarding the extent of bias of this compound due to the signaling assays used to quantify bias and differences in cellular background, as discussed in Reference 18. Evidence to support the development of G protein–biased ligands for the κ-opioid receptor stems from the observation that the analgesic activity and antipruritic efficacy of κ-opioid receptor agonists are retained in β-arrestin 2 knockout mice (144, 145). Additionally, several studies have reported that some of the adverse effects of κ-opioid receptor activation, such as aversion, are mediated via GRK-3/β-arrestin 2 recruitment and subsequent p38 phosphorylation (146–148). However, G protein signaling mediated by the κ-opioid receptor has been suggested to induce both antinociception and aversion, whereas β-arrestin 2 signaling induces motor incoordination and potentially sedation and anhedonia (145). This difference again raises important questions about our understanding of the underlying physiology and the utility of global knockout mice when attempting to identify a particular signaling pathway with a specific behavior.

A recent review of biased agonism at the κ-opioid receptor has highlighted the lack of consistency in the field with respect to the use of signaling assays and choice of reference ligand (138), similar to concerns about the μ-opioid receptor discussed above. Further, differences in the signaling profiles of κ-opioid receptors from different species have been reported (138). Therefore, there must be greater consistency with respect to the analysis and application of biased agonism and the field would benefit from a more unified approach to classifying biased agonists. For the κ-opioid receptor, there are some promising biased ligands, but the complex nature of the different behaviors and our appreciation of the different signaling pathways involved require further investigation.

Nociceptin Receptor

The NOP receptor is implicated in diverse biological functions, and activation of the receptor is thought to modulate behaviors mediated by the μ-opioid receptor, including antinociception, tolerance development, and reward (149). The analgesic effects mediated by the NOP receptor appear to be more complex than those following activation of other opioid receptors. NOP receptor activation with the endogenous peptide nociceptin/orphanin FQ peptide (N/OFQ) at the spinal level is analgesic, whereas when given supraspinally, N/OFQ reverses the analgesic effect of exogenous opioids, acting as a functional opioid antagonist (149). With respect to biased agonism, only a few studies to date have investigated the ability of ligands to differentially activate G proteins versus β-arrestins (150). Consequently, the behavioral outcome of preferential activation of one pathway over another remains largely unknown, although it has been suggested that the efficacy for β-arrestin 2 recruitment, rather than G protein activation, underlies anxiolytic activity (151). Due to the pleiotropic responses following activation of this receptor, it will be interesting to see whether biased agonism can provide a mechanism for improving the therapeutic profile or whether the complexity and diversity of physiological actions of this receptor may prove too challenging. Certainly, there is a need to develop more G protein– and β-arrestin-biased NOP ligands to aid our understanding of this receptor and its functions.

CONCLUSIONS

The key questions and suggested ways forward regarding bias at opioid receptors are summarized in **Table 3**. It can be argued that over the years the field of bias at the μ-opioid receptor has largely failed to address these questions with sufficient rigor, leading to some rather costly cul-de-sacs. Recent studies and commentaries suggest that at least some of the lessons from 20 years of study of

Table 3 Key issues concerning bias at opioid receptors and potential ways forward

Key issues concerning bias at opioid receptors	Potential ways forward
Which cell signaling pathways mediate the therapeutic versus adverse effects of opioid receptor activation? Are the signaling pathways mediating the therapeutic and adverse effects the same or different?	Localized manipulation of receptor signaling pathways in tissues and organisms, e.g., localized knockout of components of the receptor signaling pathways (37); expression of mutant receptors with biased signaling profile (33); use of agents that selectively inhibit signaling pathways (36)
Is the biased agonist really a biased agonist or instead a low-intrinsic-efficacy partial agonist at the opioid receptor?	Rigorous quantification of agonist responses and calculation of bias, preferably corroborated by experimentation in different cell systems and by different laboratories (6, 19, 28, 118)
Do different cellular levels of G proteins/GRKs/β-arrestins, as well as endogenous opioid peptides, affect cellular bias?	Manipulation of relative levels of signaling components in model systems (84); quantification of relative levels of signaling components in native tissues (154)
Which cells in the body are to be targeted by biased agonists?	Experimentation in native tissues (59, 98), e.g., localized administration of biased agonist into defined brain nuclei
Do disease, chronic pain or prior and ongoing opioid use affect the receptor signaling pathways?	Use of animal models of disease, e.g., chronic inflammatory pain (127); chronic opioid administration (83, 155)
Do other aspects of drug pharmacodynamics or pharmacokinetics explain apparently biased effects?	Determination of fundamental drug properties other than bias, including ligand binding kinetics (86) and pharmacokinetic profile including ability to cross the blood-brain barrier (80, 96)

the μ-opioid receptor are finally beginning to be learned. There are also some grounds to expect more success with biased ligands for δ- and κ-opioid receptors, particularly if the lessons learned from the μ-opioid receptor story are applied.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

LITERATURE CITED

1. Michel MC, Charlton SJ. 2018. Biased agonism in drug discovery—Is it too soon to choose a path? *Mol. Pharmacol.* 93:259–65
2. Kenakin T, Christopoulos A. 2013. Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat. Rev. Drug Discov.* 12:205–16
3. Smith JS, Lefkowitz RJ, Rajagopal S. 2018. Biased signalling: from simple switches to allosteric microprocessors. *Nat. Rev. Drug Discov.* 17:243–60
4. Che T, Dwivedi-Agnihotri H, Shukla AK, Roth BL. 2021. Biased ligands at opioid receptors: current status and future directions. *Sci. Signal.* 14:eaav0320
5. Wingler LM, Lefkowitz RJ. 2020. Conformational basis of G protein-coupled receptor signaling versatility. *Trends Cell Biol.* 30:736–47
6. Kenakin T. 2019. Biased receptor signaling in drug discovery. *Pharmacol. Rev.* 71:267–315
7. Onfroy L, Galandrin S, Pontier SM, Seguelas M-H, N'Guyen D, et al. 2017. G protein stoichiometry dictates biased agonism through distinct receptor-G protein partitioning. *Sci. Rep.* 7:7885
8. Kelly E, Bailey C, Henderson G. 2008. Agonist-selective mechanisms of GPCR desensitization. *Br. J. Pharmacol.* 153(Suppl. 1):S379–88
9. Kunselman JM, Lott J, Puthenveedu MA. 2021. Mechanisms of selective G protein-coupled receptor localization and trafficking. *Curr. Opin. Cell Biol.* 71:158–65
10. Williams JT, Ingram SL, Henderson G, Chavkin C, von Zastrow M, et al. 2013. Regulation of μ-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol. Rev.* 65:223–54

11. Weiss N, Zamponi GW. 2021. Opioid receptor regulation of neuronal voltage-gated calcium channels. *Cell. Mol. Neurobiol.* 41:839–47
12. Yudin Y, Rohacs T. 2018. Inhibitory $G_{i/o}$-coupled receptors in somatosensory neurons: potential therapeutic targets for novel analgesics. *Mol. Pain* 14:1744806918763646
13. Heinke B, Gingl E, Sandkühler J. 2011. Multiple targets of μ-opioid receptor-mediated presynaptic inhibition at primary afferent Aδ- and C-fibers. *J. Neurosci.* 31:1313–22
14. Laporte SA, Scott MGH. 2019. β-arrestins: multitask scaffolds orchestrating the where and when in cell signalling. *Methods Mol. Biol.* 1957:9–55
15. Stein C. 2016. Opioid receptors. *Annu. Rev. Med.* 67:433–51
16. Darcq E, Kieffer BL. 2018. Opioid receptors: drivers to addiction? *Nat. Rev. Neurosci.* 19:499–514
17. Skolnick P. 2018. The opioid epidemic: crisis and solutions. *Annu. Rev. Pharmacol. Toxicol.* 58:143–59
18. Mores KL, Cummins BR, Cassell RJ, van Rijn RM. 2019. A review of the therapeutic potential of recently developed G protein-biased kappa agonists. *Front. Pharmacol.* 10:407
19. Conibear AE, Kelly E. 2019. A biased view of mu-opioid receptors? *Mol. Pharmacol.* 96:542–49
20. Grim TW, Acevedo-Canabal A, Bohn LM. 2020. Toward directing opioid receptor signaling to refine opioid therapeutics. *Biol. Psychiatry* 87:15–21
21. De Neve J, Barlow TMA, Tourwe D, Bihel F, Simonin F, Ballet S. 2021. Comprehensive overview of biased pharmacology at the opioid receptors: biased ligands and bias factors. *RSC Med. Chem.* 12:828–70
22. Faouzi A, Varga BR, Majumdar S. 2020. Biased opioid ligands. *Molecules* 25:4257
23. Chang SD, Mascarella SW, Spangler SM, Gurevich VV, Navarro HA, et al. 2015. Quantitative signaling and structure-activity analyses demonstrate functional selectivity at the nociceptin/orphanin FQ opioid receptor. *Mol. Pharmacol.* 88:502–11
24. Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, Lin FT. 1999. Enhanced morphine analgesia in mice lacking β-arrestin 2. *Science* 286:2495–98
25. Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG. 2000. μ-Opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence. *Nature* 408:720–23
26. Raehal KM, Walker JK, Bohn LM. 2005. Morphine side effects in β-arrestin 2 knockout mice. *J. Pharmacol. Exp. Ther.* 314:1195–201
27. Raehal KM, Schmid CL, Groer CE, Bohn LM. 2011. Functional selectivity at the μ-opioid receptor: implications for understanding opioid analgesia and tolerance. *Pharmacol. Rev.* 63:1001–19
28. Kliewer A, Gillis A, Hill R, Schmiedel F, Bailey C, et al. 2020. Morphine-induced respiratory depression is independent of β-arrestin2 signalling. *Br. J. Pharmacol.* 177:2923–31
29. Bachmutsky I, Wei XP, Durand A, Yackle K. 2021. β-Arrestin 2 germline knockout does not attenuate opioid respiratory depression. *eLife* 10:e62552
30. Haouzi P, McCann M, Tubbs N. 2021. Respiratory effects of low and high doses of fentanyl in control and β-arrestin 2-deficient mice. *J. Neurophysiol.* 125:1396–407
31. He L, Gooding SW, Lewis E, Felth LC, Gaur A, Whistler JL. 2021. Pharmacological and genetic manipulations at the μ-opioid receptor reveal arrestin-3 engagement limits analgesic tolerance and does not exacerbate respiratory depression in mice. *Neuropsychopharmacology* 46:2241–49
32. Bateman JT, Levitt ES. 2021. Evaluation of G protein bias and β-arrestin 2 signaling in opioid-induced respiratory depression. *Am. J. Physiol. Cell Physiol.* 321:C681–83
33. Kliewer A, Schmiedel F, Sianati S, Bailey A, Bateman JT, et al. 2019. Phosphorylation-deficient G-protein-biased μ-opioid receptors improve analgesia and diminish tolerance but worsen opioid side effects. *Nat. Commun.* 10:367
34. Lohse MJ, Hofmann KP. 2015. Spatial and temporal aspects of signaling by G-protein-coupled receptors. *Mol. Pharmacol.* 88:572–78
35. Ramirez J-M, Burgraff NJ, Wei AD, Baertsch NA, Varga AG, et al. 2021. Neuronal mechanisms underlying opioid-induced respiratory depression: our current understanding. *J. Neurophysiol.* 125:1899–919
36. Montandon G, Ren J, Victoria NC, Liu H, Wickman K, et al. 2016. G-protein-gated inwardly rectifying potassium channels modulate respiratory depression by opioids. *Anesthesiology* 124:641–50
37. Wei AD, Ramirez J-M. 2019. Presynaptic mechanisms and KCNQ potassium channels modulate opioid depression of respiratory drive. *Front. Physiol.* 10:1407

38. Kelly B, Hollingsworth SA, Blakemore DC, Owen RM, Storer RI, et al. 2021. Delineating the ligand-receptor interactions that lead to biased signaling at the μ-opioid receptor. *J. Chem. Inf. Model.* 61:3696–707
39. Lee JH, Shon SY, Jeon W, Hong SJ, Ban J, Lee DS. 2021. Discovery of μ,δ-opioid receptor dual-biased agonists that overcome the limitation of prior biased agonists. *ACS Pharmacol. Transl. Sci.* 4:1149–60
40. Gillis A, Kliewer A, Kelly E, Henderson G, Christie MJ, et al. 2020. Critical assessment of G protein-biased agonism at the μ-opioid receptor. *Trends Pharmacol. Sci.* 41:947–59
41. Bubier JA, He H, Philip VM, Roy T, Hernandez CM, et al. 2020. Genetic variation regulates opioid-induced respiratory depression in mice. *Sci. Rep.* 10:14970
42. Mogil JS, Wilson SG. 1997. Nociceptive and morphine antinociceptive sensitivity of 129 and C57BL/6 inbred mouse strains: implications for transgenic knock-out studies. *Eur. J. Pain* 1:293–97
43. Crain SM, Shen K-F. 2000. Enhanced analgesic potency and reduced tolerance of morphine in 129/SvEv mice: evidence for a deficiency in GM1 ganglioside-regulated excitatory opioid receptor functions. *Brain Res.* 856:227–35
44. Bohn LM, Gainetdinov RR, Sotnikova TD, Medvedev IO, Lefkowitz RJ, et al. 2003. Enhanced rewarding properties of morphine, but not cocaine, in βarrestin-2 knock-out mice. *J. Neurosci.* 23:10265–73
45. Bohn LM, Dykstra LA, Lefkowitz RJ, Caron MG, Barak LS. 2004. Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. *Mol. Pharmacol.* 66:106–12
46. Raehal KM, Bohn LM. 2011. The role of beta-arrestin2 in the severity of antinociceptive tolerance and physical dependence induced by different opioid pain therapeutics. *Neuropharmacology* 60:58–65
47. Gillis A, Gondin AB, Kliewer A, Sanchez J, Lim HD, et al. 2020. Low intrinsic efficacy for G protein activation can explain the improved side effect profiles of new opioid agonists. *Sci. Signal.* 13:eaaz3140
48. Hill R, Canals M. 2022. Experimental considerations for the assessment of in vivo and in vitro opioid pharmacology. *Pharmacol. Ther.* 230:107961
49. Miess E, Gondin AB, Yousuf A, Steinborn R, Mosslein N, et al. 2018. Multisite phosphorylation is required for sustained interaction with GRKs and arrestins during rapid μ-opioid receptor desensitization. *Sci. Signal.* 11:eaas9609
50. Gillis A, Christie MJ. 2021. Opioid overdose and tolerance: Is the recruitment of β-arrestin to the μ-receptor involved? *Neuropsychopharmacology* 46:2226–27
51. Schmid CL, Kennedy NM, Ross NC, Lovell KM, Yue Z, et al. 2017. Bias factor and therapeutic window correlate to predict safer opioid analgesics. *Cell* 171:1165–75.e13
52. Black JW, Leff P. 1983. Operational models of pharmacological agonism. *Proc. R. Soc. B* 220:141–62
53. Kenakin T, Watson C, Muniz-Medina V, Christopoulos A, Novick S. 2012. A simple method for quantifying functional selectivity and agonist bias. *ACS Chem. Neurosci.* 3:193–203
54. Ehlert FJ. 2008. On the analysis of ligand-directed signaling at G protein-coupled receptors. *Naunyn Schmiedebergs Arch. Pharmacol.* 377:549–77
55. Rajagopal S, Ahn S, Rominger DH, Gowen-MacDonald W, Lam CM, et al. 2011. Quantifying ligand bias at seven-transmembrane receptors. *Mol. Pharmacol.* 80:367–77
56. Borgland SL, Connor M, Osborne PB, Furness JB, Christie MJ. 2003. Opioid agonists have different efficacy profiles for G protein activation, rapid desensitization, and endocytosis of mu-opioid receptors. *J. Biol. Chem.* 278:18776–84
57. McPherson J, Rivero G, Baptist M, Llorente J, Al-Sabah S, et al. 2010. μ-Opioid receptors: correlation of agonist efficacy for signalling with ability to activate internalization. *Mol. Pharmacol.* 78:756–66
58. Molinari P, Vezzi V, Sbraccia M, Gro C, Riitano D, et al. 2010. Morphine-like opiates selectively antagonize receptor-arrestin interactions. *J. Biol. Chem.* 285:12522–35
59. Rivero G, Llorente J, McPherson J, Cooke A, Mundell SJ, et al. 2012. Endomorphin-2: a biased agonist at the μ-opioid receptor. *Mol. Pharmacol.* 82:178–88
60. Connor M, Osborne PB, Christie MJ. 2004. μ-Opioid receptor desensitization: Is morphine different? *Br. J. Pharmacol.* 143:685–96
61. Kelly E. 2013. Efficacy and ligand bias at the μ-opioid receptor. *Br. J. Pharmacol.* 169:1430–46

62. Gillis A, Sreenivasan V, Christie MJ. 2020. Intrinsic efficacy of opioid ligands and its importance for apparent bias, operational analysis, and therapeutic window. *Mol. Pharmacol.* 98:410–24
63. Bailey CP, Llorente J, Gabra BH, Smith FL, Dewey WL, et al. 2009. Role of protein kinase C and μ-opioid receptor (MOPr) desensitization in tolerance to morphine in rat locus coeruleus neurons. *Eur. J. Neurosci.* 29:307–18
64. Bohn LM, Lefkowitz RJ, Caron MG. 2002. Differential mechanisms of morphine antinociceptive tolerance revealed in βarrestin-2 knock-out mice. *J. Neurosci.* 22:10494–500
65. Kelly E, Bailey CP, Henderson G. 2008. Agonist-selective mechanisms of GPCR desensitization. *Br. J. Pharmacol.* 153(Suppl. 1):S379–88
66. Kelly E, Sutcliffe K, Cavallo D, Ramos-Gonzalez N, Alhosan N, Henderson G. 2021. The anomalous pharmacology of fentanyl. *Br. J. Pharmacol.* **https://doi.org/10.1111/bph.15573**
67. de Waal PW, Shi J, You E, Wang X, Melcher K, et al. 2020. Molecular mechanisms of fentanyl mediated β-arrestin biased signaling. *PLOS Comput. Biol.* 16:e1007394
68. Podlewska S, Bugno R, Kudla L, Bojarski AJ, Przewlocki R. 2020. Molecular modeling of μ opioid receptor ligands with various functional properties: PZM21, SR-17018, morphine, and fentanyl—simulated interaction patterns confronted with experimental data. *Molecules* 25:4636
69. Winpenny D, Clark M, Cawkill D. 2016. Biased ligand quantification in drug discovery: from theory to high throughput screening to identify new biased mu opioid receptor agonists. *Br. J. Pharmacol.* 173:1393–403
70. Vasudevan L, Vandeputte M, Deventer M, Wouters E, Cannaert A, Stove CP. 2020. Assessment of structure-activity relationships and biased agonism at the Mu opioid receptor of novel synthetic opioids using a novel, stable bio-assay platform. *Biochem. Pharmacol.* 177:113910
71. DeWire SM, Yamashita DS, Rominger DH, Liu G, Cowan CL, et al. 2013. A G protein-biased ligand at the μ-opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *J. Pharmacol. Exp. Ther.* 344:708–17
72. Altarifi AA, David B, Muchhala KH, Blough BE, Akbarali H, Negus SS. 2017. Effects of acute and repeated treatment with the biased mu opioid receptor agonist TRV130 (oliceridine) on measures of antinociception, gastrointestinal function, and abuse liability in rodents. *J. Psychopharmacol.* 31:730–39
73. FDA (US Food Drug Adm.). 2020. *FDA approves new opioid for intravenous use in hospitals, other controlled clinical settings*. News Release, Aug. 7, FDA, Silver Spring, MD. **https://www.fda.gov/news-events/press-announcements/fda-approves-new-opioid-intravenous-use-hospitals-other-controlled-clinical-settings**
74. Negus SS, Freeman KB. 2018. Abuse potential of biased mu opioid receptor agonists. *Trends Pharmacol. Sci.* 39:916–19
75. Schwienteck KL, Faunce KE, Rice KC, Obeng S, Zhang Y, et al. 2019. Effectiveness comparisons of G-protein biased and unbiased mu opioid receptor ligands in warm water tail-withdrawal and drug discrimination in male and female rats. *Neuropharmacology* 150:200–9
76. Yudin Y, Rohacs T. 2019. The G-protein-biased agents PZM21 and TRV130 are partial agonists of μ-opioid receptor-mediated signalling to ion channels. *Br. J. Pharmacol.* 176:3110–25
77. Singleton S, Baptista-Hon DT, Edelsten E, McCaughey KS, Camplisson E, Hales TG. 2021. TRV130 partial agonism and capacity to induce anti-nociceptive tolerance revealed through reducing available μ-opioid receptor number. *Br. J. Pharmacol.* 178:1855–68
78. Bossert JM, Kiyatkin EA, Korah H, Hoots JK, Afzal A, et al. 2020. In a rat model of opioid maintenance, the G protein-biased mu opioid receptor agonist TRV130 decreases relapse to oxycodone seeking and taking and prevents oxycodone-induced brain hypoxia. *Biol. Psychiatry* 88:935–44
79. Fakira AK, Devi LA, Kennedy PJ. 2020. Novel application for G protein-biased mu opioid receptor agonists in opioid relapse prevention. *Biol. Psychiatry* 88:896–97
80. Soergel DG, Subach RA, Sadler B, Connell J, Marion AS, et al. 2014. First clinical experience with TRV130: pharmacokinetics and pharmacodynamics in healthy volunteers. *J. Clin. Pharmacol.* 54:351–57
81. Lambert D, Calo G. 2020. Approval of oliceridine (TRV130) for intravenous use in moderate to severe pain in adults. *Br. J. Anaesth.* 125:e473–74
82. Manglik A, Lin H, Aryal DK, McCorvy JD, Dengler D, et al. 2016. Structure-based discovery of opioid analgesics with reduced side effects. *Nature* 537:185–90

83. Hill R, Disney A, Conibear A, Sutcliffe K, Dewey W, et al. 2018. The novel μ-opioid receptor agonist PZM21 depresses respiration and induces tolerance to antinociception. *Br. J. Pharmacol.* 175:2653–61
84. Groom S, Blum NK, Conibear AE, Disney A, Hill R, et al. 2020. A novel G protein-biased agonist at the μ opioid receptor induces substantial receptor desensitisation through G protein-coupled receptor kinase. *Br. J. Pharmacol.* **https://doi.org/10.1111/bph.15334**
85. Kudla L, Bugno R, Skupio U, Wiktorowska L, Solecki W, et al. 2019. Functional characterization of a novel opioid, PZM21, and its effects on the behavioural responses to morphine. *Br. J. Pharmacol.* 176:4434–45
86. Fritzwanker S, Schulz S, Kliewer A. 2021. SR-17018 stimulates atypical μ-opioid receptor phosphorylation and dephosphorylation. *Molecules* 26:4509
87. Klein Herenbrink C, Sykes DA, Donthamsetti P, Canals M, Coudrat T, et al. 2016. The role of kinetic context in apparent biased agonism at GPCRs. *Nat. Commun.* 7:10842
88. Grim TW, Schmid CL, Stahl EL, Pantouli F, Ho JH, et al. 2020. A G protein signaling-biased agonist at the μ-opioid receptor reverses morphine tolerance while preventing morphine withdrawal. *Neuropsychopharmacology* 45:416–25
89. Kudla L, Przewlocki R. 2021. Influence of G protein-biased agonists of μ-opioid receptor on addiction-related behaviors. *Pharmacol. Rep.* 73:1033–51
90. Gutridge AM, Robins MT, Cassell RJ, Uprety R, Mores KL, et al. 2020. G protein-biased kratom-alkaloids and synthetic carfentanil-amide opioids as potential treatments for alcohol use disorder. *Br. J. Pharmacol.* 177:1497–513
91. Todd DA, Kellogg JJ, Wallace ED, Khin M, Flores-Bocanegra L, et al. 2020. Chemical composition and biological effects of kratom (*Mitragyna speciosa*): in vitro studies with implications for efficacy and drug interactions. *Sci. Rep.* 10:19158
92. Dekan Z, Sianati S, Yousuf A, Sutcliffe KJ, Gillis A, et al. 2019. A tetrapeptide class of biased analgesics from an Australian fungus targets the μ-opioid receptor. *PNAS* 116:22353–58
93. Li Y, Cazares M, Wu J, Houghten RA, Toll L, Dooley C. 2016. Potent μ-opioid receptor agonists from cyclic peptides Tyr-*c*[D-Lys-Xxx-Tyr-Gly]: synthesis, biological, and structural evaluation. *J. Med. Chem.* 59:1239–45
94. Kaserer T, Steinacher T, Kainhofer R, Erli F, Sturm S, et al. 2020. Identification and characterization of plant-derived alkaloids, corydine and corydaline, as novel mu opioid receptor agonists. *Sci. Rep.* 10:13804
95. Kennedy NM, Schmid CL, Ross NC, Lovell KM, Yue Z, et al. 2018. Optimization of a series of mu opioid receptor (MOR) agonists with high G protein signaling bias. *J. Med. Chem.* 61:8895–907
96. Yang Y, Wang Y, Zuo A, Li C, Wang W, et al. 2022. Synthesis, biological, and structural explorations of a series of μ-opioid receptor (MOR) agonists with high G protein signaling bias. *Eur. J. Med. Chem.* 228:113986
97. Wang H, Hetzer F, Huang W, Qu Q, Meyerowitz J, et al. 2022. Structure-based evolution of G protein-biased μ-opioid receptor agonists. *Angew. Chem. Int. Ed. Engl.* 61:e202200269
98. Ehrlich AT, Semache M, Gross F, Da Fonte DF, Runtz L, et al. 2019. Biased signaling of the μ opioid receptor revealed in native neurons. *iScience* 14:47–57
99. Dahan A, van Dam CJ, Niesters M, van Velzen M, Fossler MJ, et al. 2020. Benefit and risk evaluation of biased μ-receptor agonist oliceridine versus morphine. *Anesthesiology* 133:559–68
100. Goudra B, Mason KP. 2021. Emerging approaches in intravenous moderate and deep sedation. *J. Clin. Med.* 10:1735
101. Liu Y, Hu Q, Yang J. 2021. Oliceridine for the management of acute postoperative pain. *Ann. Pharmacother.* 55:1283–89
102. Finlay JE, Leslie K. 2021. Sedation/analgesia techniques for nonoperating room anesthesia: new drugs and devices. *Curr. Opin. Anaesthesiol.* 34:678–82
103. Kaye AD, Edinoff AN, Babin KC, Hebert CM, Hardin JL, et al. 2021. Pharmacological advances in opioid therapy: a review of the role of oliceridine in pain management. *Pain Ther.* 10:1003–12
104. Azevedo Neto J, Costanzini A, De Giorgio R, Lambert DG, Ruzza C, Calò G. 2020. Biased versus partial agonism in the search for safer opioid analgesics. *Molecules* 25:3870
105. Pineyro G, Nagi K. 2021. Signaling diversity of mu- and delta- opioid receptor ligands: re-evaluating the benefits of β-arrestin/G protein signaling bias. *Cell. Signal.* 80:109906

106. Nickolls SA, Waterfield A, Williams RE, Kinloch RA. 2011. Understanding the effect of different assay formats on agonist parameters: a study using the μ-opioid receptor. *J. Biomol. Screen.* 16:706–16
107. Stahl EL, Zhou L, Ehlert FJ, Bohn LM. 2015. A novel method for analyzing extremely biased agonism at G protein-coupled receptors. *Mol. Pharmacol.* 87:866–77
108. Gundry J, Glenn R, Alagesan P, Rajagopal S. 2017. A practical guide to approaching biased agonism at G protein coupled receptors. *Front. Neurosci.* 11:17
109. Stahl EL, Bohn LM. 2022. Low intrinsic efficacy alone cannot explain the improved side effect profiles of new opioid agonists. *Biochemistry* 61(18):1923–35
110. Pedersen MF, Wrobel TM, Marcher-Rorsted E, Pedersen DS, Moller TC, et al. 2020. Biased agonism of clinically approved μ-opioid receptor agonists and TRV130 is not controlled by binding and signaling kinetics. *Neuropharmacology* 166:107718
111. Onaran HO, Costa T. 2021. Conceptual and experimental issues in biased agonism. *Cell. Signal.* 82:109955
112. Benredjem B, Gallion J, Pelletier D, Dallaire P, Charbonneau J, et al. 2019. Exploring use of unsupervised clustering to associate signaling profiles of GPCR ligands to clinical response. *Nat. Commun.* 10:4075
113. Groer CE, Schmid CL, Jaeger AM, Bohn LM. 2011. Agonist-directed interactions with specific β-arrestins determine μ-opioid receptor trafficking, ubiquitination, and dephosphorylation. *J. Biol. Chem.* 286:31731–41
114. Markova V, Hejnova L, Benda A, Novotny J, Melkes B. 2021. β-Arrestin 1 and 2 similarly influence μ-opioid receptor mobility and distinctly modulate adenylyl cyclase activity. *Cell. Signal.* 87:110124
115. Shukla AK, Westfield GH, Xiao K, Reis RI, Huang LY, et al. 2014. Visualization of arrestin recruitment by a G-protein-coupled receptor. *Nature* 512:218–22
116. Cahill TJ 3rd, Thomsen AR, Tarrasch JT, Plouffe B, Nguyen AH, et al. 2017. Distinct conformations of GPCR-β-arrestin complexes mediate desensitization, signaling, and endocytosis. *PNAS* 114:2562–67
117. Zimmerman B, Beautrait A, Aguila B, Charles R, Escher E, et al. 2012. Differential β-arrestin-dependent conformational signaling and cellular responses revealed by angiotensin analogs. *Sci. Signal.* 5:ra33
118. Kolb P, Kenakin T, Alexander SPH, Bermudez M, Bohn LM, et al. 2022. Community guidelines for GPCR ligand bias: IUPHAR review 32. *Br. J. Pharmacol.* 179(14):3651–74
119. Vandeputte MM, Cannaert A, Stove CP. 2020. In vitro functional characterization of a panel of non-fentanyl opioid new psychoactive substances. *Arch. Toxicol.* 94:3819–30
120. Cong X, Maurel D, Déméné H, Vasiliauskaité-Brooks I, Hagelberger J, et al. 2021. Molecular insights into the biased signaling mechanism of the μ-opioid receptor. *Mol. Cell* 81:4165–75.e6
121. Schneider S, Provasi D, Filizola M. 2016. How oliceridine (TRV-130) binds and stabilizes a μ-opioid receptor conformational state that selectively triggers G protein signaling pathways. *Biochemistry* 55:6456–66
122. Pradhan AA, Befort K, Nozaki C, Gaveriaux-Ruff C, Kieffer BL. 2011. The delta opioid receptor: an evolving target for the treatment of brain disorders. *Trends Pharmacol. Sci.* 32:581–90
123. Codd EE, Carson JR, Colburn RW, Stone DJ, Van Besien CR, et al. 2009. JNJ-20788560 [9-(8-azabicyclo[3.2.1]oct-3-ylidene)-9*H*-xanthene-3-carboxylic acid diethylamide], a selective delta opioid receptor agonist, is a potent and efficacious antihyperalgesic agent that does not produce respiratory depression, pharmacologic tolerance, or physical dependence. *J. Pharmacol. Exp. Ther.* 329:241–51
124. Gallantine EL, Meert TF. 2005. A comparison of the antinociceptive and adverse effects of the μ-opioid agonist morphine and the δ-opioid agonist SNC80. *Basic Clin. Pharmacol. Toxicol.* 97:39–51
125. Comer SD, Hoenicke EM, Sable AI, McNutt RW, Chang KJ, et al. 1993. Convulsive effects of systemic administration of the delta opioid agonist BW373U86 in mice. *J. Pharmacol. Exp. Ther.* 267:888–95
126. Pradhan AAA, Walwyn W, Nozaki C, Filliol D, Erbs E, et al. 2010. Ligand-directed trafficking of the δ-opioid receptor in vivo: two paths toward analgesic tolerance. *J. Neurosci.* 30:16459–68
127. Conibear AE, Asghar J, Hill R, Henderson G, Borbely E, et al. 2020. A novel G protein-biased agonist at the δ opioid receptor with analgesic efficacy in models of chronic pain. *J. Pharmacol. Exp. Ther.* 372:224–36
128. Fossler MJ, Schmith V, Greene SA, Lohmer L, Kramer MS, et al. 2020. A Phase I, randomized, single-blind, placebo-controlled, single ascending dose study of the safety, tolerability, and pharmacokinetics of

subcutaneous and oral TRV250, a G protein-selective delta receptor agonist, in healthy subjects. *CNS Drugs* 34:853–65
129. Gendron L, Cahill CM, von Zastrow M, Schiller PW, Pineyro G. 2016. Molecular pharmacology of δ-opioid receptors. *Pharmacol. Rev.* 68:631–700
130. Dripps IJ, Chen R, Shafer AM, Livingston KE, Disney A, et al. 2020. Pharmacological properties of δ-opioid receptor-mediated behaviors: agonist efficacy and receptor reserve. *J. Pharmacol. Exp. Ther.* 374:319–30
131. Pradhan AA, Perroy J, Walwyn WM, Smith ML, Vicente-Sanchez A, et al. 2016. Agonist-specific recruitment of arrestin isoforms differentially modify delta opioid receptor function. *J. Neurosci.* 36:3541–51
132. Aguila B, Coulbault L, Davis A, Marie N, Hasbi A, et al. 2012. βarrestin1-biased agonism at human δ-opioid receptor by peptidic and alkaloid ligands. *Cell. Signal.* 24:699–707
133. Vicente-Sanchez A, Dripps IJ, Tipton AF, Akbari H, Akbari A, et al. 2018. Tolerance to high-internalizing δ opioid receptor agonist is critically mediated by arrestin 2. *Br. J. Pharmacol.* 175:3050–59
134. Dripps IJ, Boyer BT, Neubig RR, Rice KC, Traynor JR, Jutkiewicz EM. 2018. Role of signalling molecules in behaviours mediated by the δ opioid receptor agonist SNC80. *Br. J. Pharmacol.* 175:891–901
135. Thompson GL, Kelly E, Christopoulos A, Canals M. 2015. Novel GPCR paradigms at the μ-opioid receptor. *Br. J. Pharmacol.* 172:287–96
136. Thompson GL, Lane JR, Coudrat T, Sexton PM, Christopoulos A, Canals M. 2016. Systematic analysis of factors influencing observations of biased agonism at the mu-opioid receptor. *Biochem. Pharmacol.* 113:70–87
137. Broom DC, Guo L, Coop A, Husbands SM, Lewis JW, et al. 2000. BU48: a novel buprenorphine analog that exhibits δ-opioid-mediated convulsions but not δ-opioid-mediated antinociception in mice. *J. Pharmacol. Exp. Ther.* 294:1195–200
138. Paton KF, Atigari DV, Kaska S, Prisinzano T, Kivell BM. 2020. Strategies for developing κ opioid receptor agonists for the treatment of pain with fewer side effects. *J. Pharmacol. Exp. Ther.* 375:332–48
139. Kumagai H, Ebata T, Takamori K, Muramatsu T, Nakamoto H, Suzuki H. 2010. Effect of a novel kappa-receptor agonist, nalfurafine hydrochloride, on severe itch in 337 haemodialysis patients: a Phase III, randomized, double-blind, placebo-controlled study. *Nephrol. Dial. Transplant.* 25:1251–57
140. Endoh T, Matsuura H, Tajima A, Izumimoto N, Tajima C, et al. 1999. Potent antinociceptive effects of TRK-820, a novel κ-opioid receptor agonist. *Life Sci.* 65:1685–94
141. Endoh T, Tajima A, Suzuki T, Kamei J, Narita M, et al. 2000. Characterization of the antinociceptive effects of TRK-820 in the rat. *Eur. J. Pharmacol.* 387:133–40
142. Liu JJ, Chiu Y-T, DiMattio KM, Chen C, Huang P, et al. 2019. Phosphoproteomic approach for agonist-specific signaling in mouse brains: mTOR pathway is involved in κ opioid aversion. *Neuropsychopharmacology* 44:939–49
143. Kozono H, Yoshitani H, Nakano R. 2018. Post-marketing surveillance study of the safety and efficacy of nalfurafine hydrochloride (Remitch® capsules 2.5 mug) in 3,762 hemodialysis patients with intractable pruritus. *Int. J. Nephrol. Renovasc. Dis.* 11:9–24
144. Morgenweck J, Frankowski KJ, Prisinzano TE, Aube J, Bohn LM. 2015. Investigation of the role of βarrestin2 in kappa opioid receptor modulation in a mouse model of pruritus. *Neuropharmacology* 99:600–9
145. White KL, Robinson JE, Zhu H, DiBerto JF, Polepally PR, et al. 2015. The G protein-biased κ-opioid receptor agonist RB-64 is analgesic with a unique spectrum of activities in vivo. *J. Pharmacol. Exp. Ther.* 352:98–109
146. Bruchas MR, Land BB, Aita M, Xu M, Barot SK, et al. 2007. Stress-induced p38 mitogen-activated protein kinase activation mediates κ-opioid-dependent dysphoria. *J. Neurosci.* 27:11614–23
147. Bruchas MR, Chavkin C. 2010. Kinase cascades and ligand-directed signaling at the kappa opioid receptor. *Psychopharmacology* 210:137–47
148. Ehrich JM, Messinger DI, Knakal CR, Kuhar JR, Schattauer SS, et al. 2015. Kappa opioid receptor-induced aversion requires p38 MAPK activation in VTA dopamine neurons. *J. Neurosci.* 35:12917–31
149. Toll L, Bruchas MR, Calò G, Cox BM, Zaveri NT. 2016. Nociceptin/orphanin FQ receptor structure, signaling, ligands, functions, and interactions with opioid systems. *Pharmacol. Rev.* 68:419–57

150. Mann A, Moulédous L, Froment C, O'Neill PR, Dasgupta P, et al. 2019. Agonist-selective NOP receptor phosphorylation correlates in vitro and in vivo and reveals differential post-activation signaling by chemically diverse agonists. *Sci. Signal.* 12:eaau8072
151. Asth L, Ruzza C, Malfacini D, Medeiros I, Guerrini R, et al. 2016. Beta-arrestin 2 rather than G protein efficacy determines the anxiolytic-versus antidepressant-like effects of nociceptin/orphanin FQ receptor ligands. *Neuropharmacology* 105:434–42
152. Kruegel AC, Gassaway MM, Kapoor A, Váradi A, Majumdar S, et al. 2016. Synthetic and receptor signaling explorations of the *Mitragyna* alkaloids: mitragynine as an atypical molecular framework for opioid receptor modulators. *J. Am. Chem. Soc.* 138:6754–64
153. Kenakin T. 2017. A scale of agonism and allosteric modulation for assessment of selectivity, bias, and receptor mutation. *Mol. Pharmacol.* 92:414–24
154. Bychkov E, Zurkovsky L, Garret MB, Ahmed MR, Gurevich EV. 2012. Distinct cellular and subcellular distributions of G protein-coupled receptor kinase and arrestin isoforms in the striatum. *PLOS ONE* 7:e48912
155. Araldi D, Ferrari LF, Levine JD. 2018. Mu-opioid receptor (MOR) biased agonists induce biphasic dose-dependent hyperalgesia and analgesia, and hyperalgesic priming in the rat. *Neuroscience* 394:60–71
156. Roth BL, Chuang DM. 1987. Multiple mechanisms of serotonergic signal transduction. *Life Sci.* 41:1051–64