Trends in Pharmacological Sciences

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Review

Critical Assessment of G Protein-Biased Agonism at the μ-Opioid Receptor

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G protein-biased agonists of the μ-opioid receptor (MOPr) have been proposed as an improved class of opioid analgesics. Recent studies have been unable to reproduce the original experiments in the β-arrestin2-knockout mouse that led to this proposal, and alternative genetic models do not support the G protein-biased MOPr agonist hypothesis. Furthermore, assessment of putatively biased ligands has been confounded by several factors, including assay amplification. As such, the extent to which current lead compounds represent mechanistically novel, extremely G protein-biased agonists is in question, as is the underlying assumption that β-arrestin2 mediates deleterious opioid effects. Addressing these current challenges represents a pressing issue to successfully advance drug development at this receptor and improve upon current opioid analgesics.

Highlights

G protein-biased agonists of the μ-opioid receptor have been hypothesized to be an improved class of opioid analgesics

Early studies in the β-arrestin2-knockout mouse that suggested a separation between the signaling mediating analgesia versus side effects have not been reproduced, and a 'G protein-biased' mutant MOPr mouse does not support the original proposal.

There is now evidence for a G protein-dependent signal mediating deleterious opioid effects.

The previously observed G protein bias of many recently developed MOPr agonists has been confounded by assay amplification. Such ligands may in fact be unbiased, with low intrinsic efficacy.

Current evidence does not support the proposal that G protein-biased agonism at the MOPr will provide substantially improved therapeutic profiles.

Low intrinsic efficacy represents an alternative mechanism by which novel opioids may display wider therapeutic windows.

Current View of MOPr Biased Signaling

G protein-**biased agonists** (see Glossary) of the **μ-opioid receptor (MOPr**) have been widely proposed to be a novel, substantially improved class of analgesics [1,2]. The prototypical such agonist, oliceridine (TRV130), has proceeded to Phase III clinical trials [3], and was recently approved in the USA for use in acute pain. Existing, clinically approved opioid analgesics, such as morphine, oxycodone, and fentanyl, are MOPr agonists that provide pain relief that is unmatched by other drug classes. Current opioids have an array of adverse effects, including respiratory depression, constipation, and euphoria, as well as inducing tolerance and dependence over time. These important limitations of opioid analgesics have all been proposed to be addressed by G protein-biased MOPr agonists (Box 1). However, recent results have brought into question the hypothesis that underpins the proposed mechanism of action of this anticipated new drug class, that **β-arrestin2** mediates deleterious opioid effects. In addition, there is evidence that challenges both the extent of the G protein bias of lead compounds and the extent to which such compounds are likely to represent improved analgesics.

G Protein Signaling in Respiratory Depression and Constipation Induced by Opioids

The target of opioid analgesics, MOPr, is a **G protein coupled receptor (GPCR)** that signals predominantly through activation of the $G\alpha_{i/o}$ and βγ proteins. MOPr activation alters neuronal function through well-established G protein signaling mechanisms, including postsynaptic activation of **G protein coupled inwardly rectifying potassium channels (GIRK)**, causing hyperpolarization and inhibition of neurons [4]. Presynaptic inhibition of neurotransmission also occurs through G protein signaling of MOPr, predominantly via the inhibition of **voltage-gated calcium channels (VGCC)** [5]. G protein signaling of the MOPr can be negatively regulated via a system of intracellular C-terminal phosphorylation by various kinases, and β-arrestin binding common to most GPCRs. In addition to negative regulation of G protein signaling, the recruitment of β-arrestin to MOPr has been proposed to transduce a G protein-independent signal

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[1,2]. To date, however, the nature of this putative signal has not been described and it remains unclear how β-arrestin signaling mediates discrete physiological effects.

Central to the proposed benefit of putatively G protein-biased MOPr agonists [6–9] is the hypothesis that MOPr/β-arrestin interactions mediate opioid-induced respiratory depression and constipation [1], based on results obtained using the β-arrestin2-knockout mouse [10]. Opioid-induced respiratory depression is the major cause of overdose death, and multiple genetic knockout studies have shown that it results from activation of MOPr rather than δ- or κ-opioid receptor subtypes (DOPr and KOPr) or the opioid-related nociceptin/orphanin FQ receptor (NOPr) [7,11,12], as does inhibition of gastrointestinal transit [13].

Expression of MOPr is abundant throughout the respiratory network of the brainstem [14]. Local removal of MOPr via viral Cre delivery from either the **preBötzinger (preBötC) neurons** or **Kölliker-Fuse (KF) neurons**, regions critical in respiratory control, substantially reduced the effect of morphine on respiratory rate in mice [15]. A similar study confirmed this result, as well as reporting the abolition of both morphine- and fentanyl-induced respiratory depression following simultaneous MOPr deletion from both preBötC and KF [16], demonstrating the necessity of MOPr expression within these areas for opioid-induced respiratory depression.

There is now significant evidence that MOPr activation in these nuclei, as is established extensively throughout the nervous system, inhibits neuronal activity via G protein signaling. The preBötC has been characterized as a site critical for opioid-induced respiratory depression [17]. Morphine effects on respiratory rate have been defined via pharmacological ion channel modulation and genetic knockout to occur at least partially via MOPr-induced GIRK activation in neurons of this nucleus [18]. A presynaptic mechanism for opioid effects on preBötC activity, via VGCC inhibition, has also been suggested from electrophysiological experiments [19]. Neurons of the KF, similar to the preBötC, are hyperpolarized via MOPr activation of GIRK, suppressing inspiratory drive [20], while local antagonism of MOPr within the KF partially reversed the respiratory changes induced by systemic fentanyl application [21].

These results, demonstrating that opioid-induced respiratory depression occurs substantially via G protein signaling, are difficult to reconcile with the hypothesis that the respiratory depressant effects of morphine are β-arrestin dependent (Box 1) [1]. This hypothesis was formed after the initial report that morphine-induced respiratory depression was greatly attenuated in β-arrestin2-knockout mice [10]. However, subsequent experiments in three independent laboratories failed to observe any effect of this genotype on morphine- or fentanyl-induced respiratory depression [22] (Box 2).

Global β-arrestin2 knockout will affect all systems regulated by this ubiquitously expressed protein. A recently developed genetic model, more selective for opioid function, used mice expressing a modified MOPr, in which C-terminal serine and threonine phosphorylation sites were mutated to alanine (11S/T-A mice) [23], thereby preventing both phosphorylation and β-arrestin binding [24]. Critically, both morphine and fentanyl depressed respiration in 11S/T-A mice [23], implying, together with the lack of a β-arrestin2-knockout phenotype, that MOPr/β-arrestin interactions are not essential for opioid-induced respiratory depression.

Opioid-induced constipation is mainly mediated by MOPr expressed peripherally in the enteric nervous system. Key research in human intestine showed that opioids act to inhibit neuromuscular transmission, causing an increase in muscle tone and a decrease in propulsive motility [25]. Following β-arrestin2-knockout mouse studies [10], the involvement of β-arrestin2-mediated signaling in the gastrointestinal actions of opioids was proposed [1]. More recent work has again,

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conversely, observed persistent morphine- and fentanyl-induced constipation in both β-arrestin2-knockout [22,26] and 11S/T-A [23] mice. In the enteric nervous system of various species, opioids induce neuronal hyperpolarization and reduced neuronal excitability, as well as presynaptic inhibition of neurotransmitter release [27–29], an effect prevented by pretreatment with pertussis toxin [30]. Therefore, it is well demonstrated that opioid-induced constipation, similar to respiratory depression, is mediated by G protein signaling, including activation of GIRK and VGCC inhibition, without any evidence for the hypothesized β-arrestin-mediated signaling. Thus, it is difficult to reconcile this recent evidence with the hypothesis that G protein-biased MOPr agonists, which stimulate signaling through G proteins while sparing β-arrestin recruitment, will be safer than unbiased agonists with regard to respiratory depression and constipation.

Opioid-Induced Analgesia, Tolerance, and Dependence

G protein-biased MOPr agonists were further proposed to induce enhanced analgesia without tolerance [1], as well as resulting in less dependence with reduced rewarding effects [2]. Currently used opioids, such as the prototypical morphine, produce robust analgesia through MOPr, rather than DOPr, KOPr, or NOPr, activation [13].

Acute opioid antinociception is primarily centrally mediated, as recently shown through conditional knock out of MOPr from peripheral neurons [31,32], occurring through MOPr activation at multiple central nervous system sites. MOPr activation within the periaqueductal gray of the brainstem disinhibits a descending, endogenous analgesic pathway that suppresses ascending nociceptive sensation [33]. Additionally, activation of MOPr within pre- and postsynaptic sites of the dorsal horn directly inhibits nociception [34]. G protein-signaling mechanisms mediate MOPr agonist effects at these sites, including GIRK activation [35,36] and VGCC inhibition [37].

Substantial and protracted efforts to develop G protein-biased MOPr agonists were spurred by mouse genotype studies. Global knock out of β-arrestin2 in mice was observed to greatly enhance the acute antinociception induced by morphine [38], but not by fentanyl, methadone, or etorphine [39]. Remarkably, tolerance to the hotplate antinociceptive effect of morphine was reported not to develop in these mice [40]. This effect was again ligand specific, with no difference in the development of antinociceptive tolerance to oxycodone, methadone, or fentanyl between wild-type and knockout mice [41]. More recent work has observed no effect of β-arrestin2 knockout on either acute antinociception in response to morphine, oxycodone, or fentanyl, or to the subsequent development of tolerance to these drugs following prolonged administration [42] (Box 2).

The 11/ST-A mutant mouse, more selective for MOPr function than the global β-arrestin2 knockout, is a less confounded model of the role of the C-terminal phosphorylation and β-arrestin-binding system in acute antinociception and tolerance development. Morphine and fentanyl acute antinociceptive responses were enhanced, and tolerance to this effect developed to a reduced extent, in 11S/T-A mice compared with wild-type mice [23]. Knock out of individual G protein receptor kinase (GRK) subtypes does not substantially alter acute or chronic opioid antinociception, whereas homozygous knock out of a subtype crucial for MOPr regulation, GRK2, is embryonically lethal and, therefore, has not been studied [43]. Together, these mixed genetic results suggest some role of the GRK phosphorylation and β-arrestin-binding system in tolerance development, as distinct from β-arrestin2 recruitment *per se*. However, given the lack of consistent effect of β-arrestin2 knockout, and no effect of β-arrestin1 knockout [43], on opioid antinociception and tolerance, it cannot be predicted that a lack of β-arrestin recruitment alone will enhance antinociception and prevent tolerance. Tolerance to opioid-induced antinociception in fact occurs in an agonist-dependent manner through multiple mechanisms [e.g., there is

Glossary

129/SvJ, C57BL/6 strains: inbred laboratory mouse substrains. Each mouse within a given substrain is genetically identical to another. Mixed background animals are derived from multiple strains and share characteristics of each.
β-arrestin1 and 2 (arrestin2 and 3): membrane receptor-binding family of proteins with ubiquitous expression and scaffolding functions. β-arrestin1 and 2 bind MOPr and mediate receptor internalization, although MOPr agonists recruit β-arrestin2 more effectively than they do β-arrestin1.
μ-opioid receptor (MOPr): GPCR expressed in the nervous system that is the target of opioid analgesics.
Biased agonist: activates one signaling pathway downstream of a receptor more efficiently than it activates another, discrete signaling pathway of the same receptor. Biased agonism is relative and considered in comparison to reference ligands.
DAMGO: [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin, a MOPr-selective analog of the endogenous opioid Met-enkephalin, which is a high efficacy reference agonist.
G protein coupled inwardly rectifying potassium channel (GIRK): activated by Gβγ subunits following activation of Gi/o coupled receptors, such as MOPr, hyperpolarizing neurons.
G protein coupled receptors (GPCRs): seven-transmembrane proteins that signal to cognate G proteins throughout the body
Intrinsic efficacy: ability of a ligand to activate a target, in this case a GPCR, as distinct from affinity. True antagonists have no efficacy but substantial affinity. Biased agonists may have low intrinsic efficacy in one signaling pathway, but high efficacy in another.
Kölliker-Fuse (KF) neurons: part of the parabrachial nuclei and contribute to opioid-induced respiratory depression.
PreBötzinger (preBötC) neurons: located in the ventral respiratory group of the medulla of the brainstem; is essential for the generation of the respiratory rhythm, and is involved in opioid-induced respiratory depression.
Therapeutic window: preclinical or clinical separation in compound potency for desired drug effect (e.g., analgesia) from side effects (e.g., respiratory depression).

substantial evidence for a role of a protein kinase C (PKC)-dependent process [44]], suggesting that avoiding β-arrestin recruitment alone will not prevent antinociceptive tolerance development.

Voltage-gated calcium channel (VGCC): inhibited by Gβγ subunits, inhibiting neurotransmission.

Physical dependence, commonly characterized in non-human animals by somatic withdrawal signs, is considered a component of the formation of opioid use disorder. Opioid withdrawal signs following chronic morphine treatment were unchanged [40] or slightly reduced at one dose [41] in the mixed background β-arrestin2-knockout mouse (Box 2). Again, the reported phenotype was ligand specific, because β-arrestin2 knockout did not alter oxycodone, fentanyl, or methadone withdrawal signs [41], as well as in fact worsening morphine-induced conditioned place preference, a model of opioid reward [45]. Despite these results, G protein bias has been proposed as a mechanism underlying lesser dependence [2,46], or lesser reward signs, induced by novel MOPr agonists [8,9]. Abolished C-terminal phosphorylation sites and, therefore, β-arrestin recruitment in the 11S/T-A mouse did not alter morphine or fentanyl withdrawal signs [23]. As such, there is limited evidence that altered arrestin recruitment by biased MOPr agonists would prevent physical dependence or withdrawal, or reduce addictive liability [47].

Thus, it is clear that an absolute separation of the signaling pathways leading to the analgesic effect of opioids (i.e., G protein mediated) from those producing adverse effects (i.e., β-arrestin2 mediated), as has been hypothesized (Box 1), is unlikely. Dependence, reward, constipation, and respiratory depression, some of the most problematic effects of opioid analgesics, are not improved in genetic models of abolished MOPr/β-arrestin interactions. There is no physiological evidence for a putative β-arrestin2 signal mediating acute deleterious opioid effects, but a substantial body of literature demonstrates the manner in which G protein-dependent signaling mediates opioid effects as well as analgesia (Figure 1). Therefore, MOPr agonists biased toward G protein signaling over β-arrestin recruitment, originally suggested to be improved analgesics, cannot be predicted to have the previously claimed characteristics. Delineating MOPr agonist properties that result in safe and efficacious opioid analgesia should occur via revisiting and fully understanding the pharmacology of both existing and novel opioids, including putatively biased ligands, and the complexity of MOPr signaling.

Current Methodological Challenges in Developing Biased MOPr Agonists

Assessment of potentially G protein-biased lead MOPr compounds has been hampered by systematically confounded signaling assays. Limitations on the dynamic range of observable effects, such as pathway amplification and inefficiently coupled signals, have posed significant challenges to the accurate characterization of ligand pharmacology and their relative efficacies in different signaling endpoints.

Putatively G protein-biased compounds oliceridine [6], PZM21 [8], mitragynine pseudoindoxyl [9], and SR170178 [7], were all initially assessed to have comparable efficacy to morphine for G protein activation. Later experiments [48–53] showed these ligands to in fact have low **intrinsic efficacy** relative to morphine (Figure 2A). Importantly, highly amplified G protein assays are relatively insensitive to efficacy differences between agonists due to the presence of receptor reserve [54], causing test agonists to reach a similar maximal 'ceiling' of the assay, regardless of variation in efficacy [55]. This is a straightforward pharmacological explanation for the initial description of apparently G protein-biased MOPr compounds with low intrinsic efficacy as being highly efficacious for G protein activation [56].

Analyses of MOPr bias routinely compare an amplified assay of G protein activation to one of β-arrestin recruitment [55]. In contrast to G protein signaling, β-arrestin recruitment to the MOPr is a protein–protein interaction without amplification, measurements of which are therefore

Box 1. The G Protein-Biased MOPr Agonism Hypothesis

G protein-biased MOPr agonists maintain the eponymous G protein signal, established to mediate analgesia, while minimizing recruitment of β-arrestin to the receptor. MOPr/β-arrestin interactions are proposed to mediate, or to positively facilitate, deleterious opioid effects, including respiratory depression, constipation, tolerance, and physical dependence. Therefore, MOPr agonists, which are G protein biased are hypothesized to be dramatically improved, less addictive analgesics that induce lesser respiratory depression and constipation (Figure I). However, the initial β-arrestin2-knockout results [10,38,40] have not been repeated in later experiments [22,26,42], while studies of mice expressing a phosphorylation-deficient mutant MOPr that does not recruit β-arrestins also do not support the G protein-biased agonist hypothesis [23]. Furthermore, substantial evidence has accumulated for G protein mechanisms mediating respiratory depression and constipation, as well as analgesia.

The profile of apparently G protein-biased (see main text), minimally β-arrestin-recruiting lead compounds is not significantly improved over established opioids, such as morphine [76]. The prototypical G protein-biased MOPr agonist, oliceridine (but see [51,53]), was initially reported in rodent studies to have an improved separation in potency between antinociceptive effects and both respiratory depression and constipation [6]. Clinical trial results of this drug candidate have been mixed, with persistent respiratory depression and constipation, but a marginally improved therapeutic window observed in some measures compared with morphine [3,77,78]. In regard to abuse-related effects, volunteers rated oliceridine similarly to morphine in a drug effects questionnaire [77], and, in rodents, both the self-administration of, and the facilitation of, intracranial self-stimulation induced by oliceridine was similar to other opioids [79,80]. Additionally, oliceridine generalized to fentanyl in a drug-discrimination procedure, similarly to other MOPr agonists [69]. A second putatively G protein-biased compound, PZM21 (but see [48,51]), was initially reported not to induce respiratory depression, constipation, or to be rewarding in a conditioned-place preference test in mice [8]. However, several later studies found substantial respiratory depression to be induced by PZM21 [48,51], albeit with some variation in potency between experimenters, while the observation of minimal conditioned-place preference has been reproduced [81]. A recent examination of the reinforcing effects of PZM21 in non-human primates showed similar abuse liability to oxycodone [82], and antinociceptive tolerance comparable to morphine develops following prolonged treatment [48].

Recently, the G protein-bias factor of a family of newly developed compounds was correlated to the separation in potency between antinociception and respiratory depression [7]. Most significantly, SR17018 was reported not to induce respiratory depression [7], although again later studies have shown decreases in respiratory rate at minimally antinociceptive doses [51]. Studies of this ligand are significantly hampered by extremely poor solubility [46,51], and a recent study reported high, DAMGO-like arrestin recruitment by SR17018 [58].

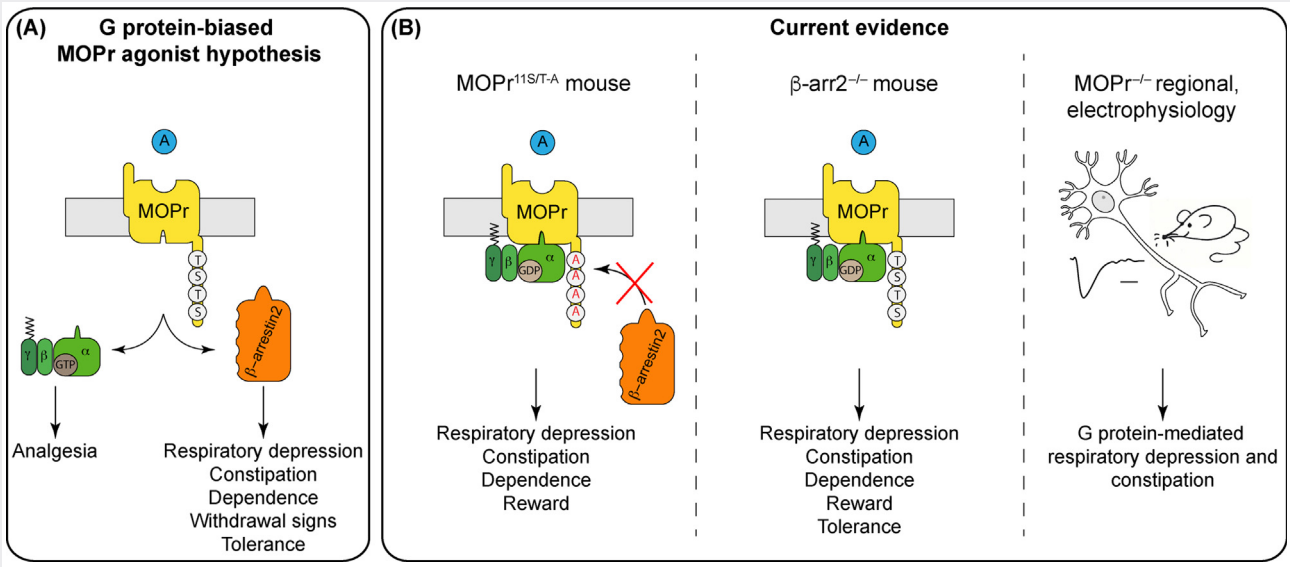

Figure I. Re-evaluation of G Protein-Biased μ-Opioid Receptor (MOPr) Agonist Hypothesis. (A) The G protein-biased MOPr agonist hypothesis proposes G protein activation mediating opioid analgesia, whereas several side effects are mediated by β-arrestin2 (β-arr2). (B) Current evidence from behavioral studies in 11S/T-A MOPr-knock-in mice, β-arrestin2-knockout mice, and direct physiological studies using regional MOPr-knockout mice and electrophysiology among other techniques show that side effects are likely to be G protein-mediated.

sensitive to differences in efficacy [54]. Thus, an agonist with low intrinsic efficacy may display a lower maximal effect relative to a high efficacy reference agonist, such as **DAMGO**, in a β-arrestin assay, despite displaying the same maximal effect in a G protein activation assay. This pattern of activity stemming from the unequal comparison made between assays with different amplification leads to apparent bias of low intrinsic efficacy agonists toward G protein signalling over β-

Box 2. Opioid Effects in β-Arrestin2-Knockout Animals

Examination of the role of β-arrestin2 in modulating, or mediating, opioid effects *in vivo* was stimulated by early experiments on the β-arrestin2 global knockout mouse. The antinociception of morphine was observed to be dramatically enhanced [38], while tolerance to that effect over time was abolished [40], in this genotype when compared with wild-type animals. This effect was ligand specific, in that there was no difference in either the acute antinociception or tolerance to that effect induced by fentanyl, methadone, or oxycodone [39,41], or etorphine acute antinociception, between wild-type and knockout mice. The lack of effect of β-arrestin2 knockout on fentanyl, methadone, or etorphine acute antinociception is surprising, given that these are high-efficacy agonists that robustly induce the recruitment of β-arrestin2 to the MOPr [7,39,51]. No mechanism underpinning the morphine-specific phenotype has yet been demonstrated, although these results suggest that β-arrestin2 recruitment does not negatively regulate the acute antinociception of fentanyl, methadone, or oxycodone, and is not critical for their antinociceptive tolerance.

Later experiments on the β-arrestin2-knockout mouse showed a dramatic reduction in morphine-induced respiratory depression and constipation [10]. This observation was surprising, suggesting a positive role of β-arrestin2 in facilitating, or mediating, opioid effects, rather than simply negatively regulating MOPr G protein activation. This led to the hypothesis that opioid-induced respiratory depression and constipation are β-arrestin2-dependent and, therefore, that G protein-biased agonists would reduce these effects while producing enhanced analgesia (Box 1).

The initial experiments by Bohn *et al.* [10,38,40,41] were performed on β-arrestin2-knockout animals with a mixed strain background, the colony being formed from the interbreeding of knockout **129/SvJ** male mice with wild-type **C57BL/6** females. Inbred laboratory mouse strains vary in nociceptive responses, and a heightened antinociceptive effect of morphine in 129/SvJ, compared with C57BL/6, mice has been demonstrated and specifically noted to be relevant to transgenic experiments on mixed background animals [83]. An absence of tolerance to morphine antinociception in a related 129 mouse substrain has also been shown in multiple studies [84]. In terms of side effects, a recent study showed that the sensitivity of 129 substrain animals to lethal morphine respiratory depression was lower than that of C57BL/6 mice [85]. This evidence of strain variation in opioid response suggests that the dramatic β-arrestin2-knockout phenotype initially observed was substantially confounded by parental 129/SvJ strain characteristics segregating with the knockout locus.

Therefore, it is crucial for the G protein-biased MOPr agonist proposal that these experiments are repeated on knockout animals without a mixed strain background. Indeed, more recent work has observed minimal effects of β-arrestin2 knockout on opioid responses in mice congenic with the C57BL/6J strain. Neither acute antinociception in response to morphine, oxycodone, or fentanyl, nor the subsequent development of tolerance to these drugs following prolonged administration, was observed to be altered by β-arrestin2 knockout [42]. Similarly, experiments in multiple independent laboratories have failed to observe any effect of the β-arrestin2-knockout genotype on morphine- or fentanyl-induced respiratory depression or constipation [22,26]. Studies of withdrawal signs and dependence in the β-arrestin2-knockout mouse on a C57BL/6J background have not yet been reported. The observed β-arrestin2-knockout phenotype of enhanced morphine antinociception, alongside reduced tolerance and profoundly attenuated side effects, led directly to the formation of the G protein-biased MOPr agonist hypothesis, but has not yet been reproduced.

arrestin recruitment [55,57]. Many MOPr agonists proposed to be biased, including newly developed leads [58], as well as buprenorphine [59,60], levorphanol [61], and desmetramadol [62], have low intrinsic efficacy relative to morphine (Figure 2A). To reiterate, the observed profile of minimal β-arrestin recruitment, but robust G protein activation, is predictable from an understanding of assay amplification, and is entirely consistent with multiple apparently G protein-biased MOPr agonists having simply low intrinsic efficacy instead [51].

One solution to this confound is to compare assays with similar amplification levels. This remains challenging but has recently been facilitated by the development of conformationally selective nanobodies [63] and soluble miniature G proteins [64] that can be used to report receptor activation and G protein recruitment, respectively. This allows the direct comparison of agonist-induced binding of G proteins or β-arrestin to MOPr (Figure 2B). Studies using these unamplified probes of MOPr activation report the spectrum of agonist efficacy, confirming the partial agonism of lead compounds oliceridine and PZM21 [51–53] (Figure 2C). Highly amplified systems can alternatively be adjusted through partial receptor inactivation with irreversible antagonists, reducing agonist maximal effect to well below the ceiling of the assay and, therefore, permitting the comparison of relative efficacy [54]. Efficacy as estimated through either recruitment of MOPr activation sensors or partial irreversible antagonism in a GIRK activation assay was consistent across a

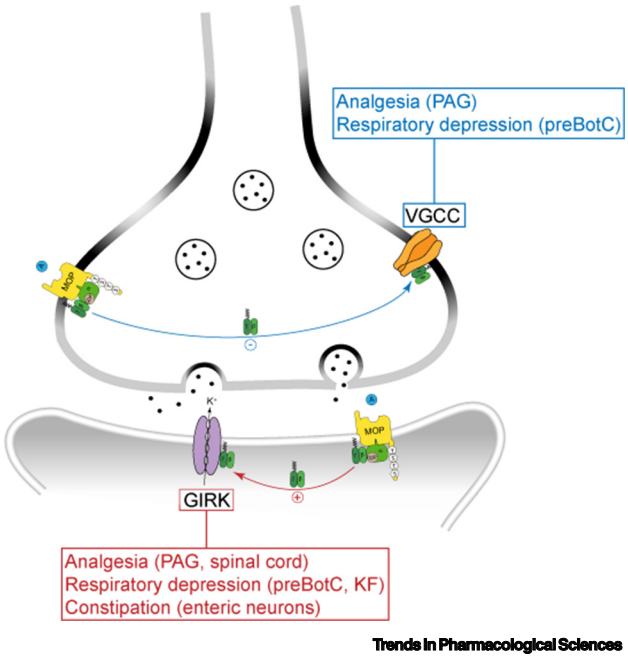

Figure 1. Illustration of G Protein Mediated Signaling after MOPr Activation. Illustration of G protein-mediated interaction after MOPr activation. Presynaptic inhibition of voltage-gated calcium channels (VGCC) via Gβγ. Postsynaptic activation of G protein coupled inwardly rectifying potassium channel (GIRK) by Gβγ. Analgesia and side effects are mediated by G protein signaling. Abbreviations: KF, Kölliker-Fuse; PAG, periaqueductal gray; preBötC, preBötzinger neurons.

family of MOPr agonists [51]. It is also important to consider that β-arrestin recruitment assays can have a limited dynamic range resulting in a 'floor' effect, wherein ligands below a certain efficacy do not give a detectable response. This can be addressed by designing experimental conditions to increase amplification, for example, by overexpression of GRK, such that all agonists produce a quantifiable response (Figure 2D) [51,65]. In assays in which these amplification confounds have been addressed, lead compounds, such as oliceridine and PZM21, as well as buprenorphine, are not observed to be significantly G protein biased [48,51,53] (Figure 2E,F), demonstrating that previous descriptions of biased agonism have been largely driven by system parameters rather than by novel ligand characteristics.

Given the role of receptor phosphorylation in the development of tolerance to opioid antinociception [23], and the limitations of β-arrestin-recruitment assays, additional descriptors for novel ligands are needed, such as phosphorylation site analysis. GRK recruitment to the active MOPr and subsequent phosphorylation are the usual prerequisites for β-arrestin binding [24]. The pattern of phosphorylation induced by agonist treatment varies between high-efficacy agonists, such as DAMGO, which induce phosphorylation at multiple sites, and lower efficacy agonists, such as morphine [66], which have a more restricted phosphorylation pattern [24]. Generally, putatively biased agonists with low efficacy for G protein activation follow this pattern, with the exception of SR17018, which, surprisingly, has been shown to induce DAMGO-like multisite phosphorylation [51].

The observations outlined earlier should prompt a re-evaluation of the pharmacological characteristics of proposed biased opioids. At present, experimental methods have been inconsistent between studies, making a systematic evaluation of efficacy and, therefore, bias, challenging. Accurate, clear quantification of intrinsic efficacy is necessary to enable robust comparison of agonists and any potential bias. Empirical measurements of ligand activity, such as agonist

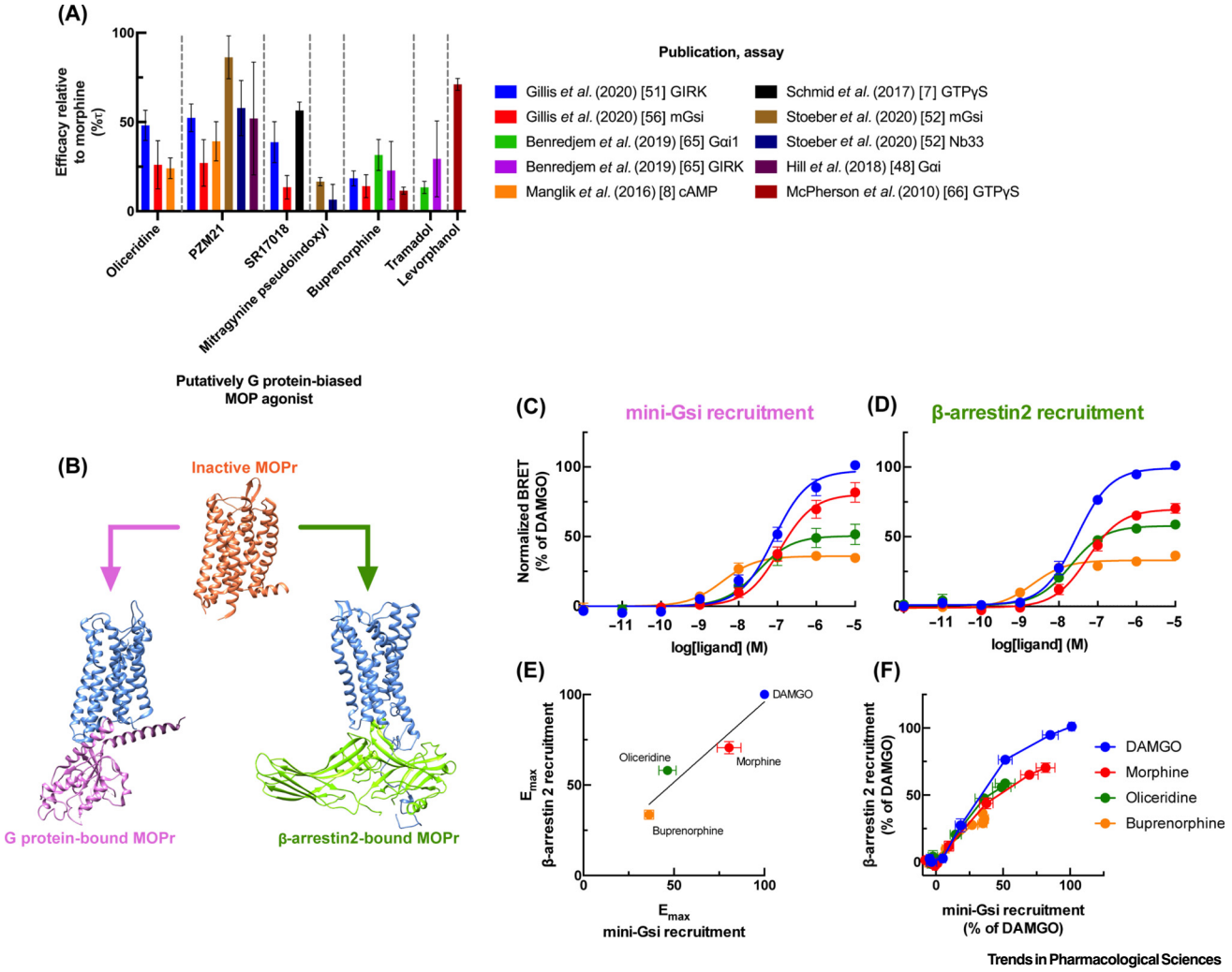

Figure 2. G Protein-biased Agonists have Low Intrinsic Efficacy. (A) Low intrinsic efficacy of putatively biased μ-opioid receptor (MOPr) agonists, reanalyzed from published data as previously described [51]. Y-axis shows operational efficacy, τ, of each agonist as a percentage of morphine efficacy within that assay. (B) Comparison of G protein to β-arrestin2 binding is a robust test of potential G protein-biased MOPr agonism. This was recently facilitated by the development of soluble, 'miniature' G proteins (mini-Gsi), which can be used to assay MOPr activation (C), and then compared with β-arrestin2 recruitment (D). Maximum effect (E) and potency (F) of these agonists is consistent between the two assays, which have comparable levels of amplification. See [8,48,51,52,56,65,66]. Data on mini-Gsi and β-arrestin2 recruitment reproduced from [51]. All data shown as mean, with bars representing standard error of the mean.

maximal effect and potency, allow quantification without making mechanistic assumptions about the receptor system [56,67]. Maximal effect and potency can be directly compared between assays (Figure 2E,F). Bias plots of ligand activity between two assays use untransformed data to highlight differences in system amplification and show the extent to which agonists may vary in activity across signaling pathways [2]. The combination of these simple analyses with assays optimized to avoid systematic confounds allows assessment of potential agonist bias in a rigorous manner (Figure 2E,F and Box 3). Deviations from the profile of a family of reference compounds should then reveal potential biased agonism.

The fact that most putative G protein-biased MOPr ligands described to date have been low efficacy agonists (Figure 2A), together with challenges to the 'β-arrestin2 hypothesis', brings

Box 3. Designing Assays for Evaluation of Opioid Ligands

Several factors should be accounted for in the systematic design of signaling assays to capture the full range of MOPr agonist efficacy. Consideration of these factors should allow analysis of potential ligand bias in a manner not confounded by system amplification or kinetics of effect.

Reference Ligands

The consistent use of multiple reference ligands that span a large efficacy range is a helpful strategy that facilitates the detection of ceiling (amplification) and floor (underamplification) effect confounds [56]. High-efficacy agonists DAMGO or met-enkephalin will define the maximum possible effect in most assays. The partial agonist morphine is an essential reference that allows for the detection of system confounds. If possible, another agonist known to be low efficacy, such as oliceridine, should also be used. If the maximum effect reached by morphine and/or oliceridine is similar to DAMGO, the amplification of the assay is very high and due to receptor reserve, will not be sensitive even to dramatic efficacy differences. Similarly, if the low efficacy agonists do not produce substantial, measurable effects that can be fitted to a curve, underamplification will confound any estimate of relative efficacy, potency, or agonist bias.

Probe Ligands

Considerations such as the active concentration range and whether maximal effect concentrations can be reached within the solubility range are key for an accurate description of the pharmacology of a ligand. If practical, half-log unit increments in concentration are recommended to obtain more accurate potency estimates. This is particularly important for low-efficacy agonists that give weak responses in poorly coupled assays.

Assay Amplification

It is important to assess each assay for relative levels of system amplification. This can be done using reference compounds with known low intrinsic efficacy as suggested earlier, for example, morphine and oliceridine. When a possible ceiling effect is observed, this can be controlled by adjusting relative expression of receptor and/or reporter, or considering irreversible antagonism/receptor inactivation. Underamplification can be adjusted by ensuring all probe agonists reach significant effect compared with vehicle, such that maximal effect and potency estimates can be made. Again, modification of receptor and reporter expression and consideration of 'bottlenecks', such as low GRK expression in β-arrestin-recruitment assays, will ensure optimized assay conditions.

Assay and Ligand-Binding Kinetics

Given that divergent kinetics of effect can alter the apparent bias profile [86], it is important to make real-time measurements to ensure a maximum effect is being captured for all agonists. When real-time measurements are not possible, variations in agonist-incubation time-courses are recommended. Additionally, it is important to consider how agonist efficacy and ligand binding kinetics may interact to alter apparent potency and maximal effect.

Analysis

A consistent analysis workflow is important for reproducible pharmacological descriptions of ligands: (i) fit simple concentration–response curves to the mean of replicates within each experimental day. If the curve is atypical (e.g., steep or two-phase) conduct robust statistical test comparing fits, for instance an extra sum-of-squares test, to check if more complex model is preferred; (ii) average maximal effect and potency from each separate experiment. Compare rank order across assays, for instance via simple scatter plot. Inspect for outliers compared with reference ligands; (iii) construct bias plots (showing the relative efficacy of a given concentration of a drug in two different assays) to visualize both assay amplification differences (skewing all curves toward one assay) and divergent agonists (potential biased agonism); and (iv) if desired, maximal effect can be converted to operational efficacy directly (E_{max} of test/E_{max} of reference = $\tau/[1+\tau]$) when assay overamplification is not a confound.

Identification of Biased Agonists

Biased agonists will diverge from a family of reference compounds, in terms of potency, as shown in a bias plot or rank order of maximal effect. The magnitude of biased agonism should be considered alongside its presence or absence, for instance via the widely used transduction coefficient, or by subtraction of maximal effect.

into question to what extent the profile of the ligands investigated (Box 1) can be explained by partial agonism rather than by ligand bias. While agonists proposed to be biased certainly produce significant respiratory depression (Box 1), there may be marginal improvements in the **therapeutic window** arising from the low efficacy of these compounds [51]. The existing opioid analgesic buprenorphine, which has extremely low intrinsic efficacy, has been noted to have a plateau of effect on respiration [68], contributing to reduced overdose risk. A recent study of

clinically used opioids found that intrinsic efficacy, rather than any G protein/β-arrestin bias, predicted the rate of reported adverse events [65]. This suggests a route toward opioid analgesics with reduced respiratory burden, and is a plausible counter explanation for the slightly improved profile of some apparently biased MOPr agonists. The reduced efficacy of oliceridine and PZM21, while not as low as that of buprenorphine (Figure 2A,E) [51], might explain the observed preclinical and clinical profile of these ligands. Additional studies are required to connect specific signaling profiles to behavioral effects. Recent work has applied fixed agonist:antagonist ratios to determine the efficacy requirements of *in vivo* opioid agonist effects [69,70]. Using this methodology may resolve the contribution of *in vitro* signaling profiles, alongside ligand pharmacokinetics, bias, and off-target interactions, to MOPr agonist behavioral effects.

Several studies have observed some MOPr agonists to be substantially biased when amplification confounds are addressed. Notably, the peptide endomorphin 2 has been described as β-arrestin-biased following characterization of efficacy using irreversible antagonism, with comparable β-arrestin recruitment to Met-enkephalin but lesser efficacy for G protein activation [71,72]. Another peptide, bilorphin, displays reduced β-arrestin2 recruitment but similar G protein efficacy to morphine, again as described using irreversible antagonism [71]. However, testing the physiological effect of biased MOPr agonism in animal models will require development of brain-penetrant agonists consistently and rigorously described to retain their bias toward or away from β-arrestin recruitment.

Concluding Remarks

G protein-biased agonists of MOPr have been widely asserted to constitute a novel and substantially improved class of analgesics. The hypothesis underlying this, being that MOPr coupling to β-arrestin2 mediates deleterious opioid effects, including respiratory depression, constipation, dependence, and withdrawal, has been recently challenged. First, a recent study was unable to repeat initial results in the β-arrestin2-knockout mice. Second, a genetic model of reduced MOPr/β-arrestin interactions, the 11S/T-A mouse, does not support a role of β-arrestin recruitment in mediating or facilitating these effects. Third, physiological studies show the manner in which well-established MOPr G protein-signaling mechanisms cause analgesia, respiratory depression, and constipation (Figure 1). As such, current evidence does not support the proposal that the absence or reduction of β-arrestin2 recruitment to the MOPr by drug candidates (i.e., G protein-biased agonism) would improve the *in vivo* profile of opioid analgesics (Box 1 and Figure 1)

Furthermore, re-evaluation of lead compounds that have been proposed to be G protein biased has led to the recognition of the low intrinsic efficacy of these compounds relative to morphine (Figure 2A). This factor, together with amplification confounds, has led to activity typical of partial agonists being described as apparent biased agonism away from β-arrestin recruitment. Very low efficacy MOPr agonists continue to be proposed to be G protein-biased (e.g., [58]) from confounded assay results. The extent to which these newly developed ligands, such as the drug candidate oliceridine, constitute substantially G protein-biased MOPr agonists is under question. Certainly, it appears that no MOPr agonist has yet been identified with high, DAMGO-like G protein efficacy and substantially less β-arrestin recruitment than would be predicted from that efficacy.

The measurement of β-arrestin recruitment to the MOPr in model cell systems has proven to be robust and amenable to high-throughput screening. However, the identification of β-arrestin-dependent MOPr-signaling pathways in neurons and their role in controlling the physiological effects of opioids is still necessary to understand the potential impact of biased MOPr agonism. The most widely proposed β-arrestin-dependent MOPr signal is activation of ERK/MAPK, but this is at least partly also G protein dependent [73]. The concept of β-arrestin-dependent

Outstanding Questions

Are MOPr agonists biased between different G protein subtypes or effectors downstream of G protein activation?

Are MOPr agonists biased between the engagement of different GRKs and/or PKC isoforms?

Are there other unappreciated signaling events that are differentially engaged upon MOPr activation by different agonists?

How does the recruitment of β-arrestins modulate MOPr activity *in vivo*?

How do the multiple systems of MOPr regulation (GRKs, β-arrestins, PKC isoforms, and internalization machinery) interact in the development of tolerance and dependence?

How does tolerance development to distinct opioid-mediated physiological effects, such as analgesia and respiratory depression, differ depending on the opioid agonist?

Can biased agonists differentially induce tolerance between two different physiological opioid effects?

Do β-arrestins mediate a yet to be discovered G protein-independent signal in nervous tissue with a physiological outcome?

Does extremely low agonist efficacy consistently reduce opioid-induced respiratory depression? To what extent is it possible to improve MOPr agonist analgesics via manipulation of intrinsic efficacy?

Might there be a MOPr agonist with high, DAMGO-like G protein efficacy and significantly less β-arrestin recruitment than would be predicted from that efficacy?

What are the efficacy requirements for the various responses to opioids in different tissues?

What is the effect of species difference on opioid signaling and behavior?

signaling has recently been challenged at related GPCRs via genetic deletion of G proteins [74,75]. A specific physiological effect of MOPr-signaling events dependent on β-arrestin recruitment has not, to date, been shown in relevant tissue. Future studies linking *in vitro* signaling events to *in vivo* behavior would benefit from physiological experiments across species, including rodents and non-human primates, because MOPr agonist effects, including antinociception and lethality, vary substantially from the most common model species, mice, to other rodents and to primates.

In regard to the theoretical effect of an extremely G protein-biased opioid ligand, while acute side effects and withdrawal are not predicted to be reduced, tolerance to opioid antinociceptive effect may be altered. The extent to which agonists can be designed that avoid C-terminal phosphorylation remains to be seen, as does how additional systems might interact with the primary desensitization pathway in this case. Multiple interacting pathways contribute to tolerance to opioid antinociception. Tolerance to opioid effects, such as respiratory depression and constipation, develops via similar systems, although how tolerance to opioid effects may differ for G protein or β-arrestin-biased agonists has not yet been resolved (see Outstanding Questions).

Recent data have challenged the relationship between MOPr/β-arrestin recruitment and opioid-induced side effects. We encourage a critical re-evaluation of descriptions of biased agonism at the MOPr that takes into consideration the confounding factors that have led to confusion in the opioid field. With the toolbox of opioid agonists and assays continuing to expand, there is a clear opportunity to understand the pharmacological and signaling characteristics that dictate the physiological effects of these opioid ligands, with a view to improved analgesics (see Outstanding Questions). However, this will only be possible through systematic and consistent characterizations of novel compounds.

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