Special Section on 50 Years of Opioid Research – Minireview

Intrinsic Efficacy of Opioid Ligands and Its Importance for Apparent Bias, Operational Analysis, and Therapeutic Window [S]

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ABSTRACT

Evidence from several novel opioid agonists and knockout animals suggests that improved opioid therapeutic window, notably for analgesia versus respiratory depression, is a result of ligand bias downstream of activation of the μ-opioid receptor (MOR) toward G protein signaling and away from other pathways, such as arrestin recruitment. Here, we argue that published claims of opioid bias based on application of the operational model of agonism are frequently confounded by failure to consider the assumptions of the model. These include failure to account for intrinsic efficacy and ceiling effects in different pathways, distortions introduced by analysis of amplified (G protein) versus linear (arrestin) signaling mechanisms, and nonequilibrium effects in a dynamic signaling cascade. We show on both theoretical and experimental grounds that reduced intrinsic efficacy that is unbiased across different downstream pathways, when analyzed without due considerations, does produce apparent but erroneous MOR ligand bias toward G protein signaling, and the weaker the G protein partial agonism is the greater the apparent bias. Experimentally, such apparently G protein–biased opioids have been shown to exhibit low intrinsic efficacy for G protein signaling when ceiling effects are

properly accounted for. Nevertheless, such agonists do display an improved therapeutic window for analgesia versus respiratory depression. Reduced intrinsic efficacy for G proteins rather than any supposed G protein bias provides a more plausible, sufficient explanation for the improved safety. Moreover, genetic models of G protein–biased opioid receptors and replication of previous knockout experiments suggest that reduced or abolished arrestin recruitment does not improve therapeutic window for MOR-induced analgesia versus respiratory depression.

SIGNIFICANCE STATEMENT

Efforts to improve safety of μ-opioid analgesics have focused on agonists that show signaling bias for the G protein pathway versus other signaling pathways. This review provides theoretical and experimental evidence showing that failure to consider the assumptions of the operational model can lead to large distortions and overestimation of actual bias. We show that low intrinsic efficacy is a major determinant of these distortions, and pursuit of appropriately reduced intrinsic efficacy should guide development of safer opioids.

Introduction

Ligand bias, variously called functional selectivity or ligand-directed signaling, refers to the now-widespread concept in the pharmacology of G protein–coupled receptors (GPCRs) of differential engagement of downstream signaling pathways. The ability of different ligands to promote a distinct signaling complement while acting at the same receptor has long been proposed as an avenue for the development of therapeutics (Luttrell et al., 2015). Accurate and critical assessment of the biological profiles of GPCR ligands is necessary to ascertain the presence and significance of true agonist bias in a given

GPCR system (Michel and Charlton, 2018). We discuss here how conclusions regarding ligand bias can be severely impacted by fundamental factors of experimental design and pharmacological analysis, which have substantial implications for G protein bias at the opioid receptor and the GPCR pharmacology field more widely.

The μ-opioid receptor (MOR), the primary target of opioid analgesics (Williams et al., 2013), is a GPCR system in which agonist bias has been extensively explored (Siuda et al., 2017). Existing clinical agonists of the MOR, in addition to being strongly analgesic, are addictive and produce an array of serious side effects, including respiratory depression, sedation, and constipation. Landmark studies of the β-arrestin 2 (also referred to as arrestin-3) knockout mouse found almost abolished respiratory depression and constipation in response to morphine while also observing enhanced acute analgesia

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ABBREVIATIONS: DAMGO, [D-Ala²; *N*-MePhe⁴, Gly-ol]-enkephalin; E_m, system maximal effect; GIRK, G protein–coupled inwardly-rectifying potassium; GPCR, G protein–coupled receptor; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; MOR, μ-opioid receptor.

[Bohn et al., 1999; Raehal et al., 2005; but see Kliewer et al., 2020]. β-Arrestin 2 is a member of the GPCR-binding arrestin family of regulatory proteins. In addition to the eponymous, desensitizing function of occluding G protein coupling, the arrestin family has been proposed to transduce signals from GPCRs independently of G proteins (Luttrell and Gesty-Palmer, 2010).

The β-arrestin 2 knockout phenotype led to the proposal of pursuing reduced arrestin recruitment via G protein–biased agonism as a strategy to reduce on-target side effects of opioid analgesics (Violin et al., 2014). The underlying assumption is that such biased agonists, by not recruiting arrestin to the receptor, would not engage a putative G protein–independent, arrestin-signaling pathway thought to mediate side effects (Siuda et al., 2017) while preserving the MOR G protein signal. G protein signaling of the MOR is well-established to be analgesic via neurons in the spinal cord, brainstem, and brain (Stein, 2016). An alternative proposed mechanism to the hypothesized arrestin-dependent signal was differential arrestin regulation or facilitation of G protein signaling between brain regions (Raehal et al., 2011).

Agonists of the MOR proposed to be G protein–biased have been identified and developed by use of concentration-response data largely from heterologous cell lines expressing MOR (DeWire et al., 2013; Manglik et al., 2016; Schmid et al., 2017), and the calculated bias has been interpreted as the basis for improved safety. That is, the quantified degree of ligand bias is compared directly with in vivo ligand profiles. MOR agonist bias is assessed almost uniformly by comparison of the level of G protein activation to that of β-arrestin 2 recruitment to the MOR, with arrestin recruitment acting as a proxy for the hypothetical G protein–independent signal. This review provides a critical analysis of the assumptions and experimental pitfalls of this approach and suggests that opioid ligand bias may not be the primary determinant of the observed improved safety in preclinical and clinical data for supposedly G protein–biased agonists. The issues we highlight regarding ligand bias at the MOR are somewhat generalizable to the many other GPCRs in which biased agonism is actively being explored for therapeutic benefit.

Operational analysis has become the de facto standard for interpretation of concentration-response data in GPCR pharmacology (Kenakin et al., 2012). The use of this model was historically due to its theoretical ability to separate functional affinity, K_A, from intrinsic efficacy, denoted τ (Black and Leff, 1983). The application and importance of these parameters in opioid pharmacology have been reviewed previously (Kelly, 2013). However, the standard analysis of opioid bias in the literature, and indeed GPCR pharmacology more generally, employs a "transduction coefficient," τ/K_A (Kenakin and Christopoulos, 2013). This parameter is compared between agonists and across signal pathways to quantify potentially interesting or novel characteristics in signaling profile—for instance, reduced or absent β-arrestin 2 recruitment compared with G protein signaling. The proposed advantage of this model is the cancellation of system-dependent effects due to internal normalization to a reference agonist (Kenakin and Christopoulos, 2013). We argue here on both theoretical and experimental grounds that this calculation is susceptible to systematic error in dynamic nonequilibrium systems and amplification differences between the two signaling pathways. As a result, errors in bias quantification may lead to errors in correlating signaling characteristics to in vivo outcomes.

Assumptions of the Operational Model

The operational model, especially as it pertains to biased agonism, has been extensively reviewed elsewhere (Kenakin, 2017). What follows is a brief overview of its derivation, as presented in Black et al. (2010), to highlight key implicit and explicit assumptions. The model is intended to relate agonist concentration, [A], to the effect produced, E, in a cellular receptor system in a mechanistic manner (Finlay et al., 2020). The effect in the system is expressed as a fraction from a basal level to a system maximum, E_m. The general reaction scheme is described by:

$$[A] + [R] \leftrightarrow [AR] \leftrightarrow [AR^*] \rightarrow E$$

in which the agonist, A, binds to receptor, R, and the resulting agonist-receptor complex, AR, isomerizes to an active state, AR^*, that produces an effect.

The first step, agonist binding to receptor, is considered to behave according the law of mass action. This step is modeled as a Hill-Langmuir system and assumes an equilibrium between the free agonist and the agonist bound to a homogenous pool of available receptors. It produces a hyperbolic relationship (eq. 1) that saturates as the agonist concentration increases to occupy the total available receptor, R_0. The "Langmuirian" affinity K_A is a measure of the attraction between the agonist and its target.

$$[AR] = \frac{[R_0][A]K_A}{1 + [A]K_A} \qquad (1)$$

The second step, the transduction of an effect by the agonist-receptor complex, AR, is also assumed to operate as an equilibrium, hyperbolic system to explain the saturation of the effect (eq. 2). As the concentration of agonist-receptor complex becomes large, the effect is assumed to approach some maximal effect, E_m. This maximal effect is the limiting full response that can possibly be produced by that receptor and is intrinsic to the system. K_E is a model parameter that quantifies the ability of the agonist-receptor complex to produce an effect. Agonist intrinsic efficacy is modeled as the efficiency of the AR complex to produce an active state, AR^*.

$$\frac{E}{E_m} = \frac{[AR]}{K_E + [AR]} \qquad (2)$$

Equation 2 is the "operating" point of the operational model. Note that it does not address the production of a distinct "ternary" complex, that is, the binding of signaling partner proteins to the agonist-receptor complex to produce an effect. The necessity of this ternary-complex formation is clear for GPCR signaling, including G protein or arrestin coupling. The impact of an operating ternary complex, rather than the assumed operating binary complex, has been previously considered. Although the rank order of affinity and efficacy of a simple ternary-complex system estimated via operational analysis is likely accurate, it was demonstrated that absolute or relative values are not estimated well (Leff and Harper, 1989). This is due to the underlying signaling reaction being dependent on ternary partner concentrations and therefore
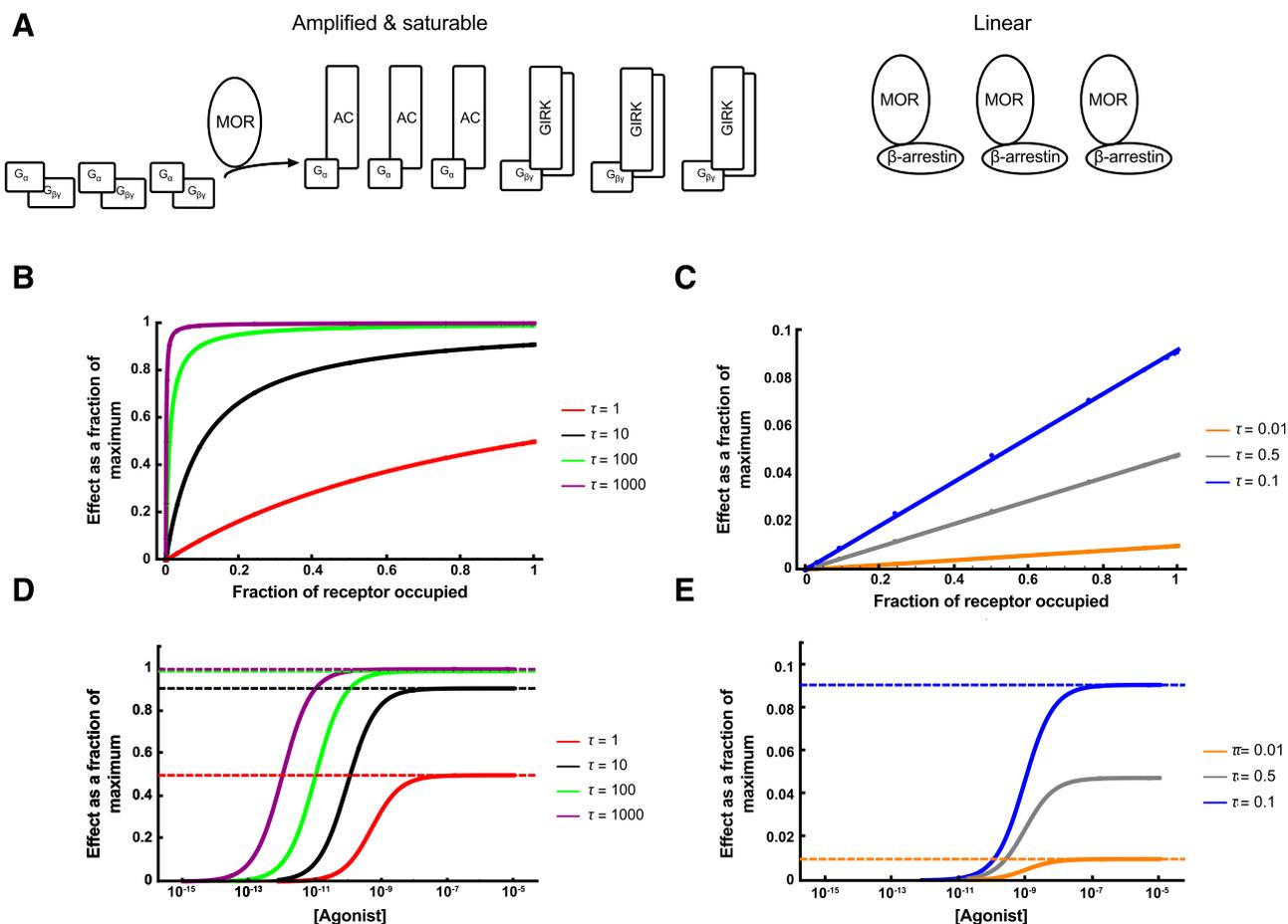
Fig. 1. (A) GPCR signaling is amplified via the production of multiple active G proteins from a single receptor. Effect can reach a maximum because of limited effector—for example, adenyl cyclase (AC) or ion channels (GIRK) completely activated/inhibited—or by a relative excess of receptor. Alternatively, linear systems, such as arrestin recruitment, have no amplification, with each receptor binding a single arrestin molecule. (B) The operational model typically assumes a hyperbolic relationship between the fraction of receptor occupied and effect. Most agonists in a highly amplified system reach close to a system maximal effect due to saturation of downstream effector proteins. (C) The operational model can be extended to a linear relationship between fractional occupancy and effect by considering a system with extremely low efficacy. In this case, no agonist reaches close to the true system maximum. Systems with $\tau \leq 0.1$ have approximately linear occupancy-response relationships. (D) In highly amplified systems, large differences in agonist efficacy (for example, between $\tau = 10$ and $\tau = 1000$) are not directly observable in changes to agonist maximal effect, α, because of the ceiling of the assay. (E) In contrast, in a linear system any difference in agonist efficacy is directly observable in agonist maximal effect. It is also notable that a linear system appears as a sigmoidal curve indistinguishable from hyperbolic systems.

second-order and not accurately modeled by this approach. This will lead to systematic error when operational parameters, K_A and τ, are to be compared between systems with different levels of ternary signaling proteins, for instance between G protein and arrestin signaling. Besides, cell types differ substantially in the level and complement of signaling partners expressed, including G proteins and GPCR kinases. Thus, the estimates of bias based on operational analysis are neither absolute nor independent of the measurement system.

The first equation can be substituted into the second, producing eq. 3:

$$\frac{E}{E_m} = \frac{\tau[A]}{K_A + [A](1 + \tau)} \qquad (3)$$

in which $\tau = \frac{R_0}{K_E}$.

The Greek tau, τ, is a measure of agonist efficacy and depends on the level of receptor in the system, R_0, as well as the ability of the agonist-receptor complex to transduce an effect, K_E. The resulting equation can be used to fit experimentally determined agonist concentration-response curves producing efficacy and affinity estimates and has been very widely employed in GPCR pharmacology.

The asymptote of an agonist's concentration-response system curve, α, is the agonist maximal effect that is efficacy-dependent. The effect produced by a full agonist reaches the system maximum and $\alpha \sim E_m$ but in the case of partial agonism, $\alpha < E_{max}$. The τ term incorporates R_0, such that "full" or "partial" agonism is therefore dependent upon receptor level. Application of the model assumes that a reference agonist reaches the system maximal effect, and it can therefore be accurately estimated. This may not be possible, especially in cases of very low system amplification (Fig. 1E).

It is also assumed in the derivation of this model that the two processes are independent, that is, that agonist activation of the receptor does not alter agonist binding and vice versa. This assumption does not hold, as the active state of the MOR (Huang et al., 2015) and other GPCRs (Lebon et al., 2012) has distinct agonist affinity and kinetics from the inactive state.

Black and Leff (1983) note that "plainly, evidence for such cooperativity prohibits the use of any of the present models as the primary assumption in all of them is a non-cooperative bimolecular interaction between agonist and receptor". Additionally, the cooperative nature of the binding of G proteins and other effectors to GPCRs (Christopoulos and Kenakin, 2002) constitutes a complex affinity-efficacy interaction. This gives rise to the concept of "functional affinity," reflecting the observed K_A term as a measure of observed agonist affinity for the active receptor (Kenakin and Christopoulos, 2013). This functional affinity produced by the application of operational analysis does not accurately estimate specific affinity for any one receptor state because of the violated assumptions of the model. The invalidation of the independence of efficacy and affinity results in inaccuracy in both K_A and τ, and therefore $\Delta\Delta\log(\tau/K_A)$, estimates.

Applicability to G Protein/Arrestin Bias at a Prototypical GPCR

Considering the model system of G protein/arrestin bias at the MOR as a clinically relevant example of active biased agonist development, we sought to investigate how pharmacological quantification could be confounded by invalid assumptions.

G protein–biased MOR ligands have been proposed to be a new class of drastically improved analgesics (Siuda et al., 2017), and one such compound is in late clinical trials (Soergel et al., 2014; Bergese et al., 2019). A number of confounding factors have not been thoroughly addressed in assessing the profile of such potentially biased compounds. Whether these compounds represent a novel signaling mechanism, as has been claimed, is wholly dependent on pharmacological quantification. The role of intrinsic efficacy, as distinct from affinity, has not been systematically considered and may contribute to both observations of bias at the MOR and to the in vivo effects of these compounds. In addition, assumptions made in operational analysis of G protein/arrestin systems may not hold, as has been previously expressed (Stott et al., 2016). Given recent criticism of the approach to biased opioid agonism (Conibear and Kelly, 2019), we here identify critical points of confounded analysis. We discuss here that the varying success of putatively G protein–biased MOR agonists in progressing from in vitro assays to preclinical and clinical testing is likely based, at least in part, on failure to fully appreciate these complications of bias quantification.

Assays of GPCR signaling are inherently limited by a dynamic range of observeable effect. This may introduce systematic error in the analysis of potential ligand bias. Here, we consider how assays of excessively amplified G protein signaling or low-amplification linear protein-protein interactions alter the quantification of agonist activity. We consider the impact of analyzing a nonequilibrium dynamic system with equilibrium models and recommend simplified analysis to reduce procedural error and eliminate the attribution of erroneous measured values to mechanistic parameters.

Efficacy Estimates in Common G Protein Assays Are Confounded by Ceiling Effects. Despite the intended purpose of the design of the operational model to explicitly account for distinct efficacy and affinity parameters, it is practically applied in the biased signaling literature as the combined signaling coefficient τ/K_A (Kenakin et al., 2012). Many studies describing novel MOR ligands do not quantify G protein efficacy explicitly. Notably, the most thorough study of efficacy for MOR G protein signaling, arrestin recruitment, and internalization found a strong correlation between efficacy in all assays for a family of agonists (McPherson et al., 2010). There were few exceptions to the robust prediction of arrestin-recruitment efficacy by G protein efficacy, a remarkable observation considering the chemical diversity of ligands studied. This suggests the active receptor state produced by most agonists equally couples to the signal pathways studied. Recent work has observed that agonist efficiency for recruitment of an active-state selective nanobody to the MOR predicts activity in all studied signaling pathways, again suggesting that most agonists produce a similar receptor "active state" (Gillis et al., 2020).

A common confound preventing straightforward efficacy assessment is the presence of significant receptor reserve, wherein agonists produce a full response while occupying only a small fraction of available receptor (Kelly, 2013). As discussed below, the G protein system acts as an amplifier with a large gain under most circumstances, such that a single activated receptor can produce multiple activated $G_\alpha/G_{\beta\gamma}$ protein subunits, each one of which can activate or inhibit enzymes or ion channel effectors (Fig. 1A). This signaling cascade, together with a limited effector pool relative to receptors, accounts for the amplification and saturation of effect and produces a hyperbolic relationship between occupied receptor and effect. G protein assays used in assessment of MOR agonists typically measure outputs from an enzyme or channel after the GPCR in question is heterologously expressed in immortalized cell lines. Because of high receptor expression levels, heterologous assays commonly have a ceiling of effect, in which most agonists reach a similar maximal response regardless of their efficacy (e.g., see the cAMP assays of Nickolls *et al.*, 2011; Thompson *et al.*, 2015; Schmid *et al.*, 2017; Gillis *et al.*, 2020). This upper limit of response is intrinsic to the experimental system.

In a circumstance wherein all agonists reach a similar maximal effect, unless an externally measured affinity is assumed to apply to the assay, as in the case of McPherson et al. (2010), it is impossible to determine relative efficacy of the agonists in question (Fig. 1D). Application of externally derived receptor-binding affinities, for example from radioligand competition, to a functional assay has been argued to be inappropriate because of the known distinct conformational receptor states (Kenakin, 2014). That is, a given agonist's affinity for the inactive state captured by a binding assay and an active state in a functional assay are not consistent. Even in cases in which test agonists do not completely reach the assay ceiling, operating close to a known ceiling reduces the sensitivity of efficacy estimates. Small errors in estimates of concentration-response curve asymptote, α, at high amplification produce large errors in operational efficacy estimates because of the hyperbolic relationship between these parameters, as observed with simple Monte-Carlo simulation in Fig. 4E.

Guanosine 5′-3-O-(thio)triphosphate (GTPγS) accumulation assays are often presumed to be a direct measure of receptor production of active G_α, and so have been assumed to have no ceiling because of an excess of effector (Ehlert, 2008). However, data presented in a rigorous study by Nickolls et al.
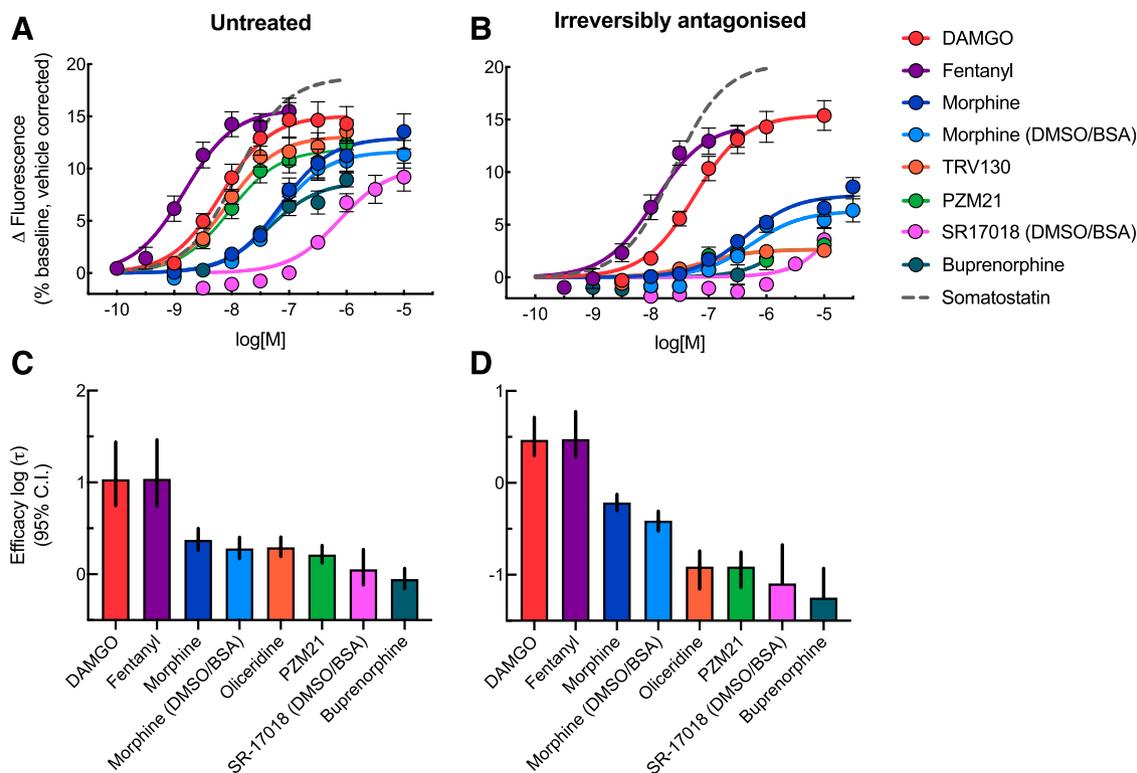
Fig. 2. (A) Concentration-response data for activation of GIRK in AtT20 cells after heterologous expression of MOR for a family of opioid agonists. Mean and S.E. of the mean of six to eight separate experiments performed in duplicate. Note that because of solubility constraints, SR-17018 was assayed in a DMSO + bovine serum albumin vehicle. (B) Data of the same experiment performed in parallel after preincubation with irreversible MOR antagonist β-chlornaltrexamine to reduce receptor reserve. Note that the maximal response of the system is defined by somatostatin acting through a different receptor on the same GIRK channels. (C) Efficacy with 95% confidence intervals as estimated before receptor reduction, with affinity estimated by fitting untreated and β– chlornaltrexamine-treated curves together. Fentanyl and DAMGO have higher efficacy than the other compounds. (D) Efficacy as estimated after irreversible antagonism. Rank order is conserved, but efficacy estimates can be made with superior confidence. Putatively biased agonists oliceridine, PZM21, and SR17018 are lower efficacy than prototypical morphine, similar to the uniquely low-efficacy analgesic buprenorphine.

(2011) contradict this assumption. The authors examined the effect of reduction in available receptor by irreversible antagonism on several common MOR functional assays. All test compounds in the author's MOR GTPγS accumulation test system reached an equal maximal effect before reduction of receptor reserve, after which differences in efficacy were clearly discernable. This is in line with a hyperbolic, saturated effect curve, as assumed by the operational model, and consistent with an assay ceiling that confounds analysis. As such, GTPγS accumulation assays may not capture low relative efficacy despite assumptions to the contrary unless performed in nonsaturating conditions (Stott et al., 2016). In short, signal windows limited by high amplification occur in a variety of functional assays of G protein activity. As a consequence, true relative efficacy of MOR agonists will not be observed in assays limited in such a way. As discussed below, G protein–receptor proximity assays provide a more direct measure of receptor activation.

A further confound is that descriptions of G protein–biased MOR agonists often do not specifically quantify efficacy relative to existing agonists (DeWire et al., 2013; Manglik et al., 2016; Schmid et al., 2017). Later experiments have found that the apparently biased agonists assumed to have high G protein efficacy, oliceridine, PZM21, and SR17018 actually exhibit low G protein efficacy relative to the prototypical MOR agonist morphine, a crucial parameter not reported in the earlier work (Burgueño et al., 2017; Hill et al.,

2018; Yudin and Rohacs, 2019; Gillis et al., 2020). Similarly, buprenorphine and levorphanol, morphinans proposed as biased (Ehrlich et al., 2019; Le Rouzic et al., 2019; Pedersen et al., 2020), also have relatively low intrinsic efficacy for G protein activation (McPherson et al., 2010) as do a set of structurally distinct, putatively biased mitragynine-based compounds (Kruegel et al., 2016).

The relevance of the low efficacy of these novel compounds to drug development is immediately obvious, as medically used compounds targeting the MOR vary extremely in their G protein efficacy (McPherson et al., 2010; Molinari et al., 2010). Morphine is consistently found to be a partial agonist of intermediate efficacy compared with the higher-efficacy analgesic fentanyl and endogenous agonist met-enkephalin or its analogs when studied under conditions avoiding excessive amplification [see Kelly (2013) for summary]. Tapentadol has lower efficacy than morphine, and buprenorphine possesses uniquely low G protein efficacy (Sadeghi et al., 2015). An experiment using irreversible antagonism of MOR in a G protein–coupled potassium (GIRK) channel assay with identical methodology to that previously described (Gillis et al., 2020) using AtT20 cells (Yousuf et al., 2015) reveals that apparently G protein–biased agonists oliceridine, PZM21, and SR17018 have low intrinsic G protein efficacy relative even to the partial agonist morphine (Fig. 2B). The same experiment performed in parallel without receptor knockdown was largely insensitive to these efficacy differences (Fig. 2A), further

confirming that the overamplification confound limits the accurate description of GPCR agonists.

If measurements of efficacy in the G protein system are confounded or not made with high confidence, all further assessments of bias between G protein and other signals are difficult to interpret. As discussed by Conibear and Kelly (2019) and shown in McPherson et al. (2010) and Benredjem et al. (2019), MOR agonists with low G protein efficacy consistently produce a very low response in assays of arrestin recruitment, and this may not reflect true ligand bias (see below). Additionally, if the efficacy and affinity parameters are not separated, their relative contribution to agonist effects in vivo cannot be independently assessed. As such, interpretations of bias and its relevance in animals can become seriously confounded by failure to consider efficacy.

Comparison of an Amplified System to a Linear System. The central issue of biased agonism is reliable comparison of agonist activity between two or more signaling assays. As such, any quantification of putative GPCR bias must be independent of inherent differences between such assays—so-called "system bias."

When comparing an assay of G protein signaling to one of arrestin recruitment, as is done routinely in analyses of MOR bias, a comparison is being made between a highly amplified system and an unamplified system (Kelly, 2013). In the case of arrestin binding to the receptor, there is no amplification step between receptor and the "effect" to be measured, as the effect of interest is simply the formation of a receptor-arrestin complex. This binding is measured commonly in fluorescence or bioluminescence resonance energy transfer or enzyme complementation (e.g., PathFinder) assays using receptor/arrestin molecules tagged with reporter proteins. This protein-protein interaction is not "operating," as there is no downstream effect, and it is not explicable by an operational model. A single agonist-receptor complex can be assumed with some reservations to cause the binding of single arrestin molecule. It has been shown that reduction of receptor level in an MOR–β-arrestin 2 interaction assay reduces agonist maximal effect in direct proportion to the loss of receptor (Nickolls et al., 2011).

As the authors conclude, this would be expected in a linear system and contrasts with the same experiment performed for G protein assays that clearly showed hyperbolic amplification at the level of receptor effect.

Black et al. (2010) describe how the operational model might encompass a linear [AR]/E relationship—"a linear system is the limiting case of the relationship $K_E >> R_0$." This could be considered simply a system with extremely low efficacy, that is $\tau << 1$, (Fig. 1C), and so $\alpha << E_m$ (Fig. 1E, all agonists reach only a small fraction of system maximum effect). Crucially, because of the hyperbolicity of the agonist-receptor occupancy curve, such a linear system would be indistinguishable from an amplified system by simple observation of concentration-response curves, as noted by Black et al. (2010) and shown experimentally by Nickolls et al. (2011). The maximal effect of any agonist in a linear system, under operational assumptions, is limited by full receptor occupation and does not approach the true system maximum (Appendix 1).

Practically, the operational model is applied by fixing a system maximum to the largest effect observed, which is in most cases the response to the highest concentration of a highly efficacious reference agonist rather than a separately established system maximum (Kenakin et al., 2012). In the case of a linear occupancy-effect relationship within the assumptions of the operational model, this is a substantial underestimation of the true system maximum. The immediate result of this is that the operational efficacy values of all agonists are overestimated (Fig. 3A; Appendix 1). Agonists reaching close to the incorrectly assumed system maximum would be calculated to have very high efficacy (Fig. 3B). Observed efficacy of partial agonists, although remaining an overestimate, is relatively less affected. In a true linear system, the efficacy of an agonist is directly proportional to the fraction of receptors activated at full occupancy and is equivalent to asymptote α of that compound's concentration-response curve (Kelly, 2013; Stott et al., 2016). Furthermore, the error in efficacy estimation means that operational analysis assuming hyperbolicity does not produce accurate affinity estimates in a linear system (Stott et al., 2016; Appendix 1).
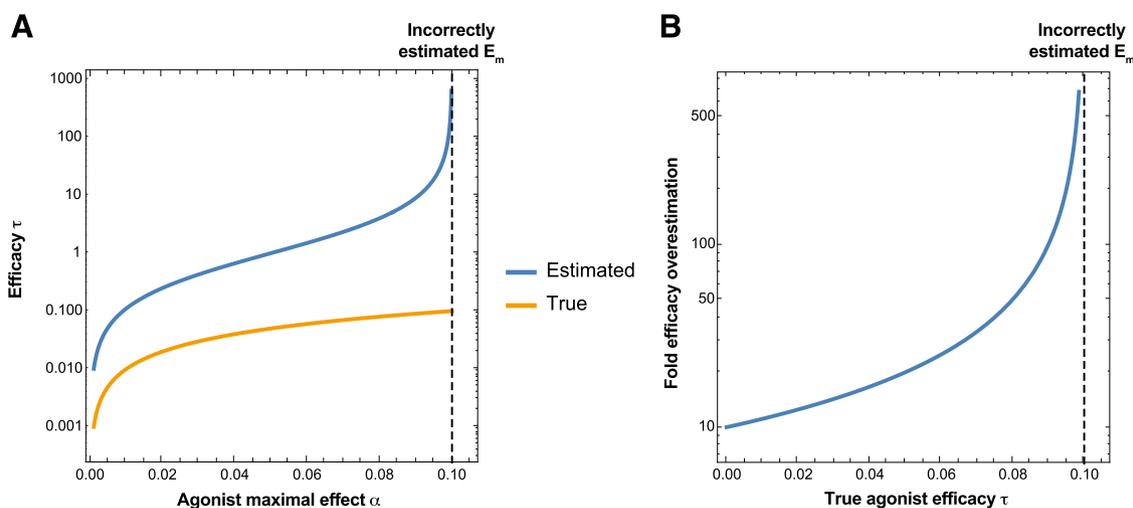

Fig. 3. (A) In an example linear pathway, wherein a reference agonist has efficacy $\tau = 0.1$, operational analysis will lead to an underestimation (dashed black line) of the true system maximum. The efficacy of all agonists will be overestimated as a result of this. (B) The relative overestimation of agonist efficacy will be greater for high-efficacy agonists. This error is propagated to K_A and log(τ/K_A) estimates.
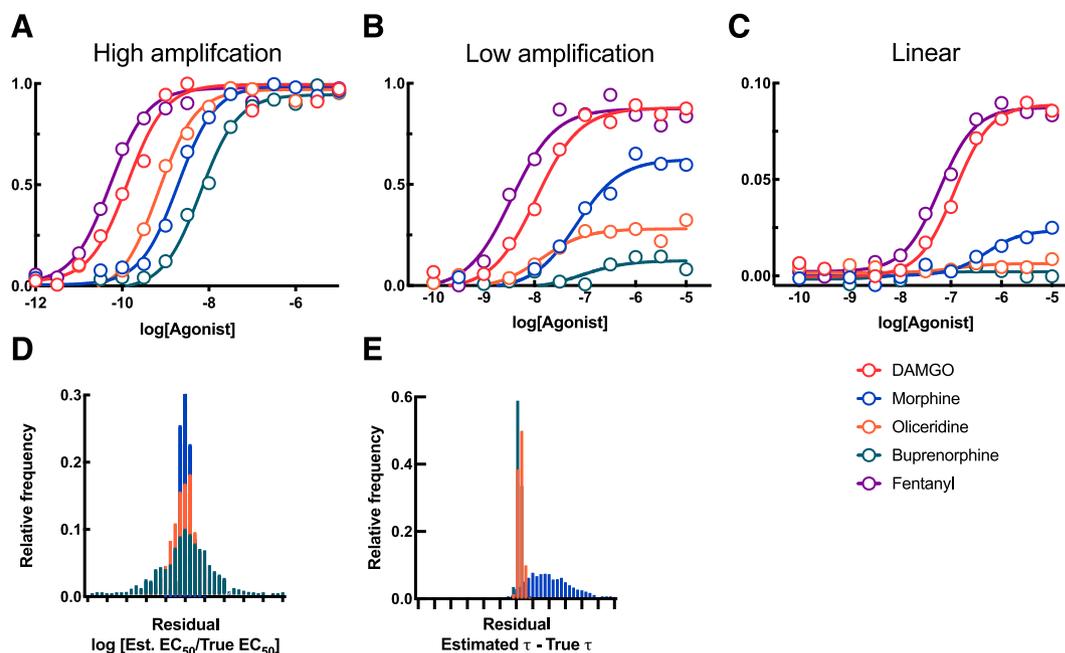
Fig. 4. Agonists with the relative efficacy of DAMGO, morphine, oliceridine, buprenorphine, and fentanyl were simulated in various operational systems with consistent S.D. (A) In a high-amplification system, even low-efficacy agonists produce close to a full response. (B) In a lower-amplification system, differences in efficacy are discernable. (C) In a linear, no-amplification system, low-efficacy agonists, such as oliceridine and buprenorphine, produce very little effect. Comparison of a linear system, such as an arrestin-recruitment assay, with a high-amplification system, such as G protein signaling, causes low-efficacy agonists to appear "biased" toward the amplified signaling pathway. (D) Monte-Carlo simulation of curve fit, with a consistent sample S.D. of 5% of the maximal effect with 1000 interations, in the low-amplification system shows how the curve-position estimate, $\log(EC_{50})$, is markedly worse for low-efficacy agonist oliceridine or very low-efficacy agonist buprenorphine than higher-efficacy ligands, such as morphine. (E) Conversely, in the same Monte-Carlo simulated system, the efficacy of higher-efficacy morphine is less accurately estimated than oliceridine- or burprenorphine-like agonists because of the hyperbolic relationship between maximal effect and operational efficacy.

When comparing a known high-amplification G protein system to a linear arrestin-recruitment systems, partial agonists will visually appear "biased" (Fig. 4, A and C), a systematic error further compounded by poor G protein–efficacy estimates arising from excessive amplification (as described above). The combined, normalized $\Delta\log(\tau/K_A)$ parameter is not dependent on accurate estimation of system maximum effect when a Hill slope is accurately measured as 1 (Appendix 1). However, it has been asserted that normalization and subtraction of the $\Delta\Delta\log(\tau/K_A)$ parameter cancels system bias (Kenakin, 2017). As the underlying affinity and efficacy estimates of operational analysis do not reflect any absolute, mechanistic values due to the invalidated model assumptions, system bias cannot be said to be canceled.

It is also important to note that $\log(\tau/K_A)$ is mathematically equivalent to $\log(\alpha/EC_{50})$ when the Hill slope is equal to 1 (Kenakin, 2017). This equivalence of $\log(\tau/K_A)$ to $\log(\alpha/EC_{50})$ for systems with Hill slopes close to 1 was borne out in a comparison of the two analyses on a large family of opioid agonists (Winpenny et al., 2016). As such, in this case, mechanistic operational analysis does not provide additional information over more simplistic analyses, such as comparison of agonist maximal effect and potency. Furthermore, as above, there is no evidence that the efficacy or affinity parameters will be system-independent. Under operational assumptions, linear systems, such as any protein-protein interaction, will have Hill slopes of 1 and, therefore, an "n" equal to 1. Given that the operational efficacy is directly approximated by α in a linear system, and EC_{50} relates directly to affinity, the utility of operational analysis

of linear systems is not clear. On the other hand, if the Hill slope, and therefore operational "n" value, is not equal to 1 in a protein-protein interaction assay, the underlying dynamics must be too complicated to be explicable by the operational model.

Biochemical assays may also have a window limited by the dynamic range of the measurement system, rather than saturation of effector proteins (Stott et al., 2016). This would produce an apparent ceiling of effect, but this is not addressed by the assumptions of the operational model and cannot produce satisfactory efficacy and affinity estimates.
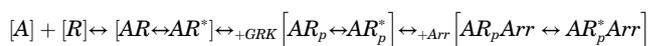
Because low-efficacy agonists in a linear pathway produce an extremely small response (Fig. 4C), quantification of effect is difficult (Stahl et al., 2015). As agonist response decreases with consistent absolute error, the relative accuracy of any curve-position estimate decreases rapidly (Fig. 4D). Estimates of operational parameters are wholly dependent on this curve-fitting process. Analysis of G protein/arrestin systems often involves curve fitting to extremely partial responses that do not reach a plateau at tested concentrations (Schmid et al., 2017). Mathematical solutions to this problem have been proposed but involve additional assumptions regarding the nature of receptor available for binding and competition (Stahl et al., 2015). Caution must be taken when generating quantitative estimates of agonist bias from such low responses (Kelly, 2013; Kenakin and Christopoulos, 2013).

Crucially, many currently identified "G protein–biased" MOR agonists have low intrinsic efficacy (Fig. 2D) and would therefore show very low activity in a linear assay. Conibear

and Kelly (2019) and others (Stott et al., 2016) have noted that few MOR ligands have been robustly demonstrated to be significantly biased with application of the operational model. Although there is a valid criticism to be made that descriptions of biased agonism without robust quantification may lead to false positives, it may be the case that operational analysis alone will not resolve the issue. If either the G protein assay remains highly amplified or the lack of amplification in the arrestin-recruitment system is not explicitly addressed, variation in agonist efficacy and affinity will present differently between assays. The error is fundamental to the comparison of different systems and cannot necessarily be canceled out by normalization (Smith et al., 2018). A major contributor to the potential misidentification of agonist bias is systems with varying amplification (Kenakin and Christopoulos, 2013; Smith et al., 2018). The widely used operational model fundamentally assumes an operating bimolecular agonist-receptor complex, which neglects ternary-complex formation in studies of protein-protein interactions and is therefore not robust.

In summary, the observed profile of many existing or novel MOR agonists proposed as G protein–biased is entirely consistent with that which would be predicted for unbiased, low–intrinsic efficacy compounds in highly amplified and linear systems (Kelly, 2013; Stott et al., 2016; Conibear and Kelly, 2019). Conclusions of G protein over arrestin bias of these MOR compounds have been driven by an assumption that their intrinsic G protein efficacy is nearly equal to morphine and other existing agonists (DeWire et al., 2013; Manglik et al., 2016; Schmid et al., 2017). Evidence presented here and elsewhere that they are, in fact, low-efficacy compounds should prompt a re-examination of their true signaling bias.

Arrestin Binding Is a Dynamic, Nonequilibrium System. A core assumption in all applications of operational analysis is a homogenous receptor population that is in equilibrium with agonist. This assumption leads to the application of Langmuirian affinity in modeling the agonist-receptor interaction. This neglects the impact of nonequilibrium systems of distributed receptor states. For instance, at the MOR and other GPCRs, covalent protein modification through intracellular receptor phosphorylation via G protein receptor kinases occurs at multiple sites within seconds of agonist stimulation (Doll et al., 2011). Phosphorylated MOR states persist for minutes even after removal of agonist and have been shown to exhibit distinct binding kinetics and functions (Doll et al., 2012; Birdsong et al., 2015). Phosphorylation is followed sequentially by arrestin binding to produce another receptor state, which, again, may have altered agonist affinity and kinetics (Gurevich et al., 1995). Finally, in assays with time courses longer than several minutes, receptor loss through internalization into the cell will occur. The progressive processes of phosphorylation, arrestin binding, and receptor internalization imply the receptor population is no longer homogenous but a dynamic system of interacting species. Although a dynamic steady state may be reached, true chemical equilibrium as assumed in operational analysis is impossible (Lauffenburger and Linderman, 1996). A simplified schematic of the reaction scheme leading to arrestin binding is presented below. Note that each state, including phosphorylated and arrestin-bound receptor, can be ligand-bound or unbound.

$$[A] + [R] \leftrightarrow [AR \leftrightarrow AR^*] \leftrightarrow_{+GRK} \left[AR_p \leftrightarrow AR_p^* \right] \leftrightarrow_{+Arr} \left[AR_pArr \leftrightarrow AR_p^*Arr \right]$$

Does operational analysis adequately account for such a system? The concept of a single "functional affinity" representing a system of at least three persistent, potentially functional receptor states available for agonist binding is clearly fraught. The operational model assumes a homogenous surface of available receptor and therefore applies a bimolecular model (eq. 1). This has been demonstrated not to be the case, and available receptor sites are remarkably heterogeneous. Therefore, the functional affinity K_A parameter is not an estimate of any particular receptor state affinity.

The operational model, as derived, assumes two independent processes: one of agonist binding and one of effect (Black and Leff, 1983). There is now clear evidence that the process of "effect" in a phosphorlyated receptor, arrestin system is not independent of agonist binding. Invalidation of the assumed independence of the binding (eq. 1) and activation (eq. 2) functions results in error in efficacy and affinity determination.

The dynamic process from agonist binding, G protein trimer dissociation, kinase recruitment, multisite phosphorylation, and, finally, arrestin binding cannot be satisfactorily modeled by a single effect hyperbola (eq. 2 of the operational model). The underlying relationships involve multiple component second-order processes that are dependent both on the amount of receptor and on the concentration of kinases and arrestins. Capturing the full range of agonist effects in a dynamic system is nontrivial and requires real-time assays.

Indeed, the "kinetic context" of bias has been robustly shown to complicate interpretation and in some cases completely alter conclusions on the nature of agonist bias (Klein Herenbrink et al., 2016). The steady-state point is agonist-dependent and the complex influence of assay factors on apparent agonist activity should be considered. Hoare et al. (2018) present an extended kinetic implementation of the operational model to address this limitation of current models. As a mechanistic model, operational analysis of bias is substantially confounded by temporal dynamics. The immediate result for the present case, in which comparison of G protein activity with arrestin recruitment is desired, is that violation of operational assumptions confounds quantified signaling parameters, which are often endowed mechanistic significance. Operational "affinity" and "efficacy" values do not reflect an underlying biochemical parameter of such a dynamic system. The application of a mechanistic, mathematical model to a biologic system is a hypothesis, which in the case of the operational model has been invalidated.

Application of the operational model, in the form of $\Delta\Delta\log(\tau/K_A)$ values, to determine agonist bias relies upon the assumption that the system-dependent bias is canceled by reference agonist normalization. Because the dynamic arrestin-recruitment system does not meet the assumptions of the operational model, it therefore cannot be said to be accurately quantified with these parameters. This will be true of any temporally evolving process. Signaling of GPCRs varies over time because of the interaction of a network of proteins. In the case that this network cannot be accurately modeled, quantification via empirical observation rather than mechanistic assumptions is preferable (Fig. 5).
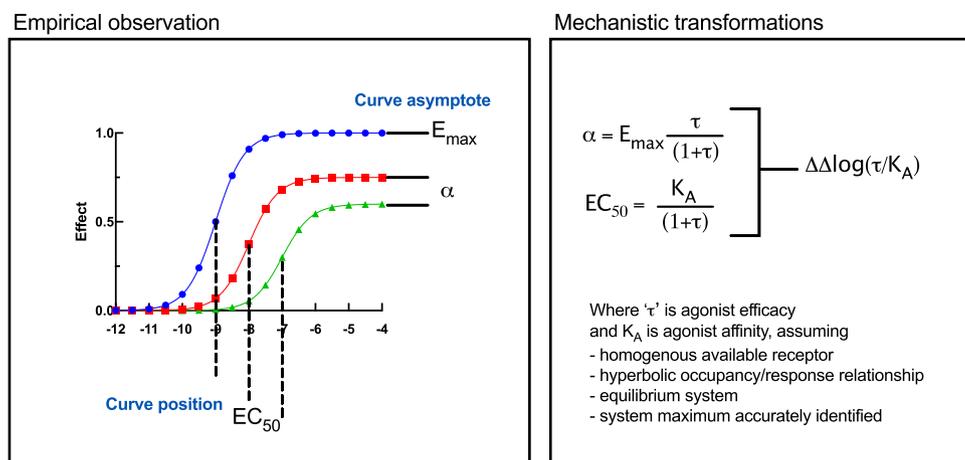
Empirical observation

Mechanistic transformations

Fig. 5. Empirical observation of agonist/effect concentration-response curves can be quantified directly with curve asymptote (α) and position (EC$_{50}$) estimates. Mechanistic transformations, such as those in operational model analysis, require additional assumptions and may be systematically confounded.

In summary, observation of apparent agonist bias, visually and upon operational analysis, between amplified G protein signaling and linear arrestin-recruitment system can be driven by amplification differences and the underlying dynamics of mutiple receptor states. Partial agonists with low efficacy relative to the reference compound, if not identified as such, will present as biased toward G protein signaling in such a set of systems in a manner not directly addressed by current pharmacological quantification methods.

The primary issues highlighted above regarding the analysis of G protein/arrestin bias concern two main elements, being amplification within systems and the dynamic nature of signaling. The null hypothesis in any examination of proposed ligand or receptor bias should be consistent activity across pathways. Given the robust prediction of rank order of arrestin-recruitment efficacy by G protein efficacy at the MOR (McPherson *et al.*, 2010; Benredjem *et al.*, 2019; Gillis *et al.*, 2020), this is a reasonable starting place. It is theoretically (Fig. 4) and empirically (Gillis et al., 2020) sound to predict that a low-efficacy agonist may still produce a full response in an amplified assay while having low activity in a low-amplification system, such as arrestin recruitment. A lack of apparent arrestin recruitment is therefore not sufficient in itself for an agonist to be considered G protein–biased (Conibear and Kelly, 2019).

Other Possible Approaches to Determine the Presence of Bias in the MOR G Protein/Arrestin System. As mechanistic operational analysis is systematically confounded in the MOR G protein arrestin system, because of invalidation of underlying assumptions, we recommend the use of empirical measurements (Fig. 5). Ligand maximal effect and potency are directly observable parameters. Rank order and shifts in σ and EC$_{50}$ should be compared between pathways to examine potential ligand bias from a skeptical perspective.

Accurate quantification of G protein efficacy, as distinct from affinity, in an MOR assay not limited by its signal window will facilitate more informed conclusions to be reached concerning actual ligand bias. Establishing the true system maximum of an amplified assay can be done via pharmacologic (Fig. 2, Kelly, 2013) or genetic (Hermans et al., 1999) methods. Alternatively, ensuring an excess of reporter relative to receptor will reduce the likelihood of effector saturation. At the MOR, the prototypical agonist morphine is well-established to have operational intrinsic efficacy around one-fifth that of

DAMGO (Kelly, 2013). Thus, if an assay is not sensitive to the partial agonism of morphine, then it will not show efficacy differences over at least that range such that inclusion of these two reference compounds is a simple test for the presence of amplification confounds. Conversely, low-amplification protein-protein interaction systems, such as arrestin recruitment, highlight efficacy differences such that partial agonists may appear biased (Fig. 1E). The window for detection of G protein–biased agonists is limited without increasing signal-to-noise ratio in a given arrestin assay, for instance by G protein receptor kinase overexpression (Dekan *et al.*, 2019; Gillis *et al.*, 2020). A high-efficacy agonist, such as DAMGO, is the most appropriate reference compound in studies of the MOR because it is the most likely to define the maximum possible effect in most assays. Well-established partial agonists, such as morphine, which has itself been presented as G protein–biased, are critical additional reference ligands to examine the level of system overamplification or underamplification across assays.

The proposed benefit of operational analysis of ligand bias is predicated on the concept that normalization cancels system bias (Kenakin and Christopoulos, 2013). Given the multiple ways in which the GPCR G protein/arrestin system does not meet the assumptions underpinning operational analysis, this cannot be confidently stated to be the case. The affinity and efficacy parameters do not reflect biochemical parameters of accurately modeled chemical processes. Furthermore, employing a single, integrated signaling coefficient without extracting a specific efficacy estimate does not adequately represent the complexity of the underlying system. In fact, the normalized signal coefficient $\Delta\log(\tau/K_A)$ representing activity in a given pathway is essentially determined by agonist potency (EC$_{50}$) in published literature on MOR G protein and arrestin assays (Supplemental Fig. 1). These and other limitations of operational analysis have previously been raised in a more general sense (Kelly, 2013; Kenakin, 2014; Stott et al., 2016; Hoare et al., 2018). Because $\Delta\log(\tau/K_A)$ values are almost entirely determined by agonist potency, the additional utility of this complex model for studies of the MOR G protein/arrestin system is questionable.

Extensions to quantitative operational bias analysis, such as those proposed by Hoare et al. (2018) and others (Roche et al., 2013), may improve analysis of concentration-response data. However, any such extension would require separate or

additional assumptions regarding the nature of the underlying system. How then should the potential agonist bias between a G protein signal and arrestin recruitment be observed? Examining receptor systems with more simplistic analysis reduces the impact of confounding factors and encourages more critical interpretation. For instance, "bias plots" of equimolar agonist comparisons show system biases, such as amplification differences as well as possible intrinsic agonist bias (Smith et al., 2018). We recently performed this analysis on a family of opioid analgesics and lead compounds to visualize amplification differences between assays (Gillis et al., 2020). It is notable that when amplification confounds were addressed, potency and the rank order of maximal effect were entirely conserved across many distinct assays of MOR activation. Although absolute quantification of ligand bias in this nonequilibrium, kinetic system is not possible, especially given varying levels of amplification and signaling proteins, relative estimates are sufficient when assay factors are controlled.

Direct comparison of rank order of agonist maximal effect and potency between assays not confounded by limited windows will show if any agonist has a truly aberrant signaling signature. The use of β-chlornaltrexamine or β-funaltrexamine partial irreversible antagonism to sufficiently reduce maxima of "full" agonists ensures true relative efficacy can be observed. If rank order of ligand maximal effect, when signal windows are not limited, and potency are conserved between assays, true ligand bias is unlikely to be present regardless of how amplification alters operational parameters. Notably, when these confounds are addressed, endomorphin 2 has been consistently observed to be arrestin-biased when compared with met-enkephalin (Rivero et al., 2012; Dekan et al., 2019), and a peptidergic agonist appears to have lower efficacy for arrestin recruitment than would be predicted from G protein–efficacy estimates (Dekan et al., 2019).

Relevance of Efficacy and Biased Signaling to the Development of Safer Opioids

The assumptions behind G protein–biased agonism as a strategy for safer opioid analgesics have recently been challenged. One study examined mice expressing MOR with mutation of the receptor C-terminal phosphorylation sites (Kliewer et al., 2019). Crucially, this animal model of a "G protein–biased" MOR that does not recruit arrestin showed more potent opioid analgesia but enhanced rather than attenuated or abolished side effects, including respiratory depression. In addition, the original result of substantially reduced morphine respiratory depression in the β-arrestin 2 knockout mouse was not able to be repeated by multiple groups working independently (Kliewer et al., 2020). Supporting physiologic evidence for β-arrestin 2 mediating or enhancing opioid side effects, aside from the original experiment on the knockout animal, is not substantial (Raehal et al., 2011; Siuda et al., 2017). There is, however, a body of physiologic evidence that opioid agonist effects on brainstem respiratory nuclei, such as the Kölliker-Fuse (Levitt et al., 2015) and pre-Bötzinger complex (Montandon et al., 2016; Wei and Ramirez, 2019), are G protein–mediated via ion channel effectors. Evidence for the underlying premise of MOR G protein–biased agonists is therefore limited.

In a recent study in which the confounding factors highlighted here were addressed, we did not observe any significant G protein bias in a family of opioid analgesics and lead compounds previously observed to be biased (Gillis et al., 2020). Despite this, there was a good correlation observed between lower intrinsic efficacy and improved safety profile in a preclinical mouse model. Rodent experiments on oliceridine (DeWire et al., 2013), PZM21 (Manglik et al., 2016, but see Hill et al., 2018), SR17018 (Schmid et al., 2017), mitragynine (Váradi et al., 2016), and levorphanol (Le Rouzic et al., 2019) have suggested these compounds may have improved respiratory profiles over morphine. An alternative explanation to bias for these improvements may lie in the low intrinsic efficacy of these compounds, which is close to the uniquely low efficacy of buprenorphine, which may have an improved therapeutic window (Wolff et al., 2012). Buprenorphine has reduced liability for overdose (Power et al., 2019) and is rarely associated with fatalities in contrast with other marketed opioids (Bell et al., 2009; Marteau et al., 2015). Buprenorphine's demonstrated ceiling effect on respiration, which is widely assumed to be driven by low efficacy and shown in both humans and rodents (Dahan et al., 2005), plausibly underlies this effect. Multiple rodent studies of buprenorphine have shown markedly reduced respiratory effects,
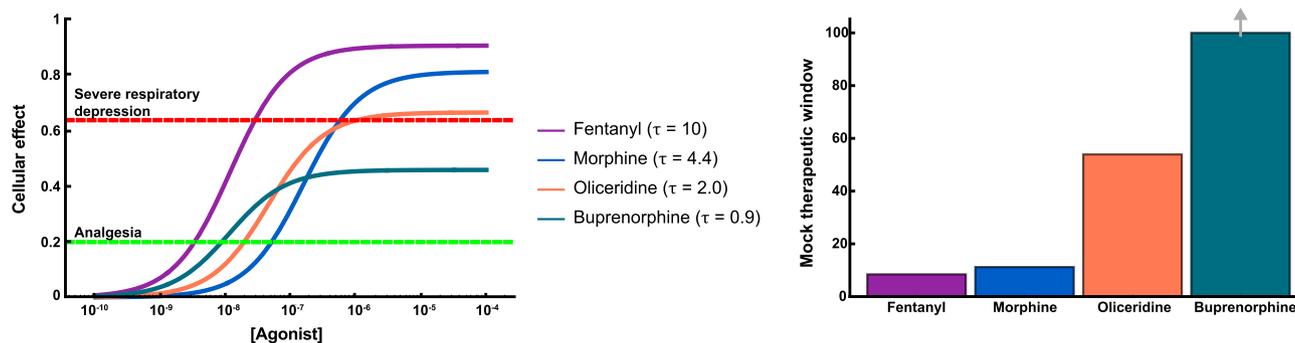

Fig. 6. (A) Agonists with the relative efficacy of fentanyl, morphine, oliceridine, and buprenorphine were simulated in an operational system. All agonists produce a full response in the theoretical high-sensitivity pathway, analgesia (green line). Buprenorphine reaches a ceiling in the low-sensitivity pathway, severe respiratory depression (red line). (B) A mock therapeutic window between the low- and high-sensitivity effects was modeled by considering the ratio of concentrations producing each effect. In the theoretical system, morphine and fentanyl reach full effects and have similar profiles, whereas oliceridine as modeled has a slightly improved therapeutic window due to its efficacy, which is half that of morphine. Buprenorphine does not produce full respiratory depression and, therefore, has an arbitrarily large therapeutic window.

Nature of Occupancy-Response Relationship	Generic: Hyperbolic	Limiting Case: Linear
Theoretical relationship between maximum agonist response, system maximal response, and the operational efficacy of a drug (Black et al. 2010)	$\alpha^X = E_m \times \left(\dfrac{\tau^X}{1+\tau^X}\right)$ (1) or $\tau^X = \dfrac{\alpha^X}{E_m - \alpha^X}$ (2)	
Anticipated experimental maximum response of reference agonist ($X = ref$)	Since $\tau^{ref} \gg 1$ in high-amplification systems, eq. 1 becomes $\widetilde{\alpha^{ref}} = E_m$ (3a)	Since $\tau^{ref} \ll 1$ in low-amplification systems, eq. 1 becomes $\widetilde{\alpha^{ref}} = E_m \times \tau^{ref}$ (3b)
Anticipated experimental maximum response of a test agonist ($X = A$)	Since $\tau_A < \tau_R \ll 1$, eq. 1 can be rewritten as $\widetilde{\alpha^A} = E_m \times \left(\dfrac{\tau^A}{1+\tau^A}\right)$ (4a)	Since $\tau_A < \tau_R \ll 1$, eq. 1 becomes $\widetilde{\alpha^A} = E_m \times \tau^A$ (4b)
Experimental implementation of operational model to determine operational efficacy	Implementation of the operational model assumes eq. 2 to be true for experimentally measured values; in other words, $\widetilde{\tau^A} = \dfrac{\widetilde{\alpha^A}}{E_m - \widetilde{\alpha^A}}$ Since operational model also assumes hyperbolicity, that is, that the reference agonist can evoke system maximal response, $E_m = \alpha^{ref}$. $\therefore \widetilde{\tau^A} = \dfrac{\widetilde{\alpha^A}}{\widetilde{\alpha^{ref}} - \widetilde{\alpha^A}}$	
Experimental determination of operational efficacy, τ^A, of a test agonist	Substituting eqs. 3a and 4a $\widetilde{\tau^A} = \dfrac{E_m \times \left(\frac{\tau^A}{1+\tau^A}\right)}{E_m - E_m \times \left(\frac{\tau^A}{1+\tau^A}\right)}$ $\therefore \widetilde{\tau^A} = \tau^A$ (5a)	Substituting eqs. 3b and 4b $\widetilde{\tau^A} = \dfrac{E_m \times \tau^A}{E_m \times \tau^{ref} - E_m \times \tau^A}$ $\therefore \widetilde{\tau^A} = \dfrac{\tau^A}{\tau^{ref} - \tau^A}$ (5b)

especially compared with morphine and fentanyl (Chevillard et al., 2009; Brown et al., 2011).

A recent paper that analyzed the signaling characteristics of existing analgesics and compounds in development found that efficacy predicted clinical outcomes in terms of reported adverse events (Benredjem et al., 2019). The authors concluded that partial agonism was a route to improved opioid safety. Reanalysis of the signaling data of that study shows that efficacy for each G protein–signaling pathway studied is robustly correlated to arrestin recuitment efficacy for the 25 test compounds (Supplemental Fig. 2), as we found in our own study of existing analgesics and lead compounds (Gillis et al., 2020) and observed by McPherson et al. (2010). Although cell types vary in signaling background, when confounding levels of system gain are adjusted, rank order of efficacy has consistently been found to be conserved for large families of MOR agonists.

Improved safety of low–intrinsic efficacy opioids is inherently plausible. Analgesia is elicited at lower doses of MOR agonists than severe side effects such that there is some window of therapeutic effect. This could be considered to be driven by the high sensitivity of the endogenous receptor system to agonist analgesic effect compared with lower sensitivity for severe respiratory depression. In a high-sensitivity pathway, even very low-efficacy agonists may stimulate a reasonable response, whereas only high-efficacy agonists are able to produce a substantial response in low-sensitivity side-effect pathways. If we consider in a simplified system that the underlying cellular mechanism producing both analgesia and respiratory depression is the same, then we can hypothesize the result of distinct ceilings of behavioral effect (Fig. 6A). Desired "analgesia" is reached quickly by even the extremely low-efficacy agonist, but this reaches a low ceiling of effect in the low-sensitivity, side-effect pathway. Even in the case in which both agonists reach "full" effect in desired and deleterious

pathways, the lower-efficacy oliceridine-like agonist shows an improved therapeutic window (Fig. 6B).

The model presented above is an overt oversimplification. There are clearly additional receptor-signaling mechanisms underlying all opioid effects, some of which are extensively characterized and some not, and multiple sites of action. Opioid agonists act at MOR at the level of the spinal cord, brainstem, and cortex in a synergistic manner, which contributes to their unparalleled activity as analgesics (Stein, 2016). In contrast, there are complex and multilayered neural compensation mechanisms that maintain respiration and blood oxygen saturation (Pattinson, 2008). It may be these circuit level nuances, rather than underlying cellular transduction differences, that drive differing sensitivities of behavioral effects. Differences in efficacies, rather than any agonist G protein/arrestin bias, are an alternative explanation for variation in opioid safety window between agonists. This potential mechanism addresses the apparent reduced overdose risk of buprenorphine and may underpin the profiles of putatively biased, low-efficacy lead compounds.

Conclusions

Proposed ligand bias at the MOR has been an area of intense and prolonged research. G protein/arrestin bias at MOR is one of the most developed areas of the maturing field surrounding biased signaling of GPCRs (Michel and Charlton, 2018), with several novel compounds in development and one having entered clinical trials. We have shown here that failure to account for intrinsic efficacy and ceiling effects in different pathways, distortions introduced by analysis of amplified (G protein) versus linear (arrestin) signaling mechanisms, and nonequilibrium effects in a dynamic signaling cascade produce erroneous bias calculations of G protein signaling. The weaker

Nature of Occupancy-Response Relationship	Generic: Hyperbolic	Limiting Case: Linear
Theoretical relationship between half-maximal drug response and the operational efficacy and affinity of a drug (Black et al. 2010)	$EC_{50}^X = \dfrac{K_A^X}{1+\tau^X}$ (6) or $K_A^X = (1+\tau^X) \times EC_{50}^X$ (7)	
Anticipated experimental EC_{50} of reference agonist	Since $\tau^{ref} \gg 1$, eq. 6 becomes $\widetilde{EC_{50}^{ref}} = \dfrac{K_A^{ref}}{\tau^{ref}}$ (8a)	Since $\tau^{ref} \ll 1$, eq. 6 becomes $\widetilde{EC_{50}^{ref}} = K_A^{ref}$ (8b)
Anticipated experimental EC_{50} of a test agonist	$\widetilde{EC_{50}^A} = \dfrac{K_A^A}{1+\tau^A}$ (9a)	Since $\tau^A < \tau^{ref} \ll 1$, eq. 6 becomes $\widetilde{EC_{50}^A} = K_A^A$ (9b)
Experimental implementation of operational model to determine affinity	Implementation of the operational model assumes eq. 7 to be valid for experimentally measured values; in other words, $\widetilde{K_A^A} = (1 + \widetilde{\tau^A}) \times \widetilde{EC_{50}^A}$ (10)	
Experimental determination of affinity, $\widetilde{K_A^A}$, of a test agonist	Substituting eqs. 5a and 9a $K_A^A = (1+\tau^A) \times \dfrac{K_A^A}{1+\tau^A}$ $\therefore \widetilde{K_A^A} = K_A^A$ (11a)	Substituting eqs. 5b and 9b $\widetilde{K_A^A} = \left(1 + \dfrac{\tau^A}{\tau^{ref}-\tau^A}\right) \times K_A^A$ $\therefore \widetilde{K_A^A} = \left(\dfrac{\tau^{ref}}{\tau^{ref}-\tau^A}\right) \times K_A^A$ (11b)

the G protein partial agonism, the greater the apparent bias is. This does not imply that biased signaling does not exist at all or is not important. Indeed, detailed analyses of rank orders of relative intrinsic efficacy when accounting properly for excessive amplification in the G protein pathway and/or weak signaling in the arrestin pathway do reveal some bias toward or away from G protein signaling. However, evidence of improved opioid safety in preclinical assays and the clinic for supposedly G protein–biased opioids is much more clearly attributable to their reduced intrinsic efficacy for G protein signaling than to actual G protein bias. Direct screening of opioid agonists for appropriately limited intrinsic efficacy is thus more likely to yield opioids safer for respiratory depression and thus reduced lethal overdose risk than pursuit of bias.

Authorship Contributions

Participated in research design: Gillis, Christie.
Conducted experiments: Gillis.
Performed data analysis: Gillis, Sreenivasan.
Wrote or contributed to the writing of the manuscript: Gillis, Sreenivasan, Christie.

Appendix 1

Incorrect Assumption of Hyperbolicity in Operational Analysis Leads to Overestimation of Efficacy (τ) and Underestimation of Affinity (*K_A*)

Notations used to represent parameters (within certain signaling system):

$E([X])$ Response at a drug concentration of $[X]$

E_m Maximum system response

α^X Maximum response of a drug X, as in, $\alpha^X = \lim\limits_{[X]\to\infty} E([X])$

K_A^X Receptor-binding affinity of a drug X, as defined by the operational model

τ^X Operational efficacy of a drug X, as defined by the operation model

EC_{50}^X Potency or drug concentration that elicits half-maximal response for that drug

K_E^X Number of receptors occupied by the agonist, X, to elicit half-maximal response

R_0 Available receptor concentration

n Slope of transducer function, set to 1 for simplicity

$\sim$ All symbols accented by a tilde represent experimentally determined values

Introduction

The response evoked by an agonist is determined by the amplification within a signaling pathway in conjunction with that agonist's intrinsic efficacy and receptor-binding affinity. The operational model defines the amplification of a signaling pathway as the fold excess of receptors available for a reference agonist to bind and elicit its half-maximal response. Practically, reference agonist is a receptor's endogenous ligand or a prototypical full agonist with high efficacy. Thus, the amplification of the system, its sensitivity to agonism, is simply the operational efficacy of a reference agonist, which can be mathematically written as $\tau^{ref} = \dfrac{R_0}{K_E^{ref}}$ (refer above for description of symbols).

As per the operational model, the amplification value determines the nature of relationship between the number of agonist-occupied receptors (occupancy) and the evoked response. For example, in a high-amplification signaling pathway (e.g., G protein–coupled signaling), the response can be saturated by an agonist occupying just a fraction of the available receptors (i.e., $K_E^{ref} < R_0$ or $\tau^{ref} > 1$). This behavior implies that

Nature of Occupancy-Response Relationship	Generic: Hyperbolic	Limiting Case: Linear
Theoretical expression for transduction coefficient	Division of eq. 1 by eq. 6 and rearrangement of terms gives rise to $$\frac{\tau^X}{K_A^X} = \frac{\alpha^X}{EC_{50}^X \times E_m}$$	
Experimental implementation of operational model to determine transduction coefficient	Implementation of the operational model assumes that above equation is valid for experimentally measured values; in other words, $$\left(\widetilde{\frac{\tau^X}{K_A^X}}\right) = \frac{\widetilde{\alpha^X}}{\widetilde{EC_{50}^X} \times E_m}$$ Since operational model also assumes hyperbolicity, that is the reference agonist can evoke system maximal response, $E_m = \alpha^{ref}$. $$\therefore \left(\widetilde{\frac{\tau^X}{K_A^X}}\right) = \frac{\widetilde{\alpha^X}}{\widetilde{EC_{50}^X} \times \widetilde{\alpha^{ref}}} \quad (12)$$	
Experimental determination of transduction coefficient of a test agonist ($X = A$)	Substituting eqs. 3a, 4a, and 9a $$\left(\widetilde{\frac{\tau^A}{K_A^A}}\right) = \frac{E_m \times \left(\frac{\tau^A}{1+\tau^A}\right)}{\frac{K_A^A}{1+\tau^A} \times E_m} \#$$ $$\therefore \left(\widetilde{\frac{\tau^A}{K_A^A}}\right) = \frac{\tau^A}{K_A^A} \quad (13a)$$	Substituting eqs. 3b, 4b, and 9b $$\left(\widetilde{\frac{\tau^A}{K_A^A}}\right) = \frac{E_m \times \tau^A}{K_A^A \times E_m \times \tau^{ref}}$$ $$\therefore \left(\widetilde{\frac{\tau^A}{K_A^A}}\right) = \frac{\tau^A}{K_A^A} \times \frac{1}{\tau^{ref}} \quad (13b)$$
Experimental determination of transduction coefficient of a test agonist ($X = ref$)	$$\left(\widetilde{\frac{\tau^{ref}}{K_A^{ref}}}\right) = \frac{\widetilde{\alpha^{ref}}}{\widetilde{EC_{50}^{ref}} \times \widetilde{\alpha^{ref}}}$$ $$\therefore \left(\widetilde{\frac{\tau^{ref}}{K_A^{ref}}}\right) = \frac{1}{\widetilde{EC_{50}^{ref}}} \quad (14)$$ Equation 12 can be rewritten for reference agonist as	
	Substituting eq. 8a $$\left(\widetilde{\frac{\tau^{ref}}{K_A^{ref}}}\right) = \frac{\tau^{ref}}{K_A^{ref}} \quad (15a)$$	Substituting eq. 8b $$\left(\widetilde{\frac{\tau^{ref}}{K_A^{ref}}}\right) = \frac{1}{K_A^{ref}} \quad (15b)$$
Relative experimental transduction coefficient of test agonist with respect to a reference	Relative transduction coefficient, as defined in Kenakin et al. (2012) $$\Delta\log\left(\widetilde{\frac{\tau}{K_A}}\right)^A = \log\left[\frac{\left(\widetilde{\frac{\tau^A}{K_A^A}}\right)}{\left(\widetilde{\frac{\tau^{ref}}{K_A^{ref}}}\right)}\right]$$	
	Substituting eqs. 13a and 15a $$\Delta\log\left(\widetilde{\frac{\tau}{K_A}}\right)^A = \log\left[\frac{\left(\frac{\tau^A}{K_A^A}\right)}{\left(\frac{\tau^{ref}}{K_A^{ref}}\right)}\right]$$ $$\therefore \Delta\log\left(\widetilde{\frac{\tau}{K_A}}\right)^A = \Delta\log\left(\frac{\tau}{K_A}\right)^A \quad (16a)$$	Substituting eqs. 13b and 15b $$\Delta\log\left(\widetilde{\frac{\tau}{K_A}}\right)^A = \log\left[\frac{\left(\frac{\tau^A}{K_A^A} \times \frac{1}{\tau^{ref}}\right)}{\left(\frac{1}{K_A^{ref}}\right)}\right]$$ $$\therefore \Delta\log\left(\widetilde{\frac{\tau}{K_A}}\right)^A = \Delta\log\left(\frac{\tau}{K_A}\right)^A$$ $$(16b)$$

the signal-transduction mechanism dictates the maximal response that is possible to any agonist. Such a signaling pathway is referred to here as *hyperbolic* in nature because of the shape of the occupancy-response relationship (Fig. 1B – main text). In contrast, in a very low-amplification signaling pathway (e.g. protein-protein interaction, such as arrestin recruitment), saturation of the response occurs because of the limited pool of available receptors (i.e., $K_E^{ref} \gg R_0$ or $\tau^{ref} \ll 1$). Such a system is referred to here as *linear* in nature because of the shape of the occupancy-response relationship (Fig. 1C – main text).

Low-amplification systems are therefore a limiting case of the operational model, in which the generic hyperbolic nature of the occupancy-response relationship collapses to linear.

Although this is mathematically satisfying, assumption of hyperbolicity can result in dramatic systematic errors while operational efficacy and affinity of drugs are estimated, even when normalized to a reference. The source of this error, as will be demonstrated below, lies in the practical difficulty of determining the hyperbolicity of a system (i.e., whether a system is linear or hyperbolic) from concentration-response curves (Fig. 1 – main text) without performing additional controls (e.g., receptor depletion). The unintended consequence of this practical challenge is the incorrect assumption that the reference agonist can elicit the system maximal response. Below, we present derivations that quantify the extent to which operational efficacy and affinity are overestimated when the operational model (Black et al., 2010) is used to analyze the

concentration-response curves measured in a linear system (i.e., when the assumption $E_m = \alpha^{ref}$ is made).

All agonists in a linear system could be considered "partial agonists" since the signal-transduction mechanism does not limit the response. Consequently, in a linear system it is practically impossible to reach the maximum possible system response by activating the receptor with available agonists. This constraint results in a theoretical failure of the operational model in accurately determining operational efficacies, affinities, and transduction coefficient.

Overestimation of Operational Efficacy in a Linear System

The set of equations eqs. 5a and 5b relate the true value of operational efficacy (τ^A, right-hand side) to that determined experimentally by applying the operational model ($\widetilde{\tau^A}$, left-hand side). It quantifies the systematic error introduced when estimating operational efficacy of drug in a linear system. Since the choice of agonist used as the reference sets the value of $(\tau^{ref} - \tau^A) < 1$, experimentally determined operational efficacy of all test agonists in a linear system is overestimated. This effect is more pronounced for relatively high-efficacy partial agonists, as overestimation increases exponentially as agonist efficacy approaches that of the reference (Fig. 3 – main text).

Underestimation of Affinity

The set of equations eqs. 11a and 11b relates the true value of operational efficacy of a drug A (K_{A_A}, right-hand side) to that determined experimentally by applying the operational model ($\widetilde{K_A^A}$, left-hand side). It quantifies the systematic error introduced when estimating affinity of a drug in a linear system. Since the choice of agonist used as the reference sets the value of $\left(\frac{\tau^{ref}}{\tau^{ref} - \tau^A}\right) > 1$, experimentally determined affinity of all test agonists in a linear system is underestimated.

Estimation of Transduction Coefficient of a Test Agonist Relative to Reference Agonist

The identity of the equations eqs. 16a and 16b state that relative transduction coefficient of a test drug with respect to a reference $\Delta \log\left(\frac{\tau}{K_A}\right)$ is robust to underestimation of E_m resulting from the incorrect assumption of hyperbolicity of a linear system to be hyperbolic, despite it resulting in overestimation of raw τ^A and K_A^A values. However, it is noteworthy that the Hill slope was set to 1 ($n = 1$) in this derivation. If, on the other hand, the system assayed is appreciably linear but is characterized by Hill slopes significantly different to unity, there must be a parameter outside of the bounds of the operational model affecting the system. When Hill slopes are equal to unity, the $\frac{\tau A}{K_A^A}$ ratio is equivalent to simply $\frac{\alpha^A}{EC_{50}^A}$.

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