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Signaling diversity of mu- and delta- opioid receptor ligands: *Re*-evaluating the benefits of β-arrestin/G protein signaling bias

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ABSTRACT

Opioid analgesics are elective for treating moderate to severe pain but their use is restricted by severe side effects. Signaling bias has been proposed as a viable means for improving this situation. To exploit this opportunity, continuous efforts are devoted to understand how ligand-specific modulations of receptor functions could mediate the different *in vivo* effects of opioids. Advances in the field have led to the development of biased agonists based on hypotheses that allocated desired and undesired effects to specific signaling pathways. However, the prevalent hypothesis associating β-arrestin to opioid side effects was recently challenged and multiple of the newly developed biased drugs may not display the superior side effects profile that was sought. Moreover, biased agonism at opioid receptors is now known to be time- and cell-dependent, which adds a new layer of complexity for bias estimation. Here, we first review the signaling mechanisms underlying desired and undesired effects of opioids. We then describe biased agonism at opioid receptors and discuss the different perspectives that support the desired and undesired effects of opioids in view of exploiting biased signaling for therapeutic purposes. Finally, we explore how signaling kinetics and cellular background can influence the magnitude and directionality of bias at those receptors.

1. Introduction

Opioids have been therapeutically used to alleviate pain for centuries. However, their medical application is hindered by adverse effects, particularly those affecting respiratory and gastrointestinal systems [1]. Moreover, these substances can lead to addiction and overdose deaths [2,3]. However, and despite such drawbacks, their unique and powerful analgesic properties make opioid analgesics an essential tool for pain relief and the development of safer opioids remains an urgent necessity.

Opioid analgesics produce their desired and undesired effects *via* G protein coupled receptors that share structural similarities [4] and downstream signaling partners [5–8]. Over the last ten years, it has become clear that ligands acting at this type of receptors may stabilize GPCRs into different active states that distinctively interact with downstream signaling partners [9,10]. Receptors mediating the effect of opioid analgesics are no exception [7,11,12]. The functional consequence of this particular signaling configuration is that ligands stabilizing different active states of the same receptor may preferentially engage distinct downstream effectors. In practical terms, this ligand-based bias in signaling could direct the pharmacological stimulus towards pathways that support desired therapeutic actions and away from those underlying undesired effects.

Not surprisingly, this concept dubbed "biased signaling" or "functional selectivity" [13] was embraced by researchers seeking to improve the side effects profile of opioid analgesics [14,15]. In particular, studies

Abbreviations: GIRK, G-protein coupled inward rectifier potassium channel; Ca_v, Voltage-gated calcium channels; cAMP, Cyclic adenosine monophosphate; AC, Adenylyl cyclase; DOR, Delta-opioid receptor; MOR, Mu-opioid receptor; JNK, c-Jun N-terminal kinase; GRK, G protein-coupled receptor kinase; ARM390, N, NDiethyl- 4-(phenylpiperidin-4-ylidenemethyl) benzamide; SNC80, (+)-4-[(alpha R)-alpha-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N, N-diethyl-benzamide; JNJ20788560, 9-(8-azabicyclo[3.2.1]oct-3-ylidene)-9H-xanthene-3-carboxylic acid diethylamide; DAMGO, [D-Ala(2), N-Me-Phe(4), Gly(5)-ol]-enkephalin; Oliceridine, TRV130; DPDPE, [D-Pen(2),D-Pen(5)]-enkephalin; TRPV1, Transient receptor potential cation channel subfamily V member 1; OIH, Opioid-induced hyperalgesia; FDA, Food and Drug Administration; GPCR, G protein-coupled receptor; CHO, Chinese hamster ovary.

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with transgenic mice had initially hinted to the fact that knock-out of β-arrestin2 could protect from respiratory depression and constipation induced by opioids analgesics [16]. These observations immediately gave place to the notion of mu opioid receptor (MOR) agonists which failed to recruit β-arrestin while maintaining G protein signaling could produce the desired profile [14]. However, the fact that the protective effects of β-arrestin2-knock-out could not be independently replicated [17], and the observation that respiratory and gastrointestinal side effects of opioids are still present in mice expressing MORs in which β-arrestin recruitment is severely compromised have weakened the hypothesis. Moreover, difficulties in distinguishing partial from biased MOR agonists [18] add another layer of complexity when trying to identify therapeutically relevant biased opioid ligands. Below we discuss these issues and complexities.

2. Opioid receptors signaling in relation to their *in vivo* actions

2.1. G protein-driven in vivo actions

Pharmacological studies have revealed the importance of opioid receptors in pain management and compounds targeting these receptors are pursued for the treatment of severe pain. To induce their analgesic effect, opioid receptors control neuronal excitability by inhibiting the flow of the nociceptive information. Gβγ subunits contribute to these actions by reducing neurotransmitter release and suppressing neuronal excitability *via* inhibition of presynaptic voltage-gated calcium (VGCC or Ca_v) channels [19,20], or by promoting hyperpolarization of post-synaptic neurons *via* activation of G protein-gated inwardly rectifying potassium (GIRK or Kir3) channels [7,8]. Thus, opioid modulation of ion channels activity through direct binding of the Gβγ subunits to the channels [7,21,22] is involved in the analgesic effect of opioids (Fig. 1).

In addition to their established efficacy in mediating distinct physiological responses through activation of the Gβγ dimer, opioid receptors are also predominantly coupled to inhibitory G-proteins, which signal by inhibiting the cyclic adenosine monophosphate (cAMP) production [23] following acute opioid stimulation. Modulation of the cAMP signaling by opioid receptors may also contribute to the analgesic effects of opioids, but the exact way in which these signals influence nociception remains unclear. Indeed, data demonstrated that elevated levels of intracellular cAMP were correlated with increased nociception [24]. Also in that context, mice lacking one or multiple AC isoforms or over-expressing those isoforms in specific brain regions have been used to demonstrate the involvement of cAMP signaling to the analgesic effects of opioids. For example, in mice lacking AC5, the ability of DOR agonists to suppress AC activity was absent and the analgesic effect of acute opioid stimulation was reduced compared to wild-type mice [25]. Conversely, pain responses following acute stimulation with morphine did not differ in mice lacking either AC1, AC8 or both isoforms

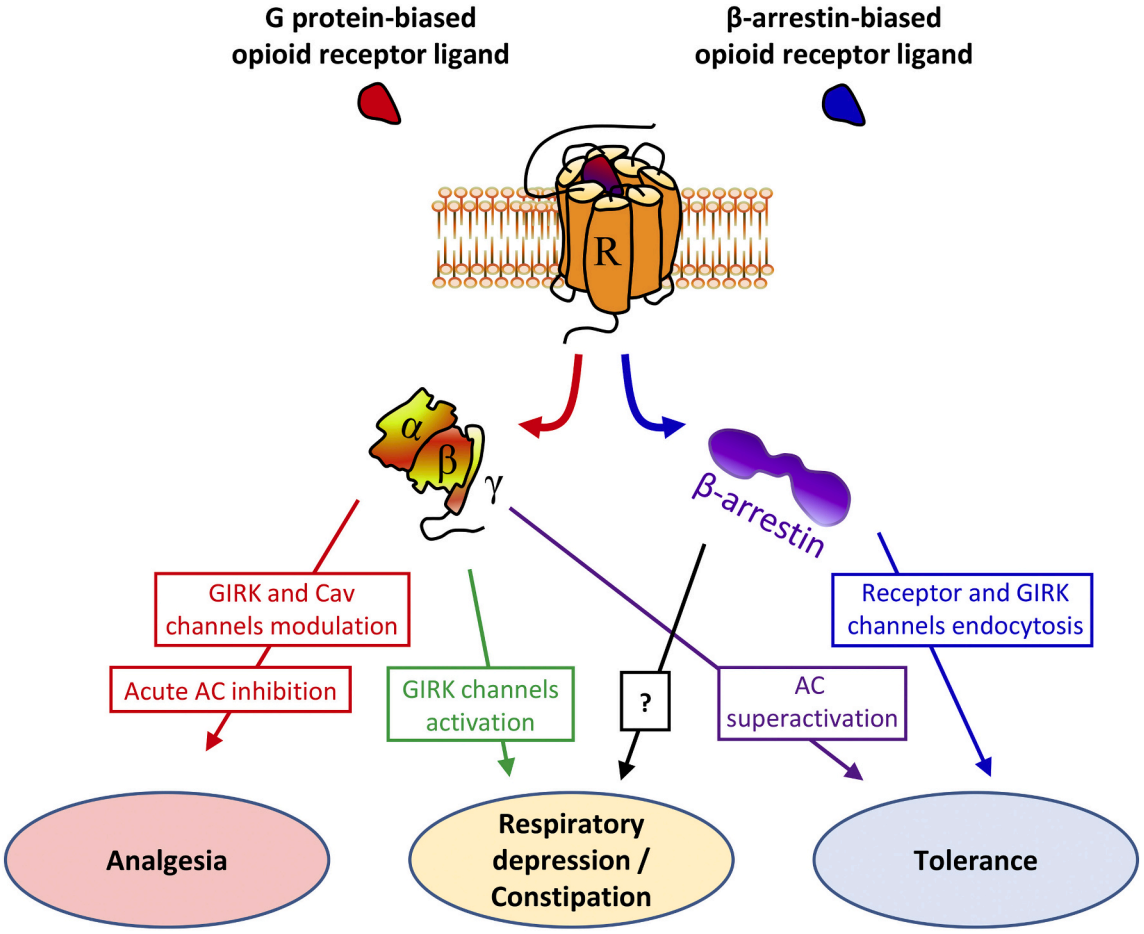

Fig. 1. Schematic representation of opioid receptors signaling.
This scheme describes the different signaling pathways that can be activated by opioid biased ligands. Initial observations associated opioid receptor activation of G protein signaling to the analgesic effect of opioids *via* modulation of ion channels (GIRK and Cav channels) activity and acute inhibition of AC, whereas β-arrestin signaling was associated with side effects such as tolerance to the analgesic effect. However, recent evidence indicated that G protein-dependent pathways could also contribute to the development of opioid side effects and questioned the involvement of β-arrestin signaling in the development of constipation and respiratory depression.
Abbreviations: G-protein coupled inward rectifier potassium channel (GIRK), Adenylyl cyclase (AC).

compared to wild-type animals [26], but enhanced in mice over-expressing AC7 in the brain [27] which demonstrate the involvement of specific AC isoforms in acute opioid antinociception. In addition, elevated levels of intracellular cAMP were shown to increase calcium channel activity in dentate granule cells [28] and dorsal root ganglion neurons [29,30]. Results also showed that opioid receptors reduce neuronal calcium currents by means of activation of the PTX-sensitive Gαo protein in rat dorsal root and nodose ganglion sensory neurons [31,32]. Furthermore, the involvement of Gα proteins in mediating the analgesic effect of opioids was also assessed by silencing specific Gα subunits through intracerebroventricular injection of antisense oligodeoxynucleotides in mice. In those studies, data showed the ability of different opioid agonists to use distinct Gα subunits in inducing their spinal antinociceptive responses [33–35].

Importantly, it should also be noted that in addition to contributing to the analgesic effect of opioids, G protein signaling may also contribute to opioid side effects. These side effects include constipation [1,36] and respiratory depression, that has been associated with Gβγ-mediated activation of GIRK channels [37,38]. Indeed, direct GIRK channel inhibition by Tertiapin-Q markedly reduced the DAMGO-induced respiratory depression in wild-type mice, while mice lacking GIRK2 channel subunits did not show respiratory depression following exposure to fentanyl or DAMGO. It is also well documented that repeated administration of opioid agonists induces adaptive changes in the cyclase pathway, a prototypical G protein effector [39]. These changes contribute to the development of tolerance and physical dependence [40–42], which limit the clinical use of opioids as therapeutic agents. Those adaptive changes are initiated by signaling cascades that involve regulation of membrane-delimited adenylyl cyclase (AC) enzymes [39,43,44] and act simultaneously with β-arrestin proteins signaling, whose implication to the development of analgesic tolerance is detailed below. Together, these studies indicate that G protein signaling does not only mediate the desired but also at least some unwanted effects of opioids.

2.2. Mechanisms associated with β-arrestin-mediated responses

The persistence and extent of opioid-dependent G protein signaling are controlled by multiple factors including opioid receptor desensitization and endocytosis. Scaffolding proteins such as β-arrestins orchestrate both processes and their recruitment to phosphorylated receptors initiate signaling termination with consequent reduction in the associated *in vivo* responses [45]. As a result, these processes have been extensively examined using a panoply of delta- (DOR) and mu-opioid receptor (MOR) ligands. Here, we will highlight the key findings in these processes and discuss their involvement in mechanisms of tolerance to the analgesic effect.

Several studies have proposed that endocytosis (also called internalization or sequestration) of opioid receptors could reduce the development of opioid tolerance. In particular, morphine-activated opioid receptors fail to promote robust endocytosis [12] and signal for long periods leading to AC superactivation [39,40] that contributes directly to the development of morphine tolerance. In this respect, cells expressing MOR mutants that undergo morphine-induced endocytosis have been shown to develop a reduced AC superactivation and reduced cellular tolerance following chronic morphine exposure compared to wild-type MOR expressing cells [46]. Together, these data demonstrate a critical role for receptor endocytosis in preventing both AC superactivation and the development of analgesic tolerance following prolonged opioid use.

However, in contrast to the previously presented mechanism, there is also evidence implicating endocytosis in the development of analgesic tolerance. In this context, endocytosis is thought to contribute directly to tolerance by decreasing the amount of functional receptors at the cell membrane. It was previously shown that chronic morphine treatment in mice lacking β-arrestin2 failed to induce MOR desensitization and to develop antinociceptive tolerance [47]. In addition, several *in vivo* studies have demonstrated that treating mice with the high-internalizing DOR agonist SNC80 desensitizes the receptor and leads to the development of acute tolerance [10,48], whereas, treatment with the low-internalizing DOR agonist ARM390 failed to generate an acute tolerance to the analgesic effect [42,49].

Based on these observations, it could be confusing to speculate whether recruiting or avoiding β-arrestin function might be beneficial in terms of avoiding the loss of analgesic actions. In this sense, recent studies have tried to clarify mechanisms underlying distinct potentials for tolerance by opioid agonists and proposed "biased agonism" as a way of explaining this diversity. This new pharmacological concept proposes that each agonist stabilizes the receptor in a specific active conformation among several others that the receptor could adopt. Each of these ligand-specific conformations supports distinct interactions with regulatory proteins and effectors leading to different patterns of functional desensitization. Such distinct patterns of desensitization are codified by ligand-specific configurations of phosphorylation that coordinate the affinity and stability of interactions with specific downstream regulatory proteins [50,51]. For example, acute analgesic tolerance to morphine was shown to be c-Jun N-terminal kinase (JNK)-dependent but not G protein receptor kinase 3 (GRK3)-dependent, whereas, other MOR agonists like fentanyl, methadone and oxycodone produce an acute analgesic tolerance that is GRK3-dependent and JNK-independent. The observed differences in desensitization could not be simply explained by the intrinsic activity of the agonist used, suggesting specific characteristics for the ligand-receptor complex in determining the ability of the receptor to interact with downstream proteins.

Several studies have shown the presence of a ligand-specific interaction of β-arrestin proteins with DORs [12,52] and revealed some of the functional consequences of such interactions on pain-relieving effects of opioids [10,49]. In primary neuronal cultures, sustained stimulation with SNC80 and DPDPE induced DOR internalization in a β-arrestin, GRK2 and PKC-dependent manner [12]. However, the conformations stabilized by each ligand were distinctively phosphorylated by the two kinases [53] which resulted in DOR phosphorylation at different residues [54]. As a consequence, DPDPE-activated DORs interacted transiently with β-arrestin2 and recycled to the membrane while those activated by SNC80 promoted a stable interaction with no recycling. The capacity of DPDPE to promote recycling and receptor resensitization [55] prevented this drug from developing acute and chronic tolerance, which was observed in SNC80-injected mice [10,56]. Furthermore, examination of brain samples prepared from DOR-eGFP mice showed that SNC, but not ARM390, was able to induce DOR phosphorylation at serine 363 [57]. Consistent with the difference in phosphorylation patterns, different agonists were also shown to trigger a distinct modulation of opioid receptors by β-arrestin proteins. In particular, DORs stimulated by SNC80 preferentially recruited β-arrestin1 in cultured cells, and mice lacking this regulatory protein (β-arrestin1 knock-outs) did not develop tolerance to the agonist. In contrast, exposure to ARM390 or JNJ20788560 preferentially recruited β-arrestin2, but developed tolerance in β-arrestin2 knock-out mice, an effect that corresponded to a reduced rate of DOR resensitization [49]. These findings highlight the importance of ligand-selective conformations in opioid receptors signaling. Therefore, further work is needed to determine how structural characteristics in opioid agonists determine receptor conformations and how the conformational information generated by each ligand is transmitted downstream to regulatory proteins to mediate distinct regulatory responses.

Receptor ability to form signaling complexes with downstream effectors and the interaction of these complexes with regulatory proteins, should also be considered. Monitoring β-arrestin recruitment following sustained DOR stimulation has revealed its implication in the removal of GIRK/Kir3 channels from the plasma membrane [58]. Both DORs and GIRK channels internalized with the same kinetics in a β-arrestin-dependent manner *via* a clathrin/dynamin-mediated endocytic path

[58]. Given the involvement of GIRK channels in inducing opioid analgesia, removing that effector from the cell membrane after chronic administration constitutes another mechanism whereby β-arrestin contributes to reducing the analgesic actions of opioids and the development of tolerance (Fig. 1).

Another mechanism by which β-arrestin modulation of channels contributes to analgesic tolerance involves the transient receptor potential cation channel TRPV1. In this case, β-arrestin2 associates with TRPV1 and scaffolds the phosphodiesterase PDE4D5, which controls the phosphorylation status of TRPV1 at the plasma membrane and contributes to its desensitization [59]. In regard to this type of modulation, chronic activation of DORs by SNC80, and not ARM390, leads to development of behavioral signs of opioid-induced hyperalgesia (OIH). Indeed as a consequence of β-arrestin2 recruitment to SNC-80-activated DORs, β-arrestin2 dissociates form TRPV1 and mediates its sensitization [60]. Similar effect on TRPV1/β-arrestin2 interaction and OIH development was observed following stimulation of MORs with morphine or DAMGO [61]. Together, these findings identify novel mechanisms for β-arrestin in modulating the pain response following chronic opioid administration and reveal new ways in which this regulatory protein may limit the clinical response to opioid analgesics.

3. Biased agonism at opioid receptors

3.1. Efficacy and biased agonism

Drug efficacy was initially defined in quantitative terms describing the capacity of a ligand to stabilize different amounts of the unique active state of the receptor. Now, the concept of efficacy has evolved to incorporate the ligand-specific quality of the response determined by the capacity to stabilize one specific subset of active conformations among several others that the same receptor can adopt. The direct consequence of this conformational diversity is the ability of a ligand to activate a specific subset of signaling pathways among all those controlled by a receptor, a signaling modality that has been dubbed “biased agonism”, “functional selectivity” or “biased signaling” [8,62]. Preferential activation of one signaling pathway over another is currently the focus of drug discovery efforts that try to identify ligands capable of directing the pharmacological stimulus towards pathways that are therapeutically beneficial while avoiding those associated with side effects [63].

3.2. G protein vs β-arrestin signaling bias and expectations of opioid analgesics with reduced side effects profile

Initial observations indicated that mice lacking β-arrestin2 displayed more effective and prolonged analgesia to a single dose of morphine, developed less analgesic tolerance over repeated morphine administration, and displayed reduced constipation and respiratory depression as compared to wild-type mice [16,47,64]. These observations gave rise to the hypothesis that β-arrestin-dependent mechanisms downstream of the MOR mediate the undesired effects of morphine [16,64] and fueled the notion that ligands capable of stabilizing the receptor in a conformation that allows it to activate G protein signaling without inducing β-arrestin recruitment (G protein-biased agonists) can provide safer analgesia [65,66]. However, a number of recent studies have challenged this hypothesis. First, the use of knock-in mice expressing phosphorylation-deficient MORs demonstrated not only persistence of constipation, but also an enhanced respiratory depression by morphine and fentanyl [38]. Since reduced phosphorylation interferes with β-arrestin recruitment to MORs [51,67], these observations are contrary to what one would expect if β-arrestins were driving these side effects. Moreover, another line of evidence pursued independently in different laboratories, could not replicate the initial observations in β-arrestin2 KO mice and reported that morphine and fentanyl similarly induced respiratory depression and constipation in β-arrestin2 knock-out and wild-type mice [17]. These recently published studies bring into question the involvement of β-arrestin as a determinant of the severity of opioid adverse effects and call for better understanding of the opioid-mediated mechanisms that alter the respiratory function and gastrointestinal motility (Fig. 1).

The proposed role of β-arrestin proteins in the unwanted effects of opioids has led to the development of MOR ligands that induce G protein activation with reduced β-arrestin2 recruitment. Oliceridine (TRV130) for example, which completed phase 3 clinical studies, was initially reported to have a profile of robust analgesia with reduced respiratory suppression and gastrointestinal dysfunction compared to morphine in rodent studies [14]. However, additional studies on rodents could not replicate these observations, reporting constipation and abuse-related effects for oliceridine [68,69]. Moreover, when tested on human subjects, this ligand produced similar constipation and respiratory adverse effects compared to morphine at equi-analgesic dose regimens [70,71]. PZM21, another G protein-biased ligand that was also initially reported not to affect respiratory frequency in mice [15], was later found to depress respiration in a manner similar to morphine [72]. At the same time, cumulative evidence indicates that G protein signaling contributes to opioid side effects (see section 1.1), further questioning the rationale of the initial hypothesis. Thus, taken together, the existing evidence indicates that G protein-biased MOR agonists are still likely able to induce severe side effects and urge for the need to develop a better strategy for identifying more effective and tolerated opioid analgesics.

A recent study tried to fulfill this need by designing a new strategy to classify ligands according to signaling similarities across a multiplicity of signaling pathways, associating the resulting categories to the frequency of undesired events as reported in the Food and Drug Administration (FDA)’s pharmacovigilance database [18]. Data showed that: a) ligands within each individual category had similar signaling properties, b) the major difference among categories was signaling efficacy at all pathways and not signaling bias, and c) ligands in different categories displayed distinct frequencies of respiratory and gastrointestinal events, where the most efficacious ligands were those with highest frequency of reported side effect. The authors also concluded that failure to recruit β-arrestin by various ligands characterized in the study was simply an indication of partial agonism and not signaling bias [18,73]. This also seems to be the case for biased MOR ligands like TRV130, PZM21 and SR compounds [74]. Thus, all biased ligands identified thus far could simply produce less side effects because they are partially effective at stimulating the receptor, and not necessarily because they preferentially target G protein over β-arrestin signaling.

4. Bias is time and cell dependent

It is now well established that the presence and magnitude of bias measures may not only vary over time (time-dependent), but may also depend on the cellular system used (cell-dependent). This raises the question of whether the value of bias estimated from *in vitro* data could predict the biased responses that ligands may display *in vivo*. Therefore, considering these variables during bias analysis may help increasing the value of bias measures for a more effective identification of signals and ligands that can support analgesia with fewer side effects.

4.1. Signaling kinetics and biased agonism

Within the same cellular background, signaling diversity of opioid receptor ligands has been interpreted as indicative of ligand-specific conformations [7,18,75]. Also, it is becoming increasingly apparent that these different active conformational states of the receptor could be influenced by the dynamics and kinetics of interaction between the ligand and the receptor (Fig. 2). In matter of fact, several studies have investigated the dynamic conformational changes in GPCRs following ligand binding and showed the presence of a significant structural variability in the receptor conformations stabilized by different biased ligands [76,77]. Those receptor conformations might change over time

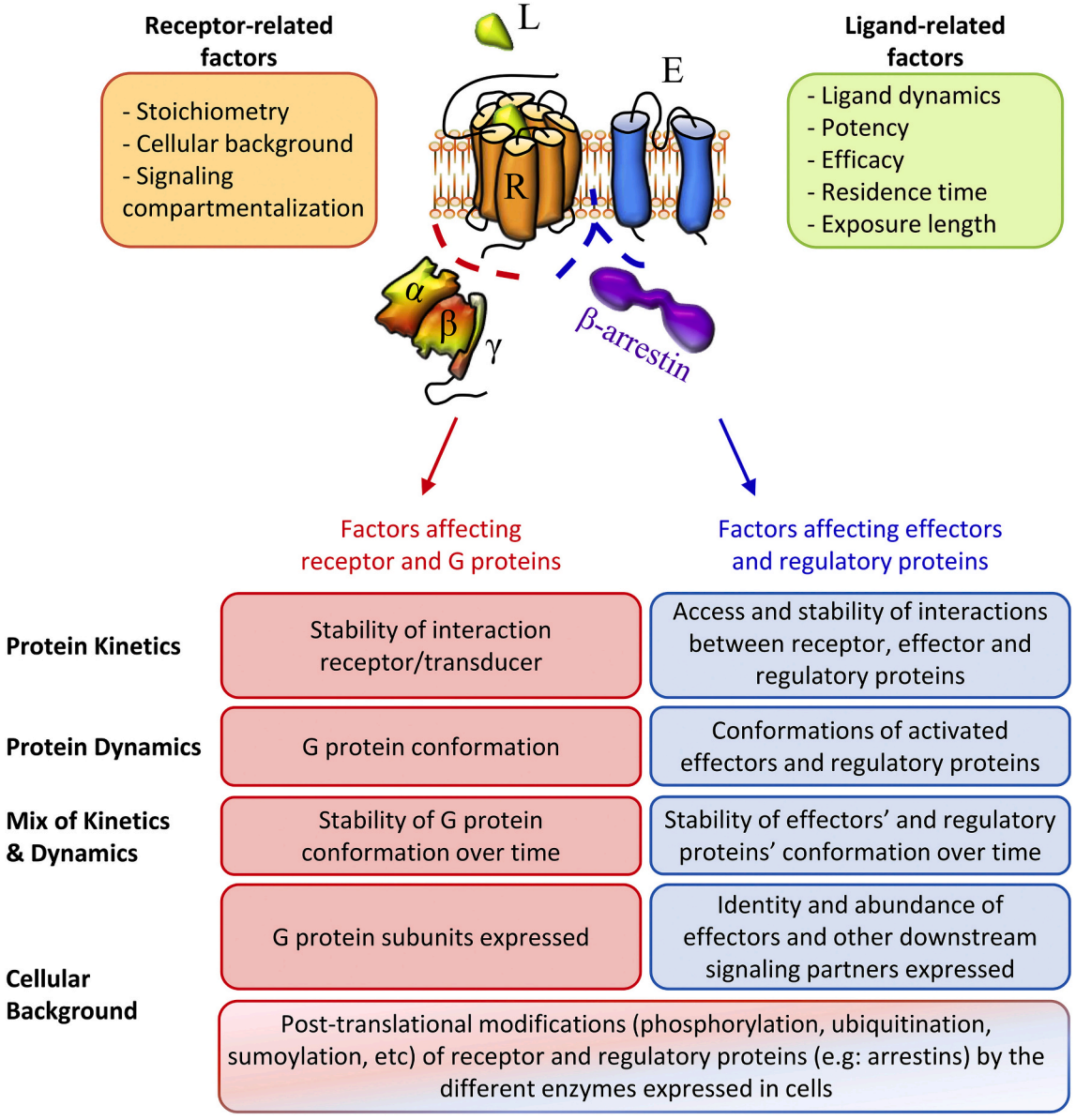

Fig. 2. Compilation of the different factors that can influence biased signaling and the estimation of bias magnitude.
The quality and stability of receptor interactions with ligands, G proteins, effectors and downstream regulatory proteins can influence bias estimation. Those interactions depend on several intra-molecular (e.g: conformation, dynamics, efficacy, potency), extra-molecular (e.g: expression, localization), and inter-molecular (e.g: residence time, oligomerization, post-translational modifications) factors in the receptor, downstream interacting proteins, and/or the binding ligand. Colored dashed lines indicate factors that can influence the receptor/transducer interaction (red) or the transducer/effector interaction and interactions between the receptor, effector and regulatory proteins (blue).
Abbreviations: Ligand (L), receptor (R) and effector (E).

and be influenced by the ligand dynamics at the receptor. In addition, the residence time on the receptor (kinetics of ligand-receptor interaction) for a range of ligands, was found to be correlated to their efficacy in stimulating distinct signaling pathways at various GPCRs [78,79].

Several models have attempted to interpret the correlation between the kinetics of ligand binding and agonist efficacy. In the original residence model, a high-efficacy ligand dissociates rapidly from the receptor, allowing another agonist to bind, whereas, a low-efficacy ligand dissociates slowly from the receptor and act as a competitive antagonist by preventing agonist binding [80]. This concept was subsequently challenged by another model that positively correlates ligand binding to efficacy, and considers long ligand residence time at the receptor as an opportunity to induce more G protein activation as a consequence of trapping the ligand with an internalized receptor. Accordingly, opioids with long-residence times can signal from endosomes [81], and are thus expected to induce more signaling from novel locations apart from the membrane.

Differences in ligand residence times were also shown to affect ligand bias and the identity of the different effectors and regulatory proteins engaged over time by the ligand-occupied receptor [82,83]. This suggests that ligands can stabilize different receptor conformations over time and therefore change bias towards different signaling pathways in a time-dependent manner. This possibility was recently tested for MOR agonists but results showed that fast dissociating agonists such as DAMGO, morphine, loperamide and oliceridine, and the slow dissociating buprenorphine [84,85] displayed potencies that remained stable over time while activating $G\alpha_{i2}$ and $G\alpha_{oA}$, as well as recruiting β-arrestin1 and β-arrestin2 [85]. Accordingly, differences in the binding

kinetics of those selected agonists were not a critical factor when bias for G protein activation and β-arrestin recruitment was compared.

Also, it is of interest to note that a receptor may adopt different conformations as it interacts with different signaling transducers or regulatory proteins. Furthermore, factors influencing ligand efficacy may extend beyond receptor conformations and expand to involve a diversity of transducers [86] and regulatory protein [87] conformations (Fig. 2). Indeed, by their ability to modify the signaling efficacy of ligands, those time-dependent events add a new dimension to signal transduction and therefore need to be considered during the identification and interpretation of biased agonism at GPCRs.

Besides protein conformations, it should be noted that every signaling pathway has different activation and desensitization kinetics that could be differentially triggered by different ligands. Thus, biased agonism detected at a specific pathway by comparing the maximal response of ligands following a precise incubation period may change at later time points. An example of such temporal shift in bias was recently demonstrated for the MOR and showed that sustained cytosolic ERK phosphorylation was detected following receptor stimulation with morphine, whereas, DAMGO only induces a transient cytosolic ERK activation [88]. Different biased responses could also be detected within the same signaling pathway while using different experimental assays that require different incubation times [82].

Thus, further studies are needed to clarify how interaction kinetics, at the level of opioid receptors, transducers or downstream proteins, could affect ligand efficacy and influence the choice of activating one pathway over another and why does kinetics affect bias for some GPCRs/ligands but not for others.

4.2. Cellular background and biased agonism

The ability of a ligand to distinguish among receptors associated with different signaling partners is the main feature that characterizes biased agonism. Therefore, modifying the cell content by altering the stoichiometry of receptors, transducers, effectors or regulatory proteins will likely affect bias (Fig. 2). However, if tested in the same cellular system, a change in ligand preferences due to the expression of signaling partners, also referred to as system bias, will equally affect all the tested ligands and could therefore be eliminated by quantifying bias relative to a reference ligand using the operational model [89,90]. Removing the influence of the cellular background during the quantitative analysis of bias in a specific cellular system is essential. However, this normalization procedure cannot be applied to compare the signaling preferences of ligands in different cellular backgrounds since any difference in the expression level of signaling partners between cells will modulate the coupling efficiencies of ligands for different signaling pathways and consequently affect both the magnitude and directionality of bias. Thus, optimization of transfection conditions will be required in order to mimic expression levels of key signaling partners in the two cellular backgrounds. For example, overexpressing specific GRK isoforms could enhance β-arrestin recruitment in cells that lack those isoforms [18,91]. But again, changing the cellular content by altering the expression of key effectors could result in affecting the expression levels of others and directing ligand signaling to certain specific pathways. Therefore, measuring the ability of a ligand to activate signaling pathways under identical conditions and in different cellular systems can be difficult to achieve and stresses the importance of stating the experimental conditions in which measures were taken when reporting biased agonism.

The effect of variations in the cellular background on biased agonism has been reported at the MOR. Studies performed in HEK293 cells showed that both endomorphins 1 and 2 were biased towards β-arrestin2 recruitment over G protein activation compared with leu-enkephalin [92,93], whereas, only endomorphin-1 was reported to be biased towards β-arrestin2 over G protein activation in Chinese hamster ovary (CHO) cells [94].

In this regard, bias calculation methods have made distinct assumptions as to how the ligand, the receptor and its downstream signaling partners influence each other. One of the methods assumes that association of the activated receptor with different signaling proteins may distinctively modify its affinity for different ligands [89,90], whereas, another method assumes independence between ligand affinity and receptor interactions with cellular partners [95]. However, these methods admit that bias is the consequence of interactions between receptor conformations stabilized by different ligands and distinct signaling partners, which raises the question as to whether bias involving internalization signals would be maintained across distinct cellular backgrounds. Experiments characterizing the internalization mechanisms of DORs with different agonists have indicated that both GRK2 and PKC are required for receptor sequestration in cortical neurons but not in HEK293 cells, with β-arrestin contributing to DOR internalization in both cell types [12]. This limitation is not exclusive to heterologous expression systems, since molecular determinants of internalization are also distinct across neuronal populations expressing opioid receptors [57,96,97]. Consistent with this notion, bias measures involving internalization as one of the responses revealed important variations in the magnitude and direction of bias depending on whether bias was evaluated in AtT20 or CHO cells [94,98].

Indeed, different cell types have diverse abundance of signaling effectors [99], which could explain the variations in the bias observed (Fig. 2). Overexpressing different signaling partners, such as GRK proteins, and measuring their effect on signaling have been intensely investigated [15,18,98]. It has been demonstrated that the expression level of GRK2, a key mediator in β-arrestin recruitment and thereby receptor internalization, can affect the degree of bias at the DOR [18] and the MOR [15,98] in HEK293 cells. Data also indicated that overexpressing GRK2 or GRK5 proteins in HEK293 cells can even promote G protein biased ligands, such as buprenorphine, to recruit β-arrestin2 proteins to the MOR [18], and promote internalization [85]. Hence, depending on the cellular contents of GRK2/5, buprenorphine's preferential activation of G proteins over β-arrestin recruitment and internalization could vary. Studies have also reported that the ability of ligands to trigger the DOR internalization can change profoundly by altering the expression level of GRK2, resulting in modification of internalization bias in neurons [12]. Moreover, in CHO cells, bias of endomorphin-1 between the inhibition of cAMP production and the β-arrestin1/2 recruitment was also shown to change when the level of GRK2 expression was increased [98]. Together, those studies indicate that biased responses of ligands are not necessarily conserved across cells with different cellular backgrounds and indicate the importance of reporting and characterizing the cellular system used when considering biased agonism.

Moreover, assessing ligand pharmacology within physiologically relevant systems should also be of great interest. Particularly, comprehending the diversity in cell backgrounds, not only between cellular systems for *in vitro* experiments, but among different neuronal populations might help in identifying new properties of biased signaling at opioid receptors. Advancements in single cell phenotyping strategies and high-throughput sequencing using barcoded oligonucleotides are making this possible. The use of such methods can provide a unique opportunity of reconstructing the intracellular signaling network by measuring protein (receptors, transducers, effectors, and regulatory proteins) abundance, state, and even interactions on a single-cell level. Indeed, monitoring changes in proteins expression and interactions following opioids stimulation could than help answering questions like: Are neurons involved in pain regulation the same as those inducing side effects such as respiratory depression? Are downstream signaling proteins mediating analgesia the same as those mediating the different opioid side effects? Answering such questions can undoubtedly aid in identifying the signaling pathways involved in the desired opioid actions and consequently, in developing biased ligands that specifically target them.

5. Concluding remarks and future avenues

The key feature of biased ligands is their ability to stabilize specific receptor conformations leading to ligand-specific activation of signaling effectors that support different physiological responses. Studies discussed in this review describe the different pathways that connect activated opioid receptors with their downstream signaling effectors. Results from such studies were used to generate hypotheses for designing opioid agonists that link discrete signaling pathways to specific physiological outcomes. However, some of these hypotheses were recently challenged, and biased opioid analgesics generated according to the proposed rationale still induce severe undesired effects, urging for an alternative strategy to develop more effective and better tolerated opioid analgesics.

Moving forward, different aspects seem essential to improve the side effects profile of opioid analgesics. In first place, establishing which signals actually mediate respiratory depression and gastrointestinal side effects, and determining if they differ from those mediating analgesia. In this sense, single-cell phenotyping of MOR-expressing neurons in brainstem and muscular/submucosal digestive tract plexuses should provide valuable information to validate *via* transgenic approaches. Second, if after such survey is completed, biased ligands could remain a viable strategy for reducing respiratory side effects and constipation, and identification of such ligands should take into account confounders such as system bias and time-dependent factors, which may have been overlooked in previous attempts.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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