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Opioid Receptor Interacting Proteins and the Control of Opioid Signaling

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Abstract

Opioid receptors are seven-transmembrane domain receptors that couple to intracellular signaling molecules by activating heterotrimeric G proteins. However, the receptor and G protein do not function in isolation but their activities are modulated by several accessory and scaffolding proteins. Examples include arrestins, kinases, and regulators of G protein signaling proteins. Accessory proteins contribute to the observed potency and efficacy of agonists, but also to the direction of signaling and the phenomenon of biased agonism. This review will present current knowledge of such proteins and how they may provide targets for future drug design.

Keywords

Opioid receptors; regulator of G protein signaling proteins; arrestins; G protein-coupled receptor kinases; protein kinase C; biased agonism; drug discovery

1. INTRODUCTION

Opioid receptors are the targets of clinically-relevant analgesic drugs that are typified by the small molecule agonist morphine. Decades of research have led to the identification and characterization of three primary opioid receptor types, mu (μ), delta (δ), and kappa (κ), as well as their endogenous peptide ligands, which are widely expressed throughout the central and peripheral nervous systems [1, 2]. Although they produce effective analgesia, drugs targeting opioid receptors also elicit several unwanted side effects, depending on the opioid receptor type involved [3]. Further limiting the clinical utility of opioid drugs is the development of adaptations such as tolerance, in which larger doses of drug are required to produce the desired effect, and dependence, which is manifested as a severe withdrawal syndrome upon drug discontinuation. Moreover, opioid receptors are also known to modulate brain reward circuitry and therefore represent important targets in the neurobiology of addiction. Current research in opioid receptor physiology focuses on identifying the cellular mechanisms that underlie the behavioral effects of opioids to identify new targets for the development of improved analgesics with reduced side effects.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

1.1. Opioid Receptor Physiology

Opioid binding sites were first identified in 1973 by three independent research groups [4–6]. Shortly thereafter, three distinct opioid receptor types were identified based on physiological observations; each receptor was named after the drug or assay system with which it was characterized: the μ receptor for morphine, the κ receptor for ketocyclazocine [7], and the δ receptor for the mouse *vas deferens* [8]. Opioid receptors were classified solely on the basis of physiology until the δ receptor was cloned in 1992 [9, 10]. This was followed by cloning and molecular characterization of all three opioid receptor types [11–13]. Later, the nociceptin receptor (NOPR) was classified as a fourth opioid receptor type [14]. Given that only a few studies have evaluated NOPR regulation [15], this receptor will not be examined in the present review.

Physiological and behavioral responses to opioid agonists have since been more fully characterized using opioid receptor-specific gene knockout strategies in mice [3]. Although all three opioid receptor types are able to modulate nociceptive transmission [16], it is clear that the behavioral effects of morphine are mediated primarily by the μ receptor, as demonstrated by a loss of morphine-mediated analgesia, dependence, and reward in μ receptor knockout mice [17]. Thus, both the analgesic effects and the addictive liability of the prototypical opioid agonist morphine are mediated by the same receptor. Similarly, although δ and κ receptor agonists produce some degree of analgesia, they are also associated with unwanted side effects, including dysphoria mediated *via* the κ receptor [18] and convulsions mediated by the δ receptor [19]. Agonists at the δ receptor have also been shown to modify mood states [20], which could be beneficial or deleterious depending on the desired outcome. Given that both the desired behavioral effects and the unwanted side effects produced by μ , δ , and κ receptor agonists cannot be differentiated on the basis of opioid receptor type, viable strategies toward developing more effective analgesics have aimed to identify differences in signaling pathways downstream of the receptor.

1.2. Opioid Receptor Signaling and Regulation

Opioid receptors belong to the family of seven-transmembrane, G protein-coupled receptors (GPCRs). GPCRs convert extracellular signals (e.g. neurotransmitters, hormones, etc.) into intracellular responses by coupling to heterotrimeric G proteins composed of a $G\alpha$ subunit bound to a $\beta\gamma$ heterodimer [21, 22]. In the inactive state, the $G\alpha$ subunit is bound to the guanine nucleotide GDP. Agonist activation of a GPCR leads to a conformational change within the receptor that is propagated to the G protein [23, 24], resulting in the exchange of GTP for GDP on the $G\alpha$ subunit. This leads to the dissociation of $G\alpha$ -GTP from the $\beta\gamma$ heterodimer and subsequent modulation of downstream effector proteins (Fig. 1a). There exist over 15 $G\alpha$ subtypes that are divided into four major families based upon structural and functional homology: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$. Each GPCR may interact with G proteins from one or more of these families [25]. In particular, μ , δ , and κ opioid receptors couple to G proteins of the $G\alpha_{i/o}$ family [26–28], which are called “inhibitory” G proteins for their classic inhibitory activity on adenylate cyclase (AC) enzymes. As such, opioid receptor stimulation has been shown to produce AC inhibition [29], as well as Ca^{2+} channel inhibition [30], G protein-coupled inwardly-rectifying K^+ channel (GIRK) activation [31],

32] phospholipase C (PLC) stimulation [33], and mitogen-activated protein kinase (MAPK) activation [34]. For a comprehensive review of opioid receptor signaling, see Law *et al.* [35].

Termination of G protein signaling is achieved *via* intrinsic hydrolysis of GTP to GDP by the GTPase domain of the $G\alpha$ subunit, which results in the reassociation of $G\alpha$ -GDP and $G\beta\gamma$. Although the intrinsic GTP hydrolysis rate for $G\alpha_{i/o}$ is slow (on the order of minutes), this process is significantly accelerated *in vivo* by regulator of G protein signaling (RGS) proteins (Fig. 1a), which enhance the GTPase activity of $G\alpha$ subunits [36–38]. Thus, RGS proteins function on a short timescale (seconds to minutes) to negatively regulate GPCR signaling, including signaling mediated by opioid receptors.

In addition to signal termination by RGS proteins, GPCRs are also subject to regulation by desensitization, which serves to dampen signaling on an extended timescale (minutes to hours) [39]. For most GPCRs, desensitization is initiated when the receptor is phosphorylated by G protein-coupled receptor kinases (GRKs) or other second messenger-regulated kinases such as protein kinase C (PKC; (Fig. 1b) [40]. Proteins known as arrestins are then recruited to the phosphorylated receptor and serve not only to block further G protein coupling, but also to facilitate receptor internalization *via* association with clathrin and clathrin-adaptor proteins (Fig. 1c)[41]. Following internalization, receptors may either be recycled back to the plasma membrane for future activation or degraded *via* one of several possible degradation pathways [42]. The mechanism of opioid receptor desensitization is similar to that described for other GPCRs, and is thought to be an initial stage in the development of analgesic tolerance [43].

It has recently begun to be appreciated that arrestin proteins play multifaceted roles in GPCR signaling beyond simply mediating desensitization and internalization. Specifically, arrestin binding to phosphorylated GPCRs can initiate signaling independently of G protein coupling (Fig. 1c, d) [44]. This is also true for the opioid receptors, which have been shown to activate various intracellular kinases through a process that requires arrestin [15, 45]. Though the behavioral consequences of arrestin-mediated signaling at opioid receptors are still being elucidated, it is possible that the characterization of such pathways will lead to new avenues for the development of effective analgesics with reduced side effects.

Although opioid receptor activation of G proteins initiates signaling, the receptor and G protein are regulated by several accessory and modifier proteins that include RGS proteins, arrestins, and kinases, among others [46, 47]. These accessory proteins contribute to the strength of opioid agonist signaling and behavior by affecting both the ability of the receptor to activate G proteins and the lifetime of activated $G\alpha$ subunits. Further, these proteins may modify the direction and/or type of signaling that occurs and so contributes to a phenomenon that has been termed “biased agonism”, “functional selectivity”, or “ligand-directed signaling”. This review will provide an overview of how RGS proteins, arrestins, and kinases modulate μ , δ , and κ opioid receptor signaling, and so contribute to opioid receptor physiology (Table 1). Lastly, the review will consider opioid receptor-interacting proteins as targets for future drug discovery.

2. RGS PROTEINS AND OPIOID RECEPTOR SIGNALING

The existence of GTPase-enhancing proteins was initially proposed when it was recognized that the intrinsic GTP hydrolysis rate for $G\alpha_{i/o}$ subunits ($\sim 1-3 \text{ min}^{-1}$) was significantly slower than that predicted from the rate of inactivation of AC inhibition ($\sim 1-3 \text{ s}^{-1}$). Such a negative regulator of G protein signaling was first described in yeast, where it was observed that a mutation in the protein Sst2p resulted in enhanced signaling through G protein-mediated pathways [48]. At the same time, De Vries *et al.* [27] identified a $G\alpha$ -interacting protein (GAIP) using a yeast two-hybrid screen, while Siderovski and colleagues [49] described G0S8, a mammalian homolog of Sst2p that shared a conserved domain (now known as the RGS homology, or RH domain) with several other mammalian proteins. It was finally appreciated that these proteins belonged to a larger superfamily of G protein regulators when it was discovered that Egl-10, a protein that negatively regulates signaling through $G\alpha_o$ in *C. elegans*, shared significant homology with Sst2p, G0S8, GAIP, and several other mammalian proteins of previously unknown function [50]. Today there are over 30 known proteins that contain the conserved RH domain, the majority of which have been shown to negatively regulate physiological responses mediated by G proteins [38].

The superfamily of RGS proteins is divided into several families based upon the structure of the RH domain and the presence of other protein-protein binding domains [37]. “Classical” RGS proteins of the R4, RZ, R7 and R12 families (Fig. 2) are best-known for their ability to serve as GTPase-accelerating proteins, or GAPs, for $G\alpha$ subunits by enhancing GTP hydrolysis and reducing the lifetime of activated $G\alpha$ (Fig. 1a). Structural characterization of $G\alpha$:RGS complexes has revealed that RGS proteins enhance GTP hydrolysis by binding to active $G\alpha$ -GTP proteins and stabilizing the transition state during GTP hydrolysis [51]. Whereas the smaller, simple RGS proteins (R4 and RZ families) function primarily as GAPs, some of the larger, complex RGS proteins (R7 and R12 families) have been shown to serve additional regulatory functions that are mediated through various protein-protein interacting domains. These activities include scaffolding, facilitation of G protein signaling, regulation of non-G protein signaling, and signal transduction at non-GPCRs [36, 52]. “Non-canonical” RGS proteins of the RA and RL families, including GRK2 and p115 RhoGEF, contain an RH domain yet do not possess GAP activity. However, these proteins are able to regulate GPCR signaling by directly interacting with and/or scaffolding G proteins [52].

2.1. Effects on Opioid Signaling

RGS proteins have been hypothesized to show varying degrees of selectivity for specific GPCRs, G proteins, and/or signaling pathways [37]. However, due to the diversity of mammalian RGS proteins, it is often difficult to identify a single RGS isoform involved in a response of interest. Therefore, some evidence demonstrating that RGS proteins regulate opioid receptor signaling comes from studies using RGS-insensitive (RGSi) mutant $G\alpha_{i/o}$ proteins [53]. A mutant $G\alpha$ protein resistant to RGS GAP activity was initially discovered in yeast [54]. This mutation, a Gly to Ser substitution at amino acid position 183 in $G\alpha_{i1}$ (or the analogous position in other $G\alpha$ subtypes), does not alter intrinsic GTPase activity yet prevents RGS protein binding and GAP activity [55]. Thus, evaluation of signaling mediated

by RGSi $G\alpha_{i/o}$ proteins in cell systems [56, 57] or *in vivo* [58] can illuminate the role of endogenous RGS proteins in a response or behavior of interest.

The majority of the work utilizing RGSi $G\alpha_{i/o}$ proteins in the context of opioid receptor signaling has evaluated cellular responses to μ receptor agonists in heterologous expression systems. In cells expressing RGSi $G\alpha_o$ subunits, endogenous RGS proteins negatively regulate μ opioid receptor signaling to AC and MAPK [59, 60]. In the absence of RGS regulation of $G\alpha_o$, the μ receptor agonists morphine and [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO) produced increased levels of cellular tolerance [61] and dependence, as measured by AC supersensitization [62], indicating that RGS-mediated inactivation of $G\alpha$ subunits is protective against these adaptations. Moreover, morphine supraspinal analgesia was significantly enhanced in a transgenic “knock-in” mouse expressing RGSi $G\alpha_o$ protein, demonstrating that RGS proteins are negative regulators of this behavioral response (Lamberts *et al.*, unpublished observations).

Although this has provided evidence that endogenous RGS proteins play a role in opioid receptor signaling, it is impossible to discern from these studies whether a specific RGS isoform is responsible for the observed changes. Thus, approaches utilizing knockdown, overexpression, or constitutive gene knockout of individual RGS proteins have further supported the importance of RGS proteins in opioid receptor signaling and behavior [63]. There is considerable experimental evidence of roles for several RGS proteins in opioid receptor signaling.

R4 Family—R4 family members are relatively small proteins (~20–30 kDa) that contain short N- and C-terminal regions flanking the RH domain (Fig. 2) [64] with an N-terminal amphipathic helix that may be necessary for membrane targeting and full biological function [65]. R4 family members function as GAPs for both $G\alpha_q$ and $G\alpha_{i/o}$ proteins [66].

RGS4 itself has been extensively studied as a modulator of opioid signaling. RGS4 is enriched in heart and central nervous system (CNS) [67–69]. Expression is especially high in CNS regions such as the cortex, thalamus, and brainstem, as well as the dorsal root ganglion, areas where there are also high levels of opioid receptors [70]. Importantly, RGS4 has been shown to interact directly with μ and δ opioid receptors both *in vitro* [71, 72] and in brain tissue [73], suggesting that RGS4 modulates opioid receptor function.

Numerous studies have demonstrated that RGS4 functions as a negative regulator of μ opioid receptor signaling in heterologous expression systems. For example, in HEK293 cells expressing the μ opioid receptor, introduction of RGS4 has been shown to reduce agonist-mediated inhibition of AC and activation of MAPK [71, 72, 74–76]. Moreover, overexpression of RGS4 in neurons of the locus coeruleus (LC) attenuated morphine-induced electrophysiological responses [77]. In contrast, opposing work suggests that μ receptor signaling is not modulated by RGS4. For instance, expression of RGS4 did not alter μ opioid receptor-induced pigment aggregation in *Xenopus* oocytes [78]. Furthermore, shRNA-mediated knockdown of RGS4 in SH-SY5Y cells did not alter morphine-induced inhibition of AC or activation of MAPK [79].

Conflicting studies have also been published regarding the role of RGS4 in δ opioid receptor activity. In SH-SY5Y cells, knockdown of RGS4 resulted in a reduction in the ability of the δ receptor-selective agonist SNC80 to inhibit AC and activate MAPK, indicating that RGS4 negatively regulates δ receptor signaling in these cells [79]. Similarly, introduction of RGS4 into HEK293 cells expressing the δ opioid receptor resulted in a loss of agonist-mediated MAPK activation [72]. In contrast, another study in HEK293 cells demonstrated that exogenously applied RGS4 did not affect δ opioid receptor-mediated inhibition of AC [76].

These differing observations concerning the role of RGS4 in opioid receptor signaling may be a consequence of the different experimental conditions used in each study, including the choice of cell type (heterologous *vs.* endogenous) and the method of RGS4 manipulation (overexpression *vs.* knockdown). It is possible that in situations where several components of a signaling pathway are overexpressed, there is reduced selectivity between receptors, G proteins and/or RGS proteins. Therefore, systems in which these components are expressed at endogenous levels, such as in SHSY5Y cells, may exhibit more physiologically-relevant behavior. Indeed, findings obtained using shRNA knockdown of RGS4 in SH-SY5Y cells (*i.e.* that RGS4 does not modify μ receptor signaling) more closely mimic behavioral studies in RGS4 knockout mice (see below).

By comparison to μ and δ opioid receptors, far less is known regarding RGS4 modulation of the κ opioid receptor. RGS4 introduced into oocytes reduced the ability of κ agonists to activate K^+ currents, suggesting that RGS4 does control κ receptor signaling [80]. Furthermore, activation of κ opioid receptors expressed in PC12 cells was shown to increase RGS4 mRNA levels; increased RGS4 protein might then inhibit $[g3]$ receptor signaling [81].

Knowledge concerning the contribution of RGS4 to μ opioid receptor physiology comes exclusively from studies in which RGS4 knockout mice were evaluated for opioid-dependent behavioral responses (Table 1). Global knockout of RGS4 does not affect morphine analgesia or analgesic tolerance, although increased withdrawal behaviors have been reported [82]. Surprisingly, RGS4 was a positive modulator of fentanyl and methadone antinociception [82]. Morphine reward, measured by conditioned place preference (CPP), was also enhanced following selective knockout of RGS4 in the nucleus accumbens (NAcc). Thus, RGS4 appears to regulate some μ opioid signaling pathways but not others, as measured using a behavioral model. Further studies are clearly warranted to understand the specific function of RGS4 in each of these behaviors.

Other R4 family members that have been shown to modulate μ opioid systems, at least in cultured cells, include RGS1, 2, and 8 [75, 76, 78]. In behavioral studies, knockdown of either RGS2 or RGS3 in mouse brain reduced morphine spinal analgesia [83].

RZ Family—Knockdown of RZ family RGS proteins (Fig. 2) in mouse brain, including RGS17, 19, and 20, resulted in the enhancement of μ opioid receptor-mediated analgesia and the facilitation of morphine analgesic tolerance [84–86]. In addition, knockdown of the RGS19 binding protein GIPC (GAIP-interacting protein C-terminus) that links RGS19 to a variety of signaling molecules also increased morphine antinociception [85]. Furthermore,

RGS19 has been shown to both interact with and modulate signaling at δ and κ receptors in cell culture systems [87, 88].

R7 Family—This family comprises large, complex RGS proteins that contain multifunctional domains for protein-protein interaction (Fig. 2)[89]. In addition to the conserved RH domain, each member of the R7 family contains a DEP (Disheveled, Egl-10, Pleckstrin) domain with a helical extension (DHEX) and a GGL (G protein gamma-like) domain. The DEP/DHEX domain has two primary functions. First, it is thought to mediate the interaction between R7 proteins and specific GPCRs [90]. Secondly, it binds the accessory protein R7BP (R7 family binding protein); this association is important for trafficking [91, 92], membrane localization [93], and overall stability [94]. The GGL domain exhibits significant homology to other $G\gamma$ subunits and binds the non-signaling $G\beta$ subunit $G\beta_5$ with high affinity [95]. The interaction between R7 RGS proteins and $G\beta_5$ is essential for RGS protein stability and biological activity [96–98]. In general, R7 RGS proteins have been shown to preferentially function at $G\alpha_o$ [95, 99, 100].

There is most evidence of a role for the R7 family member RGS9–2 in opioid receptor signaling. RGS9 exists as two variants that are alternatively spliced from a single gene: RGS9–1 is retina-specific and exhibits very little expression outside of the eye, whereas RGS9–2 is the brain-specific isoform [101, 102]. RGS9–2 is highly enriched in the striatum [68], although it is also expressed in regions associated with nociceptive processing, including the periaqueductal gray (PAG), spinal cord, and dorsal root ganglion [103]. Given that these regions also express high levels of opioid receptors, it is not surprising that RGS9–2 is able to interact with μ receptors both *in vitro* [104] and in PAG membranes [105], and functions as a negative regulator of μ opioid receptor signaling in single cell systems [76, 102].

Several studies have addressed the role of RGS9–2 in opioid receptor-mediated behaviors (Table 1). Knockdown of RGS9–2 expression in mouse brain using antisense oligodeoxynucleotides resulted in enhanced μ opioid receptor-mediated analgesia in the tail-flick assay, which is thought to be a measure of spinal nociception [83, 106, 107]. RGS9 knockout mice (traditional genetic manipulation techniques do not allow for the ablation of only one RGS9 splice variant) showed an increase in morphine-mediated analgesia in the hot-plate test, which is thought to be a measure of supraspinal nociception [108]. In addition, knockdown of RGS9–2 [83, 86, 105–107] or knockout of RGS9 [108] results in delayed analgesic tolerance to morphine, suggesting that RGS9–2 promotes rapid morphine tolerance, presumably by acting to inhibit μ opioid receptor signaling. There is also enhanced dependence to morphine in RGS9 knockout mice [108].

Signaling *via* the δ opioid receptor has also been shown to be negatively modulated by RGS9–2 *in vitro* [76], although there are no published studies that examine the effect of RGS9–2 on κ opioid receptor signaling. Surprisingly, knockdown of RGS9–2 resulted in a reduced antinociceptive effect of the δ -selective opioid [D-Pen^{2,5}]-enkephalin (DPDPE) in mice [106, 107], indicating that RGS9–2 may function as a positive regulator of δ opioid receptor signaling *in vivo* (Table 1). This was also observed with the other R7 family members RGS6 and 11 [106]. In contrast, knockdown of RGS7 enhanced δ receptor-

mediated analgesia [106]. The mechanisms underlying these contradictory effects remain to be elucidated.

R12 Family—RGS proteins of the R12 family (Fig. 2) have not been studied in great detail, although there are isolated reports. For instance, knockdown of RGS12 led to an increase in μ opioid antinociception [83]. Further, overexpression of the R12 family member RGS10 was shown to reduce μ opioid signaling in a heterologous cell system [76].

Overall, *in vitro* studies with RGS proteins are consistent in that these proteins are shown to inhibit G protein signaling by virtue of their GAP activity. In contrast, *in vivo* findings illustrate the complexity of RGS effects *in vivo* and the challenges presented in unraveling them. This is particularly highlighted by the reports of both agonist- and RGS-specific effects on the direction of opioid-mediated antinociception. These observations are possibly due to RGS effects on several transmitter systems involved in the production of complex behaviors and/or to the role of RGS proteins as scaffolding proteins as well as GAPs. Such controversies underscore the need to develop more precise techniques for the evaluation of RGS protein activity *in vivo*.

3. ARRESTINS AND OPIOID RECEPTOR SIGNALING

GPCR desensitization occurs as part of a conserved process that involves receptor phosphorylation followed by the recruitment of arrestin proteins [39]. Arrestin proteins were initially identified in the eye, where it was recognized that a 48-kDa protein known as S-antigen could enhance the inactivation of rhodopsin mediated by rhodopsin kinase phosphorylation (i.e. this protein could “arrest” rhodopsin signaling) [109, 110]. Soon after, it was observed that β_2 -adrenergic receptor desensitization mediated by β -adrenergic receptor kinase (now known as GRK2) only occurred upon addition of pure retinal arrestin, suggesting that there may be a homolog of retinal arrestin that is required for complete inactivation of non-visual GPCRs [111]. Such a protein, initially called β -arrestin, was later cloned [112].

The arrestin family is comprised of four proteins, each with a distinct profile of expression and function [41]. Arrestin-1 (visual arrestin or rod arrestin) and arrestin-4 (X-arrestin or cone arrestin) are expressed primarily in the retina and are known to regulate phototransduction [109, 110, 113]. On the other hand, arrestin-2 (β -arrestin 1) and arrestin-3 (β -arrestin 2) are ubiquitously expressed and regulate the activity of a variety of GPCRs [112, 114, 115]. In general, arrestins are recruited to phosphorylated GPCRs where they uncouple G protein signaling [114, 116]; however, there are slight differences in the effects of arrestin-2 vs. arrestin-3 at individual GPCRs [117]. Furthermore, arrestins facilitate GPCR internalization *via* clathrin-mediated endocytosis by interacting directly with clathrin and/or clathrin adaptor proteins [118, 119]. In addition to their roles in GPCR desensitization and internalization, arrestins have also recently been demonstrated to mediate G protein-independent signaling for several GPCRs [44], including the μ [120], δ [121, 122], and κ opioid receptors [45]. Thus, arrestins function as multifunctional regulators of GPCR signaling (Fig. 1c, d), as discussed later under biased agonism [123].

Arrestin-2 is expressed predominantly in the brain and spleen [114], including brain regions such as the cortex, hippocampus, and thalamus that also express opioid receptors [124]. Arrestin-2 and arrestin-3 share significant homology [114]. Like arrestin-2, arrestin-3 is expressed primarily in the brain and can be found at relatively high levels in the cortex, hippocampus, hypothalamus, and amygdala [124].

In vitro, arrestin-2 was shown to have relatively low affinity for binding to the μ opioid receptor in comparison with arrestin-3 [125]. In support of this, Cheng *et al.* [126] found that arrestin-2 did not regulate μ receptor desensitization in HEK293 cells, although others have demonstrated that arrestin-2 does facilitate μ receptor internalization in multiple cell types [127, 128]. Furthermore, arrestin-2 appears to be important for the resensitization of μ opioid receptors following agonist activation and internalization [129]. Nevertheless, morphine analgesia is unchanged in arrestin-2 knockout mice [130], which indicates that arrestin-2 does not play a major role in μ opioid receptor physiology (Table 1). Arrestin-2 has been hypothesized to desensitize the μ opioid receptor primarily in the absence of arrestin-3, suggesting that the ultimate contribution of arrestins to GPCR activity depends upon the arrestin isoform that is expressed in a particular cell type [130]. In HEK293 cells, stimulation of the μ opioid receptor promotes arrestin-3 translocation to the plasma membrane [128, 130], and arrestin-3 is able to rescue μ receptor internalization in arrestin-2/arrestin-3 knockout cells [129]. Furthermore, multiple investigations have shown that arrestin-3 mediates μ receptor desensitization as measured by agonist-induced changes in K^+ conductance in oocytes [131–134]. Finally, arrestin-3 has also been reported to mediate μ receptor activation of MAPK independently of G protein stimulation in both HEK293 cells [135] and cultured striatal neurons [136].

Introduction of the δ receptor and arrestin-2 into HEK293 cells facilitated both G protein uncoupling, as evidenced by a reduction in agonist-mediated AC inhibition [126], and δ opioid receptor recycling back to the plasma membrane following endocytosis [121]. The δ opioid receptor is also desensitized by arrestin-3 when both proteins are co-expressed in oocytes, with a significantly faster time course than that observed for the δ receptor [132–134]. Arrestin-3 modulates the ultimate fate of the δ receptor following internalization, as knockdown of endogenous arrestin-3 in HEK293 cells using RNA interference resulted in reduced recycling and degradation [121]. Both arrestin-2 and arrestin-3 have been observed to mediate G protein-independent activation of MAPK following δ receptor stimulation [137].

The κ opioid receptor has been consistently shown to undergo regulation by arrestin-2. Several groups have utilized heterologous expression systems to show that arrestin-2 facilitates κ opioid receptor desensitization [126], internalization [138], and downregulation [139]. Arrestin-3 also regulates κ receptor activity, as demonstrated by enhanced desensitization of agonist-stimulated K^+ conductance in oocytes upon introduction of arrestin-3 [140]. In addition, arrestin-3 facilitates κ opioid receptor-mediated, G protein-independent signaling to MAPKs in both neurons and astrocytes [141, 142]. Thus, arrestins, especially arrestin-3 (β -arrestin-2) play important, multifunctional roles in both signaling and regulation of μ , δ , and κ opioid receptors *in vitro*.

The role of arrestin-3 in μ opioid receptor physiology has been evaluated in several paradigms using arrestin-3 knockout mice (Table 1). This seminal group of studies has shown that knockout of arrestin-3 results in enhanced morphine analgesia in both the hot-plate and the tail-flick assay [130, 143, 144], indicating that arrestin-3 plays a critical role in desensitization of the μ receptor *in vivo*. In addition, endogenous arrestin-3 was shown to facilitate the development of morphine tolerance, as either knockdown of arrestin-3 in rats [145] or gene knockout of arrestin-3 in mice [146, 147] greatly suppresses morphine analgesic tolerance. These authors also reported that arrestin-3 knockout mice exhibit either equivalent or slightly reduced morphine withdrawal symptoms, which could indicate that arrestin-3 facilitates morphine dependence. Although these studies lend support to the idea that inhibition of arrestin-3 would improve the therapeutic potential of morphine (i.e. enhanced analgesia with reduced tolerance and dependence), arrestin-3 knockout mice also exhibit enhanced morphine reward, as measured by CPP [148]. On the other hand, arrestin-3 does not appear to regulate analgesia mediated by either the δ or κ receptor (Table 1), at least in a model of spinal nociception in mice [149]. However, arrestin-3 is hypothesized to play a role in stress-induced dysphoria mediated by endogenous dynorphins acting at the κ opioid receptor [150], which could have implications for the development of κ opioid ligands for therapeutic use in stress, analgesia, and drug dependence [45].

4. RECEPTOR PHOSPHORYLATION AND OPIOID RECEPTOR SIGNALING

Receptor phosphorylation alone is not sufficient to cause complete desensitization [111] but is required for high-affinity arrestin binding [116], and therefore phosphorylation serves to initiate the process of desensitization.

4.1. G Protein-coupled Receptor Kinases (GRKs)

Agonist-activated GPCRs are recognized and phosphorylated by a family of kinases known as GRKs (Fig. 1b), which specifically mediate homologous desensitization because phosphorylation occurs at the receptor being activated [151]. There are seven GRK isoforms, although three of these, GRK1, 4, and 7, exhibit highly restricted patterns of tissue distribution. Thus, GRK2, 3, 5, and 6, which are widely expressed throughout the body, account for the desensitization of most GPCRs, including opioid receptors [39, 123]. Consequently, GRKs have been demonstrated to play an important role in μ opioid receptor signal regulation. In particular, GRK2 has been consistently shown to mediate μ receptor desensitization in both HEK cells [128, 152, 153] and CNS neurons [154–156]. Agonist-induced desensitization of the μ receptor by GRK2, or the structurally similar GRK3, is thought to involve phosphorylation of residues Ser 375 and Thr 370 [157, 158]; however, other residues are also phosphorylated and there are qualitative and quantitative differences in the ability of μ opioid receptor ligands to induce phosphorylation (see next section on biased agonism). In addition, μ receptor desensitization may be mediated by GRK5 [132, 155] *via* phosphorylation at Ser 375 [157, 158], and by GRK6 [159], although one group did not find a role for GRK6 in DAMGO-induced desensitization of the μ receptor in LC neurons [154].

Despite several studies demonstrating a role for GRKs in μ receptor regulation *in vitro*, evaluation of transgenic mouse strains, each lacking one GRK isoform, revealed no effect of GRK2, 3, 4, 5, or 6 on morphine antinociception (Table 1) [123, 159]. In contrast, knockout of GRK3 [160, 161] or inhibition of GRK2 or GRK5 [155] was shown to reduce analgesic tolerance produced by μ receptor agonists, suggesting that *in vivo*, GRKs may play a role in the long-term regulation of the μ receptor. In addition, GRK6 knockout mice exhibited enhanced μ opioid-stimulated locomotor activity and reduced μ opioid receptor-mediated constipation [159, 162], which could indicate that GRK6 plays distinct roles in different μ receptor-mediated physiologic pathways.

GRK2/3 have been shown to phosphorylate the δ receptor in heterologous expression systems [163–165], presumably at residues Thr 358 and Ser 363 in the C-terminal tail [166]. This GRK-dependent phosphorylation facilitates desensitization and internalization of the δ receptor [163, 167]. In contrast, others have reported that GRK6, but not GRK2, mediates agonist-induced desensitization of δ receptors in NG108–15 cells [168]; this likely is due to cell-specific differences.

At the κ opioid receptor, phosphorylation of Ser 369 in the C-terminus by GRK2/3 facilitates agonist-induced desensitization, internalization and downregulation [138–140, 169, 170]. GRK5 is also able to mediate κ receptor desensitization by a mechanism that probably requires phosphorylation at Ser 369 [140]. In addition, GRK3 knockout mice exhibit reduced κ opioid agonist-mediated dysphoria [150] and analgesic tolerance (Table 1) [171].

4.2. Protein Kinase C (PKC)

GPCRs are also subject to phosphorylation by second messenger-dependent protein kinases such as PKC (Fig. 1b). This can be either homologous or heterologous, meaning that PKC may phosphorylate either receptors that are not activated or downstream pathways that are common to several receptors.

PKC has been shown to phosphorylate the μ receptor, although there is some disagreement as to which amino acid(s) are involved (e.g. Ser 363, Thr 370) [152, 157, 172]. Regulation of the μ opioid receptor by PKC mediates receptor desensitization [154, 172, 173] as well as analgesic tolerance (Table 1) [143, 155, 174]. The PKC α isoform in particular is knockout mice exhibit reduced morphine-induced desensitization of the μ receptor in LC neurons [154]. Furthermore, PKC is involved in certain μ opioid-induced signaling pathways, including the activation of MAPKs such as c-Jun N-terminal kinase (JNK) [160]. Thus, PKC may function to desensitize GPCRs and also to mediate intracellular signaling cascades.

Only the δ receptor has also been shown to be phosphorylated by PKC at Ser 344, which is hypothesized to facilitate δ opioid receptor internalization in an arrestin-3- and clathrin-dependent manner [175]. To this point, there are no reports linking PKC to κ opioid receptor function.

5. CONTRIBUTION OF ACCESSORY PROTEINS TO BIASED AGONISM AT OPIOID RECEPTORS

It is generally believed that the extent of agonist-dependent GPCR signaling and regulation is a direct consequence of the efficacy of the ligand. On the other hand, recent evidence suggests that GPCRs exhibit discrete, ligand-dependent properties that cannot be explained solely on the basis of efficacy. For example, a ligand that is classically defined as an “agonist” may actually function as an agonist for one pathway (e.g. G protein activation) and a partial agonist or even an antagonist for another pathway (e.g. arrestin recruitment). Thus, individual ligands may differentially activate signaling pathways mediated by a single GPCR, a phenomenon that has been called “biased agonism”, “functional selectivity”, “pluridimensional efficacy”, or “ligand-directed signaling” [176, 177]. Although many of the behavioral consequences of biased agonism observed *in vitro* have yet to be determined, knowledge of such pathways may have important applications in the design and/or discovery of ligands that possess therapeutic activity with reduced side effects.

Biased agonism at opioid receptors was recognized early on in studies demonstrating that μ opioid receptor internalization in HEK293 cells was agonist-dependent, with DAMGO causing significantly more internalization than morphine [178]. Since that time, agonists at μ , δ , and κ opioid receptors have been shown to exhibit ligand-dependent responses in several systems [45, 120, 179, 180]. This differential signaling is thought to arise from the stabilization of ligand-specific conformational states of the receptor that activate selective downstream signaling and/or regulatory pathways. Indeed, there is indirect evidence that opioid receptors can adopt distinct active conformations depending upon the activating ligand [181, 182]. However, in some instances biased agonism may arise from differences in the cellular environment between assay systems, including the expression of G proteins, effectors, and other regulatory proteins. For example, one agonist may poorly recruit arrestin in one cell type, but may do so efficiently in another system. Along those lines, morphine elicits internalization of the μ receptor in dendrites of NAcc neurons, but not cell bodies [183], suggesting that even regional differences within single neurons can affect the regulation of agonist-activated GPCRs.

Regardless of whether these divergent signaling responses are dependent upon ligand-induced receptor conformations or factors related to the cellular environment, it is clear that they can be regulated at multiple levels of the signaling cascade, including G protein activation, phosphorylation, desensitization, and internalization. Discussed here are examples of biased agonism at opioid receptors that are hypothesized to involve arrestins and/or kinases and RGS proteins.

The most frequent example of biased agonism at opioid receptors involves the recruitment of arrestin proteins. Although agonist activation of the μ opioid receptor may result in the recruitment of either arrestin-2 or arrestin-3, different μ agonists appear to differentially recruit individual arrestin subtypes. Specifically, while morphine only produces translocation of arrestin-3, both arrestin-2 and arrestin-3 are recruited to DAMGO-activated μ receptors [129]. With respect to arrestin-3, some μ agonists, such as etorphine, efficiently recruit this arrestin subtype to the μ receptor expressed in HEK293 cells, whereas other μ opioids,

including morphine, do not [128, 184, 185]. These ligand-dependent differences in arrestin recruitment have been hypothesized to be related to the degree of desensitization and tolerance seen *in vivo*. For example, agonists such as morphine that recruit arrestin-3 (albeit inefficiently) but do not recruit arrestin-2 produce analgesic tolerance that is blocked by either knockdown or knockout of arrestin-3, but not arrestin-2 [146, 186]. In contrast, μ agonists such as etorphine and fentanyl recruit both arrestin-2 and arrestin-3, and neither analgesia nor analgesic tolerance produced by these agonists is affected by arrestin-3 knockout [130, 147].

By evaluating panels of μ opioid agonists, several groups have demonstrated that the efficacy of a ligand to activate and/or recruit G proteins is not always directly correlated with the efficacy to recruit arrestin, [187, 188], but see McPherson *et al.* [189]. In this way, it is possible to detect ligands that are “biased” toward one pathway or another. In particular, endomorphin-2 has been hypothesized to function as an “arrestin-biased” agonist due to its ability to more efficiently recruit arrestin-3 than might be predicted solely on the basis of its efficacy to activate G proteins [190]. In contrast, the novel μ opioid agonist herkinorin is thought to be G protein-biased, given that it efficiently activates G proteins yet induces no arrestin-3 translocation [191]. Since arrestin-3 plays a role in the development of analgesic tolerance to morphine, it was hypothesized that herkinorin would not cause any analgesic tolerance. In support of this, repeated administration of herkinorin did not lead to analgesic tolerance in the formalin test in rats, and herkinorin retained its analgesic efficacy in morphine-tolerant animals [192]. Thus, the relative bias of a ligand toward G protein- or arrestin-dependent pathways, as determined using biochemical techniques, can influence the physiological or behavioral response observed.

Arrestins are also involved in the activation of G protein-independent signaling pathways at the μ opioid receptor; however, arrestin-mediated signaling has not been observed for all μ opioid agonists. Thus, fentanyl, but not morphine, strongly stimulates arrestin-dependent activation of MAPK in HEK293 cells [135] and cultured striatal neurons [136]. Arrestin-dependent activation of MAPK by fentanyl enhances dendritic spine stability in hippocampal neurons *in vitro*, while arrestin-independent MAPK activation by morphine results in decreased spine stability [193], suggesting that agonist-dependent signaling *via* arrestins has implications for μ receptor physiology *in vivo*.

Ligand-dependent recruitment of either arrestin-2 or arrestin-3 has also been demonstrated for agonists acting at the δ opioid receptor [165, 182, 194]. For instance, Audet and colleagues [182] demonstrated in HEK293 cells that DPDPE and SNC80 differentially induced arrestin-3 interactions with the δ receptor. Moreover, the interaction of G protein and receptor promoted by δ opioid agonists was not necessarily correlated with the agonist-induced recruitment of arrestin-3 [187], supporting the existence of pathway-biased signaling at the δ receptor. Biased agonism at the δ receptor has been proposed to predict the differential development of tolerance to various δ opioid agonists [182, 195, 196], although a direct mechanistic link between these two phenomena has yet to be established.

Biased agonism mediated by arrestins at the κ opioid receptor primarily involves differential mechanisms of MAPK activation. Specifically, while the prototypical κ opioid agonist

U69,593 is able to elicit arrestin-3-dependent MAPK activation leading to the proliferation of immortalized astrocytes, the novel κ opioid ligand C(2)-methoxymethyl salvinorin B produced only G protein-dependent MAPK phosphorylation and did not affect astrocyte proliferation [142]. Further, κ opioid ligands that antagonize agonist-mediated G protein signaling, such as nor-binaltorphimine (nor-BNI), have recently been shown to activate JNK, which is likely through an arrestin-dependent mechanism [197]. This G protein-independent JNK activation has been proposed to underlie the long duration of action of nor-BNI and related κ antagonists *in vivo* [160]. Ligand-dependent mechanisms of MAPK activation mediated by arrestin recruitment to the κ opioid receptor likely underlie differences in the behavioral effects of κ opioid ligands observed *vivo*.

Recruitment of kinases that phosphorylate opioid receptors is also variable depending on the agonist used [40, 198]. Several studies have demonstrated that site-specific phosphorylation of the μ receptor is agonist-dependent, with morphine inducing phosphorylation at Ser 375 and DAMGO causing the phosphorylation of both Ser 375 and Thr 370 [157, 158]. These agonist-dependent patterns of phosphorylation have been hypothesized to underlie the differential ability of morphine and DAMGO to induce μ receptor desensitization and internalization. In contrast, others have suggested that quantitative differences in the level of phosphorylation induced by such agonists are more important for μ opioid receptor regulation than the qualitative pattern of phosphorylation [199].

Several investigators utilizing a variety of model systems and biochemical endpoints have demonstrated that μ opioid receptor phosphorylation and desensitization following morphine employs a PKC-dependent mechanism, whereas other μ opioid agonists such as DAMGO or etorphine utilize a GRK-dependent mechanism [128, 152, 154, 200]. Since desensitization of the opioid receptor is thought to underlie the development of analgesic tolerance to μ opioid agonists, it is therefore not surprising that PKC has been demonstrated to mediate analgesic tolerance to morphine, while GRK3 has been shown to mediate tolerance to fentanyl [155, 160, 161, 174]. Collectively, this work has led to the hypothesis that the agonist-activated μ receptor adopts distinct conformations that are differentially recognized by kinases. In contrast, other groups have demonstrated that μ receptor regulation may depend more on the cellular environment than on the receptor conformation *per se*. In particular, Bohn and colleagues [143, 146] have shown that knockout of arrestin-3 blocks morphine tolerance in the hot-plate test (i.e. supraspinal), but only slightly reduces tolerance in the tail-flick assay (i.e. spinal) that was fully blocked upon addition of the PKC inhibitor chelerythrine. These investigators hypothesized that under physiological conditions, arrestin-3 primarily regulates μ opioid receptor desensitization, whereas the loss of arrestin-3 uncovers a PKC-dependent mechanism of regulation. Thus, the mechanism of μ receptor regulation *in vivo* likely depends, at least in part, upon the specific cellular environment within a given pathway or tissue.

Agonists acting at the δ receptor have also been shown to differentially recruit kinases. In particular, in HEK293 cells expressing the δ receptor, etorphine, but not morphine, was able to elicit GRK2-dependent phosphorylation of the receptor [165]. Using a variety of δ opioids, other studies have demonstrated agonist-dependent phosphorylation and/or desensitization of the δ receptor [201, 202], which may be due to a differential ability of δ

agonists to recruit kinases such as PKC and GRK2. However, it is not yet clear whether these differences are relevant to δ receptor physiology *in vivo*. At the κ opioid receptor, agonist activation of specific MAPK pathways has been shown to be either GRK3-independent, in the case of extracellular-regulated kinase 1/2 (ERK 1/2) activation [203], or GRK3-dependent, in the case of p38 MAPK stimulation [150]. Moreover, GRK3-dependent activation of p38 MAPK was shown to mediate the dysphoric effects of κ agonists, but not κ opioid analgesia.

Although RGS proteins are not classically viewed as regulators of diverse GPCR signaling responses, there are several examples of biased agonism at μ opioid receptors, both *in vitro* and *in vivo*, in which RGS proteins might be involved. In general, RGS proteins negatively regulate signaling responses downstream of the μ opioid receptor. However, several studies have shown that this is not the case for all signaling pathways initiated by μ opioid receptor activation. While inhibition of RGS activity at G_{α_o} in C6 glioma cells stably expressing the μ opioid receptor results in the expected enhancement of morphine-stimulated AC inhibition, it also results in a paradoxical decrease in intracellular Ca^{2+} mobilization [60], which is most likely mediated through activation of PLC. Thus, in this system, RGS proteins acting at the same receptor and G protein facilitate some signaling pathways (i.e. Ca^{2+} mobilization) while simultaneously inhibiting others (i.e. AC inhibition). This dual role of RGS proteins arises from “kinetic scaffolding” [204], and could help dictate the specificity of G protein signaling. For example, in cells expressing high levels of RGS proteins, activation of μ receptors may preferentially initiate Ca^{2+} signaling, while in low RGS-expressing cells, AC and MAPK pathways may function more efficiently. In addition, differential roles of individual RGS subtypes within downstream signaling pathways can affect the signaling outcome, as has been demonstrated for other GPCRs [205]. Altogether, these studies demonstrate that an agonist acting at a single receptor is able to produce diverse signaling responses, depending on the RGS status of the cell.

Work investigating the role of RGS proteins in μ opioid receptor physiology has also demonstrated agonist-specific behaviors. First, in RGS4 knockout mice, although there was no alteration in morphine analgesia, loss of RGS4 was reported to paradoxically reduce analgesia elicited by either methadone or fentanyl [82], suggesting that RGS4 actually functions as a positive mediator of this response. A similar observation was made in RGS9 knockout mice, in which morphine analgesia was enhanced while the analgesic effects of methadone or fentanyl were significantly reduced [206]. Together, these data led to the hypothesis that RGS proteins differentially modulate behaviors produced by various agonists acting at the μ receptor by recognizing distinct agonist-induced receptor conformations or complexes [206], demonstrating that RGS proteins do have the potential to control biased agonist responses at GPCRs.

6. OPIOID INTERACTING PROTEINS AS POTENTIAL DRUG TARGETS

Though considerable work remains to define the precise roles of accessory proteins in opioid receptor physiology, efforts thus far have highlighted the possibility of targeting such proteins for therapeutic use.

Studies demonstrating that arrestin-3 knockout resulted in enhanced morphine analgesia with reduced tolerance and side effects (e.g. constipation, respiratory depression) led to the hypothesis that inhibition of arrestin-3 could be a viable therapeutic approach for improving the therapeutic potential of opioid analgesics [144, 146, 207]. Alternatively, GRKs, which have been shown to regulate several aspects of opioid receptor physiology, may represent promising drug targets [151]. In particular, recent studies have identified selective GRK2 inhibitors, including several heterocyclic compounds developed by Takeda Pharmaceuticals (Osaka, Japan) [208] and the selective serotonin reuptake inhibitor paroxetine, that inhibit GRK2 *in vivo* [209]. Moreover, given that morphine tolerance may be mediated by PKC, inhibition of PKC has also been proposed as a mechanism to improve the therapeutic profile of morphine [174, 198]. Whilst compounds targeting arrestins, GRKs, or PKC will be useful tools to tease apart the roles of these proteins, the potential therapeutic benefit may be limited by the ubiquitous distribution and importance of these proteins for the control of diverse biological functions.

Perhaps a more likely target is the RGS protein family, since family members do show differential expression across the CNS [68, 210] and there is evidence for both receptor and G protein selectivity. RGS proteins have been proposed as drug targets for several pathophysiological states involving opioid receptors, including pain [211] and addiction [53, 212–215]. For example, given that RGS4 does not regulate morphine analgesia yet dampens morphine dependence and reward [82], therapeutics that either enhance RGS4 activity or promote $G\alpha$:RGS4 interactions may be useful as adjuncts to opioid analgesics to prevent the development of unwanted side effects. Another way to improve the activity of opioid analgesics could be to co-administer an inhibitor of RGS9–2, which is known to negatively regulate the analgesic effects of morphine [108]. However, inhibition of RGS9–2 may also enhance morphine's side effects in addition to enhancing analgesia. An ideal situation would be to use an RGS inhibitor to selectively enhance the analgesic activity of a partial agonist, such as buprenorphine [59], without increasing the unwanted effects. Finally, given that GPCRs other than the μ receptor are involved in the addictive process, it is possible that RGS proteins acting at these receptors could represent targets for the management of addiction. Future investigations will need to identify the specific function of RGS proteins in the pathways that underlie addiction in order to determine when and how these proteins could be targeted.

Small molecules that target various aspects of RGS function are being explored as ways to direct GPCR signaling [216]. In particular, significant progress has been made toward the identification and characterization of high potency inhibitors of RGS4 [217]. Alternatively, the regulation of RGS proteins is currently being explored as a way to enhance RGS function, for instance by reducing protein degradation [218].

Several investigators have proposed using biased agonists as novel therapeutics. In this way, a single drug molecule (instead of a combination therapeutic) could be used to selectively target signaling pathways that mediate the desired therapeutic effect while simultaneously avoiding pathways that lead to unwanted side effects, thereby improving the therapeutic index [176, 177, 219]. For example, a biased agonist at the μ opioid receptor could be used to dissociate analgesia from unwanted adaptations such as tolerance, dependence, and

addiction [120]. Given that arrestins appear to mediate such adaptations, an agonist that is biased against arrestin recruitment, such as the novel μ agonist herkinorin [191], may be more useful for the long term treatment of various pain conditions.

Agonists at the δ opioid receptor have been shown to induce distinct mechanisms of receptor regulation, and this may have implications for the development of tolerance *in vivo*. Thus, a δ agonist that does not induce significant analgesic tolerance could be useful for pain treatment, whereas a δ agonist that produces analgesic tolerance (but not generalized tolerance) may be useful for treating mood disorders [179]. Continued investigation of the behavioral correlates of ligand-directed δ opioid receptor regulation will likely validate such therapeutic uses for biased agonists at the δ receptor. Finally, biased agonists at the κ opioid receptor could also be useful for generating improved analgesia without appreciable side effects [45]. In particular, κ opioid-induced dysphoria has been hypothesized to be mediated by an arrestin-dependent mechanism, and therefore a G protein-biased agonist at the κ opioid receptor, such as 6'-guanidinonaltrindole [220], could be more useful as an analgesic than traditional κ opioid agonists.

7. CONCLUSION

Extensive studies to date have identified several accessory proteins that play important multifunctional roles in opioid receptor signaling and regulation. In particular, RGS proteins such as RGS4 and RGS9–2, arrestins including arrestin-2 and arrestin-3, and protein kinases such as PKC, GRK2, GRK3, GRK5 and GRK6 have all been shown to regulate the activity of μ , δ and κ opioid receptors, both *in vitro* and *in vivo*. Not only do such accessory proteins affect the extent of opioid signaling, but they also contribute to the observation of biased agonism. With such an important relationship to opioid receptor physiology, it is no wonder that these proteins have been proposed as drug targets for several disorders involving opioid receptors, including pain and addiction. Despite all we currently know regarding the function of RGS proteins, arrestins, and protein kinases, future work is necessary to precisely characterize the role of these proteins in the behavioral effects of opioid receptor agonists. Such efforts will undoubtedly reveal new contributions of these proteins to opioid pharmacology as well as novel mechanisms of targeting such molecules for therapeutic benefit.

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ABBREVIATIONS

μ	Mu
δ	Delta
κ	Kappa
AC	Adenylate cyclase

CNS	Central nervous system
CPP	Conditioned place preference
DAMGO	[D-Ala ² ,N-Me-Phe ⁴ ,Gly ⁵ -ol]-enkephalin
DEP	Disheveled, egl-10, Pleckstrin domain
DHEX	DEP domain helical extension
DPDPE	[D-Pen ^{2,5}]-enkephalin
GAIP	Gα-interacting protein
GAP	GTPase activating protein
GGL	G protein gamma-like domain
GIRK	G protein-coupled inwardly-rectifying K ⁺ channel
GPCR	G protein-coupled receptor
GRK	G protein-couple receptor kinase
JNK	c-Jun N-terminal kinase
LC	Locus coeruleus
MAPK	Mitogen-activated protein kinase
NAcc	Nucleus accumbens
NOPR	Nociceptin receptor
PAG	Periaqueductal gray
PKC	Protein kinase C
PLC	Phospholipase C
RGS	Regulator of G protein signaling
RGSi	RGS-insensitive
RH	RGS homology domain

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Fig. (1).

Schematic illustration of the basic processes involved in opioid receptor signaling and regulation. Depicted are the canonical functions performed by (a) regulator of G protein signaling (RGS) proteins, (b) the kinases G protein-coupled receptor kinases (GRKs) and protein kinase C (PKC), and (c) arrestins. Agonist stimulation of μ , δ , and κ opioid receptors (1) results in the activation of associated heterotrimeric $G_{i/o}$ proteins *via* nucleotide exchange (2). Once activated, $G\alpha_{i/o}$ -GTP dissociates from the $\beta\gamma$ heterodimer, and both subunits signal to downstream effectors (3), including adenylate cyclase (AC), Ca^{2+} channels (Ca^{2+}), G protein-coupled inwardly-rectifying K^+ channels (GIRK), mitogen-activated protein kinases (MAPKs), and phospholipase C (PLC). G protein signaling is terminated by GTP hydrolysis, which is enhanced by RGS proteins (4). Agonist activation of opioid receptors also leads to desensitization, which is initiated by receptor phosphorylation by kinases such as GRKs and PKC (5). Arrestin proteins are then recruited to phosphorylated receptors (6), and this elicits further regulatory events, including receptor internalization, downregulation, and/or recycling (7). In addition, arrestin-bound opioid receptors have the ability to signal to MAPKs (8).

Family	Structure	Members
R4		RGS1, RGS2, RGS3, RGS4 , RGS5, RGS8, RGS13, RGS16, RGS18, RGS21
RZ		RGS17 (RGSZ2) RGS19 (GAIP) RGS20 (RGSZ1)
R7		RGS6, RGS7 RGS9 , RGS11
R12		RGS10, RGS12 RGS14

Fig. (2).

Diagram of the basic domain structures for “classical” regulator of G protein signaling (RGS) proteins of the R4, RZ, R7 and R12 families. Shown for each family is the common domain structure with the N-terminus oriented to the left, as well as a list of individual family members. The R4 and RZ families contain relatively simple RGS proteins, with short N- and C-terminal extensions. R4 family members, including RGS4, contain the RGS homology (RH) domain, together with a short N-terminal amphipathic helix. The RZ family is comprised of proteins with an N-terminal cysteine string preceding the conserved RH domain. The R7 and R12 families are more complex, and contain several domains for protein-protein interaction in addition to the RH domain. Members of the R7 family, including RGS9, contain both a DEP (Disheveled, Egl-10, Pleckstrin) domain with a helical extension (DHEX) and a GGL (G protein gamma-like) domain. The R12 family contains a PDZ (PSD95, Dlg1, ZO-1) domain, a phosphotyrosine binding (PTB) domain, Ras binding domains (RBD), and a GoLoco motif that binds Gα-GDP subunits. Highlighted in bold are the two primary RGS proteins discussed in this review.

Table 1. Roles of Regulator of G Protein Signaling Proteins, Arrestins, and Kinases in the Physiology of μ , δ , and κ Opioid Receptors.

Accessory Protein	Model	Effect on opioid receptor physiology			Reference(s)							
		μ	δ	κ								
RGS proteins												
RGS4	Constitutive RGS4 knockout mouse	No change morphine analgesia	---	---	[82]							
		↑ morphine withdrawal										
		↓ methadone, fentanyl analgesia										
		↑ morphine reward										
RGS9-2	NAcc-specific RGS4 knockout mouse	↑ morphine locomotor stimulation	↓ DPDPE, [D-Ala ²]deltorphin II analgesia	---	[106,107]							
		↑ morphine, DAMGO analgesia										
	ODN knockdown of RGS9-2 in mice (i.c.v.)	↓ morphine tolerance										
		↑ morphine analgesia										
	Constitutive RGS9 knockout mouse	↓ morphine tolerance				---	[108,206]					
		↑ morphine withdrawal										
		↑ morphine reward										
		↓ methadone, fentanyl analgesia										
		Arrestins										
		Arrestin-2						siRNA knockdown of arrestin-2 in mouse PAG	↑ morphine analgesia	---	---	[186]
Constitutive arrestin-2 knockout mouse	↓ morphine tolerance											
Arrestin-3	Constitutive arrestin-2 knockout mouse	No change morphine analgesia	---	---	[130]							
		↑ morphine, heroin analgesia										
	Constitutive arrestin-3 knockout mouse	No change methadone, fentanyl analgesia	---	---	[130,143,144]							
		↓ morphine tolerance										
		↓ morphine withdrawal										
		No change methadone, fentanyl tolerance										
		↑ morphine reward										
		↓ morphine tolerance										
ODN knockdown of arrestin-3 in rats (i.t.)	↓ morphine tolerance	No change [D-Ala ²]deltorphin II analgesia	No change U50,488 analgesia	[148]								
	Antibody knockdown of arrestin-3 in mice (i.t.)											

Accessory Protein	Model	Effect on opioid receptor physiology			Reference(s)
		μ	δ	κ	
Kinases					
GRKs	Constitutive GRK3 knockout	No change morphine tolerance	---	---	[158,159]
		↓ methadone, fentanyl tolerance			

	Administration of GRK2 or GRK5 inhibitor	↓ DAMGO tolerance	---	---	[155]
		No change methadone, fentanyl tolerance			
	Constitutive GRK6 knockout	No change morphine analgesia	---	---	[157]
↑ morphine locomotor stimulation					
↓ morphine, loperamide constipation					
PKC	Administration of PKC inhibitor	↓morphine, fentanyl tolerance	---	---	[155,171]

DAMGO, [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin; DPDPE, [D-Pen^{2,5}]-enkephalin; GRKs, G protein-coupled receptor kinases; i.c.v., intracerebroventricular; i.t., intrathecal; NAcc, nucleus accumbens; PKC, protein kinase C; RGS, regulator of G protein signaling.