GRK2 Constitutively Governs Peripheral Delta Opioid Receptor Activity

Graphical Abstract

Highlights
- GRK2 naively associates with peripheral DOR, maintaining incompetent state
- GRK2 interaction, not kinase activity, underlies peripheral DOR incompetence
- Knock down of GRK2 increases peripheral DOR-mediated analgesia in vivo
- BK induces PKC-dependent RKIP sequestration of GRK2, increasing DOR competence

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In Brief
Brackley et al. demonstrate that constitutive GRK2 interaction with DOR, not kinase activity, desensitizes the receptor at the plasma membrane in peripheral pain-sensing neurons. Priming by inflammatory mediator BK induces PKC-dependent RKIP sequestration of GRK2 to the cytosol, enhancing DOR responsiveness. Knock down of GRK2 enhances peripheral DOR-mediated analgesia in vivo.
GRK2 Constitutively Governs Peripheral Delta Opioid Receptor Activity

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SUMMARY

Opioids remain the standard for analgesic care; however, adverse effects of systemic treatments contraindicate long-term administration. While most clinical opioids target mu opioid receptors (MOR), those that target the delta class (DOR) also demonstrate analgesic efficacy. Furthermore, peripherally restrictive opioids represent an attractive direction for analgesia. However, opioid receptors including DOR are analgesically incompetent in the absence of inflammation. Here, we report that G protein-coupled receptor kinase 2 (GRK2) naïvely associates with plasma membrane DOR in peripheral sensory neurons to inhibit analgesic agonist efficacy. This interaction prevents optimal Gβ subunit association with the receptor, thereby reducing DOR activity. Importantly, bradykinin stimulates GRK2 movement away from DOR and onto Raf kinase inhibitory protein (RKIP), protein kinase C (PKC)-dependent RKIP phosphorylation induces GRK2 sequestration, restoring DOR functionality in sensory neurons. Together, these results expand the known function of GRK2, identifying a non-internalizing role to maintain peripheral DOR in an analgesically incompetent state.

INTRODUCTION

Opioid agonists are essential therapeutic strategies in the treatment of pain. Opioids produce analgesia by activating G protein-coupled receptors (GPCRs) known as mu (μ, MOR), delta (δ, DOR), and kappa (κ, KOR) opioid receptors. Traditionally, MOR analgesics are prescribed for the treatment of severe pain. However, DOR agonists have reduced side effect profiles compared to MOR agonists in rodent and non-human primate models (Vanderah, 2010). Like other opioid receptors, DOR primarily signals downstream through Ga11 and Gβγ subunits (Alves et al., 2004) that inhibit neuronal depolarization by decreasing cAMP activity (Law and Bergsbaken, 1995) and inhibiting voltage-gated Ca2+ channels (VGCCs) (Ford et al., 1999). In an effort to reduce systemic side effects and abuse potential, peripherally restricted DOR agonists serve as an attractive alternative to systemic opioid therapies. However, multiple reports demonstrate that peripheral DOR analgesic competence requires an inflammatory pre-stimulus (Stein et al., 1989; Patwardhan et al., 2005, 2006; Gavériaux-Ruff et al., 2008; Rowan et al., 2009; Pettinger et al., 2013). Importantly, peripheral DOR incompetence is not well understood and could provide important insight on the role of inflammatory mediators in peripheral opioid receptor regulation.

Peripheral tissues release inflammatory mediators such as bradykinin (BK) in response to injury (Levy and Zochodne, 2000). Nociceptive responses following BK administration are mediated via Gαq/11-coupled GPCRs expressed by primary afferent neurons that co-express opioid receptors (Steranka et al., 1988; Patwardhan et al., 2005; Petcu et al., 2008). Importantly, BK induces rapid functional competence of DOR antinociception (Patwardhan et al., 2005; Rowan et al., 2009), indicating that peripheral DOR exists naïvely in a desensitized state. Inflammation-induced DOR analgesic competence at peripherally restrictive doses is known as “priming” (Patwardhan et al., 2005; Rowan et al., 2009; Pradhan et al., 2013), yet a mechanism for this phenomenon remains unknown. Recent work has identified a role for protein kinase C (PKC) (Patwardhan et al., 2005; Rowan et al., 2009), which agrees with work demonstrating that BK activation drives phospholipase C (PLC) activity to stimulate downstream PKC isoforms (Fu et al., 1989; Tippmer et al., 1994; Graness et al., 1997). Indeed, careful dissection of this mechanism would increase the application of peripherally restrictive DOR agonists to treat pain and reduce centrally mediated negative side effects associated with systemic MOR agonist administration.

Agonist-induced desensitization of DOR is dependent on hierarchical phosphorylation by G protein-coupled receptor kinase 2 (GRK2) (Kouhen et al., 2000; Guo et al., 2000). In contrast, the scaffolding protein Raf kinase inhibitory protein (RKIP) facilitates opioid receptor activity (Krosliak et al., 2001). An important regulatory feature of RKIP modulation of GPCR activity is direct...
phosphorylation by PKC, which induces RKIP dimerization and subsequent sequestration of GRK2 (Lorenz et al., 2003; Deiss et al., 2012). This represents a fundamental research effort to identify that GRK2 chronically downregulates DOR antinociception. Furthermore, we provide support for the hypothesis that BK primes DOR analgesic competency in peripheral sensory neurons via RKIP sequestration of GRK2.

RESULTS

DOR Competence in Naive and Primed Sensory Neurons

Opioids elicit their analgesic effects, in part, via receptor-mediated inhibition of VGCCs (Stein and Zöllner, 2009). In sensory neurons, transient exposure to 50 mM KCl evokes a measureable increase in intracellular Ca^{2+}, which is attributable to an influx of extracellular Ca^{2+} through VGCCs following neuronal depolarization (Khasabova et al., 2002). Activation of DOR inhibits KCl-evoked Ca^{2+} influx through L-type, N-type, and P/Q-type VGCCs, as well as KCl-induced neurotransmitter release in DRG (Khasabova et al., 2004). Multiple investigators have previously quantified opioid inhibition of VGCCs via KCl-evoked Ca^{2+} influx in cultured sensory neurons (Khasabova et al., 2004; Pettinger et al., 2013). Thus, DOR agonist inhibition of KCl-evoked Ca^{2+} influx is a validated method for quantifying DOR activity in a population of sensory neurons. Given that DOR is not expressed in all peripheral sensory neurons, this method circumvents the limitation of user bias when determining whether native DOR is functionally expressed within a given cell and eliminates potential changes to receptor activity and/or biochemistry that result from receptor-fusion proteins introduced by gene targeting.

DOR activity was assessed in adult rat TG and DRG CAP-sensitive neurons (Figures 1A and 1B). At doses ranging from 1 nM to 1 μM, the DOR agonist [D-Pen(2),D-Pen(5)]-enkephalin (DPDPE) did not significantly inhibit Ca^{2+} influx elicited by KCl in vehicle-treated TG or DRG neurons. However, at doses above 1 μM, DPDPE efficiently inhibited KCl-evoked Ca^{2+} influx in both vehicle-treated TG and DRG. Pretreatment with BK (200 nM) significantly increased the potency of DPDPE to inhibit KCl-evoked Ca^{2+} influx in both populations of TG and DRG neurons at 1 μM (also known as the BK priming effect). Importantly,
equimolar dose of DOR agonist DADLE has previously been shown to increase the population of TG capable of DOR-mediated inhibition of VGCCs (Pettinger et al., 2013). Thus, 1 μM DPDPE was the dose used for the remainder of this study in both cultured TG and DRG neurons. IC50 values for DPDPE were 6.91 x 10^-6 M versus 5.21 x 10^-6 M for vehicle-treated TG and DRG, respectively. IC50 values for DPDPE were 6.15 x 10^-6 M versus 7.39 x 10^-6 M for BK-treated TG and DRG, respectively. The efficacy or maximal response to DPDPE was unaltered by BK in both TG and DRG. Thus, the BK priming effect on DOR activity was indistinguishable between TG and DRG neurons.

The response to DPDPE (1 μM) treatment in DRG pretreated with BK (200 nM; 5 min) was blocked when co-treated with irreversible selective DOR antagonist naltrindole (10 nM, 50 × K; 5 min) (Figure 2C). This demonstrates that inhibition of KCl-evoked Ca2+ influx by DPDPE at the selected dose is mediated by DOR. SNC 80 was included as a more selective nonpeptide agonist for DOR, compared to peptide agonist DPDPE (Calderon et al., 1994). At a concentration of SNC 80 equal to the dose used for DPDPE and previously verified to inhibit VGCCs in sensory neurons (Rowan et al., 2014), SNC80 (1 μM) and DPDPE (1 μM) equally inhibited DOR activity in DRG when primed by BK (200 nM; 5 min) (Figure 1D). Furthermore, patch-clamp electrophysiology revealed that DPDPE (1 μM) significantly inhibited VGCCs in DRG neurons (20–35 pF) only following BK (200 nM) pretreatment (Figures 1E and 1F). Collectively, these studies demonstrate that DOR is functionally incompetent unless primed by BK in peripheral sensory neurons.

**GRK2 Modulation of Functional DOR Competence**

Agonist-induced DOR activation recruits GRK2, which stimulates receptor phosphorylation that induces canonical receptor desensitization and internalization (Kouhen et al., 2000; Guo et al., 2000; Hong et al., 2009; Xu et al., 2010). Recent work in immortalized cells demonstrated that GRK2 may chronically remain associated with a GPCR in the absence of agonist stimulation, thereby reducing receptor competence (Namkung et al., 2009). To explain the chronic analgesic incompetence of peripheral DOR under naive conditions, we first used co-immunoprecipitation (coIP) analyses to investigate the possibility that GRK2 might be constitutively associated with DOR in primary neuronal culture (Figure 2A). In plasma membrane (PM) preparations from serum-starved, vehicle-treated TG cultures, DOR co-immunoprecipitates with GRK2. Notably, there is a significant reduction in GRK2 co-immunoprecipitation with DOR following treatment with BK (200 nM; 5 min). These data indicate that in the absence of agonist stimulation, GRK2 is statically bound to DOR under naive conditions and provides support that BK induces a reduction in DOR association with GRK2 at the PM.

To evaluate the role of GRK2 in functional DOR competence under naive and primed conditions, we employed small interfering RNA (siRNA)-mediated knock down of GRK2 expression and assessed DOR activity using Ca2+ imaging and patch-clamp electrophysiology. To demonstrate the specificity of this molecular approach, we first assessed the efficiency of GRK2 knock down relative to β-actin and found a 76% reduction in normalized GRK2 protein expression one day post-transfection (Figure 2B). Next, we determined DOR competency in the neuronal population of primary DRG following siRNA-mediated knock down of GRK2 using Ca2+ imaging. BK pretreatment (200 nM; 5 min) significantly increased DPDPE inhibition of KCl-evoked Ca2+ influx in mock-transfected DRG neurons (Figures 2C and 2D). However, in DRG cultures transfected with FITC-GRK2 siRNA, the increase in DPDPE inhibition of KCl-evoked Ca2+ influx was independent of BK pretreatment. Similarly, DPDPE significantly inhibited VGCCs only following priming by BK (200 nM) in mock-treated small to medium DRG neurons (Figure 2F). Importantly, small to medium DRG neurons transfected with FITC-GRK2 siRNA did not require BK pretreatment to evoke significant DPDPE inhibition of VGCCs. These data indicate that GRK2 participates in functional DOR incompetence in cultured DRG neurons.

GRK2 kinase activity regulates homologous desensitization of many GPCRs through receptor-G protein uncoupling and receptor internalization following phosphorylation (Premont et al., 1995). Following DOR agonist stimulation, GRK2 hierarchically phosphorylates DOR first at Ser363 to mediate receptor desensitization through G protein uncoupling and then at Thr358 to initiate internalization of the receptor to further attenuate signaling (Guo et al., 2000; Kouhen et al., 2000; Xu et al., 2010). To determine whether BK affects GRK2 phosphorylation of DOR, we used a phosphorylation site-specific antibody for DOR at Ser363 (Figure 3A). In PM preparations from serum-starved, vehicle-treated TG cultures, DOR is phosphorylated at Ser363. Interestingly, DOR phosphorylation at Ser363 remains unchanged following pretreatment with BK (200 nM; 5 min). These data demonstrate that BK does not affect GRK2-mediated phosphorylation of DOR at its primary desensitization site.

To assess whether GRK2 kinase activity supports functional DOR incompetence in primary DRG cultures, we overexpressed GRK2 or a kinase-inactive mutant that maintains the ability to interact with GPCRs (K220R) (Kong et al., 1994) and measured DOR inhibition of KCl-evoked Ca2+ influx in vehicle-treated and BK-treated conditions. BK pretreatment (200 nM; 5 min) significantly increased DPDPE inhibition of KCl-evoked Ca2+ influx in GFP-positive DRG nucleofected with empty vector (E.V.) (Figures 3B and 3C). However, in DRG nucleofected with GRK2 or K220R, BK was unable to induce DPDPE inhibition of KCl-evoked Ca2+ influx over vehicle-treated DRG. These data demonstrate that GRK2 kinase activity is not required for GRK2 modulation of functional DOR incompetence in primary sensory neurons.

**GRK2 Modulation of DOR Analgesic Competence In Vivo**

After we identified a role for GRK2 modulation of functional DOR incompetence in sensory neurons in vitro, we measured physiologic peripheral DOR analgesic incompetence in vivo. We assessed both ipsilateral and contralateral DRG. In this study, we employed antisense-oligodeoxynucleotides (AS-ODN) against GRK2 mRNA to knock down GRK2 expression in a model of BK priming of peripheral DOR. Intrathecal (i.t.) injections of GRK2 AS-ODN over 3 days significantly reduced GRK2 expression in peripheral sensory nerves (Ferrari et al., 2012). Utilizing the same AS-ODN in this study, daily i.t. injections of GRK2 AS-ODN (30 μg/day) over 3 days nearly ablated GRK2 protein expression in both ipsilateral and contralateral DRG (Figures 4A and 4B). Next, we determined DOR competency in the neuronal population of primary DRG following siRNA-mediated knock down of GRK2 using Ca2+ imaging. BK pretreatment (200 nM; 5 min) significantly increased DPDPE inhibition of KCl-evoked Ca2+ influx in mock-transfected DRG neurons (Figures 2C and 2D). However, in DRG cultures transfected with FITC-GRK2 siRNA, the increase in DPDPE inhibition of KCl-evoked Ca2+ influx was independent of BK pretreatment. Similarly, DPDPE significantly inhibited VGCCs only following priming by BK (200 nM) in mock-treated small to medium DRG neurons (Figure 2F). Importantly, small to medium DRG neurons transfected with FITC-GRK2 siRNA did not require BK pretreatment to evoke significant DPDPE inhibition of VGCCs. These data indicate that GRK2 participates in functional DOR incompetence in cultured DRG neurons.

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To assess whether GRK2 knock down affects DOR analgesic competence, we assessed DPDPE inhibition of PGE$_2$-induced mechanical and thermal allodynia following BK priming in MM-ODN- and AS-ODN-treated rats (Figures 4C–4H). In MM-ODN-treated animals, injection of a peripherally restrictive dose of DPDPE (20 μg) (Rowan et al., 2009) into the hindpaw did not block PGE$_2$ (0.3 μg)-induced mechanical or thermal allodynia unless primed by BK (25 μg). Similar to functional data, GRK2 knock down eliminated the BK priming requirement for functional competence of the DOR in vivo. Surprisingly, both BK-induced mechanical and thermal allodynia remain unchanged with GRK2 knock down. Although GRK2 is reduced in both ipsilateral and contralateral DRG, contralateral PWTs and PWLs remained unchanged from BL. These data suggest that GRK2 impairs peripheral DOR analgesic competence in vivo.

**BK Activates PLC-PKC Pathway to Modulate DOR**

BK stimulation of B2R activates downstream PLC or cAMP signaling and leads to PKC or PKA activation, respectively (Liebmann and Böhmer, 2000). To determine whether kinases downstream of BK signaling were involved in BK-mediated GRK2 dissociation from DOR, we employed inhibitors of PLC (U73122, 10 μM), PKC (GF 109203X [GFX], 10 μM), and PKA (H-89 20 μM) (Figure S3). BK-induced GRK2 dissociation from DOR was reversed by inhibitors for PLC and PKC, but not PKA. These results demonstrate that a PLC-PKC-dependent pathway is involved in BK-mediated GRK2 dissociation from DOR in cultured TG neurons. BK-induced functional DOR competence is mediated by PKC both in vitro and in vivo (Patwardhan et al., 2005; Rowan et al., 2009). However, whether other second messengers downstream...
of BK-receptor activation mediate DOR priming remains unknown. Thus, we tested whether inhibitors for PLC (U73122, 10 μM) or PKA (H-89 20 μM) could block the BK effect on DPDPE inhibition of KCl-evoked Ca\(^{2+}\) influx in CAP-sensitive DRG neurons, using the PKC inhibitor (GFX, 10 μM) as a positive control. Inhibition of PLC and PKC, but not PKA, blocked BK priming of functional DOR competence in DRG (Figure S3). In agreement with GRK2 dissociation from DOR, these data indicate that BK priming of DOR competence in peripheral sensory neurons is mediated by the PLC-PKC pathway and not PKA.

Modulation of RKIP in Sensory Neurons

RKIP is an important signal modifier of GPCR signaling. In sensory neurons, BK evokes rapid PKC activation (Delmas et al., 2002; Cesare et al., 1999). Studies in immortalized cells have demonstrated that agonist-induced PKC phosphorylation of RKIP facilitates its self-dimerization, which is crucial for RKIP association with GRK2 (Corbit et al., 2003; Lorenz et al., 2003; Deiss et al., 2012). Dimerized RKIP can then sequester GRK2 and hinder GRK2-mediated receptor desensitization. Given its expression in intact TG and DRG tissue (Figure 5A) and sensitivity to PKC, we hypothesized that BK stimulates PKC-dependent modulation of RKIP in primary sensory neurons.

Previous studies have demonstrated that activation of G\(_{q}\)-coupled GPCRs results in PKC phosphorylation of RKIP at Ser153, followed by RKIP self-dimerization and recruitment of GRK2 in multiple cell lines (Corbit et al., 2003; Lorenz et al., 2003; Deiss et al., 2012). We sought to determine whether this mechanism occurs in sensory neurons. To determine whether BK activation of B2R leads to PKC phosphorylation of RKIP, we utilized a phosphorylation site-specific antibody for RKIP at Ser153 (Figure 5B). BK (200 nM; 5 min) nearly triples RKIP phosphorylation at Ser153 in serum-starved TG, which was blocked by pretreatment with a B2R antagonist (HOE-140; 10 μM; 5 min) or PKC inhibitor (GFX, 10 μM; 5 min). These data demonstrate that BK stimulation of B2R results in PKC phosphorylation of RKIP in sensory neurons.

We next sought to determine whether BK-induced PKC stimulation also directs RKIP dimerization and association with GRK2, using coIP analyses (Figure 5C). BK (200 nM; 5 min) induced GRK2 co-immunoprecipitation with the RKIP dimer in cytosolic lysates from serum-starved TG cultures, in a manner sensitive to PKC inhibition (GFX, 10 μM; 5 min). These data indicate that BK-induced PKC activation stimulates RKIP self-dimerization and association with GRK2 in sensory neurons, supporting PKC-dependent RKIP sequestration of GRK2.

RKIP Sequestration of GRK2 Modulates BK Priming of DOR

In immortalized cells, RKIP facilitates DOR signaling (Kroslak et al., 2001). Given that BK activation of PKC induces functional DOR competence (Figure S3) (Patwardhan et al., 2005), GRK2 dissociation from PM DOR (Figure 2A), and RKIP phosphorylation and self-dimerization resulting in association with GRK2 (Figures 5B and 5C), we hypothesized that RKIP sequestration of GRK2 governs BK priming of DOR in sensory neurons.
Figure 4. GRK2 Modulation of DOR-Mediated Antinociception

(A) Timeline for ODN injections and rat behavior protocol for BK priming of peripheral DOR antinociception.

(B) WCL from ipsilateral (ipsi-) and contralateral (contra-) DRG of rats treated with MM-ODN or GRK2 AS-ODN. *p < 0.05; ns, no significance; n = 3 independent trials; one-way ANOVA Bonferroni post hoc; mean ± SEM.

(C–F) Time course for DPDPE inhibition of PGE$_2$-induced allodynia in ipsilateral (C), mechanical; (D), thermal and contralateral (E), mechanical; (F), thermal hindpaws. Paw withdrawal readings were measured 5 min and 10 min post-intraplantar (i.pl.) injection (vehicle or BK [25 μg], and 5-min intervals for 20 min following second i.pl. injection (co-injection DPDPE [20 μg]/PGE$_2$ [0.3 μg]); BK-induced allodynia: mechanical and thermal, ***p < 0.005 versus vehicle-treated groups; DPDPE inhibition of PGE$_2$-induced allodynia: mechanical, 20 min, *p < 0.05 (MM-ODN/BK), 25 min, **p < 0.01 (AS-ODN/BK), ***p < 0.005 (AS-ODN/Veh, MM-ODN/BK); 30 min, *p < 0.05 (AS-ODN/Veh), **p < 0.01 (AS-ODN/BK, MM-ODN/BK); 35 min, ***p < 0.005 (MM-ODN/BK, AS-ODN/Veh, AS-ODN/BK) versus

(legend continued on next page)
We employed siRNA-mediated knock down of RKIP expression in sensory neuron cultures to evaluate the role of RKIP in BK priming of DOR. To demonstrate the specificity of this molecular approach in TG cultures, we assessed the efficiency of RKIP knock down relative to β-actin and found a 65% reduction in normalized RKIP protein expression 1 day post-transfection (Figure 6A).

Next, we sought to determine whether BK priming of DOR functional competence remained intact in DRG following siRNA-mediated knock down of RKIP protein expression. BK pretreatment (200 nM; 5 min) significantly increased DPDPE inhibition of KCl-evoked Ca\(^{2+}\) influx in mock-transfected DRG (Figures 6B and 6C). However, in DRG transfected with FITC-RKIP siRNA, there was no longer a BK-induced increase in DPDPE inhibition of KCl-evoked Ca\(^{2+}\) influx. Furthermore, when we re-introduced RKIP into FITC-RKIP siRNA-treated DRG (RKIP Rescue), the effect on DPDPE-inhibition of KCl-evoked Ca\(^{2+}\) influx was restored. These data indicate that RKIP expression is required for BK priming of DOR functional competence.

To determine whether PKC phosphorylation of RKIP is necessary for BK priming of functional DOR competence in cultured DRG, we overexpressed RKIP or a phospho-deficient mutant (RKIP-S153A) and measured DOR activity in vehicle- and BK-treated conditions. BK pretreatment (200 nM; 5 min) significantly increased DPDPE inhibition of KCl-evoked Ca\(^{2+}\) influx in GFP-positive DRG nucleofected with empty vector (E.V.) or RKIP (Figure S4). However, in GFP-positive DRG cultures nucleofected with RKIP-S153A, BK was unable to induce DPDPE inhibition of KCl-evoked Ca\(^{2+}\) influx. These data demonstrate that PKC phosphorylation of RKIP is required for BK-induced functional DOR competence in sensory neurons.

**GRK2 Modulation of DOR G Protein Coupling**

The primary signaling event in GPCR activation involves G protein interaction (Rodbell et al., 1971). Following agonist stimulation, DOR interacts with a heterotrimer G protein complex comprised of an αi subunit and βγ dimer (Alves et al., 2004). Upon dissociation from the receptor, Gαi and Gβγ signal as downstream effectors. Gβ subunits function to inhibit VGCCs (Herlitze et al., 1996; Ford et al., 1998). Given that BK enhances the potency of DPDPE-mediated inhibition of VGCCs in sensory neurons (Figures 1A and 1B), we investigated the possibility that BK might influence interactions between Gβ and DOR using coIP analyses. In PM preparations from serum-starved, vehicle-treated TG cultures, Gβ co-immunoprecipitates with DOR (Figure 7A). Notably, there is an increase in Gβ co-immunoprecipitation with DOR following treatment with BK (200 nM; 5 min). Conversely, there is a decrease in DOR-bound Gβ in TG cultures treated with DPDPE (1 μM; 15 min) following initial BK pretreatment (Figure 7B). These data indicate that prior to ligand binding, BK facilitates the coupling of Gβ to DOR. Taken together with our Ca\(^{2+}\) imaging data, these findings also indicate that Gβ is...
cooperatively released to potentially act on second order targets when DPDPE activates primed DOR.

Next, we hypothesized that the constitutive interaction between GRK2 and DOR may block the coupling of DOR to Gb. For this, we utilized siRNA and coIP techniques in TG cultures to validate the role of GRK2 in DOR coupling to Gb. BK pretreatment (200 nM; 5 min) increased DOR-bound Gb in serum-starved mock- and GRK2 siRNA-treated TG culture PM lysates (Figure 7). These data indicate that the absence of GRK2 alone is not sufficient to recruit Gb to the receptor. To investigate whether GRK2 overexpression impairs DOR coupling to Gb, PM coIPs were conducted from TG cultures nucleofected with GRK2 or E.V., serum-starved, and treated with vehicle or BK (200 nM; 5 min). As expected, Gb co-immunoprecipitated with DOR in vehicle-treated TG cultures nucleofected with E.V., while BK treatment increased Gb co-immunoprecipitation with DOR. GRK2 overexpression blocked the BK-dependent increase in Gb association with DOR (Figure 7D). Thus, the constitutive association between GRK2 and DOR attenuates Gb:receptor coupling in sensory neurons. Together with our DOR-GRK2 association and functional imaging studies, these findings suggest that GRK2 hinders receptor coupling to Gb, such that Gb may not inhibit VGCCs unless primed first by BK.

DISCUSSION

The phenomenon of peripheral DOR incompetence in the periphery has been observed in vitro (Patwardhan et al., 2005, 2006) and in vivo (Stein et al., 1989; Rowan et al., 2009), and pre-treatment with an inflammatory stimulus is required for DOR activation, thereby promoting analgesia. However, a major gap in knowledge existed concerning (1) why peripheral DOR remains functionally incompetent under naive conditions, and (2) a mechanism for DOR priming beyond PKC-dependence. This study identifies two important conclusions that fill this gap. First, our data illustrate that DOR responsiveness to agonist stimulation in naive afferent terminals is impaired by a constitutive interaction with GRK2 at the PM that prevents receptor coupling to Gb, which subsequently prohibits VGCC inhibition. Second, when peripheral sensory neurons undergo BK activation of B2R, PKC directly phosphorylates RKIP. This initiates RKIP self-dimerization and sequestration of GRK2. Consequently, DOR can couple to Gb to inhibit VGCCs, which results in antinociception. Within this framework we have identified pharmaceutical targets that may enhance DOR-mediated analgesia.

Numerous reports in immortalized cell lines indicate that GRK2 phosphorylation of DOR occurs following stimulation by highly efficacious agonists such as DPDPE (Guo et al., 2000; Kouhen et al., 2000; Marie et al., 2008; Bradbury et al., 2009), deltorphin II (Bradbury et al., 2009), and (+)BW373U86, as well as SNC 80 in hippocampal lysates (Pradhan et al., 2009). In response to agonist stimulation, GRK2 hierarchically phosphorylates DOR for phosphorylation site-specific receptor regulation (Kouhen et al. 2000). The initial site phosphorylation, Ser363, promotes uncoupling of activated DOR from G proteins and desensitizes PM DOR, whereas the second phosphorylation site, Thr358, regulates receptor internalization. Although BK elicits an increase in DOR competence (Figures 1A, 1B, 4C, 4D, 4G, and 4H), it does...
not affect GRK2 phosphorylation of DOR at Ser363 (Figure 3A).

With no commercially available phospho-specific antibody for DOR Thr358, we could not evaluate GRK2 phosphorylation of the internalization residue. β-arrestin-2 mediates internalization of DOR following GRK2 phosphorylation at Ser363 (Bradbury et al., 2009). We report here that DPDPE and SNC 80, a β-arrestin-2-biased agonist in primary sensory neurons (Rowan et al., 2014), equally inhibited KCl-evoked Ca\textsuperscript{2+} influx following pretreatment by BK (Figure 1D), which suggests that β-arrestin-2 neither mediates constitutive DOR incompetence nor BK-induced functional DOR competence. Furthermore, genetic ablation of β-arrestin-2 has been reported to have no effect on DOR-mediated inhibition of VGCCs or analgesia in the absence or presence of inflammation (Pradhan et al., 2013). These data suggest that the hierarchical nature of DOR phosphorylation by GRK2 does not necessarily regulate DOR activity in sensory neurons.

GRK2 regulation of GPCR coupling independent of receptor phosphorylation has been reported for G\textsubscript{a\textsubscript{q}/11}-coupled (Dicker et al., 1999), G\textsubscript{a\textsubscript{s}}-coupled (Reiter et al. 2001), and G\textsubscript{a\textsubscript{i/o}}-coupled receptors (Lembo et al., 1999; Namkung et al., 2009). In our overexpression studies, we found that GRK2 suppression of DOR signaling in DRG is not attributable to GRK2 kinase activity because the ability of a kinase-deficient mutant to attenuate DOR activity is indistinguishable from that of GRK2 (Figures 3B and 3C). Furthermore, GRK2 overexpression also attenuates optimal DOR-G protein coupling (Figure 7D). These data implicate that GRK2 protein-protein interaction with DOR, rather than kinase activity and receptor phosphorylation, governs DOR responsiveness to agonist stimulation in peripheral sensory neurons. In immortalized cells, GRK2 constitutive association with D\textsubscript{2}R attenuates receptor signaling and G protein coupling independent of receptor phosphorylation by GRK2 (Namkung et al., 2009). Whether GRK2 chronically downregulates GPCRs other than DOR at the PM in sensory neurons remains to be elucidated.

An orchestration of signaling events are likely necessary to induce peripheral functional DOR competence in physiologically relevant systems. BK receptors are fundamental to peripheral opioid analgesia following inflammatory insult or after chronic constriction injury (Cayla et al., 2012). In approximately half of small-sized TG and DRG that overexpress DOR-GFP, a mild increase in DOR trafficking to the PM is induced by BK (200 nM) within 5–10 min (Pettinger et al., 2013). We did not observe any notable differences in native DOR trafficking to the PM following BK exposure (Figures 2A and 7A–7D); however, this effect could be diluted in our cultures for biochemistry that included neurons with sizes ranging from small to large along with support cells (glial, etc.). In addition to an increase in DOR targeting to the PM in small neurons, Pettinger et al. (2013) also observed a doubling of CAP-sensitive TG neurons that respond to a DOR agonist following BK (200 nM; 15 min) treatment. We also
observed this phenomenon in DRG; the total population of neurons that responded to DPDPE rose from 22.9%–25.0% to 48.3%–52.9% following BK (200 nM; 5 min) treatment (Figure S2). Interestingly, our population data also revealed that BK increased the total population of DRG that responded to CAP (1 μM) from 50.6%–59.5% to 70.3%–79.4%. These data are consistent with another report that found that approximately 70% of DRG are sensitive to this dose of CAP (Wang et al., 2008). It has been demonstrated that BK lowers the threshold for heat-activation of TRPV1 in DRG (Sugiura et al., 2002), and our data suggest that BK also enhances CAP-sensitivity in DRG.

Comprehensively, results presented herein contribute to a collection of findings that characterize mechanisms driving DOR responsivity in multiple cellular models. For instance, allosteric modulation of DOR by sodium ions (Fenali et al., 2014) could also allosterically affect constitutive GRK2 association with the receptor. Additionally, GRK2 association with DOR, which contributes to receptor internalization, most likely facilitates reduced DOR responsiveness following biased ligand administration (Pradhan et al., 2009). However, it is difficult to determine whether STATS signalosome formation with DOR is affected by chronic GRK2 association with the receptor, since both utilize the same C-terminal amino acids (Georganta et al., 2010). Importantly, many of these studies utilize non-physiologic model cell systems, including transfected immortalized cells, which can overexpress receptor proteins relative to other endogenous regulatory proteins. Results presented here employ more physiologically relevant sensory neurons, providing analysis of endogenously expressed receptors and regulatory proteins that correlate more with behavioral measures, and hence, clinical relevance.

B2R couples to multiple classes of G proteins, including G_{q/11} and G_{q/12}, and stimulates differential signaling cascades (Liebmann and Böhrer, 2003). B2R activation of G_{q/11} primarily activates the PLC-PKC pathway, whereas G_{q/12} initiates cAMP-PKA signaling. Additionally, studies in immortalized cell lines have demonstrated that GRK2 can be directly phosphorylated by either PKC (Chuang et al., 1995; Pronin and Benovic, 1997) or PKA (Cong et al., 2001) to affect GPCR desensitization. We found that PLC and PKC, but not PKA, were involved in BK-induced GRK2 dissociation from PM DOR and BK priming of DOR in sensory neurons (Figure S3). Indeed, work in neuroblastoma cells demonstrated PKC and GRK2 mediate DOR desensitization (Marie et al., 2008). Although the concentration of BK used in our study (200 nM) activates B2R’s primary PLC-PKC pathway, it may not sufficiently activate cAMP in DRG (Wang et al., 2008). Thus, at higher concentrations of BK, it may be possible that PKA contributes to functional DOR competence. Although PKA was not investigated, Patwardhan et al. (2006) demonstrated that PKC inhibition blocks peripheral DOR competence induced by a more potent dose of BK (10 μM) in sensory neurons.

The importance of BK priming on functional DOR competence observed in vitro, by this study and others (Patwardhan et al., 2005, 2006; Sullivan et al., 2015), was recapitulated in vivo. A peripherally restrictive dose of DPDPE was unable to elicit an anti-allodynic response unless primed by BK in MM-ODN-treated rats. This finding is similar to observations in rats in the absence of i.t. ODN treatments prior to behavioral experimenta-
Animals
Adult male Sprague-Dawley rats (Charles River Laboratories) weighing 200–250 g (biochemistry, Ca\(^{2+}\) imaging) or 350–400 g (oligodeoxynucleotide-treated) were used in this study. Animals were housed in clean cages with a 12 h light/dark cycle for 1 week with food and water ad libitum before use.

Neuronal Cultures
For biochemistry, trigeminal ganglia (TG) were dissected bilaterally from male rats. TG were dissociated by 30 min collagenase (Worthington) treatment followed by 30 min trypsin (Sigma-Aldrich) treatment, with gentle rocking every 10 min. Cells were then resuspended in complete media (DMEM, Invtrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 ng/ml nerve growth factor (NGF; Harlan Laboratories), mitotic inhibitors (Sigma-Aldrich), 1% penicillin/streptomycin (Invitrogen), and 1% glucose (Sigma-Aldrich) and plated on poly-D-lysine-coated plates (Corning). Cultures were maintained at 37°C and 5% CO\(_2\) and grown for 5–6 days with media changed the following day and every 2 days thereafter. TG were utilized for biochemical experiments to satisfy NIH requirements to reduce animal use in research.

For Ca\(^{2+}\) imaging, TG or dorsal root ganglia (DRG) dissected bilaterally at L4-L6 were dissociated by 40 min co-treatment with collagenase (Worthington) and dispase (Sigma-Aldrich) with gentle rocking every 10 min. Next, cells were resuspended in complete media and plated on poly-D-lysine/laminin-coated coverslips (BD Biosciences). Media was changed the following day and experiments were conducted within 24–48 h of initial culture.

Knock down and overexpression strategies are described in the Supplemental Experimental Procedures.

Crude Membrane Preparation
Primary TG cultures were pretreated as indicated. Cells were harvested and homogenized in homogenization buffer (25 mM HEPES, 25 mM sucrose, 1.5 mM MgCl\(_2\), 50 mM NaCl [pH 7.4], 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate [Sigma-Aldrich], 1 μg/ml pepstatin [Sigma-Aldrich], 1 μg/ml leupeptin [Sigma-Aldrich], 1 μg/ml aprotenin [Sigma-Aldrich], and 100 mM phenylmethylsulphonyl fluoride [PMSF, Sigma-Aldrich]) with 20 strokes using a Potter-Elvehjem pestle and glass homogenizer tube. Homogenates were placed on ice for 15 min incubation and then centrifuged at 1,000 x g for 1 min to remove nuclei and unlysed cells from the homogenate. Resulting supernatant was centrifuged at 16,000 x g for 30 min at 4°C to separate cell membrane proteins from cytosolic proteins. Cytosolic supernatant was separated from the pellet (crude membrane fraction), which was re-suspended in 250 μl homogenization buffer containing 1% Triton X-100 (Fisher Scientific). Total protein was quantified using Bradford assay (Sigma-Aldrich) prior to co-immunoprecipitation (coIP). For coIP protocol and western blot (WB) analysis details, please see the Supplemental Experimental Procedures.

Ca\(^{2+}\) Imaging
Fura-2 AM was used to image individual neurons within a population of cultured ganglionic cells. Following 2 hr serum-starvation, cells were loaded with fura-2 AM (1 μM; Molecular Probes) in the presence of pluronic F-127 (0.04%; Molecular Probes) for 1 hr at 37°C in the dark, in standard extracellular solution (SES) containing (in mM): 140 NaCl, 10 EGTA, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, pH 7.20. Whole-cell configuration was established in extracellular solution containing (in mM): 140 TEA-Cl, 1 MgCl\(_2\), 10 HEPES, 10 D-glucose, pH 7.30, Axopatch 200B amplifier and pCLAMP9.6 software (Molecular Devices, Axon) were used to acquire and analyze data. From a holding potential of ~60 mV, VGCC currents were activated by single pulse from ~60 to 0 mV (50-ms duration). Waveform was applied repetitively every 10 s. DPDPPE (1a was applied via local application. Coverslips were incubated at room temperature for 10 min either with vehicle or with BK (200 nM). All recorded cells sized 20–35 pF were included in analysis.

Behavioral Test for BK Priming of DOR Functional Competence
This study utilized custom GRK2 antisense (AS) and mismatch (MM) oligodeoxynucleotide (ODN) sequences synthesized by Invitrogen first described by Ferrari et al. (2012). ODNs were intrathecally (i.t.) administered to rats anesthetized with 2.5% isoflurane once daily for 3 days prior to behavioral testing. On day 4, BK-induced DPDPPE inhibition of prostaglandin (PGE\(_2\))-induced thermal and mechanical alldynia was assessed in ODN-treated rats to determine the role of GRK2 in BK priming of peripheral DOR antinociception. All measurements were conducted by blinded observers. For further explanation, see the Supplemental Experimental Procedures.

Electrophysiology
Whole-cell patch-clamp recordings were used to measure DOR-mediated inhibition of VGCCs in cultured rat DRG neurons (20–35 pF). Following 2 hr incubation in 2% SEAS at 37°C, whole-cell patch-clamp configuration was performed at room temperature on neurons viewed on an upright Nikon Eclipse E600FN microscope fitted with a 40x/0.80W numerical aperture objective. Borosilicate glass patch capillaries (Sutter) were polished to resistances of 2–4 MΩ and filled with internal solution containing (in mM): 140 NaCl, 10 EGTA, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, pH 7.20. Whole-cell configuration was established in extracellular solution containing (in mM): 140 TEA-Cl, 1 MgCl\(_2\), 10 HEPES, 10 D-glucose, pH 7.30. Axopatch 200B amplifier and pCLAMP9.6 software (Molecular Devices, Axon) were used to acquire and analyze data. From a holding potential of ~60 mV, VGCC currents were activated by single pulse from ~60 to 0 mV (50-ms duration). Waveform was applied repetitively every 10 s. DPDPPE (1a was applied via local application. Coverslips were incubated at room temperature for 10 min either with vehicle or with BK (200 nM). All recorded cells sized 20–35 pF were included in analysis.

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.07.084.

AUTHOR CONTRIBUTIONS

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Supplemental Information

GRK2 Constitutively Governs Peripheral Delta Opioid Receptor Activity

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Figure S1, related to figure 1. DPDPE Inhibition of KCl-evoked Ca\(^{2+}\) influx in CAP-sensitive sensory neurons. a. Representative trace from single-cell Ca\(^{2+}\) imaging protocol for DPDPE (1 µM) inhibition of KCl (50 mM)-evoked Ca\(^{2+}\) influx from cultured DRG neuron following 5 min pretreatment with vehicle. b. Average KCl response in the presence of DPDPE compared to the average KCl response in the absence of DPDPE (from trace a). c. Representative trace from single-cell Ca\(^{2+}\) imaging protocol for DPDPE (1 µM) inhibition of KCl (50 mM)-evoked Ca\(^{2+}\) influx from cultured DRG neuron following 5 min pretreatment with BK (200 nM). d. Average KCl response in the presence of DPDPE compared to the average KCl response in the absence of DPDPE (from trace c). Area Under the Curve (AUC) from the average responses are used obtain DPDPE inhibition of KCl-evoked Ca\(^{2+}\) influx as a measure of DOR activity using the equation outlined within Methods and Materials section.
**Figure S2**

**Figure S2, related to figure 1.** DPDPE Inhibition of KCl-evoked Ca\(^{2+}\) influx at various post-dissection culturing time points. **a.** Cumulative traces and **b.** quantification of DPDPE (1 µM) inhibition of KCl (50 mM)-evoked Ca\(^{2+}\) influx in CAP-sensitive rat DRG neurons pretreated for 5 min with vehicle or BK (200 nM) at 2 h, 24 h, and 48 h following DRG dissection. Data represented as % mean ± SEM, significance determined by two-way ANOVA with Bonferroni post-hoc analyses for multiple comparisons (***p<0.01, **p<0.005, P=0.001; n = 19-27 DRG/group). **c.** Population data related to **a.** and **b.** for sensitivity to capsaicin (CAP; 1 µM; threshold: 25% above baseline) and DPDPE (1 µM; threshold 20+% inhibition) in vehicle- and BK-treated DRG cultures. Percentages; CAP+/DPDPE+: Vehicle – 4.76% (2 h), 2.86% (24 h), 2.50% (48 h); BK – 41.37% (2 h), 47.06% (24 h), 40.74% (48 h); CAP+/DPDPE-: Vehicle – 54.76% (2 h), 51.43% (24 h), 47.5% (48 h); BK – 34.48% (2 h), 32.35% (24 h), 29.63% (48 h); CAP-/DPDPE+: Vehicle – 19.05% (2 h), 20.00% (24 h), 22.50% (48 h); BK – 6.90% (2 h), 5.88% (24 h), 11.11% (48 h); CAP-/DPDPE-: Vehicle – 21.43% (2 h), 25.71% (24 h), 27.50% (48 h); BK – 17.24% (2 h), 14.71% (24 h), 18.52% (48 h).
Figure S3, related to figure 2. PLC-PKC pathway activation mediates DOR priming by BK. a. Co-immunoprecipitation of crude PM preparations from cultured rat TG neurons serum-starved for 18 h and treated for 5 min with vehicle or inhibitor [GFX (GF 109203X, 10 µM); U73122, 10 µM; H-89, 20 µM] prior to 5 min treatment with vehicle or BK (200 nM). Densitometric quantification of WB data represented as mean ± SEM, results representative of three independent trials, statistics determined by one-way ANOVA with Bonferroni post-hoc analyses (*p<0.05 vs. Vehicle/Vehicle, P=0.0023; n = 3 independent trials). b. Cumulative traces and c. quantification of DPDPE (1 µM) inhibition of KCl (50 mM)-evoked Ca²⁺ influx in rat DRG neurons pretreated for 5 min with vehicle or inhibitor [GFX (GF 109203X, 10 µM); U73122, 10 µM; H-89, 20 µM] prior to 5 min treatment with vehicle or BK (200 nM). Data represented as % mean ± SEM, significance determined by two-way ANOVA with Bonferroni post-hoc analyses for multiple comparisons (*p<0.05, P=0.002; n = 20-29 DRG/group).
Figure S4. Related to figure 5. BK priming of functional DOR competence is mediated by PKC phosphorylation of RKIP. a. Cumulative traces and b. quantification of DPDPE (1 µM) inhibition of KCl (50 mM)-evoked Ca$^{2+}$ influx in DRG nucleofected with a combination of cDNA encoding for empty vector (E.V.), RKIP, or RKIP$_{S153A}$, with a GFP-expression empty vector cDNA for positive identification of positively-nucleofected neurons. All cells on coverslips were pretreated with vehicle or BK (200 nM, 5 min). Data represent % mean ± SEM with significance determined by two-way ANOVA with Bonferroni post-hoc analyses for multiple comparisons (**)p<0.01, ns = no significance, Interaction: P=0.0186; Vehicle/BK: P=0.0002; Overexpression: P=0.004; (n = 18-29 DRG neurons).
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Co-Immunoprecipitation (Co-IP/IP)

Total protein from either plasma membrane (PM) or cytosolic fractions were quantified using Bradford assay (Sigma). For Co-IP, equal amounts of protein (125 µg) were immunoprecipitated with 1 µg anti-GRK2 (C-15, Santa Cruz Biotechnology) or anti-DOR (ab66317, Abcam) antiserum. GRK2 antibody specificity has been confirmed in inducible GRK2 sensory neuron knockout animals (Wang et al., 2011) and DOR antibody specificity for immunoprecipitation has been previously vetted (Rowan et al., 2014). Protein samples were eluted at 95°C for 5 min or 37°C for 30 min to maintain dimerization, such that the protein of interest close in molecular weight was not masked by immunoglobulin monomer band.

Western Blot (WB) Analysis

Whole cell lysates (WCL) from bilaterally dissected L4-L6 dorsal root ganglia (DRG), and WCL, plasma membrane (PM), and cytosolic immunoprecipitates from primary trigeminal ganglia (TG) cultures were resolved on SDS/PAGE (15% gels) and transferred to PVDF membrane (Millipore). Membranes were then blocked with 5% non-fat dried milk in TBS containing phosphatase inhibitor sodium orthovanadate (1 µM) for phosphorylation-specific antibodies. WBs were visualized using anti-GRK2 (C-15, Santa Cruz Biotechnology; Wang et al., 2011), anti-DOR (ab66317, Abcam; Byun et al., 2012; Rowan et al., 2014), anti-p-DORS363 (ab62152, Abcam; Byun et al., 2012; Rowan et al., 2014), anti-RKIP (D-5, Santa Cruz Biotechnology; Deiss et al., 2012), anti-p-RKIPS153 (rSer 153, Santa Cruz Biotechnology; Deiss et al., 2012), anti-β-actin (1A4, Santa Cruz Biotechnology; Santa Cruz Biotechnology; Tselnicker et al., 2010), or anti-β-actin (1-19-R, Santa Cruz Biotechnology; Por et al., 2010), followed by appropriate horseradish-peroxidase-conjugated secondary antisera (GE Healthcare) and ECL (enhanced chemiluminescence prime) detection following the manufacturer’s protocol (GE Healthcare). Antibody specificities were verified by the manufacturers, BLAST sequence analysis, and used in previous publications as indicated. Integrated density measurement values, equivalent to the product of area and mean gray value, were performed using NIH ImageJ software.

siRNA

Sequence Details

Specific siRNA and FITC-labeled siRNA duplexes custom-designed to target GRK2 or RKIP were designed and ordered from Qiagen (Valencia, CA). The sequence for the sense strand of GRK2 siRNA was 5’-GAGAGAUCAGAGGAGAUUU-3’ and antisense strand of GRK2 siRNA was 5’-AUCCUCUAUAACUCUGCUU-3’. The sequence for the sense strand of RKIP siRNA was 5’-ACACAGGUCUGCACCUGUAU-3’ and antisense strand of RKIP siRNA was 5’-UAGCGGAGCAGACCUGU-3’.

Transfection

For GRK2 siRNA experiments, primary TG or DRG cultures were transfected with siRNA or FITC-labeled siRNA (45 ng/coverslip; 2 µg/10-cm plate) for 18 h, replenished with complete media, and biochemistry or calcium imaging experiments began within 3 h following media change. For experiments utilizing RKIP siRNA, cells were transfected with siRNA or FITC-labeled siRNA (450 ng/coverslip; 20 µg/10-cm plate) for 24 h and the transfection mix was replaced with complete media for 16 h prior to experimentation. GRK2 and RKIP siRNA duplexes were transfected into cultured sensory neurons using HiPerFect (Qiagen, Valencia, CA), following manufacturer’s directions. Additional cells were treated with no siRNA (mock) or scrambled siRNA (Silencer-1, Ambion, Austin, TX), used as a negative control to determine specificity of GRK2 and RKIP siRNAs.

Knockdown in TG Cultures

**GRK2 siRNA:** TG cultures were transfected with siRNA or FITC-labeled siRNA targeted against GRK2 mRNA, or with scrambled siRNA or no siRNA (mock). Following treatment, cells were homogenized in homogenization buffer (25 mM HEPES, 25 mM sucrose, 1.5 mM MgCl₂, 50 mM NaCl (pH 7.4), 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate (Sigma), 1 µg/mL pepstatin (Sigma), 1 µg/mL leupeptin (Sigma), 1

**RKIP siRNA:** TG cultures were transfected with siRNA or FITC-labeled siRNA targeted against RKIP mRNA, or with scrambled siRNA or no siRNA (mock). Following treatment, cells were homogenized in homogenization buffer (25 mM HEPES, 25 mM sucrose, 1.5 mM MgCl₂, 50 mM NaCl (pH 7.4), 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate (Sigma), 1 µg/mL pepstatin (Sigma), 1 µg/mL leupeptin (Sigma), 1
µg/mL aprotinin (Sigma), and 100 nm phenylmethylsulphonyl fluoride (PMSF, Sigma)) containing 1% Triton X-100 (Fisher Scientific) with 20 strokes using a Potter-Elvehjem pestle and glass homogenizer tube. Homogenates were placed on ice for 15 min incubation and then centrifuged at 1000 x g for 1 min to remove nuclei and unlysed cells from the homogenate. Resulting supernatant, whole cell lysates (WCL), was collected and Western blot (WB) analysis was conducted with anti-GRK2 (C-15, Santa Cruz Biotechnology, Santa Cruz, CA; Wang et al., 2011) normalized to anti-β-actin (I-19-R, Santa Cruz Biotechnology; Por et al., 2010). Antibody specificity was previously demonstrated as referenced.

**RKIP siRNA:** Similarly, TG cultures were transfected with siRNA or FITC-labeled siRNA targeted against RKIP mRNA, or with scrambled siRNA or no siRNA (mock). Following treatment, cells were homogenized in homogenization buffer containing 1% Triton X-100 with 20 strokes using a Potter-Elvehjem pestle and glass homogenizer tube. Homogenates were placed on ice for 15 min incubation and then centrifuged at 1000 x g for 1 min to remove nuclei and unlysed cells from the homogenate. Resulting supernatant, whole cell lysates (WCL), was collected and Western blot (WB) analysis was conducted with anti-RKIP (D-5, Santa Cruz Biotechnology; Deiss et al., 2012) normalized to anti-β-actin (I-19-R, Santa Cruz Biotechnology; Por et al., 2010). Antibody specificity was previously demonstrated as referenced.

**cDNA Nucleofection**

Sensory neurons were cultured as described above and nucleofected with Effectene nucleofection reagent (Qiagen) following manufacturer’s instruction, maintaining a cDNA to enhancer reagent ratio of 1:8, for 10 h. The day prior to overexpression and rescue experiments in DRG, 500 ng/cover slip of empty vector (E.V.) pcDNA3.1, GRK2, GRK2-K220R (Dr. Jeffrey L. Benovic, Thomas Jefferson University, USA), RKIP or RKIPK220R (Dr. Kristina Lorenz, University of Wuerzburg, Germany) cDNA was co-nucleofected with 500 ng/cover slip GFP cDNA to identify positive transfection. Nucleofection efficiency was approximately sixty percent. For overexpression biochemistry experiments in TG, 4 µg E.V. or GRK2 cDNA were nucleofected onto 10-cm plates following the same protocol.

**Oligodeoxynucleotides (ODNs)**

**Sequence Details**

This study utilized custom GRK2 antisense- (AS) ODN and mismatch- (MM) ODN sequences synthesized by Invitrogen first described by Levine and colleagues (Ferrari et al., 2012). GRK2 AS-ODN, 5’-CTT TGT GAT GTC G-3’, was directed against a unique sequence of rat GRK2 mRNA. MM-ODN, 5′-GTT TAC GTA GTT CTC C-3’, is the AS sequence with boldface-denoted changes switched.

**Preparation and Administration**

MM- or AS- ODNs were reconstituted in sterile-filtered PBS at a stock concentration of 10 µg/µL and stored at -20°C. For administration of ODNs, rats were briefly anesthetized with 2.5% isoflurane before a 28½-gauge needle insulin syringe (Becton Dickinson, Franklin Lakes, NJ) was inserted intrathecally (i.t.) on the midline between vertebrae L5 and L6. Injection location at the level of cauda equine was confirmed by tail-flick (Papir-Kricheli et al., 1987; Mestre et al., 1994). 30 µg ODN (in 20 µL sterile PBS) was administered once daily for three consecutive days. Behavioral testing was completed the following day within 24 h of the final injection and ODN knockdown was confirmed as described below.

**Knockdown**

**GRK2 AS-ODN:** Following behavior and within 24 h of the final ODN administration, DRG were bilaterally harvested from L4-L6 and DRG bundles were removed from afferent branches (axons). DRG were homogenized in homogenization buffer containing 1% Triton X-100 with 40 strokes using a Potter-Elvehjem pestle and glass homogenizer tube. Homogenates were placed on ice for 15 min incubation and then centrifuged at 1000 x g for 1 min to remove nuclei and unlysed cells from the homogenate. Resulting supernatant, WCL, was collected for WB analysis. Anti-GRK2 (C-15, Santa Cruz Biotechnology, Santa Cruz, CA; Wang et al., 2011) normalized to anti-β-actin (I-19-R, Santa Cruz Biotechnology; Por et al., 2010) was utilized to confirm GRK2 protein knockdown by AS-ODN. Antibody specificity was previously demonstrated as referenced.

**Behavioral Test for BK Priming of DOR Functional Competence**

The original Electronic von Frey (IITC Life Science Inc., Woodland Hills, CA) was used to assess mechanical allodynia in rats, measured by paw withdrawal threshold (PWT; g; maximum set to 55 g) to a rigid tip. After 30 min
habitation to acclimate rats to raised von Frey mesh stand, a rigid tip was placed beneath the hindpaw. For testing, the pressure probe tip was applied to the test subject’s hindpaw. Upon reaction the electronic system displayed the reading in grams and automatically determined PWT values. Duplicate measurements were taken for each time point at least 30 s apart and averaged for statistical analysis.

In a separate cohort of rats, thermal allosthenia was assessed by readings of paw withdrawal latency (PWL; sec; maximum set to 20 s) to a thermal stimulus using a plantar test apparatus (Hargreaves et al., 1988). After 30 min habitation to acclimate to environment, a beam of radiant heat was placed under the glass floor directly beneath the hindpaw, which gradually increases in the hindpaw temperature (Dirig et al., 1997). A photoelectric cell automatically determined PWL values. Duplicate measurements were taken for each time point at least 30 s apart and averaged for statistical analysis.

A minimum of six baseline (BL) PWT and four BL PWL measurements were obtained from ipsilateral and contralateral hindpaws of each animal. In this protocol, PGE$_2$ (0.3 µg; Cayman Chemicals, Ann Arbor, MI) is used to elicit a mild and prolonged allodynia that remains for a minimum of 20 min. DOR activity was measured as DPDPE (20 µg) inhibition of PGE$_2$-induced allodynia, which is known to elicit analgesia only when primed by an inflammatory mediator, such as BK (25 µg). Following this protocol, BL PWT and PWL were collected and animals were injected with vehicle or BK 15 min prior to co-injection of PGE$_2$ and DPDPE. To determine whether GRK2 knockdown affects the magnitude and duration of BK allodynia, mechanical and thermal readings were measured on the ipsilateral hindpaw at 5 and 10 min post-pretreatment. To determine if GRK2 knockdown affects BK priming of DOR, ipsilateral PWT and PWL were also measured every 5 min for 20 min following co-injection. Drug doses and time course followed previous studies (Rowan et al., 2009; Berg et al., 2012; Rowan et al. 2014; Sullivan et al., 2015). Stock solutions were prepared in ethanol (PGE$_2$), sterile water (DPDPE), or saline (BK) and diluted in saline prior to injections. Drugs were administered via intraplantar (i.pl.) injection at a final volume of 50 µL. All measurements were conducted by blinded observers.

**Tissue Processing and Immunohistochemistry**

Male Sprague-Dawley rats were anesthetized with an intraperitoneal (i.p.) injection of pentobarbital (25 µg) and were transcardially perfused with 100 mL 0.9% saline in water followed by 250 mL of fixative (4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4). Fixed TG and DRG were removed and left intact. Tissue specimens were rinsed in 0.1 M PB for 10 min three times and then rinsed in 0.1 M PB with 15% sucrose for 1 h at room temperature. Intact TG and DRG rocked at 4°C in 30% sucrose in 0.1 M PB for 18 h. Cryoprotected ganglia were embedded in Neg-50 mounting medium (Richard-Allan Scientific, Kalamazoo, MI) and a cryostat was used to slice structures into 30-µm transverse sections that were placed onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Processed slides were dried and stored at -20°C.

For staining, all steps were performed at room temperature. Slides were rinsed in 0.1 M phosphate buffered saline (PBS) for three periods of 10 min each. To minimize non-specific binding, samples were incubated for 90 min in blocking solution (2% bovine -globulin (Sigma, St. Louis, MO), 4% normal horse serum (Sigma), and 0.3% Triton X-100 (Fisher Scientific) in 0.1 M PBS). Next, the tissue incubated in primary antibody against RKIP (07-137, Millipore; at 1:100 dilution in blocking solution) for 18 h in a humidifier. Antibody specificity has been validated with no immunoreactivity in RKIP knockout mice (Subramanian et al., 2014). Next, tissue was rinsed with 0.1 M PBS and placed in a dark humidifier for 90 min to incubate in corresponding species-specific secondary antibody Alexa Fluor 488 (Molecular Probes at 1:100 dilution in blocking solution) used visualize RKIP immunoreactivity. Nuclei marker Topro (Invitrogen; 1:50,000 dilution) was added to the secondary antibody-blocking solution. Tissue was rinsed in 0.1 M PBS, followed by a final wash in deionized water. Once dry, stained tissue was coverslipped with Vectashield (Vector Labs, Burlingame, CA) and stored at 4°C until they were evaluated by confocal microscopy. Additional control experiments were performed on tissue sections processed as described above without primary antibody. Sections were evaluated, and images were obtained using a Nikon 90i microscope equipped with a C1si laser scanning confocal imaging system (Nikon, Melville, NY). Confocal images are representative of four individual trials.

**Ca$^{2+}$ Imaging**

Primary afferent neurons were selected by (1) characteristic phenotype (bright round cell bodies with clear nuclei; Goldenberg & De Boni, 1983; Liu et al., 2013), (2) response to depolarizing solution containing 50 mM KCl (Kahsabova et al., 2004; Pettinger et al., 2013), and (3) sensitivity to capsaicin (CAP, 1 µM, 25% above baseline; Sigma) to select for TRPV1-positive neurons. CAP-sensitivity of recorded cells also indicates positive sensory neuronal phenotype within the heterogenous culture.

For opioid-inhibition studies, coverslips prepared as described above were placed in SES containing vehicle or bradykinin acetate (BK, 200 nM; Sigma) for five minutes before experiment began. For each cell imaged, basal
Ca\(^{2+}\) levels were recorded for 60 s. Perfusion valve controller (<0.1 psi) and multi-barrel glass pipette were used to apply 3 s exposures of 50 mM KCl at five time points: 60 s, 270 s, 740 s, 950 s, and 1440 s. The first and second exposures to KCl were given in the absence of a DOR agonist. At 480 s, SES bath containing vehicle or BK was replaced with a DOR agonist ([D-Pen\(^{2,5}\)]-enkephalin (DPDPE), 1 µM, Sigma; or SNC 80, 1 µM, Tocris) in SES applied via constant perfusion (<0.1 psi) by multi-barrel glass pipette. Prior to the third and fourth exposure to KCl, DOR agonist microinjection was terminated to reduce pressure-related artifacts. At 1160 s, SES containing DOR agonist was washed out and replaced with SES via bath perfusion (<0.1 psi) using ML-6 manifold apparatus prior to the fifth exposure to KCl. Finally, cells were exposed to 1 µM CAP at 1770 s to select for TRPV1-positive primary afferent neurons that can be correlated with thermal allodynia behavior.

In order to measure opioid inhibition of KCl-evoked Ca\(^{2+}\) influx, single-cell recording traces from vehicle- and BK-treated were used to obtain the average area under the curve (AUC), calculated as an integral over a time period (3 min) for the average cellular response to KCl in the absence or presence of a DOR agonist (Figure 1A-1D). The following equations were used to calculate DOR activity:

\[
\begin{align*}
KCl &= \frac{(KCl_1, KCl_2, KCl_3)}{3} \\
KCl + DOR Agonist &= \frac{(KCl_3, KCl_4)}{2}
\end{align*}
\]

\[
DOR Agonist Inhibition (\%) = 100 - \left( \frac{AUC_{KCl + DOR Agonist}}{AUC_{KCl}} \right) \times 100\%
\]

For Ca\(^{2+}\) imaging, stock solutions of DOR agonists DPDPE (1 mM) and SNC 80 (1 mM) were prepared in sterile water and DMSO, respectively. CAP (10 µM) stock solution was prepared in EtOH. Naltrindole isothiocyanate hydrochloride, GF 109203X (GFX), U73122, and H89 stock solutions were prepared in DMSO (Sigma). BK and KCl were prepared fresh in SES the day of experimentation. All drugs and appropriate vehicles were diluted in SES. For experiments that utilized irreversible antagonist naltrindole (10 nM; Sigma) or inhibitors for PKC (GFX, 10 µM), PLC (U73122, 10 µM), or protein kinase A (PKA) (H-89, 20 µM), cultures were pretreated 5 min prior to vehicle or BK treatment.
SUPPLEMENTAL REFERENCES


