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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) naively 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.

Graphical abstract

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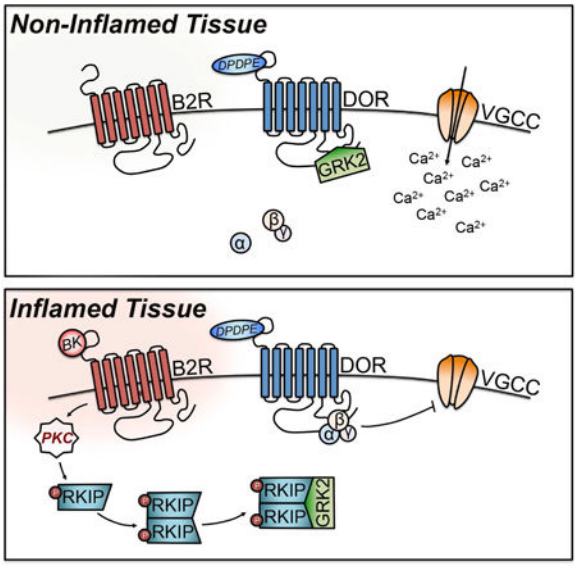

Introduction

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). 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 TW, 2010). Like other opioid receptors, DOR primarily signals downstream through Gαi and Gβγ subunits (Alvez *et al.*, 2004) that inhibit neuronal depolarization by decreasing cAMP activity (Law & Bergsbaken, 1995) and inhibiting voltage-gated Ca^{2+} channels (VGCCs) (Ford *et al.*, 1998). 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; Patwardhan *et al.*, 2006; Gaveriaux-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 & Zochodne, 2000). Nociceptive responses following BK administration are mediated via $G\alpha_{q/11}$–coupled GPCRs expressed by primary afferent neurons that co-express opioid receptors (Steranka *et al.*, 1988; Patwardhan *et al.*, 2005; Jeske *et al.*, 2006; 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 naively 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 (Kroslak *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 naïve and primed sensory neurons

Opioids elicit their analgesic effects, in part, via receptor-mediated inhibition of VGCCs (Stein & 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 (Kahsabova *et al.*, 2002). Activation of DOR inhibits KCl-evoked Ca^{2+} influx through L-, N-, and P/Q- type VGCCs, as well as KCl-induced neurotransmitter release in DRG (Kahsabova *et al.*, 2004). Multiple investigators have previously quantified opioid inhibition of VGCCs via KCl-evoked Ca^{2+} influx in cultured sensory neurons (Kahsabova *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 (Figure 1A-1B). At doses ranging from 1 nM to 1 μM, the DOR agonist 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+} accumulation 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. IC_{50} values for DPDPE were 6.91×10^{-6} M versus 5.21×10^{-6} M for vehicle-treated TG and DRG, respectively. IC_{50} values for DPDPE were 6.15×10^{-7} M versus 7.39×10^{-7} 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 X *K*i; 5 min) (Figure 2C). This demonstrates that inhibition of KCl-evoked Ca^{2+} 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 (Figure 1E-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 naïve conditions, we first used co-immunoprecipitation 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 naïve 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 naïve and primed conditions, we employed siRNA-mediated knockdown of GRK2 expression and assessed DOR activity using Ca^{2+} imaging and patch-clamp electrophysiology. To demonstrate the specificity of this molecular approach, we first assessed the efficiency of GRK2 knockdown 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 knockdown of GRK2 using Ca^{2+} imaging. BK pretreatment (200 nM; 5 min) significantly increased DPDPE inhibition of KCl-evoked Ca^{2+} influx in mock-transfected DRG neurons (Figure 2C-2D). However, in DRG cultures transfected with FITC-GRK2 siRNA, the increase in DPDPE inhibition of KCl-evoked Ca^{2+} 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 RT *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 G *et al.*, 1994) and measured DOR inhibition of KCl-evoked Ca^{+2} influx in vehicle-treated 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.) (Figure 3B-3C). However, in DRG nucleofected with GRK2 or K220R, BK was unable to induce DPDPE inhibition of KCl-evoked Ca^{2+} 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*. 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 (Figure 4A-4B). To assess whether GRK2 knockdown affects DOR analgesic competence, we assessed DPDPE inhibition of PGE_2-induced mechanical and thermal allodynia following BK priming in MM- and AS-ODN-treated rats (Figure 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 knockdown 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 knockdown. 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 & Bohmer, 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 $G_{\alpha q}$–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) treatment 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 at Ser153 in sensory neurons.

We next sought to determine whether BK-induced PKC stimulation also directs RKIP dimerization and association with GRK2, using Co-IP 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 (Figure 5B-5C), we hypothesized that RKIP sequestration of GRK2 governs BK priming of DOR in sensory neurons.

We employed siRNA-mediated knockdown 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 knockdown relative to β-actin, and found a 65% reduction in normalized RKIP protein expression one day post-transfection (Figure 6A).

Next, we sought to determine whether BK priming of DOR functional competence remained intact in DRG following siRNA-mediated knockdown of RKIP protein expression. BK pretreatment (200 nM; 5 min) significantly increased DPDPE inhibition of KCl-evoked Ca^{2+} influx in mock-transfected DRG (Figure 6B-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 neurons 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 (Alvez *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 (Figure 1A-1B), we investigated the possibility that BK might influence interactions between Gβ and DOR using Co-IP 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 Gβ. For this, we utilized siRNA and Co-IP techniques in TG cultures to validate the role of GRK2 in DOR coupling to Gβ. BK pretreatment (200 nM; 5 min) increased DOR-bound Gβ in serum-starved mock- and GRK2 siRNA-treated TG culture PM lysates (Figure 7C). These data indicate that the absence of GRK2 alone is not sufficient to recruit Gβ to the receptor. To investigate whether GRK2 overexpression impairs DOR coupling to Gβ, PM Co-IPs 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, Gβ co-immunoprecipitated with DOR in vehicle-treated TG cultures nucleofected with E.V., while BK treatment increased Gβ co-immunoprecipitation with DOR. GRK2 overexpression blocked the BK-dependent increase in Gβ association with DOR (Figure 7D). Thus, the constitutive association between GRK2 and DOR attenuates Gβ:receptor coupling in sensory neurons. Together with our DOR-GRK2 association and functional imaging studies, these findings suggest that GRK2 hinders receptor coupling to Gβ, such that Gβ 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; Patwardhan *et al.*, 2006) and *in vivo* (Stein *et al.*, 1989; Rowan *et al.*, 2009), and pretreatment 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 naïve 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 naïve afferent terminals is impaired by a constitutive interaction with GRK2 at the PM that prevents receptor coupling to Gβ, 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 Gβ 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, and 4G-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^{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 $G\alpha_{q/11}$- (Dicker *et al.*, 1999), $G\alpha_s$- (Reiter *et al.* 2001), and $G\alpha_{i/o}$-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 (Figure 3B-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_2R 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 about 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, 7A-D); 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.* 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 50-59.5% 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 suggests that BK also enhances CAP-sensivity 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 (Fenalti *et al.*, 2014) could also allosterically affect constitutive GRK2 association with the receptor. Additionally, GRK2 association with DOR, which contributes to receptor internalization, likely facilitates reduced DOR responsiveness following biased ligand administration (Pradhan *et al.*, 2009). However, it is difficult to determine whether STAT5 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 $G\alpha_q$ and $G\alpha_s$, and stimulates differential signaling cascades (Lliebmann & Böhmer, 2000). B2R activation of $G\alpha_q$ primarily activates the PLC-PKC pathway, whereas $G\alpha_s$ 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 & 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.* demonstrated that PKC inhibition blocks peripheral DOR competence induced by a more potent dose of BK (10 μM) in sensory neurons (Patwardhan *et al.*, 2006).

The importance of BK priming on functional DOR competence observed *in vitro*, by this study and others (Patwardhan *et al.*, 2005; Patwardhan *et al.*, 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

experimentation (Rowan *et al.*, 2009; Sullivan *et al.*, 2015). This experiment identified that downregulation of GRK2 eliminates the need for BK priming of DOR-mediated anti-allodynia. *Levine* and colleges found that 3 days of i.t. GRK2 AS-ODN administration produces a 39% decrease in GRK2 protein expression in the saphenous nerve (Ferrari *et al.*, 2012), yet we observed near-ablation of GRK2 protein at the level of the DRG. The difference in knockdown suggests that the support cells adjacent to distal portions of the afferent nerve were unaffected by i.t. GRK2 AS-ODN administration. Residual GRK2 that remains in fibroblasts, microglia, and other cells that run adjacent from the saphenous nerve to the peripheral terminals in the hindpaw may account for the enhanced onset of DPDPE anti-allodynia following BK priming in GRK2 knockdown animals without comparable changes in magnitude or duration of BK-induced mechanical and thermal allodynia. Because BK priming of DPDPE inhibition of PGE_2-induced allodynia is mediated by PKC (Rowan *et al.*, 2009), we theorize that BK may have been needed to induce the PKC-dependent sequestration of remaining GRK2 in order to behaviorally observe the earlier thermal anti-allodynic effects of DPDPE.

Our behavioral data also suggest that GRK2 chronically downregulates peripheral DOR anti-nociception. However, we observed a transient, enhanced thermal allodynia 5 min following DPDPE/PGE_2 co-injection in GRK2 AS-ODN-treated rats. Similarly, PGE_2-induced thermal allodynia was increased in two mouse models with reduced GRK2 expression: sensory neuron specific heterozygous GRK2 mice, and tamoxifen-treated inducible whole body heterozygous GRK2 mice (Eijkelkamp *et al.*, 2010). Another study in GRK2 AS-ODN-treated rats found that reduced GRK2 expression enhances PGE_2-induced mechanical allodynia (Ferrari *et al.*, 2012). However, our results demonstrate that PGE_2-induced mechanical allodynia is fully inhibited by DPDPE in GRK2 AS-ODN-treated rats (Figure 4C and 4G). Together with our findings, these data suggest that, although it can enhance the response to certain inflammatory mediators (including PGE_2, not BK), targeting GRK2 can also be beneficial by enhancing peripheral opioid analgesia.

In conclusion, experimental results demonstrate that GRK2 chronically downregulates DOR functional competence at the PM in peripheral sensory neurons, as well as peripheral DOR anti-nociception *in vivo*. Prior to this study, there was no identified mechanism for DOR priming by BK beyond PKC-dependence. The phenomenon of peripheral DOR incompetence in the absence of an inflammatory priming stimulus would be expected to limit the effectiveness of locally administered DOR agonists to individuals with severe inflammatory pain. Since chronic GRK2 association with DOR contributes to receptor incompetence in the absence of inflammation, we propose that peripherally targeting GRK2 in combination with DOR may improve analgesic efficacy in non-inflammatory pain conditions.

Experimental Procedures

All procedures utilizing animals were approved by the Institutional Animal Care and Use Committee of University of Texas Health Science Center at San Antonio, and were conducted in accordance with the policies for the ethical treatment of animals established by

the National Institutes of Health (NIH). Every effort was made to limit animal discomfort and the number of animals used.

Animals

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) 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 one 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, Lakewood, NJ) treatment followed by 30 min trypsin (Sigma Aldrich, St. Louis, MO) treatment, with gentle rocking every 10 min. Cells were then resuspended in complete media (Dulbecco's modified Eagle's medium, Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 100 ng/mL nerve growth factor (NGF; Harlan Laboratories Indianapolis, IN), mitotic inhibitors (Sigma), 1% penicillin/streptomycin (Invitrogen), and 1% glutamine (Sigma)) and plated on poly-D-lysine-coated plates (Corning, Glendale, AZ). Cultures were maintained at 37°C and 5% CO_2 and grown for 5-6 days with media changed the following day and every two 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) 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.

Knockdown and overexpression strategies 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), 1 μg/mL pepstatin (Sigma), 1 μg/mL leupeptin (Sigma), 1 μg/mL aprotinin (Sigma), and 100 nm phenylmethylsulphonyl fluoride (PMSF, Sigma)) 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 × g for 1 min to remove nuclei and unlysed cells from the homogenate. Resulting supernatant was centrifuged at 16000 × 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) prior to co-immunoprecipitation (Co-IP). For Co-IP

protocol and Western blot (WB) analysis details, please see Supplemental Experimental Procedures.

Ca^{2+} Imaging

Fura-2 AM was used to image individual neurons within a population of cultured ganglionic cells. Following 2 h serum-starvation, cells were loaded with fura-2 AM (1 μM; Molecular Probes, Carlsbad, CA) in the presence of pluronic F-127 (0.04%; Molecular Probes) for 1 h at 37°C in the dark, in standard extracellular solution (SES) containing (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 D-(+)-glucose, pH 7.40. Cells were viewed on an inverted Nikon Eclipse T_i-U microscope fitted with a 40×/1.35 numerical aperture Fluor objective and imaged using MetaFluor System for Ratio Fluorescence (MetaMorph, Downingtown, PA). Fluorescent images were taken alternately every 3 s with 340 and 380 nm excitation wavelengths in combination with a 510 nm emission filter with 200 ms exposure. Ratio of ΔF340/F380 was plotted for each cell versus time. Intracellular Ca^{2+} levels were analyzed as ΔF340/F380 ratios background corrected and normalized to initial value, R_0. Corresponding filters were used to select FITC-siRNA- or GFP-positive DRG.

To quantify DOR activity in sensory neurons, Ca^{2+} imaging was used to assess DOR agonist inhibition of KCl-evoked Ca^{2+} influx (Figure S1). Although time in culture varied by protocol, BK pretreatment significantly enhanced DPDPE inhibition of KCl-evoked Ca^{2+} influx at all timepoints utilized in this study (Figure S2). For details on primary afferent neuron selection, opioid inhibition of KCl-evoked Ca^{2+} influx protocol, equations, and drug stock concentrations, refer to 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 h incubation in 2% serum 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 40×/0.80W numerical aperture objective. Borosilicate glass patch clamp capillaries (Sutter, Novato, CA, USA) were polished to resistances of 2-4 MΩ and filled with internal solution containing (in mM): 140 CsCl, 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, 2 $CaCl_2$, 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 10s. DPDPE (1 s a 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 *Levine* and colleagues (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 DPDPE inhibition of prostaglandin (PGE_2)-induced thermal and mechanical allodynia 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 Supplemental Experimental Procedures.

Statistics

GraphPad Prism 5.0 was used for statistical analyses (GraphPad Software, Inc., La Holla, CA). Quantitative data expressed as mean ± S.E.M. Statistical significance was determined by student's unpaired *t*-test, one-way ANOVA, or two-way ANOVA with Bonferroni post-hoc analyses as needed. $p<0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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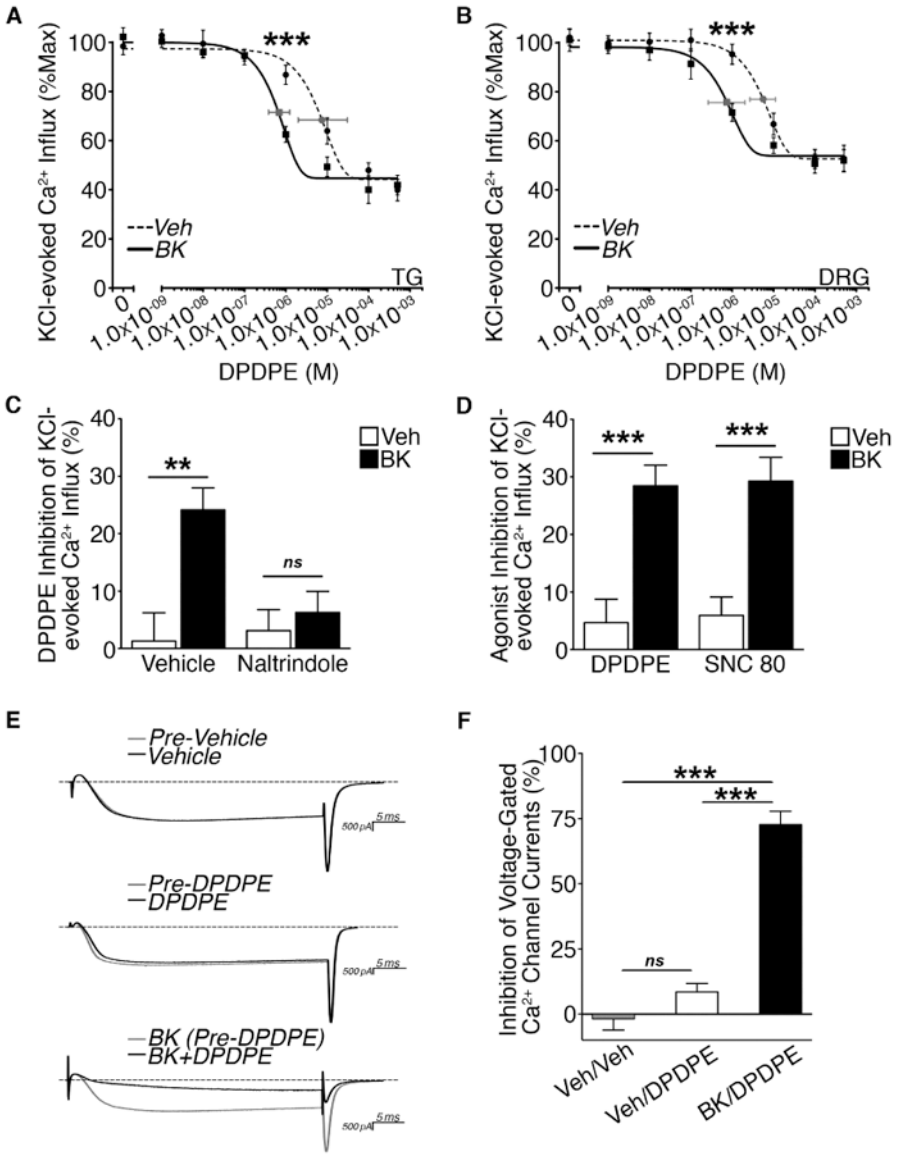

Figure 1. Functional DOR competence in sensory neurons

(**A** and **B**) Dose response for DPDPE inhibition of KCl (50 mM)-evoked Ca^{2+} influx in (**A**) TG or (**B**) DRG pretreated with vehicle or BK (200 nM, 5 min) following 2 h serum-starvation (***p<0.005 vs. vehicle; A: n=26-57 and B: n=17-39 neurons/dose; two-way ANOVA Bonferroni post-hoc; mean ± SEM). Least squares fit (best-fit) variable slope curves used to determine IC_{50} for vehicle (dotted line) and BK (solid line) and 95% confidence intervals (grey).

(**C**) Effect of naltrindole pretreatment on DPDPE (1 μM) inhibition of KCl-evoked Ca^{2+} influx in DRG neurons pretreated vehicle or naltrindole (10 nM, 5 min) prior to vehicle or BK (200 nM, 5 min) treatment following 2 h serum-starvation (**p<0.01; ns = no significance; n=14-28 DRG/group; two-way ANOVA Bonferroni post-hoc; mean ± SEM).

(**D**) Comparison of DPDPE (1 μM) versus SNC 80 (1 μM) inhibition of KCl-evoked Ca^{2+} influx in DRG neurons pretreated with vehicle or BK (200 nM, 5 min) following 2 h serum-

starvation(***p<0.005; n=24-34 DRG/group; two-way ANOVA Bonferroni post-hoc; mean ± SEM).

(**E** and **F**) (**E**) Representative traces and (**F**) quantification of DPDPE (1 μM) inhibition of VGCCs in DRG neurons (20-35 pF) pretreated with vehicle or BK (200 nM, 10 min) following 2 h serum-starvation (***p<0.005; n=4-9 DRG/group; one-way ANOVA Bonferroni post-hoc; mean ± SEM).

See also Figure S1-S2.

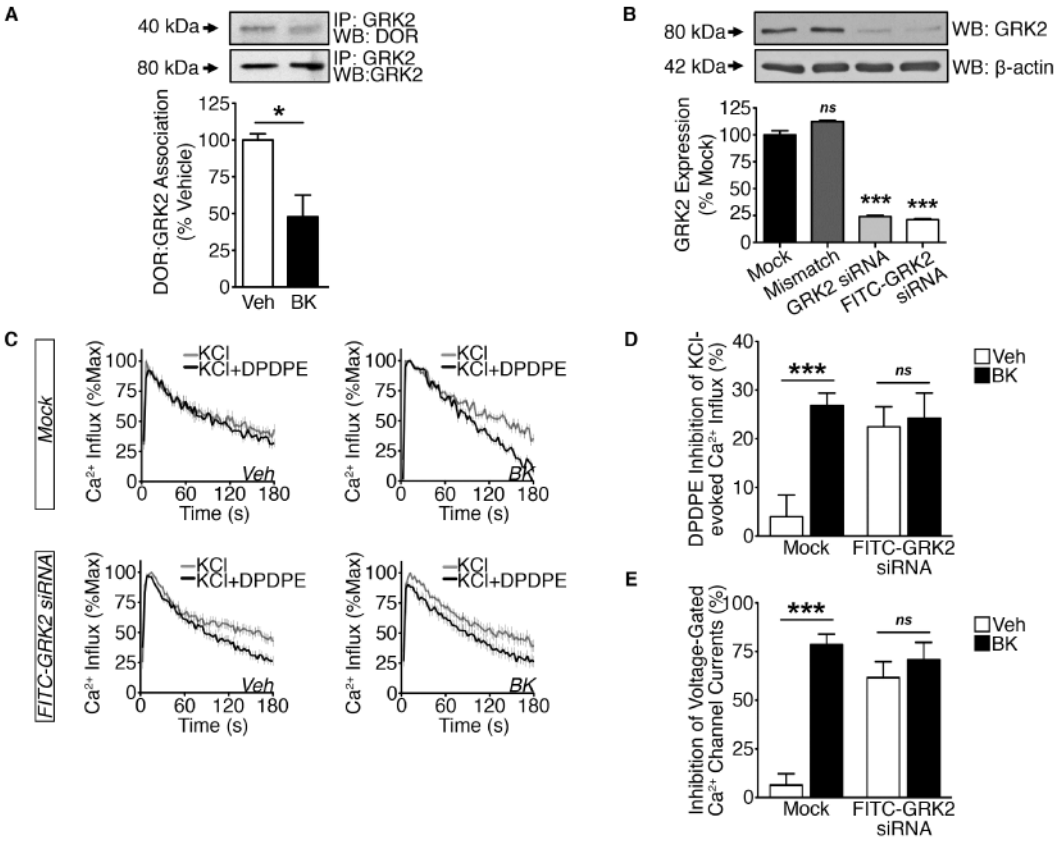

Figure 2. GRK2 modulation of DOR activity

(**A**) Crude PM Co-IP from TG cultures serum-starved for 18 h and treated with vehicle or BK (200 nM, 5 min) (*p<0.05; n=3 independent trials; unpaired two-tailed student's *t* test; mean ± SEM).

(**B**) WCL from 2 h serum-starved TG cultures transfected in mock fashion or with mismatch, GRK2, or FITC-GRK2 siRNA (***p<0.005; ns = no significance; n=6 independent trials; one-way ANOVA Bonferroni post-hoc; mean ± SEM).

(**C** and **D**) (**C**) Cumulative traces and (**D**) quantification of DPDPE (1 μM) inhibition of KCl (50 mM) -evoked Ca^{2+} influx in DRG (Mock-treated or transfected with FITC-GRK2 siRNA) pre-treated with vehicle or BK (200 nM, 5 min) following 2 h serum-starvation (***p<0.005; n=22-37 DRG/group; two-way ANOVA Bonferroni post-hoc; mean ± SEM).

(**E**) DPDPE (1 μM) inhibition of VGCCs in DRG (Mock-treated or transfected with FITC-GRK2 siRNA) pre-treated with vehicle or BK (200 nM, 10 min) following 2 h serum-starvation (***p<0.005; n=5-8 DRG/group; two-way ANOVA Bonferroni post-hoc; mean ± SEM).

See also Figure S3.

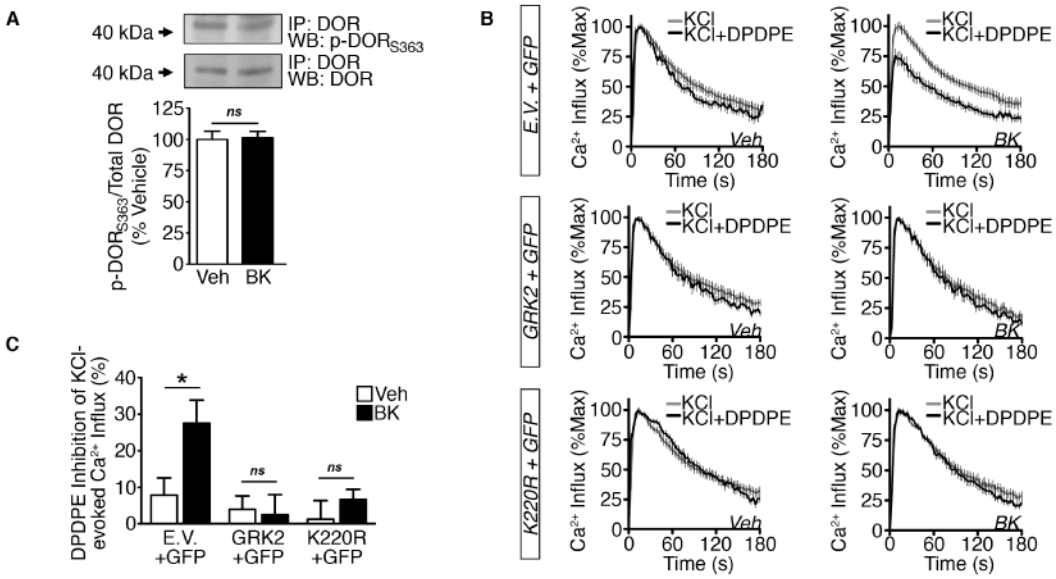

Figure 3. Constitutive DOR incompetence is independent of GRK2 kinase activity

(**A**) DOR phosphorylation at Ser 363 in crude PM immunoprecipitates from TG cultures serum-starved for 18 h and treated with vehicle or BK (200 nM, 5 min) (ns = no significance; n=3 independent trials; unpaired two-tailed student's *t* test; mean ± SEM). (**B** and **C**) (**B**) Cumulative traces and (**C**) quantification of DPDPE (1 μM) inhibition of KCl (50 mM)-evoked Ca^{2+} influx in nucleofected DRG (Overexpression: Empty Vector (E.V.) + GFP, GRK2 + GFP, K220R + GFP cDNAs) pretreated with vehicle or BK (200 nM, 5 min) following 2 h serum-starvation (*p<0.05; n=20-31 DRG/group; two-way ANOVA Bonferroni post-hoc; mean ± SEM).

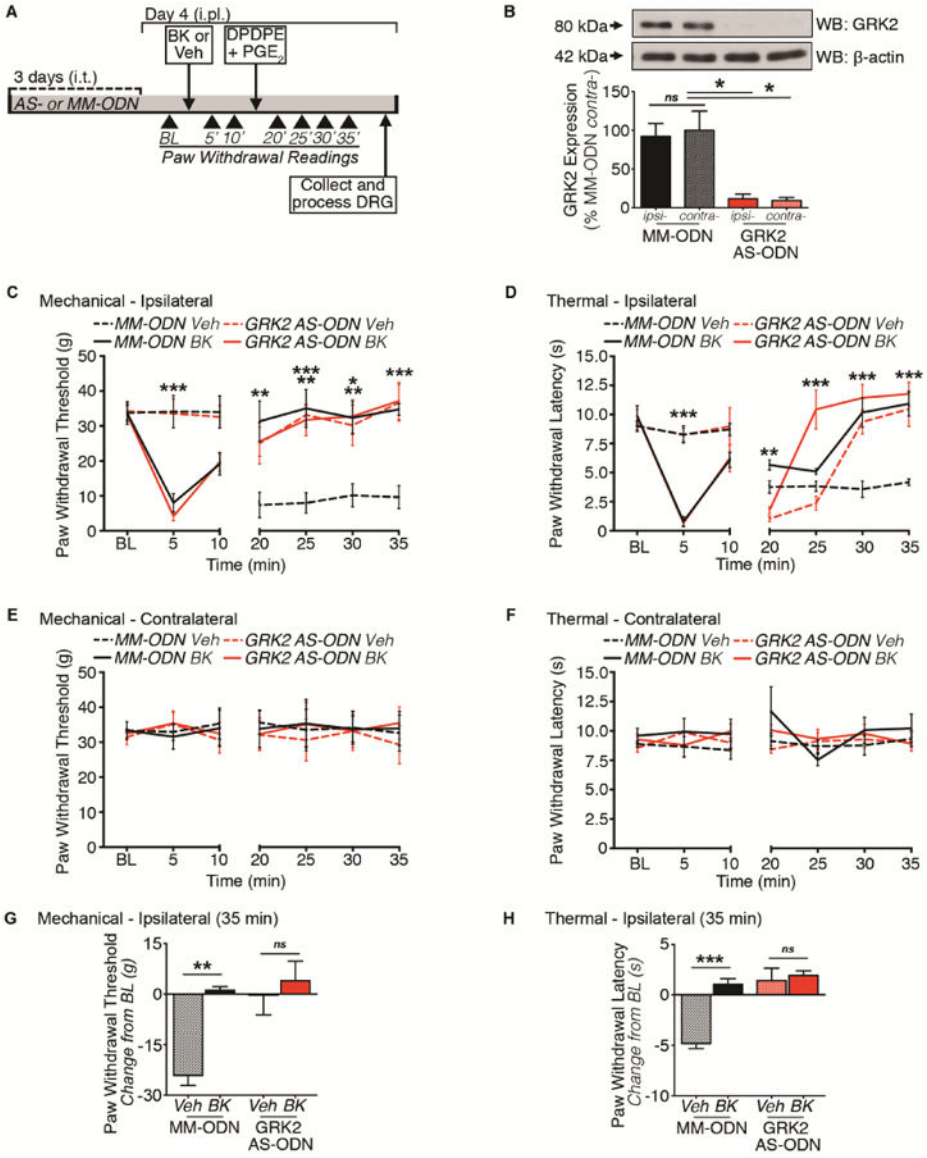

Figure 4. GRK2 modulation of DOR-mediated antinociception

(**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 vs. 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) vs. MM-ODN/Veh; *Thermal* - **p<0.01 MM-ODN/BK vs. AS-ODN/Veh; 25-35, ***p<0.005 vs. groups below baseline (BL) readings; n=6 rats per group; two-way ANOVA Bonferroni post-hoc; mean ± SEM).

(**G** and **H**) Quantified antinociceptive effect of DPDPE at 35 min for (**G**) mechanical and (**H**) thermal readings (***p<0.005; ns = no significance; n=6 rats per group; two-way ANOVA Bonferroni post-hoc; mean ± SEM).

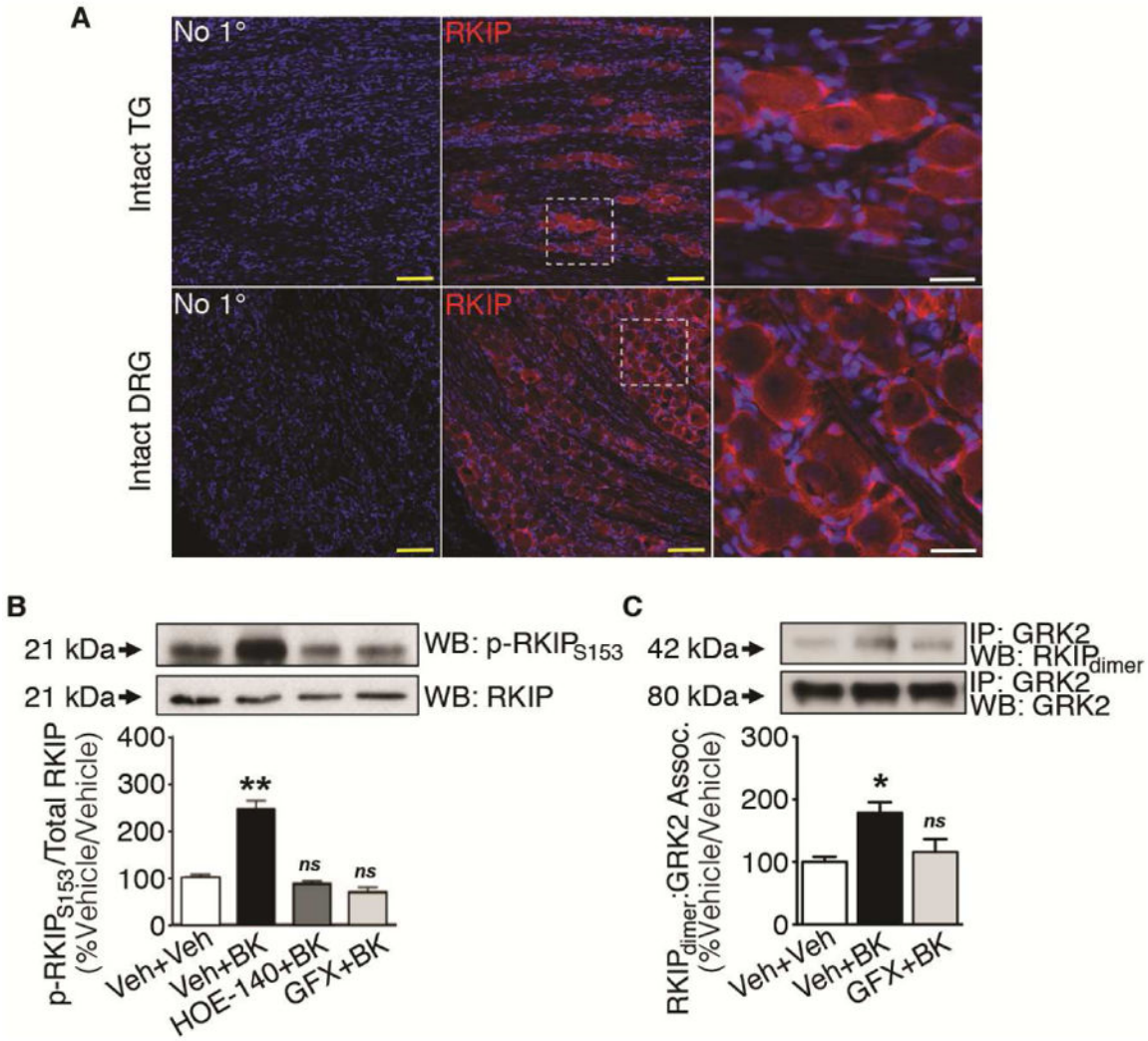

Figure 5. BK modulation of RKIP signaling in sensory neurons

(**A**) Immunohistochemical expression of RKIP (red) in rat TG and DRG with Topro (blue) to identify nuclei. Scale bars: yellow = 50 μm; white = 15 μm. Confocal images are representative of 4 independent trials.

(**B**) Cytosolic lysates from TG cultures serum-starved for 18 h and treated with vehicle (5 min)/vehicle (5 min), BK (200 nM, 5 min)/vehicle, BK/HOE-140 (10 μM, 5 min), or BK/GFX (GF 109203X, 10 μM, 5 min) (**$p<0.01$ vs vehicle/vehicle, ns = no significance; n=3 independent trials; one-way ANOVA Bonferroni post-hoc; mean ± SEM).

(**C**) Cytosolic co-IP from TG cultures serum-starved for 18 h and treated with vehicle (5 min)/vehicle (5 min), vehicle/BK (200 nM, 5 min), or BK/GFX (GF 109203X, 10 μM, 5 min) (*$p<0.05$; ns = no significance; n=3 independent trials; one-way ANOVA Bonferroni post-hoc; mean ± SEM).

See also Figure S4.

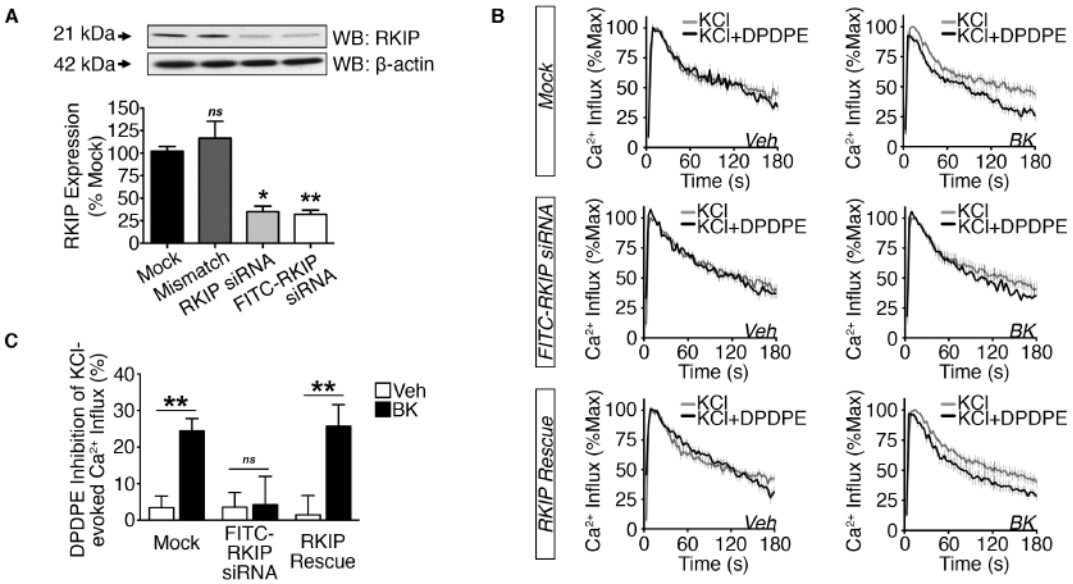

Figure 6. RKIP modulation of functional DOR competence

(**A**) WCL from 2 h serum-starved TG cultures transfected in mock fashion or with mismatch, RKIP, or FITC-RKIP siRNA (*p<0.05, **p<0.01, ns = no significance vs. mock; n=3 independent trials; one-way ANOVA Bonferroni post-hoc; mean ± SEM).

(**B** and **C**) (**B**) Cumulative traces and (**C**) quantification of DPDPE (1 μM) inhibition of KCl (50 mM)-evoked Ca^{2+} influx in DRG [Mock-treated, transfected with FITC-RKIP siRNA, or FITC-RKIP siRNA followed by nucleofection RKIP and GFP cDNAs (RKIP Rescue)] pretreated with vehicle or BK (200 nM, 5 min) following 2 h serum-starvation (**p<0.01; ns = no significance; n=20-26 DRG/group; two-way ANOVA Bonferroni post-hoc; mean ± SEM).

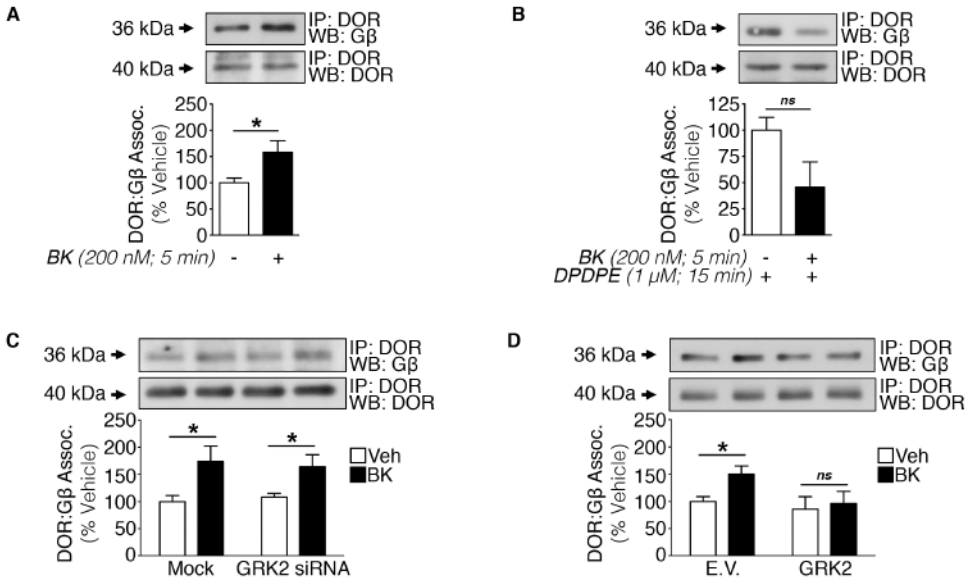

Figure 7. GRK2 hinders peripheral DOR G protein coupling

(**A** and **B**) Crude PM Co-IP from TG cultures serum-starved for 18 h and treated with (**A**) vehicle or BK (200 nM, 5 min) or (**B**) vehicle or BK (200 nM, 5 min) then DPDPE (1 μM, 15 min) (A: *p<0.05; ns = no significance; n=3 independent trials; unpaired two-tailed student's *t* test; mean ± SEM).

(**C**) Crude PM Co-IP from TG cultures serum-starved for 2 h following Mock- or GRK2-siRNA nucleofection and treated with vehicle or BK (200 nM, 5 min) (*p<0.05; n=3 independent trials; two-way ANOVA Bonferroni post-hoc; mean ± SEM).

(**D**) Crude PM Co-IP from TG cultures co-treated for 18 h with empty vector (E.V.) or GRK2 cDNA nucleofection (overexpression) in serum starved media followed by treatment with vehicle or BK (200 nM, 5 min) (*p<0.05; ns = no significance; n=3 independent trials; unpaired two-tailed student's *t* test; mean ± SEM).