Published in final edited form as:

ACS Chem Neurosci. 2020 December 16; 11(24): 4376–4386. doi:10.1021/acscchemneuro.0c00622.

GRK2 Dictates A Functional Switch of Peripheral Mu-Opioid Receptor

Yan Zhang¹, Nathaniel A. Jeske^{1,2,3}

¹Department of Oral and Maxillofacial Surgery, University of Texas Health San Antonio, 7703 Floyd Curl Dr, San Antonio, TX 78229, USA.

²Department of Pharmacology, University of Texas Health San Antonio, 7703 Floyd Curl Dr, San Antonio, TX 78229, USA.

³Department of Physiology, University of Texas Health San Antonio, 7703 Floyd Curl Dr, San Antonio, TX 78229, USA.

Abstract

Peripheral mu-opioid receptor (MOR) has been recognized as a potential target to provide safer analgesia with reduced central side effects. Although analgesic incompetence of peripheral MOR in the absence of inflammation was initially identified more than a decade ago, there has been very limited investigation into the underlying signaling mechanisms. Here we identify that G protein-coupled receptor kinase 2 (GRK2) constitutively interacts with MOR in peripheral sensory neurons to suppress peripheral MOR activity. Brief exposure to bradykinin (BK) causes uncoupling of GRK2 from MOR and subsequent restoration of MOR functionality in dorsal root ganglion (DRG) neurons. Interestingly, prolonged BK treatment induces constitutive activation of MOR through a mechanism that involves protein kinase C (PKC) activation. After silencing Raf kinase inhibitory protein (RKIP) by RNA interference, BK-induced constitutive MOR activation is completely abrogated, which agrees with previous findings that BK activates PKC signaling to initiate GRK2 sequestration by RKIP. Furthermore, we demonstrate that constitutive, peripheral MOR activity requires GRK2 uncoupling and that the FDA-approved SSRI paroxetine promotes this state of uncoupling. Collectively, these results indicate that GRK2 tightly regulates MOR functional states and controls constitutive MOR activity in peripheral sensory neurons, supporting the potential for targeting the kinase to provide safer analgesia.

Graphical Abstract

AUTHOR CONTRIBUTIONS

YZ and NAJ conceived the project, YZ performed the research, YZ and NAJ performed the analysis, YZ and NAJ wrote the manuscript.

Keywords

opioid; GRK2; pain; paroxetine; bradykinin; MOR

INTRODUCTION

Opioids are potent analgesics, yet long-term use of opioids has become a growing public health concern due to their addictive and deadly side effects. mu-opioid receptor (μ -, MOR), a principle target for opioids, is among the most studied G protein-coupled receptors (GPCR) by virtue of its critical role in mediating analgesia. However, MOR agonists produce severe systemic side effects as a result of activation of receptor targets in the CNS. Thus, a promising alternative to pain control may be to target MOR in the peripheral nervous system¹. Similar to delta (δ -, DOR) and kappa (κ -, KOR) opioid receptors, MOR is functionally inactive in peripheral afferents²⁻⁴. Inflammatory mediators acting on primary sensory neurons (nociceptors) alter MOR analgesic competency, as MOR agonists produce more pronounced analgesic effects in inflamed tissue⁵⁻⁸. In neuronal cell cultures, brief exposure of bradykinin (BK) significantly increases peripheral MOR response to agonists². This effect has been referred to as inflammatory “priming”². Given the important roles of MOR in analgesia, surprisingly little is known about how inflammation influences functional changes in peripheral afferent MOR.

MOR exhibits two activation modes (constitutive and agonist-induced). Constitutive activity of MOR results in the stimulation of G proteins in the absence of agonist⁹. The majority of studies examining constitutive MOR activity have focused on heterologous expression systems¹⁰⁻¹² and CNS¹³⁻¹⁶. However, very few studies provide evidence for the existence of constitutive MOR activity in peripheral sensory neurons¹⁷, and mechanisms for constitutive MOR activity remain largely unexplored. The first evidentiary role for constitutively active MOR in pain came from Walwyn *et al.*, who reported that constitutive MOR activation promotes the suppression of pain hypersensitivity in a latent sensitization model of inflammatory pain¹⁸. Electrophysiological data from peripheral sensory neurons have also revealed constitutive MOR inhibition of calcium channels during recovery from inflammatory pain¹⁸. But it is unclear whether MOR can transit from functionally inactive in naïve peripheral tissue, to constitutively active following extended inflammation. Therefore, understanding the regulatory mechanisms for constitutive MOR activity in native sensory neurons could identify new targets to remedy pain.

It has been reported that G protein-coupled receptor kinases (GRK) regulate MOR function by phosphorylating the carboxyl-terminal tail of the active receptor, leading to MOR desensitization and internalization^{19–21}. We recently reported an initial documentation of naïve DOR association with GRK2 in primary afferents²². Specifically, we identified that BK-induced GRK2 movement away from DOR increases receptor competence *in vitro* and *in vivo*. This has led us to explore the possibility that GRK2 can also modulate the functional output of peripheral MOR. Here, we demonstrate that GRK2 naively associates with MOR in peripheral sensory neuronal cultures, and that BK stimulates GRK2 dissociation accompanied with increased MOR response to agonist. Moreover, we report that GRK2 uncoupling from MOR through a PKC/RKIP-dependent mechanism contributes to constitutive activation of MOR. Together, these findings identify that GRK2 both physically and functionally couples peripheral MOR and determines the temporal patterns of MOR signaling in response to inflammation.

RESULTS

GRK2 negatively modulates peripheral MOR activity

MOR mediates analgesia in part by inhibition of voltage-gated Ca^{2+} channels (VGCCs) through $\text{G}_{\beta\gamma}$ subunits²³. Therefore, we performed whole-cell patch-clamp recordings to determine VGCC inhibition by the selective MOR agonist, DAMGO. MOR is enriched in a subpopulation of sensory neurons with a soma diameter of $< 30 \mu\text{m}$ ²⁴, and around 70% sensory neurons co-express MOR and BK B2 receptor². Therefore, small to medium size rat DRG neurons were chosen to maximize the fidelity of our study. MOR mainly mediates inhibition of high voltage-activated (HVA) Ca^{2+} currents composed of N-type currents in rat DRG neurons²⁵. As reported previously, depolarizing voltage steps from -60 to 0 mV elicit robust HVA currents²², and DAMGO exerts its maximal amplitude inhibition within 10 s following drug application¹⁷. In small to medium DRG neurons, we observed that DAMGO ($1 \mu\text{M}$) inhibited $26.0 \pm 2.9\%$ of VGCCs in vehicle-treated DRG neurons (Figure 1A, B), whereas BK pretreatment (200 nM , 5m) produced a significant increase in current inhibition ($46.9 \pm 3.3\%$), confirming enhanced MOR functional activity in the presence of an inflammatory mediator.

As we have previously described, GRK2 constitutively and negatively interacts with DOR in sensory neuron²². Therefore, we sought to identify whether peripheral MOR also couples GRK2 in a manner that contributes to its functional incompetence under naive conditions. We treated serum-starved DRG cultures with vehicle or BK (200 nM) for 5m and performed co-immunoprecipitation (coIP) assays. After immunoprecipitation from DRG lysates with GRK2 antibodies, MOR interaction was specifically detected in vehicle-treated samples but significantly reduced in BK-treated cultures (Figure 1C, D). These data suggest that GRK2 naively binds to MOR and BK induces GRK2 uncoupling from MOR.

To determine whether GRK2 affects MOR functional activity, small interfering RNA (siRNA) demonstrating 70–80% efficacy in knocking down GRK2 expression²² was transfected into cultured DRGs and MOR activity was assessed via electrophysiological recordings. Positively-transfected DRG neurons were identified through FITC-siRNA tagging. Application of DAMGO ($1 \mu\text{M}$) inhibited VGCCs currents with an average block of

23.9 ± 4.0% in mock-treated cells (Figure 1E, F). Yet, in DRG neurons silenced for GRK2 expression, DAMGO application resulted in a robust BK-independent inhibition of currents (42.6 ± 3.4% in FITC-GRK2 siRNA and 39.4 ± 3.0% in FITC-GRK2 siRNA/BK; $p > 0.05$), consistent with our observations in DRG neurons under BK-primed conditions (Figure 1A, B). Thus, GRK2 maintains functional incompetence of peripheral MOR, and BK-induced GRK2 uncoupling contributes to up-regulation of the receptor.

BK induces constitutive MOR activity

Previous studies provide evidence that constitutive MOR activation is involved in persistent suppression of hyperalgesia in the post-inflammation phase¹⁸. In addition, DRG neurons extracted from animals with inflammation display constitutive MOR inhibition of VGCCs¹⁸. Thus, we hypothesized that BK stimulation not only restores MOR functionality, but also generates constitutively activated MOR. To test this, we applied an established two-pulse protocol to assess constitutive MOR signaling⁹. Since inhibitory coupling of $G_{\beta\gamma}$ subunits to VGCCs is voltage-dependent²⁶, strong conditioning depolarization (+80 mV) can reverse this interaction causing an increased current amplitude, defined as “facilitation”. In the absence of agonist, the currents preceded by a conditioning pulse (post-pulse) were slightly smaller than control currents (pre-pulse) in vehicle-treated neurons (Figure 2A; black trace, left row), indicating inactivation of calcium channels by the conditioning pulse. By comparison, DAMGO (1 μ M) produced an evident agonist-dependent G-protein inhibition of VGCCs, such that current amplitudes were enhanced by a conditioning pulse (Figure 2A; green trace, left row). These results are in agreement with previously published data¹⁷. The conditioning depolarization only partially reversed the effects of DAMGO possibly due to phosphorylation of the channels²⁷. In addition, we noticed that in small to medium DRG neurons, DAMGO-responsive cells were unlikely to exhibit facilitation in basal conditions, whereas DRG neurons displayed pronounced facilitation without functional MOR expression (not responsive to DAMGO), in accordance with previous finding that endogenously expressed MOR in rat DRG neurons has little tonic VGCC inhibition under naïve conditions²⁸. The degrees of agonist-dependent and agonist-independent inhibition of VGCCs were examined by determining the facilitation ratio (post-pulse current amplitude divided by pre-pulse current amplitude; Table 1, Materials and Methods), such that higher facilitation ratios indicate higher levels of receptor activity with greater G-protein inhibition.

To determine whether inflammatory mediators increase constitutively active MOR in DRGs, we first incubated DRG neurons with BK (200 nM) for 5m as we did in previous experiments. However, no facilitation was recorded in the absence of agonist (Figure 2A, B). Application of 1 μ M DAMGO resulted in a robust inhibition of current activity by 42.6 ± 2.9% (Figure 2C) that could be partially reversed by a conditioning pulse, supporting more functional receptors available for mediating the effects of agonists following brief BK exposure (5m). Surprisingly, when BK pretreatment time was extended to 15m, we found apparent constitutive inhibition of VGCCs manifested by an increase in amplitude following a conditioning pulse (Figure 2A, black trace, right row). The mean facilitation ratios were 1.08 ± 0.03 in the absence of agonist (basal facilitation), but significantly increased to 1.25 ± 0.04 after DAMGO (Figure 2B, $p < 0.01$). However, mean DAMGO inhibition of the currents in the pre-pulse was greatly attenuated (14%), to a level comparable to that of

the vehicle-treated neurons (20%). To further investigate the mechanism behind increased facilitation by BK, we used a 15m pretreatment time for the following experiments.

To affirm that the elevated basal facilitation observed after 15m BK pretreatment was due to increased MOR constitutive activity, we investigated the effects of two antagonists on the basal activity following 15m BK pretreatment. Naloxone (NLX) blocks constitutively active MOR, showing properties of an inverse agonist with negative intrinsic efficacy³⁰. Another MOR antagonist CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂), functions as a neutral antagonist with no intrinsic activity, but shows an ability to block the effects of inverse agonist¹⁷. As shown in Figure 2C, naloxone (1 nM,²⁹) had no detectable effect on the mean basal facilitation in vehicle-treated neurons ($p > 0.05$). Yet, in BK-treated cultures, naloxone significantly reduced the mean facilitation ratio to a level comparable to vehicle control (Figure 2D – E, $p < 0.01$). In contrast, CTAP (1 μ M) had minimal effect on the mean basal facilitation recorded from BK-treated neurons (Figure 2F, $p > 0.05$). In addition, the naloxone effects were reversed by addition of CTAP (Figure 2G). The drug effects in our study are consistent with observations in sensory neurons from other studies^{17–18}. Taken together, our results indicate that prolonged BK pretreatment (15m) produces constitutively activated MOR in peripheral sensory neurons.

PKC and RKIP, but not LOX, are essential for BK-mediated constitutive activity of peripheral MOR

In previous work, we demonstrated that BK activates PKC to phosphorylate RKIP, leading to GRK2 association with RKIP instead of peripheral DOR²². We therefore investigated whether PKC activation also influenced BK-mediated MOR constitutive activity. As demonstrated in Figure 3A and B, BK-induced facilitation was reversed by inhibition of PKC with GF109203X (GFX, 10 μ M). The magnitude of current inhibition by 1 μ M DAMGO in neurons treated with GFX did not differ significantly from that seen in control neurons (Figure 3C). Additionally, we obtained similar results after knockdown of endogenous RKIP by small interfering RNA (siRNA) in BK-treated sensory neurons (Figure 3D – F). These data indicate that BK-induced MOR constitutive activity requires PKC activation and RKIP signaling, suggesting that GRK2 uncoupling could be essential in promoting constitutive MOR activation in primary sensory neurons.

A previous study implicated that a lipoxygenase (LOX)-dependent metabolite of arachidonic acid (AA) participates in regulation of peripheral opioid receptor responsiveness by BK³¹. We next tested if LOX inhibition abolished BK-induced constitutive MOR activity. The addition of LOX inhibitor NDGA (10 μ M) during the BK pretreatment did not fully abolish BK-induced facilitation. (Figure 3A, right panel). However, a trend toward decreased facilitation level was noted in the presence of NDGA, which is not significantly different from that seen in the absence of inhibitor (Figure 3B, $p > 0.05$). DAMGO (1 μ M) application induced a marked channel inhibition which is indistinguishable from those recorded in the absence of NDGA (Figure 3B, $p > 0.05$). Therefore, BK-mediated constitutive MOR activity appears independent of LOX.

GRK2 modulates constitutive activity of peripheral MOR

Next, we sought to assess the regulatory role of GRK2 on constitutive MOR signaling in cultured sensory neurons. As shown in Figure 4A and B, deletion of endogenous GRK2 by siRNA significantly enhanced the mean basal facilitation ($p < 0.05$), which mimicked the effect of BK (15m, Figure 2). When 1 μ M DAMGO was applied to GRK2-silenced neurons, the mean facilitation ratio was amplified (Figure 4B), in agreement with the idea that GRK2 down-regulates peripheral MOR function. However, unlike the effects of BK (Figure 2C), deletion of GRK2 resulted in a significant increase in the magnitude of current inhibition by DAMGO (Figure 4C, $p < 0.05$), consistent with data in Figure 1F. Among all GRK2-silenced neurons recorded, only neurons sensitive to DAMGO showed facilitation both in the absence and presence of agonist (Figure 4B).

In GRK2 siRNA-transfected cells, naloxone (1 nM,²⁹) significantly decreased mean basal facilitation levels (Figure 4D, $p < 0.01$). In contrast to naloxone, CTAP (1 μ M) produced no major effects on the mean basal facilitation (Figure 4E, $p > 0.05$). Also, the action of naloxone was blocked by CTAP (Figure 4F). Thus, these results verify MOR's involvement in constitutively activated components after GRK2 depletion by siRNA in sensory neurons. Collectively, our data show that GRK2 suppresses basal and agonist-dependent MOR activities in primary sensory neurons.

Because phosphorylation of GRK2 at Ser-685 underlies GRK2 maintenance of inactive DOR in peripheral afferents³², it is reasonable to investigate whether GRK2 governs peripheral MOR function through a similar mechanism. Mutant GRK2 with Ser-685 mutated to glycine (G), unable to be phosphorylated (phospho-deficient), or to aspartic acid (D) that mirrors constitutively phosphorylated (phospho-mimic) GRK2 were overexpressed in DRG neurons. Next, functional studies were undertaken to reveal the effects of serine mutations on MOR activity. The mutant S685D displayed no basal facilitation, with a modest increase in facilitation ratio upon DAMGO application, which closely resembles the pattern of wild-type (WT) GRK2 (Figure 5A, B). Conversely, nullifying GRK2 phosphorylation, the mutant S685G demonstrated enhanced basal facilitation and DAMGO-mediated modulation (Figure 5A, B). Furthermore, only overexpression of mutant S685G led to a distinctly greater inhibition by DAMGO (Figure 5C). These results indicate phosphorylation of GRK2 at residue Ser-685 inhibits constitutive activation of peripheral MOR.

Paroxetine as an FDA-approved GRK2 inhibitor enhances constitutive activity of peripheral MOR

The Food and Drug Administration (FDA)-approved selective serotonin-reuptake inhibitor (SSRI), paroxetine (Paxil®), has been identified as a potent and selective GRK2 inhibitor³³. Previous studies demonstrate that intrathecal paroxetine effectively increased morphine analgesia in pain models in rodents³⁴. These findings raise the possibility that paroxetine might also facilitate peripheral MOR activity by preventing GRK2/MOR interaction. First, association was tested by immunoprecipitation of GRK2 and detection by WB for the MOR. In vehicle-treated DRG cultures, there is a clear association between GRK2 and MOR (Figure 6A). Yet after 15m stimulation with paroxetine (5 μ M), GRK2 dissociates from MOR, irrespective of ligand presence (Figure 6A, B).

Next, we investigated the functional consequences of paroxetine affecting interaction between GRK2 and MOR in sensory neurons. As shown in Figure 6C and D and consistent with GRK2 deletion experiments (Figure 4), mean basal facilitation was greatly enhanced in neurons pretreated with paroxetine (5 μ M, 15m) when compared with control (vehicle) neurons. After exposure to DAMGO, a prominent increase in the mean facilitation ratio compared with the vehicle-treated cells is observed. Accordingly, paroxetine pretreatment also led to an increased DAMGO-mediated inhibition of VGCCs (Figure 6E, $p < 0.01$). Therefore, our data demonstrate that the selective GRK2 inhibitor paroxetine prevents GRK2 interaction with MOR and induces constitutive MOR activity, underlining a key role for GRK2 in the regulation of peripheral MOR function.

DISCUSSION

We describe a functional coupling between MOR and GRK2 that plays a key role in regulating peripheral MOR function. Specifically, we demonstrated that GRK2 uncoupling works as a “switch on” for active MOR in peripheral afferents, then shifts the balance toward the formation of constitutively active MOR. Along with this central information, we also demonstrated that BK-stimulated GRK2 uncoupling in peripheral sensory neurons not only yields active MOR, but also constitutively active MOR, depending on the exposure time for BK. Furthermore, BK-induced constitutive MOR activity requires activation of PKC pathway that regulates RKIP signaling to GRK2, and phosphorylation of GRK2 at residue Ser-685 blocks constitutive MOR activity. Finally, we discovered that the GRK2-specific inhibitor paroxetine inhibits constitutive GRK2/MOR interaction and facilitates constitutive activity in peripheral MOR. Our findings shed light on the functional transition of peripheral MOR programmed by inflammatory stimuli, together with the mechanisms that lead to constitutively active MOR.

Animal behavioral studies indicate that MOR exerts long-lasting inhibitory influence on pain transmission to aid in the resolution of pain. One week after complete Freund's adjuvant (CFA) inoculation, activation of peripheral MOR by MOR agonists caused more significant antinociception in inflamed tissue compared to noninflamed tissue^{7–8, 35}. This suggests that inflammatory modulation of peripheral MOR at this stage mainly aids in the analgesic effectiveness of exogenous and endogenous opioids. After tissue damage, constitutive MOR signaling is also initiated and persists for several months^{13, 36–38}, as evidenced by pharmacological blockage of constitutively active MOR-reinstated hyperalgesia^{13, 18, 38}, even though the hyperalgesia had completely resolved. A recent study using genetically modified animals in which MOR has been selectively deleted in nociceptive primary afferents, reinforced the role of peripheral MOR in the suppression of hyperalgesia in the chronic pain state³⁷. In the post-hyperalgesia state (3 weeks after CFA), the ability of agonist-activated MOR in mediating antinociceptive effects did not differ between control and inflamed DRGs, whereas constitutive MOR activity was found in DRG neurons from inflamed animals¹⁸. In our cell model, BK induces enhanced analgesic efficacy of peripheral MOR within a rapid time course (5m). In contrast, continuous availability of BK augmented constitutive MOR signaling accompanied by recovered MOR analgesic efficacy following BK priming, consistent with previous research in inflamed animals in the post-hyperalgesia state¹⁸). Despite our failure to observe basal facilitation following 5m BK pretreatment

using two-pulse protocol, we did notice that BK application caused rapid inhibition of VGCCs by a single step depolarization (see protocol in Figure 1) as described previously²⁸. Furthermore, we found this BK-induced VGCC inhibition can be reversed by naloxone (data not shown), implying that the BK-initiated inhibition effect is mediated by constitutively active MOR. These findings reveal that deactivation of some VGCCs by a conditioning pulse masks the onset of constitutive MOR activity triggered by BK, and that the degree of constitutive VGCC inhibition is underrated by a two-step voltage paradigm³⁹. Thus, it is conceivable that BK priming may produce a combination of constitutive- and agonist-activated MOR, and extended BK exposure may shift the balance toward more constitutively activated MOR.

Classically, GRK2 is characterized as a protein required for GPCR internalization upon agonist stimulation²¹. We found that, in the absence of MOR agonist, GRK2 constitutively interacted with peripheral MOR in the naïve condition. The ability of GRK2 to interact with MOR is most likely associated with impaired receptor/G-protein coupling²², resulting in a reduced MOR responsiveness. An early study reported an increase in G-protein coupling at MOR in DRG membranes of animals with inflammation for 4 days³⁵. Our data, that BK priming reduced GRK2 coupling leading to up-regulation of MOR function (Figure 1A, B), and silencing endogenous GRK2 caused enhanced MOR responsiveness (Figure 1C), support the notion that GRK2 prevents MOR coupling to G-protein, such that peripheral MOR is desensitized unless BK triggers GRK2 uncoupling. It is plausible that functional MOR competence (MOR/G-protein coupling) upon BK stimulation is only temporary. This is likely due to the mechanism by which when MOR becomes constitutively active, G-proteins separate from MOR and exert constitutive inhibition of VGCCs, and thus MOR is no longer able to mediate agonist-dependent effects. This would explain why BK gradually increased constitutive MOR activity in parallel with declined agonist-dependent MOR activity (Figure 2B, C). Considering GRK2 also contributes to MOR internalization, it is worthwhile to explore that in addition to MOR functional changes, GRK2 uncoupling by BK can also induce trafficking of MOR to the plasma membrane like other opioid receptors in sensory neurons²⁸. A-anchoring protein 79/150 (AKAP), located at the plasma membrane in primary sensory neurons, has been identified to direct PKA phosphorylation of GRK2 at Ser-685, and then increase GRK2 membrane targeting²². Thus, PKA-mediated phospho-S685 GRK2 may accumulate at membrane receptor and facilitate GRK2/MOR coupling under basal conditions.

When peripheral MOR becomes constitutively active, DAMGO exerts similar inhibitory effects on VGCCs in both control and 15m BK-treated neurons (Figure 1C), whereas enhanced DAMGO efficacy was seen in neurons in which GRK2 was silenced (Figure 4C), GRK2-S685G was overexpressed (Figure 5C) or neurons were treated with paroxetine (Figure 6E). Several possible mechanisms may account for this phenomenon. First, physiological inhibition of GRK2 by RKIP and inhibition of GRK2 catalytic activity by paroxetine are based on different molecular mechanisms⁴⁰. Thus, the differential ability to inhibit GRK2 coupling may account for varying DAMGO efficacies. Second, phosphorylation of MOR may occur during prolonged BK treatment and thus influence G-protein activation. Increased phospho-S375 MOR but without MOR internalization have been found in spinal cord sections of animals with chronic inflammatory pain¹⁸.

However, Ser-375 is typically phosphorylated by GRKs upon agonist stimulation leading to receptor internalization⁴¹. Although the functional consequence of basal phospho-S375 in the context of ongoing inflammation *in vivo* is unknown, it is possible that hierarchical and/or combinatorial MOR phosphorylation occurred during chronic inflammation might inhibit G-protein activation in response to agonist. Third, BK modulation of calcium channels is also a possible mechanism. PKC activation has been reported to increase N-type VGCC currents by disruption of $G_{\beta\gamma}$ inhibition⁴², and a mutant channel that mimics phosphorylation by PKC exhibits a reduced DAMGO-mediated inhibition⁴³, hinting at the possibility that stimulation with BK causes a PKC-dependent phosphorylation of calcium channels, and subsequent antagonism of G-protein inhibition. However, it is important to consider the possibility that both PKC and GRK2 independently regulate MOR agonist efficacy, especially when considering the outcome measure being observed. Therefore, independent signaling pathways stimulated by BK may stimulate unbiased mechanisms that act on VGCC independent from MOR, warranting future investigation to discern appropriately.

In conclusion, we propose that GRK2 plays a crucial role in the maintenance of peripheral MOR incompetency via physical interaction with the receptor. We suggest that disruption of GRK2/MOR coupling enables peripheral MOR to achieve an active state which is transient and inherently unstable but can evolve into a constitutively active state. Mechanisms for driving constitutive MOR signaling might be shared among other nociceptor-expressed, GRK2-associated opioid receptors or GPCRs, such as DOR, which is known to couple to GRK2²². Moreover, our findings suggest that the use of peripherally acting opioids together with paroxetine may effectively combat pain in the absence of inflammation.

MATERIALS AND METHODS

Animals

All procedures using animals were approved by the Institutional Animal Care and Use Committees of The University of Texas Health Science Center at San Antonio (UTHSCSA) and were conducted in accordance with policies for the ethical treatment of animals established by the National Institutes of Health (NIH) and International Association for the Study of Pain. Efforts were made to limit animal discomfort and reduce the number of animals used. Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 176–199g were used in this study.

Chemicals

Naloxone (NLX), nordihydroguaiaretic acid (NDGA) and paroxetine were from Cayman Chemical. GFX 109203X (GFX) and CTAP were from Tocris Bioscience. [D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin (DAMGO), bradykinin and common reagents were from Sigma-Aldrich.

Primary Neuronal Cultures

Sprague-Dawley rats were rapidly decapitated. For electrophysiology, dorsal root ganglia (DRG) were removed bilaterally at L4–L6 and incubated in collagenase (Worthington) and

disperse (Sigma-Aldrich) for 40m at 37°C with gentle agitation every 10m. The dissected DRG neurons were then triturated, centrifuged and re-suspended in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 ng/ml nerve growth factor (NGF, Harlan, Indianapolis, IN), 1% penicillin/streptomycin (Gibco), and 1% L-glutamine (Gibco), and then placed on coverslips coated with poly-D-lysine and laminin (Corning, Corning, NY). Cultures were maintained at 37°C, 5% CO₂ for 24 – 48h prior to electrophysiology recordings.

For co-immunoprecipitation (co-IP) experiments, L4–L6 DRGs were bilaterally removed and incubated in collagenase for 30m followed by 30m trypsin (Sigma-Aldrich) treatment, with gentle agitation every 15m. Cells were then triturated, centrifuged and re-suspended in DMEM supplemented with 10% FBS, 100 ng/ml NGF, mitotic inhibitors (Sigma-Aldrich), 1% penicillin/streptomycin, and 1% L-glutamine, and then placed on poly-D-lysine-coated 6-well plates (Corning). Cultures were maintained at 37°C, 5% CO₂ and grown for 5 – 6 days. The entire media was replaced after 24h and every 2 days thereafter.

Co-immunoprecipitation (co-IP):

Following 18h serum-starvation, neuronal cultures were treated as described. Plated neuronal cells were washed twice with ice-cold PBS and lysed in general lysis buffer (50 mM HEPES, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate [pH 7.4], 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 100 mM phenylmethylsulphonyl fluoride) with 20 strokes via a 25-gauge needle. Cell lysate was placed on ice for 15m and then centrifuged at 1,000 for 15m and then centrifuged at 1,000 for 15m and then centrifuged at 1,000 × g for 1m. Total protein in the supernatant was quantified using Bradford assay (Sigma-Aldrich). Equal amounts of protein samples (125 µg) were incubated with antibodies specific to GRK2 (1 µg, sc-562, Santa Cruz Biotechnology) for at least 2h. Protein agarose-A (Sigma Aldrich) was incubated with samples for 1h, followed by 4 washes with general lysis buffer. Protein samples were heated at 95°C for 5m in protein loading buffer, resolved in 12.5% SDS/PAGE, and transferred to PVDF membrane (Merck Millipore). After blocking with 5% nonfat milk in TBS-T (Tris-buffered saline/Tween 20: 15.35 mM Tris/HCl, 136.9 mM NaCl, pH 7.6, with 0.1% Tween 20) for 1h at 23°C, membranes were incubated 18h at 4°C with primary antibodies: anti-MOR (1:1000 in TBS-T 5% BSA; ab134054, Abcam) and anti-GRK2 (1:500 in TBS-T 5% BSA; sc-562, Santa Cruz Biotechnology) (43–45). Next, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) for 1h at 23°C. Blots were washed and incubated with ECL (GE Healthcare), exposed to X-ray film and developed for analysis. We used NIH ImageJ software for densitometry analysis.

Electrophysiology

Whole-cell patch-clamp recordings were performed under voltage-clamp mode using an EPC10 amplifier and Patchmaster software (HEKA Electronics, Lambrecht, Germany). Pipette and membrane capacitances were automatically compensated, and currents were sampled at 10 kHz. Borosilicate glass pipettes (2 – 4 MΩ resistance) were filled with an intracellular solution containing the following (in mM): 140 CsCl, 10 EGTA, 1 CaCl₂, 1

MgCl₂, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, pH 7.2 with CsOH. External recording solution contained (in mM): 140 TEA-Cl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, 10 D-glucose, pH 7.3 with TEA-OH.

Following 2h serum-starvation, voltage-gated calcium channel (VGCC) currents were recorded from small to medium size rat DRG neurons (20 – 60 pF) at room temperature. VGCC currents were elicited from a holding potential of –60 mV to 0 mV (50ms duration) at 10s intervals. Percent VGCC inhibition was determined as $[1 - (\text{peak current after DAMGO} / \text{peak current before DAMGO})] \times 100\%$. The constitutive MOR activity was investigated by a two-pulse voltage protocol, and Ca²⁺ in the external solution was replaced by Ba²⁺ to minimize Ca²⁺-dependent inactivation. Two-pulse protocol has been widely used to determine constitutive GPCR signaling^{39, 44–47}, including MOR^{17–18}. In this protocol, Ba²⁺ currents were elicited from a holding potential of –60 mV to 0 mV, followed by a 40ms strong depolarizing pulse (to +80 mV) to reverse the interaction of the $\beta\gamma$ subunits with the VGCCs, then followed by another test pulse to 0 mV after a brief return to –60 mV. The mean current amplitudes were measured between 5 and 10ms after start of the test pulse (from –60 mV to 0 mV,¹⁷). G $\beta\gamma$ -mediated VGCC inhibition was determined as facilitation ratio (post-pulse current amplitude/pre-pulse current amplitude). In our experiments, the single-pulse and two-pulse data from DRG neurons were always obtained within 5 min after rupturing the cell membrane, which avoids the “run-down” phenomenon of VGCC that is typically found approximately 10m after attaining the whole-cell recording configuration. Furthermore, DAMGO was always applied at the end of experiments to confirm MOR-expression, and recovery of the current amplitude after washout was achieved to confirm the DAMGO-reversible inhibition and monitor “run-down”. Individual experimental group results collected in Tables 1 and 2.

siRNA Silencing Experiments

FITC-labeled siRNA sequences (Qiagen) were previously described for silencing GRK2 and RKIP²². DRG neurons were transfected with FITC-labeled siRNA targeting GRK2 or RKIP, or with no siRNA (mock) using HiperFect transfection reagent (Qiagen) according to the manufacturer’s instructions. In short, cultured DRG neurons were incubated for 16h in the presence of siRNA (FITC-GRK2: 45 ng/coverslip; FITC-RKIP: 450 ng/coverslip) complexed with HiperFect (2.5 μ l/coverslip). Next day, cells were replenished with complete media prior to experiments. Knockdown efficiencies for these siRNA have been previously assessed by Western Blot analysis²².

Transfection of DRG neurons

Transient transfection of primary cultured neurons was performed using 8 μ l of Lipofectamine 2000 (Thermo Fisher Scientific) and 1 μ g cDNA for empty vector (E.V.) pcDNA3.1, GRK2-WT, GRK2-S685D or GRK2-S685G together with 0.5 μ g GFP cDNA in 500 μ l of OptiMem media (Thermo Fisher Scientific) per coverslip, media was changed 6h later⁴⁸. After transfection, the primary cultured neurons were left in complete media overnight. Transfected neurons were used for electrophysiology experiments within 2 days.

Experimental Design and Statistical Analyses

Analyses of calcium channel current amplitude were performed using Axograph X (Axograph Scientific). Only cells with a stable series resistance of $< 25 \text{ M}\Omega$ throughout the recording period were included in the analysis. Statistical analysis and plotting were performed with Prism 6 (GraphPad Software). We used the two-tailed Student's *t*-tests or ANOVA with Bonferroni *post hoc* test, as appropriate. One-way and two-way ANOVA were employed for multiple comparisons. Drug group comparisons were made using paired Student's *t*-tests. All data are presented as mean $\pm$ SEM. Specific statistical tests and sample sizes are indicated for each data set in the figures and figure legends. The number of asterisks corresponds to level of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

REFERENCES

1. Stein C; Schafer M; Machelska H, Attacking pain at its source: new perspectives on opioids. *Nat Med*2003, 9 (8), 1003–8. [PubMed: 12894165]
2. Berg KA; Patwardhan AM; Sanchez TA; Silva YM; Hargreaves KM; Clarke WP, Rapid modulation of micro-opioid receptor signaling in primary sensory neurons. *The Journal of pharmacology and experimental therapeutics*2007, 321 (3), 839–47. [PubMed: 17347322]
3. Berg KA; Rowan MP; Sanchez TA; Silva M; Patwardhan AM; Milam SB; Hargreaves KM; Clarke WP, Regulation of kappa-opioid receptor signaling in peripheral sensory neurons in vitro and in vivo. *The Journal of pharmacology and experimental therapeutics*2011, 338 (1), 92–9. [PubMed: 21487072]
4. Patwardhan AM; Berg KA; Akopain AN; Jeske NA; Gamper N; Clarke WP; Hargreaves KM, Bradykinin-induced functional competence and trafficking of the delta-opioid receptor in trigeminal nociceptors. *J Neurosci*2005, 25 (39), 8825–32. [PubMed: 16192372]
5. Stein C; Hassan AH; Lehrberger K; Giefing J; Yassouridis A, Local analgesic effect of endogenous opioid peptides. *Lancet*1993, 342 (8867), 321–4. [PubMed: 8101583]
6. Stein C; Machelska H; Binder W; Schafer M, Peripheral opioid analgesia. *Curr Opin Pharmacol*2001, 1 (1), 62–5. [PubMed: 11712537]
7. Stein C; Millan MJ; Shippenberg TS; Peter K; Herz A, Peripheral opioid receptors mediating antinociception in inflammation. Evidence for involvement of mu, delta and kappa receptors. *J Pharmacol Exp Ther*1989, 248 (3), 1269–75. [PubMed: 2539460]
8. Stein C; Millan MJ; Yassouridis A; Herz A, Antinociceptive effects of mu- and kappa-agonists in inflammation are enhanced by a peripheral opioid receptor-specific mechanism. *Eur J Pharmacol*1988, 155 (3), 255–64. [PubMed: 2853065]
9. Connor M; Traynor J, Constitutively active mu-opioid receptors. *Methods Enzymol*2010, 484, 445–69. [PubMed: 21036245]
10. Burford NT; Wang D; Sadee W, G-protein coupling of mu-opioid receptors (OP3): elevated basal signalling activity. *Biochem J*2000, 348Pt 3, 531–7. [PubMed: 10839983]
11. Sally EJ; Xu H; Dersch CM; Hsin LW; Chang LT; Prisinzano TE; Simpson DS; Giuvelis D; Rice KC; Jacobson AE; Cheng K; Bilsky EJ; Rothman RB, Identification of a novel “almost neutral” micro-opioid receptor antagonist in CHO cells expressing the cloned human mu-opioid receptor. *Synapse*2010, 64 (4), 280–8. [PubMed: 19953652]
12. Wang D; Raehal KM; Bilsky EJ; Sadee W, Inverse agonists and neutral antagonists at mu opioid receptor (MOR): possible role of basal receptor signaling in narcotic dependence. *J Neurochem*2001, 77 (6), 1590–600. [PubMed: 11413242]
13. Corder G; Doolen S; Donahue RR; Winter MK; Jutras BL; He Y; Hu X; Wieskopf JS; Mogil JS; Storm DR; Wang ZJ; McCarson KE; Taylor BK, Constitutive mu-opioid receptor activity leads to long-term endogenous analgesia and dependence. *Science*2013, 341 (6152), 1394–9. [PubMed: 24052307]

14. Heinzen EL; Booth RG; Pollack GM, Neuronal nitric oxide modulates morphine antinociceptive tolerance by enhancing constitutive activity of the mu-opioid receptor. *Biochem Pharmacol*2005, 69 (4), 679–88. [PubMed: 15670586]
15. Meye FJ; van Zessen R; Smidt MP; Adan RA; Ramakers GM, Morphine withdrawal enhances constitutive mu-opioid receptor activity in the ventral tegmental area. *J Neurosci*2012, 32 (46), 16120–8. [PubMed: 23152596]
16. Wang D; Raehal KM; Lin ET; Lowery JJ; Kieffer BL; Bilsky EJ; Sadee W, Basal signaling activity of mu opioid receptor in mouse brain: role in narcotic dependence. *J Pharmacol Exp Ther*2004, 308 (2), 512–20. [PubMed: 14600246]
17. Walwyn W; Evans CJ; Hales TG, Beta-arrestin2 and c-Src regulate the constitutive activity and recycling of mu opioid receptors in dorsal root ganglion neurons. *J Neurosci*2007, 27 (19), 5092–104. [PubMed: 17494695]
18. Walwyn WM; Chen W; Kim H; Minasyan A; Ennes HS; McRoberts JA; Marvizon JC, Sustained Suppression of Hyperalgesia during Latent Sensitization by mu-, delta-, and kappa-opioid receptors and alpha2A Adrenergic Receptors: Role of Constitutive Activity. *J Neurosci*2016, 36 (1), 204–21. [PubMed: 26740662]
19. Doll C; Poll F; Peuker K; Loktev A; Gluck L; Schulz S, Deciphering micro-opioid receptor phosphorylation and dephosphorylation in HEK293 cells. *Br J Pharmacol*2012, 167 (6), 1259–70. [PubMed: 22725608]
20. Schulz S; Mayer D; Pfeiffer M; Stumm R; Koch T; Holtt V, Morphine induces terminal micro-opioid receptor desensitization by sustained phosphorylation of serine-375. *EMBO J*2004, 23 (16), 3282–9. [PubMed: 15272312]
21. Zhang J; Ferguson SS; Barak LS; Bodduluri SR; Laporte SA; Law PY; Caron MG, Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. *Proc Natl Acad Sci U S A*1998, 95 (12), 7157–62. [PubMed: 9618555]
22. Brackley AD; Gomez R; Akopian AN; Henry MA; Jeske NA, GRK2 Constitutively Governs Peripheral Delta Opioid Receptor Activity. *Cell reports*2016, 16 (10), 2686–2698. [PubMed: 27568556]
23. Stein C; Zollner C, Opioids and sensory nerves. *Handb Exp Pharmacol*2009, (194), 495–518. [PubMed: 19655116]
24. Tan M; Groszer M; Tan AM; Pandya A; Liu X; Xie CW, Phosphoinositide 3-kinase cascade facilitates mu-opioid desensitization in sensory neurons by altering G-protein-effector interactions. *J Neurosci*2003, 23 (32), 10292–301. [PubMed: 14614088]
25. Rusin KI; Moises HC, mu-Opioid receptor activation reduces multiple components of high-threshold calcium current in rat sensory neurons. *J Neurosci*1995, 15 (6), 4315–27. [PubMed: 7540671]
26. Ikeda SR, Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits. *Nature*1996, 380 (6571), 255–8. [PubMed: 8637575]
27. Barrett CF; Rittenhouse AR, Modulation of N-type calcium channel activity by G-proteins and protein kinase C. *J Gen Physiol*2000, 115 (3), 277–86. [PubMed: 10694257]
28. Pettinger L; Gigout S; Linley JE; Gamper N, Bradykinin controls pool size of sensory neurons expressing functional delta-opioid receptors. *J Neurosci*2013, 33 (26), 10762–71. [PubMed: 23804098]
29. Raynor K; Kong H; Chen Y; Yasuda K; Yu L; Bell GI; Reisine T, Pharmacological characterization of the cloned kappa-, delta-, and mu-opioid receptors. *Mol Pharmacol*1994, 45 (2), 330–4. [PubMed: 8114680]
30. Wang Z; Bilsky EJ; Porreca F; Sadee W, Constitutive mu opioid receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. *Life Sci*1994, 54 (20), PL339–50. [PubMed: 7514710]
31. Sullivan LC; Berg KA; Clarke WP, Dual regulation of delta-opioid receptor function by arachidonic acid metabolites in rat peripheral sensory neurons. *J Pharmacol Exp Ther*2015, 353 (1), 44–51. [PubMed: 25637601]

32. Brackley AD; Sarrami S; Gomez R; Guerrero KA; Jeske NA, Identification of a signaling cascade that maintains constitutive delta-opioid receptor incompetence in peripheral sensory neurons. *J Biol Chem*2017, 292 (21), 8762–8772. [PubMed: 28381559]
33. Thal DM; Homan KT; Chen J; Wu EK; Hinkle PM; Huang ZM; Chuprun JK; Song J; Gao E; Cheung JY; Sklar LA; Koch WJ; Tesmer JJ, Paroxetine is a direct inhibitor of g protein-coupled receptor kinase 2 and increases myocardial contractility. *ACS Chem Biol*2012, 7 (11), 1830–9. [PubMed: 22882301]
34. Lee BS; Jun IG; Kim SH; Park JY, Interaction of morphine and selective serotonin receptor inhibitors in rats experiencing inflammatory pain. *J Korean Med Sci*2012, 27 (4), 430–6. [PubMed: 22468108]
35. Zollner C; Shaqura MA; Bopaiah CP; Mousa S; Stein C; Schafer M, Painful inflammation-induced increase in mu-opioid receptor binding and G-protein coupling in primary afferent neurons. *Mol Pharmacol*2003, 64 (2), 202–10. [PubMed: 12869624]
36. Campillo A; Cabanero D; Romero A; Garcia-Nogales P; Puig MM, Delayed postoperative latent pain sensitization revealed by the systemic administration of opioid antagonists in mice. *Eur J Pharmacol*2011, 657 (1–3), 89–96. [PubMed: 21300053]
37. Severino A; Chen W; Hakimian JK; Kieffer BL; Gaveriaux-Ruff C; Walwyn W; Marvizon JCG, Mu-opioid receptors in nociceptive afferents produce a sustained suppression of hyperalgesia in chronic pain. *Pain*2018, 159 (8), 1607–1620. [PubMed: 29677019]
38. Taylor BK; Sinha GP; Donahue RR; Grachen CM; Moron JA; Doolen S, Opioid receptors inhibit the spinal AMPA receptor Ca(2+) permeability that mediates latent pain sensitization. *Exp Neurol*2019, 314, 58–66. [PubMed: 30660616]
39. Beedle AM; McRory JE; Poirot O; Doering CJ; Altier C; Barrere C; Hamid J; Nargeot J; Bourinet E; Zamponi GW, Agonist-independent modulation of N-type calcium channels by ORL1 receptors. *Nat Neurosci*2004, 7 (2), 118–25. [PubMed: 14730309]
40. Sorriento D; Ciccarelli M; Cipolletta E; Trimarco B; Iaccarino G, “Freeze, Don’t Move”: How to Arrest a Suspect in Heart Failure - A Review on Available GRK2 Inhibitors. *Front Cardiovasc Med*2016, 3, 48. [PubMed: 27999776]
41. Williams JT; Ingram SL; Henderson G; Chavkin C; von Zastrow M; Schulz S; Koch T; Evans CJ; Christie MJ, Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol Rev*2013, 65 (1), 223–54. [PubMed: 23321159]
42. Hamid J; Nelson D; Spaetgens R; Dubel SJ; Snutch TP; Zamponi GW, Identification of an integration center for cross-talk between protein kinase C and G protein modulation of N-type calcium channels. *J Biol Chem*1999, 274 (10), 6195–202. [PubMed: 10037705]
43. Cooper CB; Arnot MI; Feng ZP; Jarvis SE; Hamid J; Zamponi GW, Cross-talk between G-protein and protein kinase C modulation of N-type calcium channels is dependent on the G-protein beta subunit isoform. *J Biol Chem*2000, 275 (52), 40777–81. [PubMed: 11053424]
44. Delmas P; Nomura H; Li X; Lakkis M; Luo Y; Segal Y; Fernandez-Fernandez JM; Harris P; Frischauf AM; Brown DA; Zhou J, Constitutive activation of G-proteins by polycystin-1 is antagonized by polycystin-2. *J Biol Chem*2002, 277 (13), 11276–83. [PubMed: 11786542]
45. Kammermeier PJ, Constitutive activity of metabotropic glutamate receptor 7. *BMC Neurosci*2015, 16, 17. [PubMed: 25881041]
46. Mahmoud S; Margas W; Trapella C; Calo G; Ruiz-Velasco V, Modulation of silent and constitutively active nociceptin/orphanin FQ receptors by potent receptor antagonists and Na⁺ ions in rat sympathetic neurons. *Mol Pharmacol*2010, 77 (5), 804–17. [PubMed: 20159949]
47. Pan X; Ikeda SR; Lewis DL, SR 141716A acts as an inverse agonist to increase neuronal voltage-dependent Ca²⁺ currents by reversal of tonic CB1 cannabinoid receptor activity. *Mol Pharmacol*1998, 54 (6), 1064–72. [PubMed: 9855635]
48. Dalby B; Cates S; Harris A; Ohki EC; Tilkins ML; Price PJ; Ciccarone VC, Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. *Methods*2004, 33 (2), 95–103. [PubMed: 15121163]

Figure 1.

GRK2 associates with peripheral MOR to inhibit receptor activity. (**A** and **B**) Representative traces (**A**) and analysis (**B**) of DAMGO (1 μ M) inhibition of VGCCs in DRG neurons pre-treated with vehicle or BK (200 nM, 5m). *** $p < 0.001$; unpaired two-tailed Student's t test. (**C**) *In vitro* association of GRK2 and MOR. DRG cultures were treated with vehicle or BK (200 nM, 5 m), and whole cell lysates were immunoprecipitated with anti-GRK2 and blotted with anti-MOR (upper panel) or anti-GRK2 (lower panel) from the same blot. (**D**) Averaged densitometric results of three independent experiments quantified by Image J analysis. Data were normalized to the vehicle-treated groups. * $p < 0.05$; unpaired two-tailed Student's t test. (**E** and **F**) Representative traces (**E**) and analysis (**F**) of DAMGO (1 μ M) inhibition of VGCCs in DRG neurons (mock-treated or transfected with FITC-GRK2 siRNA) treated without or with BK (200 nM, 5m). ** $p < 0.01$; One-way ANOVA Bonferroni post hoc. The number of cells or experiments is indicated inside each bar.

Figure 2.

Prolonged BK exposure increases constitutively active MOR. (A) Representative traces recorded from DRG neurons treated with vehicle or BK (200 nM, 5 min or 15 min) in the absence (black traces) or presence (green traces) of 1 μ M DAMGO, using the two-pulse voltage protocol (inset). “Pre” and “post” test pulses were obtained without and with +80 mV conditioning depolarization. (B) Summary of facilitation ratios from control (vehicle) neurons and neurons treated with BK for 5 min or 15 min. * p < 0.05, ** p < 0.01; # p < 0.05, ### p < 0.01, #### p < 0.0001 compared with corresponding baseline; two-way ANOVA Bonferroni post hoc. (C) Summary of VGCC current amplitude inhibition produced by application of DAMGO to control neurons and neurons treated with BK for 5 min or 15 min. **** p < 0.0001; One-way ANOVA Bonferroni post hoc. (D and E) Effects of inverse agonist naloxone (1 nM, ²⁹) on basal facilitation (in the absence of agonist) in control neurons (D) and neurons treated with BK for 15 min (E). ** p < 0.01; paired two-tailed

Student's t test. **(F)** Effects of neutral antagonist CTAP (1 μ M) on basal facilitation in neurons treated with BK for 15m. Paired two-tailed Student's t test. **(G)** CTAP abrogates the effects of naloxone on basal facilitation in neurons treated with BK for 15m. Paired two-tailed Student's t test. The number of cells is indicated inside each bar. ns, not significant.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3.

PKC and RKIP, but not LOX, are required for BK-mediated constitutive MOR activation. (A) Representative traces recorded from DRG neurons treated with BK (200 nM, 15m) in the absence and presence of PKC inhibitor GFX or LOX inhibitor NDGA, with (green traces) and without (black traces) 1 μ M DAMGO application. GFX (10 μ M) and NDGA (10 μ M) were added 5m prior to 15m incubation with BK (200 nM). (B) Summary of facilitation ratios from DRG neurons treated with BK (200 nM, 15m) in the absence and presence of GFX or NDGA. * $p < 0.05$, ** $p < 0.01$ compared with BK 15m; two-way ANOVA Bonferroni post hoc. (C) Summary of VGCC current amplitude inhibition produced by application of DAMGO to DRG neurons treated with BK (200 nM, 15m) in the absence and presence of GFX or NDGA. One-way ANOVA Bonferroni post hoc. (D) Representative traces recorded from DRG neurons transfected without siRNA (mock), or with FITC-RKIP siRNA in the absence (black traces) or presence (green traces) of 1 μ M DAMGO. (E)

Summary of facilitation ratios from mock-treated and FITC-RKIP siRNA-transfected DRG neurons. ** $p < 0.01$, *** $p < 0.001$; # $p < 0.05$, ### $p < 0.001$ compared with corresponding baseline; two-way ANOVA Bonferroni post hoc. (F) Summary of VGCC current amplitude inhibition produced by application of DAMGO to mock-treated and FITC-RKIP siRNA-transfected DRG neurons. Unpaired two-tailed Student's t test. The number of cells is indicated inside each bar. ns, not significant.

Figure 4.

GRK2 is critical for mediating constitutive MOR activity. (A) Representative traces recorded from DRG neurons transfected without siRNA (mock), or with FITC-GRK2 siRNA in the absence (black traces) or presence (green traces) of 1 μ M DAMGO. (B) Summary of facilitation ratios from mock-treated and FITC-GRK2 siRNA-transfected DRG neurons. *p < 0.05, ****p < 0.0001; #p < 0.05, #####p < 0.0001 compared with corresponding baseline; two-way ANOVA Bonferroni post hoc. DAMGO⁺ indicates DAMGO-responsive DRG neurons and DAMGO⁻ indicates DAMGO-nonresponsive DRG neurons. (C) Summary of VGCC current amplitude inhibition produced by application of DAMGO to mock-treated and FITC-GRK2 siRNA-transfected DRG neurons. *p < 0.05; unpaired two-tailed Student's t test. (D – F) Effects of naloxone (1 nM) alone (D), CTAP (1 μ M) alone (E), and naloxone plus CTAP (F) on basal facilitation in FITC-GRK2 siRNA-transfected DRG neurons. **p <

0.01; paired two-tailed Student's *t* test. The number of cells is indicated inside each bar. ns, not significant.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5. Phosphorylation of GRK2 at Ser-685 blocks constitutive MOR activation. **(A)** Representative traces recorded from DRG neurons transfected with either empty vector (E.V.), wild type GRK2 or serine mutants together with EGFP-C1, in the absence (black traces) or presence (green traces) of 1 μ M DAMGO. **(B)** Summary of facilitation ratios from neurons co-transfected with indicated plasmids and EGFP-C1. * $p < 0.05$, **** $p < 0.0001$ compared with E.V.; Two-way ANOVA Bonferroni post hoc. **(C)** Summary of VGCC current amplitude inhibition produced by application of DAMGO to neurons co-transfected with indicated plasmids and EGFP-C1. ** $p < 0.01$; one-way ANOVA Bonferroni post hoc. The number of cells is indicated inside each bar.

Figure 6.

Paroxetine reduces GRK2/MOR interaction and promotes constitutive MOR activation.

(A) *In vitro* association of GRK2 and MOR. DRG cultures were treated with vehicle or paroxetine (5 μ M, 15m), and whole cell lysates were immunoprecipitated with anti-GRK2 and blotted with anti-MOR (upper panel) or anti-GRK2 (lower panel) from the same blot.

(B) Averaged densitometric results of four independent experiments quantified by Image J analysis. Data were normalized to the vehicle-treated groups. * $p < 0.05$; unpaired two-tailed Student's t test. (C) Representative traces recorded from DRG neurons treated with vehicle or paroxetine (5 μ M, 15m) in the absence (black traces) or presence (green traces) of 1 μ M DAMGO. (D) Summary of facilitation ratios from control (vehicle) neurons and neurons treated with paroxetine for 15m. * $p < 0.05$, **** $p < 0.0001$; # $p < 0.05$, #### $p < 0.0001$ compared with corresponding baseline; two-way ANOVA Bonferroni post hoc. (E) Summary of VGCC current amplitude inhibition produced by application of DAMGO

to control neurons and neurons treated with paroxetine for 15m. $**p < 0.01$; unpaired two-tailed Student's t test. The number of cells or experiments is indicated inside each bar.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1:

Summary of facilitation ratios and DAMGO potencies.

Group	+PP/-PP		% Inhibition	n
	Baseline	DAMGO	(pre-pulse)	
Veh (H ₂ O)	0.94 ± 0.02	1.07 ± 0.05	20.4 ± 2.3	12
	0.97 ± 0.01	1.26 ± 0.06	42.6 ± 2.9	12
BK (in H ₂ O) 5m	1.08 ± 0.03	1.25 ± 0.04	14.4 ± 2.8	12
BK+GFX 15m	0.93 ± 0.02	1.06 ± 0.02	19.0 ± 1.3	10
BK+NDGA 15m	0.98 ± 0.02	1.17 ± 0.05	19.8 ± 3.4	10
Mock/BK 15m	1.11 ± 0.02	1.30 ± 0.05	15.3 ± 2.5	12
RKIP siRNA/BK 15m	0.92 ± 0.02	1.06 ± 0.05	18.3 ± 3.5	10
Mock	0.92 ± 0.02	1.09 ± 0.05	23.3 ± 3.4	11
GRK2 siRNA/DAMGO (+)	1.11 ± 0.02	1.45 ± 0.09	36.8 ± 4.2	12
GRK2 siRNA/DAMGO (−)	0.89 ± 0.03	0.90 ± 0.03	0.9 ± 3.6	10
E.V.	0.95 ± 0.01	1.07 ± 0.03	20.4 ± 2.9	10
GRK2 WT	0.98 ± 0.05	1.26 ± 0.08	25.7 ± 4.0	9
GRK2 S685D	1.00 ± 0.03	1.25 ± 0.10	21.8 ± 5.3	13
GRK2 S685G	1.23 ± 0.04	1.72 ± 0.08	38.7 ± 3.6	14
Veh (DMSO)	0.94 ± 0.02	1.03 ± 0.03	10.8 ± 1.4	10
PAROX (in DMSO) 15m	1.07 ± 0.04	1.30 ± 0.06	24.4 ± 3.6	10

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 2:

Effects of MOR antagonists on basal facilitation.

Group		+PP/-PP		n
		Before	After	
Control	NLX	0.94 ± 0.02	0.95 ± 0.02	9
	NLX	1.05 ± 0.02	0.96 ± 0.01	8
BK 15m	CTAP	1.08 ± 0.02	1.02 ± 0.01	6
	NLX/CTAP	1.08 ± 0.02	1.07 ± 0.02	5
	NLX	1.08 ± 0.03	1.03 ± 0.02	8
GRK2 siRNA	CTAP	1.07 ± 0.03	1.05 ± 0.02	8
	NLX/CTAP	1.13 ± 0.03	1.10 ± 0.04	8