Non-canonical opioid signaling inhibits itch transmission in the spinal cord of mice

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Non-canonical Opioid Signaling Inhibits Itch Transmission in the Spinal Cord of Mice

Graphical Abstract

Highlights
- KOR inhibits itch by attenuating GRPR function
- KOR-GRPR cross-talk is independent of G<sub>αi</sub> signaling
- KOR desensitizes GRPR via PKCδ translocation
- KOR activates PKCδ translocation via PLC signaling

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In Brief
Munanairi et al. show that the kappa opioid receptor (KOR) agonists inhibit nonhistaminergic itch transmission by attenuating the function of the gastrin-releasing peptide receptor (GRPR), an itch receptor in the spinal cord. KOR activation causes the translocation of PKCδ from plasma to membrane, which phosphorylates GRPR to dampen itch transmission.

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Non-canonical Opioid Signaling Inhibits Itch Transmission in the Spinal Cord of Mice

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SUMMARY

Chronic itch or pruritus is a debilitating disorder that is refractory to conventional anti-histamine treatment. Kappa opioid receptor (KOR) agonists have been used to treat chronic itch, but the underlying mechanism remains elusive. Here, we find that KOR and gastrin-releasing peptide receptor (GRPR) overlap in the spinal cord, and KOR activation attenuated GRPR-mediated histamine-independent acute and chronic itch in mice. Notably, canonical KOR-mediated Gαi signaling is not required for desensitizing GRPR function. In vivo and in vitro studies suggest that KOR activation results in the translocation of Ca2+-independent protein kinase C (PKC)δ from the cytosol to the plasma membrane, which in turn phosphorylates and inhibits GRPR activity. A blockade of phospholipase C (PLC) in HEK293 cells prevented KOR-agonist-induced PKCδ translocation and GRPR phosphorylation, suggesting a role of PLC signaling in KOR-mediated GRPR desensitization. These data suggest that a KOR-PLC-PKCδ-GRPR signaling pathway in the spinal cord may underlie KOR-agonists-induced anti-pruritus therapies.

INTRODUCTION

Chronic itch or pruritus may arise from dysfunction of skin, immune, nervous system, or internal organ metabolism, such as liver and kidney diseases (Ikoma et al., 2006; Paus et al., 2006). Despite recent progress in identifying signaling molecules as potential targets for anti-pruritus therapies (Bautista et al., 2014; Liu and Ji, 2013), much less is known about the central targets for itch (Barry et al., 2018; Bautista et al., 2014). The mu and kappa opioid receptor systems appear to have opposing roles in a wide range of physiological processes (Pan, 1998), including itch transmission (Ballantyne et al., 1988). Most opioids are pruritogens, and morphine-induced pruritus could be a serious unwanted effect of epidural analgesia (Ballantyne et al., 1988; Reich and Szepietowski, 2010). On the other hand, the inhibitory effect of kappa opioid receptor (KOR) agonists, e.g., butorphanol or nalfurafine (TRK-820), on a wide range of itch behaviors has made them attractive drug candidates for treating patients with uremic, cholestatic, and opioid-induced pruritus (Cowen et al., 2015; Kumagai et al., 2010; Lawhorn et al., 1991; Phan et al., 2012; Togashi et al., 2002; Wikström et al., 2005). KOR-agonist-based anti-pruritic therapies, however, may have unwanted side effects, such as insomnia, somnolence, and constipation (Land et al., 2008; Phan et al., 2012). Despite a potential for KOR agonists in anti-itch application, the underlying mechanisms remain poorly understood.
Gastrin-releasing peptide receptor (GRPR) is primarily required for relaying nonhistaminergic itch in the spinal cord (Akiyama et al., 2013; Barry et al., 2018; Liu et al., 2011; Shiratori-Hayashi et al., 2015; Sun and Chen, 2007; Sun et al., 2009). Its endogenous ligand, gastrin-releasing peptide (GRP), is expressed in a subset of dorsal root ganglion (DRG) neurons, which also overlaps with TRPV1 and substance P (SP) (Barry et al., 2016; Takanami et al., 2014; Zhao et al., 2013).

In this study, we investigated whether spinal KOR activation attenuates itch transmission by blocking GRPR signaling in mice. Using several complementary approaches, we have demonstrated that KOR activation inhibits GRPR signaling via a Ca^{2+}-independent phospholipase C (PLC)-protein kinase C (PKC)β pathway. Our studies may help design spinal KOR-GRPR cross-signaling-based therapeutic strategies to alleviate chronic itch.

RESULTS

Spinal KOR Activation Inhibits Nonhistaminergic Itch
To determine the effect of spinal activation of KOR on itch, scratching behavior was quantified in C57BL/6J mice after intrathecal (i.t.) injection of U-50,488, a selective KOR agonist (Simonen et al., 1996). Consistent with this previous study (Inan and Cowan, 2004), U-50,488 significantly attenuated scratching behavior induced by chloroquine (CQ), an anti-malaria drug with generalized pruritus (Ajayi et al., 1989). By contrast, U-50,488 had no effect on histamine-induced scratching (Figure 1A). Consistently, U-50,488 markedly reduced i.t. GIS, monkeys, and humans with chronic itch were upregulated (Choi et al., 2016; Kagami et al., 2013; Lou et al., 2017; Nattkemper et al., 2013; Tirado-Sánchez et al., 2015; Tominaga et al., 2009; Zhao et al., 2013). A blockade of GRPR or GRP markedly diminishes long-lasting itch in various types of mouse models (Lagerström et al., 2010; Shiratori-Hayashi et al., 2015; Zhao et al., 2013). These studies raise the question of whether KOR may inhibit itch in part by blocking GRPR function. Consistent with this possibility, studies have shown that spinal KOR activation inhibits GRP-induced scratching (GIS) (Kardon et al., 2014; Lee and Ko, 2015) and morphine-induced scratching (MIS) (Ko et al., 2003; Sakakihara et al., 2016).

In this study, we investigated whether spinal KOR activation diminishes long-lasting itch in mice. Using several complementary approaches, we have demonstrated that KOR activation inhibits GRPR signaling via a Ca^{2+}-independent phospholipase C (PLC)-protein kinase C (PKC)β pathway. Our studies may help design spinal KOR-GRPR cross-signaling-based therapeutic strategies to alleviate chronic itch.

KOR Inhibits GRPR in a Cell-Autonomous Manner
The finding that KOR activation inhibited GIS prompted us to examine whether KOR inhibits GIS indirectly through inhibitory neural circuits or directly in GRPR neurons, which are primarily excitatory interneurons (Wang et al., 2013). To differentiate between these two possibilities, we first examined whether KOR and GRPR are co-expressed in the spinal cord using dual-labeled RNAscope in situ hybridization (ISH) (Wang et al., 2012). Opirk1 mRNA was detected in ~50% (104/205) of Grpr neurons in the superficial dorsal horn (Figures 1E and 1F).

The co-expression of KOR and GRPR raised the possibility that KOR activation may cross-inhibit GRPR in a cell-autonomous manner rather than through activation of inhibitory neural circuits. GRPR transduces itch via the PLCβ/IP3/Ca^{2+} signaling pathway (Wang et al., 2014a). To examine this, the dorsal horn of the spinal cord was dissected and dissociated neurons were cultured for calcium imaging (Figure 2A; Video S1). To determine whether U-50,488 inhibits GRP-induced, GRPR-mediated intracellular Ca^{2+} mobilization, a two-step protocol was employed, whereby dissociated dorsal horn GRPR+ neurons can be identified by an application of GRP (20 nM) and then re-sensitized after a 30-min wash-out period (Figure 2C; Table S1; Zhao et al., 2014a). The ratio of the second GRP-induced response to the first response was used for quantitation, thereby avoiding inconsistencies that may result from GRPR neuronal heterogeneity. U-50,488 (up to 20 μM) alone did not induce Ca^{2+} responses in GRPR+ neurons (data not shown). However, incubation of U-50,488 (10 μM) attenuated Ca^{2+} responses of GRPR+ neurons to GRP (Figures 2B, 2D, and 2F; Video S2), and this inhibitory effect was reversed by norbinaltorphimine (norBNI), a selective KOR antagonist (Portoghese et al., 1987; Figures 2E and 2F). About 42% of GRPR+ neurons showed complete inhibition (52/124) by KOR activation, 26% showed partial inhibition (32/124), and 32% were resistant to U-50,488 application (40/124; Table S2). The finding that the percentage of non-responders was slightly lower than Grpr+/Oprk1− mice, as observed by RNAscope ISH (Figures 1E and 1F), could be due to several factors, such as younger age of mice used for calcium imaging study and/or different sensitivities associated with each approach. CQ itch is significantly reduced, but not abolished, in Grpr KO mice (Sun and Chen, 2007), suggesting the involvement of a GRPR-independent pathway. To test whether U-50,488 may inhibit CQ itch via GRPR-independent pathway, we examined its effect on CQ itch using Grpr KO mice and found that i.t. U-50,488 did not further reduce CQ-induced scratching (Figure S2A). This result suggests that spinal KOR activation inhibits CQ itch predominantly via GRPR-cell-autonomous mechanism.

Next, we investigated whether KOR activation inhibits GRPR signaling via the canonical opioid-mediated Gαi signaling pathway (Al-Hasani and Bruchas, 2011). Unexpectedly,
pertussis toxin (PTX) (200 ng/mL), a \( G_{\alpha_i} \) inhibitor, did not block the inhibitory effect of U-50,488 (Figure 2G), suggesting that a \( G_{\alpha_i} \)-independent pathway is involved in spinal KOR activation-mediated itch inhibition. Of the 23 GRPR neurons treated with PTX, GRP-induced calcium responses in 16/23 (70%) were completely inhibited by U-50,488, 2/23 (9%) showed partial inhibition, whereas 5/23 (21%) were resistant to U-50,488 treatment. As expected, PTX reversed U-50,488-mediated inhibition of cyclic AMP (cAMP) synthesis in HEK293 cells (Figure S3A).

According to the canonical pathway, activation of KOR recruits G-protein-coupled receptor kinases (GRK). Arrestin will then bind to the phosphorylated KOR, resulting in acute desensitization (Bruchas and Chavkin, 2010). In contrast, U-50,488-mediated desensitization lasts for at least two days in mice (Figure S1C). Furthermore, U-50,488 attenuated GIS

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**Figure 1. Intrathecal Administration of U-50,488 Attenuates Nonhistaminergic Itch**

(A) 10-min pre-injection of U-50,488 (10 nmol) significantly reduced scratching induced by CQ (200 \( \mu \)g intradermal [i.d.]) and GRP (0.3 nmol i.t.), but not Hist (200 \( \mu \)g i.d.) and NMB (1 nmol i.t.; \(*p < 0.05; ***p < 0.001; \) Student’s unpaired t test; \( n = 6–11 \)).

(B–D) Reduced spontaneous scratching in (B) BRAF\(^{Nav1.8}\), (C) ACD, and (D) dry-skin mice after U-50,488 injection (\(*p < 0.05; ***p < 0.001; \) Student’s paired t test; \( n = 7–9 \)).

(E) Representative images of RNAscope \textit{in situ} hybridization of Grpr (green) and Oprk1 (red) mRNA expression in superficial dorsal horn of transverse lumbar sections. Arrows indicate double-stained neurons. Blue represents DAPI nucleic acid stain (scale bar, 20 \( \mu \)m).

(F) Venn diagram showing the overlap in expression of Grpr and Oprk1 in dorsal horn neurons (\( n = 20 \) lumbar sections from 3 mice). Data are represented as mean ± SEM.

See also Figures S1 and S2.
in Arrb2−/− mice (Figure S3C), consistent with previous studies (Bohn et al., 2000; Morgenweck et al., 2015). Although we cannot completely exclude the involvement of arrestin signaling, due to possible genetic compensation in Arrb2−/− mice, the long-lasting effect of KOR-mediated inhibitory action on itch transmission supports the notion that KOR activation
attenuates itch through β-arrestin2 signaling-independent pathway.

Spinal KOR Activation Inhibits GRPR Function via a PKC-Dependent Mechanism

Previous in vitro studies show that GRPR is a substrate of PKC that phosphorylates and desensitizes GRPR (Ally et al., 2003). To explore the possibility that PKC activation inhibits itch, scratching behavior was examined in mice pre-injected with i.t. phorbol myristate acetate (PMA), a PKC activator (Way et al., 2000), which markedly attenuated CQ-induced scratching and GIS, mimicking the U-50,488 effect (Figure 3A). Interestingly, bisindolylmaleimide (BIM), a selective inhibitor for PKCa,b1,b2,g,d, and ε isoforms (Toullec et al., 1991), blocked the effect of U-50,488 on GIS (Figure 3B). Furthermore, PMA completely blocked spontaneous scratching behavior in BRAFNav1.8, ACD, and dry-skin mice (***p < 0.001; Student’s paired t test; n = 7–9). (D and E) Representative traces (D) and quantified data (E) show that PMA (1 μM) inhibits GRP-induced Ca2+ responses in dissociated GRPR neurons (***p < 0.001; Student’s unpaired t test; n = 12). The inhibitory effect was no further reduced by co-application of PMA (1 μM) and U-50,488 (10 μM; ***p < 0.001; Student’s unpaired t test; n = 15). (F and G) BIM (5 μM) blocked U-50,488 inhibitory effect on GRP-induced Ca2+ responses (***p < 0.001; Student’s unpaired t test; n = 43–65). Data are represented as mean ± SEM.

Next, we examined whether PKC activation could reduce GRP-induced Ca2+ responses in GRPR+ neurons by applying PMA. Consistent with behavioral studies, PMA significantly attenuated GRP-induced Ca2+ responses (Figures 3D and 3E). Co-application of PMA and U-50,488 did not further reduce GRP-induced Ca2+ responses, suggesting that KOR activation attenuates GRPR signaling via PKC (Figures 3D and 3E). Moreover, pre-incubation with BIM (5 μM) blocked the inhibitory effect of U-50,488 on Ca2+ responses of GRPR+ neurons (Figures 3F and 3G). These observations support the notion that KOR activation attenuates itch transmission via PKC-mediated inhibition of spinal GRPR function.

Activation of KOR Induces GRPR Phosphorylation via PKC

Whole-cell phosphorylation assays were performed to further elucidate the role of PKC in KOR-activation-induced inhibition of GRPR signaling. In HEK293 cells expressing FLAG-KOR and Myc-GRPR, GRPR phosphorylation increased 13-fold after a 2-min incubation in U-50,488 (10 μM; Figures 4A, 4B, and S7A). Consistent with behavior and calcium-imaging results, PKC inhibition by BIM (5 μM) blocked KOR-activation-induced GRPR phosphorylation (Figures 4C and S7B). Rapid GRPR phosphorylation was also observed within 2 min after treatment with PMA (1 μM) and decreased after 15 min (Figures 4D and S7C), in accordance with previous findings (Ally et al., 2003). Phosphorylation assays showed that U-50,488-mediated KOR activation induces rapid and robust, PKC-independent phosphorylation of GRPR, which may cause desensitization of GRPR activity.

KOR Activation Attenuates Itch via PKCδ

The PKC family consists of a variety of isoforms that can be classified into three sub-families: conventional (α, β1, β2, and γ; Ca2+-
and diacylglycerol (DAG) dependent); novel (δ, ε, η, and θ; DAG dependent); and atypical (ζ and η; Nishizuka, 1995; Steinberg, 2008). Given that U-50,488 failed to induce Ca<sup>2+</sup> responses in GRPR neurons, we postulated that Ca<sup>2+</sup>-independent PKC isoforms (PKCd or ε) may be involved in mediating KOR-dependent PKC activation. To identify the PKC isoform involved, we performed spinal PKC-isofrom-specific small interfering RNA (siRNA) knockdown studies (Liu et al., 2011). Remarkably, siRNA knockdown of Prkd not only blocked U-50,488 inhibition of GIS (Figure 5A) but also enhanced CQ-induced itch, even in the presence of U-50,488 (Figure 5B). Treatment of control siRNA did not affect U-50,488 inhibitory effect on GIS and CQ itch (Figures S4A and S4B). qRT-PCR of the lumbar spinal cord confirmed specific knockdown of Prkd, but not Prkca (Figure 5C). Consistently, U-50,488 lost effect on GRP-induced calcium responses of dorsal horn neurons isolated from Prkd<sup>−/−</sup> mice (Figure S5B). We also performed siRNA knockdown of Prkca. However, U-50,488 attenuated CQ-induced itch after siRNA knockdown of Prkca, suggesting that PKCd does not mediate KOR activation inhibition of itch (Figures S4C and S4D).

Next, we examined the role of PKCd in KOR-activation-mediated itch inhibition using Prkd<sup>−/−</sup> mice and their wild-type (WT) littermates (Leitges et al., 2001) and did not find differences in GIS between Prkd<sup>−/−</sup> and WT littermates. As predicted, the inhibitory effect of U-50,488 on GIS is lost in Prkd<sup>−/−</sup> mice relative to their WT littermates (Figure 5D). To examine whether PKCd is co-expressed with GRPR in the superficial dorsal horn, we generated a Grpr<sup>Cre</sup>/Ai9 reporter mouse line by crossing Grpr<sup>Cre</sup>-mice with a tdTomato Ai9 line. Double immunohistochemistry (IHC) studies were conducted, and PKCd was detected in Grpr<sup>Cre</sup>-tdTomato<sup>+</sup> neurons (arrows indicate overlap in expression; Figure 5E). Further, we labeled spinal GRPR neurons with enhanced yellow fluorescent protein (eYFP) by injection of AAV5-Ef1α-DIO-eYFP virus into the dorsal spinal cord of Grpr<sup>Cre</sup> mice. Consistently, we detected numerous PKCd in Grpr<sup>Cre</sup> AAV-DIO-eYFP neurons (Figures 5F and S5A).

KOR Activation Induces PKCd Translocation to the Plasma Membrane

To further evaluate the role of PKCd in KOR-activation-induced inhibition of GRPR signaling, we examined PKC translocation from the cytosol to the plasma membrane, a hallmark of PKC activation (Mochly-Rosen et al., 1990). Using GFP-tagged PKC, the dynamics of PKC translocation in response to different stimuli can be monitored in live cells and in real time (Dancea et al., 1998; Wang et al., 1999). HEK293 cells expressing KOR and GRPR were transfected with PKCd-EGFP or PKCc-EGFP. Confocal live-cell imaging was then performed to characterize spatiotemporal properties of PKCd-EGFP or PKCc-EGFP after application of U-50,488 or PMA. PKCd and PKCc were present in the cytosol without stimulation (Figure 6A; 0 min). Application of U-50,488 (10 μM) prompted translocation of PKCd, but not PKCc, to the cell membrane. PKCd-EGFP translocation from the cytosol to the plasma membrane was apparent as early as 5 min and reached a maximum after 30 min of incubation in U-50,488 (Figure 6A; Video S3). After U-50,488 treatment, translocation to the plasma membrane increased significantly (from 15% ± 4% to 62% ± 8%; Figure 6B). As expected, direct activation of PKC with PMA (100 nM) induced translocation of both PKCd-EGFP and PKCc-EGFP from the cytosol to the membrane (Figures 6C and 6D; Video S4).

To evaluate that the U-50,488-induced PKCd translocation observed in HEK293 cells mimics events in vivo, the fraction of PKCd-positive dorsal horn neurons was quantified 30 min after i.t. injection of U-50,488 in mice. We found that the fraction of dorsal horn neurons with plasma-membrane-bound PKCd nearly doubled after U-50,488 injection (from 40% ± 1% to 73% ± 2%; Figures 6E–6G). This confirmed that KOR activation stimulates PKCd activity manifested by its translocation to the plasma membrane, which subsequently phosphorylates and desensitizes GRPR signaling.

KOR Activation Stimulates PKCd via PLC

To elucidate the mechanism by which KOR activation stimulates PKCd, we tested a myriad of inhibitors on U-50,488-induced PKCd translocation in HEK293 cells expressing KOR and GRPR. Pre-incubation of U73122 (10 μM), a PLC inhibitor, for 10 min blocked U-50,488-induced PKCd-EGFP translocation to the plasma membrane. In contrast, U-50,488 treatment increased the membrane translocation of PKCc-EGFP from 12% ± 4% to 64% ± 5% in the presence of U73343 (10 μM), an inactive analog of U73122 (Figures 7A and 7B). Furthermore,
U-50,488 treatment increased the translocation of PKC\(d\)-EGFP from 11% ± 2% to 47% ± 5% and 15% ± 3% to 70% ± 9% after a prior pre-incubation in gallein (100 \(\mu\)M), a G\(_{bg}\) inhibitor, and PTX (200 ng/mL), respectively (Figures 7C and 7D), suggesting that PLC mediates PKC\(d\) activation by KOR in a G\(_{bg}\)- and G\(_{ai}\)-independent process. Whole-cell phosphorylation assays were also used to show that KOR activation induces GRPR phosphorylation (Figures 7E, 7F, and S7D). Together, these results suggest that KOR activation stimulates PLC, resulting in the translocation of PKC\(d\) from the cytosol to the plasma membrane, where it phosphorylates GRPR (Figure 7G).

To investigate whether this pathway is similarly engaged by other KOR agonists, we tested butorphanol, a mixed KOR agonist/MOR antagonist (Abeliovich et al., 1993), which has been used to treat various types of intractable pruritus in human studies (Dawn and Yosipovitch, 2006; Dunteman et al., 1996). We found that i.t. injection of butorphanol (2 nmol) did not further reduce CQ-induced scratching in Grpr KO mice (Figure S2C). Consistently, butorphanol also lost effect in Prkcd\(^{-/-}\) mice (Figure S2D). These studies provide clinically relevant evidence supporting that spinal KOR-agonists-mediated itch inhibition is dependent on the KOR-GRPR cross-talk, but not other mechanisms. In HEK293 cells expressing KOR and GRPR, butorphanol induced PKC\(d\) -EGFP, but not PKC\(a\) -EGFP, translocation from the cytosol to the plasma membrane, mimicking the U-50,488 effect (Figure S6). Consistent with U-50,488 and butorphanol results, dynorphin, an endogenous ligand for KOR (Chavkin et al., 1982), also attenuated CQ-induced itch (Figure S2E). However, mice lacking dynorphin (\(Pdyn\)^{-/-}) exhibited normal acute and chronic itch (Figures S2F and S2G). These data demonstrate that spinal KOR activation by different agonists suppresses itch transmission via PLC-PKC\(d\) pathway and endogenous dynorphin is not required for itch modulation under either normal physiological or chronic itch conditions.

**DISCUSSION**

Using a multidisciplinary and spinal-cord-specific approach, we show that a Ca\(^{2+}\)-independent KOR-PLC-PKC\(d\)-GRPR pathway is activated in response to KOR agonists, resulting in an attenuation of GRPR function, which is required for development of chronic itch in mice (Sun and Chen, 2007; Zhao et al., 2013). Consistent with previous findings showing that GRPR is minimally required for histaminergic itch (Akiyama et al., 2014; Sun et al., 2009; Zhao et al., 2013), we show that spinal KOR...
KOR agonist-induced anti-itch effect is lost in Grpr spinal cord would contribute to itch inhibition. Importantly, it is unlikely that inhibition of non-GRPR KOR neurons in the itch and pain antagonism and their distinct neuronal outputs, induced by CQ in Grpr effects. The remaining KOR-agonist-resistant scratching effect suggesting that GRPR is required for mediating anti-itch effect in Grpr- KO mice, thereby dampening PLC\(\beta\) signaling. One remarkable finding is that Ca\(^{2+}\)-independent PKC\(\delta\) activation via membrane translocation provides a major mechanism for spinal KOR-induced itch suppression in Grpr spinal cord. 

**Figure 6. KOR Activation Induces Translocation of PKC\(\delta\), but Not PKC\(\alpha\), to the Plasma Membrane**

(A) HEK293 cells expressing KOR/GRPR transfected with PKC\(\alpha\)-EGFP (upper row) and PKC\(\alpha\)-EGFP (lower row) were incubated in 10 \(\mu\)M U-50,488. Confocal images taken at indicated time points showed that U-50,488 induced the translocation of PKC\(\alpha\)-EGFP, but not PKC\(\alpha\)-EGFP, to the plasma membrane (scale bar, 20 \(\mu\)m).

(B) Percentage of PKC\(\alpha\)-EGFP and PKC\(\alpha\)-EGFP translocation to the plasma membrane in response to U-50,488 (**p < 0.01; Student’s paired t test; n = 3–4 cells per experiment).

(C) 100 nM PMA incubation induced the translocation of both PKC\(\alpha\)-EGFP and PKC\(\alpha\)-EGFP to the plasma membrane.

(D) Percentage of PKC\(\alpha\)-EGFP and PKC\(\alpha\)-EGFP translocation to the plasma membrane in response to PMA (*p < 0.05; **p < 0.01; Student’s paired t test; n = 3–4 cells per experiment).

(E) IHC images show that PKC\(\alpha\)-red is mostly distributed in the cytosol of superficial dorsal horn neurons of control mouse (upper row). After U-50,488 injection, PKC\(\alpha\) translocates to the plasma membrane (lower row).

(F) High-power images of the boxed regions in (E; scale bar, 5 \(\mu\)m; blue, NeuN).

Data are presented as mean ± SEM. See also Figure S6.
by which GRPR is phosphorylated and desensitized. Importantly, behavioral studies indicate that PKC\(\delta\)-mediated desensitization of GRPR is long lasting. Previous in vitro studies suggested that the classic GRPR-PLC-IP\(3\) Ca\(^{2+}\) signaling activates kinases other than PKC (Kroog et al., 1995), and GRPR agonist-induced Ca\(^{2+}\)-dependent desensitization is transient and lasts less than 2 min (Zhao et al., 2014a). It is possible that distinct phosphorylation sites at the C-terminal domain of GRPR may contribute to the duration of desensitization (Ally et al., 2003). Whether PKC\(\delta\) desensitizes agonist-unoccupied GRPR via direct phosphorylation or indirectly via other kinases awaits further studies (Kelly et al., 2008). Direct examination of spinal GRPR phosphorylation levels after KOR activation requires an antibody that can specifically detect phosphorylated GRPR in vivo, which contains multiple potential phosphorylation sites on its C terminus (Ally et al., 2003). Several lines of evidence fail to support the view that the endogenous dynorphin is an important modulator of itch transmission (Kardon et al., 2014): first, consistent with previous studies using either Pdyn KO mice or ablation of spinal Dyn\(^+\) neurons (Duan et al., 2014; Kardon et al., 2014), Pdyn KO mice showed normal acute and chronic itch behaviors. Second, KOR agonists attenuate GRPR function via a long-lasting phosphorylation rather than acute desensitization process, the former of which rarely occurs under normal physiological condition. These observations are in support of the notion that KOR-GRPR cross-signaling induced by exogenous KOR agonists reflects an artificial process. While there is no evidence that the endogenous dynorphin modulates itch in normal physiological context, the possibility that a dramatic down-regulation of Pdyn in chronic itch condition (data not shown) may suggest an attenuation of inhibitory circuit cannot be excluded.

In summary, we demonstrate a non-canonical opioid signaling mechanism by which GRPR activity is attenuated by KOR-mediated cross-signaling in the spinal cord of mice. The finding of the inhibitory effect of KOR activation and its downstream signaling components on distinct types of chronic itch (BRAF\(^{Nav1.8}\), ACD, and AEW) suggests a possibility for a broader application of KOR-GRPR-based anti-itch strategy to the treatment of chronic itch with various etiologies.

Figure 7. PLC Mediates KOR-Activation-Induced Translocation of PKC\(\delta\) to the Plasma Membrane

(A–D) Confocal images (A and C) and quantified data (B and D) showing U-50,488 induced translocation of PKC\(\delta\)-eGFP from the cytosol to the plasma membrane was blocked by 10 \(\mu\)M U73122 (PLC inhibitor) (A and B), U73343 (inactive analog of U73122) (A and B), 100 \(\mu\)M gallein (G\(_{bg}\) inhibitor), and 200 ng/mL PTX (G\(_{i}\) inhibitor; C and D) had no effect on U-50,488-induced translocation of PKC\(\delta\)-eGFP (\(n = 3–4\) cells per experiment; *p < 0.05; Student’s paired t test in B; one-way ANOVA followed by Dunnett’s multiple comparison test).

(E and F) Western blots (E) and quantified data (F) showing that U-50,488-induced GRPR phosphorylation is blocked by U73122, but not U73343. Data are represented as mean ± SEM. See also Figure S5.

(G) Schematic showing signaling events by which KOR activation results in GRPR desensitization.

(H) Schematic illustrates concurrent activation of two distinct subpopulations of dorsal horn interneurons by U-50,488: activation of KOR neurons without GRPR mediates anti-nociceptive output, whereas activation of KOR/GRPR neurons inhibits itch.
EXPERIMENTAL PROCEDURES

**Animals**
Behavioral tests were carried out on C57BL/6J, Oprk1−/− (Hough et al., 2000), Gpr80 KO (Hampton et al., 1998), BRAFNav1.8 (Zhao et al., 2013), Gpr85ΔC, A9 (MRRMC), Arnb−/− (Bohn et al., 1999), and Prkcd−/− (Leitges et al., 2001) male mice and their WT littermates unless indicated otherwise. All experiments conform to guidelines set by the NIH and the International Association for the Study of Pain and were reviewed and approved by the Animal Studies Committee at Washington University School of Medicine.

**Itch Behavior**
Mice were individually put into observation boxes and videotaped. The videos were played back on a computer and quantified by an observer who was blinded to the treatment or mouse genotype. A scratch is defined as a bout of scratching that occurs after the mouse lifts its hind paw to the moment the hind paw is returned to the ground or mouth (Sun and Chen, 2007). For the dry-skin model, mice were painted twice daily with a mixture of acetone and diethyl ether (1:1) followed by water. Scratching behavior directed at the neck was counted in the morning before treatment (Miyamoto et al., 2002; Zhao et al., 2013). For ACD model, mice were sensitized by applying 100 μL of 0.15% DNFB on abdominal skin. One week later, mice were challenged by nape application of 50 μL of 0.15% DNFB every 2 or 3 days. Scratching responses were measured 24 hr after applying DNFB (Zhao et al., 2013).

**RNAscope In Situ Hybridization and Immunohistochemistry**
RNAscope ISH and IHC staining were performed as described (Wang et al., 2012; Zhao et al., 2014a). Spinal sections were processed according to the manufacturer’s instructions in the RNAscope Fluorescent Multiplex Assay v2 manual for fixed frozen tissue (Advanced Cell Diagnostics).

**siRNA Studies**
Prkcd siRNA (Sigma) was delivered to the lumbar region of the spinal cord via i.t. injection as described (Liu et al., 2011; Zhao et al., 2014a). Mice were injected twice daily for 3 consecutive days, and behavior was performed 24 hr after the last injection.

**Dissociation of Dorsal Horn Neurons and Calcium Imaging**
Primary culture of spinal dorsal horn neurons was prepared from 5- to 7-day-old C57BL/6J mice and seeded onto 12-mm coverslips coated with poly-D-lysine. Calcium imaging was performed 3–5 days after seeding as described previously (Zhao et al., 2014a).

**Whole-Cell Phosphorylation Assay**
HEK293 cells expressing FLAG-KOR and Myc-GRPR were incubated in 10 μM U-50,488 or 1 μM PMA at 37°C and lysed as described (Liu et al., 2011). Proteins were incubated with mouse anti-Myc antibody (Sigma) overnight. The complex was precipitated, resolved on polyacrylamide gels, and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Proteins were detected by immunoblotting with mouse anti-phosphoserine antibody (1:2,500; Sigma) overnight, and the blot was developed by enhanced chemiluminescence (Thermo Scientific).

**PKC Translocation Assay**
KOR-GRPR HEK293 cells, transiently expressing PKCζ-EGFP or PKCd-EGFP (kindly provided by Dr. Peter M. Blumberg) were seeded in 29-mm glass bottom dishes (In Vitro Scientific). After 24 hr, the subcellular distribution of EGFP-fused protein was analyzed on a Leica TCS SPE confocal microscope.

**Statistical Analysis**
Statistical comparisons were performed with Graphpad Prism 7. Groups were considered significantly different if p < 0.05. Results are presented as the mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, two tables, and four videos and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.03.087.

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AUTHOR CONTRIBUTIONS
Conceptualization, Z.-F.C.; Investigation, A.M., X.-Y.L., D.M.B., Q.Y., J.-B.Y., H.J., Q.-T.M., J.-H.P., Z.-Y.W., J.Y., X.-Y.Z., L.W., P.M., S.K., F.-Q.H., J.J., and R.B.; Resources, J.Y.; Writing – Original Draft, A.M., X.-Y.L., and Z.-F.C.; Formal Analysis, A.M. and X.-Y.L.; Writing – Review & Editing, Z.-F.C., A.M., X.-Y.L., D.M.B., and M.R.B.; Funding Acquisition, Z.-F.C., H.L., Y.-Q.L., and M.R.B.; Supervision, Z.-F.C.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

Non-canonical Opioid Signaling Inhibits Itch Transmission in the Spinal Cord of Mice

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Supplemental Information

Figure S1. Intrathecal U-50,488 Inhibition of Itch is Long-lasting. Related to Figure 1

(A) U-50,488 lost its inhibitory effect on scratching induced by GRP and CQ in Oprk1−/− mice (*p < 0.05, **p < 0.01, Student’s unpaired t test, n = 6–11).
(B) U-50,488 significantly reduced scratching induced by CQ in female mice (**p < 0.01, Student’s unpaired t test, n = 6–7).
(C) GIS is unchanged after multiple injections. U-50,488 inhibition of GRP-induced scratching lasts 48 h. (*p < 0.05, **p < 0.01, two way ANOVA followed by Bonferroni posttest, n = 8).

Data are represented as mean ± SEM.
Figure S2. Spinal KOR Activation Inhibition of Itch Is Mediated by Cell Autonomous Effects. Related to Figures 1 and 2

(A) I.t. U-50,488 lost its inhibitory effect on scratching induced by CQ in Grpr KO mice (*p < 0.05, NS, not significant, Student’s unpaired t test or paired t test, (basal relative to U-50), n = 5–6).

(B) I.t. butorphanol (2 nmol) significantly reduced scratching induced by CQ in WT but not Oprk1−/− littermates (*p < 0.05, Student’s paired t test, n = 6–7).

(C) I.t. butorphanol loses inhibitory effects on scratching induced by CQ in Grpr KO mice (*p < 0.05, NS, not significant, Student’s unpaired t test or paired t test, (basal relative to Butor), n = 5–6).

(D) Butorphanol (2 nmol) significantly reduced scratching induced by CQ in WT but not Prkcd−/− littermates (*p < 0.05, Student’s unpaired t test or paired t test, (basal relative to Butor), n = 7–8).

(E) Dynorphin significantly reduced scratching induced by CQ (*p < 0.05, Student’s unpaired t test, n = 6-7).

Data are represented as mean ± SEM.

(F) WT littermate controls and Pdyn−/− mice exhibited no difference in scratching induced by CQ, Hist, GRP and NMB (n = 6–8).

(G) Dry skin itch was comparable in Pdyn−/− mice and WT (n = 9–14).
Figure S3. KOR Activation Suppresses GRPR Activity in $G_{\alpha S}$- and $\beta$-arrestin2-independent Manner. Related to Figure 2

(A) BRET assay showing that U-50,488 (10 µM) attenuated forskolin-induced increase in cAMP levels (purple). PTX (200 ng/ml) reversed the effect of U-50,488 (pink). RLuc-EPAC-Venus (1.0 µg) was used as a BRET sensor. Blank media was used as a control (black).

(B) 1 µM GRP or 10 µM U-50,488 or a co-incubation of U-50,488 and GRP does not induce an increase in cAMP levels (brown, red and green traces respectively).

(C) Arrb2−/− and their WT littermates mice exhibited no difference in scratching induced by GRP. I.t. U-50,488 attenuated scratching induced by GRP in both Arrb2−/− and their WT littermates (*p < 0.05, **p < 0.01, Student’s unpaired t test or paired t test, basal relative to U-50), n = 6–8).

Data are represented as mean ± SEM.
Figure S4. U-50,488 Inhibits Itch After Treatment of Prkcd Mismatch Control siRNA and Prkca siRNA. Related to Figure 5

(A) U-50,488 attenuated scratching induced by GRP (0.3 nmol) in control siRNA-treated mice (*p < 0.05, unpaired t test, n = 6).
(B) U-50,488 attenuated scratching induced by CQ (200 µg) in control siRNA-treated mice (**p < 0.01, unpaired t test, n = 6).
(C) U-50,488 attenuated scratching induced by GRP (0.3 nmol) in Prkca siRNA-treated mice (*p < 0.05, unpaired t test, n = 6).
(D) U-50,488 attenuated scratching induced by CQ (200 µg) in Prkca siRNA-treated mice (**p < 0.01, unpaired t test, n = 6).
Data are represented as mean ± SEM.
Figure S5. Expression of PKCδ in GRPR Neurons. Related to Figure 5

(A) Double IHC of PKCδ (purple) and eYFP (green) shows co-expression of GRPR and PKCδ in superficial dorsal horn neurons of Grpr<sup>Cre</sup> mice injected with AAV-DIO-eYFP. Scale bar, 50 µm.

(B) Representative traces showing U-50,488 had no effect on inhibited GRP-induced Ca<sup>2+</sup> responses in dissociated dorsal horn neurons from Prkcd<sup>−/−</sup> mice.
Figure S6. Butorphanol Induces Translocation of PKCδ but not PKCα to the Plasma Membrane. Related to Figure 6

HEK293 cells expressing KOR/GRPR transfected with PKCδ-eGFP (upper row) and PKCα-eGFP (lower row) were incubated in 20 µM butorphanol. Confocal images taken at indicated time points showed that butorphanol induced the translocation of PKCδ-eGFP but not PKCα-eGFP to the plasma membrane (scale bar, 20 µm).
Figure S7. Related to Figures 4 and 7. (A-D) Full size unedited gels for phosphorylated GRPR (pGRPR) for Figures 4A, 4C, 4D and 7E respectively. The boxed regions correspond to those shown in the cropped images within the respective figures. The numbers represent molecular weight of the respective bands (in kDa).
**Supplemental Tables**

Table S1. Characterization of GRP-induced calcium responses of GRPR neurons (control). **Related to Figure 2**

| Cell type (normalized to first GRP response) | Fractional Responses to GRP |
|---------------------------------------------|-----------------------------|
| Non-responders (0-10 %)                     | 2/32 (6 %)                  |
| Reduction (10-50 %)                         | 3/32 (9 %)                  |
| Responders (> 50 %)                         | 27/32 (84 %)                |

Table S2. Characterization of GRP-induced calcium responses of GRPR neurons after U-50,488 treatment. **Related to Figure 2**

| Cell type (normalized to first GRP response) | Fractional Responses to GRP |
|---------------------------------------------|-----------------------------|
| Non-responders (0-10 %)                     | 52/124 (42 %)               |
| Reduction (10-50 %)                         | 32/124 (26 %)               |
| Responders (> 50 %)                         | 40/124 (32 %)               |

**Supplemental Experimental Procedures**

**Animals**

All behavioral experiments conform to guidelines set by the National Institute of Health and the International Association for the Study of Pain, and were reviewed and approved by the Animal Studies Committee at Washington University School of Medicine. Mice were housed in a controlled environment with free access to food and water. The animal room was on a 12/12 h light/dark cycle with lights on at 0700 h. C57BL/6J, Oprk1−/− (Hough et al., 2000), Grpr KO (Hampton et al., 1998), BRAFNav1.8 (Zhao et al., 2013), GsprKre (its generation will be described in a separate manuscript), Ai9 (MMRRC), Arrb2−/− (Bohn et al., 1999), Prkcd−/− (Leitges et al., 2001), Pdyne− (Sharifi et al., 2001), and their WT littermates were used for this study. Male mice were used for all experiments, except Figure S1B.

**Drugs and Reagents**

Drugs were from Sigma, unless otherwise indicated. U-50,488, butorphanol (Zoetis), GRP, NMB (Bachem), CQ, histamine, BIM, PMA, norBNI, PTX (Tocris) were dissolved in sterile 0.9 % saline. Forskolin, gallein, U73122 and U73343 were from Tocris and dissolved in DMSO. Other relevant information is included in results and figure legends.

**Itch Behavior**

**Acute Itch**

Behavioral experiments were performed during the day (0800 – 1500 h). For i.t. injections, mice had their backs shaved off a day before the experiments. On the test day, mice were given at least 30 min to get accustomed to recording conditions prior to injections and recordings. For i.t. injections, a 30-gauge needle was inserted into the intervertebral space between L5 and L6. Drugs were injected in a volume of 5 μl. I.t. placement was confirmed by a swift flick of the tail on needle entry. Immediately after injections, mice were put into rectangular, transparent observation boxes (10 × 11 × 15 cm) and videotaped using SONY HDR-CX190 digital video camcorders from a side angle. The videos were played back on a computer and quantified by an observer who was blinded to the treatment or mouse genotype. For time-course analysis, scratches were recorded every 5 min immediately after injection. A scratch is defined as a bout of scratching that occurs after the mouse lifts its hind-paw to the moment the hind-paw is returned to the ground, mouth or paused (a bout usually lasts about 5 seconds). Each mouse was observed for 30 minutes, and the number of scratches recorded. For intradermal injections, briefly mice had a small part of their necks shaved, and 50 μl of the test substance was injected using a syringe attached to a SS30M3009 – 3/10 cc, 30G × 3/8” needle (Terumo). Only scratches to the injection site were counted for 30 min. These behavioral studies are based on an already published protocol (Sun and Chen, 2007).

**Chronic Itch Mouse Models**

**Dry Skin (AEW) Model:** Mice were treated with an acetone/ether mixture followed by water, inducing spontaneous scratching. Briefly, cotton socked with a mixture of acetone and diethyl ether (1:1) was applied on the nape of the neck for 15 s, followed by 30 s of cotton soaked in water. This procedure was performed twice daily for
9 days, with a 6 h window in-between. Scratching behavior directed at the neck was counted for 1 h the morning before treatment by an observer who was blinded to the treatment (Miyamoto et al., 2002; Zhao et al., 2013).

**BRAF**<sup>Nav1.8</sup> **Mice:** BRAF<sup>Nav1.8</sup> mice, at least 6 weeks old, which would have developed spontaneous scratching and their WT littermates were used in this study (Zhao et al., 2013).

**ACD Model:** C57BL/6J male mice were sensitized by applying 100 µl of 0.15 % DNFB acetone solution on ~2 cm<sup>2</sup> area of fur-shaved abdominal skin (sensitization, day 1). On day 8, 50 µl of 0.15% DNFB acetone solution was topically applied twice a week (every 2-3 days) to the clipped rostral part of mouse back for over 3 weeks (challenge). Scratching responses were measured 24 h after applying DNFB by an observer who was blinded to the treatment (Zhao et al., 2013).

**Small Interfering RNA Treatment**
Negative control siRNA (SC001) and selective duplex siRNA for mouse Prkcd mRNA (SASI_Mm02_00319898), Prkca mRNA (SASI_Mm02_00162578), and mismatch control siRNA for mouse Prkcd mRNA were purchased from Sigma. RNA was dissolved in diethyl pyrocarbonate-treated PBS and prepared immediately prior to administration by mixing the RNA solution with a transfection reagent, in vivo-jet PEI<sup>®</sup> (Polyplus-transfection SA). The final concentration of RNA was 1.25 µg/10 µl. siRNA was delivered to the lumbar region of the spinal cord. Mice were injected twice daily for 3 consecutive days as described previously (Liu et al., 2011; Liu et al., 2014; Zhao et al., 2014b). Behavioral testing was carried out 24 h after the last injection. The spinal cord tissue was collected for RT-PCR after 1 more day of siRNA injections.

**RNAscope In Situ Hybridization (ISH)**
RNAscope ISH was performed as described (Wang et al., 2012). Briefly, mice were anesthetized with a ketamine/xylazine cocktail (ketamine, 100 mg/kg and xylazine, 15 mg/kg) and perfused intracardially with 0.01 M PBS, pH 7.4, and 4 % paraformaldehyde (PFA). The spinal cord was dissected, post-fixed in 4 % PFA for 16 h, and cryoprotected in 20 % sucrose overnight at 4 °C. Tissues were subsequently cut into 18 µm-thick sections, adhered to Superfrost Plus slides (Fisher Scientific), and frozen at −20°C. Samples were processed according to the manufacturer’s instructions in the RNAscope Fluorescent Multiplex Assay v2 manual for fixed frozen tissue (Advanced Cell Diagnostics), and coverslipped with Fluoromount-G antifade reagent (Southern Biotech) with DAPI (Molecular Probes). The following probes, purchased from Advanced Cell Diagnostics, and coverslipped with Fluoromount-G antifade reagent (Southern Biotech) with DAPI (Molecular Probes). The following probes, purchased from Advanced Cell Diagnostics, and coverslipped with Fluoromount-G antifade reagent (Southern Biotech) with DAPI (Molecular Probes).

**Intra-spinal Virus Injection for Labeling GRPR Neurons**
An IRES-iCre-Neo cassette was knocked-in to the 3’UTR of Grpr locus to generate Grpr<sup>iCre</sup> mice (details of knock-in strategy will be published in a subsequent study). For spinal injection, Grpr<sup>iCre</sup> mice were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) intraperitoneally and injected with buprenorphine (BupSR, 0.5 mg/kg) for analgesia. Cervical vertebrae were exposed at C3-C6 and the vertebral column was mounted onto a stereotaxic frame with spinal adaptor (Stoelting catalog number: 51690). After removal of tissue around and between the vertebrae to expose the spinal cord, the dura was incised with a sharp needle to expose the spinal cord surface. AAV5-Ef1a-DIO-eYFP (5.6 X 10<sup>12</sup> vg/mL) was injected into the left side of the spinal cord at 2 sites between successive vertebrae at C4-C5 with a Hamilton Neuros-syringe with beveled needle (catalog number: 65458-02, 34 gauge, 20 degree angle). The syringe needle was inserted into the dorsal spinal cord at an angle of ~35 degrees at a depth of ~250 µm to target the superficial dorsal horn. The AAV was injected (~500 nL AAV per injection) at a rate of 100 nL/min with a Stoelting Quintessential Injector (QSI, catalog number: 53311) and the needle was slowly removed 5 min after the injection was complete. The surgery site was closed with nylon sutures and triple antibiotic ointment and lidocaine were applied to the skin. Antibiotics (enrofloxacin, 2.5 mg/kg) with saline were injected subcutaneously to prevent infection. Mice were monitored for recovery following surgery and were perfused 2-3 weeks later for immunohistochemistry (IHC).
ISH and Immunohistochemistry (IHC)
ISH was performed using digoxigenin-labeled cRNA probes as previously described (Chen et al., 2001). IHC staining was performed as described (Zhao et al., 2014b). Briefly, mice were anesthetized with an overdose of a ketamine/xylazine cocktail and fixed by intracardial perfusion of 0.01 M PBS (pH 7.4) and 4 % PFA. Spinal cord tissues were immediately removed, post-fixed in the same fixative for 2-4 h, and cryoprotected in 20 % sucrose solution overnight at 4 °C. Spinal cord tissues were frozen in OCT and sectioned at 20 µm thickness on a cryostat. Free floating sections were incubated in blocking solution containing 2 % donkey serum and 0.1 % Triton X-100 in PBS (PBS-T) for 2 h at room temperature. The sections were incubated with primary antibodies overnight at 4 °C, washed three times in PBS, incubated with secondary antibodies for 2 h at room temperature and washed three times. Sections were mounted on slides with Fluoromount G (Southern Biotech) and coverslips. The following primary antibodies were used: rabbit polyclonal PKCδ antibody, (1:500, catalog number: sc-213, Santa Cruz Biotechnology) and mouse monoclonal NeuN antibody (1:2000, catalog number: MAB377, Millipore). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories: FITC or Cy5 conjugated donkey anti-rabbit or anti-mouse IgG (FITC - 1.25 µg/ml; Cy5 - 0.5 µg/ml). Confocal images were taken using a Leica TCS SPE confocal microscope. Three mice per group and 18 lumbar sections across each group were used for statistical comparisons.

Dissociation of Dorsal Horn Neurons
Primary culture of spinal dorsal horn neurons was dissected and dissociated from 5-7 day-old C57BL/6J mice. Three mice were used for each experimental condition (Zhao et al., 2014a). After decapitation, laminectomy was performed and the dorsal horn of the spinal cord was dissected out with a razor blade. The dorsal horn, kept on ice during dissection was incubated in Neurobasal-A medium (Gibco) containing 30 µl papain (Worthington) at 37 °C for 20 min. The tissue was washed three times in Neurobasal-A medium, after which gentle trituration was performed using a flame polished glass pipette, and cells were filtered through a 40 µm nylon cell strainer (BD Falcon). The homogenate was centrifuged at 1,500 rpm for 5 min and the supernatant was discarded. The cell pellet was resuspended in 180 µl culture medium consisting of Neurobasal medium (Gibco, 92 % vol/vol), fetal bovine serum (Sigma, 2 % vol/vol), horse serum (Invitrogen, 2 % vol/vol), glutamax (Invitrogen, 2 mM, 1 % vol/vol), B27 (Invitrogen, 2 % vol/vol), penicillin/streptomycin (Gibco, 100 µg/ml) and plated onto 12-mm coverslips coated with poly-D-lysine. The medium was changed daily, and calcium imaging was performed 3-5 days after seeding.

Calcium Imaging
Calcium imaging was performed on a Nikon Eclipse Ti microscope using fura-2 AM (Invitrogen). Drugs were dissolved or diluted to the required concentrations (shown in Figure legends) in artificial cerebrospinal fluid (ACSF) buffer (in mM): NaCl 140, CaCl₂ 2.4, MgCl₂ 1.3, KCl 4, HEPES 10, glucose 5. Results are presented as a ratio (F340/F380) and a calcium calibration buffer kit (Invitrogen) was used to quantify intracellular Ca²⁺ concentrations.

Whole-cell Phosphorylation Assays in HEK293 Cells
HEK293 cells expressing FLAG-KOR and Myc-GRPR were incubated in 10 µM U-50,488 or 1 µM PMA at 37°C, and lysed as described (Liu et al., 2011). Cells were lysed for 30 min at 4 °C in radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl, 7.4; 1 mM EDTA; 1 % NP-40; 150 mM NaCl; 0.25 % sodium-deoxycholate; 0.1 % SDS) supplemented with protease inhibitors (0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin) and a phosphatase inhibitor cocktail (Thermo Scientific). Solubilized lysates were cleared by centrifugation at 1,000 g for 5 min to remove nuclei and debris. A portion of the supernatants was removed and used to determine protein concentrations using Pierce® BCA Protein Assay Kit (Thermo Scientific). Cell lysates (supernatant fraction, S1) were incubated with Protein A/G PLUS agarose (Santa Cruz Biotechnology) and 1 µg immunoprecipitation antibody (mouse anti-Myc, catalog number: M4439) overnight at 4 °C. The resin was then collected by centrifugation, washed 4 times with 1 % NP-40 lysis buffer (50 mM Tris-HCl, 7.4, 1 % NP-40, 150 mM NaCl) and 3 times with 0.3 % PBST-2 (PBS + 0.3 % Triton X100). Bound proteins were eluted with Laemmli sample buffer (Bio-rad) supplemented with 50 mM dithiothreitol (DTT), heated for 10 min at 95 °C, and centrifuged at 10,000 xg for 5 min. The supernatant was loaded and resolved on a 7.5 % polyacrylamide gel (Bio-rad), and proteins were transferred to PVDF membranes (Millipore). Blots were blocked with 5 % w/v nonfat dry milk in PBS with 0.1 % v/v Tween 20 (PBST-3) and incubated overnight at 4 °C with mouse anti-phosphoserine antibody, Sigma (1:2500, catalog number P5747). The blots were then washed, incubated with donkey anti-mouse IgG-HRP antibody (Santa Cruz Biotechnology) at room temperature for 1 h, and detected by enhanced chemiluminescence (Thermo Scientific). Protein bands were analyzed by densitometry using Kodak 1D 3.6.
**PKC Translocation Assay**

HEK293 cells expressing KOR and GRPR grown in 29 mm glass bottom dishes (In Vitro Scientific) were transfected with pEGFP/PKCδ-eGFP or pEGFP/PKCα-eGFP (kindly provided by Dr. Peter M. Blumberg taken) using lipofectamine 3000 (Invitrogen). After 24 h, cells were analyzed and the subcellular distribution of eGFP-fused protein was recorded under a Leica TCS SPE confocal microscope. Sequential images of the same cell were collected at 1 min intervals. Percent membrane translocation was calculated as $(I_{\text{total}} - I_{\text{cyto}})/I_{\text{total}} \times 100$, where $I_{\text{total}}$ represents the total cell fluorescence intensity and $I_{\text{cyto}}$ is the fluorescence intensity in the cytoplasm and the nucleus.

**RT-PCR**

RT-PCR was performed as previously described with Fast-Start Universal SYBR Green Master (Roche Applied Science) (Liu et al., 2011; Liu et al., 2014). All samples were assayed in duplicates (heating at 95 °C for 10 s and at 60 °C for 30 s). Data was analyzed using the Comparative CT Method (StepOne Software version 2.2.2.), and the expression of target mRNA was normalized to the expression of Actb and Gapdh. The primers used are: Actinb: forward 5’-TGTTACCACTGGGACGACA-3’; reverse 5’-GGGTTGTTGAAGGTCTCAA-3’

Gapdh: forward 5’-CCCAGCAAGGACACTGAGCAA-3’; reverse 5’-TTATGGGGGTCTGGGATGAAA-3’

Prkcd: forward 5’-AGAGGGACCTGACAAGGAG-3’; reverse 5’-GGTCTGTAGTCTGAAGGGGA-3’

Prkca: forward 5’-CTGTTGCTTGGGGTTGAATG-3’; reverse 5’-TAACTCCTGGGCTGAC-3’

**Bioluminescence Resonance Energy Transfer (BRET)**

HEK293 cells expressing KOR and GRPR were transiently transfected with RLuc-EPAC-Venus (1 µg), a cAMP BRET sensor. Emission signals from Renilla luciferase (RLuc) and Venus were measured simultaneously using a BRET1 filter set (475–30/535–30) on a Synergy H1 Hybrid Reader (BioTek) as described (Jiang et al., 2007).

**Statistical Analysis**

Behavioral tests, cell counting and molecular analysis counting were performed by observers blinded to treatments and genotypes of the mice used. Statistical comparisons were performed using one or two way ANOVA followed by post-hoc analysis when comparing three or more groups, paired or unpaired, two-tailed student’s t-test when comparing two groups with a 95% confidence interval with Graphpad Prism 7 (version 7.03, GraphPad). Groups were considered significantly different if p < 0.05. A normality test was performed to confirm the data were normally distributed. Values are presented as the mean ± standard error of the mean (SEM).

**Supplemental references**

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