Cell

Unidirectional Cross-Activation of GRPR by MOR1D Uncouples Itch and Analgesia Induced by Opioids

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DOI 10.1016/j.cell.2011.08.043

SUMMARY

Spinal opioid-induced itch, a prevalent side effect of pain management, has been proposed to result from pain inhibition. We now report that the μ-opioid receptor (MOR) isoform MOR1D is essential for morphine-induced scratching (MIS), whereas the isoform MOR1 is required only for morphine-induced analgesia (MIA). MOR1D heterodimerizes with gastrin-releasing peptide receptor (GRPR) in the spinal cord, relaying itch information. We show that morphine triggers internalization of both GRPR and MOR1D, whereas GRP specifically triggers GRPR internalization and morphine-independent scratching. Providing potential insight into opioid-induced itch prevention, we demonstrate that molecular and pharmacologic inhibition of PLCβ3 and IP3R3, downstream effectors of GRPR, specifically block MIS but not MIA. In addition, blocking MOR1D-GRPR association attenuates MIS but not MIA. Together, these data suggest that opioid-induced itch is an active process concomitant with but independent of opioid analgesia, occurring via the unidirectional cross-activation of GRPR signaling by MOR1D heterodimerization.

INTRODUCTION

Itch and pain are two fundamental sensory perceptions evoked by distinct external inputs. They are encoded and transmitted by primary nociceptive fibers and varying subpopulations of dorsal horn neurons (Davidson and Giesler, 2010; Patel and Dong, 2010). The ability to discriminate between itch and pain allows animals to employ the proper motor response (scratching versus withdrawal) so that potentially damaging stimuli from the environment can be avoided. Intriguingly, it has been well documented that itch and pain may counteract each other under some conditions. Indeed a wide range of noxious stimuli including thermal, mechanical, chemical, and electrical stimuli are able to inhibit itch (Ikoma et al., 2006). Conversely, it is widely assumed that itch may be unmasked by pain reduction, and one of the most cited examples of this antagonistic relationship is opioid-induced itch, or pruritus (Davidson and Giesler, 2010; Ikoma et al., 2006; Paus et al., 2006). In fact, pruritus is one of the most prevalent acute side effects of the spinal or epidural use of opioids in patients who undergo pain treatment or in those who receive cesarean section (Ballantyne et al., 1988; Chaney, 1995; Hales, 1980), which has hampered the use of opioids as an analgesic to their full extent. The most influential theory offered to explain the antagonism of itch and pain is perhaps the "occlusion" or selectivity hypothesis, which stipulates that pruriceptors are part of nociceptors and that inactivation of the pain signaling centrally is a prerequisite for activation of the itch signaling (Carstens, 1997; McMahon and Koltzenburg, 1992). The occlusion hypothesis has gained more support from an analysis of mutant mice lacking vesicular glutamate transporter 2 in subsets of dorsal root ganglia (DRG) neurons that displayed attenuated pain but enhanced itch (Lagerström et al., 2010; Liu et al., 2010). In the spinal cord, all spinothalamic track neurons in primates recorded to be responsive to capsaicin also responded to pruritic stimuli (Davidson et al., 2007). In addition, ablation of dorsal horn neurons expressing neurokinin 1 receptor attenuated both pain and itch in rats (Carstens et al., 2010; Nichols et al., 1999). Mice lacking neurons expressing gastrin-releasing peptide receptor (GRPR), a molecular signature for the putative itch-specific labeled line in the spinal cord, nearly eliminate their scratching response to a range of pruritic stimuli without altering normal nociceptive transmission (Sun and Chen, 2007; Sun et al., 2009). Conversely, mice lacking a subset

of neurons expressing transcription factor *Bhlhb5* during development display enhanced spontaneous scratching behavior, but their pain behavior is not reduced (Ross et al., 2010), suggesting that removal of pain signaling is not a prerequisite for induction of itch and that the central itch signaling can be induced independently of nociceptive transmission. Collectively, convincing evidence in support of the "occlusion" theory in the spinal cord is lacking.

Opioid-induced itch has been suggested to be mediated primarily through the μ-opioid receptor (MOR), a key receptor for opiates (Kieffer, 1999). Intrathecal (i.t.) injection of morphine, a prototypical opiate agonist, produces dose-dependent scratching behavior (Ko and Naughton, 2000; Kuraishi et al., 2000). Consistently opioid antagonists have been found to reduce itch and concomitantly attenuate the analgesic effects of opiates (Ballantyne et al., 1988; Ko et al., 2004). MOR1 is activated by exogenous morphine without rapid internalization in several cell types including dorsal horn neurons (Alvarez et al., 2002; Keith et al., 1996; Trafton et al., 2000). Activation of MOR1 primarily inhibits adenylyl cyclase and the cAMP/PKA signaling pathway (Law et al., 2000). As opioid-induced itch is most notable and severe when opioids are intrathecally applied, one tantalizing hypothesis is that opioids evoke itch sensation by activating GRPR signaling. The present study was designed to test this hypothesis and to determine whether activation of the itch signaling is due to a removal of pain inhibition.

RESULTS

Morphine-Induced Scratching Occurs Independent of Morphine-Induced Analgesia

To examine whether morphine-induced scratching (MIS) and morphine-induced analgesia (MIA) are correlated to each other, we studied the dose-response curve and time course of MIS and MIA after i.t. injection of morphine and found that both MIA and MIS increased in a dose-dependent manner (Figure 1A). However, when the morphine dose increased from 0.3 to 1.0 nmol, the MIA effect was enhanced by 81%, whereas MIS only had a slight increase. In addition, time course analysis at 0.3 nmol of morphine revealed obvious segregation of MIA and MIS (Figure 1B). After i.t. morphine, MIS increased dramatically within 10 min and quickly decreased. In contrast, MIA maintained at a maximal level for at least 1 hr. To further examine whether opioid-induced itch is due to pain inhibition, we employed a morphine tolerance paradigm in which the degree of tolerance to morphine is measured by the latency of tail-flick (analgesic effect) (Fairbanks and Wilcox, 1999). If pain inhibition unmasks itch, MIS would be attenuated in mice with morphine tolerance. Twenty-four hours after morphine pretreatment, tail-flick latencies of mice returned to their baseline (Figure 1C). As expected, mice pretreated with morphine developed morphine tolerance as measured by a significant reduction of MIA relative to the saline control (Figure 1D). To our surprise, despite reduced analgesic effect, MIS did not differ between the two groups (Figure 1E). Separation of MIS from MIA was also examined by a chronic morphine tolerance model. Tail immersion assay showed gradually reduced amplitude of MIA during the 5 days of induction (Figure 1F), and morphine tolerance was evident on the 6th day (Figure 1G). Again, there was not a significant difference of MIS between the control and tolerant mice (Figure 1H). Therefore, MIS occurs irrespective of the degree of MIA, indicating that MIS and MIA are mediated by distinct mechanisms.

MOR1D Is an Itch-Specific Receptor

The finding that MIS is separable from MIA prompted us to study the molecular basis of disassociation of MIS and MIA. Mice lacking the *Oprm* gene displayed loss of MIA (Loh et al., 1998; Matthes et al., 1996; Sora et al., 1997). MIS was nearly abolished in mice lacking the coding exons 2 and 3 of the *Oprm* gene (Loh et al., 1998), whereas GRP-induced scratching (GIS) was not affected (Figure 2A). Consistent with previous studies (Ballantyne et al., 1988; Ko et al., 2004), MIS was also abolished by naloxone, a nonspecific MOR antagonist (Figure 2B). In contrast, neither naloxone (see Figure S1A available online) nor beta-FNA (Figure S1B) impacted GIS. The mouse *Oprm* gene encodes 16 coding exons, comprising dozens of spliced isoforms that primarily differ at C terminus (Pan, 2005; Pasternak, 2010). For example, MOR1 consists of exons 1∼4, whereas MOR1D of exons 1∼3 and 8∼9 (Figure 2C). The multiplicity of the *Oprm* isoform system has been suggested to underlie the heterogeneity and variability of analgesic and scratching effects exerted by different agonists (Andoh et al., 2008; Pasternak, 2004; Ravindranathan et al., 2009). We postulated that different isoforms of MOR are responsible for MIS and MIA, respectively. To test this, we performed an exon-specific siRNA knockdown experiment in the spinal cord of mice followed by examining the effect of knockdown on MIS. Knockdown of either exon 1 contained by the majority of MOR isoforms including MOR1, or exon 9 contained by isoforms 1C, 1D, and 1E significantly attenuated MIS (Figure 2D). However, siRNA knockdown of exon 4 contained by MOR1 or exon 7 contained by 1C and 1E failed to reduce MIS significantly (Figure 2D). Interestingly, knockdown of exon 1 or 4 markedly attenuated MIA, whereas knockdown of exon 7 or 9 had no effect on MIA (Figure 2E). Quantitative RT-PCR tests confirmed that spinal MOR1 mRNA was selectively decreased by exon 1 or exon 4 siRNA (Figures 2F and 2G), and spinal MOR1D mRNA was significantly reduced after exon 1 or exon 9 siRNA treatment (Figures 2F and 2H). In contrast, neither MOR1 nor MOR1D expression in DRG neurons was compromised by siRNA treatments (Figures S1C and S1D). The knockdown of MOR1D protein in spinal cord by exon 9 siRNA was verified by western blot (Figures 2I and 2J), whereas MOR1 and GRPR protein level was not affected. To further exclude the possibility that exon 9 siRNA treatment might affect GRPR function, we examined i.t. GIS, and found no significant reduction of GIS after MOR isoform knockdown (Figure S1E). These results indicate that exon 9 is critical for MIS but not for MIA, whereas exon 4 is critical for MIA but not for MIS. Thus, spinal MOR1D has emerged as a MIS-specific isoform, whereas MOR1 possesses MIA-specific function.

Colocalization of GRPR and MOR1D in the Dorsal Horn of the Spinal Cord

To determine the expression pattern of MOR1D, we used the strategy previously described (Abbadie et al., 2000) to generate an antibody specifically against a unique MOR1D C terminus.

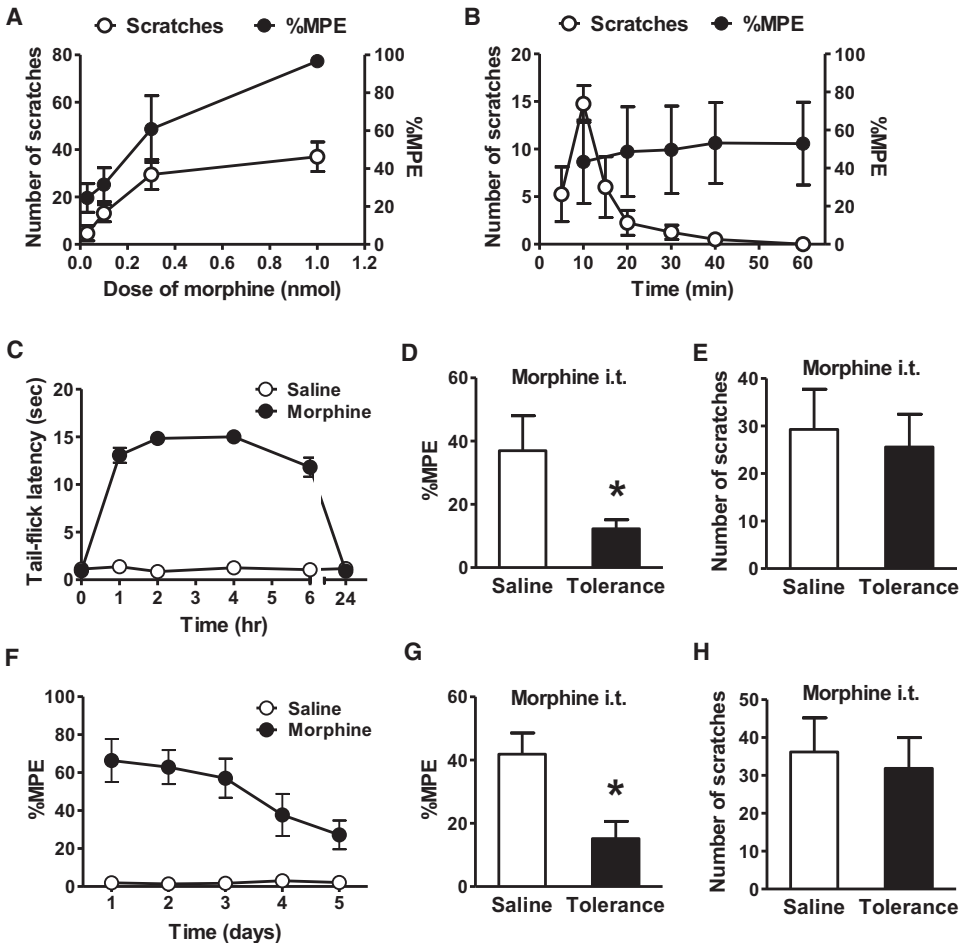

Figure 1. MIS Is Not Correlated with MIA

(A) Dose effect of i.t. morphine on MIS and MIA in 30 min.

(B) Time course of morphine on MIS and MIA.

(C) Induction of acute MIA tolerance with morphine (100 mg/kg, s.c.) or saline. Mice returned to the basal nociceptive latencies 24 hr after the morphine treatment.

(D) Acute MIA tolerance was tested with i.t. morphine 24 hr after morphine pretreatment. *$p < 0.05$.

(E) i.t. morphine induced comparable scratches in acute tolerant mice and control mice.

(F) Induction of chronic MIA tolerance by daily injection of morphine (10 mg/kg, s.c.) or saline for 5 days and MIA tolerance was examined daily.

(G) After 5 days of systemic morphine injection, i.t. morphine also showed antinociceptive tolerance. *$p < 0.05$.

(H) i.t. morphine induced comparable scratches in chronic MIA tolerant mice and control mice.

In all experiments, the dose of i.t. morphine is 0.3 nmol. n = 6–8 per group. Error bars represent SEM. s.c., subcutaneous injection.

MOR1D and MOR1 antibodies specifically recognized human embryonic kidney 293 (HEK293) cells transfected with either MOR1D or MOR1, respectively (Figure S2B), and no cross-activity was observed between the two antibodies. These data validate the specificity of MOR1D antibody. Immunostaining using MOR1D antibody indicates that MOR1D is expressed mainly in lamina I of the spinal cord (Figures 3A, 3E, and S2A), and no staining was observed in the spinal cord of MOR knockout (KO) mice (Figure S2A). In contrast, MOR1 staining is largely restricted to lamina II with a few in lamina I (Figures 3B and 3H). Importantly, no colocalization of MOR1 and MOR1D was detected (Figure 3C).

We next examined whether the expression of MOR1D and GRPR overlaps. Double-staining of MOR1D and GRPR revealed that the expression of the two receptors overlaps in lamina I cells (Figures 3D–3F). In 25 sections across the lumbar spinal cord, approximately 31% of $GRPR^+$ cells were costained with MOR1D, and approximately 65% of $MOR1D^+$ cells with GRPR. No overlapping expression between GRPR and MOR1 was observed (Figures 3G–3I). Together these data suggest that MOR1D and GRPR may function together in MIS.

Opioid-Induced Scratching Was Abolished by the Blockade of the GRPR Function in the Spinal Cord

To examine whether GRPR is important for mediating opioid-induced itch, we compared MIS between GRPR KO and wild-type mice. Strikingly, MIS was nearly abolished in GRPR KO mice (Figure 4A). In contrast, no significant difference in MIA was observed between the groups (Figure 4B). The abolition of MIS in GRPR KO mice was recapitulated when an MOR agonist

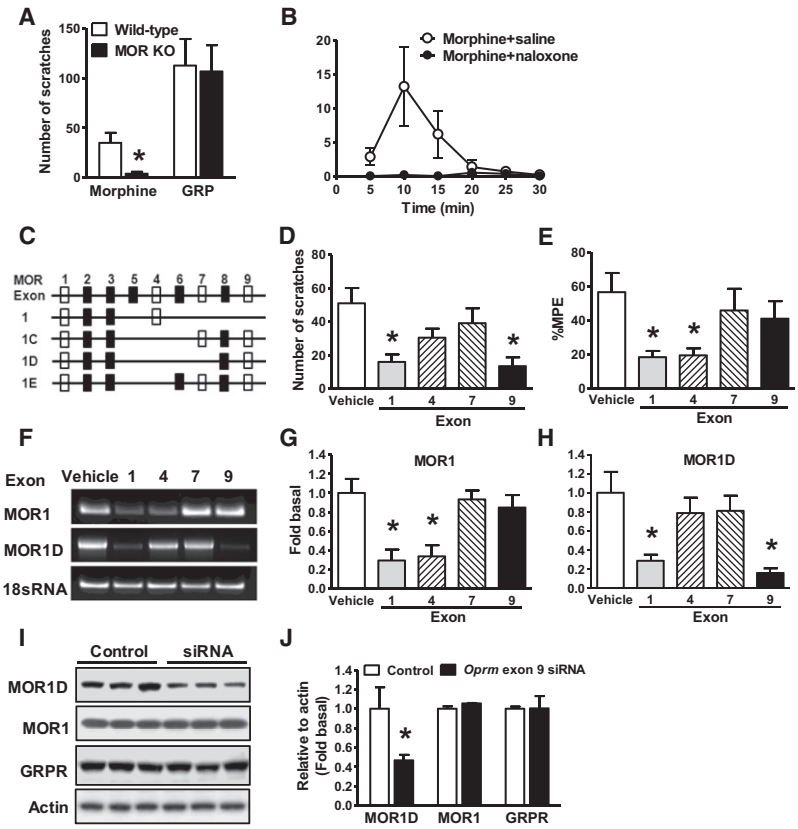

Figure 2. Identification of MIA- and MIS-Specific MOR Isoforms

(A) MIS was severely impaired in MOR KO mice, whereas GIS in MOR KO mice was comparable to that in wild-type littermate control mice. *p < 0.05.

(B) MIS was significantly reduced by naloxone (3 mg/kg, s.c.). *p < 0.05.

(C) Schematic representation of partial alternative MOR splicing in the mouse. Clear rectangles represent the targeting exons by siRNA.

(D) MIS was significantly reduced by MOR siRNA targeting at exon 1 (MOR1, 1C, 1D, and 1E) and exon 9 (MOR1C, 1D, and 1E), but not by siRNA targeting at exon 4 (MOR1) or exon 7 (MOR1C and 1E). *p < 0.05. Sequences of siRNAs are included in Extended Experimental Procedures.

(E) MOR siRNA targeting at exon 1 and exon 4, but not exon 7 or exon 9 significantly reduced MIA. *p < 0.05.

(F) Representative gel images showing decreased spinal MOR1 mRNA level after exon 1- and exon 4-specific siRNA treatments and decreased spinal MOR1D mRNA level after exon 1- and exon 9-specific siRNA treatments. 18S RNA, an internal control, was comparable among all groups.

(G) Exon 1- and exon 4-specific siRNA significantly knocked down MOR1 mRNA in spinal cord as detected by q-RT-PCR. *p < 0.05.

(H) Spinal MOR1D mRNA level was significantly reduced by siRNA specific to MOR exon 1 and exon 9 as detected by qRT-PCR. *p < 0.05.

(I and J) Western blot (I) and quantified data (J) showed that MOR exon 9 siRNA specifically reduced protein level of MOR1D but not that of MOR1 or GRPR in the spinal cord. *p < 0.05.

In all experiments, n = 5–8 per group. Error bars represent SEM. See also Figure S1.

(DAMGO or fentanyl) was intrathecally injected (Figures 4C and 4E). Analgesic effects did not differ between GRPR KO mice and their littermate controls after DAMGO or fentanyl treatment (Figures 4D and 4F). Consistently, i.t. injection of a GRPR antagonist dramatically inhibited MIS (Figure 4G), whereas MIA remained unchanged (Figures 4H and S3A). These findings demonstrate that GRPR is required for MIS but not for antinociceptive transmission. Importantly, the GRPR antagonist itself has no significant effect on acute pain as tested by tail immersion assay (Figure S3B) and von Frey (Figure S3C). Therefore, GRPR is essential for mediating opioid-induced itch, but not for opioid-mediated antinociception.

Heterodimerization and Cointernalization of MOR1D and GRPR

The coexpression of GRPR and MOR1D, along with their requirement for MIS, prompted us to ask whether GRPR and MOR1D may physically interact through receptor heterodimerization, a mechanism commonly employed by G protein-coupled receptors (GPCRs) to increase their diverse pharmacological and physiological properties (Bouvier, 2001; Milligan, 2009). Coimmunoprecipitation (co-IP) was performed using extracts of HEK293 cells stably expressing Myc-tagged GRPR together with HA-tagged MOR1D or HA-tagged MOR1. Myc-GRPR, when coexpressed with HA-MOR1D, was precipitated by anti-HA antibody (Figure 5A, L4). Conversely, precipitation with anti-Myc antibody identified a band corresponding to HA-MOR1D in cells coexpressing GRPR and MOR1D (Figure 5B, L4). This physical interaction is specific to MOR1D because HA-MOR1 and Myc-GRPR were not able to coprecipitate (Figures 5A and 5B, L3). To examine the physical interaction of MOR1D and GRPR in vivo, we performed co-IP experiments using the spinal cord membrane preparation. GRPR coprecipitated with MOR1D by anti-MOR1D antibody (Figure 5C, L3), but not by anti-MOR1 antibody (Figure 5C, L4) or an irrelevant rabbit IgG (Figure 5C, L2). Together these results indicate that physical interactions between GRPR and MOR1D exist both in vitro and in vivo.

To test whether MOR1D may cross-activate GRPR and internalize with GRPR in response to morphine, we first examined internalization of Myc-tagged GRPR in HEK293 cells stably expressing either MOR1D and GRPR or MOR1 and GRPR after morphine stimulation. Morphine failed to induce GRPR internalization in cells expressing GRPR alone (Figures 5D and 5E) or in cells coexpressing MOR1 and GRPR (Figures 5F and 5G). In contrast, GRPR internalization was significantly enhanced in HEK293 cells coexpressing MOR1D and GRPR (Figures 5F and 5G). Consistent with a previous study (Whistler et al., 1999), no internalization of HA-MOR1 by morphine was found, regardless of whether cells express MOR1 only (Figures 5D and 5E) or coexpress GRPR (Figures 5F and 5G). However, cells

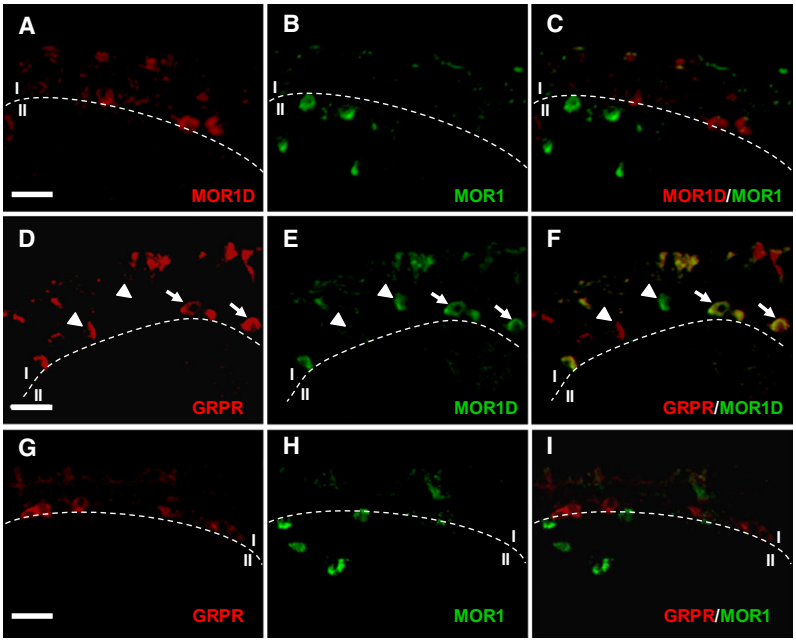

Figure 3. Coexpression of GRPR and MOR1D in Lamina I of the Spinal Cord

(A–C) Double immunostaining revealed no colocalization of MOR1D (red, lamina I) and MOR1 (green, lamina II) in the spinal cord.

(D–F) Double immunostaining of GRPR (red) and MOR1D (green) in lamina I of the spinal cord. Arrows indicate co-expression (yellow) and arrowheads indicate singular expression. Cells coexpressing GRPR (11/33) and MOR1D (11/17), which represent approximately 31% of GRPR-positive cells and approximately 65% of MOR1D-positive cells respectively, were found in 25 lumbar spinal cord sections.

(G–I) Double immunostaining revealed no colocalization of GRPR (red, lamina I) and MOR1 (green, lamina II) in the dorsal spinal cord.

Scale bar, 50 μm. See also Figure S2.

expressing MOR1D (Figures 5D and 5E) or MOR1D and GRPR (Figures 5F and 5G) showed significant MOR1D internalization in response to morphine. Both MOR1 and MOR1D were internalized in the presence of DAMGO, regardless of whether GRPR was present (Figure S4). These results suggest that the coexistence of GRPR and MOR1D is a prerequisite for morphine-mediated GRPR internalization.

Next, we assessed whether naloxone would affect morphine-induced MOR1D-GRPR internalization. Naloxone inhibited morphine-induced GRPR or MOR1D internalization in a dose-dependent manner and at a dose of 10 μM could nearly abolish MOR1D-GRPR internalization (Figure 5H). Interestingly, the GRPR antagonist inhibited morphine-induced internalization of GRPR but not MOR1D (Figure 5I). Consistently, GRP was able to internalize GRPR, regardless of whether GRPR was coexpressed with MOR1D or MOR1 (Figures 5D–5G). However, neither MOR1D nor MOR1 internalized upon GRP stimulation, regardless of whether they were coexpressed with GRPR (Figures 5D–5G). Taken together, these results indicate that despite coexpression of MOR1D and GRPR, they cannot be reciprocally activated; only MOR1D is able to cross-activate GRPR in response to morphine, not vice versa.

Cross-Activation of the GRPR Signaling Transduction Pathway by MOR1D upon Morphine Stimulation

GRPR can activate multiple signaling pathways, including the phospholipase C (PLC)/inositol 1,4, 5-trisphosphate (IP3)/Ca^{2+} signaling pathway, in response to GRPR agonists in a number of heterologous cell lines (Jensen et al., 2008; Kroog et al., 1995). To ascertain whether GRPR-dependent calcium response might be cross-activated by morphine, we examined Ca^{2+} signals in HEK293 cells expressing various combinations of MOR1, MOR1D, and GRPR. Both morphine and GRP induced calcium spikes in cells coexpressing MOR1D and GRPR (Figure 6A), suggesting an activation of GRPR by morphine or GRP. Morphine- or GRP-induced calcium signals were not affected in calcium-free extracellular buffer, indicating the endoplasmic reticulum origin of the calcium (Figure S5A). However, morphine failed to evoke Ca^{2+} spikes in cells coexpressing MOR1 and GRPR or in cells containing only GRPR; neither morphine nor GRP generated a calcium response in cells expressing MOR1D alone (Figure 6A).

To ascertain whether morphine-induced calcium spike is a consequence of a cross-activation of GRPR, we pretreated cells coexpressing MOR1D and GRPR with a GRPR antagonist or naloxone. Morphine-induced calcium spike was blocked by the GRPR antagonist and naloxone (Figure 6B). GRP-induced calcium spikes were completely blocked by the GRPR antagonist and significantly reduced by naloxone (Figures 6B and 6D). Both morphine- and GRP-evoked Ca^{2+} increase were blocked by U73122 (a selective PLC inhibitor that prevents IP3 liberation) or 2-APB (an IP3 receptor [IP3R] antagonist), whereas U73343 (an inactive structural analog control for U73122) had no effect on calcium response to morphine or GRP (Figure 6C). These data suggest that morphine cross-activates GRPR through MOR1D as well as the PLC/IP3/Ca^{2+} signaling pathway.

Coexpression of PLCβ Isoforms, IP3R3, and GRPR in the Spinal Cord

A prerequisite for PLC and IP3R signaling molecules to act downstream of GRPR is that they are coexpressed in $GRPR^{+}$ cells. To circumvent the difficulties of double-staining each individual PLC and IP3R isoform with GRPR, we took advantage of mice whose $GRPR\ neurons^{+}$ can be ablated specifically in the spinal cord by bombesin-saporin treatment (Sun et al., 2009) and used qRT-PCR to compare the mRNA change of individual isoforms in the superficial dorsal horn between mice treated with bombesin-saporin and with blank-saporin. As confirmed by the significant decrease of GRPR mRNA (Figure S5B), there was a complete loss of PLCβ3 expression and a significant decrease of PLCβ1, IP3R type 3 (IP3R3), and MOR1D mRNA in bombesin-saporin-treated tissues as compared to the control

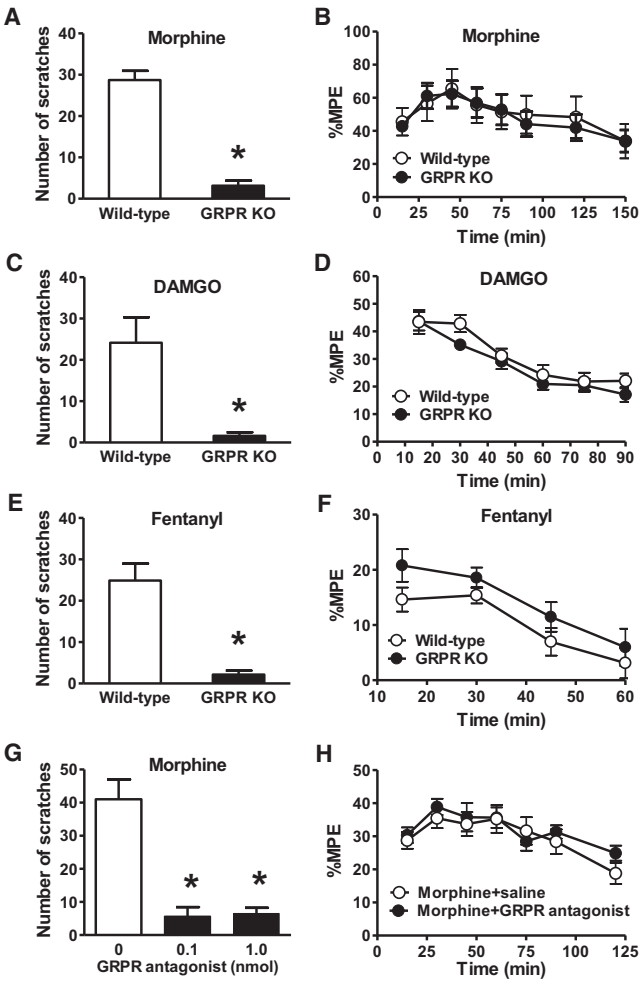

Figure 4. GRPR Is Important for Opioid-Induced Scratching Behavior

(A) MIS was nearly abolished in GRPR KO mice compared with wild-type littermate mice. *$p < 0.05$.

(B) MIA was comparable between GRPR KO and wild-type littermates.

(C) Scratching behavior induced by i.t. DAMGO (0.02 nmol) was significantly reduced in GRPR KO mice. *$p < 0.05$.

(D) Analgesic effect of i.t. DAMGO was comparable between GRPR KO and wild-type littermates.

(E and F) Scratching behavior induced by i.t. fentanyl was significantly reduced in GRPR KO mice (E), whereas the analgesic effect of fentanyl was not affected (F). *$p < 0.05$.

(G) MIS was significantly inhibited by coinjection with the GRPR antagonist (0.1, 1 nmol). *$p < 0.05$.

(H) MIA was not significantly affected by coinjection of the GRPR antagonist (1.0 nmol).

In all experiments, the dose of i.t. morphine is 0.3 nmol. n = 6~9 per group. Error bars represent SEM of the mean. See also Figure S3.

(Figures 6E, 6F, and S5B). These results reveal coexpression of PLCβ1/3, IP3R3, MOR1D, and GRPR.

Inhibition of PLC/IP3 Signaling Significantly Attenuates MIS but Not MIA

To determine the physiological relevance of morphine-induced signaling transduction in vivo, a spinal siRNA knockdown approach was employed to investigate whether PLC/IP3 signaling is important for MIS. Consistently, siRNA knockdown of PLCβ1/3 and IP3R3 in mice compromised MIS (Figure 6G). In contrast, the same treatments did not alter MIA (Figure 6H). The efficiency and selectivity of siRNA were determined by qRT-PCR. Spinal PLCβ and IP3R3 mRNA level was significantly knocked down by approximately 62% and 33%, respectively (Figure 6I). No significant knockdown of the PLCβ and IP3R3 mRNA in DRG neurons was observed (Figures S5C and S5D). The reduction of PLCβ3 and IP3R3 protein levels in spinal cord was further confirmed by western blot (Figures S5E and S5F). Interestingly, i.t. injection of both U73122 and 2-APB significantly attenuated MIS but had no impact on MIA (Figures 6J and 6K), suggesting an existence of MIS-specific PLC/IP3 signaling in vivo.

MOR1D C Terminus Is Critical for MIS and MOR1D and GRPR Heterodimeric Interaction

The difference between MOR1 and MOR1D isoforms lies in a motif consisting of seven amino acids (RNEEPSS) in MOR1D C terminus (Figure 7A). This motif is likely to be essential for MOR1D and GRPR physical interaction. To test this, a Tat-fusion peptide (Tat-MOR1D$_{CT}$) containing a Tat (YGRKKRRQRRR), a *trans*-activating domain of HIV protein that can permeate the cell membrane (Schwarze et al., 1999), and the RNEEPSS motif was synthesized (Figure 7A) and injected into the spinal cord. Introduction of Tat-MOR1D$_{CT}$ permits its competition with MOR1D for physical contacts with GRPR in vivo. Remarkably, i.t. injection of Tat-MOR1D$_{CT}$ specifically blocked MIS (Figure 7B), while leaving GIS (Figure 7B) and MIA (Figure 7C) unperturbed. Subsequent co-IP analysis using the membrane extracts of the spinal cord injected with Tat-MOR1D$_{CT}$ and the control peptide revealed that Tat-MOR1D$_{CT}$ significantly reduced the amount of GRPR precipitated by MOR1D antibody relative to the control (Figures 7D and 7E). These results demonstrate that MOR1D C terminus is critical for MOR1D-GRPR dimerization and MIS.

DISCUSSION

In this study, we present molecular, cellular, biochemical, and behavioral data that demonstrate uncoupling of opioid-induced itch and opioid-induced antinociception in the spinal cord. MOR1D is an identified MOR isoform that does not possess the cardinal function of an opioid receptor. These data argue against the prevailing view that opioid induces itch as result of pain inhibition and uncover that opioid-induced itch is an active process, independently initiated by MOR1D-mediated activation of GRPR. Coupled with the finding that MIA remains unaffected in GRPR KO mice, the present studies further support the notion that GRPR is an itch-specific receptor (Sun and Chen, 2007) and GRPR-expressing neurons represent a labeled line for itch in the spinal cord (Sun et al., 2009).

Unidirectional Cross-Activation of GRPR by MOR1D through Heterodimeric Interactions

GRP is an itch-specific peptide that is presumably released from primary afferents to activate spinal GRPR in response to pruritic

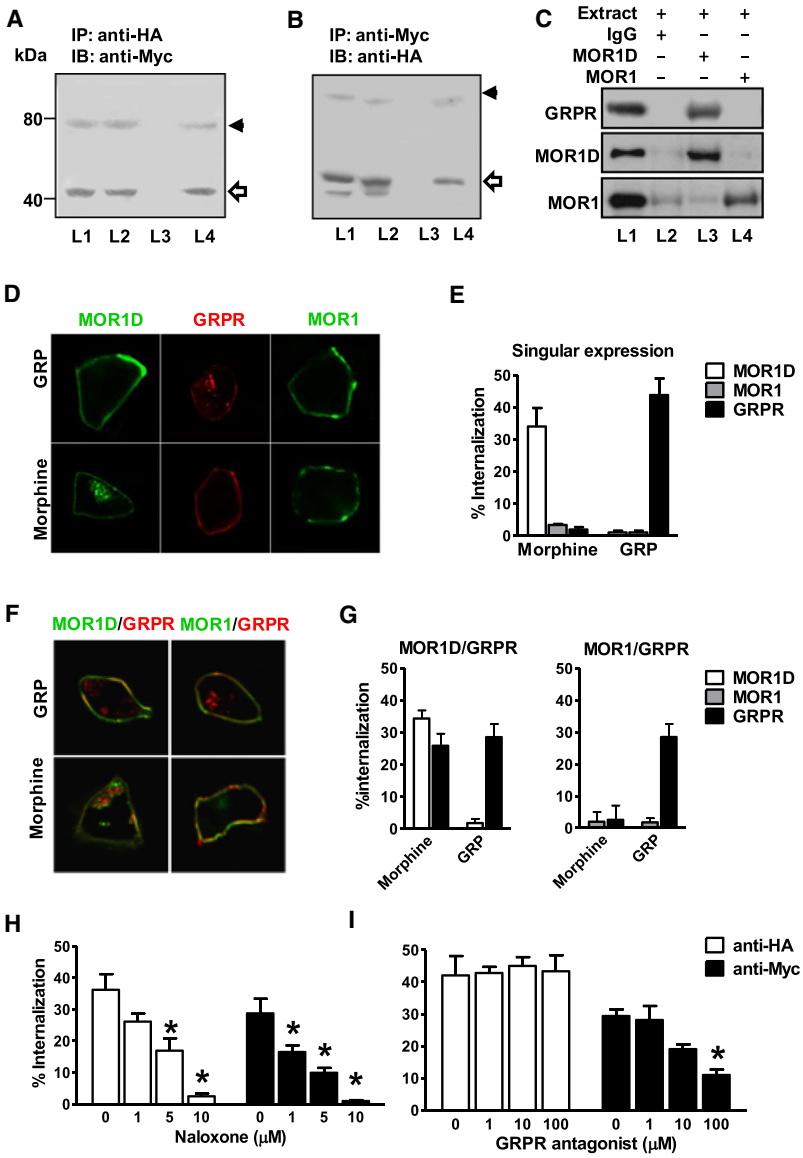

Figure 5. Coimmunoprecipitation and Cointernalization of GRPR and MOR1D

(A) Myc-GRPR (43 kDa) was detected in membrane fraction of MOR1/GRPR cells (L1) and MOR1D/GRPR cells (L2). Anti-HA antibody coprecipitated Myc-GRPR from MOR1D/GRPR cells (L4), but not from MOR1/GRPR cells (L3).

(B) Expression of HA-MOR1 (44 kDa) in HA-MOR1/Myc-GRPR cells (L1) and expression of HA-MOR1D (44 kDa) in HA-MOR1D/Myc-GRPR cells (L2) were revealed by anti-HA immunoblotting. An HA-MOR1D band (44 kDa) was precipitated by anti-Myc antibody from HA-MOR1D/Myc-GRPR cells (L4). Anti-Myc antibody failed to precipitate HA-MOR1 from cells expressing both Myc-GRPR and HA-MOR1 (L3). IP: immunoprecipitaion, IB: immunoblotting, kDa: kilodalton.

(C) GRPR, MOR1D, and MOR1 were detected in the membrane extract of dorsal horn (L1). GRPR was coprecipitated by anti-MOR1D (L3) but not by anti-MOR1 (L4) or irrelevant IgG (L2).

(D and E) Immunostaining (D) and ELISA (E) revealed endocytosis of HA-MOR1D but not HA-MOR1 or Myc-GRPR upon morphine treatment, whereas GRP induced endocytosis of GRPR but not MOR1D or MOR1.

(F and G) Immunostaining (F) and ELISA (G) revealed that Myc-GRPR, when coexpressed with HA-MOR1D but not HA-MOR1, internalized upon morphine stimulation. GRP only induced internalization of GRPP but not MOR1D or MOR1.

(H) Naloxone dose-dependently blocked morphine-induced internalization of Myc-GRPR and HA-MOR1D.

(I) The GRPR antagonist blocked morphine-induced internalization of Myc-GRPR, but not HA-MOR1D.

Data are expressed as mean and standard error of three independent experiments. Error bars represent SEM. *$p < 0.05$. See also Figure S4.

stimuli (Sun and Chen, 2007). Spinal morphine may promote presynaptic release of GRP to activate central GRPR signaling. However, our studies suggest that GRP is dispensable for morphine-induced activation of GRPR, and activation of GRPR in response to morphine is mediated via a postsynaptic mechanism. Indeed, MOR1D and GRPR dimers are detectable by co-IP in heterologous cells, and MOR1D and GRPR can also be coimmunoprecipitated from spinal cord membrane preparation. Thus, spinal opiates produce itch through MOR1D and GRPR heterodimerization. Importantly, in vivo interference with Tat-MOR1D$_{CT}$ markedly reduces co-IP of GRPR and MOR1D and blunts MIS. Taken together, these data demonstrate the importance of physical interactions between MOR1D and GRPR in MIS.

Calcium imaging studies illustrate that neither GRPR nor MOR1D alone are able to elicit a calcium response to morphine. Strikingly, a blockade of PLCβ and IP3R abolished morphine-induced calcium signaling in cells coexpressing MOR1D and GRPR. These results are in accord with previous observations that the ability of the G_i-coupled receptors to evoke calcium signaling often depends on a concomitant activation of the G_q-coupled receptors (Samways and Henderson, 2006). Distinguished from previous studies, the present study provides behavioral relevance for the PLCβ/IP3-dependent Ca^{2+} signaling evoked by morphine. Interestingly, PLCβ3 in DRG neurons has been shown to be required for MIA (Xie et al., 1999) as well as for histaminergic itch (Han et al., 2006). The fact that spinal opioid-induced itch is histamine independent (Ko et al., 2004), along with our finding that no change of PLCβ and IP3R occurs in DRG neurons by siRNA knockdown, indicates that the canonical PLCβ/IP3R3/Ca^{2+} signal transduction pathway in the spinal cord is itch specific and is different from its function in DRG neurons.

GPCR heterodimerization synergistically modulates respective receptor activity, resulting in either enhanced or inhibited ligand-binding properties or conferring novel function not originally possessed by the singular receptors (George et al., 2000; Jordan and Devi, 1999; Lopez and Salomé, 2009). In contrast

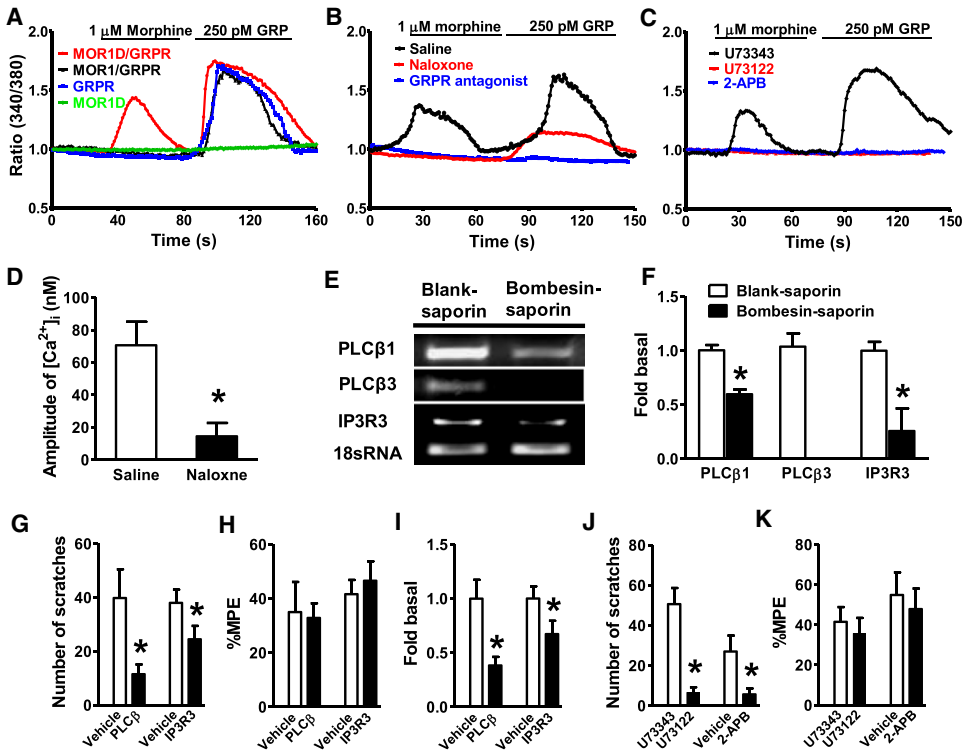

Figure 6. Cross-Activation of the GRPR Signal Transduction Pathway by MOR1D in Response to Morphine

The responses of HEK293 cells expressing vary receptors to morphine or GRP were tested using calcium imaging.

(A) HEK293 cells coexpressing MOR1D and GRPR showed calcium response to both morphine and GRP. Cells coexpressing MOR1 and GRPR were unable to respond to morphine, whereas they responded to GRP.

(B) The GRPR antagonist completely blocked morphine and GRP-induced Ca^{2+} increase in cells coexpressing MOR1D and GRPR. Naloxone blocked morphine- and reduced GRP-induced Ca^{2+} response in cells coexpressing MOR1D and GRPR.

(C) Both PLC inhibitor U73122 and IP3 receptor antagonist 2-APB blocked the response to morphine and GRP in cells coexpressing MOR1D and GRPR. U73343, an inactive structural analog of U73122, had no effect on morphine- or GRP- evoked $Ca2^{+}$ increase.

(D) Quantified data comparing peak intracellular calcium concentration. Naloxone significantly reduced GRP-induced $[Ca^{2+}]_i$ increase in cells coexpressing MOR1D and GRPR. n = 3, *p < 0.05.

(E and F) $GRPR^{+}$ cells in superficial dorsal horn were ablated by bombesin-saporin. The superficial dorsal horn was dissected for qRT-PCR. Gel image (E) and quantitative analysis (F) showed that PLCβ3 mRNA was lost in bombesin-saporin-treated group. PLCβ1 and IP3R3 mRNA were significantly decreased by bombesin-saporin treatment.

(G) Two days after the last injection of PLCβ siRNA or IP3R3 siRNA, MIS was significantly reduced. *p < 0.05.

(H) MIA was not significantly affected by PLCβ siRNA or IP3R3 siRNA.

(I) PLCβ mRNA and IP3R3 mRNA level in the superficial dorsal horn was significantly reduced by i.t. injection of PLCβ siRNA and IP3R3 siRNA, respectively. *p < 0.05.

(J) I.t. MIS was significantly inhibited by U73122, a PLC inhibitor, or 2-APB, an IP3R antagonist. *p < 0.05.

(K) Analgesic effect of i.t. morphine was not significantly affected by U73122 or 2-APB.

In all experiments, n = 6∼7 per group. Error bars represent SEM. See also Figure S5.

to reciprocal regulation of each receptor by respective agonists commonly found in GPCR heterodimerization, which allows for coincidental detection, our results uncover a unidirectional signaling model for GPCR crosstalk: whereas morphine-encoded itch information is transmitted from MOR1D to GRPR, GRP-encoded itch signaling cannot be reversely relayed to MOR1D by GRPR. Interestingly, the observation that a MOR1D-GRPR coimmunoprecipitated band from spinal cord membrane preparation is detected in the absence of morphine stimulation indicates a constitutive presence of MOR1D-GRPR heterodimeric assembly in vivo. How can GRPR be activated and internalized by morphine via MOR1D, whereas MOR1D cannot be internalized by GRP? One can envision that MOR1D and GRPR heterodimers may exist in a relatively unstable and dynamic equilibrium state that can be either strengthened/activated upon morphine stimulation, resulting in a cointernalization, or weakened in response to GRP, leading to a dissociation of heterodimers so that only GRPR internalizes. This is reminiscent of agonist-dependent dimerization and internalization of the δ-opioid receptor (Cvejic and Devi, 1997) and may also explain why the GRPR antagonist blocks morphine-mediated GRPR but not MOR1D endocytosis. Such a unidirectional signaling may ensure that opioid-encoded itch information is correctly relayed to the GRPR-signaling machinery and avoid accidental

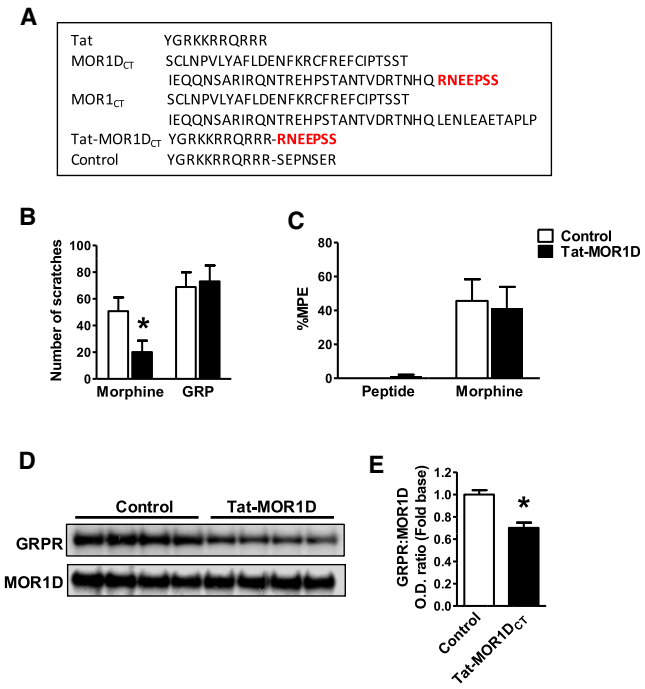

Figure 7. MOR1D C Terminus Is Critical for MIS and MOR1D/GRPR Dimerization

(A) Sequence comparison of MOR1D and MOR1 reveals a unique motif in MOR1D C terminus. Synthesized peptide Tat-MOR1D$_{CT}$ contains a Tat domain from human immunodeficiency virus-type 1 and the motif from MOR1D$_{CT}$. Control peptide contains Tat domain and scrambled sequence of MOR1D$_{CT}$.

(B) Tat-MOR1D$_{CT}$ blocked MIS without affecting GIS. *$p < 0.05$.

(C) Tat-MOR1D$_{CT}$ had no effect on MIA. *$p < 0.05$.

(D and E) Co-IP by anti-MOR1D (D) and quantified O.D. ratio of GRPR and MOR1D (E) showing Tat-MOR1D$_{CT}$ decreased GRPR/MOR1D interaction in the lumbar spinal cord.

In all experiments, n = 6∼8 per group. Error bars represent SEM.

engagement of MOR1D that may result in inappropriate signaling in a condition when GRPR is activated by GRP released from primary afferents. This one-way communication mechanism allows for added versatility to the physiological significance for GPCR heterodimerization and enables opioid receptors to carry out an unorthodox function.

Why has such a mechanism evolved to permit cross-activation of itch signaling by opioids? One plausible explanation is that opioid-induced pruritus may serve as the body's warning sign for opiate overdose or for internal metabolism disorders. For example, patients with cholestasis often suffer from terrible pruritus, which has been attributable to enhanced endogenous opioidergic signaling that is centrally mediated because opiate antagonists could ameliorate cholestasic itch, along with several other systemic itch conditions (Bergasa, 2005; Jones and Bergasa, 1990; Metze et al., 1999).

Our study cannot exclude this possibility that MOR1D may additionally regulate GRPR signaling through the Gi-coupled intracellular crosstalks. In this regard, MIS provides a reliable, unique, and unparalleled behavioral paradigm for facilitating further dissection of detailed intracellular signaling mechanisms of MOR1D and GRPR interactions and for understanding the corresponding physiological relevance.

Uncoupling of Itch and Pain: Therapeutic Implications

The identification of itch-specific MOR1D may shed light into the physiological and therapeutic relevance of the multiplicity of the MOR system. Although opiate antagonists may be used clinically to ameliorate spinal opioid-induced itch, their undiscriminating actions on both MOR1D and MOR1 might hinder opioid analgesia (Szarvas et al., 2003). Our finding, which uncouples MIS and MIA, enables us to envisage new therapeutic strategies. Pharmacological or antibody disruption of GPCR heterodimerization may be a highly cell type-specific targeting approach (Agnati et al., 2003; Hipser et al., 2010; Waldhoer et al., 2005), and the unique C terminus of MOR1D may be one of the best therapeutic targets. This heterodimeric-specific approach would not perturb the normal function of GRPR or MOR1D in other tissues where they are singularly expressed and where their physiological function may be important. Likewise, if MOR1D-GRPR signaling were involved in cholestatic itch, such a specific blockade may overcome side effects such as withdrawal-like symptoms often associated with the use of opioid antagonists in cholestatic itch (Bergasa, 2005). The present study implies that the physiological significance of multiple MOR isoforms may go beyond their normal antinociception paradigm that has been primarily restricted to the heterogeneity of opioid analgesia and patients (Pasternak, 2010). Although the disassociation of centrally mediated MIA from the nonneural tissue-mediated side effects of opioids has been reported (Ling et al., 1989; Manara et al., 1986), it is much more difficult to separate MIA from the side effect originating centrally. In this regard, an interesting question arises as to whether MOR1D may mediate other types of opiate side effects, as it is expressed in other brain areas such as the nucleus of the solitary tract, where no colocalization with MOR1 has been found (Abbadie et al., 2000). The uncoupling of MIA and MIS underscores the necessity of elucidating the function of individual MOR isoforms, which may promise novel pain therapy without debilitating side effects.

EXPERIMENTAL PROCEDURES

Animals

Generation and genotyping of GRPR KO and MOR KO were described previously (Hampton et al., 1998; Loh et al., 1998). All the experiments were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Animal Studies Committee at Washington University School of Medicine.

Drugs and Reagents

Morphine, DAMGO, fentanyl, GRP, naloxone, bombesin-saporin (Advanced Targeting), the GRPR antagonist (D-Phe-6-Bn(6-13)OMe), U73122, U73343, 2-APB, siRNA (Sigma), Tat-MOR1D$_{CT}$, and sequence-scrambled control peptide were administered intrathecally.

Behavior

Scratching behavior and tail immersion assay were performed as previously described (Sun and Chen, 2007). Morphine antinociceptive tolerance was induced as described (Fairbanks and Wilcox, 1999) (Zhao et al., 2007).

Preparation and Intrathecal Injection of siRNA

Selective siRNA duplexes for mouse *Oprm* exons, PLCβ1/3, and IP3R3 were intrathecally injected daily for 3 consecutive days. Behavior testing and tissue harvest were carried out at 48 hr after the last injection.

Laser Capture Microdissection

Laser capture microdissection (LCM) were performed as previously described (van Baarlen et al., 2009), and laminae I and II of the spinal cord were dissected using the Pix-Cell II with HS caps (Arcturus).

Quantitative RT-PCR

RNA was isolated from the LCM sample caps using the PicoPure RNA isolation kit (Arcturus). qRT-PCR amplification was performed using an Mx3000 QPCR system (Stratagene). All samples were run in triplicate.

Generation of MOR1D Antibody and Immunohistochemistry

Rabbit anti-MOR1D antibody was generated as described (Abbadie et al., 2000). Double-staining was performed using standard protocols.

Cell Culture and Transfections

To generate lines coexpressing Myc-tagged GRPR and HA-tagged MOR1D or MOR1 receptors, the cells were subjected to G418/hygromycin double selection. Clones expressing Myc-GRPR, HA-MOR1, HA-MOR1D, HA-MOR1/Myc-GRPR, and HA-MOR1D/Myc-GRPR were examined using quantitative western blot analysis to ensure that clones coexpress GRPR and MOR in 1:1 ratio.

Coimmunoprecipitation and Western Blot Analysis

HEK293 cells expressing MOR1D/GRPR or MOR1/GRPR were exposed to the crosslinking agent dithiobis-(succinimidylpropionate) (Pierce) and subsequently lysed as described (Koch et al., 2001). The receptor proteins were incubated with HA antibody (BD bioscience), or c-Myc antibody (Covance). The complex was precipitated, deglycosylated and separated on SDS gels (Invitrogen). Proteins were incubated with c-Myc antibody or HA antibody first, and then with goat horseradish peroxidase-linked secondary antibodies (Santa Cruz). Immunoblots were developed with the enhanced chemiluminescence reagents (Amersham).

Internalization Assays

The receptor internalization assay was performed as described previously (Pfeiffer et al., 2002).

Calcium Imaging

The cells were loaded with Fura 2-acetomethoxy ester (Molecular Probes) for ratiometric studies. Cells were imaged at 340 and 380 nm excitation to detect intracellular free calcium. Each experiment was done in triplicate, and at least 50 cells were analyzed each time.

Statistical Analysis

Statistical comparisons were performed with two-way analysis of variance (ANOVA) or Student's t test. All data were expressed as the mean ± standard error of the mean (SEM) and error bars represent SEM. $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and five figures and can be found with this article online at doi:10.1016/j.cell.2011.08.043.

ACKNOWLEDGMENTS

We thank J. Yin, S. Weng, Y.C. Pan, and Z.Q. Zhao for technical help and comments, C.J. Coscia, Y.X. Pan, and G.W. Pasternak for MOR1 and MOR1D plasmids, E. Wada and J. Battey for GRPR antibodies, P. Sternweis for PLCβ3 antibody, E. Liman for IP3R3 antibody, D. Coy for a GRPR antagonist, B.W. Li for qRT-PCR help, and W. Huh for LCM. We also thank M. Bruchas for comments. J.J. has been supported by a NIDA training grant. The work was supported by a NIAMS grant to Z.-F.C.

Received: January 10, 2011
Revised: July 20, 2011
Accepted: August 8, 2011
Published: October 13, 2011

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