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μ-Opioid receptors in primary sensory neurons are essential for opioid analgesic effect on acute and inflammatory pain and opioid-induced hyperalgesia

Jie Sun[1,2], Shao-Rui Chen[1], Hong Chen[1] and Hui-Lin Pan[1]

[1] *Center for Neuroscience and Pain Research, Department of Anesthesiology and Perioperative Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA*

[2] *Department of Anesthesiology, The First Affiliated Hospital/Jiangsu Province Hospital, Nanjing Medical University, Nanjing, Jiangsu 210029, China*

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Key points

- μ-Opioid receptors (MORs) are expressed peripherally and centrally, but the loci of MORs responsible for clinically relevant opioid analgesia are uncertain.
- Crossing *Oprm1*$^{flox/flox}$ and *Advillin*$^{Cre/+}$ mice completely ablates MORs in dorsal root ganglion neurons and reduces the MOR expression level in the spinal cord.
- Presynaptic MORs expressed at primary afferent central terminals are essential for synaptic inhibition and potentiation of sensory input by opioids.
- MOR ablation in primary sensory neurons diminishes analgesic effects produced by systemic and intrathecal opioid agonists and abolishes chronic opioid treatment-induced hyperalgesia.
- These findings demonstrate a critical role of MORs expressed in primary sensory neurons in opioid analgesia and suggest new strategies to increase the efficacy and reduce adverse effects of opioids.

Abstract The pain and analgesic systems are complex, and the actions of systemically administered opioids may be mediated by simultaneous activation of μ-opioid receptors (MORs, encoded by the *Oprm1* gene) at multiple, interacting sites. The loci of MORs and circuits responsible for systemic opioid-induced analgesia and hyperalgesia remain unclear. Previous studies using mice in which MORs are removed from Nav1.8- or TRPV1-expressing neurons provided only an incomplete and erroneous view about the role of peripheral MORs in opioid actions *in vivo*. In the present study, we determined the specific role of MORs expressed in primary sensory neurons in the analgesic and hyperalgesic effects produced by systemic opioid administration. We generated *Oprm1* conditional knockout (*Oprm1*-cKO) mice in which MOR expression is completely deleted from dorsal root ganglion neurons and substantially reduced in

Jie Sun received his PhD and MD degrees from the Medical School of Nanjing University in China in 2005. He has been involved in patient care and clinical research as an attending anesthesiologist in the Department of Anesthesiology at Jiangsu Province Hospital affiliated with Nanjing Medical University. He is currently working with Dr Hui-Lin Pan as a visiting scientist to study the sites and circuitry underlying opioid analgesic actions at The University of Texas MD Anderson Cancer Center in Houston, Texas, USA. His general research interest is to define the cellular and molecular mechanisms of the analgesic and anaesthetic actions.

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the spinal cord, which was confirmed by immunoblotting and immunocytochemical labelling. Both opioid-induced inhibition and potentiation of primary sensory input were abrogated in *Oprm1*-cKO mice. Remarkably, systemically administered morphine potently inhibited acute thermal and mechanical nociception and persistent inflammatory pain in control mice but had little effect in *Oprm1*-cKO mice. The analgesic effect of intrathecally administered morphine was also profoundly reduced in *Oprm1*-cKO mice. Additionally, chronic morphine treatment-induced hyperalgesia was absent in *Oprm1*-cKO mice. Our findings directly challenge the notion that clinically relevant opioid analgesia is mediated mostly by centrally expressed MORs. MORs in primary sensory neurons, particularly those expressed presynaptically at the first sensory synapse in the spinal cord, are crucial for both opioid analgesia and opioid-induced hyperalgesia.

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Corresponding author Hui-Lin Pan: Department of Anesthesiology and Perioperative Medicine, Unit 110, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030, USA. Email: huilinpan@mdanderson.org

Introduction

Opioids remain the most powerful analgesics for treating moderate and severe pain caused by cancer and tissue injury. Therapeutic doses of opioids, such as morphine, produce analgesia through μ-opioid receptors (MORs, encoded by the *Oprm1* gene; Matthes *et al.* 1996; Sora *et al.* 1997). MORs are distributed throughout the peripheral and central nervous systems, including the dorsal root ganglion (DRG), spinal cord dorsal horn and discrete brain regions (Mansour *et al.* 1986). Systemically administered opioids may produce analgesia by concomitant activation of MORs at multiple, interacting sites, but the exact sites of MORs and circuits responsible for this effect are unclear. Because intrathecal injection of MOR antagonists abolishes the analgesic effect of systemic opioids (Chen & Pan, 2006*a*), MORs in primary sensory neurons and/or spinal dorsal horn neurons may play a major role in the analgesic effect of systemic opioids. To specifically define the contribution of MORs in the DRG to the opioid analgesic effect, conditional MOR knockout mice have been used. In *Nav1.8*$^{Cre/+}$::*Oprm1*$^{flox/flox}$ mice in which MORs are removed from Nav1.8-expressing DRG neurons, the analgesic effect of systemic morphine on acute nociception is not changed although the morphine's effect on inflammatory pain is partially reduced (Weibel *et al.* 2013). Another study showed that the analgesic effect of systemic morphine is not altered in *TRPV1*$^{Cre/+}$::*Oprm1*$^{flox/flox}$ mice in which MORs are ablated from TRPV1-expressing sensory neurons, leading to the presumption that only centrally expressed MORs are involved in opioid analgesia (Corder *et al.* 2017). However, in both studies using conditional MOR knockout mice (Weibel *et al.* 2013; Corder *et al.* 2017), MORs were removed from only a subpopulation of primary sensory neurons.

In addition to the analgesic effect, opioids can paradoxically cause hyperalgesia and analgesic tolerance in both animals and humans (Mao *et al.* 1994; Celerier *et al.* 2000), leading to rapid dose escalation and diminished pain control. Opioid-induced hyperalgesia and analgesic tolerance are reduced when TRPV1-expressing DRG neurons are ablated by resiniferatoxin treatment (Chen *et al.* 2007) and in *TRPV1*$^{Cre/+}$::*Oprm1*$^{flox/flox}$ mice (Corder *et al.* 2017). However, opioid analgesic tolerance is not affected in *Nav1.8*$^{Cre/+}$::*Oprm1*$^{flox/flox}$ mice (Weibel *et al.* 2013). Nevertheless, it is unclear whether opioid-induced hyperalgesia and analgesic tolerance are solely due to stimulation of MORs expressed in DRG neurons. It is important to define the functional significance of MORs in primary sensory neurons in the actions produced by systemic opioids using an animal model in which MORs are entirely eliminated from the DRG.

In this study, we used the Cre/LoxP recombination system to generate *Oprm1* conditional knockout (*Oprm1*-cKO) mice in which MORs are completely deleted from primary sensory neurons. Using this mouse line, we provide unambiguous evidence that MORs in primary sensory neurons are essential for both analgesic and hyperalgesic effects produced by systemic opioids. This new knowledge advances our understanding of the loci of MORs and circuits involved in the actions of opioids *in vivo* and can inform the design of alternative strategies to improve the efficacy and reduce adverse effects of opioids.

Methods

Ethical approval and animal models

All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center (Approval No. 1174-RN00; Houston, TX, USA) and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals of the National Institutes of Health*. *Oprm1*$^{flox/flox}$ mice were purchased from The

Jackson Laboratory (Stock No. 030074, Bar Harbor, ME, USA), and *Advillin*$^{Cre/+}$ mice were provided by Dr F. Wang (Duke University, Durham, NC, USA). All of the mutant mice were backcrossed onto the C57BL/6J background for more than eight generations. To genetically delete MORs in peripheral sensory neurons, we crossed mice bearing alleles in which exons 2 and 3 of the *Oprm1* gene are flanked by loxP sites (*Oprm1*$^{flox/flox}$) (Weibel *et al.* 2013) with knock-in mice in which expression of Cre recombinase is driven by the promoter of the *Advillin* gene (*Advillin*$^{Cre/+}$) (Zhou *et al.* 2010*b*; da Silva *et al.* 2011). Male *Advillin*$^{Cre/+}$ mice were crossed with female *Oprm1*$^{flox/flox}$ mice to obtain male *Advillin*$^{Cre/+}$::*Oprm1*$^{flox/+}$ mice, which were crossed again to female *Oprm1*$^{flox/flox}$ mice to generate *Advillin*$^{Cre/+}$::*Oprm1*$^{flox/flox}$ mice, referred to as *Oprm1* conditional knockout (*Oprm1*-cKO) mice. Mice were ear-marked at the time of weaning (21 days after birth), and tail biopsies were used for PCR genotyping. Mice were housed at no more than 5 per cage and maintained on a 12 h light/dark cycle. They were kept in a temperature-controlled environment and received standard mouse chow and water *ad libitum*.

Age- and sex-matched adult *Oprm1*-cKO mice and their WT littermates (8—10 weeks of age) were used for all the experiments. In some mice, inflammatory pain was induced by injecting 20 μl complete Freund's adjuvant (CFA; Sigma-Aldrich, St Louis, MO, USA) into the plantar surface of the right hindpaw.

Drug administration

Morphine sulfate (West Ward Pharmaceuticals, Eatontown, NJ, USA), fentanyl (Sigma-Aldrich), clonidine hydrochloride (Sigma-Aldrich), and naloxone hydrochloride (Sigma-Aldrich) were diluted with normal saline and injected intraperitoneally (I.P.) or intrathecally. To induce opioid analgesic tolerance, morphine was injected I.P. at 10 mg/kg, twice per day, for 8 consecutive days, as described previously (Anand *et al.* 2018).

Intrathecal injection was performed using a lumbar puncture technique, as previously described (Hylden & Wilcox, 1980). Mice were briefly anesthetized with 2% isoflurane and placed in a prone position with a small tube under the abdomen to expose the lumbar vertebral space between L5 and L6. Lumbar puncture was performed with a 30.5-gauge needle connected to a 10 μl Hamilton syringe. Successful intrathecal injection (5 μl of drug solution) was indicated by brisk tail movement.

Nociceptive behavioural tests

To measure the tactile threshold, mice were placed in individual plastic boxes on a mesh floor and allowed to acclimatize for 30 min. A series of calibrated von Frey filaments was applied perpendicularly to the plantar surface of the hindpaw with sufficient force to bend the filaments for 6 s, and a brisk paw withdrawal or flinching was considered a positive response. In the absence of a response, the filament of next greater force was applied. If a response occurred, the filament of next lower force was applied. The tactile stimulus producing a 50% likelihood of withdrawal was determined using the 'up-down' method (Chaplan *et al.* 1994).

To determine the mechanical nociception, the hindpaw withdrawal threshold in response to a noxious pressure stimulus (Randall-Selitto test) was measured using a paw pressure Analgesy-Meter (Ugo Basile Biological Research, Comerio, Italy), as described previously (Chen & Pan, 2006*b*; Chen *et al.* 2007). When the mice displayed pain by either withdrawal of the hindpaw or vocalizing, the pedal was immediately released, and the mechanical threshold was read on the scale. A cut-off of 200 g was used to prevent potential pressure tissue injury.

To measure thermal nociception, mice were placed on the glass surface of a thermal testing apparatus (IITC Life Sciences, Woodland Hills, CA, USA). The temperature of the glass surface was maintained constant at 30°C. A mobile radiant heat source located under the glass was focused onto the hindpaw, and the paw withdrawal latency was recorded by a timer. A cut-off of 30 s was used to prevent potential tissue damage (Chen & Pan, 2006*a*). The investigators performing behavioural tests were blinded to the treatment and mouse genotypes.

Immunoblotting

The lumbar DRG, dorsal half of the lumbar spinal cord, brainstem, and periaqueductal gray (PAG) were obtained from mice deeply anaesthetized with isoflurane. Total proteins were extracted from the tissues after they were incubated with RIPA lysis buffer containing 1× PMSF. The protein concentration of each sample was detected by using a BCA kit. Forty microgram protein samples were subjected to SDS-PAGE (Bio-Rad, Hercules, CA, USA) electrophoresis and transferred on PVDF membranes (Immobilon-P, Millipore Corporation, Burlington, MA, USA). Membranes were washed and incubated with Tris-buffered saline and Tween 20 (TBST) containing 5% non-fat dry milk for 1 h at 25°C and then incubated with the following primary antibodies overnight at 4°C: rabbit anti-MOR (No. AB5511, 1:1000, EMD Millipore Corporation, Temecula, CA, USA) and rabbit anti-GADPH (No. 5174, 1:2000 Cell Signaling Technology, Danvers, MA, USA) diluted with TBST containing 5% bovine serum albumin. The specificity of the MOR antibody has been confirmed using brain tissues from global *Oprm1* KO mice (Kasai *et al.* 2011). After three washes in TBST, membranes were incubated

with a peroxidase-conjugated secondary antibody for 1 h at 25°C. The secondary antibody used was anti-rabbit IgG (No. 111-036-003, 1:7500 Jackson ImmunoResearch, West Grove, PA, USA). Target proteins were visualized by an ECL Plus detection kit (Thermo Fisher Scientific, Rockford, IL, USA), and the protein band intensity was quantified using an Odyssey Fc Imager (LI-COR, Lincoln, NE, USA) and normalized to the GADPH band on the same blot.

Double immunofluorescence labelling

We performed double immunofluorescence labelling of MORs and NeuN (a neuronal marker) in L4 and L5 DRGs and spinal cords from three WT and three *Oprm1*-cKO mice, as we described previously (Chen & Pan, 2006*b*; Chen *et al.* 2007). The tissue sections were rinsed in 0.1 M phosphate buffered saline and blocked in 4% normal goat serum for 1 h. The sections were then incubated with the mixture of primary antibodies (rabbit anti-MOR, No. RA10104, dilution 1:1000, Neuromics; Mouse anti-NeuN, No. AB104224, dilution 1:300, Abcam) diluted in PBS solution containing 2% normal goat serum and 0.3% Triton X-100 for 2 h at room temperature and overnight at 4°C. Subsequently, sections were rinsed in 0.1 M phosphate buffered saline and incubated with the secondary antibody mixture (Alexa Fluor 488 conjugated to goat anti-rabbit IgG, Alexa Fluor 594 conjugated to goat anti-mouse IgG; Molecular Probes, dilution: 5 μg/ml) for 1.5 h. The sections were then rinsed, mounted on slides, dried and coverslipped. Images were acquired with a confocal laser-scanning microscope (Zeiss, Jena, Germany).

Quantitative PCR

Total RNA was extracted from tissues using TRIsure (No. BIO-38032, Bioline, Taunton, MA, USA). After treatment with RNase-free DNase (No. 79254, Qiagen, Hilden, Germany), 1 μg RNA was used for reverse transcription with a RevertAid RT Reverse Transcription Kit (No. K1691, Thermo Fisher Scientific, Waltham, MA, USA). Two microlitres of cDNA diluted 5-time was added to a 20 μl reaction volume with SYBR Green PCR Mix (No. A25780, Thermo Fisher Scientific). Real-time PCR was performed using a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The thermal cycling conditions used were: 1 cycle at 95°C for 5 min and 40 cycles at 95°C for 15 s followed by 60°C for 45 s. The following primers were used: mouse *Oprm1* forward: TCT GCC ATT GGT CTG CCC GTA A; mouse *Oprm1* reverse: GAT GAG GAC CGG CAT GAT GAA GGC; mouse *Gapdh* forward: TGA AGG TCG GTG TGA ACG GA; mouse *Gapdh* reverse: ACA AGC TTC CCA TTC TCG GC. Relative mRNA levels were calculated using the $2^{-\Delta\Delta}$CT method and normalized to the GAPDH level in the same sample.

Spinal cord slice preparation and electrophysiological recordings

Under 2–3% isoflurane anaesthesia, the lumbar spinal cord of mice was quickly removed through laminectomy. The spinal cord tissues were immediately placed in ice-cold sucrose artificial cerebrospinal fluid (ACSF), which had been presaturated with 95% O_2 and 5% CO_2. Our ACSF contained (in mM): 234 sucrose, 3.6 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 1.2 NaH_2PO_4, 25.0 $NaHCO_3$, and 12.0 glucose. The spinal tissue was then glued to the stage of a vibratome (Leica, Wetzlar, Germany), and transverse slices (400 μm thick) were cut and placed in Krebs solution oxygenated with 95% O_2 and 5% CO_2 at 34°C for at least 1 h before being transferred to the recording chamber. The Krebs solution contained (in mM): 117.0 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 11.0 glucose, and 25.0 $NaHCO_3$. The spinal cord slices in a recording chamber were perfused with Krebs solution at 5.0 ml/min at 34 °C.

The lamina II outer neurons were visualized and selected for whole-cell recording since they predominantly receive nociceptive primary afferent input (Zhou *et al.* 2010*a*, Zhao *et al.* 2012). Excitatory postsynaptic currents (EPSCs) were evoked from the dorsal root using a bipolar tungsten electrode connected to a stimulator (0.2 ms, 0.6 mA). Monosynaptic EPSCs were identified on the basis of the constant latency and absence of conduction failure of evoked EPSCs in response to 20 Hz electrical stimulation. The impedance of the glass electrode was 5–8 MΩ when the pipette was filled with an internal solution containing (in mM): 135.0 potassium gluconate, 2.0 $MgCl_2$, 0.5 $CaCl_2$, 5.0 KCl, 5.0 HEPES, 5.0 EGTA, 5.0 Mg-ATP, 0.5 Na-GTP, 1 guanosine 5′-*O*-(2-thiodiphosphate) (GDP-β-S), and 10 lidocaine *N*-ethyl bromide (290–300 mosM, adjusted to pH 7.20–7.30 with 1.0 M CsOH). GDP-β-S and Cs^+ were used to inhibit the postsynaptic effect of the MOR agonist (Zhou *et al.* 2010*a*).

The postsynaptic G protein-coupled inwardly rectifying K^+ channels (GIRK) currents were recorded at a holding potential of −60 mV (Zhou *et al.* 2010*a*). The pipette internal solution contained (in mM): 135.0 potassium gluconate, 2.0 $MgCl_2$, 0.5 $CaCl_2$, 5.0 KCl, 5.0 HEPES, 5.0 EGTA, 5.0 Mg-ATP, and 0.5 Na-GTP (290–300 mosM, adjusted to pH 7.20–7.40 with 1.0 M KOH). All signals were recorded using an amplifier (MultiClamp700B; Molecular Devices, San Jose, CA, USA), filtered at 1 to 2 kHz, digitized at 10 kHz, and stored in a computer.

(D-Ala^2,*N*-Me-Phe^4,Gly^5-ol)-enkephalin (DAMGO, Sigma-Aldrich) was prepared in ACSF and delivered via

a syringe pump. Only one neuron was recorded from each spinal cord slice, and 6–8 mice were used for each recording protocol.

Statistical analysis

Data are expressed as means ± standard errors of the mean (SEM). Biochemical and electrophysiological data were compared between genotypes using one-way ANOVA followed by Tukey's *post hoc* test or repeated-measures ANOVA followed by Dunnett's *post hoc* test. The amplitude of the evoked EPSCs and GIRK currents were analysed using Clampfit software (version 10.0, Molecular Devices, San Jose, CA, USA). The behavioural data were analysed with two-way ANOVA followed by Tukey's or Bonferroni's *post hoc* test. All statistical analyses were performed using Prism software (version 7, GraphPad Software Inc., La Jolla, CA, USA). *P* values of less than 0.05 were considered to be statistically significant.

Results

MOR expression is abrogated in the DRG and reduced in the spinal cord in *Oprm1*-cKO mice

Advillin is an actin–binding protein of the gelsolin family, and its expression is almost exclusively restricted to DRG and trigeminal ganglion neurons (Hasegawa *et al.* 2007). We generated *Oprm1*-cKO mice by crossing *Oprm1*$^{flox/flox}$ (Weibel *et al.* 2013) and *Advillin*$^{Cre/+}$ mice (da Silva *et al.* 2011). To determine whether MORs in peripheral sensory ganglia are selectively removed in *Oprm1*-cKO mice, we measured MOR protein levels in the DRG, dorsal spinal cord, brainstem and periaqueductal gray (PAG). Immunoblotting analysis showed that two protein bands (50 and 55 kDa) of MORs were present in all of the tissues from wild-type (WT) mice, possibly due to glycosylation of MORs (Huang *et al.* 2015). However, the MOR protein bands were undetectable in the DRG from *Oprm1*-cKO mice (Fig. 1*A* and *B*). Also, the MOR protein level in the dorsal spinal cord was significantly lower in *Oprm1*-cKO mice than in WT mice ($P = 0.0069$, $F(7,40) = 25.22$; $n = 6$ mice per group). The MOR protein level in the brainstem and PAG tissues did not differ significantly between *Oprm1*-cKO and WT mice ($n = 6$ mice per group, Fig. 1*A* and *B*).

We also determined the mRNA level of *Oprm1* in the above tissues in *Oprm1*-cKO and WT mice. Quantitative PCR analysis showed that the mRNA level of *Oprm1* in the DRG was extremely low in *Oprm1*-cKO compared with that in WT mice (Fig. 1*C*). The mRNA level of *Oprm1* in the dorsal spinal cord was significantly lower in *Oprm1*-cKO mice than in WT mice ($P = 0.0179$, $F(7,40) = 43.35$; $n = 6$ mice per group; Fig. 1*C*). In contrast, the mRNA levels of *Oprm1* in the brainstem and PAG were similar between *Oprm1*-cKO and WT mice ($n = 6$ mice per group, Fig. 1*C*).

To determine changes in the distribution of MORs in DRG and spinal cord in *Oprm1*-cKO mice, we performed double immunofluorescence labelling in the DRG and spinal cord tissues. Confocal images show that the MOR immunoreactivity was present extensively in DRG neurons in WT mice. In contrast, the MOR immunoreactivity was not detected in the DRG in *Oprm1*-cKO mice (Fig. 2*A*). Furthermore, the MOR immunoreactivity was predominantly distributed in the superficial laminas of the spinal dorsal horn in WT mice. However, the MOR immunoreactivity in the superficial dorsal horn was substantially reduced in *Oprm1*-cKO mice (Fig. 2*B*). Together, these results indicate that MORs are selectively ablated from primary sensory neurons and their central terminals in the spinal cord of *Oprm1*-cKO mice.

MORs in primary sensory neurons are essential for the inhibitory and stimulatory effects of the MOR agonist on glutamatergic input to spinal dorsal horn neurons

Activation of MORs expressed at the central terminals of DRG neurons can profoundly inhibit nociceptive glutamatergic input to spinal dorsal horn neurons (Kohno *et al.* 1999; Zhou *et al.* 2010*a*), underlining the analgesic effect of MOR agonists. Furthermore, brief stimulation of MORs can induce a rebound increase in glutamate release from the central terminals of DRG neurons, and this rebound potentiation is involved in the initiation of opioid-induced hyperalgesia (Zhou *et al.* 2010*a*; Corder *et al.* 2017). Thus, we recorded monosynaptically evoked excitatory postsynaptic currents (EPSCs, representing glutamate release from primary sensory nerves) from the dorsal root to determine whether MORs in DRG neurons mediate the biphasic effects of the MOR agonist. In spinal cord slices from WT mice, bath application of DAMGO (1 μM), a highly specific MOR agonist, for 3 min rapidly and potently inhibited the amplitude of EPSCs in all 23 lamina II neurons examined (40.71 ± 8.52% peak inhibition, Fig. 3*A* and *B*). Fifteen to eighteen minutes after DAMGO washout, the EPSC amplitude gradually increased and significantly surpassed the pre-DAMGO baseline level in 12 of 23 neurons (53.02 ± 9.24% peak potentiation, Fig. 3*A* and *B*). In the remaining 11 neurons, the amplitude of evoked EPSCs returned to the pre-DAMGO baseline level after DAMGO washout. Strikingly, DAMGO application had no inhibitory effect on the amplitude of evoked EPSCs in any of the 14 lamina II neurons examined from *Oprm1*-cKO mice. In addition, none of the 14 neurons from *Oprm1*-cKO mice showed an increase in the EPSC amplitude 15–30 min after DAMGO washout (Fig. 3*A* and *B*). These findings indicate that both the opioid-induced inhibition and opioid-induced

stimulation of glutamatergic input to spinal dorsal horn neurons fully depend on presynaptic MORs expressed in primary sensory neurons.

MORs are expressed postsynaptically in spinal dorsal horn neurons, and stimulation of these MORs can activate G protein-coupled inwardly rectifying K^+ channels (GIRKs) to inhibit dorsal horn neurons (Marker *et al.* 2006; Zhou *et al.* 2010*a*). We recorded GIRK currents in lamina II neurons to determine whether MOR-mediated signalling is impaired in *Oprm1*-cKO mice. Bath application of DAMGO (1 μM) elicited outward GIRK currents in lamina II neurons from both WT and *Oprm1*-cKO mice (Fig. 3*C*). The amplitude of DAMGO-induced GIRK currents did not differ significantly between WT and *Oprm1*-cKO mice ($n = 11$ neurons per group, Fig. 3*D*). These data suggest that the function of postsynaptic MORs in the spinal dorsal horn is not compromised by genetic ablation of MORs in primary sensory neurons.

MORs in primary sensory neurons are required for the analgesic effect of systemic opioids

We next determined whether ablation of MORs in primary sensory neurons affects the analgesic effect produced by systemic administration of clinically used opioids. Baseline tactile, pressure and thermal withdrawal thresholds were similar between adult *Oprm1*-cKO and WT mice (Fig. 4*A*), suggesting that acute physiological nociception is not controlled by endogenous opioid tone acting at MORs in primary sensory neurons.

In WT mice, intraperitoneal (I.P.) injection of morphine at 2.5, 5 and 10 mg/kg dose-dependently increased paw withdrawal thresholds in response to von Frey filaments

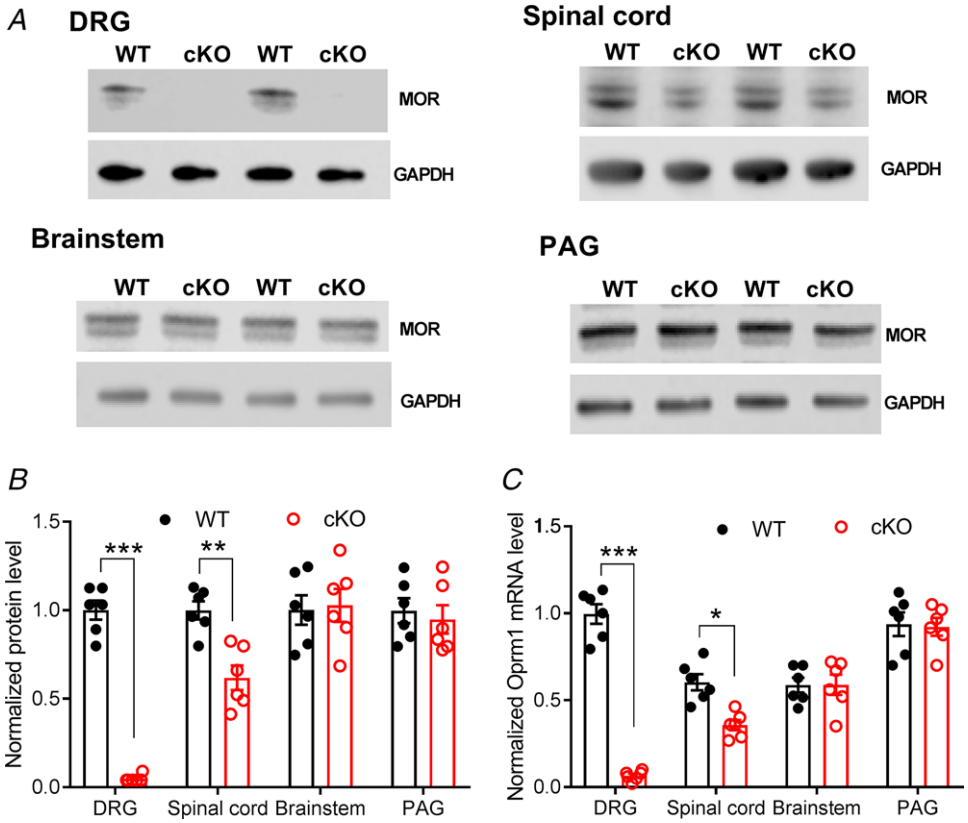

Figure 1. *Oprm1* conditional knockout selectively deletes MORs in the DRG and reduces MOR expression in the spinal cord
A and *B*, representative blot images (*A*) and quantification (*B*) of MOR protein levels in the DRG, spinal cord, brainstem and periaqueductal gray (PAG) ($n = 6$ mice per group). The amount of MOR proteins (50 and 55 kDa) was normalized to that of GAPDH (37 kDa) in the same sample, and the mean MOR level in WT mice was considered to be 1. Data are shown as means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the WT group. One-way ANOVA. *C*, quantification of mRNA levels of *Oprm1* in the DRG, spinal cord, brainstem and PAG ($n = 6$ mice in each group). The *Oprm1* mRNA level was normalized to the *Gapdh* mRNA level in the same sample, and the mean *Oprm1* level in the DRG from WT mice was considered to be 1. Data are shown as means ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with the WT group. One-way ANOVA. [Colour figure can be viewed at wileyonlinelibrary.com]

and noxious pressure and heat stimuli (n = 12 mice, Fig. 4*A*). The morphine's analgesic effect peaked 30 min after the drug injection and lasted for about 60–90 min. Remarkably, I.P. injection of morphine at 2.5, 5 and 10 mg/kg produced no analgesic effect in any *Oprm1*-cKO mice tested (n = 12 mice, Fig. 4*A*).

We also compared the analgesic effect of fentanyl, which is more lipophilic and ∼100 times more potent than morphine, in WT and *Oprm1*-cKO mice. In WT mice, I.P. injection of fentanyl (50 and 100 μg/kg) dose-dependently increased the withdrawal thresholds in response to mechanical and heat stimuli (n = 10 mice, Fig. 4*B*). In contrast, in *Oprm1*-cKO mice, I.P. injection of 50 μg/kg of fentanyl produced no analgesic effect (n = 10 mice). Only at 100 μg/kg, did fentanyl produce a small, transient effect on the pressure and heat withdrawal thresholds in *Oprm1*-cKO mice (Fig. 4*B*). These results indicate that MORs in primary sensory neurons are essential for the effect of systemically administered opioids on acute nociception.

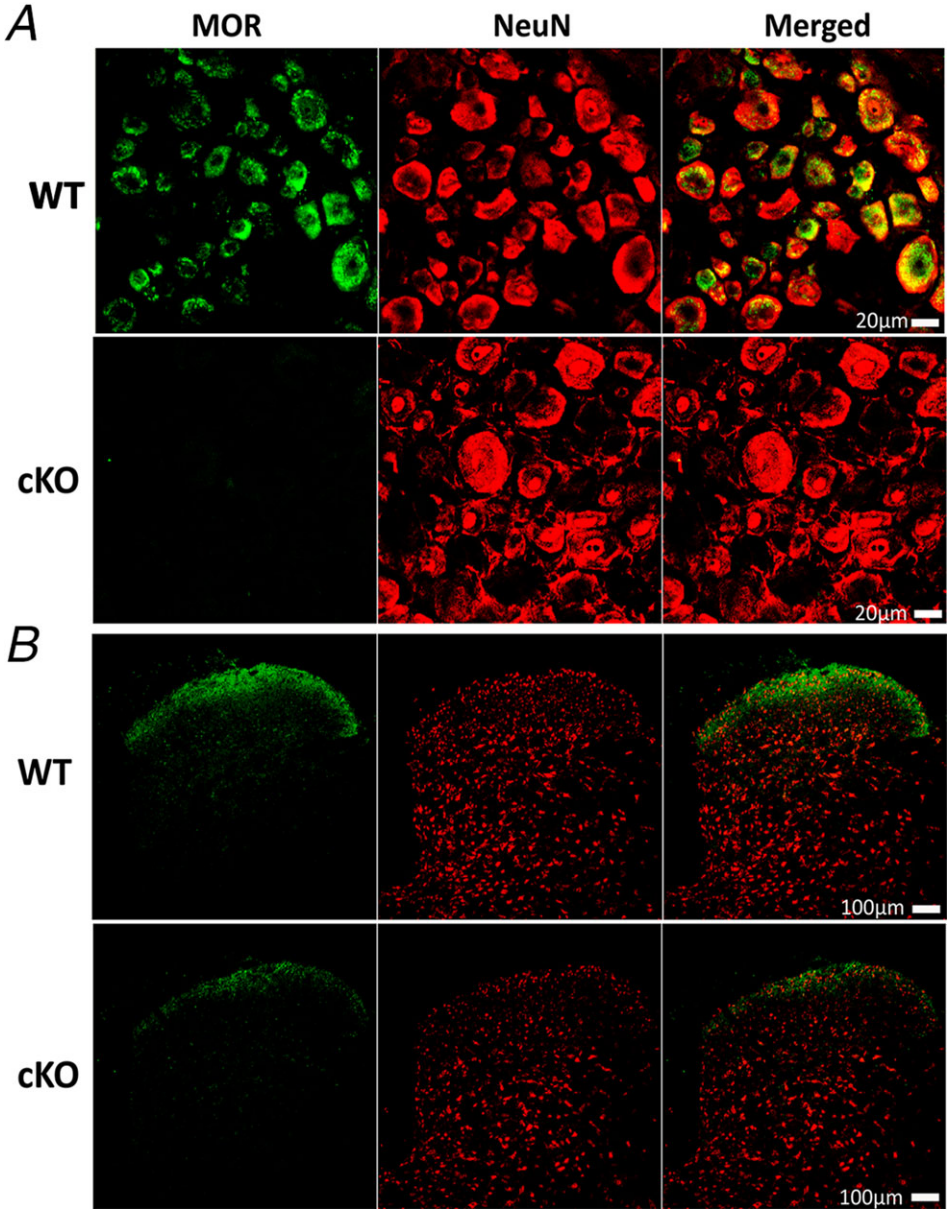

Figure 2. *Oprm1* conditional knockout ablates MORs in all DRG neurons and diminishes MOR expression in the spinal superficial dorsal horn
A and *B*, representative confocal images of double immunofluorescence labelling of MORs and NeuN in the DRG (*A*) and spinal dorsal horn (*B*) in WT and *Oprm1*-cKO mice. All images are single confocal optical sections. Scale bar, 20 μm for DRG sections and 100 μm for spinal cord sections (sections selected from 3 mice per group). [Colour figure can be viewed at wileyonlinelibrary.com]

The analgesic effect of systemic morphine on inflammatory pain depends on MORs in primary sensory neurons

Opioids produce a potent analgesic effect on persistent inflammatory pain, and the analgesic effect of morphine on pain hypersensitivity induced by complete Freund's adjuvant (CFA) is reduced in *Nav1.8*$^{Cre/+}$::*Oprm1*$^{flox/flox}$ mice (Weibel *et al.* 2013). We thus determined whether MORs in primary sensory neurons mediate the opioid analgesic effect under inflammatory pain conditions. Three days after injection of CFA into the hindpaw, there was a large reduction in paw withdrawal threshold in response to mechanical and heat stimuli in both *Oprm1*-cKO and WT mice (n = 12 mice per group, Fig. 4*C*). *Oprm1*-cKO and WT mice displayed similar

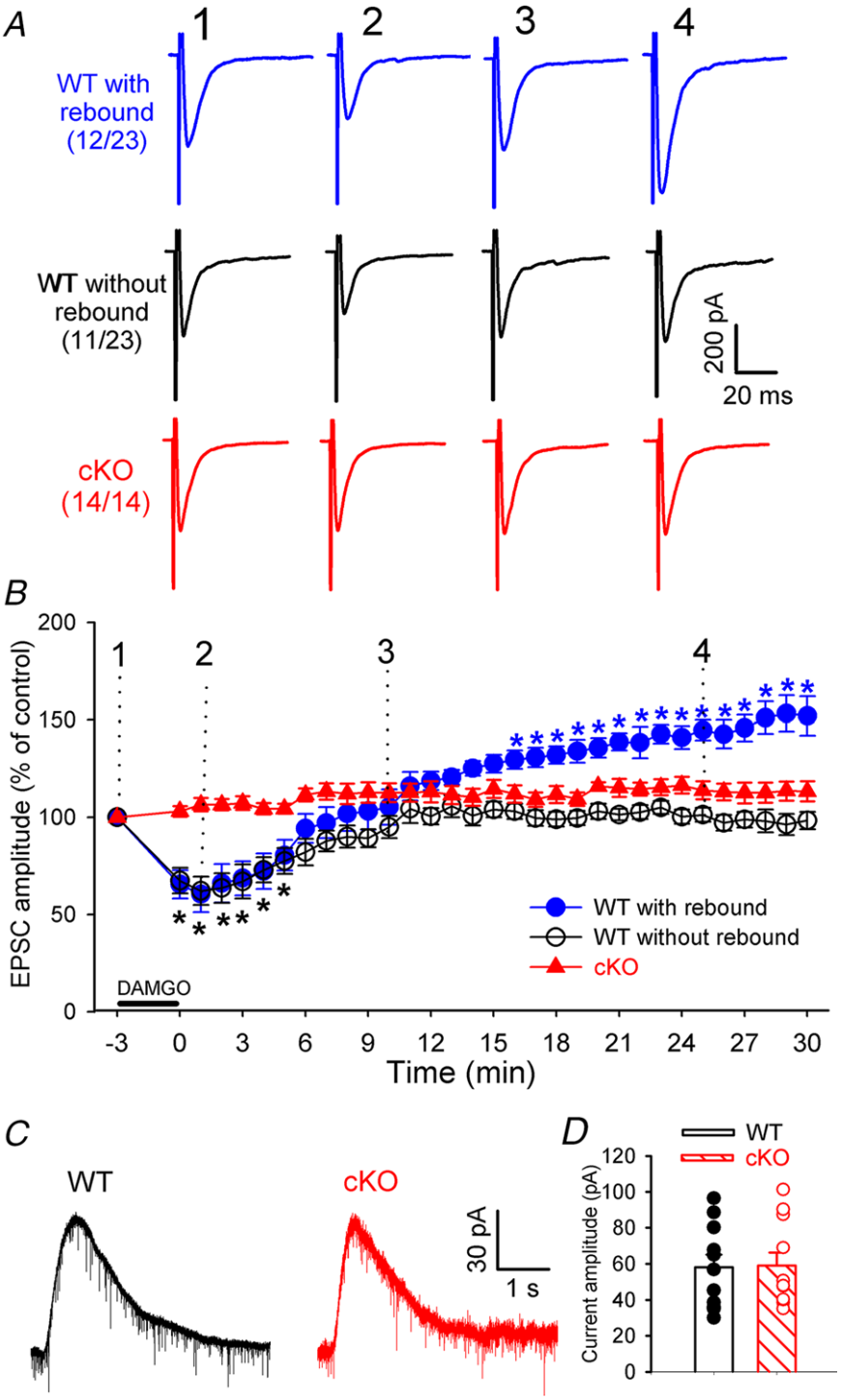

Figure 3. *Oprm1* conditional knockout in DRG neurons abrogates the presynaptic, not postsynaptic, effect of the MOR agonist in the spinal cord
A and *B*, representative recording traces (*A*) and the time course of changes (*B*) in the amplitude of evoked monosynaptic EPSCs during baseline control, during bath application of 1 μM DAMGO, and after DAMGO washout in lamina II neurons from WT mice (n = 12 neurons without a rebound increase, n = 11 neurons with a rebound increase; recorded from 8 mice) and *Oprm1*-cKO mice (n = 14 neurons from 6 mice). Current traces 1–4 were selected from the time points shown in the time course plot. Data are shown as means ± SEM. *P < 0.05 compared with the respective baseline before DAMGO application. Repeated-measures ANOVA. *C* and *D*, representative current traces (*C*) and quantitation (*D*) showed the effect of bath application of 1 μM DAMGO on the amplitude of GIRK currents of lamina II neurons from WT and *Oprm1*-cKO mice (n = 11 neurons from 8 WT mice, and n = 11 neurons from 6 *Oprm1*-cKO mice). Data are shown as means ± SEM. [Colour figure can be viewed at wileyonlinelibrary.com]

mechanical and heat hypersensitivity. In WT mice, I.P. injection of morphine (2.5, 5 and 10 mg/kg) reversed CFA-induced reduction in the paw withdrawal thresholds in a dose-dependent manner (Fig. 4*C*). In contrast, in *Oprm1*-cKO mice under the same inflammatory pain condition, I.P. injection of 2.5 and 5 mg/kg morphine produced no analgesic effect (Fig. 4*C*). I.P. injection of 10 mg/kg morphine had only a small effect on the pressure and heat withdrawal thresholds in *Oprm1*-cKO mice. These data indicate that systemically administered opioids diminish inflammatory pain through activation of MORs expressed in primary sensory neurons.

MORs in primary sensory neurons are involved in the analgesic effect produced by intrathecal morphine

Spinally administered opioids can activate presynaptic MORs at the central terminals of DRG neurons and postsynaptic MORs in dorsal horn neurons. We next determined whether genetic deletion of MORs in primary sensory neurons impacts on the analgesic effect of intrathecal morphine. We selected intrathecal doses of morphine based on our previous studies (Chen & Pan, 2006*a*,*b*; Chen *et al.* 2007; Zhang *et al.* 2016) and pilot experiments so that the peak effects produced by intrathecal and I.P. morphine were comparable in

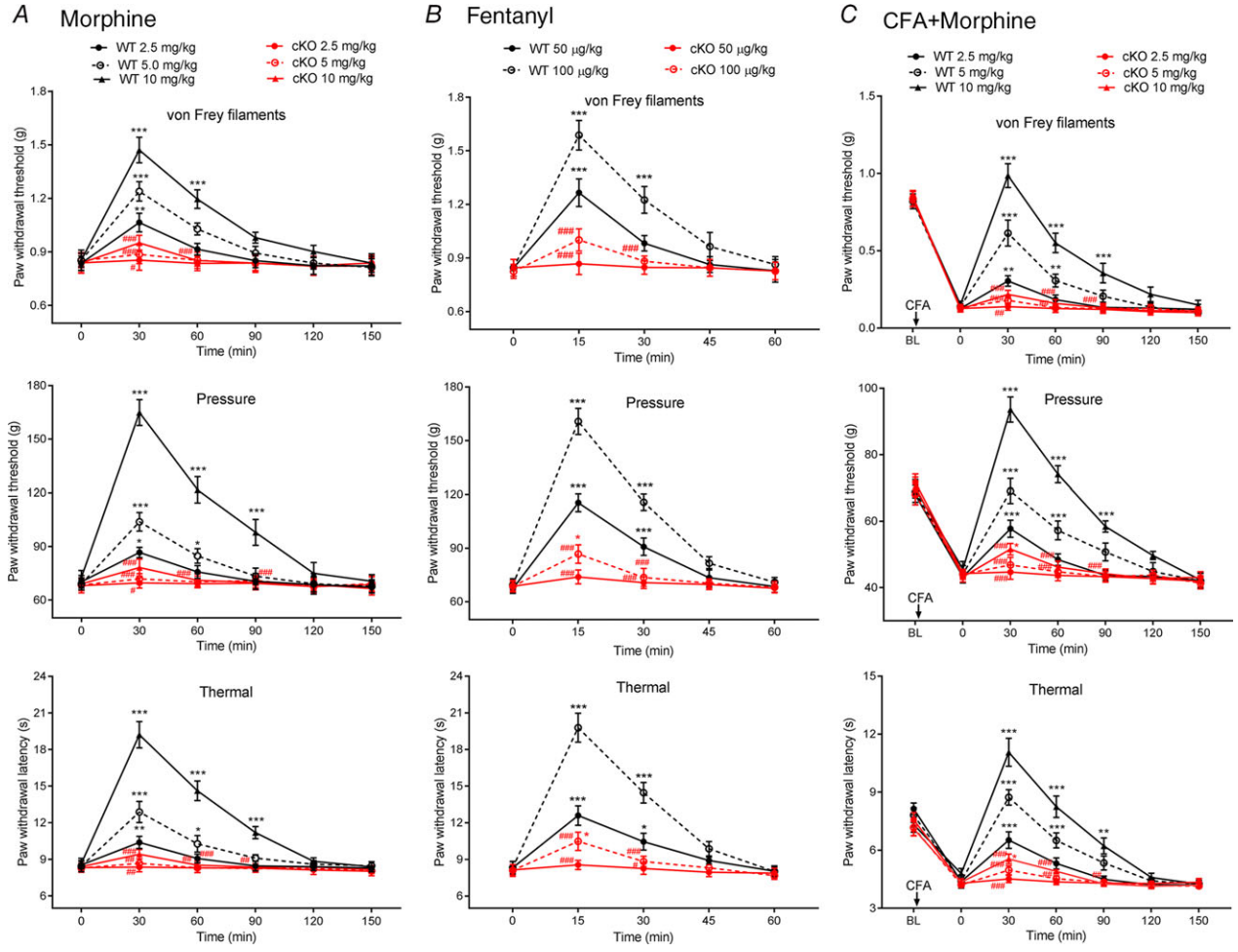

Figure 4. MORs in primary sensory neurons mediate the analgesic effects of systemically administered morphine or fentanyl on acute nociception and inflammatory pain
A, time course of the effects of intraperitoneal injection of 2.5, 5 and 10 mg/kg of morphine on the paw withdrawal thresholds in response to von Frey filaments and noxious pressure and heat stimuli in WT and *Oprm1*-cKO mice ($n = 12$ mice per group). *B*, time course of the effects of intraperitoneal injection of 50 and 100 μg/kg of fentanyl on the paw withdrawal thresholds in response to von Frey filaments and noxious pressure and heat stimuli in WT and *Oprm1*-cKO mice ($n = 10$ mice per group). *C*, time course of the effects of intraperitoneal injection of 2.5, 5 and 10 mg/kg morphine on the paw withdrawal thresholds in response to von Frey filaments and noxious pressure and heat stimuli in WT and *Oprm1*-cKO mice with CFA injected into the right hindpaw ($n = 12$ mice per group). Data are shown as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the respective baseline. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the value at the same time point in the WT group. Two-way ANOVA. [Colour figure can be viewed at wileyonlinelibrary.com]

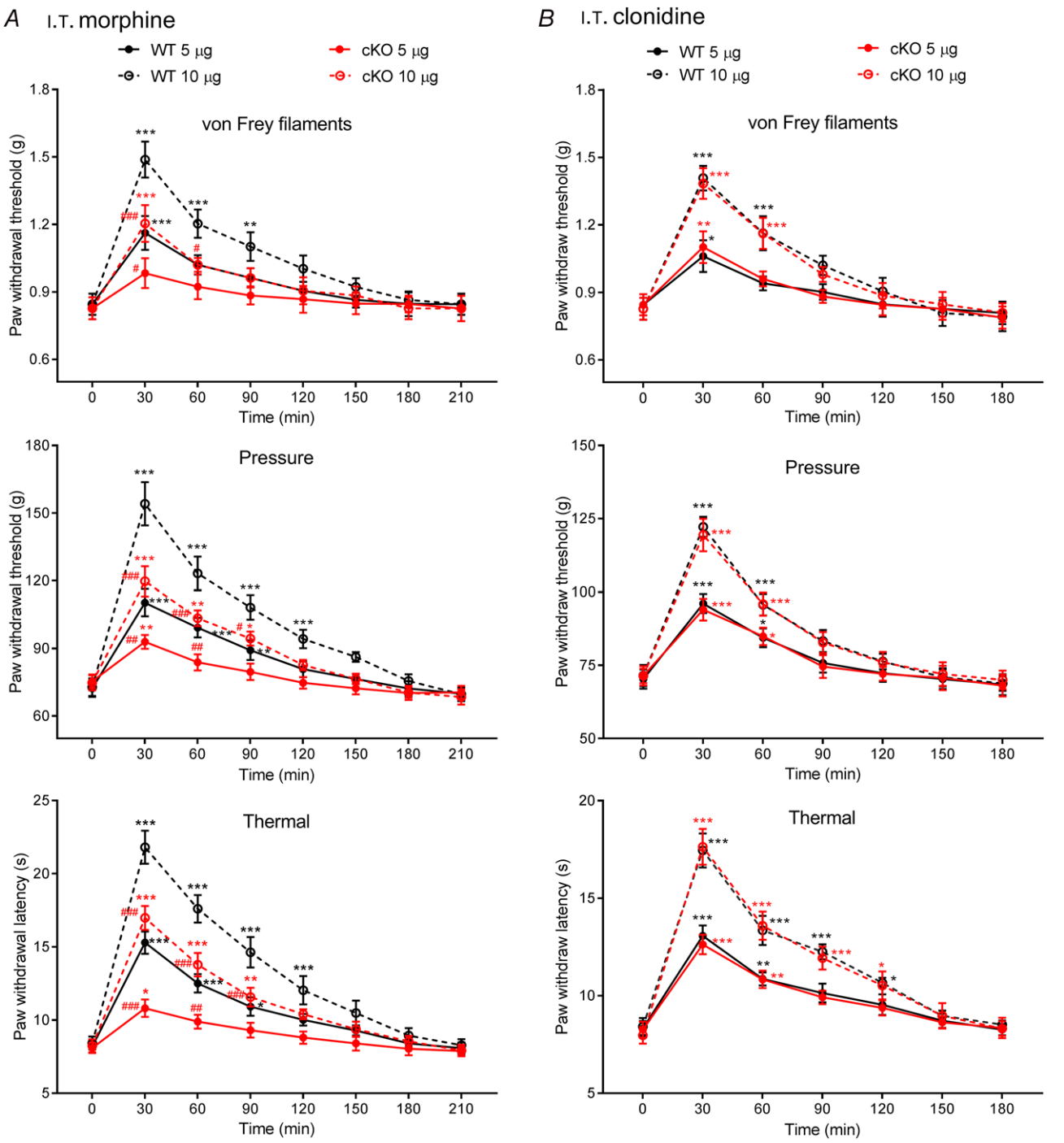

Figure 5. MORs in primary sensory neurons mediate the analgesic effect of intrathecally injected morphine but not clonidine
A, time course of the effects of intrathecal (I.T.) injection of 5 and 10 μg of morphine on the paw withdrawal thresholds in response to von Frey filaments and noxious pressure and heat stimuli in WT and *Oprm1*-cKO mice (n = 10 mice per group). Data are shown as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the respective baseline. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the value at the same time point in WT group. Two-way ANOVA. *B*, time course of the effects of intrathecal (I.T.) injection of 5 and 10 μg of clonidine on the paw withdrawal threshold in response to von Frey filaments and noxious pressure and thermal stimuli in WT and *Oprm1*-cKO mice (n = 10 mice per group). Data are shown as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the respective baseline. Two-way ANOVA. [Colour figure can be viewed at wileyonlinelibrary.com]

WT mice. In WT mice, intrathecal injection of 5 and 10 μg of morphine dose-dependently increased the paw withdrawal thresholds in response to mechanical and heat stimuli ($n = 10$ mice, Fig. 5*A*). In contrast, these effects of morphine were largely attenuated in *Oprm1*-cKO mice ($n = 10$ mice, Fig. 5*A*). These findings indicate that the full analgesic effect of intrathecally administered opioids critically depends on MORs expressed in primary sensory neurons.

We also compared the analgesic effect of intrathecal injection of clonidine between WT and *Oprm1*-cKO mice. Clonidine is an α2-adrenergic receptor agonist, and spinally administered clonidine produces potent analgesia by inhibiting nociceptive input from primary afferent nerves to dorsal horn neurons (Buerkle & Yaksh, 1998; Pan *et al.* 2002). In WT mice, intrathecal injection of 5 and 10 μg of clonidine increased the paw withdrawal thresholds in response to mechanical and heat stimuli in a dose-dependent manner ($n = 10$ mice, Fig. 5*B*). In *Oprm1*-cKO mice ($n = 10$ mice, Fig. 5*B*), intrathecal injection of clonidine at 5 and 10 μg produced an analgesic effect similar to that in WT mice. These data suggest that G protein-mediated signalling at the spinal cord level is not impaired in *Oprm1*-cKO mice.

MORs in primary sensory neurons are required for hyperalgesia caused by chronic opioid treatment

MORs in TRPV1-expressing DRG neurons are involved in the development of opioid-induced hyperalgesia and analgesic tolerance (Chen *et al.* 2007; Zhao *et al.* 2012; Corder *et al.* 2017). We therefore determined whether MORs in primary sensory neurons are required for hyperalgesia and analgesic tolerance induced by chronic morphine administration. In WT mice, I.P. administration of morphine (10 mg/kg, twice a day) for 8 consecutive days produced a gradual reduction in baseline mechanical and heat withdrawal thresholds, indicating the presence of mechanical and thermal hyperalgesia ($n = 10$ mice, Fig. 6*A*). Also, the analgesic effect of morphine was gradually lost in WT mice (Fig. 6*B* and *C*), representing opioid analgesic tolerance. In contrast, daily morphine treatment for 8 days had no effect on the baseline withdrawal thresholds in any *Oprm1*-cKO mice tested ($n = 10$ mice, Fig. 6*A*). Furthermore, I.P. injection of 10 mg/kg morphine produced no analgesic effect during chronic morphine treatment in these *Oprm1*-cKO mice (Fig. 6*B* and *C*). These findings indicate that MORs expressed in primary sensory neurons are essential for hyperalgesia induced by chronic systemic opioid treatment.

Discussion

Our study provides new evidence that MORs in primary sensory neurons play an essential role in the inhibitory effects of systemically administered opioids on both acute nociception and persistent inflammatory pain. We showed that genetic ablation of MORs in primary sensory neurons reduced the amount of MOR proteins in the dorsal spinal cord by ~41%, supporting the previous finding that a considerable portion of MORs in the spinal cord originate from DRG somas and are transported to their central terminals (Stevens & Seybold, 1995; Abbadie *et al.* 2002). Because the mRNA can also be transported from DRG somas to their nerve terminals (Bi *et al.* 2006; Jung *et al.* 2012), the similar reduction in both the mRNA and protein levels of MORs in the spinal cord of *Oprm1*-cKO mice suggests that some MOR proteins in the spinal dorsal horn may be produced via localized translation and synthesis at presynaptic terminals.

Because MORs are completely ablated in the DRG in *Advillin*$^{Cre/+}$::*Oprm1*$^{flox/flox}$ mice, the role of MORs in primary sensory neurons in the analgesic effect of systemic opioids can be adequately defined using our *Oprm1*-cKO mice. The complete absence of the opioid analgesic effect in *Advillin*$^{Cre/+}$::*Oprm1*$^{flox/flox}$ mice is surprising in light of the relatively widespread distribution (and overlap) of TRPV1- and Nav1.8-expressing DRG neurons. The lack of alteration in the analgesic effect of systemic morphine on acute nociception in *TRPV1*$^{Cre/+}$::*Oprm1*$^{flox/flox}$ and *Nav1.8*$^{Cre/+}$::*Oprm1*$^{flox/flox}$ mice (Weibel *et al.* 2013; Corder *et al.* 2017) is probably due to a partial removal of MORs in DRG neurons. The implication of our findings is that the full analgesic effect of opioids on both thermal and mechanical nociception may require MOR activity in multiple subpopulations of primary sensory neurons. Alternatively, MORs expressed in TRPV1-expressing neurons are mainly involved in opioid-induced hyperalgesia (i.e. opposing opioid analgesia), because the opioid analgesic effect is markedly augmented when TRPV1-expressing neurons are removed (Chen & Pan, 2006*b*; Chen *et al.* 2007). It is thus possible that deletion of MORs in TRPV1-expressing neurons not only fails to reduce opioid analgesia but also unmasks the opioid analgesic effect resulting from activation of MORs expressed in other subpopulations of DRG neurons. Notably, peripheral nerve injury markedly represses MOR expression in the DRG and diminishes the analgesic effect of systemically administered opioids via epigenetic mechanisms involving G9a and neuron-restrictive silencer factor (Uchida *et al.* 2010; Zhang *et al.* 2016), which corroborates our findings about the critical role of MORs in primary sensory neurons in the analgesic effect of opioids.

At the spinal cord level, MORs are expressed presynaptically at the central terminals of DRG neurons and postsynaptically in dorsal horn neurons (Stevens & Seybold, 1995; Abbadie *et al.* 2002; Marker *et al.* 2006). Activation of presynaptic MORs strongly inhibits nociceptive input from primary sensory nerves (Kohno *et al.* 1999; Zhou *et al.* 2008), which is mediated by inhibition of voltage-gated Ca^{2+} channels (Moises *et al.*

1994; Wu *et al.* 2004). Furthermore, MOR stimulation directly hyperpolarizes postsynaptic dorsal horn neurons through GIRK activation (Marker *et al.* 2006). We showed that the inhibitory effect of opioids on glutamatergic input from primary afferent nerves was abolished, whereas opioid-elicited GIRK currents in dorsal horn neurons were not affected, in *Oprm1*-cKO mice. Because GIRKs expressed in dorsal horn neurons are involved in the analgesic effect produced only by high doses of opioids (Marker *et al.* 2005), MORs in postsynaptic dorsal horn neurons may have a small contribution to the analgesic effect produced by high doses of systemically administered opioids in *Oprm1*-cKO mice. We found that the analgesic effect of intrathecal morphine was largely reduced in *Oprm1*-cKO mice. Also, blocking MORs at the spinal cord level abrogates the analgesic effect of systemic opioids (Chen & Pan, 2006*a*). Altogether, these findings suggest that presynaptic MORs expressed at the first sensory synapse in the spinal dorsal horn are particularly important for the analgesic actions of systemic opioids.

Our study clearly demonstrates that MORs in DRG neurons are required for opioid-induced hyperalgesia. It has been shown that hyperalgesia induced by morphine and its metabolite morphine-3β-D-glucuronide is absent in conventional MOR-KO mice (Roeckel *et al.* 2017). We found that selective ablation of MORs in DRG neurons eliminated the potentiation of glutamatergic input to dorsal horn neurons by an opioid agonist. Acute and chronic treatment with opioid agonists increases synaptic glutamate release through NMDA receptors at certain

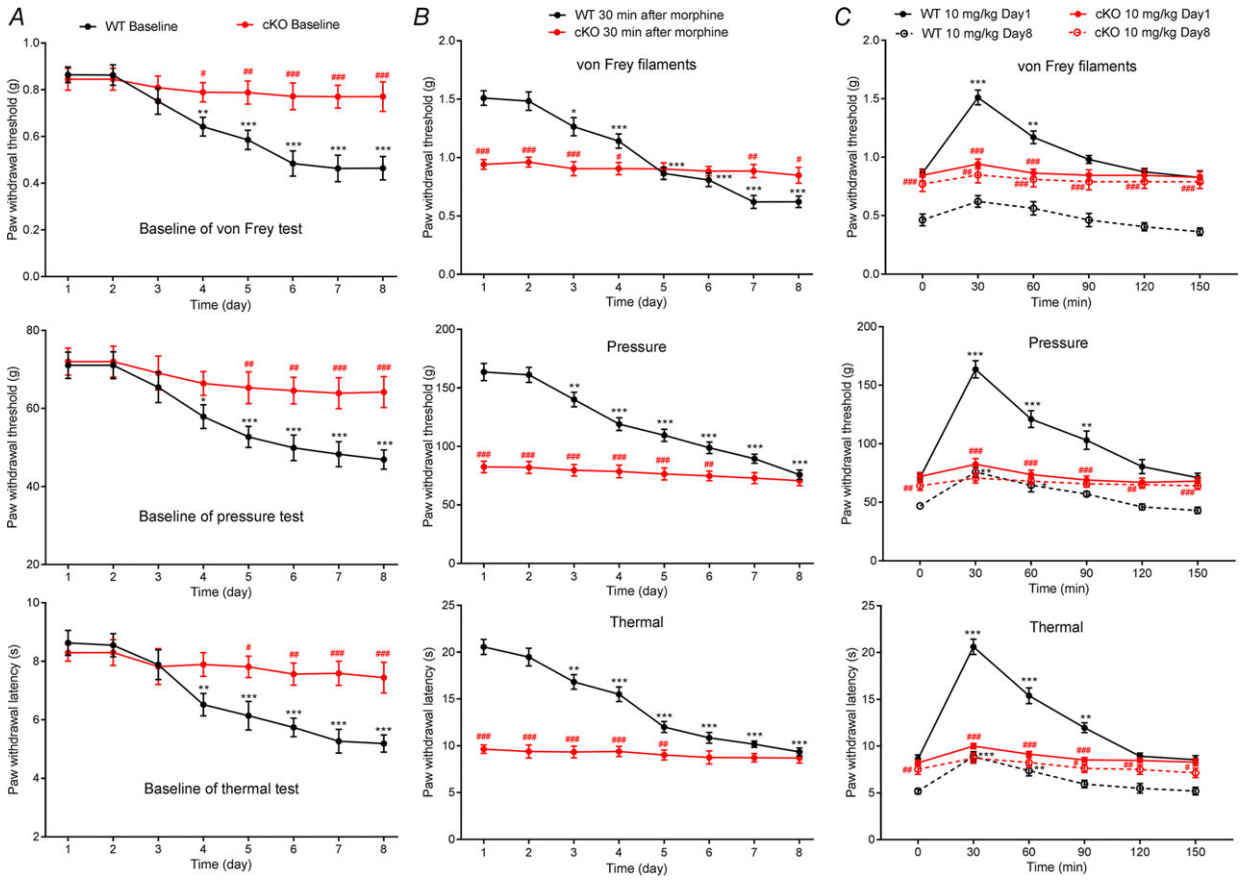

Figure 6. MORs in primary sensory neurons are required for systemic opioid-induced hyperalgesia
A, time course of changes in baseline paw withdrawal thresholds in response to von Frey filaments and noxious pressure and heat stimuli during daily intraperitoneal treatment with morphine (10 mg/kg, twice daily for 8 days) in WT and *Oprm1*-cKO mice (n = 10 mice per group). The measurements were taken 30 min before the first morphine injection daily. *B*, time course of changes in the effect of morphine on the paw withdrawal thresholds in response to von Frey filaments and noxious pressure and heat stimuli during daily intraperitoneal treatment with morphine (10 mg/kg, twice daily for 8 days) in WT and *Oprm1*-cKO mice (n = 10 mice per group). *C*, time course of the effect of intraperitoneal injection of 10 mg/kg morphine on the paw withdrawal thresholds in response to von Frey filaments and noxious pressure and heat stimuli in WT and *Oprm1*-cKO mice on the 1st and 8th days after chronic morphine treatment (10 mg/kg, twice daily for 8 days, n = 10 mice per group). Data are shown as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the respective baseline. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the value at the same time point in WT group. Two-way ANOVA. [Colour figure can be viewed at wileyonlinelibrary.com]

central terminals of DRG neurons (Zeng *et al.* 2006; Zhou *et al.* 2010*a*; Zhao *et al.* 2012). These presynaptic NMDA receptors, particularly those in TRPV1-expressing neurons, play a major role in the initiation and maintenance of opioid-induced glutamatergic input and opioid-induced hyperalgesia and analgesic tolerance (Chen *et al.* 2007; Zhou *et al.* 2010*a*; Zhao *et al.* 2012). Because the analgesic effect produced by systemic opioid administration is abrogated in *Oprm1*-cKO mice, we were unable to assess opioid analgesic tolerance in *Oprm1*-cKO mice. Nevertheless, because opioid-induced hyperalgesia and analgesic tolerance are mechanistically linked, MORs in DRG neurons probably also mediate opioid-induced analgesic tolerance.

The DRG neuronal somas are accessible to systemically administered agents (Jimenez-Andrade *et al.* 2008; Godel *et al.* 2016). However, because the central terminals of DRG neurons are embedded within the spinal dorsal horn, which is protected by the blood-brain and blood-spinal cord barriers, it would not be appropriate to consider MORs expressed in DRG neurons to be simply peripheral MORs. It has been shown that methylnaltrexone bromide, a peripherally restricted MOR antagonist, blocks hyperalgesia, not analgesia, produced by systemic morphine (Corder *et al.* 2017). This observation was interpreted to suggest that peripheral MORs mediate hyperalgesia, whereas centrally expressed MORs mediate opioid analgesia. Nevertheless, a well-controlled clinical trial shows that methylnaltrexone increases postoperative morphine consumption by about 40%, suggesting an important role of peripheral MORs in analgesia produced by systemic morphine (Jagla *et al.* 2014). Previous studies have shown that very low doses of MOR antagonists, including naloxone and naltrexone, also can reduce opioid-induced hyperalgesia and analgesic tolerance (Powell *et al.* 2002; Wang *et al.* 2005; Aguado *et al.* 2013). It is unclear how low doses of MOR antagonists selectively reduce hyperalgesia, but not analgesia, of systemic morphine and whether peripherally restricted MOR antagonists can affect MORs at the central terminals of DRG neurons in the spinal cord. MORs and their associated signalling in TRPV1-expressing neurons may be distinct from those in non-TRPV1 neurons in the DRG. For example, ablating TRPV1-expressing DRG neurons unexpectedly potentiates the analgesic effect of systemically and intrathecally administered opioids and

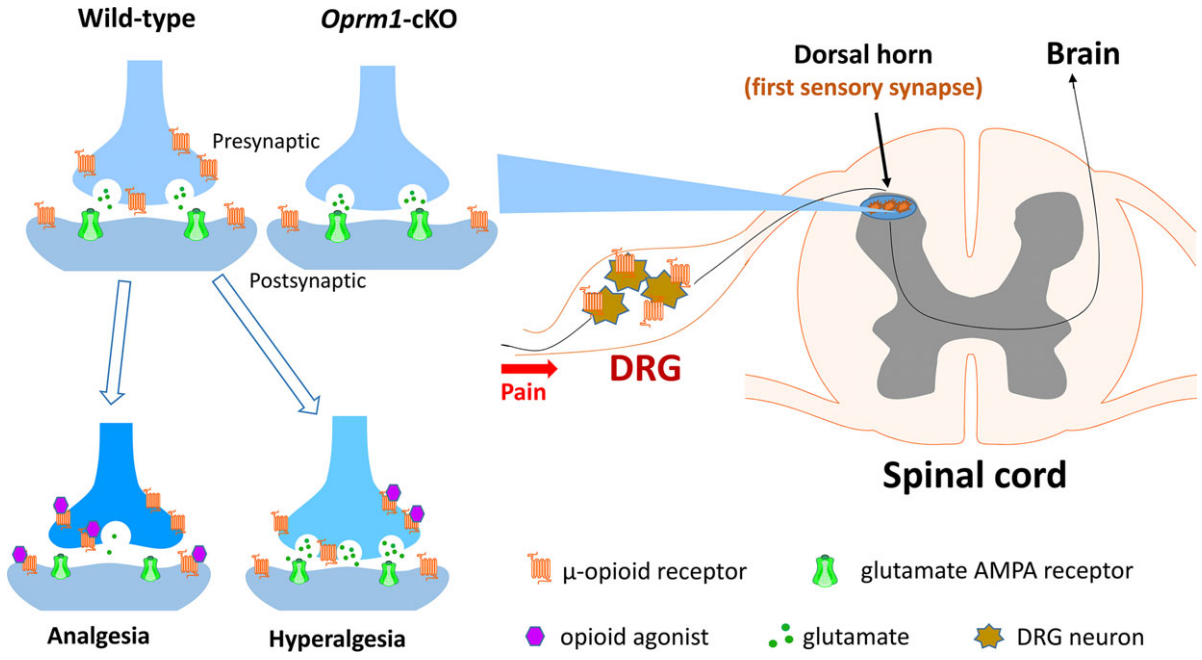

Figure 7. Schematic diagram shows the role of MORs expressed in primary sensory neurons in systemic opioid-induced analgesia and hyperalgesia
MORs are highly expressed in primary sensory neurons and presynaptically at primary afferent terminals in the spinal superficial dorsal horn in WT mice. Systemically administered opioids inhibit nociceptive transmission and produce analgesia predominantly through activation of MORs expressed in a subpopulation of primary sensory neurons and their presynaptic terminals in the spinal cord. By comparison, spinal cord postsynaptic MORs have a small contribution to the analgesic effect produced by high doses of systemic opioids. Furthermore, opioids can augment nociceptive transmission and induce hyperalgesia via stimulation of MORs expressed in a separate population (i.e. TRPV1-expressing) of primary sensory neurons and their central terminals in the spinal cord. However, in *Oprm1*-cKO mice, MORs expressed in all primary sensory neurons and their central terminals in the spinal cord are ablated, diminishing both the analgesic and hyperalgesic effects of opioids. [Colour figure can be viewed at wileyonlinelibrary.com]

the opioid inhibitory effect on glutamatergic input from primary afferent nerves (Chen & Pan, 2006*b*; Zhou *et al.* 2008). An MOR splice variant, MOR1D, in the spinal cord has been linked specifically to opioid-induced itch (Liu *et al.* 2011), and G protein-mediated signalling is preferentially involved in opioid analgesic actions, whereas arrestin-mediated signalling contributes mainly to opioid analgesic tolerance (Bohn *et al.* 2000). Further studies are needed to determine whether MOR subtypes (i.e. splice variants), MOR-interacting proteins, or MOR-mediated downstream signalling differs between TRPV1-expressing neurons and non-TRPV1-expressing neurons in the DRG.

In summary, our study provides conclusive *in vivo* evidence that MORs in primary sensory neurons play a predominant role in both analgesic and hyperalgesic actions of systemically administered opioids (Fig. 7). These opioid actions are principally mediated by MORs expressed at the first sensory synapse in the spinal cord, where they are crucially involved in regulating nociceptive input from primary sensory nerves. Because MORs in TRPV1-expressing DRG neurons are predominantly involved in opioid-induced hyperalgesia and analgesic tolerance, either identifying MOR agonists that primarily target MORs and their signalling in non-TRPV1-expressing sensory neurons or selectively blocking MORs and their signalling in TRPV1-expressing neurons may represent an appropriate strategy to improve opioids' analgesic efficacy and minimize opioids' adverse effects.

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Additional information

Competing interests

The authors declare that they have no conflicts of interest with the contents of this study.

Author contributions

J.S., S.R.C. and H.C. conducted experiments. J.S., S.R.C. and H.-L.P. performed data analysis. H.-L.P. conceived the project and wrote the manuscript with input from the other authors. All authors approved the final version of the manuscript submitted for publication and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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