ORIGINAL ARTICLE

Mu-Opioid Receptors Expressed in Glutamatergic Neurons are Essential for Morphine Withdrawal

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Received: 29 November 2019 / Accepted: 11 February 2020 / Published online: 25 May 2020
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Abstract Although opioids still remain the most powerful pain-killers, the chronic use of opioid analgesics is largely limited by their numerous side-effects, including opioid dependence. However, the mechanism underlying this dependence is largely unknown. In this study, we used the withdrawal symptoms precipitated by naloxone to characterize opioid dependence in mice. We determined the functional role of mu-opioid receptors (MORs) expressed in different subpopulations of neurons in the development of morphine withdrawal. We found that conditional deletion of MORs from glutamatergic neurons expressing vesicular glutamate transporter 2 (Vglut2⁺) largely eliminated the naloxone-precipitated withdrawal symptoms. In contrast, conditional deletion of MORs expressed in GABAergic neurons had a limited effect on morphine withdrawal. Consistently, mice with MORs deleted from Vglut2⁺ glutamatergic neurons also showed no morphine-induced locomotor hyperactivity. Furthermore, morphine withdrawal and morphine-induced hyperactivity were not significantly affected by conditional knockout of MORs from dorsal spinal neurons. Taken together, our data indicate that the development of

morphine withdrawal is largely mediated by MORs expressed in Vglut2⁺ glutamatergic neurons.

Keywords Morphine · Mu-opioid receptor · Naloxone-precipitated withdrawal · Dorsal spinal cord · Locomotor hyperactivity

Introduction

Opioids have been used in pain management for a long time, but their chronic use has been limited by numerous side-effects. Among these, opioid-induced dependence causes drug craving and prolongs medication, leading to a public health problem. Multiple maladaptive behaviors induced by the termination of opioid drugs, termed opioid withdrawal, are often used to characterize opioid dependence. Mu-opioid receptors (MORs), encoded by *Oprm1* [1–3], are responsible for opioid withdrawal, as MOR-knockout mice do not express naloxone-precipitated opioid withdrawal behavior [4]. Like other opioid receptors, MORs are coupled with inhibitory G proteins, which in turn activate several intracellular effectors [5–7]. Repeated opioid administration impairs the homeostasis of the nervous system, as reflected by adaptive changes at both the cellular and synaptic levels [8]. These adaptations can lead to dysfunction of excitatory and inhibitory synaptic transmission [8–10].

Recent studies have started to examine the contribution of MORs expressed in different neuronal populations to morphine analgesia and other effects, including withdrawal, using genetic approaches [11–13]. As forebrain GABAergic neurons are an important substrate for opioid reward and drug addiction [14], several studies have examined the functional role of MORs in forebrain

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12264-020-00515-5>) contains supplementary material, which is available to authorized users.

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GABAergic neurons [15–17], and found that these MORs play essential roles in locomotor and motivational effects, as well as alcohol reward [15, 16]. Although MORs in striatal GABAergic neurons play an important role in opioid reward, these MORs are not involved in morphine withdrawal [17]. Thus, MORs expressed in forebrain GABAergic neurons are not the key component for the aversive effects in opioid adaptation. The involvement of excitatory transmission during opioid dependence has also been examined in several studies. It has been shown that the excitatory projection from the paraventricular nucleus of the thalamus to the nucleus accumbens [18], and the projection from the lateral habenula to the raphe nucleus [10] are engaged in aversive behaviors during morphine withdrawal. The brain areas with both dense expression of MORs and essential roles in aversive behaviors are potentially involved in naloxone-precipitated withdrawal. A recent study found that MORs expressed in the medial habenula significantly contribute to aversive effects in opioid withdrawal, but not the analgesic, locomotor, or reward effects of morphine [19]. Although the involvement of multiple neuronal populations in morphine withdrawal has been investigated, the mechanism underlying opioid dependence and the precise contribution of MORs in different neuronal subpopulations to withdrawal symptoms need to be further investigated.

MORs are widely expressed in the dorsal spinal cord [20], and they are important for morphine analgesia [21, 22]. Previous studies have focused on microglial adaptation in the spinal cord during opioid dependence, and found chronic morphine-induced activation [23–26]. In addition, morphine withdrawal also induces long-term synaptic facilitation in the superficial dorsal spinal cord [27], which is a result of the ATP release induced by activation of the spinal microglial pannexin-1 channel [28]. However, the potential involvement of MORs expressed in dorsal spinal neurons in morphine withdrawal is largely unknown.

To characterize the functional role of MORs expressed in different neuronal populations in morphine withdrawal, we investigated the contribution of MORs in distinct neuronal subpopulations to morphine withdrawal in MOR conditional-knockout mouse lines.

Materials and Methods

Animals

Vglut2-ires-Cre (JAX #016963, referred to as Vglut2-Cre), Vgat-ires-Cre (JAX #016962, referred to as Vgat-Cre), and Lbx1-Cre [29] mice were used for experiments. We generated an MOR conditional-knockout line, referred to

as *Oprm1*^{fl/fl}, in which two *loxP* sites flanking *Oprm1* exon 2 and exon 3 were inserted to an *Oprm1* allele of *Oprm1*^{fl/fl} mice (Fig. 1A), allowing for conditional deletion of MORs in neurons with Cre recombinase. *Oprm1*^{fl/fl} mice were crossed with Vglut2-Cre, Vgat-Cre or Lbx1-Cre mice to obtain the MOR conditional-knockout mice, referred to as Vglut2-Cre/*Oprm1*^{fl/fl}, Vgat-Cre/*Oprm1*^{fl/fl}, and Lbx1-Cre/*Oprm1*^{fl/fl} mice. Male mice (8 weeks–12 weeks old) were used for behavioral experiments. All mice were raised under a 12-h light/dark cycle (lights on at 07:00) with *ad libitum* food and water. All procedures were approved by the Animal Care and Use Committee of the Institute of Neuroscience, Chinese Academy of Sciences, Shanghai, China.

Tissue Preparation

Each mouse was anesthetized with pentobarbital sodium (0.1 mg/g) and perfused transcardially with saline followed by 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO). The brain and spinal cord (lumbar segments) were dissected. The brain was weighed and post-fixed overnight in 4% PFA at 4°C, followed by cryoprotection in 30% sucrose in phosphate-buffered saline (PBS) at 4°C for further experiments.

In Situ Hybridization

In situ hybridization was performed as previously described [30]. The *Oprm1* probe was made using digoxigenin (DIG)-labeled nucleotides. Brain sections were cut at 40 µm on a cryostat (Leica CM 1950, Buffalo Grove, IL) and floated in diethyl pyrocarbonate (DEPC)-PBS. The sections were washed once in DEPC-PBS and then twice in DEPC-PTw (PBS-Tween, 0.1% Tween-20 in PBS) for 5 min each. After immersion in 2 × saline sodium citrate (SSC) with 0.5% Triton for 30 min and washing twice in DEPC-PTw for 5 min, the sections were treated with proteinase K (4 µg/mL) for 5 min, and then fixed in 4% PFA for 10 min. After washing once in DEPC-PTw, followed by acetylation in triethanolamine (0.1 mol/L) with 0.25% acetic anhydride for 10 min, sections were washed twice in DEPC-PBS for 5 min. Sections were pre-hybridized for 3 h at 65°C in hybridization buffer, then hybridized in hybridization buffer containing 1 µg/mL *Oprm1* probe for 12 h–16 h at 65°C. A series of washes were performed sequentially as follows: pre-hybridization buffer and pre-hybridization/TBST (1% Tween-20, 500 mmol/L NaCl, 20 mmol/L Tris) at 65°C, each for 30 min; 2 × 5 min in TBST at room temperature; one rinse in TBST/Tris-acetate-EDTA (TAE); and 3 × 5 min in TAE at room temperature. To remove the probe, sections were collected into wells in 2% agarose/TAE gel, and electrophoresed at

Fig. 1 Generation and verification of the MOR conditional-knockout mouse line. **A** Targeting strategy for generating the *Oprm1*^{fl/fl} mouse line. **B** Representative images of *Oprm1* expression in *Oprm1*^{fl/fl} and *Vglut2-Cre/Oprm1*^{fl/fl} mice determined by *in situ* hybridization (scale bar, 500 μ m). **C** Representative images of MOR expression in *Oprm1*^{fl/fl} and *Vglut2-Cre/Oprm1*^{fl/fl} mice determined by fluorescent immunostaining (scale bar, 200 μ m). Cpu, striatum; NAc, nucleus

accumbens; AcbC, accumbens nucleus, core; MHb, medial habenula; PV, paraventricular thalamic nucleus; CM, central medial thalamic nucleus; CeA, central amygdala; BLA, basolateral amygdala; IPN, interpeduncular nucleus; VTA, ventral tegmental area; PBN, parabrachial nucleus; scp, superior cerebellar peduncle; LPB, lateral parabrachial nucleus.

60 V for 2 h. After washing twice in TBST, the sections were next incubated with sheep anti-DIG-alkaline phosphatase (AP; 1:2000 in 0.5% blocking reagent) overnight at 4°C. The next day, after 3 $\times$ 30 min washes in TBST and 2 $\times$ 30 min in AP buffer (100 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.1% Tween-20, 50 mmol/L MgCl₂), sections were visualized in AP buffer containing 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium and fixed in 4% PFA after colorization.

MOR Immunofluorescent Staining

Brain sections were cut at 40 μ m on a cryostat for immunofluorescent staining. The sections were blocked for

1 h at room temperature in PBST (0.3% Triton X-100) with 5% normal donkey serum, followed by incubation with primary antibody at 4°C overnight and secondary antibody at room temperature for 2 h in PBST (0.3% Triton X-100) with 1% normal donkey serum. The primary antibody used was anti-MOR [17, 31] (rabbit, 1:500, ab134054 Abcam, Cambridge, MA), and the secondary antibody was donkey anti-rabbit IgG-Cy3 (1:200, 711-165-152, Jackson ImmunoResearch Laboratories, West Grove, PA).

Behavioral Procedure for Morphine Withdrawal

The procedure for morphine withdrawal was adapted from previous studies [26, 28]. Briefly, mice were randomly

divided into groups and received escalating doses of morphine (7.5 mg/kg–50 mg/kg, i.p.) or saline as the control twice daily for four consecutive days with an 8-h interval (day 1, 7.5 and 15 mg/kg; day 2, 20 and 25 mg/kg; day 3, 30 and 35 mg/kg; and day 4, 40 and 45 mg/kg). On day 5, the mice received a single morphine (50 mg/kg) or saline injection and were challenged with naloxone (2 mg/kg, i.p.) 2 h after the last injection to induce acute withdrawal. Next, each mouse was placed in a transparent box and somatic signs of withdrawal were videotaped and scored across 30 min by an observer blinded to the treatment. Jumping, teeth-chattering, headshakes, wet-dog shakes, and grooming behaviors were evaluated and scored from 0 to 3 based on behavioral bouts (0 = absent; 1, 1–3 bouts; 2, 4–6 bouts; 3, ≥ 7) at 5-min intervals. Piloerection, paw tremor, and twitching were also evaluated; one point was given when each sign appeared in the 5-min interval. The total bouts of each behavior and the global withdrawal scores were calculated.

Open Field Test

Locomotor activity was evaluated in the open field test. Mice were placed in the testing room for ~ 1 h for habituation. In the baseline test, each mouse was placed in an open field box ($40 \times 40 \times 40$ cm³) and videotaped for 10 min. Then the mouse was removed from the box and subcutaneously injected with 10 mg/kg morphine. The test was run again 40 min after morphine injection. Total travel distance and average speed in a 10-min period were recorded. The track was automatically analyzed using LabState (AniLab, Ningbo, China) or EthoVision XT (Noldus, Wageningen, Netherlands) software.

Elevated Plus Maze Test

The elevated plus maze test was used to assess anxiety-like behavior [32]. The maze contained two opposing closed arms (30 cm $\times$ 5 cm $\times$ 15 cm), two open arms (30 cm $\times$ 5 cm), and a center zone (5 cm $\times$ 5 cm). The platform was 50 cm above the floor. At the beginning of the test, each mouse was placed in the central zone, facing one of the open arms. In the subsequent 5 min, the total time spent in the open arms, closed arms, and center zone, and the number of entries into the open arms and closed arms were recorded. The track was automatically analyzed using EthoVision XT (Noldus) software.

Tail Suspension Test

The tail suspension test was used to assess depression-like behavior [33]. During the test, each mouse was suspended by tape ~ 1.5 cm from the tail tip, and ~ 20 cm above the

floor. The mice were videotaped for 6 min, and the time spent immobile was scored manually. The suspension test was repeated once if a mouse climbed up its tail.

Novel Object Test

The novel object test was used to evaluate the exploration behaviors and anxiety levels [34]. A circular wooden cone (base diameter, 5 cm; height, 8 cm) was placed in the center of an open field arena (40×40 cm²) and the entries into and time spent exploring the object in a 13.3×13.3 cm² square in the center of the arena were recorded in a 10-min period. The track was automatically analyzed using EthoVision XT (Noldus) software.

Sucrose Preference Test

The sucrose preference test was used to assess anhedonia behavior. We adapted the procedure from previous studies [35, 36]. Mice were housed individually and habituated before the test. The experimental mice were habituated with two identical tubes, one containing 1% sucrose and the other containing water in their home cages for 1 day. The positions of the tubes were switched once to reduce position preference. Then, these mice were deprived of both water and food for 24 h. During the test, two identical tubes containing 1% sucrose and water were placed in the cage and left for 2 h, and the positions of the tubes were switched and weighed after 1 h. The consumption of each fluid in the 2-h interval was measured by weight, then the sucrose preference was calculated as the percentage of sucrose intake over the total fluid intake in the first hour and the whole 2 h.

Image Acquisition and Statistical Analysis

Images were captured using an Olympus VS120 microscope (Olympus, Tokyo, Japan). Statistical analysis was performed using Prism 6 (GraphPad Software, San Diego, CA). The data were analyzed using two-way ANOVA, one-way ANOVA followed by Bonferroni *post hoc* analysis, and the two-tailed unpaired *t*-test. The cut-off for significance was set at $P = 0.05$. All data are presented as the mean $\pm$ SEM.

Results

MORs Expressed in Vglut2⁺ Glutamatergic Neurons are Essential for Morphine Withdrawal

MORs are widely expressed in the nervous system and play an important role in mediating opioid dependence [4].

Opioid dependence is commonly characterized by the withdrawal symptoms at the termination of drug use. We investigated the functional role of MORs expressed in different subpopulations of neurons in morphine withdrawal by selectively deleting MOR in specific neuronal subpopulations using the conditional-knockout strategy. To achieve this, we generated a *Oprm1^{fl/fl}* mouse line, in which two *loxP* sites were inserted to flank exon 2 and exon 3 of the *Oprm1* gene (Fig. 1A), allowing for conditional deletion of MOR with Cre recombinase. We first investigated the functional role of MORs expressed in glutamatergic neurons, most of which express *Vglut2*. We thus crossed *Vglut2-Cre* mice with *Oprm1^{fl/fl}* mice to obtain *Vglut2-Cre/Oprm1^{fl/fl}* mice, which would lead to deletion of MORs from most glutamatergic neurons. We found that both the body weight and brain weight were comparable in *Oprm1^{fl/fl}* and *Vglut2-Cre/Oprm1^{fl/fl}* littermates (Fig. S2A, B). Next, we assessed *Oprm1* expression in the brains of *Vglut2-Cre/Oprm1^{fl/fl}* mice using *in situ* hybridization, and found that its expression in the thalamus, medial habenula, and parabrachial nucleus (PBN), where most neurons are glutamatergic, was largely abolished compared to that in *Oprm1^{fl/fl}* mice. In contrast, expression of *Oprm1* in the striatum, nucleus accumbens (NAc), central amygdala (CeA), and interpeduncular nucleus (IPN), where most neurons are GABAergic, was comparable to that in *Oprm1^{fl/fl}* mice (Figs. 1B and S1A, B). We also used immunohistochemistry to confirm the selective deletion of MORs in *Vglut2-Cre/Oprm1^{fl/fl}* mice. The immunostaining results also showed that the expression of MORs was selectively abolished in the glutamatergic neuron-enriched brain regions of *Vglut2-Cre/Oprm1^{fl/fl}* mice (Figs. 1C and S2C, D). These results showed that the expression of MORs was selectively abolished from glutamatergic neurons in *Vglut2-Cre/Oprm1^{fl/fl}* mice.

We next examined the functional role of MORs expressed in glutamatergic neurons in morphine withdrawal using *Vglut2-Cre/Oprm1^{fl/fl}* mice and littermate *Oprm1^{fl/fl}* mice. The mice were given escalating doses of morphine (7.5 mg/kg–50 mg/kg, i.p.) for 5 consecutive days to establish dependence, and another batch of mice was given saline as controls with the same schedule (Fig. 2A). Acute morphine withdrawal was induced by injection of the opioid receptor antagonist, naloxone, 2 h after the last morphine/saline injection. The mice were video-taped for 30 min after naloxone injection for behavioral analysis (Fig. 2A). Consistent with previous reports [28], naloxone induced robust withdrawal signs. The withdrawal score of the *Oprm1^{fl/fl}* mice in the morphine group was significantly higher than that in the control group (Fig. 2B, C). In contrast, the withdrawal score of *Vglut2-Cre/Oprm1^{fl/fl}* mice treated with morphine was comparable to that of mice treated with saline. In

addition, *Vglut2-Cre/Oprm1^{fl/fl}* mice treated with morphine showed a significantly lower global withdrawal score than *Oprm1^{fl/fl}* littermates treated with morphine (Fig. 2B, C), indicating that opioid dependence was largely abolished after selective deletion of MORs in glutamatergic neurons. Specifically, naloxone significantly increased the jumping, teeth-chattering, headshaking, piloerection, and twitching behaviors compared to the saline-treated control group (Fig. 2D, E, G, I, K), and the paw tremor behavior in *Oprm1^{fl/fl}* mice with morphine treatment was slightly elevated (Fig. 2J). However, the changes of withdrawal symptoms in *Vglut2-Cre/Oprm1^{fl/fl}* mice were variable. *Vglut2-Cre/Oprm1^{fl/fl}* mice exhibited a significant reduction in jumping, teeth-chattering, headshaking, and paw tremor (Fig. 2D, E, G, J), while the expression of wet-dog shakes, piloerection, and twitching were not significantly affected (Fig. 2F, I, K). The number of grooming bouts was slightly higher in *Vglut2-Cre/Oprm1^{fl/fl}* mice than in *Oprm1^{fl/fl}* littermates (Fig. 2H), and the decrease of grooming behavior in *Oprm1^{fl/fl}* mice might have resulted from the increases in other withdrawal symptoms.

As morphine can also lead to locomotor hyperactivity in mice, we evaluated the functional role of MORs expressed in glutamatergic neurons on morphine-induced locomotor activity using *Vglut2-Cre/Oprm1^{fl/fl}* mice. In the open field test, *Oprm1^{fl/fl}* and *Vglut2-Cre/Oprm1^{fl/fl}* mice showed comparable basal locomotor activity (Fig. 2L, M). Morphine significantly elevated the speed of movement and travel distance in *Oprm1^{fl/fl}* mice, whereas the morphine-induced locomotor hyperactivity was abolished in *Vglut2-Cre/Oprm1^{fl/fl}* mice (Fig. 2L, M). We also assessed anxiety/depression-associated behaviors in *Vglut2-Cre/Oprm1^{fl/fl}* mice. In the novel object test, the frequency and time spent on novel object exploration by *Vglut2-Cre/Oprm1^{fl/fl}* mice were comparable to those in *Oprm1^{fl/fl}* mice (Fig. S2E). There was also no difference between *Oprm1^{fl/fl}* and *Vglut2-Cre/Oprm1^{fl/fl}* mice in the elevated plus maze, tail suspension, and sucrose preference tests (Fig. S3F–H), suggesting that *Vglut2-Cre/Oprm1^{fl/fl}* mice maintained normal anxiety-like behaviors indistinguishable from those of their *Oprm1^{fl/fl}* littermates. Taken together, these data indicate that morphine withdrawal and morphine-induced locomotor hyperactivity largely rely on MORs expressed in *Vglut2⁺* glutamatergic neurons.

MORs Expressed in GABAergic Neurons Play a Negligible Role in Morphine Withdrawal

To determine the involvement of MORs expressed in GABAergic neurons in morphine withdrawal, we generated *Vgat-Cre/Oprm1^{fl/fl}* mice by crossing *Vgat-Cre* mice with *Oprm1^{fl/fl}* mice to conditionally knockout MORs from GABAergic neurons. We found that the body weight of

Fig. 2 Deletion of MORs in glutamatergic neurons alleviates naloxone-precipitated withdrawal symptoms. **A** Experimental timeline for chronic morphine delivery and naloxone-precipitated withdrawal. **B**, **C** Time course of scores and total scores for naloxone-precipitated withdrawal in *Oprm1^{fl/fl}* and *Vglut2-Cre/Oprm1^{fl/fl}* mice ($n = 6-8$ mice/group). **D-K** Bar graphs showing bouts and scores for each withdrawal symptom in *Oprm1^{fl/fl}* and *Vglut2-Cre/Oprm1^{fl/fl}* mice. Jumping, teeth-chattering, wet-dog shakes, headshakes, grooming, piloerection, paw tremor, and twitching behaviors were analyzed from recorded videos. **L**, **M** Effects of morphine on locomotor activity of *Oprm1^{fl/fl}* and *Vglut2-Cre/Oprm1^{fl/fl}* mice. Travel distance and average speed were measured in the open field test before and after morphine administration ($n = 9-12$ mice/group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s., not significant (one-way ANOVA in C-K; two-way ANOVA between genotypes or morphine treatment in L and M, followed by a *post hoc* Bonferroni test). Data are presented as the mean $\pm$ SEM.

Vgat-Cre/Oprm1^{fl/fl} mice was slightly higher than their *Oprm1^{fl/fl}* littermates, while their brain weights were

comparable (Fig. S3A, B). We assessed both the *Oprm1* and MOR expression in *Vgat-Cre/Oprm1^{fl/fl}* mice, and found that they were largely abolished in GABAergic neuron-enriched brain areas such as the striatum, NAc, CeA, and IPN, while the MOR expression in non-GABAergic regions, such as the thalamus and PBN, was intact (Figs. 3, S1A, C, S3C, D). Thus, the expression of MORs was selectively abolished from GABAergic neurons in *Vgat-Cre/Oprm1^{fl/fl}* mice.

We next examined the functional role of MORs in GABAergic neurons in opioid dependence using the withdrawal procedure in *Vgat-Cre/Oprm1^{fl/fl}* mice (Fig. 4A). Naloxone administration induced robust withdrawal signs in *Oprm1^{fl/fl}* mice of the morphine group compared to the saline-treated control group (Fig. 4B-K). Deletion of MORs from GABAergic neurons had no significant effect on the naloxone-precipitated withdrawal symptoms, as evidenced by the comparable global

Fig. 3 MOR expression is abolished in multiple brain regions of *Vgat-Cre/Oprm1^{fl/fl}* mice. **A** Representative images of *Oprm1* expression in *Oprm1^{fl/fl}* and *Vgat-Cre/Oprm1^{fl/fl}* mice (scale bar, 500 μ m). **B** Representative images of MOR expression in *Oprm1^{fl/fl}* and *Vgat-Cre/Oprm1^{fl/fl}* mice determined by fluorescent immunostaining (scale bar, 200 μ m). NAc, nucleus accumbens; MHb, medial habenula; CeA, central amygdala; IPN, interpeduncular nucleus; PBN, parabrachial

nucleus. Cpu, striatum; NAc, nucleus accumbens; AcbC, accumbens nucleus, core; MHb, medial habenula; PV, paraventricular thalamic nucleus; CM, central medial thalamic nucleus; CeA, central amygdala; BLA, basolateral amygdala; IPN, interpeduncular nucleus; VTA, ventral tegmental area; PBN, parabrachial nucleus; scp, superior cerebellar peduncle; LPB, lateral parabrachial nucleus.

Fig. 4 Deletion of MORs in GABAergic neurons has limited effects on naloxone-precipitated withdrawal symptoms. **A** Experimental timeline for chronic morphine delivery and naloxone-precipitated withdrawal. **B, C** Time course of scores and total scores for naloxone-precipitated withdrawal in *Oprm1^{fl/fl}* and *Vgat-Cre/Oprm1^{fl/fl}* mice ($n = 6-8$ mice/group). **D-K** Bar graphs showing bouts and scores for each withdrawal symptom in *Oprm1^{fl/fl}* and *Vgat-Cre/Oprm1^{fl/fl}* mice. Jumping, teeth-chattering, wet-dog shakes, headshakes, grooming, piloerection, paw tremor, and twitching behaviors were analyzed from recorded videos. **L, M** Effects of morphine on the locomotor activity of *Oprm1^{fl/fl}* and *Vgat-Cre/Oprm1^{fl/fl}* mice. Travel distance and average speed were measured in the open field test before and after morphine administration ($n = 9-10$ mice/group). $^{**}P < 0.01$, $^{***}P < 0.001$, n.s., not significant (one-way ANOVA in **C-K**; two-way ANOVA between genotypes or morphine treatment in **L** and **M**, followed by a *post hoc* Bonferroni test). Data are presented as the mean $\pm$ SEM.

withdrawal scores in *Oprm1^{fl/fl}* and *Vgat-Cre/Oprm1^{fl/fl}* mice (Fig. 4B, C). Most withdrawal behaviors were consistent with each other, as *Vgat-Cre/Oprm1^{fl/fl}* mice showed almost intact withdrawal symptoms relative to *Oprm1^{fl/fl}* mice except for decreased jumping bouts (Fig. 4D-K). Although the jumping bouts in *Vgat-Cre/Oprm1^{fl/fl}* mice were significantly decreased, they were not totally blocked (Fig. 4D). In addition, morphine-induced locomotor hyperactivity was slightly but significantly lower in *Vgat-Cre/Oprm1^{fl/fl}* mice than in *Oprm1^{fl/fl}* mice, while the two groups showed similar basal locomotor activity (Fig. 4L, M). We also applied anxiety/depression-associated behavioral tests in *Vgat-Cre/Oprm1^{fl/fl}* mice, and found that the behavioral expression of *Oprm1^{fl/fl}* and *Vgat-Cre/Oprm1^{fl/fl}* mice were comparable (Fig. S3E-H), indicating normal anxiety-like behaviors in the *Vgat-Cre/Oprm1^{fl/fl}* mice. These results suggest that MORs expressed in GABAergic neurons play a minor role in mediating the development of morphine withdrawal and morphine-induced locomotor hyperactivity.

MORs Expressed in Dorsal Spinal Neurons Are Not Involved in Morphine Withdrawal

MORs are also highly expressed in the dorsal spinal cord, which exhibits adaptive changes during withdrawal [25, 28]. We thus explored the functional role of MORs expressed in the dorsal spinal cord in morphine withdrawal. To achieve this, we generated *Lbx1-Cre/Oprm1^{fl/fl}* mice by crossing *Lbx1-Cre* mice with *Oprm1^{fl/fl}* mice (Fig. 5A), which led to the selective deletion of MORs from dorsal spinal neurons. The expression of *Oprm1* was assessed by *in situ* hybridization, and the results showed that the *Oprm1* signal was lost in the dorsal spinal cord of *Lbx1-Cre/Oprm1^{fl/fl}* mice (Fig. 5B, C). Next, we determined the morphine withdrawal in *Lbx1-Cre/Oprm1^{fl/fl}* mice

(Fig. 5D), and found significant naloxone-precipitated withdrawal symptoms in both *Oprm1^{fl/fl}* and *Lbx1-Cre/Oprm1^{fl/fl}* mice with chronic morphine treatment compared to the control group (Fig. 5E, F). Furthermore, the behavioral expression of *Lbx1-Cre/Oprm1^{fl/fl}* mice was indistinguishable from *Oprm1^{fl/fl}* littermates, as both the global withdrawal score and the behavioral symptoms were comparable in these groups (Fig. 5E-N). In addition, the reduction of grooming bouts in the two morphine groups might be a result of the appearance of other withdrawal behaviors (Fig. 5K). We also examined the functional role of these MORs in morphine-induced locomotor hyperactivity, and found that *Oprm1^{fl/fl}* and *Lbx1-Cre/Oprm1^{fl/fl}* mice showed comparable travel distances and average speeds in the open field test both at baseline and after morphine injection (Fig. 5O, P). These results indicate that MORs expressed in dorsal spinal neurons are not involved in the development of morphine withdrawal and morphine-induced locomotor hyperactivity.

Discussion

Due to the wide distribution of opioid receptors, their activation gives rise to numerous side-effects besides analgesia, including opioid dependence. Both spinal and supraspinal mechanisms have been implicated in opioid withdrawal [9]. In this study, we determined the involvement of MORs expressed in different neuronal populations during morphine withdrawal using genetic approaches. We demonstrated that MORs expressed in *Vglut2⁺* glutamatergic neurons are essential for the development of morphine withdrawal and the morphine-induced locomotor hyperactivity, while those expressed in GABAergic neurons are not.

Using genetic approaches, we investigated the functional roles of MORs expressed in glutamatergic and GABAergic neurons in the opioid dependence that is characterized by morphine withdrawal. Our results showed that deletion of MORs expressed in *Vglut2⁺* glutamatergic neurons almost abolished the naloxone-precipitated withdrawal symptoms, indicating that these MORs play an important role in opioid dependence. The reduction of behaviors associated with morphine withdrawal in *Vglut2-Cre/Oprm1^{fl/fl}* mice is likely due to less neuronal adaptation. In wild-type mice, the absence of opioid receptor activation induced by naloxone unmasks the neuronal adaptation resulting from continuous opioid application, leading to acute naloxone-precipitated withdrawal. Accordingly, the activation of MORs in glutamatergic neurons by morphine results in long-term inhibition, and termination of the action of morphine may result in rebound activation of these neurons. Thus, the absence of MORs in

Fig. 5 Deletion of MORs in the dorsal spinal cord has no significant effect on naloxone-precipitated withdrawal symptoms. **A** Schematic of the protocol for deleting MORs from the dorsal spinal cord. Lbx1-Cre mice were crossed with *Oprm1*^{fl/fl} mice, and exons 2 and 3 of *Oprm1* were excised. **B, C** Representative images of *Oprm1* expression in the dorsal cord of *Oprm1*^{fl/fl} and Lbx1-Cre/*Oprm1*^{fl/fl} mice (scale bar, 200 μ m). **D** Experimental timeline for chronic morphine delivery and naloxone-precipitated withdrawal. **E, F** Time course of scores and total scores for naloxone-precipitated withdrawal in *Oprm1*^{fl/fl} and Lbx1-Cre/*Oprm1*^{fl/fl} mice ($n = 6$ –7 mice/group). **G–N** Bar graphs showing bouts and scores for each withdrawal symptom in *Oprm1*^{fl/fl} and Lbx1-Cre/*Oprm1*^{fl/fl} mice. Jumping, teeth-chattering, wet-dog shakes, headshakes, grooming, piloerection, paw tremor, and twitching behaviors were analyzed from recorded videos. **O, P** Effect of morphine on locomotor activity of *Oprm1*^{fl/fl} and Lbx1-Cre/*Oprm1*^{fl/fl} mice. Travel distance and average speed were measured in the open field test before and after morphine administration ($n = 6$ mice/group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s., not significant (one-way ANOVA in F–N; two-way ANOVA between genotypes or morphine treatment in O and P, followed by a *post hoc* Bonferroni test). Data are presented as the mean $\pm$ SEM.

glutamatergic neurons dramatically eliminates the withdrawal symptoms. Our results are consistent with a recent study showing that deletion of MORs from the MHB, in which most neurons are glutamatergic, decreases the withdrawal symptoms [19]. Thus, the reduction of withdrawal symptoms in our study may be partially due to the loss of MORs in the MHB. Interestingly, the influences of loss of MORs in Vglut2⁺ neurons on withdrawal symptoms are divergent. It is likely that different withdrawal symptoms are mediated by MORs expressed in brain structures that are differentially involved in aversive or anxiety circuits [37]. Thus, further studies are needed to dissect the role of MORs expressed in distinct glutamatergic neuronal subpopulations and neural circuits. MORs are also likely expressed in glutamatergic neurons labeled with other molecular markers, such as Vglut1⁺ and Vglut3⁺. However, the contribution of those MORs is likely to be minimal, as most withdrawal symptoms were largely reduced in mice lacking MORs in Vglut2⁺ glutamatergic neurons. Although several previous studies have demonstrated the involvement of the excitatory circuits for aversive behaviors during morphine withdrawal [10, 18], further studies are still necessary to further dissect the circuit mechanism underlying opioid withdrawal. In contrast, our study demonstrated that MORs expressed in GABAergic neurons play a negligible role in mediating morphine withdrawal, which is consistent with a previous study showing that selective expression of MORs in striatal neurons does not rescue the morphine withdrawal symptoms [17]. This has also been further confirmed in several recent studies showing that deletion of MORs from forebrain GABAergic neurons does not change the behavioral expression during morphine withdrawal [15]. Here,

we deleted MORs from all GABAergic neurons, thus extending investigations of the functional role of MORs expressed in all GABAergic neurons in morphine withdrawal. In addition, some of the withdrawal symptoms were intact with both the MOR conditional-knockout of Vglut2⁺ glutamatergic and GABAergic neurons, such as piloerection, suggesting that MORs expressed in other cell populations are also engaged during withdrawal.

Our results also demonstrated that MORs expressed in the dorsal spinal cord are not involved in morphine withdrawal. We selectively abolished their expression in the dorsal cord, and found that naloxone-precipitated withdrawal symptoms were not affected, suggesting that MORs expressed in the dorsal cord are not involved in the development of morphine withdrawal. As MORs are densely expressed in the ventral spinal cord, we cannot exclude their possible role in morphine withdrawal. However, recent studies have indicated that the spinal cord exhibits plasticity induced by ATP release from microglia during morphine withdrawal [28, 38], and the expression of MORs in microglia is very limited [13]. Further investigation is needed to address the functional role of spinal microglia in morphine withdrawal, including the action of opioids on microglia and neuron–glia interactions.

In summary, our study revealed the major neuronal subpopulation that mediates morphine withdrawal. These results provide important insights into the mechanism of morphine withdrawal, and lay a foundation for further dissection of the circuit mechanisms of opioid dependence. A better understanding of the mechanisms underlying opioid dependence will benefit the development of novel opioid analgesics with fewer side-effects.

Acknowledgements We thank Yan-Jing Zhu for technical support. This work was supported by the National Natural Science Foundation of China (31825013 and 61890952) and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB32010200).

References

1. Chen Y, Mestek A, Liu J, Hurley JA, Yu L. Molecular cloning and functional expression of a mu-opioid receptor from rat brain. *Mol Pharmacol* 1993, 44: 8–12.
2. Thompson RC, Mansour A, Akil H, Watson SJ. Cloning and pharmacological characterization of a rat mu opioid receptor. *Neuron* 1993, 11: 903–913.
3. Wang JB, Imai Y, Eppler CM, Gregor P, Spivak CE, Uhl GR. mu opiate receptor: cDNA cloning and expression. *Proc Natl Acad Sci U S A* 1993, 90: 10230–10234.
4. Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, *et al.* Loss of morphine-induced analgesia, reward

- effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* 1996, 383: 819–823.
5. Al-Hasani R, Bruchas MR. Molecular mechanisms of opioid receptor-dependent signaling and behavior. *Anesthesiology* 2011, 115: 1363–1381.
 6. Stein C. Opioid receptors. *Annu Rev Med* 2016, 67: 433–451.
 7. Corder G, Castro DC, Bruchas MR, Scherrer G. Endogenous and exogenous opioids in pain. *Annu Rev Neurosci* 2018, 41: 453–473.
 8. Williams JT, Christie MJ, Manzoni O. Cellular and synaptic adaptations mediating opioid dependence. *Physiol Rev* 2001, 81: 299–343.
 9. Burma NE, Kwok CH, Trang T. Therapies and mechanisms of opioid withdrawal. *Pain Manag* 2017, 7: 455–459.
 10. Valentinova K, Tchenio A, Trusel M, Clerke JA, Lalive AL, Tzanoulinou S, *et al.* Morphine withdrawal recruits lateral habenula cytokine signaling to reduce synaptic excitation and sociability. *Nat Neurosci* 2019, 22: 1053–1056.
 11. Maldonado R, Banos JE, Cabanero D. Usefulness of knockout mice to clarify the role of the opioid system in chronic pain. *Br J Pharmacol* 2018, 175: 2791–2808.
 12. Weibel R, Reiss D, Karchewski L, Gardon O, Matifas A, Filliol D, *et al.* Mu opioid receptors on primary afferent nav1.8 neurons contribute to opiate-induced analgesia: insight from conditional knockout mice. *PLoS One* 2013, 8: e74706.
 13. Corder G, Tawfik VL, Wang D, Sypek EI, Low SA, Dickinson JR, *et al.* Loss of mu opioid receptor signaling in nociceptors, but not microglia, abrogates morphine tolerance without disrupting analgesia. *Nat Med* 2017, 23: 164–173.
 14. Fields HL, Margolis EB. Understanding opioid reward. *Trends Neurosci* 2015, 38: 217–225.
 15. Charbogne P, Gardon O, Martin-Garcia E, Keyworth HL, Matsui A, Mechling AE, *et al.* Mu opioid receptors in gamma-aminobutyric acidergic forebrain neurons moderate motivation for heroin and palatable food. *Biol Psychiatry* 2017, 81: 778–788.
 16. Ben Hamida S, Boulos LJ, McNicholas M, Charbogne P, Kieffer BL. Mu opioid receptors in GABAergic neurons of the forebrain promote alcohol reward and drinking. *Addict Biol* 2019, 24: 28–39.
 17. Cui Y, Ostlund SB, James AS, Park CS, Ge W, Roberts KW, *et al.* Targeted expression of mu-opioid receptors in a subset of striatal direct-pathway neurons restores opiate reward. *Nat Neurosci* 2014, 17: 254–261.
 18. Zhu Y, Wienecke CF, Nachtrab G, Chen X. A thalamic input to the nucleus accumbens mediates opiate dependence. *Nature* 2016, 530: 219–222.
 19. Boulos LJ, Ben Hamida S, Bailly J, Maitra M, Ehrlich AT, Gaveriaux-Ruff C, *et al.* Mu opioid receptors in the medial habenula contribute to naloxone aversion. *Neuropsychopharmacology* 2020, 45: 247–255.
 20. Wang D, Tawfik VL, Corder G, Low SA, Francois A, Basbaum AI, *et al.* Functional divergence of delta and mu opioid receptor organization in CNS pain circuits. *Neuron* 2018, 98: 90–108 e105.
 21. Kline RH, Wiley RG. Spinal mu-opioid receptor-expressing dorsal horn neurons: role in nociception and morphine antinociception. *J Neurosci* 2008, 28: 904–913.
 22. Abbadie C, Lombard MC, Besson JM, Trafton JA, Basbaum AI. Mu and delta opioid receptor-like immunoreactivity in the cervical spinal cord of the rat after dorsal rhizotomy or neonatal capsaicin: an analysis of pre- and postsynaptic receptor distributions. *Brain Res* 2002, 930: 150–162.
 23. Raghavendra V, Rutkowski MD, DeLeo JA. The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats. *J Neurosci* 2002, 22: 9980–9989.
 24. Song P, Zhao ZQ. The involvement of glial cells in the development of morphine tolerance. *Neurosci Res* 2001, 39: 281–286.
 25. Mattioli TA, Leduc-Pessah H, Skelhorne-Gross G, Nicol CJ, Milne B, Trang T, *et al.* Toll-like receptor 4 mutant and null mice retain morphine-induced tolerance, hyperalgesia, and physical dependence. *PLoS One* 2014, 9: e97361.
 26. Ferrini F, Trang T, Mattioli TA, Laffray S, Del’Guidice T, Lorenzo LE, *et al.* Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl(-) homeostasis. *Nat Neurosci* 2013, 16: 183–192.
 27. Drdla R, Gassner M, Gingl E, Sandkuhler J. Induction of synaptic long-term potentiation after opioid withdrawal. *Science* 2009, 325: 207–210.
 28. Burma NE, Bonin RP, Leduc-Pessah H, Baimel C, Cairncross ZF, Mousseau M, *et al.* Blocking microglial pannexin-1 channels alleviates morphine withdrawal in rodents. *Nat Med* 2017, 23: 355–360.
 29. Sieber MA, Storm R, Martinez-de-la-Torre M, Muller T, Wende H, Reuter K, *et al.* Lbx1 acts as a selector gene in the fate determination of somatosensory and viscerosensory relay neurons in the hindbrain. *J Neurosci* 2007, 27: 4902–4909.
 30. Gao ZR, Chen WZ, Liu MZ, Chen XJ, Wan L, Zhang XY, *et al.* Tac1-expressing neurons in the periaqueductal gray facilitate the itch-scratching cycle via descending regulation. *Neuron* 2019, 101: 45–59 e49.
 31. Lupp A, Richter N, Doll C, Nagel F, Schulz S. UMB-3, a novel rabbit monoclonal antibody, for assessing mu-opioid receptor expression in mouse, rat and human formalin-fixed and paraffin-embedded tissues. *Regul Pept* 2011, 167: 9–13.
 32. Zhou W, Jin Y, Meng Q, Zhu X, Bai T, Tian Y, *et al.* A neural circuit for comorbid depressive symptoms in chronic pain. *Nat Neurosci* 2019, 22: 1649–1658.
 33. Guo QH, Tong QH, Lu N, Cao H, Yang L, Zhang YQ. Proteomic analysis of the hippocampus in mouse models of trigeminal neuralgia and inescapable shock-induced depression. *Neurosci Bull* 2018, 34: 74–84.
 34. Anthony TE, Dee N, Bernard A, Lerchner W, Heintz N, Anderson DJ. Control of stress-induced persistent anxiety by an extra-amygdala septohypothalamic circuit. *Cell* 2014, 156: 522–536.
 35. Liu MY, Yin CY, Zhu LJ, Zhu XH, Xu C, Luo CX, *et al.* Sucrose preference test for measurement of stress-induced anhedonia in mice. *Nat Protoc* 2018, 13: 1686–1698.
 36. Li Y, Liu J, Liu X, Su CJ, Zhang QL, Wang ZH, *et al.* Antidepressant-like action of single facial injection of botulinum neurotoxin A is associated with augmented 5-HT levels and BDNF/ERK/CREB pathways in mouse brain. *Neurosci Bull* 2019, 35: 661–672.
 37. Lutz PE, Kieffer BL. Opioid receptors: distinct roles in mood disorders. *Trends Neurosci* 2013, 36: 195–206.
 38. Trang T, Al-Hasani R, Salvemini D, Salter MW, Gutstein H, Cahill CM. Pain and poppies: the good, the bad, and the ugly of opioid analgesics. *J Neurosci* 2015, 35: 13879–13888.