Cellular/Molecular

Brief Opioid Exposure Paradoxically Augments Primary Afferent Input to Spinal Excitatory Neurons via α2δ-1–Dependent Presynaptic NMDA Receptors

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Treatment with opioids not only inhibits nociceptive transmission but also elicits a rebound and persistent increase in primary afferent input to the spinal cord. Opioid-elicited long-term potentiation (LTP) from TRPV1-expressing primary afferents plays a major role in opioid-induced hyperalgesia and analgesic tolerance. Here, we determined whether opioid-elicited LTP involves vesicular glutamate transporter-2 (VGluT2) or vesicular GABA transporter (VGAT) neurons in the spinal dorsal horn of male and female mice and identified underlying signaling mechanisms. Spinal cord slice recordings revealed that μ-opioid receptor (MOR) stimulation with DAMGO initially inhibited dorsal root–evoked EPSCs in 87% VGluT2 neurons and subsequently induced LTP in 49% of these neurons. Repeated morphine treatment increased the prevalence of VGluT2 neurons displaying LTP with a short onset latency. In contrast, DAMGO inhibited EPSCs in 46% VGAT neurons but did not elicit LTP in any VGAT neurons even in morphine-treated mice. Spinal superficial laminae were densely innervated by MOR-containing nerve terminals and were occupied by mostly VGluT2 neurons and few VGAT neurons. Furthermore, conditional *Grin1* knockout in dorsal root ganglion neurons diminished DAMGO-elicited LTP in lamina II neurons and attenuated hyperalgesia and analgesic tolerance induced by repeated treatment with morphine. In addition, DAMGO-elicited LTP in VGluT2 neurons was abolished by protein kinase C inhibition, gabapentin, *Cacna2d1* knockout, or disrupting the α2δ-1–NMDA receptor interaction with an α2δ-1 C terminus peptide. Thus, brief MOR stimulation distinctively potentiates nociceptive primary afferent input to excitatory dorsal horn neurons via α2δ-1–coupled presynaptic NMDA receptors, thereby causing hyperalgesia and reducing analgesic actions of opioids.

Key words: **DRG neuron; electrophysiology; NMDA receptor; opioid; pain; synaptic plasticity**

Significance Statement

Opioid drugs are potent analgesics for treating severe pain and are commonly used during general anesthesia. However, opioid use often induces pain hypersensitivity, rapid loss of analgesic efficacy, and dose escalation, which can cause dependence, addiction, and even overdose fatality. This study demonstrates for the first time that brief opioid exposure preferentially augments primary sensory input to genetically identified glutamatergic excitatory, but not GABAergic/glycinergic inhibitory, neurons in nociceptive dorsal horn circuits. This opioid-elicited synaptic plasticity is cell type specific and mediated by protein kinase C–dependent and α2δ-1–dependent activation of NMDA receptors at primary sensory nerve terminals. These findings elucidate how intraoperative use of opioids for preemptive analgesia paradoxically aggravates postoperative pain and increases opioid consumption and suggest new strategies to improve opioid analgesic efficacy.

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S.-R.C., H.C., D.J., and H.-L.P. are employees of the University of Texas System, which currently holds a patent for targeting α2δ-1–bound glutamate receptors for treating diseases and disorders.

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Introduction

The μ-opioid receptor (MOR) agonists remain the most efficacious and indispensable analgesic agents for treating moderate and severe pain caused by cancer, surgery, and trauma. However, MOR agonists often cause rapid loss of analgesic efficacy (tolerance) and dose escalation, which can result in addictive and abuse liability and even overdose death. Opioid analgesic tolerance can develop within hours or days in both animals and humans after acute opioid exposure (Vinik and Kissin, 1998; Chia et al., 1999; Célérier et al., 2000; Guignard

et al., 2000; Van Elstraete et al., 2005). Also, treatment with morphine or fentanyl can paradoxically induce long-lasting hyperalgesia (enhanced pain responses to noxious stimuli) in animals and in patients (Mao et al., 1994; Guignard et al., 2000; Chen et al., 2007; Van Elstraete et al., 2008; Zhao et al., 2012). Despite reports that intraoperative administration of opioids worsens postoperative pain and increases postoperative opioid consumption (Chia et al., 1999; Guignard et al., 2000; Joly et al., 2005; Kim et al., 2013), opioids are still commonly used during general anesthesia. The exact cellular and signaling mechanisms underlying opioid-induced hyperalgesia and tolerance remain elusive.

Both the analgesic and hyperalgesic effects of opioids are predominantly mediated by MORs expressed in primary sensory neurons, particularly MORs at primary afferent central terminals (Chen and Pan, 2006a,b; Zhao et al., 2012; Sun et al., 2019). Brief opioid exposure not only inhibits primary afferent input but also induces a rebound and sustained increase in glutamatergic input [i.e., long-term potentiation (LTP)] from TRPV1-expressing primary afferents to spinal dorsal horn neurons (Zhou et al., 2008, 2012; Sun et al., 2019). Neurons in the spinal dorsal horn are molecularly and functionally heterogenous, and they can be broadly classified into glutamatergic excitatory neurons and GABAergic/glycinergic inhibitory neurons (Grudt and Perl, 2002; Santos et al., 2007; Peirs et al., 2020; Sullivan and Sdrulla, 2022). In the superficial dorsal horn, most excitatory neurons express vesicular glutamate transporter 2 (VGluT2; Zhou et al., 2009; Wang et al., 2018), whereas inhibitory neurons express vesicular GABA transporter (VGAT, also known as vesicular inhibitory amino acid transporter; Chaudhry et al., 1998; Saito et al., 2010). Furthermore, VGluT2 neurons robustly facilitate, whereas VGAT neurons tonically inhibit nociceptive transmission from primary sensory nerves to the spinal cord (Koga et al., 2017; Wang et al., 2018; Huang et al., 2022). Although the phenotype of primary afferent nerves in opioid-triggered LTP has been identified, it is unclear how excitatory and inhibitory neurons in the spinal dorsal horn are involved in processing opioid-triggered primary afferent input.

NMDA receptors (NMDARs) at the spinal cord level play a key role in opioid-induced hyperalgesia and tolerance. Repeated treatment with MOR agonists augments NMDAR activity in the spinal cord (Zhao et al., 2012; Deng et al., 2019a,b), whereas blocking NMDARs reduces opioid-induced hyperalgesia and tolerance in animal models and in humans (Mao et al., 1994; Grande et al., 2008; Loftus et al., 2010; Zhao et al., 2012). In the spinal dorsal horn, opioid-elicited rebound LTP is mediated by presynaptic NMDARs, which counteract the analgesic effect of opioids (Chen and Pan, 2006b; Chen et al., 2007; Zhao et al., 2012; Zhou et al., 2012). However, little is known about the signaling mechanisms leading to rapid activation of presynaptic NMDARs by brief opioid exposure. In addition, gabapentinoids (gabapentin and pregabalin), frequently used for treating neuropathic pain, also reduce opioid-induced hyperalgesia and tolerance in animal models and in patients (Eckhardt et al., 2000; Gilron et al., 2003; Van Elstraete et al., 2008; Deng et al., 2019c). Gabapentinoids mainly bind to $\alpha2\delta$-1 proteins (encoded by the *Cacna2d1* gene; Gee et al., 1996; Marais et al., 2001; Fuller-Bicer et al., 2009), which interact directly with NMDARs via the C terminus of $\alpha2\delta$-1 to promote synaptic trafficking of NMDARs in the spinal cord (Chen et al., 2018, 2019; Zhang et al., 2021). At present, it is uncertain whether $\alpha2\delta$-1 is required for presynaptic NMDAR activation in the spinal cord initiated by brief opioid exposure.

In this study, we tested the hypothesis that brief MOR stimulation triggers LTP preferentially in excitatory spinal dorsal horn neurons via $\alpha2\delta$-1–bound NMDARs at primary afferent central terminals. Our study demonstrates for the first time that MOR stimulation elicits LTP predominantly in VGluT2-expressing excitatory neurons in the spinal cord. Also, we found that this opioid-triggered LTP requires protein kinase C (PKC) and $\alpha2\delta$-1–coupled presynaptic NMDARs. These findings shed new light in our holistic understanding of how opioids acutely elicit hyperalgesia and tolerance.

Materials and Methods

Animals. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center and conducted according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Male and female adult mice (25–32 g, 8–12 weeks of age) were used in this study and were maintained in pathogen-free conditions. These mice were housed at 24°C on a 12 h light/dark cycle with food and water *ad libitum*. *Cacna2d1* knock-out (KO) mice were obtained by crossing *Cacna2d1* heterozygous mice (catalog #6900, Medical Research Council). VGluT2-ires-Cre knock-in mice (catalog #028863), VGAT-ires-Cre knock-in mice (catalog #028862), and tdTomato$^{flox/flox}$ reporter mice (catalog #007909) were purchased from The Jackson Laboratory. VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ mice were obtained by mating male VGluT2-ires-Cre mice with female tdTomato$^{flox/flox}$ mice as described previously (Wang et al., 2018; Huang et al., 2022). Similarly, male VGAT-ires-Cre knock-in mice and female tdTomato$^{flox/flox}$ mice were crossed to generate VGAT$^{Cre/+}$::tdTomato$^{flox/flox}$ mice. The inhibitory neuronal marker Pax2 labels mostly VGAT-tagged neurons in the spinal dorsal horn of VGAT$^{Cre/+}$::tdTomato$^{flox/flox}$ mice (Browne et al., 2020). Conversely, Pax2 is not colocalized with VGluT2-tagged neurons in the spinal dorsal horn of VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ mice (Wang et al., 2018).

Grin1$^{flox/flox}$ mice (catalog #005246) were purchased from The Jackson Laboratory, and *Avil*$^{Cre/+}$ mice (da Silva et al., 2011) were provided by Fan Wang (Duke University). In brief, female *Grin1*$^{flox/flox}$ mice were crossed with male *Avil*$^{Cre/+}$ mice to obtain male *Avil*$^{Cre/+}$::*Grin1*$^{flox/+}$ mice, which were then crossed with female *Grin1*$^{flox/flox}$ mice to generate *Avil*$^{Cre/+}$::*Grin1*$^{flox/flox}$ mice, referred to as *Grin1* conditional knock-out (*Grin1*-cKO) mice (Huang et al., 2020; Zhang et al., 2021). Adult mice (9–11 weeks of age, sex and age matched) were used for final experiments. Cre-negative littermates were used as wild-type (WT) control mice, and all the mice were maintained on a C57BL/6J genetic background and were housed at no more than five per cage. Behavioral and electrophysiological data were pooled from males and females because no evident sex difference was detected during the course of our study.

Morphine treatment and nociceptive behavioral tests. For induction of opioid analgesic tolerance, mice were intraperitoneally injected with morphine (10 mg/kg, twice per day; catalog #0641-6127-25, West Ward Pharmaceuticals) for 8 d (Sun et al., 2019; Jin et al., 2022). On each day, we measured baseline nociceptive thresholds before morphine treatment and the peak analgesic effect produced 30 min after the first injection of 10 mg/kg morphine.

To determine tactile sensitivity, mice were individually placed in suspended chambers on a mesh floor. After acclimation for 30–40 min, a series of calibrated von Frey filaments was applied perpendicularly to the plantar surface of the hindpaw with sufficient force to bend them for 6 s. Brisk withdrawal or flinching of the paw was considered a positive response. In the absence of a response, the filament was applied at the next greater force. If the mouse showed a response, the filament was applied at the next lower force. The tactile stimulus producing a 50% likelihood of withdrawal response was calculated using the up-down method (Chaplan et al., 1994; Chen et al., 2019; Huang et al., 2020). Each test was repeated two to three times at 2 min intervals, and the mean value was used as the tactile withdrawal threshold.

Mechanical nociception in mice was measured using a digital Randall Selitto paw pressure testing device (catalog #2500, IITC Life

Science), as we described previously (Zhang et al., 2018; Jin et al., 2022). In brief, the mouse hindpaw was placed on the device, and the pointed end of a pressure applicator was applied to the midplantar glabrous surface of the hindpaw to generate a constantly increasing force. The force was immediately terminated when the animal withdrew the hindpaw or vocalized, and the value at that point was recorded as the pressure withdrawal threshold.

A thermal testing apparatus (catalog #390G, IITC Life Science) was used to quantify the nociceptive heat sensitivity as described previously (Chen and Pan, 2006a; Chen et al., 2014). Mice were placed individually in the transparent chamber on the glass surface maintained at 30°C and allowed to acclimate for 30–40 min. A radiant heat light source was positioned under the glass and targeted the midplantar surface of the hindpaw. The paw withdrawal latency was recorded by an electronic stopwatch that switched off the heat light source when the animal withdrew its hindpaw.

Spinal cord slice preparation and electrophysiological recordings. Under 2–3% isoflurane anesthesia, the lumbar segment of the mouse spinal cord was quickly removed via laminectomy. The spinal cord tissue was immediately placed in ice-cold, sucrose-based artificial CFS presaturated with 95% O_2 and 5% CO_2, and transverse spinal cord slices (400 μm) were cut using a vibratome. The sucrose artificial CFS contained the following (in mM): 206 sucrose, 10.0 glucose, 2.8 KCl, 1.0 $MgCl_2$, 2 $MgSO_4$, 1.0 $CaCl_2$, 1.25 NaH_2PO_4, and 26.0 $NaHCO_3$. The spinal cord slices were then preincubated in Krebs solution oxygenated with 95% O_2 and 5% CO_2 at 34°C for at least 1 h before recording. The Krebs solution contained the following (in mM): 117.0 NaCl, 3.6 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 1.2 NaH_2PO_4, 11.0 glucose, and 25.0 $NaHCO_3$.

EPSCs of lamina II neurons were recorded in spinal cord slices using the whole-cell voltage-clamp mode, as we described previously (Li et al., 2002; Pan and Pan, 2004; Zhou et al., 2012). In brief, the spinal cord slice was perfused continuously with Krebs solution at 5.0 ml/min at 34°C. The lamina II in the slice was identified as a distinct translucent band across the superficial dorsal horn on a fixed-stage microscope, and tdTomato-tagged VGluT2 or VGTA neurons in lamina II were identified and visualized using a combination of epifluorescence illumination and infrared and differential interference contrast optics. EPSCs were recorded at a holding potential of −60 mV with a glass electrode (impedance, 5–8 MΩ), which was filled with the following internal solution (in mM): 135 potassium gluconate, 5 KCl, 2.0 $MgCl_2$, 0.5 $CaCl_2$, 5.0 HEPES, 5.0 EGTA, 5.0 ATP-Mg, 0.5 Na-GTP, 1 guanosine 5′-O-(2-thiodiphosphate), and 10 lidocaine *N*-ethyl bromide that had been adjusted to pH 7.2–7.3 with 1 M CsOH (290–300 mOsm). EPSCs of lamina II neurons were evoked by electrical stimulation (0.6 mA and 0.2 ms) of the attached dorsal root in the slice, and monosynaptic EPSCs were identified on the basis of the constant latency and the absence of conduction failure of evoked EPSCs in response to a 20 Hz electrical stimulation as we described previously (Li et al., 2010; Zhou et al., 2012). Signals were recorded using an amplifier (MultiClamp 700B; Molecular Devices), filtered at 1–2 kHz, digitized at 10 kHz, and stored in a computer.

(D-Ala^2,*N*-Me-Phe^4,Gly-ol^5)-enkephalin (DAMGO; catalog #E7384) and gabapentin (catalog #1287303) were purchased from Millipore Sigma. Gö6983 (catalog #22851) was obtained from Tocris Bioscience. $\alpha 2\delta$-1 C terminus peptide (VSGLNPSLWSIFGLQFILLWLVSGSRHYLW) and scrambled control peptide (FGLGWQPWSLSFYLVWSGLILSVLHLIRSN), both fused to the Tat domain (YGRKKRRQRRR), were synthesized by SynPeptide Co and validated using liquid chromatography and mass spectrometry. All agents were freshly dissolved in artificial CFS before the recording.

Immunocytochemical labeling. *VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$* and *VGAT$^{Cre/+}$::tdTomato$^{flox/flox}$* mice were deeply anesthetized with intraperitoneal injection of Beuthanasia-D (0.1 ml/kg) and then transcardially perfused with 4% paraformaldehyde in 0.1 M PBS. The spinal cords at levels L3–L5 were dissected and postfixed for 2 h with the same fixative followed by 30% sucrose solutions in PBS for 24 h. The spinal cord sections were cut (25 µm thick) using a Leica microtome (model CM1860) and collected free floating in 0.1 M PBS buffer. Before antibody incubation, the sections were rinsed in 0.1 M PBS for 30 min and then blocked with 4% normal donkey serum for 60 min at 22°C. For double MOR and NeuN immunolabeling, sections were incubated in the mixture of rabbit anti-MOR antibody (dilution 1:1000; catalog #RA10104, Neuromics) and mouse anti-NeuN antibody (dilution 1:300; catalog #ab104224, Abcam) diluted in 0.1 M PBS solution containing 4% normal donkey serum for 2 h at 22°C and then 24 h at 4°C. The specificity of the MOR antibody has been validated using *Oprm1*-cKO mice (Sun et al., 2019, 2020). Subsequently, all sections were rinsed in 0.1 M PBS and were incubated with a secondary antibody (Alexa Fluor 488 conjugated to donkey anti-rabbit IgG, dilution 1:200; catalog #A-21206, Invitrogen; Alexa Fluor 647 conjugated to donkey anti-mouse IgG, dilution 1:200; catalog #A31571, Invitrogen) diluted in 4% normal goat serum in PBS for 2 h at 22°C.

For double IB4 and NeuN labeling, sections were incubated with mouse anti-NeuN (dilution 1:300; catalog #ab104224, Abcam) diluted in 0.1 M PBS solution containing 4% normal donkey serum for 2 h at 22°C and then 24 h at 4°C. After being rinsed in 0.1 M PBS for 30 min, sections were incubated with a secondary antibody (Alexa Fluor 647 conjugated to donkey anti-mouse IgG, dilution 1:200; Invitrogen) diluted in 4% normal goat serum in PBS for 2 h at 22°C. Then, tissue sections were rinsed in 0.1 M PBS for 20 min and incubated with Alexa Fluor 488 conjugated to IB4 (isolectin GS-IB4 from *Griffonia simplicifolia*, dilution 1:500; catalog #I21411, Invitrogen) for 2 h at 22°C. All sections were rinsed with 0.1 M PBS again and were then mounted on slides, dried, and coverslipped with an antifade mounting medium. The sections on slides were examined on a Zeiss laser scanning confocal microscope, and areas of interest were photodocumented. We randomly selected nine high-magnification images from three mice per group and used the ImageJ Cell Counter plug-in for quantitative cell counting.

Study design and data analysis. Data are presented as mean ± SEM. Data collection was randomized, and the investigators were blinded to the mouse genotypes and experimental treatments. For spinal cord slice recordings, at least six mice were used in each recording protocol, and only one neuron was recorded from each slice. Sample sizes, although not predetermined, were similar to those generally accepted in the field (Chen et al., 2007; Zhao et al., 2012; Zhou et al., 2012; Huang et al., 2022). The amplitude of evoked EPSCs was analyzed by averaging 2–3 consecutive EPSCs using Clampfit 11.0 software (Molecular Devices). Neurons with LTP were defined as those showing the increased amplitude of EPSCs at least 20% above the baseline and lasting >15 min after DAMGO washout (Zhou et al., 2012; Sun et al., 2019; Huang et al., 2022). The cell capacitance, input resistance, and series resistance were continuously monitored during recordings, and the recording was abandoned if any of these parameters changed by >15%. A two-tailed Student's *t* test was used to compare two groups. One-way or two-way ANOVA followed by Tukey's or Dunnett's *post hoc* test was used to determine the differences between more than two groups. A chi-square test was used for determining the difference in the proportion of neurons displaying LTP, a categorical variable, between the experimental groups, and $p < 0.05$ was considered statistically significant.

Results

NMDARs expressed in primary sensory neurons mediate opioid-induced hyperalgesia and analgesic tolerance

Prolonged treatment with morphine increases the activity of presynaptic NMDARs at the central terminals of dorsal root ganglion (DRG) neurons (Zhao et al., 2012; Deng et al., 2019a,c). The role of NMDARs at the spinal cord level are known to have a role in opioid-induced hyperalgesia and tolerance, as shown by intrathecal administration of NMDAR antagonists (Mao et al., 1994; Zhao et al., 2012). However, because intrathecally administered agents readily access both the DRG and spinal cord neurons (Cai et al., 2009), the relative contribution of NMDARs in primary sensory neurons to the development of opioid-induced hyperalgesia and tolerance remains uncertain. To this end, we generated *Avil$^{Cre/+}$::Grin1$^{flox/flox}$* (*Grin1*-cKO) mice in which GluN1, an obligatory subunit of NMDARs, is ablated in primary sensory neurons, as we described previously (Huang et al., 2020;

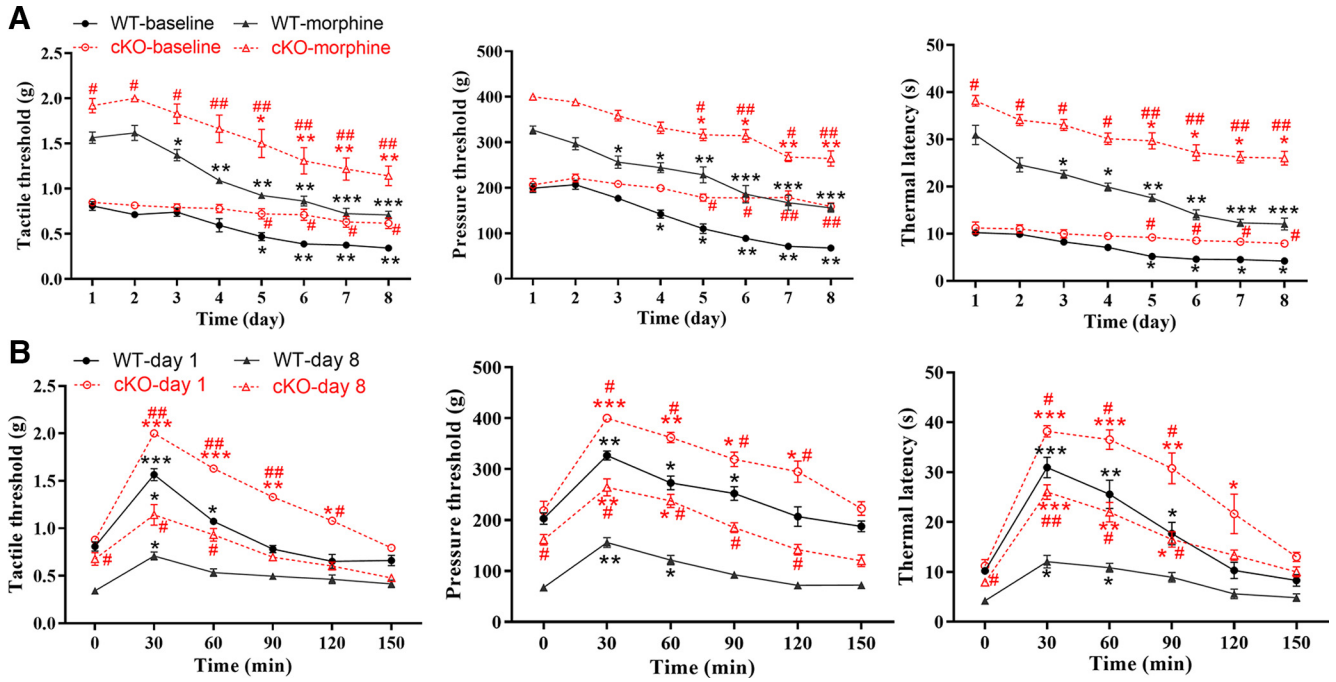

Figure 1. NMDARs in primary sensory neurons mediate opioid-induced hyperalgesia and analgesic tolerance. ***A***, Time course of changes in baseline paw withdrawal thresholds and the analgesic effect of morphine during daily intraperitoneal treatment with morphine (10 mg/kg, twice daily) for 8 d in WT and *Grin1*-cKO mice ($n = 8$ mice per group). The baseline thresholds were measured with von Frey filaments (tactile) and noxious pressure and heat stimuli 30 min before the first morphine injection daily; the acute analgesic effect of morphine was tested 30 min after the first morphine injection daily. ***B***, Comparison of the acute analgesic effect of the first morphine injection between WT and *Grin1*-cKO mice on day 1 and day 8 ($n = 6$ mice per group). Data are shown as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the respective initial time point (day 1 in ***A***; time 0 in ***B***); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the value at the same time point in the respective WT group. Two-way ANOVA followed by Tukey's *post hoc* test.

Zhang et al., 2021; Huang et al., 2022). Ablation of GluN1 in the DRG of *Grin1*-cKO mice has been validated in our previous study (Huang et al., 2020). Before treatment with morphine, the baseline withdrawal thresholds in response to tactile (von Frey filaments) and noxious heat and pressure stimuli applied to the hindpaw did not differ significantly between untreated WT and *Grin1*-cKO mice ($n = 8$ mice per group; Fig. 1*A*).

Intraperitoneal injection of morphine (10 mg/kg, twice per day) for 8 d in WT mice caused a gradual reduction in the baseline withdrawal thresholds (before the first injection of the day), indicating the presence of hyperalgesia ($n = 8$ mice; Fig. 1*A*). Repeated treatment with morphine also diminished the inhibitory effect of morphine (analgesic tolerance) over an 8 d period in the same WT mice (WT-morphine vs WT-baseline, Fig. 1*A*). In *Grin1*-cKO mice, repeated treatment with morphine did not induce a significant reduction over time in the baseline withdrawal thresholds ($n = 8$ mice; Fig. 1*A*). In addition, the reduction in the analgesic effect of morphine (30 min after the first injection of the day) was attenuated significantly in these *Grin1*-cKO mice (Fig. 1*A*,*B*). The inhibitory effect of morphine on the withdrawal thresholds over time on both day 1 and day 8 was greater in *Grin1*-cKO mice than in WT mice (Fig. 1*B*). These findings demonstrate directly that NMDARs expressed in primary sensory neurons play a major role in the development of opioid-induced hyperalgesia and tolerance.

Brief MOR stimulation triggers rebound LTP in the spinal dorsal horn via NMDARs expressed in primary sensory neurons

Opioid-elicited rebound LTP in the spinal dorsal horn is abolished when MORs in DRG neurons are genetically ablated (Sun et al., 2019). Because LTP triggered by acute opioid exposure is blocked by bath application of AP5 (a specific NMDAR antagonist), but not by postsynaptic dialysis with MK-801, an open-channel NMDAR blocker, presynaptic NMDARs are implicated in this event (Zhou et al., 2012). Next, we used spinal cord slices from WT and *Grin1*-cKO mice to corroborate the important role of presynaptic NMDARs expressed in primary sensory neurons in opioid-elicited LTP. We conducted whole-cell voltage-clamp recordings of lamina II neurons in perfused live spinal cord slices. In WT mice, bath application of 1 μM DAMGO, a highly specific MOR agonist (Kitchen et al., 1997; Zhou et al., 2010), for 3 min initially inhibited the amplitude of EPSCs in 40 of 47 lamina II neurons monosynaptically evoked from the dorsal root (Fig. 2*A*). DAMGO application had no effect on EPSCs in the remaining seven lamina II neurons tested. Among the 40 lamina II neurons showing initial inhibition of EPSCs by DAMGO, the amplitude of EPSCs of 19 (48%) neurons gradually returned to the baseline level 12–15 min after DAMGO washout. Notably, the other 21 (53%) lamina II neurons showed a persistent increase in the EPSC amplitude (i.e., LTP) ~12 min after DAMGO washout (Fig. 2*A*), which is similar to our previous reports (Zhou et al., 2010; Sun et al., 2020).

In *Grin1*-cKO mice, bath application of DAMGO initially inhibited evoked EPSCs in 23 of 28 lamina II neurons but had no effect on EPSCs in the remaining five lamina II neurons (Fig. 2*B*). Among 23 lamina II neurons showing an initial response to DAMGO, the amplitude of EPSCs in most (20/23, 87%) neurons returned to the baseline level after DAMGO washout. The remaining three (13%) lamina II neurons still showed a rebound LTP after DAMGO washout (Fig. 2*B*). However, the proportion of lamina II neurons with LTP was much smaller in *Grin1*-cKO mice than in WT mice [$p = 0.008$, χ^2 (2, $n = 75$) = 9.666; Fig. 2*C*]. The residual LTP in *Grin1*-cKO mice likely results from an incomplete removal of GluN1 in DRG neurons because *Advillin*-

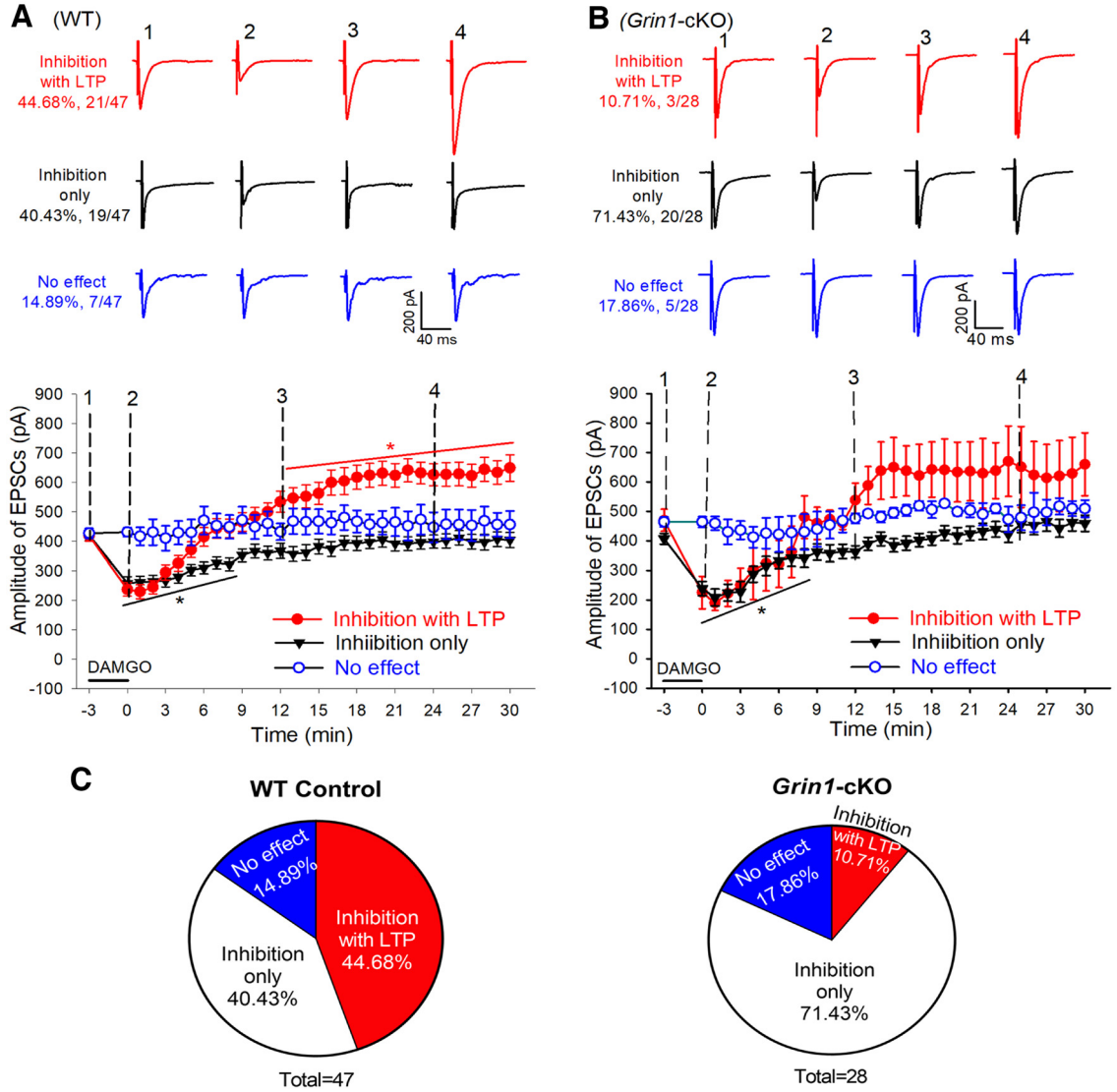

Figure 2. Acute MOR stimulation triggers rebound LTP in the spinal dorsal horn via NMDARs in primary sensory neurons. ***A***, ***B***, Representative recording traces and time course of changes in the amplitude of monosynaptic EPSCs evoked by dorsal root stimulation during baseline control, during bath application of 1 μM DAMGO, and after DAMGO washout in lamina II neurons from wild-type mice ($n = 47$ neurons from 10 mice, ***A***) and *Grin1*-cKO mice ($n = 28$ neurons from 8 mice, ***B***). Calibration: 200 pA, 40 ms. Current traces 1–4 were selected from the time points shown in the time course plot. ***C***, Pie charts show the proportion of spinal lamina II neurons displaying differential responses to DAMGO application in WT and *Grin1*-cKO mice. Data are shown as mean ± SEM; $^*p < 0.05$ compared with the respective baseline (−3 min in the same group) before DAMGO application. Repeated-measures ANOVA followed by Dunnett's *post hoc* test.

Cre–induced target gene KO occurs in 84% of DRG neurons (Zappia et al., 2017). These data indicate that presynaptic NMDARs play a major role in the opioid-elicited rebound increase in primary afferent input to spinal dorsal horn neurons.

Spatial distribution of MORs and VGluT2-expressing and VGAT-expressing neurons in the superficial spinal dorsal horn

In the spinal cord, most MOR proteins originate from DRG neurons and are expressed in the superficial dorsal horn (Chen and Pan, 2006b; Sun et al., 2019). To determine the spatial relationship between MORs and VGluT2- and VGAT-expressing neurons in the spinal cord, we conducted immunofluorescent labeling of MORs and NeuN, a neuronal marker, in the spinal cord from VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ and VGAT$^{Cre/+}$::tdTomato$^{flox/flox}$ mice. Confocal microscopic images showed that MOR-immunoreactive nerve terminals (Fig. 3, green) were densely distributed in laminae I and II. Also, tdTomato-tagged VGluT2 neurons were extensively distributed in spinal cord laminae I–IV, particularly laminae I and II (Fig. 3). In contrast, tdTomato-tagged VGAT neurons were mainly present in laminae III and IV, and only a small number of tdTomato-tagged VGAT neurons were present in laminae I and II (Fig. 3).

Furthermore, confocal images showed that IB4, a cellular marker for nonpeptidergic DRG neurons (Pan et al., 2003; Vydyanathan et al., 2005), was present at nerve terminals (Fig. 4, green) and was predominantly distributed in spinal laminae I and II. These IB4-positive terminals were in close contact mainly with tdTomato-tagged VGluT2 neurons and with only a few tdTomato-tagged VGAT neurons in laminae I and II (Fig. 4). Using IB4 fluorescent labeling as a boundary marker for laminae I and II, we quantified the number of VGluT2-expressing and VGAT-expressing neurons in this region. VGluT2 neurons were much more numerous than VGAT neurons in laminae I and II (77.89 ± 2.29 vs 9.44 ± 0.78 cells per high-magnification imaging field; $p < 0.001$, $t_{(8)} = 25.51$). These results indicate that

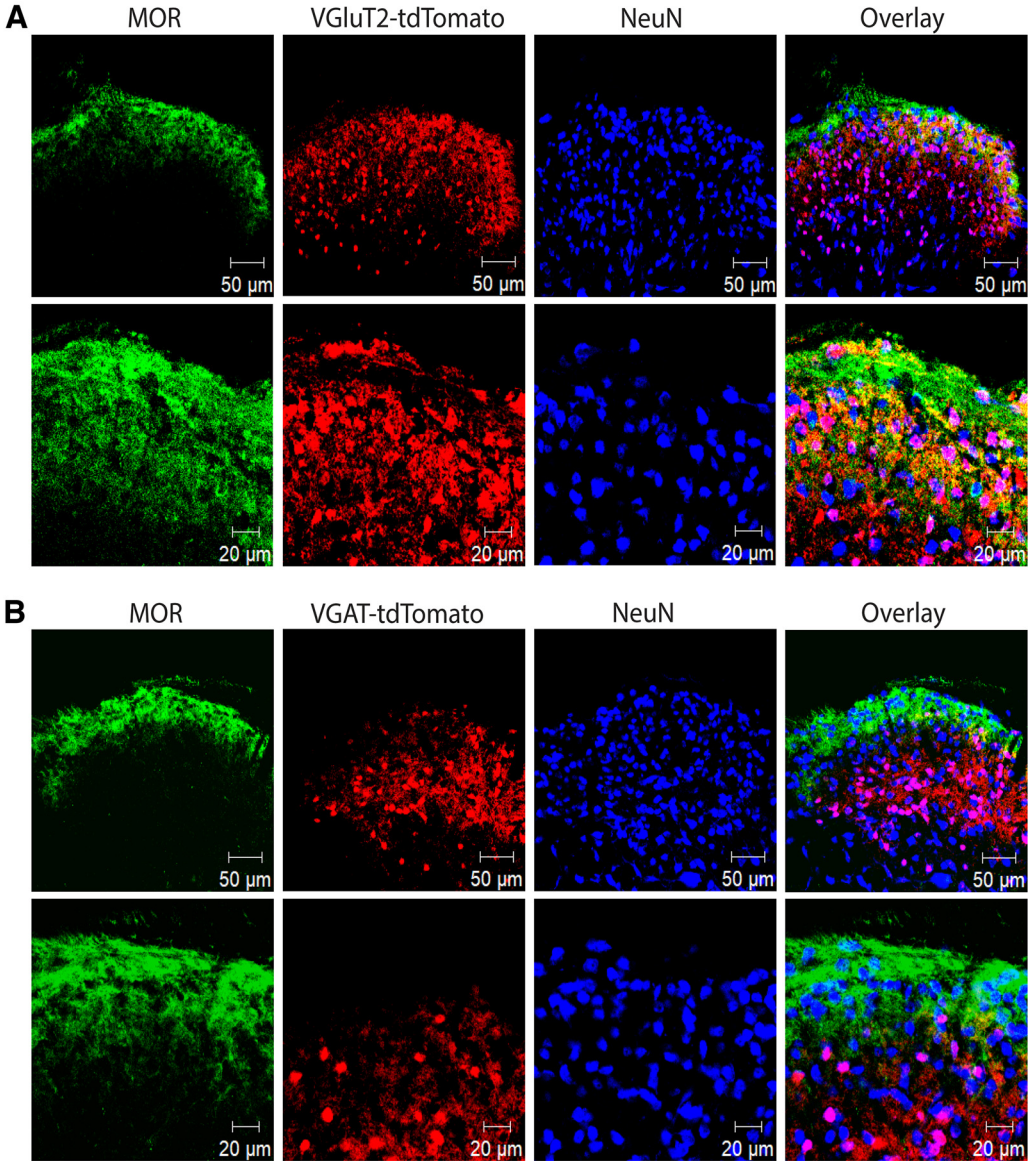

Figure 3. Spatial distribution of MORs and VGluT2- and VGAT-expressing neurons in the superficial spinal dorsal horn. ***A***, ***B***, Confocal microscopic images show the spatial relationship of MOR (green) immunoreactivity with tdTomato-labeled VGluT2-expressing neurons (red, ***A***) or tdTomato-labeled VGAT-expressing neurons (red, ***B***) in the superficial spinal dorsal horn of mice. NeuN, a neuronal marker, is shown in blue in ***A*** and ***B***. All images are single confocal optical sections. Scale bars: (in ***A***) 50 μm, top; 20 μm, bottom; (in ***B***) 50 μm, top; 20 μm, bottom.

spinal laminae I and II contain predominantly glutamatergic excitatory neurons, which are broadly innervated by MOR-expressing nerve terminals.

Brief MOR stimulation distinctively elicits rebound LTP in VGluT2-expressing, but not VGAT-expressing, neurons in the spinal dorsal horn

VGluT2-expressing neurons in the spinal dorsal horn play a crucial role in the relay of nociceptive information from primary sensory neurons (Wang et al., 2018). Also, increased primary afferent nerve activity triggers LTP predominantly in VGluT2-expressing dorsal horn neurons (Huang et al., 2022). We thus used VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ mice to determine how acute MOR stimulation affects glutamatergic input from primary afferents to VGluT2-expressing neurons. We visually identified and recorded tdTomato-tagged VGluT2 neurons in lamina II in perfused spinal cord slices. Bath application of 1 μM DAMGO for 3 min markedly inhibited the baseline amplitude of monosynaptic EPSCs evoked from the dorsal root in most (39 of 45, 87%) VGluT2 neurons (Fig. 5*A*). DAMGO had no effect on the baseline amplitude of evoked EPSCs in the remaining six VGluT2 neurons. Among the 39 VGluT2 neurons with initial inhibition of EPSCs by DAMGO, the amplitude of EPSCs in 20 (51%) neurons gradually returned to the baseline level after washout of DAMGO. In the other 19 (49%) VGluT2 neurons showing initial EPSC inhibition by DAMGO, there was a profound and persistent increase in the amplitude of EPSCs after DAMGO washout (Fig. 5*A*). The proportion of VGluT2 neurons displaying opioid-induced rebound LTP was similar to that of unlabeled lamina II neurons in rats and WT mice (Fig. 2; Zhou et al., 2010; Sun et al., 2019).

VGAT-expressing neurons tonically inhibit nociceptive transmission in the spinal cord (Koga et al., 2017). Next, we determined how MOR stimulation affects primary afferent input to tdTomato-tagged VGAT neurons in the spinal dorsal horn of VGAT$^{Cre/+}$::tdTomato$^{flox/flox}$ mice. Bath application of 1 μM DAMGO for 3 min profoundly reduced the baseline amplitude of evoked monosynaptic EPSCs in 21 of 46 (46%) VGAT-

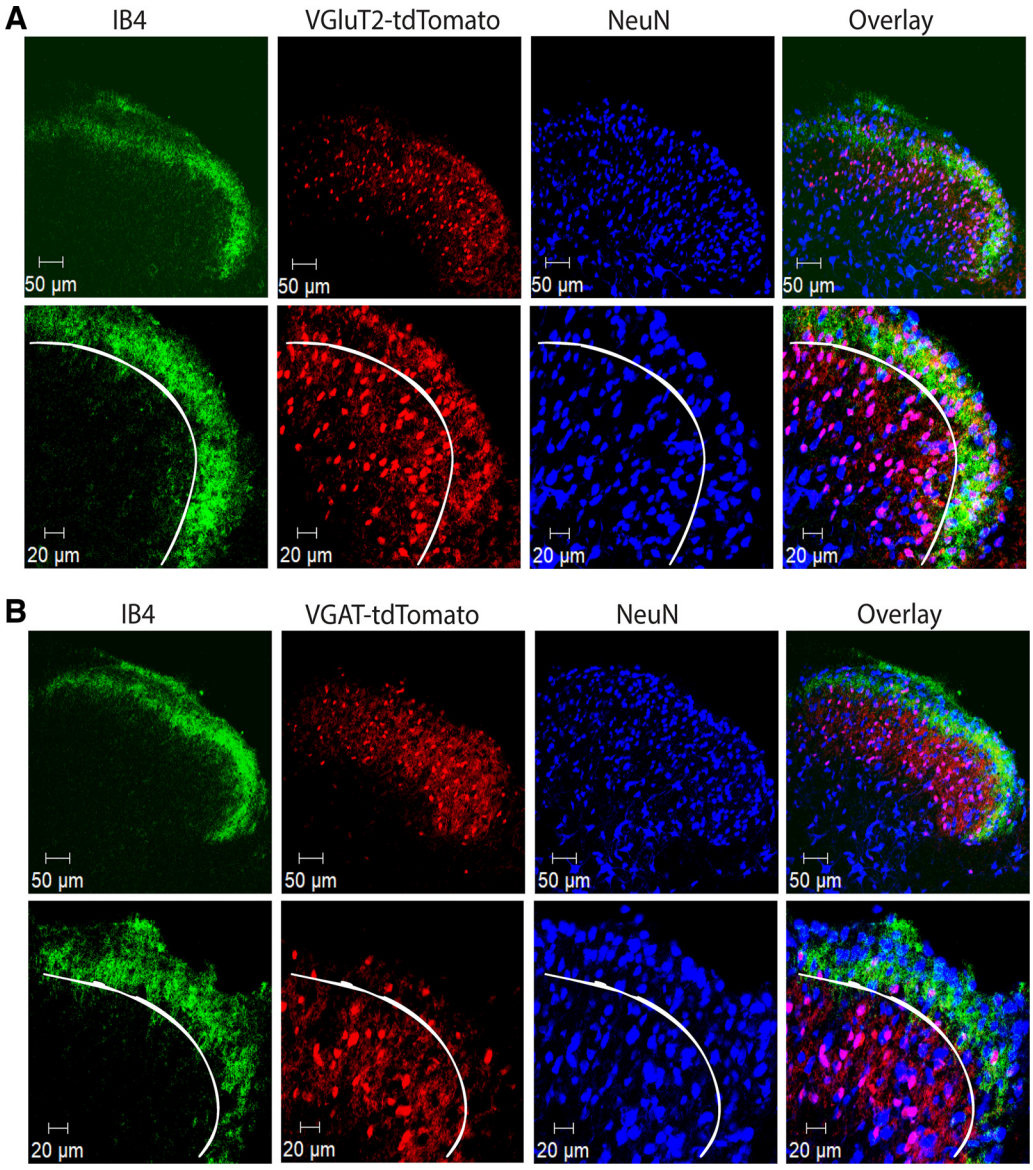

Figure 4. Spatial distribution of IB4 and VGluT2- and VGAT-expressing neurons in the superficial spinal dorsal horn. ***A***, ***B***, Confocal microscopic images show the spatial relationship of IB4 labeling (green) with tdTomato-labeled VGluT2-expressing neurons (red, ***A***) or tdTomato-labeled VGAT-expressing neurons (red, ***B***) in the mouse superficial dorsal horn. NeuN, a neuronal marker, is shown in blue in ***A*** and ***B***. White lines indicate the corresponding boundary of IB4 labeling. All images are single confocal optical sections. Scale bars: (in ***A***) 50 μm, top; 20 μm, bottom; (in ***B***) 50 μm, top; 20 μm, bottom.

expressing neurons in lamina II (Fig. 5*B*). The proportion of VGAT neurons inhibited by DAMGO was much smaller than that of VGluT2 neurons [46 vs 87%; $p < 0.001$, χ^2 (1, $n = 91$) = 17.036]. DAMGO had no effect on the amplitude of evoked EPSCs in the other 25 (54%) VGAT neurons in lamina II (Fig. 5*B*). No rebound LTP was observed in any VGAT neurons within 30 min of recordings after DAMGO washout. These findings demonstrate that brief MOR stimulation preferentially elicits a rebound increase in primary afferent input to glutamatergic excitatory neurons in the spinal dorsal horn.

Prolonged treatment with morphine increases the prevalence of spinal VGluT2 neurons with opioid-triggered LTP

Next, we determined whether repeated treatment with morphine alters the inhibitory and stimulatory effect of DAMGO on evoked monosynaptic EPSCs of tdTomato-tagged VGluT2 neurons in lamina II. We first treated VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ mice with intraperitoneal injection of morphine (10 mg/kg, twice per day) for 8 d, a protocol that consistently causes hyperalgesia and analgesic tolerance in mice (Sun et al., 2019; Jin et al., 2022). In morphine-treated VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ mice, bath application of DAMGO inhibited the amplitude of evoked EPSCs in 28 of 31 tdTomato-tagged VGluT2 neurons and had no effect on EPSCs in the remaining three VGluT2 neurons (Fig. 6*A*). Among the 28 VGluT2 neurons showing initial EPSC inhibition by DAMGO, the amplitude of EPSCs returned to the baseline in six (21%) neurons after DAMGO washout. Strikingly, 22 of these 28 (79%) VGluT2 neurons displayed a rebound LTP after DAMGO washout (Fig. 6*A*). This proportion of VGluT2 neurons with LTP was significantly greater in the morphine-treated mice than in the naive mice in the previous experiments [$p = 0.0408$, χ^2 (2, $n = 76$) = 6.396; Fig. 6*C*]. Also, the rebound LTP after DAMGO washout appeared more quickly in the 22 VGluT2 neurons from morphine-treated mice, compared with that in the 19 VGluT2 neurons with LTP recorded from naive mice (onset

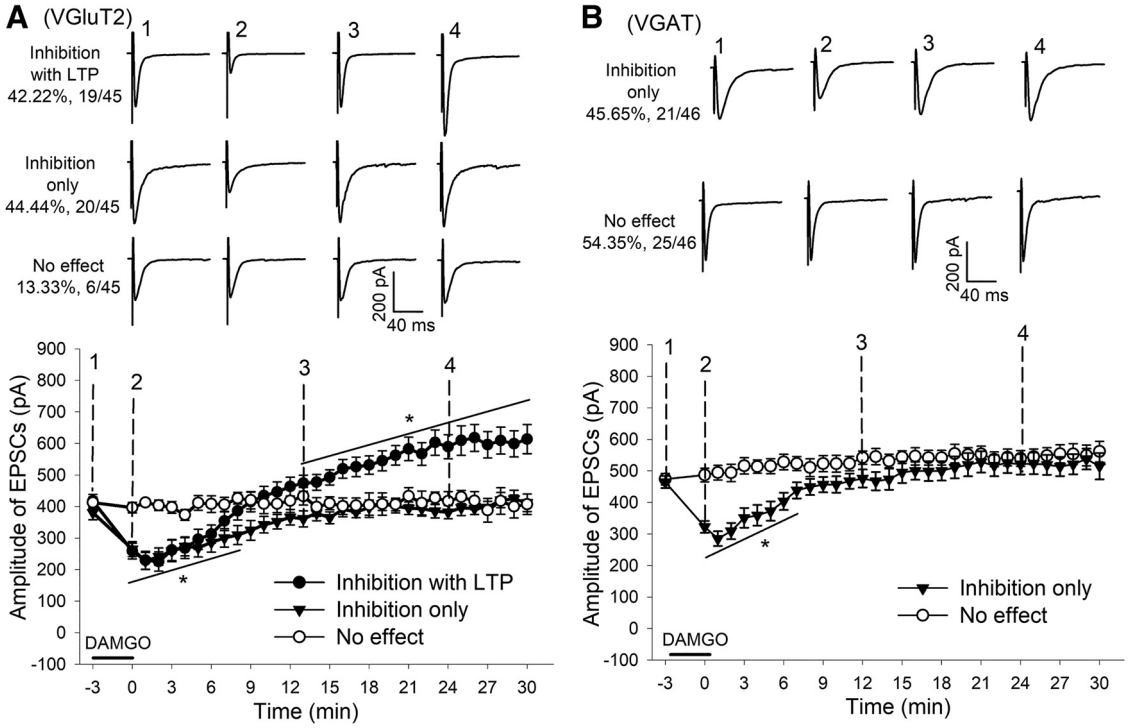

Figure 5. Brief MOR stimulation elicits rebound LTP in VGluT2-expressing, but not VGAT-expressing, neurons in the spinal dorsal horn. ***A***, ***B***, Representative recording traces and time course of changes in the amplitude of EPSCs monosynaptically evoked by dorsal root stimulation during baseline control, during bath application of 1 μM DAMGO, and after DAMGO washout in tdTomato-labeled VGluT2 neurons (***A***, $n = 45$ neurons from 10 mice) and tdTomato-labeled VGAT neurons (***B***, $n = 46$ neurons from 10 mice) in lamina II in spinal cord slices obtained from VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ and VGAT$^{Cre/+}$::tdTomato$^{flox/flox}$ mice. Calibration: 200 pA, 40 ms. Current traces 1–4 were selected from the time points shown in the time course plot. Data are shown as mean ± SEM; *$p < 0.05$ compared with the respective baseline (–3 min in the same group) before DAMGO application. Repeated-measures ANOVA followed by Dunnett *post hoc* test.

latency of LTP, 9.33 ± 0.371 min vs 13.18 ± 0.286 min; $p < 0.001$, $t_{(39)} = 8.025$; Figs. 5*A*, 6*A*). In addition, the baseline amplitude of EPSCs in these VGluT2 neurons with LTP was significantly larger in the morphine-treated mice than in the naive mice (538.7 ± 17.94 pA vs 417.4 ± 20.95 pA; $p < 0.001$, $t_{(39)} = 4.424$), likely because of tonic activation of presynaptic NMDARs caused by prolonged treatment with morphine (Zhao et al., 2012; Deng et al., 2019c).

We then determined whether repeated treatment with morphine has an impact on the effect of DAMGO on evoked EPSCs of tdTomato-tagged VGAT neurons in lamina II. In VGAT$^{Cre/+}$::tdTomato$^{flox/flox}$ mice subjected to repeated systemic treatment with morphine for 8 d, bath application of 1 μM DAMGO inhibited the amplitude of EPSCs in 8 of 25 VGAT neurons and had no effect on EPSCs in the other 17 VGAT neurons (Fig. 6*B*). No LTP was observed after DAMGO washout in any VGAT neurons examined from morphine-treated mice. The proportion of VGAT neurons with EPSCs inhibited by DAMGO did not differ significantly between morphine-treated mice and naive mice [$p = 0.264$, χ^2 (1, $n = 71$) = 1.249; Fig. 6*D*]. These data indicate that repeated treatment with opioids augments opioid-triggered primary afferent input to excitatory, but not inhibitory, spinal dorsal horn neurons.

PKC activity is required for opioid-triggered rebound LTP in VGluT2-expressing dorsal horn neurons

Increased PKC activity at the spinal cord level plays an important role in opioid-induced hyperalgesia and tolerance (Mao et al., 1994; Granados-Soto et al., 2000) and in the control of phosphorylation and activity of synaptic NMDARs in the spinal cord (Zhao et al., 2012; Xie et al., 2016; 2017; Zhou et al., 2021). Also, MOR stimulation leads to downstream PKC-mediated signaling in DRG neurons (Xie et al., 1999; Hong et al., 2010). We subsequently determined the signaling mechanism responsible for presynaptic NMDAR activation induced by DAMGO application. Because opioid-elicited LTP occurred only in VGluT2-expressing neurons, we focused the following experiments on tdTomato-tagged VGluT2 neurons in lamina II of VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ mice.

We treated the spinal cord slices of VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ mice with 1 μM Gö6983, a highly specific PKC inhibitor (Gschwendt et al., 1996; Zhou et al., 2021), for 25–30 min immediately before whole-cell recording of tdTomato-tagged VGluT2 neurons in lamina II. Bath application of 1 μM DAMGO for 3 min caused initial inhibition of monosynaptic EPSCs evoked from the dorsal root in 21 of 24 (88%) VGluT2 neurons and had no effect on the amplitude of EPSCs in the other three VGluT2 neurons (Fig. 7). PKC inhibition did not significantly change the percentage of VGluT2 neurons initially inhibited by DAMGO. After DAMGO washout, none of the 24 VGluT2 neurons tested showed LTP in spinal cord slices pretreated with Gö6983. These results suggest that PKC activity is required for the opioid-triggered rebound increase in primary afferent input to glutamatergic spinal dorsal horn neurons.

α2δ-1 Is essential for opioid-triggered rebound LTP in VGluT2-expressing dorsal horn neurons

α2δ-1 is a newly discovered NMDAR-interacting protein and promotes synaptic trafficking and activity of NMDARs in the brain and spinal cord (Chen et al., 2018; Luo et al., 2018; Zhou et al., 2018). We next used gabapentin, an α2δ-1 inhibitory ligand (Gee et al., 1996; Chen et al., 2018), to determine whether α2δ-1 plays a role in opioid-triggered LTP. We treated spinal cord slices

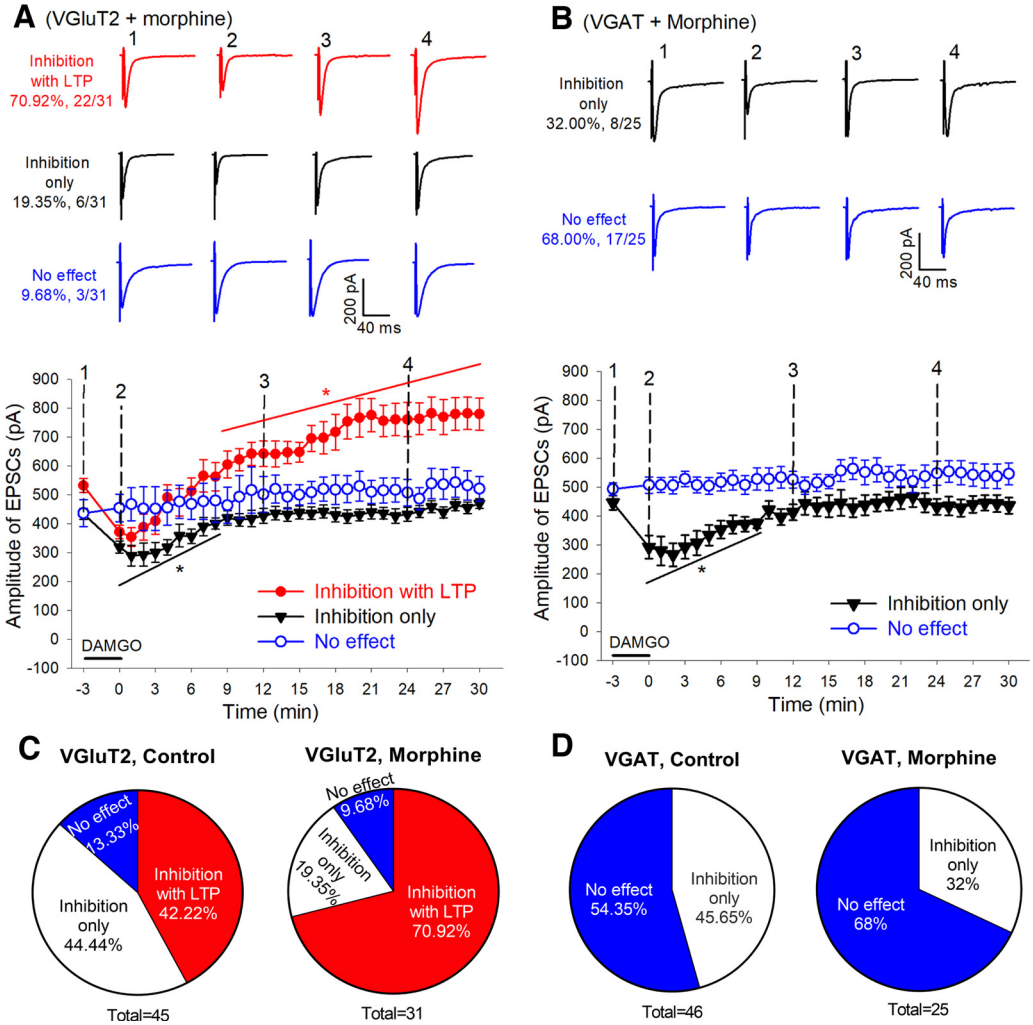

Figure 6. Prolonged treatment with morphine increases the prevalence of spinal VGluT2 neurons with opioid-triggered LTP. ***A***, ***B***, Representative recording traces and time course of changes in the amplitude of dorsal root–evoked EPSCs during baseline control, during bath application of 1 μM DAMGO, and after DAMGO washout in tdTomato-labeled VGluT2 neurons (***A***, $n = 31$ neurons from 7 mice) and VGAT neurons (***B***, $n = 25$ neurons from 7 mice) in lamina II. Spinal cord slices were obtained from VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ and VGAT$^{Cre/+}$::tdTomato$^{flox/flox}$ mice treated with intraperitoneal injection of morphine (10 mg/kg, twice daily) for 8 d. Calibration: 200 pA, 40 ms. Current traces 1–4 were selected from the time points shown in the time course plot. ***C***, ***D***, Pie charts show the effect of prolonged treatment with morphine on the proportion of spinal cord VGluT2 neurons (***C***) and VGAT neurons (***D***) displaying differential responses to DAMGO application. Data are shown as mean ± SEM; *$p < 0.05$ compared with the respective baseline (–3 min in the same group) before DAMGO application. Repeated-measures ANOVA followed by Dunnett's *post hoc* test.

from VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ mice with 100 μM gabapentin for 25–30 min immediately before electrophysiological recording. In spinal cord slices treated with gabapentin, bath application of DAMGO for 3 min produced an initial inhibition of EPSCs in 21 of 24 (88%) VGluT2 neurons and had no effect on the baseline amplitude of EPSCs in the other three VGluT2 neurons. None of the 24 VGluT2 neurons recorded in lamina II displayed LTP after DAMGO washout (Fig. 8*A*).

Because gabapentinoids bind to both $\alpha2\delta$-1 and $\alpha2\delta$-2 (Gee et al., 1996; Marais et al., 2001), we then used conventional *Cacna2d1* KO mice (Fuller-Bicer et al., 2009) to validate the role of $\alpha2\delta$-1 in DAMGO-triggered LTP. Bath application of DAMGO for 3 min inhibited EPSCs in 26 of 31 (84%) lamina II neurons and had no effect on the baseline amplitude of EPSCs in the other five lamina II neurons (Fig. 8*B*). No LTP was detected after DAMGO washout in all 31 lamina II neurons recorded from *Cacna2d1* KO mice. $\alpha2\delta$-1 inhibition or *Cacna2d1* KO did not change significantly the percentage of VGluT2 neurons initially inhibited by DAMGO. These data provide strong evidence showing that $\alpha2\delta$-1 is essential for opioid-triggered potentiation in primary afferent input to spinal excitatory dorsal horn neurons.

Opioid exposure triggers rebound LTP in VGluT2-expressing dorsal horn neurons via α2δ-1–coupled NMDARs

Finally, we determined whether opioid-triggered LTP in the spinal cord requires $\alpha2\delta$-1–bound NMDARs. We have previously shown that $\alpha2\delta$-1 forms a protein complex with NMDARs via its C terminus (an intrinsically disordered protein region) and that the $\alpha2\delta$-1 C terminus–interfering peptide fused with Tat ($\alpha2\delta$-1CT peptide) effectively disrupts the $\alpha2\delta$-1–NMDAR interaction (Chen et al., 2018; Luo et al., 2018). Furthermore, $\alpha2\delta$-1 functions as a phospho-binding protein and mainly interacts with phosphorylated NMDARs by PKC activation (Zhou et al., 2021). We treated spinal cord slices from VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ mice with 1 μM $\alpha2\delta$-1CT peptide or 1 μM Tat-fused control peptide for 25–30 min immediately before slice recording. In spinal cord slices pretreated with the control peptide, bath application of DAMGO for 3 min initially inhibited EPSCs in 26 of 30 (87%) tdTomato-tagged VGluT2 neurons and

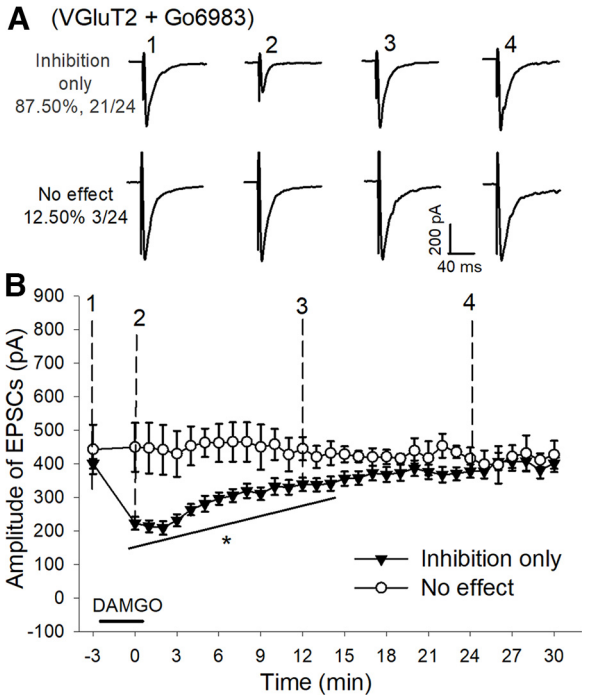

Figure 7. PKC activity is required for opioid-triggered rebound LTP in VGluT2-expressing dorsal horn neurons. ***A***, ***B***, Representative recording traces (***A***) and time course of changes (***B***) in the amplitude of dorsal root–evoked EPSCs during baseline control, during bath application of 1 μM DAMGO, and after DAMGO washout in tdTomato-labeled VGluT2 neurons in lamina II ($n = 24$ neurons from 6 mice). Spinal cord slices were obtained from VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ mice and were pretreated with 1 μM Gö6983 before recordings. Calibration: 200 pA, 40 ms. Current traces 1–4 were selected from the time points shown in the time course plot. Data are shown as mean ± SEM; $^{*}p < 0.05$ compared with the respective baseline (−3 min in the same group) before DAMGO application. Repeated-measures ANOVA followed by Dunnett's *post hoc* test.

induced LTP after DAMGO washout in 13 of 26 (50%) DAMGO-responsive VGluT2 neurons in lamina II (Fig. 9*A*). By contrast, in spinal cord slices pretreated with the $\alpha2\delta$-1CT peptide, DAMGO application produced initial inhibition of EPSCs in 20 of 24 (83%) VGluT2 neurons. After DAMGO washout, no LTP was recorded in any VGluT2 neurons pretreated with $\alpha2\delta$-1CT peptide (Fig. 9*B*). Treatment with $\alpha2\delta$-1CT peptide had no significant effect on the percentage of VGluT2 neurons initially inhibited by DAMGO. These data suggest that opioid-triggered potentiation in primary afferent input to excitatory dorsal horn neurons depends on $\alpha2\delta$-1–bound NMDARs.

Discussion

Our study provides the first direct evidence that NMDARs expressed in primary sensory neurons mediate opioid-induced hyperalgesia and tolerance. Chronic opioid treatment induces a progressive reduction in the nociceptive thresholds, which occurs in parallel with the development of analgesic tolerance (Mao et al., 1994; Célèrier et al., 2000; Guignard et al., 2000; Chen et al., 2007; Zhao et al., 2012; Deng et al., 2019b). Some evidence suggests a strong link between hyperalgesia and analgesic tolerance induced by opioids, but the unifying cellular and molecular mechanisms for these two important phenomena are poorly understood. Prolonged treatment with morphine mainly increases presynaptic NMDAR activity at primary afferent central terminals (Zhao et al., 2012; Deng et al., 2019c). In the present study, we showed that genetic ablation of *Grin1* in primary sensory neurons in mice not only diminished hyperalgesia but also attenuated analgesic tolerance caused by repeated treatment with morphine. Notably, *Grin1*-cKO mice were largely resistant to the development of hyperalgesia but still manifested evident tolerance after repeated treatment with morphine. Repeated treatment with opioids may still produce analgesic tolerance via other signaling mechanisms, including impaired MOR synaptic trafficking and MOR–G-protein coupling. For example, previous studies using cell lines expressing MORs or cultured brain neurons suggest that the loss of the analgesic effect of opioids may result from MOR endocytosis, increased cAMP levels, augmented activity of G-protein-coupled receptor kinases, and/or biased arrestin-MAPK signaling (Clark and Traynor, 2005; Dang and Christie, 2012; Williams et al., 2013; Gomes et al., 2020). However, the relevance of these *in vitro* findings to opioid tolerance is uncertain. Because our results in *Grin1*-cKO mice are similar to the effects of intrathecal injection of NMDAR antagonists (Mao et al., 1994; Zhao et al., 2012), these findings indicate a crucial role of presynaptic NMDARs in the spinal cord in the development of opioid-induced hyperalgesia and analgesic tolerance.

By using mice in which functional NMDARs are ablated in DRG neurons, we provide complementary evidence that reinforces the notion that opioid-elicited LTP results from activation of presynaptic NMDARs expressed at primary afferent central terminals (Zhou et al., 2012). In this study, we used spinal cord slices in which native MORs are expressed in a well-defined cellular circuitry that is crucially involved in the analgesic and hyperalgesic actions of opioids (Chen and Pan, 2006b; Zhao et al., 2012; Sun et al., 2019). The first sensory synapse formed by primary afferent terminals and second-order neurons in the spinal dorsal horn are integral to nociceptive transmission and regulation (Grudt and Perl, 2002; Pan and Pan, 2004; Sun et al., 2019; Sullivan and Sdrulla, 2022). NMDARs at primary afferent terminals in the spinal dorsal horn are normally latent but become tonically activated to amplify nociceptive input in neuropathic pain and opioid-induced hyperalgesia (Zhao et al., 2012; Xie et al., 2016; Deng et al., 2019a, c). We showed that DAMGO-induced rebound LTP was diminished, whereas DAMGO-induced initial inhibition of EPSCs was not affected in *Grin1*-cKO mice. Because DAMGO-triggered LTP can be used as a real-time measure of activation of NMDARs at primary afferent central terminals, our study suggests that these presynaptic NMDARs become tonically activated within ~12 min after DAMGO removal or withdrawal. MOR activation typically attenuates neurotransmitter release via inhibition of voltage-gated Ca^{2+} channels in primary sensory neurons (Wu et al., 2004). It is possible that initial inhibition of voltage-gated Ca^{2+} channels by DAMGO may mask the early appearance of LTP. From a homeostatic point of view, NMDAR-mediated rebound LTP could be considered a compensatory response to restore intracellular Ca^{2+} levels after initial profound Ca^{2+} channel–mediated inhibition of neurotransmitter release at primary afferent central terminals. We found in this study that the analgesic effect of morphine on both day 1 and day 8 was potentiated in *Grin1*-cKO mice, supporting the concept that this presynaptic NMDAR-mediated LTP serves to antagonize the analgesic action of opioids.

Our study reveals the divergence between excitatory and inhibitory spinal dorsal horn neurons in opioid-induced LTP, showing the prominent role of NMDARs at primary afferent–excitatory neuron synapses in opioid-induced hyperalgesia and tolerance. In this study, we specifically determined the effects of DAMGO on VGluT2-expressing excitatory and VGAT-expressing inhibitory

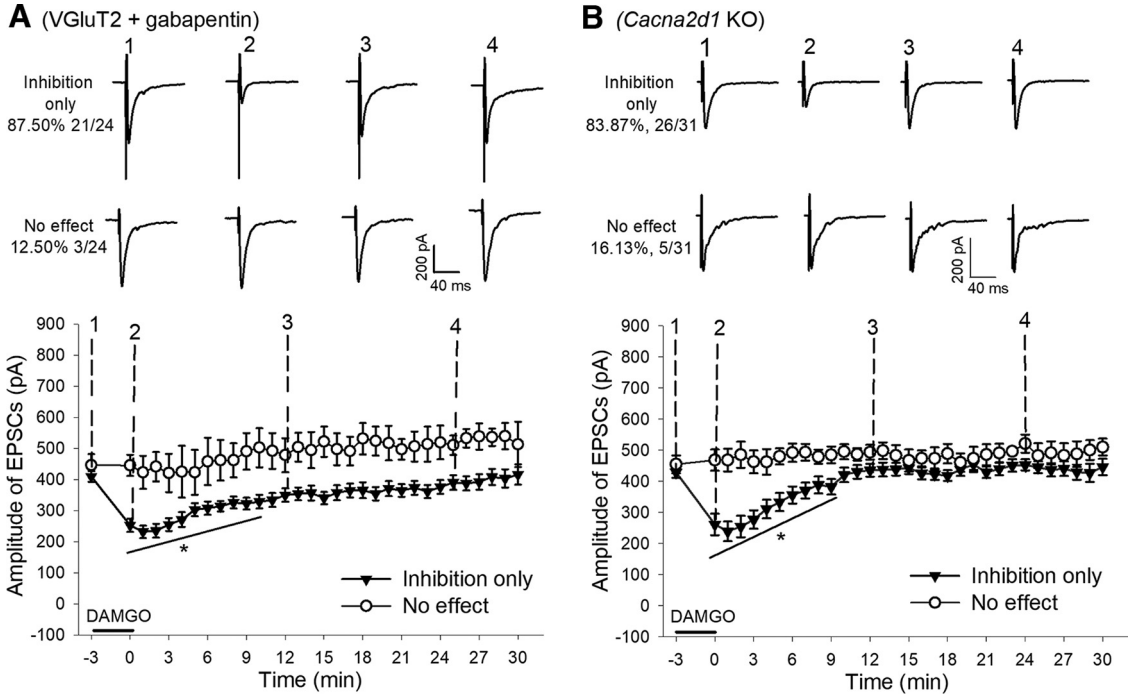

Figure 8. $\alpha2\delta$-1 is essential for opioid-triggered rebound LTP in VGluT2-expressing dorsal horn neurons. ***A***, ***B***, Representative recording traces and time course of changes in the amplitude of dorsal root–evoked EPSCs during baseline control, during bath application of 1 μM DAMGO, and after DAMGO washout in lamina II neurons. Spinal cord slices were obtained from VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ mice (***A***, pretreated with 100 μM gabapentin; $n = 24$ neurons from 6 mice) and *Cacna2d1* KO mice (***B***, $n = 31$ neurons from 6 mice). Calibration: 200 pA, 40 ms. Current traces 1–4 were selected from the time points shown in the time course plot. Data are shown as mean ± SEM; *$p < 0.05$ compared with the respective baseline (–3 min in the same group) before DAMGO application. Repeated-measures ANOVA followed by Dunnett's *post hoc* test.

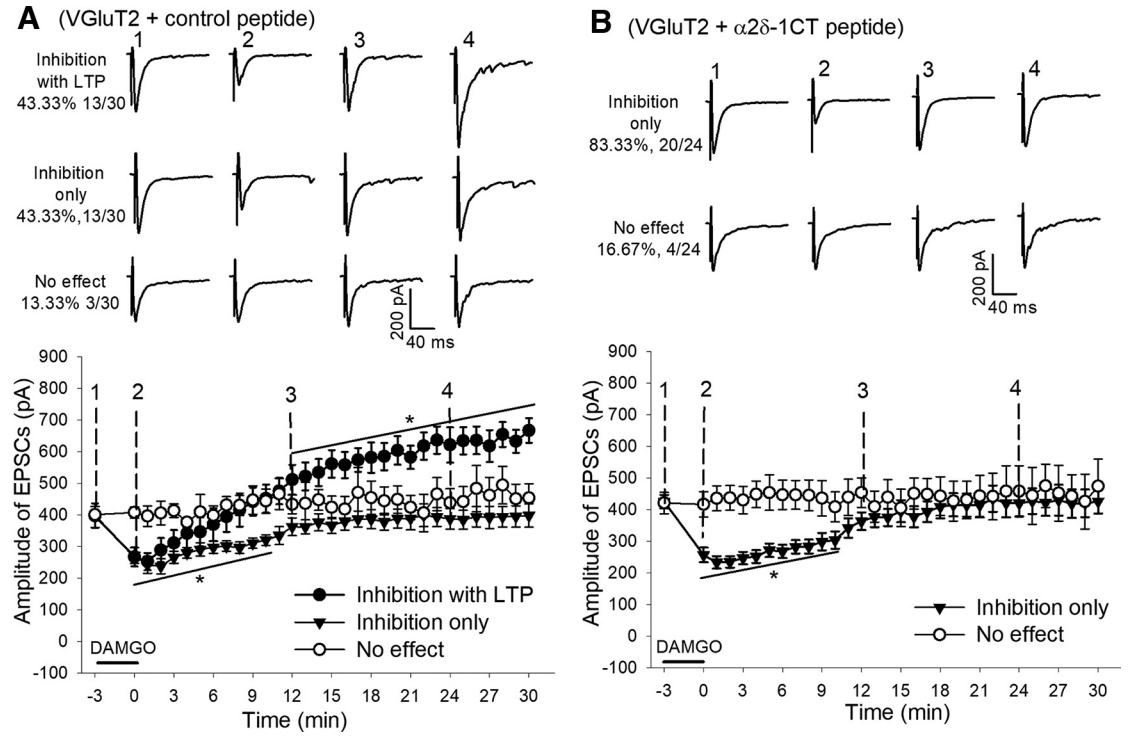

Figure 9. Brief opioid exposure triggers rebound LTP in VGluT2-expressing dorsal horn neurons via $\alpha2\delta$-1–coupled NMDARs. ***A***, ***B***, Representative recording traces and time course of changes in the amplitude of dorsal root–evoked EPSCs during baseline control, during bath application of 1 μM DAMGO, and after DAMGO washout in lamina II neurons. Spinal cord slices were obtained from VGluT2$^{Cre/+}$::tdTomato$^{flox/flox}$ mice and pretreated with 1 μM Tat-fused control peptide (***A***, $n = 30$ neurons from 6 mice) or 1 μM $\alpha2\delta$-1CT peptide (***B***, $n = 24$ neurons from 6 mice). Calibration: 200 pA, 40 ms. Current traces 1–4 were selected from the time points shown in the time course plot. Data are shown as mean ± SEM; *$p < 0.05$ compared with the respective baseline (–3 min in the same group) before DAMGO application. Repeated-measures ANOVA followed by Dunnett's *post hoc* test.

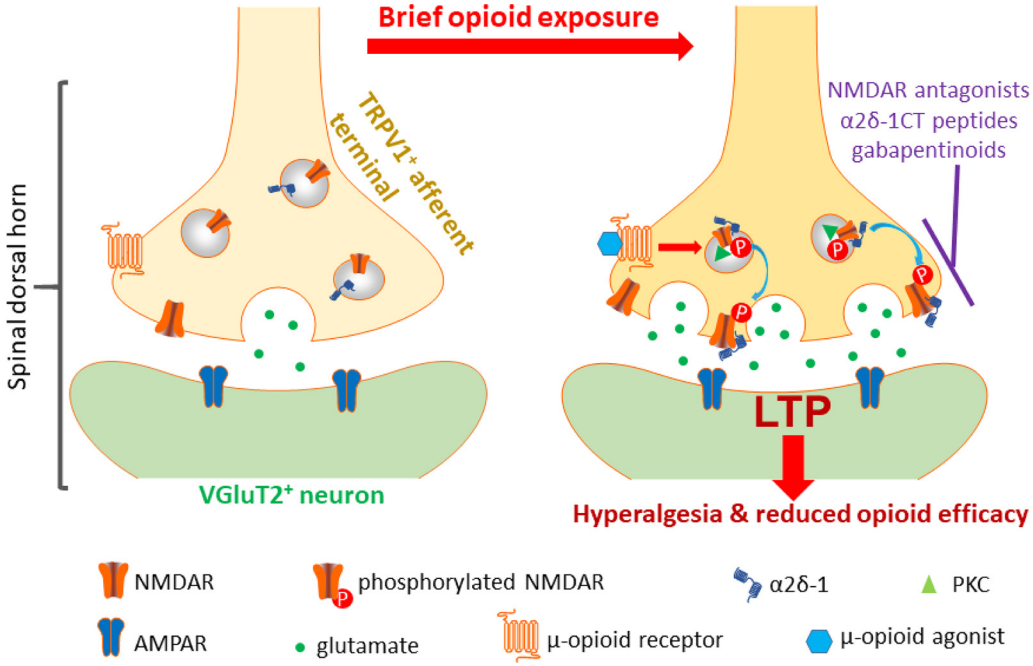

Figure 10. Schematic showing the signaling mechanism involved in opioid-elicited LTP in synaptic transmission from TRPV1-expressing primary afferent terminals to VGluT2 neurons in the spinal dorsal horn. Under normal conditions, most NMDARs are not phosphorylated or associated with $\alpha2\delta$-1 at primary afferent central terminals. Stimulation of μ-opioid receptors rapidly induces PKC activation and NMDAR phosphorylation. $\alpha2\delta$-1 then physically interacts with phosphorylated NMDARs to promote their synaptic trafficking, increasing presynaptic NMDAR activity. Consequently, glutamate release from TRPV1-expressing presynaptic terminals to VGluT2 excitatory neurons in the spinal dorsal horn is persistently augmented. This presynaptic LTP mediates opioid-elicited hyperalgesia and reduced analgesic efficacy, which could be attenuated by NMDAR antagonists, $\alpha2\delta$-1CT peptides, and gabapentinoids. AMPAR, AMPA receptor.

neurons in the spinal dorsal horn. We found that the number of VGluT2-expressing neurons was much more than that of VGAT-expressing neurons in laminae I and II innervated densely by MOR-containing nerve terminals. Consistent with this observation, direct paired-neuronal recordings in spinal cord slices indicate that the vast majority of lamina II neurons are excitatory and glutamatergic (Santos et al., 2007). Remarkably, we showed that DAMGO preferentially elicited rebound LTP in VGluT2 neurons, but not VGAT neurons, in the spinal dorsal horn. Furthermore, we found that repeated treatment with morphine increased the prevalence of VGluT2 neurons with LTP and caused a more rapid rebound LTP after DAMGO washout, which may be because of the reduced initial inhibition of Ca^{2+} channels by opioids. Because repeated treatment with opioids promotes rebound LTP in more excitatory dorsal horn neurons, this recurrent potentiation in nociceptive primary afferent input likely contributes to central sensitization associated with opioid-induced hyperalgesia and tolerance. Notably, we found that the initial inhibition of EPSCs by DAMGO was still present in many VGluT2 neurons after opioid tolerance had developed, which suggests that opioids given over time may still maintain their inhibitory effect on primary afferent input, but the concurrent increase in NMDAR-mediated LTP could oppose the analgesic effect of opioids, producing an effect that mimics tolerance (Chen and Pan, 2006b; Chen et al., 2007; Zhao et al., 2012).

Interestingly, we found that although VGAT-expressing dorsal horn neurons showed no rebound LTP, DAMGO inhibited EPSCs in many VGAT neurons even in mice subjected to repeated treatment with morphine. This finding is somewhat unexpected because primary afferent input to inhibitory neurons in the spinal dorsal horn is supposed to inhibit spinal nociceptive transmission, according to the gate control theory (Melzack and Wall, 1965). Nevertheless, the overall number of VGAT neurons that are in direct contact with MOR-expressing nerve terminals in the superficial dorsal horn is small. VGAT neurons are mainly present in spinal laminae III–IV, whereas TRPV1-expressing primary afferent terminals predominantly innervate neurons in laminae I and II (Chen and Pan, 2006b; Chen et al., 2007; Zhou et al., 2010). Thus, there are very few TRPV1-expressing primary afferents that form synapses with VGAT neurons in the spinal dorsal horn. Although inhibiting spinal VGAT neurons causes opioid-resistant pain hypersensitivity (Koga et al., 2017), further studies are needed to define whether inhibition of primary afferent input to VGAT dorsal horn neurons by MOR agonists is involved in the analgesic or hyperalgesic effect of opioids.

The observed diverse effects (inhibition only, inhibition followed by LTP, and no effect) of DAMGO on EPSCs evoked from primary afferents likely result from differential expression of MORs, MOR splice variants, and/or distinct signaling pathways in various types of DRG neurons and their central terminals. For example, DRG neurons with evident T-type Ca^{2+} channel currents have no expression of MORs (Wu et al., 2009). Importantly, when nociceptive TRPV1-expressing DRG neurons are ablated, DAMGO-triggered LTP is abolished (Zhou et al., 2008, 2010). Also, ablating TRPV1-expressing DRG neurons profoundly potentiates the analgesic effect of opioids and attenuates opioid tolerance (Chen and Pan, 2006b; Chen et al., 2007). Accordingly, DRG neurons expressing TRPV1 are mainly involved in counteracting the analgesic effect of opioids. In addition, the six-transmembrane-domain *Oprm1* variants mediate morphine-induced hyperalgesia and tolerance (Marrone et al., 2017). At present, it is unknown whether these *Oprm1* variants are involved in opioid-elicited LTP in the spinal cord.

Another salient finding of our study is that opioid-triggered LTP in excitatory dorsal horn neurons involves PKC-dependent and $\alpha2\delta$-1–dependent NMDARs present at primary afferent central terminals. Although repeated opioid treatment leads to tonic activation of presynaptic NMDARs that are coupled to $\alpha2\delta$-1 (Deng et al., 2019b), how presynaptic NMDARs in the

spinal dorsal horn become tonically activated after initial opioid exposure remains elusive. We found that inhibiting PKC activity or inhibiting $\alpha2\delta$-1 with gabapentin abolished DAMGO-triggered rebound LTP in VGluT2 neurons. Furthermore, DAMGO application failed to induce rebound LTP in dorsal horn neurons in $\alpha2\delta$-1 KO mice. In addition, we showed that disrupting the $\alpha2\delta$-1–NMDAR interaction prevented LTP in VGluT2 neurons induced by DAMGO. Because the C terminus of $\alpha2\delta$-1 is required for the *in vivo* actions of $\alpha2\delta$-1 in neuropathic pain and opioid-induced hyperalgesia and tolerance (Chen et al., 2018; Deng et al., 2019b; Li et al., 2021), the therapeutic effect of gabapentinoids is independent of voltage-gated calcium channels and thrombospondins, which interact with the von Willebrand A domain near the N terminus of $\alpha2\delta$-1 (Taylor and Harris, 2020). It seems that the PKC-dependent and $\alpha2\delta$-1–dependent NMDAR signaling is either not present or efficiently coupled to MORs in non-TRPV1 primary afferents that form synapses with VGluT2 dorsal horn neurons. Thus, MOR activation predominantly inhibits input from non-TRPV1 primary afferents to VGluT2 dorsal horn neurons via inhibition of voltage-gated Ca^{2+} channels. Our data are consistent with *in vivo* studies demonstrating the roles of PKC activity and $\alpha2\delta$-1–bound NMDARs in the development of opioid-induced hyperalgesia and tolerance (Mao et al., 1994; Granados-Soto et al., 2000; Deng et al., 2019b). Collectively, our findings suggest that presynaptic NMDARs at synapses formed by TRPV1-expressing primary afferent terminals and VGluT2 dorsal horn neurons could be rapidly phosphorylated by augmented PKC activity after opioid exposure and withdrawal, which leads to increased interaction of phosphorylated NMDARs with $\alpha2\delta$-1. This increased $\alpha2\delta$-1 and NMDAR association can subsequently augment synaptic trafficking and activity of NMDARs (Chen et al., 2018; Zhou et al., 2021), thereby causing hyperalgesia and counteracting the inhibitory effect of opioids on primary afferent input to spinal excitatory dorsal horn neurons (Fig. 10).

In summary, our study provides new insight into the cell types in the spinal cord and signaling mechanisms involved in opioid-elicited LTP, a critical substrate associated with opioid-induced hyperalgesia and tolerance. Opioid-elicited rebound LTP occurs preferentially in excitatory neurons in the spinal cord, and PKC-dependent and $\alpha2\delta$-1–dependent presynaptic NMDARs are integral to this opioid-elicited LTP. Thus, our findings elucidate how brief use of opioids can paradoxically amplify pain and reduce opioid analgesic efficacy. Increased activity of NMDARs at primary afferent central terminals by brief opioid exposure elicits hyperalgesia, which offsets the initial inhibitory effect of opioids on nociceptive transmission and opposes the opioid analgesic effect. This information not only advances our mechanistic understanding of the complex opioid action in nociceptive dorsal horn circuits but also has important clinical implications for improving pain management with opioids. Accordingly, the rationale for preemptive analgesia with opioids needs to be reconsidered, and routine intraoperative use of opioids should be minimized. Concurrent treatment with NMDAR antagonists, gabapentinoids, or $\alpha2\delta$-1CT–targeting agents perioperatively could prevent or reduce the development of opioid-induced hyperalgesia and tolerance.

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