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# Signaling Cascades for δ-Opioid Receptor-Mediated Inhibition of GABA Synaptic Transmission and Behavioral Antinociception

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## ABSTRACT

Membrane trafficking of the δ-opioid receptor (DOR) from intracellular compartments to plasma membrane in central neurons, induced by various pathological conditions such as long-term opioid exposure, represents unique receptor plasticity involved in the mechanisms of long-term opioid effects in opioid addiction and opioid treatment of chronic pain. However, the signaling pathways coupled to the newly emerged functional DOR in central neurons are largely unknown at present. In this study, we investigated the signaling cascades of long-term morphine-induced DOR for its cellular and behavioral effects in neurons of the rat brainstem nucleus raphe magnus (NRM), a key supraspinal site for opioid analgesia. We found that, among the three phospholipase $A_2$ ($PLA_2$)-regulated arachidonic acid (AA) metabolic pathways of lipoxygenase, cyclooxygenase, and epoxygenase, 12-lipoxygenase of the lipoxygenase pathway primarily mediated DOR inhibition of GABA synaptic transmission, because inhibitors of 12-lipoxygenase as well as lipoxygenases and $PLA_2$ largely blocked the DOR- or AA-induced GABA inhibition in NRM neurons in brainstem slices in vitro. Blockade of the epoxygenase pathway was ineffective, whereas blocking either 5-lipoxygenase of the lipoxygenase pathway or the cyclooxygenase pathway enhanced the DOR-mediated GABA inhibition. Behaviorally in rats in vivo, NRM infusion of 12-lipoxygenase inhibitors significantly reduced DOR-induced antinociceptive effect whereas inhibitors of 5-lipoxygenase and cyclooxygenase augmented the DOR antinociception. These findings suggest the $PLA_2$-AA-12-lipoxygenase pathway as a primary signaling cascade for DOR-mediated analgesia through inhibition of GABA neurotransmission and indicate potential therapeutic benefits of combining 5-lipoxygenase and cyclooxygenase inhibitors for maximal pain inhibition.

## Introduction

Opioids are commonly used for treating chronic pain, but they also have strong reinforcing effects that induce addiction after prolonged exposure (Woolf and Hashmi, 2004). Opioid effects are mediated mostly by three classic opioid receptor subtypes: μ-opioid receptor (MOR), δ-opioid receptor (DOR), and κ-opioid receptor. Both the analgesic and reinforcing effects of opioids are mediated primarily by MOR, which is readily expressed on surface membrane of central neurons (Matthes et al., 1996; Contet et al., 2004; Waldhoer et al., 2004). In contrast, the function of DOR in opioid analgesia and opioid addiction remains elusive at present (Contet et al., 2004; Fields, 2004).

Membrane trafficking of G protein-coupled receptors (GPCRs) is a highly regulated dynamic process involved in neuronal adaptations to changing environmental stimuli in brain functions and in chronic diseases (Tan et al., 2004; Hanyaloglu and von Zastrow, 2008). Constitutively localized in intracellular compartments, DOR, a GPCR, normally lacks cellular effects in brain neurons, but translocates to surface membrane and becomes functional under various pathological conditions, including long-term opioid exposure, chronic pain, tissue inflammation, and behavioral stress (Hurley and Hammond, 2000; Commons, 2003; Hack et al., 2005; Ma et al., 2006). Thus, DOR may play a more important role in neuronal adaptations to pathological conditions than in basal opioid functions in the brain. These findings of adaptive DOR translocation not only provide a unique plat-

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**ABBREVIATIONS:** MOR, μ-opioid receptor; DOR, δ-opioid receptor; GPCR, G protein-coupled receptor; AA, arachidonic acid; $PLA_2$, phospholipase A2; COX, cyclooxygenase; LOX, lipoxygenase; P450, cytochrome P450; NRM, nucleus raphe magnus; IPSC, inhibitory postsynaptic current; NDGA, nordihydroguaiaretic acid; AA-861, 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-*p*-benzoquinone; PD146176, 11-dihydro[1]benzothiopyrano[4,3-*b*]indole; mIPSC, miniature inhibitory postsynaptic current; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acids; PKA, protein kinase A; DAMGO, [D-Ala$^2$,*N*-Me-Phe$^4$,Gly$^5$-ol]-enkephalin.

form for studying the neurobiological mechanisms of GPCR trafficking but also serve as a valuable model for understanding of DOR functions in chronic opioid-related diseases such as drug dependence and drug addiction (Cahill et al., 2007; Bie et al., 2010). Because the DOR trafficking occurs both in pain-modulating neurons in the brain and spinal cord and in drug reward-related amygdala neurons (Ma et al., 2006; Cahill et al., 2007; Bie et al., 2009), understanding how this newly emerged DOR signals via intracellular pathways to exert its cellular effects is important to reveal its significance in the pathogenesis of such chronic opioid-related diseases.

Opioid inhibition of GABAergic synaptic transmission is one of the main cellular mechanisms for opioid-induced analgesia (Pan et al., 1997; Fields, 2004). Previous studies have shown important roles of arachidonic acid (AA) metabolites in MOR-mediated GABA inhibition and analgesic effects under opioid-naive conditions. In response to various pathological stimuli including inflammatory cytokines, AA is released from phospholipids of cell membrane under regulation primarily by cytosolic phospholipase $A_2$ ($PLA_2$) and is converted to signaling molecules of eicosanoids through three main metabolic pathways of cyclooxygenase (COX), lipoxygenase (LOX), and epoxygenase/P450 (Shimizu and Wolfe, 1990; Harizi et al., 2008). The COX pathway is well known for generating inflammatory molecules, prostaglandins, whereas the LOX pathway mediates MOR inhibition of presynaptic GABA release in central neurons in vitro (Vaughan et al., 1997). A recent behavioral study in vivo shows that the MOR agonist morphine induces antinociception through the epoxygenase/P450 pathway (Conroy et al., 2010). However, under long-term opioid conditions, the signaling of these opioid receptors has yet to be characterized. We have shown recently that long-term opioid-induced new DOR produces inhibition of GABA synaptic transmission and an analgesic effect in a $PLA_2$-dependent manner (Zhang and Pan, 2010). Nevertheless, the intracellular signaling pathways to which the emerged DOR couples to produce its cellular effects are largely unknown at present.

In this study with cellular recording of synaptic activities in vitro and system analysis of pain behaviors in vivo, we determined the detailed signaling cascades responsible for DOR inhibition of GABA neurotransmission and for DOR-mediated behavioral antinociception in brainstem neurons of the nucleus raphe magnus (NRM), an important supraspinal structure for opioid modulation of pain (Pan et al., 1997; Fields, 2004).

## Materials and Methods

**Animals and Long-Term Morphine Treatment.** All procedures involving the use of animals conformed to the guidelines of The University of Texas MD Anderson Cancer Center Animal Care and Use Committee. Male Wistar rats were randomly divided into two groups, morphine group and saline or placebo control group, and were treated with long-term morphine or saline/placebo as described previously (Zhang and Pan, 2010). For whole-cell recordings, neonatal rats (9–14 days old) in the morphine group were injected (intraperitoneal) with increasing doses of morphine twice daily for 6 days with the last morphine injection at approximately 5:00 PM on day 6. The morphine dose was 10 mg/kg on day 1 and was increased by 5 mg/kg each day to reach the maximum dose of 30 mg/kg on days 5 and 6. Rats in the saline group were similarly injected with saline for controls. The injection volume was 0.1 to 0.3 ml. Neonatal rats were used for whole-cell recording because of limited cell visibility and quality for visualized recording in NRM slices from older rats. Both similarities and differences are recognized between neonatal and adult rats in pain responses of NRM neurons for data interpretation (Zhang and Hammond, 2010). Nonetheless, we have successfully used findings of cellular recording from neonatal rats as a useful and efficient model with recognized limitations to help understand the cellular mechanisms of pain behavior in adult rats (Pan et al., 1997; Bie et al., 2005; Ma and Pan, 2006; Zhang and Pan, 2010; Zhang et al., 2011). For behavioral experiments, rats (200–300 g) in the morphine group were subcutaneously implanted with one morphine pellet (75 mg) on day 1 and two more morphine pellets on day 4. In the placebo group, rats were similarly implanted with placebo pellets. Experiments for whole-cell recordings and behavioral tests were performed on day 7. For long-term morphine treatment, the methods of daily morphine injections in neonatal rats and pellet implantations in adult rats were selected primarily because of technical difficulties for pellet implantation in neonatal rats and too large an amount of morphine required for injections in adult rats. As shown in our previous studies, no detectable difference was observed between the two methods in terms of adaptive responses of NRM neurons and pain behaviors with our experimental protocols, because data were collected after the completion of the morphine treatments (Pan, 2003; Bie et al., 2005).

**Brian Slice Preparations and Whole-Cell Recording.** The general methods of visualized whole-cell voltage-clamp recording have been described previously (Bie et al., 2005; Zhang and Pan, 2010). NRM slices (200 $\mu$m thick) were cut, and a single slice was perfused in a recording chamber with preheated (35°C) physiological saline containing the following: 126 mM NaCl, 2.5 mM KCl, 1.2 mM $NaH_2PO_4$, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 11 mM glucose, and 25 mM $NaHCO_3$, saturated with 95% $O_2$ and 5% $CO_2$, pH 7.2 to 7.4. Visualized whole-cell recordings were obtained from identified NRM neurons with a glass pipette (resistance, 3–5 MΩ) filled with a solution containing the following: 126 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES, 2 mM ATP, and 0.25 mM GTP, pH adjusted to 7.3 with KOH and osmolarity to 280 to 290 mOsM. Electrical stimuli of constant current (0.25 ms, 0.2–0.4 mA) were used to evoke GABA-mediated inhibitory postsynaptic currents (IPSCs) with a bipolar stimulating electrode placed within the nucleus. With a KCl-filled pipette and a holding potential of −70 mV, GABA IPSCs were in an inward (downward) direction (Pan et al., 1990). GABA IPSCs were recorded in the presence of glutamate receptor antagonists D-(−)-2-amino-5-phosphonopentanoic acid (50 $\mu$M) and 6-cyano-7-nitroquinoxaline-2,3-dione (10 $\mu$M) and were completely blocked by the $GABA_A$ receptor antagonist bicuculline (10 $\mu$M). Miniature IPSCs were obtained in 60-s epochs in the presence of tetrodotoxin (1 $\mu$M) and a sliding IPSC template custom-defined with the acquisition software was used to detect and analyze the frequency and amplitude of miniature IPSCs. Recordings were typically made 1 to 3 h after slice cutting, and no morphine was added to the bath solution so that neurons in slices of the morphine group were likely under a condition of early withdrawal (Bie et al., 2005). We did not observe a time-dependent withdrawal effect on neuronal properties in our experimental conditions. Inhibitors nordihydroguaiaretic acid (NDGA) (10 $\mu$M), baicalein (10 $\mu$M), 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-*p*-benzoquinone (AA-861) (10 $\mu$M), indomethacin (10 $\mu$M), miconazole (20 $\mu$M), and 11-dihydro[1]benzothiopyrano[4,3-*b*]indole (PD146176) (10 $\mu$M) were applied through the bath for at least 10 min before data collection. $Ca^{2+}$ channel inhibitors were administered through the bath 3 min before deltorphin application. The target effects of the above-mentioned inhibitors at the concentrations used have been reported in previous studies (Sendobry et al., 1997; Vaughan et al., 1997; Metea and Newman, 2006). At these inhibitor concentrations, no effect was observed on the amplitude of evoked IPSCs by the inhibitors themselves, nor was it reported previously.

**Microinjection and Behavioral Experiments.** NRM microinjection was performed as described previously (Ma et al., 2006; Zhang and Pan, 2010). A rat was implanted with a 26-gauge double-guide cannula (Plastics One, Roanoke, VA) aiming at the NRM

(anteroposterior, −10.0 from the bregma; lateral, 0; and dorsoventral, −10.5 from the dura) (Kroon and Riley, 1986). The pain threshold was measured every 5 min by the tail-flick test on a freely moving rat with a Hargreaves analgesia instrument (Stoelting, Kiel, WI). The heat intensity was set to elicit stable baseline latencies with a cutoff time of 10 s. Drugs were microinjected into the NRM in a total volume of 0.4 μl through a 33-gauge double injector with an infusion pump at a rate of 0.2 μl/min. The confined effect of an injected drug within the NRM with this microinjection method has been demonstrated in our previous studies (Ma et al., 2006; Zhang and Pan, 2010). All NRM microinjection sites were histologically verified after the experiment by injecting a blue dye and controls of off-site injections were performed as shown before (Bie et al., 2005). Indomethacin was injected intraperitoneally in consideration of this widely used administration route in previous pain research and the known permeability of indomethacin through the blood-brain barrier. The behavioral effect of a microinjected inhibitor was measured 20 to 30 min after its administration.

**Statistical Analyses and Materials.** Numerical data of GABA IPSCs from control groups and treatment groups were statistically analyzed and compared with the Student's *t* test (paired or unpaired, two-tailed). Data are presented as means ± S.E.M. Behavioral results were statistically analyzed by an analysis of variance for repeated measures and the Tukey-Kramer test of post hoc analysis. $P < 0.05$ was considered as statistically significant. Deltorphin, morphine sulfate, and morphine and placebo pellets were kindly supplied by the drug program of the National Institute on Drug Abuse (Bethesda, MD). All other drugs were purchased from Sigma-Aldrich (St. Louis, MO) or Tocris Bioscience (Ellisville, MO).

## Results

**Long-Term Morphine Induces New Functional DOR on Presynaptic GABA Terminals.** Under whole-cell voltage-clamp recording in brainstem slices in vitro, the selective DOR agonist deltorphin (1 μM), ineffective in control neurons from saline-treated rats, inhibited the GABAergic IPSC in NRM neurons from morphine-treated rats, and the deltorphin inhibition was completely blocked by the DOR antagonist naltriben (10 μM) (Fig. 1A). This inhibition of evoked GABA IPSCs by long-term morphine-induced DOR is consistent with the results in our previous report (Zhang and Pan, 2010). We determined effects of the DOR on action potential-independent GABAergic miniature IPSCs (mIPSCs) to further identify the synaptic site of this DOR action. Deltorphin (1 μM) significantly decreased the frequency but not the amplitude of mIPSCs in neurons ($n = 10$) from morphine-treated rats but had no apparent effect on either the frequency or the amplitude of mIPSCs in neurons ($n = 6$) from saline-treated rats (Fig. 1, B and C). These results indicate that long-term morphine induces functional DOR on presynaptic GABA terminals and activation of this DOR inhibits presynaptic GABA release in NRM neurons.

**The 12-LOX Pathway Mediates DOR Inhibition of GABA IPSCs.** Our recent study shows that inhibition of GABA IPSCs by the emerged DOR is dependent on both $PLA_2$ and cAMP/protein kinase A (PKA) (Zhang and Pan, 2010). In NRM neurons from long-term morphine-treated rats, we investigated the detailed signaling cascades downstream from $PLA_2$ that were involved in the DOR inhibition of GABA synaptic transmission, focusing on the $PLA_2$-regulated AA metabolic pathways. As illustrated in Fig. 2A, we used selective inhibitors to pharmacologically block each of the three major AA signaling pathways of COX, LOX, and epoxygenase/P450 in the following experiments on NRM neurons from morphine-treated rats. We have reported previously that arachidonyltrifluoromethyl ketone (10 μM), a se-

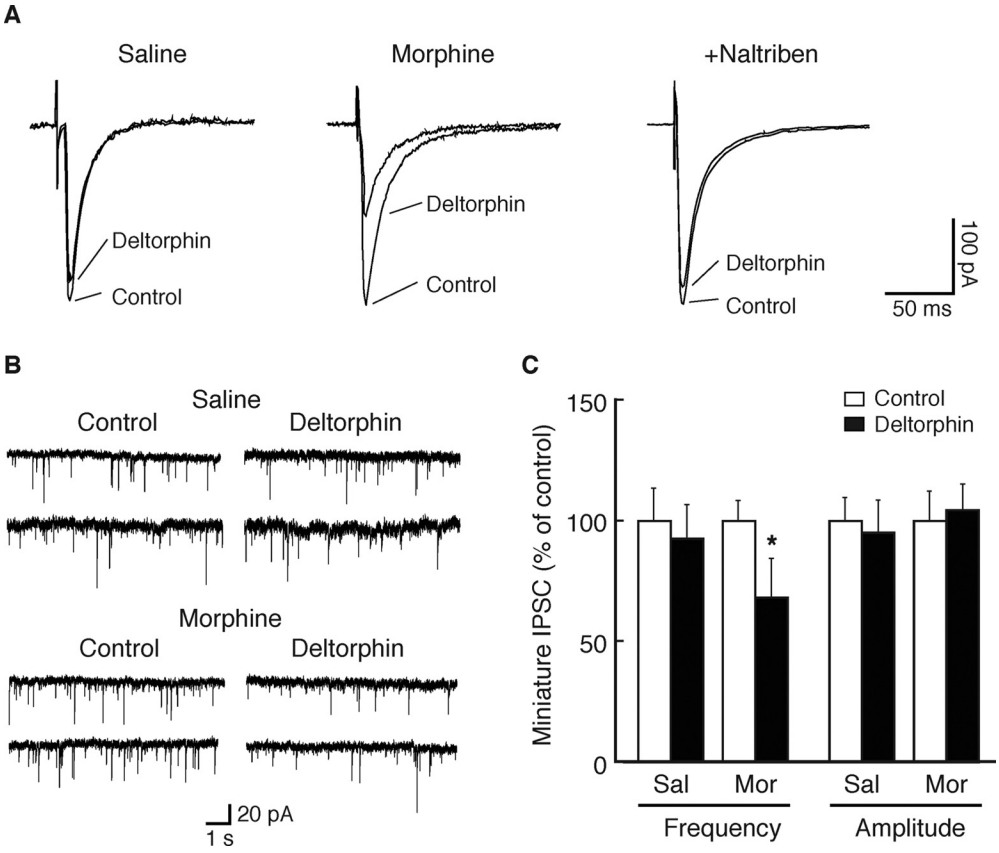


**Fig. 1.** Long-term morphine-induced DOR inhibits GABA synaptic transmission by reducing presynaptic GABA release. A, representative GABAergic IPSCs in the absence (control) and presence of the selective DOR agonist deltorphin (1 μM) in neurons of the NRM from a saline-treated rat and a morphine-treated rat without and with addition of the DOR antagonist naltriben (10 μM). On average, deltorphin inhibited the GABA IPSCs to 53.6 ± 5.6% of control in neurons ($n = 15$) from morphine-treated rats [the figure is based on the findings in Zhang and Pan (2010).] B, representative current traces of miniature IPSCs with and without deltorphin in an NRM neuron from a saline- and a morphine-treated rat. C, summarized data of deltorphin effects on the frequency and amplitude of miniature IPSCs in neurons of the saline (Sal) group ($n = 6$) and of the morphine (Mor) group ($n = 10$). *, $p < 0.05$.

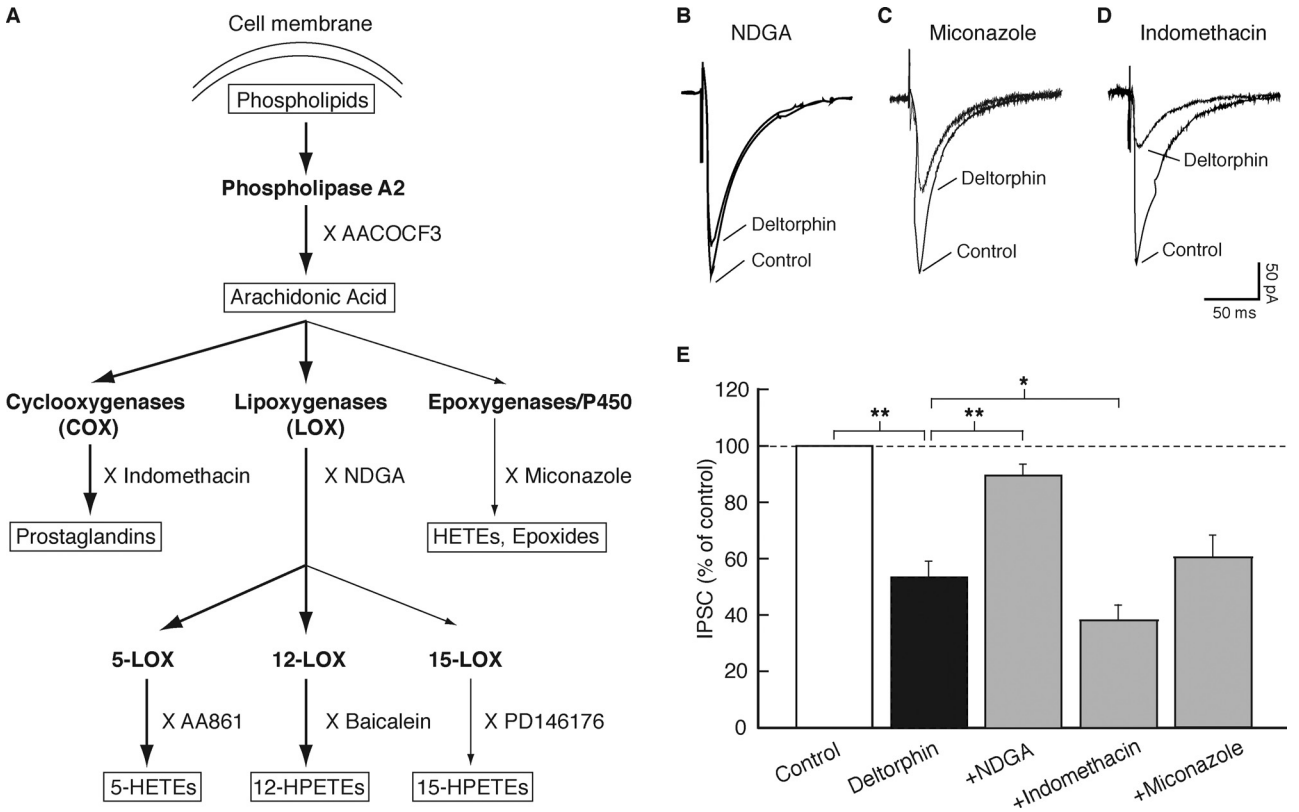


**Fig. 2.** DOR inhibition of GABA synaptic transmission is mediated by the LOX pathway. A, signaling pathways of AA metabolism. Drug names after an X indicate the selective inhibitor that blocks that specific metabolic pathway. The thicker arrows depict relatively more dominant pathways involved in DOR-mediated effects (see *Results* for details). B–D, representative GABA IPSCs in control and in deltorphin (1 $\mu$M) in NRM slices from morphine-treated rats and pretreated in vitro by bath application of the LOX inhibitor NDGA (10 $\mu$M, B), the epoxygenase/P450 inhibitor miconazole (20 $\mu$M, C), or the COX inhibitor indomethacin (10 $\mu$M, D) for 10 to 15 min. E, normalized effects of the indicated inhibitors on deltorphin inhibition of GABA IPSCs in NRM neurons of the morphine group. $n$ = 6 to 9 cells for each group. *, $p < 0.05$; **, $p < 0.01$.

lective inhibitor of $PLA_2$ that mediates the production of AA from membrane phospholipids (Harizi et al., 2008), significantly inhibits the deltorphin (1 $\mu$M)-induced inhibition (to 53.6 ± 5.6% of control) of GABA IPSCs (Zhang and Pan, 2010). In the presence of the LOX inhibitor NDGA (10 $\mu$M) (Vaughan et al., 1997), we found that deltorphin inhibition of IPSCs was largely blocked (to 85.1 ± 7.2%, $p < 0.01$, $n = 8$) (Fig. 2, B and E). The remaining DOR inhibition in the presence of NDGA is probably mediated, at least partially, by the cAMP/PKA pathway as we showed before (Zhang and Pan, 2010). Blocking the epoxygenase/P450 pathway with the selective inhibitor miconazole (20 $\mu$M) (Metea and Newman, 2006) did not significantly affect the deltorphin effect on IPSCs (to 60.8 ± 7.8%, $p > 0.05$, $n = 8$) (Fig. 2, C and E). In contrast to the blocking effect of NDGA, the COX inhibitor indomethacin (10 $\mu$M) augmented the deltorphin effect, increasing the inhibition of IPSCs to 38.0 ± 5.2% ($p < 0.05$, $n = 7$) (Fig. 2, D and E). Thus, it appears that the LOX pathway primarily mediates DOR inhibition of GABA synaptic release in these neurons.

Next, we determined which LOX subtype was mainly responsible for the DOR inhibition of IPSCs in NRM neurons from long-term morphine-treated rats. We found that blocking 12-LOX with baicalein (10 $\mu$M) (Vaughan et al., 1997) significantly antagonized the deltorphin effect (to 81.3 ± 10.3%, $p < 0.01$, $n = 8$) (Fig. 3, A and F), whereas blockade of 15-LOX with PD146176 (10 $\mu$M) (Sendobry et al., 1997) had no effect on the DOR inhibition (to 52.1 ± 8.7%, $p > 0.05$, $n = 6$) (Fig. 3, B and F). Similar to the indomethacin effect, the 5-LOX inhibitor AA-861 (10 $\mu$M) (Vaughan et al., 1997) also enhanced the deltorphin effect, increasing DOR inhibition of IPSCs to 35.2 ± 2.8% ($p < 0.05$, $n = 9$) (Fig. 3, C and F). In addition, application of AA (150 $\mu$M) produced a significant inhibition on the IPSC amplitude in these neurons from morphine-treated rats (to 63.5 ± 14.2%, $p < 0.01$, $n = 5$), which was mostly reversed by the 12-LOX inhibitor baicalein (10 $\mu$M, to 85.2 ± 8.6%, $p < 0.05$, $n = 6$) (Fig. 3, D and G). To further validate the involvement of 12-LOX in the DOR effect, we examined the effect of AA metabolites, hydroperoxyeicosatetraenoic acids (HPETEs) and hydroxyeicosatetraenoic acids (HETEs), converted by the three LOX subtypes (Fig. 2) (Vaughan et al., 1997; Harizi et al., 2008). Consistent with the above experiments using enzyme inhibitors, bath application of 12-HPETE (100 nM) significantly suppressed the IPSC amplitude to 65.0 ± 4.5% of control ($n = 8$, $p < 0.01$); in contrast, 5-HETE (100 nM) did not alter the IPSC amplitude ($n = 8$, $p > 0.05$) nor did 15-HPETE (50 $\mu$M, $n = 5$, $p > 0.05$) (Fig. 3, E and G).

Taken together, these results suggest that activation of long-term morphine-induced DOR on GABAergic terminals inhibits presynaptic GABA release through the $PLA_2$-AA-12-LOX pathway. The DOR-facilitating effect of blocking COX and 5-LOX is presumably due to shunting of more endogenous AA metabolites to the 12-LOX pathway, thus augmenting the DOR effect.

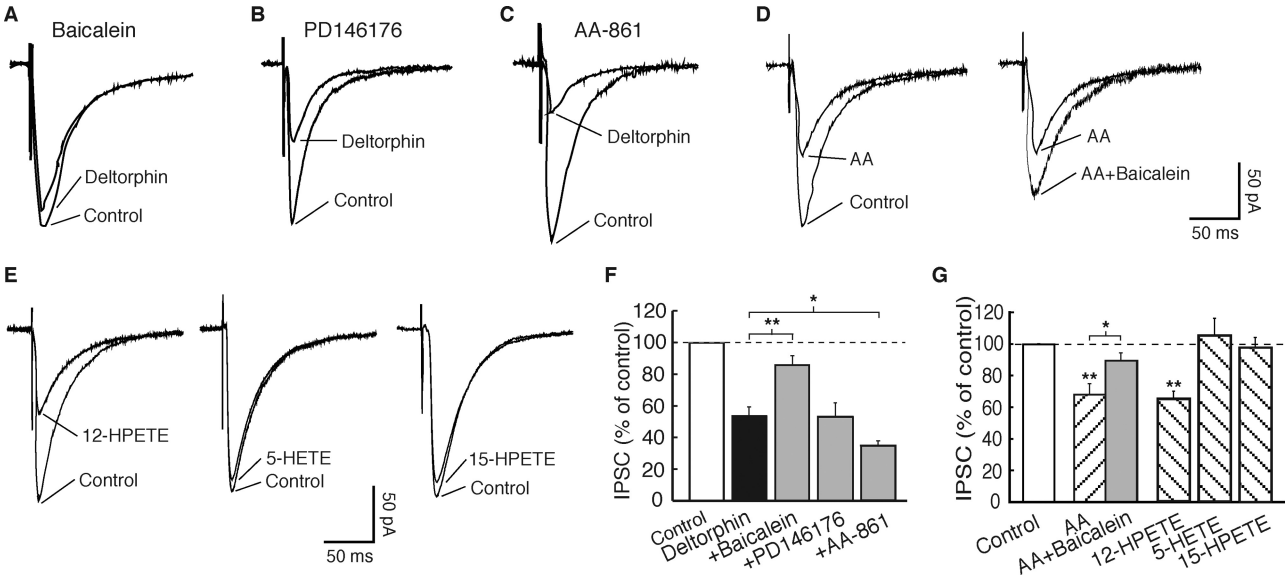


**Fig. 3.** The 12-LOX pathway mediates DOR inhibition of GABA synaptic transmission. A–C, representative GABA IPSCs in control and in deltorphin in the presence of the 12-LOX inhibitor baicalein (10 μM, A), the 15-LOX inhibitor PD146176 (10 μM, B) or the 5-LOX inhibitor AA-861 (10 μM, C). D, GABA IPSCs in control and in the presence of AA (150 μM) or AA plus baicalein (10 μM). E, GABA IPSCs in control and in the presence of 12(*S*)-HPETE (100 nM), (±)-5-HETE (100 nM) or 15(*S*)-HPETE (5 μM). F and G, summarized data of deltorphin effects shown in A–C (F) and effects of AA metabolites shown in D and E (G) on GABA IPSCs in the absence and presence of the indicated inhibitors. $n = 5$ to 8 cells for each group. *, $p < 0.05$; **, $p < 0.01$.

**Signaling Pathway for MOR Inhibition of GABA IPSCs.** We have shown previously that the long-term morphine-induced DOR emerges on GABAergic terminals on which MOR is readily expressed in NRM neurons, producing functional synergism of DOR and MOR (Zhang and Pan, 2010). We examined the $PLA_2$-dependent signaling pathways for MOR inhibition of GABA IPSCs under long-term morphine conditions. In neurons from long-term morphine-treated rats, the selective MOR agonist DAMGO (1 μM) inhibited the amplitude of GABA IPSCs to 47.9 ± 4.3% of control ($n = 16$, $p < 0.01$). This MOR inhibition was significantly antagonized by the 12-LOX inhibitor baicalein (10 μM) (to 68.2 ± 8.7%, $n = 8$, $p < 0.05$) (Fig. 4). Of interest, blockade of epoxygenases/P450 with miconazole (20 μM) also significantly reduced the DAMGO inhibition (to 69.0 ± 4.9%, $n = 8$, $p < 0.01$) (Fig. 4). Similar to the DOR inhibition, the remaining MOR inhibition is probably mediated partially by the cAMP/PKA pathway (Zhang and Pan, 2010). Consistent with the indomethacin effect reported in a previous study under naive conditions (Vaughan et al., 1997), the COX inhibitor (10 μM) enhanced the DAMGO inhibition of IPSCs in NRM neurons from long-term morphine-treated rats (data not shown). These results support the notion that, under long-term opioid conditions, both the 12-LOX and epoxygenase/P450 pathways mediate MOR inhibition of GABA synaptic transmission in these neurons. Thus, it appears that both DOR and MOR share the same 12-LOX pathway in their presynaptic inhibition of GABA synaptic transmission under long-term opioid conditions.

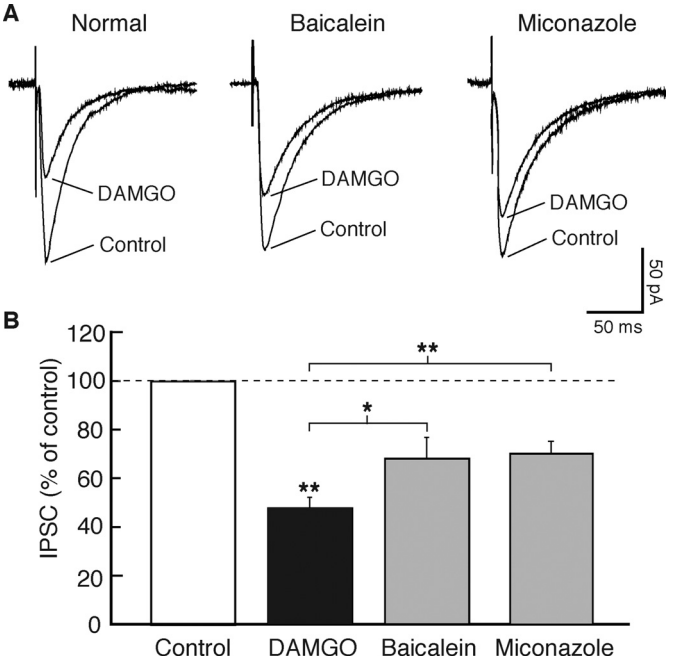


**Fig. 4.** Both the 12-LOX and epoxygenase/P450 pathways mediate the inhibition of GABA synaptic transmission by the MOR under long-term opioid conditions. A, effects of the selective MOR agonist DAMGO (1 μM) on GABA IPSCs in the absence (normal) and presence of baicalein (10 μM) or miconazole (20 μM) in NRM neurons from morphine-treated rats. B, summarized data of the effects of DAMGO and the indicated inhibitors on GABA IPSCs. $n = 8$ to 16 cells for each group. *, $p < 0.05$; **, $p < 0.01$.

**Involvement of $Ca^{2+}$ Channels in DOR Inhibition of GABA IPSCs.** We investigated whether $Ca^{2+}$ channels, commonly involved in presynaptic neurotransmitter release (Catterall and Few, 2008), were involved in the DOR inhibition of GABA release. In slices from long-term morphine-treated rats, we found that deltorphin inhibition of GABA IPSCs was significantly reduced by pretreatment of the slice with ω-conotoxin GVIA (0.5 μM) (Fig. 5), a selective N-type $Ca^{2+}$ channel inhibitor (Andrade et al., 2010). In contrast, similar pretreatment with the L-type $Ca^{2+}$ channel inhibitor nifedipine (10 μM) or the P/Q type $Ca^{2+}$ channel inhibitor ω-agatoxin TK (0.2 μM) (Momiyama and Fukazawa, 2007) failed to alter the deltorphin inhibition

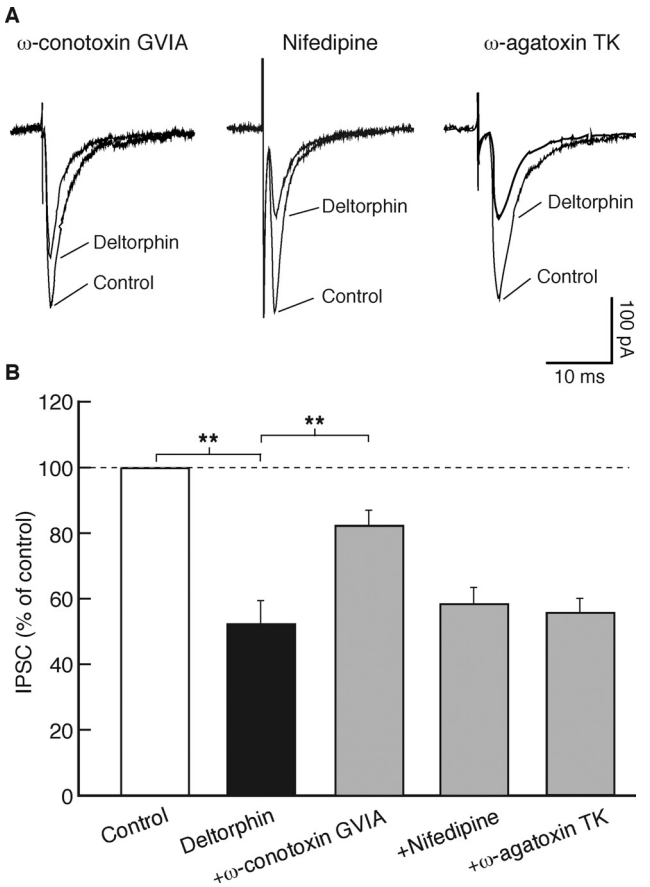


**Fig. 5.** DOR inhibition of GABA neurotransmission involves the N-type $Ca^{2+}$ channel. A, representative GABA IPSCs in control and in deltorphin (1 $\mu$M) in NRM neurons in slices from morphine-treated rats and pretreated in vitro with the N-type $Ca^{2+}$ channel inhibitor $\omega$-conotoxin GVIA (0.5 $\mu$M), the L-type $Ca^{2+}$ channel inhibitor nifedipine (10 $\mu$M), or the P/Q-type $Ca^{2+}$ channel inhibitor $\omega$-agatoxin TK (0.2 $\mu$M). B, normalized effects of $\omega$-conotoxin GVIA ($n$ = 8 cells), nifedipine ($n$ = 10), and $\omega$-agatoxin TK ($n$ = 5) on deltorphin inhibition of GABA IPSCs ($n$ = 7) in slices as in A. **, $p < 0.01$.

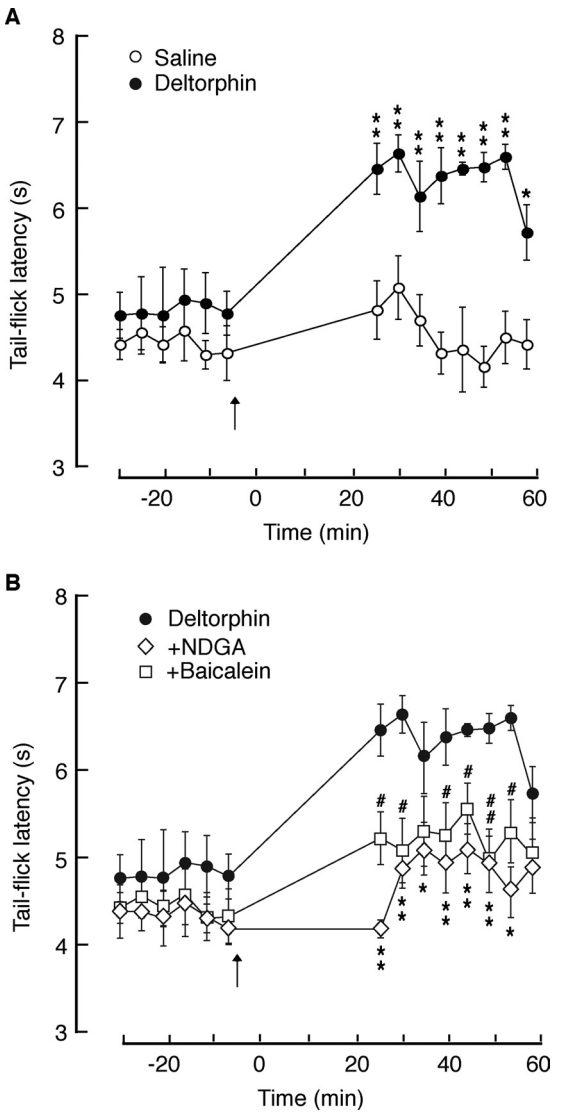


**Fig. 6.** The 12-LOX pathway is involved in DOR-mediated antinociception in vivo. A, effects of a single microinjection (arrow) of saline ($n$ = 5 rats) or deltorphin (1 $\mu$g, $n$ = 5 rats) into the NRM on pain threshold measured by the tail-flick test in long-term morphine-treated rats in vivo. B, antagonizing effect of a pretreatment (1 h before) with NRM microinjection of the LOX inhibitor NDGA (1 $\mu$g, $n$ = 5 rats) or the 12-LOX inhibitor baicalein (1 $\mu$g, $n$ = 5 rats) on the deltorphin-induced antinociception. * and #, statistical significance of the NDGA group and the baicalein group, respectively, compared with the group of deltorphin alone. * and #, $p < 0.05$; ** and ##, $p < 0.01$.

(Fig. 5). This result indicates the involvement of N-type $Ca^{2+}$ channels in DOR inhibition of GABA release.

**The 12-LOX Pathway Mediates DOR-Induced Antinociception.** Inhibition of central GABA synaptic transmission, consequently causing disinhibition (or activation) of central pain-inhibiting neurons, is one of the main mechanisms for opioid-induced analgesia (Pan et al., 1997; Fields, 2004). Thus, activation of the emerged DOR in the NRM produces behavioral antinociception in vivo (Ma et al., 2006; Zhang and Pan, 2010). Based on the cellular results above, we hypothesized that the 12-LOX metabolic pathway was important for the DOR-induced antinociception. To demonstrate that, we conducted behavioral experiments with site-specific microinjections in long-term morphine-treated rats in vivo. A single microinjection of deltorphin (1 $\mu$g) into the NRM produced a significant antinociceptive effect in morphine-treated rats ($n$ = 5 rats) (Fig. 6A). We have shown that this deltorphin effect is blocked by comicroinjection of the DOR antagonist naltriben, suggesting a specific DOR-mediated effect (Pan et al., 1997; Ma et al., 2006). Consistent with our hypothesis, a pretreatment with NRM microinjection of the LOX inhibitor NDGA (1 $\mu$g, $n$ = 5) 1 h before deltorphin infusion blocked the deltorphin-induced antinociceptive effect ($n$ = 5) (Fig. 6B). In addition, the deltorphin effect was also significantly attenuated by a similar NRM preinfusion of the 12-LOX inhibitor baicalein (1 $\mu$g, $n$ = 5) (Fig. 6B). These findings provide behavioral evidence showing an important role of the 12-LOX signaling pathway in DOR-mediated antinociception under long-term opioid conditions.

Because blockade of the COX pathway or the 5-LOX pathway augmented DOR inhibition of GABA IPSCs, we reasoned that this pharmacological treatment should enhance the DOR-mediated antinociceptive effect in long-term morphine-treated rats in vivo. As expected, we found that systemic administration of the COX inhibitor indomethacin (20 mg/kg, i.p., $n$ = 5) 1 h before NRM microinjection of deltorphin significantly increased the deltorphin-induced antinociceptive effect and so did NRM preinfusion of the 5-LOX inhibitor

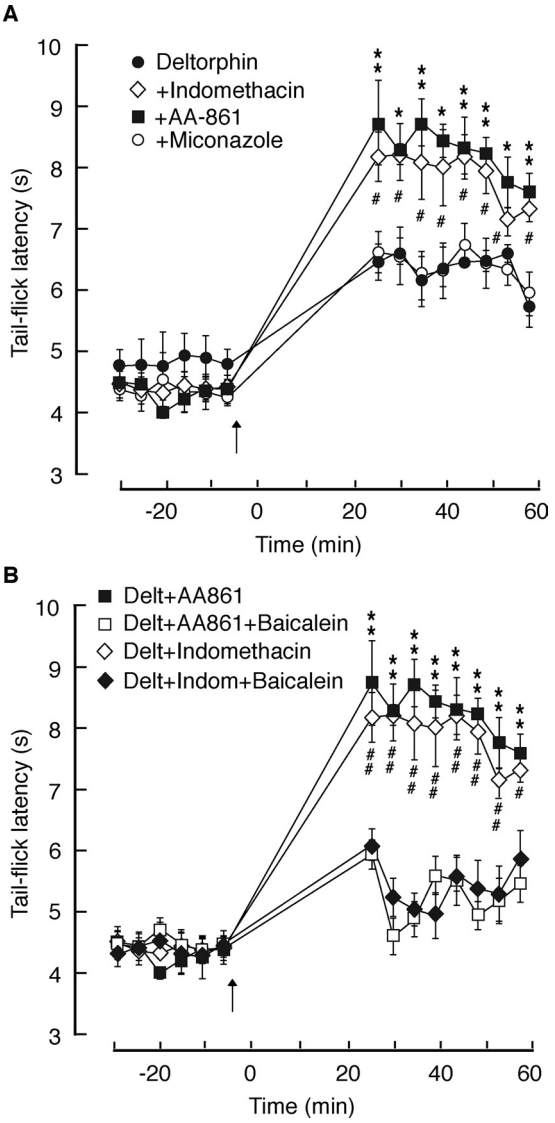


**Fig. 7.** Inhibition of COX or 5-LOX augments DOR-mediated antinociception through 12-LOX. A, behavioral effects of a pretreatment (1 h before) with indomethacin (20 mg/kg i.p., $n = 5$ rats) or with NRM microinjection of AA-861 (1 $\mu$g, $n = 5$ rats) or miconazole (1 $\mu$g, $n = 5$ rats) on the deltorphin-induced antinociception. $*$ and #, statistical significance of the AA-861 group and the indomethacin group, respectively, compared with the group of deltorphin alone. B, behavioral effects of baicalein (1 $\mu$g) by NRM comicroinjection on facilitatory interaction in the antinociception induced by NRM microinjection of deltorphin plus AA-861 (1 $\mu$g) or deltorphin (Delt) plus indomethacin (Indom, 20 mg/kg, i.p.). $n = 5$ rats for each group. $*$, statistical significance for comparisons between the two groups of deltorphin plus AA-861 without and with baicalein; #, results of statistical comparisons between the two groups of deltorphin plus indomethacin without and with baicalein. The arrows indicate NRM microinjection. #, $p < 0.05$; $**$ and ##, $p < 0.01$.

AA-861 (1 $\mu$g, $n = 5$) (Fig. 7A). Consistent with its lack of effect on DOR inhibition of IPSCs, the epoxygenase/P450 inhibitor miconazole (1 $\mu$g, $n = 5$) preinfused into the NRM failed to alter the deltorphin antinociception (Fig. 7A). Finally, we determined whether this enhanced DOR antinociception by blocking 5-LOX or COX was dependent on the 12-LOX pathway. In morphine-treated rats, pretreatment with NRM infusion of baicalein (1 $\mu$g) plus indomethacin or AA-861 1 h before deltorphin microinjection nearly abolished the enhanced antinociceptive effect ($n = 5$ rats for each group) (Fig. 7B). This result further supports the notion that the enhanced DOR effect is probably due to shunting of AA metabolites from the COX pathway and the 5-LOX pathway to the 12-LOX pathway, which mediates the DOR antinociception.

## Discussion

We have demonstrated cellular and behavioral evidence showing that the 12-LOX pathway functionally couples to the newly emerged DOR induced by long-term opioids and primarily mediates DOR inhibition of GABA synaptic transmission in NRM neurons, leading to a DOR-mediated antinociceptive effect in vivo. Of interest, this 12-LOX pathway, shared by existing MOR on the same GABA terminals, is also involved in the augmentation of DOR-mediated antinociception by blocking the COX and 5-LOX pathways. Thus, the $PLA_2$-AA-12-LOX cascade is a key signaling pathway for the cellular effect and behavioral antinociception of DOR as well as MOR under long-term opioid conditions.

**Signaling of DOR and MOR.** Opioid receptors couple to the release of AA from membrane phospholipids via a G protein-regulated, $PLA_2$-mediated signal transduction pathway (Fukuda et al., 1996). The LOX pathway, or specifically 12-LOX, was first discovered to mediate MOR inhibition of presynaptic GABA release in midbrain periaqueductal gray neurons in the naive condition (Vaughan et al., 1997). Our previous study in central amygdala neurons suggests that MOR inhibition of presynaptic glutamate release is also mediated by a $PLA_2$-regulated mechanism in normal conditions (Zhu and Pan, 2005). Under long-term opioid conditions, the present study shows that both the newly emerged DOR and existing MOR on GABAergic terminals couple to the $PLA_2$-AA-12-LOX pathway, of which the metabolic product 12-HPETE and possibly other metabolites inhibit presynaptic GABA release directly or indirectly by opening the 4-aminopyridine-sensitive potassium channels (Vaughan et al., 1997; Zhu and Pan, 2005). Therefore, it appears that, whereas MOR signaling remains unchanged after long-term opioid treatment, the translocated DOR couples to the same signaling pathway as MOR on GABAergic terminals in NRM neurons. The current study also demonstrates that this DOR signaling is required for its antinociceptive effect in vivo. It should be noted that both the DOR inhibition and MOR inhibition of GABA IPSCs were partially blocked by inhibiting the 12-LOX pathway. As reported in our previous study (Zhang and Pan, 2010), the remaining inhibition is probably mediated, at least partially, by the cAMP/PKA pathway that also couples to the translocated DOR and is recruited to the MOR signaling by long-term opioids. In addition to the AA metabolite-gated potassium channels, our results indicate that DOR inhibition of presynaptic GABA release is also partially mediated by inhibition of the N-type $Ca^{2+}$ channel, a mechanism also shared by MOR inhibition of glutamate synaptic transmission in spinal neurons under naive conditions (Heinke et al., 2011). It is also worth noting that our results were based on pharmacological inhibitors, which, although reasonably selective, have limits in selectivity for blockade of their intended targets. Alternative methods are warranted to further validate the relative weight of the signaling pathways for the DOR effects.

An interesting topic related to receptor signaling is the heteromerization of opioid receptors and particularly, MOR-

DOR heteromers. Recent evidence suggests that, in the rostroventral medulla that contains NRM, long-term morphine increases the abundance of MOR-DOR heteromers, which display altered properties in ligand binding, G protein coupling, and agonist-induced receptor activity (Rozenfeld and Devi, 2011). It is not known whether MOR-DOR heteromerization occurs in NRM neurons after long-term morphine treatment under the experimental conditions of this study. In a preliminary study using functional analysis in NRM neurons, we found no significant changes in the kinetics and potency of MOR and DOR agonist-induced inhibition of presynaptic GABA synaptic release and activation of postsynaptic potassium channels after long-term morphine treatment (J. Ma and Z. Z. Pan, unpublished observations). Further studies with various functional assays are necessary to detect potential MOR-DOR heteromers and examine their signaling properties in the brainstem neurons.

**Interaction of AA Signaling Pathways.** Because long-term opioid-induced DOR appears on MOR-expressing GABA terminals and both opioid receptors share the same 12-LOX pathway, understanding of the functional interaction between DOR and MOR and among the AA signaling pathways is important to reveal the adaptive role of DOR in opioid analgesia under long-term opioid conditions for which analgesic tolerance occurs to MOR agonists. Our observation that blockade of 5-LOX as well as of COX, but not of 15-LOX or epoxygenases/P450, enhanced both the synaptic and behavioral effects of DOR may indicate that the AA-12-LOX, AA-5-LOX, and AA-COX pathways are metabolically more active or dominant than the AA-epoxygenase/P450 and AA-15-LOX pathways so that blocking 5-LOX or COX pathways shunts AA metabolites to the 12-LOX pathway, therefore enhancing the DOR effects. The ineffectiveness of blocking 15-LOX or epoxygenases/P450 on the DOR effects may reflect low metabolic activity of these two pathways in the DOR-coupled AA metabolism. This result is consistent with AA metabolic activities coupled to MOR in naive conditions (Vaughan et al., 1997).

The role of epoxygenases/P450 in the signaling and function of opioid receptors is just emerging. A recent behavioral study using transgenic mice shows that genetic reduction in P450 activity partially blocks analgesia induced by morphine, a primarily MOR agonist (Conroy et al., 2010). However, the underlying cellular mechanisms are unknown. The present results in NRM neurons provide synaptic evidence for the epoxygenase/P450 involvement in MOR inhibition of GABA IPSCs, an opioid effect that accounts for opioid-induced behavioral analgesia (Pan et al., 1997; Fields, 2004). Of interest, our data also show that the synaptic signaling of emerged DOR, in contrast to MOR, does not appear to involve the epoxygenase/P450 pathway, perhaps representing some differences in the DOR and MOR signaling in regulation of GABA release.

**Behavioral Significance of DOR Signaling.** In addition to identifying the 12-LOX pathway responsible for the synaptic action of DOR, the present study also provides evidence for the behavioral significance of this DOR signaling in DOR-mediated antinociception in animals. Thus, DOR signaling through the AA-12-LOX pathway appears to mainly mediate the DOR-induced antinociceptive effect, supporting the synaptic inhibition of GABA release by DOR through the same pathway as a likely underlying mechanism for DOR inhibition of pain. The analgesic effects of nonsteroidal anti-inflammatory drugs, primarily COX inhibitors such as aspirin and indomethacin, are well documented and have been partially attributed to their shunting of endogenous AA metabolites from the COX pathway to the 12-LOX pathway (Williams, 1997). However, whether 5-LOX inhibitors, which also enhance synaptic effects of opioids as shown in this study, are behaviorally analgesic has not been demonstrated. The current study shows that blocking 5-LOX produces a significant augmentation of the DOR-mediated antinociceptive effect that is at least equally potent to the augmentation by blocking COX in long-term opioid conditions. Furthermore, both of the two analgesia-augmenting effects of 5-LOX and COX inhibitors are sensitive to 12-LOX inhibitors, further supporting the notion that the augmentation is at least partly due to shunting of AA metabolites to the 12-LOX pathway.

In summary, the present study has identified the $PLA_2$-AA-12-LOX pathway as a primary signaling cascade for long-term opioid-induced DOR inhibition of GABA synaptic transmission and, consequently, DOR-mediated antinociceptive effect. It also suggests that the combination of DOR-induced 12-LOX metabolites and 5-LOX inhibitors may serve as a promising therapeutic strategy in long-term opioid treatment of chronic pain that has been tolerant to conventional MOR-based analgesics.

### Authorship Contributions

*Participated in research design:* Zhang and Pan.
*Conducted experiments:* Zhang.
*Performed data analysis:* Zhang and Pan.
*Wrote or contributed to the writing of the manuscript:* Zhang and Pan.

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