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Emergence of Functional δ-Opioid Receptors Induced by Chronic Morphine

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Running Title: Emergence of functional delta-opioid receptors

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Abstract	249
Introduction	454
Discussion	1371

Abbreviations:

GAD, glutamic acid decarboxylase; IPSC, inhibitory post-synaptic current; MPE, maximum possible effect; NRM, nucleus raphe magnus.

Abstract

Opioid analgesics remain the choice for the treatment of moderate to severe pain. Recent research has established that the mu-opioid receptor is predominantly responsible for mediating many opioid actions, including analgesia and opioid tolerance. However, the function of delta-opioid receptors is rather puzzling at present with inconsistent reports of system effects by agonists of delta-opioid receptors. The functional interaction between u-opioid receptors and delta-opioid receptors is also poorly understood. In this study, we demonstrate that in a brainstem site critically involved in opioid analgesia, agonists of delta-opioid receptors, ineffective in opioid naïve rats, significantly inhibit presynaptic GABA release in the brainstem neurons from morphine-tolerant rats. In membrane preparation from control brainstem tissues, Western blot detected no proteins of delta-opioid receptors, but consistent delta-opioid receptor proteins were expressed in membrane preparation from morphine-tolerant rats. Immunohistochemical studies reveal that chronic morphine significantly increases the number of delta-opioid receptor-immunoreactive varicosities that appose the postsynaptic membrane of these neurons. The co-localization of delta-opioid receptor-immunoreactive varicosities with the labeling of the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) is also significantly increased. Behaviorally, activation of delta-opioid receptors in the brainstem nucleus, lacking an effect in opioid naïve rats, becomes analgesic in morphine-tolerant rats and significantly reduces morphine tolerance. These findings indicate that chronic morphine induces the emergence of functional delta-opioid receptors and delta-opioid receptor-mediated analgesia likely through receptor translocation to surface membrane in

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GABAergic terminals. They also suggest that opioid drugs with preference for delta-opioid receptors may have better therapeutic effect in a mu-opioid-tolerant state.

Introduction

Opioids are still the most effective analgesics for the treatment of moderate to severe pain, such as cancer pain. Recent studies, particularly those using opioid receptor knockout mice, have clearly established that the μ-opioid receptor is predominantly responsible for mediating major opioid actions, including analgesia, reward and the development of opioid tolerance and dependence (Matthes et al., 1996; Sora et al., 1997; Kieffer, 2000). However, the function of the δ-opioid receptor remains puzzling at present (Fields, 2004). Abundant immunoreactivity for δ-opioid receptors has been illustrated in brain areas important for opioid analgesia (Arvidsson et al., 1995; Cheng et al., 1995; Commons et al., 2001), but no cellular effect of δ-opioid receptors on neurons in these brain areas has been demonstrated in normal conditions. Behaviorally, agonists of δ-opioid receptors produce little to weak analgesic effects under normal conditions in animal studies and in clinical applications (Inturrisi, 2002; Fields, 2004). Some of the δ-opioid receptor agonist-induced analgesia has been argued to result from the effect of recruited μ-opioid receptors (Kieffer, 2000; Scherrer et al., 2004). Other δ-opioid receptor agonist-mediated analgesia may be attributed to normally functional δ-opioid receptors in the spinal cord. The reasons for the lack of significant brain functions of δ-opioid receptors in pain modulation have been unknown. The functional interaction between δ-opioid receptors and μ-opioid receptors in chronic opioid conditions also is poorly understood.

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A significant clinical problem in pain management with current opioid therapies is the development of analgesic tolerance to and dependence on repeatedly used agonists of μ-opioid receptors such as morphine. As agonists of μ-opioid receptors appear to remain the primary and widely used analgesics for pain in the foreseeable future, understanding of functions of δ-opioid receptors in acute and chronic opioid conditions is of great significance for improvement of current opioid therapies by increasing analgesic efficacy and reducing tolerance. The nucleus raphe magnus (NRM) in the medulla is a crucial brainstem site for opioid-induced analgesia. Neurons in the NRM directly modulate pain transmission at the spinal cord via their descending projections to the dorsal horn (Scholz and Woolf, 2002; Fields, 2004). We have previously characterized the cellular mechanisms for μ-opioid receptor-mediated analgesia in the NRM and its functional interactions with the κ-opioid receptor (Pan et al., 1990; Pan et al., 1997). In this study, we examined functions of δ-opioid receptors in morphine naïve rats and in chronic morphine-treated, morphine-tolerant rats in both NRM slice preparations *in vitro* and a rat model of morphine tolerance *in vivo*. We focused on a class of NRM neurons that lacks postsynaptic μ-opioid receptors, is activated by agonists of analgesic μ-opioid receptors through disinhibition (inhibition of GABA synaptic inputs), and presumably inhibits spinal pain transmission through descending inhibition (Pan et al., 1997; Fields, 2004).

Materials and Methods

All procedures involving the use of animals conformed the guidelines set by the University of Texas-MD Anderson Cancer Center Animal Care and Use Committee.

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Chronic morphine treatment. Male, Wistar rats were treated with chronic morphine to induce morphine tolerance as previously described (Pan, 2003). For whole-cell recordings, neonatal rats (9-14 days) were randomly divided into two groups. One group was injected (i.p.) with increasing doses of morphine twice daily for 6 days. The morphine dose was 10 mg/kg on day 1 and increased by 5 mg/kg each day to reach the maximum does of 30 mg/kg on day 5 and 6. The other group was injected with saline for controls. The injection volume was 0.1-0.3 ml. Neonatal rats were used for better visualization of neurons in brain slices for visualized whole-cell recording. It has been shown that the physiological and pharmacological properties of neurons from these young rats are indistinguishable from those of adult rats (Pan et al., 1997). For molecular, immunohistochemical and behavioral experiments, rats (200-300 g) were treated with morphine by subcutaneous implantation of morphine pellets. One morphine pellet (75 mg) was implanted on day 1 and two more morphine pellets were implanted on day 4. Same numbers of placebo pellets were implanted on the same schedule in a separate group of rats as controls. On day 7, brain slice preparations were made for whole-cell recordings, or behavioral experiments were conducted, or NRM tissues were taken for analysis. While developmental differences exist, our previous studies have shown that those cellular studies in slices from neonatal rats serve well as guidelines for mechanisms underlying behavioral effects observed in adult rats (Pan et al., 1997; Bie et al., 2005).

Brain slice preparations. The rat brain was cut in a vibratome in cold ($4^{o}C$) physiological saline to obtain brainstem slices (200 μm thick) containing the NRM. A single slice was submerged in a shallow recording chamber and perfused with preheated ($35^{o}C$) physiological saline (in mM: NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2;

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$CaCl_2$, 2.4; glucose, 11; $NaHCO_3$, 25, saturated with 95% O_2 and 5% CO_2, pH 7.2-7.4). Slices from morphine-treated rats (tolerant group), as well as slices from saline-treated rats (control group), were maintained in 1 μM morphine *in vitro* throughout the recording experiment to prevent morphine withdrawal as previously described (Ingram et al., 1998; Bie and Pan, 2005). A third group of slices (normal group) from saline-treated rats and kept in morphine-free solution was used as controls for the acute morphine.

Whole-cell recordings and data analysis. Visualized whole-cell voltage-clamp recordings were obtained from identified NRM neurons with a glass pipette (resistance 3-5 MΩ) filled with a solution containing (mM): KCl, 126; NaCl, 10; $MgCl_2$, 1; EGTA, 11; Hepes, 10; ATP, 2; GTP, 0.25; pH adjusted to 7.3 with KOH; osmolarity 280-290 mosmol/L. An AxoPatch-1D amplifier and AxoGraph software (Axon Instruments, Inc.) were used for data acquisition and on-line/off-line data analyses. A seal resistance of 2 GΩ or above and an access resistance of 15 MΩ or less were considered acceptable. Series resistance was optimally compensated. The access resistance was monitored throughout the experiment. All NRM cells included in this study were identified as a cell type that lacks the μ-opioid receptor according to the criteria described in our previous study (Pan et al., 1990). Electrical stimuli of constant current (0.25 ms, 0.2-0.4 mA) were used to evoke GABA-mediated inhibitory post-synaptic current (IPSC) with bipolar stimulating electrodes placed close to the recorded cell. With KCl-filled electrodes, GABA IPSCs were in inward direction (Pan et al., 1990). Miniature IPSCs were obtained in 60-sec epochs in the presence of tetrodotoxin (TTX, 1 μM). The AxoGraph software was used to detect and measure the amplitude and intervals of synaptic events, and to analyze their distribution data. On average, cells displayed 128 $\pm$ 29 synaptic events of

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GABA IPSCs during a 60-sec period in normal conditions (n=11). All GABA IPSCs were recorded in the presence of glutamate receptor antagonists D-(-)-2-Amino-5-phosphonopentanoic acid (AP5, 30 μM) and 6-Cyano-7-nitroquinoxaline-2,3dione (CNQX, 10 μM) with a holding potential of –60 mV. Drugs were generally applied through the bath solution unless specified otherwise.

Relative quantitative real-time PCR and Western blotting. Both methods have been published previously (Bie et al., 2005). NRM Tissues were taken from morphine- or placebo-treated rats (n=9 in each group). The primer sequences for PCR were: δ-opioid receptor (TGGGTCTTGGCTTCAGGTGT), (CGTGCATACCACTGCTCCAT) and GAPDH (TGCACCACCAACTGCTTAGC), (GGCATGGACTGTGGTCATGAG). Real-time PCRs were performed using SYBR Green RT-PCR Reagents Kit and the ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The GAPDH gene was used as an internal normalizer. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (Livak and Schmittgen, 2001). For Western blotting, NRM tissues from morphine- (n=10) and placebo- (n=8) treated rats were homogenized and divided into two parts to extract total and membrane proteins separately. Total proteins were prepared after tissue lysis and centrifugation for SDS-PAGE. Membrane protein was extracted with a Membrane Protein Extraction Reagent Kit (Pierce, Rockford, IL). Samples were incubated overnight with a primary antibody for δ-opioid receptors (1:2000, Chemicom, Temecula, CA), and for GAPDH (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive proteins were detected by enhanced chemiluminescence (ECL Advance Kit, Amersham Pharmacia). The intensity of bands was quantitatively analyzed with the software Kodak 1D v.3 (Kodak Co., Rochester,

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NY). For Endo F treatment, total protein samples were incubated with 1 μl Endo F1, F2 or F3 and respective 5x reaction buffer (Native Protein Deglycosylation Kit NDEGLY, Sigma) at 37 ^{o}C for 2 to 5 hours. The reaction products were analyzed on SDS-PAGE.

Quantitative immunohistochemistry. The general methods are similar to those reported previously (Kalyuzhny et al., 1996; Kalyuzhny and Wessendorf, 1998). Serial sections (10 μm) containing the NRM was cut from the brainstem of fixed rats pre-treated with morphine (n=4) or placebo (n=4). Sections were processed for double-labeling immunofluorescence using rabbit antisera directed against the cloned δ-opioid receptor (1:1000 dilution; a gift from Dr. Bob Elde) (Arvidsson et al., 1995), and mouse monoclonal antibodies against GAD-6 (1:200 dilution) (Kalyuzhny and Wessendorf, 1998). For apposition experiments, sections were incubated with δ-opioid receptor antiserum and then counterstained with Fluoro Nissl Green (Quinn et al., 1995). Quantitative analysis of confocal microscopic images was conducted on 408 x 136 μm area within the NRM on four randomly selected sections per rat in each group. The number of single-labeled δ-opioid receptors and GAD varicosities, and the number of double-labeled δ-opioid receptors/GAD varicosities were quantified with custom-designed image analysis software (MVS Pacific, MN). Profiles were defined as double-labeled for δ-opioid receptors and GAD if both appeared in profiles overlapping (yellow color) after merging of pseudo-colored images of δ-opioid receptors (red color) and GAD (green color). NRM neurons were considered apposed by δ-opioid receptor-immunoreactive profiles if there was no conceivable distance observed between δ-opioid receptor-immunoreactive varicosities and cell membrane counterstained with Fluoro Nissl Green in confocal images.

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Microinjection and behavioral experiments. For NRM microinjection, a rat was anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and restrained in a stereotaxic apparatus. A 26-gauge double-guide cannula (Plastics One, Roanoke, VA) was inserted into the brain and aimed at the NRM (AP: -10.0 from the Bregma; L: 0; V: -10.5 from the dura) (Paxinos and Watson 1986). The guide cannula was then cemented in place to the skull and securely capped. The rat was allowed to recover for 5 days from the surgery before implantation of morphine or placebo pellets for tolerance induction and control. Morphine tolerance was assessed by constructing cumulating dose-response curves for acute morphine in both control and morphine-treated groups. The morphine doses used were 0.1, 0.3, 0.6, 1 and 3 mg/kg for control rats and 1, 3, 6, 10 and 30 mg/kg for chronic morphine-treated rats. The time between morphine doses was 45 minutes. Pain threshold was measured by the tail-flick test on a freely moving rat with a Hargreaves analgesic instrument (Stoelting Co., Wood Dale, IL). Tail-flick latency to a heat stimulus was measured every 5 min. The heat intensity was set to elicit stable baseline latencies with a cutoff time of 10 sec. The antinociceptive effect of acute morphine was measured 30 minutes after morphine injection and expressed as maximum possible effect (MPE) defined by: MPE=(test latency-baseline)/(cutoff-baseline). The EC_{50} values were obtained by fitting the morphine dose-response curve with the logistic equation and Kaleidagraph software. After development of morphine tolerance, single NRM microinjection of deltorphin (0.5 μg) or deltorphin plus naltriben (20 ng) was made 15 min before the first morphine dose through a 33-gauge double-injector with an infusion pump at a rate of 0.2 μl/min. The total volume for microinjection was 1 μl. The injection sites for the NRM were histologically verified afterward by injecting 0.5 μl of a

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blue dye through the injector. The confined effect within the NRM of this microinjection method has been demonstrated in our previous study (Bie et al., 2005).

Statistical analyses and materials. General numerical data were statistically analyzed with Students' t tests and presented as standard error of means. Statistic analysis of miniature IPSCs was performed using the Statview software with the Kolmogorov-Smirnov test. Behavioral results were statistically analyzed by an ANOVA for repeated measures and the Tukey-Kramer test of *post hoc* analysis. Morphine and placebo pellets were kindly supplied by the National Institute on Drug Abuse. All other drugs were purchased from Sigma-Aldrich or Tocris Cookson (Ellisville, MO).

Results

We first examined the effect of δ-opioid receptor agonists in NRM neurons that lack μ-opioid receptors, using whole-cell voltage-clamp recording in NRM slices *in vitro*. Focal electrical stimulation evoked an inhibitory postsynaptic current (IPSC) in the presence of glutamate receptor antagonists AP5 (30 μM) and CNQX (10 μM), and glycine receptor antagonist strychnine (10 μM). The IPSC was completely abolished by the $GABA_A$ receptor antagonist bicuculline (30 μM, control, 217.6 $\pm$ 48.1 pA, bicuculline, 12.9 $\pm$ 2.1 pA, n=6, $p<0.01$, Fig. 1A). In normal slices from saline-treated rats under normal, morphine-free conditions (normal group), bath application of selective δ-opioid receptor agonist deltorphin (1 μM) failed to change the holding current in any cell tested (n=8), indicating a general lack of functional postsynaptic δ-opioid receptors in this type of NRM neurons. Deltorphin (1 μM) also failed to alter the IPSC amplitude in these neurons of normal slice group (control, 239.0 $\pm$ 46.9 pA, deltorphin, 240.5 $\pm$ 40.0

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pA, n=18, p>0.05, Fig. 1C). In control slices from saline-treated rats and maintained in 1 μM morphine *in vitro* (control group), deltorphin (1 μM) did not change the amplitude of evoked GABA IPSCs in every cell tested (control, 224.2 ± 30.2 pA, deltorphin, 209.1 ± 27.9 pA, n=16, p>0.05, Figs. 1A and 1C). This indicates a general absence of functional δ-opioid receptors on GABAergic terminals innervating these neurons in control conditions.

However, in slices from chronic morphine-treated rats and maintained in 1 μM morphine *in vitro* to prevent morphine withdrawal (tolerant group), deltorphin at the same concentration (1 μM) significantly reduced the amplitude of GABA IPSCs in 9 of 20 (45%) neurons surveyed (control, 298.1 ± 52.9 pA, deltorphin, 178.5 ± 25.1 pA, n=9, p<0.01, Figs. 1B and 1C). The maximum inhibition was 36.1 ± 8.2% of controls. Addition of the selective δ-opioid receptor antagonist naltriben (10 μM) blocked this deltorphin effect (control, 217.4 ± 31.3 pA, deltorphin, 163.7 ± 24.5 pA, deltorphin plus naltriben, 210.5 ± 25.9 pA, n=6, p>0.05 when compared with control, Figs. 1B and 1C). The μ-opioid receptor-selective antagonist CTAP (1 μM) did not alter the deltorphin-induced inhibition (CTAP, 288.3 ± 29.9 pA, CTAP plus deltorphin, 209.0 ± 21.8 pA, n=5, p<0.05), neither did the selective κ-opioid receptor antagonist nor-Binaltorphimine dihydrochloride (norBNI,1 μM) (norBNI, 235.1 ± 58.6 pA, norBNI plus deltorphin, 170.8 ± 44.5 pA, n=4, p<0.05). These results suggest that the emerged deltorphin inhibition of GABA IPSCs is selectively mediated by δ-opioid receptors.

To determine the synaptic site of this action of δ-opioid receptors, we examined deltorphin effect on GABAergic miniature IPSCs in the presence of tetrodotoxin (1 μM). In slices of control group, deltorphin (1 μM) had no effect on either the frequency or

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amplitude of GABA miniature IPSCs (Figs. 2A, 2B and 2F). However, in identified neurons whose evoked IPSCs were inhibited by deltorphin in slices of tolerant group, deltorphin (1 μM) also significantly decreased the miniature IPSC frequency (control, 1.47 $\pm$ 0.36 Hz, deltorphin, 0.85 $\pm$ 0.17 Hz, n=5, $p<0.05$, Figs. 2C, 2D and 2F), without altering the IPSC amplitude (Figs. 2E and 2F). No deltorphin effect was observed on either frequency or amplitude of GABA miniature IPSCs in the normal slice group (Frequency: control, 2.14 $\pm$ 0.49 Hz, deltorphin, 2.30 $\pm$ 0.55 Hz; amplitude: control, 41.0 $\pm$ 3.1 pA, deltorphin, 40.9 $\pm$ 3.0 pA, n=11, $p>0.05$). These observations indicate that the emerged inhibition of GABA IPSCs by δ-opioid receptors is caused by reduction of presynaptic GABA release from GABAergic terminals.

This emerged functional δ-opioid receptors could be due to an increased expression of δ-opioid receptors in the nucleus through transcriptional upregulation and/or upregulated protein translation. To determine if that was the case, we used real-time RT-PCR to determine changes in expression level of mRNA for δ-opioid receptors in the NRM. The amplification plots of signals for δ-opioid receptors showed that the averaged cycle threshold (Ct) in NRM tissues taken from placebo-treated rats and from morphine-treated rats was 33.91 and 33.51, respectively ($\Delta Ct=0.4$, Fig. 3A). After normalization to internal reference GAPDH, relative quantitative analysis showed that there was a 0.74 fold increase in δ-opioid receptor mRNA in the NRM from morphine-treated rats, but that increase did not reach statistical significance ($p=0.18$, Fig. 3B). We then used Western blot to determine changes in both total proteins of δ-opioid receptors and membrane proteins of δ-opioid receptors in the NRM after chronic morphine treatment. We found no detectable δ-opioid receptor protein in membrane preparations

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from placebo-treated rats (n=8), but in NRM tissues from morphine-treated rats (n=10), membrane proteins of δ-opioid receptors were consistently present in the NRM from all 10 rats (Fig. 3C). In contrast, no significant difference was detected in total proteins of δ-opioid receptors, normalized to GAPDH, between NRM tissues from placebo- and morphine-treated rats (Figs. 3C and 3D). To examine the contribution of protein glycosylation to the molecular weight of the δ-opioid receptor, total protein samples were incubated in endoglycosidases F1, F2 and F3 to remove potential N-linked oligosaccharides on the receptor protein. As shown in Fig. 3E, the molecular weight of the protein was still close to 72 KD, indicating that glycosylation does not significantly contribute to the molecular weight of δ-opioid receptors in this NRM. Thus, it appears that chronic morphine induces only a moderate, if any, upregulation of δ-opioid receptor mRNA, but largely increases δ-opioid receptor proteins in surface membrane, with no significant change in total proteins of δ-opioid receptors as determined by the Western blot technique in a morphine-dependent state.

G-protein-coupled receptors, including opioid receptors, are dynamically regulated through the mechanism of receptor trafficking and membrane insertion (Tan et al., 2004). We next used immunohistochemistry and quantitative confocal microscopy to examine expression and trafficking of δ-opioid receptors. We found that the total number of varicosities immunoreactive for δ-opioid receptors in the NRM from morphine-treated rats significantly increased by 73.4% ($p<0.01$), while there was no significant change in total labeling for the glutamic acid decarboxylase (GAD) (average in 16 images from 4 rats in each treatment group, Fig. 4). Co-localization of δ-opioid receptor- and GAD-immunoreactive varicosities also was significantly increased from 0 to 1.75 $\pm$ 0.30 per

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examined NRM area of the same images (Figs. 4B and 4C). To examine potential synaptic contacts of presynaptic δ-opioid receptors on postsynaptic cell membrane, we determined changes in δ-opioid receptor-immunoreactive varicosities apposing the membrane of NRM neurons. Quantitative analysis revealed a significant increase in the number of δ-opioid receptor-immunoreactive varicosities that apposed on the membrane of a NRM neuron in slices from morphine-treated rats (Fig. 5). The number of neurons apposed by δ-opioid receptor-immunoreactive varicosities was also significantly increased. This increase was observed in a comparable total number of cells in each treatment group (Fig. 5C).

We have shown previously that a primary action of μ-opioid agonists producing behavioral analgesia in the NRM is inhibition of GABA synaptic transmission through presynaptic μ-opioid receptors (Pan et al., 1997). Then, the emergence of δ-opioid receptor-mediated inhibition of GABA IPSCs should lead to δ-opioid receptor-mediated analgesia in morphine-treated rats *in vivo*. In our next behavioral experiments, single microinjection of deltorphin (1 μg) into the NRM did not significantly alter the baseline pain threshold measured by the tail-flick test on a freely moving rat in the placebo-treated group (n=4 rats). However, in chronic morphine-treated rats (n=5), the same dose and same NRM microinjection of deltorphin produced a significant antinociceptive effect that lasted about an hour (Fig. 6A). Co-NRM microinjection of deltorphin with the δ-opioid receptor antagonist naltriben (20 ng) largely blocked this deltorphin effect (n=6 rats), indicating a selective δ-opioid receptor-mediated effect. Further verifying this effect of δ-opioid receptors, NRM co-microinjection of the μ-opioid receptor antagonist CTAP (200 ng) abolished the antinociceptive effect induced by the μ-opioid receptor agonist

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DAMGO (1 μg, n=3 rats in each group), but it did not significantly alter the deltorphin effect (n=3, Fig. 6B). NRM microinjection of a higher dose of deltorphin (3 μg) in morphine-tolerant rats produced stronger analgesia, but it was only partially blocked by naltriben (data not shown).

Finally, we examined deltorphin effect on chronic morphine-induced analgesic tolerance. Concerning the small residual effect of 1 μg deltorphin that was resistant to δ-opioid receptor antagonist naltriben at a dose of 20 ng (Fig. 6A), we used a smaller dose of deltorphin (0.5 μg) to make sure that only the δ-opioid receptor was activated by deltorphin in examining its effect on morphine tolerance. In the groups of placebo-treated rats and morphine-treated rats with single microinjection of saline into the NRM prior to morphine analgesia tests, the morphine dose-response curve in the morphine-treated rats was significantly shifted to the right by >8 fold when compared to that in the placebo-treated rats, as measured by their estimated EC_{50} values (placebo treated, 1.34 $\pm$ 0.15 mg/kg, n=6, morphine treated, 10.90 $\pm$ 1.25 mg/kg, n=6, $p<0.01$). This shift represents strong analgesic tolerance with much higher morphine concentrations required to produce comparable analgesic effects in morphine–treated rats (Fig. 6C). NRM microinjection of deltorphin (0.5 μg) had no apparent effect in the placebo-treated rats, but significantly shifted the morphine dose-response curve to the left in the morphine-treated rats (placebo-treated, EC_{50}=1.19 $\pm$ 0.28 mg/kg, n=4, morphine-treated, EC_{50} = 6.42 $\pm$ 1.00 mg/kg, n=4), indicating a 41% reduction in the chronic morphine-induced tolerance measured by change in the EC_{50} values (morphine-treated plus saline, 10.90 $\pm$ 1.25 mg/kg, morphine-treated plus deltorphin, 6.42 $\pm$ 1.00 mg/kg, $p<0.01$, Fig. 6C). While NRM microinjection of δ-opioid receptor antagonist naltriben (20 ng) did not

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significantly change the morphine dose-response curve in the placebo-treated rats (EC_{50}=1.43 ± 0.21 mg/kg, n=3), it completely reversed the deltorphin effect when co-injected with deltorphin in the morphine-treated rats (EC_{50}=15.76 ± 4.97 mg/kg, n=5, $p<0.05$ when compared to 6.42 ± 1.00 mg/kg for the group of NRM deltorphin alone), indicating a specific effect mediated by δ-opioid receptors (Fig. 6D).

Discussion

We have demonstrated that functional δ-opioid receptors, absent in morphine naïve rats, appears on GABAergic terminals to inhibit presynaptic GABA release in NRM neurons from morphine-tolerant rats. This emergence of δ-opioid receptor function is likely mediated by translocation of δ-opioid receptors to surface membrane on GABA terminals. This notion is supported by the appearance of δ-opioid receptors in NRM preparations of membrane proteins, by an increased co-localization of δ-opioid receptors and GAD, and by the significant increase in the number of δ-opioid receptor-immunoreactive profiles that appose NRM neurons. The function of the emerged δ-opioid receptors is further supported by behavioral observations that activation of δ-opioid receptors becomes analgesic and relieves analgesic tolerance in morphine-tolerant rats.

Previous anatomical studies at light and electron microscopy levels have shown abundant immunoreactivity of δ-opioid receptors in brain areas involved in pain modulation, including several brainstem nuclei such as the periaqueductal gray, the NRM and other raphe nuclei (Arvidsson et al., 1995; Cheng et al., 1995; Commons et al., 2001). The δ-opioid receptor in all these areas is predominantly present in presynaptic

axon terminals, associated with large dense-core vesicles in the intracellular compartments, but not on plasma membrane of synaptic boutons (Commons et al., 2001). Despite the presence of the δ-opioid receptor illustrated by immunocytochemistry in these areas, no δ-opioid receptor-mediated cellular action has been reported in those brainstem areas in normal conditions (Chieng and Christie, 1994; Fields, 2004). This indicates that these intracellularly localized δ-opioid receptors are not functional in normal conditions. While a large proportion of immunoreactive δ-opioid receptors co-localize with enkephalin immunoreactivity in the brainstem (Arvidsson et al., 1995; Cheng et al., 1995), no co-localization of δ-opioid receptors and GABA immunoreactivity was observed in the periaqueductal gray under normal conditions (Commons et al., 2001). We also found no co-localization of δ-opioid receptors and GAD in the NRM from morphine naïve rats, consistent with our electrophysiological observation of a lack of δ-opioid receptor-mediated effect on presynaptic GABA release.

In the spinal cord including the dorsal horn, immunoreactivity of δ-opioid receptors also has been shown predominantly in axon terminals with some labeling for δ-opioid receptors on cell bodies (Arvidsson et al., 1995; Cheng et al., 1995). In contrast to the lack of δ-opioid receptor-mediated cellular effect in the brainstem, activation of spinal δ-opioid receptors inhibits glutamate synaptic current, with no effect on GABA synaptic transmission (Glaum et al., 1994; Kohno et al., 1999). Behaviorally, activation of spinal δ-opioid receptors produces significant analgesia under normal conditions (Standifer et al., 1994; Cahill et al., 2001).

It is increasingly recognized that opioid receptors, like other G protein-coupled receptors, undergo rapid and dynamic regulation by the mechanisms of receptor

trafficking (Tan et al., 2004). As an initial step of receptor trafficking, receptor internalization has been extensively studied and a mechanism involving G protein-coupled receptor kinase and β-arrestin has been demonstrated (Krupnick and Benovic, 1998). Several recent studies have reported the trafficking of opioid receptors stimulated by various conditions. In the spinal cord, density of postsynaptic δ-opioid receptors on cell bodies and δ-opioid receptor-mediated analgesic action are increased by 48 hours morphine exposure (Cahill et al., 2001). Stress and inflammation increase the expression of δ-opioid receptors and the analgesic action of δ-opioid receptor agonists (Hurley and Hammond, 2000; Commons, 2003). As for the mechanisms for δ-opioid receptor insertion into surface membrane, recent studies have demonstrated in dorsal root ganglion neurons that interaction of the third luminal domain of δ-opioid receptors with the substance P domain of protachykinin sorts the δ-opioid receptor into large dense-core vesicles, leading to Ca^{++}-dependent δ-opioid receptor insertion into surface membrane upon activation of vanilloid and $P2Y_1$ receptors (Bao et al., 2003; Guan et al., 2005). In addition, withdrawal from morphine induces δ-opioid receptor-mediated inhibition of GABA synaptic current in neurons of the periaqueductal gray (Hack et al., 2005). cAMP analogs induce β-arrestin-dependent inhibition of GABA IPSCs by μ-opioid receptors in dorsal motor nucleus of the vagus neurons (Browning et al., 2004). The current study further demonstrates with anatomical, molecular, cellular and behavioral evidence that in an opioid-tolerant state, functional δ-opioid receptors appear on surface membrane of cell-apposing GABAergic terminals, inhibits GABA release, produces analgesia and relieves morphine tolerance. The mechanism underlying chronic morphine-induced δ-opioid receptor trafficking is currently unknown.

The evidence from the current study supports the increase in membrane δ-opioid receptors on presynaptic GABAergic terminals as the primary mechanism for the emerged cellular and behavioral actions of δ-opioid receptors. This does not exclude a possible mechanism of increased mRNA expression and protein synthesis for δ-opioid receptors, as our real-time RT-PCR experiment detected a moderate, though not statistically significant, increase (74%) in δ-opioid receptor mRNA level after morphine treatment. The failure of the Western blotting experiment to detect any change in total proteins of δ-opioid receptors in the NRM may reflect the fact that the change was too small to be detected by the technique in our experimental conditions. Consistent with the PCR experiment for δ-opioid receptor mRNA, immunocytochemical analysis showed a significant and comparable increase (73.4%) in total immunoreactivity of δ-opioid receptors in the NRM of morphine-tolerant rats. The source of the translocated δ-opioid receptors on terminal membrane is currently unclear. They may come primarily from the intracellular pool of δ-opioid receptors. As discussed above, newly synthesized δ-opioid receptors may also contribute. Another possibility is that they are transported externally from the periaqueductal gray, as 50% of neurons in the periaqueductal gray projecting directly to the NRM express δ-opioid receptor mRNA (Wang and Wessendorf, 2002). It is noteworthy that the chronic morphine-induced cellular action of δ-opioid receptors was observed only in about 45% of NRM neurons. The reason for this partial surface expression of δ-opioid receptors among NRM cells is unclear at present. However, it is interesting to note that under opioid withdrawal conditions, the distribution of this functional δ-opioid receptors is increased to >90% of NRM cells (Ma and Pan, unpublished observations). This indicates that all these GABAergic terminals are capable

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of expressing the functional δ-opioid receptors and the regulating mechanism is related to the magnitude of chronic morphine-induced adaptations that are further augmented during opioid withdrawal.

The function of δ-opioid receptors in pain modulation has been particularly perplexing. agonists of δ-opioid receptors generally produce little to weak analgesic effect under normal conditions in both animal studies and clinical applications (Kovelowski et al., 1999; Inturrisi, 2002; Hurley et al., 2003; Fields, 2004; Scherrer et al., 2004). This can be largely attributed to the lack of δ-opioid receptor-mediated cellular actions in pain-modulating neurons in the brain and only the spinal δ-opioid receptor-mediated analgesia. Synergism of δ-opioid receptors and μ-opioid receptors in producing analgesia has been reported (Kovelowski et al., 1999; Schmidt et al., 2002; Gomes et al., 2004). In δ-opioid receptor knockout mice, spinal δ-opioid receptor-mediated analgesia is nearly abolished, but agonists of δ-opioid receptors retain their supraspinal analgesic potency, which is insensitive to antagonists of δ-opioid receptors (Zhu et al., 1999). This retained analgesia mediated by agonists of δ-opioid receptors in δ-opioid receptor knockout mice has been found later to be abolished by antagonists of μ-opioid receptors (Kieffer, 2000; Scherrer et al., 2004). In δ-opioid receptor-deficient mice, δ-opioid receptor agonist-mediated analgesia is attenuated (Matthes et al., 1998). These findings question the receptor selectivity of δ-opioid receptor agonists applied systemically *in vivo* without confirmation by δ-opioid receptor antagonists and argue that some of the analgesia induced by agonists of δ-opioid receptors is actually mediated by μ-opioid receptors. The current study shows that chronic morphine induces δ-opioid receptor-

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mediated analgesia likely through inhibition of GABA synaptic transmission via emerged δ-opioid receptors on GABAergic terminals in the NRM neurons. This disinhibition mechanism for analgesia by μ-opioid receptor agonists in the NRM has been demonstrated in our previous studies (Pan et al., 1990; Pan et al., 1997). As expected, the emerged analgesia mediated by δ-opioid receptors adds to the analgesic effect of morphine and relieves morphine tolerance. The findings of this study reveal a compensatory analgesic function of δ-opioid receptors and its functional interaction with μ-opioid receptors in a condition of chronically stimulated μ-opioid receptors. With enhanced analgesia and reduced tolerance in an opioid-tolerant state, agonists of δ-opioid receptors may hold great promise as better analgesics or adjuvant analgesics to improve current opioid therapies based on agonists of μ-opioid receptors for the treatment of chronic pain.

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Figure Legends

Fig. 1. Chronic morphine induces δ-opioid receptor-mediated inhibition of evoked GABA IPSCs in neurons of the nucleus raphe magnus (NRM). A, Representative evoked IPSCs recorded with KCl-filled pipettes in controls and in the presence of $GABA_A$ receptor antagonist bicuculline (30 μM, left) or in the presence of the selective agonist of δ-opioid receptors deltorphin (1 μM, right) in NRM slices from saline-treated rats. B, GABA IPSCs in control and in deltorphin without (left) or with (right) the addition of selective antagonist of δ-opioid receptors naltriben (10 μM) in NRM neurons from chronic morphine-treated rats. C, Summarized data of deltorphin effects in NRM slices from saline-treated rats and maintained *in vitro* in morphine-free solution (normal group) or in 1 μM morphine (control group), and in NRM slices in 1 μM morphine from chronic morphine-treated tolerant rats with or without naltriben. Numbers in columns indicate cell numbers. Scales in A apply to both A and B. Error bars are standard error of means. ** $p<0.01$.

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Fig. 2. Chronic morphine induces deltorphin inhibition of presynaptic GABA release. A, B, Plots of cumulative distribution of frequency (A) and amplitude (B) of GABA miniature IPSCs in NRM neurons from a saline-treated rat. C-E, Representative current traces of miniature IPSCs (C) and distribution plots of miniature IPSC frequency (D) and amplitude (E) in control and in the presence of deltorphin in NRM neurons from a morphine-treated rat. F, Summarized data of deltorphin effects on miniature IPSCs in neurons from the two groups of rats. * $p<0.05$.

Fig. 3. Chronic morphine increases membrane proteins of δ-opioid receptors in the NRM. A, B, Applications plot of real-time RT-PCR for δ-opioid receptor mRNA (A) and normalized fold change in δ-opioid receptor mRNA (B) in placebo-treated rats and morphine-tolerant rats. C, Representative lanes of Western blots of membrane proteins of δ-opioid receptors (top panel), total proteins of δ-opioid receptors (middle panel) and total GAPDH proteins (bottom panel) in the NRM from placebo-treated rats and morphine-tolerant rats. The molecular weight was 72 KD for δ-opioid receptors and 37 KD for GAPDH. D, Normalized percent change of total proteins of δ-opioid receptors in the NRM from the two groups of rats. E. Total proteins of δ-opioid receptors in the NRM from placebo and tolerant rats after incubation with endoglycosidases F1, F2 and F3. The molecular weight after the Endo F treatment was 72 KD. Plac, placebo.

Fig. 4. Chronic morphine increases the total number of δ-opioid receptor-immunoreactive varicosities and co-localization of δ-opioid receptors and GAD in the NRM. A-C, Confocal microscopic images of labeling for δ-opioid receptors (red), GAD (green) and

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co-localization of δ-opioid receptors and GAD (yellow, circled) in the NRM from a placebo-treated rat (A) and from a morphine-tolerant rat (B, C). Scale bar in A is 50 μm for both A and B. Scale bar in C is 5 μm. D, Summarized data of total δ-opioid receptors and GAD from the two groups of rats. ** $p<0.01$.

Fig. 5. Chronic morphine increases the number of δ-opioid receptor-immunoreactive varicosities apposing NRM neurons. A, A representative confocal microscopic image showing δ-opioid receptor-immunoreactive varicosities (red, arrowheads) that do not appose to an Fluoro Nissl Green-labeled (green) NRM neuron from a placebo-treated rat. B, A confocal image showing δ-opioid receptor-immunoreactive varicosities that appose to a NRM neuron from a morphine-tolerant rat. Scale bar is 10 μm and applies to both A and B. C, Summarized data of cell apposition of δ-opioid receptor-immunoreactive varicosities from the two groups of rats. ** $p<0.01$.

Fig. 6. Chronic morphine induces δ-opioid receptor-mediated analgesia and reduction of morphine tolerance in rats *in vivo*. A, Effects of deltorphin microinjected into the NRM (arrow) on pain thresholds in placebo-treated rats (open circles) and in chronic morphine-treated rats without (filled circles) or with (filled squares) NRM co-microinjection of naltriben. B, Effects of NRM microinjection (arrow) with DAMGO (open circles), CTAP plus DAMGO (filled circles) and CTAP plus deltorphin (filled squares) on pain thresholds in morphine-tolerant rats. Statistical comparison was made between groups of CTAP plus DAMGO and CTAP plus deltorphin. C, D, Morphine dose-response curves in placebo-treated rats (open symbols) and chronic morphine-treated rats (filled symbols)

with NRM microinjection of saline (circles, same data in C and D). Deltorphin was microinjected into the NRM in placebo-treated rats (C, open squares) and in morphine-treated rats (C, filled squares). Naltriben was microinjected into the NRM in placebo-treated rats (D, open squares) and naltriben plus deltorphin was microinjected into the NRM in morphine-treated rats (D, filled squares). MPE, maximum possible effect (see Materials and Methods). * $p<0.05$, ** $p<0.01$.

Figure 1

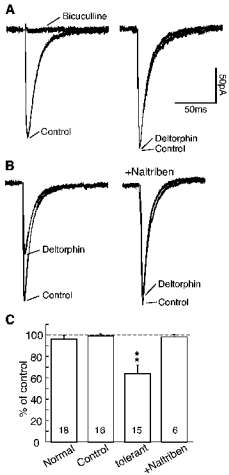
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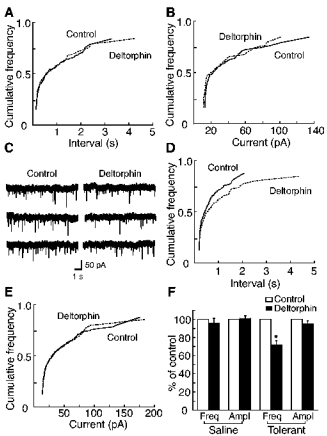
Figure 2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Deltorphin
Control
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100
140
Current (pA)
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Control
Deltorphin
50 pA
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D
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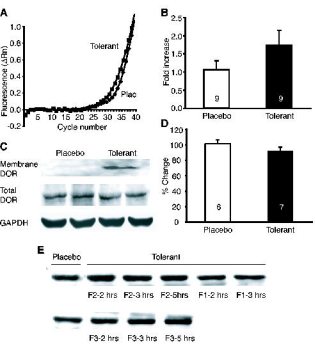
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Figure 4

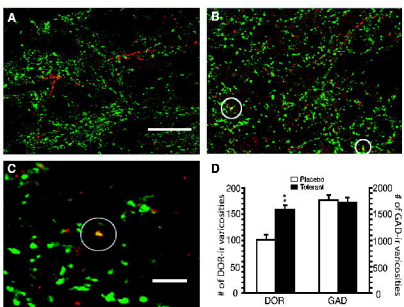
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Figure 5

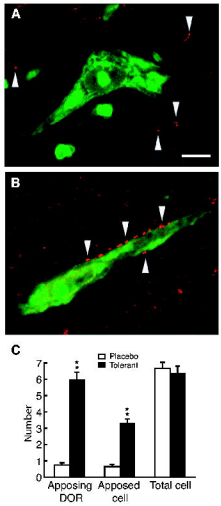
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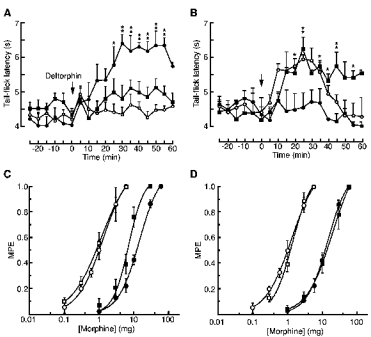
Figure 6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