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Co-administration of ultra-low dose naloxone attenuates morphine tolerance in rats via attenuation of NMDA receptor neurotransmission and suppression of neuroinflammation in the spinal cords

Shinn-Long Lin [a,b], Ru-Yin Tsai [b], Ching-Hui Shen [a,c], Fu-Huang Lin [a], Jhi-Joung Wang [d], Shih-Tai Hsin [e], Chih-Shung Wong [a,b,f,*]

[a] Graduate Institute of Medical Science, National Defense Medical Center, Taipei, Taiwan
[b] Department of Anesthesiology, Tri-Service General Hospital and National Defense Medical Center, Taipei, Taiwan
[c] Department of Anesthesiology, Taichung Veterans General Hospital, Taichung, Taiwan
[d] Department of Medical Research and Anesthesiology, Chi-Mei Medical Center, Tainan, Taiwan
[e] Department of Anesthesiology, Chang-Hua Hospital, Department of Health, Taiwan
[f] Department of Anesthesiology, Cathay General Hospital, Taipei, Taiwan

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ABSTRACT

Although mechanisms underlying ultra-low dose naloxone-induced analgesia have been proposed, possible interactions with glutamatergic transmission and glial cell activation have not been addressed. In the present study, we examined the effect of ultra-low dose naloxone on spinal glutamatergic transmission and glial cell activity in rats chronically infused with morphine.

In male Wistar rats, intrathecal morphine infusion (15 μg/h) for 5 days induced (1) antinociceptive tolerance, (2) downregulation of glutamate transporters (GTs) GLT-1, GLAST, and EAAC1, (3) increasing of NMDA receptor (NMDAR) NR1 subunit expression and phosphorylation, (4) upregulation of protein kinase C gamma (PKCγ) expression, and (5) glial cell activation. On day 5, morphine challenge (15 μg/10 μl) caused a significant increase in the concentration of the excitatory amino acids (EAAs) aspartate and glutamate in the spinal CSF dialysates of morphine-tolerant rats. Intrathecal co-infusion of ultra-low dose naloxone (15 pg/h) with morphine attenuated tolerance development, reversed GTs expression, inhibited the NMDAR NR1 subunit expression and phosphorylation, and PKCγ expression, inhibited glial cell activation, and suppressed the morphine-evoked EAAs release. These effects may result in preservation of the antinociceptive effect of acute morphine challenge in chronic morphine-infused rats. Ultra-low dose naloxone infusion alone did not produce an antinociceptive effect. These findings demonstrated that attenuation of glutamatergic transmission and neuroinflammation by ultra-low dose naloxone co-infusion preserves the lasting antinociceptive effect of morphine in rats chronically infused with morphine.

1. Introduction

Opioids are the mainstay treatment for moderate to severe pain in the clinic, but their use is hampered by the development of tolerance after long-term administration (Ossipov et al., 2004). We previously demonstrated that opioid receptor uncoupling and downregulation are involved in the development of opioid tolerance (Wong et al., 1992). Activation of central glutamatergic receptors, in particular N-methyl-D-aspartate receptor (NMDAR), and protein kinase C gamma (PKCγ) also plays an important role in the development of opioid tolerance (Lim et al., 2005; Mao et al., 1994; Mao et al., 1995; Mayer et al., 1999; Sanchez-Blazquez et al., 2009; Trujillo and Akil, 1991). Prolonged exposure to morphine was demonstrated to activate NMDAR and downregulate spinal glutamate transporters (GTs) (Mao et al., 2002). In a rat spinal model, we found that both competitive and non-competitive NMDAR antagonists not only inhibit the development of tolerance, but also prevent the decrease in mu-opioid receptor (MOR) high-affinity binding sites in chronic morphine-infused rats (Wong et al., 1996). Furthermore, we also observed an increase in the ^{3}H-MK-801 binding affinity of NMDAR in morphine-tolerant rats (Wong et al., 2000b). NMDAR NR1 subunit, specifically, was shown to be a target for phosphorylation by PKC at serine 896 (Tingley et al., 1997) and the phosphorylation of NR1 subunit was found to potentiate NMDAR activity, which facilitated nociceptive input transmission in inflammatory and neuropathic pain models (Gao et al., 2005; Zhang et al., 2008). Additionally, NMDAR antagonist MK-801 was demonstrated to prevent PKCγ recruitment

* Corresponding author. Department of Anesthesiology, Cathay General Hospital, No. 280, Renai Road, Section 4, Taipei, Taiwan. Tel.: +886 2 27082121x3510; fax: +886 2 87924835.
E-mail address: w82556@gmail.com (C.-S. Wong).

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to MORs and serine phosphorylation of the receptors following intracerebroventricular (icv) morphine (Rodriguez-Munoz et al., 2008). In patients receiving long-term intrathecal (i.t.) morphine for cancer pain control, we have observed an increase in the levels of glutamate and aspartate in the cerebrospinal fluid (CSF), accompanied by a reduction in the analgesic effect of morphine (Wong et al., 2002). Intriguingly, we have also observed an increase in CSF EAA levels in morphine-tolerant rats following morphine challenge (Tai et al., 2006; Wen et al., 2004). Based on these findings, an increase in glutamatergic transmission due to either a high synaptic glutamate concentration or NMDAR hypersensitivity is implicated in the development of opioid tolerance.

Glial cells were originally considered to merely play a supporting role in the central nerve system, but are now recognized as playing a critical role in the development of morphine tolerance (Song and Zhao, 2001). Chronic morphine treatment has been shown not only to increase spinal microglial reactivity (Raghavendra et al., 2002; Tawfik et al., 2005), but also to enhance microglial cell migration (Horvath and DeLeo, 2009; Inoue, 2006). Thus, the targeting of glial cell activity is a promising strategy for the treatment of morphine insensitivity, including treatment of neuropathic pain and prevention of development of tolerance.

Co-administration of a second drug with an opioid is a strategy for enhancing the antinociceptive effect and attenuating the development of tolerance (Chavooshi et al., 2009; Haghparast et al., 2008; Jinsmaa et al., 2008; Pinardi et al., 2005). Ultra-low dose naloxone, used as an effective adjuvant with opioids to suppress opioid tolerance and dependence, has been shown to be associated with changes in MOR-G coupling and Gbetagamma subunit signaling in chronic morphine-treated rats (Wang et al., 2005). Moreover, ultra-low dose naloxone prevents the coupling switch of Gi/Gs protein, thus attenuating opioid tolerance, via the high-affinity interaction of naloxone with a pentapeptide in the C-terminal region of filamin A, a scaffolding protein interacting with the mu-opioid receptor (Wang and Burns, 2009). Furthermore, i.t. morphine (2 mg) combined with naloxone (20 ng) has been reported to produce a dramatic improvement in pain management in humans with severe chronic low back pain (post-laminectomy syndrome) (Hamann et al., 2008).

On the basis of the evidence for increased glutamatergic transmission and glial cell activation during the development of morphine tolerance and the modulating effect of ultra-low dose naloxone on the antinociceptive effect of morphine, in the present study, we investigated whether the effect of ultra-low dose naloxone in maintaining the antinociceptive effect of morphine during tolerance development was through regulation of glutamatergic transmission and glial cell activation in the spinal cord. Thus, the goal of present study is to determine the effects of ultra-low dose naloxone on glutamatergic transmission and glial cell activation in morphine tolerance.

2. Materials and methods

This experiment was approved by our Animal Use and Care Ethics Committee according to the 3R principle (replacement, reduction, and refinement), and the use of rats in this study conformed to the Guiding Principles in the Care and Use of Animals of the American Physiology Society and was approved by the National Defense Medical Center Animal Care and Use Committee.

2.1. Animal preparation and microdialysis probe implantation

Pathogen-free male Wistar rats (350–400 g) were anesthetized with phenobarbital (65 mg/kg, intraperitoneally; Sigma) and implanted with two i.t. catheters (8.5 cm ± 0.5 cm) via the atlantooccipital membrane down to the lumbar enlargement (L1–L2) of the spinal bony structure. In rats used for CSF EAA dialysate collection, a microdialysis loop was also implanted into the intrathecal space. Levels of spinal bony structure L1–L2 correspond to spinal cord segments L5, L6, and S1–S3 which are responsible for the tail-flick reflex (Grossman et al., 1982). The ends of the catheters and the dialysis probe were externalized and fixed to the dorsal aspect of the head. One i.t. catheter was connected to a mini-osmotic pump and used for a period of 5 days for infusion with morphine (15 μg/h), naloxone (15 pg/h), saline (1 μl/h), or morphine (15 μg/h) plus naloxone (15 pg/h), and the other catheter was preserved for morphine challenge on day 5. Microdialysis probe assembly was adapted and modified from that in previous studies (Marsala et al., 1995; Wen et al., 2004). The probe was assembled from two polyethylene tubes (PE5; 7 ± 0.5 cm in length, 0.008 in. ID, 0.014 in. OD, Spectranetics, Colorado Springs, CO, USA) with an active dialysis region, a 4.2 cm cuprophan hollow fiber (Hospal, M100 Pre Set, France), and two 3.5 cm silastic tubes. A Nichrome-Formvar wire (0.0026 in. diameter; A-M System, Everret Inc., WA, USA) was passed through a polycarbonate tube (102 μm ID, 194 μm OD; 0.7 cm in length) and the cuprophan hollow fiber (active dialysis region), which was then connected to a PE5 catheter with epoxy resin. The middle portion of the cuprophan hollow fiber was bent to form a U-shaped loop, and both ends of the dialysis loop, which consisted of silastic tubes, were sealed with silicone rubber. The dead space of the dialysis probe was 8 μl and the recovery rate of the probe around 43% at an infusion rate of 5 μl/min during in vitro measurements (Marsala et al., 1995). With this setting, it is feasible to measure CSF amino acids for up to 12 days after implantation. After catheterization, all rats were returned to their home cages for recovery, each rat being housed individually and maintained on a 12 h light/dark cycle with food and water available ad libitum. Motor function was quantified by assessment of ambulation using the hindlimbs placing/stepping reflex after recovery (Marsala and Yaksh, 1994). Rats with neurological deficits were excluded.

2.2. Drug delivery and antinociceptive test

One group of rats received continuous morphine infusion for 5 days for tolerance induction, while another received morphine plus ultra-low dose naloxone to examine the effect on morphine tolerance development. Rats receiving saline or ultra-low dose naloxone alone were used as controls. Animals were assigned in a randomized manner to each group. All drug infusions were at a rate of 1 μl/h via a mini-osmotic pump (Alzet, Cupertino, CA) implanted in the interscapular region. Tail-flick latency, using the hot water immersion test (52 ± 0.5 °C), was examined before drug infusion and daily after the start of infusion for 5 days. The tail-flick latencies of all rats had an average baseline of 2 ± 0.5 s, and a latency of 10 s was set as the cut-off time to avoid tail damage. A morphine challenge test (15 μg/10 μl, i.t.) was performed after the 5 day infusion period; the rats were placed in plastic restrainers for drug injection and antinociception measurement. The peak antinociceptive response to morphine challenge has been demonstrated to occur 30 min after injection in our previous studies (Tai et al., 2006; Wen et al., 2005). The purpose of using the tail-flick test was to allow an assessment of the effect of morphine on the noxious thermal test, and further examined the reversal effect of ultra-low dose naloxone on morphine's antinociceptive effect. The percentage of the maximal possible antinociceptive effect (% MPE) was calculated as (maximum latency − baseline latency)/(cut-off latency − baseline latency) × 100. Animals were also habituated to the restrainer 1 h per day for three days prior to catheterization surgery in all groups.

2.3. CSF sample collection and measurement of EAAs

On day 5, after termination of drug infusion, the rats were placed in restrainers without anesthesia, and the environment was kept quiet

and dim for microdialysis collection. One of the externalized silastic tubes of the microdialysis probe was connected to a syringe pump (CMA-100; Acton, MA, USA), and perfused with Ringer's solution (0.33 mg/ml of calcium chloride, 8.6 mg/ml of sodium chloride, and 0.3 mg/ml of potassium chloride). CSF dialysates were collected from the other externalized silastic tube of the microdialysis probe. CSF dialysates were collected using a standard procedure of a 30-min washout period, followed by a 30-min sample collection period at a flow rate of 5 μl/min; samples were collected in a polypropylene tube on ice, then frozen at −80 °C until assayed. The dialysis samples were analyzed for amino acids by phenylisothiocyanate derivatization using an HPLC (Agilent 1100; Agilent Technologies, Santa Clara, CA, USA) equipped with a reverse-phase ZORBAX Eclipse AAA column (4.6×150 mm^2, 3.5 μm) and fluorescence detector (Gilson model 121, set at 428 nm) as described previously (Wen et al., 2004). External standards (authentic amino acids at concentrations of 156.25, 312.5, 625, 1250, and 2500 μM) were run before and after each sample group. Peak heights were normalized to the standard peaks and quantified based on the linear relationship between the peak height and amount of the corresponding standard. The experimenter was blind to the each group during testing in all studies.

2.4. Spinal cord sample preparation

On day 5, after the experiments, the rats were killed by exsanguination under isoflurane anesthesia (ABBOTT, Abbott Laboratories Ltd., Queenborough, Kent, England). Laminectomy was performed at the lower edge of the twelfth thoracic vertebra, and the L5–S3 segment of the spinal cord was removed and embedded in optimal cutting temperature compound (Sakura Finetec Inc., USA) for immunohistochemistry or immediately separated into the ventral and dorsal parts, the former being discarded and the latter being used for Western blotting and real-time PCR analysis.

2.5. Western blotting analysis

The dorsal part of the lumbar spinal cord was homogenized in ice-cold RIPA lysis buffer (Upstate Inc., Lake Placid, NY, USA), the homogenates centrifuged at $100{,}000\times g$ for 30 min at 4 °C, and the supernatants used for Western blotting. Samples were denatured by heating at 95 °C for 10 min with an equal volume of sample buffer, then separated on a 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated overnight at 4 °C with polyclonal guinea-pig anti-rat GLT-1 (1:5000) or anti-rat GLAST (1:2500) or polyclonal rabbit anti-rat EAAC1 antibodies (1:2500; all from Chemicon, Temecula, CA) or polyclonal rabbit anti-phospho-NR1 (Serine 896, 1:800, Millipore) or polyclonal rabbit anti-NR1 (1:1000, Cell Signaling Technology) or polyclonal rabbit anti-PKCγ (1:1000, Abcam) antibodies diluted in 5% non-fat milk in TBST. After washing with TBST buffer, the membrane was incubated for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated donkey anti-guinea-pig or anti-rabbit IgG antibodies (both from Chemicon) diluted in TBST. After reaction with ECL solution (Amersham, Arlington Heights, IL, USA), bound antibodies were visualized using a chemiluminescence imaging system (Syngene, Cambridge, UK). Finally, the blots were incubated at 56 °C for 18 min in stripping buffer (0.0626 M Tris–HCl, pH 6.7, 2% SDS, 0.1 M mercaptoethanol) and re-probed with a monoclonal mouse anti-recombinant human epidermal growth factor receptor (EGFR) antibody (MBL, Japan) as the loading control. All Western blots were repeated three times. The optical density of each specific band was measured using a computer-assisted imaging analysis system (Gene Tools Match software; Syngene, Cambridge, UK).

2.6. Immunohistochemistry and image analysis

Spinal cord sections (5 μm) were fixed by immersion in ice-cold acetone/methanol (1:1) for 5 min. After three washes in ice-cold phosphate-buffered saline (PBS), sections were labeled by incubation overnight at 4 °C with FITC-labeled mouse monoclonal anti-rat CD11b/c antibody (OX42; for microglia; Serotec, Oxford, UK), FITC-labeled mouse monoclonal anti-GFAP antibody (for astrocytes; Molecular Probes, Oregon, USA), or FITC-labeled mouse monoclonal anti-neuronal nuclei (for neurons; Chemicon, Temecula, CA) diluted in 1% normal goat serum in PBS. After three PBS rinses, images were captured using an Olympus BX 50 fluorescence microscope (Olympus, Optical, Tokyo, Japan) and a Delta Vision disconsolation microscopic system operated by SPOT software (Diagnostic Instruments Inc., USA). The quantification measurement, referred to as the 'activation grade', was defined as the ratio between immunolabeling densities in different groups. All the sections were analyzed by a pathologist blinded to the treatment. The grading of activation of cells morphology was based on the immunolabeling density of each kind of cells in the dorsal horns graded as: + basal density; ++ median density; and +++ high density. The score for each rat was the mean for the right and left dorsal horns in three consecutive sections.

2.7. Quantitative real-time PCR analysis

2.7.1. Total RNA extraction and cDNA synthesis

Total RNA was extracted from the dorsal horn using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and treated with DNase (Invitrogen) to remove contaminating DNA. The concentration and purity were determined by measuring the absorbance at 260 and 280 nm on a spectrophotometer. Total RNA was reverse-transcribed into cDNA using the Superscript II First strand Synthesis system from Invitrogen. cDNA was synthesized using 1 μg of total RNA, 50 ng of random hexamer primer, 0.5 mM dNTP mix, 10 mM dithiothreitol, and 200 U of Superscript II reverse transcriptase made to a volume of 20 μl with 1× RT buffer. The reaction was carried out at 42 °C for 50 min and terminated by deactivation of the enzyme at 70 °C for 15 min. Control reactions, lacking either reverse transcriptase or template, were included to assess carryover of genomic DNA.

2.7.2. Quantitative real-time PCR

cDNA amplification was performed using the Applied Biosystems Prism 7000 Sequence Detection system and TaqMan® universal PCR master mix (SYBR Green I) according to the manufacturer's instructions (Applied Biosystems Inc., Foster City, CA). The gene-specific probes were labeled at the 5′ end with the reporter dye FAM and the GAPDH internal control probe was labeled at the 5′ end with the reporter dye VIC. A non-fluorescent quencher and a minor groove binder were added to the 3′ end of each probe as quenchers. The thermal cycle conditions were: 10 min at 95 °C, two-step PCR for 40 cycles of 95 °C/15 s, then a final incubation at 60 °C for 1 min. All samples were tested in triplicate. The amplification data were analyzed using Applied Biosystems Prism Sequence Detection Software version 1.1 (Applied Biosystems). To compare the relative expression level using different treatments, the expression of the gene of interest was normalized to that of the GAPDH control using the $\Delta\Delta C_T$ method recommended by the manufacturer. The primer sequences were designed using Primer Express software and were: GLT-1 (forward: 5′-TTGTCTATCACCTTTCCAAGTC-3′, reverse: 5′-AGTTGCTTTCCCTGTGGTT-3′); GLAST (forward: 5′-CGGGATTGTTGAACACTTGTC-3′, reverse: 5′-ACGGGTTTCTCTGGTTCATTG-3′); EAAC1 (forward: 5′-CAGGTGGATGTCTCCTCTGAAGT-3′, reverse: 5′-GAACGAGATGGTGTCAGATTTGTCTA-3′); GAPDH (forward: 5′-CATTGACCTCAACTACATGG-3′, reverse: 5′-GATGCCAAAGTTGTCATGGA-3′).

2.8. Statistical analysis

All data are presented as the mean ± SEM and were analyzed using SigmaStat 3.0 (SYSTAT Software Inc., San Jose, CA). The tail-flick latency data were analyzed using two-way analysis of variance (ANOVA) with a post hoc Bonferroni test. Real-time PCR $\Delta\Delta C_T$ and Western blot density data were analyzed by one-way ANOVA, followed by multiple comparisons with the Student–Newman–Keuls post hoc test. Differences in immunolabeling density data were analyzed by the non-parametric Kruskal–Wallis test. A significant difference was defined as a P value less than 0.05.

3. Results

3.1. Number of rats used in the experiments

In this study design, all rats ($n=119$) were intrathecally implanted with either a catheter connected with mini-osmotic pump only ($n=75$), or in combination with another catheter for morphine challenge ($n=20$), or in combination with a microdialysis probe ($n=24$) for the following experiments. Animals connected with mini-osmotic pump only were used for nociceptive hot water immersion test, and some of these rats were killed for western blot ($n=35$), IHC ($n=12$), and RT-PCR ($n=12$). For EAAs measurement, rats were implanted with another microdialysis probe, in addition to the intrathecal catheter, for CSF dialysates collection. Rats with either neurological deficit, or obstructed catheters, or catheter loss were excluded from the study, it was approximately 22%, including 20% of rats were excluded after two catheters catheterization and 29% of rats were excluded after four catheters catheterization.

3.2. Ultra-low dose naloxone co-infused with morphine attenuates development of morphine tolerance

As shown in Fig. 1A, i.t. administration of ultra-low dose naloxone (15 pg/h) alone (NLX) did not produce an antinociceptive effect over the 5-day infusion period. In morphine-infused rats (MO), the maximum antinociceptive effect was seen on day 1, with morphine tolerance being observed from day 2 and being maximal at days 3 to 5. The antinociceptive effect of morphine was maintained over the whole 5 days when ultra-low dose naloxone (15 pg/h) was co-infused during tolerance induction (MO + NLX). As shown in Fig. 1B, morphine challenge (15 μg/10 μl, i.t.) on day 5 at 4 h after discontinuation of drug infusion produced a significant antinociceptive effect in both the saline (CONT) and ultra-low dose naloxone (NLX) infusion groups, but not in the morphine-infused group (MO); however, co-infusion of ultra-low dose naloxone (MO + NLX) prevented the decline in the antinociceptive effect of morphine. As shown in Fig. 1C, the MPE (%) was 97.6 ± 1.2 in saline-treated rats, 9.1 ± 0.9 in morphine-tolerant rats, 94.5 ± 1.2 in ultra-low dose naloxone-infused rats, and 72.3 ± 1.3 in the co-infused group. MPE (%) $F(3, 20) = 1257.969$, $P<0.0001$.

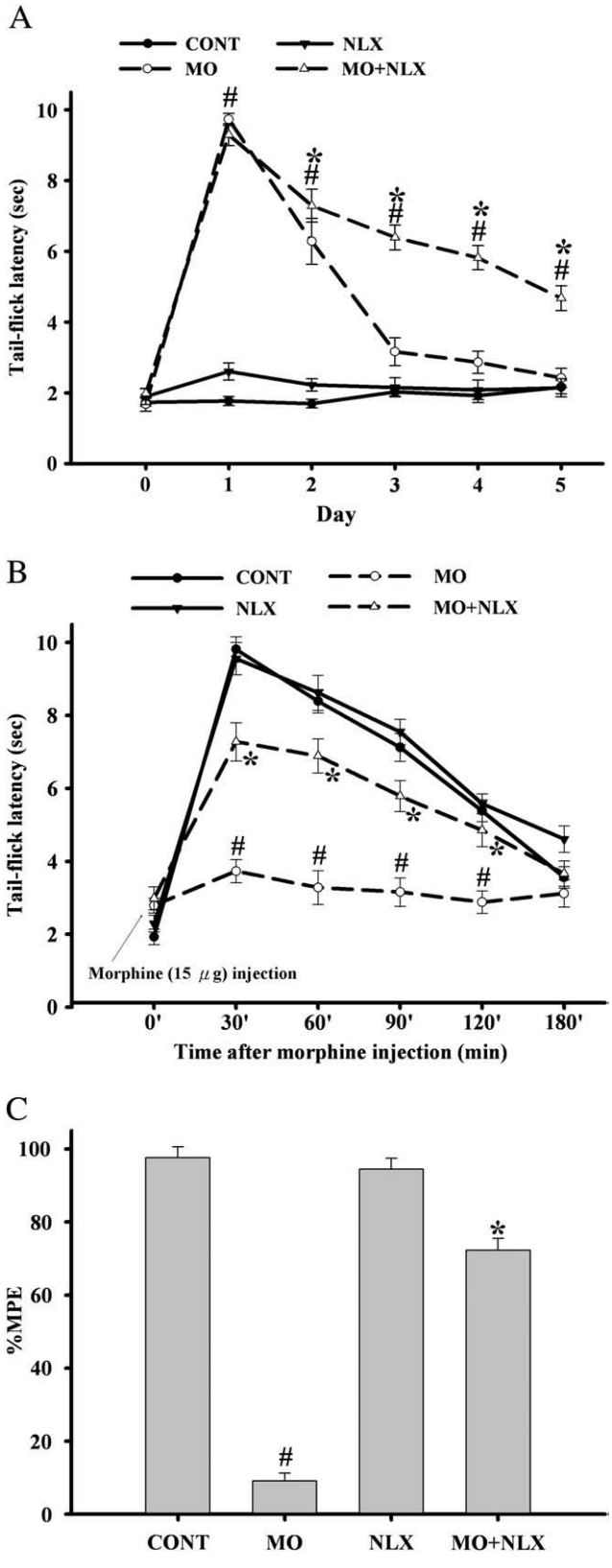

Fig. 1. Effect of intrathecal morphine and ultra-low dose naloxone co-infusion on the antinociceptive effect of morphine in chronic morphine-infused rats. (A) Co-infusion of ultra-low dose naloxone plus morphine attenuates development of morphine tolerance. Time-course of tail-flick latencies over the 5-day period of i.t. infusion of saline (1 μl/h) or morphine (15 μg/h) with/without ultra-low dose naloxone (15 pg/h). CONT, saline infusion ($n=6$); MO, morphine infusion ($n=8$); NLX, naloxone (15 pg/h) infusion ($n=5$); MO + NLX: morphine (15 μg/h) plus naloxone (15 pg/h) co-infusion ($n=9$). #$P<0.05$ compared to the control group; *$P<0.05$ compared to the morphine-infused group. All data points are expressed as the mean ± SEM. (B) Antinociceptive effect of a single challenge dose of morphine administrated in the rats on day 5. A single dose of morphine (15 μg) was administrated at 4 h after discontinuing drug infusion via mini-osmotic pump (when the basal tail-flick latencies had declined below 3 s before morphine challenge in all test rats). The antinociceptive effect in the hot water immersion (52 °C) test was measured every 30 min for 180 min after morphine challenge (15 μg) ($n=5$ in each group). (C) The maximal antinociceptive effect of morphine (15 μg) challenge in each group is expressed as the % change in the MPE compared to the saline control group (100% MPE). #$P<0.05$ compared to the control group; *$P<0.05$ compared to the morphine-infused group.

3.3. Ultra-low dose naloxone co-infused with morphine suppresses the morphine-evoked EAA increase in the CSF dialysate in tolerant rats

Chronic morphine infusion had no significant effect on release of the EAAs aspartate and glutamate or other amino acids (glycine, aspartate, glutamine, citrulline, arginine, alanine, histidine, taurine, and serine) during the 5-day infusion period compared to the other groups (data not shown). Single morphine challenge (15 μg, i.t.) had no effect on the EAA concentrations in saline- and ultra-low dose naloxone-infused rats, but induced a significant increase in aspartate (Fig. 2A) and glutamate (Fig. 2B) levels in morphine-tolerant rat CSF dialysates, as in our previous reports (Tai et al., 2006; Wen et al., 2004). Co-infusion of ultra-low dose naloxone not only blocked this morphine-evoked aspartate and glutamate increase, but resulted in levels of EAAs below the baseline and this pattern corresponded to the restoration of the antinociceptive effect of morphine (Fig. 1B).

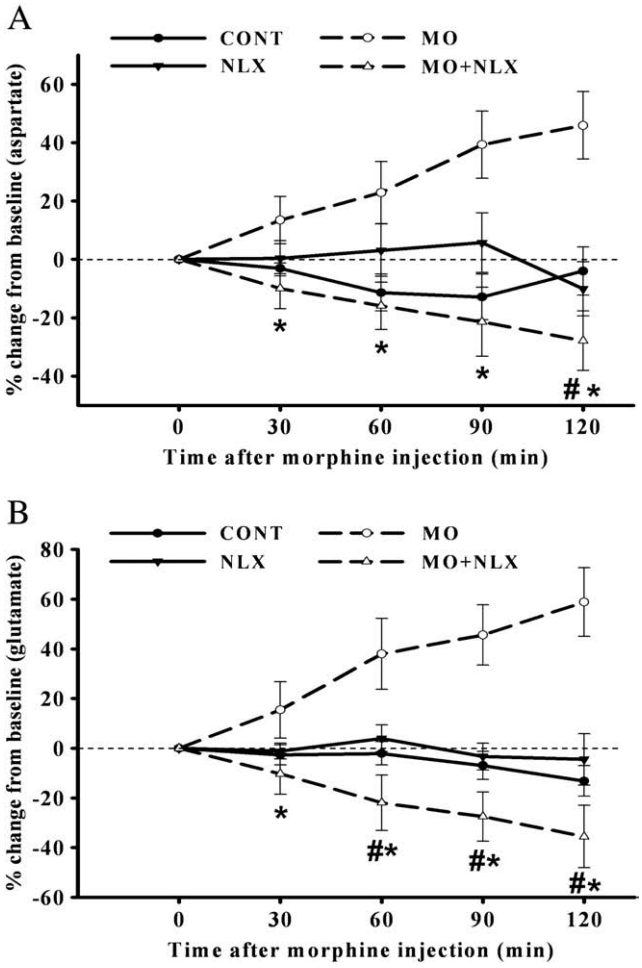

Fig. 2. Co-infusion of ultra-low dose naloxone plus morphine suppresses the morphine-evoked EAA increase in the spinal CSF dialysate after continuous intrathecal morphine infusion. Drug infusion was discontinued on day 5, then morphine challenge (15 μg/10 μl, i.t.) was given 4 h later, when the baseline latency had returned to about 2 s. CSF dialysates were collected for measurement of aspartate (A) and glutamate (B). The concentrations in two consecutive CSF dialysates (30 min each) just before morphine challenge were averaged as the basal level (100%), and another four dialysates were collected at 30, 60, 90, and 120 min after morphine challenge. All data points are expressed as the mean ± SEM ($n = 6$ in each group) for the % change compared to the control. #$P<0.05$ compared to the control group; *$P<0.05$ compared to the morphine-infused group.

3.4. Effect of ultra-low dose naloxone co-infusion on glutamate transporter protein and mRNA levels in the chronic morphine-infused rat spinal cords

Fig. 3 shows GLT-1, GLAST, and EAAC1 expression in the spinal cord dorsal horn of treated rats at day 5 of treatment expressed relative to that in saline-infused rats. Spinal cords from chronic morphine-infused rats showed downregulation of all three GT proteins by approximately 50%, while i.t. co-administration of ultra-low dose naloxone not only prevented this effect, but also resulted in significant upregulation of EAAC1 expression compared to the control group. Ultra-low dose naloxone infusion alone had no effect on spinal GT protein expression. As shown in Fig. 4, i.t. infusion of morphine, ultra-low dose naloxone, or the combination of these two drugs had no significant effect on spinal GT mRNA levels.

3.5. Effect of ultra-low dose naloxone co-infusion on NMDAR NR1 subunit phosphorylation and expression, and PKCγ expression in the chronic morphine-infused rat spinal cords

Fig. 5 shows increasing of NMDAR NR1 subunit phosphorylation and expression, and PKCγ expression in the rat spinal cord dorsal horn after 5 days continuous i.t. morphine infusion, it was associated with morphine tolerance at day 5. Intrathecal co-administration of ultra-low dose naloxone inhibited this morphine-infused effect ($P<0.05$; $n = 5–6$). It suggests that the increase of glutamatergic neurotransmission could be due to NMDAR hyperactivity rather than the insignificant increase of EAAs accumulation at synaptic cleft. Ultra-low dose naloxone infusion alone did not affect the phosphorylation and expression levels of the spinal NR1, and the PKCγ expression.

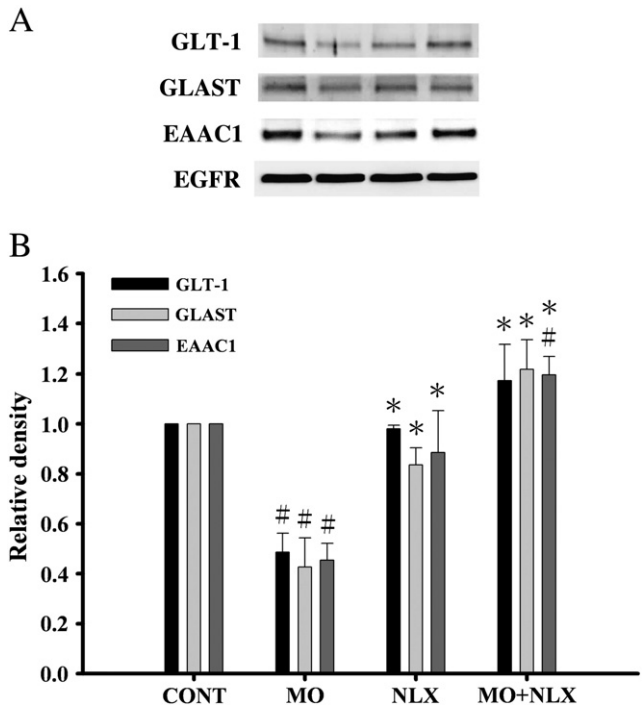

Fig. 3. Co-infusion of ultra-low dose naloxone plus morphine prevents the down-regulation of spinal GT expression seen following chronic morphine infusion. On day 5 after drug infusion without morphine challenge, the L5–S3 segment of the rat spinal cord dorsal horns was taken for Western blot analysis. (A) Expression of GLT-1 (73 kDa), GLAST (65 kDa), and EAAC1 (69 kDa) proteins in the four groups. CONT, saline infusion; MO, morphine infusion; NLX, naloxone (15 pg/h) infusion; MO + NLX, morphine (15 μg/h) plus naloxone (15 pg/h) co-infusion. All data points are expressed as the mean ± SEM ($n = 3$ in each group). EGFR (180 kDa) was used as the loading control (also in other figures). (B) Relative density compared to control levels and controls were set as 1. #$P<0.05$ compared to the control group; *$P<0.05$ compared to the morphine-infused group.

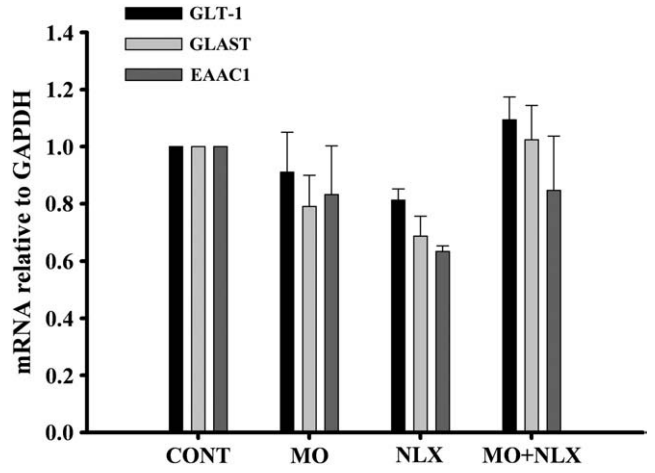

Fig. 4. Effect of ultra-low dose naloxone plus morphine co-infusion on GT mRNA levels. On day 5 after drug infusion without morphine challenge, the L5–S3 segment of the rat spinal cord dorsal horn was used for quantitative real-time PCR ($n=3$ in each group). The abbreviations for the groups are the same as in previous figures.

3.6. Ultra-low dose naloxone co-infused with morphine prevents neuroinflammation following chronic intrathecal morphine infusion

In control rats, weak staining for quiescent, non-activated astrocytes (GFAP, seen in green) and microglia (OX42, seen in green) was distributed throughout the spinal cord, and the cells had a quiescent, fine ramified shape (Fig. 6B, C). In morphine-tolerant rats, strong GFAP and OX42 staining was observed throughout the section and the stained cells had the classic amoeboid shape of activated astrocytes and microglia (Fig. 6E, F). Fewer cells with an amoeboid shape and less GFAP and OX42 staining were observed in the spinal cords of rats co-infused with ultra-low dose naloxone and morphine (Fig. 6H and I), supporting the idea that activation of astrocytes and microglia induced by chronic morphine infusion might play an important role in tolerance formation and that ultra-low dose naloxone inhibited this activation. Quantification of changes of above immunoreactivity is shown in Fig. 7.

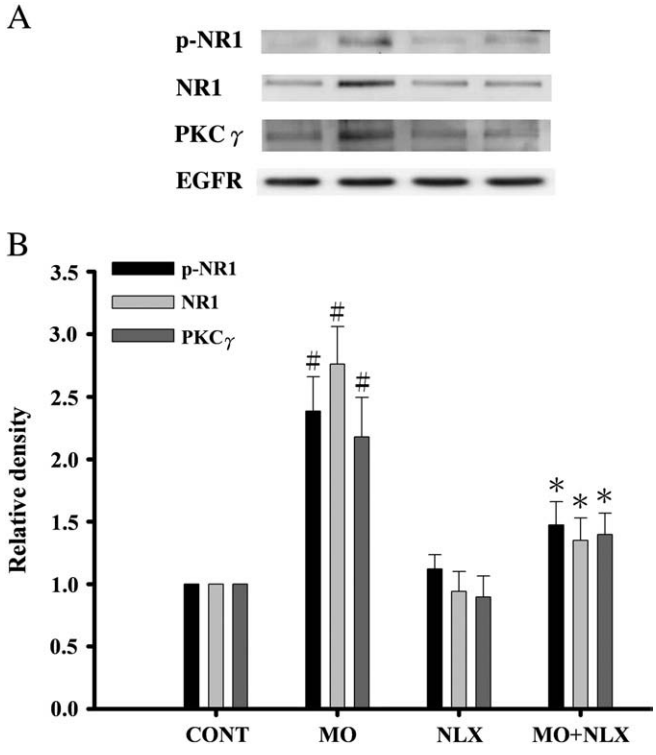

Fig. 5. Co-infusion of ultra-low dose naloxone with morphine inhibits the phosphorylation and expression of spinal NMDAR NR1 subunit, and PKCγ expression following chronic intrathecal morphine infusion. The preparation of spinal cords sample for Western blot analysis was the same as GTs experiment. (A) Expression of NR1 phosphorylation (p-NR1, 120 kDa), NR1 (100 kDa), and PKCγ (80 kDa) proteins of different treatment in the four groups. The abbreviations for the groups are the same as in previous figures. All data points are expressed as mean ± SEM ($n=5$ in saline infusion group, $n=6$ in other groups). (B) Relative density compared to control levels. #$P<0.05$ compared to the control group; *$P<0.05$ compared to the morphine-infused group.

4. Discussion

In the present study, co-infusion of ultra-low dose naloxone with morphine maintained the antinociceptive effect of morphine and this was accompanied by attenuation of the morphine-evoked EAAs accumulation in the CSF dialysates, GTs downregulation, increasing of NMDAR NR1 subunit phosphorylation and expression, and PKCγ expression thus decreasing glutamatergic transmission and morphine tolerance. The antinociceptive effect (MPE,%) of morphine on day 5 of chronic morphine infusion was restored from 9.1 ± 0.9% in morphine-infused rats to 72.3 ± 1.3% in rats co-infused with ultra-low dose naloxone plus morphine and this might be explained by the suppression of excess spinal glutamatergic transmission by ultra-low dose naloxone co-infusion with morphine.

Downregulation of GLT-1, GLAST, and EAAC1 protein expression, enhancement of NMDAR NR1 subunit phosphorylation and expression, and upregulation of PKCγ expression were observed in the dorsal horn of the lumbar segment in morphine-tolerant rat spinal cords, and this phenomenon was prevented by ultra-low dose naloxone co-infusion, which even led to upregulation of EAAC1 protein expression. Moreover, glial cell activation (astrocytes and microglia) was observed in the tolerant rat spinal cord, and this was also inhibited by ultra-low dose naloxone co-infusion.

In our previous studies (Tai et al., 2006; Wen et al., 2005; Wong et al., 2000a), we demonstrated that the addition of a second drug to morphine infusion is an effective strategy for attenuating morphine tolerance and maintaining the antinociceptive efficacy of morphine in chronic morphine-infused rats. We found that morphine tolerance can be attenuated by amitriptyline co-infusion by upregulation of GLAST, GLT-1, and EAAC1 protein (Tai et al., 2006) and the IL-10–p38–MAPK–HO1 signaling pathway (Tai et al., 2009). Modulation of morphine-evoked EAA release after morphine challenge and GT expression was also seen after co-infusion of the glucocorticoid dexamethasone in morphine-tolerant rats (Wen et al., 2005). The NMDAR antagonist MK-801 is reported to lower CSF EAA levels, thus contributing to the preservation of the antinociceptive effect of morphine (Wen et al., 2004). Thus, the addition of these drugs to morphine has been shown to preserve the antinociceptive effect of morphine, at least in part, by modulating spinal glutamatergic transmission. Furthermore, we also found that i.t. injection of naloxone (15 ng) reverses the insensitivity of morphine analgesia in pertussis toxin-treated rats through upregulation of GTs (Tsai et al., 2008). Since amitriptyline, MK-801, dexamethasone, and ultra-low dose naloxone are effective in preserving the antinociceptive effect of morphine and since all these drugs have a modulating effect on spinal glutamatergic transmission by inhibiting the morphine-evoked EAA increase and upregulation of GT protein expression in different rat models, we infer that ultra-low dose naloxone co-infusion can modulate spinal glutamatergic transmission in chronic morphine-infused rats and thus restore the antinociceptive effect of morphine.

In our present study, ultra-low dose naloxone infusion alone had no antinociceptive effect. Ultra-low dose naloxone has been demonstrated to selectively antagonize the excitatory effect of morphine on sensory neurons at picomolar to nanomolar concentrations (Crain and Shen, 1995, 1996). In the present study, a significant antinociceptive

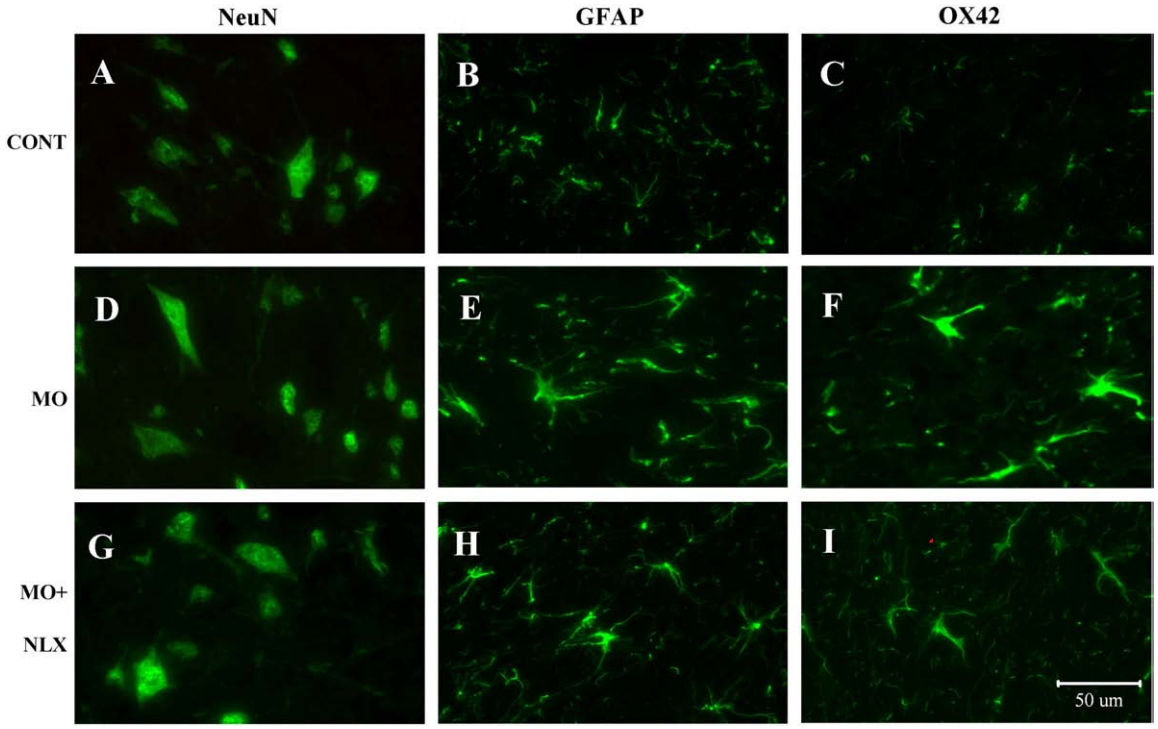

Fig. 6. Co-infusion of ultra-low dose naloxone plus morphine inhibits the glial cell activation seen in chronic morphine-infused rat spinal cords. On day 5, after drug infusion, the rats were killed under isoflurane anesthesia and the dorsal horn of the L5–S3 segment of the rat spinal cord used for immunohistochemical analysis. CONT, saline infusion control (top row); MO, morphine infusion (middle row); MO + NLX, co-infusion of morphine (15 μg/h) plus naloxone (15 pg/h) (bottom row). Staining for cell type is shown in green; Neu-N for neuron (left panels), GFAP for astrocytes (center panels), and OX-42 for microglia (right panels). Strong immunoreactivity and cell morphology transition to ameboid cell shape represented glial cells activation following the 5-day chronic morphine infusion (E and F), and ultra-low dose naloxone co-infusion inhibited the immunoreactivity of glial cells and the inactive glial cells were observed as ramified cell shape (H and I). Scale bar represents 50 μm.

effect was observed with co-infusion of femtomolar levels of naloxone (15 pg/μl) with morphine in rats. Although previous studies demonstrated that naloxone (0.001–0.1 μg/kg) has an analgesic effect in animal models (Crain and Shen, 1995, 2000), it had no obvious analgesic effect when the combination of morphine 1 mg/ml plus naloxone 0.6 μg/ml was given intravenously in humans (Cepeda et al., 2004). In addition to its potent antioxidant properties (Ebrahimkhani et al., 2006; Mikawa et al., 2006), naloxone also produces excess sympathetic outflow when applied to human or rats (Kaye et al., 2003; Ward, 1996). These diverse results might be due to differences in the dosage of naloxone. In our present study, infusion of ultra-low dose naloxone (15 pg/h, equal to about 41 fmol/h) for 5 days had no intrinsic antinociceptive effect or neurotoxicity.

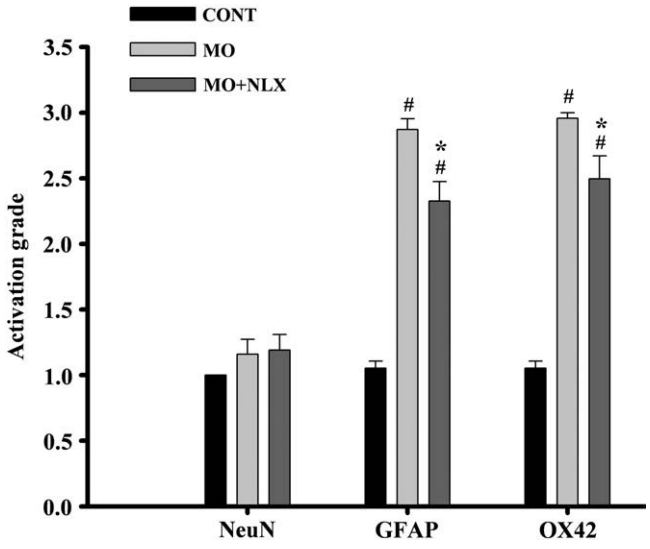

Fig. 7. Quantification of immunolabeling density of the neuron, astrocyte and microglia cell responses to the 5-day drug infusion. On day 5 after continue infusion, dorsal horn of lumbar segment of the rat spinal cord was used for immunolabeling density analysis in saline or morphine or ultra-low dose naloxone plus morphine treatment groups. The abbreviations for these three groups are the same as previous figures. All data points are expressed as the mean ± SEM ($n = 4$ in each group). Relative density compared to control levels. #$P<0.05$ compared to the control group; *$P<0.05$ compared to the morphine-infused group.

Glutamate, the principal excitatory neurotransmitter in the neuronal circuitry, is known to play a critical role in pain modulation and opioid tolerance, dependence, and withdrawal (Tai et al., 2006; Wong et al., 1998). Two major mechanisms are responsible for protecting cells from glutamate-induced neurotoxicity: (1) the removal of synaptic glutamate by high-affinity uptake by GTs and (2) the metabolism and recycling of glutamate by synaptic astrocytes through glutamine synthetase (Danbolt, 2001). To ensure a high signal/noise ratio for synaptic signaling and to protect neurons, the extracellular concentrations of EAAs in the synaptic junction have to be maintained at a low level (<1 μM), and GTs, especially the glial GTs, play an important role in maintaining synaptic EAA concentrations at physiologic levels (Danbolt, 2001). Intriguingly, we have also demonstrated a correlation between morphine analgesia and CSF EAA concentrations in terminal cancer pain patients, with lower levels of EAAs being observed in the CSF at an effective analgesic dose of morphine and an increase in EAA levels in the CSF being observed when morphine loses its analgesic effect after long-term i.t. administration (Wong et al., 2002). Accordingly, the discrepancies between the present study (no obvious increase of EAAs concentration during tolerance formation) and the result from the terminal cancer patients might be due to several possible mechanisms: firstly,

the increase of glutamate neurotransmission could be due to other changes (i.e., NMDAR hyperactivity) rather than the increase of EAAs accumulation at synaptic cleft; secondly, as the spare receptor theory (Zhu, 1993), constitutive spare GTs may be enough for maintaining the CSF EAAs concentration during chronic morphine infusion, however, the down-regulated GT is not enough for the drastic EAA elevation in the CSF by the morphine challenge in morphine-tolerant rats; thirdly, the increased spinal EAAs release might be not secondary to the loss of morphine analgesia in cancer patients; finally, the loss of morphine analgesia in cancer patients might be directly related to an increase in spinal EAAs raised by tumor related nociceptive inputs such as nerve compression instead of chronic morphine treatment itself. Moreover, previous studies found that development of morphine insensitivity and formation of neuropathic pain might be linked to an increase in spinal EAA concentrations after PTX or lipopolysaccharide treatment and subsequent NMDAR activation (Liu et al., 2000; Wen et al., 2003). Similarly, in the present study, chronic i.t. morphine infusion induced an increase in EAA level in rat spinal CSF dialysates by morphine challenge and it was accompanied by loss of the antinociceptive effect of morphine; this might be due to down-regulation of GTs, which decreases the re-uptake of synaptic EAAs. Additionally, a single dose of 10 nmol morphine has been demonstrated to promote the recruitment of PKCγ to phosphorylate the MOR (Rodriguez-Munoz et al., 2008). Spinal NMDAR and PKCγ activation have also been suggested to play a role in the development of morphine tolerance (Lim et al., 2005; Narita et al., 2001; Zeitz et al., 2001). Furthermore, an enhancement of NMDAR2A subunits phosphorylation, via a PKCγ activation, has also been demonstrated by icv morphine administration (Sanchez-Blazquez et al., 2009). In the present study, our data showed that ultra-low dose naloxone co-infusion inhibited not only the spinal NMDAR NR1 subunit phosphorylation and expression, but PKCγ expression after chronic i.t. morphine infusion. Besides, the insensitivity of morphine in the present study, before challenge, without a significant increase of CSF EAAs concentration suggested that NMDAR might be primed (i.e., increased NMDAR NR1 subunit phosphorylation) by long-term morphine infusion via activation of PKCγ. Therefore, an increase of glutamatergic transmission produced by increasing of NMDAR NR1 subunit expression and phosphorylation, via PKCγ activation, which may contribute to morphine tolerance formation although no significant increase of CSF EAAs in the spinal CSF dialysates under down-regulated GTs (Jhamandas et al., 1996; Mao et al., 1995; Mao et al., 2002; Rodriguez-Munoz et al., 2008). Our data indicate that ultra-low dose naloxone co-infusion inhibited not only the morphine challenge-induced EAA increase in chronic morphine-infused rat spinal CSF via preserving the spinal GTs expression but an inhibition of NMDAR NR1 subunit expression and phosphorylation via PKCγ activation, thus restoring the antinociceptive effect of morphine.

Downregulation of GT protein expression contributes to the development of morphine tolerance (Mao et al., 2002; Sung et al., 2005; Tai et al., 2006), inflammatory pain (Sung et al., 2004), and dependence (Ozawa et al., 2001). A reduction in GLT-1 mRNA levels was demonstrated in the brain of naloxone-precipitated morphine withdrawal rats (Ozawa et al., 2001). Moreover, chronic morphine treatment was demonstrated to induce post-transcriptional down-regulation of EAAC1 in C6 glioma cells with a concurrent decrease in glutamate uptake and increase in ubiquitin–proteasome activity (Yang et al., 2008). In our present study, neither chronic morphine infusion nor ultra-low dose naloxone co-infusion altered GT mRNA levels. This strongly supports the idea that chronic morphine infusion or ultra-low dose naloxone co-infusion might only affect GT expression at the translational or post-translational level, but not the transcriptional level. Consistent with previous studies (Mao et al., 2002; Tai et al., 2006; Wen et al., 2005), both the neuronal (EAAC1) and glial (GLT-1 and GLAST) GTs were down-regulated in chronic morphine-infused rat spinal cords. Furthermore, we found that ultra-low dose naloxone co-infusion with morphine reversed this effect and even increased neuronal EAAC1 expression, thus enhancing the re-uptake of synaptic EAAs, preventing the accumulation of synaptic EAAs, and restoring the antinociceptive effect of morphine in chronic morphine-infused rats. These results provide direct evidence for a relationship between the expression of GTs and the antinociceptive effect of morphine. Taken together, the present results suggest that ultra-low dose naloxone co-infused with morphine modulates glutamatergic transmission in the rat spinal cord.

Chronic morphine exposure leads to glial cell activation and enhances pro-inflammatory cytokine expression in the rat spinal cords (DeLeo et al., 2004; Johnston et al., 2004; Raghavendra et al., 2002). In response to diverse noxious stimuli, activated astrocytes and microglia in the spinal cord release pro-inflammatory cytokines, including TNFα, IL-1β, and IL-6. Pro-inflammatory cytokines act in a paracrine manner and influence cells far away from their site of release and promote the release of other pain-related transmitters or substances (e.g. nitric oxide, prostaglandins, and EAA) from primary afferent nerve terminals (Watkins et al., 2003). Strategies for inhibiting glial cell activation and the downstream cytokine signaling, including administration of glial metabolic inhibitors, cytokine antagonists, the anti-inflammatory cytokine IL-10, or the IL-1 receptor antagonist IL-1ra, have been shown to attenuate development of morphine tolerance and prevent hyperalgesia and allodynia (Raghavendra et al., 2002; Song and Zhao, 2001). Interestingly, a synergistic effect of opioid administration and neuropathy on glial cell activation and cytokine production has been demonstrated in L5 spinal nerve-transected rats (Raghavendra et al., 2002).

Recent studies have revealed that the opioid receptor antagonists naloxone and naltrexone have a novel anti-inflammatory effect. Naloxone treatment inhibits activation of microglia and resident macrophages in the brain and the production of TNF-α, IL-1β, and superoxide and decreases cerebral ischemic injury by reducing neutrophil accumulation and chemokine expression (Liao et al., 2003). The protective effect of naloxone might not be directly related to opioid receptors, as the naloxone stereoisomer (+)-naloxone, an ineffective opioid receptor antagonist, has a similar anti-inflammatory effect. In additional, (+)-naloxone and (−)-naloxone have been demonstrated to act as toll-like receptor 4 (TLR4) antagonists in vitro (transfected HEK-TLR4 cells and microglial cell lines) and in vivo (sciatic nerve chronic constriction injury in rats) (Hutchinson et al., 2008). Recently, we discovered an interesting mechanism by which ultra-low dose naloxone co-infusion with morphine beneficially upregulates anti-inflammatory cytokine IL-10 production to counter the expression of the pro-inflammatory cytokines TNFα, IL-1β, and IL-6 and suppress neuroinflammation, thus restoring the antinociceptive effect of morphine (Lin et al., 2010). In agreement with previous studies, our present study revealed that i.t. ultra-low dose naloxone co-infusion effectively attenuated morphine infusion-induced glial cell activation, which may be responsible for the significant effect in preventing morphine tolerance.

In conclusion, our present study demonstrates that i.t. co-infusion of ultra-low dose naloxone with morphine not only attenuates the development of morphine tolerance, but also maintains the anti-nociceptive efficacy of morphine. These effects of ultra-low dose naloxone co-infusion are thought to be mediated by prevention of the downregulation of GTs (even increasing EAAC1 expression), which result in increased synaptic EAAs uptake and a reduction in EAA levels in the CSF of morphine-tolerant rats, attenuation of NMDAR-PKCγ related glutamatergic transmission, and inhibition of glial cell activation. Based on the present study, we believe that i.t. ultra-low dose naloxone may be a good analgesic adjuvant in patients who need long-term opioid administration for pain management. This may explain why ultra-low dose naloxone has been reported to produce satisfactory analgesia with i.t. morphine in human chronic low back pain (Hamann et al., 2008).

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pbb.2010.05.012.

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