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N-Methyl-d-aspartate receptor antagonist MK-801 suppresses glial pro-inflammatory cytokine expression in morphine-tolerant rats

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ABSTRACT

Chronic opioid therapy induces tolerance and hyperalgesia, which hinders the efficacy of opioid treatment. Previous studies have shown that inhibition of neuroinflammation and glutamatergic receptor activation prevents the development of morphine tolerance. The aim of the present study was to examine whether N-Methyl-D-aspartate receptors are involved in the regulation of chronic morphine-induced neuroinflammation in morphine-tolerant rats.

Morphine tolerance was induced in male Wistar rats by intrathecal infusion of morphine (15 μg/h) for 5 days. Tail-flick latency was measured to estimate the antinociceptive effect of morphine. Morphine challenge (15 μg, intrathecally) on day 5 at 3 h after discontinuation of morphine infusion produced a significant antinociceptive effect in saline-infused rats, but not in morphine-tolerant rats. Pretreatment with MK-801 (20 μg, intrathecally) 30 min before morphine challenge preserved its antinociceptive effect in morphine-tolerant rats. Morphine-tolerant rats expressed high levels of the pro-inflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α and the increase in interleukin-1β and interleukin-6, and tumor necrosis factor-α levels was prevented by MK-801 pre-treatment at both the protein and mRNA levels.

The results show that a single dose of MK-801 reduces the increase in pro-inflammatory cytokines in the spinal cord, thus re-sensitizing neurons to the antinociceptive effect of morphine in morphine-tolerant rats. This study provides a piece of theoretical evidence that NMDA antagonist can be a therapeutic adjuvant in treating morphine tolerant patients for pain relief.

1. Introduction

Morphine is the most widely used analgesic for the treatment of moderate to severe pain. Long-term morphine administration tends to induce tolerance and hyperalgesia, which hinder the efficacy of opioid treatment. The mechanisms that have been postulated to explain morphine tolerance include G protein uncoupling (Chakrabarti et al., 2005a,b), opioid receptor internalization (Zuo, 2005), down-regulation of opioid receptors and glutamate transporters (Lim et al., 2005; Mao et al., 2002), and upregulation of N-methyl-D-aspartate (NMDA) receptors (Liu et al., 2009; Xu et al., 2007). Recently, it has been suggested that spinal cord glia, mainly astrocyte and microglia, contribute to the development of morphine tolerance and tolerance-associated pain sensitization and that, upon activation by opioids, glial cells affect pain sensitization by releasing proinflammatory cytokines (tumor necrosis factor, interleukin IL-1, and IL-6) and excitatory amino acids (EAAs), nitric oxide, and prostaglandins (Watkins et al., 2005).

The involvement of ionotropic glutamate NMDA receptors in the development of morphine tolerance has been discussed. Trujillo and Akil (1991) first showed that co-treatment with the NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate (MK-801) and morphine attenuated the development of opiate tolerance and dependence. Competitive and non-competitive NMDA antagonists were then shown to preserve the antinociceptive effect of morphine in both morphine tolerance and neuropathic pain models (Bilsky et al., 1996; Manning et al., 1996; Yamamoto and Yaksh, 1992). In addition, overexpression of the NR2B subunit of the NMDA receptor was demonstrated to enhance inflammatory pain (Wei et al., 2001), while the use of antisense oligonucleotides to knock down NMDA receptor expression prevented formalin-induced pain (Garry et al., 2000). These studies provide several lines of evidence that spinal NMDA receptors are critical in the development and maintenance of neural plasticity in pain-related behaviors (e.g. tolerance, hyperalgesia, and allodynia).

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In agreement with these reports, we previously demonstrated that intrathecal co-infusion of either a competitive NMDA receptor antagonist, D-(−)-2-amino-5-phosphonovaleric acid (D-AP5), or a non-competitive NMDA receptor antagonist, MK-801, with morphine, inhibits the development of tolerance (Wong et al., 1996) and attenuates the increase in EAA levels induced by chronic morphine treatment in the cerebrospinal fluid (CSF) (Wen et al., 2004). We also observed that the tricyclic antidepressant amitriptyline prevents the development of morphine tolerance by lowering EAA and pro-inflammatory cytokine levels in the spinal cords in chronic morphine-infused rats (Tai et al., 2006). Whether NMDA receptors regulate the chronic morphine-induced pro-inflammatory cytokines expression is still not clear. MK-801 acts as a channel blocker by binding inside the NMDA receptor, at which magnesium ion binds and blocks NMDA receptor activation in the resting state and thus prevents the flow of ions, such as calcium, through the channel and the subsequent downstream signaling. Studies on the effect and mechanism of acute NMDA receptor antagonist treatment in morphine-tolerant rats have yielded conflicting results (Bilsky et al., 1996; Grass et al., 1996; Yamamoto and Yaksh, 1992), probably due to the use of different NMDA receptor-targeted drugs, animal models, and routes of drug delivery. The aim of the present study was to examine the effect of acute MK-801 treatment on morphine antinociception in morphine-tolerant rats, and to elucidate the mechanisms involved in influencing the chronic morphine-induced neuroinflammation in the spinal cord.

2. Materials and methods

2.1. Animal preparation and drug administration

All animal experiments were performed on 8-week-old male Wistar rats (350–400 g) maintained on a 12-h light/dark cycle with food and water freely available. Their use was approved by the National Defense Medical Center Animal Care and Use Committee (NDNC IACUC), and conformed to the Guiding Principles in the Care and Use of Animals of the American Physiological Society. Animals were assigned in a randomized manner to each group. Animal preparation and intrathecal catheter implantation were performed as described in our previous study (Wen et al., 2004). Briefly, rats were anesthetized by intraperitoneal injection with pentobarbital (50 mg/kg) and two catheters were implanted via the atlantooccipital membrane into the intrathecal space at the lumbar enlargement (L1–L2) of the spinal bony structure corresponding to spinal cord segments L5, L6, and S1–S3, which are responsible for the tail-flick reflex. One catheter was connected to a mini-osmotic pump and was used to infuse saline (1 μl/h) or morphine (15 μg/h) for 5 days, while the other catheter was used for acute drug injection. Animals exhibiting neurological deficits after catheter implantation were excluded from experiments.

On day 5, after morphine tolerance had developed, morphine infusion was discontinued for 3 h and the rats were injected intrathecally with MK-801 (20 μg), fluorocitrate (0.826 μg, 1 nmol, a glia metabolic inhibitor) (Song and Zhao, 2001) or saline (as control), then, 30 min later, a single dose of morphine (15 μg/5 μl) was injected intrathecally. The antinociceptive effect of morphine at 30, 60, 90, or 120 min post-challenge was assessed in the tail-flick latency test. All drugs were purchased from Sigma, and were delivered intrathecally in a total volume of 5 μl, followed by a 10 μl saline flush.

2.2. Antinociception test

Rats subjected to the different treatments were placed in plastic restrainers and their tails were subjected to a hot water immersion test (52 ± 0.5 °C) for tail-flick latency measurement to evaluate the antinociceptive effect of morphine. To rule out stress-induced interference as possible, before tail-flick latency measurement, we put rats in the container and waited for them calming down for 30 min. The observers were blinded to group assignment when the tail flick analysis was performed. Without performing any surgery, twelve naïve rats constituted a group in the tail-flick latency test. The rat's tail was immersed in the 52 °C water bath, and the time for the rat to flick its tail away from the hot water was recorded by a timer for 3 times in each animal. The results were taken as a baseline latency data in the present study. The average baseline for the tail-flick latency test in naïve rats was 2.18 ± 0.15 s. A cut-off time of 10 s was used to prevent damage. The data were expressed as the maximal possible effect (MPE%), calculated as the [(experimental latency) − (baseline latency)]/[(cut-off latency) − (baseline latency)] × 100.

2.3. Spinal cord sample preparation

On day 5, 60 min after morphine challenge, the rats were sacrificed by exsanguination under isoflurane anesthesia, then laminectomy was performed, and the L5–S3 segment of the spinal cord removed. The dorsal portion of the spinal lumbar enlargement was then separated immediately and stored at −80 °C until used for further study.

2.4. Western blot analysis

Spinal cord dorsal horn samples were homogenized in ice-cold RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA; Upstate) containing protease and phosphatase inhibitors and the homogenate centrifuged at 15,000 *g* for 30 min at 4 °C and the supernatant kept. After heating in reducing SDS sample buffer at 95 °C for 5 min, the samples were separated on a SDS-PAGE gel and transferred to PVDF membranes (Millipore). The membranes were blocked for 1 h at room temperature with blocking buffer (5% milk in phosphate-buffered saline containing 0.05% Tween 20) and incubated overnight at 4 °C with rabbit polyclonal antibodies against rat pP44/42, p44/42, pJNK, JNK, pp38, or p38 (1:1000 in blocking buffer; Cell Signaling), then for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (1:7000 in blocking buffer; anti-rabbit IgG). After incubation for 2 min in ECL solution (Amersham, Arlington Heights, USA), signals were visualized using a chemiluminescence imaging system (Syngene, Cambridge, UK). Finally, the blots were incubated for 3 min at room temperature in stripping reagent (Bionovas, Canada) and reprobed with a monoclonal mouse anti-β-actin antibody (1:80000; Sigma, Missouri, USA) as loading control. The Western blot analysis of each animal

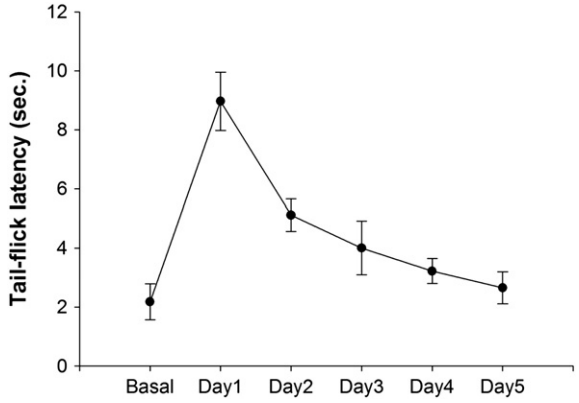

Fig. 1. Morphine tolerance is developed in 5-day intrathecal morphine infusion. The antinociceptive effect of morphine was examined by tail-flick latency test every day for 5 days. The data points are expressed as the mean ± SD (n = 12).

sample was repeated three times. The density of each specific band was measured using a computer-assisted imaging analysis system (Gene Tools Match software, Syngene, Cambridge, UK).

2.5. Measurement of cytokine levels

After homogenization and centrifugation as for Western blotting, the supernatant was analyzed for four cytokines in a simultaneous multiplexed format using a microbead-based and flow-based protein detection system (Bio-Plex Suspension Array System, Bio-Rad Laboratories Inc., Hercules, CA) following the manufacturer's guidelines. Each sample was first incubated with a mixture of the different color-coded beads conjugated to specific antibodies against target cytokines. After 3 washes, a secondary biotinylated detection antibody specific for a different epitope on the cytokine was added to the beads, resulting in a sandwich of antibodies around the cytokine, then streptavidin-coupled phycoerythrin was added. After suspension in assay buffer, the reaction mixture was drawn up into the flow-based Bio-plex suspension array system, which identifies and quantifies each specific reaction based on bead color and fluorescence. A minimum of 100 colored beads per cytokine was analyzed in each spinal cord sample. All spinal cord lysates were assayed in triplicate. The concentrations of each target cytokine were calculated using a standard curve set up using a recombinant rat cytokine standard.

2.6. Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The first-strand cDNA synthesis reaction was carried out using 3 μg of DNase-treated total RNA, 50 ng of random hexamers, 0.5 mM dNTP mix, 10 mM DTT, 1× RT buffer, and 200 U of Superscript III™ reverse transcriptase (Invitrogen) in a final volume of 20 μl and reaction was performed at 25 °C for 10 min, followed by 50 °C for 50 min, and terminated at 85 °C for 5 min. Real-time PCRs were performed in an Applied Biosystems Prism 7500 Sequence Detection system with FastStart Universal SYBR Green Master mix (ROX) (Roche Applied Science, Mannheim, Germany), gene-specific primers, and diluted cDNA. Thermocycle conditions were initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Fluorescent data were acquired during each extension phase.

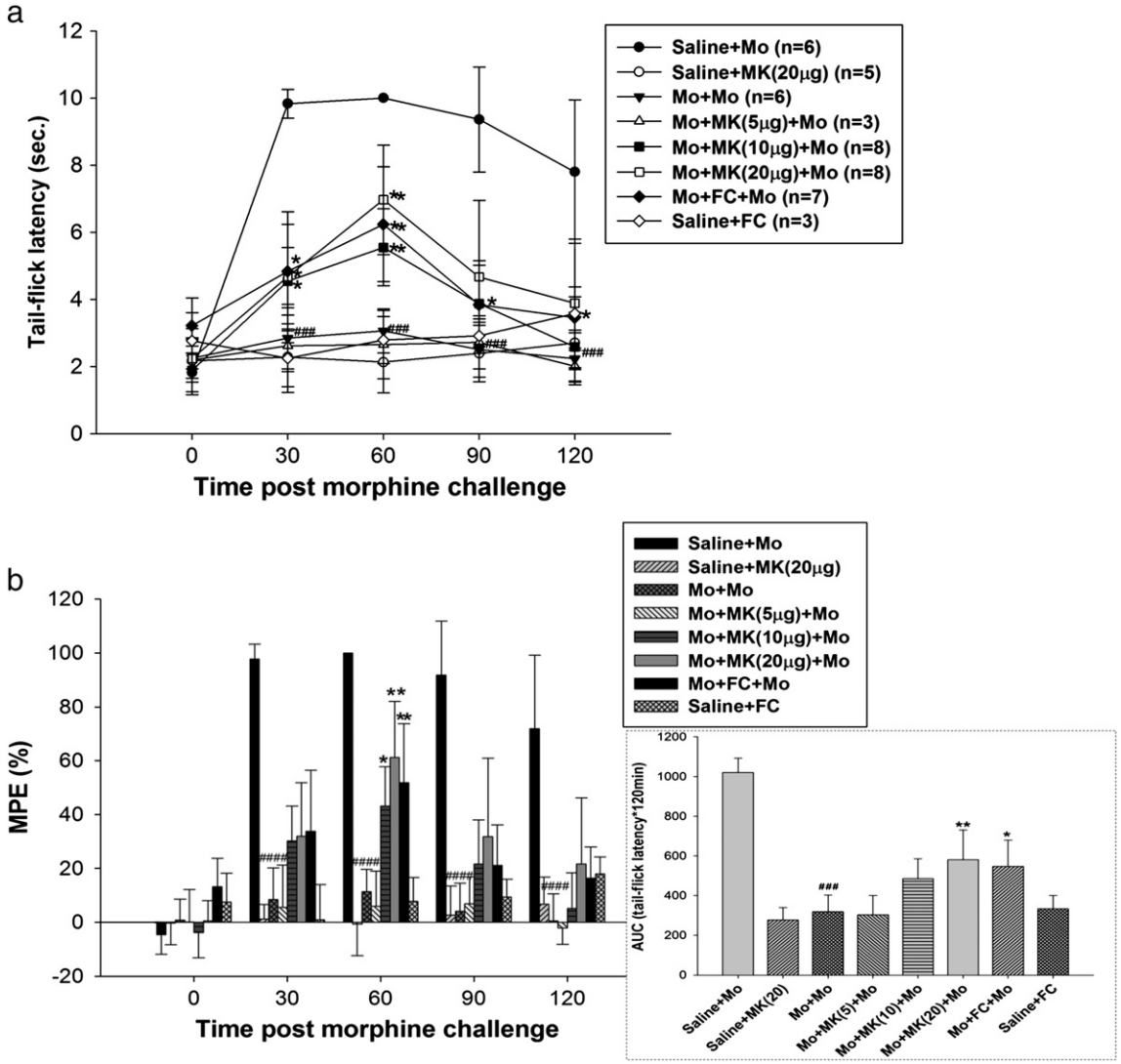

Fig. 2. MK-801 restores the antinociceptive effect of morphine in morphine-tolerant rats. (a) The antinociceptive effect of morphine was examined on day 5 after intrathecal infusion of saline or morphine (15 μg/h, i.t.). At 3 h after discontinuation of infusion, the rats were injected with either saline or MK-801 (5, 10, or 20 μg, i.t.) 30 min before morphine challenge (15 μg, i.t.), then the tail-flick latency was measured every 30 min for 120 min. (b) The data are expressed as the percentage of the maximal possible effect (MPE%) calculated as follows: [(Experimental latency) − (baseline latency)]/[(cut-off latency) − (baseline latency)] × 100. A 10 s tail-flick latency was defined as 100% MPE in saline-infused rats receiving morphine challenge. The areas under the curve (AUC) from time 0 to 120 min for tail-flick latency were obtained from (a). All data points are expressed as the mean ± SD. $^{###}P<0.005$ compared to the Saline + Mo group; $^{*}P<0.05$, $^{**}P<0.01$, compared to the Mo + Mo group.

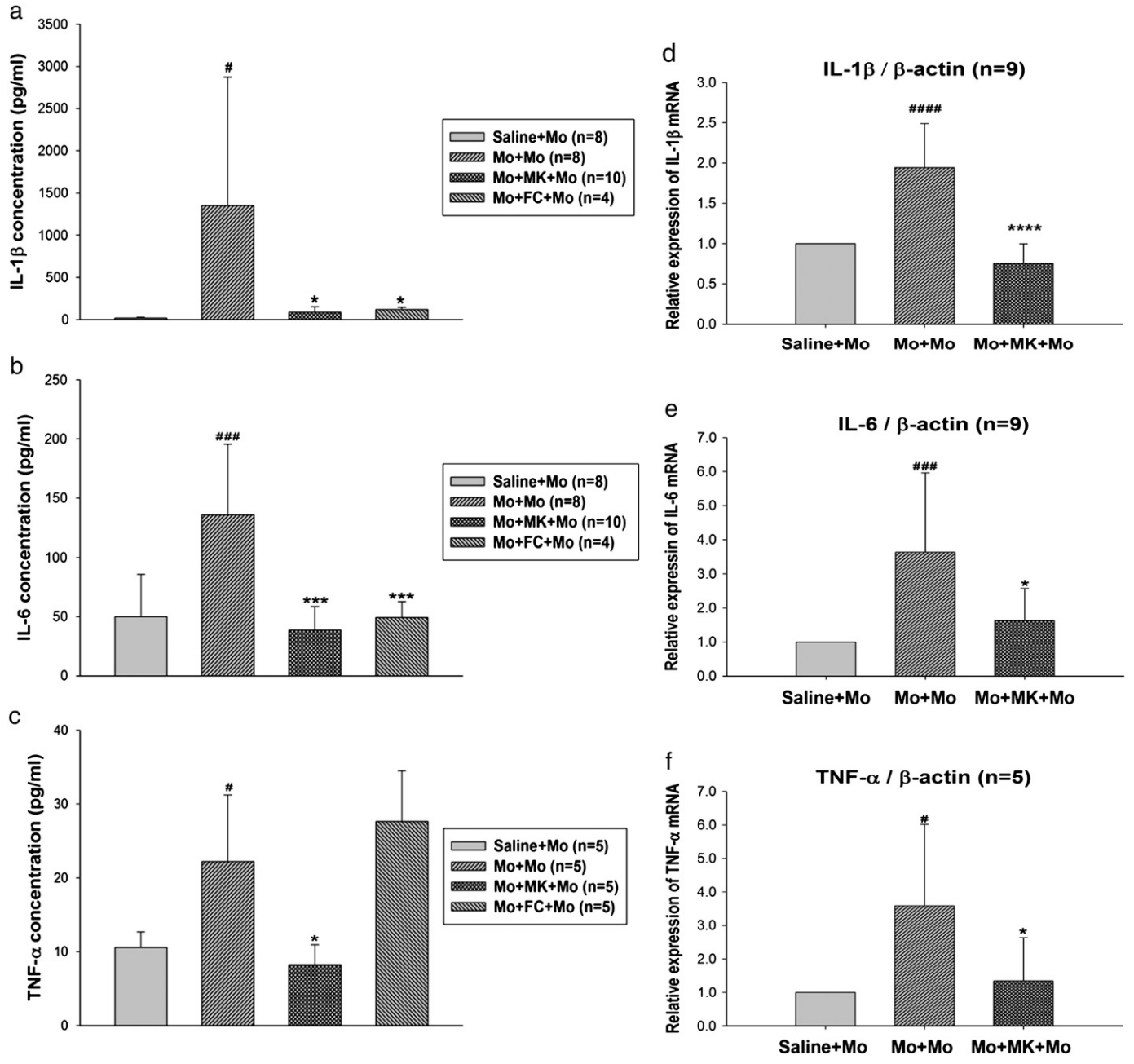

Fig. 3. MK-801 suppresses pro-inflammatory cytokine expression in the morphine-tolerant rat spinal cord. On day 5 morphine on which tolerance developed, at 3 h after discontinuation of morphine infusion, MK-801 was injected intrathecally 30 min before morphine challenge (15 μg) and samples were collected at 60 min post challenge. (a–c) Total protein extracts from the different treatment groups were assayed for IL-1β (a), IL-6 (b), or TNF-α (c) using a Bio-plex system. (d–f) Samples for real-time PCR analysis were collected as indicated, and IL-1β (d), IL-6 (e) or TNF-α (f) mRNA levels were measured using an Applied Biosystems Prism 7500 Sequence Detection system, with β-actin mRNA as the loading control. Saline + Mo: saline control rats challenged with morphine; Mo + Mo: morphine-infused rats challenged with morphine; Mo + MK + Mo: morphine-infused rats treated with 20 μg of MK-801, then challenged with morphine; Mo + FC + Mo: morphine-infused rats treated with 1 nmol of fluorocitrate, then challenged with morphine. All data points are expressed as the mean ± SD. $^{\#}P<0.05$, $^{\#\#\#}P<0.005$, $^{\#\#\#\#}P<0.001$ compared to the Saline + Mo control; $^{*}P<0.05$, $^{***}P<0.005$, $^{****}P<0.001$ compared to the Mo + Mo group.

After 40 cycles, a melting curve was generated to verify primer specificities. All samples were tested in triplicate. To rule out DNA contamination of the RNA preparations, qRT-PCR was performed with the RNA templates and did not show any amplification. The fluorescent data were converted into cycle threshold (CT) measurements and analyzed using Applied Biosystems Prism Sequence Detection Software (version 1.1). The expression of the target gene was normalized to that of the β-actin control using the $\Delta\Delta C_T$ method. The relative expression of each target gene in the saline-treated and drug-treated rats was calculated as the fold change $= 2^{-\Delta\Delta C_T}$, where ΔC_T represents the C_T for the target gene − the C_T for the housekeeping gene and $^{\Delta\Delta}C_T$ represents the ΔC_T for the drug-treated sample − the ΔC_T for the saline control sample. The primer sequences used were: IL-1β (NM_031512, forward: 5′-GCATCCAGCTTCAAATCTCA-3′, reverse: 5′-ATCATCCCACGAGTCACAGA-3′); IL-6 (NM_012589, forward: 5′-TGATGGATGCTTCCAAACTG-3′, reverse: 5′-TGCTCTGAATGACTCTGGCT-3′); TNF-α (NM_012675, forward: 5′-CTCTTCTCATTCCCGCTCGTG-3, reverse: 5′-GGAACTTCTCCTCCTTGTTGGG-3′); β-actin (NM_031144, forward: 5′-AGTGTGACGTTGACATCCGT-3′, reverse: 5′-CAGAGTACTTGCGCTCAGGA-3′).

2.7. Immunohistochemistry and image analysis

On day 5, 60 min after morphine challenge, the rats were sacrificed, laminectomy performed, and the L5–S3 segment of the spinal cord immediately removed and embedded in optimal cutting temperature compound (Sakura Finetec Inc, USA) and frozen at −20 °C. Sections (5 μm) were cut, air-dried on microscope slides for 30 min at room temperature, and fixed by immersion in ice-cold acetone/methanol (1:1) for 10 min. After three washes in ice-cold phosphate-buffered saline (PBS), the sections were pre-incubated at room temperature with 4% fetal bovine serum diluted in PBS containing 0.01% Triton X-100. For immunohistochemical studies, the sections were double-labeled by incubation overnight at 4 °C with fluorescein isothiocyanate (FITC)-labeled mouse monoclonal antibody against rat CD11b (OX42; for microglia; Serotec, Oxford, UK) or FITC-labeled mouse monoclonal anti-GFAP antibody (1:400, Molecular Probe, Oregon, USA) and unlabeled goat polyclonal antibodies against rat IL-1β or IL-6 (both from R&D System, Minneapolis, USA). The sections were then incubated sequentially for 1 h at room temperature with biotin-conjugated donkey anti-goat $F(ab')_2$ secondary antibody (1:100, Jackson ImmunoResearch Laboratories, Inc., Baltimore, USA), then with Cy™3-conjugated streptavidin (1:800, Jackson ImmunoResearch Laboratories, Inc., Baltimore, USA). Finally, the sections were counterstained in 1% methanol containing 20 μg/ml of DAPI (Sigma-Aldrich, St. Louis, MO, USA) and 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. Sterling Heights, MI).

2.8. Data and statistical analysis

The data for the tail flick withdrawal latency are presented as the mean ± SD All data were analyzed by one-way ANOVA, followed by Tukey's pairwise comparison test. A significant difference was defined as a P value < 0.05.

3. Results

3.1. Acute treatment with MK-801 attenuates morphine tolerance

In the morphine tolerance model, rats receiving morphine (15 μg/h) intrathecal infusion for 5 days developed morphine tolerance, showing changes of nociceptive thresholds in the tail-flick latency

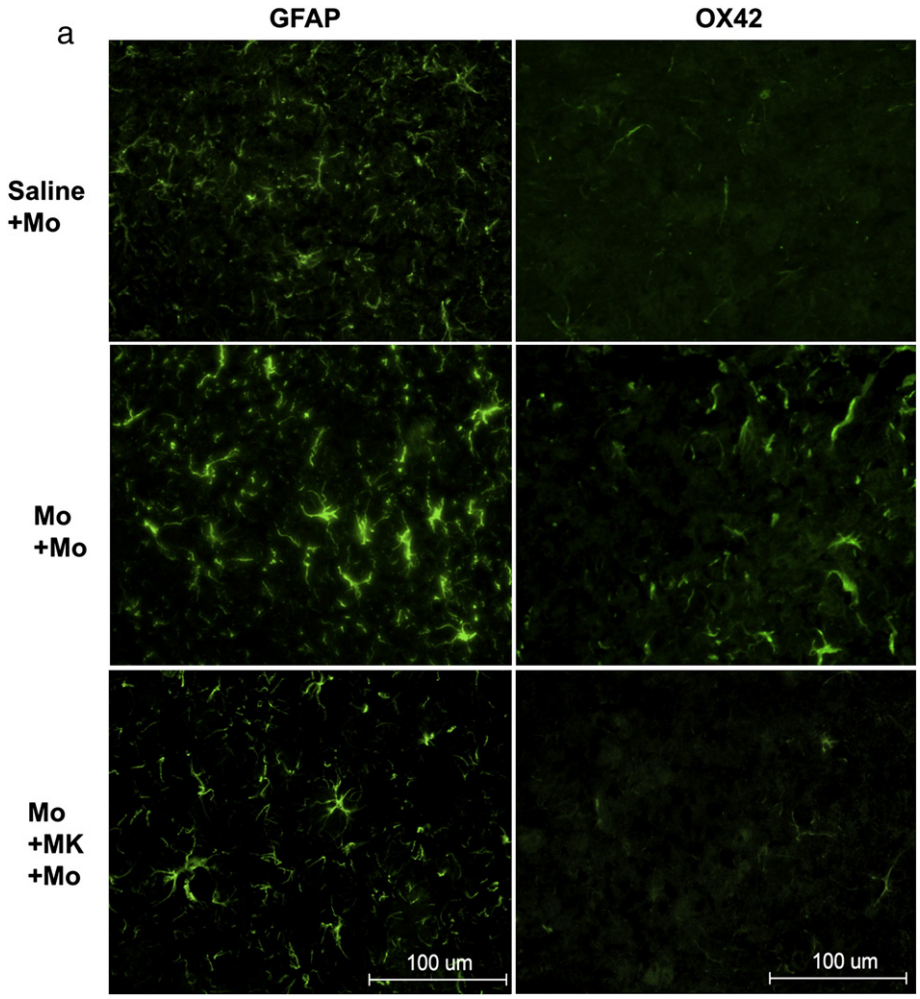

Fig. 4. MK-801 suppresses the neuroinflammation caused by chronic morphine infusion. On day 5, morphine tolerance developed, then at 3 h after discontinuation of morphine infusion, MK-801 was injected intrathecally 30 min before morphine challenge (15 μg) and samples were collected 60 min post-challenge. Saline + MO: saline control rats challenged with morphine; center panels, Mo + Mo: morphine-infused rats challenged with morphine; bottom panels, Mo + MK + Mo: morphine-infused rats treated with 20 μg MK-801, then challenged with morphine (a). Cells stained for astrocytes (left panels) or microglia (right panels). (b and c) Double-staining for IL-1β (red) and either the astrocyte marker GFAP or the microglia cell marker OX42 (green); in the large panel, the arrows indicate co-localization. (d) Double-staining for IL-6 (red) and the microglia marker OX42 (green); the arrow indicated co-localization. The pictures are representatives of those for samples from 3 rats.

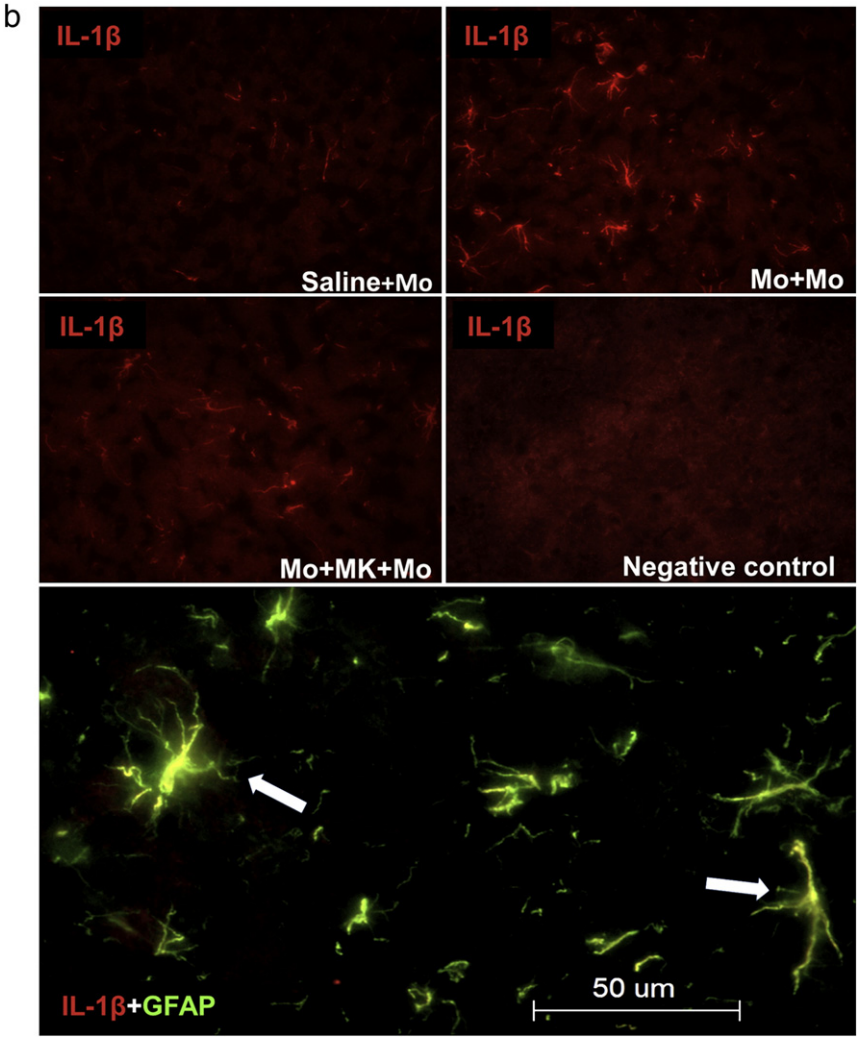

Fig. 4 (*continued*).

test (Fig. 1). To examine the effect of acute MK-801 treatment on morphine antinociception in morphine-tolerant rats and its possible mechanism, we decided to apply MK-801 on day 5 after morphine discontinuation, then to see the antinociceptive response of tolerant rats toward morphine. Based on the work of Chow et al. (2004), an antinociceptive effect was obtained with 30 μg of MK-801 intrathecal injection and neurotoxic effects were observed with 40 μg of MK-801, therefore the dose response of intrathecal MK-801 was constructed with doses of 5, 10 and 20 μg to study its acute effect on morphine analgesia in morphine-tolerant rats. Briefly, male Wistar rats were given saline (1 μl/h) or morphine (15 μg/h) for 5 days. On day 5 on which tolerance developed, at 3 h after discontinuation of morphine infusion, MK-801 or saline was injected intrathecally 30 min before morphine challenge (15 μg). As in our previous study (Tai et al., 2006), morphine challenge on day 5 produced a significant antinociceptive effect in saline-infused rats, but not in morphine-tolerant rats (Fig. 2a). MK-801 (20 μg) alone had no antinociceptive effect in either saline-infused controls (Fig. 1a) or morphine-tolerant rats (data not shown). However, pretreatment with MK-801 before morphine challenge preserved its antinociceptive effect in morphine-tolerant rats in a dose-dependent manner, with a maximal effect at 60 min. The dose of 10 μg of MK-801 resulted in only slight preservation of morphine-induced antinociception, while 5 μg of MK-801 had no effect. As shown in Fig. 2b, injection of 20 μg of MK-801 significantly improved morphine-induced antinociception, with a maximal effect (MPE%) of up to 61%, with a 10 s tail-flick latency being defined as 100% MPE in saline-infused rats receiving morphine challenge. Morphine-tolerant rats showed an MPE of only 11% after morphine challenge. Treatment with the glial metabolic inhibitor fluorocitrate (FC, 1 nmole) was used to examine the role of glia in the antinociceptive effect of morphine and was found to result in a preservation of the antinociceptive effect of morphine comparable to that of acute MK-801 treatment in morphine-tolerant rats. Fluorocitrate alone had no antinociceptive effect. The inset is constructed from the results of tail-flick latency measurement and presented by area-under-curve (AUC) (Fig. 2b).

At time zero just before morphine administration, there existed the differences in nociceptive thresholds from different experimental groups. We assume that it might be due to the individual variation. All animals were randomly assigned to each group in our study. Even though we did see differences in the nociceptive threshold at time zero, the differences were not statistically different. MPE (%) were calculated as the [(experimental latency) − (baseline latency)]/[(cut-off latency) − (baseline latency)] × 100. Different basal nociceptive thresholds may affect the calculation of MPE (%). However, the changes in nociceptive thresholds at time zero didn't affect the results of MPE shown here.

Since the most promising behavioral improvement was seen at 20 μg of MK-801, this dosage was used in all subsequent studies unless specified otherwise. In the following experiment, samples were

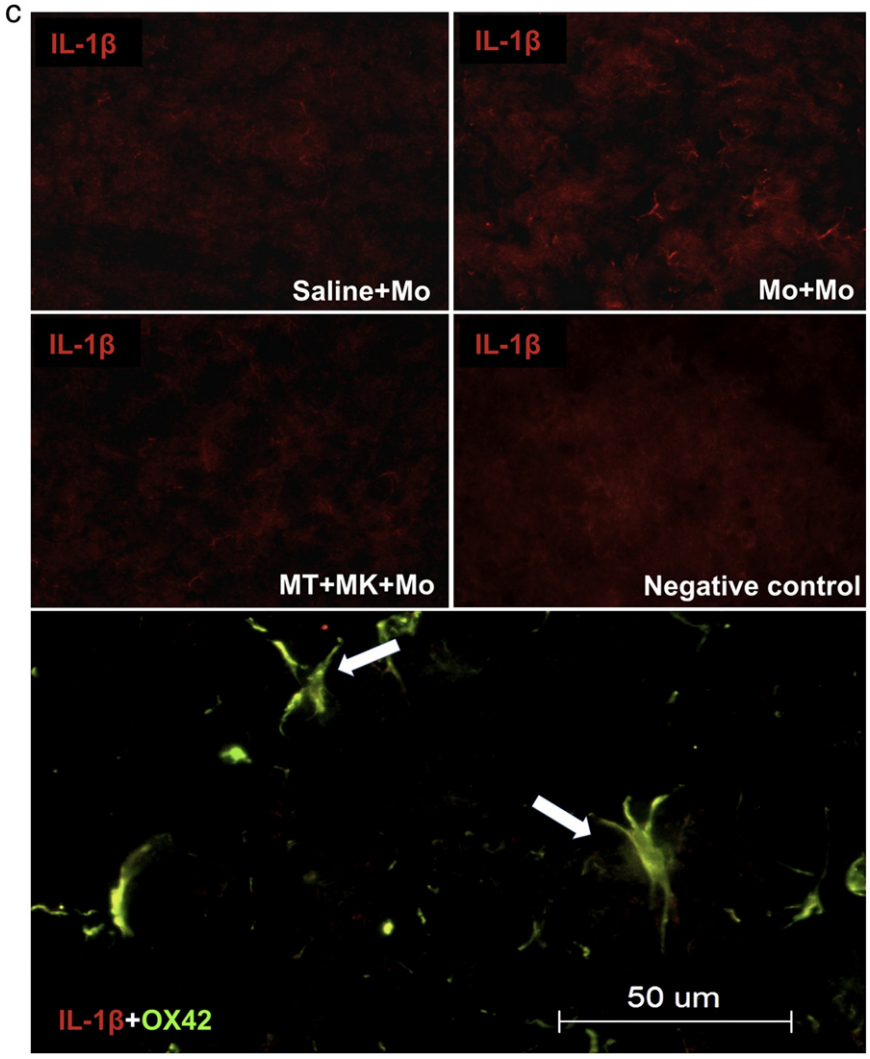

Fig. 4 (*continued*).

collected at 60 min post-morphine challenge when morphine's antinociception reached its peak.

3.2. Acute MK-801 treatment significantly reduces chronic morphine-induced pro-inflammatory cytokine expression

Samples from rats either saline or MK-801 or fluorocitrate pre-treatment were collected at 60 min after morphine challenge and analyzed for cytokine expression using a Bio-plex system. Compared to the saline control, chronic morphine infusion induced expression of the three pro-inflammatory cytokines IL-1β (Fig. 3a), IL-6 (Fig. 3b), and TNF-α (Fig. 3c) in the dorsal spinal cord and these increases were suppressed by the treatment of 20 μg of MK-801. Similarly, fluorocitrate treatment also suppressed the expression of IL-1β (Fig. 3a) and IL-6 (Fig. 3b) in response to morphine challenge in tolerant rats. Consistent with this, real-time PCR analysis demonstrated that mRNA levels of IL-1β (Fig. 3d), IL-6 (Fig. 3e) and TNF-α (Fig. 3f) were also markedly reduced to by acute MK-801 treatment before morphine challenge.

3.3. Acute MK-801 treatment inhibits the activation of astrocytes and microglia induced by chronic morphine treatment

Glial cell activation appears as a cell morphology transition from a ramified shape to ameboid shape. In immunohistochemical studies, we observed strong immunoreactivity for the astrocyte marker GFAP in the dorsal spinal cords of morphine-tolerant rats, indicating that astrocytes in the dorsal spinal cord were more activated than those in the saline control group (Fig. 4a), and that, after MK-801 pre-treatment, some activated astrocytes were reversed to the resting state. Weaker staining for the microglia marker OX42 (CD11b) also suggested that microglia were less activated in the saline control and in the MK-801 pre-treated group (Fig. 4a). Acute MK-801 treatment was not able to completely reverse the astrocyte and microglia activation induced by chronic morphine treatment. Staining for IL-1β and IL-6 co-localized with GFAP and OX42, indicating that those pro-inflammatory cytokines were expressed by astrocytes and microglia, and they can be inhibited by the MK-801 (20 μg, i.t.) pretreatment (Fig. 4b, c, and d).

3.4. The effect of acute MK-801 treatment in reducing chronic morphine-induced neuroinflammation does not involve the ERK, JNK, and p38 signaling pathways

As shown above, MK-801 reduced chronic morphine-induced pro-inflammatory cytokines (IL-1β and IL-6) levels in morphine-tolerant rats, implying that somehow MK-801 interferes with the inflammation pathways either directly or indirectly. Evidence is accumulating that all three MAPK pathways, extracellular signal regulated kinases (ERK), p38, and c-Jun N-terminal kinase (JNK), contribute to pain

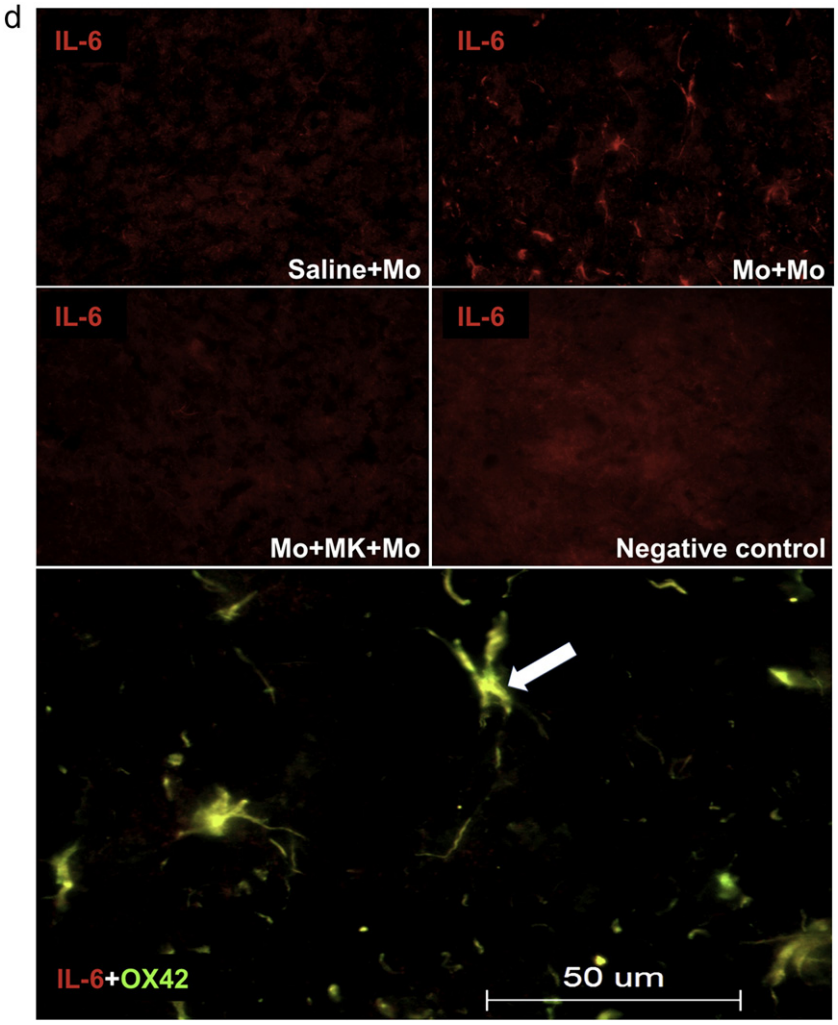

Fig. 4 (*continued*).

sensitization after tissue and nerve injury (Ji et al., 2009). Chronic morphine treatment is also reported to activate glia cells (astrocytes and microglia) to express pro-inflammatory cytokines (Tai et al., 2006) or to turn on inflammatory signaling. However, Western blot analysis did not show any significant difference in levels on phosphorylation of JNK (Fig. 5a), ERK (Fig. 5b), or p38 (Fig. 5c) between saline control, morphine-tolerant, or MK801-treated rats in our study.

4. Discussion

In this study, pretreatment with 20 μg of MK-801 significantly preserved the antinociceptive effect of morphine in morphine-tolerant rats in the 52 °C tail-flick latency test, and more than 60% of the antinociceptive effect was restored compared to that of saline control group (MPE = 100%). However, the calculation of AUC indicated a less efficacy of MK-801, showing only about 40% improvement. The area under curve (AUC) takes the variable "time/duration of action" into consideration. MK-801 (20 μg) reached a maximal effect (MPE%) at 60 min post morphine challenge. With single injection of MK-801, rats at other time point did not show significant improvement in response to morphine, compared with Mo + Mo group. This probably is due to the pharmacokinetic issue. Therefore, the acute MK-801 effect would be diluted when we calculated with AUC. Similar to MK-801, pretreatment with the glia-specific metabolic inhibitor fluorocitrate also restored the antinociceptive effect (MPE = 51.9 ± 8.26%), showing that acute suppression of glial cell activity was effective enough to influence pain behavior.

As reported previously (Johnston et al., 2004; Raghavendra et al., 2004), IL-1β, IL-6, and TNF-α levels were elevated in morphine-tolerant rats. Here, we observed that this elevation could be prevented by MK-801 pre-treatment at both the mRNA and protein levels, suggesting that MK-801 may be involved in transcriptional regulation of cytokine expression by indirect interaction with certain transcription factors or related signaling pathways. Moreover, MK-801 pre-treatment partially reversed the morphology of activated astrocytes and microglia in morphine-tolerant rats to a less active ramified state, which was reflected by the reduction in pro-inflammatory cytokine expression in glia cells. However, MK-801, at the dose of 20 μg, could not completely reverse the activation of all activated glia cells, as glial cell staining and the increased glia protein levels seen on Western blots could not be completely reversed (data not shown). These results suggest that MK-801 interferes with the inflammatory pathway to a certain extent. Besides MK-801, other NMDA receptor antagonists (e.g. dextromethorphan and ketamine) are also reported to have an anti-inflammatory effect. Dextromethorphan protects dopaminergic neurons from LPS-induced degeneration by inhibition of microglial activation, and decreases the release of nitric oxide, TNF-α, prostaglandin E2, and superoxide from microglia (Li et al., 2005; Liu et al., 2003). Ketamine attenuates the induction and maintenance of the

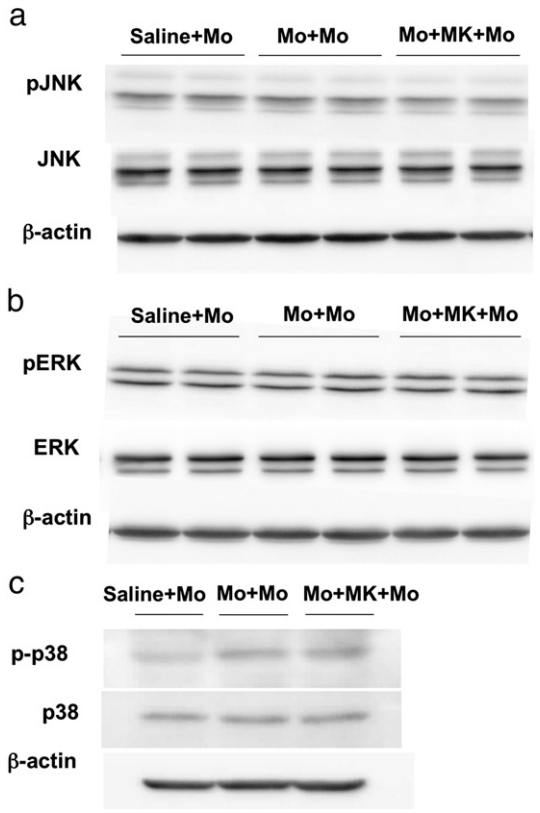

Fig. 5. The MAPK signaling pathways (ERK, JNK, and p38) are not affected by MK-801 treatment. On day 5 when morphine tolerance developed, at 3 h after discontinuation of morphine infusion, MK-801 was injected intrathecally 30 min before morphine challenge (15 μg) and samples were collected 60 min post challenge and total protein extracts subjected to Western blot analysis. (a) pJNK and JNK expressions. (b) pERK and ERK expressions. (c) p-p38 and p38 expressions. Each blot is representative of the results from 5 rats. β-Actin was used as the loading control.

inflammatory responses of hyperalgesia behavior and joint swelling in a chronic antigen-induced arthritis model (Boettger et al., 2010). Moreover, in a mouse laparotomy model, ketamine suppressed TNF and IFN-γ secretion after LPS and *E. coli* challenge (Takahashi et al., 2010). Together, the previous results and our present data show that these clinically used NMDA receptor antagonists have an anti-inflammatory effect, probably by interfering with inflammation through the NMDA receptor signaling pathway rather than having a specific effect on inflammation. Many studies have shown the importance of MAPK pathways in pain (Ji et al., 2009) and our previous study showed that chronic morphine treatment induces significant activation of p38, but not ERK and JNK (Tai et al., 2009). Nevertheless, in the present study, acute MK-801 treatment was unable to suppress the spinal activation of the p38 signaling pathway in morphine-tolerant rats.

NMDA receptors are expressed on both astrocytes and microglia (Aicher et al., 1997; Pocock and Kettenmann, 2007). Moreover, astrocytes in the brain have been shown to express functional NMDA receptors (Krebs et al., 2003; Schipke et al., 2001). MK-801 is an NMDA receptor antagonist and blocks NMDA receptors both on neurons and glia cells. Whether neuronal and/or glial NMDA receptors are responsible for this regulatory effect of MK-801 on pro-inflammatory cytokine expression is an interesting question. One possible mechanism is MK-801 works on neuronal NMDA receptors to regulate neuron plasticity in response to morphine challenge, and, in some way, influences downstream pro-inflammatory cytokine expression. By measuring NMDA-evoked currents, Lalo et al. (2006) suggested that chemical transmission between neurons and glial cells in the mouse cortex is mediated through NMDA receptor signaling. Thus, it is also possible that MK-801 interferes with neuroinflammation by blocking neuron-to-glia signaling through neurotransmitters such as glutamate, or substance P. In a preliminary study, we observed that toll-like receptor 2 (TLR2) mRNA levels were decreased in response to MK-801 pretreatment and morphine challenge in tolerant rats (data not shown). The blockade of glial cell activation by decreasing the expression of toll-like receptors could be an alternative way to explain the interference with neuron-to-glia signaling.

In summary, our study demonstrated that MK-801 pretreatment restored the antinociceptive effect of morphine and suppressed chronic morphine-induced neuroinflammation in morphine-tolerant rats. This is the first study to show that, in the presence of morphine, acute MK-801 treatment can suppress the increase in IL-1β and IL-6 and TNF-α expressions in morphine-tolerant rats. MK-801 might not only simply affect post-synaptic neuron plasticity by blocking NMDA receptor signaling, but also interfere with chronic morphine-induced neuroinflammation. Accordingly, this study provides new evidence that NMDA receptor antagonists are a useful analgesic adjuvant in providing the morphine-tolerant patient with pain relief. However, the detailed underlying mechanism requires further study.

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