

Suppressing HMGB1-TLR4-Mediated Neuroinflammation Alleviates Morphine Tolerance via Inhibiting AMPK-HO-1 Pathway in The Spinal Cord of Mice

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Abstract

Background and objectives: A major unresolved issue in treating pain is the analgesic tolerance produced by opioids. Neuroinflammation was thought to be important in the development of morphine tolerance. The role of High mobility group box-1 (HMGB1) in morphine tolerance is elusive.

Methods: ICR mice were used for tail-flick test to evaluate morphine tolerance. SD rats were used to collect the CSF to investigate whether morphine could induce the efflux of HMGB1 into extracellular environment. The neural cell line SH-SY5Y and the microglial cell line BV-2 were used to investigate the pharmacological effects and the mechanism of morphine-induced neuroinflammation. The activation of microglia was assessed by immunofluorescence staining. Neuroinflammation-related cytokines were measured by western blot and real-time PCR. The level of HMGB1 and related signaling pathway were evaluated by western blot and immunofluorescence staining.

Results: Morphine induces the release of HMGB1 from neurons. The released HMGB1 activated microglia and triggered TLR4-mediated inflammatory response, leading to the phosphorylation of nuclear factor- κ B (NF- κ B) p65 and the upregulation of IL-1 β . The secretion of HMGB1 was under the control of AMPK-HO-1 pathway. AMPK inhibitor and HO-1 inhibitor inhibited the release of HMGB1 and suppressed HMGB1-TLR4 mediated neuroinflammation.

Conclusion: Our study indicated that morphine-induced extracellular HMGB1 was critical for morphine tolerance. The release of HMGB1 was regulated by AMPK-HO-1 pathway. Our findings may represent a bright prospect for the improvement of morphine tolerance with HMGB1 inhibitor and/or the inhibitors for AMPK-HO-1 axis.

Background

Opioids, such as morphine, are the most potent drugs utilized to control severe pain [1]. Long-term use of morphine leads to addiction and tolerance, which significantly hinder its application. Tolerance is defined as described efficacy of the opioid and a need for higher and more frequent doses to achieve the same analgesic effect. For several decades, numerous studies have been devoted to understanding the mechanisms underlying opioid tolerance.

Despite intensive research into the neurobiological mechanisms of morphine tolerance, including endocytosis of μ -opioid receptor (MOR), upregulation of N-methyl- D-aspartic acid (NMDA) receptor, and downregulation of γ -aminobutyric acid (GABA) receptor, the activation of neuroinflammation was thought to be important in the development and maintenance of morphine tolerance [2, 3]. In the central nervous system (CNS), microglia plays a major role in neuroinflammation. Persistent activation of dorsal horn by the release of damage-associated molecular patterns (DAMPs) from neurons stimulates microglia to release inflammatory mediators [4]. During the process of microglia activation, toll-like receptor 4 (TLR4) predominantly expressed on microglia is considered as one of the most critical signaling receptor [5]. TLR4 is a pattern-recognition receptor that recognizes specific DAMPs and subsequently initiates

immune response [6]. It was reported that morphine could trigger TLR4-mediated neuroinflammation by binding with MD-2, a TLR4 accessory protein [7]. Blockade of TLR4 inhibited the activation of microglia and attenuated morphine tolerance, which suggested that TLR4 is very important in the development of morphine tolerance [8].

There are several endogenous TLR4 agonists, such as heat shock proteins (HSPs), high-mobility group box 1 (HMGB1), heparan sulfate (HS) [9] and fibrinogen (Fg) [10]. Our previous study indicated that morphine could induce the release of HSP70 from neurons. As an important DAMPs, HSP70 induced the activation of TLR4-NLRP3 inflammasome signal pathway and led to neuroinflammation [11]. Furthermore, it was reported that disulfide high mobility group box-1 (ds-HMGB1) could mimic the effects of morphine induced-persistent sensitization through TLR4 receptor (Grace et al., 2016). The released extracellular HMGB1 could increase the expression of proinflammatory cytokines, such as IL-1 β , IL-6 and TNF- α [12]. HMGB1, a non-histone DNA binding protein, mainly located in the cell nucleus could stabilize nucleosome formation, and regulate the interaction of transcription factors with DNA in the physiological state [13]. It was reported that significant amount of HMGB1 was released from the nucleus into the extracellular space after neuronal injury immediately [14]. HMGB1, as an important DAMPs has been shown to participate in neuroinflammation, modulate autophagy and apoptosis as well as regulate gene transcription [15, 16]. It is considered as a critical factor in several pathophysiological conditions. Active HMGB1 secretion by inflammatory cells and passive release by dying neurons affects cell viability and apoptosis, and contributes to neuroinflammation and neurodegeneration associated with Parkinson disease [13]. The release of HMGB1 from astrocytes through a pannexin-1 and P2 \times 7 receptor signaling cascade could then contribute to the development of major depressive disorder (MDD) [17].

These information give us a hint that HMGB1 might be released by chronic morphine administration and the extracellular HMGB1 could be another critical morphine-induced inflammatory mediator contributing to the development of morphine tolerance. Here, we investigate the role of HMGB1 in pathogenesis of morphine tolerance as well as its underlying mechanisms.

Methods

Animals

Adult male ICR mice (18-22 g) and adult male Sprague-Dawley rats (200–250 g) were provided by the Experimental Animal Center at Nanjing Medical University, Nanjing, China. Animals were housed 5 to 6 per cage under pathogen-free conditions with soft bedding under controlled temperature (22 \pm 2 $^{\circ}$ C) and a 12-h light/dark cycle (lights on at 8:00 am). Behavioral testing was performed during the light cycle (between 9:00 am and 5:00 pm). The animals were allowed to acclimate to these conditions for at least 2 days before starting experiments. For each group of experiments, the animals were matched by age and body weight.

Chemicals and Reagents

Morphine hydrochloride was purchased from Shenyang First Pharmaceutical Factory, Northeast Pharmaceutical Group Company (Shenyang, China). Glycyrrhizin was purchased from Sigma Aldrich (St. Louis, MO, USA). Compound C and TAK242 were purchased from MedChemExpress (New Jersey, USA). Zinc protoporphyrin IX was purchased from Sigma Aldrich (St. Louis, MO, USA). Recombinant HMGB1 was purchased from Sino Biological (Beijing, China). Antibody for β -actin was from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for AMPK, p-AMPK (Thr172), NF- κ B p65 (Ser536), p-NF- κ B p65 (Ser536) were from Cell Signaling Technology (Beverly, MA, USA). HMGB1 and Transferrin were from Abcam (Cambridge, MA, USA). HO-1 was purchased from Affinity Biosciences (Cincinnati, OH, USA). Secondary antibodies for western blot were from Sigma-Aldrich (St. Louis, MO, USA). Antibody for IL-1 β was purchased from R&D Systems (Minneapolis, MN, USA). Immunofluorescent antibody for ionized calcium-binding adapter molecule1 (Iba-1) was from Abcam (Cambridge, MA, USA). Antibodies for glial fibrillary acidic protein (GFAP) and neuronal nuclear protein (NeuN) were from Millipore (Billerica, MA, USA). Secondary antibodies for immunofluorescence were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and Abcam (Cambridge, MA, USA). Anti-HMGB1 neutralizing antibody was from Novus Biologicals (Littleton, Colorado, USA). Normal IgG was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fetal bovine serum (FBS), cell culture media and supplements were purchased from Gibco (Grand Island, NY, USA).

Tolerance model and behavioral analysis

We habituated animals in the testing environments for 2 days and carried out behavioral testing in a blinded manner. For the test of chronic tolerance, mice were intrathecally injected with vehicle or morphine (10 μ g/10 μ L) once daily for seven consecutive days. Behavioral testing was performed 30 min after morphine administration by tail-flick assay every morning. The tail-flick test was performed using a water bath with temperature maintained at 52°C. Each animal was gently wrapped in a cloth by the experimenter. The distal one-third of tail was immersed in a water bath, and mice rapidly removed their tail from the bath at the first sign of the discomfort. Stop the chronometer as soon as the mouse withdraws its tail from the hot water and record the latency time (in sec). A cut-off time of 10 s was set to avoid tissue damage. Different doses of Glycyrrhizin (25, 50, 100 mg/kg) were administered by intragastric injection 15 min before morphine administration. Different doses of Compound C (1, 3 or 10 μ g/10 μ L) were administered by intrathecal injection 15 min before morphine administration. Data were calculated as a percentage of maximal possible effect (%MPE), which was calculated by the following formula: $100\% \times [(Drug\ response\ time - Basal\ response\ time) / (10\ s - Basal\ response\ time)] = \%MPE$. Rats were intrathecally injected with morphine (20 μ g/10 μ L) daily for 7 consecutive days.

Cell Cultures

SH-SY5Y cells were maintained in humidified 5% CO₂ at 37°C in Modified Eagle Media: F-12 (MEM/F12, Gibco, NY, USA) supplemented with 10% (v/v) FBS (Gibco), 80 U/mL penicillin and 0.08 mg/mL streptomycin. For further experiments, SH-SY5Y cells were plated in 6-well plate overnight and then treated with morphine (200 μ M) in the following morning with or without Zinc protoporphyrin IX (Znpp) (2

μM), Compound C (10 μM) for 12 h. The cell extracts and precipitated supernatants were analyzed by immunoblot assay.

BV-2 cells were maintained in humidified 5% CO₂ at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM, KenGEN BioTECH, China) supplemented with 10% (v/v) FBS (Gibco), 80 U/mL penicillin, and 0.08 mg/mL streptomycin. For further experiments, BV-2 cells were plated in 6-well plate overnight and then treated with recombinant HMGB1 protein or conditional medium from SH-SY5Y cells. Then the cell extracts and precipitated supernatants were analyzed by immunoblot assay.

Intrathecal injection procedure

To perform intrathecal (*i.t.*) injections, the mice were placed in a prone position and the midpoint between the tips of the iliac crest was located. A Hamilton syringe with a 30-gauge needle was inserted into the subarachnoid space of the spinal cord between the L5 and L6 spinous processes. Proper intrathecal injection was systemically confirmed by observation of a tail flick. Intrathecal injection did not affect baseline responses, compared with latencies recorded before injection.

Western blot

Samples (cells or spinal cord tissue segments at L1-L6) were collected and washed with ice-cold PBS before being lysed in radio immunoprecipitation assay (RIPA) lysis buffer and then sample lysates were separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were blocked with 10 % low-fat dry powdered milk or with 5% BSA and 5% low-fat dry powdered milk in TBST (Tris–HCl, NaCl, Tween 20) for 2 h at room temperature, and then probed with primary antibodies at 4°C for overnight. Finally, the horseradish peroxidase (HRP)-coupled secondary antibodies were utilized for detecting corresponding primary antibody. The primary antibodies utilized included β -actin (1:5000), AMPK (1:1000), p-AMPK (1:1000) (Thr172), HO-1 (1:1000), NF- κ B p65 (1:1000), p- NF- κ B p65 (Ser536) (1:1000), HMGB1 (1:1000), Transferrin (1:1000) and IL-1 β (1:300). The bands were then developed by enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA). Data were analyzed with the Molecular Imager and the associated software Image J (NIH, USA).

Immunohistochemistry

For fluorescence immunohistochemistry, mice were anaesthetized and transcardially perfused with 4% cold paraformaldehyde on day 7. Lumbar spinal cords were harvested, post-fixed for 4 h at 4°C in 4% paraformaldehyde, and then cryo-protected sequentially in 10, 20 and 30% sucrose overnight for 3 days. Frozen sections (15 μm) were cut on a cryostat and air-dried on microscope slides for 30 min at room temperature. For dual antibody immunofluorescence, primary antibodies against HMGB1 (1:200), Iba1 (1:200), NeuN (1:200) and GFAP (1:200) were incubated with the tissue section in 10% normal donkey serum and 0.01% Triton-X-100 overnight at 4°C. For c-fos and CGRP fluorescence immunohistochemistry, primary antibodies against c-fos (1:300) and CGRP (1:300) were incubated with the tissue section in 10% normal donkey serum and 0.01% Triton-X-100 overnight at 4°C. The appropriate fluorescent secondary

antibody (1:300, Alexa Fluor 488 or 567) was used for each primary antibody. Confocal microscopy of immunofluorescence in the dorsal horn was performed with a confocal microscopy (Zeiss LSM710, Germany).

RNA interference

HO-1 siRNA (EHU051241), AMPK siRNA (AMPK α 1, EHU074041; AMPK α 2, EHU042081) and control siRNA (EHUEGFP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Control siRNA was used as a negative control. For the transfection of siRNA, SH-SY5Y cells were cultured in 6-well plates with antibiotic-free medium the day before transfection. The transfection was conducted when cells reached 50 ~ 70% confluence using Lipofectamine 2000 (Invitrogen, USA) and serum-free medium according to the manufacturer's instructions. After 5 h, the transfection medium was replaced with the culture medium containing 10% FBS and then incubated at 37 °C.

Collection of cerebrospinal fluid (CSF)

Adult male Sprague-Dawley rats (200–250 g) were housed under a 12 light/dark cycle, with food and water available ad lib. The animals were anesthetized with pentobarbital sodium (50 mg/kg, *i.p.*). The CSF was carefully collected from the cisterna magna of each rat and inspected for blood contamination. Contaminated samples were discarded. Approximately 80 μ L of CSF was collected from each animal. After a short centrifugation step (5 min at 5000 g, 4 °C), the samples were dissolved in 2 \times SDS loading buffer, boiled, and analyzed by SDS-PAGE followed by western blotting.

Statistical Analysis

GraphPad Prism 7 software (GraphPad Software, San Diego, Calif.) was used to conduct all the statistical analyses. The differences between two groups were evaluated by one-sample t-test. The data from more than two groups were evaluated by one-way ANOVA or two-way ANOVA. Results were represented as mean \pm SEM of the independent experiments. Results described as significant were based on a criterion of $P < 0.05$.

Results

Morphine induces the release of HMGB1 in vivo and in vitro

We established morphine tolerance model with SD rats collecting the CSF to investigate whether morphine could induce the efflux of HMGB1 into extracellular environment. As shown in Figure 1A, the immunoblot data indicated that morphine markedly caused HMGB1 to be released into CSF. To further investigate the cellular mechanism underlying morphine-induced release of HMGB1, analysis of the cellular distribution of HMGB1 by confocal microscopic scanning was performed. It was showed that, in naive mice, HMGB1 mainly co-localized with NeuN (neuronal marker), but not with Iba1 (microglia marker) or GFAP (astrocyte marker) in the spinal cord (Fig. 1B). Therefore, we utilized human neuroblastoma cell line SH-SY5Y cells, which express both mu- and delta-opioid receptors (ratio

approximately 4.5:1) [18], to further confirm the release of HMGB1 induced by morphine in neuron. We incubated SH-SY5Y cells with different concentrations (50, 100, 200 μ M) of morphine for 12 h (the cells were stimulated as described) [2], then the supernatants of SH-SY5Y cells were collected and analyzed by western blot. MTT assay indicated that the different concentrations (50, 100, 200 μ M) of morphine did not affect cell proliferation (Additional file 1: Figure S1). We found that morphine promoted the efflux of HMGB1 into extracellular environment in a concentration-dependent manner (Fig. 1C). Furthermore, the immunofluorescence results showed that morphine caused the migration of HMGB1 firstly from nucleus to cytoplasm before releasing to extracellular environment (Fig. 1D).

Glycyrrhizin attenuates chronic morphine tolerance

According to the data mentioned above, morphine induced the release of HMGB1. Then we questioned whether the released HMGB1 was important for the development of morphine tolerance. As a selective HMGB1 inhibitor, glycyrrhizin was utilized to investigate the therapeutic effects to the released HMGB1 in morphine tolerance. Glycyrrhizin did not affect the acute analgesic effect of morphine (Fig. 2A), and the behavioral test results showed that glycyrrhizin attenuated chronic morphine tolerance in dose-dependent manner (Fig. 2B). At day 7, the MPE at 30 min after morphine administration decreased to 14.5% in chronic morphine-treated mice, whereas mice with co-administration of glycyrrhizin (25, 50, and 100 mg/kg) and morphine displayed the MPE of 26.3%, 35.9% and 54.7%, respectively (Fig. 2B). Furthermore, the expression of c-fos and CGRP protein, two of the immediate early genes rapidly expressed in neurons after a noxious stimulus, were increased in the dorsal horn of the spinal cord after morphine tolerance, and these increases were then suppressed by glycyrrhizin (Fig. 2C).

Extracellular HMGB1 triggers inflammatory response dependent on TLR4 in microglia.

Next, we investigated the role of extracellular HMGB1 in neuroinflammation. Microglia, accounting for 10%~15% of all central nerve cells, are natural immune cells in the central nervous system. When exposed to a variety of damaging stimuli, such as ischemia/hypoxia, trauma and infection, microglia cells can be activated rapidly and release a large number of proinflammatory cytokines, including TNF- α , IL-1 β and IL-6 [19-21]. TLR4 can be activated by DAMPs, triggering its downstream signaling pathway, consequently evoking innate immune response through the maturation of IL-1 β . As HMGB1 is an important TLR4 agonist, we investigated the effect of extracellular HMGB1 on inflammatory response and immortalized murine microglial cell line BV-2 was utilized [22, 23]. Firstly, we found that repeated morphine administration led to the phosphorylation of NF- κ B p65 and the upregulation of proinflammatory cytokines IL-1 β in the spinal cord (Fig. 3A and B). Glycyrrhizin significantly attenuated the phosphorylation of NF- κ B p65 and the expression of IL-1 β in the spinal cord (Fig. 3A and B). Furthermore, Immunofluorescence data showed that repeated morphine treatment (10 μ g/10 μ L, once daily for 7 days) led to the activation of microglia (Iba-1 as microglia marker), and glycyrrhizin significantly inhibited morphine-induced activation of microglia (Fig. 3C). In order to verify whether the extracellular HMGB1-triggered inflammatory response is dependent on TLR4 in microglia, we found that recombinant HMGB1 (25 nM) significantly upregulated the phosphorylation of NF- κ B p65 in BV-2 cells. In

addition, the administration of TLR4 antagonist (TAK242, 10 μ M) suppressed the upregulation of the phosphorylation of NF- κ B p65 caused by recombinant HMGB1 in BV-2 cells (Fig. 3D). The data indicated that TLR4 was essential for the inflammatory response caused by HMGB1. In order to further confirm the role of HMGB1 in inducing inflammatory response, we utilized conditional medium (CM) from morphine-treated (200 μ M, 12 h) SH-SY5Y cells to activate BV-2 cells. Anti-HMGB1 antibody (2 μ g/ μ L) suppressed CM-induced upregulation of phosphorylation of NF- κ B p65 and the expression of IL-1 β (Fig. 3E and F). Normal IgG (2 μ g/ μ L) did not show an inhibitory effect (Fig. 3E and F).

The release of HMGB1 induced by morphine depends on AMPK/HO-1 pathway

The next question addressed how morphine induced the release of HMGB1 from neurons. HO-1, a stress-responsive protein, serves a vital metabolic function as the rate-limiting step in the degradation of heme [24]. It may function as a pleiotropic regulator during inflammation. Increased HO-1 expression in tissue is commonly associated with increased inflammation or oxidative stress. HO-1 can be up-regulated by a variety of factors, including hypoxia, hyperoxia, heat shock, cytokines, heavy metals, hydrogen peroxide, UV irradiation, and its substrate, heme [25, 26]. It was reported that HO-1 signal could prevent HMGB1-mediated activation of NLRP3 inflammasomes. HMGB1-mediated inflammatory and immune reactions in nervous system played an essential role in the generation of neuroinflammation.[27, 28]. However, despite these observations, deleterious consequences of HO-1 overexpression have been reported in vitro and in vivo. Song W et al. reported that in rat astrocytes, HO-1 over-expression resulted in significant oxidative damage to mitochondrial lipids, proteins, and nucleic acids, partial growth arrest, and increased cell death [29]. Besides, glial HO-1 over-expression was considered as a possible cause of pathological iron deposition in the aging and degenerating mammalian CNS [30]. Furthermore, HO-1 activity inhibitors displayed the therapeutic value in Alzheimer's disease and related neurodegenerative disorders [31].

Therefore, these conflicting viewpoints remind us that the role of HO-1-HMGB1 axis in the development of morphine tolerance need further exploration. Our data showed that HO-1 was significantly elevated in SH-SY5Y cells after morphine (200 μ M) administration (Fig. 4A). And Znpp (HO-1 inhibitor, 2 μ M) , given 12h before morphine (200 μ M, 12 h) administration inhibited the morphine-induced release of HMGB1 (Fig. 4B). Furthermore, HO-1 siRNA downregulated the level of HO-1 and inhibited the morphine-induced release of HMGB1 from SH-SY5Y cells (Fig. 4C and D).

Recent studies indicated that HO-1 can be regulated under several mechanisms, such as, SIRT-1, AMPK and Nrf-2 [32, 33]. Among them, AMPK, a key regulator of energy homeostasis, plays a fundamental role in the process of chronic pain occurrence, development and maintenance [34]. AMPK has recently emerged as a novel target for the treatment of pain with the exciting potential for disease modification [35]. Zhang et al. demonstrated that morphine activated AMPK pathway, induced epithelial-mesenchymal transition (EMT) via upregulating Snail and Slug levels, and increased oxidative stress in esophageal carcinoma cells [36]. In addition, morphine could also inhibit PINK1/Parkin-mediated mitophagy in spinal cord neurons and increase the phosphorylation of AMPK [37]. Hence, we speculated that HO-1-HMGB1 activation was downstream to AMPK. Firstly, we found morphine induced the phosphorylation of AMPK

in a concentration-dependent manner in SH-SY5Y cells and Compound C significantly decreased the level of HO-1 and released HMGB1 induced by morphine in SH-SY5Y cells (Fig. 5A-C). Then we utilized AMPK siRNA, it downregulated the level of phosphorylation of AMPK, HO-1 and released HMGB-1 of SH-SY5Y cells (Fig. 5D-F).

Furthermore, to further confirm our pharmacological results, repeated i.t. injection of Compound C was performed to evaluate the effect of AMPK in chronic morphine tolerance in vivo. Compound C co-administration with morphine significantly potentiated acute morphine analgesic effect and attenuated chronic morphine tolerance in mice (Fig. 6A and B). At day 7, the MPE at 30min after morphine administration decreased to 14.39% in chronic morphine-treated mice, whereas mice co-administration with Compound C (1, 3, 10 $\mu\text{g}/10 \mu\text{L}$) and morphine displayed the MPE of 28.79%, 51.52% and 70.05% respectively (Fig. 6B). Furthermore, western blot data showed that Compound C could decrease the release of HMGB1 in CSF (Fig. 6C) and suppress the level of p-AMPK/HO-1. Furthermore, Compound C inhibited the phosphorylation of p65 induced by morphine in the spinal cord (Fig. 6D-F). These data provided sufficient evidence that morphine induced the release of HMGB1 via AMPK/HO-1 pathway and the suppression of this pathway could effectively improve chronic analgesic tolerance induced by morphine. **Figure 6**

Discussion

In the present study, the principal findings are: (1) administration of morphine induces the release of HMGB1, an endogenous TLR4 ligand, from neurons; (2) morphine induces the release of HMGB1 to extracellular environments via AMPK/HO-1 pathway; (3) extracellular HMGB1 activates microglia and causes inflammatory response, upregulating the phosphorylation of p65 and the levels of IL-1 β .

The mechanisms underlying morphine tolerance is complex and far beyond having a clear explanation. Several mechanisms are involved in the tolerance to analgesic opioids, including desensitization or internalization of the opioid receptor, elevation of cAMP levels and downregulation of spinal glutamate transporters [38]. Especially, compelling evidence has demonstrated that chronic morphine exposure induced sterile neuroinflammation in the spinal cord. HMGB1 has been implicated as a key factor in the mediation of neuroinflammation processes in several pathophysiological conditions. For example, HMGB1 is massively released into the extracellular space immediately after ischemic insult and it subsequently induces neuroinflammation in the postischemic brain [39]. HMGB1-Mac1-NADPH oxidase signaling axis bridges chronic neuroinflammation and progressive dopaminergic neurodegeneration in PD progression [40]. In addition, the persistent release of HMGB1 contributes to tactile hyperalgesia in a rodent model of neuropathic pain [41]. Anti-HMGB1 monoclonal antibody (mAb) has been shown to be effective for the treatment of a wide range of CNS diseases when modeled in animals, including stroke, traumatic brain injury, epilepsy and Alzheimer's disease [42]. Recently, Qian J et al. discovered that morphine-mediated upregulation of HMGB1 in the spinal cord which contributed to analgesic tolerance and hyperalgesia [43]. In our study we not only confirmed that morphine could induce the efflux of HMGB1 into extracellular environment via AMPK-HO-1 axis. But also, we illustrated that the released

HMGB1 triggered TLR4 signaling which consequently induced the upregulation of the phosphorylation of NF- κ B p65 and proinflammatory cytokines. Our immunoblot data gave the evidence that morphine markedly caused HMGB1 released into CSF under the tolerance model with SD rats (Fig. 1A). Furthermore, the immunofluorescence results and immunoblot data showed that morphine induced the release of HMGB1 from SH-SY5Y cells in a concentration-dependent manner (Fig. 1C and 1D). Therefore we provided a hypothesis that the released HMGB1 induced by morphine probably played an important role in neuron-microglia crosstalk, especially in the development of neuroinflammation. Previous studies revealed that HMGB1 presented its activities by serving as a ligand for several different receptors, including TLR2, TLR4, TLR5, TLR9, and RAGE[12]. Among them, HMGB1-triggered TLR4 signaling is a strong inducer of proinflammatory cytokine production. In our study, we demonstrated that extracellular HMGB1 upregulated the phosphorylation of NF- κ B p65 and increased the transcription of IL-1 β in the spinal cord (Fig. 3A and 3B). Recombinant HMGB1 protein significantly upregulated the phosphorylation of NF- κ B p65 in BV-2 cells and the administration of TLR4 antagonist suppressed the upregulation of the phosphorylation of NF- κ B p65 in BV-2 cells (Fig. 3D). In addition, anti-HMGB1 antibody could significantly inhibit the upregulation of the phosphorylation of NF- κ B p65 and IL-1 β in BV-2 cells in protein level (Fig. 3E). Our in vitro study showed a direct effect of morphine in inducing HMGB1 release from neurons and the released HMGB1 displayed its ability in triggering neuroinflammation mediated by TLR4. Furthermore, glycyrrhizin (HMGB1 inhibitor, 100 mg/kg) could increase MPE by 39.88% comparing with that in the morphine-treated group at day 7 (Fig. 2B). Immunofluorescence staining data revealed glycyrrhizin could decreased the level of CGRP (Fig. 2C), which was a peptide released by primary afferents and was able to mediate the activation of NMDA receptor in neurons. And glycyrrhizin also downregulated c-fos (Fig. 2C), which were implicated in pain transmission and morphine tolerance.

Next, we explored the mechanism of HMGB1 releasing from neurons caused by morphine. It is increasingly recognized that HO-1 down-regulates the inflammatory response. In a rat model of pleurisy, up-regulation of HO-1 significantly attenuates acute cellular inflammation [44]. In addition, numerous studies indicated that HO-1 decreased the expression of HMGB1 during inflammatory response. Although many preclinical research studies have pointed to anti-inflammatory effects of HO-1 in tissue injury, recent studies also propose a pro-pathogenic effect of HO-1 in the propagation of chronic inflammation. HO-1 has been considered to be among the strongest positive predictors of metabolic disease in humans [45]. For example, although previous studies have shown that systemic induction of HO-1 by chemical inducers reduces adiposity and improves insulin sensitivity. Huang JY et al. reported that HO-1 overexpression in adipocytes does not protect against high fat diet induced obesity and the development of insulin resistance in mice [46]. In addition, hepatocyte and macrophage conditional HO-1 deletion in mice conferred protection against diet-induced insulin resistance and inflammation, dramatically reducing secondary disease such as steatosis and liver toxicity [47]. Heterozygous Hmox1 knockout mice were also shown to be protected against high fat diet induced insulin resistance by reducing macrophage migration [48].

Therefore, the role of HO-1-HMGB1 axis in the development of morphine tolerance remains complex and incompletely understood. Our results indicated that morphine induced the release of HMGB1 via the

activation of HO-1 and the utilization of Znpp and HO-1 siRNA significantly decreased the release of HMGB1 (Fig. 4B and D) induced by morphine. Targeting HO-1 remains an attractive therapeutic strategy for the treatment of inflammatory conditions. In the unfolded protein response, activated AMPK could boost the Nrf2/HO-1 signaling axis [49]. Esculentoside A has protective potential against APAP toxicity in acute liver failure by potentiating the Nrf2/HO-1-regulated survival mechanism through the AMPK/Akt/GSK3 β pathway [50]. Here, we focus on AMPK based on its important role in the regulation of pain, including neuropathic pain, incision-induced acute and chronic pain [51–53]. According to the mentioned above, we inferred that the release of HMGB1 induced by morphine probably depends on AMPK-HO-1 axis. Our data demonstrated that Compound C and AMPK siRNA could suppress the elevation of HO-1 and the releasing of HMGB1 induced by morphine in SH-SY5Y cells (Fig. 5). In vivo experiments, Compound C displayed the ability to effectively improve morphine tolerance through the suppression of HMGB1 releasing to CSF.

Conclusion

Our study indicated that morphine-induced extracellular HMGB1 was critical for morphine tolerance. We demonstrated that morphine could induce the release of HMGB1 from neurons via AMPK-HO-1 pathway. HMGB1 mediates morphine-induced activation of TLR4 in microglia. The extracellular HMGB1 increased the phosphorylation of NF- κ B p65 via TLR4 and led to the expression of proinflammatory cytokine IL-1 β (Fig. 7). Therefore, our findings may represent a bright prospect for the improvement of morphine tolerance with HMGB1 inhibitor and/or the inhibitors for AMPK-HO-1 axis and lay the groundwork for treatment of patients with chronic pain.

Abbreviations

AMPK

Amp-activated protein kinase; CGRP: Calcitonin gene-related peptide; CM: Conditional medium; CNS: Central nervous system; CSF: Collection of cerebrospinal fluid; DAMP: damage-associated molecular pattern; DMSO: Dimethyl sulfoxide; FBS: Fetal bovine serum; GFAP: Glial fibrillary acidic protein; HMGB1: High-mobility group box 1; HSP70: Heat shock protein 70; HO-1: Hemeoxygenase - 1; i.t.: Intrathecal; Iba1: Ionized calcium-binding adapter molecule-1; IL-1 β : Interleukin-1 β ; MOR: μ -Opioid receptor; MPE: Maximal potential effect; NF- κ B: Nuclear factor- κ B; NMDA: N-methyl-D-aspartic acid; PBS: Phosphate-buffered saline; RIPA: Radio immunoprecipitation assay; TLR4: Toll-like receptor 4; TNF- α : Tumor necrosis factor- α

Declarations

Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author upon reasonable request.

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Authors' contributions

Participated in research design: Chun-Yi Jiang, Wen-Tao Liu. Conducted experiments: Tong-Tong Lin, Lei Sheng, Li Wan, Lu-Lu Ji, Jin-Can Li, Xiao-Di Sun and Yin-Bing Pan. Performed data analysis: Tong-Tong Lin, Chen-Jie Xu, Liang Hu, Xue-Feng Wu and Yuan Han. Wrote or contributed to the writing of the manuscript: Chun-Yi Jiang, Tong-Tong Lin and Lei Sheng. All of the authors reviewed the manuscript and approved the final version.

Ethics approval and consent to participate

All procedures were strictly performed in accordance with the regulations of the ethics committee of the International Association for the Study of Pain and the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006). All animal experiments were approved by the Nanjing Medical University Animal Care and Use Committee. Meanwhile, the experiments we did were designed to minimize suffering and the number of animals used.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

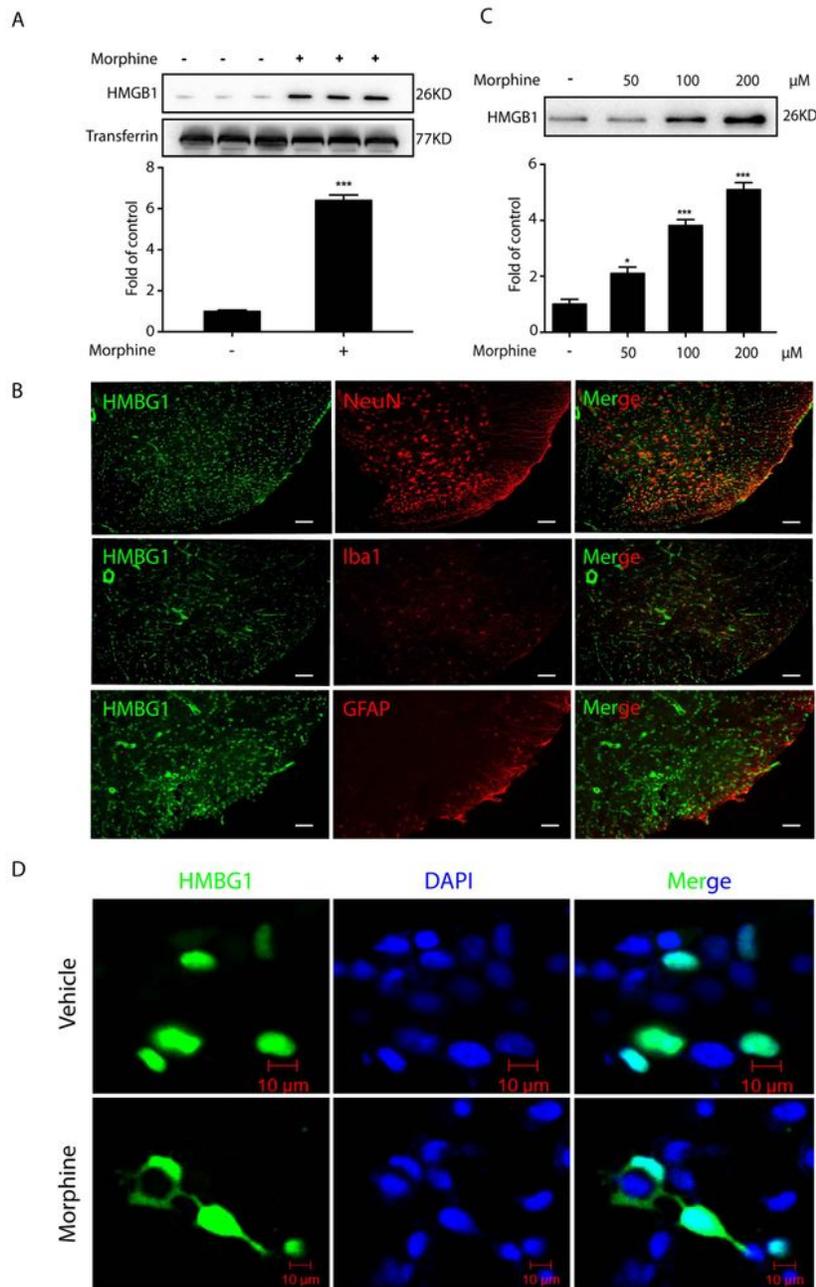


Figure 1

Morphine induces the release of HMGB1 (A) Rats were intrathecally injected with morphine (20 $\mu\text{g}/10 \mu\text{L}$) for 7 consecutive days. Consecutive administration of morphine induced the release of HMGB1 in CSF. CSF was collected from rats 1 h after the last administration and determined by western blot (n = 6). (B) Distribution and cellular localization of HMGB1 in the dorsal horn of the spinal cord from mice without morphine administration. Double immunostaining of HMGB1 and specific markers showed that

HMGB1 was co-localized with NeuN (red) not with astrocytic maker GFAP (red) or microglial maker Iba1 (red). Scale bar: 100 μ m. (C) Morphine induced the efflux of HMGB1 into extracellular environment in a concentration-dependent manner in SH-SY5Y cells. Supernatants were collected 12 h after morphine (50, 100, 200 μ M) and analyzed by western blot (n = 3). Significant difference was revealed following one-sample t-test (*P < 0.05, ***P < 0.001 vs. control). (D) Morphine (200 μ M, 12 h) induced the efflux of HMGB1 (green fluorescence) into extracellular environment in SH-SY5Y cells. (n = 4). Scale bar: 10 μ m.

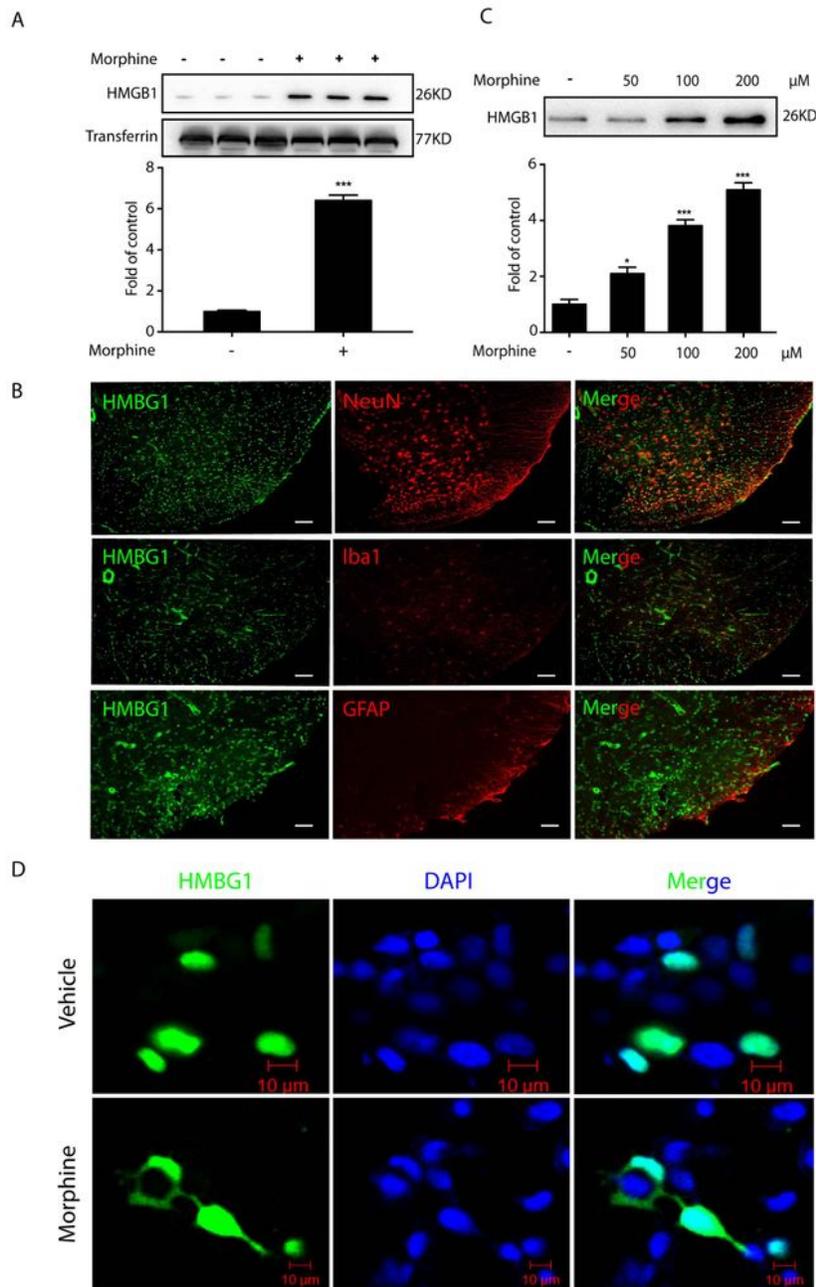


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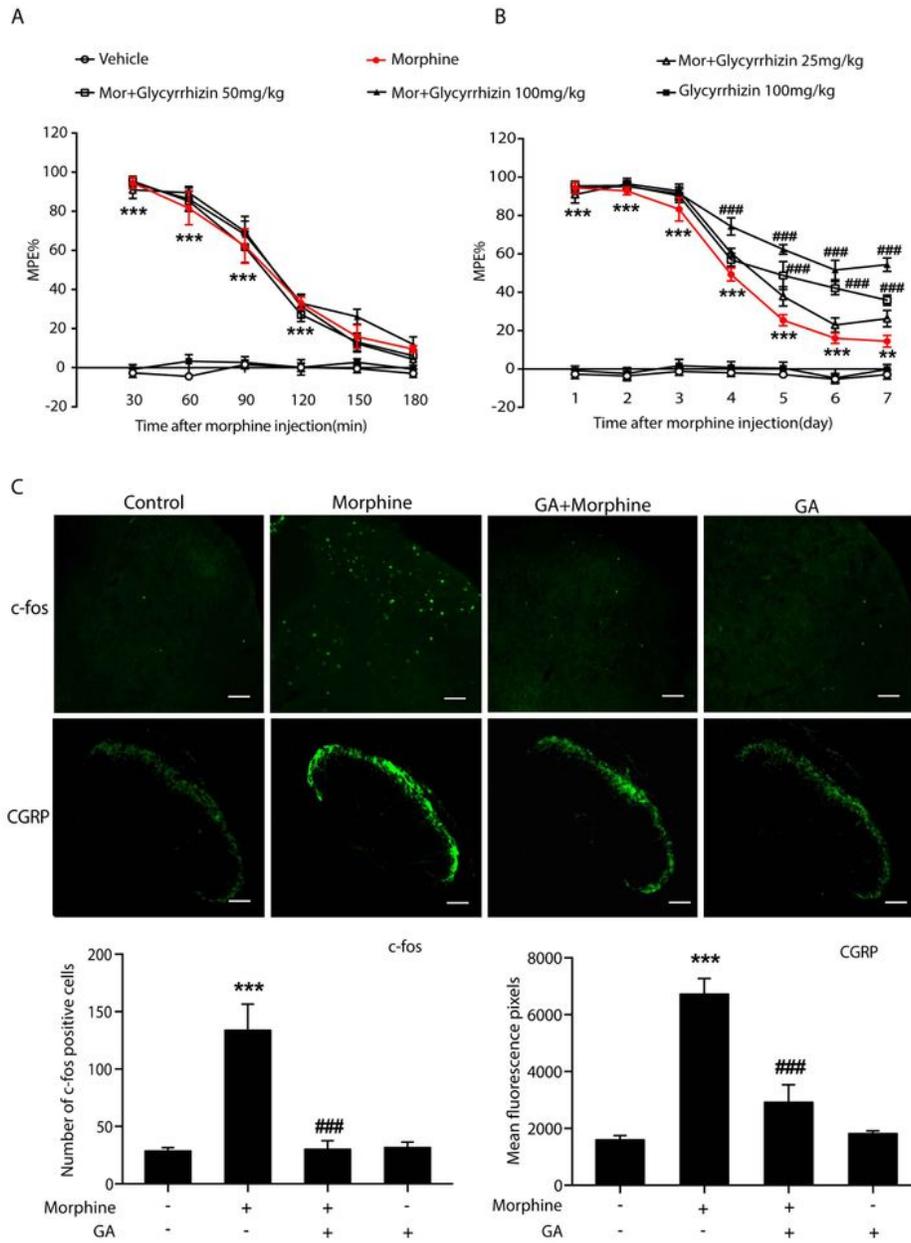


Figure 2

Glycyrrhizin attenuates chronic morphine tolerance Tail-flick method was performed to evaluate the effect of glycyrrhizin on the morphine tolerance. Data was shown as percentage of maximal possible effect (%MPE). (A) Glycyrrhizin did not affect the acute analgesic effect of morphine. Before the treatment of morphine, mice were pre-treated with different doses of glycyrrhizin (25, 50, 100 mg/kg) for 15 min. (B) Glycyrrhizin improved chronic morphine tolerance in mice (n = 8). Morphine (10 μ g/10 μ L) was

intrathecally injected with different doses of glycyrrhizin (25, 50, 100 mg/kg) once daily, and the MPE was measured 1 h after the first injection of each day. (C) Immunofluorescence analysis showed glycyrrhizin markedly inhibited the activation of neuronal c-fos and CGRP after morphine administration in the spinal cord (n = 4). The quantification of c-fos and CGRP immunofluorescence was respectively represented as number of c-fos positive cells and mean fluorescence pixels in the superficial dorsal horns. Scale bar: 75 μ m. A, B data were analyzed by two-way ANOVA; C data was analyzed by one-way ANOVA (**P < 0.01, ***P < 0.001 vs. vehicle, ###P < 0.001 vs. morphine-treated group).

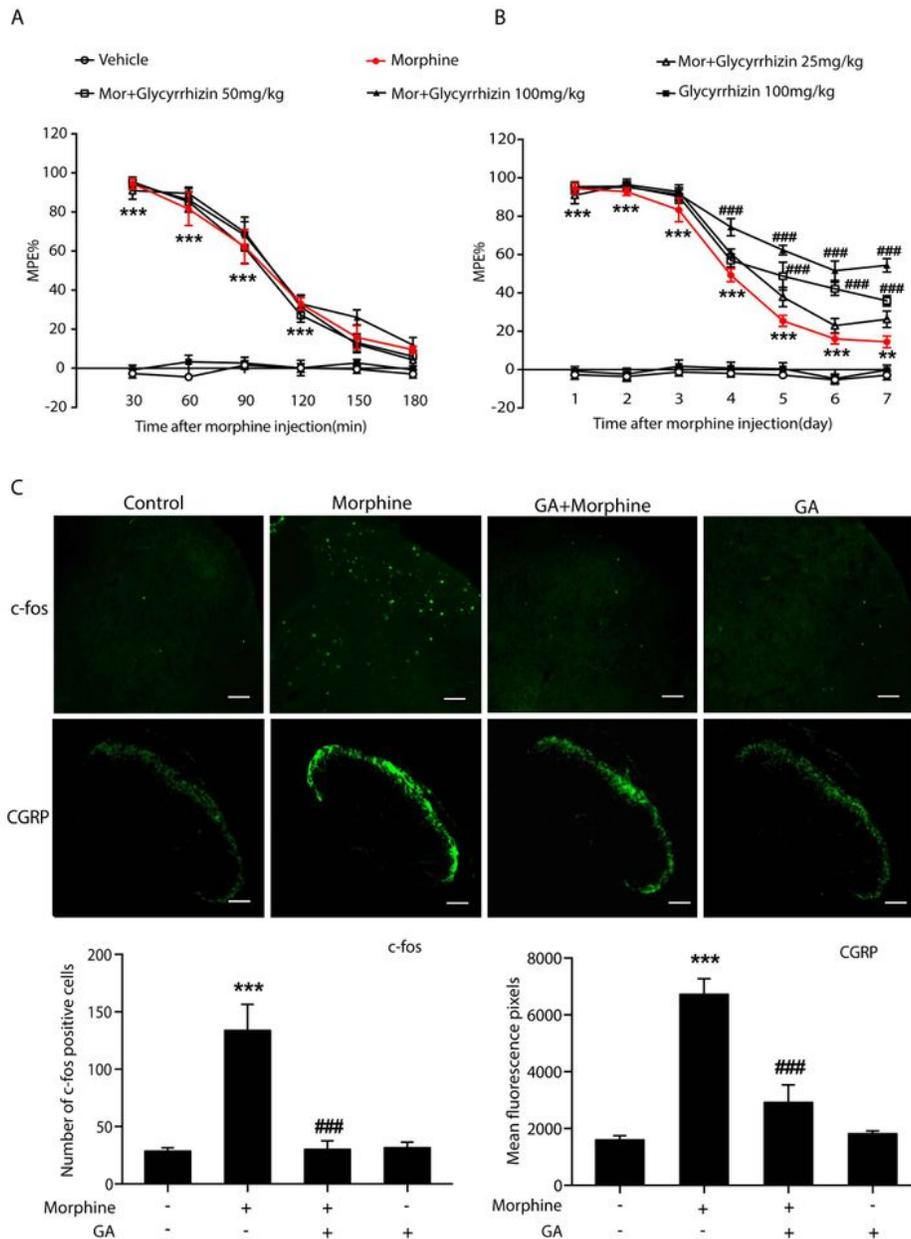


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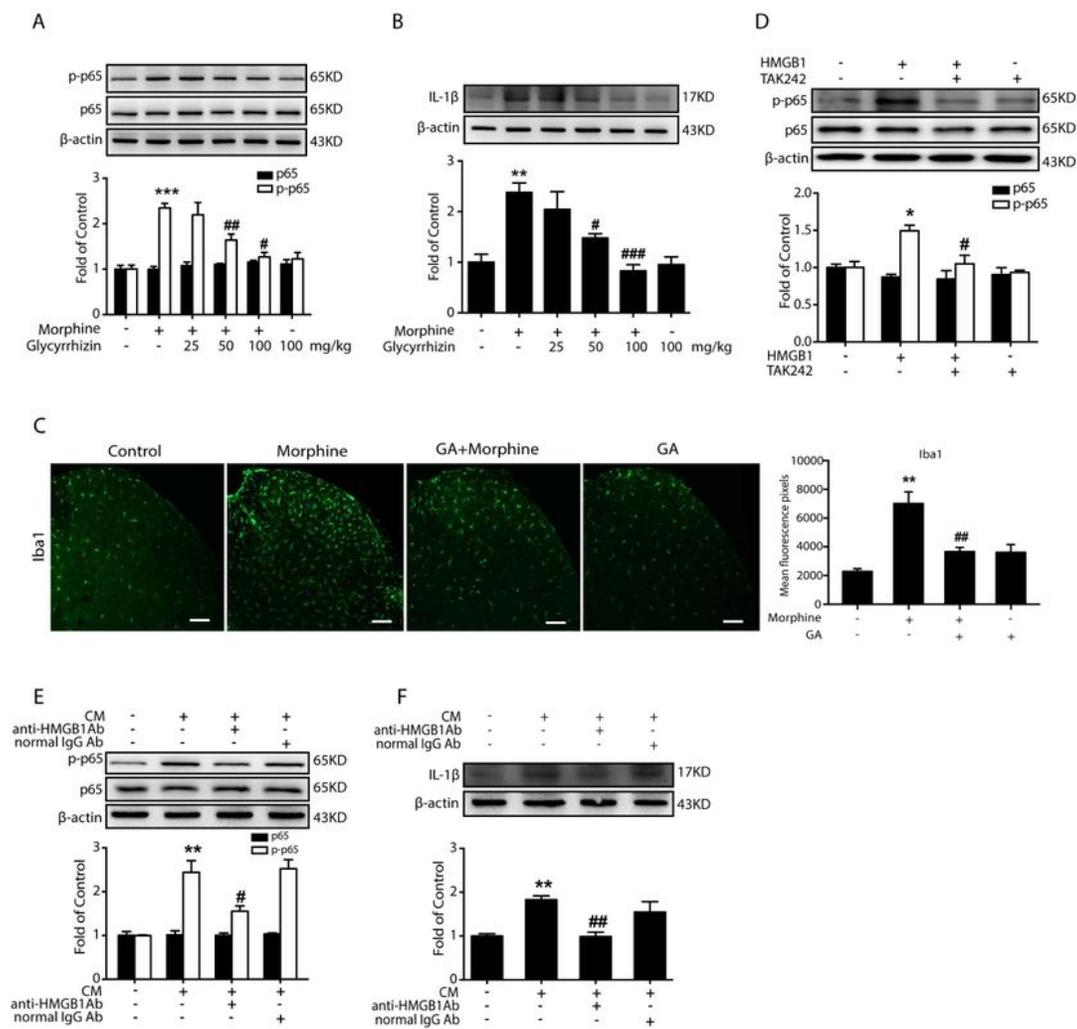


Figure 3

Extracellular HMGB1 triggers inflammatory response dependent on TLR4 in microglia. (A) Glycyrrhizin (25, 50, 100 mg/kg) inhibited the morphine-induced upregulation of p-p65 in the spinal cord (n = 4). (B) Glycyrrhizin (25, 50, 100 mg/kg) markedly decreased the level of mature IL-1 β evoked by morphine in the spinal cord (n = 4). (C) Immunofluorescence analysis showed that glycyrrhizin attenuated the activation of Iba1 evoked by morphine in the spinal cord (n = 4). (D) The level of phosphorylation of NF- κ B p65 in

response to HMGB1 under treatment of TLR4 antagonist was assessed in BV-2 cells. Cells were pretreated with TLR4 antagonist (TAK242, 10 μ M) for 15 min, followed by recombinant HMGB1 (25 nM) treatment. Then, cell extracts were collected 12 h after HMGB1 treatment and analyzed by western blot (n = 3). (E-F) BV-2 cells was incubated with conditional medium collected from morphine-treated (200 μ M, 12 h) SH-SY5Y cells for 12 h in presence of anti-HMGB1 antibody (2 μ g/mL) or normal IgG (2 μ g/mL), and then, the cell extracts were collected and analyzed by western blot (n = 3). A-C data were analyzed by one-way ANOVA (**P < 0.01, ***P < 0.001 vs. vehicle, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. morphine-treated group); D-F data were analyzed by one-way ANOVA (*P < 0.05, **P < 0.01 vs. control, #P < 0.05, ##P < 0.01 vs. HMGB1-treated group).

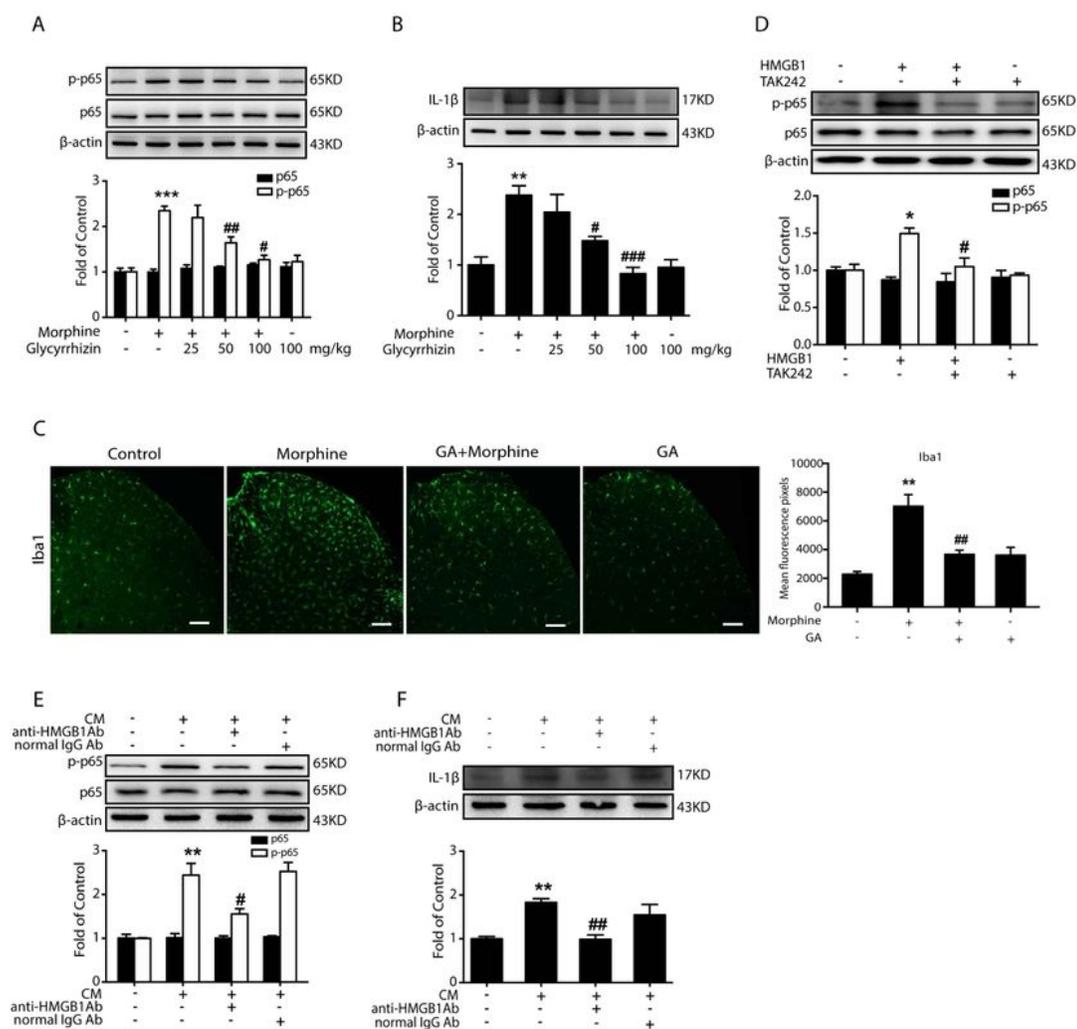


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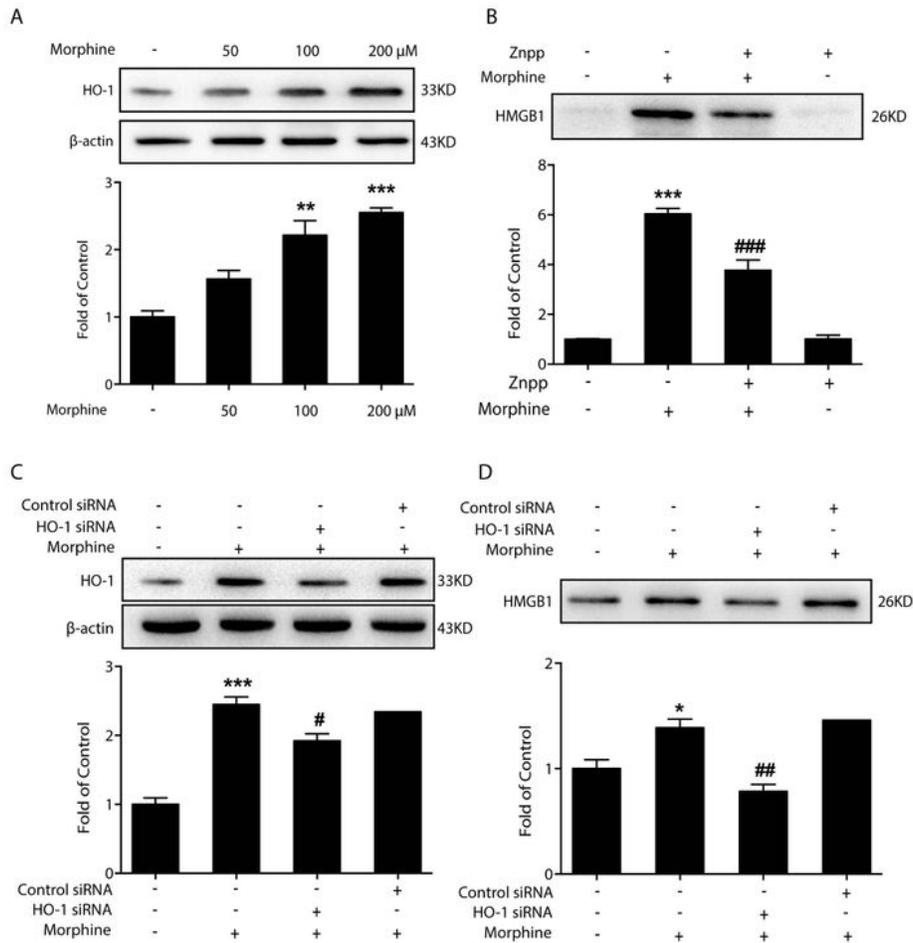


Figure 4

The release of HMGB1 induced by morphine is under the control of HO-1 (A) Morphine decreased HO-1 in a concentration-dependent manner in SH-SY5Y cells. Supernatants were collected 12 h after morphine (50, 100, 200 μ M) treatment and analyzed by western blot (n = 3). (B) Znpp decreased the level of HMGB1 induced by morphine in SH-SY5Y cells. Znpp (HO-1 inhibitor, 2 μ M) was given 12h before morphine (200 μ M, 12 h) administration. Cells were collected 12h after morphine treatment and analyzed by western blot

(n = 3). (C) HO-1 siRNA downregulated the level of HO-1 in SH-SY5Y cells. (D) HO-1 siRNA decreased the level of HMGB1 induced by morphine. Supernatants were collected 12 h after morphine (200 μ M) treatment and analyzed by western blot (n = 3). A data was analyzed by one-sample t-test (**P < 0.01, ***P < 0.001 vs. control); B-D data were analyzed by one-way ANOVA (*P < 0.5, ***P < 0.001 vs. control, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. morphine-treated group).

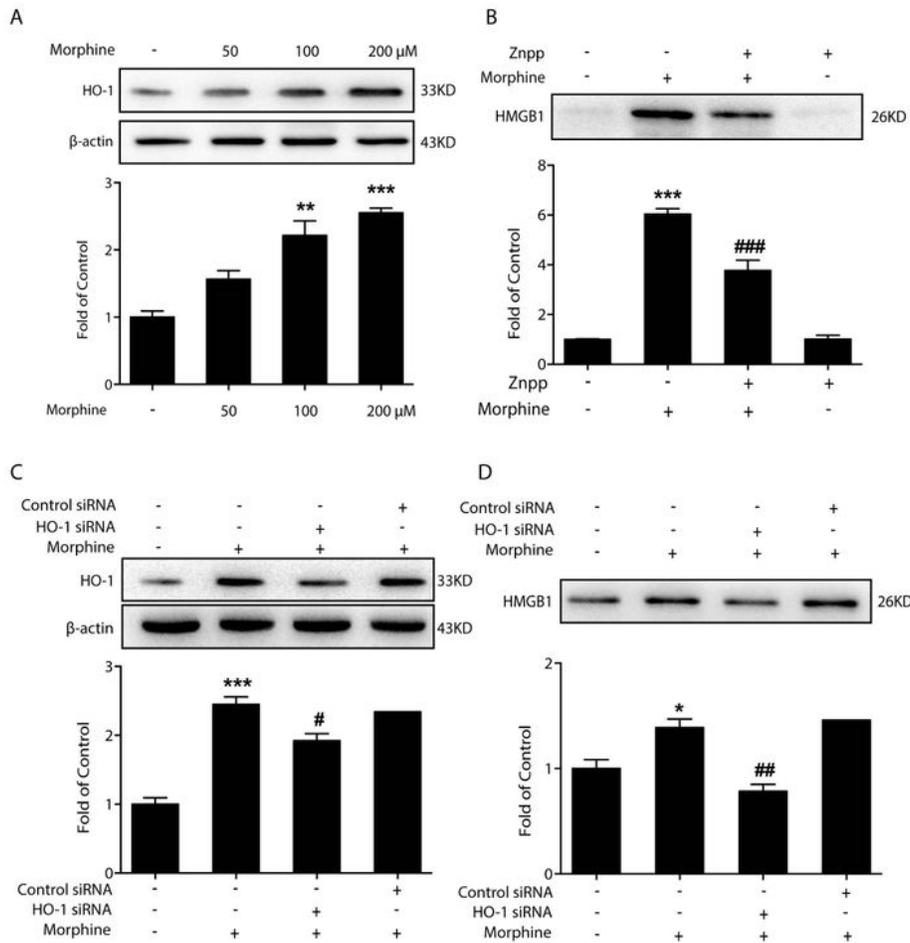


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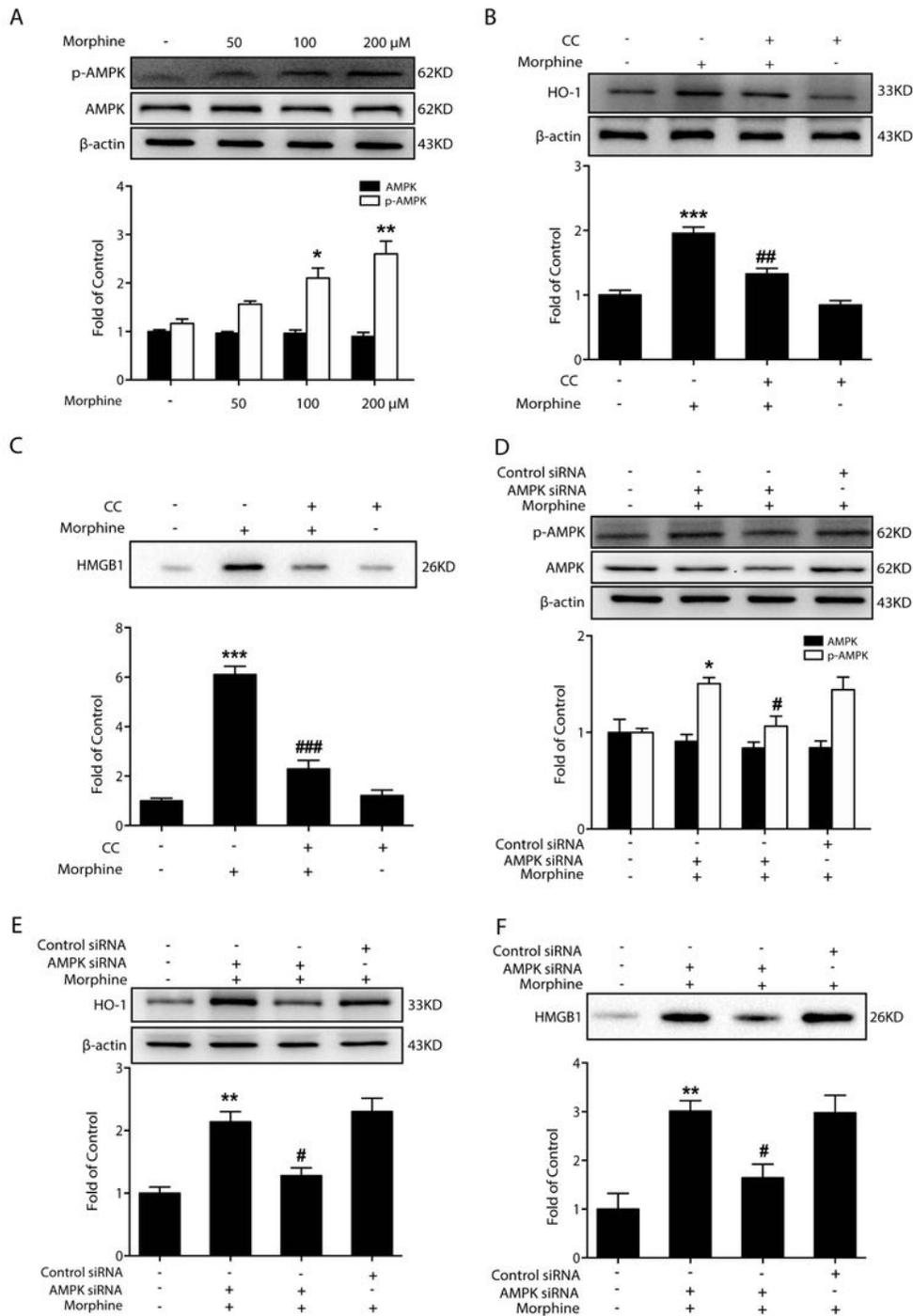


Figure 5

The phosphorylation of AMPK induced by morphine increases the release of HMGB1 through the upregulation of HO-1 (A) Morphine promoted the phosphorylation of AMPK in a concentration-dependent manner in SH-SY5Y cells. Cells were collected 12 h after morphine treatment and analyzed by western blot (n = 3). (B) Compound C decreased the level of HO-1 induced by morphine in SH-SY5Y cells. Compound C (AMPK inhibitor, 10 μ M) was given 2h before morphine (200 μ M, 12 h) administration. Cells

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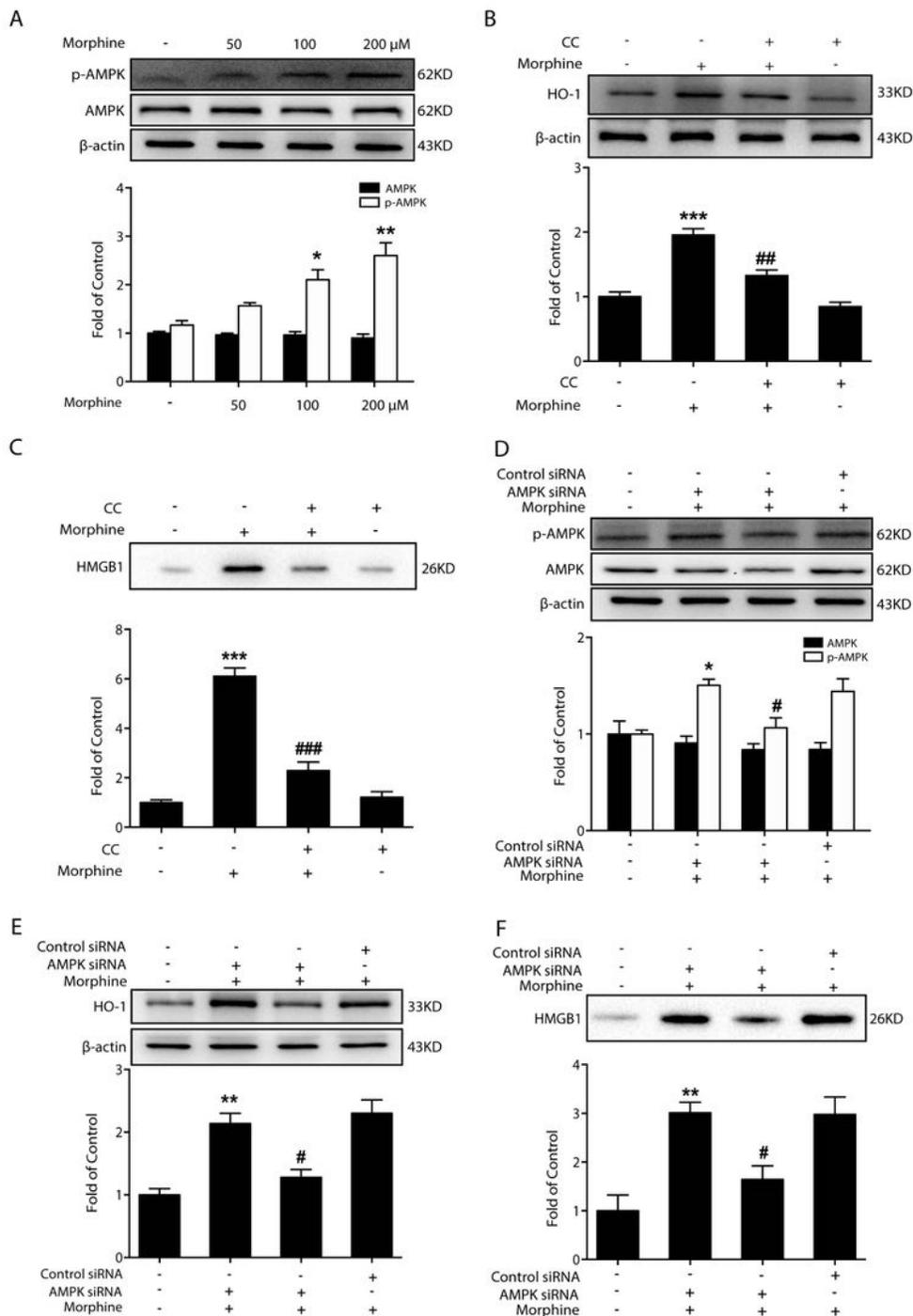


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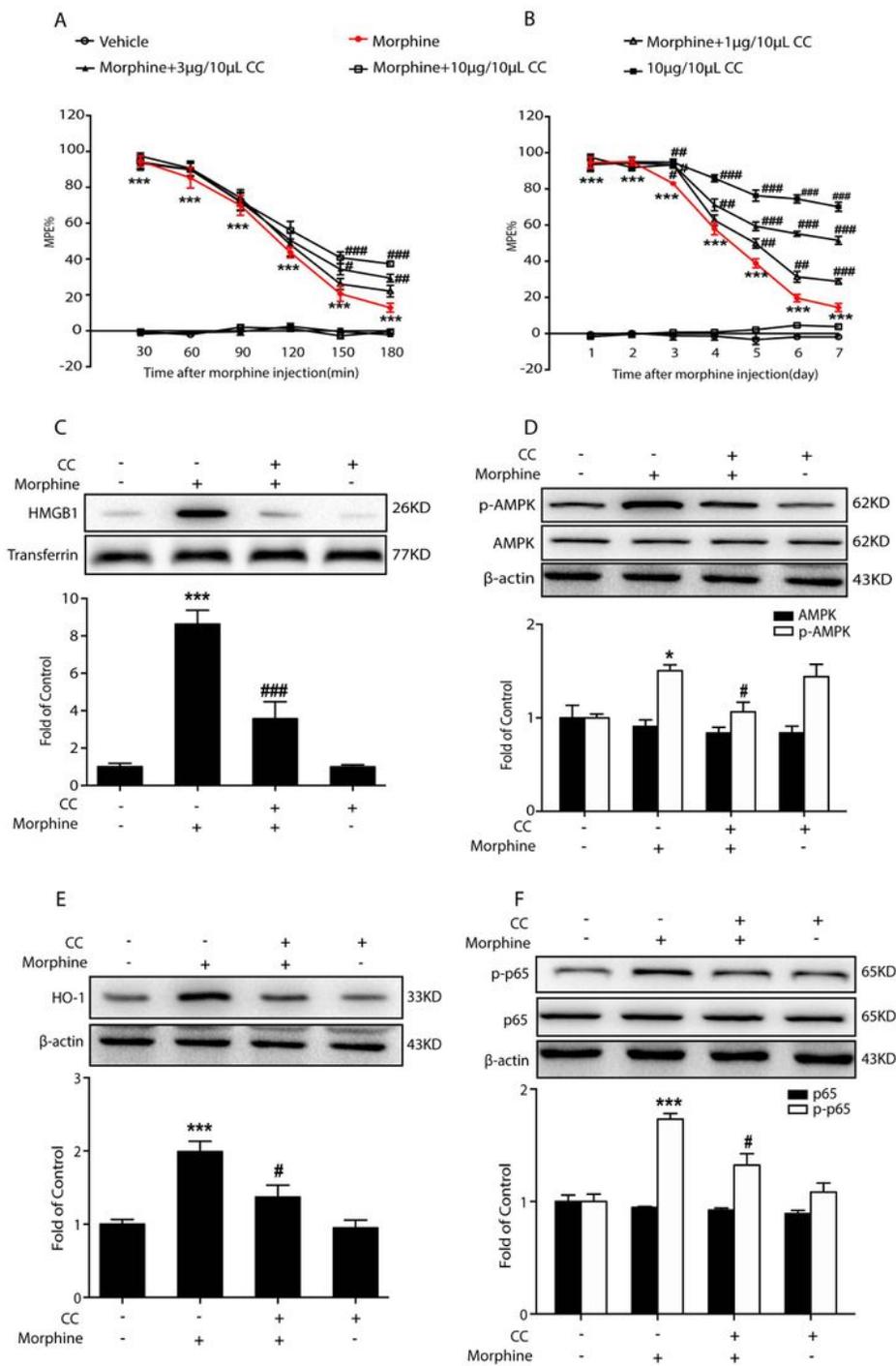


Figure 6

Compound C potentiates acute morphine analgesic effect and suppresses chronic morphine tolerance. Tail-flick method was performed to evaluate the effect of Compound C on the morphine tolerance. Data was shown as percentage of maximal possible effect (%MPE). (A) Compound C potentiated acute morphine analgesic effect. Before the treatment of morphine, mice were pre-treated with different doses of Compound C (1, 3 or 10 µg/10 µL) and MPE was measured after morphine (10 µg/10 µL).

administration at the first day. (B) Compound C improved chronic morphine tolerance in mice (n = 8). Morphine (10 µg/10 µL) was intrathecally injected with different doses of Compound C (1, 3 or 10 µg/10 µL) once daily and the MPE was measured 30 min after the first injection of each day (n = 8). (C) Consecutive administration of Compound C (10 µg/10 µL, i.t.) for 7 days inhibited the release of HMGB1 induced by morphine (10 µg/10 µL, i.t.) in CSF. CSF was collected from rats 1 h after the last administration and determined by western blot (n = 4). Transferrin was used as a loading control. (D-F) Consecutive administration of Compound C (10 µg/10 µL, i.t.) for 7 days inhibited the phosphorylation of AMPK, HO-1 and p-p65 induced by morphine (10 µg/10 µL, i.t.) in the spinal cord (n = 4). A-B data were analyzed by two-way ANOVA; C-F data were analyzed by one-way ANOVA (*P < 0.05, ***P < 0.001 vs. vehicle, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. morphine-treated group).

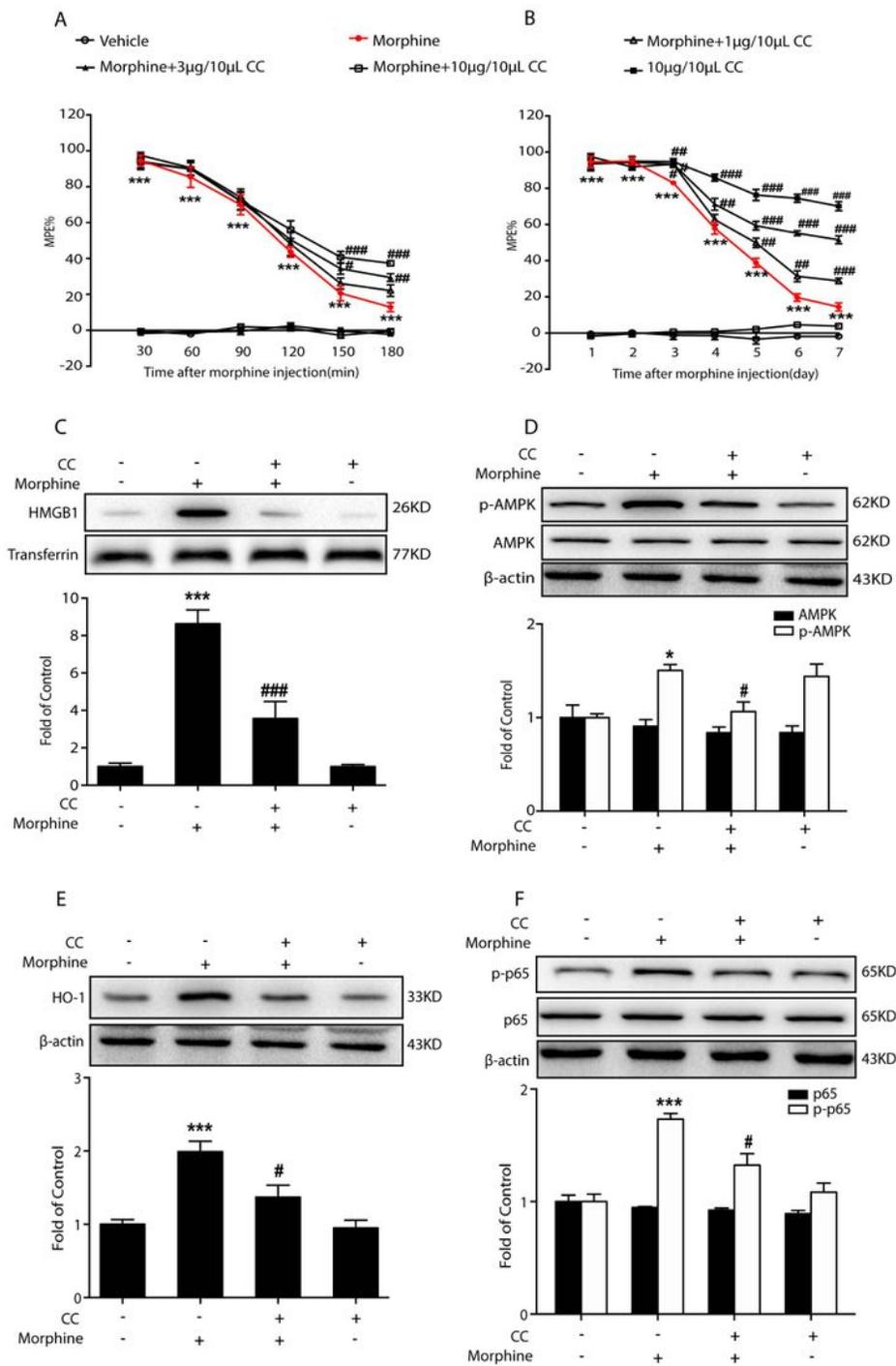


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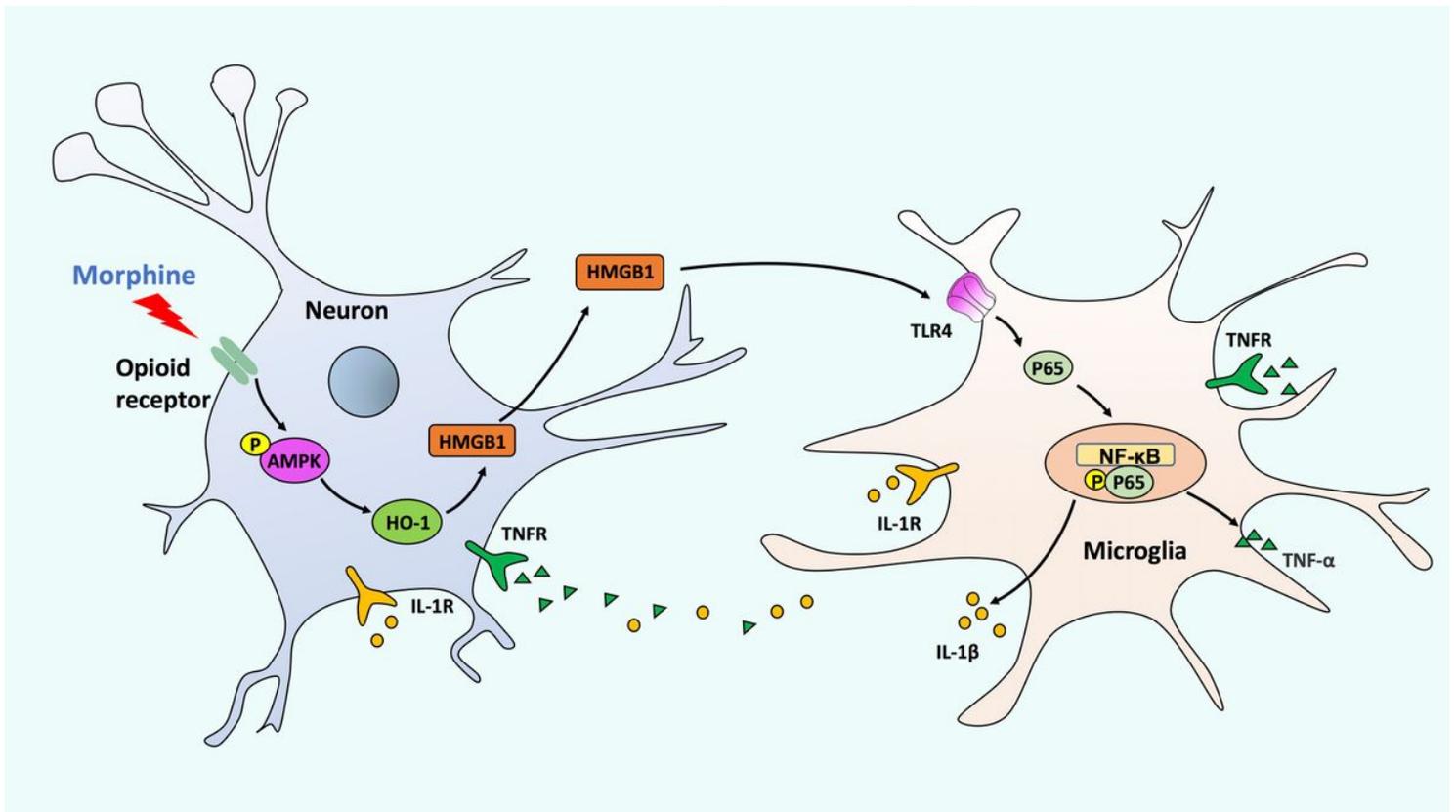


Figure 7

Schematic model indicates that suppressing HMGB1-TLR4-mediated neuroinflammation alleviates morphine tolerance via inhibiting AMPK-HO-1 pathway in the spinal cord in rodents. Morphine activates TLR4 by inducing the release of HMGB1. The secretion of HMGB1 is under the control of AMPK-HO-1 signal pathway. Extracellular HMGB1 induces the activation of microglia and upregulation of neuroinflammatory cytokine IL-1 β via TLR4 receptor. Inhibiting the release of HMGB1 induced by morphine suppresses the neuroinflammation and consequently alleviates morphine tolerance.

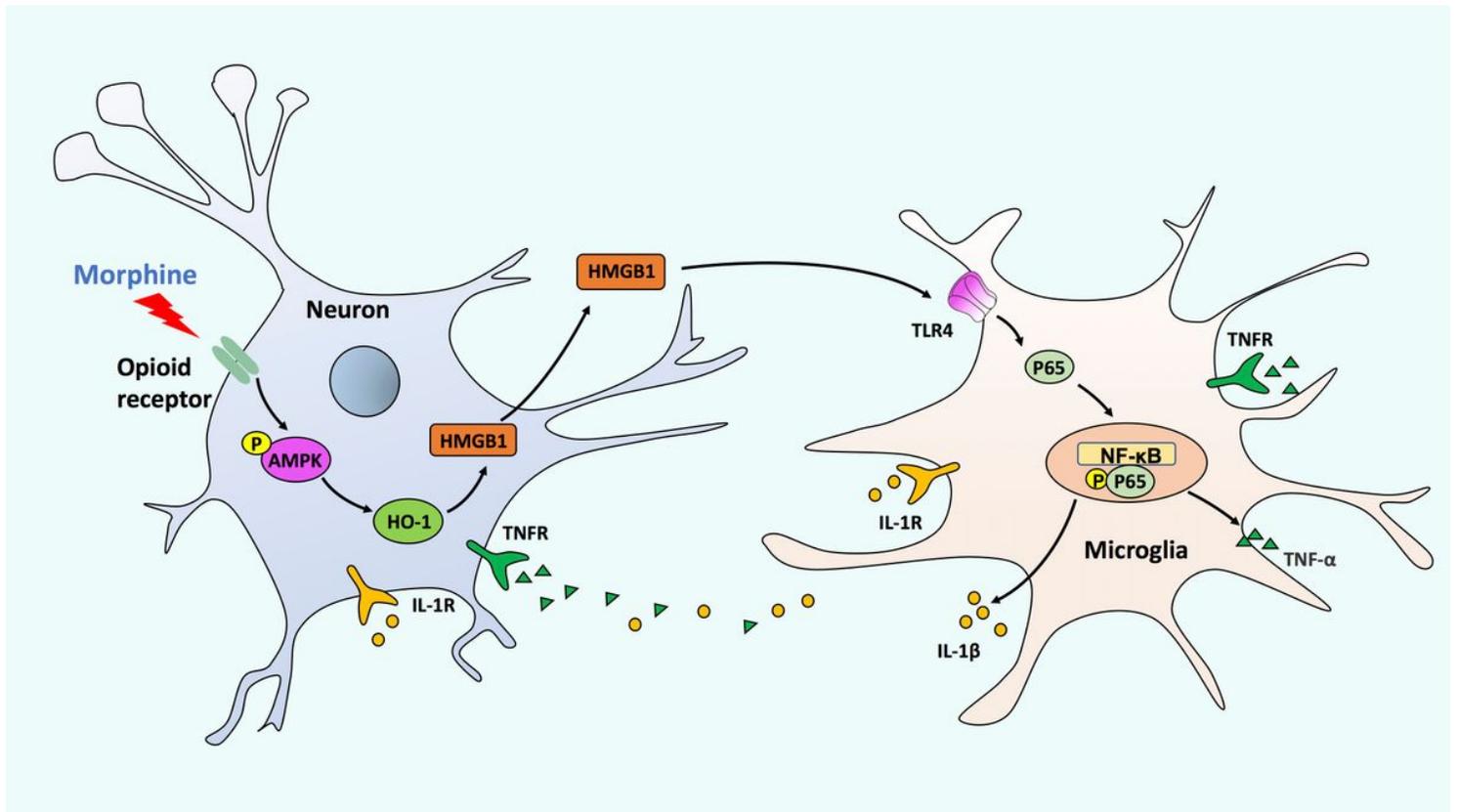


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