

# Aprepitant Inhibits JNK and p38/MAPK to Attenuate Inflammation in Microglia and Suppresses Inflammatory Pain

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## Research

**Keywords:** Aprepitant, JNK, p38, NF- $\kappa$ B, Inflammatory pain

**Posted Date:** May 5th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-445852/v1>

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**Version of Record:** A version of this preprint was published at Frontiers in Pharmacology on January 11th, 2022. See the published version at <https://doi.org/10.3389/fphar.2021.811584>.

# Abstract

**Background:** Substance P (SP) contributes to the pathogenesis of pain by acting on neurokinin-1 receptor (NK-1R), specialized sensory neurons that detect noxious stimuli. Aprepitant, an antagonist of NK-1R widely used to treat chemotherapy-induced nausea and vomiting. In this study, we assessed aprepitant's analgesic effect on inflammatory pain in mice and its mechanism of action in spinal cord.

**Methods:** The excitability of DRG neurons treatment with aprepitant was measured using whole-cell patch-clamp recordings. The inflammatory pain model was induced in adult ICR mice by a single dose intraplantar injection of formalin and carrageenan. The mice were treated with aprepitant, and the behavioral tests were measured after the intraperitoneal injection of aprepitant. The morphological changes on inflamed paw tissues were determined by using hematoxylin eosin staining. The mRNA expressions of MCP-1, TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and NF- $\kappa$ Bp65 were measured by real-time quantitative PCR analysis. Changes in the protein expression levels were assayed using enzyme linked immunosorbent assay. Western blotting and immunohistochemistry were used to assay the cell signaling.

**Results:** Aprepitant treatment showed a significantly higher AP threshold *in vitro*. *In vivo*, the aprepitant showed a significant anti-inflammatory and analgesia effect in the mice with inflammatory pain. After the administration of aprepitant, the paw tissues inflammatory damage was significantly relived. The mRNA levels of MCP-1, TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and NF- $\kappa$ Bp65 were down-regulate following aprepitant treatment. Meanwhile, aprepitant significantly suppressed over-expression of proinflammatory cytokines, as well as the phosphorylation of JNK and p38 MAPK in the spinal cord.

**Conclusions:** The present study suggests that the extract of aprepitant attenuates inflammatory pain in mice via suppressing the phosphorylation of JNK and p38, and inhibiting the NF- $\kappa$ B signaling pathway.

## Introduction

Inflammatory pain is a common chronic pain in clinical, which adversely impacts the quality of life[1, 2]. Although to relieve inflammatory pain, significant progress has been achieved in existing analgesics, such as nonsteroidal anti-inflammatory drugs (NSAIDs), opioid analgesics, the insufficient efficacy and adverse side effects limit the curative effect[1–3]. Consequently, promising analgesic agents against inflammatory pain are imminently required.

Mechanisms of inflammatory pain are complex and involve many factors. It has long been generally accepted that microglia in spinal cord plays an important role in the pathogenesis of inflammatory pain. Spinal cord microglia are persistently activated in inflammatory pain conditions[4, 5]. Upon activation, microglia secrete numerous inflammatory mediators, including chemokine and proinflammatory cytokines such as monocyte chemoattractant protein 1 (MCP-1), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [4, 6, 7]. These proinflammatory mediators can damage surrounding neurons and enhance the hyperactivity of neurons, resulting in central sensitization and enhanced pain sensitivity[6, 8, 9]. Therefore, inhibiting the inflammatory microglial response will be beneficial for

inflammatory pain therapy. Numerous studies have recently indicated that intrathecal injection of minocycline (a microglial inhibitor) significantly suppresses the behavioral and neurochemical signs of chronic pain[7], including neuropathic pain[10] and inflammatory pain[11–13]. However, minocycline, as a broad-spectrum antibiotic, its prolonged use may cause severe side effects including systemic lupus erythematosus-like syndrome and autoimmune hepatitis [14]. Therefore, a novel, potent and safe drug is urgently needed for the prevention of inflammatory pain through microglial inhibition.

Aprepitant (5-[[[(2R, 3S)-2-[(1R)-1-[3,5-bis(trifluoromethyl) phenyl] ethoxy]-3-(4-fluorophenyl)-4-morpholinyl]methyl]-1,2-dihydro-3 H-1,2,4-triazol-3-one), a highly selective and non-peptide neurokinin-1 receptor (NK-1R) antagonist, was approved for the prevention of chemotherapy-induced nausea and vomiting by the U.S. Food and Drug Administration. Aprepitant is safe and well tolerated for currently used in clinical practice. Furthermore, it has been reported that aprepitant plays specific anti-inflammatory roles in HIV infection[15], osteoarthritis pain[16], rheumatoid arthritis[17], prurigo nodularis[18]. This drug exerts anti-inflammatory, analgesic, antiviral, antiemetic, antioruritic and antitumor effects[19, 20].

A previous study has reported that aprepitant could act as an anti-inflammatory agent and attenuate lipopolysaccharide (LPS)-induced inflammatory responses in macrophages[21]. However, there is currently no research on the role of aprepitant in neuroinflammation and inflammatory pain. Therefore, we hypothesized that aprepitant might provide benefit for treatment of inflammatory pain, and interfere with LPS-stimulated microglia activation, as well as the production of inflammatory cytokines in microglia. Hence, this research provides a novel view of the role of aprepitant in anti-inflammatory responses, which in turn provides a potential therapeutic approach for neuroinflammation and inflammatory pain.

## Materials And Methods

### Animals

Adult male ICR mice (weighing 18-22 g) were obtained from the Qinglongshan Animal Center of Nanjing, and kept in an animal care facility with controlled temperature, mild humidity, a 12 h natural light-dark cycle, and free access to fresh food and water. Every effort was made to minimize the suffering and number of animals used during the experiments. All experimental procedures were performed in accordance with the guidelines for laboratory animal care of the Nanjing Medical University and China Pharmaceutical University.

### Cell culture and viability assay

Primary murine microglia BV-2 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM, Hyclone/Thermo, Rockford, IL, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, NY, USA), penicillin and streptomycin (Hyclone/Thermo) in 5% CO<sub>2</sub> at 37°C. To assess cell viability, the BV2 cells (5 × 10<sup>4</sup> cells/mL) were seeded in 96-well plates. After treatment for 24h, 10 μL of MTT solution (5 mg/ml,

Sigma-Aldrich, Germany) was added to each well and the cells were incubated for 4 h. The supernatant was removed and 150 µl of dimethylsulfoxide (DMSO, Sigma-Aldrich) was added to dissolve formazan crystals in each well. After 30 min of incubation, absorbance levels for formazan at 490 nm were measured using a microplate reader (Bio-Rad Inc., Hercules, CA, USA).

### **Mice lumbar dorsal root ganglion cell culture preparation**

Primary DRG neurons were isolated from male ICR mice lumbar DRGs, as previously described. Briefly, DRG neurons were quickly removed and placed into ice-cold DMEM, then digested in collagenase (1 mg/ml, type II; Worthington, USA), and 0.25% trypsin, followed by addition of 0.25% trypsin inhibitor. Then the DRG neurons were triturated with Pasteur pipettes and centrifuged through 15% BSA. The cell pellet was resuspended in 1 mL of Neurobasal media. Isolated neurons were plated onto poly-D-lysine (100 g/mL) and laminin (1 mg/mL)-coated 35-mm tissue culture dishes containing B27 supplement (Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin, in the incubator at 37°C with 5% carbon dioxide.

### **Preparation of DRG neurons**

DRG were isolated from spine and then were enzymolyzed in normal Tyrode solution containing 1 mg/ml collagenase II (Worthington, USA) for 60 min at 37 °C. DRG were plated onto poly-D-lysine coated glass coverslips. Single cells were grown in DMEM (high glucose) supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg /ml streptomycin. Neurons were cultured at 37 °C in 5% CO<sub>2</sub> overnight. Cells were used for patch clamp recordings at 24 h post dissociation.

### **Electrophysiology**

The excitability of DRG neurons were measured using whole-cell patch-clamp recordings. Small DRG neurons (<25µm) were chosen in our study. Voltages were recorded by using a HEKA EPC 10 amplifier (HEKA Instruments, Germany). The data were obtained and analyzed by Pulse software (HEKA Instruments, Germany). Signals were filtered at 3 KHz and sampled at 10 KHz. Electrodes were drawn from borosilicate capillary glass (Institute of Biophysics, Chinese Academy of Sciences, China) using a P-97 puller (Sutter Instrument, USA). Electrodes had a resistance of 2–5 MW when filled with intracellular solution. The pipette solution contained (in mM) KCl 140, MgCl<sub>2</sub> 1, EGTA 5, Na<sub>2</sub>ATP 2, and HEPES 5 (pH =7.2, adjusted with KOH). The external solution contained (in mM): NaCl 137, MgCl<sub>2</sub> 1.2, KCl 5.4, NaH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 1, HEPES 10, and glucose 10 (pH =7.4, adjusted with NaOH). The action potentials (Aps) was recorded under the current-clamp mode. APs were evoked by 1 s depolarizing current pulses from -10 up to 100 pA at 5 pA steps. The minimum current that triggered the first AP was defined as rheobase. To observe the excitability, DRG neuron was stimulated by 2×rheobase.

### **Formalin Test**

20 microliter of saline or 2% formalin (in saline) were subcutaneously injected into the hind paw. Mice were randomly divided into 5 experimental groups: control (Ctrl), formalin, formalin + 5 mg/kg aprepitant,

formalin + 10 mg/kg aprepitant, formalin + 20 mg/kg aprepitant. Mice were intraperitoneal injected aprepitant, followed by a subcutaneous injection of 2% formalin. Mice were monitored for time spent licking paw, and number of lick bouts, for 60 min post-injection by researchers blinded to experimental condition. All sessions were video-recorded. The time spent paw licking and biting was calculated in 5-minute and recorded for 60 min. Additionally, acute (0-5 min) and inflammatory (15-45 min) phase pain responses were quantified.

### **Carrageenan-induced inflammatory pain**

To establish the inflammatory pain model, 1% carrageenan (25  $\mu$ L) was injected into the hind paw. Mice were allocated to following groups: control, carrageenan, carrageenan + 5 mg/kg aprepitant, carrageenan + 10 mg/kg aprepitant, carrageenan + 20 mg/kg aprepitant. Aprepitant was intraperitoneal injected 30 minutes before injection of 25  $\mu$ L of carrageenan.

### **Hot-plate test**

The hot plate test was done at  $55.0 \pm 0.1^\circ\text{C}$ . Mice were placed separately on the heated surface, and the latency between placement and the first withdrawal response was recorded. A cut-off time of 25 s was set to avoid tissue damage. All testing was conducted blindly with respect to group assignment.

### **Mechanical allodynia**

Mechanical allodynia was indicated by von Frey filaments ranging from 0.02 to 6 g. After 30 min of adaptation, filaments were used to stimulate the plantar surface of each hindpaw. The filaments were applied to six designated loci distributed over the plantar surface of the hindpaw. The minimal value that induced licking, paw withdrawal, and trembling was recorded as the threshold of mechanical withdrawal.

### **RNA extraction and real-time PCR**

Under deep anesthesia, the L4-L5 spinal cord segments of mice were quickly removed and analyzed. Total RNA was extracted from tissues or cells with TRIzol reagent and reversely transcribed using a reverse transcription kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. The subsequent real-time PCR was performed with the SYBR® Green qPCR Kit (Vazyme Biotech Co., Ltd. Nanjing, China). Differential expression was calculated according to the  $2^{-\Delta\Delta\text{CT}}$  method and statistically evaluated.

MCP-1, The forward primer 5'-CTT CTG GGC CTG CTG TTC A-3' and reverse primer of 5'-CAG CCT ACT CAT TGG GAT CA-3'.

TNF- $\alpha$ , The forward primer 5'-CAT CTT CTC AAA ATT CGA GTG ACA A-3' and reverse primer of 5'-TGG GAG TAG ACA AGG TAC AAC CC-3'.

IL-6, The forward primer 5'-CCC TAC TTC ACA AGT CCG GAG AGG AGA-3' and reverse primer of 5'-GGT AGC ATC CAT CAT TTC TTT GTA TCT CT-3'.

IL-1 $\beta$ , The forward primer of 5'-CCT GTG TCT TTC CCG TGG ACC TTC CAG G-3' and reverse primer of 5'-CAT CAT CCC ATG AGT CAC AGA GGA TGG G-3'.

NF- $\kappa$ Bp65, The forward primer of 5'-TGA TGG TGC TGA GGG ATG CTG-3' and reverse primer of 5'-ATT GCT GTG CCT ACC CGA AAC-3'.

GAPDH, The forward primer of 5'-ACC ACA GTC CAT GCC ATC AC-3' and reverse primer of 5'-TCC ACC ACC CTG TTG CTG TA-3'.

## **Western blotting**

Total proteins were isolated from the spinal cord at L4-L5 segments or the cell lines. Protein lysates were separated by SDS PAGE gels and then transferred to PVDF membrane. The membrane was incubated in 5% nonfat milk or 3% bovine serum albumin and then blotted with specific primary antibody. After incubation in the horseradish peroxidase-conjugated secondary antibody, the membrane was detected with chemiluminescence western blot detection system (Bio-Rad Laboratories, CA, USA).

## **ELISA**

The spinal cord at L4-L5 segments and the paw tissue samples were quickly removed from deeply anesthetized mice. Cell supernatants were collected after treatment with reagents. TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and MCP-1 were analyzed by enzyme linked immunosorbent assay (ELISA) kits (Neobioscience, Shenzheng, China) following the manufacturer's instructions. Concentrations were determined by measuring absorbance at 450 nm (Bio-Rad Inc., Hercules, CA, USA).

## **Immunohistochemistry and inflammation scoring**

Paw tissues were quickly excised, and then fixed in 10% formalin at 4°C. Briefly, sections were cut using a microtome, stained with HE, and visualized by using a light microscope. Immunohistochemistry was performed using immunohistochemistry kit (Key-GEN, Nanjing, China). The degree of inflammation was quantified using a 0 to 5 scoring system. The scores were defined as follows: 0 = no inflammation, 1 = mild inflammation, 2 = mild/moderate inflammation, 3 = moderate inflammation, 4 = moderate/severe inflammation and 5 = severe inflammation.

## **Statistical analysis**

The results were analyzed by GraphPad Prism and expressed as means  $\pm$  SEM. Alteration of expression of the proteins or mRNA detected and the behavioral responses to mechanical stimuli over time among groups were tested with 1-way and 2-way ANOVA followed by Bonferroni post hoc tests, respectively. Statistical results are considered significant if  $p < 0.05$ .

# **Results**

## **Aprepitant inhibits formalin-induced nociceptor-like cultured dorsal root ganglion neurons and suppresses pain behavior when injected intraplantarly in vivo**

Substance P has been shown to participate in the inflammatory process[22, 23], and NK-1R is expressed in DRG neurons[24]. Therefore, we thought to determine whether aprepitant, one of the NK-1R inhibitors, modulated the activity of dissociated nociceptive neurons (about 25  $\mu\text{m}$  diameter) *in vitro*. To investigate the effect of aprepitant on the excitability of DRG neurons, we observed the characteristics of APs. As shown in Fig. 1A, aprepitant treatment showed a significantly higher AP threshold compared with control group. There were no significant differences in resting membrane potential, rheobase, and overshoot. These results demonstrated that aprepitant could decrease the excitability of DRG neurons. Formalin injection into the mouse's hind paw produces a reliable and widely used model of inflammatory pain when tested with analgesic drugs. Intraplantar injection of formalin induces distinct acute (0–5 min) and long-term inflammatory (15–45 min) phases in nocifensive behavior, while responses to a hotplate or von Frey filaments are acute and transient. We discovered that aprepitant treatment attenuated the pain behavior (Fig. 1B–D,  $p < 0.01$ ) of inflammatory paw licking/biting after injection of aprepitant. Conversely, aprepitant had no effect on the acute phase response to formalin injection (Fig. 1E,  $p > 0.05$ ). In addition, aprepitant alleviated the response to thermal (Fig. 1F,  $p < 0.05$ ) or mechanical (Fig. 1G,  $p < 0.01$ ) pain.

## **Aprepitant relieved formalin-induced inflammation in vivo**

To investigate the effect of aprepitant on proinflammatory cytokines, we measured the levels of MCP-1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 following formalin injection in mice. Administration of aprepitant (10 mg/kg, and 20 mg/kg) significantly inhibited the formalin-induced expression of MCP-1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 in the inflamed paw (Fig. 2A,  $p < 0.05$ ). Histopathological examination showed that the control group fed saline had normal paw tissue (Fig. 2BI). In contrast, compared to the control group, the left hind paws of mice that received formalin injections showed massive accumulation of infiltrated cells (Fig. 2BII). However, treatment with aprepitant (10 mg/kg, and 20 mg/kg) remarkably decreased inflammatory cell infiltration (Fig. 2BIII–V). The degree of inflammation was evaluated by scoring of inflammation from 0 to 5. The inflammation scores indicated that co-treatment with aprepitant (10 mg/kg, and 20 mg/kg) significantly reduced formalin-induced inflammation (Fig. 2C,  $p < 0.05$ ). To further explore the anti-inflammatory effects of aprepitant, the levels of MCP-1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 in spinal cord were evaluated using ELISA and qRT-PCR. Compared with the control group, the various cytokines, MCP-1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 protein and mRNA expressions were significantly increased in the formalin group (Fig. 2D and E,  $p < 0.05$ ). However, aprepitant reduced the expressions of these cytokines (Fig. 2D and E,  $p < 0.05$ ).

## **Aprepitant suppresses LPS-induced microglia activation and inflammation in BV-2 microglial cells**

It has been indicated that activation of microglia is crucial for the inflammatory stimuli, and involved in pain facilitation[5, 8, 25]. To investigate the effects of aprepitant on LPS-induced microglia activation *in vitro*, we used the immortalized murine microglial cell line BV-2, which were derived from primary mouse microglial cells. We investigated the effects of aprepitant on the viability of BV-2 microglia. Aprepitant showed little cytotoxic effect at concentration ranging from 1 to 8  $\mu\text{M}$  (Fig. 3A,  $p > 0.05$ ). Subsequently,

we studied the effect of aprepitant on LPS-induced microglia activation in BV-2 cells. BV-2 cells were co-cultured with LPS (1  $\mu\text{g/ml}$ ) and various concentrations of aprepitant for 16 h. Pretreatment with aprepitant significantly suppressed LPS-induced over-expression of IBA-1 (Fig. 3B,  $p < 0.01$ ), in BV-2 microglia. We investigated the effect of aprepitant on the LPS-caused expression and secretion of proinflammatory cytokines by BV-2 microglia. In contrast with treatment with LPS alone, aprepitant significantly suppressed LPS-induced MCP-1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 mRNA expression in BV-2 microglial cells (Fig. 3C,  $p < 0.01$ ). In addition, aprepitant decreased the LPS-induced secretion of MCP-1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 in BV-2 (Fig. 3D,  $p < 0.01$ ). However, aprepitant (2  $\mu\text{M}$ ) alone showed no marked effects on the mRNA and protein expression of MCP-1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 in BV-2 cells (Fig. 3C and D,  $p > 0.05$ ). These results demonstrate that aprepitant inhibits LPS-induced neuroinflammation.

### **Aprepitant inhibits LPS-induced p38 and JNK phosphorylation, but not ERK1/2 phosphorylation in BV2 Microglia**

To clarify the mechanisms underlying the anti-inflammatory effects of aprepitant, we assessed the effects of aprepitant on LPS-induced phosphorylation of MAPK in BV-2 cells. The results revealed that aprepitant significantly suppressed LPS-induced up-regulated phosphorylation of p38 mitogen-activated protein kinase (p38) and c-Jun NH2 terminal protein kinase (JNK) (Fig. 4A,  $p < 0.05$ ), in BV-2 microglia. However, we did not confirm that treatment with aprepitant reduces LPS-induced up-regulated phosphorylation of extracellular signal-regulated kinase-1/2 (ERK1/2) in BV-2 (Fig. 4A,  $p > 0.05$ ). Furthermore, LPS treatment induced the translocation from the cytoplasm to the nucleus (Fig. 4B) and increased of nuclear factor  $\kappa\text{B}$  p65 (NF- $\kappa\text{B}$ p65) mRNA expression (Fig. 4C,  $p < 0.05$ ), compared with the LPS-treated group, co-incubation with aprepitant significantly reduced the effect (Fig. 4B and C,  $p < 0.01$ ).

### **The JNK, p38/MAPK and NF- $\kappa\text{B}$ p65 signal pathways involved in the analgesic mechanisms of aprepitant**

To determine whether the analgesia effects of aprepitant on mouse paw injected with formalin were associated with inhibition of the MAPK and NF- $\kappa\text{B}$ p65 signaling pathway, we evaluated phosphorylation of ERK1/2, JNK, p38 MAPK and the expression of NF- $\kappa\text{B}$ p65 in mouse spinal cord. Compared to the control group, the phosphorylation of ERK1/2, JNK, p38 MAPK and the activation of microglia in the formalin group were significantly increased (Fig. 5A,  $p < 0.05$ ). Aprepitant effectively inhibited the formalin-induced phosphorylation of JNK, p38 MAPK and over-activation of microglia (Fig. 5A-C,  $p < 0.05$ ). Simultaneously, the administration of aprepitant suppressed the mRNA expression of NF- $\kappa\text{B}$ p65 (Fig. 5D,  $p < 0.01$ ).

We also evaluated the effects of aprepitant on pain behavior, phosphorylation of p38 MAPK and the mRNA expression of NF- $\kappa\text{B}$ p65 in mice with carrageenan-induced inflammatory pain (Fig. 6). As shown in Fig. 6A, the pain behavioral test demonstrated that treatment with aprepitant via the intrathecal (2 nmol, i.t.) and intraperitoneal (i.p.) route remarkably alleviated the mechanical withdrawal threshold induced by carrageenan ( $p < 0.05$ ). To verify the suppressive effect of aprepitant on mechanical allodynia, we evaluated whether the p38 inhibitor (SB203580) and JNK inhibitor (SP600125) showed similar effects to that of aprepitant in carrageenan-induced inflammatory pain. The results showed that SB203580 (10

nmol, i.t.) and SP600125 (5  $\mu$ g, i.t.) reduced carrageenan-induced decreased of mechanical withdrawal threshold (Fig. 6A,  $p < 0.01$ ). Aprepitant markedly reduced phosphorylation of JNK and p38 MAPK and the mRNA expression of NF-kBp65 in the spinal cord in mice with carrageenan-induced inflammatory pain (Fig. 6B-D,  $p < 0.01$ ). In addition, the administration of aprepitant suppressed the expression of pro-inflammatory cytokines induced by carrageenan in the spinal cord (Fig. 6E,  $p < 0.05$ ).

## Discussion

In the present study, we found that aprepitant suppressed formalin-induced nociceptor-like cultured dorsal root ganglion neurons and alleviated formalin-induced pain behavior. Furthermore, aprepitant application suppressed the LPS-induced inflammatory responses and proinflammatory cytokines by inhibiting p38/MAPK and NF-kBp65 activation in BV-2 microglial cells. Simultaneously, the administration of aprepitant inhibited microglial activation and proinflammatory cytokines in mice with inflammatory pain. Therefore, aprepitant is a potential therapeutic strategy to alleviate the neuroinflammation and inflammatory pain mediated by overactivation of microglia. Model of the mechanism underlying inflammatory pain and aprepitant-induced inhibition on inflammatory pain is illustrated in Fig. 7.

Inflammatory pain refers to pain associated with tissue damage and inflammation of any cause, such as trauma, infection, heat, and so on. The most common clinical treatments for inflammatory pain include NSAIDs and opioids[1, 2]. Although these drugs are therapeutically effective at some time, they are associated with a number of side effects, such as liver damage, kidney damage and severe gastrointestinal reactions[2, 26]. Moreover, treatment of pain is often a long-term and complex process. Aprepitant is widely used for the prevention of chemotherapy-induced nausea and vomiting in clinic. Evidence revealed that, in the electrical hyperalgesia model in human, aprepitant has no therapeutical effects on measures of central sensitization[27]. However, a growing number of studies indicated that aprepitant has analgesic, anti-inflammatory effects[16, 19, 20]. It is a controversial issue, meanwhile, there is little research on the influences of aprepitant in inflammatory pain. Thus, we studied the effects of aprepitant on inflammatory pain, and explored the underlying mechanisms.

The data we presented from dissociated DRG neurons show that application of aprepitant leads to inhibition of the neuronal excitatory effects. Using the mice model with formalin-induced inflammatory pain, we found that the time of licking/biting was significantly decreased in formalin mice with aprepitant administration compared with the mice with formalin application alone. In addition, the threshold of mechanical allodynia, was markedly reduced in formalin mice, but increased with aprepitant administration, indicating that aprepitant treatment relieves pain. We observed that mice treated with formalin had high level of IL-1 $\beta$ , IL-6, and TNF $\alpha$  in the injected paw, while after aprepitant administration, the levels of them were down-regulated. Based on the potent anti-inflammatory effects of NK-1R antagonist, aprepitant, on peripheral inflammatory conditions[28, 29], and its remarkable ability to inhibit mechanical allodynia, it is reasonable to believe that aprepitant may also alleviate inflammatory pain through reduction of central inflammation. Then, we used the hotplate test, which is a useful tool for screening analgesic drugs that producing central effects, to explore this hypothesis. The data showed

that the latency to paw withdrawal from hotplate markedly reduced in formalin mice, but increased with aprepitant treatment. Studies have shown that central changes in inflammatory pain are key contributors to the initiation and maintenance of chronic pain.

Microglia is an important component that plays a critical role in the initiation, maintenance, and resolution of chronic pain in the central nervous system[4, 5, 25]. Microglia activation is initiated in response to various extracellular stimuli such as LPS and inflammatory cytokines[30]. Activated microglia cells release abundant inflammatory mediators, including IL-1 $\beta$ , IL-6, TNF $\alpha$ , and MCP-1, which can activate nociceptors directly or increase the excitability of neurons[25, 31, 32]. Neuroinflammation mediated by microglia involves in the pathogenesis of inflammatory pain[8, 25, 33]. We observed that mice treated with formalin had high level of IL-1 $\beta$ , IL-6, TNF $\alpha$ , and MCP-1 in the injected paw and in the spinal cord, while after aprepitant administration, the levels of IL-1 $\beta$ , IL-6, TNF $\alpha$ , and MCP-1 were down-regulated. We also observed the local inflammatory cell infiltration of the hind paw following formalin injection by histopathological examination, the results showed that treatment with aprepitant remarkably decreased formalin-induced inflammatory cell infiltration.

We then explored the mechanisms underlying the analgesic effect of aprepitant and the suppressive effect of aprepitant in microglial activation. As all known, exposure to LPS led to the activation of BV-2 microglial cells and induced inflammatory responses[34–36]. In this study, aprepitant suppressed the LPS-induced expression of IBA1. Consistently, aprepitant also inhibited LPS-induced production and expression of IL-1 $\beta$ , IL-6, TNF $\alpha$ , and MCP-1 in BV-2. These results demonstrate that aprepitant inhibited LPS-induced activation of microglia and neuroinflammation in BV-2 microglial cells. MAPK signaling pathway is activated to induce inflammation and the expression of proinflammatory mediators[8, 37]. The MAPK family consists of p38 mitogen-activated protein kinase (p38), c-Jun NH2 terminal protein kinase (JNK), extracellular signal-regulated kinase-1/2 (ERK1/2) and ERK5[5], participates in chronic pain. Interestingly, MAPK subtype activation has a cell-type specific distribution in the spinal cord of rodents with chronic pain. Microglial p38 MAPK is primarily and dramatically activated in the spinal dorsal horn[38]. In addition, SB203580, a specific p38 MAPK inhibitor, significantly attenuates pain behaviors and proinflammatory cytokines synthesis[38–40]. Meanwhile, JNK MAPK plays an important role in inflammatory response in microglia[41, 42]. It has been reported that JNK inhibitor can significantly alleviate persistent inflammatory pain and microglial activation[42–44]. We found that aprepitant treatment alone did not influence the phosphorylation of JNK and p38 MAPK in BV-2 cells, but remarkably down-regulated the LPS-induced increased phosphorylation of JNK and p38 MAPK. These data suggest that the p38 MAPK is involved in the effects of aprepitant. Many studies have proved that the activation of p38 MAPK can initiate the NF- $\kappa$ Bp65 signaling. Once activated, NF- $\kappa$ Bp65 translocates into the nucleus from cytoplasm to trigger the transcription of various mediators, which are involved in proinflammatory responses[40, 45, 46]. In addition, the inhibition of NF- $\kappa$ Bp65 can reverse the production and expression of pro-inflammatory mediators following carrageenan or LPS treatment[47, 48]. These data suggest that the activation of NF- $\kappa$ Bp65 may play an important role in the expressions of proinflammatory cytokine in inflammatory pain. Thus, based on our results demonstrating that aprepitant inhibits LPS-induced JNK and p38 MAPK phosphorylation in BV-2, we further explored the effect of

aprepitant on NF- $\kappa$ Bp65 activation. Our results revealed that aprepitant effectively reversed the LPS-induced over-translocation into nucleus of NF- $\kappa$ Bp65. Hence, it appears that inhibition of NF- $\kappa$ Bp65 activation by aprepitant may also be a possible mechanism underlying its inhibitory effect in microglia, which is critical for the suppression of the production of proinflammatory cytokine.

We further investigated whether aprepitant could inhibit microglial activation in inflammatory pain *in vivo*. We detected the IBA1 and the phosphorylation of p38 MAPK in the spinal cord of mice with formalin-induced inflammatory pain. The results showed that IBA1, and the JNK phosphorylation, as well as the p38 MAPK phosphorylation were significantly increased in the spinal cord of formalin model mice. In addition, aprepitant could effectively inhibit the up-regulation of IBA1 and the p38 MAPK phosphorylation in the spinal cord. As established previously, the protein levels of IL-1 $\beta$ , IL-6, TNF $\alpha$ , and MCP-1 and the mRNA expressions of IL-1 $\beta$ , IL-6, TNF $\alpha$ , MCP-1, and NF- $\kappa$ Bp65 in mice spinal cord were considerably suppressed by aprepitant. Consistently, aprepitant markedly alleviated the pain behavior and reduced phosphorylation of p38 MAPK and the mRNA expression of NF- $\kappa$ Bp65 in the spinal cord in mice with carrageenan-induced inflammatory pain, further confirming that the activation of p38 MAPK and NF- $\kappa$ Bp65 in the spinal cord were involved in the pain regulation of aprepitant in inflammatory pain.

## Conclusions

In conclusion, this study demonstrated a novel role for aprepitant in the therapy of inflammatory pain through mechanisms associated with the inhibition of multiple targets in activated microglia in the spinal cord. Our results support the potential for repurposing this agent for the treatment of inflammatory pain.

## List Of Abbreviations

AP, action potential

DRG, dorsal root ganglia

ERK1/2, extracellular signal-regulated kinase-1/2

IL-1 $\beta$ , interleukin-1 $\beta$

IL-6, interleukin-6

JNK, c-Jun NH2 terminal protein kinase

LPS, lipopolysaccharide

MCP-1, monocyte chemoattractant protein 1

NF- $\kappa$ Bp65, nuclear factor  $\kappa$ B p65

NK-1R, neurokinin-1 receptor

NSAIDs, nonsteroidal anti-inflammatory drugs

p38, p38 mitogen-activated protein kinase

SP, substance P

TNF- $\alpha$ , tumor necrosis factor- $\alpha$

## **Declarations**

### **Ethics approval and consent to participate**

Yes

### **Consent for publication**

Not applicable

### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available through the corresponding author on reasonable request.

### **Competing interests**

The authors have no conflicts of interest to declare.

### **Funding**

This project was supported by the National Natural Science Foundation of China, Grant/Award Number: 81801105, the Natural Science Foundation of Jiangsu Province, Grant/ Award Number: BK20181092, the National Natural Science Foundation of China, Grant/Award Number: 81903338, the National Key Research and Development Program “precision medicine research”, Grant/ Award Number: 2017YFC0908304, and the Project of Jiangsu Provincial Key medical talents in Jiangsu Province, Grant/ Award Number: ZDRCA2016034.

### **Authors' contributions**

X. D. designed research. Y. Y., W. Z., X.Q. X., J.G. S., X.X. G., F. W., and G.Q. Z. researched data. Y. Y., L. M., and X. D. analyzed data. X. D. and L. M. wrote the manuscript. All authors approved the final manuscript.

### **Acknowledgements**

We thank the staffs at the Central Laboratory of the Second Affiliated Hospital of Nanjing Medical University for providing experimental space, facilities, and technical services.

## Footnotes

Yang Yang, Wei Zhou, Xiuqi Xu, and Jiugao Sang contributed equally to this work.

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## Figures

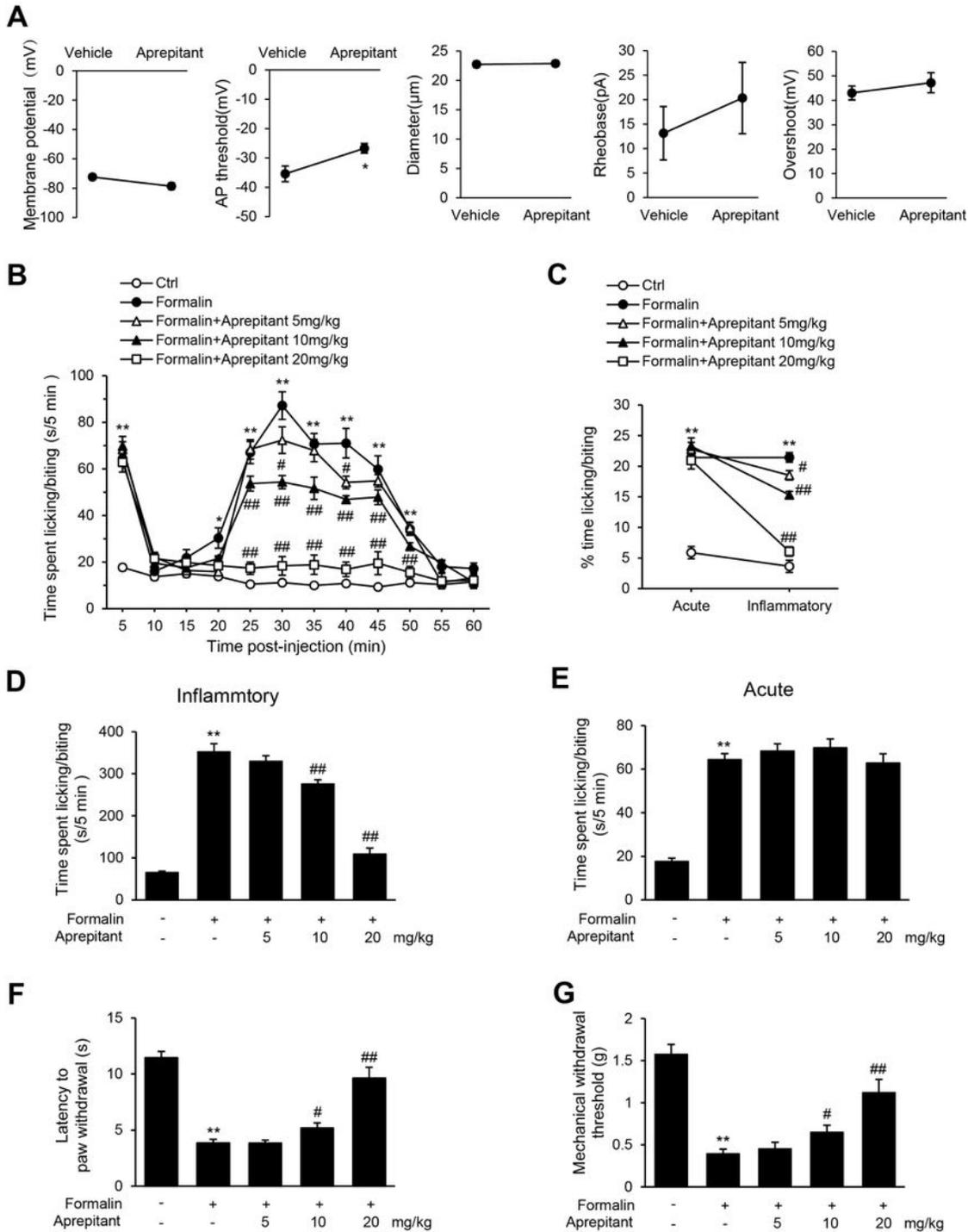


Figure 1

## Figure 1

Aprepitant attenuates response to inflammatory pain. (A) Membrane properties of DRG neurons in control and aprepitant treatment groups. AP, action potential; Rheobase, the minimum current required to trigger an AP. Statistical significance was evaluated by the unpaired Student's t test. (B) Time spent licking paw following formalin injection displayed in 5-min time bins after single injection of aprepitant. (C) % time spent paw licking during acute and inflammatory phases of formalin test with aprepitant treatment. (D)

Time spent paw licking during the inflammatory phase of formalin test. (E) Time spent paw licking during the acute phase of formalin test. (F) Latency to withdraw paw in aprepitant treatment groups versus control mice during hotplate test. (G) Withdrawal threshold (Von Frey filament at which mouse responded to >50% of trials) in aprepitant treatment groups versus control mice. Eleven to twelve mice were included in each of the groups. Data are expressed as mean  $\pm$  SEM. Two-way ANOVA, \* $p$  < 0.05, \*\* $p$  < 0.01 versus vehicle or control, # $p$  < 0.05, ## $p$  < 0.01 versus formalin.

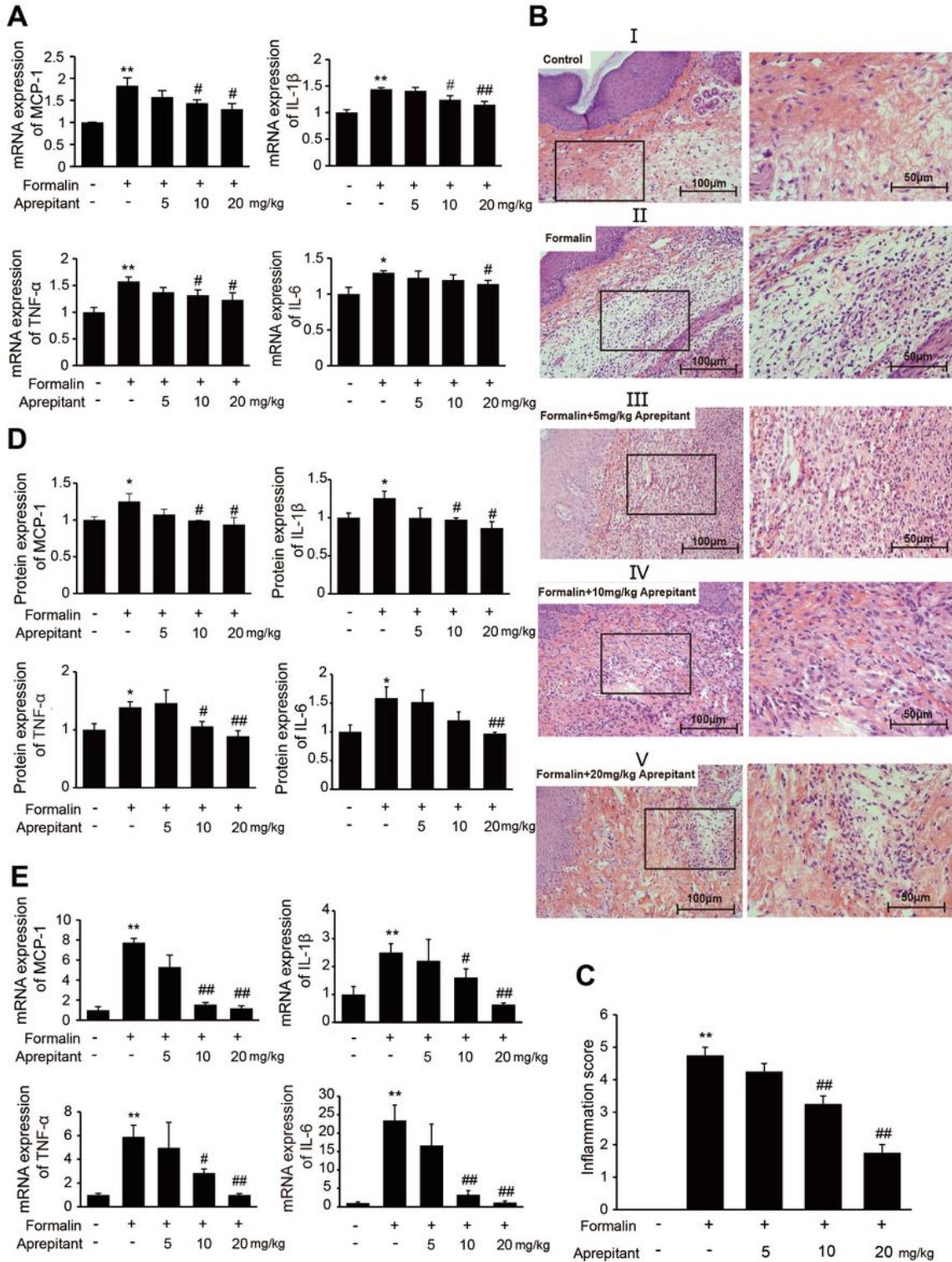


Figure 2

Figure 2

Anti-inflammatory effects of Aprepitant in mice with formalin-induced inflammatory pain. (A) mRNA expression of MCP-1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 in the inflamed paw after administration of Aprepitant. (B) Hematoxylin eosin (HE) staining of paw tissues of mice. (C) Scores of inflammation in mouse paw tissues by HE staining. Each photo is representative of six specimens for each group. (D, E) The protein (D) and mRNA (E) levels of MCP-1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 in the spinal cord of formalin treated mice after administration of Aprepitant. Six samples were included in each of the groups. Data are expressed as mean  $\pm$  SEM. One-way ANOVA, \* $p$  < 0.05, \*\* $p$  < 0.01 versus vehicle or control, # $p$  < 0.05, ## $p$  < 0.01 versus formalin.

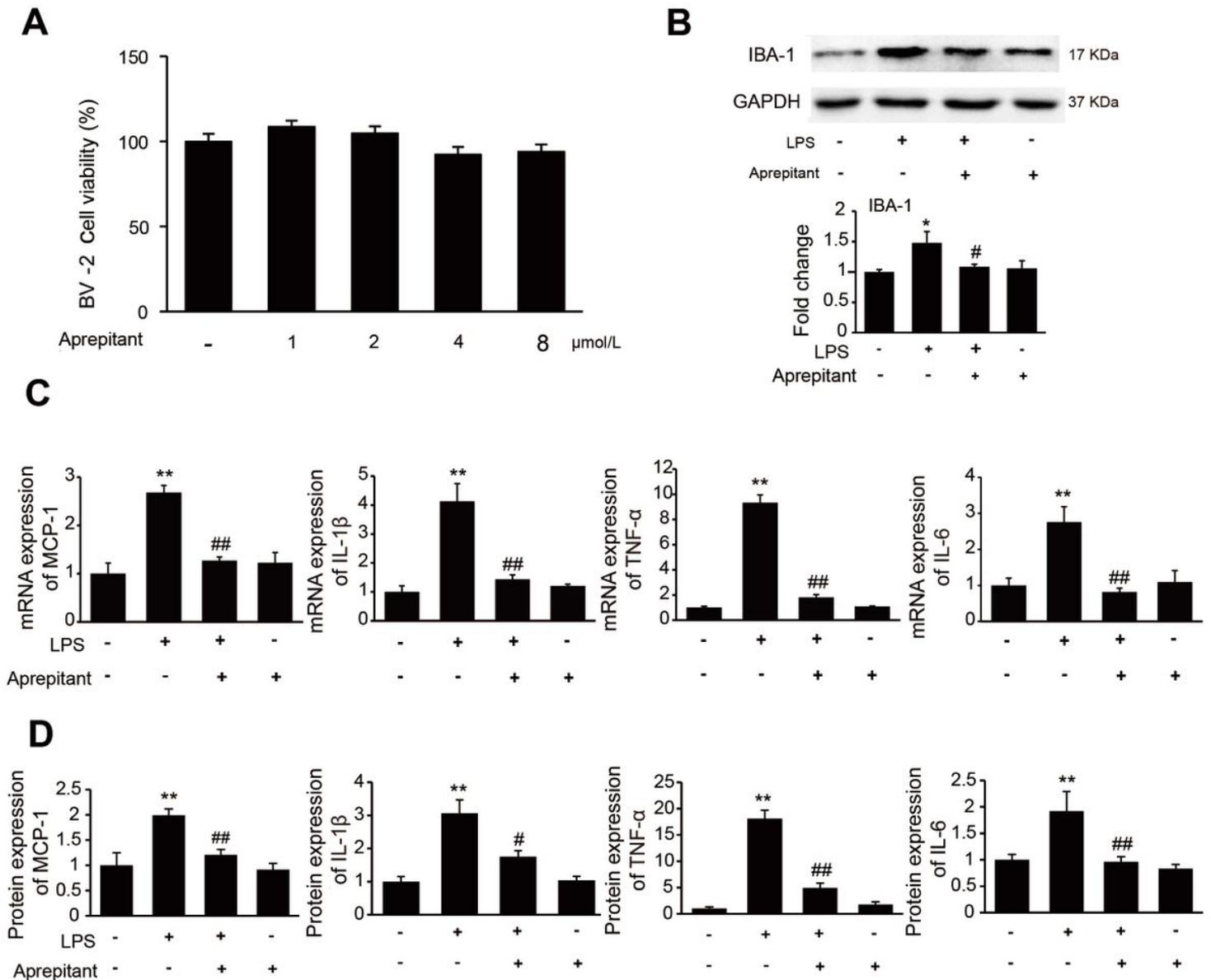


Figure 3

### Figure 3

Effect of Aprepitant on lipopolysaccharide (LPS)-induced BV-2 microglial activation. (A) Cell viability was measured using an MTT assay. (B) The representative immunoblots and graphic representation of

relative expression of IBA-1. (C) The mRNA expression of MCP-1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 in BV-2 microglia. (D) ELISA showed that aprepitant decreased the LPS-induced secretion of MCP-1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 in BV-2 microglia. Data are expressed as mean  $\pm$  SEM. One-way ANOVA, \* $p$  < 0.05, \*\* $p$  < 0.01 versus vehicle or control, # $p$  < 0.05, ## $p$  < 0.01 versus LPS.

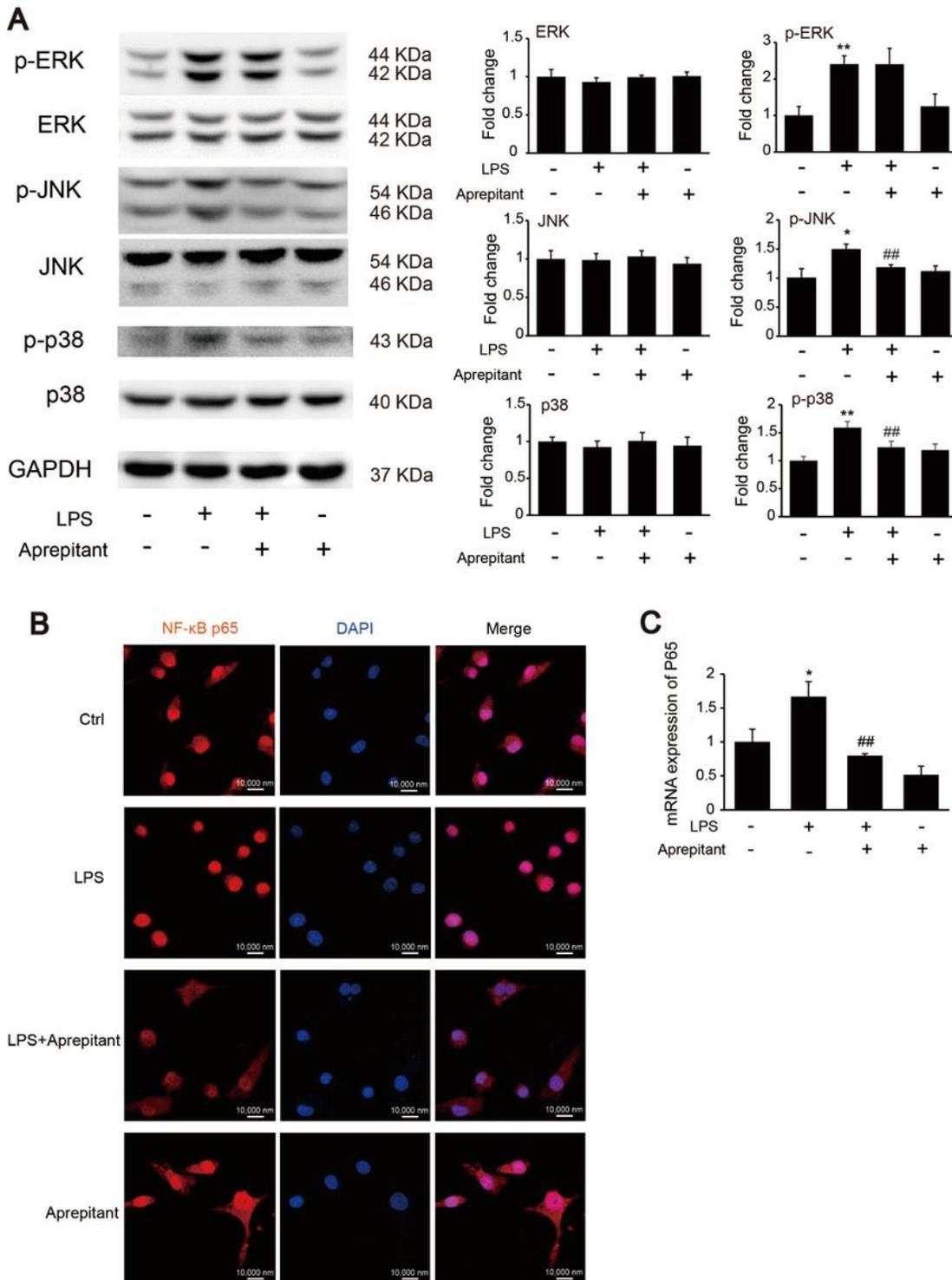


Figure 4

Figure 4

Effects of aprepitant on the protein expression of MAPK and nuclear translocation of NF- $\kappa$ Bp65 in BV-2 microglia. (A) The protein and phosphorylation of ERK, JNK and p38 MAPK in BV-2 microglia. (B) Immunofluorescence was used to analyze NF- $\kappa$ B p65 nuclear translocation. (C) The mRNA expression of NF- $\kappa$ B p65 in BV-2 microglia was assessed by real-time PCR. Data are expressed as mean  $\pm$  SEM. One-way ANOVA, \* $p$  < 0.05, \*\* $p$  < 0.01 versus vehicle or control, # $p$  < 0.05, ## $p$  < 0.01 versus LPS.

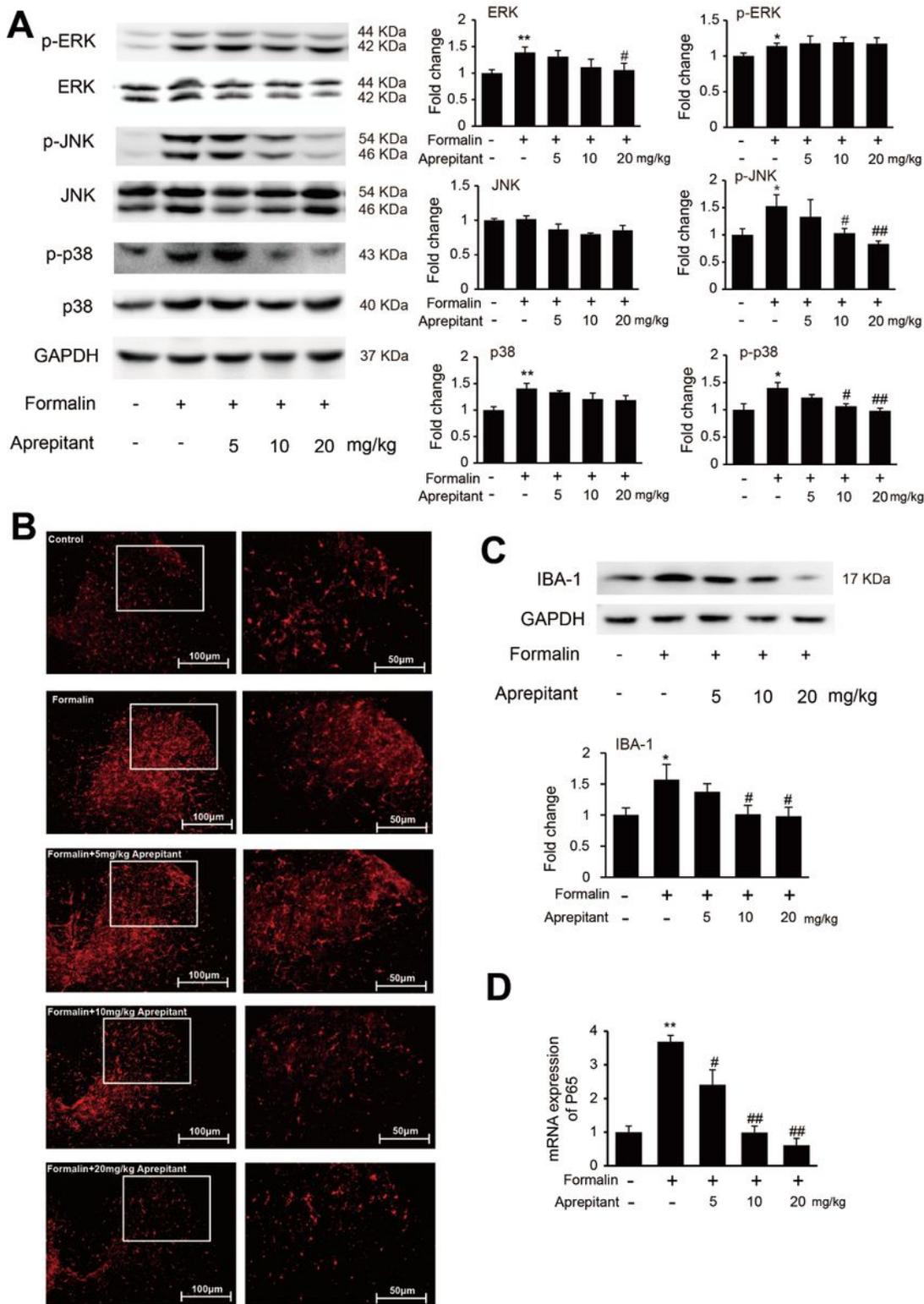


Figure 5

Effects of aprepitant on the protein expression of MAPK and microglial activation in spinal cord of mice with formalin-induced inflammatory pain. (A) The protein and phosphorylation of ERK, JNK and p38 MAPK in mouse spinal cord. (B, C) Immunofluorescence staining (B) and western blotting (C) showed the effect of aprepitant on activation of microglia in mouse spinal cord. (D) The mRNA expression of NF-kBp65 in spinal cord was assessed by real-time PCR. Data are expressed as mean  $\pm$  SEM. One-way ANOVA, \* $p$  < 0.05, \*\* $p$  < 0.01 versus control, # $p$  < 0.05, ## $p$  < 0.01 versus formalin.

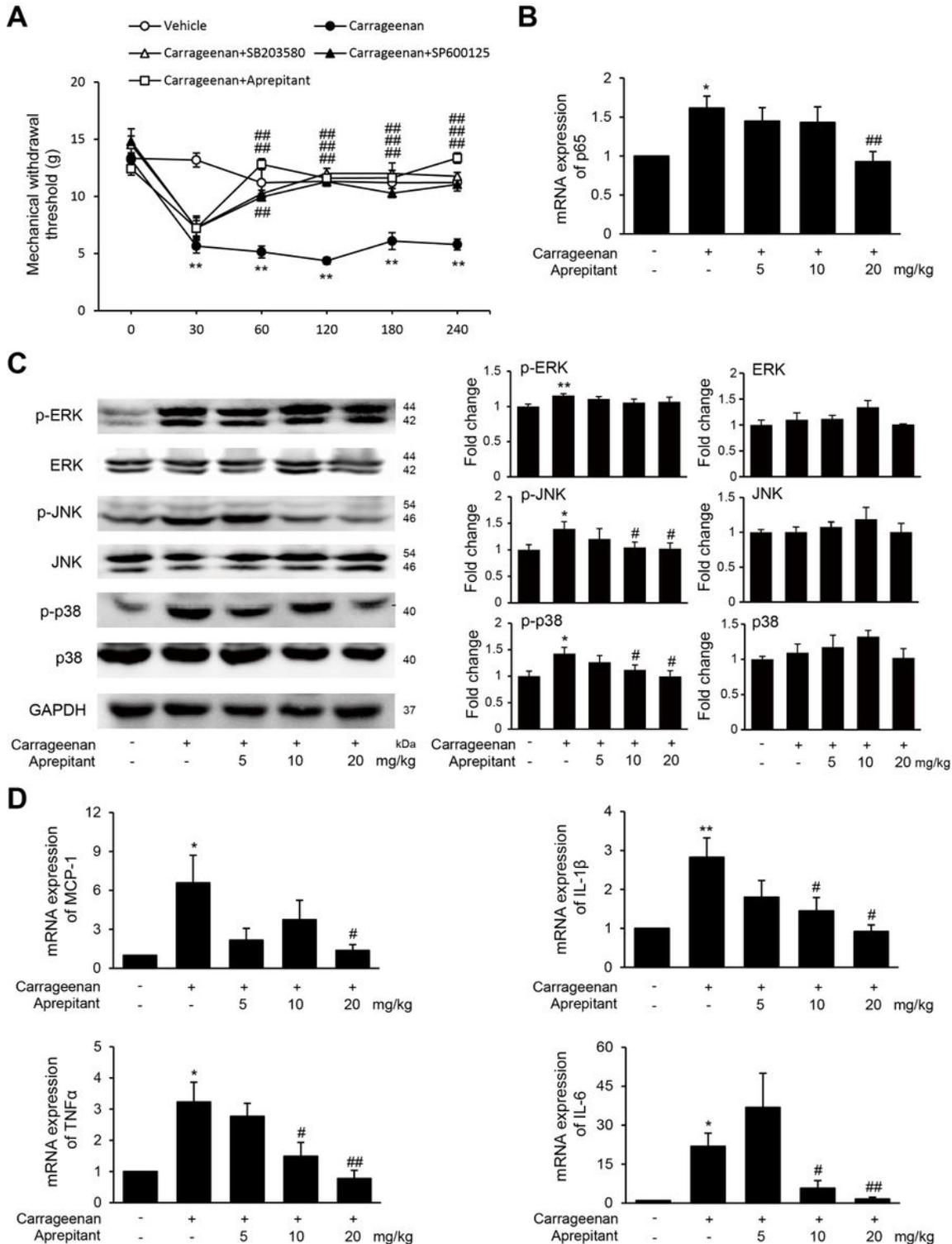


Figure 6

Figure 6

Effect of aprepitant on carrageenan-induced mechanical allodynia in mice and carrageenan-induced microglial activation and pro-inflammatory cytokine expression in the spinal cord. (A) Withdrawal threshold in aprepitant treatment groups versus control mice. Eleven to twelve mice were included in each of the groups. Data are expressed as mean  $\pm$  SEM. One-way ANOVA, \*\*p < 0.01 versus control, ##p < 0.01 versus carrageenan. (B) The mRNA expression of NF-kBp65 in spinal cord was assessed by real-time PCR. (C) The representative immunoblots and graphic representation of relative protein and phosphorylation of ERK, JNK and p38 MAPK in spinal cord. (D) The mRNA expression of MCP-1, IL-1 $\beta$ , TNF- $\alpha$  and IL-6 in spinal cord of mice with carrageenan-induced inflammatory pain. Data are expressed as mean  $\pm$  SEM. Two-way ANOVA, \*p < 0.05, \*\*p < 0.01 versus control, #p < 0.05, ##p < 0.01 versus carrageenan.

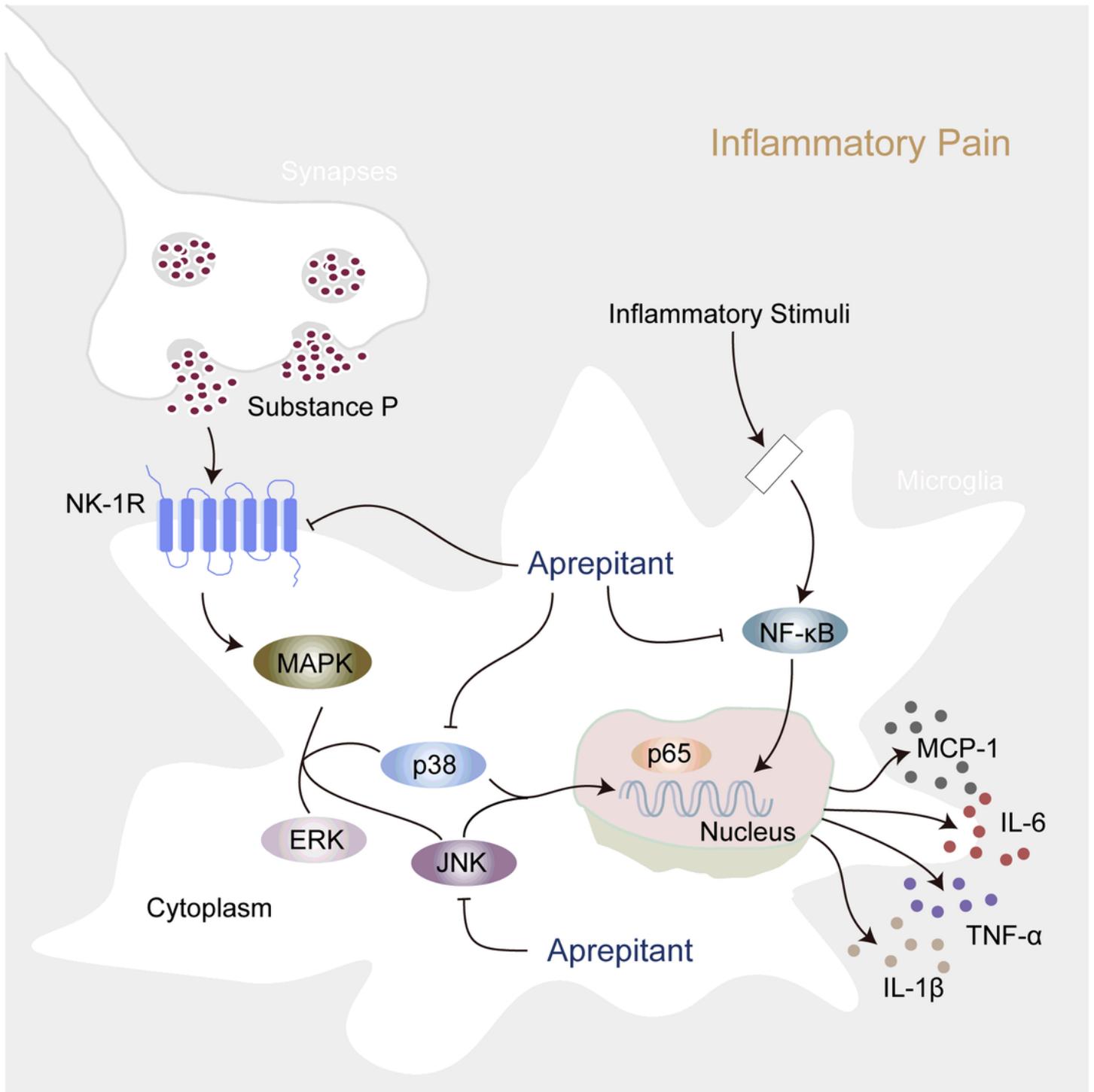


Figure 7

**Figure 7**

Proposed mechanisms of action by which aprepitant inhibited inflammation in mice with inflammatory pain.