

Involvement of Interleukin-18-Mediated Microglia/Astrocyte Interaction in Experimental Models of Migraine

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Research

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Abstract

Background: The exact molecular mechanisms of migraine were not fully understood. Emerging evidence indicated that inflammation had a significant role in pathophysiological mechanisms of migraine. The present study aimed to investigate the involvement of Interleukin-18-Mediated Microglia/Astrocyte Interaction in the development of hyperpathia or allodynia induced by migraine.

Methods: Experimental rat model of migraine was established by repetitive inflammatory soup (IS) dural infusions. The expression of Interleukin-18 (IL-18) and IL-18 receptor (IL-18R) was examined by quantitative real-time polymerase chain reaction or Western blot. The expression of activated microglia and astrocytes was examined by Western blot or immunofluorescence. The expression of nuclear factor-kappa B (NF- κ B) was examined by Western blot. TAK-242 and anti-IL-18 antibody were used to inhibit the activation of toll-like receptor 4 (TLR4) and IL-18 pathway, respectively.

Results: IS dural infusions elicited microglial activation and astrocytic activation. Meanwhile, IS dural infusions induced the upregulation of IL-18 and IL-18R in microglia and astrocytes, respectively. Blocking the IL-18 signaling pathway attenuated nociceptive behavior. Meanwhile, blocking IL-18 signaling also suppressed astrocytic activation, as well as activation of NF- κ B. And IL-18 dural infusions induced nociceptive behavior and glia activation.

Conclusions: IL-18, a product of microglial toll-like receptor 4 (TLR4) activation, acted on IL-18R expressed in astrocyte, and subsequently stimulated activation of NF- κ B, leading to the astrocytic activation. Together, the present results suggest that IL-18-mediated microglia/astrocyte interactions in the medullary dorsal horn may participate in the development of hyperpathia or allodynia induced by migraine.

Background

Migraine, a severe and disabling neurologic disorder with quite high morbidity (approximately 15%), has a great detrimental influence on society and individuals. It is characterized by multiple attacks of throbbing and often unilateral severe head pain, usually accompanied with nausea, vomiting, photophobia or phonophobia, and aggravated by physical activity or head movement^[1]. Despite decades of clinical and scientific research, the exact molecular mechanisms of migraine were not fully understood. Recently, emerging evidence indicates that inflammation has a significant role in pathophysiological mechanisms of migraine. ^[2,3] Glial cells are critical in the inflammation process. When activated, various substances, such as proinflammatory cytokines, were released. They amplify the nociceptive signals and take part in the sensitization process, leading to enhanced responsiveness of nervous tissue. In this process, glial cells, like microglia and astrocytes, were found in close proximity to each other, facilitating cell-cell communication through ligand-receptor pairings elevated in target tissues^[4-6].

IL-18, a member of the IL-1 family, is produced as an inactive precursor (pro IL-18), activated by cleavage of cysteine protease caspase-1^[7]. Related closely with IL-1 β , IL-18 was extensively involved in immunity^[8],

tumor growth^[9], inflammation^[10], and even the pain process^[11, 12]. In various rodent models, IL-18 was found primarily expressed in microglia, and was demonstrated to mediate microglia and astrocytes interaction, contributing to neuropathic pain^[11, 12] and cancer pain^[13, 14]. However, there has been no study investigating the role of IL-18 signaling pathways in migraine models.

In this work, we show that IL-18 is upregulated in microglia of migraine rat brain, while its receptor (IL-18R) upregulated in astrocytes. Here, we provide first evidence for the involvement of IL-18 signaling pathways mediating microglia/astrocyte interaction in development and maintenance of migraine.

Materials And Methods

Animals.

Male Sprague–Dawley (SD) rats weighting 200–250 g were used. All rats were housed under a 12 h light/dark cycle at 22 ± 2 °C, with free access to food and water. They were habituated in experimental environment for one week before each experimental procedure. All experiments were approved by the Committee on Animal Use for Research and Education of the Laboratory Animals Center, Renmin Hospital of Wuhan University (Wuhan, PR China), and followed the recommendations of the International Association for the Study of Pain in Conscious Animals^[15]. The number and suffering of animals were minimized as much as possible.

Surgical procedure.

The experimental procedures were carried on according to a previous study^[16]. Briefly, a midline incision was performed under pentobarbital anesthesia (60 mg/kg; i.p). A guide cannula was implanted in a previously drilled cranial window adjacent to the superior sagittal sinus, to delivery IS or PBS to the dura mater in all rats. We take special care to prevent infection and to minimize the effect of inflammation.

Repetitive dural infusions.

Rats were divided into two groups randomly: the control (CON) group and the migraine model (IS) group. The migraine models were built via repeated IS (30 ul, including 2 mM histamine, 2 mM serotonin, 2 mM bradykinin, and 0.2 mM prostaglandin E2 in PBS)^[16] dural infusions for at least 4 days. Rats in the control group received the same dose of PBS.

Drug administration.

A total of 0.8 ml anti-IL-18 antibody (100 ng/ μ l; R & D Systems) was first administered intraperitoneally 1.5 h prior to the each dural IS infusion [IS + Anti-IL-18(i.p) group]. Then, the intracerebroventricular injection of 5 ul anti-IL-18 antibody was conducted as described previously^[17]. Briefly, anti-IL-18 antibody (100 ng/ μ l; R & D Systems) was injected into the lateral ventricle (-1.0 mm rear from the Bregma and + 1.5 mm lateral, 4.0 mm from the skull plane) 24 h after the 4th IS infusion[IS + Anti-IL-18(i.c.v) group],

under adequate anesthesia. TAK (3 mg/kg, Millipore, Bedford, MA), a specific TLR4 antagonist, was administered intraperitoneally 1.5 h prior to the dural IS infusion based on the previous study (TAK group). The 30 μ l of IL-18 (5 μ g; R & D Systems) was delivered to the dura mater as described above (IL-18 group). TAK was diluted in PBS. The anti-IL-18 antibody and IL-18 were both dissolved in PBS. Rat in the control group received the equivalent volume of PBS. The dosages of above drugs were determined according to previous studies. Random grouping was used.

Nociceptive behavior

The headache behavior of rats was evaluated by a nociception threshold and face rubbing. A von Frey filament (vFF) was used when measuring the facial mechanical withdraw threshold, as described previously^[16]. The nociceptive threshold was taken as the least force applied to make rats quickly retract its head at least three of five stimulus. Face rubbing was a nociceptive behavior when rats received IS. In process testing, the experimenter was blinded to the grouping.

Quantitative real-time polymerase chain reaction (q-PCR)

Total RNA was extracted from rat medullary dorsal horn using Trizol reagent (Invitrogen) as previously described^[18]. A NanoDrop ND1000 Spectrometer (NanoDrop Technologies) was used to measure the RNA concentration. Then, 1 μ g of total RNA was reverse-transcribed into cDNA using PrimerScript RT Reagent Kit (Takara, Kyoto, Japan) according to manufacturer's protocols. Real-time PCR amplification was conducted using SYBR Premix Ex Taq™ II (Takara, Kyoto, Japan) according to manufacturer's protocols. The following primers were used: 5'-GACAAAAGAAACCCGCCTG-3' (IL-18 forward); 5'-ACATCCTTCCATCCTTC-ACAG-3' (IL-18 reverse); 5'-TGGAGTCTACTGGCGTCTT-3' (GAPDH forward); 5'-TGTCATATTTCTCGTGGTTCA-3' (GAPDH reverse). Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH), a housekeeping gene, was used to normalize expression. The relative mRNA level was represented as fold change of control by $2^{-\Delta\Delta C_t}$ method.

Immunofluorescence staining

After deep anesthesia, rats were perfused transcardially with 9% saline followed by 4% formaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4). After the perfusion, the rat brain tissues were removed and postfixed in 4% formaldehyde. Medullary dorsal horn sections (paraffin-embedded, 5 μ m) were manufactured. Then, the sections were placed in xylene for dewaxing, and followed by decreasing grades of alcohol for rehydrating. Sections were blocked by 5% BSA for 60 min at RT, and then incubated in primary antibody diluted in 1% BSA overnight at 4 °C. The dilution concentration of the primary antibody was applied as follows: IL-18 (1:500; Abcam), IL-18R (1:100; Abcam), Iba1 (1:600; Abcam), GFAP (1:500; Gene Tex), p-p38 (1:50; Santa Cruz). After cleaning with PBS, the sections were incubated with respective secondary antibodies. Anti-mouse (488 nm, green), anti-rabbit (594 nm, red), anti-rabbit (Cy3, red), anti-goat (FITC, green) and anti-goat (CY3, red) antibodies were used. DAPI (servicebio) was used to stain nuclei. Images were captured under a 20 \times or 40 \times objective with an ECLIPSE Ti-U microscope using the

NLS-ElementsBR.3.0software (Nikon, Melville, NY). The immunofluorescence intensity was measured using ImageJ software (Version1.52, National Institutes of Health).

Western blot analysis

Tissue from medullary dorsal horn homogenized in lysis buffer (consisting of RIPA, cocktail PMSF and phosphorylase inhibitor). Then, protein concentrations were measured using a BCA Protein Assay Kit (Beyotime). After heated in loading buffer for 10 min at 100°C, equal amounts of protein were loaded and separated on 10% SDS PAGE gels (Bio-Rad), and subsequently transferred to PVDF membranes (Millipore). After blocked with 5% nonfat milk for 90 min at RT, the membrane was incubated in primary antibody overnight at 4 °C. The dilution concentration of the primary antibody was applied as follows: IL-18 (1:1000; Abcam), IL-18R (1:50; Abcam), GFAP (1:2500; Gene Tex), p65(1:3000, Abcam), p-p65 (1:50; Santa Cruz) and NAPDH (1:1500, servicebio). The membrane then was washed three times in TBST and incubated with respective secondary antibodies for 60 min at RT. The antibody-reactive bands were visualized using Odyssey CLx Image Studio 3. The gray-scale value of straps was quantified using Image J software (Version1.52, National Institutes of Health). All Western blot analysis were conducted at least three times, and consistent results were obtained.

Statistical analysis

All data are presented as the mean \pm SEM. GraphPad Prism 8 (GraphPad Software, San Diego, CA) were used to perform statistical analysis and to generate the graphs. T-test was used to evaluate differences between two independent groups. $P < 0.05$ was considered significant.

Results

IS dural infusions induced the activation of microglia and astrocyte in the medullary dorsal horn

Glial fibrillary acidic protein (GFAP, astrocytic marker) and ionized calcium binding adapter molecule 1 (Iba1, microglial marker) were examined by immune-fluorescence. Rats in IS group showed higher expression of GFAP (Fig.2B, D) and Iba1 (Fig.2A, C) in the medullary dorsal horn than that in control group.

IS dural infusions induced the upregulation of IL-18/IL-18R in the medullary dorsal horn

The q-PCR results revealed a significantly increase of the IL-18 mRNA level in the medullary dorsal horn of IS group (Fig.3A). Next, the expression of IL-18 protein was detected by Western blot analysis. Consistent with changes in IL-18 mRNA levels, IL-18 protein was upregulated in the medullary dorsal horn of IS group (Fig.3B, C). Then, IL-18R upregulation after IS dural infusions was also confirmed by Western blot analysis (Fig.3B, D).

IL-18 activated in microglia, IL-18R activated in astrocyte

To identify the cell types expressing IL-18 and IL-18R after IS dural infusions, we performed double Immuno-fluorescence staining of the IL-18 or IL-18R with Iba1 and GFAP in the medullary dorsal horn of IS group. Results showed that IL-18 immunoreactivity was predominantly double-labeled with Iba1, but not with GFAP (Fig.4A). Moreover, IL-18R immunoreactivity was predominantly double-labeled with GFAP, but not with Iba1 (Fig.4B), suggesting that IS dural infusions induced IL-18 upregulation in medullary microglia, while IL-18R upregulation in medullary astrocyte.

IS dural infusions induced microglia-derived IL-18 upregulation via TLR4/p38 MAPK in the medullary dorsal horn

To examine whether TLR4 regulates IL-18 expression in microglia of medulla oblongata after IS dural infusions, we injected TAK (the TLR inhibitor). The result showed that microglia-derived IL-18 induced by IS dural infusions was significantly alleviated by blocking TLR4 (Fig.5A). Moreover, we found p-p38 entries into nucleus of IL18-immunopositive cells (Fig.5B), indicating that IS dural infusions induce microglia-derived IL-18 upregulation through TLR4/p38 MAPK pathway in the medullary dorsal horn.

IL-18 dural infusions produced nociceptive behavior

It was revealed that IL-18 occupied a key position in neuropathic pain, cancer pain and morphine tolerance^[22,11-14]. To further examine the impact of IL-18 on headache behavior, we conducted repeated IL-18 dural infusions. After the 4th repetitive dural infusions of IL-18, the number of face rubbing in five hours and the facial mechanical withdraw threshold were recorded. We found that IL-18 dural infusions induced face rubbing (Fig.6A) and produced tactile allodynia (Fig.6B).

IL-18 dural infusions induced the activation of microglia and astrocyte in the medullary dorsal horn

Rats in IL-18 group showed higher expression of GFAP (Fig.7B, D) and Iba1 (Fig.7A, C) by immunofluorescence in the medullary dorsal horn than that in control group. Furthermore, the results of Western blot analysis verified the GFAP protein upregulation in the IL-18 group (Fig.7E).

IL-18 blockade attenuated nociceptive behavior induced by repeated IS dural infusions

Next, we tested the effect of anti-IL-18 antibody on the development of IS-induced cutaneous allodynia and hyperalgesia. The injection of anti-IL-18 antibody once daily for 4d with the first injection 1.5h before IS dural infusion significantly inhibited the development of face rubbing (Fig.8A) and tactile allodynia (Fig.8B). And, intrathecal injection of anti-IL-18 antibody after the 4th IS infusion reduced face rubbing induced by repeated IS dural infusions (Fig.8C), but failed to obviously reverse the tactile allodynia induced by IS dural infusions (Fig.8D).

Microglia-derived IL-18 mediated astrocytic activation via a NF- κ B-dependent manner

A previous study revealed the TLR4-NF- κ B signaling pathway an important role in the progress of hyperalgesia following IS dural infusions^[21]. In this work, IS dural infusions induced an increase of NF- κ B

phosphorylation in the medullary dorsal horn (Fig.9C). Next, to investigate the effect of IL-18 on NF- κ B p65 activation, we compared the level of p-NF- κ B in the control and anti-IL-18 group. Western blot analysis showed that IL-18 blockade significantly inhibited NF- κ B p65 activation induced by IS dural infusions (Fig.9D), accompanied with the inhibition of astrocytic activation (Fig.9B).

Discussion

Accumulating evidence supported the glial cell is critical in pain hypersensitivity induced by migraine. Glial cells were activated in the status of experimental migraine, and subsequently released various proinflammatory cytokines like IL-1 β and TNF- α , causing hyperpathia or allodynia^[2, 21].

The present study revealed microglia-derived IL-18 signaling a vital role in experimental migraine (Figure.1). First, IS dural infusions elicited the activation of microglia, followed by increments of IL-18 via TLR4-derived p38 MAPK pathway. Secondly, IL-18 induced nociceptive behavior and activation of microglia and astrocyte. Then, microglia-derived IL-18 acted on IL-18R predominantly expressed in astrocytes, increasing NF- κ B phosphorylation, subsequently activating astrocytes. Blockage of IL-18 attenuated nociceptive behavior induced by repetitive IS dural infusions and inhibited the activation of NF- κ B phosphorylation and astrocyte. IL-18 might activate microglia and astrocytes in an autocrine or paracrine manner, then synthesized and released pro-inflammatory cytokines, neurotransmitters and neuromodulators, amplifying neuronal excitability^[4, 5, 11, 14] and inducing migraine attacks. It indicated a new mechanism underlying experimental migraine that IL-18 signaling mediating microglia-astrocyte interactions, contributing to hyperpathia or allodynia induced by IS dural infusions.

Consistent with previous studies^[2, 21], repetitive IS dural infusions induced the activation of glial cells. Activated microglia and astrocytes released various substances, including pro-inflammatory cytokines like IL-1 β , IL-6 and TNF- α , subsequently activating the trigemino-vascular system and evoking hyperalgesia and allodynia^[21, 23, 24]. Presently we found that IL-18, a member of the IL-1 family, was upregulated by IS dural infusions. And, double-labelling immunofluorescence indicated microglia is the predominant cell expressing IL-18, similar to previous studies. Multiple studies confirmed that IL-18 has a vital role in the neuropathic pain, cancer pain and morphine tolerance^[22, 11-14]. However, there was lack of evidence suggested the role of the IL-18 signaling pathway in experimental migraine. Toll-like receptor 4 (TLR4) plays a crucial role in the innate immunity, and is expressed in microglia^[19, 20]. A previous study reported that TLR4 had a critical role in experimental migraine^[21]. Intriguingly, Miyoshi and co-workers found that nerve injury induced the expression of IL-18 in spinal microglia through TLR4/p38MAPK pathway^[11]. In a similar vein, the present study hypothesized the molecular events that microglia-derived IL-18 was regulated via a TLR4/p38 MAPK-dependent manner. Blockage of TLR4 attenuated the upregulation of IL-18 induced by IS dural infusions. Furthermore, double-labelling immunofluorescence revealed p-p38 entries into nucleus of IL-18-immunopositive cells, confirming the above hypothesis.

IL-18 bound to IL-18R to play a biological role. In line with prior studies^[11, 14], we found that elevation of IL-18R induced by IS dural infusions were primarily expressed in astrocytes. IL-18/IL-18R signaling

pathway mediated microglia-astrocyte interactions and hence participated in the development of hyperpathia or allodynia induced by IS dural infusions. We pretreated rats with anti-IL-18 antibody to block the IL-18 signaling before each IS dural infusion. Blockage of IL-18 significantly inhibited face rubbing and tactile allodynia produced by IS dural infusions. And, the increments of astroglial markers caused by IS dural infusion was similarly reduced. Inversely, IL-18 dural infusions produced both headache behaviors and astrocytic activation. Such findings indicated that experimental migraine induced the synthesis of IL-18 from microglia through the activation of TLR4/p38 MAPK pathway. Microglia-derived IL-18 stimulated IL-18R expressed on neighboring astrocytes, triggering astrocytic activation.

We subsequently attempted to explore the molecular mechanisms under IL-18/IL-18R mediated astrocytic activation. Previous studies revealed that NF- κ B p65 activation was involved in the experimental migraine and targeting NF- κ B p65-driven transcription or downstream expression, which provided a potential therapeutic strategy for migraine^[21, 25, 26]. In the present study, IS dural infusions induced an increase of NF- κ B phosphorylation in the medullary dorsal horn. Moreover, activation of IL-18 signaling in neuropathic pain promoted the phosphorylation of NF- κ B in the astrocytes and activated those cells^[11]. So, we detected the effects of neutralizing IL-18 activity on NF- κ B phosphorylation. The results shown that suppression of IL-18 bioactivity significantly inhibited the phosphorylation of NF- κ B, accompanied by the downregulation of astrocytes, indicating a further establishing linkage between IL-18, NF- κ B and astrocytic activation.

Attentionally, we chose peripheral administration intervention to suppress IL-18 bioactivity before each IS dural infusion, by considering the particularity of our animal models. Western blot analysis verified the blockade of IL-18 in the medullary dorsal horn, which could be explained by blood-brain barrier (BBB) breakdown due to repeated IS dural stimulation^[27]. Glial activation occupied a key position in the BBB modulation^[28, 29]. In present works, microglia and astrocyte were activated by IS dural infusion, providing further evidence for the above. It is noteworthy that IL-18 might also play a role through peripheral mechanisms, similar to role of calcitonin gene-related peptide (CGRP) in Migraine^[30]. Then posttreatment with anti-IL-18 antibody could not obviously reverse the hyperpathia or allodynia induced by IS dural infusions, which might be explained by that only one dose of anti-IL-18 antibody after successful modeling could not effectively block the IL-18 bioactivity.

Conclusion

The present study revealed a new molecular mechanism for migraine. IL-18-mediated microglia/astrocyte interactions in the medullary dorsal horn may participate in the development of hyperpathia or allodynia induced by migraine.

Abbreviations

IS: inflammatory soup; IL-18: Interleukin-18; IL-18R: IL-18 receptor; NF- κ B: nuclear factor-kappa B; TLR4: toll-like receptor 4; vFF: von Frey filament; q-PCR: quantitative real-time polymerase chain reaction; GAPDH: Glyceraldehyde-3-phosphate Dehydrogenase; PBS: phosphate buffer; GFAP: Glial fibrillary acidic protein; Iba1: ionized calcium binding adapter molecule 1; BBB: blood-brain barrier; CGRP: calcitonin gene-related peptide.

Declarations

Author Contributions

QG designed the experiments for the manuscript. QG and YL conducted the experiments and analysis for the manuscript. QG wrote the paper. ZL and ZX reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable

Ethics approval and consent to participate

All experiments were approved by the Committee on Animal Use for Research and Education of the Laboratory Animals Center, Renmin Hospital of Wuhan University (Wuhan, PR China).

Disclosure of conflicts of interest

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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Figures

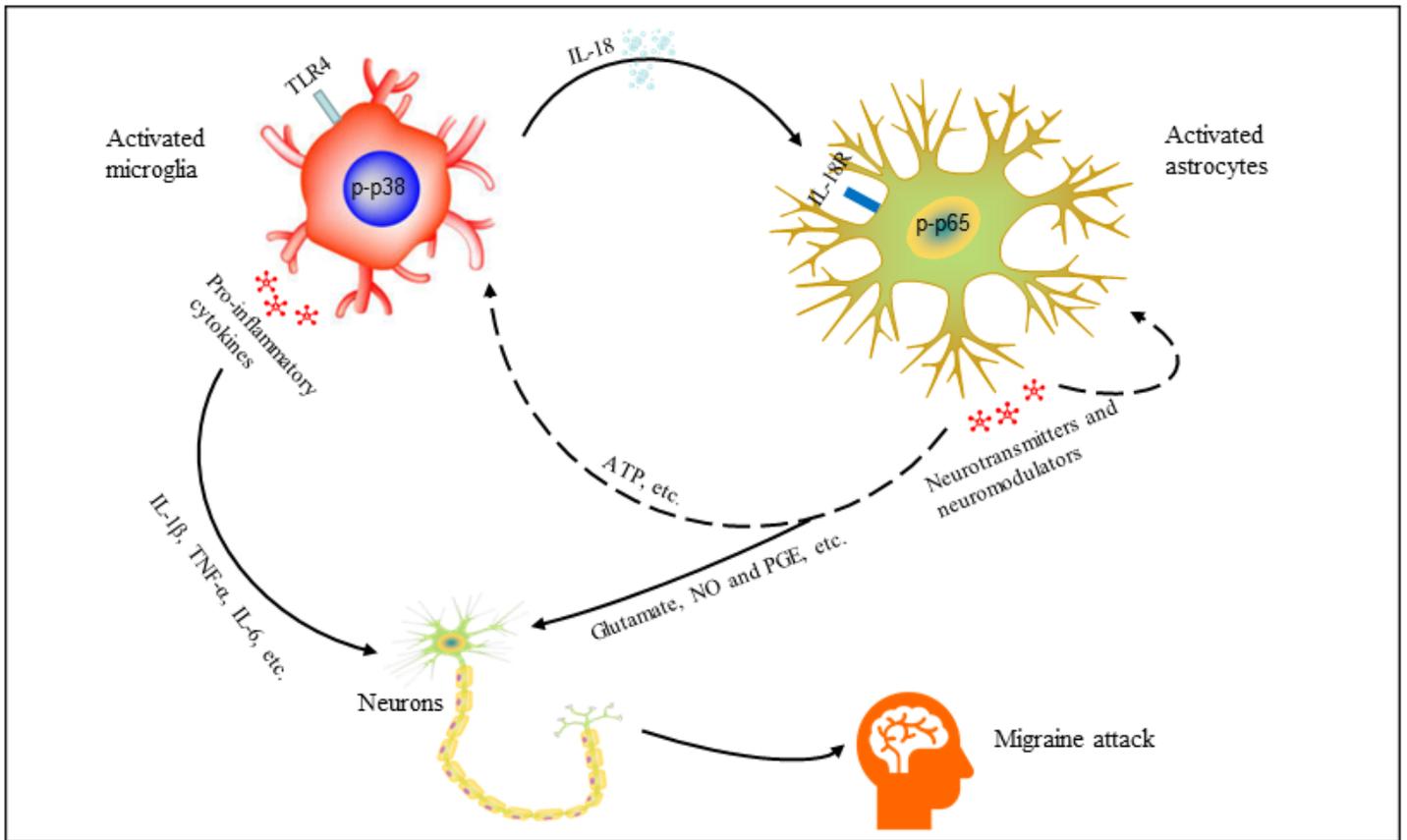


Figure 1

Schematic illustration of IL-18-mediated microglia/astrocyte interaction in the in the medullary dorsal horn in experimental migraine. First, IS dural infusions induced activated microglia, synthesizing and releasing IL-18 via TLR/ p38MAPK pathway. Next, microglia-derived IL-18 bound to IL-18R, mediating astroglial activation via a NF-κB-dependent manner. Then, activated microglia and astrocytes synthesized and released pro-inflammatory cytokines, neurotransmitters and neuromodulators, amplifying neuronal excitability and inducing migraine attacks.

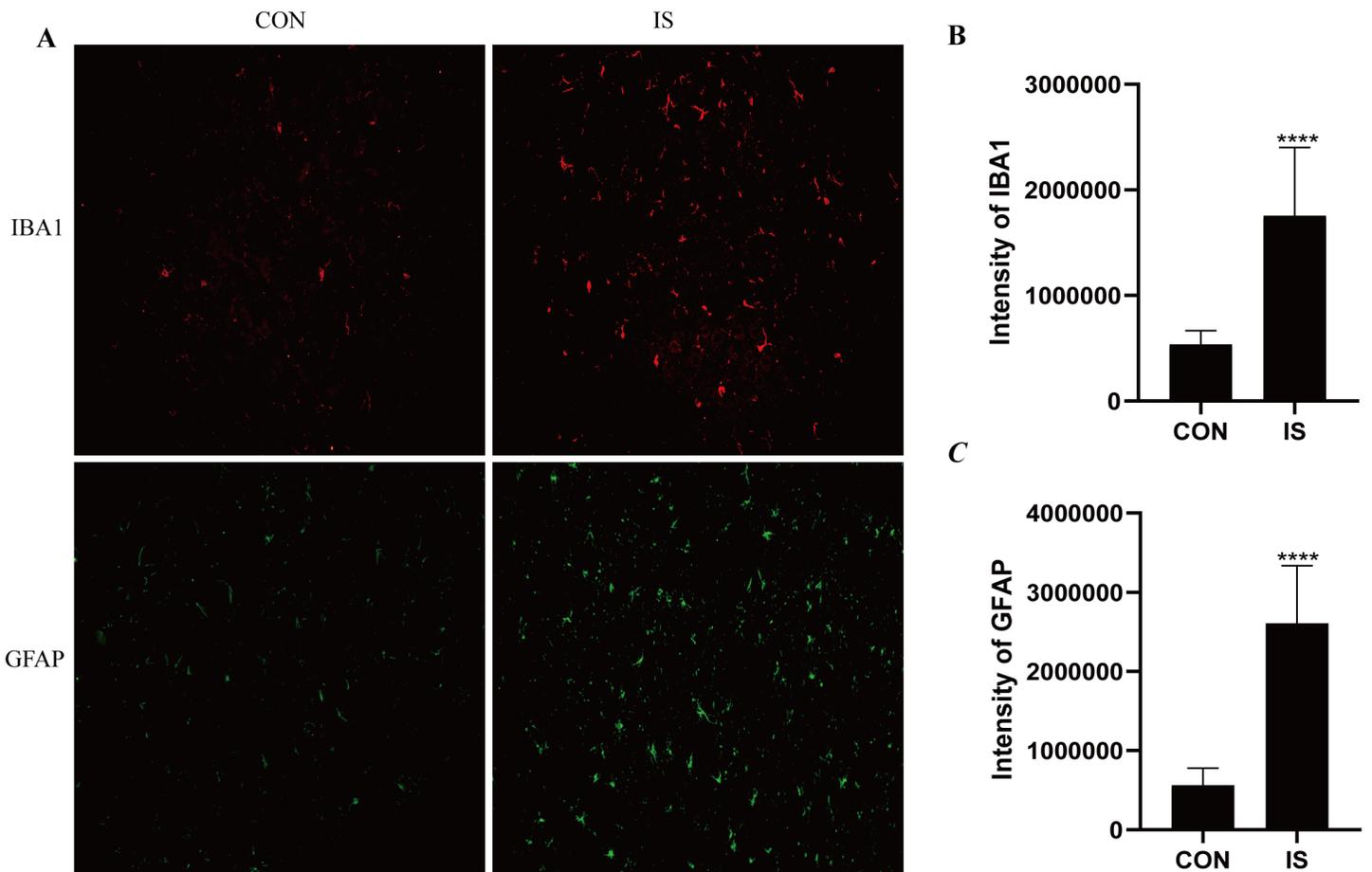


Figure 2

IS dural infusions induced the activation of microglia and astrocyte in the medullary dorsal horn. A, Immunofluorescence of IBA1(a microglial marker) and GFAP (an astroglial marker) expression in the medullary dorsal horn. C, Immunofluorescence intensity of IBA1 determined as the total pixel density in the medullary dorsal horn. D, Immunofluorescence intensity of GFAP determined as the total pixel density in the medullary dorsal horn. Values given were the mean \pm SD. **** $p < 0.0001$ versus control group. **** $p < 0.0001$ versus control group.

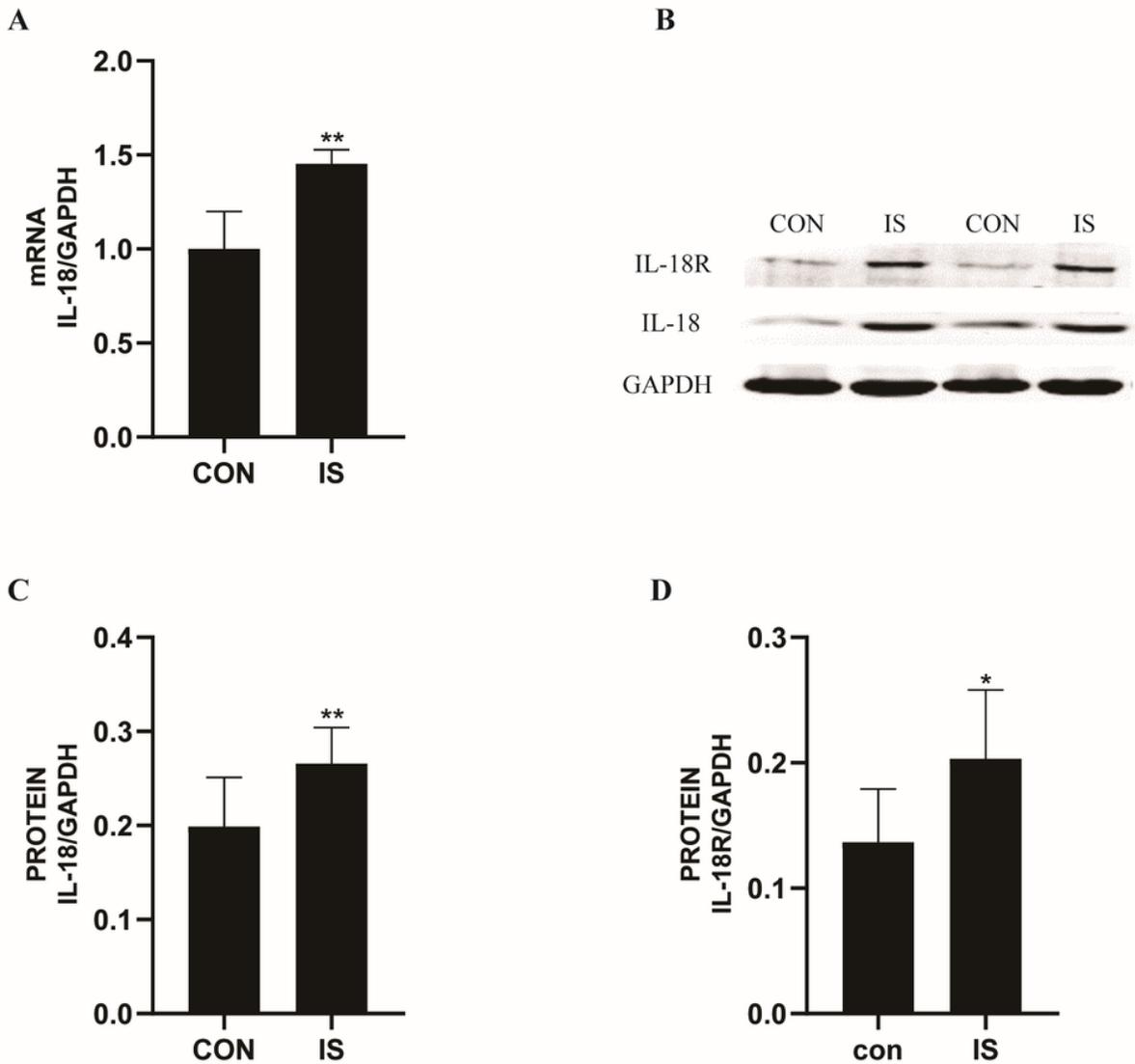


Figure 3

IS dural infusions induced the upregulation of IL-18/IL-18R in the medullary dorsal horn. A, qRT-PCR analysis showing that IS dural infusions induced the upregulation of IL-18 mRNA. B, Western blot analysis of the IL-18R and IL-18 protein levels in the medullary dorsal horn. C, Quantification data showing that IS dural infusions enhanced the IL-18 protein level in the medullary dorsal horn. D, Quantification data showing that IS dural infusions enhanced the IL-18R protein level in the medullary dorsal horn. Values given were the mean \pm SD. ** $p < 0.01$ versus control group. * $p < 0.05$ versus control group.

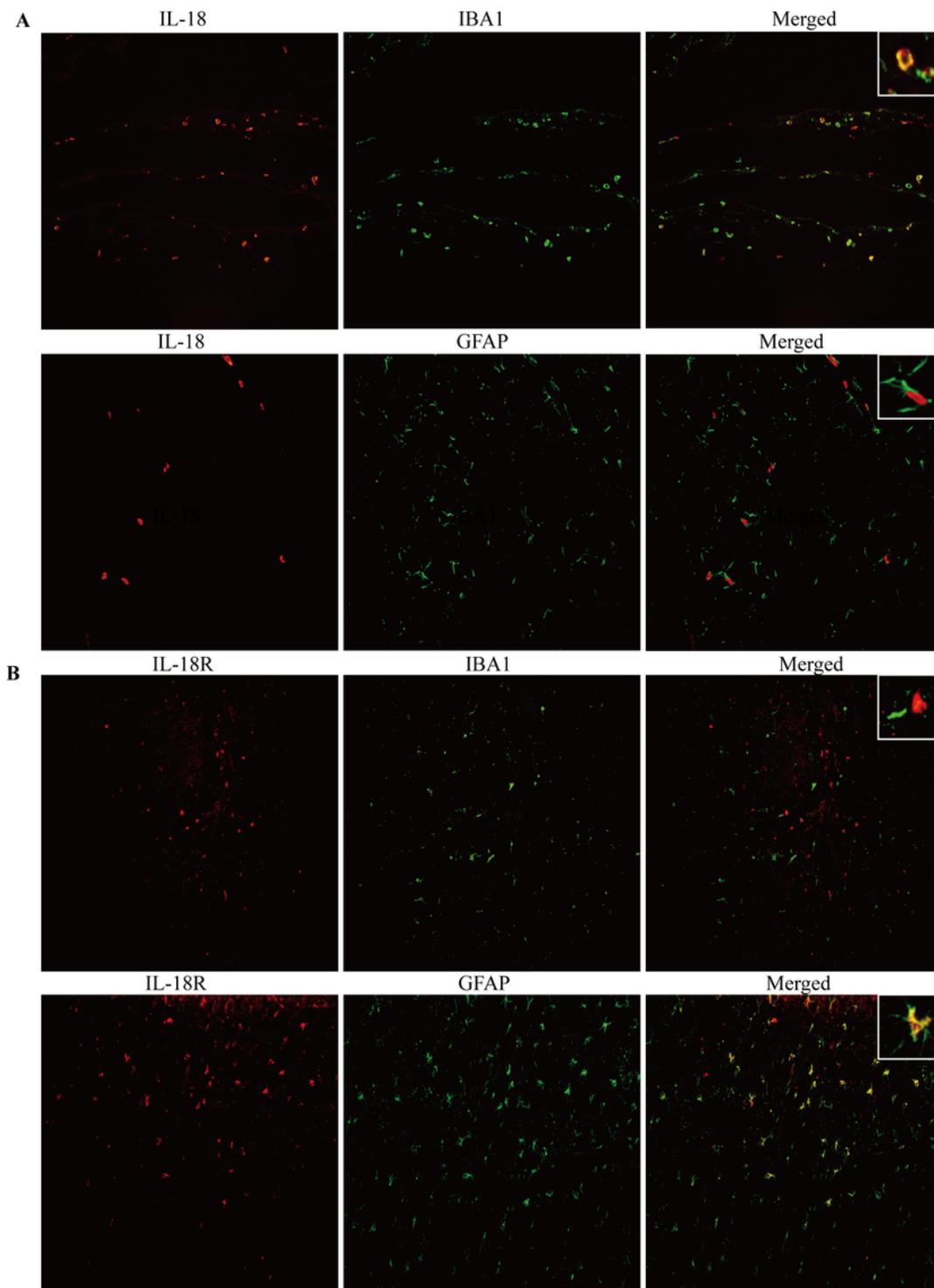


Figure 4

IL-18 expressed in microglia, IL-18R expressed in astrocyte. A, Double immunofluorescence reveals that IL-18 was expressed in Iba1-labeled microglia, but not in GFAP-labeled astrocyte. B, Double immunofluorescence reveals that IL-18R was expressed in GFAP-labeled astrocyte, but not in Iba1-labeled microglia.

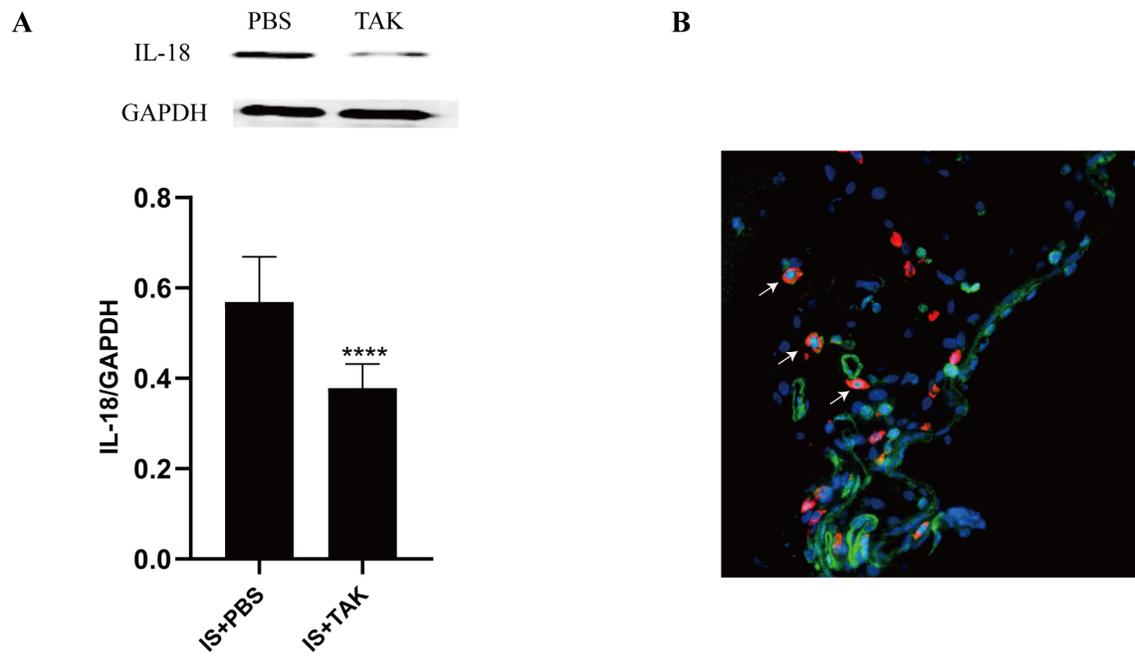
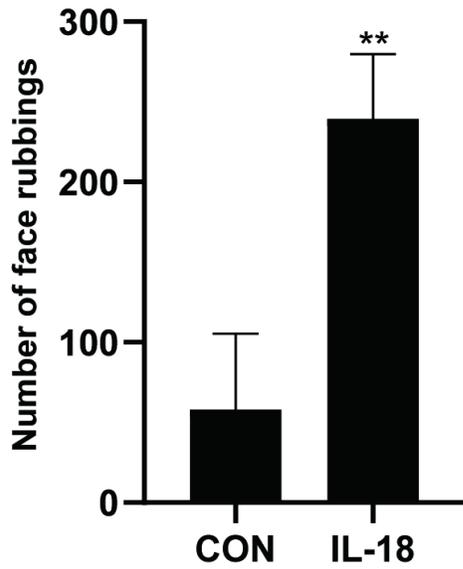
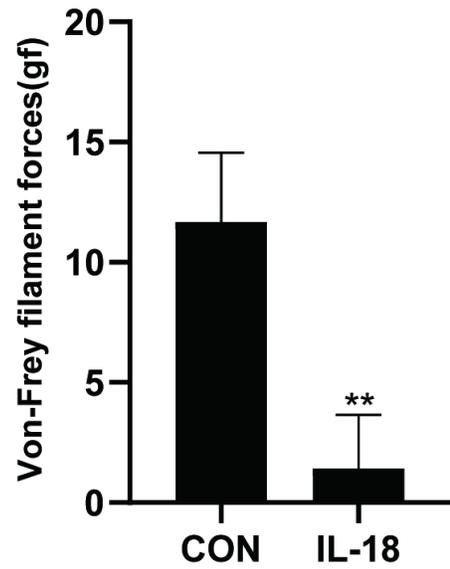


Figure 5

IS dural infusions induced microglia-derived IL-18 upregulation via TLR4 in the medullary dorsal horn. A, Western blot analysis of the IL-18 protein levels in the medullary dorsal horn and quantification data showing that blockade of TLR4 by TAK (a specific TLR4 antagonist) significantly suppressed IS dural infusions-induced IL-18 in the medullary dorsal horn. Values given were the mean \pm SD. **** $p < 0.0001$ versus control group. B. Double immunofluorescence showing p-p38 (green) entries into nucleus (blue) of IL18-immunopositive cells (red).

A**B****Figure 6**

IL-18 dural infusions produced nociceptive behavior. A, Analysis of the number of face rubbings showing a significantly increase in the IL-18 group. B, Analysis of the von Frey filament forces showing a significantly decrease in the IL-18 group. Values given were the mean \pm SD. ** $p < 0.01$ versus control group.

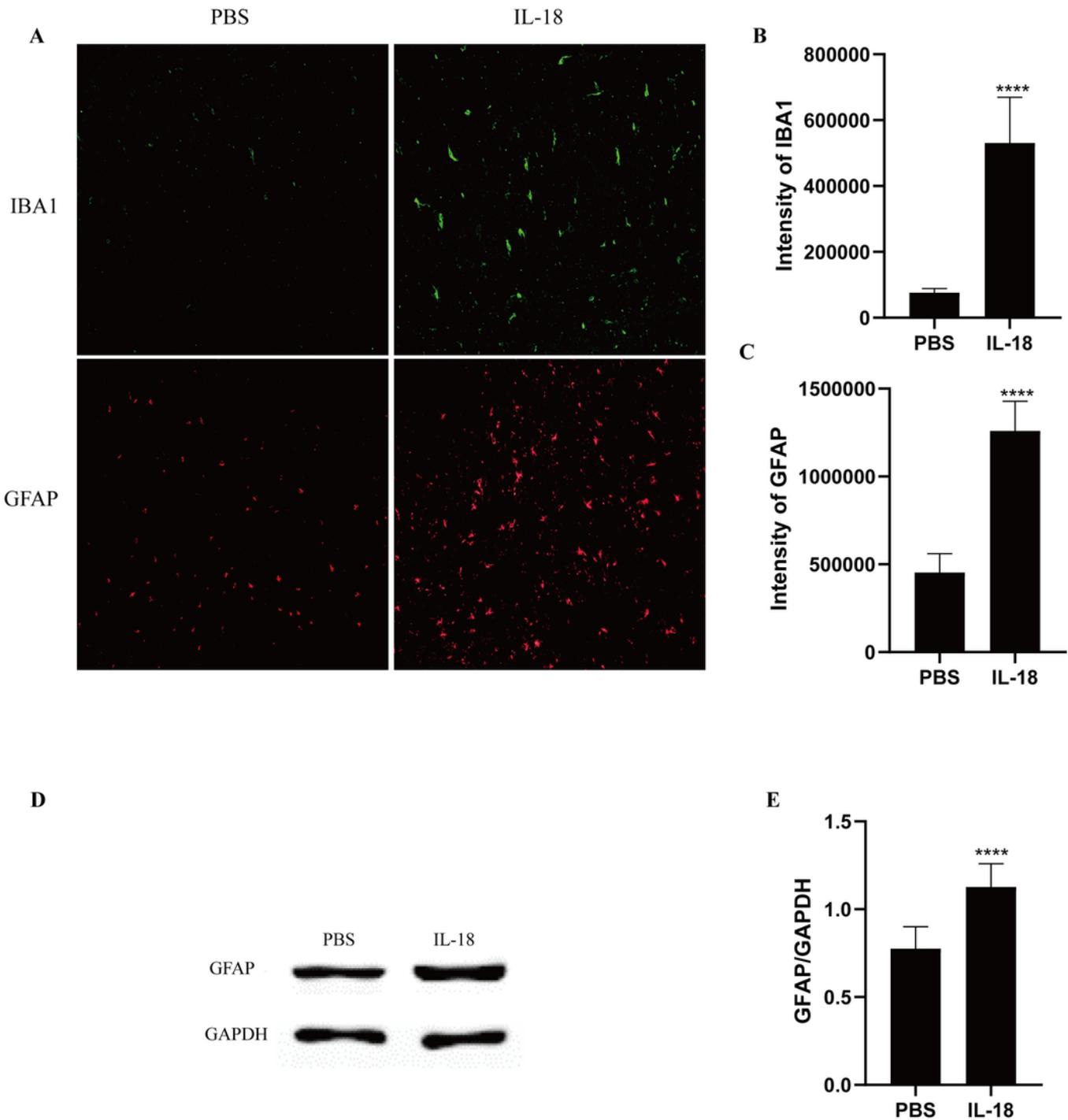


Figure 7

IL-18 dural infusions induced the activation of microglia and astrocyte in the medullary dorsal horn. A, Immunofluorescence of IBA1(a microglial marker) and GFAP (an astroglial marker) expression in the medullary dorsal horn. B, Immunofluorescence intensity of IBA1determined as the total pixel density in the medullary dorsal horn. C, Immunofluorescence intensity of GFAP determined as the total pixel density in the medullary dorsal horn. D, Western blot analysis of the GFAP protein levels in the medullary dorsal

horn. E, Quantification data showing a significantly increase of GFAP in the IL-18 group. Values given were the mean \pm SD. **** $p < 0.0001$ versus control group.

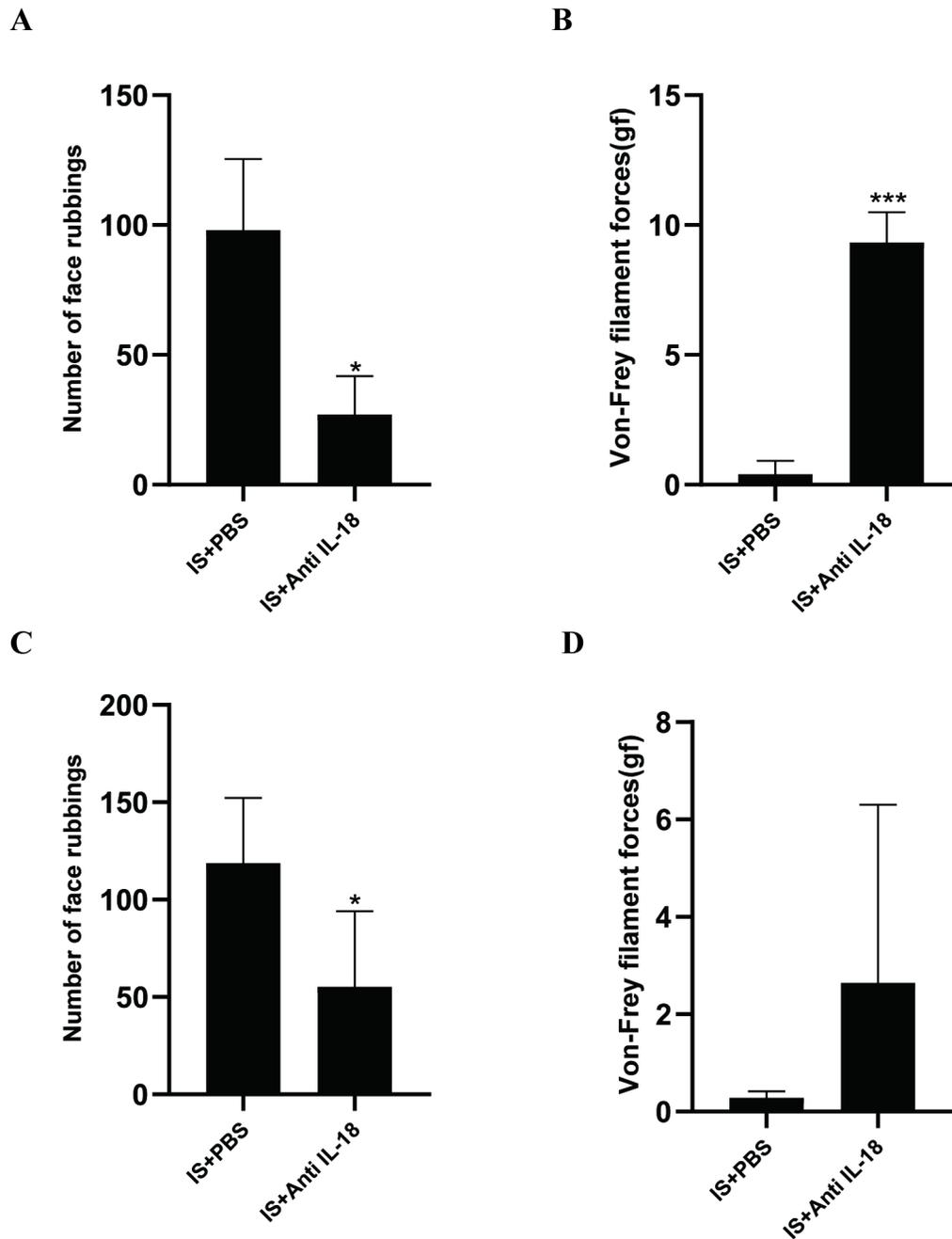


Figure 8

IL-18 blockade attenuated nociceptive behavior induced by repeated IS dural infusions. A, Analysis of the number of face rubbings showing a significantly decrease in the group pretreated with anti-IL-18 antibody (i.p). B, Analysis of the von Frey filament forces showing a significantly increase in the group pretreated

with anti-IL-18 antibody (i.p). C, Analysis of the number of face rubbings showing a significantly decrease in the group with posttreatment of anti-IL-18 antibody (i.c.v). Values given were the mean \pm SD. * $p < 0.05$ versus control group. *** $p < 0.001$ versus control group. D, Analysis of the number of the von Frey filament forces showing no significantly decrease in the group with posttreatment of anti-IL-18 antibody (i.c.v). Values given were the mean \pm SD. $P > 0.05$.

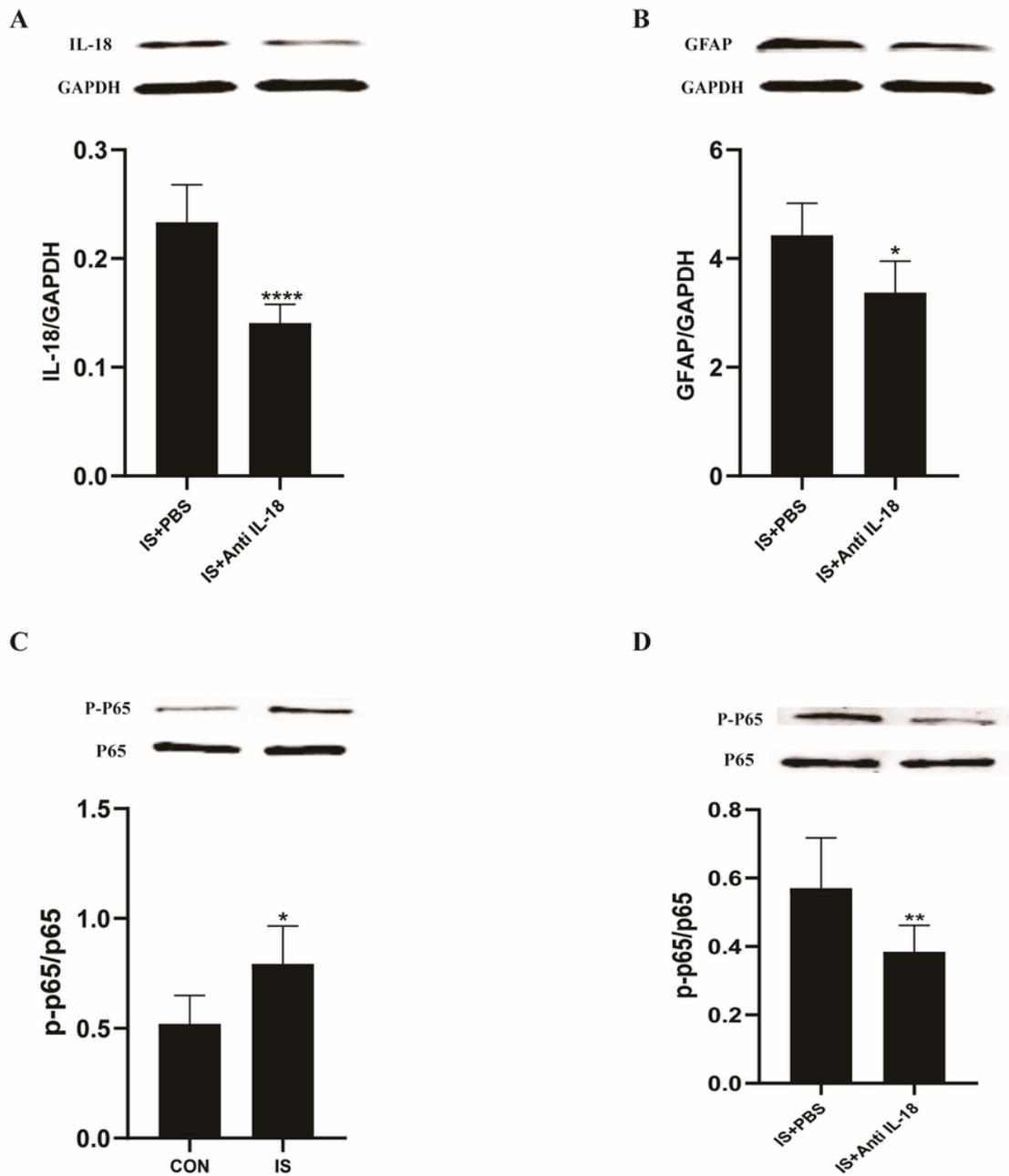


Figure 9

Microglia-derived IL-18 in the medullary dorsal horn mediated astroglial activation via a NF- κ B-dependent manner. A, Western blot analysis of IL-18 showing a significantly decrease in the group pretreated with anti-IL-18 antibody. B, Western blot analysis showing that IL-18 blockade suppressed the upregulation of GFAP (an astroglial marker) caused by IS dural infusions. C, Western blot analysis of p-p65 showing a

significantly increase in the IS group. D, Western blot analysis showing that IL-18 blockade suppressed the upregulation of p-p65 caused by IS dural infusions. Values given were the mean \pm SD. **** $p < 0.0001$ versus control group. * $p < 0.05$ versus control group. ** $p < 0.01$ versus control group.