Opening K_{ATP} Channels Induces Inflammatory Tolerance and Prevents Chronic Pain

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Research

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Abstract

Background: Current treatments for chronic pain are not satisfactory, prompting a frantic search for new therapeutics and new therapeutic targets. Our previous study indicates K_{ATP} channel opener has analgesic effect, but the mechanism has not been elucidated. We speculated that K_{ATP} channel opener may increase suppressor of cytokine signaling (SOCS)-3 expression to induce inflammatory tolerance and attenuate chronic pain.

Methods: The plantar incision (PI) surgery-induced postoperative pain was performed to establish chronic pain model. Growth arrest–specific 6 $(Gas6)^{-/-}$ and $Axl^{-/-}$ mice were used for signaling research. The microglia cell line BV-2 was cultured for *in vitro* experiments.

Results: K_{ATP} channel opener significantly attenuated incision-induced mechanical allodynia in mice, associated with the up-regulated expression of SOCS3. Opening K_{ATP} channels induced the expression of SOCS3 dependent on Gas6/Axl signaling pathway in microglia. Opening K_{ATP} channels inhibits incision-induced mechanical allodynia by activating Gas6/Axl-SOCS3 signaling pathway. Opening K_{ATP} channels induces inflammatory tolerance to relieve neuroinflammation and postoperative pain.

Conclusions: We demonstrated that K_{ATP} channel opening activated Gas6/Axl/SOCS3 signaling to induce inflammatory tolerance and relief chronic pain. We explored a new target for anti-inflammatory and analgesia by regulating the innate immune system, and provide a theoretical basis for clinical preemptive analgesia.

Background

Chronic pain is a serious long-term complication developing as a result of persistent stimulation or sensitization of nociceptors due to local tissue damage from surgery, disease and acute injury[1]. However, currently treatments for chronic pain rarely lead to complete elimination of symptoms, such as the standard drugs for pain management, nonsteroidal anti-inflammatory drug (NSAID), morphine, opioid and their derivatives show poor clinical outcome in treating chronic pain, along with severe side effects[2]. Thus, it is urgent to investigate new treatment.

Accumulating studies have indicated central sensitization, an essential mechanism underlying the increased excitability of nociceptive pathways[3], plays a crucial part in the development of chronic pain, following neuroinflammation major induced by glia, especially microglia [4-6]. As the resident cells of central innate immune system, activated microglia produce proinflammatory cytokines to improve the chronic neuroinflammation, which further activates microglia, creating a vicious cycle of inflammation to microglial activation to inflammation [7] and contributing to chronic pain states. These prompt that inhibition of neuroinflammatory and microglia seems to be efficacious.

Numerous pathophysiological adaptations are found to ease over-exuberant inflammation. Endotoxin tolerance is a classic phenomenon characterized by a progressive hyporesponsiveness to endotoxin following a first exposure to low concentrations of endotoxin [8, 9]. Whether this mechanism can be used for pain relief is rarely reported.

The ATP-sensitive potassium channel (K_{ATP} channel) is inhibited by intracellular ATP, plays key physiological roles in many tissues [10, 11]. Previous studies have shown that the loss of K_{ATP} channels current in DRG and Schwann cells can increase cell membrane excitability and cause neuropathic pain [12, 13]. Iptakalim (K_{ATP} openner) inhibit the activation of BV-2 cells and nerve destruction [14]. Our previous research demonstrated that preadministration of K_{ATP} channel opener cromakalim could improve gap junctions' function and attenuate CCI-induced neuropathic pain, but the mechanism has not been elucidated [15]. These above studies suggested that opening K_{ATP} channels before surgery may be a preemptive analgesic strategy for chronic pain.

Suppressor of cytokine signaling (SOCS)-3, a "powerful brake" of cytokine signaling pathways[16], negatively regulates inflammatory response via inhibiting TLR4, IL-1R and TNF-R signaling [17, 18]. SOCS3 overexpression could prevent the development of rheumatoid arthritis in mouse models [19], and is effective for various types of inflammation and septic shock [20]. We speculated that upregulation of SOCS3 by K_{ATP} channel opener may be beneficial for abirritation through mechanisms similar to endotoxin tolerance.

Here we provide the first evidence that opening K_{ATP} channel increases SOCS3 expression via Axl (an efferocytosis receptor)-mediated signaling to induce inflammatory tolerance and prevent chronic pain.

Methods

Ethical 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 Nanjing Medical University Animal Care and Use Committee and were approved by the Ethics Committee of Nanjing Medical University (No. IACUC-1908026).

Chemicals and Reagents

Cromakalim (Catalog No. 94470-67-4), Minoxidil (Catalog No.38304-91-5), Diazoxide (Catalog No. 364-98-7), Nicorandil (Catalog No. 65141-46-0), LPS (Catalog No. SMB00610), IL-1β (Catalog No. SRP8033) and IL-6 (Catalog No. SRP3330) was purchased from Sigma-Aldrich (St. Louis, MO, USA). R428 (HY-15150) was purchased from MedChemExpress (Pudong New Area, Shanghai, China). Recombinant Mouse Gas6 Protein (rGas6, Catalog No. 986-GS-025/CF). Kir6.1 small interfering RNA (siRNA) designed and constructed by GenePharma Corporation (Shanghai, China). SOCS3 siRNA (Catalog No. sc-41001),

Axl siRNA (Catalog No. sc-29770), Gas6 siRNA (Catalog No. sc-35451) and Control siRNA (Catalog No. sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The lentiviral vectors (LV-SOCS3 or LV-EGFP) were purchased from Obio Technology (Shanghai) Corp.,Ltd. Antibody for Axl (Catalog No. ab215205), IBA-1 (Catalog No. ab178847) SOCS3 (Catalog No. ab16030) was purchased from Abcam (Cambridge, MA, USA). DAPI (Catalog No. D9542) was purchased from Merck Corporation (Darmstadt, Germany). Antibody for c-Fos (Catalog No. 2250), CGRP (Catalog No. 14959), Gas6 (Catalog No. 67202), p-Axl (Catalog No. 44463), STAT3 (Catalog No. 12640) and p-STAT3(Catalog No. 9145) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody for Transferrin (Catalog No. 17435-1-AP) were purchased from proteintech Corporation. Antibody for β-actin (Catalog No. A1978) was purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco, and other cell culture media and supplements were purchased from KenGEN (KenGEN BioTECH, China). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals and treatment

Adult male C57BL/6J mice (18-22 g) at 8 weeks of age were provided by the Experimental Animal Center at Nanjing Medical University, Nanjing, China. Animals had free access to food and water and were housed in groups of five to six per cage under pathogen-free conditions with soft bedding under controlled temperature (22 ± 2 °C) and a 12-h light/dark cycle (lights on at 8:00 a.m.). All procedures were conducted in accordance with the guidelines and regulations of the National Institutes of Health (NIH) and were approved by the Ethics Committee of Nanjing Medical University (No. IACUC-1908026).

Surgery

The plantar incision surgery was performed as previously described (Brennan et al., 1996). All surgeries were done under anesthesia induced by 1 % sodium amobarbital. The plantar aspect of the left hind paw was sterilized with a 10% povidone-iodine solution before and after surgeries and was placed through a hole in a sterile drape. A 1-cm longitudinal incision was made through skin and fascia of the plantar aspect of the foot, starting 0.5 cm from the proximal edge of the heel and extending toward the toes. The plantaris muscle was elevated and longitudinally incised through, leaving muscle origin and insertion intact. After hemostasis with gentle pressure, the skin was opposed with 2 mattress sutures of 5-0 nylon. The animals were allowed to recover. The incision was checked daily, and any sign of wound infection or dehiscence excluded the animal from the study.

Behavioral analysis

Animals were habituated to the testing environment daily for at least 5 days before baseline testing. Mechanical withdrawal threshold was detected by Von Frey Hairs (Woodland Hills, Los Angeles, CA, USA) test. Animals were placed in boxes set on an elevated metal mesh floor and were allowed 30 min for habituation before testing. The plantar surface of each hind paw was stimulated with a series of von Frey hairs with logarithmically incrementing stiffness perpendicularly to the plantar surface. Each mouse was tested for three times and the average of the threshold was measured.

Cell preparation and treatment

BV-2 cells were purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's Medium (DMEM, KenGEN Bio TECH, China) supplemented with 10% (v/v) FBS (Gibco), penicillin (100 U/ml), and streptomycin (100 U/ ml). All cells were kept in a humidified chamber with 5% CO_2 at 37 °C. For further experiments, BV-2 cells were seeded in 6-well plate at a density of 1 x 10^5 cells/well. After 24 hours, cells were treated with different tool drugs, and then collected the cells and supernatants to detect the expression SOCS3, Gas6, etc.

Quantitative PCR

Quantitative PCR was performed on BV-2 cell samples and on spinal cord samples obtained from mice. Total RNA was isolated by a standard method with TRIZOL reagent (Invitrogen Life Technologies). Isolated RNA was reverse-transcribed into cDNA using PrimeScript™ RT Reagent Kit (TaKaRa) following standard protocols. Real-time quantitative PCR (qPCR) was performed with synthetic primers and SYBR Green (TaKaRa) with a QuantStudio 5 Real-Time PCR Detection System (Thermo Fisher Scientific). The relative expression level of SOCS3 and SOCS1 was calculated and quantified with the $2^{-\Delta\Delta Ct}$ method after normalization with the reference β-actin. All primers used are listed as follows:

SOCS1: Forward CTGCGGCTTCTATTGGGGAC

Reverse AAAAGGCAGTCGAAGGTCTCG;

SOCS3: Forward GCGGGCACCTTTCTTATCC

Reverse CTGGAGGCGGCATGTAGTG;

β-actin: Forward GGCTGTATTCCCCTCCATCG

Reverse CCAGTTGGTAACAATGCCATGT

Western blotting

Samples (cells or spinal cord) were collected and washed with PBS before being lysed in radio immunoprecipitation assay (RIPA) lysis buffer. The protein concentrations were determined by BCA Protein Assay (Thermo Fisher, Waltham, MA, USA) and 40-80 μg of proteins were loaded and separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA, USA). The membranes were blocked with 5% bovine serum albumin for 2 h at room temperature, probed with antibodies overnight at 4 °C with the primary antibodies and then incubated with HRP-coupled secondary antibodies. The primary antibodies used included Axl (1:1000), p-Axl (1:1000), Gas6 (1:1000), SOCS3 (1:1000), p-STAT3 (1:1000), STAT3 (1:1000), and Transferrin (1:1000). For loading control, the blots were probed with antibody for β-actin (1:1000). The filters were then developed by enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA) with secondary

antibodies (Chemicon, Billerica, MA). Data were acquired with the Molecular Imager (Gel DocTM XR, 170-8170) and analyzed with Quantity One-4.6.5 (Bio-Rad Laboratories, Berkeley, CA, USA).

Immunofluorescence assay

After deep anesthesia, the animals were perfused transcardially with normal saline followed by 4% paraformaldehyde in 0.1 M PB, pH 7.4, each for 20 min. Then L4 and or L5 lumbar segment was dissected out and post-fixed in 4% paraformaldehyde. The embedded blocks were sectioned as 25 μm thick. Sections from each group (five animals in each group) were incubated with rabbit antibodies for c-Fos (1:200), CGRP (1:200) and SOCS3 (1:200), goat antibodies for IBA-1 (1:200). Then the free-floating sections were washed with PBS, and incubated with the secondary antibody (1:300, Jackson Laboratories, USA) for 2 h at room temperature. After washing out three times with PBS, the samples were studied under a confocal microscope (Olympus FV1000 confocal system, Olympus, Japan) for morphologic details of the immunofluorescence staining. Examination was blindly carried out.

NF-κB Activation Assay

BV-2 cells were plated in class bottom cell culture dishes and pretreated with cromakalim (100 μM) 6 h, and then co-cultured with LPS (1 μg/ml) or IL-6 (10 ng/ ml) for 3 h. BV-2 Cells were fixed with 4% paraformaldehyde for 30 min, and then fixed with ice-cold methanol and were permeabilized with 0.25 % Triton X-100/PBST. After blocking with 1 % bovine serum albumin (BSA) in PBST for 1 h, the coverslips with BV-2 cells were incubated for 2 h at room temperature with the p65/RelA antibody diluted in 1 % BSA (1:50). Then the coverslips were exposed to the fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:100, at room temperature for 1 h) and then were rinsed three times with PBS. Finally, the coverslips were stained with 1 μg/ml DAPI (4',6-diamidino-2-phenylindole, a fluorescent DNA dye to mark nucleus) for 1 min. Confocal microscopy analyze was carried out using Olympus FV1000 confocal system

RNA interference

3.3 nmol siRNA was dissolved in 330 μl RNase-free water. Control siRNA was used as a negative control. For the transfection of siRNA, cells were cultured in six-well plates with antibiotic-free medium the day before transfection. The transfection was conducted when cells reached 60~80% confluence using Lipofectamine 2000 (Invitrogen, USA) and serum-free medium according to the manufacturer's instructions. After 6 h, the transfection medium was replaced with the culture medium containing 10% FBS and then incubated at 37 °C in 5% CO_2. For the animals' experiments, *i.t.* administration of siRNA into mice for 48 h just before plantar incision surgery. The mechanical thresholds were measured to determine whether interference with SOCS3, Gas6 and Axl genes could abolish the analgesia of cromakalim.

SOCS3 overexpression Assay

The lentiviral vectors (LV-SOCS3 or LV-EGFP) were purchased from Obio Technology (Shanghai) Corp.,Ltd. The titer of lentiviral vectors (LV-SOCS3) was 3.00E + 10 PFU/ml. LV-EGFP is as a negative

control. 10 μl of lentiviral vectors (LV-SOCS3 or LV-EGFP) was intrathecally injected into mice three days before plantar incision surgery.

Statistical analyses

GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA) was used to conduct all the statistical analyses. Alteration of expression of the proteins detected and the behavioral responses were tested with one-way ANOVA, and the differences in latency over time among groups were tested with two-way ANOVA. Bonferroni post hoc tests were conducted for all ANOVA models. Results were represented as mean ± SEM of three independent experiments. $P < 0.05$ was deemed to be statistically significant.

Results

Opening K_{ATP} channels significantly attenuates incision-induced mechanical allodynia in mice

To verify the effect of K_{ATP} opener on chronic pain, the plantar incision surgery-induced postoperative pain was performed to establish chronic pain model. The results showed that intrathecal (*i.t.*) administration of different doses of K_{ATP} channel opener cromakalim (2.5 or 5 μg/ 10 μl) significantly attenuated plantar incision-induced mechanical allodynia (Fig. 1A). Besides, we also found that cromakalim (5 μg/ 10 μl, *i.t.*) could reduce the activity of MMP-2 and MMP-9 in mice subjected to plantar incision (Fig. 1B). Moreover, immunofluorescence results also found *i.t.* administration of cromakalim (5 μg/ 10 μl) also markedly reduced the expression of c-Fos and CGRP (pain markers) in spinal cord in mice (Fig. 1C and D). These data suggested that opening K_{ATP} channels can alleviate chronic pain.

Opening K_{ATP} channels induces the expression of SOCS3 in microglia

Our previous studies have found that preemptive inducing the up-regulation of SOCS3 in spinal cord can relieve neuropathic pain [21]. Here, we further explore the relationship among K_{ATP}-SOCS3, inflammatory tolerance and postoperative pain. Firstly, *i.t.* administration of different doses of cromakalim (2.5 and 5 μg / 10 μl) in mice, and then collect the samples at different time points (2 and 4 h) for real-time quantitative PCR testing, and it was found that cromakalim (2.5 and 5 μg) could significantly increase the SOCS3 mRNA level (Fig. 2A), but not SOCS1 mRNA level in spinal cord in mice (Fig. 2B). Confocal images also indicated that cromakalim (5 μg / 10 μl) could promote the up-regulation of SOCS3 protein in microglia in mice (Fig. 2C). Moreover, further study has found that BV-2 cells treatment with cromakalim (100 μM) for 2 or 4 h could induce an increase in SOCS3 mRNA levels *in vitro* (Fig. 2D), but had no effect on SOCS1 (Fig. 2E). Western blot results also indicated that *i.t.* administration of cromakalim (5 μg/ 10 μl) for 4 or 6 h could significantly promote the expression of SOCS3 in the spinal cords *in vivo* (Fig. 2G), as well as in BV-2 cells *in vitro* (Fig. 2F and 2H). We further investigated whether cromakalim induce the up-regulation of SOCS3 *in vitro* and *in vivo* is through opening K_{ATP} channels, or it is only the specific

pharmacodynamic effect of cromakalim. Three K_{ATP} channel openers, Minoxidil (30 μM), Diazoxide (200 μM) and Nicorandil (1 mM) were added into BV-2 cells respectively, and then after 6 h, collected the samples for western blots, and the results showed that all the three K_{ATP} channel openers can increase the expression of SOCS3 in BV-2 cells *in vitro* (Fig. 2I), while conditionally knockout the Kir6.1 channel can reverse the effect of cromakalim on BV-2 cells *in vitro* (Fig. 2J). These data suggest that opening K_{ATP} channels can significantly increase the expression of SOCS3 *in vitro* and *in vivo*.

Opening K_{ATP} channels inhibits incision-induced mechanical allodynia by up-regulating SOCS3 expression

As shown in Figure 1, we have demonstrated that *i.t.* administration of cromakalim can produce a preemptive analgesia state in mice. Here, we investigate whether local application of lentiviral-mediated SOCS3 overexpression could induce preemptive analgesia in mice. Three days before the plantar incision modeling, the lentiviral vectors (LV-SOCS3 and LV-EGFP) were injected into the spinal cord of mice by *i.t.* injection, and the western blot were used to detect the expression of SOCS3 in spinal cord. Results showed that *i.t.* administration of lentiviral vectors (LV-SOCS3) three days before plantar incision surgery markedly promoted the expression of SOCS3 in spinal cords (Fig. 3A) and inhibited plantar incision-induced mechanical allodynia in mice (Fig. 3B). These indicated that cromakalim is capable of producing analgesic effects similar to those of SOCS3 overexpressing lentiviral vectors. Moreover, we further investigate whether the analgesic effect of cromakalim depends on the expression of SOCS3 in spinal cord in mice. Alternatively, *i.t.* administration of SOCS3 shRNA 48 h before surgery could significantly inhibit the analgesic effect of cromakalim (Fig. 3D) by interfering with SOCS3 expression in the spinal cord (Fig. 3C). Taken together, these results suggest that opening K_{ATP} channels could markedly inhibit plantar incision-induced mechanical allodynia by up-regulating SOCS3 expression in spinal cord in mice.

Opening K_{ATP} channels induces the expression of SOCS3 dependent on Gas6/Axl signaling pathway in vitro

Previous studies have found that TAM (Tyro3/Axl/Mer) receptors can be activated when they in response to the TAM ligands (Gas6 and ProS), and inducing the expression of SOCS3 protein [22, 23]. Here, the elisa kit were used to detect the expression level of Gas6 in the supernatant of BV-2 cells at different time (0, 0.5, 1, 2 h) after cromakalim (100 μM) treatment. It was shown that the protein concentration of Gas6 were quickly increased at 0.5 h and peaked during the 1 hour after cromakalim treatment (Fig. 4A). Additionally, cromakalim can also induce the activation of Axl receptor *in vitro* (Fig. 4B). Our results also found that the exogenous recombinant Gas6 (rGas6) protein (500 ng/ml) treatment with BV-2 cells for 2 h can also significantly induce the activation of Axl receptor (Fig. 4C) and the up-regulation of SOCS3 protein *in vitro* (Fig. 4D), whereas these effects can be abrogated by the Axl inhibitor (R428, 1 μM) (Fig. 4D). Moreover, pretreatment with Axl inhibitor (R428, 1 μM) also markedly inhibited the effect of cromakalim on SOCS3 in BV-2 cells *in vitro* (Fig. 4E). Both Axl siRNA and Gas6 siRNA can also significantly cancel cromakalim-mediated the up-regulation of SOCS3 *in vitro* (Fig. 4F). The data

indicated that opening K_{ATP} channels induces the up-regulation of SOCS3 dependent on Gas 6/Axl signaling pathway.

Opening K_{ATP} channels inhibits incision-induced mechanical allodynia by activating Gas6/Axl-SOCS3 signaling pathway

As shown in Fig. 5A, mice were treatment with cromakalim (5 μg / 10 μl, *i.t.*) for 6 h before plantar-incision surgery, and then the von Frey hairs were used to detect the mechanical threshold, the results showed that cromakalim significantly inhibited incision-induced mechanical allodynia in mice, while knocking out Gas6 gene could abolish the analgesic effect of cromakalim. Moreover, we also found that *i.t.* administration of Axl inhibitor (R428, 1 μM) or knockout Axl gene both can cancel the analgesic effect of cromakalim in mice (Fig. 5B and C). Further studies also found that cromakalim (5 μg / 10 μl, *i.t.*) could increase the level of Gas6 in cerebrospinal fluid (Fig. 5D), and induce the activation of Axl in spinal cord in mice (Fig. 5E). We also investigated whether *i.t.* administration of cromakalim can induce the up-regulation of SOCS3 in the spinal cord of Gas6 and Axl knockout mice. The results showed that, compared with wild-type mice, both knocking out Gas6 or Axl could significantly reverse cromakalim-mediated the up-regulation of SOCS3 (Fig. 5F and G). These data suggest that *i.t.* administration of K_{ATP} channel opener (cromakalim) can alleviate postoperative pain by activating the Gas6/Axl-SOCS3 pathway.

Opening K_{ATP} channels induces inflammatory tolerance to relieve neuroinflammation and postoperative pain

As shown in Fig. 6A, compared with the control group, LPS (1 μg/ ml) could significantly mediate the nuclear translocation of NF-κB p65 in BV-2 cells *in vitro*, while pretreatment with cromakalim (100 μM) 6 h before LPS treatment could reverse this effect (Fig. 6A). Cromakalim could also markedly inhibit IL-6-mediated the increase of STAT3 phosphorylation in BV-2 cells *in vitro* (Fig. 6B). Moreover, pretreatment with cromakalim (100 μM) could also inhibit IL-1β induce the nuclear translocation of NF-κB p65 *in vitro* (Fig. 6C), and alleviate IL-1β mediated mechanical allodynia in mice (Fig. 6D). The above data suggest that opening K_{ATP} channels could promote the production of SOCS3, and mediate inflammation tolerance, inhibit neuroinflammation and attenuate postoperative pain.

Discussion

In this study, our major findings are as follows: (1) K_{ATP} channel opener significantly attenuated incision-induced mechanical allodynia in mice, associated with the up-regulated expression of SOCS3. (2) Opening K_{ATP} channel inhibits incision-induced mechanical allodynia via Gas6/Axl-SOCS3 signaling pathway. (3) Opening K_{ATP} channel induces inflammatory tolerance to relieve neuroinflammation and postoperative pain.

We proposed that reopening of K_{ATP} channels regulated astroglial gap junctions in the rat spinal cord and reduced neuropathic pain [15], but the mechanism was not clarified. Recently, the close relationship between K_{ATP} channel and pain has been further verified. Melatonin relieves pain via L-arginine/nitric oxide/cyclic GMP/K_{ATP} channel signaling pathway [24], Zerumbone alleviates neuropathic pain through the involvement of L-Arginine/nitric oxide/cGMP/K_{ATP} channel pathways in chronic constriction injury in mice model [25], Our research suggested that regulation of the K_{ATP}-JNK gap junction signaling pathway by immunomodulator astragaloside IV attenuated neuropathic pain [26]. However, these studies have not clarified the analgesia mechanism of K_{ATP} opening, and provided a detailed theoretical basis for the clinical application of K_{ATP} openers. With the in-depth study of microglia's activation in neuropathic pain and the review of our previous study, we found that K_{ATP} openers could significantly inhibit the activation of microglia. Therefore, we speculated that the analgesic effect of K_{ATP} channel opener is not only related to astrocytes, but also may be related to the inflammatory state of microglia.

Firstly, we verified the analgesic effect of K_{ATP} channel openers in mice. K_{ATP} openers could significantly relieve the incision-induced chronic pain, and inhibit the expression of pain-related indicators c-Fos and CGRP in the spinal cord (Fig. 1). Considering the strong anti-inflammatory effect of SOCS3, is the abirritation effect of K_{ATP} channel openers related to SOCS3? We found that SOCS3 expression was increased and most was co-localizated with the microglia in the mice spinal cord of K_{ATP} channel openers group (Fig. 2). We further verified the importance of SOCS3 though local application of lentiviral-mediated SOCS3 overexpression or SOCS3 shRNA. Overexpression of SOCS3 inhibited plantar incision-induced mechanical allodynia in mice. Administration of SOCS3 shRNA 48 h before surgery could significantly inhibit the analgesic effect of cromakalim (Fig. 3). These results suggest that the analgesic effect of K_{ATP} channel opener is related to the high expression of SOCS3.

How does K_{ATP} channel opener induce SOCS3 expression? The TAM family (Tyro3, Axl and Mer) of receptor protein tyrosine kinases interest us, which play pivotal roles in numerus major cellular processes: cell survival and proliferation, immunomodulation and phagocytosis [27]. TAM receptor mediates efferocytosis of apoptotic cells, but also induces the activation of anti-inflammatory signaling pathways [28]. SOCS3 is an important downstream target of TAM receptor participating anti-inflammatory process [29]. Study has shown that Gas6/Axl signaling attenuates alveolar inflammation in ischemia-reperfusion-induced acute lung injury by up-regulating SOCS3-mediated pathway [30]. Our research found that K_{ATP} channel opener cromakalim could significantly increase the expression of Gas6 (a substrate of Axl). Inhibition of Gas6 and Axl decreased SOCS3 expression (Fig. 4). Knockout of Gas6 and Axl abolished the analgesic effect of cromakalim and inhibit the expression of SOCS3. These results suggest that the analgesic effect of K_{ATP} channel opener depends on the up-regulation of Gas6/Axl/SOCS3 (Fig. 5).

Finally, we investigated whether K_{ATP} channel opening could induce immune tolerance. LPS was used to establish a bacterial inflammation model. K_{ATP} channel opening could inhibit LPS-induced p65 nuclear translocation and inflammatory response. IL-6 and IL-1β were used to establish a sterile inflammatory

model, K_{ATP} channel opening significantly decreased IL-6-induced STAT3 phosphorylation, inhibited IL-1β -induced p65 nuclear translocation and pain response (Fig. 6).

Conclusions

In summary, for the first time we demonstrated that K_{ATP} channel opening activated Gas6/Axl/SOCS3 signaling to induce inflammatory tolerance and relief chronic pain (Fig. 7). We explored a new target for anti-inflammatory and analgesia by regulating the innate immune system, and provide a theoretical basis for clinical preemptive analgesia.

Abbreviations

Cro: cromakalim; DMEM: Dulbecco's modified Eagle's medium; IBA-1: anti-ionized calcium-binding adaptor protein 1; IL-1β: interleukin-1β; IL-6: interleukin-6; JNK: c-Jun N-terminal kinase; LPS: Lipopolysaccharide; MMPs: matrix metalloproteases; PI: plantar incision; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SOCS3: suppressor of cytokine signaling (SOCS)-3; STAT3: signal transducer and activator of transcription 3

Declarations

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Availability of data and materials

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

Authors' contributions

CQ and YX F designed and performed the experiments, analyzed the results, and drafted the manuscript. L-L J, LW and RM J carried out the behavioral measures, gelatin zymography and immunofluorescence. XM Q and YW carried out the western blotting analysis and RT-PCR. QW, CM and LH carried out the cell

cultures and NF-κB activation assay. LH, X-Y T and W-T L conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethical 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 Nanjing Medical University Animal Care and Use Committee and were approved by the Ethics Committee of Nanjing Medical University (No. IACUC-1908026).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

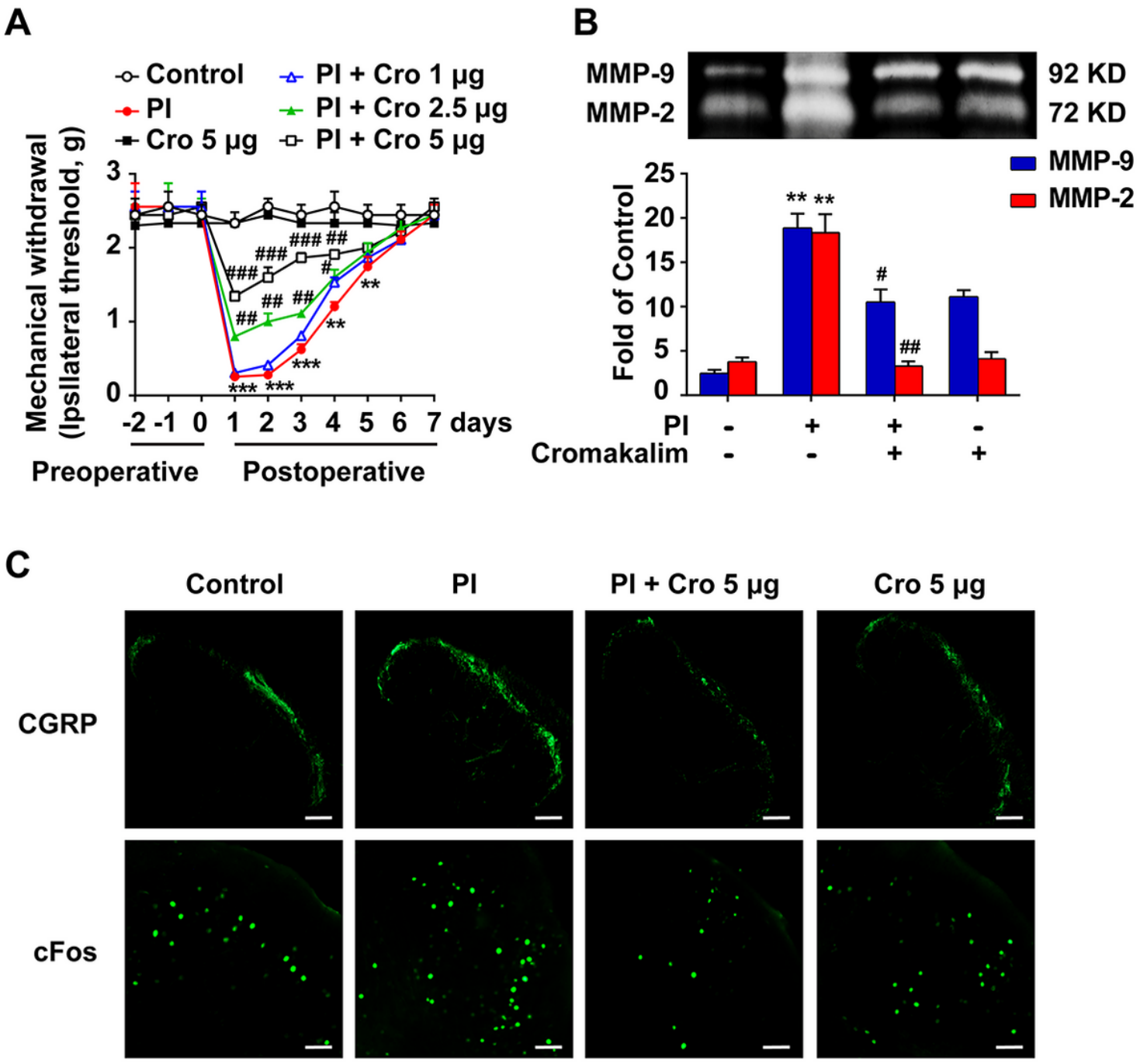

Figure 1

Opening KATP channels significantly attenuates incision-induced mechanical allodynia in mice. (A) i.t. administration of different concentration of cromakalim (1, 2.5 and 5 μg / 10 μl) 30 min before plantar-incision surgery in mice, and consecutive administration of cromakalim for 7 days, mechanical pain threshold was tested by Von Frey Hairs at 6 hours after each administration (n = 6). (B) Consecutive administration of cromakalim (5 μg/ 10 μl, i.t.) for 7 days significantly attenuated plantar incision-induced the activation of MMP-2/9 in the spinal cords in vivo. Gelatin zymography was performed to determine MMPs' activity. The spinal samples (n = 4) were collected 6 h after the last cromakalim treatment. Confocal images showing c-Fos (C), CGRP (D) in the dorsal horns (n = 4 images per animal). Magnification: 200×. Scale bar: 75 μm. Significant difference was revealed following one-way or two-way

ANOVA (**p < 0.01, ***p < 0.001 vs. naive control; #p < 0.05, ##p < 0.01, ###p < 0.001 vs. plantar incision group; Bonferroni post hoc tests).

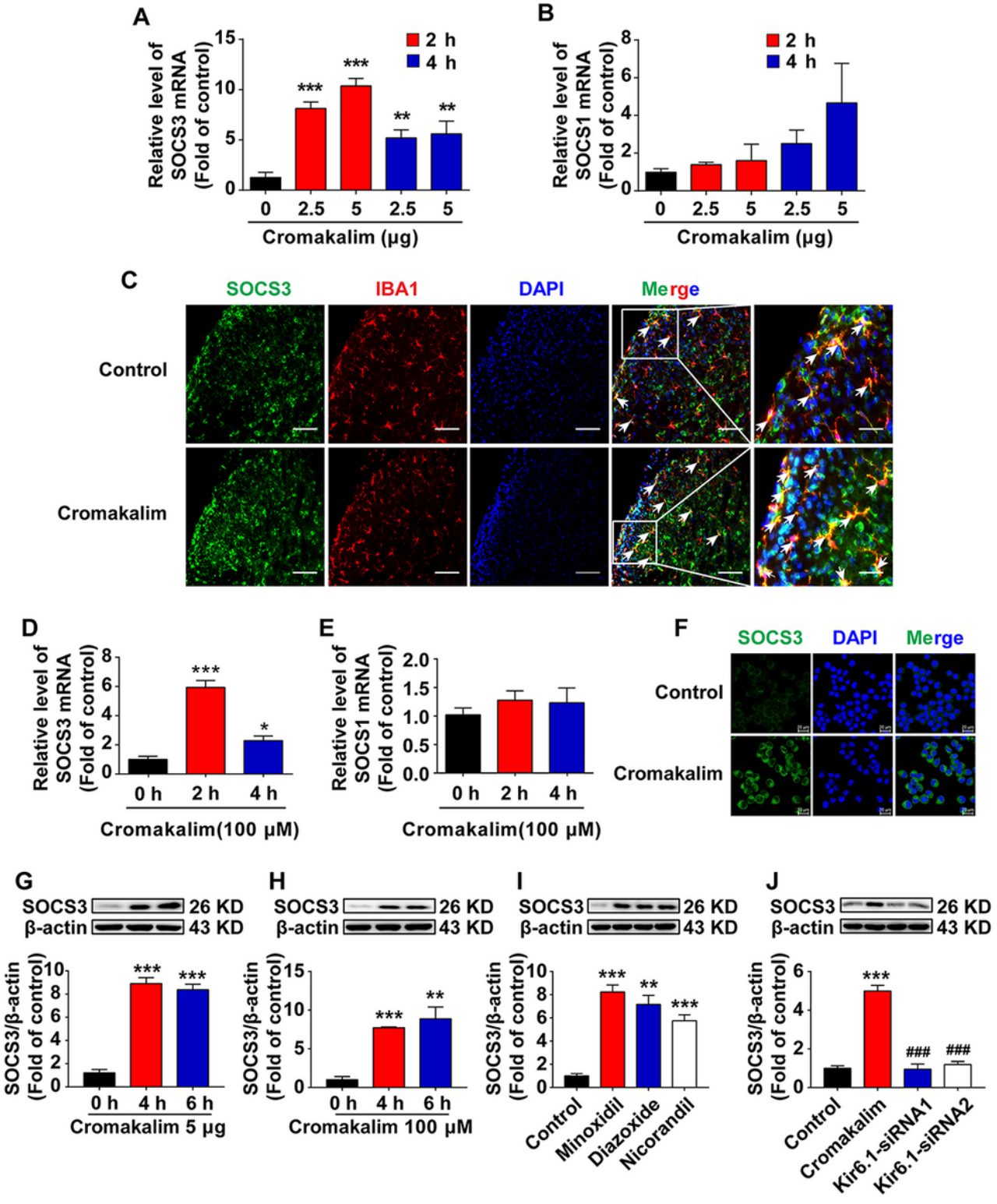

Figure 2

Opening KATP channels induces the expression of SOCS3 in microglia. (A-B) The effect of cromakalim on SOCS3 and SOCS1 mRNA level in spinal cord were detected by real-time quantitative PCR. i.t. administration of different doses of cromakalim (2.5 and 5 μg / 10 μl) into mice, and collected the spinal

samples (n = 4) at 2 or 4 h after cromakalim treatment. β-actin was used as an invariant control. (C) Confocal images of SOCS3 (green) were co-stained with microglia (IBA1, red) and DAPI (blue) by immunofluorescence assay, indicated by white arrows in the superficial dorsal horns. The samples (n = 3) were collected at 6 h after the last administration of cromakalim (5 μg / 10 μl, i.t.). Magnification: 200×. Scale bar: 75 μm. (D-E) The effect of cromakalim on SOCS3 and SOCS1 mRNA levels in BV-2 cells were determined using real-time quantitative PCR. BV-2 cells treatment with cromakalim (100 μM) for 2 or 4 h and then collected the samples (n = 4). (F) Confocal images showing BV-2 cells treatment with cromakalim (100 μM) for 6 h could increase the expression of SOCS3 (green) in vitro (n = 4). Magnification: 400×. Scale bar: 20 μm. (G) Representative Western blot images showed the levels of SOCS3 in spinal cords from mice treatment with cromakalim (5 μg / 10 μl, i.t.) for 4 or 6 h, respectively. The western blot samples were collected and analyzed (n = 3). (H) Representative Western blot images showed BV-2 cells treatment with cromakalim (100 μM) for 4 or 6 h could increase the expression of SOCS3 in vitro. The western blot samples were collected and analyzed (n = 3). (I) Representative Western blot images showed BV-2 cells treatment with three KATP channel openers, Minoxidil (30 μM); Diazoxide (200 μM) and Nicorandil (1 mM) for 6 h, and then collected the samples for western blots (n = 3). (J) Transfection reagent Lipofectamine® RNAiMAX was encapsulated with two Kir6.1 siRNA and one Control siRNA for 48 h, BV-2 cells were cultured with 100 μM cromakalim for 6 h. Representative bands and a data summary (n = 4) are shown. Significant difference was revealed following one-way or two-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. naive control; ###$p < 0.001$ vs. cromakalim-treated group; Bonferroni post hoc tests).

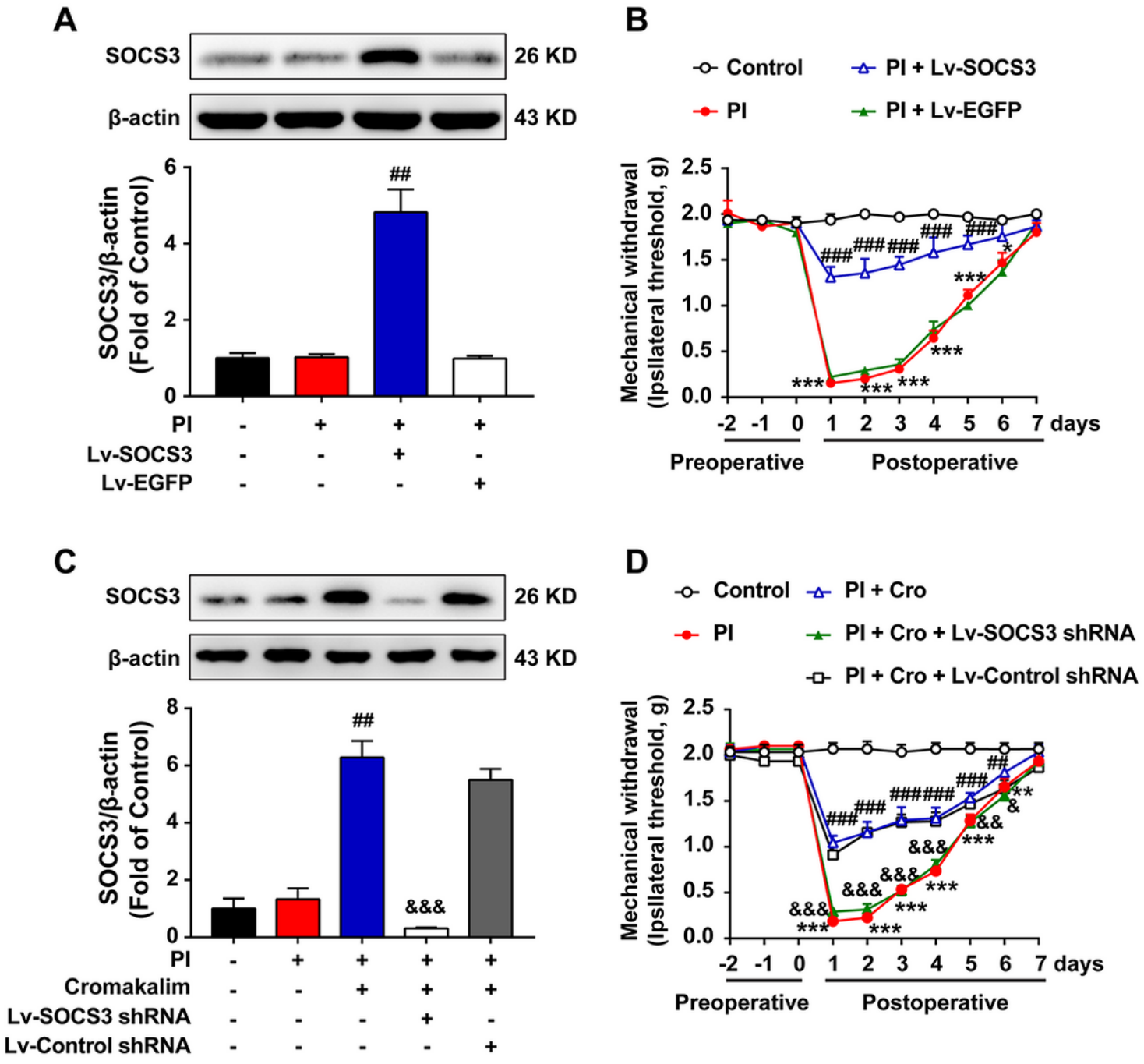

Figure 3

Opening KATP channels inhibits incision-induced mechanical allodynia by up-regulating SOCS3 expression. (A) Representative Western blot images showed the expression of SOCS3 in spinal cords from mice treatment with LV-SOCS3 and LV-EGFP lentiviral vectors, respectively. Three days before the plantar incision surgery, the lentiviral vectors (LV-SOCS3 and LV-EGFP, 3.00E + 10 PFU/ml. 10 μl) were injected into mice by i.t. injection, and the spinal samples were collected for western blot (n = 3). (B) i.t. administration of lentiviral vectors (LV-SOCS3, 10 μl) three days before plantar incision surgery significantly attenuated plantar incision-induced mechanical allodynia in mice (n = 6). (C) Representative Western blot images showed the efficiency of SOCS3 knockdown in spinal cord in mice. i.t.

administration of SOCS3 shRNA and Control shRNA (330 nM / 10 μl) 48 h before plantar incision surgery, and then collected the spinal samples (n = 3) from mice after cromakalim (5 μg / 10 μl, i.t.) treatment for 6 h. (D) The analgesic effect of cromakalim was abrogated by SOCS3 siRNA in mice (n = 6). i.t. administration of SOCS3 shRNA and Control shRNA (330 nM / 10 μl) 48 h before plantar incision surgery, and then subject to cromakalim (5 μg / 10 μl, i.t.) pretreatment for 30 min, and consecutive administration of cromakalim for 7 days, mechanical pain threshold was tested by Von Frey Hairs at 6 hours after each administration. Significant difference was revealed following one-way or two-way ANOVA (**p < 0.01, ***p < 0.001 vs. naive control; ##p < 0.01, ###p < 0.001 vs. plantar incision group; &p < 0.05, &&p < 0.01, &&&p < 0.001 vs. cromakalim-treated group; Bonferroni post hoc tests).

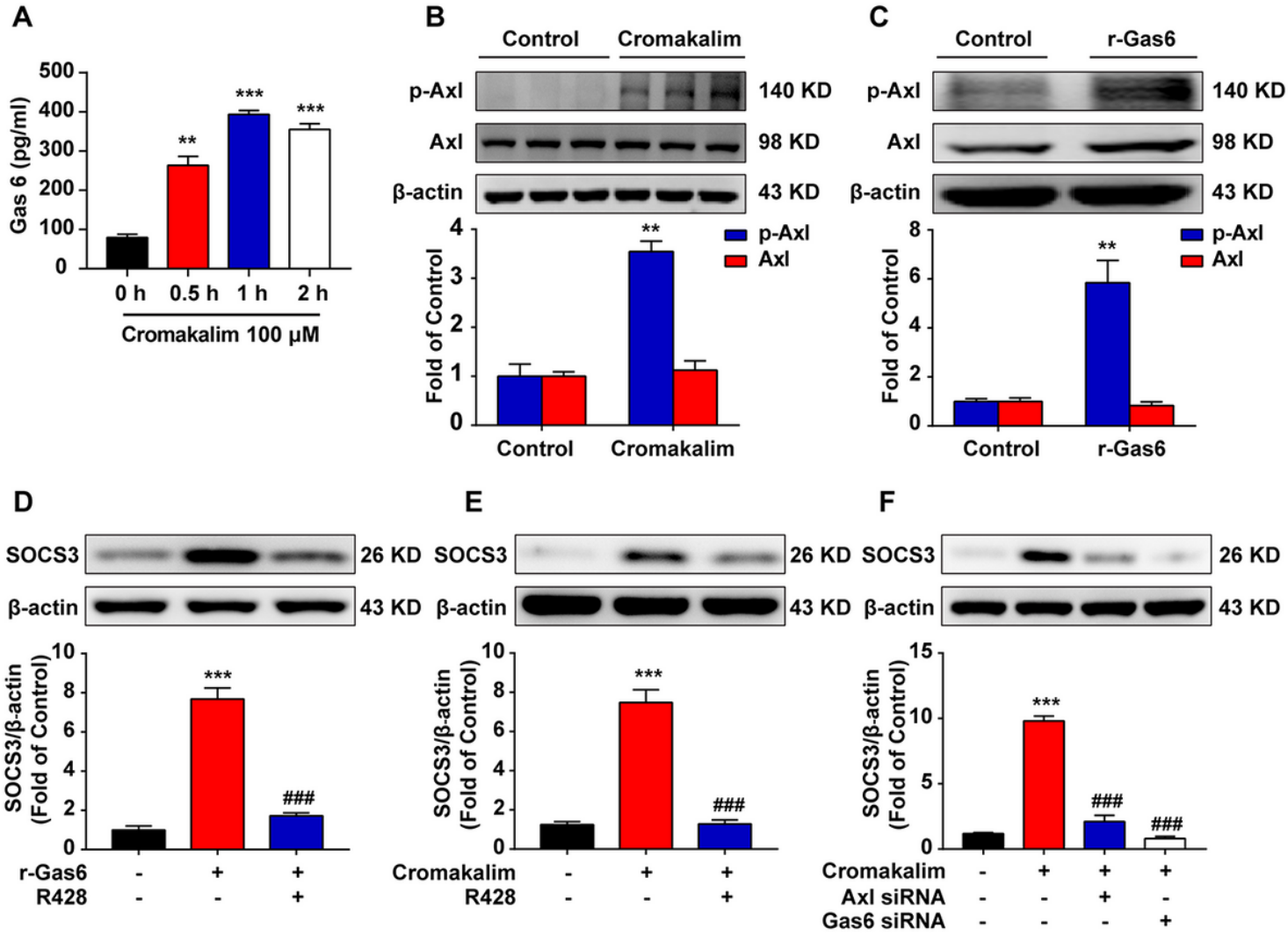

Figure 4

Opening KATP channels induces the expression of SOCS3 dependent on Gas6/Axl signaling pathway. (A) Cromakalim significantly induce the upregulation of Gas6 in the supernatant of BV-2 cells. The supernatant of BV-2 cells was collected at 0, 0.5, 1 or 2 h after cromakalim (100 μM) treatment, respectively (n = 3). (B) Representative Western blot images showed the expression of p-Axl and Axl in BV-2 cells in vitro. BV-2 cells were treatment with cromakalim (100 μM) for 6 h and then collected the samples for western blots (n = 3). (C) BV-2 cells treatment with recombinant Gas6 (rGas6) protein (500

ng/ml) for 2 h can significantly induce the activation of Axl receptor, The western blot samples were collected and analyzed (n = 3). (D) Representative Western blot images showed Axl inhibitor (R428, 1 μM) could reverse rGas6 (500 ng/ml) induced the up-regulation of SOCS3 protein in vitro. BV-2 cells were pretreated with Axl inhibitor (R428, 1 μM) for 30 min and co-cultured with rGas6 for 6 h. The western blot samples were collected and analyzed (n = 3). (E) Representative Western blot images showed Axl inhibitor (R428, 1 μM) could abolish the effect of cromakalim (100 μM) on SOCS3 in BV-2 cells in vitro. (F) Representative Western blot images showed the expression of SOCS3 in vitro. BV-2 cells were transfected with 100 pM Axl siRNA, Gas6 siRNA or control siRNA for 24 h, and then subjected to cromakalim (100 μM) for 6 h. The western blot samples were collected and analyzed (n = 3). Significant difference was revealed following one-way or two-way ANOVA (**p < 0.01, ***p < 0.001 vs. naive control; ###p < 0.001 vs. cromakalim-treated group; Bonferroni post hoc tests).

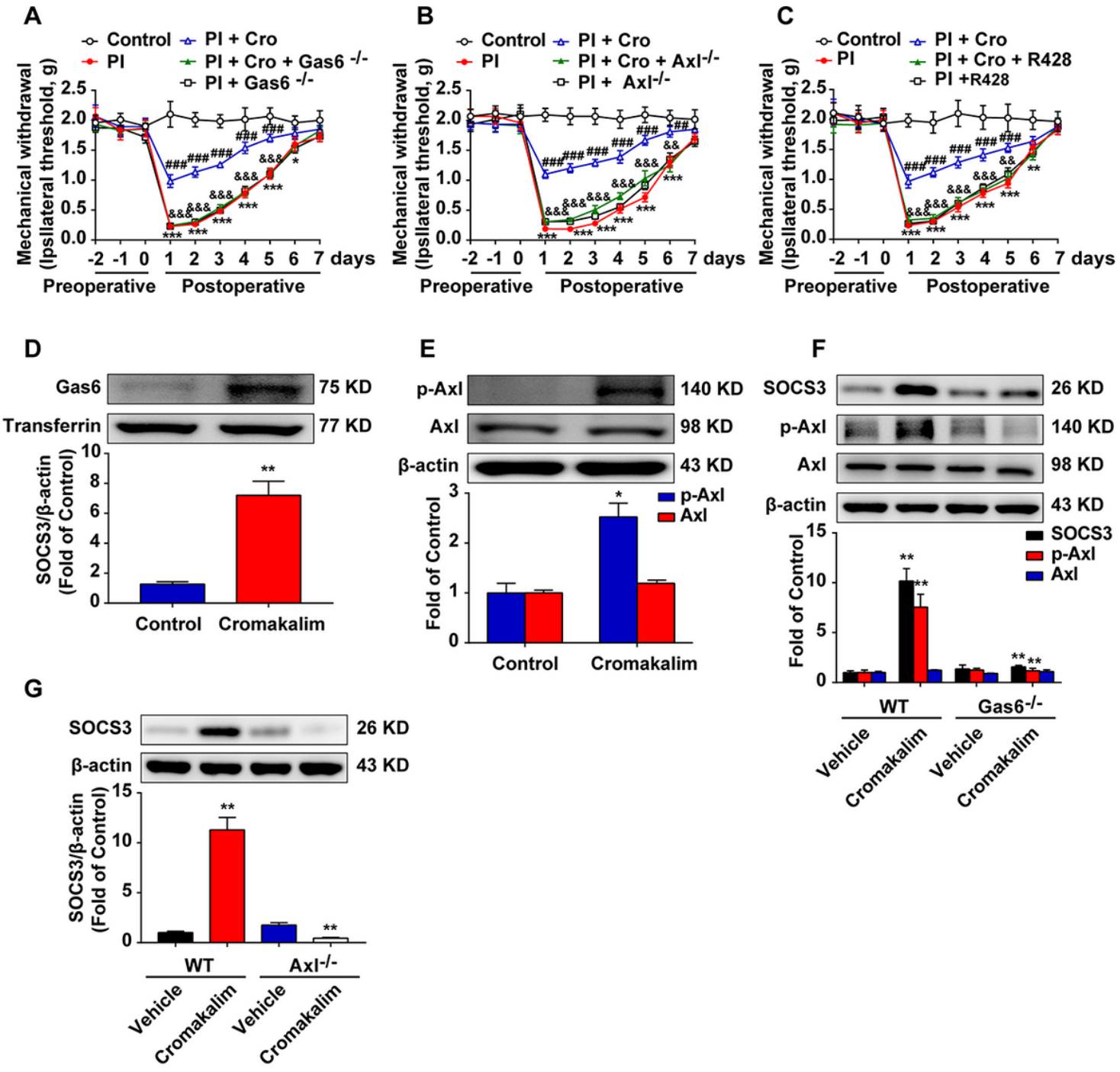

Figure 5

Opening KATP channels alleviates incision-induced mechanical allodynia by activating Gas6/Axl-SOCS3 signaling pathway. (A-B) i.t. administration of cromakalim (5 μg / 10 μl) into Gas6-/- or Axl-/- mice for 7 consecutive days, mechanical pain threshold was tested by Von Frey Hairs at 6 h after each administration (n = 6). (C) Axl inhibitor (R428, 1 μM, i.t.) was given 1 hour before plantar incision surgery, and then i.t. administration of cromakalim (5 μg / 10 μl) 30 min later, and consecutive administration of these drugs for 7 days, mechanical pain threshold was tested by Von Frey Hairs at 6 h after each cromakalim administration (n = 6). (D) Representative Western blot images showed the expression of Gas6 in cerebrospinal fluid from mice treatment with cromakalim. i.t. administration of cromakalim (5 μg

/ 10 μl) into mice for 2 h, and then collect the samples (cerebrospinal fluid) for western blots (n = 3). Transferrin is used as a loading control. (E) Representative Western blot images showed the expression of p-Axl and Axl in spinal cord from mice treatment with cromakalim (5 μg / 10 μl, i.t.) in vivo. The western blot samples were collected and analyzed (n = 3). (F) Representative Western blot images showed the expression of SOCS3, p-Axl and Axl in spinal cords from wild type (WT) and Gas6-/- mice treatment with cromakalim (5 μg / 10 μl, i.t.) in vivo. The western blot samples were collected and analyzed (n = 3). (G) Representative Western blot images showed the expression of SOCS3 in spinal cords from WT and Axl-/- mice treatment with cromakalim (5 μg / 10 μl, i.t.) in vivo. The western blot samples were collected and analyzed (n = 3). Significant difference was revealed following one-way or two-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001 vs. naive control; ##p < 0.01, ###p < 0.001 vs. plantar incision group; &&p < 0.01, &&&p < 0.001 vs. cromakalim-treated group; Bonferroni post hoc tests).

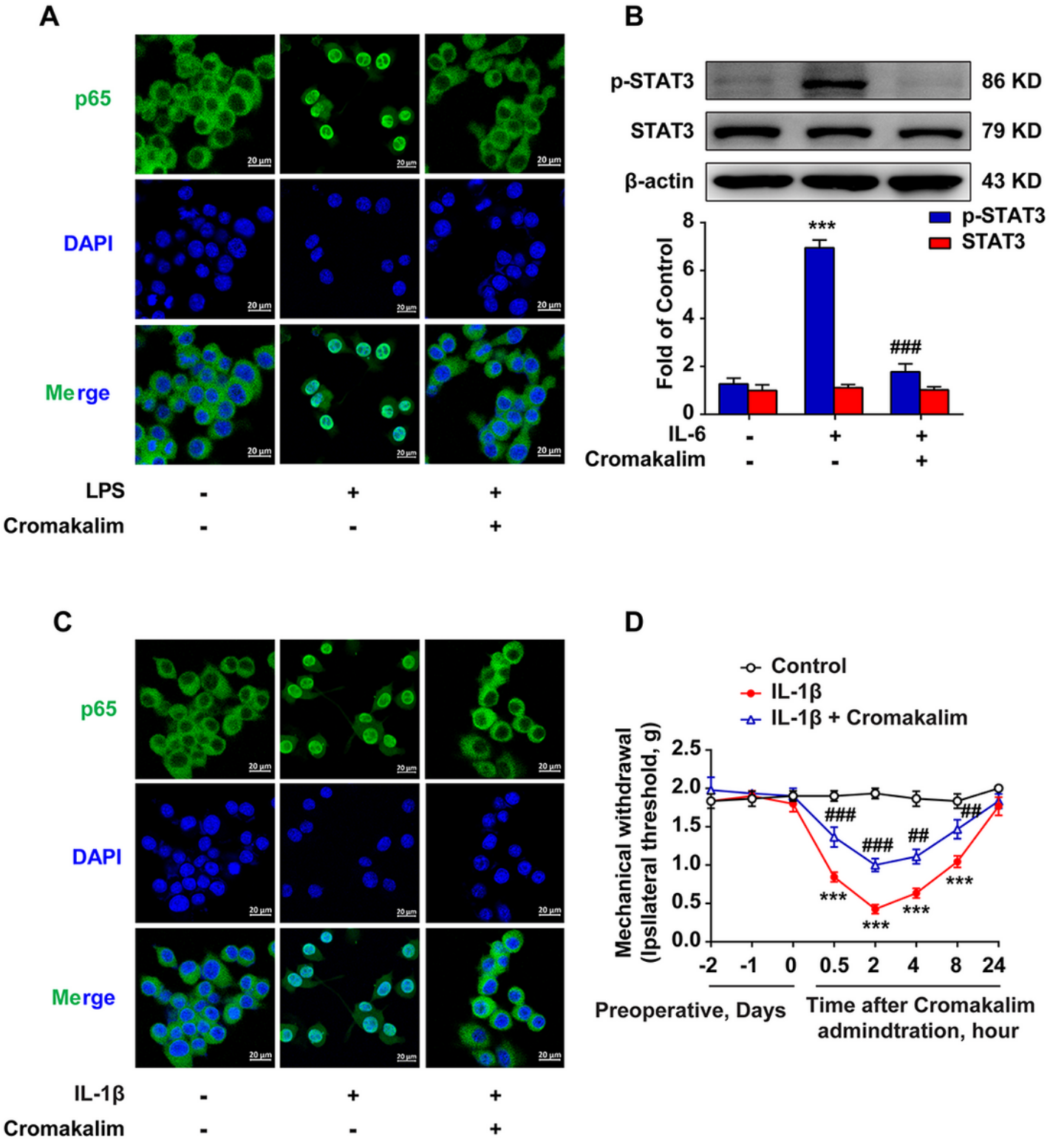

Figure 6

Opening KATP channels induces inflammation tolerance to decrease neuroinflammation and ameliorate postoperative pain. (A) Cromakalim inhibited the translocation of NF-κB p65 from the cytosol to the nucleus after LPS treatment in vitro. BV-2 cells were pretreated with cromakalim (100 μM) 6 h and then co-cultured with LPS (1 μg/ml) for 3 h. Magnification: 400×. Scale bar: 75 μm. (B) Representative Western blot images showed the expression of p-STAT3 and STAT3 in BV-2 cells in vitro. BV-2 cells were

pretreated with cromakalim (100 μM) 6 h and then co-cultured with IL-6 (10 ng/ ml) for 30 min. The western blot samples were collected and analyzed (n = 3). (C) Cromakalim inhibited the translocation of NF-κB p65 from the cytosol to the nucleus after IL-1β treatment in vitro. BV-2 cells were pretreated with cromakalim (100 μM) 6 h and then co-cultured with IL-6 (10 ng/ ml) for 3 h. Magnification: 400×. Scale bar: 75 μm. (D) Pretreatment with cromakalim significantly inhibited IL-1β mediated mechanical allodynia in mice. i.t. administration of cromakalim (5 μg / 10 μl, i.t.) before IL-1β (25 ng/10 μl, i.t.) treatment, and then using the Von Frey Hairs to detect the mechanical pain threshold of mice at different time points (0, 0.5, 2, 4, 8, 24 h) after cromakalim administration. Significant difference was revealed following one-way or two-way ANOVA (***p < 0.001 vs. naive control; ##p < 0.01, ###p < 0.001 vs. IL-6 or IL-1β group; Bonferroni post hoc tests).

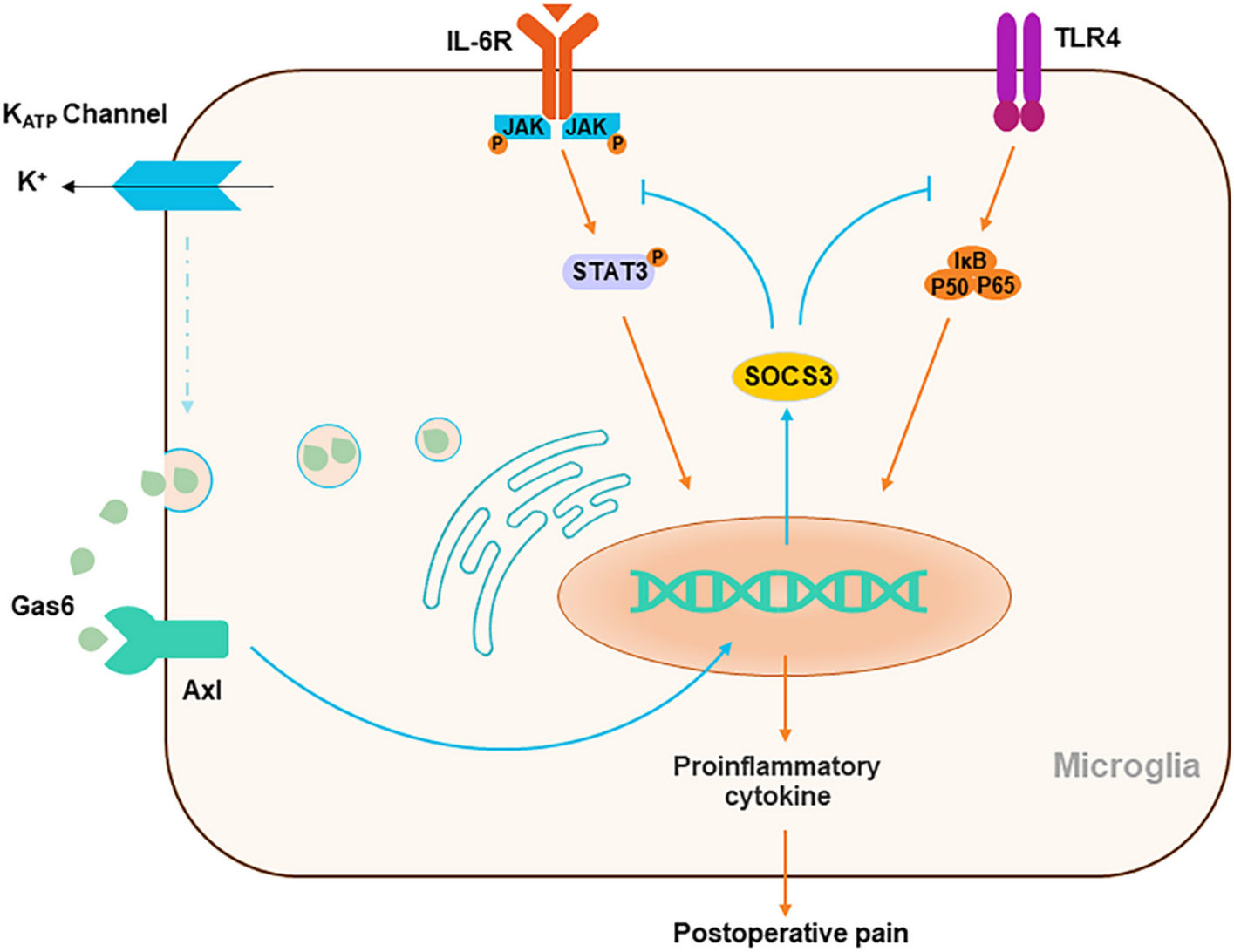

Figure 7

Schematic indicating that KATP channel opening activated Gas6/Axl/SOCS3 signaling to induce inflammatory tolerance and relief chronic pain. Opening KATP channels increases Gas 6 expression to activate Axl receptor, induces SOCS3-mediated inflammatory tolerance and ameliorates chronic pain.