Opening $K_{ATP}$ channels induces inflammatory tolerance and prevents chronic pain

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ABSTRACT

Current treatments for chronic pain are unsatisfactory, therefore, new therapeutics are urgently needed. Our previous study indicated that $K_{ATP}$ channel openers have analgesic effects, but the underlying mechanism has not been elucidated. We speculated that $K_{ATP}$ channel openers might increase suppressor of cytokine signaling (SOCS)-3 expression to induce inflammatory tolerance and attenuate chronic pain. Postoperative pain was induced by plantar incision to establish a chronic pain model. Growth arrest–specific 6 (Gas6)−/− and Axl−/− mice were used for signaling studies. The microglia cell line BV-2 was cultured for the in vitro experiments. The $K_{ATP}$ channel opener significantly attenuated incision-induced mechanical allodynia in mice associated with the upregulated expression of SOCS3. Opening $K_{ATP}$ channels induced the expression of SOCS3 in the Gas6/Axl signaling pathway in microglia, inhibited incision-induced mechanical allodynia by activating the Gas6/Axl-SOCS3 signaling pathway, and induced inflammatory tolerance to relieve neuroinflammation and postoperative pain. We demonstrated that opening of the $K_{ATP}$ channel opening activated Gas6/Axl/SOCS3 signaling to induce inflammatory tolerance and relieve chronic pain. We explored a new target for anti-inflammatory and analgesic effects by regulating the innate immune system and provided a theoretical basis for clinical preemptive analgesia.

1. Introduction

Chronic pain is defined by the International Association for the Study of Pain (IASP) as an unpleasant sensory and emotional experience associated with or resembling that associated with actual or potential tissue damage (Raja et al., 2020), and includes three main categories: nociceptive (from tissue injury), neuropathic (from nerve injury), or nociceptive (from a sensitized nervous system) (Cohen et al., 2021). Unfortunately, current treatments for chronic pain rarely lead to complete elimination of symptoms, such as the standard drugs for pain management, nonsteroidal anti-inflammatory drugs (NSAID), morphine, opioids, and their derivatives, which show poor clinical outcomes in treating chronic pain, along with severe side effects (Maiaru et al., 2018). Thus, it is urgent to develop new treatments.

Neuroinflammation is a major cause of chronic pain (Ji et al., 2016; Topham et al., 2020; Zhou et al., 2022). Neuroinflammation in the...
spinal cord drives chronic pain via neuron–glial interactions (Ji et al., 2014). After peripheral nerve damage, glial cells around the lesion produce IL-1 and promote neurons in the dorsal root ganglia to release colony-stimulating factor-1 (CSF-1 or M-CSF), which stimulates and activates microglia in the dorsal horn of the spinal cord (Guan et al., 2016). The diffusion of neuroinflammation in the central nervous system is underpinned by microglial activation (Vergne-Salle and Bertin, 2021). Activated microglia produce proinflammatory cytokines, such as IL-1α and TNF-β, to improve neuroinflammation, which further activates microglia, creating a vicious cycle of inflammation to microglial activation to inflammation, and contributes to chronic pain states (Nayak et al., 2010). This suggests that targeting excessive neuroinflammation may offer new therapeutic opportunities for chronic pain.

ATP-sensitive potassium channels (KATP channels) are inhibited by intracellular ATP and play key physiological roles in many tissues (Nichols, 2006; Sattiraju et al., 2008). Previous studies have shown that loss of KATP channel currents in DRG and Schwann cells can increase cell membrane excitability and cause neuropathic pain (Kawano et al., 2009a,b). Iptakalim (KATP opener) inhibits the activation of BV-2 cells and nerve destruction (Zhou et al., 2007). Our previous research demonstrated that preadministration of the KATP channel opener cromakalim improved gap junction function and attenuated CCI-induced neuropathic pain; however, the mechanism was not elucidated (Wu et al., 2016). The diffusion of neuroinflammation in the central nervous system is underpinned by microglial activation (Vergne-Salle and Bertin, 2021). After peripheral nerve damage, glial cells around the lesion develop and migrate to the lesion site, and contribute to chronic pain states (Nayak et al., 2010). The diffusion of neuroinflammation in the central nervous system is underpinned by microglial activation (Vergne-Salle and Bertin, 2021). Activated microglia produce proinflammatory cytokines, such as IL-1α and TNF-β, to improve neuroinflammation, which further activates microglia, creating a vicious cycle of inflammation to microglial activation to inflammation, and contributes to chronic pain states (Nayak et al., 2010). This suggests that targeting excessive neuroinflammation may offer new therapeutic opportunities for chronic pain.

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To verify the analgesic mechanism of the KATP opener, we used cell RNA sequencing (RNA-Seq) to investigate gene alterations in mice treated with cromakalim. Gene ontology (GO) functional enrichment analysis showed that the biological functions of the altered genes were mainly related to immunity and inflammation, and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway enrichment analysis demonstrated that the differentially expressed genes were enriched in inflammation-related signaling pathways. Among the significantly altered genes, we found that the suppressor of cytokine signaling (SOCS)-3 gene was significantly upregulated after cromakalim administration. SOCS-3, a “powerful brake” of cytokine signaling pathways (Choi et al., 2021), negatively regulates inflammatory responses by inhibiting TLR4, IL-1R, and TNF-R signaling (Frobose et al., 2006; Yoshimura et al., 2007). SOCS3 overexpression can prevent the development of rheumatoid arthritis in mouse models (Shouda et al., 2001) and is effective against various types of inflammation and septic shock (Jo et al., 2005). Thus, we speculated that upregulation of SOCS3 by KATP channel opening might be beneficial for ablation.

Here, we provide the first evidence that opening the KATP channel increases SOCS3 expression via Axl (an effecroytosis receptor)-mediated signaling to provide preemptive analgesia and alleviate chronic pain.

2. Materials and methods

2.1. Ethical approval and Consent to participate

All procedures were performed in strict accordance with the regulations of the Ethics Committee of the International Association for the Study of Pain and the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006). All animal experiments were approved by the Nanjing Medical University Animal Care and Use Committee and the Ethics Committee of Nanjing Medical University (No. IACUC-1908026).

2.2. 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), lipopolysaccharide (LPS) (Catalog No. SMB00610), IL-1β (Catalog No. SRP0833), IL-6 (catalog no. SRP3330), and TNF-α (catalog no. 17539) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glibenclamide (catalog no. HY-15206) and R428 (Catalog No. HY-15150) were purchased from MedChemExpress (Shanghai, China). Re-combinant mouse Gas6 protein (rGas6, Catalog No. 986-GS-025/CF). Kir6.1 small interfering RNA (siRNAs) were designed and constructed by GenePharma Corporation (Shanghai, China). SOCS3 siRNA (catalog no. sc-41001), Axl siRNA (catalog no. sc-29770), and Gas6 siRNA (catalog no. sc-35451), control siRNA (catalog no. sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lentiviral vectors (LV-SOCS3 and LV-EGFP) were purchased from Ohio Technology Corp., Ltd. (Shanghai, China). Antibodies against Axl (Catalog No. ab215205), IBA-1 (catalog no. ab178847), SOCS3 (catalog no. ab16030), and SUR1 (catalog no. ab216733) was purchased from Abcam (Cambridge, MA, USA). DAPI (Catalog No. D9542) was purchased from Merck (Darmstadt, Germany). Antibodies against c-Fos (Catalog No. 2250), CGRP (Catalog No. 14959), Gas6 (Catalog No. 67202), p-Axl (Catalog No. 44463), STAT3 (Catalog No. 12640), p-STAT3 (Catalog No. 9145), p-p38 (Catalog No. 9211), p-ERK (Catalog No. 9101), and p-JNK (Catalog No. 9251) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against GFAP (Catalog No. SAB5700611) and NeuN (catalog no. ABN78) was purchased from Millipore (Billerica, MA, USA). Antibodies against SUR2 (LS-C590902) was purchased from LifeSpan Biosciences. Antibodies against Kir6.1 (Catalog No. 14954–1-AP) and transferrin (Catalog No. 17435–1-AP) were purchased from Proteintech. Antibodies against β-actin (Catalog No. A1978) were purchased from Sigma-Aldrich (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).

2.3. Animals and treatment

Adult male C57BL/6J mice (18–22 g) at 8 weeks of age were provided by the Experimental Animal Center of 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).

2.4. Surgery

Plantar incision surgery was performed as previously described (Brennan et al., 1996). All surgeries were performed under anesthesia induced with 1 % sodium amobarbital. The plantar aspect of the left hind paw was sterilized with a 10 % povidone-iodine solution before and after surgery and was placed through a hole in a sterile drape. A 1-cm longitudinal incision was made through the skin and fascia of the foot, starting 0.5 cm from the proximal edge of the plantar aspect of the foot, and extending toward the toes. The plantaris muscle was elevated on the plantar aspect of the foot, starting 0.5 cm from the proximal edge of the plantar aspect of the foot, and extending toward the toes. The plantaris muscle was elevated on the plantar aspect of the foot, starting 0.5 cm from the proximal edge of the plantar aspect of the foot, and extending toward the toes. The plantaris muscle was elevated on the plantar aspect of the foot, starting 0.5 cm from the proximal edge of the plantar aspect of the foot, and extending toward the toes. The plantaris muscle was elevated on the plantar aspect of the foot, starting 0.5 cm from the proximal edge of the plantar aspect of the foot, and extending toward the toes.

2.5. Behavioral analysis and treatment

Animals were habituated to the testing environment daily for at least five days before baseline testing. The mechanical withdrawal threshold was detected using von Frey hairs (Woodland Hills, Los Angeles, CA, USA). Thirty minutes before plantar incision surgery, intrathecal (i.t.)
administration of cromakalim (1, 2.5, and 5 μg) and glibenclamide (20 μg) under brief inhalational anesthesia was performed by means of lumbar puncture at the intervertebral space of L₄₅. Using a stainless-steel needle (27 gauge) attached to a 25μL Hamilton syringe. Cromakalim and glibenclamide were dissolved in Dimethyl sulfoxide (DMSO) and diluted in PBS (final concentration of DMSO: 1 %). The control group was administered the same volume of the vehicle. Behavioral tests were performed 6 h after each dose. Specifically, the animals were placed in boxes set on an elevated metal mesh floor and 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 increasing stiffness perpendicular to the plantar surface. Each hind paw was tested three times at intervals of 5- to 6-minutes. The threshold of mechanical withdrawal in each mouse was calculated by averaging three readings, and the force was converted to millinewtons.

2.6. Cell preparation and treatment

BV-2, Neuro-2a (N2A), and 293 T 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 % CO₂ at 37 °C. For Elisa experiments, BV-2 and N2A cells were seeded in six-well plates at a density of 1 × 10⁶ cells/well. After 24 h, the cells were treated with different tool drugs, such as cromakalim and rGas6 and the cells and supernatants were collected to detect the expression of SOCS3, Gas6, and protein S.

2.7. Gelatin zymography

The spinal cord segments (L₄₅) were rapidly dissected and homogenized by 1 % NP40 lysis. Then, 250–300 μg of protein per lane was loaded into the wells of precast gels (8 % polyacrylamide gel containing 0.1 % gelatin). After electrophoresis, each gel was incubated with 50 μl of developing buffer for 48 h (37.5 °C) in a shaking bath. The gels were then stained with Coomassie brilliant blue (1 %, with 10 % acetic acid and 10 % isopropyl alcohol, diluted with ddH₂O).

2.8. RNAseq

Total RNAs was extracted from vehicle- or cromakalim-treated mouse spinal cords and subjected to poly(A) RNA sequencing (LC Sciences). The library was prepared according to Illumina’s TruSeq Stranded mRNA sample preparation protocol. RNA integrity and purity were analyzed using a Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA, 5067–1511), and only RNA samples with RIN (RNA integrity) > 7 were used for sequencing. Paired-ended sequencing was performed on an Illumina NovaSeq 6000 Sequencing System, following the vendor’s recommended protocol. The clean reads were filtered using Cutadapt (https://cutadapt.readthedocs.io/en/stable/; version: cutadapt-1.9). HISAT2 (https://dewghankimlab.github.io/hisat2/; version: hisat2-2.2.1) was used to map reads to the mouse genome and StringTie (https://ccb.jhu.edu/software/stringtie/) was used to assemble the mapped reads. Differential gene expression analysis was performed using DESeq2 software between two different groups (and edgeR between two samples). Genes with a false discovery rate (FDR) < 0.05 and absolute fold change ≥ 2 were considered as differentially expressed genes.

2.9. Quantitative PCR

Quantitative PCR was performed on BV-2 cell and spinal cord samples obtained from mice. Total RNA was isolated using the standard method with TRIzol reagent (Invitrogen Life Technologies). The isolated RNA was reverse-transcribed into cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa) following standard protocols. Real-time quantitative PCR (qPCR) was performed using synthetic primers and SYBR Green (TaKaRa) on a QuantStudio 5 Real-Time PCR Detection System (Thermo Fisher Scientific). The relative expression levels of SOCS3 and SOCS1 were calculated and quantified using the 2^–ΔΔCT method after normalization with the reference β-actin. The following primers were used:

- SOCS3: Forward: CTGCGGCTCTTATTGGGGAC. Reverse: AAAAAAGGCTCGAGGTTCTG;
- SOCS1: Forward GCGGGACCTCTTCTTATCC. Reverse: CTGGAGGCGGATGTATGT;
- β-actin: Forward: GCCGTATTTCCTCCCATCG. Reverse: CCAGTGGTAACATGCGATGT.

2.10. Western blotting

Samples (cells or spinal cord) were collected and washed with PBS before being lysed in radioimmunoprecipitation assay (RIPA) lysis buffer. Protein concentrations were determined by BCA Protein Assay (Thermo Fisher, Waltham, MA, USA) and 40–80 μg of protein was 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 were Axl (1:1000), p-Axl (1:1000), p-ERK (1:1000), Gas6 (1:1000), p-JNK (1:1000), p-STAT3 (1:1000), p-PI3K (1:1000), p-JNK (1:1000), and transferrin (1:1000). For the loading control, the blots were probed with antibodies against β-actin (1:1000). The filters were then developed using enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA, USA) with secondary antibodies (Chemicon, Billerica, MA, USA). Data were acquired using a Molecular Imager (Gel DocTM XR, 170–8170) and analyzed with Quantity-One-4.6.5 (Bio-Rad Laboratories, Berkeley, CA, USA).

2.11. Immunofluorescence assay

After deep anesthesia, the animals were perfused transcardially with normal saline followed by 4 % paraformaldehyde in 0.1 M PB at pH 7.4, each for 20 min. Then, L4 and or L5 lumbar segments were dissected and post-fixed in 4 % paraformaldehyde. The embedded blocks were sectioned at a thickness of 25-μm. Sections from each group (five animals in each group) were incubated with rabbit antibodies against c-Fos (1:200), p-CREB (1:1000), p-p38 (1:1000), SOCS3 (1:1000), p-STAT3 (1:1000), p-PI3K (1:1000), and p-JNK (1:1000); mouse antibodies against IBA-1 (1:200); and chicken antibodies against GFAP for 2 h at room temperature before being rinsed in radioimmunoprecipitation assay (RIPA) lysis buffer. Protein concentrations were determined by BCA Protein Assay (Thermo Fisher, Waltham, MA, USA) and 40–80 μg of protein was 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 were Axl (1:1000), p-Axl (1:1000), p-ERK (1:1000), Gas6 (1:1000), p-JNK (1:1000), p-PI3K (1:1000), p-JNK (1:1000), and transferrin (1:1000). For the loading control, the blots were probed with antibodies against β-actin (1:1000). The filters were then developed using enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA, USA) with secondary antibodies (Chemicon, Billerica, MA, USA). Data were acquired using a Molecular Imager (Gel DocTM XR, 170–8170) and analyzed with Quantity-One-4.6.5 (Bio-Rad Laboratories, Berkeley, CA, USA).

2.12. NF-κB activation assay

BV-2 cells were plated in cell culture dishes and pretreated with LV-SOCS3 plasmid for 48 h, cromakalim (10 μM) for 6 h, and then co-cultured with LPS (1 μg/ml) or IL-1β (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 antibodies diluted in 1 % BSA (1:50).
The coverslips were then exposed to fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:100, at room temperature for 1 h) and 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 the nucleus) for 1 min. Confocal microscopy was performed using an Olympus FV1000 confocal microscope.

2.13. RNA interference

First, 3.3 nmol siRNA was dissolved in 330 μl RNase-free water. Control siRNA was used as a negative control. For siRNA transfection, cells were cultured in six-well plates in 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 a culture medium containing 10% FBS and then incubated at 37°C in 5% CO2. For animal experiments, i.t. administration of siRNA into mice was performed for 48 h just before plantar incision surgery. The mechanical thresholds were measured to determine whether interference with the SOCS3, Gas6, and Axl genes could abolish the analgesia of cromakalim.

2.14. SOCS3 overexpression assay

Lentiviral vectors (LV-SOCS3 and LV-EGFP) were purchased from Obio Technology Corp., ltd. (Shanghai, China). LV-EGFP was used as a negative control. The titer of the lentiviral vectors (LV-SOCS3) was 3.00E + 10 PFU/ml. Ten microliters of lentiviral vectors (LV-SOCS3 or LV-EGFP) were i.t. injected into mice three days before plantar incision surgery.

2.15. Statistical analyses

GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA) was used to conduct all the statistical analyses. Alteration of the 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 the mean ± SEM of at least three independent experiments. P < 0.05 was deemed to be statistically significant.

3. Results

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

The K_{ATP} channels are composed of eight protein subunits. Four of these are members of the inwardly rectifying potassium channel family Kir6.x (Kir6.1 or Kir6.2), whereas the other four are sulfonylurea receptors (SUR1, SUR2A, SUR2B, and SUR2C) (Inagaki et al., 1996; Inagaki et al., 1995). Our previous study reported that the K_{ATP} channel subunits SUR1, SUR2, and Kir6.1 were expressed in the spinal cord; however, the Kir6. subunit was not detectable in the spinal cord but was detected in the DRG and longissimus muscles (Wu et al., 2011). In this study, we further found that the K_{ATP} channel subunit Kir6.1 was colocalized primarily with microglia (IBA) and a small number of neurons (NeuN) and astrocytes (GFAP) (Fig. S1A). In addition, we also found that...
the K\textsubscript{ATP} channel subunits SUR1, SUR2, and Kir6.1, were significantly downregulated after plantar incision surgery on day 3, and slowly restored to the basic level on day 7 (Fig. S1 B–E). To verify the effect of K\textsubscript{ATP} channel opening on postoperative pain, consecutive i.t. administration of different doses of the K\textsubscript{ATP} channel opener cromakalim (1, 2.5, or 5 \(\mu\)g/10 \(\mu\)l) for 7 days, demonstrated that cromakalim (2.5 or 5 \(\mu\)g/10 \(\mu\)l) significantly attenuated plantar incision-induced mechanical allodynia (Fig. 1A). A single injection of cromakalim (5 \(\mu\)g/10 \(\mu\)l, i.t.) also markedly ameliorated pain from approximately 2 h, which peaked at 6–8 h, and the pain returned within 24 h (Fig. S1 F). In addition, i.t. administration of the K\textsubscript{ATP} channel blocker, glibenclamide (20 \(\mu\)g/10 \(\mu\)l), significantly reversed the analgesic effect of cromakalim (Fig. S1 G).

SOCS3 mRNA levels in BV-2 cells were determined using real-time quantitative PCR. BV-2 cells were treated with cromakalim (100 \(\mu\)M) for 2 or 4 h and then the samples were collected (n = 4). (K) Representative western blot images showing that BV-2 cells were treated with three K\textsubscript{ATP} channel openers, minoxidil (30 \(\mu\)M), diazoxide (200 \(\mu\)M), and nicorandil (1 mM) for 6 h, and then the samples were collected for western blotting (n = 3). (L) Transfection reagent Lipofectamine\textsuperscript{®} RNAiMAX was encapsulated with two Kir6.1 siRNA and one control siRNA for 48 h. BV-2 cells were cultured with 100 \(\mu\)M cromakalim for 6 h. Representative bands and a data summary (n = 4) are shown. Significant differences were obtained using 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).
incision surgery (Fig. 1B), but not on day 7 (Fig. S1H). Interestingly, this phenomenon was abolished by glibenclamide treatment (Fig. S1I). In addition, immunofluorescence results also showed that i.t. administration of cromakalim (5 μg/10 μl) markedly reduced the expression of c-Fos and CGRP (pain markers) in the spinal cord of mice (Fig. 1C and D). These data suggest that opening K\textsubscript{ATP} channels can alleviate postoperative pain.

3.2. Opening K\textsubscript{ATP} channels induces the expression of SOCS3 in microglia

To verify the analgesic mechanism of K\textsubscript{ATP} channel opening, cell RNA sequencing (RNA-Seq) was used to investigate gene alterations in mice treated with cromakalim. Volcano plots and heatmap clustering analysis of the effect of cromakalim relative to vehicle controls in the spinal cord of mice revealed changes in gene expression, with 398 and 21 genes up- or downregulated, respectively (Fig. 2A and B). Gene Ontology (GO) functional enrichment analysis showed that the biological functions of these altered genes were mainly related to immunity and inflammation (Fig. 2C). Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway enrichment analysis demonstrated that the differentially expressed genes were enriched in inflammation-related signaling pathways, such as the TNF signaling pathway, cytokine-cytokine receptor interaction, IL-17 signaling pathway, and NF-kappa B signaling pathway (Fig. 2D). We also found that SOCS3 expression was significantly upregulated after cromakalim administration. SOCS3 is an important negative regulator of the body and can inhibit inflammatory signals by regulating various signaling pathways (Croker et al., 2003; Frobose et al., 2006). Our previous study also found that overexpression of

Fig. 3. Opening K\textsubscript{ATP} channels inhibits incision-induced mechanical allodynia by upregulating SOCS3 expression. (A) 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). (B) Representative western blot images showed the expression of SOCS3 in spinal cords from mice treated with LV-SOCS3 and LV-EGFP lentiviral vectors, respectively. Three days before the plantar incision surgery, the lentiviral vectors (LV-SOCS3 or LV-EGFP, 3.00E + 10 PFU/ml. 10 μl, i.t.) were injected into mice, and the spinal samples were collected for western blotting (n = 3). (C) The analgesic effect of cromakalim was abrogated by SOCS3 shRNA in mice (n = 6). i.t. administration of SOCS3 shRNA and Control shRNA (330 nM/10 μl) into mice 48 h before plantar incision surgery, and the mice were then subjected to cromakalim (5 μg/10 μl, i.t.) for 7 days, and the mechanical pain threshold was tested by von Frey hairs 6 h after each administration. (D) Representative western blot images showing the efficiency of SOCS3 knockdown in spinal cord in mice. i.t. administration of SOCS3 shRNA and Control shRNA (330 nM/10 μl) was conducted 48 h before plantar incision surgery, and then the spinal samples were collected (n = 3) from mice after cromakalim (5 μg/10 μl, i.t.) treatment for 6 h. Significant differences were obtained using one-way or two-way ANOVA (\( ^* p < 0.01, \quad ^{**}p < 0.001 \) vs naive control; \( ^{##}p < 0.01, \quad ^{###}p < 0.001 \) vs plantar incision group; \( ^{&}p < 0.05, \quad ^{&&}p < 0.01, \quad ^{&&&}p < 0.001 \) vs cromakalim-treated group; Bonferroni post hoc tests).
3.3. Opening K<sub>ATP</sub> channels can significantly increase the expression of SOCS3 in vitro and in vivo.

After cromakalim (2.5 and 5 μM), the expression of SOCS3 was markedly increased at 2, 4, and 6 h in BV-2 cells (Fig. 2F). Furthermore, treatment of microglia (Fig. 2G) and neurons (Fig. S2C) but not in astrocytes (Fig. 2H) with cromakalim also induced an increase in SOCS3 protein (Fig. 2I) and mRNA (Fig. 2J) levels in vitro but had no effect on the pain threshold in naïve mice (Fig. S3C). Taken together, these results indicated that opening K<sub>ATP</sub> channels induces the expression of SOCS3 in vitro and in vivo by opening K<sub>ATP</sub> channels or whether it is only the specific pharmacodynamic effect of cromakalim. Three K<sub>ATP</sub> channel openers, minoxidil (30 μM), diazoxide (200 μM), and nicorandil (1 mM), were added to BV-2 cells, and after 6 h, samples were collected for western blotting. The results showed that all three K<sub>ATP</sub> channel openers can increase the expression of SOCS3 in BV-2 cells. Moreover, we further investigated whether cromakalim induces the upregulation of SOCS3 protein expression in microglia (Fig. 2G) but not in astrocytes (Fig. S2D) in the spinal cord of mice. Furthermore, treatment of BV-2 cells with cromakalim (100 μM) also induced an increase in SOCS3 protein (Fig. 2H) and mRNA (Fig. 2J) levels in vitro but had no effect on SOCS1 mRNA (Fig. S2B). We further investigated whether cromakalim induces the expression of SOCS3 in vitro and in vivo by opening K<sub>ATP</sub> channels or whether it is only the specific pharmacodynamic effect of cromakalim. Three K<sub>ATP</sub> channel openers, minoxidil (30 μM), diazoxide (200 μM), and nicorandil (1 mM), were added to BV-2 cells, and after 6 h, samples were collected for western blotting. The results showed that all three K<sub>ATP</sub> channel openers can increase the expression of SOCS3 in BV-2 cells in vitro (Fig. 2K), while conditionally knocking out the Kir6.1 channel can reverse the effect of cromakalim on BV-2 cells in vitro (Fig. 2L). These data suggest that opening K<sub>ATP</sub> channels can significantly increase the expression of SOCS3 in vitro and in vivo.

3.4. Opening K<sub>ATP</sub> channels inhibits incision-induced mechanical allodynia by upregulating SOCS3 expression

As shown in Fig. 1, we demonstrated that i.t. administration of cromakalim can produce a preemptive analgesic state in mice. Pretreatment with the K<sub>ATP</sub> channel blocker, glibenclamide, reversed the analgesic effect of cromakalim (Fig. S1B) and inhibited the expression of SOCS3 in mice (Fig. S3A). Here, we further investigated whether the local application of lentiviral-mediated SOCS3 overexpression could induce pre-emptive analgesia in mice. The results showed that i.t. administration of lentiviral vectors (LV-SOCS3) three days before plantar incision surgery markedly promoted the expression of SOCS3 in the spinal cord (Fig. 3A) and inhibited plantar incision-induced mechanical allodynia in mice (Fig. 3B). The transfection efficiency of the lentiviral vectors (LV-EGFP) was confirmed in 293 T cells (Fig. S3B). These results indicated that cromakalim produces analgesic effects similar to those of SOCS3-overexpressing lentiviral vectors. Moreover, we investigated whether the analgesic effect of cromakalim was dependent on the expression of SOCS3 in the spinal cord of mice. Alternatively, i.t. administration of SOCS3 shRNA 48 h before surgery significantly inhibited the analgesic effect of cromakalim (Fig. 3D) by interfering with SOCS3 expression in the spinal cord (Fig. 3C). However, i.t. administration of SOCS3 shRNA alone had no effect on the pain threshold in naïve mice (Fig. S3C). Taken together, these results suggest that opening K<sub>ATP</sub> channels markedly inhibits plantar incision-induced mechanical allodynia by upregulating SOCS3 expression in the spinal cord of mice.
and analyzed (n = 3). Significant differences were observed 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).

cells (Fig. S4A), but did not alter the protein S levels (Fig. S4B and 4C). Additionally, cromakalim induced Axl receptor activation in vitro (Fig. 4B). Our results also showed that the exogenous recombinant Gas6 protein (rGas6, 500 ng/ml) treatment of BV-2 cells for 2 h can also significantly induce the activation of the Axl receptor (Fig. 4C) and the upregulation of SOCS3 protein in BV-2 cells (Fig. 4D), whereas these effects were abrogated by an Axl inhibitor (R428, 1 μM) in vitro (Fig. 4D). Moreover, pretreatment with Axl inhibitor (R428, 1 μM) markedly inhibited the effect of cromakalim on SOCS3 in BV-2 cells in vitro (Fig. 4E). Both Axl siRNA and Gas6 siRNA significantly inhibited the cromakalim-mediated upregulation of SOCS3 in vitro (Fig. 4F). The data indicated that opening K_{ATP} channels induces the upregulation of SOCS3, which is dependent on the Gas6/Axl signaling pathway.

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

As shown in Fig. 5A, mice were treated with cromakalim (5 μg/10 μl, i.t.) for 6 h before plantar incision surgery, and then 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 could abolish the analgesic effect of cromakalim. Moreover, we also found that i.t. administration of Axl inhibitor (R428, 1 μM) or knockout of the Axl gene 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 the cerebrospinal fluid (Fig. 5D) and induce the activation of Axl in the spinal cord of mice (Fig. 5E). We also investigated whether i.t. administration of cromakalim could induce the upregulation of SOCS3 in Gas6- and Axl-knockout mice. The results showed that, compared with wild-type mice, both knocking out Gas6 or Axl significantly reversed the cromakalim-mediated upregulation of SOCS3 in the spinal cord of mice (Fig. 5F and G). These data suggest that i.t. administration of the K_{ATP} channel opener cromakalim could alleviate postoperative pain by activating the Gas6/Axl-SOCS3 pathway.

3.6. 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) significantly mediated the nuclear translocation of NF-κB p65 in BV-2 cells in vitro, whereas pretreatment with cromakalim (100 μM) 6 h
before LPS treatment could reverse this effect. In addition, pretreatment with LV-SOCS3-shRNA for 48 h could cancel the inhibitory effect of cromakalim in the LPS-treated group in vitro (Fig. 6A). Cromakalim also markedly inhibited the IL-6-mediated increase in STAT3 phosphorylation in BV-2 cells in vitro, and this effect was dependent on SOCS3 (Fig. 6B). In addition, pretreatment with cromakalim (100 μM) for 6 h, and further co-cultured with LPS (1 μg/ml) for 3 h. Magnification: 400 × . Scale bar: 20 μm. (B) Representative western blot images showing the expression of p-STAT3 and STAT3 in BV-2 cells in vitro. BV-2 cells were pretreated with LV-SOCS3 shRNA for 48 h, and then treated with cromakalim (100 μM) for 6 h, and further co-cultured with IL-6 (10 ng/ml) for 30 min. The western blot samples were collected and analyzed (n = 3). (C) Cromakalim can induce the expression of SOCS3 and inhibit the translocation of NF-κB p65 from the cytosol to the nucleus after LPS treatment in vitro. BV-2 cells were pretreated with LV-SOCS3 shRNA for 48 h, and then treated with cromakalim (100 μM) for 6 h, and further co-cultured with IL-6 (10 ng/ml) for 30 min. The western blot samples were collected and analyzed (n = 3). (D) Pretreatment with cromakalim significantly inhibited IL-1β-mediated mechanical allodynia in mice, and this effect can be reversed by small interfering RNA targeting SOCS3. IL-6 administration of SOCS3 shRNA and Control shRNA (330 nM) also conducted 48 h before behavioral testing, and then subjected to cromakalim (5 μg/10 μl, i.t.) before IL-1β (25 ng/10 μl, i.t.) treatment. von Frey hairs was then used to detect bacterial pain threshold of mice at different time points (0, 0.5, 2, 4, 8, 24 h) after cromakalim administration. Significant differences were obtained using 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).

4. Discussion

The major findings of this study were as follows: (1) KATP channel opening significantly attenuated incision-induced mechanical allodynia in mice, and was associated with the upregulated expression of SOCS3. (2) KATP channel opening inhibited incision-induced mechanical allodynia via the Gas6/Axl-SOCS3 signaling pathway. (3) KATP channel opening induced inflammatory tolerance to relieve neuroinflammation and postoperative pain.

We proposed that the regulation of the KATP gap junction signaling pathway by atraguloside IV attenuated neuropathic pain (Dai et al., 2020; Wu et al., 2011), but we did not clarify the analgesic mechanism of KATP opening. Neuroinflammation plays an important role in the development of chronic pain, and microglial activation is the basis for the expansion of the neuroinflammation cascade. A previous study found that plantar incisions can induce the activation of microglia in the spinal cord and lead to further exacerbation of pain (Wen et al., 2009). In our study, intrathecal administration of the KATP opener cromakalim significantly inhibited central inflammation and exerted an analgesic effect. Additionally, we also investigated its peripheral effect, and found that consecutive intrathecal injections of cromakalim for 3 days did not relieve plantar inflammation compared to the plantar-incision surgery group (Fig. S6). These data suggest that the analgesic effect of the KATP channel opener is related to the regulation of central neuroinflammation.

Since the KATP channel opener cromakalim was intrathecally injected into mice 30 min before plantar incision surgery, we proposed the idea of preemptive analgesia. RNA-Seq was used to investigate the alterations in genes in the spinal cord of mice treated with cromakalim, and we found that the SOCS-3 gene was significantly upregulated. SOCS3 expression was increased, and mostly co-localized with microglia in the spinal cord of the KATP channel openers group (Fig. 2). We further
verified the importance of SOCS3 through the local application of lentiviral-mediated SOCS3 overexpression or SOCS3 shRNA (Fig. 3). These results demonstrated that the preemptive analgesia of cromakalim induced SOCS3 expression in such a way as to induce inflammatory tolerance in advance to fight against subsequent damage.

How does the $K_{\text{ATP}}$ channel opener induce SOCS3 expression? The efferocytosis receptor TAM family (Tyro3, Axl, and Mer) mediates efferocytosis of apoptotic cells and induces activation of anti-inflammatory signaling pathways (Myers et al., 2019). SOCS3 is an important downstream target of TAM receptors, which participate in anti-inflammatory processes (Vago et al., 2021). A previous study showed that Gas6/Axl signaling attenuates alveolar inflammation in ischemia–reperfusion-induced acute lung injury by upregulating the SOCS3-mediated pathway (Peng et al., 2019). Our research found that the $K_{\text{ATP}}$ channel opener cromakalim significantly increased the expression of Gas6 (a substrate of Axl). Inhibition or knockout of Gas6 and Axl abolished the analgesic effect of cromakalim and inhibited the expression of SOCS3 (Figs. 4 and 5). These results suggested that the analgesic effect of the $K_{\text{ATP}}$ channel opener depends on upregulation of the Gas6/Axl/SOCS3 signaling pathway (Fig. 5).

Our study also found that cromakalim not only promoted the release of Gas6 from microglia, but also from mouse Neuro2a (N2a) cells (Fig. S4 D). Gas6 (possibly from microglia or neurons) activates Axl/SOCS3 signaling in microglia to inhibit microglial activation, decrease the production of proinflammatory cytokines (such as IL-1α and TNF-β), reduce the damage caused by proinflammatory cytokines to neurons, and improve the neuroinflammatory microenvironment. Cromakalim exerts anti-inflammatory and analgesic effects through the crosstalk between microglia and neurons.

In addition, we explored whether the SOCS3-dependent mechanism of cromakalim was applicable to other nociceptive models. We found that cromakalim alleviated IL-1β-induced mechanical allodynia in mice and inhibited the IL-1β-induced nuclear translocation of NF-κB p65 in vitro. These effects were abolished by treatment with LV-SOCS3-shRNA (Fig. 6 D). These results indicated that cromakalim-induced SOCS3 production and inflammatory tolerance and not only ameliorated postoperative pain mediated by plantar incision surgery but also alleviated IL-1β-mediated inflammatory pain.

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

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability
Data will be made available on request.

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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. CM, I-I 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, Lx H and W-T L conceived the study, participated in its design and coordination, and helped to draft the manuscript. LJ Z, M and Lx H helped revised the manuscript. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2022.09.017.

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