Research Article

Phosphorylation at Ser 727 Increases STAT3 Interaction with PKCε Regulating Neuron–Glia Crosstalk via IL-6-Mediated Hyperalgesia In Vivo and In Vitro

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Background and Aim. Interleukin-6 (IL-6) modulates neurons–glia crosstalk and subsequently triggers hyperalgesia. This study is aimed at investigating whether the interaction between protein kinase C epsilon (PKCε) and signal transducer and activator of transcription 3 (STAT3) mediated IL-6-induced hyperalgesia and neurocyte activation.

Methods. A rat hyperalgesia model was induced using an intraplantar injection of Freund’s complete adjuvant (FCA) or an intrathecal injection of IL-6. Mechanical allodynia was evaluated using von Frey filament tests after intrathecal injections of T-5224 (c-Fos/AP-1 inhibitor), minocycline (Mino, a specific microglia inhibitor), L-2-aminoadipic acid (LAA, an astroglial toxin), PKCε inhibitor peptide, APTSTAT3-9R (STAT3 inhibitor), or anti-IL-6 antibody. The c-Fos, GFAP, Iba-1, PKCε, STAT3, pSTAT3 Tyr705 and pSTAT3 Ser727, and IL-6 expression at the spinal cord level was assessed by Western blot analysis. The interactive effects of PKCε and STAT3 were determined using immunofluorescence staining and immunoprecipitation in vivo and in vitro. Interleukin-6 promoter activity was examined using luciferase assays. Results. T-5224, Mino, and LAA attenuated FCA- or IL-6-mediated inflammatory pain, with a decrease in c-Fos, GFAP, Iba-1, PKCε, and IL-6 expression. PKCε inhibitor peptide and APTSTAT3-9R (STAT3 inhibitor), or anti-IL-6 antibody. The c-Fos, GFAP, Iba-1, PKCε, STAT3, pSTAT3Tyr705 and pSTAT3Ser727, and IL-6 expression at the spinal cord level was assessed by Western blot analysis. The interactive effects of PKCε and STAT3 were determined using immunofluorescence staining and immunoprecipitation in vivo and in vitro. Interleukin-6 promoter activity was examined using luciferase assays. Results. T-5224, Mino, and LAA attenuated FCA- or IL-6-mediated inflammatory pain, with a decrease in c-Fos, GFAP, Iba-1, PKCε, and IL-6 expression. PKCε inhibitor peptide and APTSTAT3-9R (STAT3 inhibitor), or anti-IL-6 antibody.

Conclusion. STAT3 phosphorylation at Ser 727 and the interaction with PKCε contribute to hyperalgesia via the IL-6-mediated signaling pathway, thus regulating neuron–glia crosstalk during inflammatory pain.

1. Introduction

Inflammatory mediators play important roles in pain development by interfering with nociceptive cellular signal transduction and transmission. The proinflammatory cytokine interleukin-6 (IL-6) is secreted by astrocytes and microglia in the central nervous system [1, 2]. It might play an important role in the development and maintenance of
2.3. Inflammatory Pain and IL-6-Induced Hyperalgesia Model. Freund’s complete adjuvant (FCA; Sigma–Aldrich Corp.) consisting of heat-killed Mycobacterium tuberculosis (1 mg/ml) in paraffin oil (150 μL) was injected into the plan tar area of the right hind paws of the rats under 1%–3% isoflurane anesthesia delivered with oxygen at a flow rate of 1 L/min. The left hind paw was not injected. Physical signs (e.g., redness and swelling) and pain behavior were monitored for 24 h after FCA injection. A model of hyperalgesia was established using naïve rats given an intrathecal injection of IL-6 (20 ng/50 μL) through the L5–L6 lumbar interspace identified by the tail-flick reflex 10 min before drug administration under isoflurane anesthesia.

2.4. Experiments In Vivo. Figure 1 shows the experimental protocol in vivo.

2.4.1. Experiment 1. The rats were randomly assigned to the following groups (n = 6/group): untreated (control naïve rats), FCA, FCA + T-5224 500 μg/50 μL (T-5224), FCA + Mino 100 μg/50 μL (Mino), FCA + LAA 1 mg/50 μL (LAA), and PBS 50 μL (vehicle).

2.4.2. Experiment 2. We investigated the roles of PKCε, STAT3, and IL-6 in the inflammatory process by randomizing rats to the following groups (n = 6/group): untreated (control naïve rats), FCA, FCA + PKCε inhibitor peptide 100 μg/50 μL (PKCε inhibitor), FCA + APTSTAT3-9R 20 μg/50 μL (APTSTAT3-9R), FCA + anti-IL-6 antibody 100 ng/50 μL (anti-IL-6), and 1% DMSO 50 μL (vehicle).

2.4.3. Experiment 3. We evaluated the effects of neuron-glial activity inhibitors on IL-6-induced hyperalgesia by randomizing rats to the following groups (n = 6/group): untreated (control naïve rats), IL-6, IL-6 + T-5224 500 μg/50 μL (T-5224), IL-6 + Mino 100 μg/50 μL (Mino), IL-6 + LAA 1 mg/50 μL (LAA), and 1% DMSO 50 μL (vehicle). All inhibitors were injected intrathecally 10 min before IL-6 (20 ng/50 μL).

2.4.4. Experiment 4. We assessed the effects of PKCε and STAT3 on IL-6-induced hyperalgesia by randomizing rats to the following groups (n = 6/group): untreated (control naïve rats), IL-6, IL-6 + PKCε inhibitor 100 μg/50 μL, IL-6 + APTSTAT3-9R 20 μg/50 μL, IL-6 + anti-IL-6 antibody 100 ng/50 μL, and PBS 50 μL (vehicle). All drugs were injected intrathecally 10 min before IL-6 (20 ng/50 μL).

The paw withdrawal mechanical threshold (PWMt) was measured daily after the intraplantar injection of FCA (Figure 1). All agents described earlier were injected intrathecally on days 4 and 6 after the intraplantar injection of FCA. The spinal cords were harvested on day 7. In the IL-6-induced hyperalgesia model, the pain thresholds after chemical administration were assessed as the PWMt up to 120 min after IL-6 administration.

2.5. Von Frey Filament Tests. We measured PWMT using von Frey filament tests (Stoelting Co., IL, USA) using the up-and-down method [18]. The rats were habituated to a wire mesh platform for at least 1 h/d for 3 days before starting experiments. All groups (n = 6 each) were tested daily.
before drug application to determine baseline levels. Briefly, the positive and negative data were tabulated as follows: X = withdrawal and 0 = no withdrawal. The 50% response threshold was interpolated using the following formula:
\[
\text{50\% threshold} = \frac{10^{-\left(x_f + k\delta\right)/10,000}}{10,000},
\]
where \(x_f\) is the last value (in log units) of the von Frey filament test, \(k\) is the tabular value for positive/negative responses, and \(\delta\) is the mean difference (in log units) between stimuli [18]. The PWMT was defined as the means of six animals before and after chemical injections. Areas under receiver operator characteristic curves (AUC) were calculated to determine the effects of the injected chemicals.

2.7. Immunofluorescence Staining. After transcardial perfusion 7 days after FCA administration with 4% paraformaldehyde, the spinal cords were removed from the rats, postfixed, and dehydrated. Transverse frozen sections (10\(\mu\)m) prepared from OCT-embedded tissues were incubated overnight with rabbit polyclonal anti-PKC\(\varepsilon\) (Abcam) and mouse monoclonal anti-STAT3 (Cell Signaling Technology). The proteins in the tissues were visualized using a DML LED scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany). Primary or secondary antibodies were omitted to ensure staining specificity. The data from three to four sections per rat (\(n = 6\)/group) were analyzed.
2.8. Immunoprecipitation Assays. Naive rats were euthanized under isoflurane anesthesia. The lumbar segments L3–L5 from whole spinal cords were ultrasonicated in ice-cold RIPA buffer (Beyotime, Shanghai, China). The supernatant after low-speed centrifugation was diluted with lysis buffer and adjusted to 2 mg/mL. The cell suspensions passed through agarose resin served as controls. The sonicates were incubated overnight at 4°C with PKCe and IgG primary antibody (5 μL) to form immune complexes that were captured with fresh elution buffer and then incubated at 95°C for 6–8 min in RIPA buffer. The mixture was centrifuged, and then, endogenous PKCe/STAT3 immune complexes in the supernatant were analyzed by Western blot analysis.

2.9. Cell Culture and Transfection. HEK293 cells (Shanghai Institutes for Biological Sciences, Shanghai, China) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific Inc., Waltham, MA USA) at 37°C in a 5% CO2 incubator (Thermo Fisher Scientific Inc.). Various constructs were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific Inc.) as described by the manufacturer.

Full-length PKCe and STAT3 were, respectively, cloned into pEGFP-C1 (Clontech Laboratories Inc., Mountain View, CA, USA) and pECMV-3X Flag-N (Biofeng, Beijing, China), then transfected into HEK293 cells. Two phosphorylation sites were mutated using point mutation technology to construct phenylalanine and alanine mutants STAT3Y705F and STAT3Y705A, respectively, which mimic dephosphorylated STAT3. The aspartic acid mutants STAT3Y705D and STAT3 mimic phosphorylated STAT3.

Extracts of transfected cells were quantified using BCA assay kits (Beyotime). Supernatants were diluted with lysate buffer and adjusted to 2 mg/mL of protein; then, 300 μL portions were incubated overnight with 20 μL of anti-Flag magnetic beads (Beyotime) at 4°C. Immune complexes with magnetic beads were washed three times with lysis buffer, precipitated, then eluted from the beads by boiling with 30 μL of SDS-PAGE loading buffer for 6–8 min. The eluate was centrifuged; then, proteins in the supernatant were analyzed by western blotting.

2.10. IL-6 Promoter Activity. The IL-6 promoter region (-1500 to +19) was amplified by PCR and ligated into the pGL3 Basic vector (Biofeng). Full-length PKCe and STAT3 were cloned into pRL-null cells (Biofeng), then transfected into cultured HEK293 cells. The pGL3-IL-6 promoter (0.5 μg), pRL-null (Renilla, 0.5 μg), pRL-STAT3 (1 μg), and pRL-PKCe (1 μg) expression vectors were cotransfected overnight into HEK293 cells. The transfected cells were incubated with 1 μg/mL lipopolysaccharide (LPS) for 12 h, and then, IL-6 promoter activity was assayed using dual luciferase kits (Jikai Gene Chemical Technology Co., Ltd., Shanghai, China).

2.11. Statistical Analysis. All data were presented as means ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni tests using SPSS 19.0 (IBM Corp., NY, USA). The graphs were constructed using the GraphPad Prism 7.0 software (GraphPad Software Inc., CA, USA). The statistical significance was set at P < 0.05.

3. Results

3.1. Inhibiting Activated Neurocytes Alleviated Inflammatory Pain and IL-6-Induced Hyperalgesia. Two injections each of T-5224, Mino, and LAA, respectively, inhibited the activation of neurons, astrocytes, and microglia cells and generated immediate and prolonged anti-FCA-induced inflammatory pain in the model (Figure 2(a)). The AUC of T-5224, Mino, and LAA in the ipsilateral side paw of the FCA-induced inflammatory pain model was, respectively, 0.94 ± 0.02, 0.91 ± 0.03, and 0.94 ± 0.02 (Figure 2(b)). These agents did not affect the mechanical threshold in the paw that was not injected (Figure 2(c)).

A single intrathecal injection of IL-6 (20 ng/50 μL) evoked transient, but significant, mechanical allodynia in the right hind paws of naïve rats. This was similar to the FCA-induced mechanical hypersensitivity and was reversed by T-5224, Mino, and LAA (Figure 2(d)). The AUC of T-5224, Mino, and LAA was 0.87 ± 0.05, 0.87 ± 0.05, and 0.88 ± 0.05, respectively, in the right hind paws of the rats under IL-6-mediated hyperalgesia (Figure 2(e)). The analogous effects of T-5224, Mino, and LAA were similar in the left hind paw under IL-6-induced hyperalgesia (Figure 2(f)).

3.2. T-5224, Mino, and LAA Decreased the Expression of c-Fos, GFAP, Iba-1, PKCe, and IL-6. T-5224, Mino, and LAA significantly decreased the expression of c-Fos, GFAP, and Iba-1, which were, respectively, the FCA-induced markers of neurons, astrocyte, and microglia activation in the spinal cord (Figures 3(a)–3(d)). The expression of PKCe and IL-6 in the spinal cord was also upregulated in FCA-treated rats (Figures 3(e) and 3(f)), whereas that of STAT3 did not differ among the groups (Figure 3(g)), indicating that the effects of T-5224, Mino, and LAA were exerted through the PKCe and IL-6 pathways, but not via the STAT3 signaling pathway.

3.3. Roles of PKCe, STAT3, and IL-6 in Inflammatory Pain and IL-6-Induced Hyperalgesia. We blocked the corresponding cellular signaling pathways using PKCe inhibitor peptide, APTSTAT3-9R, and anti-IL-6 antibodies to determine the roles of PKCe, STAT3, and IL-6 in the pain process. Anti-IL-6 significantly increased mechanical threshold inflammatory pain on the ipsilateral, but not the contralateral, hind paw from the start of drug injection for up to 7 days, whereas PKCe inhibitor peptide and APTSTAT3-9R increased mechanical pain threshold from days 5–7 (Figure 4(a)). The AUC of PKCe inhibitor peptide, APTSTAT3-9R, and anti-IL-6 antibody was 0.73 ± 0.05, 0.66 ± 0.06, and 0.91 ± 0.03 in the ipsilateral side hind paw of the FCA-induced inflammatory pain model, respectively (Figure 4(b)). The intrathecal administration of T-5224, Mino, and LAA did not affect the FCA-induced mechanical threshold in the contralateral noninflamed paw (Figure 4(c)).
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Figure 2: Continued.
When these three conditions were applied to rats with IL-6-induced hyperalgesia, only anti-IL-6 improved the hypersensitivity and response to pain in the right paw (Figure 4(d)). The AUC of the PKCe inhibitor peptide, APTSTAT3-9R, and anti-IL-6 antibody was 0.64 ± 0.08, 0.64 ± 0.08, and 0.95 ± 0.03 in the right hind paw under IL-6-mediated hyperalgesia, respectively (Figure 4(e)). The analgesic effect of anti-IL-6 was similar in the left hind paw (Figure 4(f)).

3.4. PKCe Inhibitor Peptide, APTSTAT3-9R, and Anti-IL-6 Antibody Decreased the Expression of IL-6, c-Fos, GFAP, and Iba-1. The PKCe inhibitor peptide reduced PKCe expression, and APTSTAT3-9R downregulated the spinal level of STAT3 (Figures 5(a)–5(c)). The PKCe inhibitor peptide, APTSTAT3-9R, and anti-IL-6 antibodies significantly decreased the spinal levels of phosphorylated STAT3\(^{\text{Ser727}}\) but not of STAT3\(^{\text{Thr705}}\) and IL-6 (Figures 5(d)–5(f)). The expression of c-Fos, GFAP, and Iba-1 in the spinal cord was also decreased by the PKCe inhibitor peptide, APTSTAT3-9R, and anti-IL-6 antibody in rats with FCA-induced inflammatory pain (Figures 5(g)–5(i)).

3.5. Phosphorylation of STAT3\(^{\text{Ser727}}\) Increased STAT3 Interaction with PKCe. The expression of PKCe/STAT3 in the dorsal horn of the spinal cord was significant decreased by PKCe or STAT3 inhibitor but not by anti-IL-6 antibody compared with that in the control and FCA groups (Figures 6(a)–6(f)).

Endogenous PKCe/STAT3 immunocomplexes in spinal cord tissues were assessed (Figure 7(a)). After incubating HEK293 cells with lipopolysaccharide (LPS), STAT3 increased IL-6 promoter activity, which was also enhanced in the presence of PKCe (Figure 7(b)). The STAT3\(^{\text{Ser727D}}\) phosphomimetic mutant had more affinity for PKCe, whereas the other mutants generated results similar to those of wild-type STAT3, indicating that the phosphorylation at Ser727 increased the ability of STAT3 to bind to PKCe (Figures 7(c) and 7(d)).

4. Discussion

Our results showed that FCA-induced inflammatory pain and IL-6-induced hyperalgesia were alleviated by inhibiting neurocyste activation or by anti-IL-6 therapy, indicating that IL-6 participated in the maintenance of inflammation-induced nociception. This study was novel in demonstrating that T-5224, Mino, and LAA inhibited FCA-induced inflammatory pain and IL-6-induced hyperalgesia, despite previous findings of the therapeutic effects of Mino against chronic bone cancer pain and chronic pain [19, 20].
PKCε (84kDa)
IL–6 (24kDa)
c-Fos (50kDa)
GFAP (50kDa)
Iba-1 (17kDa)
PKCe (84kDa)
STAT3 (86kDa)
GAPDH (34kDa)

Western blot

(a)

IOD of c–Fos (%)

(b)

IOD of GFAP (%)

(c)

IOD of Iba-1 (%)

(d)

Figure 3: Continued.
The present study used an inflammatory pain model created by unilateral injections of FCA or intrathecal injections of IL-6 that induced hyperalgesia in rats. The mechanical paw withdrawal threshold was significantly reduced for up to 7 days by FCA and up to 60 min by IL-6. An intraplantar injection of FCA induced central sensitization and increased the levels of pain mediators, including IL-6, peripherally and centrally, whereas the intrathecal injection of IL-6 likely resulted in transient central sensitization due to a local increase in pain mediators per se [6, 21].

We found that T-5224, Mino, and LAA decreased the expression levels of c-Fos, GFAP, Iba-1, PKCe, and IL-6 but did not alter STAT3 levels during the FCA-induced inflammatory process (Figure 3). Our results indicated that the inhibition of neurocyte activation reduced IL-6-mediated pain sensitivity, which was in line with previous findings of the crosstalk between activated neurocytes and IL-6-induced pain [3, 6, 16, 22, 23]. However, the analgesic effects of T-5224, Mino, and LAA were associated with other cytokines such as IL-1β and TNF-α [24], suggesting that activated neurocytes comprised a control mechanism of inflammatory pain [25].

We further examined the roles of PKCe, STAT3, and IL-6 in FCA-induced inflammatory pain and IL-6-induced hyperalgesia. Anti-IL-6 immediately alleviated inflammatory pain for an extended period and reversed the hyperalgesic effects of IL-6 (Figure 4), suggesting that IL-6 was a potent pain mediator. In contrast, the PKCe inhibitor peptide and

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**Figure 3**: Western blot analysis of the proteins related to activated neurocytes during inflammatory pain. Examples (a) and mean values (b-g) of c-Fos, GFAP, Iba-1, PKCe, STAT3, and IL-6 proteins in the spinal cord. Intrathecally injected T-5224 (c-Fos/AP-1 inhibitor, 500 μg/50 μL), minocycline (Mino, 100 μg/50 μL), and L-2-aminoadipic acid (LAA, 1 mg/50 μL) reduced c-Fos, GFAP, Iba-1, PKCe, and IL-6 protein levels in FCA-treated rats, whereas STAT3 expression did not change between groups (P > 0.05). Data were normalized against GAPDH and are expressed as ratios (%) of control. Data are shown as means ± SD (n = 4–5). *P < 0.05, **P < 0.01; ***P < 0.001, one-way ANOVA with Bonferroni tests.
Figure 4: Continued.
induced mechanical hyperalgesia, whereas anti-IL-6 antibody (100 ng/50 μL) in cells in the spinal cord (Figure 6), suggesting that PKCε contributed to IL-6 production and pSTAT3 Tyr705, IL-6, c-Fos, and neurocytes [26–30]. PKCε and STAT3 regulated the formation of IL-6, and pSTAT3 Ser727 formed complexes with PKCε and enhanced STAT3 localization to the IL-6 promoter, thus increasing IL-6 expression [31]. The decrease in the levels of pSTAT3 and IL-6 in cells incubated with APTSTAT3-9R and anti-IL-6 suggested that STAT3 was involved in a negative feedback loop in the IL-6-induced signaling pathway per se.

PKCε and STAT3 interacted under physiological and pathological conditions [11, 31]. Our immunofluorescence results also revealed the coexpression of PKCε and STAT3 in cells in the spinal cord (Figure 6), suggesting that PKCε together with STAT3 contributed to IL-6 production and the activation of neurons, astrocytes, and microglia during inflammation. Alone, PKCε did not affect the activity of the IL-6 promoter; but IL-6 promoter activity was increased more in the presence of both PKCε and STAT3 than that in the presence of STAT3 alone under LPS stimulation (Figure 7), suggesting that PKCε increased the ability of STAT3 to bind to the IL-6 promoter. The immunoprecipitation results in vitro showed that the pSTAT3Ser727 but not pSTAT3Tyr705 affected the interactions between PKCε and STAT3 (Figure 7), suggesting that pSTAT3Ser727 regulated the formation of PKCε/STAT3 complexes, thus influencing IL-6-mediated inflammatory pain.

Given the analgesic effect of T-5224, Mino, and LAA, they may serve as the potential therapeutic agents for inflammatory pain-related disease. This needs further discussion to understand the potential pharmacological characteristics of these compounds. T-5224 was first designed to inhibit the arthritis upstream of inflammatory cytokine and matrix metalloproteinase action [32]. It can be used in human articular chondrocytes, resulting in the inhibition of transactivation of downstream matrix metalloproteinases and inflammatory cytokines (including IL-6, IL-1β, and TNF-α) and effectively preventing cartilage destruction and osteophyte formation in an osteoarthritis-induced mouse model [33]. T-5224 was also found to be
Western blot

(a)

(b)

(c)

(d)

Figure 5: Continued.
used in acute myeloid leukemia [34], mast cell [35], and triple negative breast cancer [36], which might serve as a synergistic therapeutic strategy for the clinical diseases; however, T-5224 is still in the preclinical stage. Hence, the data related to pharmacokinetics are lacking; more research and evidence are needed in the future. Mino is a semisynthetic tetracycline antibiotic with anti-inflammatory properties, which is used to treat multiple inflammatory diseases and could be safely applied in the clinical setting, such as Parkinson’s disease [37] and neurodegenerative and psychiatric diseases [38] as well as the cerebral ischaemia [39]. It is generally well tolerated, and skin-related complaints, nausea, and dizziness are the most common patient-reported side effects [40]. LAA, a selective astrocytic toxin, has been demonstrated to exert some regulatory effects on tibia fracture [41], myotubes [42], and retina [43], thus contributing to the fracture-induced nociceptive, cell autophagy in myotubes, and retinal vascular responses. It has not yet been clinically applied due to its unusual astroglial toxin, which may trigger locomotor network damage. How to reduce the toxicity of LAA to central and peripheral nerves to the minimum is worth exploring [44].

Figure 5: Expression of proteins related to activated neurocytes detected by Western blot analysis. (a–i) Protein expression of pSTAT3 (Ser727), IL-6, c-Fos, GFAP, and Iba-1 in the spinal cords of FCA-treated rats significantly decreased (P < 0.05) after intrathecal injections of PKCε inhibitor peptide (100 μg/50 μL), APTSTAT3-9R (20 μg/50 μL), and anti-IL-6 antibody (100 ng/50 μL). Values were normalized against GAPDH and are expressed as ratios (%) of control values. Data are shown as means ± SD (n = 4–5). *P < 0.05, **P < 0.01, ***P < 0.001; one-way ANOVA followed by Bonferroni tests.
Figure 6: Continued.
PKCε inhibitor

APTSTAT3-9R

(c)

(d)

Figure 6: Continued.
Figure 6: Detection of PKCε and STAT3 coexpression in vivo and their immune complexes in vitro. (a–f) Immunofluorescence staining for PKCε (red) and STAT3 (green) coexpression (yellow) and DAPI (blue in merged image) in spinal cord sections (a–e). Ratios of cells with immunoreactive PKCε-/STAT3 among total cells. (f) Inhibitors of PKCε and STAT3 significantly decreased the coexpression of PKCε/STAT3 after APTSTAT3-9R administration. Bar = 40 μm. Data are shown as means ± SD (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001; one-way ANOVA followed by Bonferroni tests.
This study had some limitations. It focused only on IL-6-induced pain, and thus, its clinical relevance is debatable. However, early and delayed IL-6 elevation is associated with chronic neuropathic pain [45]. Interleukin-6 plays a key role in the chronic inflammation associated with rheumatoid arthritis (RA), and blocking IL-6 signaling is an important strategy in treating RA-associated diseases clinically [46]. Moreover, targeting IL-6 might be an option for treating other chronic inflammatory diseases [47]. Therefore, an in-depth understanding of how IL-6 induces cellular signaling that causes pain, and the development of new analgesic strategies associated with IL-6, have theoretical and clinical significance for pain management.

Figure 7: Ser727 of STAT3 increased its interaction with PKCe. (a) Endogenous PKCe was immunoprecipitated from cell lysates, and immune complexes and total cell lysates were analyzed by Western blot analysis with PKCe and STAT3 antibodies. Endogenous immune PKCe/STAT3 complexes were detected in the rat spinal cord tissues. (b) IL-6 promoter-firefly luciferase reporter plasmid (0.5 μg), PKCe (1 μg), and STAT3 (1 μg) were cotransfected overnight into HEK293 cells. The transfected cells were incubated with 1 μg/mL lipopolysaccharide (LPS) for 12h. Interleukin-6 promoter activity increased by STAT3 was further enhanced by PKCe and STAT3, indicating that PKCe improved the ability of STAT3 to bind to IL-6 promoter. Data are shown as means ± SD (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001; one-way ANOVA followed by Bonferroni tests. (c, d) HKE293 cells were transfected with GFP, GFP-PKCε, Flag, Flag-STAT3, and phosphomimetic and dephosphomimetic mutants of STAT3, and then, immunoprecipitants were assayed. Protein complexes were detected using an anti-GFP antibody (c), and then, relative PKCe binding to STAT3 was quantified (d). Data are presented as means ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001; one-way ANOVA followed by Bonferroni tests.
In this study, we found that the phosphorylation at Ser727 increased STAT3 interaction with PKC\(\varepsilon\). This increased IL-6 promoter activity and upregulated IL-6 expression, thus enhancing neuron–glia activation during the development of inflammatory pain. In addition, the PKC\(\varepsilon\) inhibitor peptide and STAT3 inhibitor (APTSTAT3-9R) attenuated FCA-induced nociceptive behavior via IL-6 downregulation (Figure 8).

5. Conclusions

In summary, pSTAT3\(^{\text{Ser727}}\) interaction with PKC\(\varepsilon\) contributes to FCA-induced inflammatory pain and IL-6-mediated hyperalgesia via IL-6-modulating crosstalk among neurons, astrocytes, and microglia and their activation. The translational value of our findings warrants further investigation.

Abbreviations

APTSTAT3-9R: STAT3 inhibitor
AUC: Area under the receiver operating characteristic curve

DMEM: Dulbecco’s modified Eagle’s medium
FCA: Freund’s complete adjuvant
GFAP: Glial fibrillary acidic protein
IF: Immunofluorescence
IL-6: Interleukin-6
LAA: L-2-Aminoadipic acid
LPS: Lipopolysaccharide
Mino: Minocycline
PKC\(\varepsilon\): Protein kinase C epsilon
PWMT: Paw withdraw mechanical threshold
ROC: Receiver operator characteristics curve
STAT3: Signal transducer and activator of transcription 3.

Data Availability

The data used to support the findings of this study are available from the corresponding upon request.

Conflicts of Interest

The authors have no competing interests to declare.
**Authors’ Contributions**

XL and BZ designed and implemented the experiments, curated the data, and wrote and edited the original draft of the manuscript. HY, XY, ZZ, ZP, XL, WJ, and YL collected and statistically analyzed the data. HL, QX, YL, BY, and HH helped to conduct the study and prepared the experimental materials. XL, DM, and ZL supervised and reviewed the manuscript. HY, XY, ZZ, ZP, XL, WJ, and YL contributed equally to this work.

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