

Involvement of Spinal CCR5/PKC γ Signaling Pathway in the Maintenance of Cancer-Induced Bone Pain

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Abstract Cancer-induced bone pain (CIBP) is a challenging medical problem that considerably influences cancer patients' quality of life. Currently, few treatments have been developed to conquer CIBP because of a poor understanding of the potential mechanisms. Our previous work has proved that spinal RANTES (a major ligand for CCR5) was involved in the maintenance of CIBP. In this study, we attempted to investigate whether spinal CCR5 and its downstream PKC γ pathway is involved in the maintenance of CIBP. Inoculation of Walker 256 cells into the tibia could induce a marked mechanical allodynia with concomitant upregulation of spinal CCR5 and p-PKC γ expression from day 6 to day 15 after inoculation. Spinal CCR5 was prominently expressed in microglia, and mechanical allodynia was attenuated by intrathecal injection of DAPTA (a specific antagonist of CCR5) with downregulation of spinal CCR5 and p-PKC γ expression levels at day 15 in inoculated rats. Pre-intrathecal injection of RANTES could reverse the anti-allodynia effects of DAPTA. Intrathecal administration of GF109203X (an inhibitor of PKC) could alleviate mechanical allodynia as well as decrease of spinal p-PKC γ expression level, but no influence on spinal CCR5 level. Our findings suggest that CCR5/PKC γ signaling pathway in microglia may contribute to the maintenance of CIBP in rats.

Keywords Cancer-induced bone pain · CCR5 · PKC γ · Signaling pathway

Introduction

With the development of cancer diagnoses and treatments, the life expectancy of cancer patients has been markedly increased. However, cancer-induced bone pain (CIBP) is a challenging medical problem that considerably influences cancer patients' quality of life [1–3]. It has been reported that more than one-third of patients with advanced-stage cancer will undergo skeletal metastases and experience severe CIBP [4, 5]. Currently, about 45% of patients with CIBP have inadequate pain control because current therapies are lack of efficacy or associate with dose-limiting side effects [6, 7]. Thus, understanding the potential molecular and cellular mechanisms underlying CIBP is necessary for the development more effective therapies to conquer CIBP.

Recent studies indicated that several chemokines and chemokine receptors were involved in modulating pain responses [8, 9]. CC chemokine receptor 5 (CCR5) is one of the chemokine receptors, which belongs to the family of seven-transmembrane G protein coupled receptor. The RANTES (regulated on activation normal T cell expressed and secreted; also named as CCL5) is a major ligand for CCR5. Our previous work has proved that the antinociceptive effects of triptolide correlated with inhibition of spinal RANTES expression in a rat model of CIBP [10]. The analgesic effects of triptolide also have been proved in inflammatory and neuropathic pain models [11, 12]. The established mechanical allodynia could be attenuated by intrathecal injection of anti-RANTES neutralizing antibody in CIBP rats [13]. These results indicated that spinal RANTES might participate in the CIBP processing.

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However, the potential mechanism of CCR5 in the maintenance of CIBP remains unknown.

In vitro study has shown that CCR5 can be coupled to Gαq, Gas, and Ga12/13 protein [14]. Gαq activates the phospholipase C-γ, leading to the activation of protein kinase C (PKC). Activation of Gas is able to activate PKA signaling pathway through increased adenylyl cyclase activity. Ga12/13 can activate RhoA/Rho kinase (ROCK) signaling pathway. A few researches have indicated that involvement of spinal PKC pathway in inflammatory and neuropathic pain [15–17]. If spinal CCR5 participates in the maintenance of CIBP, whether its downstream PKC signaling pathway is also involved in the CIBP still remains unknown. In the current study, we attempted to investigate whether spinal CCR5/PKCγ pathway is involved in the maintenance of CIBP.

Methods

Animals

All animal studies were approved by the Animal Care and Use Committee of the Jiangsu University, and complied with the National Institutes of Health guidelines for Care and Use of Laboratory Animals. Female Wistar rats, weighing 150–180 g, were used in this study. Rats were kept in a temperature-controlled room (22 ± 2 °C) with free access to food and water.

Drug Application

DAPTA (D-Ala-peptide T-amide), a specific antagonist of CCR5, and recombinant rat RANTES, an agonist of CCR5 were obtained from R&D Systems (Minneapolis, USA). GF109203X was purchased from Sigma-Aldrich (Shanghai, China), and dissolved in 1% DMSO. The dosage of drugs was determined by the results of pilot experiments.

A Rat Model of CIBP

A rat model of CIBP was conducted as described previously [18]. In brief, under anesthesia with sodium pentobarbital (50 mg/kg, i.p), the toe pinch test was performed to ensure an adequate state of anesthesia for animal. The rat was placed with the supine position, and the left tibia was prepared for inoculation. After iodine disinfection, a small incision was cut on the anterior-medial surface to expose the tibial plateau. The needle of 23-gauge was used to drill a hole in the tibial plateau, then, Walker 256 mammary gland carcinoma cells ($10 \mu\text{l}$, 1×10^5 cells, supplied by the Soochow University) or heat-killed cells (for sham group) was inoculated into the intramedullary space of the tibia.

After inoculation, the medical glue was used to close the hole. Finally, the incision was dusted with penicillin powder and sutured using silk thread. After fully awake, the rats returned to their individual cages.

Intrathecal Cannula Implantation

Intrathecal cannula implantation was carried out 3 days after inoculation [19]. Under anesthesia, a PE-10 tube was inserted through the L5 and L6 space, and the tip of the catheter was put near the lumbar enlargement. The position of the catheter was identified by intrathecal administration of lidocaine (2%, 10 μl).

Pain Behavior

Mechanical allodynia was assessed by von Frey hairs [19]. In brief, animals were put in individual acrylic boxes ($15 \times 20 \times 25$ cm) with a metal mesh floor and permitted 30 min to acclimatize to the chamber. A series of von Frey hairs with logarithmically incremental force was tested on the plantar surface of left hind-paw to measure the paw withdrawal threshold (PWT). Paw flinching or quick withdrawal was defined as a positive response. The investigators for behavioral test were blinded to the experimental protocol.

Immunohistochemistry

Rats were transcardially perfused with 0.9% normal saline followed by 200 ml of 4% paraformaldehyde under deeply anesthesia with pentobarbital sodium. Spinal dorsal horns (L4 and L5) were quickly removed on ice and postfixed with the same fixative for 24 h. The tissues were sectioned at 30 μm and every fifth section was collected in PBS. The sections were incubated for 60 min in blocking buffer (0.3% Triton X-100 and 2% goat serum), then incubated with one of the following primary antibodies overnight at 4 °C: anti-CCR5 antibody (1:500; Abcam, USA), anti-GFAP antibody (a marker for astrocyte; 1:500; Abcam, USA), anti-OX42 antibody (a marker for microglia; 1:500; Abcam, USA), and anti-NeuN antibody (a marker for neuron; 1:500, Abcam, USA). The DyLight® 488 or 594 conjugated secondary antibodies (1:500, Abcam, USA) were used for detection. In double label immunofluorescence experiments, the sections were incubated with a cocktail of two primary antibodies and the corresponding secondary antibodies. Finally, images in laminae I and II were captured by a fluorescence microscope. The Image J software was used to measure the staining intensity. The average intensity from four rats was presented as group data.

Western Blot

The tissue samples (dorsal horn of L4-L5) were solubilized in ice-cold RIPA buffer plus phosphatase inhibitors, and centrifuged at 10,000g for 30 min at 4 °C. The Bradford method was used to determine the protein concentrations in the supernatant. Fifty micrograms of proteins were loaded for each lane, separated using 12% SDS-PAGE gel, and electrophoretically transferred onto PVDF membrane (Millipore, USA). Then, the blots were incubated with anti-CCR5 antibody (1:1000; Abcam, USA), anti-phospho PKC γ antibody (1:1000; Abcam, USA) or anti-PKC γ antibody (1:1000; Abcam, USA) overnight at 4 °C, and then for 1 h with HP-conjugated secondary antibody. The blots were visualized by the ECL detection system. Expression of CCR5 and p-PKC γ was normalized to the β -actin.

Statistical Analysis

All data are expressed as mean \pm SEM. Repeated-measures (RM) ANOVA followed by Bonferroni test was used to analyze the behavioral data. One-way ANOVA followed by Bonferroni test was used to analyze western blot and immunofluorescence data. $p < 0.05$ was considered significant. Statistical analysis was carried out with the SPSS16.0 software.

Results

Pain Behavior Over Time

The baseline PWT values were not statistically different between sham and CIBP rats ($n=8$, $p > 0.05$). Compared with sham rats, the PWT values of the ipsilateral hind paw were markedly reduced from day 6 to day 15 in inoculated rats ($p < 0.05$ or 0.01; Fig. 1), suggesting the progressive development of mechanical allodynia. However, within the 15-day observation time, the PWT values of contralateral hind paw kept relatively unaltered.

Upregulation of Spinal CCR5 and p-PKC γ Expression in CIBP Rats

To examine whether CCR5 participates in the maintenance of CIBP, CCR5 expression levels were checked by immunohistochemistry and Western blot. The results of immunostaining showed that CCR5 expression in the ipsilateral spinal dorsal horn was markedly increased at days 6, 12, and 15 in inoculated rats (Fig. 2a–d). From day 6 to day 15 after inoculation, a statistically significant increase in the intensity of CCR5 was found (Fig. 2k). Moreover, progressively increased protein expression levels of CCR5

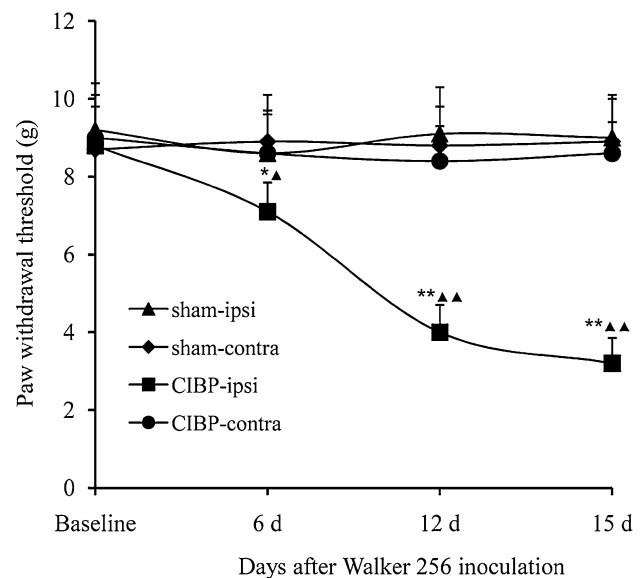


Fig. 1 Mechanical allodynia was induced by Walker 256 cells intramedullary inoculation. Compared with sham rats, the paw withdrawal threshold (PWT) values of the ipsilateral hind paw were markedly reduced on days 6, 12 and 15 at the same time-point in inoculated rats. The baseline PWT values were not statistically different between sham and CIBP rats. Within the 15-day observation time, the PWT values of contralateral hind paw kept relatively unaltered. (▲ $p < 0.05$, ▲▲ $p < 0.01$ versus baseline, * $p < 0.05$, ** $p < 0.01$ versus sham group. Data were expressed as the mean \pm SEM, each group contained eight rats.)

(Fig. 2m) and p-PKC γ (Fig. 2n) were found by Western blot analysis in inoculated rats.

To investigate the cellular distribution of CCR5 in spinal dorsal horn, double immunostaining was used. The images showed that CCR5 was mainly colocalized with OX-42 (microglial marker; Fig. 2h), but not with NeuN (neuronal marker; Fig. 2g) or GFAP (astrocytic marker; Fig. 2f), suggesting expression of CCR5 by microglia. Compared with CIBP 6 d group (Fig. 2i), the number of CCR5/OX-42 double positive cells was markedly increased in CIBP 15 d group (Fig. 2h, l). Taken together, these results suggested that the close relationship between the number of microglia and CCR5 expression level.

Intrathecal of DAPTA Attenuated Mechanical Allodynia and Decreased CCR5 and p-PKC γ Expression Levels in CIBP Rats

To further check the roles of CCR5 in the maintenance of CIBP, DAPTA (5 or 10 μ g, a CCR5 antagonist) was injected intrathecally in CIBP rats at 15 days after inoculation. Intrathecal DAPTA could significantly decrease the PWT values at 1, 2, and 3 h in a time- and dose-dependent manner ($p < 0.05$ or 0.01). Intrathecal RANTES (0.2 μ g, a CCR5 ligand) had no effects on the values of PWT.

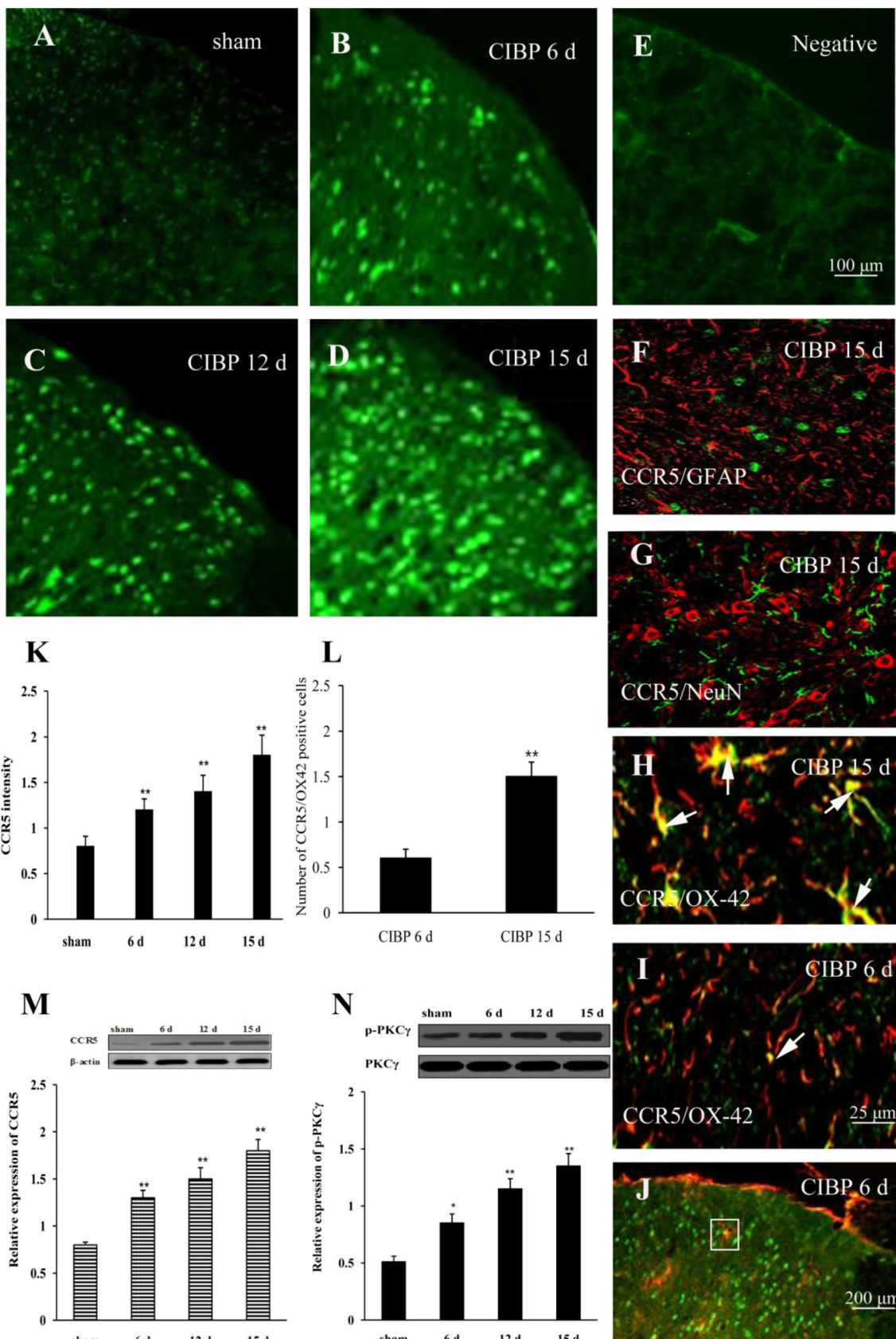


Fig. 2 Upregulation of CCR5 and p-PKC γ expression in CIBP rats. **a–d** The results of immunostaining showed that the CCR5 expression in the ipsilateral spinal dorsal horn was markedly increased at days 6, 12, and 15 in inoculated rats. The intensities of immunostaining were calculated in laminae I and II of the ipsilateral spinal dorsal horn. **e** Control tissues that were immunostained only with secondary antibody. Scale bar 100 μ m. The images of double immunostaining revealed that CCR5 (green) was mainly colocalized with microglial marker OX-42 (**h** and **i**, yellow, white arrows), but not colocalized with the astrocytic marker GFAP (red, **f**) or neuronal marker NeuN (red, **g**). Scale bar 25 μ m. **j** The white box indicates the location of panel I on the lower power image. Scale bar 200 μ m. Compared with CIBP 6 d group, the number of CCR5/OX-42 double positive cells was markedly increased in CIBP 15 d group (**i**, **h**, and **I**). From day 6 to day 15 after inoculation, a statistically significant increase in the intensity of CCR5 was found (**k**). Moreover, progressively increased protein expression levels of CCR5 (**m**) and p-PKC γ (**n**) were found by western blot analysis in inoculated rats. (* $p < 0.05$, ** $p < 0.01$ versus sham rats. Data were presented as mean \pm SEM, each group contained four rats.) (Color figure online)

However, the anti-allodynia effects of DAPTA (10 μ g) was reversed by pre-intrathecal injection of RANTES (0.2 μ g) 15 min before intrathecal DAPTA (Fig. 3a). These data suggested that CCR5 might play a key role in the maintenance of CIBP.

To examine the effects of intrathecal DAPTA on the spinal CCR5 and p-PKC γ expression levels, the Western blot analysis was used. The tissues of spinal dorsal horn were harvested at 3 h after intrathecal injection. Compared with sham group, the CCR5 and p-PKC γ expression levels were higher in the NS group of inoculated rats. Intrathecal DAPTA (10 μ g) could significantly attenuate spinal CCR5 and p-PKC γ levels in inoculated rats (Fig. 3b).

Intrathecal of GF109203X Attenuated Mechanical Allodynia and Reduced p-PKC γ Expression in CIBP Rats

To further check whether PKC γ is a downstream molecule of CCR5 for maintenance of CIBP, GF109203X, an inhibitor of PKC, was chosen to verify this hypothesis. The results showed that intrathecal injection of GF109203X (0.18 and 0.36 μ g) could dose-dependently attenuate mechanical allodynia at 15 days in inoculated rats. The peak effect was at the 1.5 h after intrathecal administration ($p < 0.01$, Fig. 4a). Meanwhile, intrathecal GF109203X (0.36 μ g) could significantly attenuate spinal p-PKC γ expression ($p < 0.01$). However, intrathecal administration of GF109203X had no effects on the expression of spinal CCR5 (Fig. 4b). Taken together, these findings suggested that PKC was an important downstream molecule of CCR5 in the maintenance of CIBP in rats.

Compared with sham rats, the expression of OX-42 was higher in NS group. Intrathecal DAPTA (10 μ g) or GF109203X (0.36 μ g) could significantly attenuate spinal

OX-42 expression level ($p < 0.01$). The results indicated that a positive correlation between microglial activation and the expression level of CCR5 or p-PKC γ .

Discussion

More recent studies have indicated that a few chemokines and chemokine receptors were involved in central sensitization in CIBP models [20–22]. RANTES, which is the main ligand for CCR5, is a member of the CC chemokine subfamily. In addition to RANTES, other CC chemokines, such as CCL3 and CCL4, may also contribute to the CCR5 activation [23]. Recently, some studies have confirmed that CCL3/CCR5 or CCL4/CCR5 signaling pathway might participate in neuropathic pain [24–26]. Our previous study verified that RANTES was involved in the alleviation of nociceptive responses of triptolide in the CIBP rats [10]. The established mechanical allodynia could be abolished by intrathecal injection of anti-RANTES neutralizing antibody in inoculated rats [13]. In the present study, the results showed that inhibition of spinal CCR5 expression was associated with the attenuated mechanical allodynia in CIBP rats. These results suggested that RANTES/CCR5 signaling pathway might contribute to the maintenance of CIBP. Lee and colleagues found that CCR5 knockout mice to chemical and inflammation stimuli were significantly attenuated, and intracerebroventricular infusion of DAPTA could markedly reduce chemical and inflammatory pain responses in wild-type mice [27]. Peritoneal administration of Met-RANTES, a CCR5 antagonist, could reduce nociceptive responses in a partial sciatic nerve ligation (PSNL) model [28]. Similarly, oral administration of RAP-103, a CCR2 and CCR5 antagonist, could attenuate mechanical allodynia and thermal hyperalgesia in PSNL rats [29]. Taken together, CCR5 may play an important role in the modulation of nociceptive perception.

The peak effect of DAPTA was at 2 h after intrathecal administration. However, the effects of intrathecal administration of DAPTA on the expression levels of CCR5 and p-PKC γ were measured at 3 h after intrathecal, owing to the fact that variation of protein expression lags behind pain behavioral changes. In pharmacology, a receptor antagonist is defined as a molecule binding to the specific receptor can inhibit the function of the agonist or inverse agonist to its receptor, which has affinity but no efficacy for its cognate receptor. The efficacy of a receptor depends on its expression level [30]. DAPTA, a specific antagonist of CCR5, can reduce the efficacy of the CCR5. So, decrease of the CCR5 expression level was observed after intrathecal of DAPTA.

CCR5/PKC signaling pathway has been proved in some studies [14, 31]. PKC is a family of serine/threonine kinases, which comprises more than ten members. PKC γ

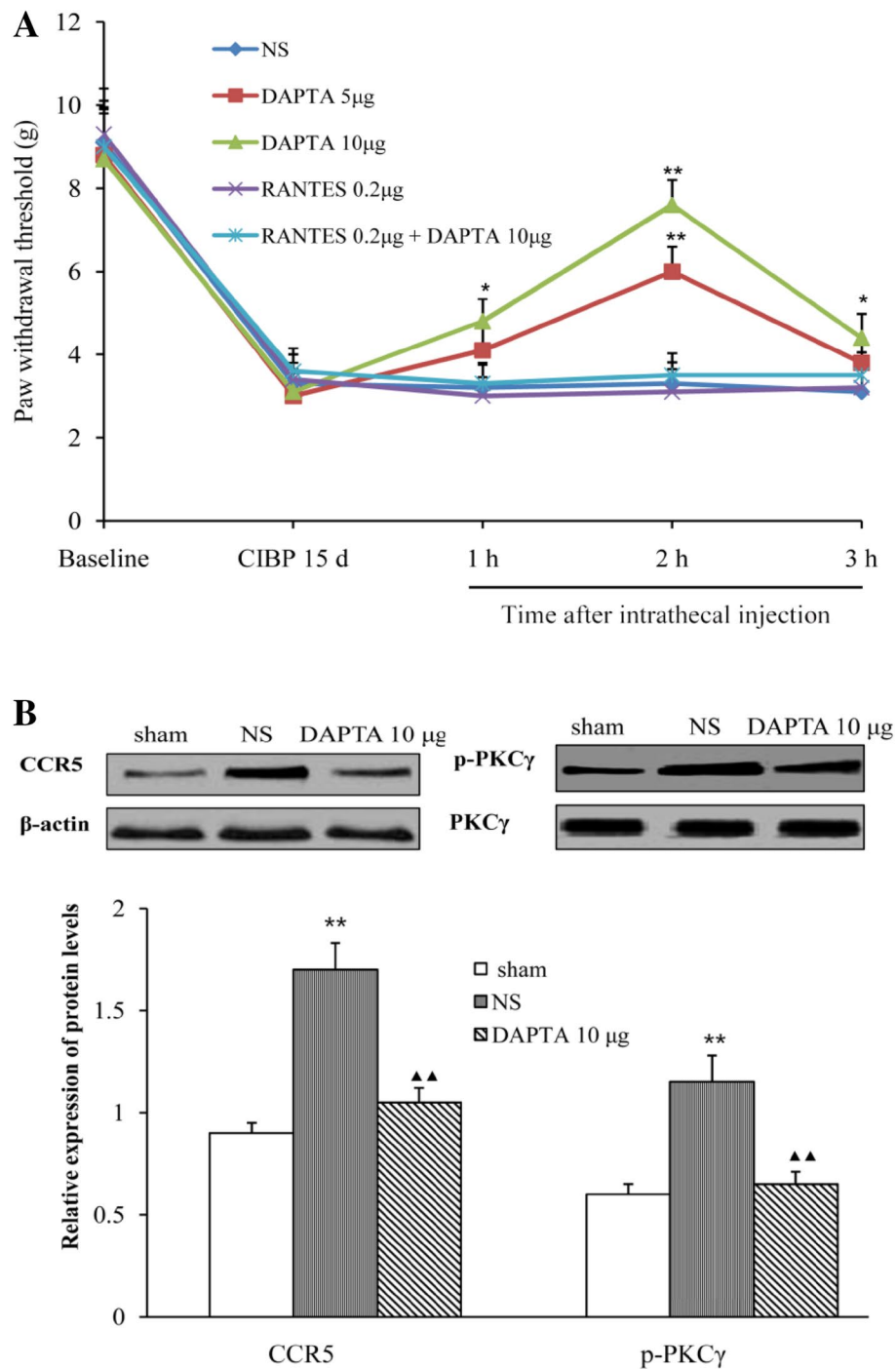
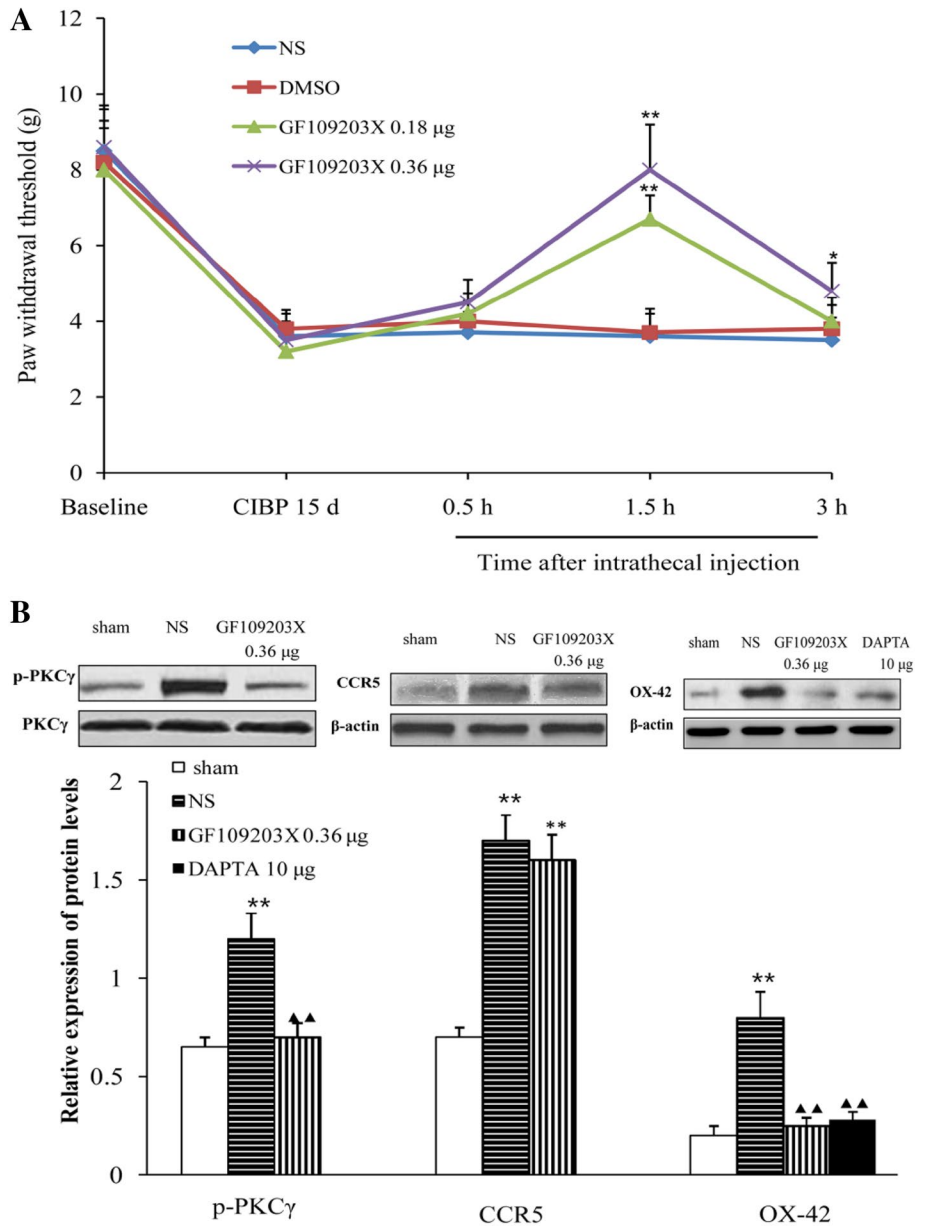


Fig. 3 Intrathecal administration of DAPTA attenuated mechanical allodynia and decreased CCR5 and p-PKC γ expression in CIBP rats. **a** Intrathecal DAPTA could significantly increase the PWT values at 1, 2, and 3 h in a time- and dose-dependent manner ($p < 0.05$ or 0.01). Intrathecal RANTES (0.2 μ g, a CCR5 ligand) had no effects on the values of PWT. However, the anti-allodynia effects of DAPTA (10 μ g) was reversed by pre-intrathecal injection of RANTES (0.2 μ g) 15 min before intrathecal DAPTA. NS group indicates intrathecal administration of normal saline. ($*p < 0.05$,

$**p < 0.01$ versus NS group. Data were presented as the mean \pm SEM, each group contained eight rats.). **b** Compared with sham group, the CCR5 and p-PKC γ expression levels were higher in the NS group of inoculated rats. Intrathecal DAPTA (10 μ g) could significantly attenuate spinal CCR5 and p-PKC γ levels in inoculated rats. The tissues of spinal dorsal horn were collected at 3 h after intrathecal injection. ($\blacktriangle p < 0.01$ versus NS group, $**p < 0.01$ versus sham rats. Data were presented as mean \pm SEM, each group contained four rats.)

Fig. 4 Intrathecal of GF109203X attenuated mechanical allodynia and blocked PKC γ activation in CIBP rats. **a** Intrathecal GF109203 \times (0.18 and 0.36 μ g) could dose-dependently attenuate mechanical allodynia at 15 days in inoculated rats. The peak effect was at the 1.5 h after intrathecal administration ($p < 0.01$, Fig. 4a). (** $p < 0.01$ versus NS group. Data were expressed as the mean \pm SEM, each group contained 8 rats.) **b** Intrathecal of GF109203 \times (0.36 μ g) could significantly attenuate spinal p-PKC γ expression ($p < 0.01$). However, intrathecal administration of GF109203X had no effects on the expression of CCR5. Compared with sham rats, the expression of OX-42 was higher in NS group. Intrathecal DAPTA (10 μ g) or GF109203 \times (0.36 μ g) could significantly attenuate spinal OX-42 expression level ($p < 0.01$). (▲▲ $p < 0.01$ versus NS group, ** $p < 0.01$ versus sham rats. Data were presented as mean \pm SEM, each group contained four rats.)



is widely distributed in the central nervous system (CNS). Immunocytochemical studies showed PKC γ was mainly localized in the inner part of lamina II of spinal cord [32], suggesting PKC γ might be involved in the modulation of pain processing. A reduced neuropathic pain behavior was observed in the mice lack of PKC γ [33]. Inflammatory pain induced by complete Freund’s adjuvant (CFA) could be attenuated by intra-arcuate nucleus injection of a PKC inhibitor [34]. These findings indicated that PKC γ participated in neuropathic and inflammatory pain. However, CIBP is different from neuropathic and inflammatory pain, and has its distinguishing feature [35]. Interestingly, in the current study, we found that only p-PKC γ expression level

was increased in inoculated rats, but not total PKC γ . Similarly, some studies found that there was no upregulation of spinal PKC γ in a mouse model of CIBP [35, 36]. These results indicated that PKC γ participated in CIBP processing through its phosphorylation. Intrathecal DAPTA could reduce mechanical allodynia with concomitant downregulation of spinal CCR5 and p-PKC γ expression at day 15 after inoculation. Moreover, intrathecal injection of GF109203X could alleviate mechanical allodynia as well as decrease of spinal p-PKC γ expression level, but no influence on spinal CCR5 level. These findings indicated that CCR5/PKC γ signaling pathway was involved in the maintenance of CIBP.

Recently, it is increasingly recognized that microglia plays a key role in the initiation and maintenance of CIBP [37, 38]. Inhibition of microglia activation by minocycline could alleviate CIBP in rats [39]. Furthermore, inhibition of microglia signaling pathway, such as CX3CR1/p38 and TLR4/p38 signaling, could also attenuate CIBP [40, 41]. In the present study, we found CCR5 was mainly expressed in microglia, and intrathecal DAPTA or GF109203X could significantly attenuate microglial activation. These studies supported that spinal microglia might play an important role in CIBP. It was reported that RANTES was secreted mainly by neurons in the CNS [42]. So, we speculate that the RANTES-CCR5- PKC γ pathway may contribute to the maintenance of CIBP via neuronal-microglial interaction in the spinal cord.

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