

# PDTC ameliorates neuropathic pain by inhibiting microglial activation *via* blockage of the TNF $\alpha$ -CX3CR1 pathway

Xilei Li,<sup>1</sup> Zhi Ye,<sup>1,2</sup> Qulian Guo,<sup>1,2</sup> E Wang,<sup>1,2</sup> Yundan Pan<sup>1,2</sup>

<sup>1</sup>Department of Anesthesiology, Xiangya Hospital, Central South University, Changsha, Hunan

<sup>2</sup>National Clinical Research Center for Geriatric Disorders, Central South University, Changsha, Hunan, China

## ABSTRACT

Previous studies have suggested that pyrrolidine dithiocarbamate (PDTC), a nuclear factor  $\kappa$ B (NF- $\kappa$ B) inhibitor, plays a role in deterring nerve injury-induced neuropathic pain (NP). The activation of NF- $\kappa$ B pathway may contribute to spinal microglial activation, CX3CR1 and tumor necrosis factor-alpha (TNF- $\alpha$ ) up-regulation. The aim of this study was to clarify whether PDTC could inhibit the development of neuropathic pain *via* decreasing TNF- $\alpha$ -induced CX3CR1 up-regulation. Sprague-Dawley rats were randomly divided into sham group and NP group. Rats in each group were treated with intrathecal infusion of PDTC (100 or 1000 pmol/d) or saline. The sciatic nerve chronic constriction injury (CCI) model was used to induce NP in rats. Mechanical stimuli and radiant heat were used to evaluate mechanical allodynia and thermal hyperalgesia. Spinal microglial marker OX42 and TNF- $\alpha$  were detected by immunohistochemistry. *In vitro* BV-2 microglia activation was induced by TNF- $\alpha$  incubation, and the levels of CX3CR1 were assessed by western blot and reverse transcription-polymerase chain reaction. Pain behavior and immunohistochemistry results showed that intrathecal infusion of PDTC at 100 or 1000 pmol/d prevented the development of mechanical and thermal hyperalgesia, spinal microglial activation and TNF- $\alpha$  expression induced by sciatic nerve CCI in rats. *In vitro* experiment results showed that PDTC inhibited the TNF- $\alpha$ -induced CX3CR1 up-regulation in BV-2 microglial cells. In conclusion, intrathecal infusion of PDTC could attenuate the pain-related behaviors induced by sciatic nerve CCI through suppressing the spinal microglia activation and TNF- $\alpha$  up-regulation in rats. The NF- $\kappa$ B activation might be responsible for TNF- $\alpha$ -induced CX3CR1 up-regulation in microglia.

**Key words:** Neuropathic pain; NF- $\kappa$ B; tumor necrosis factor- $\alpha$ ; microglia; CX3CR1.

**Correspondence:** Dr. Yundan Pan, Department of Anesthesiology, Xiangya Hospital, Central South University, Changsha 410008, Hunan, China. E-mail: kenumb@163.com

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## Introduction

Neuropathic pain is a common symptom of the peripheral nerve injury with characteristic allodynia, hyperalgesia, and spontaneous pain. Treatment options of neuropathic pain remain limited. Previous studies suggested that the nuclear factor kappa B (NF- $\kappa$ B) pathway plays an important role in regulating the gene expression of multiple pain-related cytokines, including IL-1 $\beta$ , IL-6, TNF- $\alpha$ , in the central nervous system (CNS).<sup>1,2</sup> Pyrrolidine dithiocarbamate (PDTC), a potent NF- $\kappa$ B inhibitor, inhibits factor I- $\kappa$ B ubiquitination and subsequent degradation, then decreases the translocation of NF- $\kappa$ B p65 and the transcription of target genes. It has been confirmed that intrathecal PDTC can inhibit nerve injury-induced pain and spinal neuroinflammatory activation.<sup>3</sup> Exploring the regulatory mechanism of PDTC on cell-specific inflammatory molecules in neuropathic pain may open up a new way for clinical pain treatment. This study aimed to investigate the regulatory mechanism of PDTC in the development of neuropathic pain, spinal microglia activation and inflammatory mediator expression.

The contribution of spinal proinflammatory cytokines to pain modulation attracted substantial attention recently. TNF- $\alpha$ , a spinal proinflammatory cytokine, is mainly produced and released from activated microglia and astrocytes and is involved in pain modulation. An elevated level of TNF- $\alpha$  was detected in the CNS in animals of neuropathic pain.<sup>4,5</sup> NF- $\kappa$ B pathway interacts with TNF- $\alpha$  in spinal cord, which is related to the production of pathological pain and neuroinflammation. A likely signal transduction pathway for TNF- $\alpha$  involves the phosphorylation of the inhibitor factor I- $\kappa$ B and the subsequent translocation of NF- $\kappa$ B subunit p65 to the nucleus. Studying the effect of PDTC on the expression of proinflammatory factors in specific inflammatory cells induced by TNF- $\alpha$  will provide a new theoretical basis for neuroinflammatory regulation of pain.

Spinal cord microglia, the main immune cell in CNS, plays a key role in the genesis of neuropathic pain. In several pain models, including chronic constriction injury (CCI), spinal nerve ligation (SNL), and spinal cord injury, the development of hyperalgesia was associated with spinal microgliosis.<sup>6,7</sup> It has been accepted that microglia releases proinflammatory mediators and propagates the immune response, leading to the neuropathic pain. Studies also proved that chronic intrathecal infusion of microglia inhibitor, minocycline, could prevent the development of SNL-induced pain in rats and reduce the proinflammatory cytokine expression in rat models.<sup>8,9</sup> Chemokine receptor CX3CR1 is specifically expressed in microglia, which plays a key role in regulating the activation of microglia in the spinal cord. After peripheral inflammation or nerve injury, the expression of CX3CR1 in spinal microglia in pain related areas increased in sciatic neuritis, CCI, SNL, monoarthritis and vincristine-induced neuritis models. Blocking CX3CR1 could attenuate and delay the development of neuropathic pain.<sup>10,11</sup> Exploring the effects of PDTC on TNF- $\alpha$ -induced CX3CR1 expression and microglia activation is of great significance for elucidating the mechanism of pain regulation.

In our *in vivo* study, we investigated the effects of intrathecal PDTC on the development of neuropathic pain, spinal microglial activation and TNF- $\alpha$  expression in CCI rats. The BV-2 cell line is an immortal cell line of mouse microglia. It has the morphology, phenotype and various functional characteristics of the primary cultured microglia. The aim of our *in vitro* study was to determine the expression of CX3CR1 and the morphological changes in BV-2 microglial cells upon treatment with PDTC or TNF- $\alpha$ . We investigated the effects of NF- $\kappa$ B pathway on TNF- $\alpha$ -induced CX3CR1 expression in BV-2 microglial cells.

## Materials and Methods

### Animals

Male Sprague-Dawley rats weighing 250-350 g at the time of surgery were housed individually in bedded cages on a natural 12-h light/12-h dark cycle (7am/7pm) in a temperature (21 $\pm$ 1°C) and humidity-controlled specific pathogen-free room with food and water provided *ad libitum*. All rats were acquired from Laboratory Animal Center of Xiangya Hospital, Central South University. These experiments were approved by the Institutional Animal Care and Use Committee in Central South University.

### Surgical procedures

Rats were anesthetized with sevoflurane. For repeated intrathecal injections, chronic catheters were constructed and implanted by lumbar approach according to a method previously described.<sup>12</sup> The indwelling catheters were used to microinject drugs or vehicle into the cerebrospinal fluid space surrounding the lumbosacral spinal cord. Briefly, a sterile PE-10 tube filled with saline was inserted through the L5/L6 intervertebral space, and the tip of the tube was placed at the spinal lumbar enlargement level. The cannulated rats were allowed to recover for 5 days and were housed individually. Rats that showed any neurologic deficit resulting from the surgical procedure during the whole experiment were excluded from the experiment and euthanized by carbon dioxide inhalation.

PDTC (P8765, Sigma-Aldrich) was dissolved in normal saline. The doses for intrathecal PDTC were selected according to a previous study and our preliminary experiments.<sup>12,13</sup> Ten  $\mu$ l PDTC (100 or 1000 pmol/d) or saline was administered followed by a flush of 5  $\mu$ l saline to ensure that drugs were delivered into the sub-arachnoid space. In PDTC treated rats, PDTC was injected daily for 4 consecutive days from 1 day before to 2 days after CCI or from 3 to 6 days after CCI (n=8 per group). After anesthesia with sevoflurane, the catheter placement was verified after the behavioral test was completed by visual inspection.

### Chronic constriction injury

Neuropathic pain was induced following the method reported by our previous studies.<sup>12,13</sup> An incision was made on the left thigh with the left sciatic nerve exposed. Four loosely tied ligatures (4-0 chromic catgut) were applied 1 mm apart around the left sciatic nerve above its trifurcation. For rats in the sham-operated groups, the left sciatic nerve was exposed but not ligated.

### Behavioral assessment-mechanical threshold and thermal threshold

The hind-paw withdrawal threshold (PWT) to an Electrovonfrey (IITC/Life Science Instruments, Woodland Hills, CA, USA) probe and paw withdrawal latency (PWL) to noxious radiant heat using a Hargreaves apparatus (Ugo Basile, Comerio, Italy) were determined before surgery (baseline, day 0) and once daily on each post-surgery day. PWTs and PWLs were measured according to the methods reported by our previous studies.<sup>12,13</sup> All the behavioral tests were performed between 10 am and 3 pm by an examiner blinded with respect to the treatment rendered. Five PWTs or PWLs were collected with 5-min intervals, and the average of the middle 3 thresholds was used for the following analysis.<sup>14,15</sup>

### Immunohistochemistry

After the last behavioral assessment, on days 3 and 7 after CCI surgery, the spinal cord around L4 and L5 was removed. The tissue was transected into a 10  $\mu$ m floating section with a cryostat and

fixed it according to the previously mentioned method.<sup>12,13</sup> Then sections were incubated at room temperature (RT) overnight with primary mouse monoclonal antibody (anti-OX42, 1:200, sc-53086, Santa Cruz Biotechnology, Santa Cruz, CA, USA; anti-TNF- $\alpha$ , 1:200, FNab08821, FineTest, Palm Coast, FL, USA) in PBST plus 3% normal goat serum. Sections were washed with PBST 3 times for 10 min each and incubated with goat anti-mouse biotinylated secondary antibody (1:1000; 14709s, Cell Signaling Technology Corp., Danvers, MA, USA) for 1 h at RT. After sections were washed further with PBST 3 times, avidin-biotin-horseradish peroxidase complex (29994, Pierce™ Avidin HRP, Thermo Fisher Scientific, Waltham, MA, USA) in PBST was applied in 1:160 dilution to sections for 1 h. Antigens were visualized by combining equal volumes of an ammonium nickel sulfate solution (30 mg/mL in 0.1 M sodium acetate, pH 6.0) and a diaminobenzidine solution (4 mg/mL in PBS) in the presence of 0.01% hydrogen peroxide. According to the previously reported methods,<sup>12,13</sup> the floating sections were laid flat on glass slides, air-dried, rinsed and dehydrated, and then covered with Permount fixed medium. Images were acquired with a Leica 4000 light microscope (Leica Microsystems GmbH, Wetzlar, Germany). Assessments of OX42-positive or TNF- $\alpha$ -positive cells were performed in 3 sections chosen randomly from L4/5 spinal cord of rats. A manual method was employed to measure the total number of OX42 and TNF- $\alpha$  IR cells in ipsilateral spinal dorsal horn with the microscope under 250x magnification.

### Cell culture and treatment

BV-2 microglial cells were obtained from German collection of microorganisms. Both were adopted as the substitute for microglia and were plated ( $4 \times 10^4$  cells/cm<sup>2</sup>) in culture flasks. Cells were cultured in RPMI1640 (Gibco), supplemented with 10% fetal calf serum, in a humidified atmosphere with 5% CO<sub>2</sub>. The homogenous cells between the fourth and eighth passages were used. TNF- $\alpha$  (P06804, R&D systems, 20 ng/mL) and PDTC (100 mmol/L) were applied to BV-2 cells. Serum-free medium was used as the negative control. Immunocytochemistry, RT-PCR and western blotting were used to assess cell NF- $\kappa$ B p-p65 protein expression, and CX3CR1 protein and mRNA expression.

### Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 20 min at RT and washed three times with phosphate-buffered saline (PBS). Then cells were incubated with 10% goat serum in PBS containing 0.2% Trion X-100 at RT for 20 min to block the unspecific binding of antibodies. After the blocking solution had been washed out, cells were incubated overnight with primary antibody (NF- $\kappa$ B p-p65, 1:100, ab16502, Abcam, Cambridge, UK). Then cells were washed and incubated with a secondary antibody (horseradish peroxidase-polymer-goat anti-rabbit immunoglobulin G; 1:1000) for 20 min. After cells were washed further with PBS 3 times, antigens were visualized by combining equal volumes of an ammonium nickel sulfate solution (30 mg/mL in 0.1 M sodium acetate, pH 6.0) and a diaminobenzidine solution (4 mg/mL in PBS) in the presence of 0.01% hydrogen peroxide. For nuclear staining, the cells were washed and incubated with hematoxylin. Images were acquired with a Leica 4000 light microscope (Leica Microsystems GmbH). Assessments of NF- $\kappa$ B p-p65-positive cells were performed in 3 slides chosen randomly.

### RT-PCR

Total RNA was isolated from cells using Trizol reagent (10296010, Thermo Fisher) and quantified by spectrophotometry. First strand cDNA was synthesized from 1  $\mu$ g of total RNA *via* the ReverTra Ace- $\alpha$ TM reverse-transcription system (TRT-101, TOY-

OBO). PCR products were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide. GAPDH gene expression was used as a standard. CX3CR1 was amplified using CX3CR1 primers and the sequences of the forward and reverse primers are: 5-ACGATGTCTGGGTGACTAC-3 and 5-GTATG-GTGTCAGAAAGAGGA-3, respectively. The sequences of GAPDH forward and reverse primers are: 5-ACCACAGTCCAT-GCCATCAC-3 and 5-TCCACCACCCTGTTGCTGTA-3, respectively.

### Western blotting analysis

The method was described previously.<sup>16</sup> Western blot analysis was performed to evaluate CX3CR1 expression in BV-2 cells after cells were incubated with various drugs for 2, 4 or 6 h. Cultured cells were treated with lysis buffer and then mechanically digested to release the proteins. Lysate was centrifuged at 9000 rpm for 10 min and the supernatant was taken for western blotting. Equal amount of protein (30  $\mu$ g) was loaded on each lane, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 10% nonfat dry milk for 2 h at RT and incubated overnight at 4°C with rabbit anti-CX3CR1 antibody (1:500; 14-6093-81, eBioscience, San Diego, CA, USA); or anti-GAPDH antibody (1:8000; BW0277, Cell Signaling). The blots were incubated for 2 h at room temperature with horseradish peroxidase-conjugated mouse anti-rabbit (1:2000, sc2004, Santa Cruz Biotechnology). Signals were visualized using enhanced chemiluminescence (21050, Pierce™ Western Blot Signal Enhancer Kit, Thermo Fisher Scientific) and exposed onto X-ray films for 1 to 10 mins. All western blotting analysis was performed at least 3 times, and parallel results were obtained. X-ray films with blotting bands for each sample from different rats were scanned and analyzed using the digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation, Orem, UT, USA). Concentration of protein was determined by densitometric analysis and expressed as relative densitometric unit to that of GAPDH.

### Statistical analysis

All experiments were performed in triplicate, and data were expressed as mean $\pm$ SEM. Data analysis was performed using SPSS 16.0 software. Two-way analysis of variance (ANOVA) with *post hoc* Tukey test was used to compare the behavioral data of different experimental groups. Image-analysis data were compared between groups with one-way ANOVA with *post hoc* Bonferroni test. A value of  $p < 0.05$  was considered statistically significant.

## Results

### The effects of PDTC on behavioral tests

In order to further investigate the role of NF- $\kappa$ B pathway in the modulation of neuropathic pain, the PDTC was administrated intrathecally to block spinal NF- $\kappa$ B pathway both in the early and late phases of CCI-induced neuropathic pain. Figure 1 shows the effects of PDTC [100 or 1000 pmol/d, intrathecal (i.t.) 4 days] on mechanical allodynia and thermal hyperalgesia of ipsilateral hind paws in sham and CCI rats. In the sham group, no difference was observed in the mechanical thresholds and thermal latency for different doses of PDTC. In the CCI group, the neuropathic pain exhibited remarkable mechanical allodynia and thermal hyperalgesia. Paw withdrawal threshold (PWT) to electrovolfrey filaments and paw withdrawal latency (PWL) to a radiant heat source of CCI-rats decreased over time. Behavior results showed that intrathecal administration of PDTC in the early phase (on 1 day

before to 2 day after surgery) alleviated the CCI-induced thermal hyperalgesia on day 1-3 after surgery and inhibited the mechanical hyperalgesia on day 2-3 after surgery (Figure 1A). Meanwhile, administration of PDTC in the late phase (on day 3-6 after surgery) relieved both the thermal and mechanical hyperalgesia on day 4-7 after surgery (Figure 1B). Intrathecal administration of PDTC delayed the onset of neuropathic pain and reversed the pain behaviors in rats of sciatic CCI model.

### The effects of PDTC on spinal microgliosis and TNF- $\alpha$ protein expression

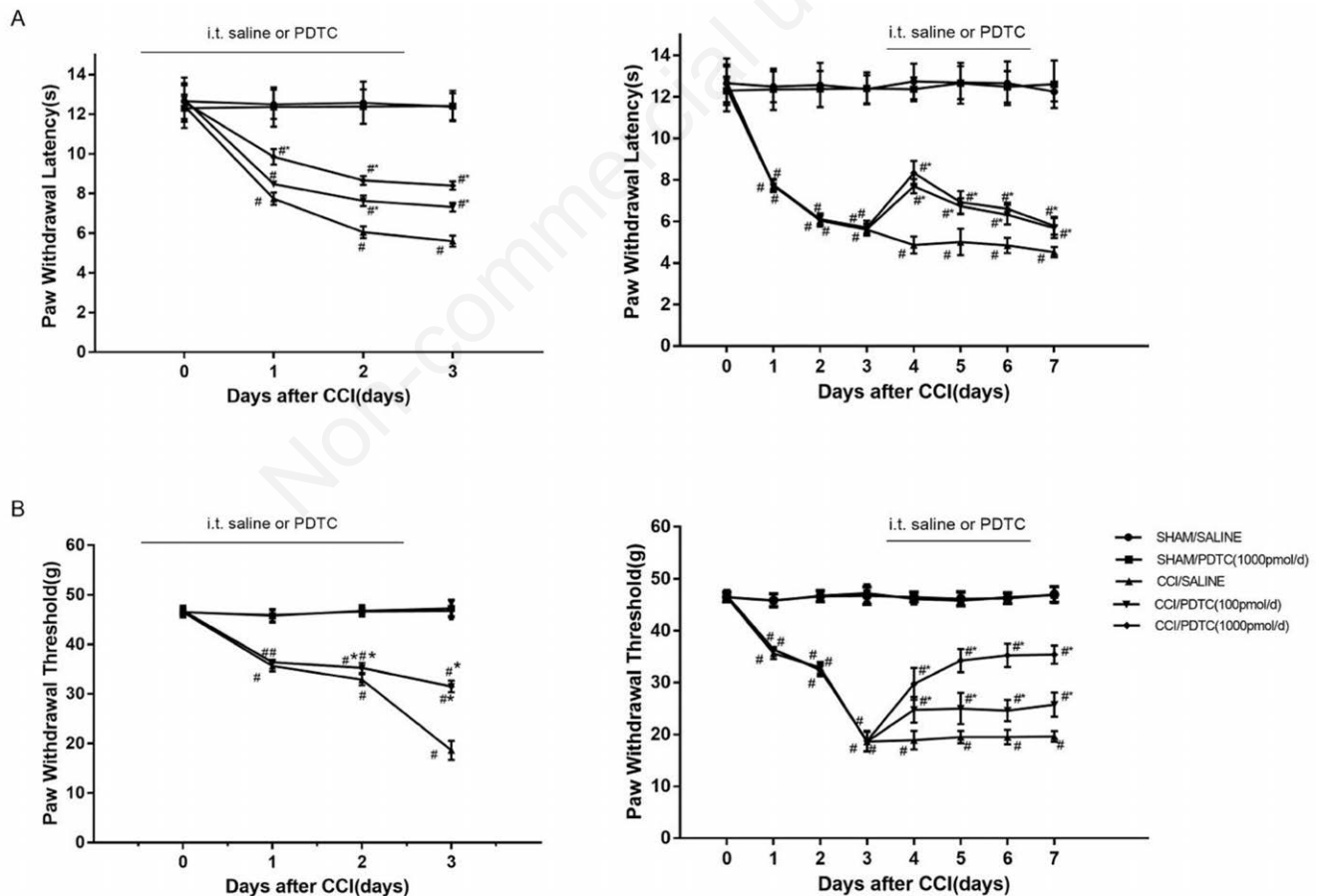
Immunohistochemistry analysis showed that PDTC had inhibitory effect on spinal spinal microgliosis both early and late phases of CCI (Figure 2). OX-42 expression in the spinal cord was measured to evaluate the influence of PDTC (100 or 1000 pmol/d, i.t.) on microglial activation after 4 days of treatment. As shown in Figure 2, OX-42-expressed cells increased significantly in the ipsilateral spinal cord in CCI rats. PDTC decreased the expression of OX-42 and the number of ipsilateral spinal OX-42 immunoreactive (IR) cells in a dose-dependent manner. The number of OX-42 IR cells decreased at both doses ( $p < 0.05$ ).

Additionally, the increase of relevant proinflammatory cytokines (TNF- $\alpha$ ) was prevented by PDTC, which was adminis-

tered both in the early and late phase of CCI (Figure 3). TNF- $\alpha$  level in spinal cord was measured to evaluate the influence of PDTC on proinflammatory factor generation after repeated infusion of PDTC (100 or 1000 pmol/d, i.t.) for 4 days. As shown in Figure 3, TNF- $\alpha$  expression increased significantly in the ipsilateral spinal cord in CCI rats ( $p < 0.05$ ). PDTC decreased the expression of TNF- $\alpha$  in a dose-dependent manner, compared to that of CCI group ( $p < 0.05$ ). The number of TNF- $\alpha$  IR cells decreased at both doses ( $p < 0.05$ ).

### PDTC inhibited TNF- $\alpha$ -induced p-p65 expression in microglial cells

To determine whether TNF- $\alpha$  mediate microglial activation *via* the NF- $\kappa$ B p-p65 signaling pathway, microglial BV-2 cells were pretreated with PDTC (100  $\mu$ mol/L) for 1 h and then incubated with TNF- $\alpha$  (20 ng/ml) for 1 h. The expression of NF- $\kappa$ B p-p65 was examined by immunocytochemistry. Very low level of nuclear p-p65 expression existed in the untreated cells. After TNF- $\alpha$  induction, nuclear p-p65 expression increased and reached the peak in 1 h (Figure 4). TNF- $\alpha$ -dependent phosphorylation of NF- $\kappa$ B p65 was inhibited when cells were pretreated with PDTC (100  $\mu$ mol/l), while PDTC cannot alter the constitutive p-p65 expression in microglial cells.

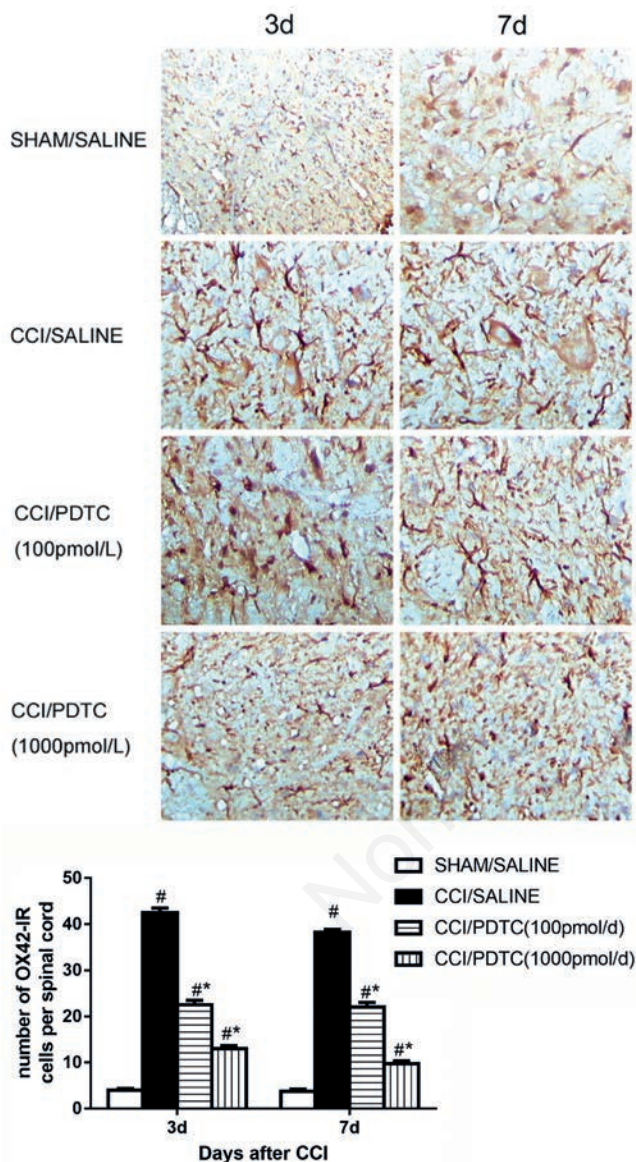


**Figure 1.** The effects of repeated intrathecal administration of saline or PDTC (100 or 1000 pmol/d, i.t. 4 days) on pain behaviors of ipsilateral hind paw in CCI rats. PWT to electrovonfrey filaments and PWL to radiant heat source of CCI-rats decreased over time. PDTC increased PWT (B) and PWL (A) in a dose-dependent manner in CCI rats. Results are expressed as mean  $\pm$  SEM (n=8). Bar above the x-axis represents intrathecal treatment with saline or PDTC. # $p < 0.05$  vs saline treated sham rats; \* $p < 0.05$  vs saline treated CCI rats.

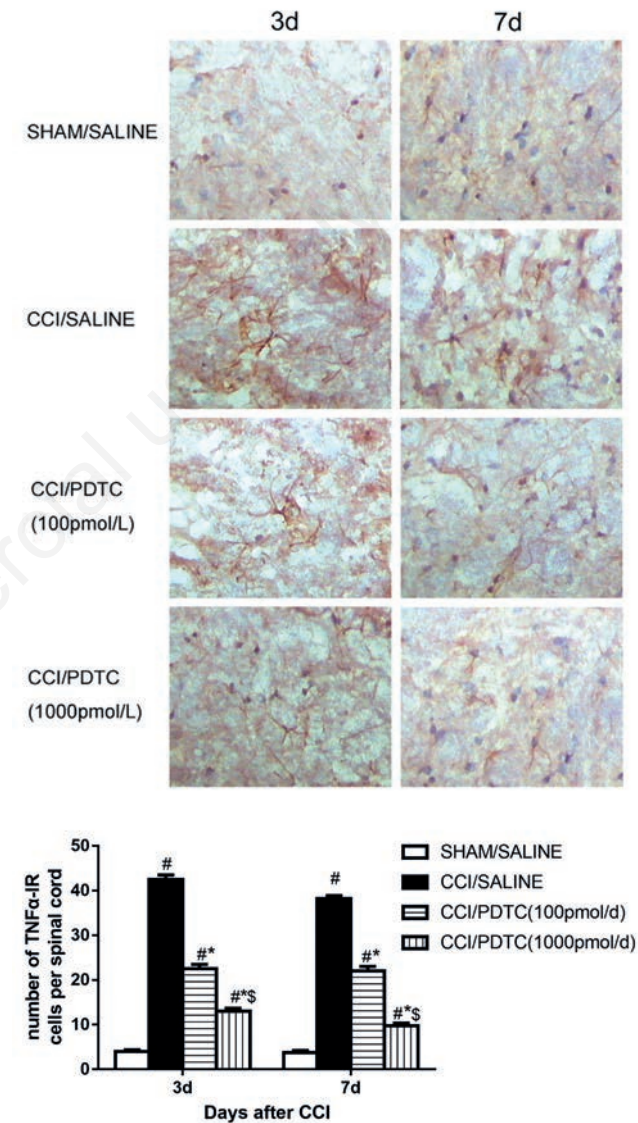
### PDTC inhibited TNF- $\alpha$ -induced microglial CX3CR1 up-regulation *in vitro*

To investigate the possible relationship between TNF- $\alpha$  and microglial CX3CR1 expression *in vitro*, extracts from microglial BV-2 cells stimulated with TNF- $\alpha$  were examined by western blotting analysis and RT-PCR. Microglial BV-2 cells were pre-treated with PDTC (100  $\mu\text{mol/l}$ ) for 1 h and then incubated with TNF- $\alpha$  (20 ng/mL). TNF- $\alpha$  incubation induced an increase in CX3CR1 mRNA in a time-dependent manner. With the presence of TNF- $\alpha$ ,

CX3CR1 mRNA expression was observed as early as 30 min and peaked in 2 h (Figure 5). The increase in CX3CR1 protein expression was noted after cells were treated with TNF- $\alpha$  for 2 h, and CX3CR1 protein expression reached the peak at hour 4 (Figure 6). The control group failed to demonstrate a meaningful increase in the expression of CX3CR1, although a constitutive expression was observed. These results indicated that PDTC inhibited the expression of constitutive and TNF- $\alpha$ -induced CX3CR1 mRNA and protein.



**Figure 2.** The effects of repeated intrathecal administration of PDTC (100 or 1000 pmol/d, i.t. 4 days) on spinal microglial activation in rats. Representative photomicrographs of OX-42 immunoreactivity (IR) of ipsilateral L4/5 spinal dorsal cord shows prominent microglial activation in CCI rats, and CCI-induced microglial activation was remarkably suppressed by PDTC. The number of ipsilateral spinal OX-42 IR cells decreased with PDTC treatment on days 3 and 7 after CCI. Results are expressed as mean  $\pm$  SEM (n=4). <sup>#</sup>p<0.05 vs saline treated sham rats; \*p<0.05 vs saline treated CCI rats.



**Figure 3.** The effects of repeated intrathecal administration of PDTC (100 or 1000 pmol/d, i.t. 4 days) on spinal TNF- $\alpha$  protein expression in CCI rats. Representative photomicrographs of TNF- $\alpha$  immunoreactivity of ipsilateral L4/5 spinal dorsal cord of rats show higher TNF- $\alpha$  expression in saline treated CCI rats, and PDTC remarkably suppressed TNF- $\alpha$  expression in CCI rats. The number of ipsilateral spinal TNF- $\alpha$  IR cells remarkably decreased with PDTC treatment on days 3 and 7 after CCI. Results are expressed as mean  $\pm$  SEM (n=4). <sup>#</sup>p<0.05 vs saline treated sham rats; \*p<0.05 vs saline treated CCI rats; <sup>§</sup>p<0.05 vs PDTC-100 pmol/d treated CCI rats.

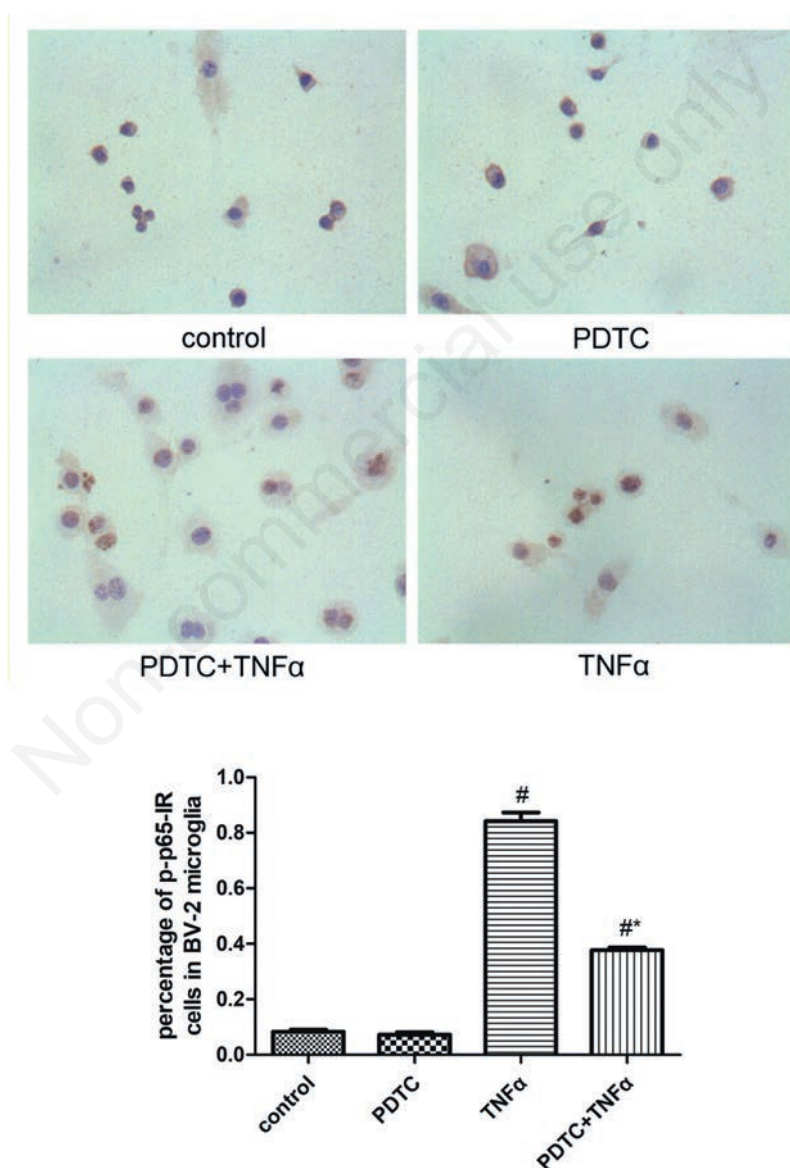
## Discussion

In this study, we established a classic model of nerve injury-induced neuropathic pain *via* chronically constricting the left sciatic nerve of male rats. Similar to the results reported by Bennett *et al.*,<sup>17</sup> sciatic constriction induced pain-related behavioral signs of mechanical allodynia and thermal hyperalgesia. The characteristic changes in pain threshold indicated that the model was successfully established for this study.

Here, we demonstrated an important but previously unrecognized role of NF- $\kappa$ B in CCI-induced neuropathic pain. Daily intrathecal infusions of NF- $\kappa$ B inhibitor PDTC (100 and 1000 pmol/d) for 4 consecutive days delayed the onset of neuropathic pain and the activation of spinal microgliosis and TNF- $\alpha$  production in a dose-dependent manner. Moreover, repeated intrathecal administration of PDTC 3 days after CCI reversed the established mechanical allodynia and thermal hyperalgesia, and reduced the

activation of spinal microglia and the production of TNF- $\alpha$  in rats. No obvious neurotoxicity was observed after repeated intrathecal infusion of PDTC. The inhibition of the NF- $\kappa$ B signaling pathway was accompanied with decreased injury-induced pain behavior and neuroinflammation, suggesting the correlation between spinal NF- $\kappa$ B and neuropathic pain. Therefore, NF- $\kappa$ B is a promising therapeutic target for the prevention of nerve injury-induced neuropathy and neuroinflammation.

TNF- $\alpha$  is a member of the superfamily of type II proteins containing full-length membrane TNF- $\alpha$  (mTNF- $\alpha$ ) that is cleaved by the TNF- $\alpha$  converting enzyme and is released as diffusible peptides, soluble TNF- $\alpha$  (sTNF- $\alpha$ ). TNF- $\alpha$  may enhance the excitability of sensory neurons and activation of microglia in the spinal dorsal horn of neuropathic pain animals.<sup>4,5</sup> Consistent with our results, recent study showed that nerve injury induced an increase in mTNF- $\alpha$  measured by immunohistochemistry or western blot, while the sTNF- $\alpha$  was unable to be detected in spinal cord.<sup>18</sup> The



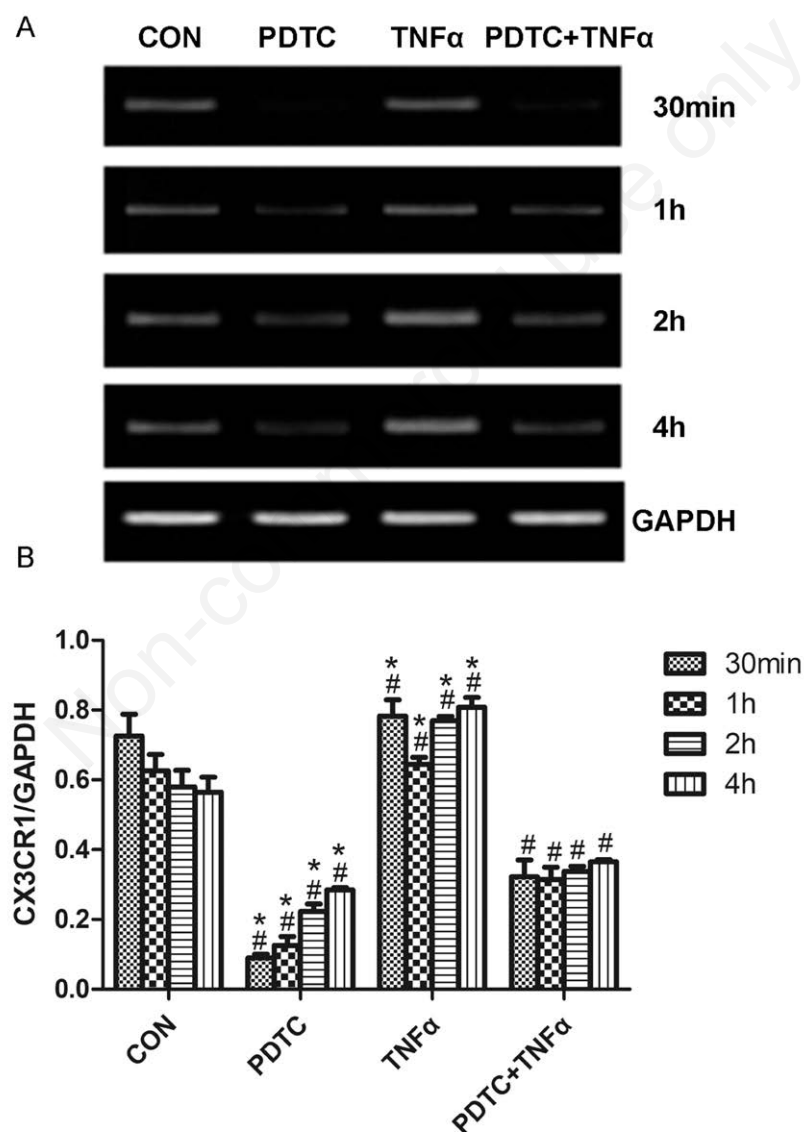
**Figure 4.** Effect of PDTC on p-p65 protein expression in TNF $\alpha$ -induced BV-2 microglial cells. Representative photomicrographs of p-p65 immunoreactivity in BV-2 microglia show increased p-p65 expression in TNF- $\alpha$  treated cells. PDTC-pre-treated group had lower p-p65 protein expression than TNF $\alpha$  treated groups did. Results are expressed as mean  $\pm$  SEM (n=4). The percentage of p-p65 IR cells in BV-2 microglia decreased substantially in PDTC-treatment cells. #p<0.05 *vs* control cells; \*p<0.05 *vs* TNF $\alpha$  treated cells.

mTNF- $\alpha$  might serve to mediate increased neuroinflammation in injury-induced neuropathic pain. However, other study proved sTNF- $\alpha$  played a role in initiating inflammation in paclitaxel-induced pain.<sup>19</sup> Further research might be needed to illuminate the involvement of two distinct forms of TNF- $\alpha$  in pain development. In this study, we observed the dose-dependent suppressive effect of intrathecal PDTC on mTNF- $\alpha$  expression induced by CCI, therefore, our data demonstrated that NF- $\kappa$ B activation could mediate spinal mTNF- $\alpha$  production. However, PDTC was also reported to reduce the levels of CX3CR1, COX-2, IL-1b and IL-6 in the rats of pain models.<sup>12,20</sup> Hence, the inhibitory effects of PDTC on these proinflammatory factors may be additional reasons for its analgesic activity observed in this study.

It is confirmed that the increased expression of CX3CR1 in spinal microglia is directly related to neuropathic pain and inflammation, although the factors involved in the regulation of CX3CR1 expression are unclear. In BV-2 microglial cells, we found that

TNF- $\alpha$  induced an increase in CX3CR1 mRNA and protein expression and the nuclear translocation of NF- $\kappa$ B p-65 in BV-2 microglial cells may be the critical step for upregulating CX3CR1 expression. In the CNS, the target genes of NF- $\kappa$ B signaling pathway are still incompletely understood. NF- $\kappa$ B signaling pathway can also regulate the expressions of inducible NO-synthase (iNOS), and catechol-o-methyltransferase (COMT) in neuronal tissue.<sup>21,22</sup> Our data and these studies suggested that increased activation of NF- $\kappa$ B pathway enhances transcription of genes that cause pain (*e.g.*, iNOS, CX3CR1) and decreases ones that ease pain (*e.g.*, COMT). Therefore, the inhibition of NF- $\kappa$ B signaling pathway may provide an effective way for clinical pain treatment.

It was shown in the present study that PDTC pretreatment did not completely inhibit the production of CX3CR1 induced by TNF- $\alpha$ , which suggested that NF- $\kappa$ B may not be the only transcription factor involved in the TNF- $\alpha$ -induced up-regulation of CX3CR1 gene. Several other transcription factors were reported to



**Figure 5.** Effect of PDTC on the expression of CX3CR1 mRNA in TNF $\alpha$ -induced BV-2 microglial cells. PDTC-treatment group had lower CX3CR1 mRNA expression than other groups did. A) Bands of RT-PCR of CX3CR1 mRNA expression. B) Statistical analysis of relative density of RT-PCR bands is shown between different groups. Results are expressed as mean  $\pm$  SEM (n=4). #p<0.05 vs control cells; \*p<0.05 vs PDTC and TNF $\alpha$  treated cells.

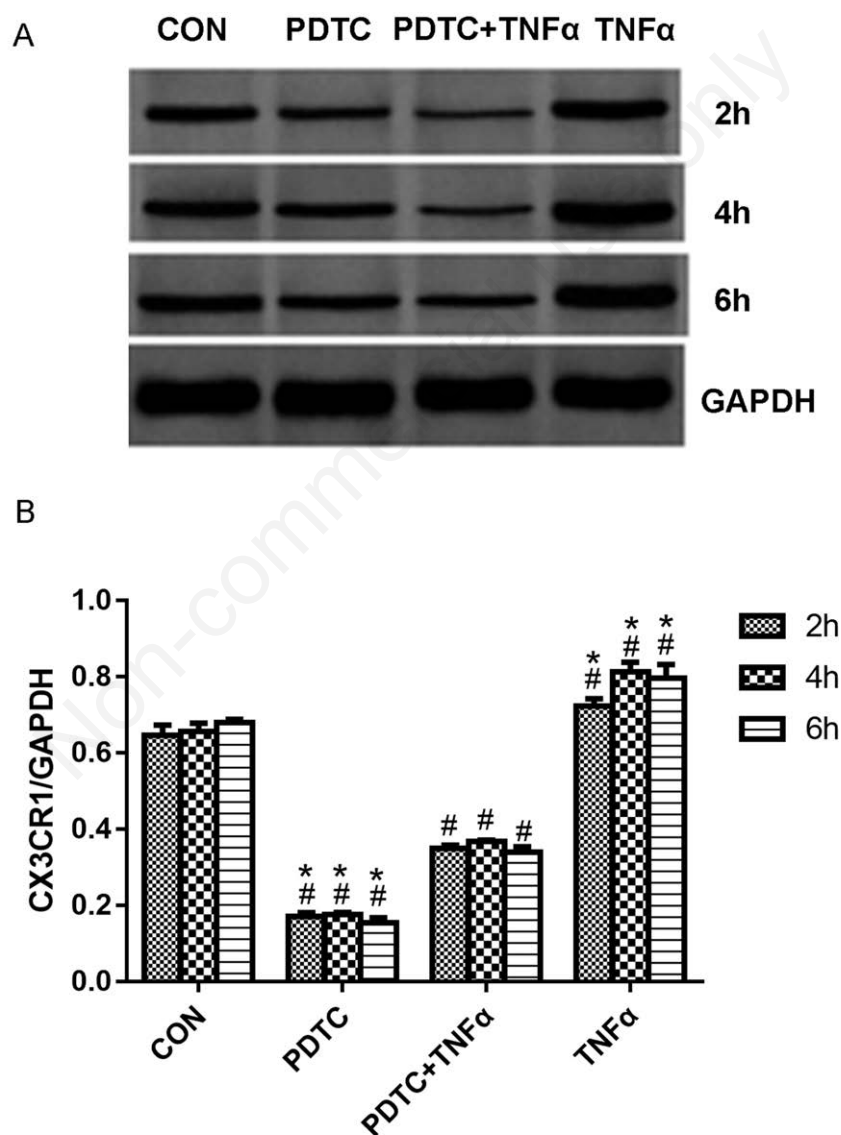
regulate CX3CR1 expression in response to TNF- $\alpha$  in various cells, such as HIF-1, AP-1 and STAT1/3, and a change on the DNA methylation status of CX3CR1 gene promoter can also change its gene expression.<sup>23</sup> Therefore, the involvement of those transcription factors in transcriptional regulation of CX3CR1 remains to be evaluated in further studies.

There are still some limitations of our study. Although we found PDTC inhibited CCI-induced spinal microglial activation and TNF- $\alpha$  upregulation, we didn't study the effects of PDTC on other cells and molecules in neural tissue and investigate the mechanisms between them. Some study shows co-localization of microglia CX3CR1 and extracellular signal-regulated protein kinase 5 (ERK5) in the spinal cord and suggests that CX3CR1 enhances nerve injury-induced pain hypersensitivity through the ERK5 signaling pathway.<sup>24</sup> Further study is needed to explore the direct target molecules for NF- $\kappa$ B and CX3CR1 in spinal microglia and effects of PDTC on other nerve cells, pain pathways

and mediators in CNS.

PDTC is largely used as an NF- $\kappa$ B inhibitor by inhibiting factor I- $\kappa$ B ubiquitination, however, it can also regulate other cell signalings, such as anti-apoptotic signaling. Recent studies have shown the embryonic lethality in mice with completely knockout of p65,<sup>25</sup> and another study indicated an inhibitory effect of p65-siRNA on transcription of COX-2.<sup>26</sup> Therefore, drugs that selectively inhibit pain-promoting NF- $\kappa$ B activity while leaving its physiological functions unaffected would be beneficial to clinical pain management and the efficacy and safety of PDTC in clinical setting still need to be explored in future research.

We have shown the protective effect of intrathecal PDTC on the development of nociceptive behaviors induced by CCI in rats. The activation of NF- $\kappa$ B pathway may contribute to spinal microglial activation and TNF- $\alpha$  up-regulation. We also demonstrated that the phosphorylation of NF- $\kappa$ B p65 was responsible for TNF- $\alpha$ -induced CX3CR1 expression in BV-2 microglial cells.



**Figure 6.** Effect of PDTC on CX3CR1 protein expression in TNF $\alpha$ -induced microglial cells. PDTC-pre-treatment group had lower CX3CR1 protein expression than other groups did. A) Bands of western blotting of CX3CR1 protein expression. B) Statistical analysis of relative density of western blotting bands is shown between different groups. Results are expressed as mean  $\pm$  SEM (n=4). #p<0.05 vs control cells; \*p<0.05 vs PDTC and TNF $\alpha$  treated cells.



These results enhanced our understanding of molecular mechanism underlying increased expressions of CX3CR1 in TNF- $\alpha$ -induced microglia cells and the inhibition of NF- $\kappa$ B signaling pathway by PDTC may provide an effective method for clinical treatment of neuropathic pain.

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