

## Original Article

# The role of chemokine CX3CL1 in the anterior cingulate cortex in a rat model of chronic pathological pain

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Received June 28, 2018; Accepted July 27, 2018; Epub November 15, 2018; Published November 30, 2018

**Abstract:** Purpose: To investigate the role of chemokine in the anterior cingulate cortex (ACC) in a rat model of chronic neuropathic pain. Methods: 80 male Sprague-Dawley rats were randomly assigned into 4 groups (n = 20): the sham group, the control group, the PBS treatment group and the anti-CX3CL1 treatment group. In the sham group, the unilateral infraorbital nerve was only exposed, but not ligated; in the control group, the unilateral infraorbital nerve was exposed and ligated. In the sham and the control group, the behavioral test was undertaken and the protein expression of CX3CL1 and CD11b was compared. Results: Compared with the sham group, there was a significant reduction in the feeling threshold in the ipsilateral ION territory from 3 d to 14 d after CCI-ION in the control group (P<0.05). Rats given antibody in the anti-CX3CL1 treatment group showed an evident increase of the feeling threshold compared with the PBS group (P<0.05). Conclusion: The ACC may take part in the chronic neuropathic pain via associating with the activated microglial cells and increased expression of CX3CL1.

**Keywords:** Chronic neuropathic pain, CCI-ION ACC, microglial cell, CX3CL1, CD11b

## Introduction

Chronic neuropathic pain can be contrasted to nociceptive pain, which is the type of pain which occurs when someone experiences an acute injury, such as smashing a finger with a hammer or stubbing a toe when walking barefoot. This type of pain is typically short-lived and usually quite responsive to common pain medications in contrast to neuropathic pain, has the potential to drastically alter a person's health and quality of life, and, unfortunately, it could not be relieved by the application of traditional drugs [1]. The mechanisms of chronic neuropathic pain, which manifest as hyperalgesia or hyperalgesia, include neurogenic inflammatory reactions, peripheral and central sensitization, and neuronal plasticity [2]. Numerous studies have demonstrated that the activation of glial cells in the spinal cord contributes to the development and progression of chronic neuropathic pain [3-5].

Activated glial cells can release a variety of neurotransmitters and extracellular signaling

molecules that influence neuronal cells, causing neuronal abnormalities (e.g., discharge that leads to neuropathic pain) [6, 7]. Verge et al. [8] found that neurons in the spinal cord can express the chemokine CX3CL1, which plays an important role in signal transduction between neurons and glial cells [9]. Little is known, however, about the role of glial cells in different regions of the brain.

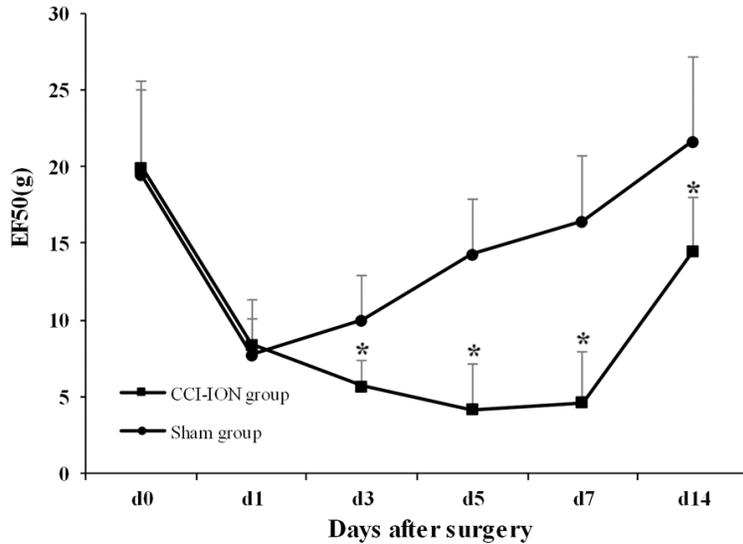
Accordingly, the present study focused on the effects of glial cells and CX3CL1 in chronic neuropathic pain in the anterior cingulate cortex (ACC).

## Materials and methods

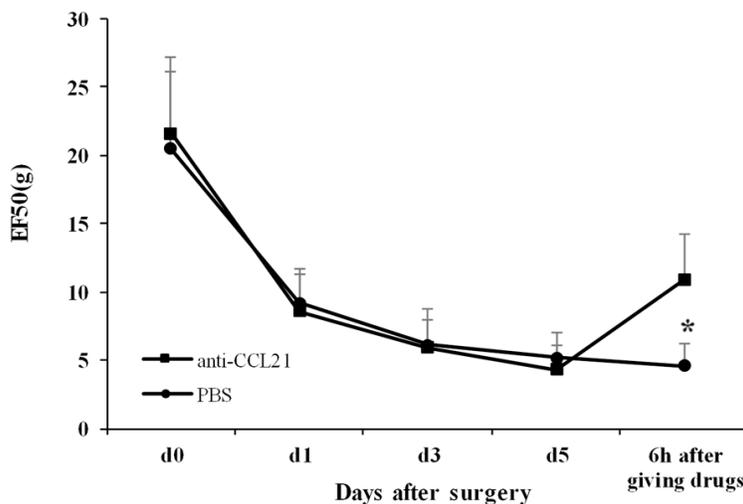
### Experimental animals and grouping

The present study was conducted using 80 clean-grade, adult male SD rats (body weight 180~220 g) from the comparative medical experimental section of Jinling Hospital. The rats were randomly divided into four groups: sham group (sham group), pain model group

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**Figure 1.** Charges of EF50(g) in all rats. Comparison of EF50(g) in rats two different treatments: \* $P<0.05$  vs Sham group.



**Figure 2.** Charges of EF50(g) in all rats. Comparison of EF50(g) in rats two different treatments: \* $P<0.05$  vs PBS group.

(CCI-ION group), PBS treatment control group (PBS group), and CX3CL1 neutralizing antibody treatment group (anti-CX3CL1 group).

**Unilateral infraorbital nerve (CCI-ION):** The rats were injected intraperitoneally with 2% sodium pentobarbital sodium 50 mg/kg. The anesthetized rats were supine with their head and limbs secured. A 1 cm longitudinal incision of about 1.5 mm thickness was made along the left side of the first molar gingival margin. Following the proximal incision, the infraorbital nerve was carefully separated from the distal orbital hole

using infraorbital nerve ligation. The diameter was made slightly thinner, but was not reduced to a degree that would block membrane blood circulation. The two ligation points were 2 mm apart, with 5.0 postoperative suture wounds. The sham operation group only had their infraorbital nerve exposed with no ligation performed, which resulted in the same postoperative wound. All experiments were performed in accordance with International Pain Research Council guidelines and were approved by the Animal Ethics Committee.

### *Anterior cingulate gyrus injection*

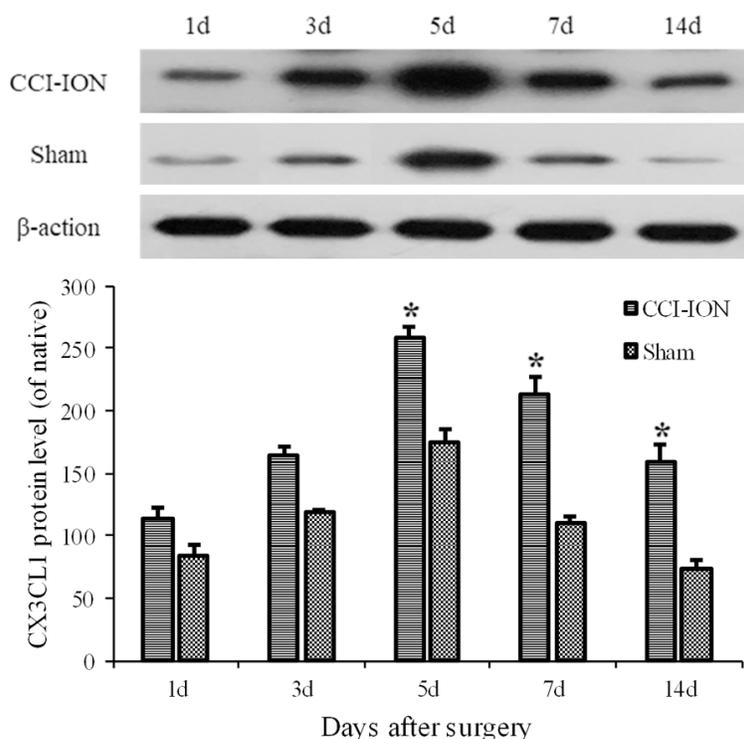
Rats were injected intraperitoneally with 2% sodium pentobarbital sodium 50 mg/kg. After anesthesia, they were fixed on a stereotactic instrument with the lower edge of a tooth rod below 3.3 mm. Following hair disinfection, the cranial fascia was cut, exposing the Bregma point. The drug was injected at a rate of 10 min into the left anterior cingulate gyrus of the ACC at the point of Bregma (before 2.6 mm, to the left by 0.6 mm) using a 5  $\mu$ L microinjector with a needle depth of 1.6 mm. The needle was withdrawn at the end of 5 min of dosing. After

administration, alcohol was used to disinfect the wound; the wound was then wrapped in paraffin and sutured at the skin.

### *Dosing regimens*

CX3CL1 neutralizing antibody is a monoclonal antibody that specifically binds to CX3CL1 in the ACC region to reduce the exposure of CX3CL1 to microglia and thereby attenuate the effect of CX3CL1 on microglia. Following CCI-ION surgery, rats in the anti-CX3CL1 group were each given a single dose of CX3CL1 neutraliz-

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**Figure 3.** Expression of CX3CL1 in the ACC. On the 5 d, 7 d, 14 d, compared with Sham group, the expression of CX3CL1 in the ACC of rats was significantly increased in CCL-ION group. However, CX3CL1 protein in the ACC of rats reached peak value on d5, and then decreased gradually. (\* $P < 0.05$  vs Sham group).

ing antibody (50 mmol; R & D Systems) in the ACC using a 5  $\mu$ L microinjector. Prior to administration, the neutralizing antibodies were dissolved in 0.9% sterile saline and tested via experimental dosing. Each rat was given 0.1 pmol of the neutralizing antibody using a single administration [10]. The PBS group was given the same dose of PBS solution as the control. The antibodies produced the desired inhibitory effect. Antibodies were stored in a refrigerator at  $-80^{\circ}\text{C}$  when not in use.

### Behavioral determination

The sham group and were fixed at 9:00 am on the 1, 3, 5, 7 and 14 d after CCI-ION, and the rats were placed in a quiet room for 30 minutes. After being quiet, Von Frey fiber (Stoeling, USA) was used to stimulate the left infraorbital V2 region (ie, Tentacles Department of skin and its surrounding long hair parts) for 3 to 5 s. One of the following three kinds of behavior, can be regarded as positive reaction: 1) rapid retreat action, curling up the body to move closer to

the cage wall, or head and face hidden in the body, To avoid the stimulus; 2) aggressive behavior: fast grasping stimulus; 3) asymmetric facial scratch: at least 3 consecutive scratching movements on the surface of the stimulus area, usually combined with retreat action. Anti-CX3CL1 group and PBS group were tested with Von Frey fiber at 6 hours after administration, and the pain threshold of left V2 area was measured. The EF50 (frequency at which rats emitted 50% of maximal responding) was calculated using Prism software (sigmoidal-dose response, variable slope; Graph Pad, San Diego, CA).

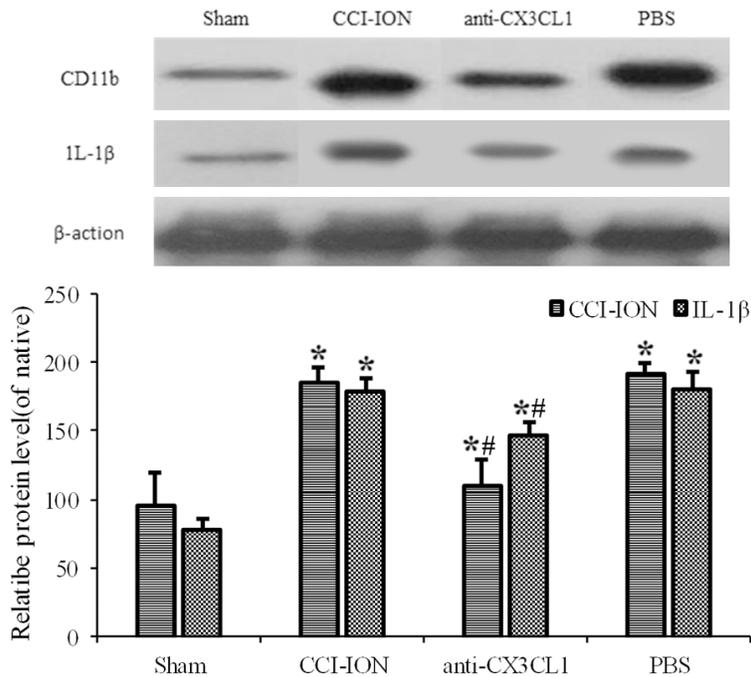
### Specimen collection

The sham group and CCI-ION group were sacrificed at 1, 3, 5, 7, and 14 d after intraperitoneal injection of 2% sodium pentobarbital sodium 80 mg/kg. The specimens were washed with 300-400 mL of saline. The brain was removed from the cortex of the anterior cingulate gyrus and stored in liquid nitrogen. The samples used for immunofluorescence were treated with 400-500 mL 4% paraformaldehyde. The brain tissue of the ACC area was removed and placed in 4% paraformaldehyde for 3 h, then transferred to 30% sucrose for 2 d. The anti-CX3CL1 group and PBS group were sacrificed 6 h after the behavioral test; the ACC area brain tissue was then removed. The specimens used for Western blotting were those stored in liquid nitrogen.

### Western blot

The ACC was homogenized with RIPA lysate containing protease inhibitor, phosphatase inhibitor, and PMSF. SDS-PAGE gel, 50  $\mu$ g (10  $\mu$ L) per sample, was used for electrophoresis. The separated proteins, transferred to a membrane and placed in 5% degreased milk at room temperature for 1 h for blocking. Rabbit anti-rat CX11XL1 (1:1000, Abcam), rabbit anti-rat

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**Figure 4.** Expression of CX3CL1 and IL-1 $\beta$  in ACC. Compared with the Sham group, the expression of CX3CL1 and IL-1 $\beta$  was significantly increased in CCI-ION group ( $P<0.05$ ). Also both of them were significantly decreased in anti-CX3CL1 group after given antibody ( $P<0.05$ ). \* $P<0.05$  vs Sham group, # $P<0.05$  vs CCI-ION group.

CD11b (1:800, Abcam), and rabbit anti-rat IL-1 $\beta$  (1:400, R & D) were incubated overnight at 4°C (D 2, TBST 5 min  $\times$  6 times). Sheep anti-rabbit secondary antibodies, with an internal reference of  $\beta$ -actin (1:1000, Cell Signaling Technology), were incubated at room temperature for 1 h, followed by a TBST wash (5 min  $\times$  6 times). The antibodies were then treated with a coloring substrate and stored in a dark room prior to color exposure and scanning. After scanning, the protein bands were analyzed using Image J software, and the relative level of target protein expression was reflected in the ratio of target band gray value to reference protein. Semi-quantitative analysis was performed.

### Statistical analysis

Statistical analysis was performed using SPSS16.0 software. The measurement data were expressed as  $x \pm s$ . The results of the behavior test were analyzed by repeated measurement of variance. A one-way ANOVA was used to compare the two groups.

## Results

### Behavioral tests in rats

No significant difference in preoperative behavior among the four groups was observed ( $P>0.05$ ). On d1, both group had a lower pain threshold. From d3, the CCI-ION group had a significant lower pain threshold than the sham group (**Figure 1**). Additionally, significant differences were found between the anti-CX3CL1 group and the sham group ( $P<0.05$ ) (**Figure 2**).

### The expression of chemokine CX3CL1 and CD11b in pre-anterior cingulate cortical area

On postoperative d5, in ACC, the expression of CX3CL1 in the CCI-ION group was significantly increased compared with the sham group ( $P<0.05$ ) (**Figure 3**). After given neutralization antibodies, the expression of CD11b and IL-1 $\beta$  were significantly decreased in the anti-CX3CL1 group compared with the PBS group ( $P<0.05$ ) (**Figure 4**).

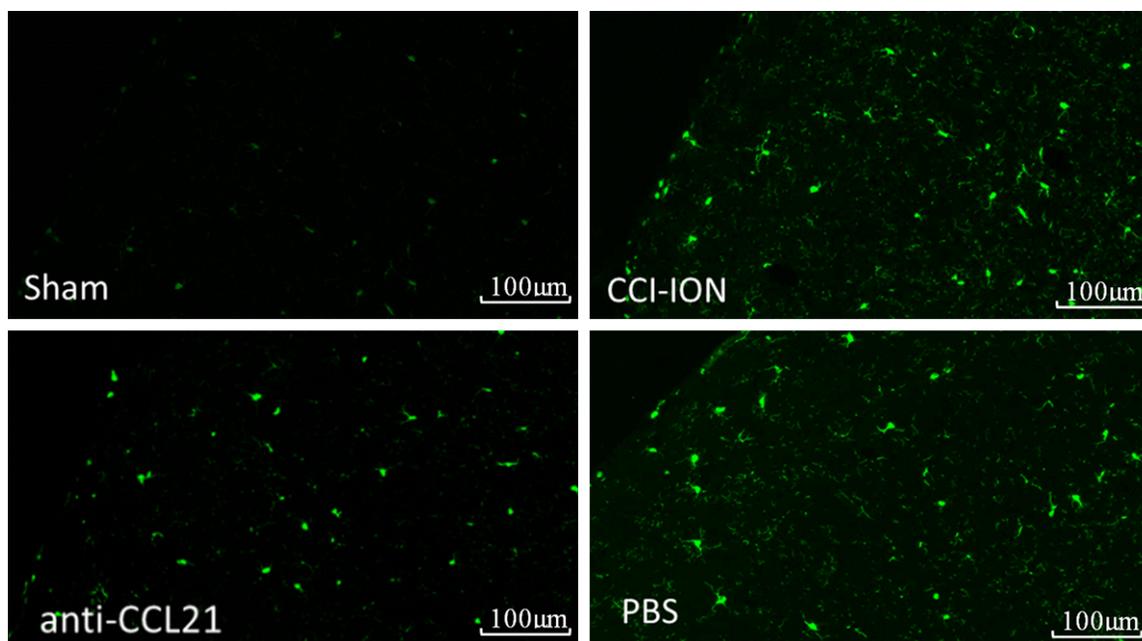
### Immunofluorescence assay of microglia in the anterior cingulate cortex of rats

The immunofluorescence results showed that CD11b expression in activated microglia of the ACC area was lower in the sham group; the expression of CD11b and number of activated microglia were significantly increased in the CCI-ION group; and the expression of CD11b in the ACC of the anti-CX3CL1 group was significantly lower in the anti-CX3CL1 group than that in the PBS group (**Figure 5**).

## Discussion

In recent years, studies have shown that chemokines play an important role in the activation of glial cells and neuropathic pain [11, 12]. Milior et al. [13] found that, in the rat model of chronic pain, neural cells in the spinal dorsal horn can release CX3CL1, thereby directly regulating the activity of microglia and promoting its

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**Figure 5.** The different expression of CD11b in ACC of rats. Compared with the Sham group, the immunoreactivity of CD11b in ACC was markedly increased in CCI-ION group, anti-CCL21 group and PBS group; Compared with CCI-ION group, the immunoreactivity was markedly decreased in anti-CCL21 group.

activation. These activated microglia can then activate p38 MAPK [14, 15], further releasing additional inflammatory factors that stimulate neurons and result in hyperalgesia [16]. This indicates that, chronic pathologic stimulation can promote neurons release of CX3CL1 and microglial activation, which can lead to hyperalgesia.

This study sought to determine the effect of ACC chemokine CX3CL1 on rats model of neuropathic pain. The researchers used the infra-orbital nerve ligation model, which requires less time for wound healing, and evokes less inflammatory response. Compared with other neuropathic pain models, it does not influence mobility of the rats, and is associated with a higher survival rate. It may also lead to a reduction in the individual difference caused by great trauma, and improve the accuracy of the behavioral test.

The behavioral test results indicated that 3 days after the acute pain period, the CCI-ION group had a significantly lower pain threshold than the sham group. While the CCI-ION group's pain threshold began to increase slowly after d5, this difference between the two groups continued to d14. The Western blot results

showed that the expression of the CX3CL1 protein in the ACC of rats on d3 had increased significantly, reached peak value on d5, and then decreased gradually. These results suggested that increased expression of ACC CX3CL1 may be involved in chronic pathological pain states.

To explore the relationship between CX3CL1 and the activation of microglia in the ACC, and whether CX3CL1 is involved in the development of chronic neuropathic pain, the CCI-ION group was classified into two additional groups: one group was treated with a CX3CL1 neutralizing antibody and the other was given PBS solution as a control. We recorded the time point when the expression of CX3CL1 reached peak value, and the behavioral test was performed 6 h after the drug was administered. The results showed that the pain threshold in the PBS group did not change significantly after administration, but the pain threshold of the anti-CX3CL1 group increased significantly, indicating that the increased expression of CX3CL1 in rats is related with increased pain sensitivity.

It's already known that normal neurons can also express CX3CL1, and that its receptor, CX3CR1, is expressed in microglia [17]. The abundant expression of CX3CL1 can lead to

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microglia activation. Sheridan et al. [18] found that CX3CL1 is a very important pathway for neuronal modulation of microglia. After knock-out of the CX3CR1 gene, neurons can no longer modulate the morphology of microglia. Thus, it is plausible that the role of ACC CX3CL1 in chronic pathological pain is also associated with microglia. The results of immunofluorescence in the present study suggested that the number of activated microglia in the ACC region had significantly decreased after administration of neutralizing antibody, indicating that increased expression of CX3CL1 may activate microglia [16].

The large number of inflammatory mediators released by microglia can stimulate the expression of prostaglandin E2 (PGE2), which helps facilitate the transportation of central glutamate as well as the reuptake of neuronal synapses [19]. As these substances can increase the pain sensitivity, the expression of IL-1 $\beta$  in the ACC region was assessed in the present study. Western blot analysis showed that, compared with the PBS group, the expression of CX3CL1, CD11b, and IL-1 $\beta$  decreased significantly in the CX3CL1 antibody group 6 h after neutralization. These results indicated that the inhibition of CX3CL1 expression in rats can reduce the activation of microglia and microglial release of inflammatory mediators, such as IL-1 $\beta$ , thereby relieving chronic neuropathic pain states.

In summary, the ligation of the infraorbital nerve in rats can increase the expression of CX3CL1 while simultaneously promoting microglial activation in the ACC region, which release inflammatory mediators and contribute to neuropathic pain. The use of antagonists to block the expression of CX3CL1 and, by extension, weaken the activation of microglia, have the potential to become a new tool in the clinical treatment of chronic neuropathic pain.

### Acknowledgements

Funded project of Jiangsu Natural Science Foundation (No. BK20131328) and Jiangsu Provincial Department of Health (No. H2015-08).

### Disclosure of conflict of interest

None.

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### References

- [1] Abuaisha B, Costanzi J and Boulton A. Acupuncture for the treatment of chronic painful peripheral diabetic neuropathy: a long-term study. *Diabetes Res Clin Pract* 1998; 39: 115-121.
- [2] Woolf CJ and Mannion RJ. Neuropathic pain: aetiology, symptoms, mechanisms, and management. *Lancet* 1999; 353: 1959-1964.
- [3] Watkins LR, Milligan ED and Maier SF. Glial activation: a driving force for pathological pain. *Trends Neurosci* 2001; 24: 450-455.
- [4] Gwak YS, Kang J, Unabia GC and Hulsebosch CE. Spatial and temporal activation of spinal glial cells: role of gliopathy in central neuropathic pain following spinal cord injury in rats. *Exp Neurol* 2012; 234: 362-372.
- [5] Noor S, Sanchez JJ, Vanderwall AG, Sun MS, Maxwell JR, Davies S, Jantzie LL, Petersen TR, Savage DD and Milligan ED. Prenatal alcohol exposure potentiates chronic neuropathic pain, spinal glial and immune cell activation and alters sciatic nerve and DRG cytokine levels. *Brain Behav Immun* 2017; 61: 80-95.
- [6] Belsky J and Pluess M. Beyond diathesis stress: differential susceptibility to environmental influences. *Psychol Bull* 2009; 135: 885.
- [7] de Jong EK, Dijkstra IM, Hensens M, Brouwer N, van Amerongen M, Liem RS, Boddeke HW and Biber K. Vesicle-mediated transport and release of CCL21 in endangered neurons: a possible explanation for microglia activation remote from a primary lesion. *J Neurosci* 2005; 25: 7548-7557.
- [8] Verge GM, Milligan ED, Maier SF, Watkins LR, Naeve GS and Foster AC. Fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. *Eur J Neurosci* 2004; 20: 1150-1160.
- [9] Milligan E, Sloane E and Watkins L. Glia in pathological pain: a role for fractalkine. *J Neuroimmunol* 2008; 198: 113-120.
- [10] Wei F, Guo W, Zou S, Ren K and Dubner R. Supraspinal glial-neuronal interactions contribute to descending pain facilitation. *J Neurosci* 2008; 28: 10482-10495.
- [11] Smith B, Whang J, Butovsky O and Scholz J. (272) Intraspinal chemokine signaling in the

## The role of chemokine in the ACC in a rat model of chronic neuropathic pain

- spared nerve injury model of neuropathic pain. *The Journal of Pain* 2016; 17: S44.
- [12] Chen G, Park CK, Xie RG, Berta T, Nedergaard M and Ji RR. Connexin-43 induces chemokine release from spinal cord astrocytes to maintain late-phase neuropathic pain in mice. *Brain* 2014; 137: 2193-2209.
- [13] Milior G, Lecours C, Samson L, Bisht K, Poggini S, Pagani F, Deflorio C, Lauro C, Alboni S and Limatola C. Fractalkine receptor deficiency impairs microglial and neuronal responsiveness to chronic stress. *Brain Behav Immun* 2016; 55: 114-125.
- [14] Zhuang ZY, Kawasaki Y, Tan PH, Wen YR, Huang J and Ji RR. Role of the CX3CR1/p38 MAPK pathway in spinal microglia for the development of neuropathic pain following nerve injury-induced cleavage of fractalkine. *Brain Behav Immun* 2007; 21: 642-651.
- [15] Ji RR and Suter MR. p38 MAPK, microglial signaling, and neuropathic pain. *Mol Pain* 2007; 3: 33.
- [16] Chang EY, Chen X, Sandhu A, Li CY and Luo ZD. Spinal 5-HT<sub>3</sub> receptors facilitate behavioural hypersensitivity induced by elevated calcium channel alpha-2-delta-1 protein. *Eur J Pain* 2013; 17: 505-513.
- [17] Limatola C and Ransohoff RM. Modulating neurotoxicity through CX3CL1/CX3CR1 signaling. *Front Cell Neurosci* 2014; 8: 229.
- [18] Sheridan GK and Murphy KJ. Neuron-glia crosstalk in health and disease: fractalkine and CX3CR1 take centre stage. *Open Biol* 2013; 3: 130181.
- [19] Yang S, Liu ZW, Wen L, Qiao HF, Zhou WX and Zhang YX. Interleukin-1 $\beta$  enhances NMDA receptor-mediated current but inhibits excitatory synaptic transmission. *Brain Res* 2005; 1034: 172-179.