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CXCR3 signalling partially contributes to the pathogenesis of neuropathic pain in male rodents

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Abstract

Background: Currently, there is a lack of effective therapy for chronic pain. Increasing evidence has shown that chemokines and their correlative receptors involved in the neuron–glial cell cross-talk could contribute to the pathogenesis of neuropathic pain. Our previous studies suggested that CXCR3 expression was elevated in the spinal dorsal horn after nerve injury.

Objectives: In this study, we aimed to explore the role of CXCR3 signalling in chronic pain modulation.

Methods: Reverse transcription quantitative PCR and Western blotting were used to measure the expression of CXCR3 and its ligands in the spinal cord following chronic constriction injury (CCI) of the sciatic nerve. Cxcr3 -knockout mice were used to observe the effect of the receptor on pain-related behaviour and microglial activation. Immunohistochemistry was used to investigate the expression of two activation markers for spinal microglia, Iba-1 and phosphorylated-p38 (p-p38) in these mice.

Results: The expression of CXCR3 and its ligand CXCL11 was upregulated in the lumbar dorsal horn of the spinal cord in CCI models. In Cxcr3 -knockout mice, CCI-induced tactile allodynia and thermal hyperalgesia were observed to be alleviated during the early stage of pain processing. Meanwhile, the expression of the glial activation markers, namely, Iba-1 and p-p38, was decreased.

Conclusion: Our results demonstrate that CXCR3 could be a key modulator involved in pain modulation of the spinal cord; therefore, CXCR3-related signalling pathways could be potential targets for the treatment of intractable pathological pain.

KEYWORDS

chronic constriction injury (CCI), CXCR3, microglia, neuropathic pain, phosphorylated-p38

1 | BACKGROUND

Chronic pain, commonly recognised as the hallmark symptom of some oral dysfunctions such as temporomandibular disorders (TMDs) and atypical facial pain, presents intractable problems and challenges in contemporary medicine. Increasing evidence suggests that neuroimmune interactions and neuroinflammation are involved in the development and maintenance of chronic neuropathic pain. Cytokines and chemokines—as crucial neuromodulators—play a pivotal role in neuroinflammatory processes.[1]

Chemokines, also known as 'chemotactic cytokines', interact with their receptors and have varied effects on the development, homeostasis and function of the immune system.[2] Apart from their biological activity, the regulatory effects of chemokines have been elucidated in the interactions between nervous and immune systems. In recent years, increasing number of chemokines and their

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receptors have been confirmed to be overexpressed in the DRG or the spinal cord of various chronic pain models.[3–5] Accumulating evidences have revealed that chemokines are key mediators of the neuron-glial cell crosstalk in some neuropathic pain conditions.[6,7] Moreover, enhancing or inhibiting the expression of these ligands and receptors could affect the process of pain development. Therefore, drug-induced modulation of chemokine receptor signalling is being considered as a novel therapeutic strategy.[8]

Neuron-glial cell crosstalk is considered to play an active role in synaptic plasticity leading to chronic pain. The chemokines secreted by neurons and non-neuronal cells—or recruited from the peripheral nervous system—interact with different types of nerve cells via specific membrane receptors expressed on their surfaces. This communication may lead to glial activation or enhanced excitatory synaptic transmission.[6] The source of C-X3-C motif chemokine ligand 1 (CX3CL1; fractalkine) in the spinal cord has been well demonstrated. CX3CL1 from the DRG neurons is affected by the spinal microglia and feeds back onto them via its unique receptor, C-X3-C motif chemokine receptor 1 (CX3CR1). Furthermore, p38, a member of mitogen-activated protein kinases (MAPK), is activated in response to the ligand-receptor signal in microglial cells, and subsequently induces the production of pro-inflammatory factors that activate neurons, resulting in pain facilitation.[9,10]

CXCR3—a chemokine membrane receptor—is a member of the G protein-coupled receptor family. Several chemokines can bind to this receptor, including CXCL9 (Mig), CXCL10 (IP-10), and CXCL11 (I-TAC), and CCL21 in rodents.[11] Previous studies have demonstrated the role of CXCL10/CXCR3 signalling in several neuropathic models such as chronic itch, cortex lesion and allergic contact dermatitis.[12–14] In our pilot study, we detected the presence of *Cxcr3* mRNA in the spinal dorsal horn after nerve ligation in chronic constriction injury (CCI) pain models. Therefore, in this study, we investigated the transcript-level expression of *Cxcr3* and its potential ligands in CCI murine models, observed the pain behaviours and microglial activation in *Cxcr3*-knockout (KO) mice, and attempted to explore the role played by CXCR3 signalling in chronic pain modulation.

2 | MATERIALS AND METHODS

2.1 | Animals and surgical procedure

Experiments were performed on adult male Sprague Dawley (SD) rats (weight, 200–220 g; $n = 40$) and *Cxcr3*-knockout (KO) mice ($n = 17$) and wild-type (WT) mice (age, 8 weeks; $n = 38$). SD rats were obtained from Vital River Laboratory Animal Technology Co., Ltd. and *Cxcr3*-KO mice (Cxcr3$^{-/-}$ mice; B6.129P2-Cxcr3^{tm1Dgen}/J; stock #005796) and WT littermate mice were obtained from the Jackson Laboratory. Animals were housed in specific-pathogen-free facilities at a temperature of 23°C (±1°C) under a 12-h light/dark cycle with free access to food and water. All animal procedures performed in this study were approved by the Animal Care and Use Committee of the Peking University Health Science Center. The care and use of animals conformed to the applicable national & international guidelines. Experimental planning, conduction and reporting were performed according to the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines. CCI models were generated by tying four (for rats) or three (for mice) loose ligations of the common sciatic nerve on the right side according to the method proposed by Bennett and Xie.[15] For sham operations, the nerve was exposed but not ligated.

TABLE 1 The primers for specific genes used in RT-qPCR

Genes	Primer sequences
Actin-Forward	5′-CGTTGACATCCGTAAAGAC–3′
Actin-Reverse	5′-TAGGAGCCAGGGCAGTA–3′
Ccxr3-Forward	5′-GCACATCTCCCTACGATT–3′
Cxcr3-Reverse	5′-GAGGCTGTAGAGGACTGG–3′
Cxcl9-Forward	5′-ATGAAGTCCGTTGCTCTATT–3′
Cxcl9-Reverse	5′-AGGTCTTTGAGGGATTTGT–3′
Cxcl10-Forward	5′-GAACAGACGCTGAGACCC–3′
Cxcl10-Reverse	5′-GCTCACCGCTTTCAATAA–3′
Cxcl11-Forward	5′-GTTCCAGGCTTCGTTATG–3′
Cxcl11-Reverse	5′-AGGCACCTTTGTCCTTTAT–3′

2.2 | Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Transcript-level expression of *Cxcr3*, *Cxcl9*, *Cxcl10* and *Cxcl11* in the L4–5 spinal dorsal horn was measured in CCI and sham rats using RT-qPCR after the operation on days 1, 3, 7 and 14 (5 rats in each group). Total L4–5 spinal cord RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed into cDNA using the M-MLV reverse transcriptase (Takara, Japan). For RT-qPCR, template cDNA (1 μL) was amplified using Taq DNA polymerase (Takara) in a total reaction volume containing 0.5 μM PCR primer as indicated (all primer sequences are listed in Table 1). RT-qPCR was performed on an ABI 7500 Fast real-time PCR system (Applied Biosystems) with SYBR Premix Ex Taq II (Takara), as per the manufacturer's instructions. β-actin was used as an internal control. Final data were normalised to those of the control group.

2.3 | Western blotting

Animals ($n = 4$/group) were deeply anaesthetised and euthanised using transcardiac perfusion. The spinal cord lumbar segments (L4–5) ipsilateral to the treatment were rapidly removed and homogenised in a lysis buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). Protein samples were separated on

SDS–PAGE gels and transferred onto polyvinylidene fluoride filters (Millipore). After being blocked, the membranes were incubated overnight with the CXCR3 antibody (anti-mouse, 1:300, Santa Cruz) or α-tubulin antibody (1:1000, Abcam) at 4°C. Then, the membranes were probed with HRP-conjugated secondary antibodies (1:1000; Jackson) and detected using an enhanced chemiluminescence system (ECL, Millipore). The density of the detected protein bands was quantified using ImageJ v.1.38 (NIH), normalised to the density of the protein band corresponding to the internal control (CXCR3/α-tubulin) and expressed as a fold change relative to the control (sham) group.

2.4 | Behavioural testing

Mice were habituated to the test environment for three consecutive days (30 min/day) before baseline testing. The investigator was blinded to the treatments received by the mice. The hind paw mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were measured to evaluate tactile allodynia and thermal hyperalgesia, respectively, at various time points before and after the surgery in KO and WT mice groups. The methods of testing were based on the classical protocols as described previously.[16,17] The adult male *Cxcr3*-KO mice and WT (C57BL/6J) controls were randomly divided into sham and CCI operation groups, respectively ($n = 5–7$ mice/group). All tests were conducted before and after the surgery on days 1, 3, 7, 10 and 14 under blinded conditions.

2.5 | Immunohistochemistry

Animals ($n = 3$ mice/group) were anaesthetised with an overdose of pentobarbital sodium on Day 7 after the operation and euthanised via transcardiac perfusion (phosphate-buffered saline followed by 4% paraformaldehyde). After the perfusion, the lumbar spinal cords (L4–5) were removed and postfixed, and 30 μm thick tissue sections were cut transversely on a cryostat for free-floating immunohistochemistry of CXCR3 (1:200, Santa Cruz), Iba-1 (1:400, AbD Serotec) and phosphorylated-p38 (p-p38, 1:400, Cell Signaling Technology). The stained sections were examined under a fluorescence microscope (Olympus), and the captured images were analysed using ImageJ.

2.6 | Statistical analysis

All data are presented as mean ±SEM. SPSS software (v.16.0; SPSS, Inc.) was used to perform all statistical calculations. All data were normally distributed (tested using the Kolmogorov-Smirnov test). Student's *t*-tests or analysis of variance (ANOVA) and post hoc tests were used to determine the significance. Differences were considered significant when the critical values reached $p < .05$.

3 | RESULTS

3.1 | CCI induced the transcript-level expression of *Cxcr3* and its related ligands in the lumbar dorsal horn

Pain behaviours such as mechanical allodynia and heat hyperalgesia developed several days after the CCI and persisted for weeks. To determine whether CXCR3 and its correlative ligands are involved in the maintenance of neuropathic pain, we performed RT-qPCR to determine the transcript-level expression of *Cxcr3*, *Cxcl9*, *Cxcl10* and *Cxcl11* after sciatic nerve ligation in the L4–5 spinal dorsal horn of rats. *Cxcr3* mRNA levels were significantly increased on Day 3 and maintained till Day 14 in CCI rats when compared with those in the sham-operated group (Figure 1A). Additionally, we found that the mRNA expression of *Cxcl11*, a potential ligand of CXCR3, was increased within 1 week after hyperalgesia development (Figure 1D). Contrastingly, the mRNA levels of *Cxcl9* and *Cxcl10* were not found to be elevated; in fact, *Cxcl10* mRNA levels decreased on Day 3 post-CCI (Figure 1B,C).

3.2 | CXCR3 protein levels were increased persistently in the spinal dorsal horn post-CCI surgery

We quantitatively analysed CXCR3 expression in the spinal dorsal horn by Western blotting. The CXCR3 protein levels in the L4–5 dorsal horn were found to increase significantly from Day 3 post-CCI and continued until the end of the observation period (Figure 2A,B). Additionally, we observed that CXCR3 expression was increased in *Iba-1*$^+$ microglia (Figure 2C).

3.3 | *Cxcr3* KO alleviated mechanical allodynia and thermal hyperalgesia at the early stage of CCI pain processing

Cxcr3 KO altered the baseline pain threshold in mice (data not shown); therefore, we calculated the percentage change from the baseline threshold for each animal to evaluate the behavioural changes. Mechanical allodynia was induced in 1–3 days post-CCI in the WT mice group, whereas the decrease in pain threshold reduced within 7 days after CCI in the KO group (Figure 3A). Similarly, thermal hyperalgesia was observed to be attenuated in *Cxcr3*-KO mice on days 3–5 post-CCI (compared with that in the WT mice); however, after 1 week, the variation tendency was observed to coincide with that of the WT group (Figure 3B).

3.4 | *Cxcr3* KO downregulated Iba-1 and p-p38 MAPK expression in the lumbar dorsal horn

In accordance with previous reports,[18,19] the expression levels of p-p38 MAPK (activated form of p38) and the counts of *Iba-1*$^+$ cells

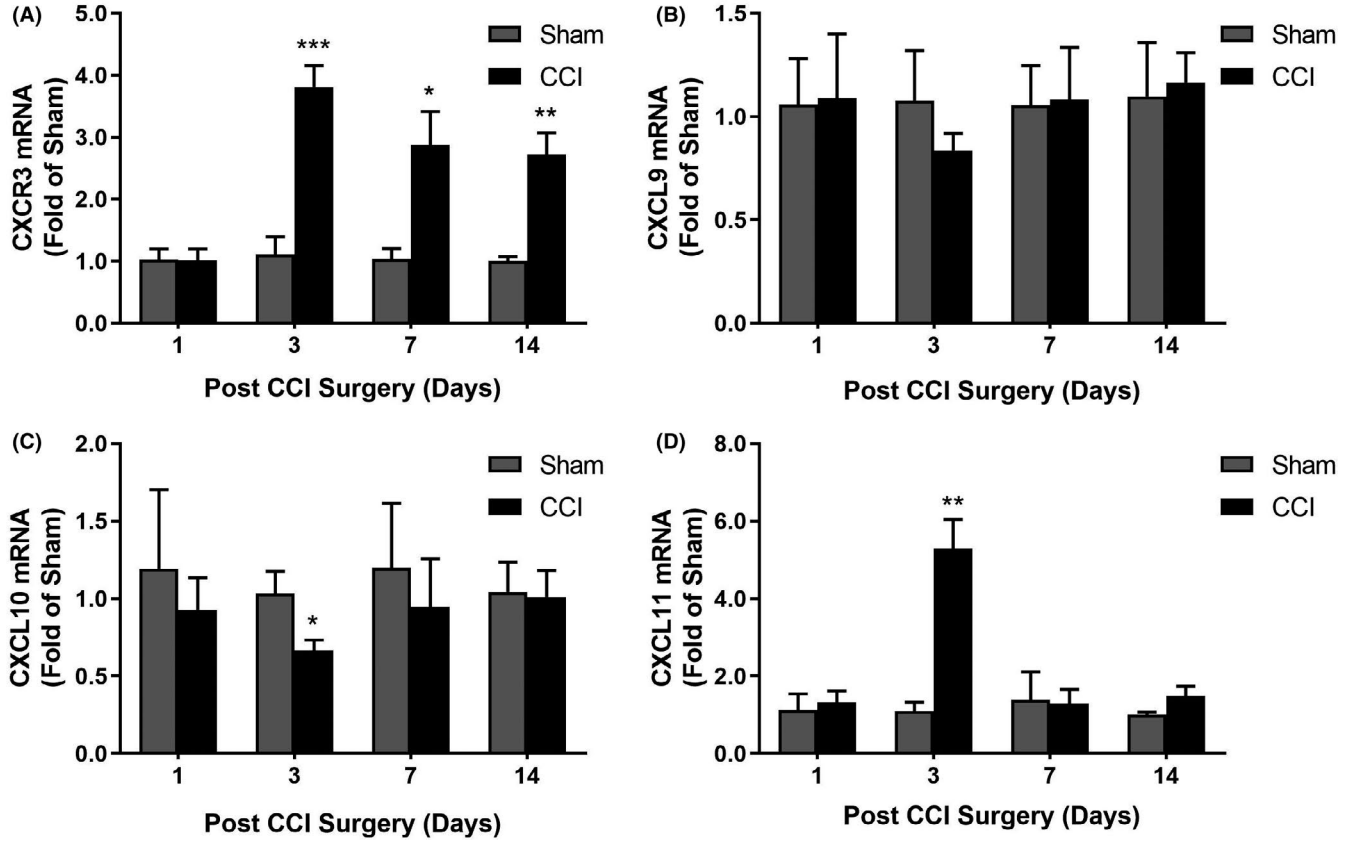

FIGURE 1 Gene expression pattern of *Cxcr3* and its ligands after CCI surgery in the lumbar spinal cord of rats. RT-qPCR results showed that the gene expression of *Cxcr3* was significantly increased from Day 3 post-CCI injury (A). There were no obvious changes in the transcript-level expression of the ligands *Cxcl9* (B) and *Cxcl10* (C). *Cxcl10* expression levels reduced by only a small amount on Day 3 after CCI. However, *Cxcl11* mRNA expression was elevated sharply on Day 3 post-CCI surgery. One-way ANOVA and Tukey's post hoc test were used to test statistical significance ($^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $n = 5$)

were increased post-CCI. Therefore, using immunofluorescence, we evaluated these markers in the dorsal horn of the spinal cord of WT and KO mice on Day 7 post-CCI. *Iba-1*$^{+}$ cells were observed to be equally distributed in the contralateral and ipsilateral dorsal horn of the sham group, whereas in the WT mice group, the *Iba1*$^{+}$ cell counts were increased in the ipsilateral area of CCI injury. The *Iba-1*$^{+}$ cell counts were found to decrease in the ipsilateral dorsal horn of the *Cxcr3*-KO CCI group compared with those in ipsilateral dorsal horn of WT mice (Figure 4). Similarly, *Cxcr3* KO resulted in the downregulation of p-p38 in the ipsilateral lumbar dorsal horn (Figure 5).

4 | DISCUSSION

In the present study, we showed that the expression of CXCR3 and its ligand CXCL11 was upregulated in the lumbar dorsal horn of the spinal cord in a murine CCI model. We used *Cxcr3*-KO models to explore the role of CXCR3 signalling in neuropathic pain and its effect on pain behaviour. In the *Cxcr3*-KO CCI group, CCI-induced mechanical allodynia and thermal hyperalgesia were abated during the early stage of pain processing, whereas microglia-activated p-p38 expression was found to be downregulated.

CXCR3 is a member of the CXC chemokine receptor family and is known to be involved in immune cell recruitment and cell proliferation. In recent years, CXCR3 has been demonstrated to play an important role in neuroinflammation and neurodegeneration. In the present study, we showed that CXCR3 expression was upregulated at both mRNA and protein levels in the lumbar dorsal horn of the spinal cord in murine CCI models. Jiang et al.[20] reported similar results in SNL models. In *Cxcr3*-KO mice, we found that the pain behaviours induced by CCI were partially inhibited in early stages of hyperalgesia. These data suggest that CXCR3 may contribute to the establishment of neuropathic pain at the initial stage.

The role of CXCL10/CXCR3 axis in some neurological diseases has been widely studied in recent years.[21] CXCL10/CXCR3 expression was found to be increased in multiple sclerosis (MS) models; thus, indicating that identification of molecules that block CXCR3 activity could serve as a novel approach for treating MS.[22] Studies revealed that CXCL10/CXCR3 signalling was enhanced in rat bone cancer pain models, and blocking the signal transduction through these chemokines using anti-CXCL10 antibody or CXCR3 antagonist could prevent the development of neuropathic pain and microglial activation.[23,24] Recently, it was reported that the CXCL10/CXCR3 signalling pathway was involved in some types

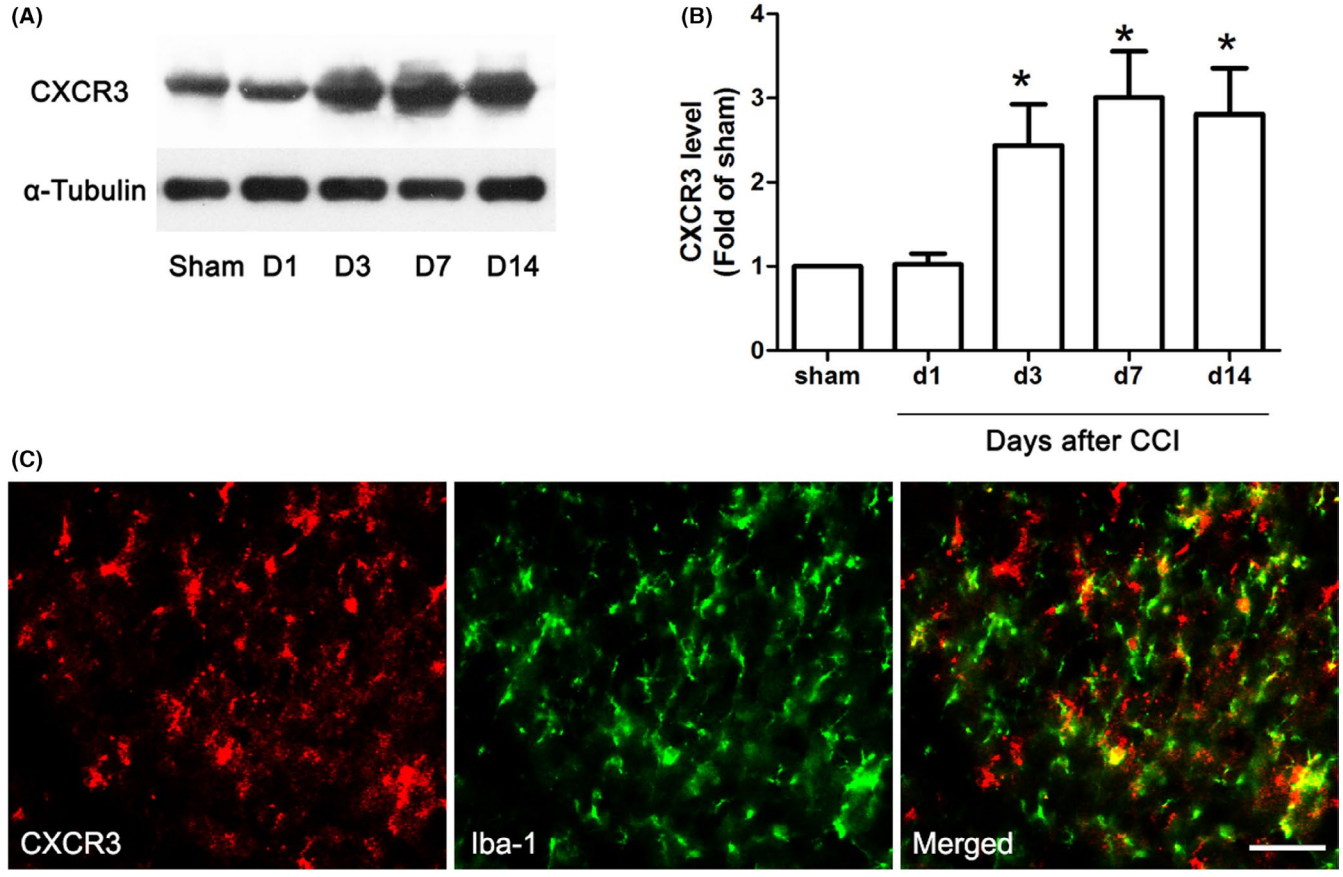

FIGURE 2 Representative bands (A) and quantification (B) of Western blot analysis show a persistent increase in CXCR3 protein levels in the lumbar spinal cord after CCI injury. CXCR3 level was quantified after normalising its expression level against a control protein, α-Tubulin. Double immunostaining (C) showed that a number of CXCR3 were expressed in *Iba-1*$^+$ cells (microglia). Scale bar: 50 μm; $^*p < .05$, one-way ANOVA and Tukey's post hoc test, compared with the sham surgery control, $n = 4$

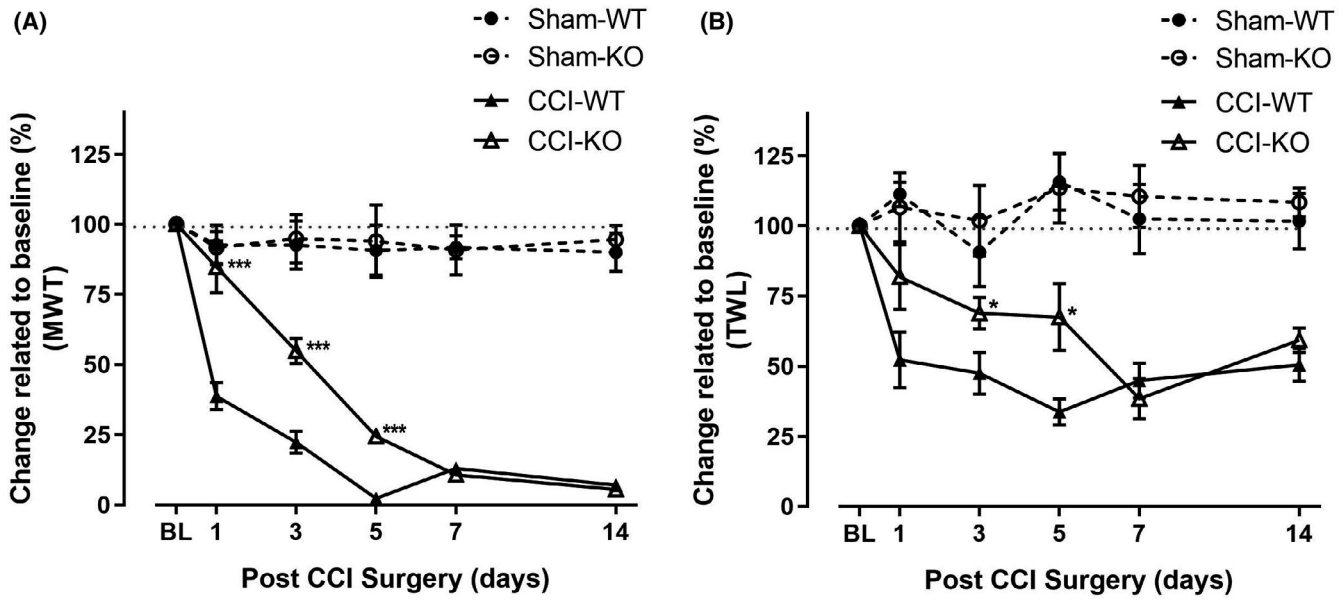

FIGURE 3 Behaviour test for tactile allodynia (A) and thermal hyperalgesia (B) after CCI surgery in the WT and KO mice. The result was showed as a percentage change from the baseline values on days 1, 3, 5, 7 and 14 after CCI or sham surgery. Sham-WT, sham-operated wild-type mice; Sham-KO, sham-operated CXCR3 knockout mice; CCI-WT, wide-type CCI mice; CCI-KO, CXCR3 knockout CCI mice. $n = 5$ or 7, two-way ANOVA, followed by Student's *t*-tests test. $^*p < .05$, $^{***}p < .001$. BL, baseline

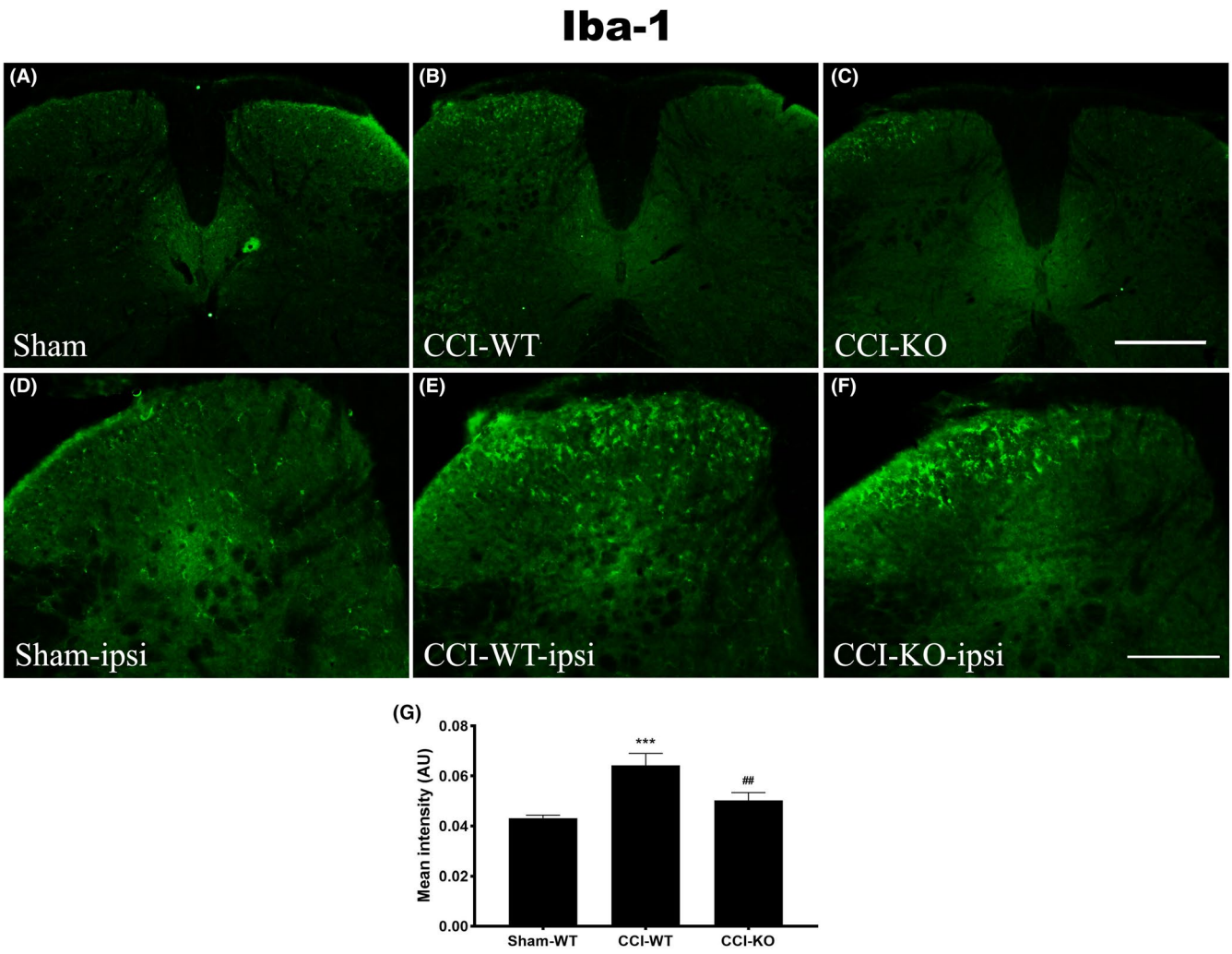

FIGURE 4 *Cxcr3* knockout results in the downregulated expression of microglia marker, Iba-1 in the lumbar spinal cord. Representative immunostaining images (A–F) of Iba-1 expression in the spinal dorsal horn from the WT and KO groups on Day 7 after CCI surgery. (A) Sham-WT, (B) CCI-WT, (C) CCI-KO group, (D) ipsilateral side of Sham-WT mice, (E) ipsilateral side of CCI-WT mice and (F) ipsilateral side of CCI-KO mice. (A–C), Scale bar (in C): 100 μm; (D–F), Scale bar (in F): 50 μm. $^{***}p < .001$ (vs. sham), $^{\#\#}p < .01$ (vs. CCI-WT), $n = 3$

of neuropathic pain.[25] Some early investigations have reported that IFN-γ alone or together with IL-1 could stimulate *CXCL11* expression in human astrocytes[26] and foetal human microglia.[27] In addition, CXCL11 is predominantly expressed by astrocytes in the central nervous system (CNS) and plays a role in experimental autoimmune encephalomyelitis of rats.[28] Here, we measured the expression levels of possible CXCR3 ligands, namely CXCL9, CXCL10 and CXCL11, and found that CXCL11 expression levels were increased; however, a significant increase in expression was observed only at Day 3 after the injury. Meanwhile, the mRNA and protein levels of CXCR3, a high-affinity receptor of CXCL11, were elevated on day 3 post-CCI. Furthermore, the inhibition of CCI-induced nociceptive behaviour to a certain extent in the early stages was demonstrated in *Cxcr3* KO murine models. Therefore, we believe that CXCL11/CXCR3 signalling may play an important role in the early stages of nociception processing induced by nerve injury.

A previous study showed that CXCR3 is constitutively expressed in neurons and glial cells in the CNS.[20] Moreover, CXCR3 is highly expressed by microglia during some physiological and pathological processes.[29,30] Microglia are considered to be the resident immune cells of the CNS. The activation of microglia in the spinal cord is a common feature of pathological pain, and microglia has been reported to be involved in the development of inflammatory and neuropathic pain.[31,32] Moreover, many studies have suggested that spinal microglial p-p38 expression is pivotal for the development and maintenance of chronic hypersensitivity in rodent models of pain.[33–35] In the present study, we demonstrated that the upregulated CXCR3 expression was partially observed in microglial cells; therefore, we used the KO mice to test whether CXCR3 is expressed in microglia and whether CXCR3 plays a role in the activation of p38 in the spinal cord after the establishment of chronic pain. In *Cxcr3*-KO models, we observed a decrease in the expression of the microglia marker Iba-1 and the phosphorylation of p38 MAPK in the lumbar spine. The chemokine receptor

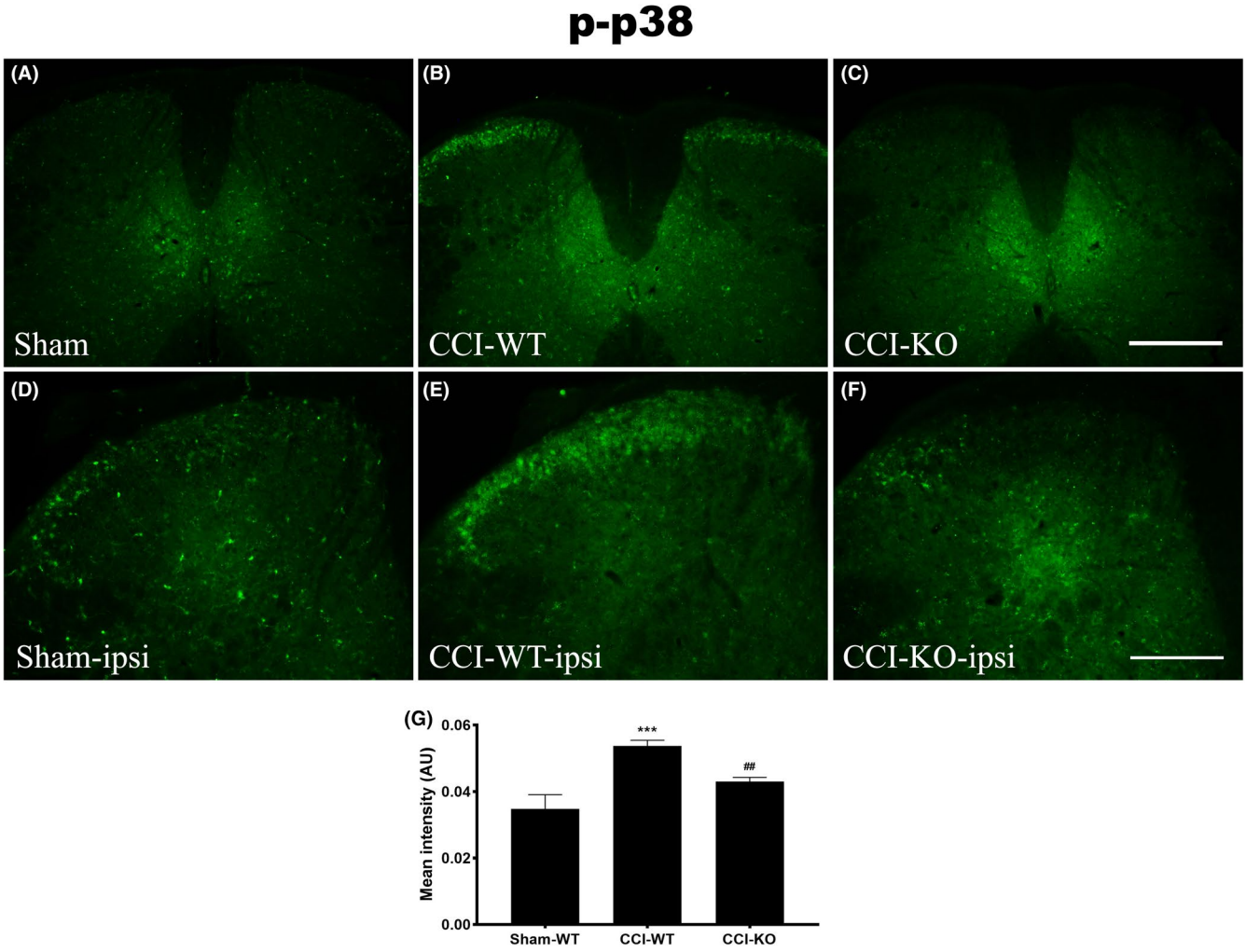

FIGURE 5 P-p38 immunostaining in the dorsal horn on Day 7 post-operation in different groups. (A and D) Sham-WT, (B and E) CCI-WT, and (C and F) CCI-KO group. (A–C), Scale bar (in C): 100 μm; (D–F), Scale bar (in F): 50 μm. $^{***}p < .001$ (vs. sham), $^{\#\#}p < .01$ (vs. CCI-WT), $n = 3$

CXCR3 has been shown to be linked with several signalling pathways, including Src, PI3K and MAPK signalling cascades.[21] Therefore, we believe that CXCR3 signalling in microglia could contribute to pain modulation of the spinal level by mediating the upstream activation of the intracellular p38 MAPK cascade in pain signalling transduction.

Chemokine and chemokine receptors are key immune regulators that participate in the pathogenesis of neuroinflammation and neurodegeneration in many neurological diseases. Currently, some chemokine receptor-targeting drugs have been tested in humans. Here, we concluded that the receptor CXCR3 could play an important role in the development of chronic pain. Therefore, blocking the receptor could offer novel approaches for designing therapeutic strategies to alleviate neuropathic pain in humans.

4.1 | Study limitations

This study explored the role of CXCR3 signalling in the modulation of spinal pain processing. Although the study provided some acceptable results for pain behaviour and spinal molecular signalling changes in *Cxcr3*-KO mice, we could not completely eliminate the impact of systemic gene KO on the performance. In addition, CXCR3 and its corresponding ligand-expressing cells have not yet been identified. Moreover, the study lacks the pharmacological evidence of the CXCR3 signalling pathway. Hence, the cellular location and regulatory mechanism of the chemokine signalling need to be clarified in further studies.

5 | CONCLUSION

Our study indicated that the expression of CXCR3 and one of its ligands, CXCL11, was upregulated in the spinal cord after CCI and that CXCR3 signalling was involved in pain modulation, thereby suggesting that CXCR3 might be a potential therapeutic target for intractable chronic pain.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare that there are no potential conflicts of interest.

AUTHOR CONTRIBUTION

K. Li participated in the design of the study, carried out the experiments and drafted the manuscript. Y.-H. Tan and S.-Y. Feng performed experiments, analysed the data and interpreted the results. K.-Y. Fu had contributions to conception and design, manuscript writing and revising, financial support and final approval of the manuscript. K. Li and Y.-H. Tan contributed equally to this work. All authors have read and approved the final version of the manuscript.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

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

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