

CXCL10 and CXCR3 in the Trigeminal Ganglion Contribute to Trigeminal Neuropathic Pain in Mice

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Yuan-Yuan Ju^{1,2}
Ming Jiang²
Feifei Xu^{1,3}
Dongqin Wang^{1,3}
Bixiao Ding^{1,3}
Ling-Jie Ma²
Hao Wu¹

¹Department of Otolaryngology, Head, and Neck Surgery, Affiliated Hospital of Nantong University, Nantong, Jiangsu, People's Republic of China; ²Institute of Pain Medicine, Institute of Nautical Medicine, Nantong University, Nantong, Jiangsu, People's Republic of China; ³Medical School of Nantong University, Nantong, Jiangsu, People's Republic of China

Purpose: Trigeminal neuropathic pain is very common clinically, but effective treatments are lacking. Chemokines and their receptors have been implicated in the pathogenesis of chronic pain. This study explored the role of the chemokine CXCL10 and its receptor, CXCR3, in trigeminal neuropathic pain in mice.

Materials and Methods: Trigeminal neuropathic pain was established by partial infraorbital nerve ligation (pIONL) in wild-type and *Cxcr3*^{-/-} mice. Facial mechanical allodynia was evaluated by behavioral testing. A lentivirus containing *Cxcr3* shRNA (LV-*Cxcr3* shRNA) was microinjected into the trigeminal ganglion (TG) to knock down *Cxcr3* expression. Quantitative polymerase chain reaction assays and immunofluorescence staining were used to examine *Cxcl10/Cxcr3* mRNA expression and protein distribution. Western blotting was performed to examine activation of extracellular signal-regulated kinase (ERK) and AKT in the TG. Intra-TG injection of an AKT inhibitor was performed to examine the role of AKT in trigeminal neuropathic pain.

Results: pIONL induced persistent trigeminal neuropathic pain, which was alleviated in *Cxcr3*^{-/-} mice. Intra-TG injection of LV-*Cxcr3* shRNA attenuated pIONL-induced mechanical allodynia. Furthermore, pIONL increased the expression of CXCR3 and its major ligand, CXCL10, in TG neurons. Intra-TG injection of CXCL10 induced pain hypersensitivity in wild-type mice but not in *Cxcr3*^{-/-} mice. CXCL10 also induced activation of ERK and AKT in the TG of wild-type mice. Finally, pIONL-induced activation of ERK and AKT was reduced in *Cxcr3*^{-/-} mice. Intra-TG injection of the AKT inhibitor alleviated pIONL-induced mechanical allodynia in WT mice but not in *Cxcr3*^{-/-} mice.

Conclusion: CXCL10 acts on CXCR3 to induce ERK and AKT activation in TG neurons and contributes to the maintenance of trigeminal neuropathic pain.

Keywords: CXCR3, CXCL10, trigeminal ganglion, neuropathic pain, mice

Correspondence: Hao Wu
Department of Otolaryngology, Head, and Neck Surgery, Affiliated Hospital of Nantong University, Xisi Road 20#, Nantong, Jiangsu Province 226001, People's Republic of China
Email entwuhao@163.com

Ling-Jie Ma
Institute of Pain Medicine, Institute of Nautical Medicine, Nantong University, Seyuan Road 9#, Nantong, Jiangsu Province 226019, People's Republic of China
Email malingjie@ntu.edu.cn

Introduction

Trigeminal neuropathic pain is the most common form of refractory craniofacial neuropathic pain following injury to the trigeminal system, including facial trauma, compression, and virus infection.^{1,2} Trigeminal neuropathic pain has a high prevalence, ranging from 4 to 29 people per 100,000;¹ however, it is difficult to treat in clinical practice, and the pathophysiological mechanisms are poorly understood.²

Accumulating evidence indicates that chemokine-mediated neuroinflammation plays an important role in the development and maintenance of chronic pain.³⁻⁵ Several chemokines, including CXCL1, CCL2, CXCL1, CXCL13, and CXCL10,

are increased in the spinal cord after tissue inflammation or nerve injury and contribute to inflammatory and neuropathic pain via different forms of neuron-glial interaction.^{6–10} In addition, recent studies have demonstrated that CCL2, CCL4, CX3CL1, and CXCL12 and their cognate receptors in the dorsal root ganglion (DRG) are involved in neuropathic pain via neuron-to-neuron interactions.^{11–15} In contrast to the well-studied role of chemokines in the DRG and spinal cord in chronic pain, the involvement of chemokines and their receptors in the trigeminal ganglion (TG) in orofacial neuropathic pain has been less frequently investigated.

The chemokines CXCL9, CXCL10, and CXCL11 belong to the same subfamily of chemokines and bind to CXCR3 to exert functions. CXCR3 is expressed in various cells, including endothelial cells, monocytes, T cells, and dendritic cells.¹⁶ Meanwhile, CXCR3 is involved in a variety of human diseases, including chronic inflammation, immune dysfunction, cancer, metastasis, and pruritus.^{16,17} Recent studies have shown that CXCR3 is expressed in spinal neurons and increased after spinal nerve ligation (SNL)⁸ or inoculation of cancer cells into the tibia.¹⁸ CXCL10 is also increased in spinal astrocytes after SNL.^{8,19} Perfusion of spinal cord slices with CXCL10 enhances excitatory synaptic transmission of lamina II neurons in the dorsal horn via CXCR3.^{8,19} Importantly, inhibition of CXCR3 by gene knockout, intraspinal injection of lentivirus expressing shRNA, or intrathecal injection of the antagonists NBI-774330 or AMG487 attenuates neuropathic pain.^{19,20} However, the role of CXCR3 and CXCL10 in trigeminal neuropathic pain and the involved mechanisms remain unknown.

Chemokine receptors are seven transmembrane G-protein coupled receptors. The binding of chemokines with their cognate receptors leads to activation of intracellular signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol-3 kinase (PI3K) pathway.⁵ MAPKs, including extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) are activated in the DRG and spinal cord by peripheral nerve injury.²¹ The PI3K/AKT pathway in the central and peripheral nervous system also mediates pain hypersensitivity induced by nerve injury, incision, or inflammation.^{22,23} Previous studies have demonstrated that CXCR3 activates several intracellular kinases, such as Ras/ERK and PI3K/AKT.^{24,25} Intrathecal injection of CXCL10 induces rapid ERK activation in the spinal cord.⁸ However, whether MAPKs and

AKT can be activated by CXCL10/CXCR3 in the TG has not been investigated.

In this study, using the partial infraorbital nerve ligation (pIONL) model, we studied the role of CXCL10/CXCR3 in the TG in the pathogenesis of trigeminal neuropathic pain. Our results demonstrated that CXCR3 was activated by CXCL10 and contributed to the maintenance of trigeminal neuropathic pain via activation of ERK and AKT in the TG.

Materials and Methods

Animals and Surgery

ICR and C57BL/6 mice (6–8 weeks, male) were purchased from the Experimental Animal Center of Nantong University. *Cxcr3*^{−/−} mice were purchased from Jackson Laboratory (stock No. 005796). All animals were free to access food and water and housed in the animal facility at a room temperature of 23 ± 1 °C on a 12:12 h light-dark cycle. All experimental procedures were performed in accordance with the UK animals Scientific Procedures Act (1986) and approved by the Animal Care and Use Committee of Nantong University.

The pIONL surgery was performed as described previously.^{26,27} In brief, the mice were anesthetized with an anesthetic compound (components: 4.25% chloral hydrate, 0.886% sodium pentobarbital, 14.25% anhydrous alcohol, 33.8% propylene glycol, and 2.12% MgSO₄) and placed in a supine position. The oral cavity was opened to locate the left tendon of the masseter muscle on the top wall of the oral cavity. A 1-mm incision was made in front of the tendon to expose the infraorbital nerve (ION), and one-half of the ION was ligated with 6–0 silk suture. For the sham operation, an incision was made on the mucous membrane, and the ION was exposed but not ligated. Facial pain behavior was assessed the day before the surgery and at 3, 7, 10, 14, and 21 days after surgery.

Lentiviral Vector Production and Intra-TG Microinjection

Recombinant lentiviruses containing *Cxcr3* shRNA (LV-*Cxcr3* shRNA, 5'-CTG AAC TTT GAC AGA ACC T-3') or negative control shRNA (LV-NC, 5'-TTC TCC GAA CGT GTC ACG T-3') were produced using the pGCSIL-GFP vector by Shanghai GeneChem. Recombinant CXCL10 (murine) was purchased from PeproTech (Rocky Hills, NJ, USA). AKT inhibitor IV was purchased from Sigma-Aldrich (St Louis, MO, USA).

For intra-TG microinjection, the mice were deeply anesthetized with isoflurane. The head was stabilized with one hand, and the needle was positioned at an approximately 10° angle relative to the midline of the head and was inserted medial (1–2 mm) to the palpated portion of the zygomatic process through the infraorbital foramen, infraorbital canal, and foramen rotundum and reached the medial wall of the fossa where the TG is located.²⁸ LV-*Cxcr3* shRNA (2 µL), CXCL10 (100 ng in 5 µL), or AKT inhibitor IV (0.2 µg in 5 µL) was slowly injected.

Facial Pain Behavioral Test

Before assessing the facial pain baseline, the mice were habituated to the behavioral test cage for 30 min every day for 3 days in the behavioral test environment. As previously reported,^{29,30} two von Frey filaments (0.02 g and 0.16 g) were used to stimulate the whisker pad innervated by the ION, and the response of the mice was recorded. The filaments were applied to the ipsilateral whisker pad three times. The mean nocifensive behavior score of three measurements was calculated according to the following criteria: 0 points, no response; 1 point, exploratory behavior - the mouse detected the von Frey filament; 2 points, slight withdrawal response - the mouse slowly moved its face from the stimulation; 3 points, quick and intense withdrawal response with paw lifting; 4 points, the mouse wiped its face with the forepaw toward the stimulated facial area less than three times; 5 points, the mouse wiped its face with the forepaw toward the stimulated facial area more than three times.^{29,30}

Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from the TG using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as described previously.⁶ Quantitative polymerase chain reaction (qPCR) analysis was performed using a real-time detection system (RotorGene 6000, Qiagen) with SYBR green I dye detection (Takara, Japan). The following primers were used: *Cxcr3* forward, 5'-TAC CTT GAG GTT AGT GAA CGT CA-3'; *Cxcr3* reverse, 5'-CGC TCT CGT TTT CCC CAT AAT C-3'; *Cxcl10* forward, 5'-TGA ATC CGG AAT CTA AGA CCA TCA A-3'; *Cxcl10* reverse, 5'-AGG ACT AGC CAT CCA CTG GGT AAA G-3'; *Gapdh* forward, 5'-AAA TGG TGA

AGG TCG GTG TGA AC-3'; *Gapdh* reverse, 5'-CAA CAA TCT CCA CTT TGC CAC TG-3'. PCR amplification was performed at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. *Gapdh* was used as an internal control for normalization. The mRNA levels were calculated using the $-\Delta\Delta C_t$ method ($2^{-\Delta\Delta C_t}$).

Immunohistochemistry

For immunofluorescence staining, mice were deeply anesthetized and transcardially perfused with 4% paraformaldehyde. TG sections (15 µm) were cut with a cryostat and processed for immunostaining as previously described.³¹ The following primary antibodies were used: CXCR3 (rabbit, 1:200, Boster Biological Technology), CXCL10 (goat, 1:100, R&D Systems), β 3-tubulin (mouse, 1:500, R&D Systems), GFAP (mouse, 1:5000, Millipore), CD11b (mouse, 1:50, AbD), and IBA-1 (rabbit, 1:3000, Wako). The sections were then incubated with Cy3- or Alexa 488-conjugated secondary antibodies (1:1000, Jackson). For the negative control, the primary antibody was omitted, and the sections were incubated with the secondary antibody only. A Leica SP8 confocal microscope was used to examine the stained sections and capture images.

Western Blotting

The TG tissues were homogenized in a lysis buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). A BCA protein assay was used to determine the protein concentrations. Sodium dodecyl polyacrylamide gel electrophoresis and Western blotting were performed as previously described.²⁷ The following antibodies were used: CXCR3 (rabbit, 1:200, Boster Biological Technology), pERK (rabbit, 1:1000, Cell Signaling), ERK (rabbit, 1:1000, Cell Signaling), pp38 (rabbit, 1:1000, Cell Signaling), p38 (rabbit, 1:1000, Cell Signaling), pJNK (rabbit, 1:1000, Cell Signaling), JNK (rabbit, 1:1000, Cell Signaling), pAKT (rabbit, 1:1000, Cell Signaling), AKT (rabbit, 1:1000, Cell Signaling), and IRDye 800CW donkey anti-rabbit secondary antibody. Images were captured using the Odyssey Imaging System (LI-COR Biosciences).

Quantification and Statistics

All results are presented as the mean \pm SEM. The behavioral data were analyzed by two-way repeated measures (RM) ANOVA followed by the Bonferroni test. The qPCR data

were analyzed by one-way ANOVA followed by the Bonferroni test. The density of specific bands on Western blots was measured with Image J software (NIH). Differences between two groups were compared using Student's *t*-test. GraphPad Prism v5.0 was used for statistical analyses, and $P < 0.05$ was considered to be significant.

Results

Deletion or Inhibition of *Cxcr3* Alleviated pIOLN-Induced Mechanical Allodynia

As previously reported,²⁷ pIOLN induced persistent mechanical allodynia, which started at 3 days and was maintained for more than 21 days (Treatment, $F_{1, 60} = 28.79$, $P < 0.001$; Time, $F_{5, 60} = 12.37$, $P < 0.001$; Interaction, $F_{5, 60} = 3.822$, $P < 0.01$, two-way RM ANOVA, Figure 1A). However, pIOLN-induced pain hypersensitivity was significantly attenuated in *Cxcr3*^{-/-} mice compared with wild-

type (WT) mice (Treatment, $F_{1, 80} = 28.31$, $P < 0.001$; Time, $F_{5, 80} = 29.43$, $P < 0.001$; Interaction, $F_{5, 80} = 19.30$, $P < 0.001$, two-way RM ANOVA, Figure 1B). To investigate whether CXCR3 in the TG is involved in trigeminal neuropathic pain, we microinjected a LV-*Cxcr3* shRNA or LV-NC into the TG. Behavioral results showed that LV-*Cxcr3* shRNA markedly attenuated pIOLN-induced mechanical allodynia at day 7, 10, and 14 after pIOLN (Treatment, $F_{1, 64} = 28.67$, $P < 0.001$; Time, $F_{4, 64} = 29.96$, $P < 0.001$; Interaction, $F_{4, 64} = 8.574$, $P < 0.001$, two-way RM ANOVA, Figure 1C). qPCR showed that LV-*Cxcr3* shRNA significantly reduced the *Cxcr3* mRNA level ($22 \pm 14\%$ of LV-NC, $P < 0.01$, one-way ANOVA, Figure 1D), confirming the knockdown effect of LV-*Cxcr3* shRNA in the TG. Collectively, the results suggest that CXCR3 in the TG is involved in the pathogenesis of trigeminal neuropathic pain.

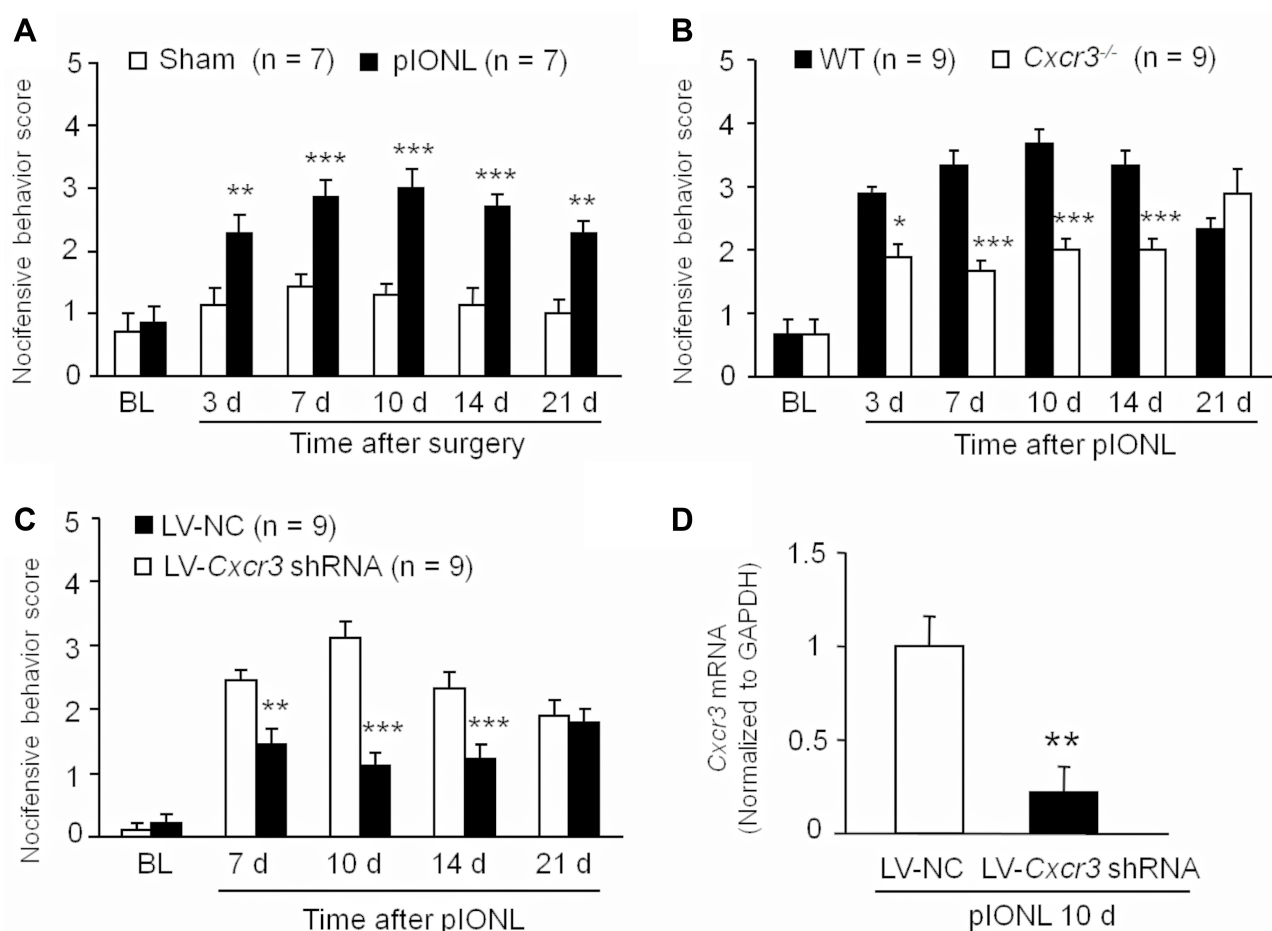


Figure 1 Inhibition of CXCR3 in the TG attenuates trigeminal neuropathic pain. **(A)** pIOLN induced mechanical allodynia which started from 3 days and maintained for more than 21 days. ** $P < 0.01$, *** $P < 0.001$. Two-way RM ANOVA followed by the Bonferroni test. **(B)** In *Cxcr3*^{-/-} mice, the nocifensive behavioral scores were significantly reduced from Day 3 to Day 14. * $P < 0.05$, *** $P < 0.001$. Two-way RM ANOVA followed by the Bonferroni test. **(C)** Intra-TG injection of LV-*Cxcr3* shRNA lentivirus alleviated pIOLN-induced mechanical allodynia. ** $P < 0.01$, *** $P < 0.001$ compared with LV-NC group. Two-way RM ANOVA followed by the Bonferroni test. **(D)** LV-*Cxcr3* shRNA injection reduced *Cxcr3* mRNA compared to the LV-NC group. ** $P < 0.01$, Student's *t*-test.

pIONL Increased CXCR3 Expression in TG Neurons

We then investigated the time-course of *Cxcr3* mRNA expression in the TG after pIONL by qPCR. Compared with naïve mice, *Cxcr3* mRNA was significantly increased at day 3, 10, and 21 (day 3, $P < 0.05$; day 10, $P < 0.01$; day 21, $P < 0.05$. pIONL vs sham, Figure 2A). Western blots further showed that CXCR3 protein was significantly increased in the TG at 10 days after pIONL ($P < 0.01$, Student's *t*-test, Figure 2B–C). We then examined the CXCR3 distribution in the TG by immunofluorescence staining. CXCR3 exhibited very low expression in the TG of naïve mice (Figure 2D) or sham-operated mice (Figure 2E) but was markedly increased 10 days after pIONL (Figure 2F). To characterize the cellular localization of CXCR3 in the TG, double staining of CXCR3 with the neuronal marker

β 3-tubulin, satellite cell marker GFAP, or macrophage marker CD11b was performed. The results showed that CXCR3 was largely co-localized with β 3-tubulin (Figure 2G), but not with GFAP (Figure 2H) or CD11b (Figure 2I), suggesting neuronal expression of CXCR3 in the TG.

pIONL Increased CXCL10 Expression in TG Neurons

CXCL10 is the major ligand of CXCR3. qPCR showed that *Cxcl10* mRNA was increased at day 3 and 10 after pIONL (day 3, $P < 0.01$; day 10, $P < 0.05$; day 21, $P > 0.05$, pIONL vs sham, Figure 3A). We further examined CXCL10 expression and its distribution by immunostaining. No signal was found when the CXCL10 antibody was omitted (Figure 3B). CXCL10 immunoreactivity was low in the TG of naïve mice (Figure 3C) and sham mice (Figure 3D) but increased in the

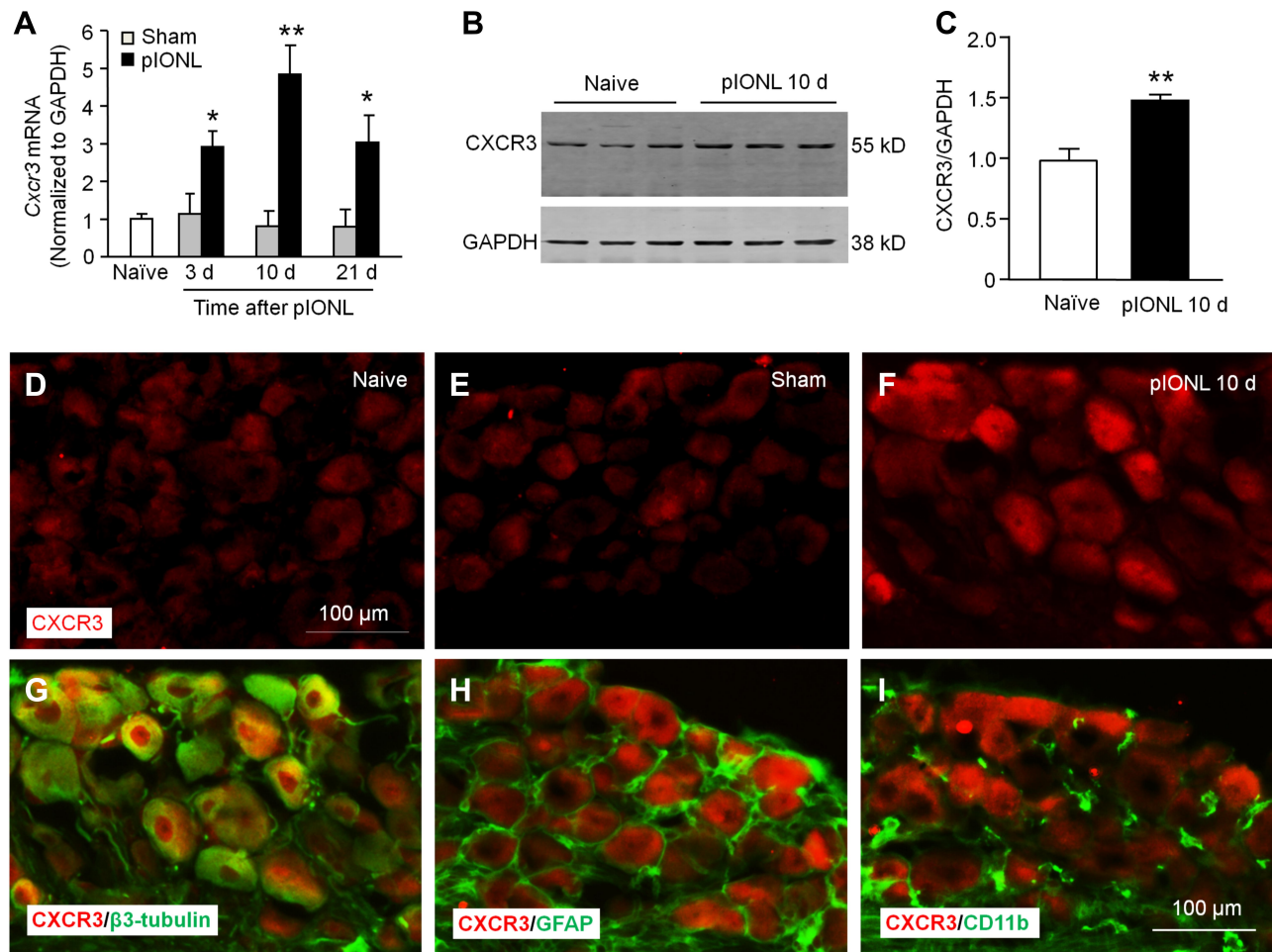


Figure 2 pIONL increases CXCR3 expression in TG neurons. (A) The time-course of *Cxcr3* mRNA expression in the TG from naïve, sham, and pIONL-operated mice. The mRNA expression of *Cxcr3* was increased Day 3, 10, and 21 after pIONL. $n = 5$ mice per group. * $P < 0.05$, ** $P < 0.01$, compared with the corresponding sham group. Student's *t*-test. (B–C) Western blot showed that CXCR3 protein was significantly increased 10 days after pIONL. ** $P < 0.01$, Student's *t*-test. (D–F) Representative images of CXCR3 immunofluorescence in the TG from naïve (D), sham (E), and pIONL (F) mice. (G–I) Double immunofluorescence staining shows that CXCR3 was mainly colocalized with the neuronal marker β 3-tubulin (G), not with satellite marker GFAP (H) or macrophage marker CD11b (I) in the TG 10 days after pIONL.

TG of pIONL mice (Figure 3E), confirming the upregulation of CXCL10 after pIONL.

To examine the distribution of CXCL10 in the TG, we performed double staining for CXCL10 and β 3-tubulin, GFAP, or IBA-1 (macrophage marker). The immunostaining results showed that CXCL10 was colocalized with β 3-tubulin (Figure 3F) but not with GFAP (Figure 3G) or IBA-1 (Figure 3H), indicating the predominant expression of CXCL10 in TG neurons.

CXCL10 Was Sufficient to Induce Pain Hypersensitivity and ERK/AKT Activation in the TG

To examine whether CXCL10 is sufficient to induce pain, we performed intra-TG injection of different

doses of CXCL10. Behavioral data showed that 10 ng of CXCL10 did not affect the nociceptive behavioral score. A higher dose (100 ng) increased the nociceptive score at 3 h and 6 h post-injection (Treatment, $F_{2, 63} = 12.88$, $P < 0.001$; Time, $F_{3, 63} = 17.58$, $P < 0.001$; Interaction, $F_{6, 63} = 5.397$, $P < 0.001$, two-way RM ANOVA, Figure 4A). Pain hypersensitivity was diminished 24 h after CXCL10 (100 ng) injection. In contrast, the nocifensive behavioral scores in *Cxcr3*^{-/-} mice after CXCL10 injection were much lower than those in WT mice (Treatment, $F_{1, 48} = 26.91$, $P < 0.001$; Time, $F_{3, 48} = 30.13$, $P < 0.001$; Interaction, $F_{3, 48} = 13.57$, $P < 0.001$, two-way RM ANOVA, Figure 4B). The data suggest that CXCL10 in the TG is sufficient to induce mechanical allodynia via CXCR3.

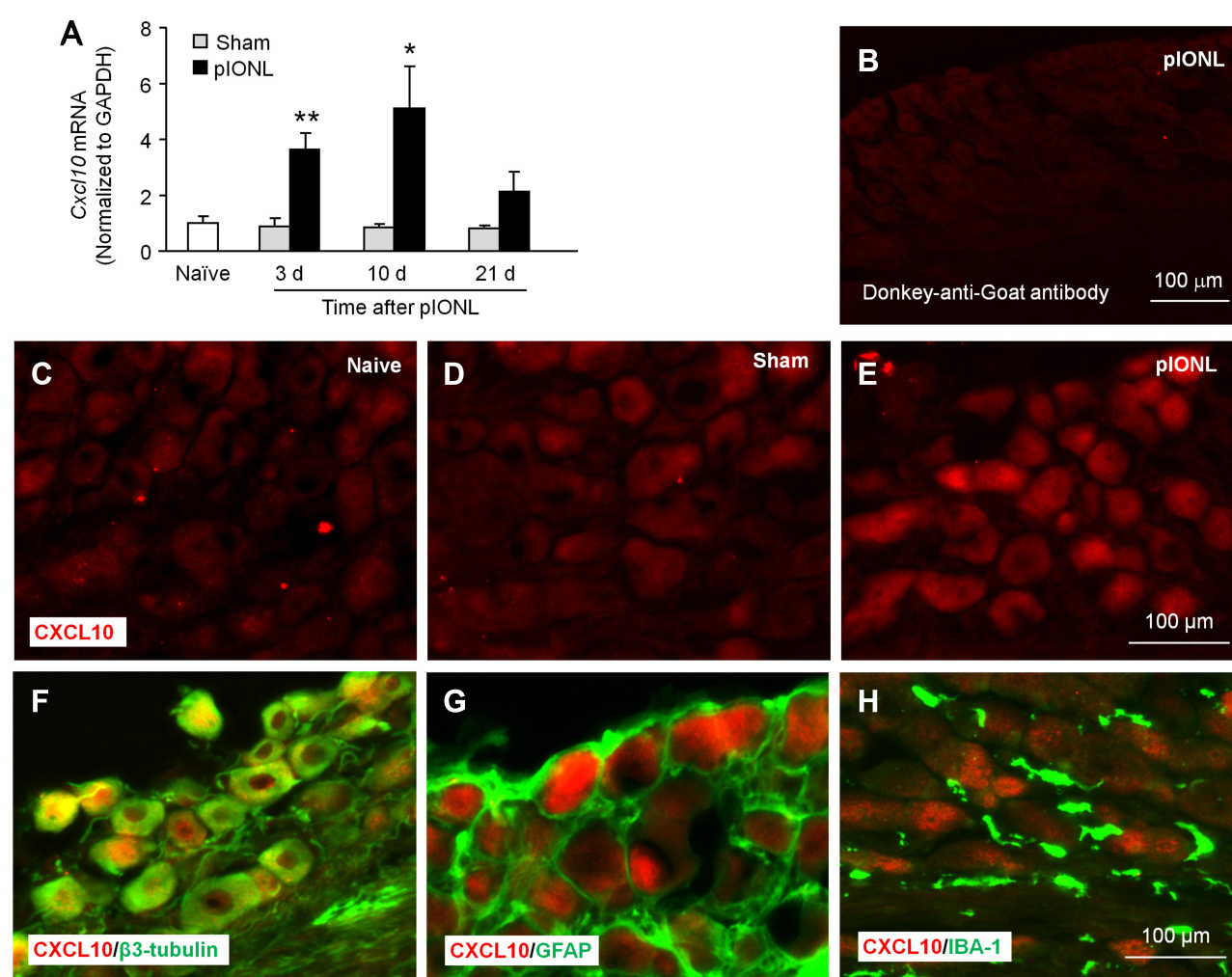


Figure 3 pIONL increases CXCL10 expression in TG neurons. (A) The time-course of *Cxcl10* mRNA expression in the TG in naïve, sham- and SNL-operated mice. $n = 5-6$ mice per group. * $P < 0.05$, ** $P < 0.01$ compared with the corresponding sham group. Student's *t*-test. (B) The staining of TG sections with secondary antibody only. (C-E) Fluorescence immunostaining of CXCL10 in the TG of naïve (C), sham (D), and pIONL (E) mice. (F-H) Double immunostaining of CXCL10 with β 3-tubulin, GFAP, and IBA-1. CXCL10 was colocalized with β 3-tubulin (F), not with GFAP (G) or IBA-1 (H).

MAPKs and AKT are important kinases for mediating chronic pain.²¹ To determine whether CXCL10 can activate these kinases, we performed intra-TG injection of CXCL10 (100 ng) and examined the phosphorylation (p) of MAPKs (ERK, p38, and JNK) and AKT. Three hours after CXCL10 injection, the pERK level was significantly increased in the TG of WT mice ($P < 0.01$, Student's *t*-test) but not in *Cxcr3*^{-/-} mice ($P > 0.05$, Student's *t*-test, Figure 4C). However, CXCL10 did not affect the activation of p38 or JNK in either WT or *Cxcr3*^{-/-} mice ($P > 0.05$, Student's *t*-test, Figure 4D-E). In contrast, CXCL10 increased the pAKT level in WT mice ($P < 0.05$, Student's *t*-test) but not in *Cxcr3*^{-/-} mice ($P > 0.05$, Student's *t*-test, Figure 4F). These data indicate that CXCL10 activates ERK and AKT via CXCR3.

pIONL Induced ERK and AKT Activation via CXCR3

We then compared pERK and pAKT levels in the TG 10 days after pIONL in WT and *Cxcr3*^{-/-} mice. As shown in Figure 5A, the pERK level was significantly lower in *Cxcr3*^{-/-} mice than in WT mice ($P < 0.05$, Student's *t*-test). In addition, the pAKT level in the TG of *Cxcr3*^{-/-} mice was also lower than that in WT mice ($P < 0.05$, Student's *t*-test, Figure 5B). These results suggest that CXCR3 plays a role in pIONL-induced ERK and AKT activation in the TG. Inhibition of ERK has been reported to attenuate pIONL-induced pain hypersensitivity.²⁶ To further examine the role of AKT in the maintenance of pIONL, we performed intra-TG injection of an AKT inhibitor at 10 days after pIONL. The behavioral data showed that AKT inhibitor IV significantly attenuated the nocifensive behavioral score at 1 h, 3 h, and 6 h post-injection (Treatment, $F_{1,50} = 75.29 = 89.80$, $P < 0.001$; Interaction, $F_{5,50} = 15.70$, $P < 0.001$, Figure 5C). However, intra-TG injection of AKT inhibitor IV in *Cxcr3*^{-/-} mice did not affect mechanical allodynia (Figure 5D). These data indicate that AKT is involved in pIONL-induced trigeminal neuropathic pain.

Discussion

In the present study, we found that global deletion or specific inhibition of *Cxcr3* in the TG alleviated pIONL-induced mechanical allodynia in the orofacial area. In addition, pIONL persistently increased CXCR3 and CXCL10 expression in TG neurons. Intra-TG injection of CXCL10 induced pain hypersensitivity and activated ERK

and AKT via CXCR3. Finally, pIONL-induced activation of ERK and AKT was reduced in *Cxcr3*^{-/-} mice, and AKT inhibition attenuated pIONL-induced orofacial mechanical allodynia. Thus, our data revealed an important role of CXCL10/CXCR3 in the TG in regulating intracellular kinase activation and trigeminal neuropathic pain.

The Involvement of CXCR3 in the Pathogenesis of Trigeminal Neuropathic Pain

CXCR3 is constitutively expressed in neurons of the neocortex, hippocampus, striatum, and spinal cord, where it plays a vital role in the pathogenesis of a variety of neuroinflammatory and neurodegenerative diseases, including Alzheimer's disease, bipolar disorder, multiple sclerosis, and chronic itch.^{32–35} Recent studies have shown that CXCR3 expression is increased in neurons, microglia, and astrocytes in the spinal cord in animal models of neuropathic pain and cancer pain.^{8,18} CXCR3 is also increased in DRG neurons in a bone cancer pain model in rats or a chronic constriction injury (CCI) model in mice.^{18,20} Here, low CXCR3 expression levels were observed in TG neurons of naïve mice, and these levels were persistently increased after pIONL. Although the mechanisms underlying CXCR3 upregulation were not investigated in this study, a previous report showed that decreased methylation of the *Cxcr3* promoter and increased binding of the transcriptional factor C/EBP α with *Cxcr3* caused upregulation of *Cxcr3* in the spinal dorsal horn after SNL,⁸ indicating epigenetic regulation of CXCR3 under neuropathic pain conditions.

Previous studies have shown that intrathecal injection of the CXCR3-specific antagonist NBI-74330 alleviates chronic pain symptoms induced by SNL, CCI, or bone cancer.^{8,18,20} *Cxcr3*^{-/-} mice show normal basal pain thresholds and motor function,⁸ but neuropathic pain induced by SNL is alleviated from day 3 to day 28.⁸ Intraspinal injection of LV-*Cxcr3* shRNA to specifically inhibit CXCR3 in the spinal cord attenuates SNL-induced mechanical allodynia and heat hyperalgesia,⁸ indicating the role of spinal CXCR3 in the maintenance of neuropathic pain. In the present study, pIONL-induced mechanical allodynia was attenuated in *Cxcr3*^{-/-} mice in the first 2 weeks, but not on day 21, suggesting that CXCR3 may not contribute to the maintenance of the late phase of trigeminal neuropathic pain. Lentivirus-expressing shRNA can rapidly and persistently reduce mRNA expression.^{6,7} Our

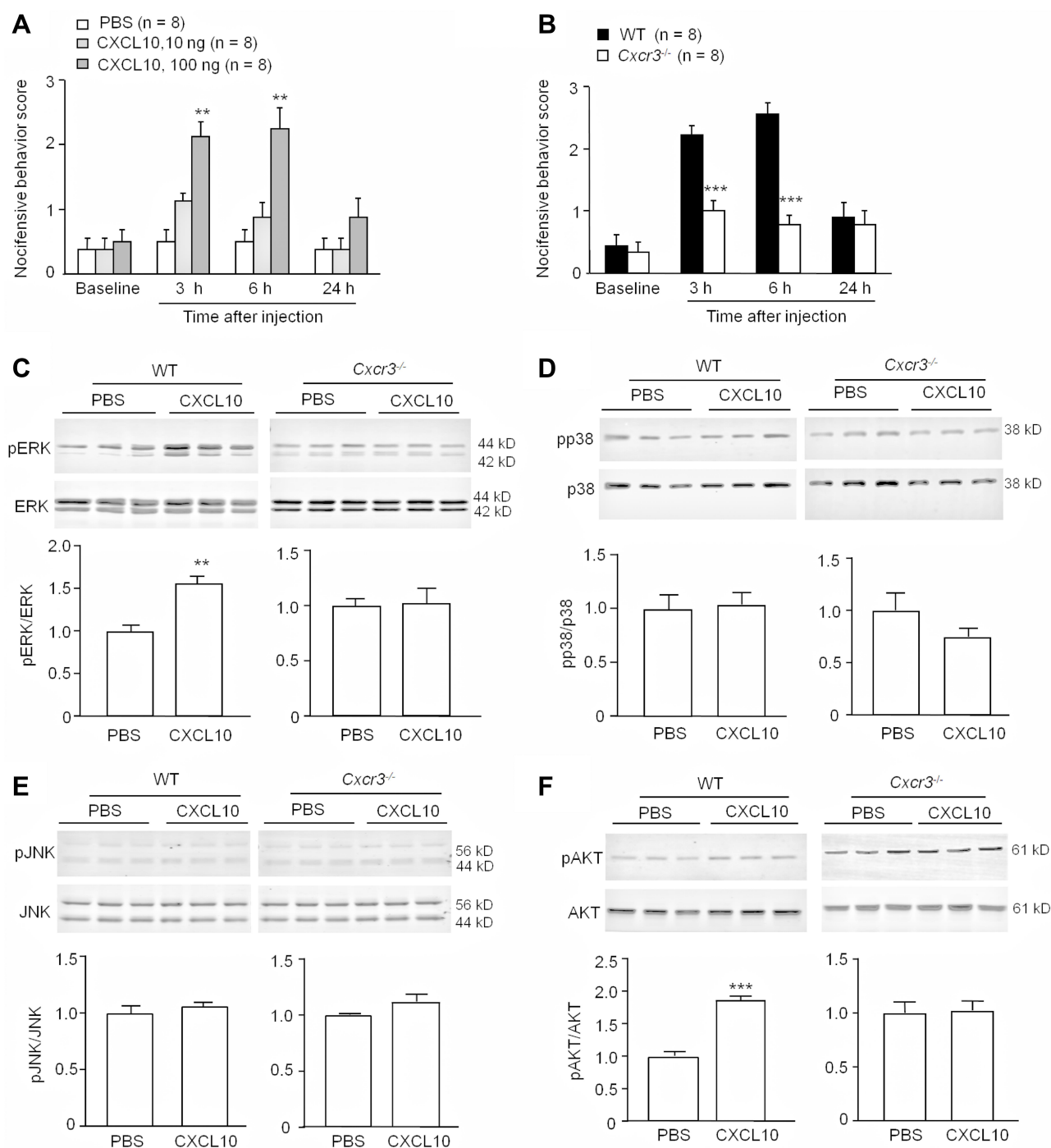


Figure 4 CXCL10 induces mechanical allodynia and increases ERK and Akt activation via CXCR3. **(A and B)** Intra-TG injection of CXCL10 (100 ng) induced mechanical allodynia in WT mice **(A)**, which was reduced in *Cxcr3*^{-/-} mice **(B)**. ** $P < 0.01$, *** $P < 0.001$, compared with PBS group or WT group. Two-way RM ANOVA followed by the Bonferroni test. **(C)** Intra-TG injection of CXCL10 (100 ng) increased pERK level in WT mice but not in *Cxcr3*^{-/-} mice. $n=3/\text{group}$. ** $P < 0.01$ compared with PBS group. Student's *t*-test. **(D, E)** Intra-TG injection of CXCL10 did not affect pp38 **(D)** or pJNK **(E)** level in either WT or *Cxcr3*^{-/-} mice. $n=3/\text{group}$. **(F)** Intra-TG injection of CXCL10 induced the activation of AKT in WT mice, but not in *Cxcr3*^{-/-} mice. $n=3/\text{group}$. *** $P < 0.001$, compared with PBS group. Student's *t*-test.

data also showed that specific knockdown of *Cxcr3* in the TG attenuated pIONL-induced mechanical allodynia from 7 to 14 days, supporting the role of CXCR3 in the TG in the maintenance of neuropathic pain. Given that CXCR3

in the spinal dorsal horn plays an important role in mediating neuropathic pain, and the medullary dorsal horn (MDH) is important in transmitting and mediating noxious signals from the orofacial area to the brain, we cannot

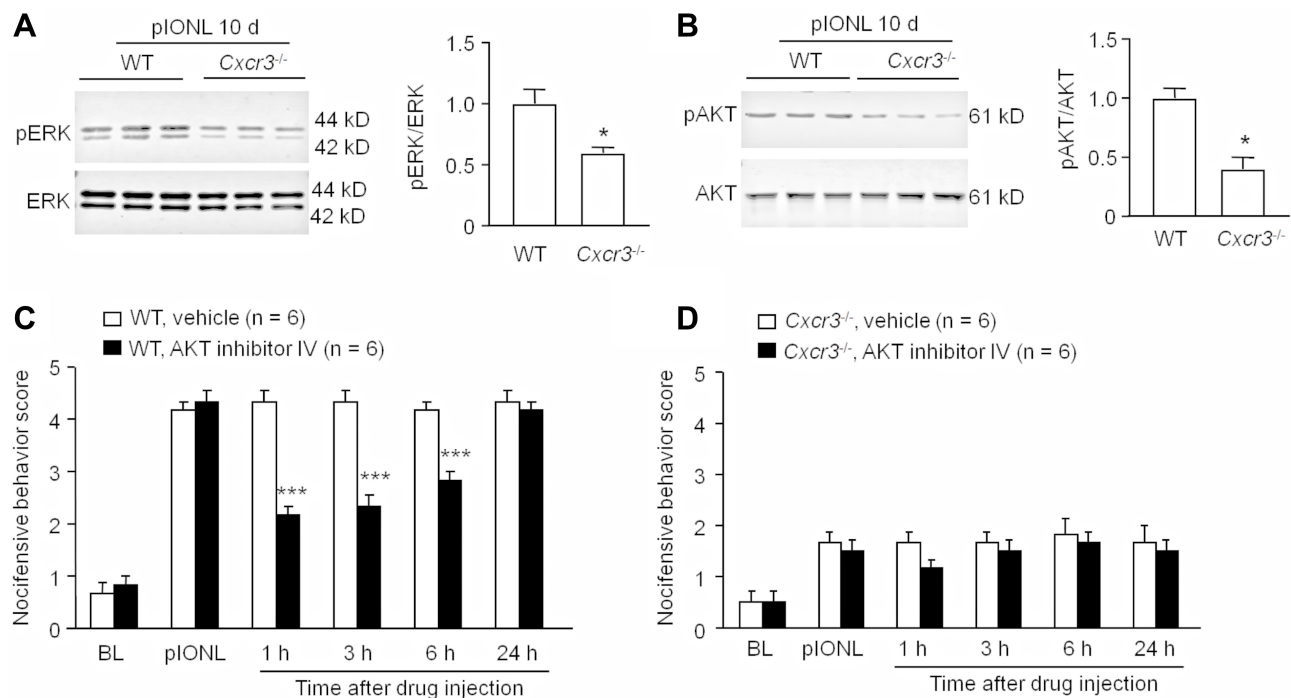


Figure 5 pIONL induces ERK and AKT activation via CXCR3. **(A)** pIONL-induced ERK activation was reduced in *Cxcr3*^{-/-} mice than that in WT mice. * $P < 0.05$. Student's *t*-test. **(B)** AKT activation was reduced in *Cxcr3*^{-/-} mice than that in WT mice. * $P < 0.05$. Student's *t*-test. **(C)** Intra-TG injection of AKT inhibitor IV in WT mice attenuated pIONL-induced mechanical allodynia at 1 h, 3 h, and 6 h. *** $P < 0.001$, compared to vehicle. Two-way RM ANOVA followed by the Bonferroni test. **(D)** Intra-TG injection of AKT inhibitor IV in *Cxcr3*^{-/-} mice did not affect the mechanical allodynia. Two-way RM ANOVA followed by the Bonferroni test.

exclude the possible role of CXCR3 in the MDH in mediating trigeminal neuropathic pain.

CXCL10/CXCR3 Induces Trigeminal Neuropathic Pain via Activation of ERK and AKT in the TG

A microarray assay showed that CXCL10 is a highly upregulated chemokine in the spinal cord after SNL.⁸ In addition, CXCL10 is constitutively expressed in dorsal horn neurons but induced in astrocytes after SNL; however, CXCR3 is expressed and upregulated in neurons. Thus, CXCL10 and CXCR3 mediate neuropathic pain via astrocyte-neuron interactions in the spinal cord.⁸ In contrast, CXCL10 and CXCR3 were both expressed in TG neurons after pIONL, suggesting that CXCL10/CXCR3 signaling may have an autocrine/paracrine function within the TG. Recent studies have demonstrated that chemokine pairs such as CCL2/CCR2 and CXCL13/CXCR5 are also expressed in TG neurons and contribute to the development and maintenance of trigeminal neuropathic pain.^{26,36} Notably, deletion of *Cxcr5* attenuated pIONL-induced mechanical allodynia for more than 21 days,²⁶ suggesting that different chemokines may have distinct roles in various phases of neuropathic pain.

CXCL10/CXCR3 induced ERK activation in dorsal horn neurons.⁸ Ligation of the ION has been shown to induce activation of ERK and p38, but not JNK, in the TG.^{26,27} ERK activation in TG neurons is also induced by migraine or lingual nerve crush.^{26,37,38} Our results showed that intra-TG injection of CXCL10 induced CXCR3-dependent activation of ERK, but not p38 or JNK, in WT mice, suggesting that ERK is a downstream kinase of CXCL10/CXCR3. Inhibition of ERK activation by PD98059 (MEK inhibitor) has been shown to attenuate pIONL-induced mechanical allodynia and lingual nerve crush-induced pain hypersensitivity.^{26,38} PD98059 also decreases pIONL-induced upregulation of TNF- α and IL-1 β in the TG.²⁶ Furthermore, intra-TG injection of the TNF- α inhibitor etanercept or the IL-1 β inhibitor diacerein attenuates pIONL-induced mechanical allodynia for up to 6 h.²⁶ In addition, activated ERK increases expression of the Nav1.8 sodium channel and regulates TRPV1 activity in peripheral sensory neurons.^{39,40} Proinflammatory cytokines upregulate Nav1.7 and Nav1.6 expression, further contributing to peripheral sensitization and neuropathic pain.^{41,42} Therefore, activation of CXCL10/CXCR3 may increase neuroinflammation and further regulate the excitability of TG neurons.

AKT is an important downstream target of PI3K and regulates multifarious cellular processes, including cell survival and death.⁴³ Evidence has shown that the PI3K/AKT signaling pathway is required for central spinal sensitization after nerve injury.⁴⁴ Inhibition of PI3K/AKT signaling pathway activation exerts analgesic effects in neuropathic pain models.^{45,46} Our present results showed that CXCL10/CXCR3 induced AKT activation, and inhibition of AKT attenuated pIONL-induced mechanical allodynia, indicating that CXCL10/CXCR3 contributes to trigeminal neuropathic pain via activation of ERK and AKT.

In summary, we explored the role of CXCR3 signaling in the TG in trigeminal neuropathic pain. Our results demonstrated that CXCR3 is activated by CXCL10 and induces downstream ERK/AKT activation, which may increase neuroinflammation and neuronal excitability and further contribute to the maintenance of neuropathic pain. Because of the important role of CXCL10/CXCR3 in the spinal cord in central sensitization, the CXCL10/CXCR3 pathway may be a promising target for neuropathic pain treatment.

Conclusion

CXCL10 acts on CXCR3 to induce ERK and AKT activation in TG neurons and contributes to the maintenance of trigeminal neuropathic pain.

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Disclosure

All authors report no conflicts of interest for this work.

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