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Chemokine contribution to neuropathic pain: respective induction of CXCL1 and CXCR2 in spinal cord astrocytes and neurons

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ABSTRACT

Recent studies have indicated an important role of chemokines such as CCL2 in the development of chronic pain. However, the distinct roles of different chemokines in the development and maintenance of neuropathic pain and in their interactions with neurons have not been clearly elucidated. We found that spinal nerve ligation (SNL) not only induced persistent neuropathic pain symptoms, including mechanical allodynia and heat hyperalgesia, but also produced sustained CXCL1 upregulation in the spinal cord. Double staining of immunofluorescence and in situ hybridization revealed that CXCL1 was primarily induced in spinal astrocytes. In cultured astrocytes, tumor necrosis factor-α induced robust CXCL1 expression via the activation of the c-jun N-terminal kinase. Intrathecal administration of CXCL1 neutralizing antibody transiently reduced SNL-induced pain hypersensitivity, suggesting an essential role of CXCL1 in neuropathic pain sensitization. In particular, intraspinal delivery of CXCL1 shRNA lentiviral vectors, either before or after SNL, persistently attenuated SNL-induced pain hypersensitivity. Spinal application of CXCL1 not only elicited pain hypersensitivity but also induced rapid neuronal activation, as indicated by the expression of phosphorylated extracellular signal-regulated kinase and cAMP response element binding protein, and c-Fos in spinal cord neurons. Interestingly, CXCR2, the primary receptor of CXCL1, was upregulated in dorsal horn neurons after SNL, and the CXCR2 antagonist SB225002 completely blocked the CXCL1-induced heat hyperalgesia. SB225002 also attenuated SNL-induced pain hypersensitivity. Collectively, our results have demonstrated a novel form of chemokine-mediated glial-neuronal interaction in the spinal cord that can drive neuropathic pain. Inhibition of the CXCL1-CXCR2 signaling may offer a new therapy for neuropathic pain management.

1. Introduction

Neuroinflammation has been recently recognized for its pivotal role in the pathogenesis of neuropathic pain as well as in inflammatory pain [34,74]. Nonneuronal cells such as glial cells (astrocytes and microglia) become reactive following peripheral nerve injuries and contribute to the enhancement and maintenance of neuropathic pain by releasing growth factors (eg, brain-derived neurotrophic factor and basic fibroblast growth factor) and inflammatory mediators (eg, proinflammatory cytokines and chemokines) [1,2,18,26,49,58]. Chemokines are a family of functionally related small secreted molecules with established roles in the modulation of numerous biological functions, including leukocyte migration and activation, cell adhesion, and T-cell activation, and have been demonstrated to regulate neuroinflammation at different anatomical locations, including nerve, dorsal root ganglion (DRG), spinal cord, and brain [46,47,63,74]. Increasing evidence has implicated chemokines in chronic pain processing following nerve injury [2,18].

CXCL1 belongs to the CXC family and is also known as keratinocyte-derived chemokines (KC), growth-related oncogene (GRO), or cytokine-induced neutrophil chemoattractant-1 (CINC-1). CXCL1 in rodents plays similar biological roles as interleukin-8 (IL-8) in humans [70]. In the peripheral tissue, CXCL1 is involved in neutrophil chemotaxis and degranulation at early phase of inflammation. Recent studies have shown that spinal nerve ligation and localized inflammation of DRG induced rapid upregulation of CXCL1 in the DRG [40,75]. CXCL1 modulates neuronal excitability of DRG neurons by increasing sodium currents, potassium currents, and the function of transient receptor potential V1 channels [14,71,77]. It also stimulates calcium influx and calcitonin gene-related peptide

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release in sensory neurons [56]. Intraplantar or intraarticular injection of CXCL1 produced mechanical hyperalgesia in rodents [10,22,56] (but see [14]). These studies suggest a pronociceptive role for CXCL1 in the peripheral nervous system. But it is virtually unknown whether and how central (spinal) CXCL1 would play a role in the genesis of neuropathic pain.

The biological effects of chemokine are mediated via G-protein-coupled chemokine receptors. Chemokines and their respective receptors are often detected in different cell types to mediate cell–cell interactions. In the spinal cord, chemokines have been strongly implicated in neuron-to-microglia signaling after nerve injury. For example, CX3CL1 (fractalkine) and its receptor CX3CR1 are expressed by neurons and microglia, respectively [23,41,69,80], and CCL2 and CCR2 are localized to primary sensory neurons and microglia, respectively [3,12,66,78]. Chemokines can also mediate glia-to-neuron signaling, as indicated by respective expression of CCL2 and its receptor CCR2 in astrocytes and neurons [20,21,36]. Distinct expression of chemokines and their receptors is critical to determine whether a particular chemokine is important for neuron-to-glia signaling or glia-to-neuron signaling. CXCR2 is the primary receptor of CXCL1 and has been detected on neurons, microglia, and oligodendrocyte progenitors in the brain [24,51,55,68]. The distinct cellular localization of CXCL1 and CXCR2 in the spinal cord after nerve injury remains unclear. In the present study, we used spinal nerve ligation (SNL) neuropathic pain model to address this issue. Our findings provide new evidence for astrocyte–neuron interactions in the spinal cord and also reveal important roles of CXCL1/CXCR2 in the development and maintenance of neuropathic pain.

2. Materials and methods

2.1. Animals and surgery

Adult ICR (Imprinting control region) mice (outbred, male, 25–35 g) were purchased from the Experimental Animal Center of Nantong University. All animal procedures in this study were performed according to the guidelines of the International Association for the Study of Pain, and were approved by the Animal Care and Use Committee of Nantong University. To produce an SNL, animals were anesthetized with sodium pentobarbital (40–50 mg/kg, intraperitoneally [i.p.]) and the L6 transverse process was removed to expose the L4 and L5 spinal nerves. The L5 spinal nerve was then isolated and tightly ligated with 6–0 silk thread [35]. For sham-operated mice, the L6 transverse process was similarly removed and the L5 spinal nerve was exposed and isolated, but no nerve ligation was carried out.

2.2. Drugs and administration

The mitogen-activated protein kinase (MAPK) inhibitors SP600125, SB203580, and U0126 were purchased from Calbiochem (EMD Millipore, Billerica, MA, USA). SB225002, a potent and selective antagonist of CXCR2, was purchased from Tocris Bioscience (Bristol, UK). CXCL1 was purchased from R & D Systems (Minneapolis, MN, USA). Etanercept, a tumor necrosis factor (TNF)α inhibitor, was purchased from Pfizer (New York, NY). For intrathecal (i.t.) injection, spinal cord puncture was made with a 30-G needle between the L5 and L6 levels to deliver the reagents to the cerebral spinal fluid [25].

2.3. Lentiviral vectors production and intraspinal injection

Three shRNAs targeting the sequence of mice CXCL1 (Gene Bank Accession NM_008176) were designed (shRNA1: 5′-AGT AAC GGA GAA AGA AGA CAG-3′; shRNA2: 5′-GAA GCT CCC TTG GTT CAG AAA-3′; shRNA3: 5′-TCAAGAATGGTCGCGAGGCTT-3′). An additional scrambled sequence was also designed as a negative control (NC) (5′-TTC TCC GAA CGT GTC ACG T-3′). Replication-deficient self-inactivating lentiviral-expressing vectors pGCSIL-GFP (LV-shCXCL1 and LV-NC) were generated by Shanghai GeneChem. The final titers of LV-shCXCL1-1, LV-shCXCL1-2, LV-shCXCL1-3, and LV-NC were 8×10^8 TU/mL, 8×10^8 TU/mL, 1×10^9 TU/mL, and 2×10^9 TU/mL, respectively.

The knockdown effect of the 3 shRNAs was examined on cultured astrocytes, and the shRNA with the best knockdown effect was chosen for intraspinal injection. For the injection, animals were anesthetized with pentobarbital sodium (40–50 mg/kg, i.p.) and underwent hemilaminectomy at the L1-L2 vertebral segments. Intraspinal injection was performed unilaterally on the left side. After exposure of the spinal cord, each animal received 2 injections (0.4 μL; 0.8 mm apart and 0.5 mm deep) of LV-shCXCL1 or LV-NC (1×10^5 TU) along the L4-L5 dorsal root entry zone using a glass micropipette (diameter 80 μm). The tip of glass micropipette reached to the depth of lamina II-IV of the spinal cord. The dorsal muscle and skin were then sutured.

2.4. Primary astrocytes cultures

Astrocytes cultures were prepared from cerebral cortexes of neonatal mice (P2). The cerebral hemispheres were isolated and transferred to ice-cold Hank's buffer and the meninges were carefully removed. Tissues were then minced into ~1-mm pieces, triturated, filtered through a 100-μm nylon screen, and collected by centrifugation at ~3000 g for 5 minutes. The cell pellets were dispersed with a pipette and resuspended in a medium containing 15% fetal bovine serum in low-glucose Dulbecco's Modified Eagle's Medium. After trituration, the cells were filtered through a 10-μm screen and then plated into 6-well plates at a density of 2.5×10^5 cells/cm^2, and cultured for 10–12 days. The medium was replaced twice a week with 10% fetal bovine serum. Once the cells were grown to 95% confluence, 0.15 mM dibutyryl cAMP (Sigma, St. Louis, MO, USA) was added to induce differentiation. Astrocytes used for immunocytochemistry were cultured onto cover glasses at a density of 2.5×10^4 cells/cm^2, and differentiation was induced when cells reached 50% confluence. Prior to stimulation with TNFα, Opti-MEM (Life Technologies, Grand Island, NY, USA) was replaced. Astrocytes were incubated with TNFα for different time periods ranging from 15 minutes to 4 hours, depending on the experiment. The treatment of the MAPK inhibitor SP600125 (10, 20, 50 μM), U0126 (20, 50 μM), or SB203580 (10, 20, 50 μM) was started 30 minutes prior to TNFα treatment. To test the knockdown effect of LV-shCXCL1, astrocytes were incubated with lentiviral vectors for 3 days and then stimulated with TNFα for 1 hour. After the treatment, the astrocytes were collected for enzyme-linked immunosorbent assay (ELISA) or real-time polymerase chain reaction (RT-PCR).

2.5. ELISA

Mouse CXCL1 ELISA kit was purchased from R & D Systems. For in vivo experiments, animals were transcardially perfused with phosphate-buffered saline (PBS) and the lumbar spinal cord segments were dissected. For in vitro experiments, cultured astrocytes were collected after treatment. Spinal cord tissues or astrocytes were homogenized in a lysis buffer containing protease and phosphatase inhibitors. Protein concentrations were determined by BCA Protein Assay (Pierce Biotechnology, Rockford, IL, USA). For each reaction in a 96-well plate, 100 μg of proteins were used, and ELISA was performed according to manufacturer's protocol. The standard curve was included in each experiment.

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2.6. Western blot

Protein samples were prepared in the same way as for ELISA analysis, and 30 μg of proteins were loaded for each lane and separated on SDS-PAGE gel (10%). After the transfer, the blots were incubated overnight at 4°C with polyclonal antibody against CXCR2 (1:50, rabbit; Boster, Fremont, CA, USA). For loading control, the blots were probed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:20,000, mouse; Sigma). These blots were further incubated with horseradish-peroxidase-conjugated secondary antibody, developed in enhanced chemiluminescence solution, and exposed onto Hyperfilm (Amersham Biosciences, Piscataway, NJ, USA) for 1–10 minutes. Specific bands were evaluated by apparent molecular size. The intensity of the selected bands was analyzed using Image J software (National Institutes of Health [NIH], Bethesda, MD, USA).

2.7. Immunohistochemistry and immunocytochemistry

After appropriate survival times, animals were deeply anesthetized with isoflurane and perfused through the ascending aorta with PBS followed by 4% paraformaldehyde with 1.5% picric acid in 0.16 M PB. After the perfusion, the L4-L5 spinal cord segments were removed and postfixed in the same fixative overnight. Spinal cord sections (30 μm, free-floating) were cut in a cryostat and processed for immunofluorescence as we described previously [20]. The sections were first blocked with 2% goat or donkey serum for 1 hour at room temperature and then incubated overnight at 4°C with the following primary antibodies: CXCL1 (rabbit, 1:100; Boster, Wuhan, Hubei, China), CXCR2 (rabbit, 1:100; Boster), glial fibrillary acidic protein (GFAP) antibody (mouse, 1:5000; Millipore), OX-42 antibody (mouse, 1:5000; Serotec, Raleigh, NC, USA), neuronal nuclei (NeuN) antibody (mouse, 1:5000; Millipore), phosphorylated extracellular signal-regulated kinase (ERK) antibody (pERK1/2, rabbit, 1:500; Cell Signaling, Boston, MA, USA), phosphorylated cAMP response element binding protein (CREB) (pCREB, rabbit, 1:1000; Cell Signaling), c-Fos antibody (rabbit, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and protein kinase C (PKC)γ (mouse, 1:500; Santa Cruz). The sections were then incubated for 1 hour at room temperature with Cy3- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:1000; Jackson ImmunoResearch, West Grove, PA, USA). For double immunofluorescence, sections were incubated with a mixture of mouse and rabbit primary antibodies followed by a mixture of FITC- and Cy3-congugated secondary antibodies. The stained sections were examined with a Leica fluorescence microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA), and images were captured with a CCD SPOT camera (SPOT Imaging Solutions, Sterling Heights, MI, USA). The specificity of CXCL1 and CXCR2 primary antibodies was tested by preabsorption experiment. The spinal cord sections were incubated with a mixture of CXCL1 or CXCR2 primary antibody and the corresponding blocking peptide for CXCL1 (25 μg/mL; Boster) or CXCR2 (25 μg/mL; Boster) overnight, followed by secondary antibody incubation. CXCL1 and CXCR2 immunostaining signal was abolished after absorption (Supplementary Fig. 1).

For immunocytochemistry, cultured astrocytes, after incubation with TNFα for 1 hour, were fixed with 4% paraformaldehyde for 20 minutes, and processed for immunofluorescence with CXCL1 (rabbit, 1:500; Santa Cruz) and GFAP (mouse, 1:5000; Millipore) antibody as shown above. After immunostaining, DAPI (Sigma, 0.1 μg/mL) was added for 5 minutes at room temperature to stain all the nuclei of cells in the cultures.

2.8. In situ hybridization

The CXCL1 cDNAs were obtained by RT-PCR and cloned into pGEM-T Easy Vector System I (Promega Corporation, Madison, WI, USA). As previously described by Pineau et al. [54], primers used for the cloning were 5′-GAA CGC TGG CTT CTG ACA AC-3′ and 5′-CGA GAC GAG ACC AGG AGA AA-3′. The sequences were verified by Genbank BLAST analysis. The antisense and sense digoxigenin-labeled RNA probes were synthesized using the Riboprobe Combination System SP6/T7 (Roche Applied Science, Indianapolis, IN, USA).

In situ hybridization was performed according to a method described previously [20]. Briefly, the slides were washed in PBS (0.01 M, pH 7.4) and then incubated in proteinase K (1 mg/L) at 37°C for 10 minutes. After being washed in PBS, the sections were prehybridized at 42°C for 2 hours, then hybridized with the digoxigenin-labeled probe (1 μg/mL) at 42°C overnight. After hybridization, slides were washed 3 times with 50% formamide in sodium chloride-sodium citrate buffer and then treated with antidigoxigenin antibody-conjugated rhodamine according to the manufacturer's protocol (Roche Applied Science). The corresponding sense probe was also incubated as control.

To identify the cell types expressing CXCL1 mRNAs, these slides were then incubated with the mouse anti-GFAP antibody overnight at 4°C, and then incubated with FITC-conjugated goat antimouse antibody. The signal was detected with a Leica fluorescence microscope, and images were captured with a CCD SPOT camera.

2.9. Real-time PCR

Total RNA was extracted from L5 spinal cord with the TRIzol reagent (Invitrogen, Life Technologies). One microgram of total RNA was converted into cDNA using a PrimeScript RT reagent kit (Takara, Otsu, Shiga, Japan). The cDNA was amplified using the following primers: CXCL1-forward (5′-GCT TGA AGG TGT TGC CCT CAG-3′) and CXCL1-reverse (5′-AGA AGC CAG CGT TCA CCA GAC-3′), TNFα-forward (5′-GTT CTA TGG CCC AGA CCC TCA C-3′), and TNFα-reverse (5′-GGC ACC ACT AGT TGG TTG TCT TTG-3′), GAPDH-forward (5′-TGT TCC TAC CCC CAA TGT G-3′) and GAPDH-reverse (5′-GTG TAG CCC AAG ATG CCC T-3′). The SYBR Premix Ex Taq II kit (Takara) was used for all PCR reactions. The PCR reactions were run on a Rotor-Gene 6000 RT-PCR machine (Corbett Research, Qiagen Inc., Valencia, CA, USA). The melting curves were performed to validate the utility and specificity of each PCR product. The data were analyzed using Rotor-Gene 6000 series software, and evaluated using the Comparative CT Method ($2^{-\triangle\triangle CT}$).

2.10. Behavioral analysis

Animals were habituated to the testing environment daily for at least 2 days before baseline testing. The room temperature and humidity remained stable for all experiments. For testing mechanical sensitivity, animals were put in boxes on an elevated metal mesh floor and allowed 30 minutes for habituation before examination. The plantar surface of each hind paw was stimulated with a series of von Frey hairs with logarithmically incrementing stiffness (0.02–2.56 g; Stoelting, Wood Dale, IL, USA), presented perpendicular to the plantar surface (2–3 seconds for each hair). The 50% paw withdrawal threshold was determined using Dixon's up-down method [7]. For testing heat sensitivity, animals were put in plastic boxes and allowed 30 minutes for habituation before examination. Heat sensitivity was tested by radiant heat using Hargreaves apparatus (IITC Life Science Inc., Woodland Hills, CA, USA) and expressed as paw withdrawal latency. The radiant heat intensity was adjusted so that basal paw withdrawal latency is between 10 and 14 seconds, with a cutoff of 18 seconds to prevent tissue damage.

2.11. Quantification and statistics

The density of specific bands from Western blotting was measured with a computer-assisted imaging analysis system (Image

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J, NIH). The size of the rectangle was fixed for each band and the background near that band was subtracted. The numbers of pERK-, pCREB-, and c-Fos-labeled cells were counted in laminae I-VI of the spinal cord. Three nonadjacent sections were randomly selected, and 3-4 mice were included in each group. Differences between groups were compared using one-way analysis of variance followed by Newman-Keuls post hoc test or using Student's *t*-test if only 2 groups were applied. All data were expressed as mean ± SEM. The criterion for statistical significance was $P < 0.05$.

3. Results

3.1. CXCL1 is upregulated in spinal cord astrocytes after nerve injury

SNL produces rapid and persistent neuropathic pain, starting from 1 day and lasting for more than 3 weeks in rats [35] and mice [32]. To examine CXCL1 expression in the spinal cord, we performed immunostaining in naïve and SNL-operated mice. As shown in Fig. 1, CXCL1 was constitutively expressed in the superficial dorsal horn (Fig. 1A and B). SNL induced a marked increase of CXCL1 expression and many more CXCL1-immunoreactive (IR) cells were found in the ipsilateral than the contralateral spinal cord at 3 days (Fig. 1C and D) and 10 days (Fig. 1E and F). ELISA results showed that CXCL1 expression increased from day 3, peaked at day 10, and was maintained at day 21 after SNL ($P < 0.05$, SNL vs naïve). CXCL1 expression was also significantly higher in SNL animals than in sham-operated animals at 10 days after operation ($P < 0.05$, SNL vs sham, Fig. 1G). RT-PCR showed a parallel and significant increase in CXCL1 mRNA at 3 days, 10 days, and 21 days in SNL animals ($P < 0.05$ or $P < 0.01$, SNL vs sham, Fig. 1H).

To define the cellular distribution of CXCL1, we performed double staining of CXCL1 with different cell markers. CXCL1-IR was primarily colocalized with the astrocytic marker GFAP (Fig. 1I), but not with neuronal marker NeuN (Fig. 1J) or microglial marker OX-42 (Fig. 1K).

To further define the cellular localization of CXCL1 in the spinal cord, we performed in situ hybridization using an antisense probe of CXCL1. Because the expression of CXCL1 protein and mRNA is peaked at 10 days after SNL, we picked this time point to check CXCL1 mRNA expression. As shown in Fig. 1L–N, CXCL1 mRNA expression was colocalized with GFAP. Collectively, these results suggest that 1) SNL induced persistent CXCL1 increase, and 2) CXCL1 was primarily produced by astrocytes.

3.2. TNFα drives spinal CXCL1 expression after nerve injury

We next explored the possible upstream trigger for CXCL1 upregulation after SNL. The proinflammatory cytokine TNFα has been implicated in triggering an inflammatory cascade that underlies the development of neuropathic pain after nerve injury [39,62,65,76]. We tested whether CXCL1 upregulation was induced by TNFα. RT-PCR analysis showed that SNL induced rapid TNFα mRNA upregulation at 1 day. The TNFα mRNA expression was declined from the peak at 3 days, maintained at 10 days, and recovered at 21 days after operation (Fig. 2A). In contrast, CXCL1 mRNA expression was not significant at 1 day but significantly increased at 3 days, 10 days, and 21 days (Fig. 1H). These data suggest that TNFα mRNA increase precedes CXCL1 mRNA increase following SNL.

We then checked whether blocking TNFα could reduce SNL-induced CXCL1 mRNA upregulation. Etanercept, a TNFα inhibitor was injected i.t. either before or 10 days after the SNL surgery. A single bolus injection of etanercept (10 μg) before SNL blocked SNL-induced CXCL1 mRNA upregulation at 3 days after SNL ($P < 0.05$, etanercept vs PBS; $P > 0.05$, etanercept vs naïve; Fig. 2B). SNL-induced mechanical allodynia and heat hyperalgesia were also significantly attenuated by etanercept (Fig. 2C and D). However, i.t. injection of same-dose etanercept at 10 days after SNL had no significant effect on SNL-induced CXCL1 mRNA expression (Fig. 2E), but mildly attenuated mechanical allodynia (Fig. 2F) and heat hyperalgesia (Fig. 2G). These data suggest the spinal TNFα is required for the induction of CXCL1 upregulation and the development of SNL-induced neuropathic pain.

To test whether TNFα is sufficient to induce CXCL1 upregulation in the spinal cord, we injected i.t. TNFα and checked CXCL1 expression. Our previous study showed that i.t. injection of TNFα induced mechanical allodynia and heat hyperalgesia in mice [20]. CXCL1 level in the spinal cord was significantly increased after TNFα application, as shown by ELISA (Fig. 2H). TNFα also increased CXCL1-IR at 3 hours, as indicated by immunostaining (Fig. 2I). These results suggest that TNFα is both required and sufficient for the CXCL1 upregulation in the spinal cord.

3.3. JNK pathway is required for CXCL1 upregulation in cultured astrocytes and spinal cord

To further determine the intracellular signaling involved in TNFα-induced CXCL1 expression, we prepared primary astrocyte cultures from cerebral cortexes of neonatal mice (P2). TNFα incubation for 1 hour increased CXCL1 expression (Fig. 3A). ELISA test further showed that TNFα (10 ng/mL) induced rapid increase of CXCL1 expression, with 1.4-fold increase at 15 minutes and 8- and 4.7-fold increase at 1 hour and 4 hours, respectively (Fig. 3B).

MAPKs are important cellular signaling components and include 3 major members: JNK (c-jun N-terminal kinase), ERK (extracellular signal-regulated kinase), and p38. To define the role of MAPKs in TNFα-induced CXCL1 expression, we examined the effects of SP600125 (JNK inhibitor), U0126 (MEK inhibitor), and SB203580 (p38 inhibitor) on CXCL1 expression by ELISA. Pretreatment of SP600125 30 minutes before TNFα treatment decreased CXCL1 expression by 55%, 78%, and 80% at the doses of 10, 20, and 50 μM, respectively ($P < 0.001$, compared to TNFα treatment, Fig. 3C). In contrast, U0126 only suppressed TNFα-induced CXCL1 expression at a high concentration (50 μM, $P < 0.01$), whereas SB203580 had no effect at all concentrations (Fig. 3D). These data suggest JNK is critical for mediating TNFα-induced CXCL1 production.

To investigate whether JNK would also be involved in SNL-induced CXCL1 upregulation and pain hypersensitivity, we injected i.t. SP600125 (20 nmol) daily for 3 days (from day 8 to day 10 after SNL). SP600125 reversed SNL-induced mechanical allodynia ($P < 0.05$, Supplementary Fig. 2A) and heat hyperalgesia ($P < 0.05$, Supplementary Fig. 2B), and also decreased CXCL1 expression by 36 ± 6% (Supplementary Fig. 2C). Taken together, these data suggest that the TNFα/JNK pathway is involved in the SNL-induced CXCL1 upregulation in spinal astrocytes.

3.4. Intrathecal injection of CXCL1 neutralizing antibody transiently attenuates SNL-induced neuropathic pain

To investigate the role of endogenous CXCL1 in the SNL-induced neuropathic pain, we injected i.t. a CXCL1 neutralizing antibody. As CXCL1 expression is peaked at 10 days after SNL, we first checked the effect of CXCL1 neutralizing antibody at 10 days. The behavioral results showed a dose-dependent inhibition of neuropathic pain by CXCL1 neutralization. CXCL1 neutralizing antibody, at the dose of 4 μg but not 1 μg, transiently (1 hour) reduced SNL-induced mechanical allodynia ($P < 0.01$, compared to control serum, Fig. 4A) and heat hyperalgesia ($P < 0.05$, compared to control serum, Fig. 4B). In contrast, i.t. injection of CXCL1-neutralizing antibody (4 μg) at 1 day after SNL did not show effect on either mechanical allodynia (Fig. 4C) or heat hyperalgesia (Fig. 4D), suggesting that CXCL1 is not involved in the early phase of SNL-induced neuropathic pain.

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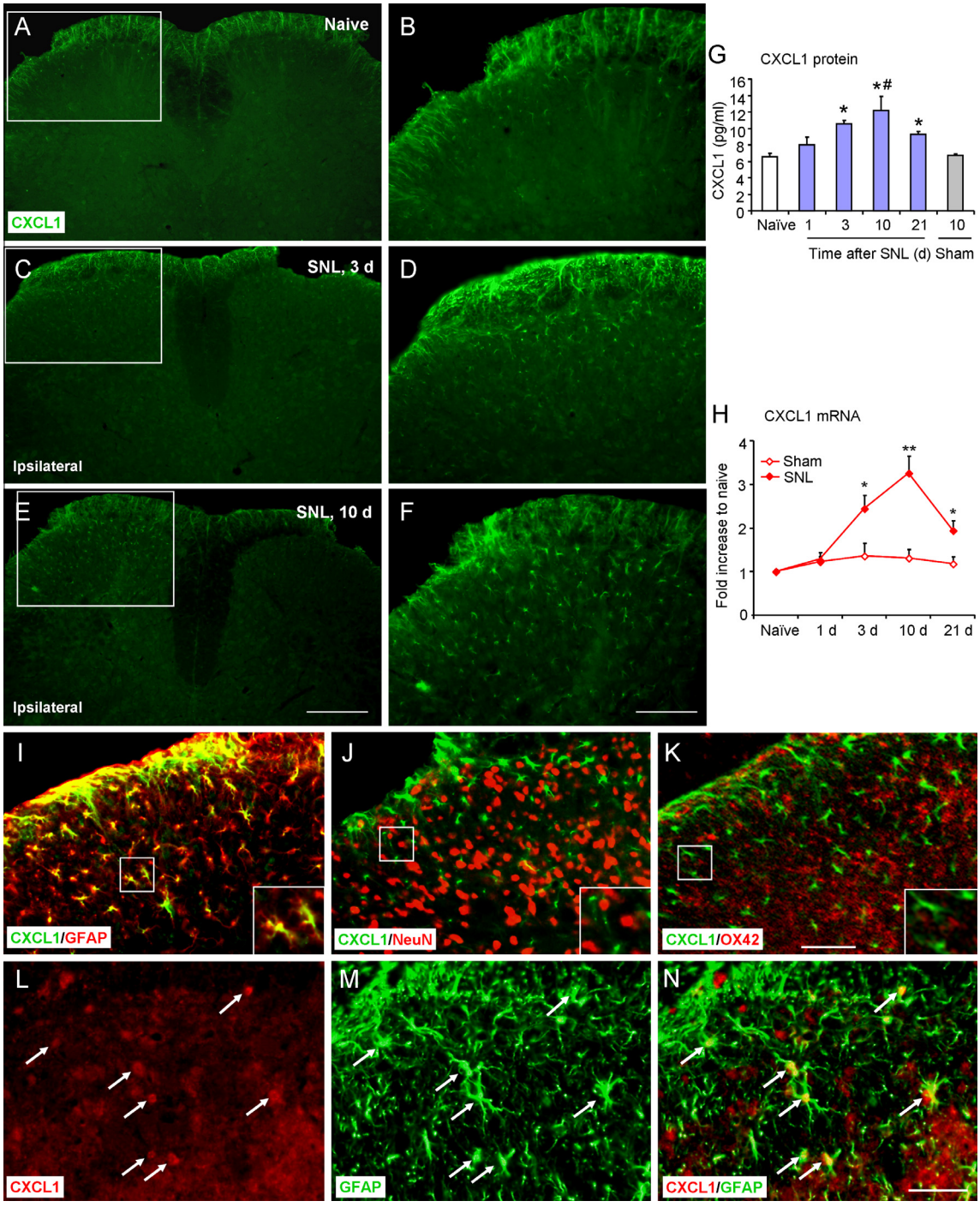

Fig. 1. Spinal nerve ligation (SNL) induces CXCL1 upregulation in spinal cord astrocytes. (A-F) CXCL1 expression in the spinal cord of naïve animals (A, B) and SNL at 3 days (C, D) and 10 days (E, F). (B, D, F) High-magnification images of A, C, E indicated in the white boxes. Scale bar, 200 μm in E, 100 μm in F. (G) Enzyme-linked immunosorbent assay results show time course of CXCL1 expression in the L5 spinal cord after SNL. $^{*}P < 0.05$, compared to naïve; $^{\#}P < 0.05$, compared to sham-operated control, Student's *t*-test. (H) Real-time polymerase chain reaction results show time course of CXCL1 mRNA expression in the spinal cord after SNL or sham operation. $^{*}P < 0.05$, $^{**}P < 0.01$, SNL vs sham, Student's *t*-test. (I-K) Double staining shows that CXCL1 is colocalized with glial fibrillary acidic protein (GFAP), a marker for astrocytes (I), but not with neuronal nuclei (NeuN), a marker for neurons (J) or OX42, a marker for microglia (K). Scale bar, 50 μm. (L-N) In situ hybridization of CXCL1 mRNA (L) and immunofluorescence staining of GFAP (M) showed that CXCL1 mRNA is colocalized with GFAP (N). Scale bar, 25 μm.

3.5. Intraspinal injection of recombinant lentivirus-CXCL1 shRNA persistently suppresses SNL-induced neuropathic pain

To test the long-term effect of inhibiting CXCL1 on neuropathic pain, we produced CXCL1 shRNA lentivirus vectors to persistently knockdown CXCL1 expression. We first tested the inhibitory effect of the lentiviral vector-expressed shRNAs on TNFα-induced CXCL1 expression in cultured astrocytes. The astrocytes were preincubated with different strands of LV-shCXCL1 or LV-NC for 3 days and followed by incubation with TNFα for 1 hour. The results showed that the 3 LV-shCXCL1 differently decreased TNFα-induced CXCL1 mRNA upregulation by 55%, 71%, and 50% (Supplementary Fig. 3A). Based on these data, we chose the LV-shCXCL1-2 for the following in vivo experiments.

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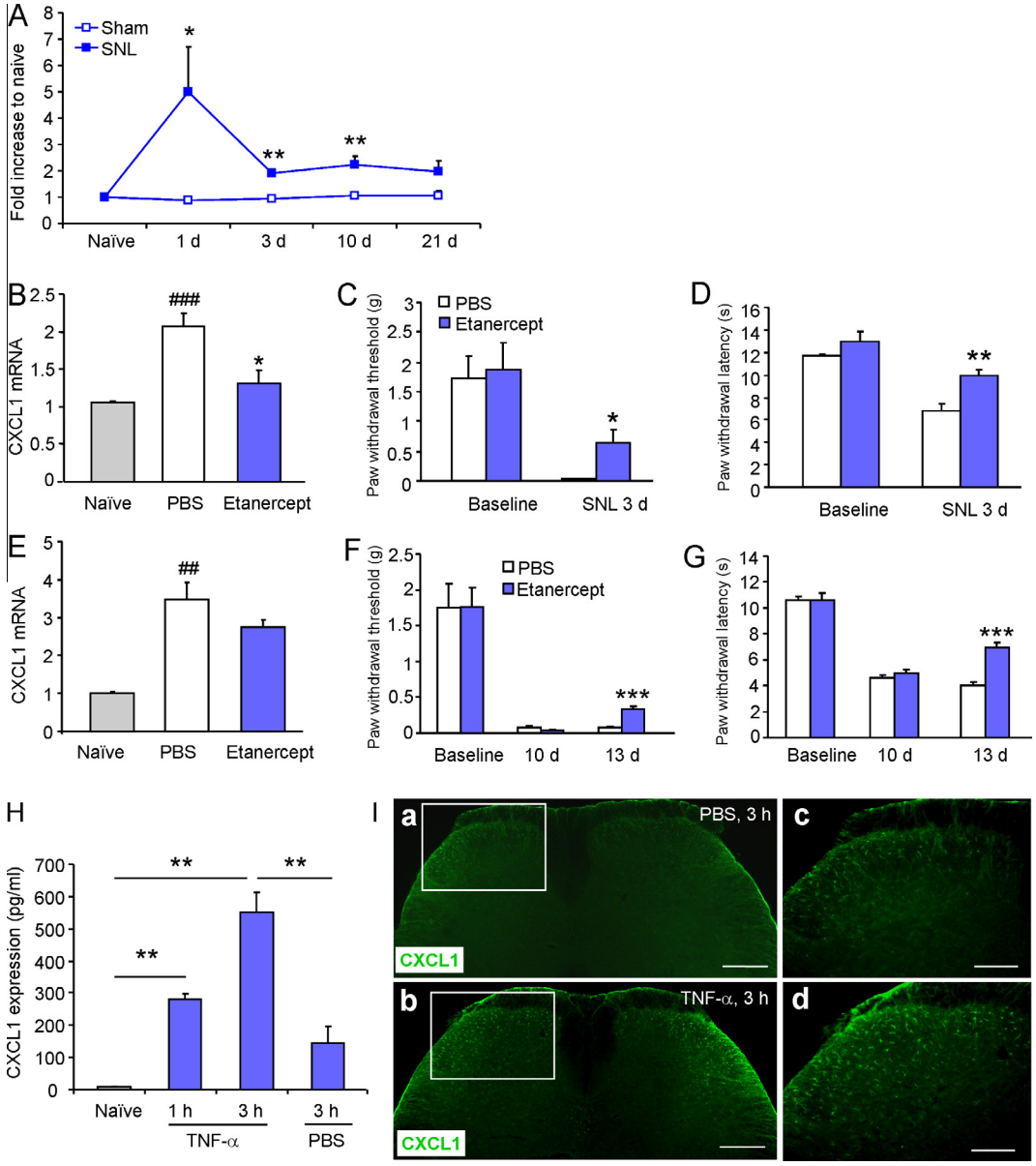

Fig. 2. Tumor necrosis factor (TNF)α triggers the expression of CXCL1 in the spinal cord. (A) Spinal nerve ligation (SNL) rapidly increases TNFα mRNA expression in the spinal cord. $^{*}P < 0.05$, $^{**}P < 0$ 0.01, SNL vs sham, Student's *t*-test. (B) Intrathecal (i.t.) injection of etanercept before SNL blocks SNL-induced CXCL1 mRNA increase in the spinal cord at 3 days after SNL. $^{###}P < 0.001$; compared with naïve; $^{*}P < 0.05$, etanercept vs phosphate-buffered saline (PBS), Student's *t*-test. (C, D) Etanercept reduces SNL-induced mechanical allodynia (C) and heat hyperalgesia (D). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with PBS control, Student's *t*-test. (E-G) i.t. injection of etanercept at 10 days after SNL does not decrease CXCL1 mRNA expression (E), but attenuated mechanical allodynia (F) and heat hyperalgesia (G). $^{##}P < 0.01$, compared with naïve; $^{***}P < 0.001$, compared with PBS control, Student's *t*-test. (E) Enzyme-linked immunosorbent assay (ELISA) shows that i.t. injection of TNFα rapidly and dramatically increases CXCL1 expression in the spinal cord at 1 hour and 3 hours after injection. $^{**}P < 0.01$, Student's *t*-test. (F) Immunostaining shows CXCL1 expression in the spinal cord at 3 hours after injection of PBS (a, c) and TNFα (b, d). Scale bar, a, b, 200 μm; c, d, 50 μm.

We intraspinally infused this LV-shCXCL1 and LV-NC into the L5 spinal cord. Seven days later, green fluorescent protein (GFP) expression was observed in the injected side of the dorsal horn (Supplementary Fig. 3B). To examine the cell types that expressed the GFP, the sections were immunostained with GFAP, ionized calcium binding adaptor molecule 1 (IBA-1), and NeuN. Throughout the dorsal horn of spinal cord, GFP was primarily localized to GFAP-positive astrocytes (Supplementary Fig. 3C). Some GFP expression was observed in IBA-1-positive microglia (Supplementary Fig. 3D) or in NeuN-positive neurons (Supplementary Fig. 3E).

We checked whether pretreatment with LV-shCXCL1 via intraspinal infusion can block the development of SNL-induced pain hypersensitivity. Injection of the vector alone did not affect the baseline paw withdrawal threshold and paw withdrawal latency tested at 7 days after the infusion (Fig. 5A and B). We then performed SNL on these animals and examined mechanical allodynia and heat hyperalgesia. LV-shCXCL1 partly prevented SNL-induced mechanical allodynia: the effect started at 3 days after SNL and was maintained for more than 21 days (Fig. 5A). Meanwhile, LV-shCXCL1 reduced SNL-induced heat hyperalgesia for 21 days (Fig. 5B). To confirm the knockdown effect of CXCL1 shRNA lentiviral vector in vivo, we checked CXCL1 mRNA expression at 10 days after SNL (17 days after vectors injection). The CXCL1 mRNA expression was reduced by 45% following the LV-shCXCL1 treatment, compared to the LV-NC control.

We then checked the reversal effect of LV-shCXCL1. The same amount of LV-shCXCL1 was infused at 5 days after SNL. As shown in Fig. 5C and D, SNL-induced mechanical allodynia was attenuated

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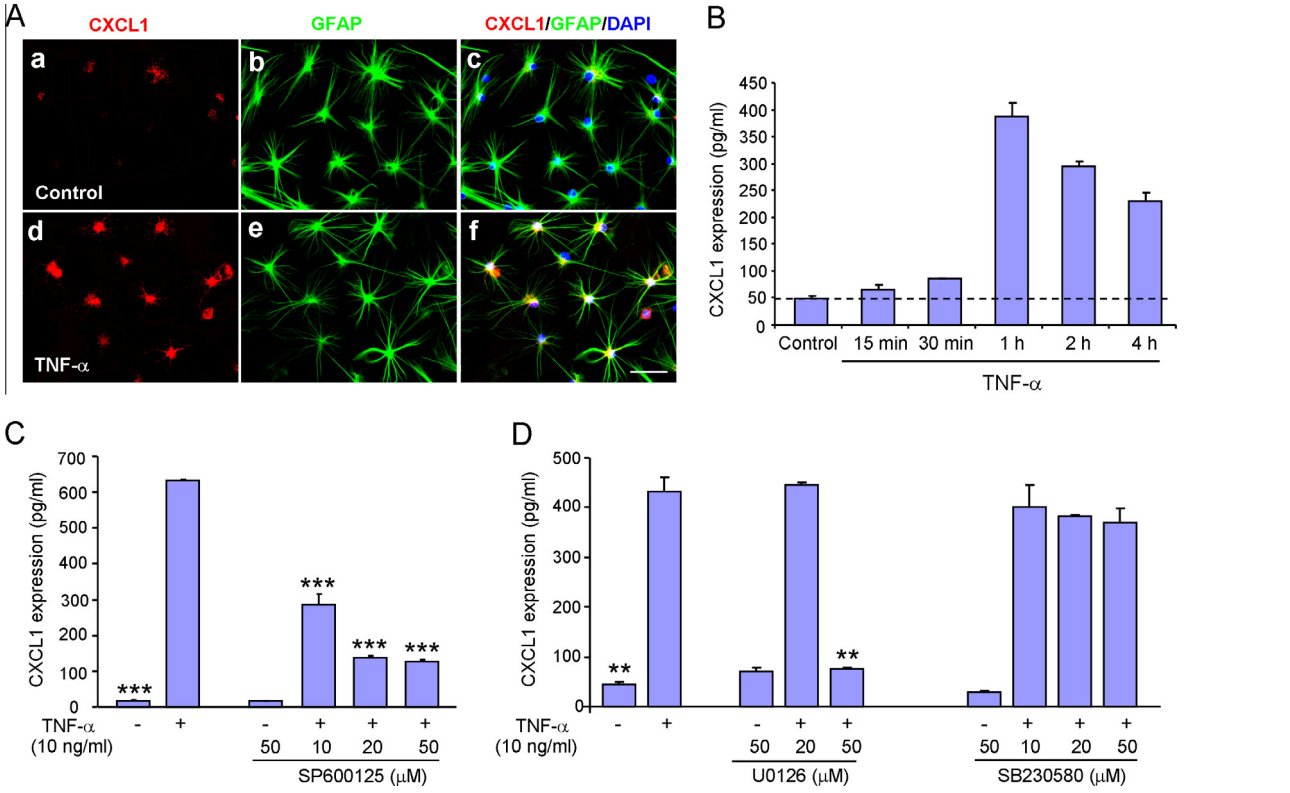

Fig. 3. Tumor necrosis factor (TNF)α induces c-jun N-terminal kinase (JNK)-dependent CXCL1 upregulation in cultured astrocytes. (A) Triple staining of CXCL1 (a, d), GFAP (b, e), and DAPI (c, f) in control (a) and TNFα-stimulated (d) astrocytes. (c, f) show that all DAPI$^+$ cells also express glial fibrillary acidic protein (GFAP). Scale bar, 50 μm. (B) TNFα induces time-dependent upregulation of CXCL1 in astrocytes. (C) JNK inhibitor SP600125 at the doses of 10 μM, 20 μM, and 50 μM reduces TNFα-induced CXCL1 upregulation. (D) MEK inhibitor PD98059 at the dose of 50 μM reduces TNFα-induced CXCL1 upregulation, whereas p38 inhibitor SB203580 at all 3 doses has no effect on TNFα-induced CXCL1 upregulation, $^{**}P < 0.01$, $^{***}P < 0.001$, vs TNFα treatment, Student's *t*-test.

from 7 to 21 days. The heat hyperalgesia was also reversed from 7 to 14 days and fully recovered at 21 days. Taken together, these behavioral data suggest that CXCL1 is critical for SNL-induced neuropathic pain sensitization.

3.6. Spinal injection of exogenous CXCL1 induces heat hyperalgesia and activation of spinal cord neurons through CXCR2

We investigated whether CXCL1 is sufficient to produce pain and how it is involved in the modulation of pain hypersensitivity. We first injected i.t. CXCL1 (10 ng or 100 ng) and tested heat hyperalgesia at different times after injection. As shown in Fig. 6A, CXCL1 produced heat hyperalgesia in a dose-dependent manner. The paw withdrawal latency was decreased at 1 hour, maintained at 7 hours, and recovered after 24 hours by CXCL1 at the dose of 100 ng.

CXCR2 is a major receptor of CXCL1 [46,73]. To test whether CXCL1-induced pain hypersensitivity is mediated by CXCR2, we injected i.t. SB225002, a potent and selective CXCR2 antagonist, 30 minutes before CXCL1 injection and tested heat hyperalgesia. At a low dose (5 μg), SB225002 partially reversed the CXCL1-induced heat hyperalgesia. But at a high dose (20 μg), SB225002 completely blocked the hyperalgesia. SB225002 itself did not change the paw withdrawal latency at all the time points (Fig. 6B).

ERK and c-Fos are important markers for neuronal activation and central sensitization following noxious stimulation [17]. In addition, phosphorylated ERK (pERK) can be translocated into the nucleus to activate several transcriptional factors, such as cAMP-response element binding protein (CREB) that is required for the transcription of several pain-related genes. We examined the expression of pERK, pCREB (phosphorylated CREB), and c-Fos at 30 minutes or 2 hours after spinal injection of CXCL1 (100 ng). As shown in Fig. 7, CXCL1 increased pERK (Fig. 7A) and pCREB (Fig. 7B) expression in the dorsal horn of the spinal cord at 30 minutes after injection, and c-Fos expression at 2 hours after injection (Fig. 7C). Additionally, i.t. injection of SB225002 (20 μg) 30 minutes before CXCL1 injection blocked CXCL1-induced pERK and pCREB expression and reduced c-Fos expression (Fig. 7D–F). The double immunofluorescence staining showed high colocalization of pERK, pCREB, or c-Fos with NeuN, suggesting the dominant expression of these markers by neurons (Fig. 7Ad, Bd, and Cd).

3.7. SNL induces CXCR2 upregulation in spinal cord neurons

We further investigated CXCR2 expression and distribution in the spinal cord in a neuropathic pain condition. In naïve animals, CXCR2 was constitutively expressed in the dorsal horn (Fig. 8A). At 3 days after SNL, CXCR2-IR was significantly increased in both superficial and deep dorsal horn (Fig. 8B). Western blot results further showed CXCR2 was significantly increased in the ipsilateral dorsal horn at 3 days after SNL ($P < 0.05$, SNL vs sham; Fig. 8C). To identify the lamina distribution of CXCR2 expression, we did CXCR2 and PKCγ double staining, because PKCγ is expressed mainly in neurons of the inner part of lamina II [43,50]. Most CXCR2-positive cells were found in lamina I and laminae IIi-V (Fig. 8D), but the cells in the outer part of lamina II are fewer. To further test whether CXCR2 was expressed in neurons, we did immunofluorescence double staining of CXCR2 and NeuN and found that many CXCR2-IR cells were also NeuN-positive (Fig. 8E and F).

To investigate the role of CXCR2 in the SNL-induced neuropathic pain, CXCR2 antagonist, SB225002 (10 μg and 20 μg) was

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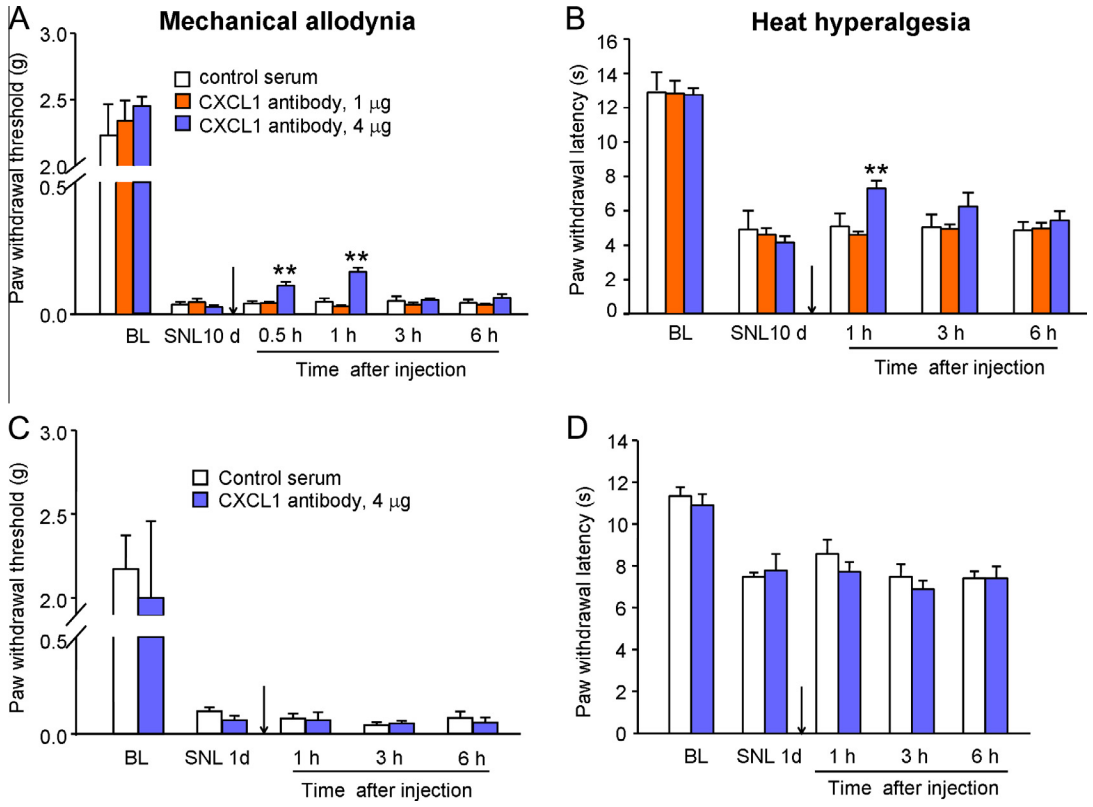

Fig. 4. Intrathecal (i.t.) injection of CXCL1 neutralizing antibody, at 10 days after spinal nerve ligation (SNL), partially and transiently reverses SNL-induced mechanical allodynia (A) and heat hyperalgesia (B). $^{**}P < 0.01$, vs control serum, one-way analysis of variance followed by Newman-Keuls post hoc test. However, i.t. injection of CXCL1 neutralizing antibody, at 1 day after SNL, has no effect on either mechanical allodynia (C) or heat hyperalgesia (D). Student's *t*-test. BL, Baseline.

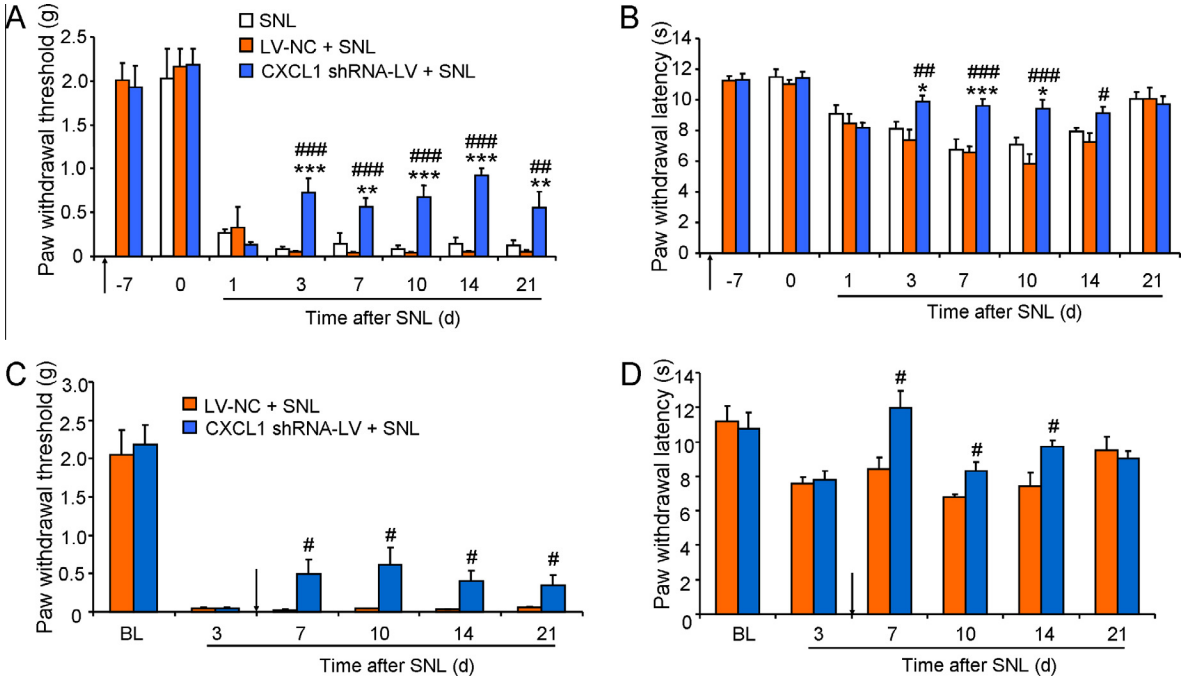

Fig. 5. Intraspinal injection of recombinant lentivirus-CXCL1 shRNA persistently suppresses spinal nerve ligation (SNL)-induced neuropathic pain. Intraspinal infusion of CXCL1 shRNA lentiviral vectors 7 days before SNL persistently reduces SNL-induced mechanical allodynia (A) and heat hyperalgesia (B). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, vs SNL. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$, vs LV-NC + SNL, one-way analysis of variance followed by Newman-Keuls post hoc test. Intraspinal infusion of CXCL1 shRNA lentiviral vectors 5 days after SNL also reduces SNL-induced mechanical allodynia (C) and heat hyperalgesia (D). $^{\#}P < 0.05$, vs LV-NC + SNL, Student's *t*-test.

injected i.t. 3 days after SNL. SB225002 dose-dependently reduced mechanical allodynia and heat hyperalgesia with the effect maintained for more than 3 hours (Fig. 8G and H). The animals with sham operation did not show pain hypersensitivity at 3 days.

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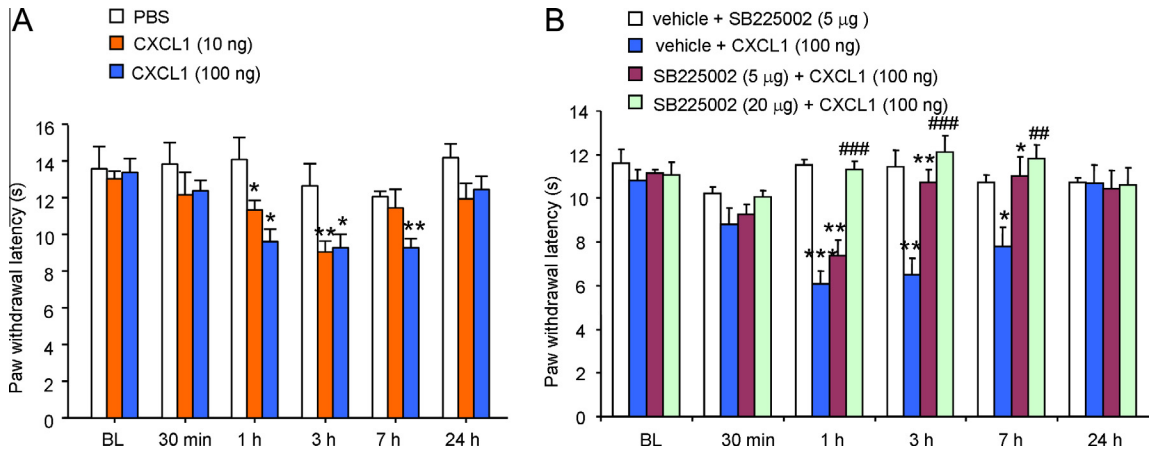

Fig. 6. Spinal injection of CXCL1 induces heat hyperalgesia via CXCR2. (A) Intrathecal injection of CXCL1 (10 or 100 ng) induces a dose-dependent heat hyperalgesia. $^{*}P < 0.05$; $^{**}P < 0.01$, vs phosphate-buffered saline (PBS), one-way analysis of variance followed by Newman-Keuls post hoc test. (B) Intrathecal injection of CXCR2 antagonist, SB225002, 30 minutes before CXCL1 injection dose-dependently prevented CXCL1-induce heat hyperalgesia. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs corresponding BL (baseline). $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs vehicle + CXCL1 (100 ng), one-way analysis of variance followed by Newman-Keuls post hoc test.

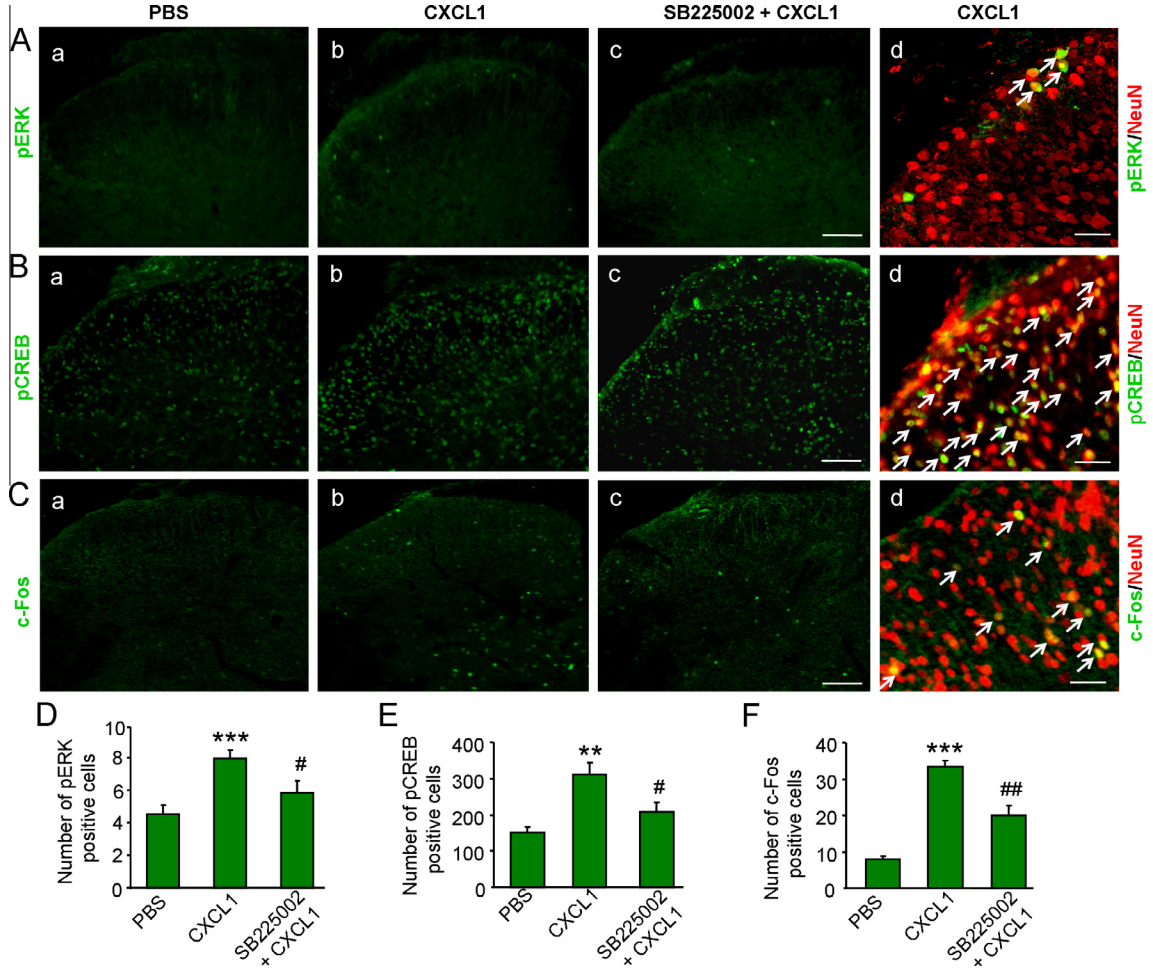

Fig. 7. CXCL1 increases the expression of phosphorylated extracellular signal-regulated kinase (pERK), phosphorylated cAMP response element binding protein (pCREB), and c-Fos in spinal cord neurons. Intrathecal injection of CXCL1 (100 ng) increases pERK expression (A, D) and pCREB expression (B, E) at 30 minutes and c-Fos expression (C, F) at 2 hours after injection, which is blocked by pretreatment with SB225002 (20 μg). Majority of pERK-, pCREB-, or c-Fos immunoreactive cells in spinal cord express the neuronal marker neuronal nuclei (NeuN) (d). Scale bar, 100 μm in c, 50 μm in days. $^{**}P < 0.01$; $^{***}P < 0.001$ vs phosphate-buffered saline (PBS). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs CXCL1, one-way analysis of variance followed by Newman-Keuls post hoc test.

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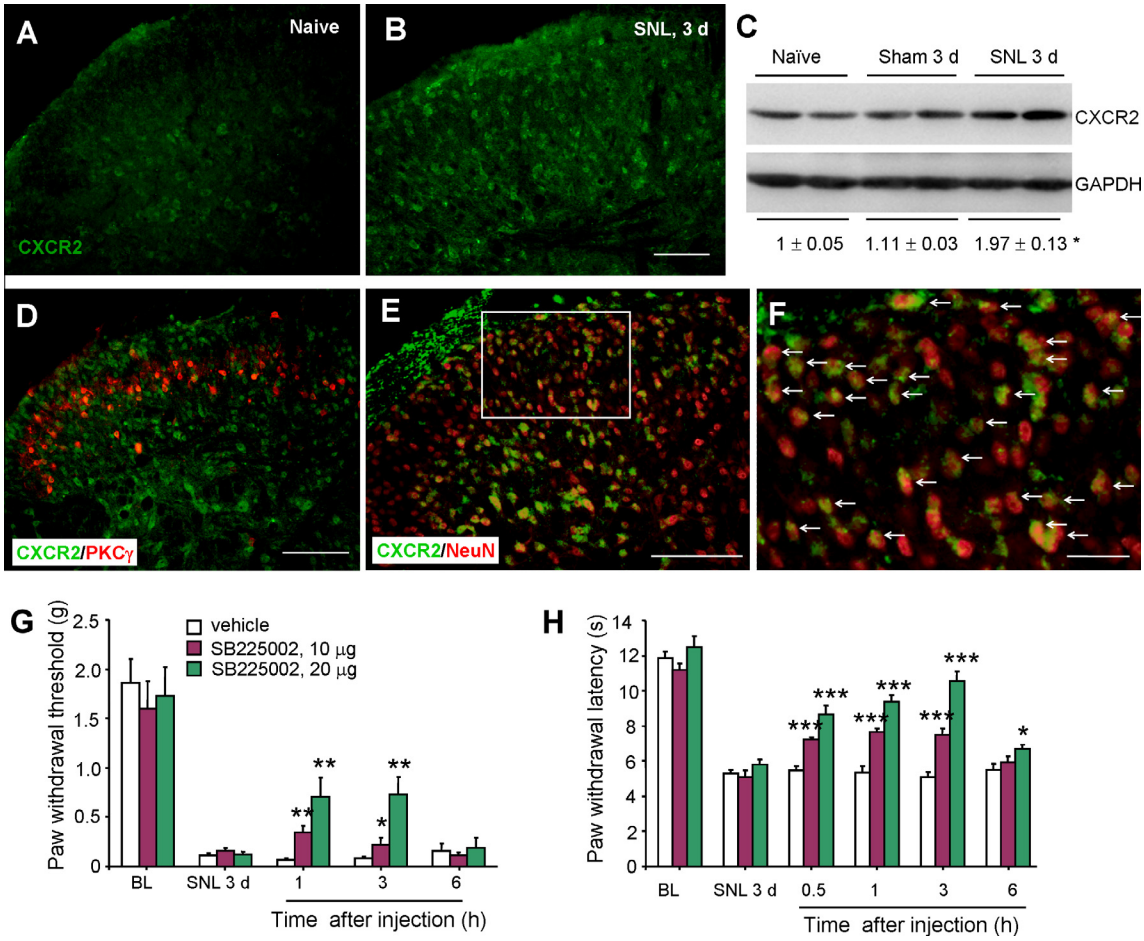

Fig. 8. Spinal nerve ligation (SNL) induces CXCR2 upregulation in spinal cord neurons. CXCR2 is expressed in the spinal cord of naïve animals (A) and increased at 3 days after SNL (B). Scale bar, 100 μm. (C) Western blot shows the expression of CXCR2 in the spinal cord in naïve, sham, and SNL animals. SNL increases CXCR2 expression compared to sham control. $^{*}P < 0.05$, SNL vs sham, Student's *t*-test. (D) Double staining of CXCR2 and protein kinase C (PKC)γ showing the lamina distribution of CXCR2-positive cells. Scale bar, 100 μm. (E) Double staining of CXCR2 and neuronal nuclei (NeuN) showing the expression of CXCR2 in neurons. Scale bar, 100 μm. (F) High-magnification image of E. Scale bar, 25 μm. (G, H) Intrathecal injection of SB225002, at 3 days after SNL, dose-dependently reversed SNL-induced mechanical allodynia (G) and heat hyperalgesia (H). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs vehicle, one-way analysis of variance followed by Newman-Keuls post hoc test.

In addition, i.t. injection of SB225002 at the dose of 20 μg at 3 days after sham operation did not affect either mechanical allodynia or heat hyperalgesia (Supplementary Fig. 4A and B), suggesting the specific role of CXCR2 in SNL-induced neuropathic pain.

4. Discussion

Neuroinflammation in the spinal cord has been implicated in the development and maintenance of central sensitization and pain hypersensitivity [74]. Accumulating evidence demonstrates that chemokines such as CCL2 and CX3CL1 are involved in neuroinflammation in the spinal cord and contribute to neuropathic pain processing [2,18]. In addition, astrocytes serve as a main source of inflammatory mediators [58,72]. In this study, we have shown that CXCL1, produced by astrocytes, plays an important role in the maintenance of central sensitization and neuropathic pain (Fig. 9). We have made the following findings. First, SNL induced slow but persistent CXCL1 upregulation in spinal astrocytes, which is dependent on the TNFα/JNK pathway. Second, spinal inhibition of CXCL1 partly reversed SNL-induced pain hypersensitivity. However, intraspinal injection of CXCL1-shRNA lentivirus vector either before or after SNL persistently attenuated SNL-induced pain hypersensitivity. Third, spinal application of CXCL1 induced heat hyperalgesia via the activation of CXCR2. CXCL1 also induced CXCR2-dependent neuronal activation and central sensitization (eg, increase of pERK, pCREB, and c-Fos) in dorsal horn neurons. Finally, SNL induced upregulation of CXCR2 in spinal neurons. Spinal posttreatment of a selective CXCR2 antagonist, SB225002, effectively attenuated SNL-induced heat hyperalgesia and mechanical allodynia.

4.1. CXCL1 upregulation in spinal astrocytes and the involvement in neuropathic pain

It has been reported that CXCL1 expression is regulated in pathological pain conditions. CXCL1 expression is increased in the DRG at 3 days but not at 7 days after SNL and localized inflammation [40,75]. In addition, McTigue et al. showed that contusion injury of spinal cord induced 30-fold increase of CXCL1 mRNA in the spinal cord at 6 hours post injury, which decayed rapidly thereafter [45]. In contrast, our data showed that SNL induced a slow (3 days) but persistent (>21 days) CXCL1 increase in the spinal cord, indicating CXCL1 may play a distinct role in neuropathic pain.

It was reported that CXCL1 is expressed in brain neurons after soman-induced status epilepticus in rats [29]. In our study, both the fluorescence double staining and in situ hybridization combining with immunofluorescence showed that CXCL1 protein and mRNA were predominantly expressed in spinal astrocytes. Several studies also demonstrated an induction of CXCL1 by astrocytes in

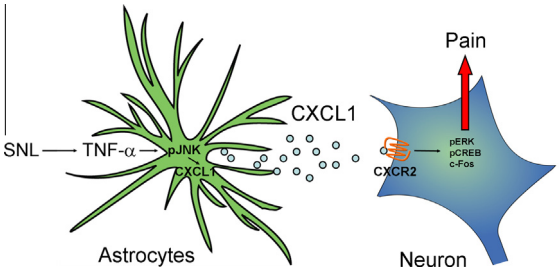

Fig. 9. Schematic shows how CXCL1 and CXCR2 in the spinal cord regulate neuropathic pain. Spinal nerve ligation (SNL) rapidly increases tumor necrosis factor (TNF)α expression, which acts on astrocytes to induce JNK activation, leading to the production of CXCL1. Upon release from astrocytes, CXCL1 acts on CXCR2 in neurons and induces the expression of phosphorylated extracellular signal-regulated kinase (pERK), phosphorylated cAMP response element binding protein (pCREB), and c-Fos, leading to the expression of late-response genes and subsequent maintenance of neuropathic pain.

the central nervous system (CNS) in pathological conditions. Pineau et al. reported that CXCL1 mRNA is upregulated in the spinal astrocytes after spinal cord injury in mice [54]. CXCL1 is induced in brain astrocytes by neuronal injury and intracerebroventricular administration of endothelin-1 [31,37]. In humans, CXCL1 is selectively expressed in hypertrophic astrocytes after active multiple sclerosis lesions [52,53]. In addition, several studies show that CXCL1 is produced in cultured astrocytes after incubation with lipopolysaccharide, NOV/CCN3 (nephroblastoma overexpressed), IL-1β, or infection with the murine encephalomyelitis virus [16,38,52,60].

RNA interference (RNAi), which can induce loss-of-function phenotypes by posttranscriptional silencing of gene expression, has shown potential as a therapeutic strategy [64]. Viral vector-mediated RNAi can induce stable and long-term gene silencing [44] and has been shown effective in inhibiting gene expression in a number of diseases [8]. In this study, i.t. injection of CXCL1 neutralizing antibody only transiently and partly attenuated SNL-induced mechanical allodynia and heat hyperalgesia. In contrast, LV-shCXCL1 showed a markedly and persistent antihyperalgesia and antiallodynia effect in SNL mice, suggesting that CXCL1 plays an important role in the maintenance of neuropathic pain. It is noteworthy that LV-shCXCL1 alleviated, not completely reversed, the SNL-induced pain hypersensitivity, which may be attributed to 2 possibilities. First, intraspinal infusion LV-shCXCL1 0.8 μL (640 TU) did not completely block CXCL1 expression (about 45% knockdown); second, CXCL1 plays a partial role in the maintenance of neuropathic pain. Our previous studies have shown that SNL induces chemokine CCL2 production, which has been demonstrated to be involved in central sensitization and neuropathic pain [20].

4.2. TNFα/JNK is the upstream of CXCL1 production in astrocytes in neuropathic pain

Our results showed that CXCL1 upregulation started from 3 days after SNL. However, the pain hypersensitivity was induced at 1 day, suggesting that CXCL1 upregulation may be secondary to the change of other mediators. Indeed, SNL induced rapid (1 day) TNFα mRNA increase, and TNFα inhibitor blocked SNL-induced CXCL1 mRNA increase at 3 days after SNL. Intrathecal injection of TNFα also increased CXCL1 expression at the spinal cord. These data indicate that TNFα is necessary and sufficient to the CXCL1 upregulation in the spinal cord, especially in the early phase of SNL. Earlier reports demonstrated that antigen-induced hyperalgesia and the increase of CXCL1 was reduced in TNF receptor 1 knockout mice [11], further supporting TNFα as a trigger of CXCL1 production in both spinal cord and peripheral tissue.

JNK is one of the members of MAPK and has been shown to be expressed in astrocytes and upregulated in neuropathic pain conditions [42,81]. TNFα-induced JNK activation regulates the production of various inflammatory mediators that may directly regulate the neuronal excitability [18,19]. Here we showed that CXCL1 is one of the chemokines that was produced by astrocytes following JNK activation in cultured astrocytes. In vivo data further showed that JNK inhibitor reduced SNL-induced pain and CXCL1 expression in the spinal cord. These results indicate that CXCL1 is an important downstream of JNK activation in mediating the process of neuropathic pain.

4.3. CXCL1/CXCR2 signaling in central sensitization and neuropathic pain

Chemokines act through a family of 7 transmembrane G protein-coupled receptors to exert their biological effects. CXCL1 acts through CXCR2 receptor, which also binds other chemokines, such as CXCL2 and CXCL3 [5,61]. The CXCR2 receptor has been detected on neurons [24,68], oligodendrocyte progenitors [51,53], and microglia [15,55] in brain. Several studies suggest that CXCL1 acting through CXCR2 on oligodendrocyte progenitors promotes oligodendrogenesis in the CNS of rodents and humans [15,51,59,67]. In the DRG, CXCR2 are expressed in neurons and CXCL1 increases the sodium currents, potassium currents in small-diameter rat sensory neurons [71,77], suggesting the direct pronociceptive effects of CXCL1. In this study, i.t. injection of CXCL1 induced heat hyperalgesia, which was blocked by pretreatment with CXCR2 antagonist, SB225002. Additionally, CXCR2 was expressed on neurons of the spinal cord. Both immunostaining and Western blot showed that SNL increased CXCR2 expression in the spinal cord. Behavioral results further demonstrated that CXCR2 antagonist attenuated SNL-induced pain hypersensitivity at 3 days after SNL in a dose-dependent manner, suggesting the involvement of CXCR2 in neuropathic pain.

Accumulating evidence suggests that proinflammatory cytokines and chemokines may be directly involved in regulating neuronal activity in the dorsal horn [20,21,33]. For example, perfusion of spinal slices with IL-1β or chemokine CCL2 increases the frequency and amplitude of spontaneous postsynaptic currents and markedly enhances *N*-methyl-d-aspartate-induced currents in dorsal horn neurons [20,33]. Here, i.t. injection of CXCL1 induced rapid ERK and CREB activation and c-Fos expression, mainly in spinal cord neurons. The ERK pathway plays an important role in neuronal plasticity and central sensitization [27,30]. The translocated pERK activates transcription factors including CREB, and regulates gene transcription to maintain central sensitization and nerve-injury pain [28]. CREB binding sites are shown in the promoter regions of the genes encoding c-Fos, cyclooxygenase (COX)-2, neurokinin-1, prodynorphin, and TrkB, which are important for the genesis of chronic pain. We observed that CXCL1 induced CXCR2-dependent mRNA increase of COX-2 in the spinal cord (unpublished observation), suggesting that CXCL1 may be involved in the maintenance of central sensitization and neuropathic pain, in part through upregulation of pain-related proteins.

Besides the central effect of CXCL1/CXCR2 on pain, it has been reported that CXCL1/CXCR2 interaction is involved in neutrophil recruitment in the peripheral tissue and mediates carrageenan-induced inflammatory pain [9,10] and paw incision-induced postincisional pain [4]. Whether SNL could induce the CXCL1/CXCR2-mediated neutrophil accumulation in local tissue and further induce the induction of neuropathic pain needs to be investigated in the future.

4.4. Conclusions

Nerve injury has been shown to upregulate several chemokines in the spinal cord to enhance neuropathic pain via neuron–glia

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interactions. It is of great interest that different chemokines mediate distinct neuron–glia interactions. In the CNS, chemokines and their respective receptors are often detected in different cell types to mediate cell–cell interactions. In the spinal cord, neurons and microglia have been shown to express chemokines (CX3CL1/fractalkine, CCL2, CCL21) and chemokine receptors (CX3CR1, CCR2, CCR7/CXCR3), respectively [3,12,20,21,23,36,41,47,66,69,78,79], indicating an important role of chemokine signaling in neuron-initiated microglial activation. However, astrocytes and neurons have also been shown to express CCL2 and CCR2, respectively [20,21,36], suggesting distinct signaling from astrocytes to neurons. In this study we demonstrated another mechanism of chemokine-mediated astrocyte-to-neuron signaling, by which astrocyte-produced CXCL1 acts on CXCR2-expressing dorsal horn neurons to elicit central sensitization and maintain neuropathic pain. This is also consistent with the studies demonstrating distinct role of microglia and astrocytes for the development and maintenance of neuropathic pain [6,13,48,57,78,81]. Of interest, nerve injury-induced CXCL1 increase in astrocytes is more persistent than that of CCL2 [20]. Thus, targeting different chemokine signaling could differentially regulate neuropathic pain in different phases. Given an important role of CXCL1/CXCR2 in the maintenance of neuropathic pain, inhibiting CXCL1/CXCR2 and astrocyte signaling may offer a novel therapeutic strategy for treating chronic neuropathic pain.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pain.2013.07.002.

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