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The Role and Mechanism of Spinal NF-κB-CXCL1/CXCR2 in Rats with Nucleus Pulposus–induced Radicular Pain

Fengjiao Gao, MD,[a] Ming Wei, MD,[a] Meiyue Wang, BD,[a] Yongting Yang, MD,[a] Xuan Duan, BD,[b] Lin Yang, MD,[b] and Laibao Sun, MD[a]

Study Design. Experimental study of the role and mechanism of spinal NFκB-CXCL1/CXCR2 in rats with nucleus pulposus-induced radicular pain.

Objective. This study investigated the role and mechanism of spinal NFκB-CXCL1/CXCR2 in autologous nucleus pulposus-induced pain behavior in rats and to clarify the involvement and regulation of spinal NFκB as an upstream molecule of CXCL1 in autologous nucleus pulposus-induced radicular pain in rats.

Summary of Background Data. The inflammatory response of nerve roots is an important mechanism for the occurrence of chronic pain. NFκB-CXCL1/CXCR2 pathway plays an important role in the development of radicular pain, but its regulatory mechanism in the model of radicular pain induced by autologous nucleus pulposus is still unclear.

Materials and Methods. We established a rat model of autologous medullary nucleus transplantation. We observed and recorded the changes in 50% mechanical withdrawal threshold and thermal withdrawal latency before and after the administration of CXCL1-neutralizing antibodies, CXCR2 inhibitor, and NFκB inhibitor in each group of rats and evaluated the expression of NFκB, CXCL1, and CXCR2 in the spinal dorsal horn using immunofluorescence and Western blot. To compare differences between groups in behavioral testing, analysis of variance was employed. Dunnett's method was used to compare differences at different time points within a group and between different groups at the same time point. A comparison of the relative concentration of protein, relative concentration of mRNA, and semiquantitative data from immunofluorescence staining was conducted utilizing one-way ANOVA and Dunnett's pairwise comparison.

Results. Autologous nucleus pulposus transplantation can induce radicular pain in rats and upregulate the expression of CXCL1, CXCR2, and NFκB in the spinal cord. CXCL1 is co-expressed with astrocytes, CXCR2 with neurons, and NFκB with both astrocytes and neurons. The application of CXCL1 neutralizing antibodies, CXCR2 inhibitors, and NFκB inhibitors can alleviate pain hypersensitivity induced by autologous nucleus pulposus transplantation in rats. Inhibitors of NFκB could downregulate the expression of CXCL1 and CXCR2.

Conclusions. We found that spinal NFκB is involved in NP-induced radicular pain in rats through the activation of CXCL1/CXCR2, enriching the mechanism of medullary-derived radicular

From the [a]Department of Anesthesiology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China; and [b]Department of Anesthesiology, Guangzhou Panyu Central Hospital, Guangzhou, Guangdong, China.

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F.G. and M.W. contributed equally to this work and should be considered co-first authors.

The device(s)/drug(s) that is/are the subject of this manuscript is/are not intended for human use.

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Address correspondence and reprint requests to Lin Yang, MD, Department of Anesthesiology, Guangzhou Panyu Central Hospital, No.8, Fuyu East Road, Panyu District, Guangzhou 511400, Guangdong, China; E-mail: salinyangyl@hotmail.com; Laibao Sun, MD, Department of Anesthesiology, The First Affiliated Hospital, Sun Yat-sen University, No.58, Zhongshan 2th Road, Guangzhou 510080, Guangdong, China; E-mail: sunlaibao66@hotmail.com

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pain and providing a possible new target and theoretical basis for the development of more effective anti-inflammatory and analgesic drugs for patients with chronic pain following LDH.

Key words: radicular pain, NFκB, CXCL1, CXCR2, astrocytes
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Chronic low back pain with radicular features frequently occurs as a consequence of lumbar disc herniation (LDH). Currently, the mechanical compression and chemical nerve root inflammation induced by herniated nucleus pulposus (NP) represent the primary mechanisms that induce radicular pain in LDH[1] and produce significant peripheral hyperalgesia in the rat model of LDH.[2,3] However, many patients still experience significant pain after relieving nerve compression, so reducing nerve root inflammation remains an urgent problem.

CXCL1 is a chemotactic cytokine released during the inflammatory process, and it contributes to different pain mechanisms through binding and activating the corresponding receptor, CXCR2.[4] The CXCL1/CXCR2 pathway plays an essential role in the development of a range of neural pain mechanisms.[5] Nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) is a critical transcription factor involved in different cell responses to a variety of stimuli. Studies have indicated that NFκB participates in nociceptive sensitization through the activation of the CXCL1/CXCR2 signaling pathway in rat models of bone cancer pain and chemotherapy-induced pain.[6,7]

In a study on a rat model of nerve root pain, autologous spinal cord activation of NFκB and administration of pyrrolidine dithiocarbamate, an inhibitor of NFκB, significantly improved pain behavior in rats. In addition, it significantly downregulated nitric oxide, tumor necrosis factor α (TNF-α), and inflammatory factors such as interleukin 6.[8] This suggests that NFκB is likely to be an important molecule involved in chronic pain. Furthermore, NFκB has been demonstrated to serve as a significant transcriptional regulator that controls the levels of CXCL1 protein expression.[9] Our earlier observations propose that in a rat model of NP-induced radicular pain, the expression of spinal CXCL1 is upregulated, and an investigation into whether the transcriptional behavior of NFκB regulates this specific upregulatory mechanism is warranted.

This study investigated the changes in NFκB expression in rats with autologous NP-induced pain behavior, astrocyte activation, and CXCL1/CXCR2 expression levels to elucidate the mechanism of NFκB in autologous NP-induced pain behavior in rats.

MATERIALS AND METHODS

Animal and Model Building

Adult male SPF grade Sprague-Dawley rats (200-280g) were supplied by the Animal Experiment Centre of Sun Yat-sen University (National Animal Experiment Center), license number SCXK (Guangdong) 2016-0029. All experimental procedures were conducted following the animal welfare guidelines at the Sun Yat-sen University Animal Experiment Centre. Autologous NP (about 0.4 mg) was harvested from the two near-end intervertebral spaces of each tail, the left L5 partial laminectomy was performed, and NP was applied to the L5 nerve root in rats. This model was designed based on Nobuhisa Sasaki's autologous medullary nucleus transplantation animal model (Fig. 1, Supplemental Fig. 1, Supplemental Digital Content 1, http://links.lww.com/BRS/C353).[10]

Experimental Grouping

Blank group (Blank): no surgery; Sham group (Sham): Except for the NP not placed around the dorsal root ganglion other procedures were the same as the NP group (n = 15); NP group (NP): autologous NP was taken and placed in L5 dorsal root ganglion.

According to the different drugs added on postoperative d3, d4, and d5 in the Sham and NP groups, they were further divided into the Sham+DMSO group, Sham +CXCL1 antibody group, Sham+SB group, NP+DMSO group, NP+CXCL1 antibody group, NP+SB group, Sham +BAY group, NP+DMSO group, and NP+BAY group (n = 12), where BAY is NFκB inhibitor BAY11-7082 (A4210, APEXBIO, USA), CXCL1 antibody is CXCL1 antibody neutralizing (A00533, BOSTER, China), and SB is CXCR2 antagonist SB225002 (B8200, APEXBIO, USA).

50% MWT Measurement

The measurement of the 50% mechanical withdrawal threshold (50% MWT) was conducted using the up-down method.[11] Various von Frey filaments were used to stimulate the rats, and the withdrawal of the rat hind limbs was considered a positive response. Starting at 4g, each force was applied five times, and the minimum von Frey fiber force with more than three positive responses, with at least 15 seconds between stimuli, was set as 50%MWT of the rat. The UpDownReader software was used to derive the corresponding 50% MWT value from the collected data as Rafael Gonzalez-Cano.[12]

TWL Measurement

Thermal withdrawal latency (TWL) for measuring the latency of thermal painful paw contraction: Rats were situated inside a Plexiglas box (5.5 cm × 10 cm) with a transparent glass base. A thermal light source was projected through the clear glass, aimed at the underside of the rat's hind paw. The light intensity selected was enough to induce a withdrawal response in typical rats for around 15 seconds. When the withdrawal response occurred, the thermal light source shut off automatically, and the apparatus automatically recorded the latency of the withdrawal response.

Tissue Immunofluorescence Staining

After anesthesia with 2% pentobarbital (60-80 mg/kg), rats were perfused through the aorta with 150 to 200 mL saline at 37°C, followed by 200 mL 4% paraformaldehyde at 4°C

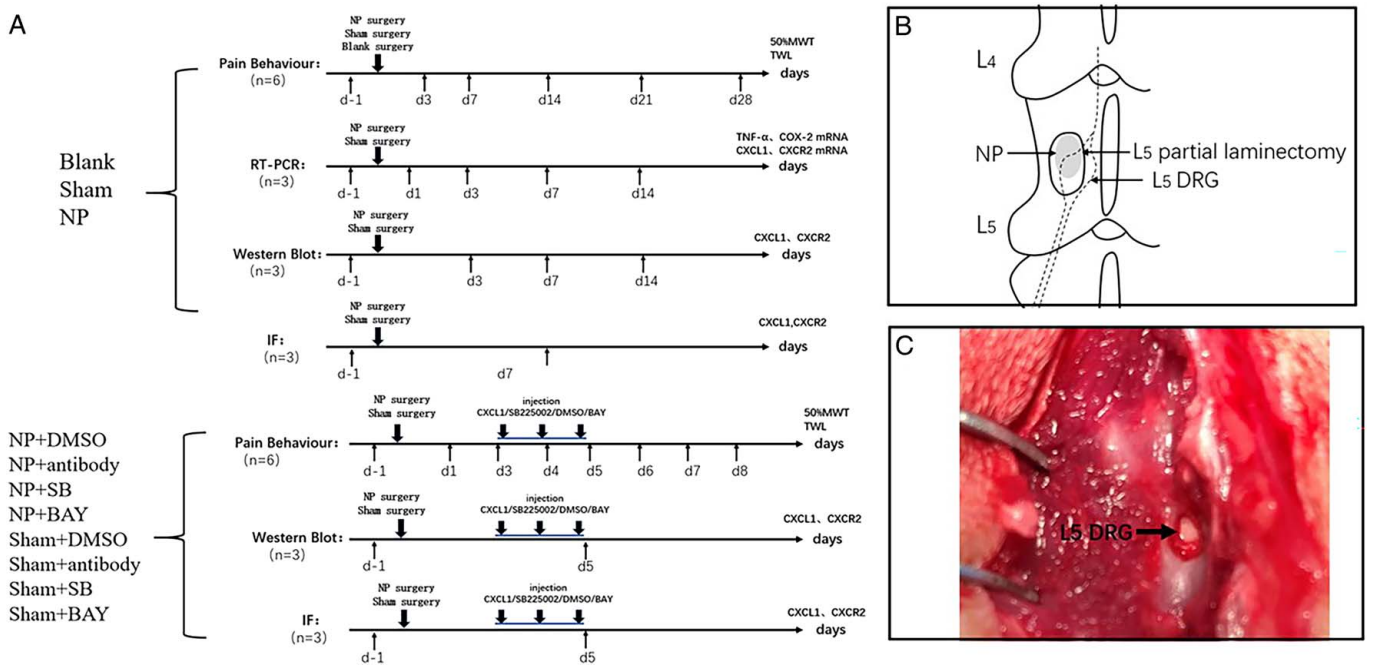

Figure 1. A, Experimental paradigms. B, Schematic diagram of surgical methods. C, Surgery procedure. NP+DMSO, NP+antibody, NP+SB: Continuous intrathecal injection of 2%DMSO(10 ul), 5 ug CXCL1 antibody(10 ul), 10 ug SB225002(10 ul), and BAY11-7082(10 ul),respectively, on the 3rd, 4th, and 5th days after surgery.

for perfusion and fixation and then switched to slow perfusion (2-3 drops/s) when the limbs began to stiffen, with a total perfusion time of 20 min. The spinal cord was removed immediately after perfusion and placed in 4% paraformaldehyde. The spinal cord was fixed in 4% paraformaldehyde for 24 h and then transferred to 30% sucrose solution (4°C). After the tissue block had settled, the L5 spinal tissues were harvested and cryosectioned at 20 μm. Sections were rinsed, sealed, and incubated with the following primary antibodies: CXCL1 (1:400, rabbit, Affinity), CXCR2 (1:400, rabbit, Abcam), NFκB (1:500, rabbit, Affinity), glial fibrillary acidic protein(GFAP) (1:1000, mouse, Abcam), Neun (1:500, mouse, Abcam) and CD11b (1:500, mouse, Abcam) at 4°C for 12h, rinsed and incubated with Alexa Fluor 488 donkey anti-rabbit IgG (1:1000, Invitrogen) and Alexa Fluor 548 donkey anti-Mouse IgG (1;10000, Invitrogen) for 2h in the greenhouse. Sections were rinsed, mounted, blocked with a fluorescence blocker, and observed under an inverted fluorescence microscope.

Western Blot

The L5 spinal tissues were homogenized with ice-cold lysis buffer containing 150 mMNaCl, 20 mMTris-HCl (PH7.6), 1 mM EDTA, 1% NP-40, 1 mM PMSF, protease inhibitor cocktail (#78439, Thermo Fisher, USA), and phosphatase inhibitor cocktail (#78439, Thermo Fisher, USA). Protein concentrations were determined by the BCA Protein assay (Pierce, Rockford, IL). Equal amounts of proteins were separated on 10% sodium dodecyl sulfate gels. Polyvinylidene difluoride membranes were used for protein transfer. Following 2h of blocking with 5% skimmed milk, the membrane was then incubated with the following primary antibodies: GAPDH(1:1000, mouse, Abcam), CXCL1(1:400, rabbit, Affinity), CXCR2 (1:400, rabbit, Abcam) and NFκB(1:500, rabbit, Affinity). The polyvinylidene difluoride membrane was rinsed three times with TBST, and the peroxidase-conjugated rabbit anti-rat (1:100, BOSTER) was added and incubated for 2h. Grey-scale analysis was then performed on the bands through Image J software.

Real-Time PCR

Total RNA was extracted from the spinal cord using the TRIZOL extraction method, and the RNA concentration and purity were measured. Gene-specific primers were designed using Primer 5.0, and cDNA was pre-denatured, denatured, annealed, and extended according to the appropriate amplification conditions of the PCR amplification kit. Each sample's cycle threshold (Ct value) was then calculated. The Bio-Rad CFX Manager Software 1.6 was utilized to compute the proportional amounts of CXCL1 mRNA, CXCR2 mRNA, COX-2 mRNA, and TNF-α mRNA using the 2-ΔΔCt technique (Table 1).

Intrathecal Drug Injection

After anesthetizing the rats, they were positioned in the prone position within the L4-L5 interval. The skin was then prepared, disinfected, and toweled before being punctured with a thick needle. Subsequently, the needle was gradually inserted into the intervertebral space using a microinjector. Successful positioning was indicated by a tail twitch or flutter, at which point the needle was maintained in its position, and the drug was slowly injected.

TABLE 1. Primers used for Real-time Polymerase Chain Reaction

Genes	Forward (5′-3′)	Reverse (5′-3′)
CXCL1	GCAGACAGTGGCAGGGATTC	CGACCATTCTTGAGTGTGGCTAT
CXCR2	GTTCTTTGCCCTGACCTTGCC	TGTACTTGTGGCGTGGACGAT
TNF-α	GCCACCACGCTCTTCTGTCTA	CGCTTGGTGGTTTGCTACGA
GAPDH	CTGGAGAAACCTGCCAAGTATG	GGTGGAAGAATGGGAGTTGCT
COX-2	ACACTCTATCACTGGCATCC	GAAGGGACACCCTTTCACAT

Statistical Analysis

All experimental results were analyzed using SPSS 20.0 software (SPSS Inc, Chicago, USA). To compare differences between groups in behavioral testing, analysis of variance was employed. Dunnett's method was used to compare differences at different time points within a group and between different groups at the same time point. A comparison of the relative concentration of protein, relative concentration of mRNA, and semiquantitative data from immunofluorescence staining was conducted utilizing one-way ANOVA and Dunnett's pairwise comparison. A statistically significant difference is indicated by $P < 0.05$.

RESULTS

Autologous NP Induces Pain in Rats and Activates the COX-2 and TNF-α in Spinal Cord

Determination of mechanical pain threshold and thermal pain threshold before and after modeling in rats shows that the 50%MWT and TWL of the blank group are both at a high level; the 50%MWT (15.3 ± 1.76 g) and TWL (11.7 ± 2.16S) of sham group decreased after surgery and finally returned to the preoperative level. The 50%MWT (4.9 ± 0.54 g) and TWL (5.5 ± 1.26S) of the NP group decreased significantly at d3 and persisted until d28 after surgery, indicating that the autologous NP transplantation induced pain behavior in rats and could produce stable radicular pain (Fig. 2).

Detecting spinal inflammatory factors COX-2 and TNF-α at different time points in rats treated with different groups, compared with the sham group, the NP group had TNF-α mRNA significantly increased on day 1 ($P < 0.001$) and continued until day 14 ($P < 0.001$); The COX-2 mRNA in the NP group began to increase 1 day after surgery ($P < 0.001$) and then decreased slightly, but both were significantly higher than the sham group ($P < 0.001$) (Fig. 3).

The CXCL1 and CXCR2 Are Persistently Increased in Spinal Astrocytes of Rats with NP-induced Radicular Pain, and CXCL1 was Co-stained With Astrocytes and CXCR2 With Neurons

We found that the fluorescence density of GFAP was increased in the spinal cord in the NP group, and astrocytes were significantly activated in the NP group *versus* the sham group by immunofluorescence on day 7 (Fig. 4). The mRNA and protein of CXCL1 were significantly higher in the spinal cord on postoperative d3, d7, and d14 versus the sham group ($P < 0.05$) (Fig. 5). Co-staining of CXCL1 with neuronal marker protein NeuN, microglial marker protein CD11b, and astrocytic marker protein GFAP using immunofluorescence double labeling showed that spinal CXCL1 was expressed only in astrocytes (Fig. 6), while CXCL1 expression was not observed in neurons and microglia. The results suggest that autologous NP placed in the dorsal root nerve can induce upregulation of CXCL1 expression in rat spinal astrocytes.

The RT-PCR and Western blot assay detected increased CXCR2 expression in the spinal cord of rats with medullary-derived radiculopathy. The NP group exhibited significantly higher mRNA and protein levels of CXCR2 on d3, d7, and d14 *versus* the Sham group ($P < 0.05$) (Fig. 7). CXCR2 was co-stained through the immunofluorescence double-labeling method with the NeuN, CD11b, and GFAP. The results indicate that spinal CXCR2 was solely expressed in neurons (Fig. 8) and not observed in astrocytes and microglia.

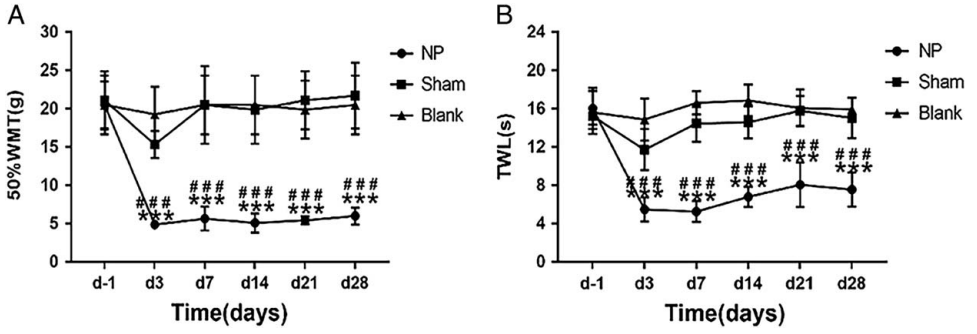

Figure 2. Behavioral changes of pain before and after surgery in rats. A, Changes in mechanical pain thresholds in rats. B, Changes in thermal pain foot reduction thresholds in rats. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus NP group of d-1; $^{\#\#\#}P < 0.001$ *versus* Sham group, the difference was statistically significant. n = 6.

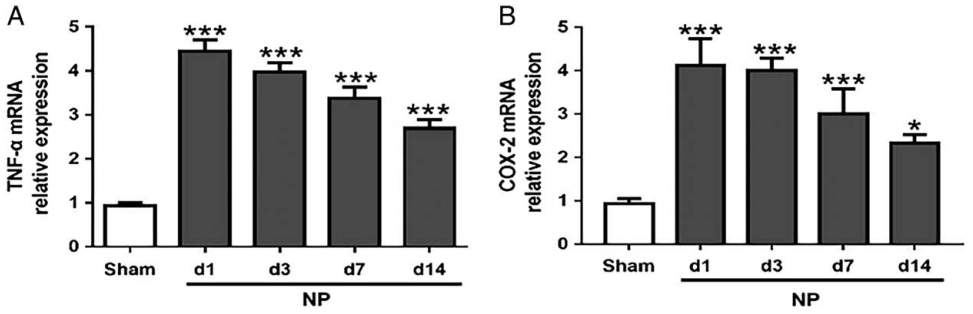

Figure 3. The mRNA levels of spinal TNF-α and COX-2 in different groups of rats. A, Expression of TNF-α mRNA in rat spinal cord. B, Expression of COX-2 mRNA in rat spinal cord. $^{*}P < 0.05$,$^{***}P < 0.001$, *versus* the Sham group, the difference was statistically significant.

Intrathecal Injection of CXCL1-neutralizing Antibody and CXCR2 Inhibitor Relieves NP-induced Radicular Pain

CXCL1 was significantly upregulated in the NP group. The role of spinal cord CXCL1 in rats with NP-induced radicular pain was observed by downregulating its expression. Compared with the NP+DMSO group, 50% MWT and TWL in the NP+CXCL1 antibody group were significantly increased after intrathecal administration of 5 ug (10 ul) of CXCL1-neutralizing antibody on d3, d4, and d5 after surgery ($P < 0.01$), and when the administration was stopped on d6 and d7 after surgery, 50% MWT and TWL were increased ($P < 0.05$), and there was no significant change on d8 ($P > 0.05$) (Fig. 9). After the injection of SB225002, the pain behavior of rats were altered. Compared with the NP+DMSO group, 10 ug(10 ul) of SB225002 was administered intrathecally on d3, d4, and d5 after surgery, and the 50% MWT and TWL of the NP+SB group were significantly increased ($P < 0.01$) (Fig. 10). The results showed that downregulation of CXCL1/CXCR2 expression in the spinal cord could significantly inhibit the NP-induced radicular pain in rats.

CXCL1-neutralizing Antibody Inhibits Autologous NP-induced Spinal Astroglial Activation and CXCR2 Activation

The activation of spinal astrocytes and the expression level of CXCR2 in the NP+DMSO, NP+CXCL1 antibody, Sham +DMSO, and Sham+CXCL1 antibody groups on d5 were examined by immunofluorescence and Western Blot. The results showed that the fluorescence density of GFAP in the spinal cord was increased and astrocytes were activated in the NP+DMSO group compared with the Sham+DMSO and Sham +CXCL1 antibody groups, and the fluorescence density of GFAP was decreased, and the activated morphology of astrocytes was suppressed in the NP+CXCL1 antibody group *versus* the NP+DMSO group (Fig. 11). Compared to the NP+DMSO group, the CXCR2 fluorescence density of the NP+CXCL1 antibody group decreased. The protein expression of CXCR2 in the spinal cord of the NP+CXCL1 antibody group significantly decreased compared to that of the NP+DMSO group. (Fig. 12).

SB225002 Inhibits CXCR2 Activation in Rats With NP-induced Radicular Pain but Does not Reverse the Activation of CXCL1

Western blot showed that CXCR2 expression was increased in the NP+DMSO group compared with the Sham+DMSO and Sham+CXCL1 antibody groups and decreased in the spinal cord of the NP+CXCL1 antibody group compared with the NP+DMSO group. CXCL1 expression was increased in the spinal cord of the NP+DMSO group compared to the Sham+DMSO and Sham+CXCL1 antibody groups; CXCL1 expression was not significantly changed in the spinal cord of the NP+CXCL1 antibody group compared to the NP+DMSO group (Fig. 13).

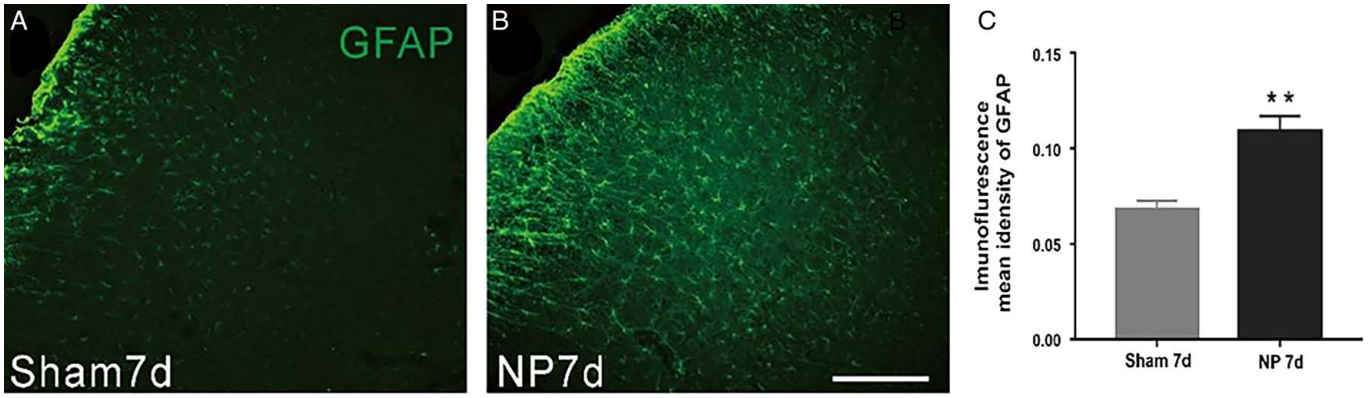

Figure 4. Autologous nucleus pulposus induces spinal astrocyte activation in rats with nucleus pulposus-induced radicular pain. A, Astrocyte activation in the Sham group. B, Astrocyte activation in the NP group. C, Intensity statistics show GFAP immunofluorescence intensity in the Sham and NP groups. Scar Bar in (B):100 μm. $^{**}P < 0.01$ *versus* the Sham group, the difference was statistically significant. n = 3.

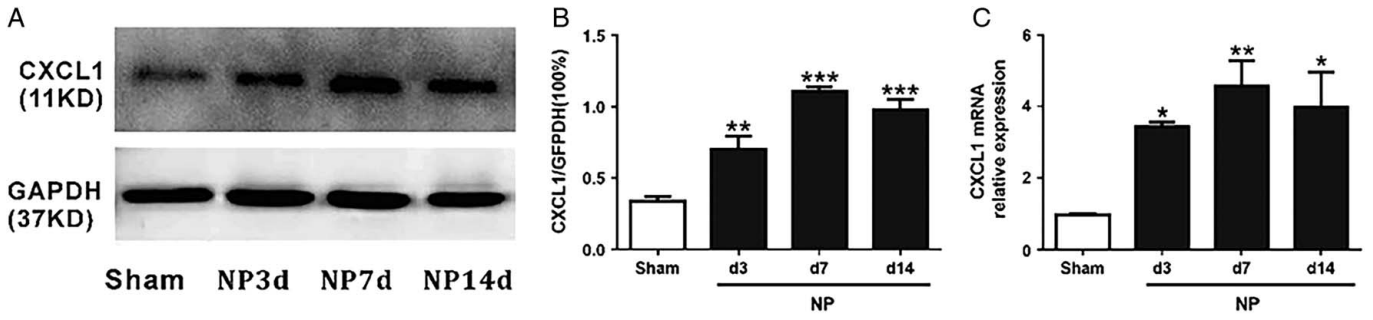

Figure 5. Autologous nucleus pulposus increased spinal CXCL1 in rats with nucleus pulposus-induced radicular pain. A, Expression of spinal CXCL1 in different groups. B, Quantitative analysis of spinal CXCL1 of Western Blot. C, CXCL1 mRNA expression of the spinal cord in rats. $^{*}P < 0.05$,$^{***}P < 0.001$ *versus* the Sham group, the difference was statistically significant. n = 3.

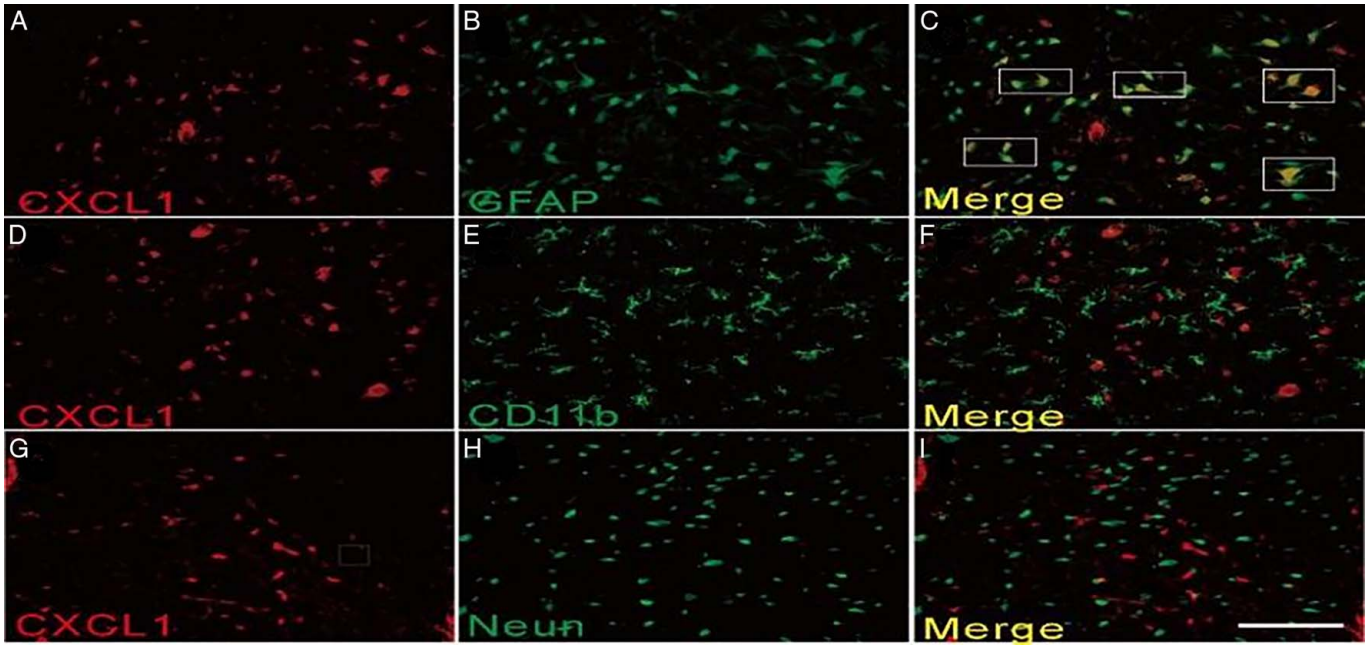

Figure 6. Cellular localization of spinal CXCL1 after autologous nucleus pulposus-induced radicular pain. A、D、G: Fluorescence staining of spinal CXCL1 on day 7 after operation in NP group. B, E, and H: Fluorescence staining of spinal GFAP, CD11b, and Neun, respectively, on day 7 after operation in the NP group. C, F, and I: Immunofluorescence double staining of CXCL1 (A, D, G) and GFAP (B), CD11b(E), Neun(H). Scale bar in (I): 100 μm. n = 3.

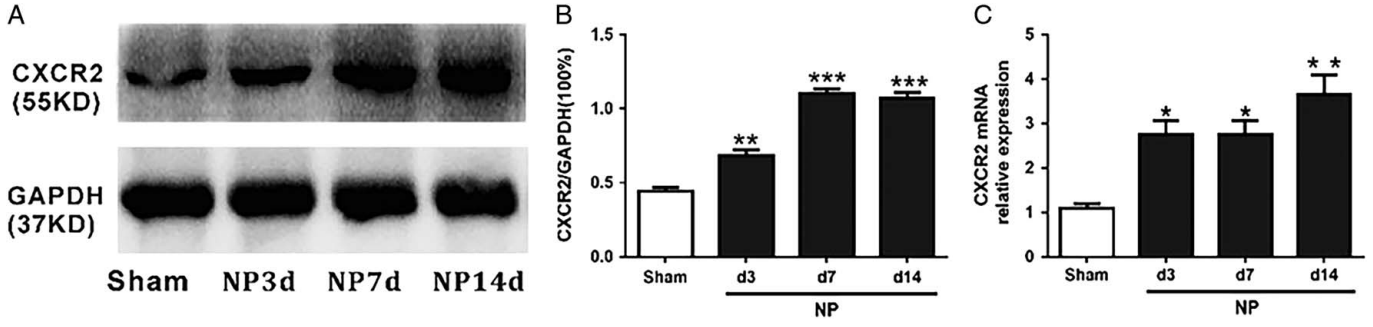

Figure 7. Autologous nucleus pulposus-induced inflammation increased CXCR2 expression in the spinal cord of rats. A, Expression of spinal CXCR2 in different groups. B, Quantitative analysis of spinal CXCR2 of Western Blot. C, CXCR2 mRNA expression of the spinal cord in rats. $^{**}P < 0.01$ *versus* the Sham group, the difference was statistically significant. n = 3.

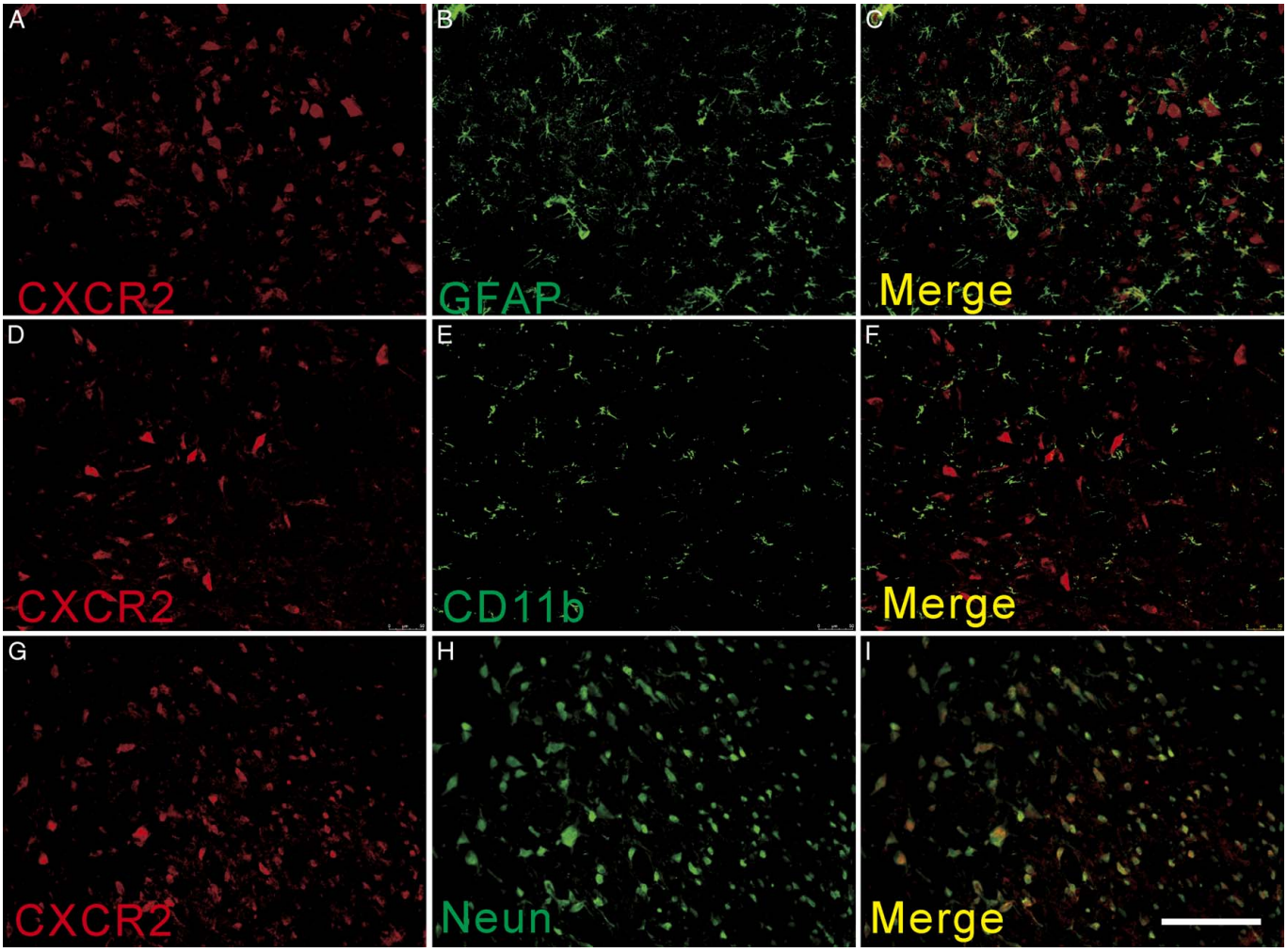

Figure 8. Cellular localization of spinal CXCR2 after autologous nucleus pulposus-induced radicular pain. A, D, G, CXCR2 fluorescence staining of spinal cord on day 7 after operation in NP group. B, E, H, GFAP, CD11b, and Neun, respectively, fluorescence staining of the spinal cord on day 7 after operation in NP group. C, F, I, Immunofluorescence double staining of CXCR2 (A, D, G) and GFAP (B), CD11b (E), Neun (H). Scale bar in (I): 100 μm. n = 3.

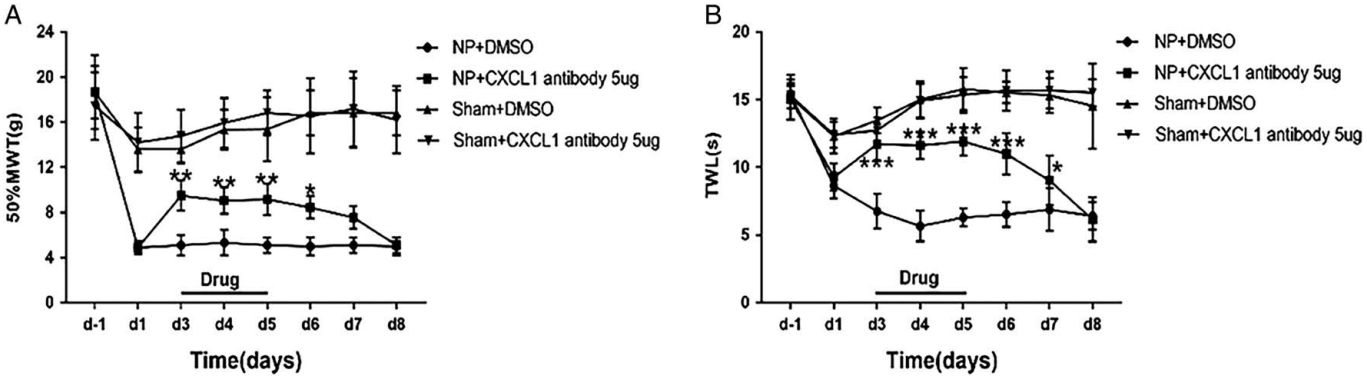

Figure 9. Behavioral changes of pain before and after drug administration in rats. A, Changes of 50% WMT before and after drug administration in rats. B, Changes of TWL before and after drug administration in rats. $^{*}P < 0.05$,$^{**}P < 0.01$,$^{***}P < 0.001$, *versus* NP+DMSO group, the difference was statistically significant. n = 6.

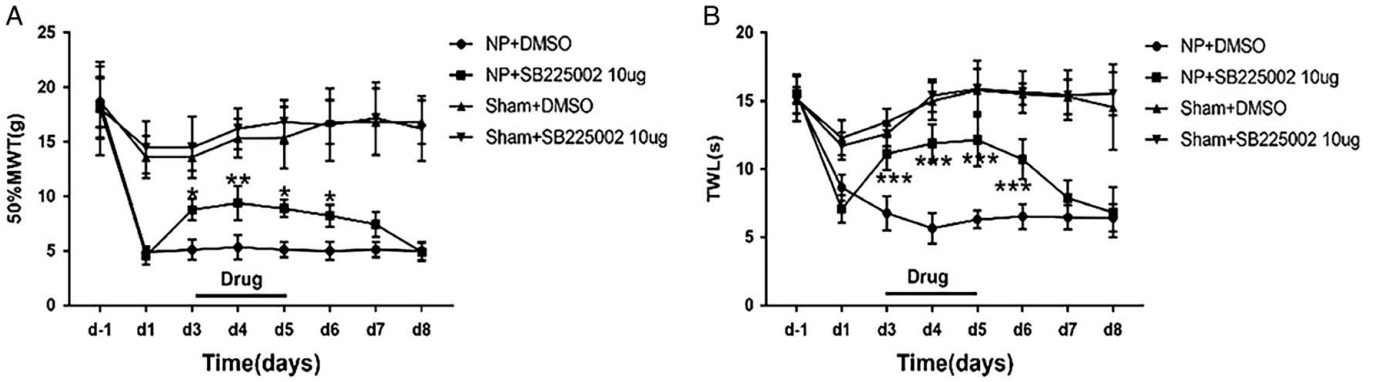

Figure 10. Behavioral changes before and after drug administration in rats. A, Changes of 50% WMT before and after drug administration in rats. B, Changes of TWL before and after drug administration in rats. $^{*}P < 0.05$,$^{**}P < 0.01$,$^{***}P < 0.001$, *versus* NP+DMSO group, the difference was statistically significant. n = 6.

Increased Spinal NFκB Expression and Co-staining of NFκB With Neurons and Astrocytes in the Spinal Cord of Rats With NP-induced Radicular Pain

The results of both Western blot and immunofluorescence showed that NFκB expression in the spinal cord was significantly increased in the NP group VS sham group on d3, d7, and d14 (Fig. 14). To clarify the expression site of spinal NFκB, NFκB was co-stained with the NeuN, and GFAP (Fig. 15).

BAY11-7082 can Significantly Alleviate Hyperalgesia in Rats With NP-induced Radicular Pain

Administration of BAY11-7082 showed that the down-regulation of NFκB at the spinal cord level significantly inhibited NP-induced radicular pain(Fig. 16A). Changes in 50% MWT after administration of BAY11-7082: On postoperative d3, d4, d5, and d6, intrathecal administration of BAY11-7082 5 ug (10 ul) significantly increased TWL in the NP+BAY group

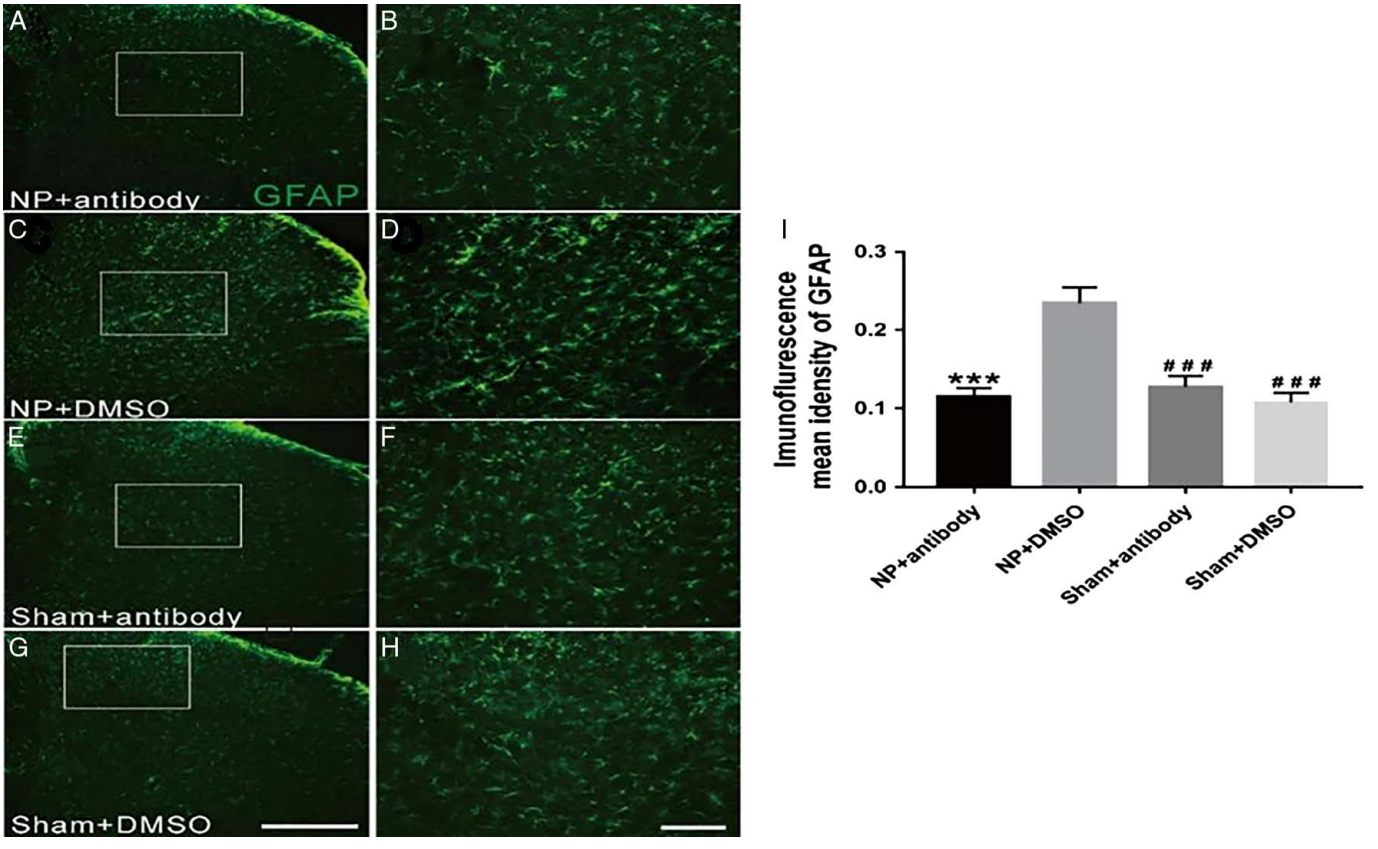

Figure 11. astrocyte activation in rat spinal cord after giving different drugs. A, C, E, and G: Fluorescence staining (20x) of GFAP in NP+CXCL1 antibody、 NP+DMSO, Sham+CXCL1 antibody, and Sham+DMSO group. B, D, F, and H: Fluorescence staining(40x) of GFAP in NP+CXCL1 antibody, NP+DMSO, Sham+CXCL1 antibody, and Sham+DMSO group. I: Quantitative analysis of spinal GFAP fluorescence staining. Scar Bar in (G) :100 μm, Scar Bar in (H) :50 μm. $^{*}P < 0.05$,$^{**}P < 0.01$,$^{***}P < 0.001$, *versus* NP+DMSO group:$^{\#\#\#}P < 0.05$, versus NP+DMSO group, the difference was statistically significant. n = 3.

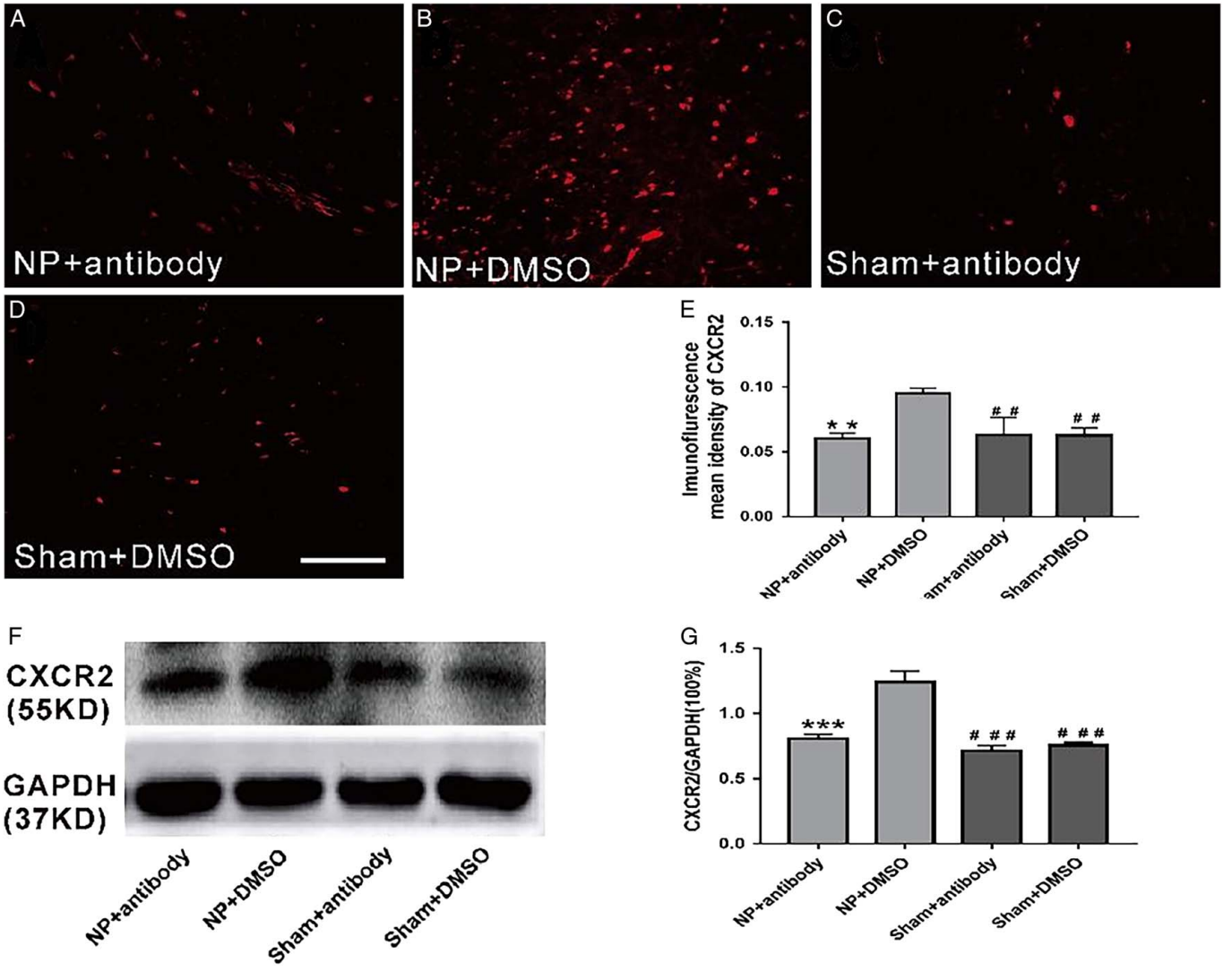

Figure 12. CXCR2 expression of rat spinal cord in different groups. A, B, C, and D, CXCR2 expression in the different groups of NP+CXCL1 antibody、 NP+DMSO, Sham+CXCL1 antibody, and Sham+DMSO. E, Intensity statistics show GFAP immunofluorescence intensity in the different groups of NP+CXCL1 antibody, NP+DMSO, Sham+CXCL1 antibody, and Sham+DMSO. F, Expression of CXCR2 in different groups of Western Blot. G, Quantitative analysis of spinal cord CXCR2 of Western blot. Scar Bar in (D) :100 μm. $^{*}P<0.05$,$^{**}P<0.01$,$^{***}P<0.001$, *versus* NP +DMSO group;$^{\#\#\#}P<0.05$, *versus* NP+DMSO group, the difference was statistically significant. n = 3.

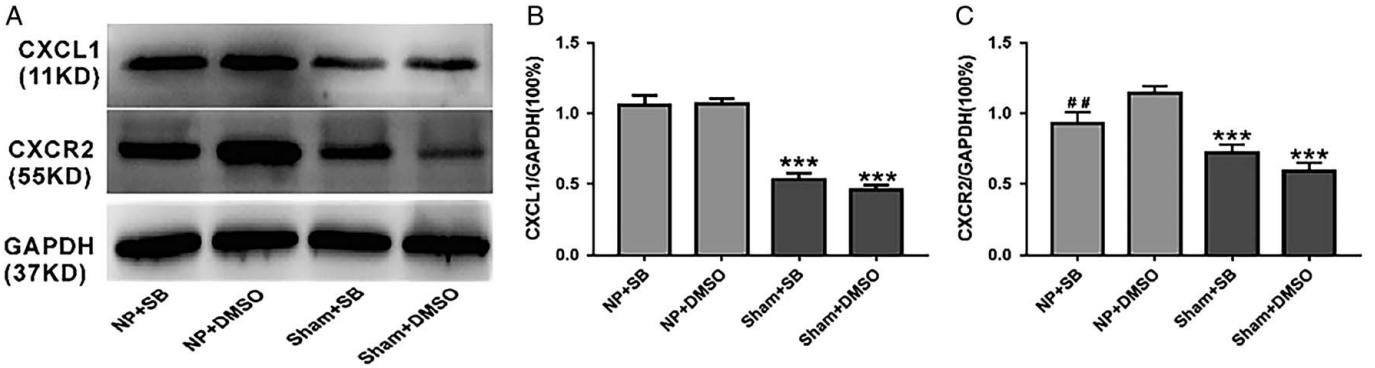

Figure 13. CXCL1 and CXCR2 expression of rat spinal cord in different groups. A, Expression of CXCL1 and CXCR2 in different groups of western blot. B and C, Quantitative analysis of spinal cord CXCL1 and CXCR2 of western blot, respectively. $^{*}P<0.05$,$^{**}P<0.01$,$^{***}P<0.001$ *versus* NP +DMSO group. $^{\#\#}P<0.01$ *versus* NP+DMSO group, the difference was statistically significant. n = 3.

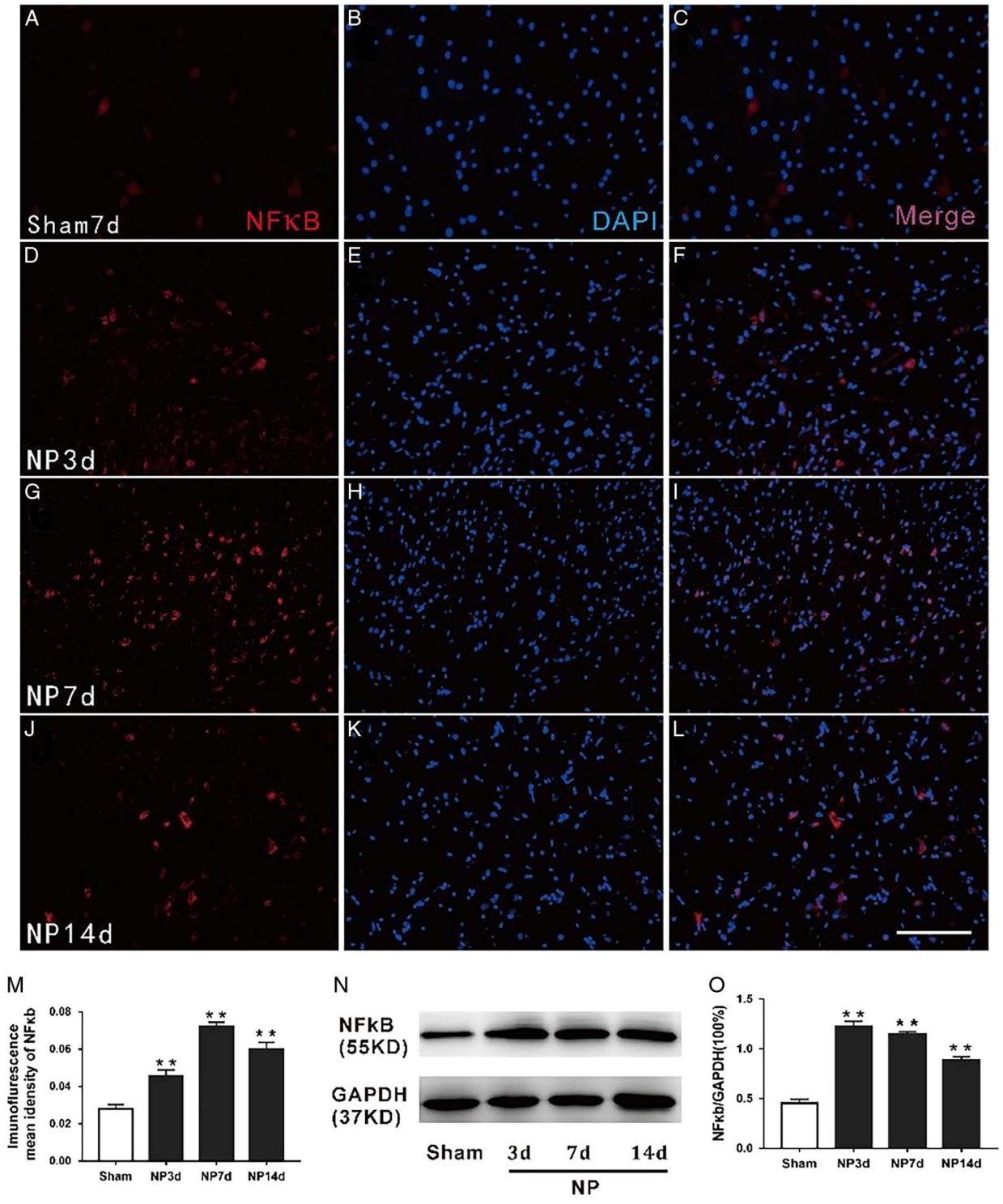

Figure 14. Expression of spinal NFκB in rats with nucleus pulposus-induced radicular pain. A, D, G, J, Fluorescence staining of spinal NFκB after 7 days of Sham group and after 3 days,7 days, and 14 days of NP group, respectively. B, E, H, and K: Fluorescence staining of DAPI after 7 days of the Sham group and after 3 days, 7 days, and 14 days of the NP group, respectively. C, F, I, and L, Immunofluorescence double staining of DAPI and NFκB after 7 days of the Sham group and after 3 days, 7 days, and 14 days of the NP group, respectively. M, Intensity statistics shows NFκB immunofluorescence intensity after 7 days of the Sham group and after 3 days, 7 days, and 14 days of NP group. N, Expression of NFκB in different groups of Western Blot. O, Quantitative analysis of spinal NFκB of western blot. Scar Bar in (L) :100 μm.*$P < 0.05$, **$P < 0.001$, *versus* Sham group, the difference was statistically significant. n = 3.

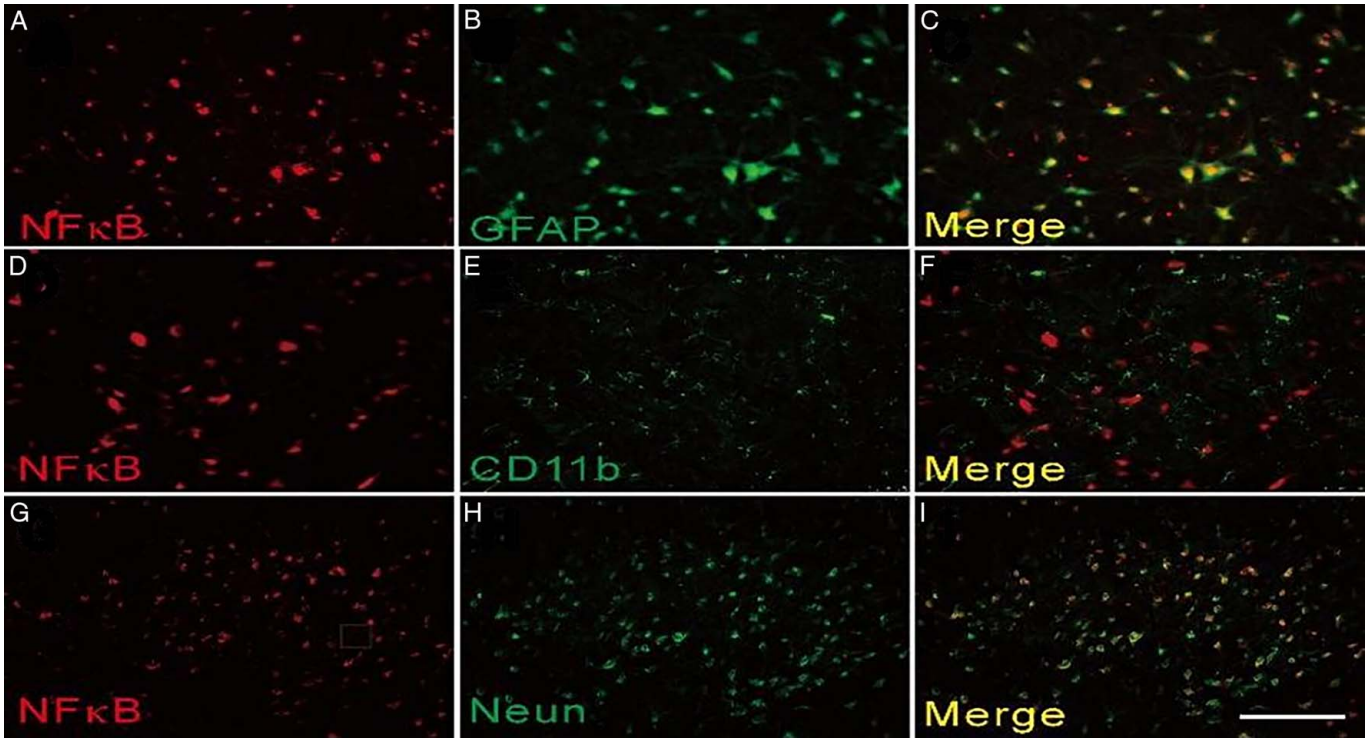

Figure 15. Cellular localization of spinal NFκB expression in rats with autologous nucleus pulposus-induced radicular pain. A, D, G, Fluorescence staining of spinal NFκB on day 7 after operation in NP group. B, E, and H, Fluorescence staining of spinal GFAP, CD11b, and Neun, respectively, on day 7 after operation in the NP group. C, F, I, Immunofluorescence double staining of NFκB (A, D, and G) and GFAP (B), CD11b(E), and Neun (H). Scale bar in (I): 100 μm. n = 3.

compared to the NP+DMSO group ($P < 0.001$). On postoperative d7, TWL was increased in the NP+BAY group *versus* the NP+DMSO group ($P < 0.05$). On postoperative d8, there was no significant change in TWL in the NP+BAY group *versus* the NP+DMSO group ($P > 0.05$). The results showed that the downregulation of NFκB in the spinal cord could significantly inhibit radicular pain hypersensitivity in our model. (Fig. 16B).

Intrathecal Injection of BAY11-7082 Inhibits the Increase in CXCL1/CXCR2 Expression Induced by Autologous NP

Western Blot assay detected the expression levels of CXCL1 and CXCR2 in the spinal cord of rats after administration of BAY11-7082, and the results showed that CXCL1 and CXCR2 expression was increased in the spinal cord of the NP+DMSO group compared with the Sham+DMSO group and the Sham+BAY group; Compared to the NP+DMSO group, the CXCR2 in the spinal cord of the NP+BAY group noticeably increased. In contrast, the CXCL1 was lower in the NP+DMSO group *versus* the Sham+BAY group. (Fig.17).

DISCUSSION

In a rat model of NP-induced radicular pain, astrocyte activation was found, which is consistent with many studies that astrocyte activation is present in chronic pain and is involved in pain onset and progression.[13,14] Among the

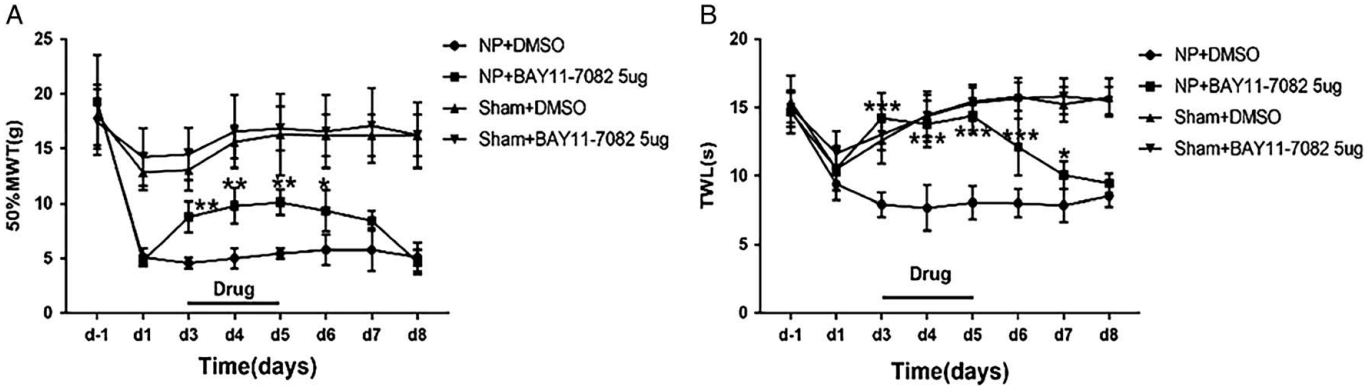

Figure 16. Behavioral changes of pain before and after drug administration in rats. A, Changes of 50% WMT before and after drug administration in rats. B, Changes of TWL before and after drug administration in rats. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* NP+DMSO group, the difference was statistically significant. n = 6.

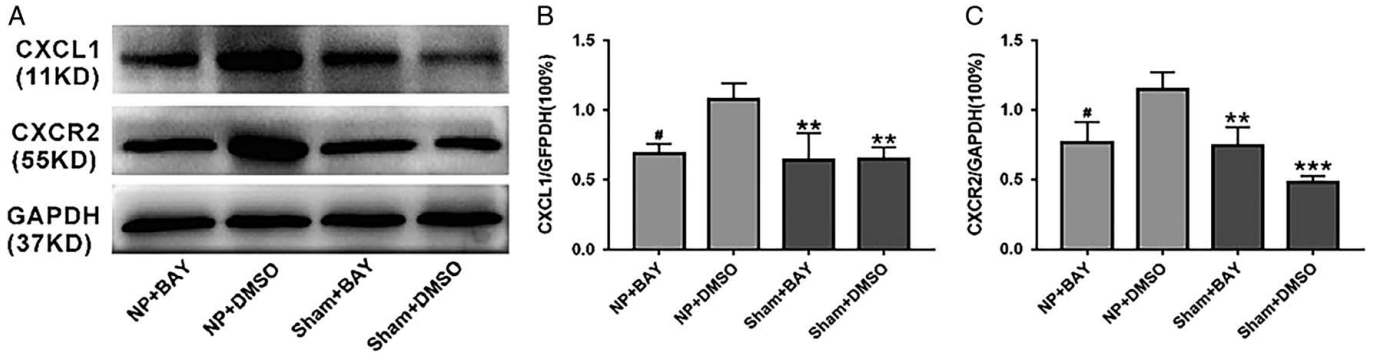

Figure 17. Expression of spinal CXCL1 and CXCR2 in different groups. A, Expression of CXCL1 and CXCR2 in different groups of western blot. B and C, Quantitative analysis of spinal cord CXCL1 and CXCR2 of western blot, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* NP+DMSO group; $^{\#}P < 0.05$ *versus* NP+DMSO group, the difference was statistically significant. n = 3.

inflammatory chemokines, the role of CXCL1 in pain behavior has attracted considerable attention.[15–17]

In addition, we found that spinal CXCR2 proteins were significantly upregulated in rats with medullary radicular pain on d3, d7, and d14, and fluorescent double staining showed co-staining with spinal neurons, which is consistent with many neuropathic pain models and radicular models of LDH causing chronic pain.[5,18,19] In this experiment, a neutralizing antibody to CXCL1 and an inhibitor of CXCR2 were used to intervene at the spinal level in rats with NP-induced radicular pain. The results showed that both drug interventions significantly inhibited nociceptive hyperalgesia in rats, but the duration of action was only maintained for five to six days after three days of continuous administration, after which it returned to pre-drug levels. This suggests that CXCL1 and CXCR2 are involved in the mechanism of autologous NP-induced radicular pain, and this is similar with neuropathic pain.[17] CXCL1 plays an important role in the development of peripheral nerve injury-induced neuropathic pain,[18] acute pain[20] and pathological pain.[21] Intraplantar injection of CXCL1 produced hyperalgesia to thermal and mechanical stimulation in rats.[22] Chemokine CXCL1 dimer is a potent agonist for the CXCR2 receptor in tissue injury,[4,23] by coupling to distinct G protein-coupled receptor kinases to regulate leukocyte functions.[24,25]

NFκB can be involved in the development of neuropathic pain by promoting CXCL1 production,[6,26,27] which has not been reported in NP-induced radicular pain models. In the present study, we found that spinal NFκB was expressed in astrocytes and neurons. However, activation of NFκB in the spinal dorsal horn occurred only in astrocytes in the mouse pain model induced by bone cancer;[27] whereas in the ventral peripheral gray matter in this model, activation of NFκB occurred in astrocytes and neurons.[6] The above results suggest that different stimulation conditions may lead to different cell types expressing NFκB, which in turn are involved in different chronic pain through different pathways and mechanisms. Chemotherapy drugs, vincristine and paclitaxel injection, cause neuropathic pain, inducing NFκB activation and expression of pro-inflammatory factors and the activation of spinal astrocyte.[7,28] GRO/KC has important effects on inflammatory processes through its direct actions on sensory neurons, and the activation of NFkB is involved in the GRO/KC-induced enhancement of K currents.[26] In the present experiment, based on the finding of the upregulation of spinal NFκB, we administered its inhibitor BAY11-7082 intrathecally and showed that NFκB inhibitor could alleviate nociceptive hyperalgesia and significantly downregulated the expression of spinal CXCL1 and CXCR2, suggesting that spinal NFκB is involved in CXCL1 expression through the regulation of pain initiation in rats with NP-induced radicular pain. Given our study design, these findings should be considered hypothesis-generating and prompt further research in both the basic science and clinical realms.

In conclusion, the study demonstrates that spinal NFκB is involved in NP-induced radicular pain in rats through the activation of CXCL1/CXCR2, enriching the mechanism of medullary-derived radicular pain and providing a possible new target and theoretical basis for the development of more effective anti-inflammatory and analgesic drugs for patients with chronic pain following LDH (Supplemental Fig. 2, Supplemental Digital Content 1, http://links.lww.com/BRS/C353).

➢ Key Points

- ❑ Autologous nucleus pulposus transplantation can induce radicular pain in rats.
- ❑ NF-κB and CXCL1/CXCR2 were upregulated in the spinal cord of this rat model.
- ❑ CXCL1 co-expressed with astrocyte and CXCR2 co-expressed with neurons.
- ❑ Both CXCL1-neutralizing antibody and CXCR2 inhibitor can alleviate autologous nucleus pulposus-related pain.
- ❑ NF-κB inhibitor alleviates radicular pain and inhibits the increase in CXCL1/CXCR2 expression.

References

1. Murai K, Sakai D, Nakamura Y, et al. Primary immune system responders to nucleus pulposus cells: evidence for immune response in disc herniation. *Eur Cell Mater*.. 2010;19:13–21.

2. Luo J, Feng J, Liu S, Walters ET, Hu H. Molecular and cellular mechanisms that initiate pain and itch. *Cellu Mol Life Sci*. 2015; 72:3201–23.
3. Kim SJ, Park SM, Cho YW, et al. Changes in expression of mRNA for interleukin-8 and effects of interleukin-8 receptor inhibitor in the spinal dorsal horn in a rat model of lumbar disc herniation. *Spine*. 2011;36:2139–46.
4. Ravindran A, Sawant KV, Sarmiento J, Navarro J, Rajarathnam K. Chemokine CXCL1 dimer is a potent agonist for the CXCR2 receptor. *J Biolo Chem*. 2013;288:12244–52.
5. Yang LH, Xu GM, Wang Y. Up-regulation of CXCL1 and CXCR2 contributes to remifentanil-induced hypernociception via modulating spinal NMDA receptor expression and phosphorylation in rats. *Neurosci Lett*. 2016;626:135–41.
6. Ni H, Wang Y, An K, et al. Crosstalk between NFkappaB-dependent astrocytic CXCL1 and neuron CXCR2 plays a role in descending pain facilitation. *J Neuroinflammation*. 2019;16:1.
7. Zhou L, Hu Y, Li C, et al. Levo-corydalmine alleviates vincristine-induced neuropathic pain in mice by inhibiting an NF-kappa B-dependent CXCL1/CXCR2 signaling pathway. *Neuropharmacology*. 2018;135:34–47.
8. Wang D, Pan H, Zhu H, Zhu L, He YJ, Wang J, Jia GY. Upregulation of nuclear factor-kappaB and acid sensing ion channel 3 in dorsal root ganglion following application of nucleus pulposus onto the nerve root in rats. *Mol Med Rep*. 2017;16:4309–14.
9. Acosta JC, O'Loghlen A, Banito A, et al. Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell*. 2008;133: 1006–18.
10. Sasaki N, Kikuchi S, Konno S, Sekiguchi M, Watanabe K. Anti-TNF-alpha antibody reduces pain-behavioral changes induced by epidural application of nucleus pulposus in a rat model depending on the timing of administration. *Spine (Phila Pa 1976)*. 2007;32:413–6.
11. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods*. 1994;53:55–63.
12. Gonzalez-Cano R, Boivin B, Bullock D, Cornelissen L, Andrews N, Costigan M. Up-Down Reader: An Open Source Program for Efficiently Processing 50% von Frey Thresholds. *Frontiers in pharmacology*. 2018;9:433.
13. Ueda H, Neyama H, Nagai J, Matsushita Y, Tsukahara T, Tsukahara R. Involvement of lysophosphatidic acid-induced astrocyte activation underlying the maintenance of partial sciatic nerve injury-induced neuropathic pain. *Pain*. 2018;159:2170–8.
14. Lv J, Li Z, She S, Xu L, Ying Y. Effects of intrathecal injection of rapamycin on pain threshold and spinal cord glial activation in rats with neuropathic pain. *Neurol Res*. 2015;37:739–43.
15. Cao DL, Zhang ZJ, Xie RG, Jiang BC, Ji RR, Gao YJ. Chemokine CXCL1 enhances inflammatory pain and increases NMDA receptor activity and COX-2 expression in spinal cord neurons via activation of CXCR2. *Exper Neurol*. 2014;261:328–36.
16. Chen G, Park CK, Xie RG, Berta T, Nedergaard M, Ji RR. Connexin-43 induces chemokine release from spinal cord astrocytes to maintain late-phase neuropathic pain in mice. *Brain: J Neurol*. 2014;137(Pt 8):2193–209.
17. Zhang ZJ, Cao DL, Zhang X, Ji RR, Gao YJ. Chemokine contribution to neuropathic pain: respective induction of CXCL1 and CXCR2 in spinal cord astrocytes and neurons. *Pain*. 2013; 154:2185–97.
18. Cao L, Malon JT. Anti-nociceptive Role of CXCL1 in a Murine Model of Peripheral Nerve Injury-induced Neuropathic Pain. *Neuroscience*. 2018;372:225–36.
19. Cao DL, Qian B, Zhang ZJ, Gao YJ, Wu XB. Chemokine receptor CXCR2 in dorsal root ganglion contributes to the maintenance of inflammatory pain. *Brain Res Bull*. 2016;127:219–25.
20. Deftu AF, Filippi A, Shibsaki K, Gheorghe RO, Chiritoiu M, Ristoiu V. Chemokine (C-X-C motif) ligand 1 (CXCL1) and chemokine (C-X-C motif) ligand 2 (CXCL2) modulate the activity of TRPV1+/IB4+ cultured rat dorsal root ganglia neurons upon short-term and acute application. *J Physiol Pharmacol*. 2017;68: 385–95.
21. Silva RL, Lopes AH, Guimarães RM, Cunha TM. CXCL1/CXCR2 signaling in pathological pain: role in peripheral and central sensitization. *Neurobiol Dis*. 2017;105:109–16.
22. Qin X, Wan Y, Wang X. CCL2 and CXCL1 trigger calcitonin gene-related peptide release by exciting primary nociceptive neurons. *J Neurosci Res*. 2005;82:51–62.
23. Tsai HH, Frost E, To V, et al. The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. *Cell*. 2002;110:373–83.
24. Molteni R, Crespo CL, Feigelson S, et al. Beta-arrestin 2 is required for the induction and strengthening of integrin-mediated leukocyte adhesion during CXCR2-driven extravasation. *Blood*. 2009;114:1073–82.
25. Raghuwanshi SK, Su Y, Singh V, Haynes K, Richmond A, Richardson RM. The chemokine receptors CXCR1 and CXCR2 couple to distinct G protein-coupled receptor kinases to mediate and regulate leukocyte functions. *J Immunol (Baltimore, Md: 1950)*. 2012;189:2824–32.
26. Yang RH, Strong JA, Zhang JM. NF-kappaB mediated enhancement of potassium currents by the chemokine CXCL1/growth related oncogene in small diameter rat sensory neurons. *Mol Pain*. 2009;5:26.
27. Xu J, Zhu MD, Zhang X, Tian H, Zhang JH, Wu XB, et al. NFkappaB-mediated CXCL1 production in spinal cord astrocytes contributes to the maintenance of bone cancer pain in mice. *J Neuroinflamm*. 2014;11:38.
28. Segat GC, Manjavachi MN, Matias DO, Passos GF, Freitas CS, Costa R, et al. Antiallodynic effect of β-caryophyllene on paclitaxel-induced peripheral neuropathy in mice. *Neuropharmacology*. 2017;125:207–19.