

CXCR4 antagonist AMD3100 elicits analgesic effect and restores the GlyR α 3 expression against neuropathic pain

Xiaoming Liu
Hongjun Liu
Lihua Dai
Bingjie Ma
Ke Ma

Pain Management Center, Xinhua
Hospital, School of Medicine, Shanghai
Jiao Tong University, Shanghai, People's
Republic of China

Objective: Chemokine CXCL12 and its receptor CXCR4 have been reported to play a critical role in neurogenesis and neuronal differentiation. Recently, some reports have implicated this chemokine signaling in the pathogenesis of many kinds of pain. However, its role in neuropathic pain (NP) is still largely unclear. This study explored the distribution and function of CXCR4 in spinal cord (SC) dorsal horn (DH) in a rat L5 spinal nerve ligation (SNL) model.

Methods: Rats received repeated intrathecal injection of CXCR4 antagonist AMD3100. Behavioral assessments were conducted using a traditional “up–down” method. The spinal CXCL12 contents were measured by enzyme linked immunosorbent assay. The expression and distribution of CXCR4 in the SC were determined by immunofluorescence and Western blot. GlyR α 3 expressions were also measured by Western blot or immunofluorescence.

Results: SNL induced CXCL12–CXCR4 activation in the spinal DH. Intrathecal administration of AMD3100 alleviated the chronic NP against SNL ($P < 0.01$). CXCR4 was colocalized with GlyR α 3-positive neurons in the spinal DH at ratio $> 97\%$. Meanwhile, AMD3100 rescued the decrease of GlyR α 3 expression ($P < 0.01$ vs the SNL group on Day 14 and Day 21).

Conclusion: CXCR4 antagonist can elicit analgesic effects and restore the inhibitory neurotransmission such as GlyR α 3 against NP.

Keywords: neuropathic pain, CXCL12, CXCR4, GlyR α 3, L5 spinal nerve ligation

Introduction

Neuropathic pain (NP) is a pain caused by a lesion or disease of the somatosensory system.¹ It is an intractable clinical disorder mainly characterized by hyperalgesia, allodynia, and spontaneous pain.² The current therapy for NP is not satisfactory. Although pharmacological management (such as antidepressants, anticonvulsants, and opioids) is widely used, more than two-thirds of NP patients obtain insufficient pain relief.²

After peripheral nerve injuries, nonneuronal cells such as microglial cells or astrocytes release numerous chemical mediators, which modulate the pain sensation by acting on neurons or other glial cells.³ Chemokines are a family of small secreted molecules (8–14 kDa) with verified roles in the many biological functions modulation, such as leukocyte chemoattractant and cytokine activation.^{4,5} CXCL12 and its major receptor CXCR4 are widely existed in the nervous system.⁶ Recently, some reports have implicated this chemokine signaling in the pathogenesis of inflammation pain,⁷ opiate-induced hyperalgesia,⁸ diabetic neuropathy,⁹ and bone cancer pain.¹⁰ CXCL12/CXCR4 signaling was demonstrated to mediate the release of proinflammatory cytokine¹¹ and neurotransmitters¹² (such as glutamic acid and prostaglandins) from glial cells, which

Correspondence: Ke Ma
Pain Management Center, Xinhua
Hospital, School of Medicine, Shanghai
Jiao Tong University, 1665# Kongjiang
Road, Shanghai 210092, People's Republic
of China
Tel +86 21 2507 8707
Fax +86 21 2507 8922
Email marke72@163.com

are involved in the maintenance of NP. GlyR α 3 is distinctly expressed in superficial spinal lamina and selectively involved in pathological pain.^{13,14} GlyR α 3 is regarded as a typical element in the inhibitory neurotransmission circuit.¹⁴ Whether CXCL12/CXCR4 affects GlyR α 3 expression and contributes to the allodynia after peripheral nerve injury remains largely unknown. The present study therefore was performed to study 1) the distribution and expression of CXCR4 in spinal cord (SC) in a rat model of L5 spinal nerve ligation (SNL) model; 2) whether administration of CXCR4 antagonist AMD3100 would alleviate the allodynia against SNL; and 3) whether AMD3100 would affect the expression of GlyR α 3 expression after SNL.

Methods

Animals

Adult male Sprague Dawley (SD) rats (weight: 150–180 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd, Shanghai Branch, Experimental Animal Center of Chinese Academy of Sciences (Shanghai, People's Republic of China). The rats were acclimated in a SPF room and provided with a standard diet at the animal center in Shanghai Xinhua Hospital for 5 days at baseline. All animal procedures in this study were performed according to the Guide for the Care and Use of Laboratory Animals of the International

Association for the Study of Pain. The whole protocol was approved by the Animal Care and Use Committee of Xinhua Hospital Experimental Animal Center.

Study design

Rats were performed SNL surgery on Day 1. AMD3100 was administrated once daily from Days 1 to 14. This study consisted of three phases. In Phase I (total of 34 rats), the distribution (n=3 for every point in time) and expression (n=5 for every point in time) of CXCR4 in the ipsilateral SC after SNL were studied at baseline on Day 14 and Day 21. And the ipsilateral spinal CXCL12 expressions were assayed at baseline on Day 7 and Day 10 (n=5 for every point in time). In Phase II (total of 18 rats), CXCR4 antagonist AMD3100 was used to assess the effects against NP. Rats were randomly assigned to three experimental groups, the sham group, the control (SNL) group, and the AMD group. Behavioral assessments were performed at baseline, on Day 7, Day 14, and Day 21, separately (n=6 for every group). In Phase III (total of 63 rats), SC specimen was collected and the protein expressions of GlyR α 3 were measured by Western blot at baseline on Day 14 and Day 21 (n=5 for every time point in every group). The distribution and signals of GlyR α 3 were studied at baseline and on Day 14 by immunofluorescence (n=3 for each time point in every group). The schematic diagram for study design is shown in Figure 1.

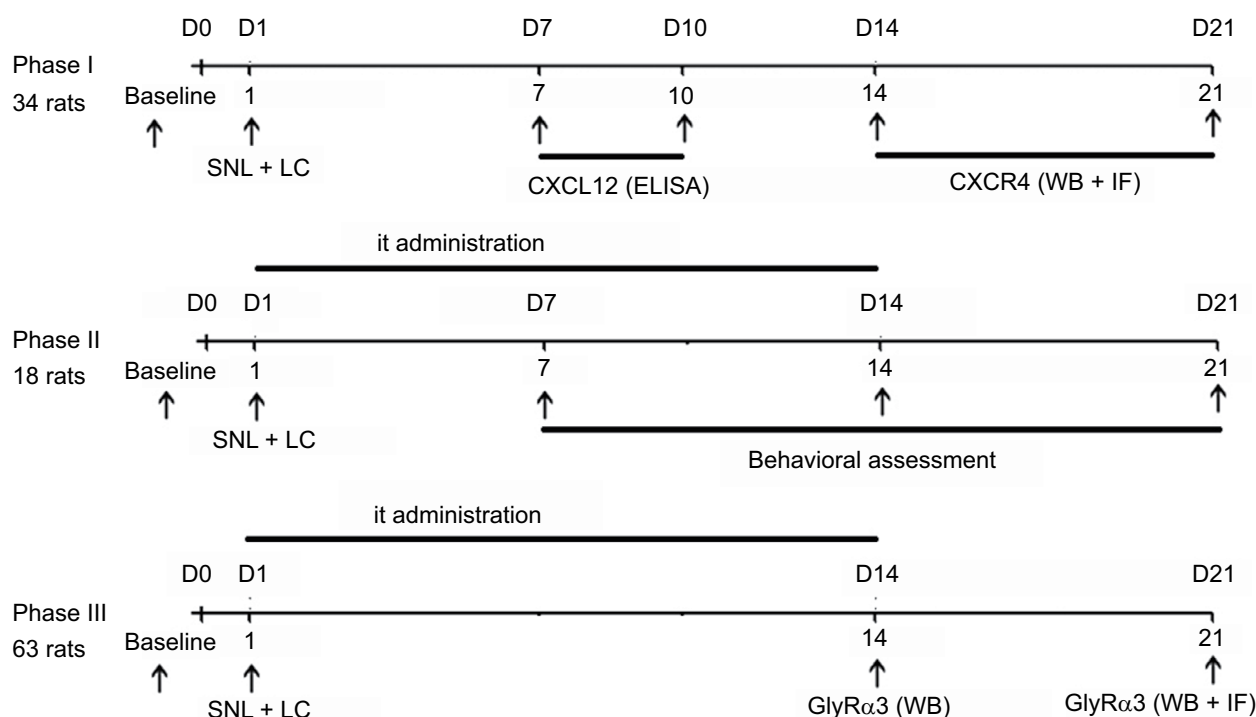


Figure 1 The schematic diagram for study schedule.

Notes: This study consisted of three phases. The baseline was defined as Day 0. The day of L5 SNL surgery and LC was referred to as Day 1. AMD3100 was administrated once daily from Days 1 to 14 at the dose of 1 μ g rat⁻¹ day⁻¹.

Abbreviations: ELISA, enzyme linked immunosorbent assay; IF, immunofluorescence; it, intrathecal; LC, lumbar catheterization; SNL, spinal nerve ligation; WB, Western blot.

Drugs and drugs' administration

Specific CXCR4 antagonist AMD3100 was purchased from Sigma-Aldrich (St. Louis, MO, USA). It was dissolved in phosphate-buffered saline (PBS) at the concentration of $0.5 \mu\text{g } \mu\text{L}^{-1}$ and injected ($1 \mu\text{g rat}^{-1} \text{ day}^{-1}$, intrathecally [it]) once daily from Days 1 to 14 using a lumbar catheterization technique. The day of SNL and lumbar catheterization surgery was referred to as Day 1. The doses of the drugs were chosen according to the results of our preliminary experiments and previous studies in the literature.^{8–10}

The drug administration using a lumbar catheterization technique was carried out in accordance with previous studies described.^{15,16} Briefly, rats were anesthetized with pentobarbital sodium (50 mg kg^{-1} intraperitoneally). The lumbar region was disinfected with 75% (v/v) ethanol after hair shaving. An incision was made over L4–L5 lumbar vertebrae. The intervertebral foramen was exposed after cutting the intervertebral ligament. Then, we inserted a PE-10 catheter filled with sterile 0.9% saline into the subarachnoid space. The correct intrathecal localization was confirmed by easy insertion of a catheter, by a tail-flick or a paw retraction, or occasionally by leakage of cerebrospinal fluid. After finishing the experiments, lidocaine ($15 \mu\text{L}$, $20 \mu\text{g } \mu\text{L}^{-1}$) followed by $10 \mu\text{L}$ of saline was injected through the catheters to verify an intraspinal location. An immediate motor paralysis of the hind part of the animal (within 15 s) lasting for 20–30 min indicated a correct intraspinal location. Finally, the catheter was secured. The paravertebral muscles and the skin incision were sutured. The external portion of the catheter was coiled into a protector made of tinsel plates.¹⁶ Animals were returned to the individual cages for recovery after surgery.

Surgical procedures of L5 SNL

Surgical L5 SNL model for NP was performed just like our previous study.¹⁴ Rats received anesthesia by 50 mg kg^{-1} sodium pentobarbital (injected intraperitoneally). A midline incision was made along the rat's back. After blunt separation of the paraspinal muscles, the L5 nerve was exposed and tightly ligated with a 3–0 silk thread. Thereafter, the incision was cleansed with saline and was sutured. Sham-operated animals received the lumbar catheterization procedure and the same operation of paraspinal muscles incision and the L5 spinal nerve exposure without nerve ligation.

Behavioral test

Behavioral assessments were performed at baseline (before SNL) on Day 7, Day 14, and Day 21. The test chamber consisted of six Plexiglas box ($30 \text{ cm} \times 30 \text{ cm} \times 30 \text{ cm}$) with a

clear floor. Each rat was placed in its own testing chamber. About 30 min after acclimation, rats were assessed on test days. The mechanical withdrawal thresholds were determined using the “up–down method” by a series of Von Frey filaments (Stoelting Co, Wood Dale, IL, USA). Generally, we started the first test with the filament of 2 g force. If paw withdrawal was observed, the next lesser force was applied. Otherwise, if no movement was observed, the next greater force was used. Every force was applied three times with 5 s intervals. A 50% mechanical threshold was calculated as described previously.¹⁷ The range of scores was between 0.25 g and 26 g. All behavioral assessments were performed by the same author (LD) who was not aware of the group assignment.

Western blot analysis

Animals were sacrificed with 10% chloral hydrate and, then, transcardially perfused with PBS, and the L5 lumbar SC segments were dissected. Then, the protein samples were quantified by a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). A total of $40 \mu\text{g}$ ipsilateral L5 spinal sample proteins were electrophoresed through a 10% sodium dodecyl sulfate-polyacrylamide gel and electrotransferred to 0.4 mm polyvinylidene fluoride membranes. The blots were first incubated overnight at 4°C with one of the following primary antibodies (CXCR4: 1:1000, rabbit; Abcam, Cambridge, UK; GlyR α 3: 1:1000, rabbit; EMD Millipore, Billerica, MA, USA) and then with a horseradish peroxidase-conjugated secondary antibody (1:4000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). An enhanced chemiluminescence detection system (Pierce Biotechnology) was applied for protein detection. An anti-GAPDH antibody (1:1000, rabbit; Cell Signaling Technology, Danvers, MA, USA) was used for normalization. The intensity of protein bands was analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescent detection

After deep anesthesia with pentobarbital sodium, rats were sequentially perfused with saline and 4% paraformaldehyde (pH 7.2–7.4, 4°C) through the heart. The L5 spinal segments were postfixed overnight and dehydrated at 4°C . After mounted in optical coherence tomography compound, the SP sections ($30 \mu\text{m}$) were cut using a cryostat (Leica CM1900 UV; Leica Microsystems, Wetzlar, Germany) for further immunofluorescent detection. Free-floating SP sections were first incubated overnight at 4°C with the following primary antibodies: neuronal nuclei (NeuN): 1:500, mouse (EMD Millipore); GFAP: 1:500, mouse (EMD Millipore);

CXCR4: 1:500, rabbit (Abcam); and GlyR α 3: 1:500, rabbit (EMD Millipore). After washing, sections were incubated with conjugated secondary antibodies (1:250; Jackson ImmunoResearch Laboratories, Inc.) in the dark for 60 min. Images were photographed by confocal microscope (Leica TCS SP5 II). The fluorescent images were analyzed by Leica AF. For the assessment of immunofluorescence, six sections from L5 SP segments were randomly selected in every animal (n=3 in each group).

Statistical analyses

All data were presented as mean \pm SD. SPSS 10.0 was used for statistical analyses. A $P<0.05$ was considered statistically significant. ELISAs and Western blot assays were analyzed by a one-way analysis of variance (ANOVA) followed by the Tukey test. Behavioral data were analyzed first by a two-way repeated measures ANOVA and, then, by multivariate ANOVA for further comparison among groups at the same time.

Results

SNL induces CXCL12–CXCR4 activation in the spinal dorsal horn (DH)

SNL induced significant CXCL12 activation (Figure 2A, main effect of SNL $F_{(2,14)}=204.95$, $P<0.01$). The expressions of CXCR4 in DH after SNL were examined using

immunofluorescence and Western blot. There was a low basal CXCR4 protein expression in the spinal DH at baseline, which significantly increased on Day 14 and Day 21 after SNL (Figure 2B and C, main effect of SNL $F_{(2,14)}=66.9$, $P<0.01$). Figure 2D–F shows representative immunofluorescent pictures of CXCR4 signals in the L5 spinal DH after SNL.

Double immunofluorescence of CXCR4 with different cell markers was then performed to define its distribution in spinal DH. CXCR4 was primarily coexpressed with NeuN (a neuronal marker, Figure 3A–C) but not GFAP (an astrocytic marker, Figure 3D–F).

Intrathecal administration of CXCR4 antagonist AMD3100 alleviates the chronic NP induced by SNL

Behavioral assessments were performed on Days 0, 7, 14, and 21 separately. After the sphericity assumption test, we got an epsilon coefficient >0.7 , so the Bonferroni test was used for post hoc comparisons. The results showed that ipsilateral 50% mechanical threshold substantially decreased in the SNL and AMD groups than in the sham group (main effect of drug $F_{(2,17)}=61.69$, $P<0.01$, interaction $F_{(6,60)}=11.52$, $P<0.01$, $n=6$ every group, Figure 4). AMD3100 at $1\text{ }\mu\text{g day}^{-1}$ repeated for 2 weeks remarkably alleviated the allodynia induced by SNL ($P=0.02$, Figure 4).

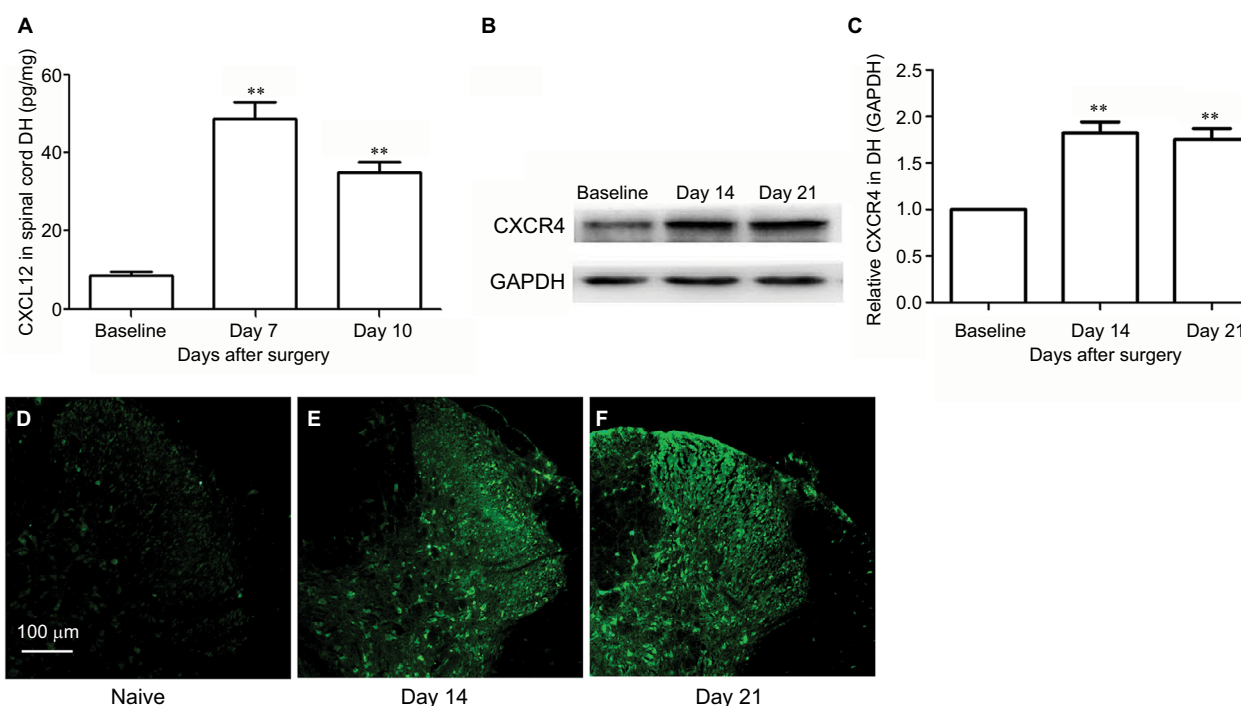


Figure 2 SNL induces CXCL12 and CXCR4 upregulation in spinal cord.

Notes: (A) CXCL12 content in the ipsilateral spinal cord DH. ** $P<0.01$ vs baseline. (B and C) CXCR4 protein content in the ipsilateral spinal cord DH. ** $P<0.01$ vs baseline. CXCR4 signals in the spinal cord of naive animals (D) and SNL animals at Day 14 (E) and Day 21 (F). Magnification $\times 100$.

Abbreviations: DH, dorsal horn; SNL, spinal nerve ligation.

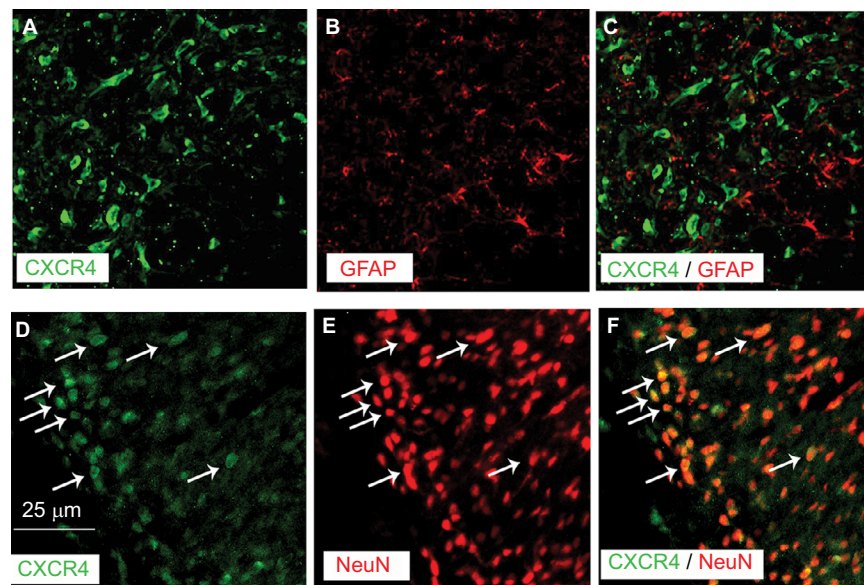


Figure 3 Double staining of immunofluorescence shows that CXCR4 is predominantly colocalized with NeuN, a marker for neurons (D–F) but not with GFAP, a marker for astrocytes (A–C) in SNL rats.

Notes: All pictures were taken under confocal microscope. Arrows indicate co-expression of CXCR4 and neurons. Magnification $\times 200$.

Abbreviations: GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclei; SNL, spinal nerve ligation.

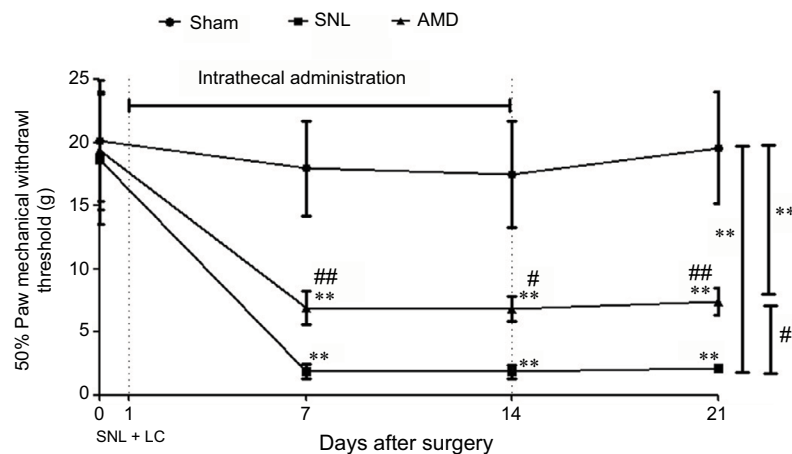


Figure 4 Effects of intrathecal injection of CXCR4 antagonist AMD3100 on SNL-induced mechanical allodynia.

Notes: Drugs were administered intrathecally after SNL and LC from Day 1 to Day 14, repeated for 14 days. AMD3100 was given at dosage of $1 \mu\text{g day}^{-1}$. General effect analyses were performed using a repeated measures two-way ANOVA. The Bonferroni test was used for post hoc comparisons. $**P < 0.01$ vs the sham group and $\#P < 0.05$ vs the SNL group. Further comparisons among groups at the same time were performed using multivariate ANOVA followed by Tukey tests. $**P < 0.01$ vs the sham group at the same point in time. $\#P < 0.05$ and $###P < 0.01$ vs the SNL group at the same point in time.

Abbreviations: ANOVA, analysis of variance; LC, lumbar catheterization; SNL, spinal nerve ligation.

Intrathecal administration of AMD3100 rescues the decrease of GlyR $\alpha 3$ expression in spinal DH

According to our results, SNL significantly decreased the GlyR $\alpha 3$ signals in the ipsilateral spinal DH without affecting the contralateral DH (Figure 5A). The Western blot assays also revealed that the GlyR $\alpha 3$ protein expression was remarkably inhibited on Day 14 and Day 21 in the SNL group than in the sham group. Furthermore, the decrease of GlyR $\alpha 3$ expression was dramatically rescued by administration of

CXCR4 antagonist AMD3100 (main effect of treatment on Day 14 $F_{(2,14)} = 123.43$, $P < 0.01$; main effect of treatment on Day 21 $F_{(2,14)} = 162.83$, $P < 0.01$, Figure 5B). Another interesting finding was that CXCR4 was colocalized with GlyR $\alpha 3$ in the spinal DH at ratio $> 97\%$ (Figure 5C–H).

Discussion

We reported the distribution, expression, and possible function of the receptor of chemokine CXCL12, CXCR4, in the SC DH in an in vivo rat SNL model. Our results reveal that

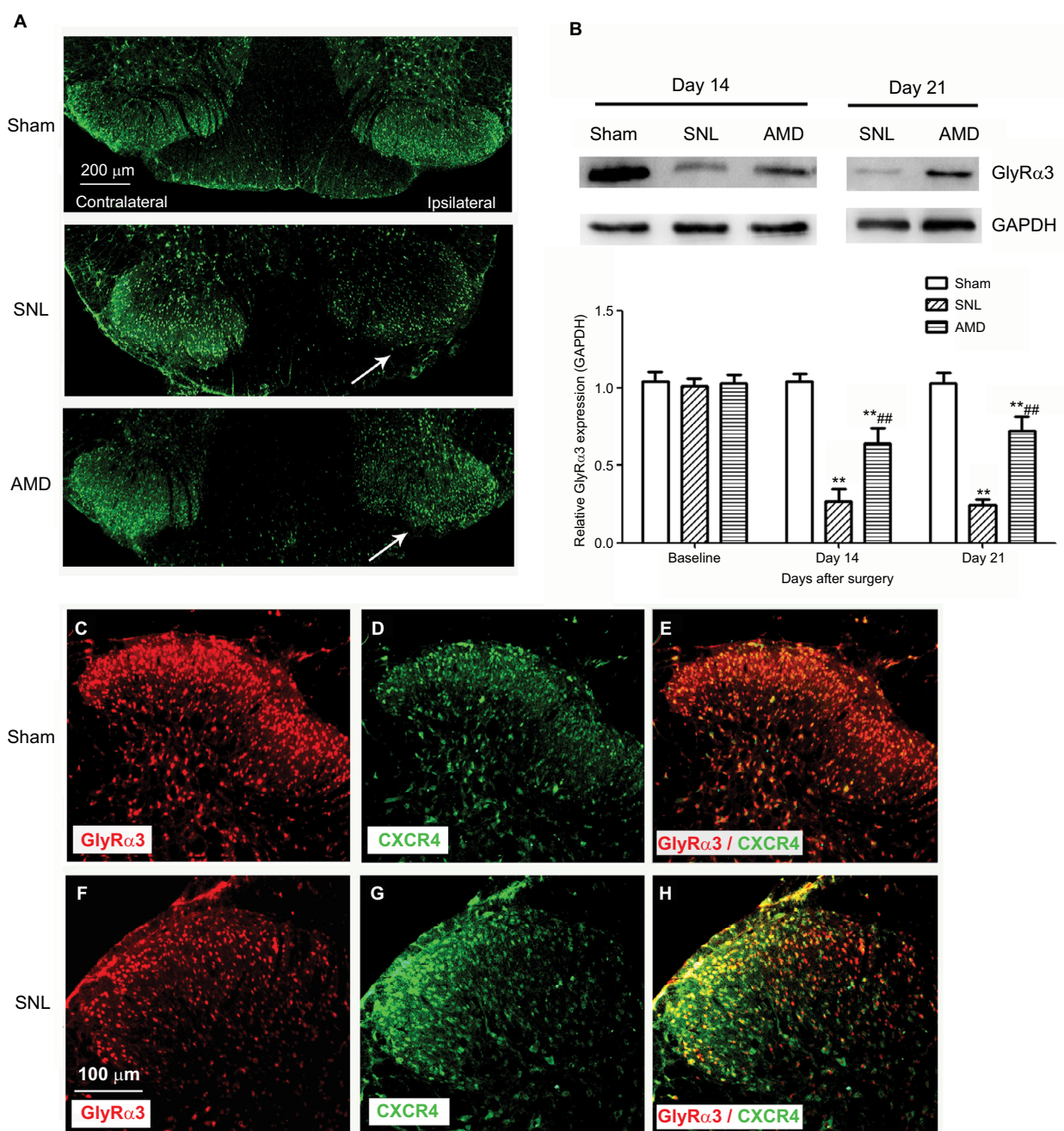


Figure 5 Effects of AMD3100 on GlyRα3 expression in spinal DH.

Notes: (A and B) SNL induced remarkable GlyRα3 decrease in the ipsilateral dorsal horn without affecting the contralateral GlyRα3 expression in the spinal cord (** $P < 0.01$ vs the sham group at the same point in time). Furthermore, administration of AMD3100 rescued the GlyRα3 expression decrease (*** $P < 0.01$ vs the SNL group at the same point in time). Arrows indicate ipsilateral spinal dorsal horn of SNL. Magnification $\times 50$. CXCR4 was colocalized with GlyRα3 in the spinal DH ([C–E] on Day 14 in the sham group and [F–H] on Day 14 in the SNL group). Magnification $\times 100$.

Abbreviations: DH, dorsal horn; SNL, spinal nerve ligation.

1) SNL induces both CXCL12 and CXCR4 upregulation in spinal DH, and CXCR4 antagonist AMD3100 can elicit analgesic effect against allodynia and decrease CXCR4 expression induced by SNL and 2) CXCR4 is predominantly expressed in neurons but not astrocytes in SC DH and co-expressed with GlyRα3. SNL induced depression of GlyRα3, which could be rescued by CXCR4 antagonist AMD3100.

These findings suggest that CXCL12/CXCR4 might play a cardinal role in the development of NP.

Increasing evidence implied CXCL12/CXCR4 to be important in nociceptive signal processing. Miller and his colleagues reported that CXCL12 and glycoprotein 120 elicited allodynia after paw injection.¹⁸ CXCR4 antagonists may be important for therapeutic interventions in the pain

that is associated with HIV-1 infection and inflammation.¹⁸ According to Bai et al's¹⁹ and Shen et al's¹⁰ studies, CXCL12/CXCR4 sensitized neurons and activated glial cells, which substantially contributed to the maintenance of NP and bone cancer pain. CXCL12/CXCR4 signaling was also reported to mediate enhanced calcium influx and excitability in DRG neurons and pain hypersensitivity in diabetic⁹ and toxic neuropathy.²⁰ Our present study studied the distribution and expression of CXCR4 in spinal DH after SNL in a rat in vivo model. According to our results, SNL induces both CXCL12 (7–10 days after SNL) and CXCR4 (14–21 days after SNL) upregulation in SC DH and CXCR4 antagonist AMD3100 could elicit analgesic effect against mechanical allodynia and decrease CXCR4 expression induced by SNL.

AMD3100 is a synthetic and highly specific inhibitor of the CXCR4 receptor. It disrupts binding of CXCL12 to CXCR4 by competing binding site, thus blocking the physiological function of CXCL12/CXCR4 axis.²¹ Hatse et al²¹ showed that inhibition by AMD3100 is strictly restricted to CXCR4 but not to any other “CXC” or “CC” chemokine receptors. Currently, there are two major kinds of CXCR4 antagonist. One is T140 and its analogs, and the other is non-T140-related such as AMD3100. CXCR4 is a G-protein-coupled receptor with seven transmembrane regions. AMD3100 and its analogs bind to the amino acid residues in the central hydrophobic core of the receptor, whereas T140 binds to the residues in extracellular domains of the hydrophobic core.^{21–23} In our study, we used AMD3100 as CXCR4 antagonist because it was widely used in other CXCR4 studies. The dose in other studies was 1–5 µg intrathecal. In our preliminary study, normal control rats showed mechanical thresholds between 15 and 26 g. AMD3100 at the dose of 5 µg for >5 days would cause pain hypersensitivity itself in rats. Therefore, we used AMD3100 at the dose of 1 µg for a longer time (14 days) that low dose of AMD3100 did not affect the pain sensation compared with the normal rats.

According to recent studies, there are several possible mechanisms of CXCL12/CXCR4 involved in NP. The upregulation of CXCR4 triggers the downstream GPCR transduction pathway, induces an increase in Ca²⁺ in neurons, which leads to hyperalgesia.²⁴ CXCL12/CXCR4 signaling activates an ERK-dependent Nav1.8 upregulation.²⁵ CXCL12 stimulates the astrocytes to release the glutamic acid and prostaglandins, which are involved in the process of sensitization.¹² CXCL12 also participates the opioids tolerance, decreasing the endogenous analgesic effects.^{26,27} Concomitant with an increased activation of excitatory neurotransmitter

receptors after NP, there is a decline in inputs of inhibitory neurotransmitters such as gamma-aminobutyric acid (GABA) and glycine.²⁸ Previous studies mostly focused on the effects of CXCR4 on microglial cells.^{8–10} However, no one has reported the effect of CXCL12/CXCR4 on spinal inhibitory neurotransmitters.

Glycine is a ubiquitous amino acid, and it is the second most important fast inhibitory neurotransmitter in the central nerve system.²⁹ There are four α subunits (Gly α 1–4) and one β subunit of glycine receptor. Subunit α 1 (GlyR α 1) is the most abundant in the adult nervous system, which is more localized to deeper layers of the spinal DH (laminae III–IV).³⁰ GlyR α 3 is not a widely expressed subunit, but it is distinctly expressed in the more superficial laminae of the spinal DH, where nociceptive A δ - and C-fiber afferents terminate.^{13,30} GlyR α 1 serves in the control of spinal motor circuit, whereas GlyR α 3 is more specifically involved in spinal nociceptive processing.^{13,30,31} All GlyR α 3 subunits' immunoreactivity was found to colocalize with gephyrin, which functions with GlyRs and GABA_A at postsynaptic sites.^{13,14} Therefore, GlyR α 3 is regarded as a typical element in the inhibitory neurotransmission circuit. Furthermore, a recent study of Lv found that this Gly α 3-associated inhibitory circuit serves as a gate control, which is the prime factor to separate the innocuous mechanoreceptive pathway to the nociceptive pathway.¹⁴ These studies suggested that nerve injury-induced inhibition of the GlyR α 3 inhibitory is vital in mechanical allodynia in NP. In our study, we found that SNL induced inhibition of GlyR α 3 expression in the ipsilateral spinal DH but not affected the contralateral one. We report for the first time that CXCR4 is colocalized with GlyR α 3 and further shows that an administration of CXCR4 antagonist AMD3100 can rescue the inhibition of GlyR α 3 in ipsilateral spinal DH. It remains unclear how exactly CXCL12/CXCR4 signaling affects the GlyR α 3 or inhibitory neurotransmission circuit. This protective effect may be direct, indirect, or even both. A further study on this topic may need coimmunoprecipitation to clarify the relationship between CXCL12/CXCR4 and GlyR α 3.

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Disclosure

The authors report no conflicts of interest in this work.

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