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ORIGINAL PAPER

CX3CR1 Mediates Nicotine Withdrawal-Induced Hyperalgesia via Microglial P38 MAPK Signaling

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Abstract Previously, we reported that nicotine withdrawal (NT) significantly increased pain sensitivity in rats. Recent reports suggest that fractalkine is involved in the spinal cord neuron-to-microglia activation via CX3CR1 signaling. However, its contribution to NT-induced hyperalgesia and the underlying mechanisms have yet to be elucidated. In the present study, a rat model of NT was used to test the changes in CX3CR1 expression in the spinal cord. We also evaluated the effect of the CX3CR1 neutralizing antibody on spinal microglial activity, the expression of phosphorylated p38-mitogen-activated protein kinase (p-p38-MAPK) and heat-induced pain responses. We established a NT model via subcutaneous injection of pure nicotine (3 mg/kg), three times daily for 7 days. The expression of CX3CR1 was studied by Western blot and immunofluorescence staining. Following NT, the rats received daily intrathecal injections of CX3CR1 neutralizing antibody for 3 days. The change in paw withdrawal latency (PWL) was observed. The activation of microglia and the expression of p-p38-MAPK were investigated by Western blot and immunofluorescence staining. The expression of CX3CR1 was significantly increased after NT and co-localized with IBA-1. NT rats treated with CX3CR1 neutralizing antibody showed significantly increased PWL on day 4 after NT. Furthermore, the activation of microglia and the expression of p-p38-MAPK in the spinal cord were suppressed. These results indicate that microglial CX3CR1/p38MAPK pathway is critical for the development of pain hypersensitivity after NT.

Keywords Nicotine withdrawal · Hyperalgesia · CX3CR1 · Microglia · p38 MAP kinase

Abbreviations

NT	Nicotine withdrawal
CX3CR1	Chemokine CX3C motif receptor
SD	Sprague–Dawley
p-p38-MAPK	Phosphorylation of p38-mitogen-activated protein kinase
CPT	Cold pressor test
PKA	Protein kinase A
PKC	Protein kinase C
CaMKII	Calcium/calmodulin-dependent protein kinase II
p38 MAPK	p38-mitogen-activated protein kinase
IBA1	Ionized calcium binding adapter molecule-1
NeuN	Neuronal nucle
GFAP	Glial fibrillary acidic protein
PWL	Paw withdrawal latency
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PVDF	Polyvinylidene fluoride membrane
SNI	Spared nerve injury
FKN	Fractalkine
TLR4	Toll-like receptor 4

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Background

Tobacco addiction is the leading avoidable cause of death in the United States [1]. In fact, it is a global public health challenge, with an estimated one-third of all adults smoking, worldwide. Clinically, long-term smokers manifest an increased risk of lumbago and back pain. For instance, it has been reported that smokers require higher number of analgesics than non-smokers following surgery including coronary bypass and gynecological procedures [2]. Sudden discontinuation of smoking leads to a nicotine withdrawal syndrome. Our previous study has confirmed that thermal hyperalgesia was significantly increased after nicotine withdrawal in rats [3]. Nicotine withdrawal is associated with nociceptive hypersensitivity and pain. In fact, smokers undergoing withdrawal showed lower pain tolerance to cold pressor test (CPT) than non-smokers. Nicotine withdrawal may affect neurophysiologic mechanisms linked with pain, that may render abstinent smokers more sensitive to physical discomfort [4]. A better understanding of the mechanisms underlying nicotine withdrawal could potentially enable the development of new treatments.

Numerous studies have shown that chemokines play a pivotal role in pain via neuronal–glial communication, resulting in central sensitization. Fractalkine (FKN) is a structurally unique chemokine and the only member of the CX3C family of chemokines. FKN has a monogamous relationship with its receptor CX3CR1 [5]. In the dorsal horn of the spinal cord, FKN is expressed on neurons binding to its specific receptor, while CX3CR1 is mainly expressed in microglia. CX3CR1 has recently been proposed to be a neuron-to-glia signal in the spinal cord leading to microglial activation [6]. FKN activates the signaling of neuronal–microglial communication in the mediation of neuropathic and inflammatory pain processing [6, 7]. On the other hand, activated microglia in the spinal cord release a variety of mediators, including neurotransmitters, proinflammatory cytokines and chemokines [8, 9]. Microglia cells are regarded as a major source of proinflammatory cytokines [9]. Furthermore, CX3CL1/CX3CR1 signaling has been shown to mediate the induction of proinflammatory cytokines, such as TNF-α, NF-κB and IL-6 [5]. Spinal nociceptive neurons are excited by these mediators, resulting in central sensitization [10].

The central sensitization is mediated by a complex biochemical cascade following the activation of primary afferent fibers. Several protein kinases, including protein kinase A (PKA), protein kinase C (PKC), and calcium/calmodulin-dependent protein kinase II (CaMKII) play a vital role in the initiation, development and maintenance of central sensitization [11, 12]. Recent studies have established the role of p38 mitogen-activated protein kinase (p38 MAPK), a member of MAPK family, in hypersensitivity and central sensitization [13]. However, the role of p38 MAPK in the development of pain hypersensitivity after nicotine withdrawal is still unknown.

Our previous study has confirmed that thermal hyperalgesia was significantly increased after nicotine withdrawal in rats, in line with our present reports [3]. In the present study, we utilized a rat model of nicotine withdrawal, as previously described [3]. We investigated the altered expression of CX3CR1 in the spinal cord. Furthermore, we tested whether intrathecal injection of anti-CX3CR1 neutralizing antibody attenuated pain behavior and inhibited the activation of spinal microglia as well as the expression of p38 MAPK in the spinal cord induced by nicotine withdrawal.

Methods

Animals

Healthy male Sprague–Dawley (SD) rats (Experimental Animal Center, Xuzhou Medical College, Xuzhou, China) weighing 180–200 g were housed in plastic cages and maintained on a 12:12 h light/dark circle under conditions of 24 ± 1 °C, with ad libitum access to food and water. Animals were habituated to the testing environment for at least 3 days prior to the experiment. All procedures were approved by the Animal Care and Use Committee of Xuzhou Medical College, and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Reagents

Nicotine hydrogen tartrate salt ([2]-1-methyl-2-[3-pyridil]pyrrolidine) and mecamylamine hydrochloride were purchased from Sigma Aldrich (St Louis, MI, USA). Anti-NeuN antibody was purchased from Millipore (Billerica, MA, USA). Anti-IBA1 antibody, anti-CX3CR1 antibody, anti-CX3CR1 neutralizing antibody and Anti-IgG antibody (for control) were purchased from Abcam (Cambridge, MA, USA). Antibodies for phosphorylated-p38 MAPK and GFAP were obtained from Cell Signaling Technology (Cell Signaling Technology, USA). Anti-CX3CR1 neutralizing antibody and anti-IgG antibody injections were performed intrathecally (10 μl/10 μg). The doses of all drugs were based on the results of preliminary experiments.

Model of Nicotine Withdrawal

As described previously, we induced a rat model of nicotine withdrawal [3]. The rats were subcutaneously injected with 3 mg/kg nicotine at 7:00, 15:00 and 23:00 h daily for 7 consecutive days. In addition, 1 mg/kg of mecamylamine was subcutaneously injected 60 min after the last injection on the 7th day, in order to trigger nicotine withdrawal.

Intrathecal Infusion

The method described by Xu et al. [14] was used for the intrathecal drug injection. Briefly, the rats were anesthetized with isoflurane. The hair in the lumbar region was shaved and disinfected with 75 % (v/v) ethanol. The intervertebral spaces were widened by placing the animal on a plexiglass tube. Next, animals were injected at the L5-6 interspace using a 29-gauge microinjection syringe needle filled with CX3CR1 neutralizing antibody or anti-IgG antibody (for control), at a dose of 10 μg/10 μl, daily from day 1 to day 3 after nicotine withdrawal. The correct subarachnoid positioning of the tip of the needle was verified by a tail- or paw-flick test, that is an abrupt flick of the tail or paw when the tip of the needle reached subarachnoid space could be visualized. Rats with signs of motor dysfunction were excluded from the experiments. Motor function was evaluated by the presence of two specific behaviors [15]. (1) The placing/stepping reflex was evoked by drawing the dorsum of either hind paw across the edge of the table. This stimulus elicits an upward lifting of the paw from the surface of the table. (2) In the righting reflex, a rat placed supine shows an immediate and coordinated twisting of the body around its longitudinal axis with crossed extension of its fore and hind paws to regain its normal crouching posture.

Measurement of Thermal Hyperalgesia

Thermal hyperalgesia was determined by measuring the paw withdrawal latency (PWL) in response to radiant heat stimulation. A plantar analgesia meter (IITC Life Science Inc., Victory Blvd Woodland Hills, CA, USA) was used to provide a heat source. The method was similar to the published one [16]. Briefly, each rat was left to adapt to the testing environment for at least 1 h prior to any stimulation. The high-intensity, movable radiant heat source was placed underneath the glass and focused onto the plantar surface of each hind paw. The nociceptive endpoint in the radiant heat test was lifting or licking of the hind paw. The time from onset of radiant heat to endpoint was considered as the PWL. An automatic 30-s cutoff was used to prevent tissue damage. Thermal stimulus was delivered three times to each hind paw at 5-min intervals. The heat was maintained at a constant intensity throughout this study.

Immunohistochemistry

In deep anesthesia, rats were intracardially perfused with saline followed by 4 % paraformaldehyde (PFA). After the perfusion, the L_{4-6} segments of the spinal cord were removed, fixed in 4 % PFA for 12 h at 4 °C, and immersed in 30 % sucrose in phosphate buffer at 4 °C for cryoprotection. Transverse spinal sections (30 μm) were cut in a cryostat and processed for immunofluorescence. All of the sections were blocked with 10 % donkey serum in 0.01 M PBS (pH 7.4), with 0.3 % Triton X-100 for 2 h at room temperature, and incubated overnight at 4 °C with the following primary antibodies: rabbit anti-CX3CR1 (1:100), rabbit anti-phosphorylated-p38MAPK (1:50), mouse anti-NeuN (1:400), mouse anti-GFAP (1:400), goat anti-IBA1 (1:400). The sections were then washed three times with PBS and incubated with the specific secondary antibodies raised in donkey serum [conjugated to Alexa Fluor 488 (1:200) or 594 (1:200), Invitrogen, Carlsbad, CA, USA] for 2 h at room temperature. For double immunofluorescence staining, the sections were incubated with a mixture of anti-CX3CR1 (1:100) antibodies with the IBA-1, microglial marker (1:400), the GFAP, astrocytic marker (1:400), and the NeuN, neuronal marker (1:400) overnight at 4 °C. Double immunolabeling of p-p38MAPK/IBA1 sections was performed by incubation in a mixture of p-p38 MAPK (1:50) with the IBA1 (1:400) overnight at 4 °C, followed by incubation with a mixture of Alexa 594-conjugated rabbit IgG, Alexa 488-conjugated mouse IgG and Alexa 488-conjugated goat IgG (1:200; Invitrogen, Carlsbad, CA, USA). All sections were cover-slipped with a mixture of 90 % glycerin in 0.01 M PBS, followed by confocal microscopy (FV1000, Olympus Co. Ltd, Tokyo, Japan).

Western Blot

The L_{4-6} spinal cords from studied rats were isolated, and frozen in liquid nitrogen or stored at 80 °C for subsequent procedures. The tissues were homogenized in a lysis buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing a cocktail of protease inhibitor and phosphatase inhibitors (Sigma Aldrich, St Louis, MI, USA). The homogenates were centrifuged at 12,000×g for 15 min at 4 °C. The supernatants were used as cytosolic proteins for Western blot. Equal amounts of protein (80 μg) were loaded in each lane and separated by 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene fluoride membrane (PVDF, Millipore, Billerica, MA, USA). The membranes were blocked in 3 % bovine serum albumin (BSA) for 2 h at

room temperature and rinsed three times in Washing Buffer for 5 min each, and incubated overnight at 4 °C with rabbit anti-CX3CR1 (1:500), rabbit anti-p-p38 MAPK (1:1000), mouse anti-β-actin (1:1000) antibody, respectively. They were further incubated with HRP-conjugated goat anti-rabbit IgG (1:1500) or goat anti-mouse secondary antibody IgG (1:1500), respectively, for 1 h at room temperature. The blots were rinsed in Washing Buffer four times for 8 min, followed by visualization using electrochemiluminescence (ECL) and exposure to X-ray film (Roche, Indianapolis, IN, USA) for 1–10 min. The scanned images were imported into Adobe Photoshop software (Adobe, CA, USA). Scanning densitometry was used for semi-quantitative analysis of the data.

Experimental Groups

Part 1: The rats were randomized into three groups (n = 24): normal group (normal), saline group (saline) and nicotine withdrawal group (NT). Animal behavior was observed for 7 consecutive days after nicotine withdrawal. After behavioral assessments, from the first day of nicotine withdrawal, three rats in each group were sacrificed for 7 consecutive days, with the L_{4-6} spinal cords isolated for Western blot, while the other three rats in each group were sacrificed only on day 4 after nicotine withdrawal, followed by the removal of L_{4-6} spinal cords for immunofluorescence assay.

Part 2: To study the effect of intrathecal injection of anti-CX3CR1 neutralizing antibody on nicotine withdrawal-induced pain and p-p38MAPK expression, rats were divided into four groups (n = 12) as following: normal group (normal), nicotine withdrawal group (NT), nicotine withdrawal + anti-IgG antibody (for control) group (NT + control IG), and nicotine withdrawal + anti-CX3CR1group (NT + anti-CX3CR1). Behavioral assessment was performed for 7 consecutive days following nicotine withdrawal. On day 4 after behavioral evaluation, six rats in each group were sacrificed, and their L_{4-6} spinal cord were isolated for either Western blot or immunofluorescence analysis (n = 6 each).

Statistical Analysis

The data are expressed as the mean ± standard deviation (SD). Time courses measured for PWL was analyzed by two-way ANOVA with Bonferroni's post hoc test. Western blot and immunofluorescence analysis were performed by one-way ANOVA, followed by Dunnett's multiple comparison post hoc test. Data were analyzed using SPSS 16.0 (Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Fig. 1 Expression levels of CX3CR1s were increased, and exclusively co-localized in microglia in the spinal cord. **a** Western blot using rabbit anti-CX3CR1 antibody (1:500) showed an increase in the level of CX3CR1 protein in the spinal cord at different times after nicotine withdrawal. The increase commenced on day 1 and peaked by day 4. The times course of change in CX3CR1s protein was similar to that of PWL (n = 3,$^{*}P < 0.05$, $^{**}P < 0.01$ vs normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs saline group). **b** There was a significant increase in the number of CX3CR1 immunoreactive cells in the dorsal horn of nicotine withdrawal rats compared with those in normal and saline rats on day 4 (n = 3, $^{**}P < 0.01$ vs normal group; $^{\#\#}P < 0.01$ vs saline group; *Scale bar* 50 μm). **c** Double staining shows that CX3CR1 (*red*) is co-localized with IBA1 (*green*), a marker for microglia, but not with NeuN (*green*), a marker for neurons, or GFAP (*green*), a marker for astrocytes. IBA1/CX3CR1 dual-labeled cells are shown in *yellow*. *Scale bars* 50 μm. The antibody concentrations for confocal imaging are as follows: rabbit anti-CX3CR1 (1:100), mouse anti-NeuN (1:400), mouse anti-GFAP (1:400), and goat anti-IBA1 (1:400)

Results

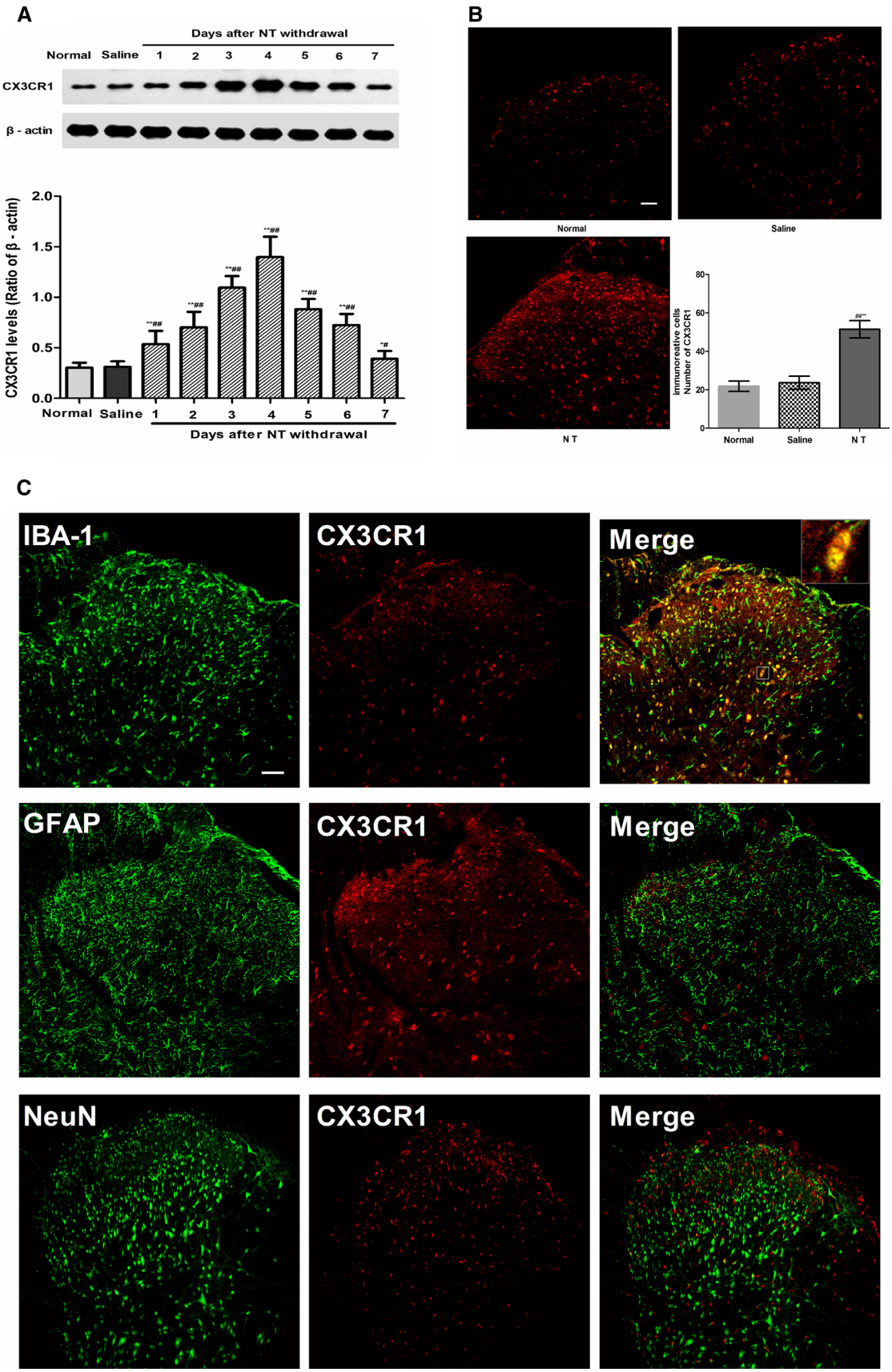
Spinal CX3CR1 is Upregulated in Microglia After Nicotine Withdrawal

Western blot showed that the CX3CR1 expression was significantly increased in the spinal cord from day 1 to 7 after nicotine withdrawal (Fig. 1a), compared with that of normal and saline rats. The increase started on day 1, peaked on day 4. Our immunofluorescence result revealed that CX3CR1 was predominantly distributed in the superficial layers (laminae I-II), and the expression of CX3CR1 was increased in the nicotine withdrawal rats compared with normal and saline rats on day 4 (Fig. 1b).

To investigate the cell distribution of CX3CR1 in the dorsal horn following nicotine withdrawal, we performed double immunostaining of CX3CR1 with three major spinal nerve cell-specific markers, including NeuN (neuronal marker), GFAP (astrocyte marker), and IBA-1 (microglial marker). Confocal images identified that CX3CR1 was co-localized with IBA-1 only (Fig. 1c). These results demonstrated that the spinal CX3CR1 was upregulated after nicotine withdrawal, and was predominately expressed in activated microglia.

Anti-CX3CR1 Antibody Injection Attenuates Thermal Hyperalgesia Caused by Nicotine Withdrawal

We found that the animal models manifested significantly decreased PWL from day 1 to day 4 after nicotine withdrawal. PWL of animal models was significantly reduced at day 4 after nicotine withdrawal compared with that of normal rats ($P < 0.01$). To further confirm if the suppression of spinal CX3CR1 inhibited pain-related behavior


A
Days after NT withdrawal
Normal Saline 1 2 3 4 5 6 7
CX3CR1
β - actin
CX3CR1 levels (Ratio of β - actin)
2.0
1.5
1.0
0.5
0.0
Normal Saline 1 2 3 4 5 6 7
Days after NT withdrawal
B
Normal
Saline
N T
Immunoreative cells Number of CX3CR1
80
60
40
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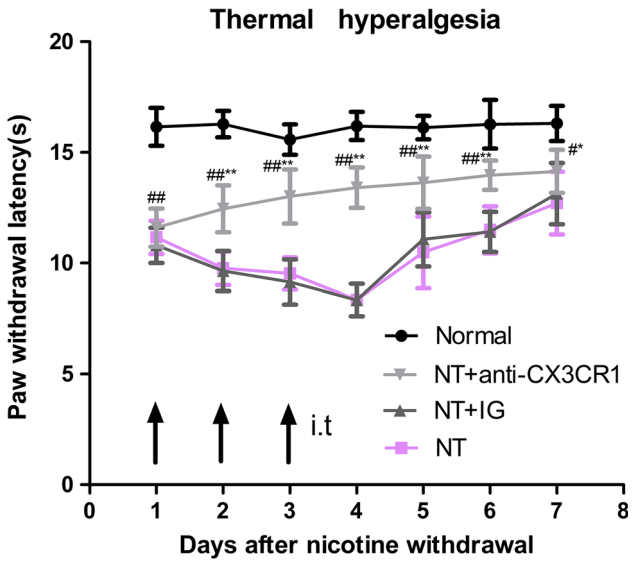


Fig. 2 Intrathecal administration of anti-CX3CR1 neutralizing antibody (10 μg/10 μl) attenuated nicotine withdrawal-induced hyperalgesia. The PWL of nicotine withdrawal rats was increased after injection of CX3CR1 neutralizing antibody. Treatment of control IgG (10 μl) failed to alter PWL in nicotine withdrawal rats. Six rats were included in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus NT + control IgG group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus normal group. Each *arrow* shows the corresponding time points of each administration

following nicotine withdrawal, we intrathecally injected a CX3CR1-neutralizing antibody (10 μg/10 μl) or anti-IgG antibody (10 μg/10 μl) daily from day 1 to day 3 after nicotine withdrawal. Compared with NT + control IG group, NT rats treated with CX3CR1-neutralizing antibody, at a dose of 10 μg/10 μl, significantly attenuated nicotine withdrawal-induced pain-related behaviors simultaneously (Fig. 2). These results indicate that CX3CR1 may be involved in the maintenance of nicotine withdrawal-induced pain-related behaviors.

Intrathecal Injection of Anti-CX3CR1 Antibody Inhibits the Activation of Microglia in the Spinal Cord after Nicotine Withdrawal

On the day 4 after nicotine withdrawal, confocal images showed that IBA-1 immunoreactivity in the spinal cord was significantly increased in NT rats compared with that in normal controls (Fig. 3A-a, A-b). Activated microglial cells exhibited hypertrophic morphological changes such as cellular hypertrophy and retraction of cytoplasmic processes (Fig. 3A-b). After intrathecal injection of anti-CX3CR1 antibody, (10 μg/10 μl), on days 1, 2 and 3 after nicotine withdrawal, the number of IBA-limmunoreactive cells was dramatically decreased in NT + anti-CX3CR1 rats compared with NT rats that received anti-IgG on day 4 (Fig. 3A-c, A-d). The morphology of spinal microglia restored to baseline structure, showing small, compact somata bearing long, thin, and ramified processes (Fig. 3A-c).

Intrathecal Injection of Anti-CX3CR1 Antibody Suppresses p-p38 Expression in the Spinal Cord after Nicotine Withdrawal

Our immunofluorescence results revealed that the number of p-p38 positive cells was increased in the dorsal horn of NT rats compared with that in normal rats on day 4. The number of p-p38 immunoreactive cells was significantly decreased by intrathecal treatment of neutralizing CX3CR1 antibody compared with that in control group by day 4 (Fig. 4a). The Western blot showed that the p-p38 was increased in NT rats compared with that in normal rats on day 4 after withdrawal. Following neutralizing CX3CR1 antibody injection from day 1 to day 3, the level of p-p38 protein expression was significantly reduced in the spinal cord of NT rats on day 4 (Fig. 4c). Furthermore, we also found that p-p38 (red) staining was co-localized with IBA1-positive cells and CX3CR1 (green) (Fig. 4e and f).

Discussion

The current study demonstrates that (a) CX3CR1 is upregulated in microglia of the spinal cord from NT rats after nicotine withdrawal; (b) Intrathecal injection of anti-CX3CR1 neutralizing antibody significantly attenuated nicotine withdrawal-induced nociceptive behaviors; (c) Blockade of CX3CR1 suppressed nicotine withdrawal-induced spinal microglial activation as well as p-p38 MAPK expression in the spinal cord. The findings confirm that neurophysiological mechanisms modulate the pain associated with nicotine withdrawal.

Over 4000 different chemical substances have been identified in tobacco. Animal experiments and clinical studies suggested that nicotine is the main component involved in pain modulation [17, 18]. Nicotine is capable of modulating the activity of sensory neurons and the transmitter substances from sensory neurons [19]. Previous studies suggested that nicotine reduced the number of in vitro cultured microglia [20, 21]. Nicotine plays a robust anti-inflammatory role through alpha-7-nicotinic acetylcholine receptors (α7nAchR) in microglia and provides significant neuroprotection [21]. Our results indicated that nicotine withdrawal was associated with nociceptive hypersensitivity and the activated microglia cells in the spinal cord of rats were significantly increased in number. Clinically, various types of chronic pain, including back pain, carpal tunnel syndrome and complex regional pain

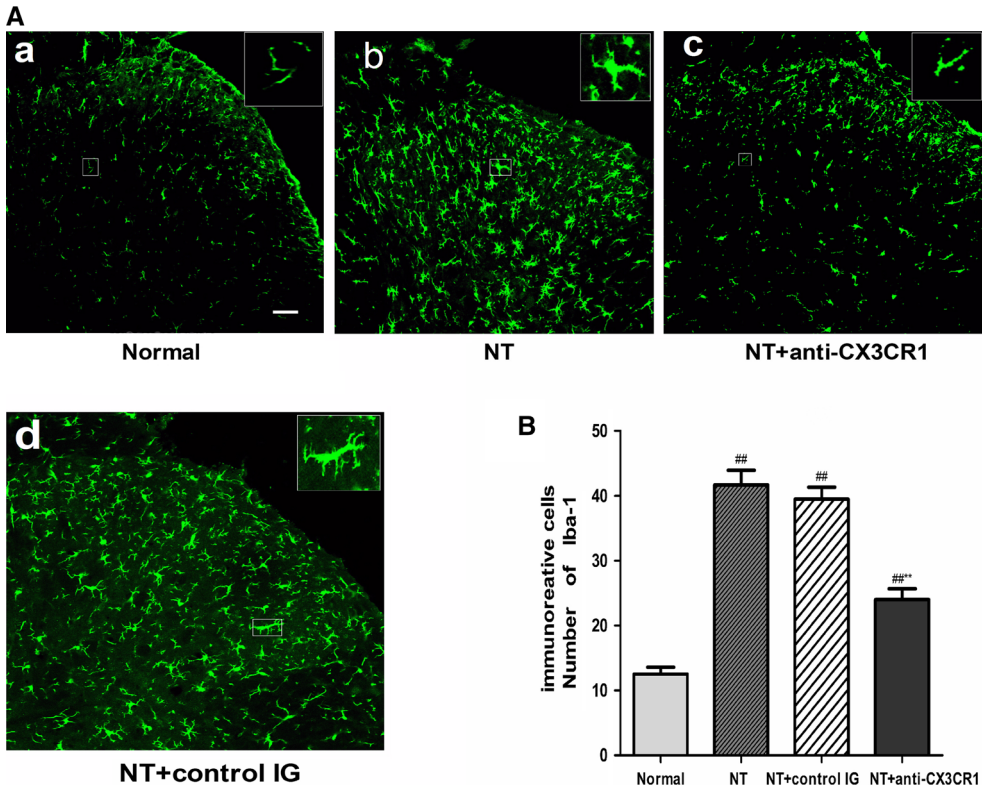
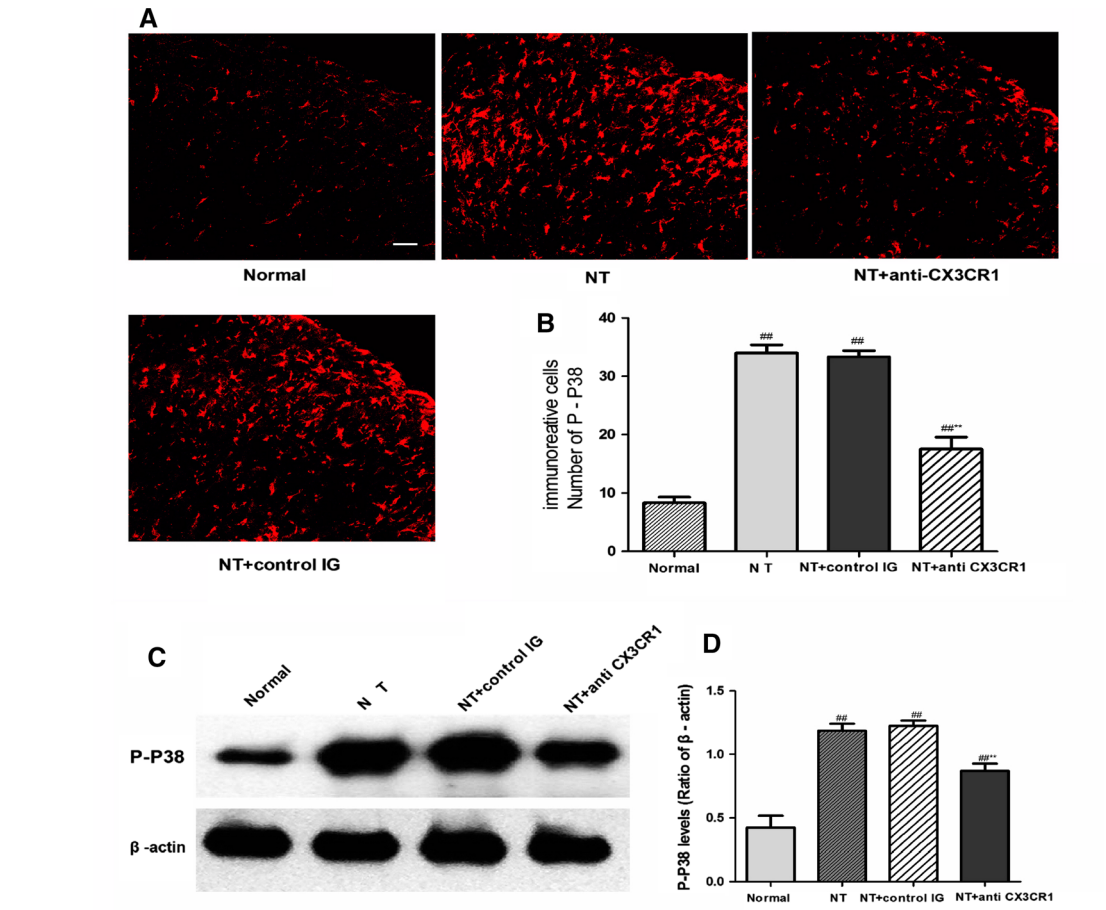


Fig. 3 Intrathecal injection of anti-CX3CR1 neutralizing antibody (10 μg/10 μl) suppressed the expression of IBA-1 (**A**). Immunofluorescence revealed an extensive decrease in microglial (IBA-1) immunoreacivity in the dorsal horn of NT rats by intrathecal anti-CX3CR1 neutralizing antibody (**A-c**). Treatment of control IgG failed to alter the expression of microglia (IBA-1) (**A-d**). In Normal and NT + anti-CX3CR1 rats, the morphology of spinal microglia displayed resting state features showing small, compact somata bearing long, thin, and ramified processes (**A-a**, **A-c**). However, in NT and NT + control IG rats, spinal microglia exhibited activated phenotype showing cellular hypertrophy and retraction of processes (**A-b**, **A-d**). **B** Quantification of IBA-1 immunoreactive cells in the dorsal horn (n = 3) using goat anti-IBA1 ab (1:400). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus normal group; $^{**}P < 0.01$ versus control IgG group. *Scale bar* 50 μm

syndrome, are worsened in chronic smokers [22]. After smoking cessation, the postoperative pain in these patients is more severe compared with non-smokers. Subsequently, they need more painkillers for pain management. The reason for the discrepancy in these results is unclear. It is plausible that this result is associated with the anti-nociceptive effect of nicotine. However, long-term exposure to nicotine induces upregulation and inactivation of nAChRs, resulting in a decreased release of inhibitory neurotransmitters. Therefore, pain sensitivity is increased in long-term smokers who quit smoking.

Our results indicate that CX3CR1 is upregulated after nicotine withdrawal, which is consistent with previous studies involving other models, including neuropathic and inflammatory pain. In the present study, Western blot data demonstrated that CX3CR1 protein expression was gradually increased from day 1 to day 4 in the spinal cord following nicotine withdrawal and peaked on day 4. The time course of the CX3CR1 upregulation in the spinal cord is consistent with the development of hyperalgesia behavioral response. PWL was significantly reduced on day 4 after nicotine withdrawal. In addition, our study demonstrated that CX3CR1 was expressed in spinal microglia, and that the number of CX3CR1-positive cells was increased in the NT rats. Blockade of CX3CR1 significantly attenuated the existing pain hypersensitivity induced by nicotine withdrawal. These data suggest that CX3CR1 upregulation may be involved in the maintenance of nicotine withdrawal induced pain-related behaviors.

Microglia are the major immune cells in the central nervous system. Accumulating evidence suggests that microglial cells in the spinal cord play a crucial role in facilitating pain states [23]. Activated microglia release a variety of mediators, such as proinflammatory cytokines and the neuronal chemokine FKN. FKN acts on microglial CX3CR1 receptors, further leading to the activation of microglia in the spinal dorsal horn. Such reciprocal interactions between glia and neurons contribute to the activation of a neuro–glial amplification loop, leading to central sensitization and exaggeration of pain signals [24].


A
Normal
NT
NT+anti-CX3CR1
NT+control IG
B
immunoreative cells
Number of P - P38
40
30
20
10
0
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##**
Normal
N T
NT+control IG
NT+anti CX3CR1
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Normal
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NT+control IG
NT+anti CX3CR1
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P-P38 levels (Ratio of β - actin)
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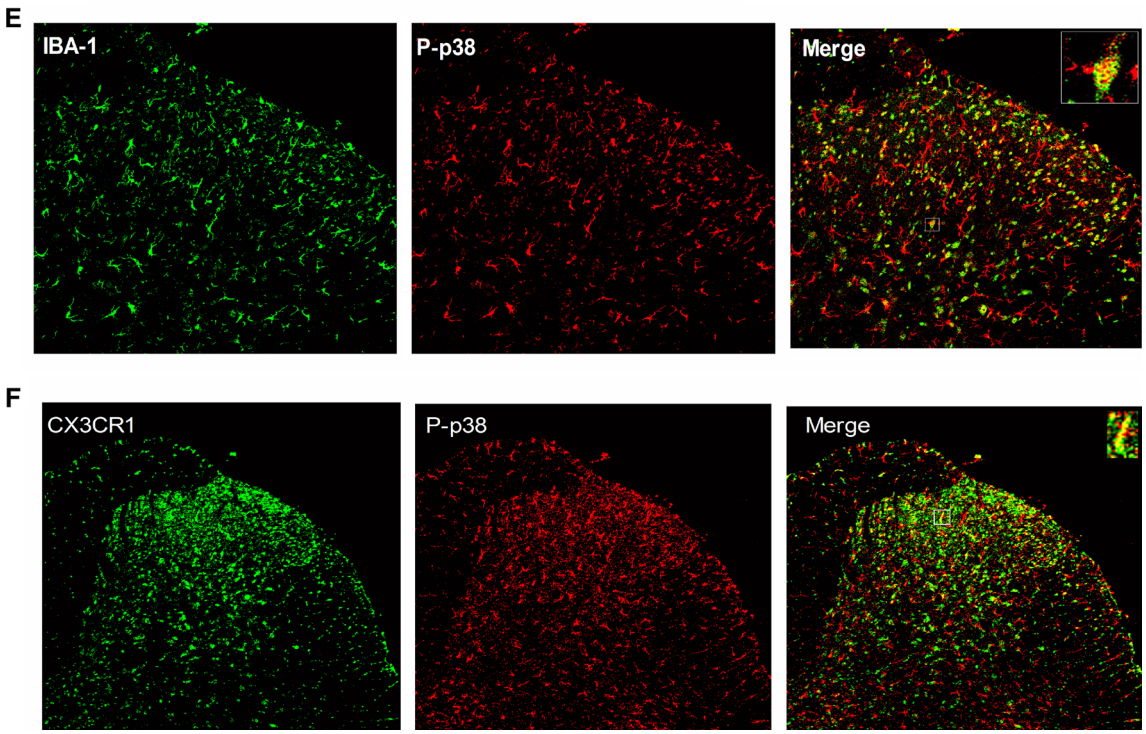
E
IBA-1
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CX3CR1
P-p38
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◀**Fig. 4** Expression levels of p-p38MAPK were inhibited by intrathecal injection anti-CX3CR1 neutralizing antibody (10 μg/10 μl). **a** Confocal imaging shows the expression of p-p38 immunoreactive cells was suppressed by intrathecal anti-CX3CR1 neutralizing antibody compared with the intrathecal injection of the control IgG (rabbit anti-p-p38MAPK (1:50). **b** Quantification of p-p38 immunoreative cells in the dorsal horn (n = 3). **c** Western blot shows a clear upregulation of p-p38 protein level in NT rats. Blockade of CX3CR1 with a neutralizing antibody (rabbit anti-p-p38MAPK (1:1000) reduced the p-p38 protein level in NT rats on day 4. **d** Quantification of p-p38 protein level in the spinal cord. p-p38 levels were normalized against β-actin (n = 3). **e** Double staining shows that p-p38 (*red*) co-localized with IBA1 (*green*), a marker for microglia. **f** Co-localization of the *green signal* (CX3CR1) and the *red signal* (p-p38). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus normal group; $^{**}P < 0.01$ versus control IgG group. *Scale bar* 50 μm. The antibody concentrations for confocal imaging are as follows: rabbit anti-p-p38 MAPK (1:50) and goat anti-IBA1 (1:400)

According to several studies using somatosensory behavioral tests, the intrathecal injection of FKN evokes dose-dependent thermal hyperalgesia in naïve rats [23]. In the spinal cord, concomitant with microglial activation, CX3CR1 expression is up-regulated after chronic constriction injury to the sciatic nerve [7]. Recently, FKN administration has been shown to increase abdominal electromyographic responses to noxious colorectal distension in control rats, and this hypersensitivity was further prevented by intrathecal injection of minocycline [24]. Thus, it is plausible that blocking CX3CR1 can inhibit microglia activation, and reduce the release of neuronal and glial-excitatory substances, and eventually, attenuate nociceptive hypersensitivity of the rats after nicotine withdrawal. Intrathecal injection of FKN leads to pain hypersensitivity in naïve rats, which can be reversed by intrathecal injection of an antibody blocking either CX3CR1 or FKN [7, 25], and CX3CR1 knock-out mice fail to develop nociception induced by FKN [7]. These results showed that FKN induces pain hypersensitivity by acting on its receptor CX3CR1. On the contrary, Holmes et al. [26] reported that intra-neural injection of FKN into the sciatic nerve of mice robustly delayed the development of allodynia following spared nerve injury (SNI). The CX3CR1 knockout mice displayed increased allodynia after SNI. The reason for these different results is unclear. It is possible that differences in strains and sites of FKN action might contribute to the results obtained.

It has been reported that p38 is activated in spinal microglia under different pain conditions. Importantly, p38 activation also contributes to the development of pain hypersensitivity [27]. Several studies explored the upstream mechanisms associated with p38 activation in spinal microglia. Although microglial receptor TLR4 (toll-like receptor 4) was reported to be one of the cellular events upstream of p38 MAPK, it is unlikely that TLR4 was the only microglial receptor associated with p38 MAPK activation [28]. Remarkably, a recent study has established that intrathecal infusion of FKN not only induces hyperalgesia behavior but also activates p38 in spinal microglia. FKN activated p38MAPK in the microglia of spinal cord samples [28]. Conversely, a neutralizing antibody against CX3CR1 reduces both p38 activation and nociceptive hypersensitivity [28]. In the present study, we observed a similar effect of intrathecal administration of anti-CX3CR1- neutralizing antibody which suppressed p-p38 MAPK activation in nicotine withdrawal rats. These findings suggest that FKN-CX3CR1-p38 cascade mediates the development of hyperalgesia after nicotine withdrawal.

Conclusions

We have provided novel evidence that spinal CX3CR1 may play an important role in the maintenance of nicotine withdrawal-induced pain-related behaviors. We observed an increase of nociceptive sensitivity, CX3CR1 protein expression and immunoreactivity, microglia and p-p38 levels in spinal cord sections from rats following nicotine withdrawal. Intrathecal injection of anti-CX3CR1 antibody attenuates thermal hyperalgesia induced by nicotine withdrawal, possibly through a p-p38 pathway. CX3CR1 and p-p38 were co-localized with the microglial marker IBA-1, which suggests that the activated microglia paralleled the activation of the CX3CR1/p38 signaling pathway. These findings open new perspectives in the understanding of the mechanisms underlying hyperalgesia associated with nicotine withdrawal in rats. Thus, the blockade of this chemokine signaling in the spinal cord may play a vital role in the management of pain hypersensitivity following nicotine withdrawal.

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Authors' Contributions ZWZ conceived and designed the study. YHD performed the animal surgery, behavioral testing and data analysis. WHS carried out the immunohistochemistry and Western blot. GNX, ALY and QHW participated in behavioral testing and immunohistochemistry experiments. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors report no financial or other conflict of interest relevant to the subject of this manuscript.

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