Pain 129 (2007) 64–75

PAIN

www.elsevier.com/locate/pain

New evidence for the involvement of spinal fractalkine receptor in pain facilitation and spinal glial activation in rat model of monoarthritis

Sun Shan [1], Cao Hong [1], Han Mei [2], Li Ting-Ting [2], Pan Hai-Li [2], Zhao Zhi-Qi, Zhang Yu-Qiu *

Institutes of Brain Science, Institute of Neurobiology, Fudan University, 138 Yixueyuan, Shanghai 200032, China
Shanghai Research Center of Acupuncture and Meridian, Shanghai, China

Received 14 March 2006; received in revised form 23 August 2006; accepted 28 September 2006

Abstract

Fractalkine, a chemokine binding to only one known receptor CX3CR1, has recently been proposed to be a neuron-to-glia signal in the spinal cord leading to microglial activation and glially dependent pain facilitation. The previous studies explored that blockade of endogenous fractalkine, using anti-CX3CR1 neutralizing antibody, dose-dependently attenuated neuropathic pain. The present study examined the role of endogenous fractalkine in inflammatory pain. Intra-articular injection of complete Freund's adjuvant (CFA)-induced rat ankle joint monoarthritis (MA) model was used. Western blot analysis revealed that CX3CR1 expression in the spinal cord was significantly increased following CFA-induced MA. Intrathecal injection of anti-CX3CR1 neutralizing antibody both delayed the development of mechanical allodynia and thermal hyperalgesia, and reversed established pain facilitation. Furthermore, blockade of CX3CR1 significantly suppressed activation of spinal glia, especially microglia, evoked by MA. These data provided new evidence for the contribution of endogenous fractalkine to the initiation and early maintenance of inflammatory pain facilitation via activating spinal microglia.

Keywords: CX3CR1; Microglia; Hyperalgesia; Allodynia; Monoarthritis; Rat

1. Introduction

Recent evidence strongly supports the involvement of spinal glia in diverse enhanced pain states. Following inflammation and damage of peripheral tissues, peripheral nerves and spinal nerves, spinal glia become activated, and the degree and time course of glial activation correlate with pain facilitory behaviors (Milligan et al., 2003; Watkins and Maier, 2003; Ledeboer et al., 2005; Twining et al., 2005). Activated glia release various substances, such as pro-inflammatory cytokines and other mediators, which can enhance pain transmission (Watkins et al., 2001; Watkins and Maier, 2003; Deleo et al., 2004; McMahon et al., 2005). However, what causes glial activation in response to peripheral inflammation and damage? One alternative is classical "pain" neurotransmitters/neuromodulators including glutamate, ATP, substance P, calcitonin-gene-related

* Corresponding author. Tel.: +86 21 54237635; fax: +86 21 54237647.
E-mail address: yuqiuzhang@fudan.edu.cn (Z. Yu-Qiu).
[1] These two authors contributed equally.
[2] These three authors contributed equally.

0304-3959/$32.00
doi:10.1016/j.pain.2006.09.035

peptide (CGRP), nitric oxide (NO), and prostaglandins (PGs) (Tsuda et al., 2005; Watkins et al., 2005). Another is novel neuron-to-glia signals, such as fractalkine (Milligan et al., 2004; Verge et al., 2004; Watkins et al., 2005).

Fractalkine is a member of the chemokine superfamily and the sole member of the CX3C chemokine class (Combadiere et al., 1998; Asensio and Campbell, 1999; Bajetto et al., 2002; Hughes et al., 2002). In the central nervous system (CNS), fractalkine is a unique chemokine, which acts exclusively on the fractalkine receptor (CX3CR1) (Chapman et al., 2000). Intriguingly, CX3CR1 is expressed predominantly by spinal microglia, suggesting that fractalkine might prime microglia to become activated in response to peripheral injury or inflammation (Verge et al., 2004; Lindia et al., 2005). A series of studies from Watkins' group demonstrated that (1) intrathecal (i.t.) fractalkine induces thermal hyperalgesia and mechanical allodynia in naive rats; (2) i.t. CX3CR1 neutralizing antibody or the microglia inhibitor, minocycline, blocks fractalkine-induced pain enhancement; (3) blockade of spinal fractalkine receptor, CX3CR1, both delays the initial development of neuropathic pain and attenuates established neuropathic pain (Milligan et al., 2004, 2005). Thus, fractalkine may represent a new target for the treatment of neuropathic pain. However, the role of this chemokine in inflammatory pain has not been well understood.

In clinical pathological pain, arthritic pain is another major health care burden besides neuropathic pain, with increasing incidence. Currently, the major recommended strategy for arthritis treatment includes non-steroidal anti-inflammatory drugs, steroids and opiates, while these are associated with serious side effects (Altman et al., 2000). Therefore, a safe and effective therapeutic strategy in arthritis is desired. Complete Freund's adjuvant (CFA) has been utilized to induce rat arthritic immunopathological disease that displays many of the pathological features of human rheumatoid arthritis (Colpaert, 1987). The purpose of the present study is to examine whether the blockade of spinal CX3CR1 could antagonize activation of spinal glia, delay or attenuate thermal hyperalgesia and mechanical allodynia in CFA-induced ankle joint monoarthritic rats.

2. Materials and methods

2.1. Animals

Experiments were performed on adult male Sprague–Dawley rats (Experimental Animal Center, Shanghai Medical College of Fudan University, China) weighing 200–250 g. Rats were housed in groups of 2 in suspended cages and maintained on a 12:12 h light–dark cycle and a constant room temperature of 21 °C with free access to food and water. Prior to experimental manipulation, animals were handled daily at least for 3 days. All experimental protocols and animal handling procedures were approved by Animal Care and Use Committee (ACUC) of Fudan University, 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.

2.2. Induction of monoarthritis (MA)

Monoarthritis (MA) was induced by an injection of complete Freund's adjuvant (CFA, Sigma) into the unilateral ankle articular cavity. The rat was briefly anesthetized with isoflurane (Shanghai Chemical Reagent (SCR) Co., Shanghai, China). The skin around the site of injection was sterilized with 75% alcohol. The left leg of the rat was held and the fossa of the lateral malleolus of the fibula was located. A 28 gauge needle was inserted vertically to penetrate the skin, and turned distally to insert into the articular cavity from the gap between the tibiofibular and tarsus bone until a distinct loss of resistance was felt. A volume of 50 μl CFA was then injected. Sham MA control animals were similarly injected with sterile normal saline (NS).

2.3. Intrathecal surgery and drug injection

As previous description (Zhang et al., 2002), an intrathecal catheter (PE-10 tube) was inserted through the gap between the L_4 and L_5 vertebrae and extended to the subarachniod space of the lumbar enlargement (L_4 and L_5 segments) under Chloral Hydrate (300 mg/kg, intraperitoneal (i.p.), SCR Co., Shanghai, China) anesthesia. The catheter was filled with sterile endotoxin free 0.01 M phosphate buffered saline (PBS, approximately 4 μl), and the outer end was plugged. The cannulated rats were allowed to recover for 3–4 days and were housed individually. Rats that showed any neurological deficit resulting from the surgical procedure were excluded from the experiment.

Endotoxin-free goat anti-CX3CR1 (Santa Cruz Biotechnology, CA, USA) was diluted in 0.01 M sterile PBS. Endotoxin-free rat recombinant fractalkine (chemokine domain amino acid residues 25–100; R& D Systems) was diluted in sterile PBS containing 0.1% bovine serum albumin (BAS, SERVA). The anti-CX3CR1 antibody, normal goat IgG (control, Santa Cruz Biotechnology, CA, USA), vehicle (sterile PBS containing 0.1% BAS), or fractalkine was injected over a period of 2 min via the catheter at a volume of 10–15 μl, followed by 5 μl PBS for flushing. The location of the distal end of the i.t. catheter was verified at the end of each experiment by visual inspection after sacrifice.

2.4. von Frey test for mechanical allodynia

The hindpaw withdrawal threshold (PWT) was determined using a calibrated series of von Frey hairs (Stoelting, IL, USA) ranging from 1 to 26 g. Animals were placed individually into Plexiglass chambers with customized platform that contains 1.5 mm diameter holes in a 5 mm grid of perpendicular rows throughout the entire area of the platform (Pitcher et al., 1999). The protocol used in this study was a variation of that described by Takaishi et al. (1996). After acclimation to the test chamber, a series of nine calibrated von Frey hairs were

applied to the central region of the plantar surface of one hindpaw in ascending order (1, 1.4, 2, 4, 6, 8, 10, 15, and 26 g). A particular hair was applied until buckling of the hair occurred. This was maintained for approximately 2 s. The hair was applied only when the rat was stationary and standing on all four paws. A withdrawal response was considered valid only if the hindpaw was completely removed from the customized platform. Each hair was applied five times at 5 s intervals. If withdrawal responses did not occur more than twice during five applications of a particular hair, the next ascending hair in the series was applied in a similar manner. Once the hindpaw was withdraw from a particular hair three out of the five consecutive applications, the paw was re-tested with the next descending hair until less than three withdrawal responses occurred in five applications. The paw withdrawal threshold (PWT) was defined as the lowest hair force in grams that produced at least three withdrawal responses in five tests. After the threshold was determined for one hindpaw, the same testing procedure was repeated on the other hindpaw at 5-min interval.

2.5. Hargreaves' test for thermal hyperalgesia

After acclimation to the test chamber, thermal hyperalgesia was assessed by measuring the latency of paw withdrawal in response to a radiant heat source. Rats were placed individually into Plexiglass chambers on an elevated glass platform, under which a radiant heat source (model 336 combination unit, IITC/life Science Instruments, Woodland Hill, CA, USA) was applied to the glabrous surface of the paw through the glass plate. The heat source was turned off when the rat lifted the foot, allowing the measurement of time from onset of radiant heat application to withdrawal of the rat's hindpaw. This time was defined as the hindpaw withdrawal latency (PWL). The heat was maintained at a constant intensity, which produced a stable withdrawal latency of approximately 10–12 s in the absence of arthritis. A 20 s cut-off was used to prevent tissue damage in the absence of a response. Both hindpaws were tested for three trials at each time period with 10 min intervals between each trial. The average of the three trials was then determined.

2.6. Western blot analysis

After defined survival times, rats were killed by overdose of urethane (SCR Co., Shanghai, China). The L_5 spinal cord from naive, sham MA, or MA rats were rapidly removed. The L_5 spinal cord segment was dissected according to the termination of the L_4 and L_5 dorsal roots, and then split into a left (ipsilateral to MA side) and right (contralateral) half from the ventral midline. After dissection, all tissues were rapidly frozen in liquid nitrogen and stored at −70 °C until further processing. In order to assess the development of arthritic pain, rats were tested for both mechanical allodynia and hyperalgesia immediately before sacrificed.

Frozen spinal cords were directly homogenized in a lysis buffer (12.5 μl/mg tissue) containing a cocktail of protease inhibitors and PMSF (Sigma). Supernatant, after 10,000 rpm centrifugation for 15 min, was used for Western blotting.

Equal amount of protein (20 μg) was loaded on each lane and separated by 10% SDS–PAGE for the CX3CR1 detection. The resolved proteins were transferred onto PVDF membranes. The membranes were blocked in 10% nonfat dry milk for 2 h at room temperature (RT), and incubated overnight at 4 °C with goat anti-CX3CR1 (1:2000, Santa Cruz Biotechnology) or mouse anti-Tubulin (1:10,000, Sigma) primary antibody. The blots were incubated for 2 h at RT with horseradish peroxidase (HRP)-conjugated donkey anti-goat or donkey anti-mouse secondary antibody (1:5000, Santa Cruz Biotechnology). Signals were finally visualized using enhanced chemiluminescence (ECL, Pierce) and exposed onto X-films for 1–10 min. Pre-absorption of the primary antibodies with the blocking peptide (Santa Cruz Biotechnology) served as specificity control. All Western blot analysis was performed at least three times, and parallel results were obtained. X-ray films with blotting bands for each sample from different rats were scanned, and the density of band area was quantified with a method described by Zhuang et al. (2005). The same size square was drawn around each band to measure the density and background near that band was subtracted. Tubulin expression was used as loading control for protein expression, and CX3CR1 level was normalized against tubulin level. The expression level for protein is an average of densities per band area from different treatment rats.

2.7. Immunohistochemistry

After defined survival times, rats were given an overdose of urethane (2 g/kg, i.p.) and perfused intracardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The L4/5 segments of spinal cord and bilateral L4/5 DRGs were then removed, post-fixed in the same fixative for 4 h at 4 °C, and immersed from 10% to 30% gradient sucrose in PB for 24–48 h at 4 °C for cryoprotection. Transverse spinal and DRG sections (20–30 μm) were cut in a cryostat and processed for immunofluorescence. All the sections were blocked with 10% donkey serum in 0.01 M phosphate buffered saline (PBS, pH 7.4) with 0.3% Triton X-100 for 1 h at RT and incubated overnight at 4 °C with mouse anti-OX-42 (1:3000, Serotec), mouse anti- glial fibrillary acidic protein (GFAP, 1:1000, Sigma), or rabbit anti-ATF3 (1:200, Santa Cruz Biotechnology) primary antibody in PBS with 1% normal donkey serum and 0.3% Triton X-100. Following three 15 min rinses in 0.01 M PBS, the sections were incubated in fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (1:200, Jackson Immunolab) or rhodamine-conjugated donkey anti-rabbit IgG (1:200, Jackson Immunolab) for 90 min at 4 °C, then washed in PBS. Omission of primary antibody served as negative control. All sections were coverslipped with a mixture of 50% glycerin in 0.01 M PBS, and then observed with a Leica fluorescence microscope, and images were captured with a CCD spot camera. Because the morphology of either microglia or astrocytes is complex and immunoreactive staining includes both cell bodies and their processes, cell counts may not sufficiently quantify activation. Therefore, the optical density of immunoreactive staining for OX-42 or GFAP was measured with the Leica Qwin 500 image analysis system (Germany). The relative density of images was determined by subtracting the background density in each image. For each animal, six sections of spinal L_{4-5} were randomly

selected for quantitative evaluation. These corrected density values of six sections were averaged to provide a mean density for each animal.

All the testing (including behavior, Western blot and immunohistochemistry) was performed blinded.

2.8. Statistical analysis

Data were expressed as means ± SEM. Pre-MA baseline measures were analyzed by one-way analysis of variance (ANOVA) for von Frey and Hargreaves' tests. Post-drug time course measures for allodynia and hyperalgesia were analyzed by two-way ANOVA (treatment × time)] followed by Newman–Keuls post hoc test. Western blot analysis was performed by one-way ANOVA followed by Dunnett multiple comparisons. Immunohistochemical analysis was performed by student's *t*-test. $p < 0.05$ was considered statistically significant.

2.9. Experimental procedures

2.9.1. Experiment 1: effects of MA on CX3CR1 expression in the spinal cord

After baseline behavioral assessments, rats received intra-articular injection of 50 μl CFA (day 0). On day 1, 3, and 11 after CFA, rats were sacrificed and the L_5-spinal cord was removed for Western blot analysis. Sham MA rats received an intra-articular injection of sterile NS (50 μl) and were killed at day 3 after NS injection. Naive rats could move freely in their home cages before they were killed. In order to assess the development of allodynia and hyperalgesia, behaviors were tested immediately before sacrifice.

2.9.2. Experiment 2: effects of i.t. CX3CR1 neutralizing antibody on the onset of MA-induced mechanical allodynia and thermal hyperalgesia

After baseline behavioral assessments, rats received twice i.t. injections of either CX3CR1 neutralizing antibody (0.3, or 3 μg) or control IgG (3 μg) at 12 and 1 h prior to intra-articular injection of CFA. Behaviors were tested 4, 8, 12 h and 1–3 days later.

2.9.3. Experiment 3: effects of i.t. CX3CR1 neutralizing antibody on established mechanical allodynia and thermal hyperalgesia

After baseline behavioral assessments, rats received an intra-articular injection of either CFA or equivolume sterile NS (day 0). Behaviors were assessed 24 h later to confirm development of mechanical allodynia and thermal hyperalgesia in MA rats. Directly after behavioral testing at 24 h, rats received an i.t. injection of either CX3CR1 neutralizing antibody (3 μg) or control IgG (3 μg). von Frey and Hargreaves' tests were then performed 1, 2, 3, 4, 6, 8, 12, 24, and 48 h later.

2.9.4. Experiment 4: effects of i.t. CX3CR1 neutralizing antibody on MA-induced glial activation in the lumbar spinal cord

Rats from Experiment 2 were perfused for OX-42 and GFAP immunohistochemical analysis on day 1, 3, and 11 after intra-articular injection of CFA.

2.9.5. Experiment 5: effects of i.t. CX3CR1 neutralizing antibody on thermal hyperalgesia induced by i.t. fractalkine

After baseline behavioral assessment, rats received an i.t. injection of either 30 ng fractaline or vehicle. Behavioral response to thermal stimulation was assessed for 100 min. Some additional rats received twice i.t. injection of either anti-CX3CR1 neutralizing antibody (3 μg) or control IgG (3 μg) at 12 and 1 h (as Experiment 2) prior to i.t. fractalkine (25 ng). Assessment of Hargreaves' test was performed before i.t. CX3CR1 neutralizing antibody (or control IgG) and i.t. fractalkine, as well as a period of 100 min after i.t. fractalkine.

3. Results

3.1. Monoarthritis induces up-regulation of CX3CR1 expression in the spinal cord

The goat polyclonal antibody was directed to the N-terminus of CX3CR1 of mouse origin. This antiserum was first identified by Western blotting of rat spinal preparation. A single immunoreactive band was detected at 31 kDa, consistent with the size of the native mouse CX3CR1. Pre-absorption of CX3CR1 antibody with the blocking peptide abolished the immunolabeling (Fig. 1).

Following intra-articular injection of CFA, Western blot analysis revealed an increase in the level of CX3CR1 on both sides of the spinal cord after MA. In the ipsilateral spinal cord, CX3CR1 levels were significantly increased at all time points examined after MA ($F_{4,15} = 6.752$, $p < 0.01$). On the contralateral spinal cord, the up-regulation of CX3CR1 level occurred from day 3 to 11 after MA. The difference in the level of CX3CR1 among the different treated groups was significant ($F_{4,15} = 4.628$, $p < 0.05$) (Fig. 2).

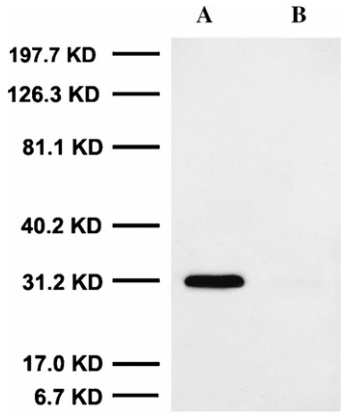

Fig. 1. Identification of CX3CR1 by Western blotting of rat spinal cord preparation. A single immunoreactive band (lane A) was detected at 31 kDa, corresponding to the size of the native mouse CX3CR1. Pre-absorption of CX3CR1 antibody with the blocking peptide abolished the immunoreactive band (lane B).

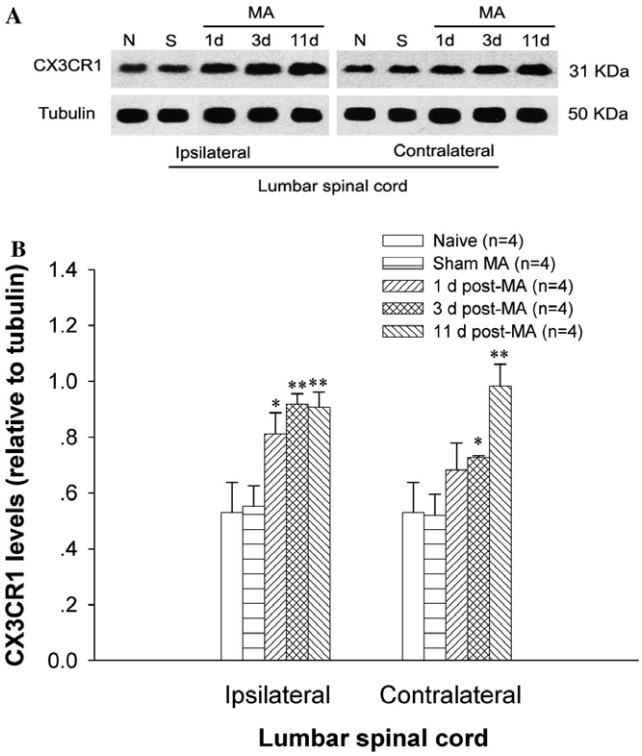

Fig. 2. Time course of CX3CR1 expression on the lumbar spinal cord following intra-articular injection of CFA. (A) Western blot analysis shows an increase in the level of CX3CR1 on the lumbar spinal cord of CFA-induced monoarthritis (MA) rats. Tubulin served as loading control. (B) Quantification of CX3CR1 level on both sides of the spinal cord. *$p < 0.05$, **$p < 0.01$ vs sham MA.

3.2. Intrathecal injection of anti-CX3CR1 neutralizing antibody delays the onset of MA-induced mechanical allodynia and thermal hyperalgesia

Baseline measures of the paw withdrawal threshold (PWT) to von Frey hairs and the paw withdrawal latency (PWL) to radiant heat stimulation did not differ across groups prior to intra-articular injection of CFA (One-way ANOVA, PWTs: $F_{11,81} = 1.511$, $p > 0.05$; PWLs: $F_{11,81} = 1.294$, $p > 0.05$). As in our previous study (Sun et al., 2006), unilateral intra-articular injection of CFA-induced profound mechanical allodynia and thermal hyperalgesia within 4 h and persisted for more than 3 days in the ipsilateral hindpaw to CFA injection (Fig. 3).

To address the role of CX3CR1 in the development of allodynia and hyperalgesia during arthritic pain, anti-CX3CR1 neutralizing antibody (0.3 or 3 μg) or control IgG (3 μg) was injected intrathecally 12 and 1 h prior to intra-articular injection of CFA. I.t. CX3CR1 neutralizing antibody dose-dependently blocked MA-induced mechanical allodynia and thermal hyperalgesia. A higher dose of CX3CR1 neutralizing antibody (3 μg) delayed the development of pain hypersensitivity over 12 h. Two-way ANOVA revealed significant effect of intrathecal CX3CR1 neutralizing antibody (3 μg) treatment (PWTs: $F_{1,84} = 47.254$, $p < 0.01$; PWLs: $F_{1,84} = 45.188$, $p < 0.01$) and interaction between CX3CR1 antibody treatment and time (PWTs: $F_{5,84} = 9.369$, $p < 0.01$; PWLs: $F_{5,84} = 8.372$, $p < 0.01$). Neither PWTs nor PWLs of the contralateral intact hindpaw in MA rats and both hindpaws in sham MA rats were affected by i.t. CX3CR1 antibody. Control IgG (3 μg) did not alter PWTs and PWLs of either the ipsilateral or contralateral hindpaw in MA rats (Fig. 3).

3.3. Intrathecal injection of anti-CX3CR1 neutralizing antibody reverses the established mechanical allodynia and thermal hyperalgesia

To assess CX3CR1 was involved in the maintenance of the MA-induced allodynia and hyperalgesia, the effects of i.t. CX3CR1 neutralizing antibody (3 μg) on the PWTs to von Frey hairs and PWLs to thermal stimuli were examined 24 h after intra-articular injection of CFA when the mechanical allodynia and thermal hyperalgesia stably occurred. I.t. CX3CR1 neutralizing antibody (3 μg) completely reversed established allodynia and hyperalgesia from 6 to 12 h. After 24 h, the mechanical allodynia and thermal hyperalgesia were fully restored. Two-way ANOVA revealed significant effect of intrathecal CX3CR1 antibody treatment (PWTs: $F_{1,168} = 386.335$, $p < 0.01$; PWLs: $F_{1,168} = 82.208$, $p < 0.01$) and interaction between CX3CR1 antibody treatment and time (PWTs: $F_{11,168} = 24.382$, $p < 0.01$; PWLs: $F_{11,168} = 10.038$, $p < 0.01$). Control IgG (3 μg) had no effect on the PWTs and PWLs of either the ipsilateral or contralateral hindpaw (Fig. 4).

3.4. Intrathecal injection of anti-CX3CR1 neutralizing antibody inhibits MA-induced glial activation in the spinal cord

To examine whether blockade of CX3CR1 prevents MA-induced microglial and astrocytic activation, OX-42 (microglial marker) and GFAP (astrocytic marker) immunoreactivity was assessed on the lumbar spinal cord of MA and sham MA rats. As expected (Raghavendra et al., 2004; Tanga et al., 2004), unilateral intra-articular injection of CFA produced a robust increase in the expression for OX-42 immunoreactivity on both sides of the lumbar spinal cord with the more prominent increase being on the ipsilateral spinal cord on day 1, 3, and 11 post-MA (Fig. 5). As shown in Fig. 5A, activated microglia showed short, thickened, or even absent processes and large soma (ameboid). The mean optical density of OX-42 immunoreactivity in the spinal cord was extremely increased in MA rats compared with that in sham MA or naive rats. Pre-administered intrathecally CX3CR1 neutralizing antibody before MA significantly suppressed OX-42 expression on both sides of the spinal cord on day 1 and 3 post-

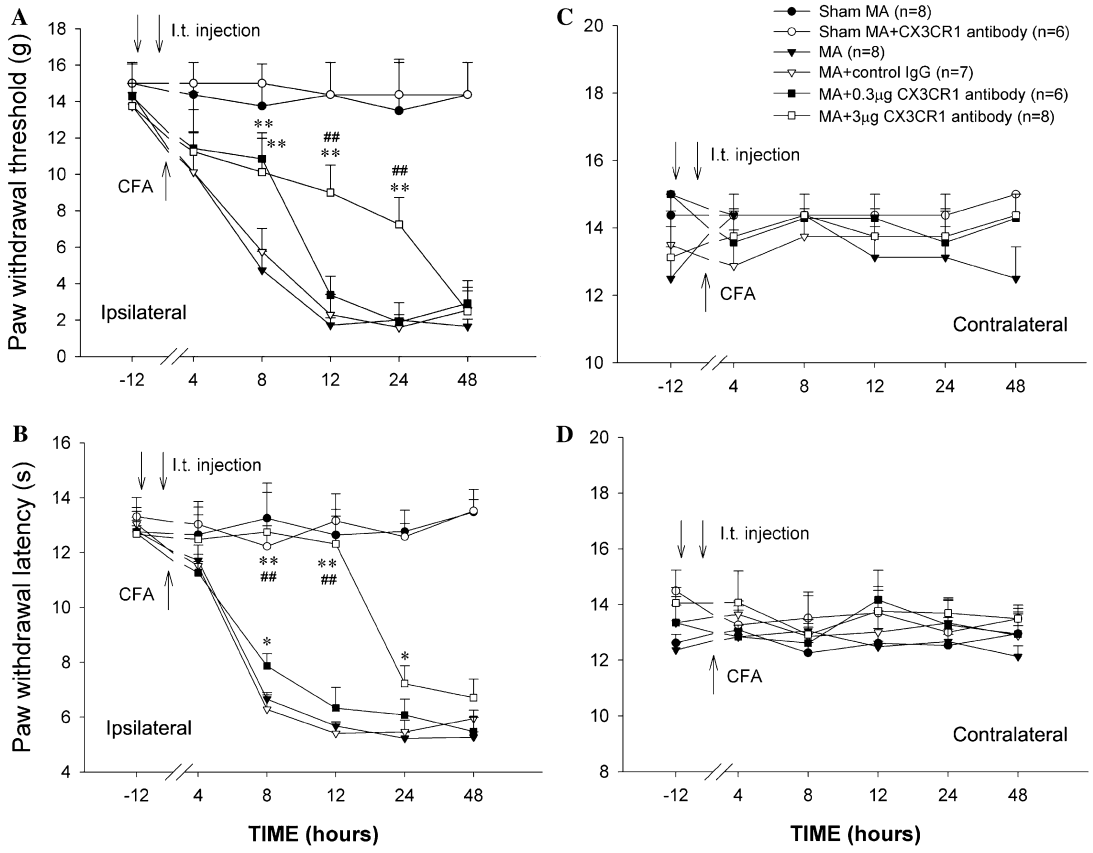

Fig. 3. Intrathecal pre-administration of anti-CX3CR1 neutralizing antibody (3 μg) blocked the onset of mechanical allodynia (A) and thermal hyperalgesia (B) in the ipsilateral hindpaw of monoarthritis (MA) rats, but did not alter the PWTs and PWLs in the contralateral hindpaw (C and D) of MA rats and both hindpaws of sham MA rats. Anti-CX3CR1 neutralizing antibody or control IgG was injected intrathecally 12 and 1 h prior to MA. $^{*}p < 0.05$, $^{**}p < 0.01$ vs control IgG; $^{\#\#}p < 0.01$ vs 0.3 μg CX3CR1 antibody.

MA. Eleven days after MA, inhibition of CX3CR1 neutralizing antibody on microglial activation was completely eliminated. Pre-administered control IgG failed to affect MA-induced microglial activation (Fig. 5A and B).

Unlike microglial activation, a slight increase in activated astrocytes was seen on the lumbar spinal cord by 3 days, and a robust increase by 11 days after MA. I.t. control IgG had no effect on MA-induced up-regulation of GFAP immunoreactivity, whereas pre-administered CX3CR1 neutralizing antibody prior to MA significantly attenuated MA-induced GFAP expression on the ipsilateral spinal cord on day 3 but not on day 11 (Fig. 6A and B). The difference in GFAP expression on the contralateral spinal cord between IgG- and anti-CX3CR1 antibody-treated groups was not statistically significant (Fig. 6B).

3.5. Intrathecal injection of anti-CX3CR1 neutralizing antibody blocks intrathecal fractaline-induced thermal hyperalgesia

To determine whether the neutralizing antibody for CX3CR1 blocks CX3CR1-mediated biological actions of fractalkine, the effect of fractalkine on behavioral response to thermal stimulation was first examined. I.t. fractalkine (25 ng) produced robust thermal hyperalgesia on the Hargreaves' test. Two-way ANOVA revealed significant effect of i.t. fractalkine treatment (left: $F_{1,127} = 129.231$, $p < 0.01$; right: $F_{1,127} = 106.607$, $p < 0.01$) and interaction between fractalkine treatment and time (left: $F_{7,127} = 8.543$, $p < 0.01$; right: $F_{7,127} = 8.543$, $p < 0.01$; 11.293). I.t. vehicle did not alter bilateral PWLs (Fig. 7).

Then, the blockade of CX3CR1 neutralizing antibody on fractalkine-induced thermal hyperalgesia was observed. Anti-CX3CR1 neutralizing antibody (3 μg) or control IgG (3 μg) was injected intrathecally 12 and 1 h prior to i.t. fractalkine. Pretreatment with anti-CX3CR1 neutralizing antibody, but not control IgG, fully prevented fractalkine-induced thermal hyperalgesia (Fig. 7). Thus, fractalkine-induced thermal hyperalgesia is mediated via activation of the fractalkine receptor, CX3CR1.

4. Discussion

A new finding of the present study is upregulation of CX3CR1 level on the spinal cord following

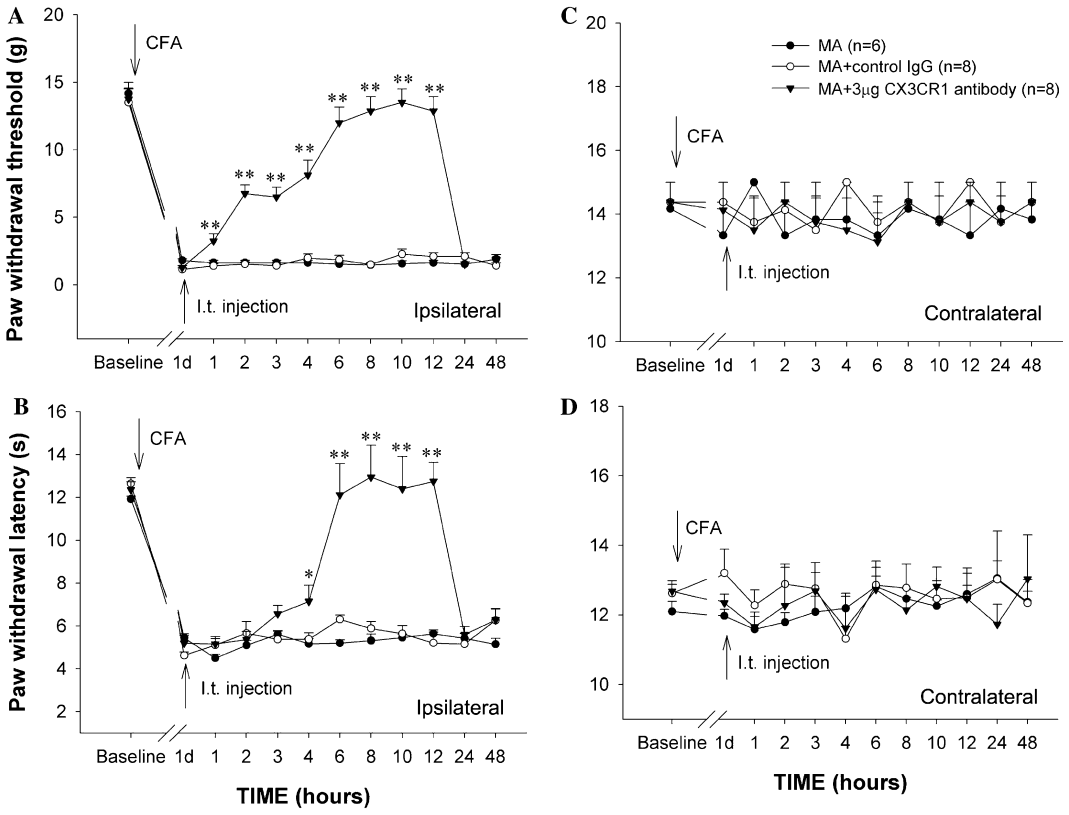

Fig. 4. Intrathecal injection of anti-CX3CR1 neutralizing antibody (3 μg) inhibited established mechanical allodynia (A) and thermal hyperalgesia (B) in the ipsilateral hindpaw of monoarthritis (MA) rats, whereas the PWTs and PWLs in the contralateral hindpaw were not altered (C and D). Anti-CX3CR1 antibody or control IgG was given one day after MA. *$p < 0.05$, **$p < 0.01$ vs control IgG.

CFA-induced MA. The time course of the upregulated CX3CR1 on the ipsilateral spinal cord followed by intra-articular injection of CFA paralleled the microglial activation and the initiation and maintenance of monoarthritic pain. This result partly supported and extended an earlier finding that both zymosan-induced sciatic inflammatory neuropathy (SIN), and chronic constriction injury (CCI)-induced trauma increased CX3CR1 mRNA expression on the ipsilateral spinal cord, and the most robust signal enhancement was observed at 24 h and still apparent 2 weeks later (Verge et al., 2004). With respect to the ipsilateral, a delayed up-regulation of CX3CR1 was observed on the contralateral spinal cord, suggesting that contralateral increase in CX3CR1 may be secondary. Fractalkine, as a neuron-to-glia signal, releases from inflamed sensory neurons and dorsal horn neurons in response to joint inflammation. Microglial binding of fractalkine rapidly induces the release of the glial-excitatory substances that then act on other glia. Via gap junctions and propagated calcium waves, excitation of glia at the ipsilateral dorsal horn can reach the contralateral one, causing release of neuro- and glial-excitatory substances, leading to up-regulation of contralateral CX3CR1 level (Hassinger et al., 1995; Innocenti et al., 2000; Parri et al., 2001). These are supported to some extent by the findings that CX3CR1 mRNA and OX-42-IR increased on both ipsilateral and contralateral spinal cord in the inflammation models (Sweitzer et al., 1999; Verge et al., 2004).

A different result from the present study was reported by Lindia et al. (2005), who found that the upregulation of CX3CR1 on the spinal cord only occurred in the nerve injury condition, but not the peripheral inflammatory model. The reason for the conflicting result is not very clear, but the differences in technical terms and inflammatory model may be primary. For example, in the study by Lindia et al. (2005), CX3CR1 expression was assessed by immunohistochemistry in a cutaneous tissue inflammatory model, whereas in the present study, CX3CR1 expression level on the spinal cord from deep tissue inflammatory (joint inflammation) rats was analyzed by Western blot. As we know, nociceptive afferent fibers terminate predominantly on the dorsal horn of the spinal cord. The dorsal horn is subdivided into six distinct layers (laminae) on the basis of the cytological features and its resident neurons. Cutaneous nociceptive Aδ- and C-fibers terminate predominately in laminae I and II, comparatively weak input to laminae V and X. Unmyelinated nociceptive afferents from the joints and muscle project primarily to laminae I, V, and VI

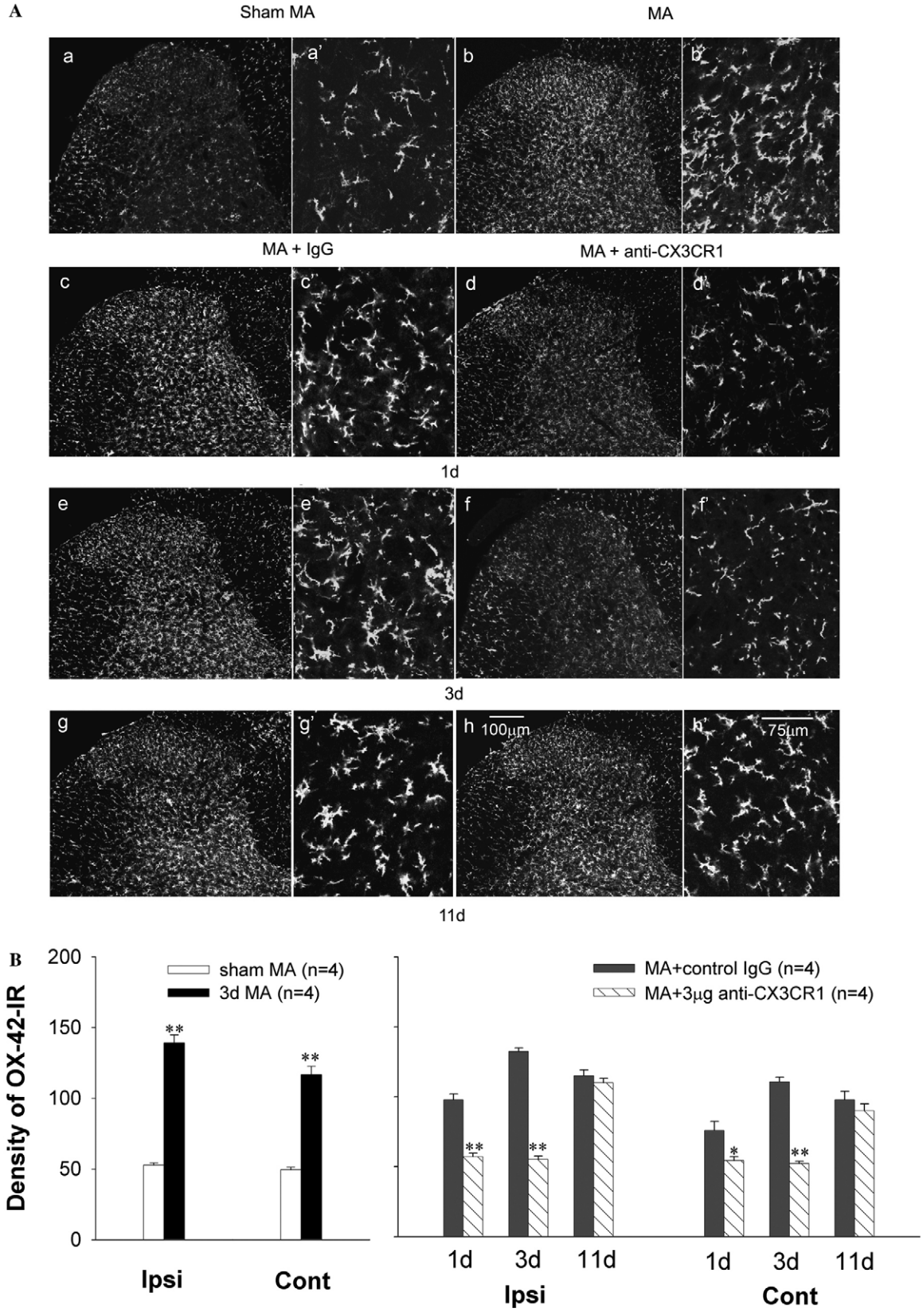

Fig. 5. (A) Photomicrographs showing OX-42 (microglial marker) immunoreactivity on the ipsilateral spinal cord. (Aa) sham MA; (Ab) MA; (Ac, e, g) control IgG (3 μg) + MA on day 1 (Ac), day 3 (Ae) and day 11 (Ag) after MA; (Ad, f, h) CX3CR1 neutralizing antibody (3 μg) + MA on day 1 (Ad), day 3 (Af) and day 11 (Ah) after MA. (Aa–h) 10×; (Aa′–h′) 40×. (B) Quantification of OX-42 immunoreactivity on both sides of the spinal cord. Anti-CX3CR1 antibody or control IgG was given 12 and 1 h prior to MA. $^{**}p < 0.01$ vs sham MA; $^{\#\#}p < 0.01$ vs control IgG.

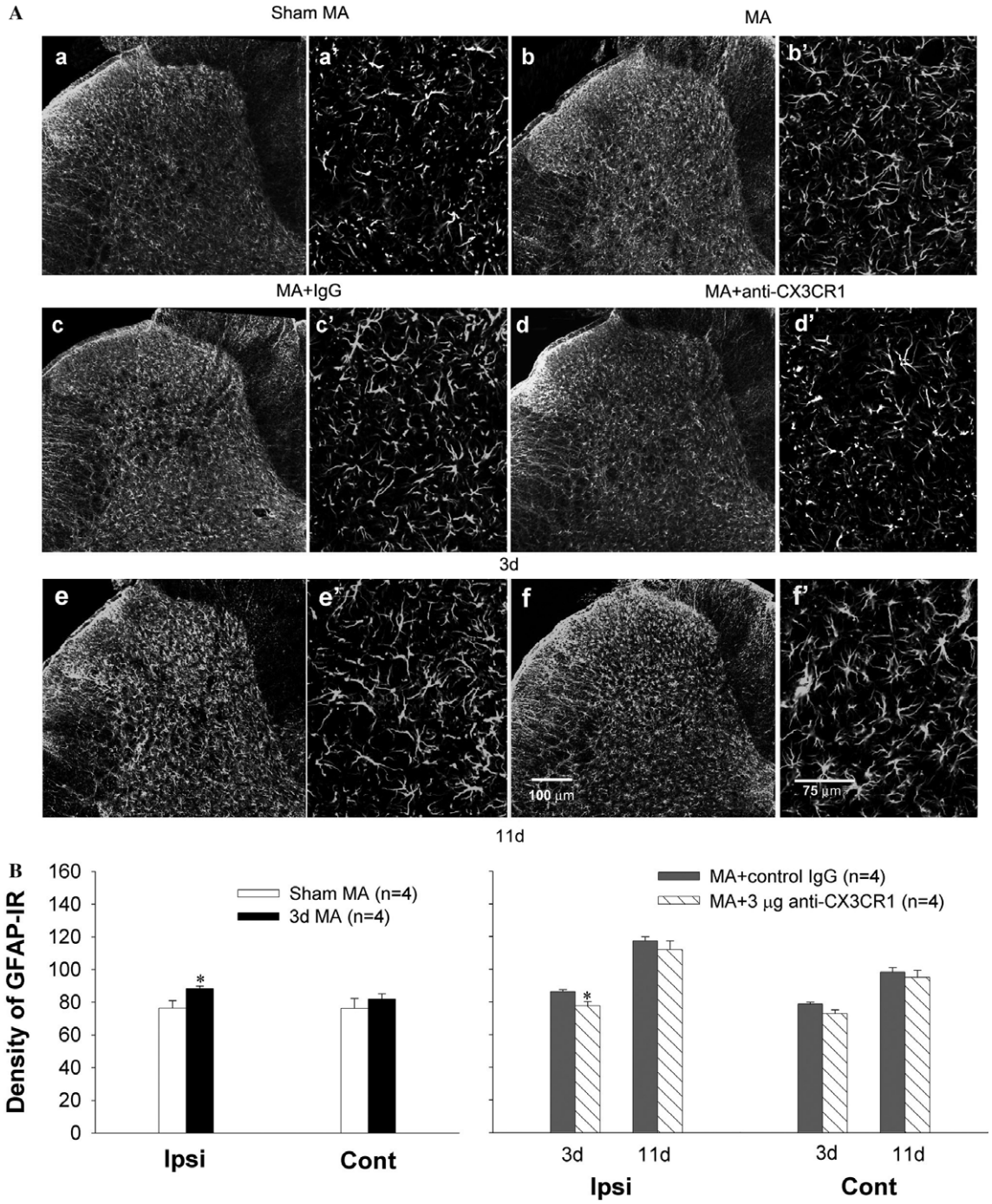

Fig. 6. (A) Photomicrographs showing GFAP (astrocytic marker) immunoreactivity on the ipsilateral spinal cord. (Aa) sham MA; (Ab) MA; (Ac, e) control IgG (3 μg) + MA on day 3 (Ac) and day 11 (Ae) after MA; (Ad,f) CX3CR1 neutralizing antibody (3 μg) + MA on day 3 (Ad) and day 11 (Af) after MA. (Aa–f) 10×; (Aa′–f′) 40×. (B) Quantification of GFAP immunoreactivity on the both sides of the spinal cord. Anti-CX3CR1 antibody or control IgG was given 12 and 1 h prior to MA. $^{*}p < 0.05$ vs sham MA; $^{\#}p < 0.05$ vs control IgG.

(Haevner et al., 2003). The different spinal targets of primary afferent nociceptive information from cutaneous and joint inflammation may be one of the explanations. In addition, it is possible that the inflammation of tissue could damage primary sensory neurons, which in turn leads to an activation of microglia on the dorsal horn. To address this issue, we examined whether MA causes damage of primary afferent sensory neurons using immunohistochemistry of ATF3, a marker of damage neurons. Unexpectedly, no ATF3 positive cells were found on the L4/5 DRG from either sham MA or MA rats (Fig. 8), suggesting that damage of primary afferent neurons has not happened within the time window examined after MA.

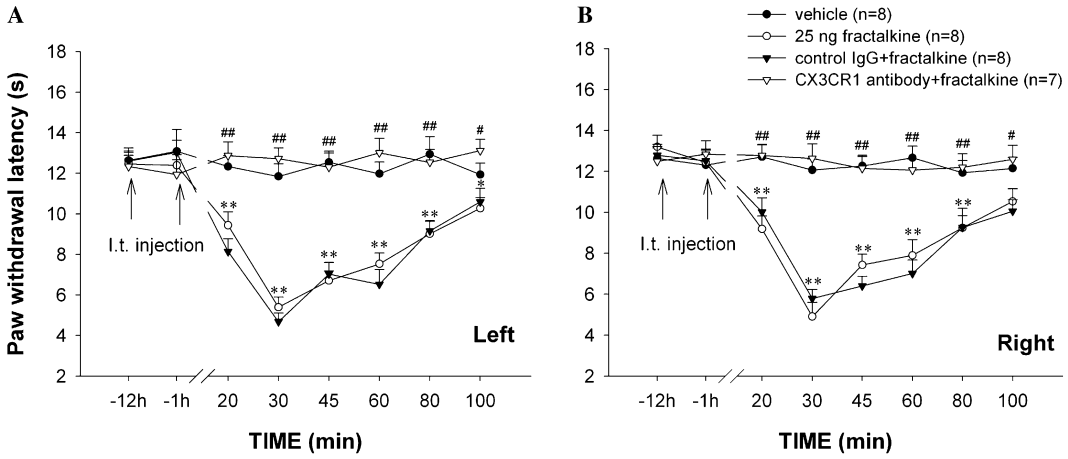

Fig. 7. Intrathecal injection of fractalkine (25 ng) produced thermal hyperalgesia in the bilateral hindpaws of naïve rats. Pre-administration of anti-CX3CR1 neutralizing antibody (3 μg) prevented fractalkine-induced thermal hyperalgesia. Anti-CX3CR1 neutralizing antibody or control IgG was injected intrathecally 12 and 1 h prior to intrathecal fractaline. $^{*}p < 0.05$, $^{**}p < 0.01$ vs vehicle; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs control IgG.

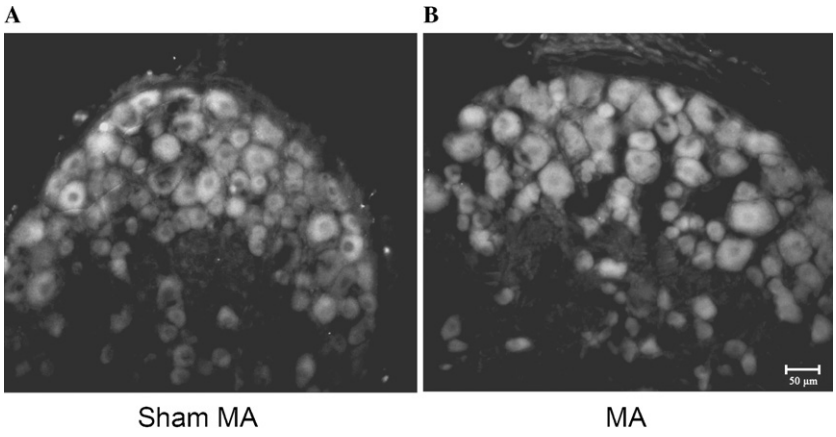

Fig. 8. Photomicrographs showing ATF3 immunoreactivity on L4 DRG. No positive nuclei were seen on the ipsilateral DRGs by 3 days after sham MA (A) and MA (B).

The previous studies from Waktins' group have shown that blockade of endogenous fracktalkine, using neutralizing antibody against rat fractalkine receptor, CX3CR1, either delayed the development of mechanical allodynia and thermal hyperalgesia or attenuated the established hypersensitive behaviors in two neuropathic pain models: CCI and SIN (Milligan et al., 2004). As an effort to elucidate a unifying role for fracktalkine in neuropathic and inflammatory pain, we further examined whether peripheral inflammation-induced mechanical allodynia and thermal hyperalgesia can be blocked by anti-CX3CR1 neutralizing antibody in CFA-induced ankle joint monoarthritis model. In agreement with the previous study on neuropathic pain model (Milligan et al., 2004), the present study showed that intrathecal administration of anti-CX3CR1 neutralizing antibody both delayed the onset of MA-induced pain hypersensitivity and attenuated established mechanical allodynia and thermal hyperalgesia in early phase of inflammation, suggesting that endogenous fracktalkine contributes to either the development or at least the early maintenance of monoarthritic pain.

Another intriguing result in the current study is that intrathecal pretreatment of rats with the anti-CX3CR1 neutralizing antibody led to a marked decrease in MA-induced microglial and astrocytic activation (as determined by OX-42 and GFAP immunoreactivity), especially the microglial activation, on the spinal cord for 3 days post-MA. This result strongly supported the proposal that fractalkine on the spinal cord may potentially be a glial-excitatory signal leading to glial activation. It has been demonstrated that, in the spinal dorsal horn, fractalkine was expressed by intrinsic neurons and sensory afferents in naive rats (Verge et al., 2004), even also by astrocytes in spinal nerve ligation (SNL) rats (Lindia et al., 2005), whereas fractalkine receptor, CX3CR1, was predominantly expressed by microglia (Verge et al., 2004; Lindia et al., 2005). In parallel with this, Milligan et al. (2005) reported that blockade of microglial activation with minocycline, a

microglial inhibitor, prevented intrathecal fractalkine-induced mechanical allodynia and thermal hyperalgesia. Thus, fractalkine-induced pain hypersensitive behaviors were mediated via the activation of spinal microglia.

It should be mentioned that pretreatment of anti-CX3CR1 antibody prevented MA-induced up-regulation of OX-42-IR for 3 days, but blocked behavioral hypersensitivity for less than 48 h. This contradiction may be explained that OX-42-IR is not a sensitive indicator of behavioral hypersensitivity. Dissociation between OX-42-IR and mechanical allodynia was reported in some neuropathic pain models (Colbum et al., 1997; Winkelstein and DeLeo, 2002). More sensitive markers of nociceptive behavioral changes are needed and further experiments are still required.

As mentioned in introduction, fractalkine is a chemokine that is tethered to the extracellular surface of neurons and can be released forming a diffusible signal (Chapman et al., 2000). Its only receptor, CX3CR1, is expressed by microglia (Schall, 1997; Hesselgesser and Horuk, 1999; Verge et al., 2004; Lindia et al., 2005). Prior works have led to the concept that fractalkine may play a role in signaling between neuron and microglia (Harrison et al., 1998; Nishiyori et al., 1998; Maciejewski-Lenoir et al., 1999). In vitro exposure of neuronal mixed cultures and brain slices to glutamate, tumor necrosis factor or interferon gamma was sufficient to release fractalkine (Chapman et al., 2000; Erichsen et al., 2003). Fractalkine could exert a pro-inflammatory role in that fractalkine induces microglial migration and activation. This activation, if occurred in the spinal cord, in turn would lead to pain facilitation. We speculate that, in response to peripheral inflammation, strong neuronal activation may release fractalkine that attracts CX3CR1-expressing microglia. Microglia binding of fractalkine leads to release of the glial-excitatory substances that then induce astrocytic activation (Milligan et al., 2004; Verge et al., 2004). Activated microglia and astrocytes release a variety of neuro- and glial-excitatory substances, i.e., nitric oxide, prostaglandin, and pro-inflammatory cytokines (Tikka and Koistinaho, 2001; Watkins and Maier, 2002; Sommer, 2003; Raghavendra et al., 2004; Wieseler-Frank et al., 2004). These substances have each been implicated in initiation and maintenance of pain hypersensitivity. This may explain why prevention of CX3CR1 activation is able to block the development and maintenance of MA-induced mechanical allodynia and thermal hyperalgesia.

In conclusion, the present study demonstrated for the first time that CX3CR1 expression level was significantly increased in the spinal cord following CFA-induced rat monoarthritis. Intrathecal injection of anti-CX3CR1 neutralizing antibody not only delayed or blocked the development and early maintenance of mechanical allodynia and thermal hyperalgesia, but also suppressed MA-induced spinal microglial and astrocytic activations. Taken together, these data indicate that endogenous fractalkine may contribute to the development and maintenance of MA-induced pain hypersensitivity via activation of spinal glia.

Acknowledgements

This work was supported by National Natural Science Fund of China (NSFC) (30570594, and 30370471), NSFC for Distinguished Young Scholars (30425022), National Basic Research Program of China (2006CB500807), Program for Changjang Scholars and Innovative Research Team in University (PCSIRT), Research Fund for Doctoral Program of Higher Education (20050246017), and Science Foundation of Shanghai Municipal Commission of Science and Technology (02DZ19150-4).

References

Altman RD, Hochberg MC, Moskowitz RW, Schnitzer TJ. Recommendations for the medical management of osteoarthritis of the hip and knee: 2000 update. American College of Rheumatology Subcommittee on Osteoarthritis Guidelines. Arthritis Rheum 2000;43:1905–15.

Asensio VC, Campbell IL. Chemokines in the CNS: plurifunctional mediators in diverse states. Trends neurosci 1999;22:504–12.

Bajetto A, Bonavia R, Barbero S, Schettini G. Characterization of chemokines and their receptors in the central nervous system: physiopathological implications. J Neurochem 2002;82:1311–29.

Chapman GA, Moores K, Harrison D, Campbell CA, Steward BR. Strijbos PJLM. Fractalkine cleavage from neuronal membranes represents an acute event in the inflammatory response to excitotoxic brain damage. J Neurosci 2000;20(RC87):81–5.

Colbum RW, DeLeo JA, Richman AJ, Yeager MP, Kwon P, Hickey WF. Dissociation of microglial activation and neuropathic pain behaviors following peripheral nerve injury in the rat. J Neuroimmunol 1997;79:163–75.

Colpaert FC. Evidence that adjuvant arthritis in the rat is associated with chronic pain. Pain 1987;28:201–22.

Combadiere C, Salzwedel K, Smith ED, Tiffany EA, Berger HL, Murphy PM. Identification of CX3CR1. J Bio Chem 1998;273:23799–804.

Deleo JA, Tanga FY, Tawfik VL. Neuroimmune activation and neuroinflammation in chronic pain and opioid tolerance/hyperalgesia. Neuroscientist 2004;10:40–52.

Erichsen D, Lopez AL, Peng H, Niemann D, Willisms C, Bauer M, et al. Neuronal injury regulates fractalkine: relevance for HIV-1 associated dementia. J Neuroimmunol 2003;138:144–55.

Haevner JE, Willis WD. Pain pathways: anatomy and physiology. In: Raj PP, editor. Pain medicine: a comprehensive review. 2nd ed. St. Louis: Mosby; 2003, pp. 10–5.

Harrison JK, Jiang Y, Chen S, Xia Y, Maciejewski-Lenoir D, McNamara RK, et al. Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1 expressing microglia. Proc Natl Acad Sci USA 1998;95:10896–901.

Hassinger TD, Atkinson PB, Strecker GJ, Whalen LR, Dudek FE, Kossel AH, et al. Evidence for glutamate-mediated activation of hippocampal neurons by glial calcium waves. J Neurobiol 1995;28:159–70.

Hesselgesser J, Horuk R. Chemokine and chemokine receptor expression in the central nervous system. J Neurovirol 1999;5:13–26.

Hughes PM, Batham MS, Frentzel S, Mir A, Perry VH. Expression of fractalkine (CX3CL1) and its receptor (CX3CR1), during acute and chronic inflammation in the rodent CNS. Clia 2002;37:314–27.

Innocenti B, Parpura V, Haydon PG. Imaging extracellular waves of glutamate during calcium signaling in cultured astrocytes. J Neurosci 2000;20:1800–8.

Ledeboer A, Sloane EM, Milligan ED, Frank MG, Mahony JH, Maier SF, et al. Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. Pain 2005;115:71–83.

Lindia JA, McGowan E, Jochnowitz N, Abbadie C. Induction of CX3CL1 expression in astrocytes and CX3CR1 in microglia in the spinal cord of a rat model of neuropathic pain. J Pain 2005;6:434–8.

Maciejewski-Lenoir D, Chen S, Feng L, Maki R, Bacon KB. Characterization of fractalkine in rat brain cells: migratory and activation signals for CX3CR1 expressing microglia. J Immunol 1999;163:1628–35.

McMahon SB, Cafferty WBJ, Marchand F. Immune and glial cell factors as pain mediators and modulators. Exp Neurol 2005;192:444–62.

Milligan ED, Twining C, Chacur M, Biedenkapp J, O'Connor K, Poole S, et al. Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. J Neurosci 2003;23:1026–40.

Milligan ED, Zapata V, Chacur M, Schoeniger D, Biedenkapp J, O'Connor KA, et al. Evidence that exogenous and endogenous fractalkine can induce spinal nociceptive facilitation in rats. Eur J Neurosci 2004;20:2294–302.

Milligan E, Zapata V, Schoeniger D, Chacur M, Green P, Poole S, et al. An initial investigation of spinal mechanisms underlying pain enhancement induced by fractalkine, a neuronally released chemokine. Eur J Neurosci 2005;22:2775–82.

Nishiyori A, Minami M, Ohtani Y, Takami S, Yamamoto J, Kswaguchi N, et al. Localization of fractalkine and CX3CR1 mRNAs in rat brain: does fractalkine play a role in signaling from neuron to microglia? FEBS Lett 1998;429:167–72.

Parri HR, Gould TM, Crunelli V. Spontaneous astrocytic Ca^{2+} oscillations in situ drive NMDAR-mediated neuronal excitation. Nat Neurosci 2001;4:803–12.

Pitcher GM, Ritchie J, Henry JL. Paw withdrawal threshold in the von Frey hair test is influenced by the surface on which the rat stands. J Neurosci Method 1999;87:185–93.

Raghavendra V, Tanga FY, Deleo JA. Complete Freunds adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. Eur J Neurosci 2004;20:467–73.

Schall T. Fractalkine – a strange attractor in the chemokine landscape. Immunol Today 1997;18:147.

Sommer C. Painful neuropathies. Curr Opin Neurol 2003;16:623–8.

Sun S, Chen WL, Wang PF, Zhao ZQ, Zhang YQ. Disruption of glial function enhances electroacupuncture analgesia in arthritic rats. Exp Neurol 2006; in press.

Sweitzer SM, Colburn RW, Rutkowski M, DeLeo JA. Acute peripheral inflammation induces moderate glial activation and spinal IL-1beta expression that correlates with pain behavior in the rat. Brain Res 1999;829:209–21.

Takaishi K, Eisele Jr JH, Carstens E. Behavioral and electrophysiological assessment of hyperalgesia and changes in dorsal horn responses following partial sciatic nerve ligation in rats. Pain 1996;66:297–306.

Tanga FY, Raghavendra V, DeLeo JA. Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. Neurochem Int 2004;45:397–407.

Tikka TM, Koistinaho JE. Minocycline provides neuroprotection against *N*-methyl-D-aspartate neurotoxicity by inhibiting microglia. J Immunol 2001;166:7527–33.

Tsuda M, Inoue K, Salter MW. Neuropathic pain and spinal microglia: a big problem from molecules in 'small' glia. Trends Neurosci 2005;28:101–7.

Twining CM, Sloane EM, Schoeniger DK, Milligan ED, Martin D, Marsh H, Maier SF, Watkins LR. Activation of the spinal cord complement cascade might contribute to mechanical allodynia induced by three animal models of spinal sensitization. J Pain 2005;6:174–83.

Verge GM, Milligan ED, Maier SF, Watkins LF, Naeve GS, Foster AC. Fractaline (CX3CL1) and fractalkine receptor (CX3CR1) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. Eur J Neurosci 2004;20:1150–60.

Watkins LR, Maier SF. Beyongd neurons: evidence that immune and glial cells contribute to pathological pain states. Physiol Rev 2002;82:981–1011.

Watkins LR, Maier SF. Glia: a novel drug discovery target for clinical pain. Nat Rev Drug Discov 2003;2:973–85.

Watkins LR, Milligan ED, Maier SF. Glial activation: a driving force for pathological pain. Trends Neurosci 2001;24:450–5.

Watkins LR, Hutchinson MR, Johnston IN, Maier SF. Glia: novel counter-regulators of opioid analgesia. Trends Neurosci 2005;28:661–9.

Wieseler-Frank J, Maier SF, Watkines LR. Glial activation and pathological pain. Neurochem Int 2004;45:389–95.

Winkelstein BA, DeLeo JA. Nerve root injury severity differentially modulates spinal glial activation in a rat lumbar radiculopathy model: considerations for persistent pain. Brain Res. 2002;956:294–301.

Zhang YQ, Ji GC, Wu GC, Zhao ZQ. Excitatory amino acid receptor antagonists and electroacupuncture synergetically inhibit carrageenan-induced behavioral hyperalgesia and spinal fos expression in rats. Pain 2002;99:525–35.

Zhuang ZY, Gerner P, Woolf CJ, Ji RR. ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. Pain 2005;114:149–59.