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Intra-neural administration of fractalkine attenuates neuropathic pain-related behaviour

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Abstract

There is increasing evidence that a number of cytokines and their receptors are involved in the processes that lead to the development and maintenance of neuropathic pain states. Here we demonstrate that levels of CX3CR1 (the receptor for the chemokine fractalkine) mRNA in lumbar dorsal root ganglia (DRG) increase 5.8-fold 7 days after sciatic nerve axotomy, and 1.7- and 2.9-fold, 3 and 7 days respectively, after the spared nerve injury (SNI) model of neuropathic pain. In contrast, no significant change in the levels of fractalkine mRNA is apparent in the DRG after axotomy or SNI. The increase in CX3CR1 mRNA is paralleled by a 3.9- and 2.1-fold increase in the number of CX3CR1-positive macrophages in the DRG 7 days after axotomy and SNI, respectively. Expression of CX3CR1 in macrophages is also markedly increased in the sciatic nerve proximal to site of injury, by 25.7-fold after axotomy and 16.2-fold after SNI, 7 days after injury. Intra-neural injection into the sciatic nerve of 400 ng or 100 ng of fractalkine in adult 129OlaHsd mice significantly delayed the development of allodynia for 3 days following SNI. Further, CX3CR1 knockout (KO) mice display an increase in allodynia for three weeks after SNI compared to strain-matched Balb/c controls. Taken together, these results suggest an anti-allodynic role for fractalkine and its receptor in the mouse.

Keywords

CX3CL1; CX3CR1; dorsal root ganglia; fractalkine; nerve injury; neuropathic pain

Damage or injury to the peripheral nervous system (PNS) induces alterations in many of the properties of sensory neurons and their central connections, which may lead to allodynia

(the perception of pain from a normally innocuous stimulus), hyperalgesia (an exaggerated response to any given pain stimulus) and the development of chronic (neuropathic) pain. Neuropathic pain is hugely detrimental to an individual's quality of life, causes considerable disability and is an increasing economic burden to health-care systems. Despite intensive research over a number of decades, the mechanisms that cause chronic pain states are still poorly understood (reviewed in Baron 2006).

One approach to elucidate the mechanisms that underlie the response of the PNS to injury is to identify and study factors and/or their receptors whose levels and distribution in the DRG are altered by nerve injury. Using degenerate primer PCR-screening we have previously demonstrated that CX3CR1, the receptor for fractalkine (CX3CL1), is expressed in the mouse DRG and that the levels of this chemokine receptor are markedly up-regulated after sciatic nerve section (axotomy) (Holmes *et al.* 2004). Fractalkine is the only ligand known to bind and activate the CX3CR1 receptor, and does not bind any other known mammalian chemokine receptors (Stievano *et al.* 2004). Thus, the normal promiscuity that typifies the chemokine superfamily is absent, suggesting that the roles played by fractalkine and CX3CR1 are non-redundant and may be of critical importance to the nervous system.

Fractalkine and CX3CR1 are widely expressed in the nervous system (Harrison *et al.* 1994, 1998; Bazan *et al.* 1997), with fractalkine being constitutively expressed by neurons and CX3CR1 predominantly, but not exclusively, by microglia (Bazan *et al.* 1997; Nishiyori *et al.* 1998; Tarozzo *et al.* 2003), and it has been suggested that fractalkine might act as a specific signal between neurons and glia (Harrison *et al.* 1998). Recent studies in rat have demonstrated that fractalkine is expressed by most, if not all, neurons of the DRG and dorsal horn of the spinal cord, and levels do not change after peripheral nerve injury or inflammation. In contrast, CX3CR1 seems to be exclusively expressed by microglia in the spinal cord and macrophages in the DRG, and markedly up-regulates in the cord, but apparently not in the rat DRG after nerve injury, and variably after inflammation (Verge *et al.* 2004; Lindia *et al.* 2005).

The functional significance of the fractalkine and CX3CR1 interaction in the spinal cord has recently been studied (Milligan *et al.* 2004, 2005). Acute intrathecal (IT) administration of fractalkine to intact adult rats produced pain facilitation (mechanical allodynia and thermal hyperalgesia), whereas IT injection of an immunoneutralising antiserum to CX3CR1 attenuated the development and maintenance of neuropathic pain (Milligan *et al.* 2004). Similarly, injection of fractalkine into the periaqueductal grey (PAG) region of the midbrain is also pro-nociceptive (Chen *et al.* 2007). In contrast, here we show that peripheral intraneural administration of fractalkine inhibits allodynia in the spared nerve injury (SNI) model of neuropathic pain. Further, CX3CR1 KO mice have increased allodynia in the SNI model of neuropathic pain. These results suggest an anti-allodynic role for fractalkine, and its receptor CX3CR1, in the mouse.

Materials and methods

Animals

Adult 12-14 week old male mice of the 129OlaHsd strain were used in all experiments unless otherwise specified. Animals heterozygous for the loss-of-function mutation in the CX3CR1 gene (Combadière *et al.* 2003) were backcrossed for six generations with wildtype Balb/c mice. Heterozygotes were crossed and KO and wildtype lines were then derived and bred at the University of Bristol. Animals were housed in standard conditions and fed standard chow and water *ad libitum*. Repeat serological testing confirmed that the animals used in this study were negative for murine cytomegalovirus. All procedures conformed to the principles of UK animal legislation and animals were humanely sacrificed at the end of

each experiment. Tissue from CX3CR1^{+/GFP} mutant mice on the C57BL/6 strain (Jung *et al.* 2000) were obtained from the colony at OSU, Ohio, USA.

Surgery

For all surgical procedures mice were anaesthetised with Hypnorm (Fentanyl citrate 0.315 mg/mL + Fluanisone 10 mg/mL, VetaPharma Ltd):Hypnovel (Midazolam 5 mg/mL, Roche):water at a ratio of 1:1: 2 at 4 µL/g. For axotomy the sciatic nerve was exposed at lower-thigh level and a ~3 mm segment of nerve resected. For intra-neural injections the sciatic nerve was exposed as above as previously described (Zelenka *et al.* 2005), and mouse fractalkine (chemokine domain, amino acid residues 25-105, R and D Systems) or vehicle [phosphate-buffered saline (PBS)/0.01% bovine serum albumin] in a volume of 4 µL was slowly injected perineuronally over a 5 min period into the nerve using a pulled microcapillary pipette attached to a Hamilton syringe. In some experiments intrasciatic injections were performed immediately prior to SNI (see below). A modification of the SNI model (Decosterd and Woolf 2000), as previously described (Holmes *et al.* 2003; Shields *et al.* 2003) was used as a model of neuropathic pain. An incision was made in the lower thigh, just above the level of the knee, exposing the three terminal branches of the sciatic nerve: the common peroneal, tibial and sural nerves. The common peroneal and sural nerves were tightly ligated with 7/0 silk and sectioned distal to the ligation, leaving the tibial branch intact. Following these procedures the overlying muscle and skin was sutured and the animals allowed to recover. In sham-operated animals the sciatic nerve was exposed at lower thigh level, but not lesioned.

Quantitative RT-PCR

CX3CR1 expression was originally detected in mouse DRG poly(A)⁺ RNA during screening with the degenerate primers 2F [5'-GC ACC G(CT)I AT(GCT) AG(CT) GTI GAC CG(AC) TAT-3', where I is deoxyinosine] and 1R [5'-G CAG GAA G(GC)C (AG)TA GA(GT) (AG)A(GCT) GGG GTT-3'], designed to amplify G-protein coupled receptor sequences encoding transmembrane domains 3-7 (TM3-TM7) (N.K., unpublished). Quantitative RT-PCR was performed as described previously (Kerr *et al.* 2004). Briefly, ipsilateral L4 and L5 DRGs were collected from three groups of six animals after sciatic axotomy, SNI or sham surgery. Total RNA was purified using a Qiagen RNeasy Mini Kit, and treated with 10 units of RQ1 RNase-free DNase (Promega) which was inactivated during re-extraction of RNA following RNA cleanup. For each RNA sample, duplicate reactions with reverse transcriptase (RT+) and one without enzyme (RT-), were performed with 1 µg RNA per 50 µL reverse transcription (RT) reaction using Applied Biosystems TaqMan Reverse Transcription Reagents Kit. Triplicate PCRs were performed on each of these samples. The sequence detection primers were designed using Primer Express software (Applied Biosystems). Primers to detect CX3CR1 were forward primer 5'-TCCGCAACTCGGAAGTCAAC-3' and reverse primer 5'-GAAGTAGCAAAGCTCATGATAAGCA-3' corresponding, respectively, to nt 791-810 and 858-833 of the published CX3CR1 cDNA sequence (AF074912) (Combadière *et al.* 1998a). Primers to detect fractalkine were forward primer 5'-CTCATCCGCTATCAGCTAAACCA-3' and reverse primer 5'-CCTTCGGGTCAGCACAGAAG-3' corresponding, respectively, to nt 227-249 and 320-301 of the published fractalkine cDNA sequence (NM_009142). SYBR green was used as reporter dye. Endogenous control gene GAPDH cDNA primers were forward primer 5'-GCAGTGGCAAAGTGGAGATTG-3' and reverse primer 5'-CTGGAACATGTAGACCATGTAGTTGA-3' and TaqMan probe (VIC label, plus TAMRA quencher) 5'-CCATCAACGACCCCTTCATTGAC-3', that correspond, respectively, to nt 111-131, 184-159 and 135-157 (M32599). Relative expression levels were determined by the comparative C_t method. Results are presented as mean of three

separate groups of DRG \pm SEM of log transformed data. Levels of expression in ipsilateral L4 and L5 DRG after axotomy and SNI were compared to that of ipsilateral L4 and L5 DRG after sham surgery.

Behavioural testing

In all tests the examiner was blind to the treatment group or genotype of the mice and the animals were tested in constant conditions. Thermal thresholds were measured according to the method of Hargreaves *et al.* (Hargreaves *et al.* 1988). Animals were habituated to the testing environment for at least 30 min. The hind paws were exposed to a beam of radiant heat through a transparent Perspex holding box (Ugo Basile) and the latency of withdrawal recorded automatically. Each hind paw was measured three times with an interval of at least 5 min between measurements.

Mechanical thresholds were measured with a series of calibrated von Frey filaments from 0.005 g to a maximum of 3.63 g. Animals were placed in Perspex enclosures placed on an elevated grid (Ugo Basile) and habituated for 30 min prior to testing. Mechanical sensitivity was assessed on each mid-plantar hind paw, employing the up-down testing paradigm to determine the threshold force required to elicit a withdrawal response to 50% of stimulations (Dixon 1980; Chaplan *et al.* 1994). Mice were tested on three separate days prior to surgery (with the third set of measurements used as baseline values), and then at regular intervals after surgery.

Immunohistochemistry

Mice were intracardially perfused with 4% paraformaldehyde/PBS and the spinal columns and sciatic nerves removed and post-fixed for 4 h at 22°C. Spinal cords, L4 and L5 DRG were dissected and all tissues equilibrated in 20% sucrose overnight at 4°C, embedded in Tissue-Tek[®] OCT mounting medium, frozen on dry ice and then cryostat sectioned (8 μ M sections) onto slides. Sections were blocked and permeabilised in 10% normal donkey serum/PBS 0.2% Triton-X-100 (PBST) for 1 h at 22°C. Sections were then incubated in rabbit anti-CX3CR1 (Nanki *et al.* 2004) (1 : 1000) overnight at 22°C. Sections were then washed 3 \times 10 min in PBS and incubated in donkey anti-rabbit FITC-conjugated secondary antibody (Jackson Labs) for 3 h at 22°C. After washing, sections were mounted in Vectashield (Vector Labs). For double labelling, sections were washed as above then incubated in either goat anti-Iba-1 (1 : 500) (Abcam), mouse anti-gial fibrillary acidic protein (1 : 400) (Sigma) or mouse anti-neuronal nuclei (NeuN) (1 : 500; Chemicon), overnight at 22°C. Sections were washed as above then incubated in the appropriate Cy3-conjugated donkey secondary antibody. Images were taken using a Leica fluorescent microscope with DC500 camera and IM50 software. Double labelled, high magnification images were taken using a Leica TCS-NT confocal laser-scanning microscope. For quantification of the numbers of CX3CR1-positive cells, 8-12 sections from 129OlaHsd L5 DRG and sciatic nerve were imaged and captured (using the Leica image capture system described above) at x200 magnification, imported into Adobe Photoshop (v7 for Windows) and then further magnified 4-fold. The number of CX3CR1-positive profiles/500 μ m² for each section was then counted (two areas per section). At least 770, 450 and 280 positive profiles were counted for each axotomised, SNI and sham operated animal, respectively ($n = 4$ for each group) and for sciatic nerves, cells were counted in 8-12 sections from each nerve, in an area of 500 μ m² mid nerve, proximal to the lesion site. At least 1000, 700 and 100 positive profiles were counted for each axotomised, SNI and sham operated animal, respectively ($n = 4$ for each group).

Statistics

Data are presented as means \pm SEM. Quantitative PCR and CX3CR1-positive cell count data were analysed by Student's *t*-test. Behavioural data were analysed by one-way repeated measures of ANOVA (Kruskal-Wallis test), followed by Dunn's multiple comparison *post-hoc* test (using version 4 of GraphPad Prism software, San Diego, USA). A *p* value of < 0.05 was taken to be significant.

Results

Quantification of CX3CR1 and fractalkine mRNA in the DRG after nerve injury

The CX3CR1 cDNA was cloned from an adult mouse (129OlaHsd) DRG cDNA library, and using quantitative RT-PCR (Taqman) analysis we found that the mRNA levels in the DRG increased 5.8 ± 0.5 -fold at 7 days after sciatic nerve axotomy, compared to sham-operated DRG. Similarly, DRG expression of CX3CR1 rose 1.7 ± 0.1 - and 2.9 ± 0.2 -fold, at 3 and 7 days after the SNI model of neuropathic pain compared to sham-operated DRG (Fig. 1a). In contrast, levels of fractalkine mRNA in the DRG did not significantly change after axotomy or SNI, compared to sham-operated DRG.

Distribution of CX3CR1-positive cells in the sciatic nerve, DRG and spinal cord after nerve injury

The upregulation of CX3CR1 mRNA in the DRG after axotomy and SNI was reflected in a marked increase in CX3CR1 protein, as determined by immunohistochemistry. A significant increase in CX3CR1 expression was also observed in the sciatic nerve, especially proximal to the site of injury, and in the dorsal horn of the spinal cord, albeit to a lesser extent than the sciatic nerve.

Sciatic nerve—Longitudinal sections of sciatic nerve from 129OlaHsd mice demonstrated a marked increase in the numbers of CX3CR1-like immunoreactive (LI) expressing cells after axotomy and SNI (Fig. 2b and c, respectively) compared to the uninjured nerve (Fig. 2a), particularly proximal to the injury site. Quantification of CX3CR1-LI positive cells showed a 25.7 ± 1.3 -fold increase in the number of CX3CR1-LI positive cells in the sciatic nerve proximal to the injury site 7 days after axotomy (Fig. 1b, CX3CR1 positive profiles/ $500 \mu\text{m}^2$: sham 6 ± 2 vs. axotomy 154 ± 8). Similarly a 11.1 ± 0.3 , 11.9 ± 1.2 and 16.2 ± 2.0 -fold increase was observed in the sciatic nerve proximal to the injury site 2, 3 and 7 days after SNI, respectively, compared to shamoperated sciatic nerve (Fig. 1, CX3CR1 positive profiles/ $500 \mu\text{m}^2$: sham 6 ± 2 vs. SNI 67 ± 2 , 71 ± 7 , and 97 ± 12 at 2, 3 and 7 days, respectively). The majority of the CX3CR1-LI-positive cells co-localised with the macrophage marker Iba-1 (Ito *et al.* 1998) (Fig. 3 top panel). No significant differences were noted in the number of CX3CR1-LI-positive cells in the sciatic nerve of sham operated and naïve animals.

DRG—Immunohistochemistry for CX3CR1 in 129OlaHsd mice demonstrated that both the staining intensity and number of CX3CR1-LI-positive cells in the DRG increased after axotomy (Fig. 2e), and SNI (Fig. 2f). This was confirmed by analysis of green fluorescent protein (GFP)-labelled cells in CX3CR1^{+/GFP} mutant mice, in which a GFP reporter gene was inserted into the CX3CR1 locus (Jung *et al.* 2000) (data not shown). Quantification of CX3CR1-LI cells revealed a 3.9 ± 0.5 - and 2.2 ± 0.2 -fold increase in the number of CX3CR1-LI positive cells in the DRG 7 days after axotomy and SNI respectively, compared to sham-operated DRG (Fig. 1c, CX3CR1-LI-positive profiles/ $500 \mu\text{m}^2$: sham 12 ± 1 vs. axotomy 49 ± 5 vs. SNI 25 ± 2). CX3CR1 was solely expressed in non-neuronal cells adjacent to neuronal soma in the DRG, and co-localised with Iba-1 (Fig. 3, bottom panel) but not with the neuronal marker NeuN (data not shown). No significant differences were

noted in the number of CX3CR1-LI-positive cells in the DRG of sham operated and naïve animals.

Spinal cord—An increase in CX3CR1-LI cells was seen in 129OlaHsd animals after axotomy and SNI, ipsilateral to the side of the lesion, particularly in the medial two thirds of laminae I-III where the majority of injured primary afferents terminate (Fig. 2h and i, respectively). This finding was identical to that observed in the GFP-positive cells in the lumbar spinal cord of CX3CR1^{+/GFP} mice (data not shown).

No specific CX3CR1 staining was observed in the sciatic nerve or DRG of CX3CR1 KO mice (Combadière *et al.* 2003), 7 days after axotomy (Fig. 2j and k). The upregulation in CX3CR1 expression in the sciatic nerve, DRG and spinal cord of Balb/c mice 7 days after SNI, was very similar to that observed in the 129OlaHsd mice (data not shown). No specific staining was observed in sections in which the primary antibody was omitted (data not shown).

Effect of intra-neural injection of fractalkine into the sciatic nerve on allodynia

In order to study the nociceptive effects of peripheral administration of fractalkine, we first performed intra-neural injection of the chemokine or vehicle into the sciatic nerve at the mid-thigh level of uninjured adult 129OlaHsd mice. The previous studies had used central administration of fractalkine varying between 30-100 ng (Milligan *et al.* 2005; Chen *et al.* 2007), and we therefore used a similar dose range (30-400 ng) by intra-neural injection. Baseline responses were measured prior to and at 2 days after 400 ng fractalkine injection and no significant effects on mechanical or thermal withdrawal thresholds were noted (1.69 ± 0.21 g vs. 1.48 ± 0.57 g; and 16.8 ± 4.5 vs. 15.9 ± 1 s, pre-injection and day 2, respectively). Similarly, no effects were noted on mechanical or thermal withdrawal thresholds in intact mice after an injection of 100 ng or 30 ng fractalkine (data not shown).

We then repeated the fractalkine or vehicle intra-sciatic injections immediately prior to performing the SNI model of neuropathic pain. In all experiments vehicle-injected mice developed significant mechanical allodynia by 2 days post-SNI/injection (Fig. 4, $p < 0.05$, one-way repeated measures ANOVA followed by Dunn's multiple comparison test). However, when 400 ng or 100 ng of fractalkine were administered into the sciatic nerve at the same time as SNI surgery, there was a significant delay in the development of allodynia by 3 days, when compared to the vehicle-injected group (Fig. 4a and b). When 30 ng of fractalkine was injected into the sciatic nerve at the same time as SNI surgery, no significant difference in allodynia was observed (data not shown). As expected, the sham SNI group did not develop allodynia (data not shown). Of note, mice do not develop significant or consistent thermal hyperalgesia following SNI (Holmes *et al.* 2003).

These results imply that peripheral CX3CR1 activation by fractalkine can play an anti-nociceptive role after nerve injury when the expression of the receptor in the sciatic nerve and DRG is up-regulated.

Development of allodynia in CX3CR1 KO mice

Since the intra-neural administration of exogenous fractalkine attenuates neuropathic pain-related behaviour, we next examined the behavioural nociceptive phenotype of CX3CR1 KO compared to sex- and age-matched Balb/c animals. Baseline thermal and mechanical withdrawal thresholds were measured prior to SNI surgery and neither were significantly different between genotypes. Thermal withdrawal thresholds were 14.0 ± 0.8 s and 12.9 ± 0.8 s for Balb/c and CX3CR1 KO animals, respectively. Mechanical withdrawal thresholds were 1.31 ± 0.19 g and 1.32 ± 0.25 g for Balb/c and KO animals, respectively (Fig. 4c, BL).

Following SNI both groups developed significant allodynia by day 2 ($p < 0.05$ wild-type mice and $p < 0.001$ CX3CR1 KO mice, one-way repeated measures ANOVA followed by Dunn's multiple comparison test). The CX3CR1 KO mice developed greater allodynia compared to Balb/c animals at all time points after day 2, for the 3 weeks of testing (Fig. 4c). Genotype had a significant effect on withdrawal thresholds ($p < 0.0001$, one-way ANOVA). It was only at the later time points once the allodynia had become chronic, that the differences were significant using Dunn's multiple comparison test. Of note, the Balb/c mice developed allodynia at day 1 post-SNI surgery compared to day 2 for the 129OlaHsd animals (Fig. 4). This strain variability in responses to nociceptive stimuli is in keeping with previous work (Mogil *et al.* 1999).

Discussion

There is an increasing body of published work demonstrating that a number of chemokines are involved in the development of neuropathic pain states. They are often proinflammatory and increase pain-related behaviour in a number of different neuropathic and/or inflammatory pain models, reviewed in (Abbadie 2005; White *et al.* 2005; Scholz and Woolf 2007). The chemokine fractalkine, acting via its cognate G-protein coupled receptor CX3CR1, has recently been proposed to be involved in signalling between neurons and glia. Unlike other chemokines, fractalkine is a relatively large multimodular protein consisting of a N-terminal chemokine domain with a mucin-like stalk connected to a transmembrane domain (which tethers the molecule to the plasma membrane), and an intracellular cytoplasmic domain (Bazan *et al.* 1997; Pan *et al.* 1997). The soluble, secreted, chemotactic form of fractalkine is produced by cleavage from the transmembrane domain (Garton *et al.* 2001; Tsou *et al.* 2001). Both forms of fractalkine activate CX3CR1 (Imai *et al.* 1997; Combadière *et al.* 1998b; Harrison *et al.* 1998; Meucci *et al.* 2000).

The present study has focused on the potential effect of intra-neural administration of fractalkine in the modulation of pain-related behaviour, following injury to the PNS. Here we show that there is an increase in CX3CR1 expression in the DRG, spinal cord and, most markedly, in the sciatic nerve after nerve injury. The CX3CR1-positive cells in the spinal cord most likely represent resident microglia as previously described in the rat (Verge *et al.* 2004; Lindia *et al.* 2005; Cardona *et al.* 2006), though it is possible that invading peripheral macrophages may also contribute to the observed staining, as suggested by Hu *et al.* (Hu *et al.* 2007). The majority of CX3CR1-positive cells in the DRG and sciatic nerve appear to be macrophages, though the possibility that a minority might represent Schwann and/or satellite cells cannot at present be excluded. We observed no evidence for neuronal expression of CX3CR1 in either the DRG or spinal cord, similar to that reported in the rat (Verge *et al.* 2004). However, it has previously been shown that fractalkine can invoke an immediate (within milliseconds) increase in the levels of intracellular calcium and electrical excitability in a subpopulation of cultured (and thus axotomised) neonatal DRG neurons (Oh *et al.* 2001), implying that some sensory neurons may express the receptor, at least in the neonatal period. In contrast to CX3CR1, levels of fractalkine mRNA did not change after axotomy or SNI in the DRG, which is also in keeping with previous findings in the rat (Verge *et al.* 2004).

The functional significance of the fractalkine/CX3CR1 interaction in the spinal cord has recently been studied by Watkins and co-workers. They demonstrated that acute injection (IT) of 30 ng fractalkine induced a rapid and short lived alteration in pain-related behaviour characterised by a reduction in thermal and mechanical thresholds in intact adult rats, whereas IT injection of 0.3 ng fractalkine only decreased mechano-sensory withdrawal thresholds (Milligan *et al.* 2004). The pro-nociceptive effect of fractalkine was blocked by the simultaneous IT administration of an anti-CX3CR1 antibody (Milligan *et al.* 2004). This

antibody also reduced the allodynia and thermal hyperalgesia that occurred after chronic constriction injury or sciatic inflammatory injury (Milligan *et al.* 2004). Two groups have shown that the pro-nociceptive effects of IT fractalkine administration were blocked by the co-administration of minocycline (an inhibitor of microglial activation), an IL-1 receptor antagonist, immunoneutralisation of IL-6, or a nitric oxide (NO) synthase inhibitor (Milligan *et al.* 2005; Owolabi and Saab 2006). Most recently, injection of fractalkine into the periaqueductal grey (PAG) region of the midbrain has been shown to be pro-nociceptive (Chen *et al.* 2007) similar to that observed when injected IT. Of note, the endogenous membrane-bound form of fractalkine has been shown to be released by cathepsin S, a cysteine protease expressed by activated microglia, after peripheral nerve injury. Furthermore, pharmacological inhibition of cathepsin S can reverse neuropathic pain-like behaviour (Clark *et al.* 2007).

Taken together, these results suggest that central administration of fractalkine, or release of membrane-bound fractalkine by cathepsin S, appears to induce a pro-nociceptive effect in the intact rat and after peripheral nerve injury by activating microglia in the spinal cord.

In contrast to the above, our findings suggest that exogenous fractalkine exerts an anti-allodynic effect when administered intra-neurally after SNI, and that this effect is mediated via activation of CX3CR1-bearing macrophages. The absence of effect of exogenous fractalkine in the intact adult animal presumably reflects the low levels of CX3CR1 expression in the uninjured sciatic nerve and DRG. The source of the CX3CR1-expressing cells is most likely a combination of an influx of non-resident macrophages into the sciatic nerve in response to injury, which is further increased by the injection of fractalkine, since it is a leukocyte chemotactic factor (Imai *et al.* 1997; Nanki *et al.* 2004). In addition, it is possible that the exogenously administered fractalkine either passively diffuses, or is retrogradely axonally transported, to the cell bodies of the sensory neurons, where the chemokine may be released and in turn activate the increased number of CX3CR1-bearing macrophages in the DRG.

The work of Watkins and others demonstrates that a single IT or PAG injection of fractalkine modulates nociception for up to 90 min (Milligan *et al.* 2004, 2005; Chen *et al.* 2007; Sun *et al.* 2007). Similarly, the effects of exogenous fractalkine in a range of other paradigms that include synaptic plasticity (Bertollini *et al.* 2006), neuroprotection (Limatola *et al.* 2005), microglial cell migration (Lauro *et al.* 2006) and leukocyte adhesion (Kerfoot *et al.* 2003) are also short lived, with effects usually lasting less than 2 h. Compatible with these findings, a number of chemokines have short half-lives (Saccani *et al.* 2000; Hillyer and Male 2005), though the half-life of fractalkine has not been reported. These data therefore imply that the prolonged antiallodynic effects observed after an intra-neural injection of fractalkine are likely to be indirect and mediated by the release from peripheral CX3CR1-positive macrophages of down-stream effectors, which in combination inhibit allodynia for a prolonged period. A diverse range of cytokines and other inflammatory mediators are known to be secreted by activated glia, many of which have been shown to modulate nociception/allodynia (reviewed in Scholz and Woolf 2007; White *et al.* 2007). These include the pronociceptive cytokines IL-1b, IL-12, IL-18, IFN γ and TNF α (Verri *et al.* 2005, 2007; Wolf *et al.* 2006; Xu *et al.* 2006; Liu *et al.* 2007) and the anti-nociceptive cytokines IL-2, IL-4, and IL-10 (Yao *et al.* 2002; Vale *et al.* 2003). To date, there is no direct evidence that fractalkine can mediate the release of any of the above cytokines from glia/macrophages in the peripheral nervous system. In contrast in the central nervous system, the effects of fractalkine appear to be dependent upon the release of IL-1, IL-6 or NO from the spinal cord (Milligan *et al.* 2005). Further, fractalkine inhibits the release of the pro-nociceptive cytokine TNF α from cultured glia obtained from newborn rat brain (Zujovic *et al.* 2000; Mizuno *et al.* 2003). Other examples of chemokines modulating the release or

activity of known pain-mediators include the regulation of calcitonin gene-related peptide by CCL2 and CXCL1 (Qin *et al.* 2005) and transient receptor potential vanilloid I by CCL3 (Zhang *et al.* 2005). In addition to the above mechanisms by which fractalkine might modulate allodynia it is also possible that the chemokine might recruit opioid-containing immune cells to the site of injury and/or DRG, which would induce local opioid release and would in turn reduce allodynia (Szabo *et al.* 2002; Brack *et al.* 2004; Rittner and Stein 2005).

In the context of previous studies, our results suggest that fractalkine may play opposing and site-dependent nociceptive roles dependent on the route of administration, though the summation of the two seems to be inhibitory, at least in the mouse, on the basis of the increased allodynia in the CX3CR1 KO animals. Consistent with this, Chen found an unequivocal pro-nociceptive response with a single PAG injection of 100 ng of fractalkine (Chen *et al.* 2007), whilst we found the opposite effect when the same dose was administered by intra-neural injection. It should however be noted that the experiments by the Watkins and Chen groups were performed in rat whereas ours were undertaken in the mouse. Although the distribution of the receptor appears to be similar in the spinal cord in both species, the absence of an upregulation of CX3CR1 in the rat DRG after nerve injury (Verge *et al.* 2004), may in part explain the observed differences in nociception. Further, it is possible that there may be differences in the immunomodulatory systems between these two species which might alter the behavioural effects of the fractalkine/CX3CR1 interaction. Compatible with this, distinct inflammatory reactions after spinal cord injury have been noted in mice and rats (Sroga *et al.* 2003).

In summary, we have demonstrated that the expression of the fractalkine receptor CX3CR1 is markedly up-regulated after nerve injury in macrophages, in the adult mouse sciatic nerve and DRG. Intra-neural administration of fractalkine delays the development of allodynia in the SNI model of neuropathic pain, and consistent with this, CX3CR1 KO mice have an increase in allodynia in the same pain model. Further investigations are now warranted to confirm that the effects of exogenous fractalkine on allodynia are mediated by CX3CR1, and to identify the down-stream effectors of CX3CR1 activation that modulate allodynia.

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Abbreviations used

DRG	dorsal root ganglia
GFP	green fluorescent protein
IT	intrathecal
KO	knockout
PAG	periaqueductal grey
PBS	phosphate-buffered saline
SNI	spared nerve injury

References

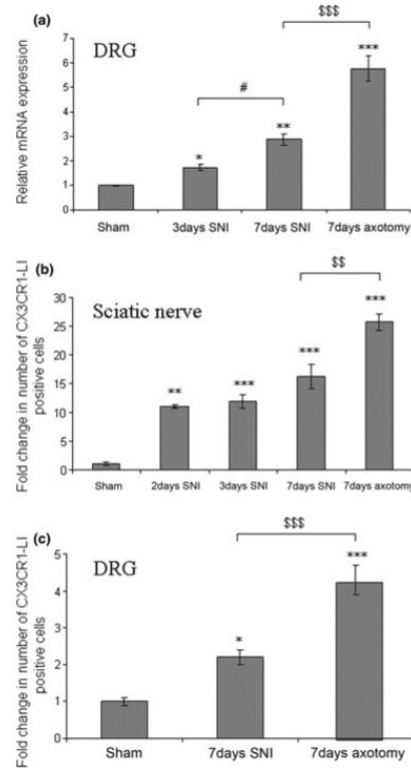
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**Fig. 1.**

Up-regulation of CX3CR1 expression in the 129OlaHsd DRG and sciatic nerve after peripheral nerve injury. (a) Quantitative RT-PCR of CX3CR1 mRNA in L4/L5 DRG after SNI or axotomy, normalised to sham operated (at day 7) L4/L5 DRG ($n = 3$). Sham vs. 3 day SNI, $p < 0.05$ (*); sham vs. 7 day SNI, $p < 0.01$ (**); sham vs. axotomy, $p < 0.001$ (***); 7 day SNI vs. 7 day axotomy, $p < 0.001$ (\$\$\$); 3 day SNI vs. 7 day SNI, $p < 0.05$ (#). (b) Fold change in the number of CX3CR1-LI-cells in $500 \mu\text{m}^2$ sciatic nerve proximal to the injury site after SNI or axotomy, normalised to equivalent sham operated (at day 7) sciatic nerve ($n = 4$), Sham vs. 2 day SNI, $p < 0.01$ (**); sham vs. 3 day and 7 day SNI, $p < 0.001$ (***); sham 7 day axotomy, $p < 0.001$ (***). 7 day SNI vs. 7 day axotomy, $p < 0.01$ (\$\$). (c) Fold change in the number of CX3CR1-LI-cells in the ipsilateral L5 DRG after SNI or axotomy, normalised to sham operated (at day 7) L5 DRG ($n = 4$). Sham vs. 7 day SNI, $p < 0.05$ (*); sham vs. 7 day axotomy, $p < 0.001$ (***); 7 day SNI vs. 7 day axotomy, $p < 0.001$ (\$\$\$).

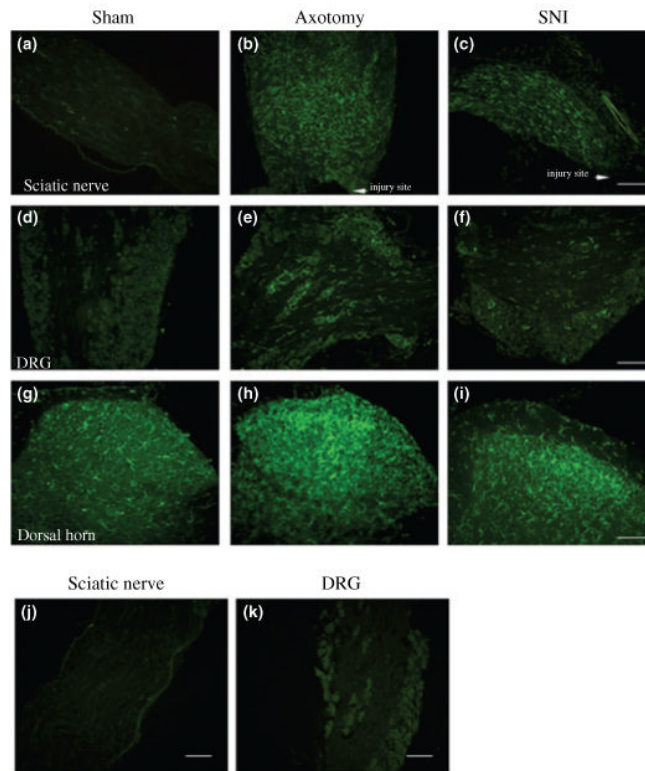


Fig. 2. CX3CR1 in 129OlaHsd mice is present at very low levels in the intact adult sciatic nerve (a), with higher expression observed in the intact DRG (d) and dorsal horn of the spinal cord (g). 7 days after axotomy or SNI, CX3CR1-LI staining is markedly increased in the sciatic nerve (b and c) and DRG (e and f), and to a lesser extent in the ipsilateral dorsal horn of the spinal cord (h and i), as compared to sham operated controls. Bars = 100 μ m. No CX3CR1 staining was noted in the sciatic nerve (j) or DRG (k) of CX3CR1 KO animals, 7 days after axotomy.

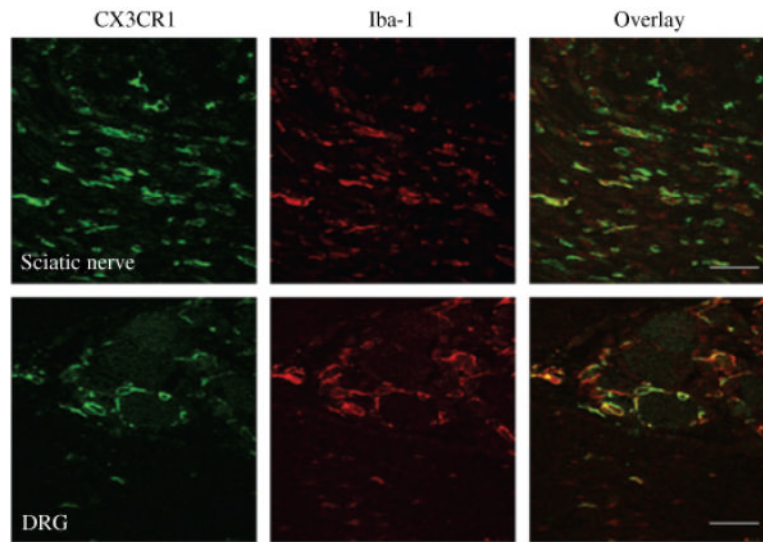
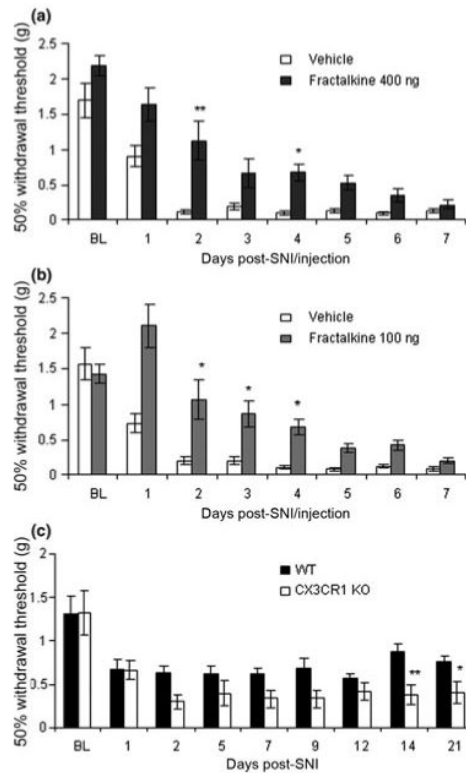


Fig. 3. Co-localisation of CX3CR1-LI (green) with Iba-1 (red), a macrophage marker, is noted in the sciatic nerve (top panel) and DRG (bottom panel), one week after SNI in 129OlaHsd mice. Bars = 25 μ m.

**Fig. 4.**

Intra-sciatic fractalkine delays the development of allodynia in 129OlaHsd mice. Responses to mechanical stimuli were assessed prior to (baseline, BL), and after SNI surgery in which (a) vehicle ($n = 8$) or fractalkine at 400 ng ($n = 8$) and (b) vehicle ($n = 10$) or 100 ng fractalkine ($n = 9$) was injected into the sciatic nerve at the time of SNI surgery. In both groups vehicle but not fractalkine-treated mice developed significant allodynia by 2 days post-SNI ($p < 0.01$ repeated measures ANOVA with Dunn's multiple comparison *post-hoc* test). The injection of fractalkine had a highly significant effect in both groups ($p < 0.0001$, Kruskal-Wallis, one-way ANOVA), and the development of allodynia was significantly delayed by 3 days in both the 400 ng and 100 ng fractalkine-treated groups (using Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison *post-hoc* test). Key: * and ** refer to $p < 0.05$ and $p < 0.01$. (c) CX3CR1 KO mice have increased allodynia after SNI. Withdrawal thresholds to mechanical stimulation were measured before (baseline values, BL) and after SNI. Both Balb/c ($n = 12$) and CX3CR1 KO mice ($n = 13$) developed significant allodynia by 2 days post-SNI ($p < 0.05$ Balb/c and $p < 0.001$ CX3CR1 KO, using one-way repeated measures ANOVA with Dunn's multiple comparison *post-hoc* test). Genotype had a highly significant effect on withdrawal thresholds ($p < 0.0001$, one-way ANOVA Kruskal-Wallis test). The KO mice had reduced mechanical withdrawal thresholds compared to Balb/c mice for the duration of the experiment, but it was only at the later time points, once the allodynia had become chronic, that the differences were significant using Dunn's multiple comparison *post-hoc* test. Key: * and ** refer to $p < 0.05$ and $p < 0.01$.