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CCL2 is a key mediator of microglia activation in neuropathic pain states

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ABSTRACT

While neuroimmune interactions are increasingly recognized as important in nociceptive processing, the nature and functional significance of these interactions is not well defined. There are multiple reports that the activation of spinal microglia is a critical event in the generation of neuropathic pain behaviors but the mediators of this activation remain disputed. Here we show that the chemokine CCL2, produced by both damaged and undamaged primary sensory neurons in neuropathic pain states in rats, is released in an activity dependent manner from the central terminals of these fibres. We also demonstrate that intraspinal CCL2 in naïve rats leads to activation of spinal microglia and neuropathic pain-like behavior. An essential role for spinal CCL2 is demonstrated by the inhibition of neuropathic pain behavior and microglial activation by a specific neutralising antibody to CCL2 administered intrathecally. Thus, the neuronal expression of CCL2 provides a mechanism for immune activation, which in turn regulates the sensitivity of pain signaling systems in neuropathic pain states.

1. Introduction

Neuropathic pain is a common corollary to peripheral nerve injury and often proves refractory to existing therapies. Many pathophysiological mechanisms underlie the development of neuropathic pain states. The site of these mechanisms include not only the damaged nerve and dorsal root ganglia (DRG), but also changes in the central processing of sensory information, most notably at the level of the spinal cord. This phenomenon has traditionally been considered a neuronally mediated response. However, growing evidence suggests that microglia, the resident macrophages of the central nervous system (CNS), are crucial contributors to these processes (Watkins and Maier, 2003; Marchand et al., 2005). Microglia release a variety of mediators including pro-inflammatory cytokines and chemokines that contribute to pain signaling. Many studies have shown that microglial inhibition, as well as impairment of microglia mediators, can modulate neuropathic pain states (Watkins and Maier, 2003; Marchand et al., 2005). However, there has been no direct identification of the factors that initiate the reactive sequelae of microglia, and the relative importance of the particular factors released by microglia remains to be established.

Chemokines are a large family of secreted proteins that are chemotactic for leukocytes. CCL2, a member of the CC chemokine family, is able to recruit and activate monocytic cell types, including macrophages and microglia, to sites of inflammation or injury. Moreover, these effects are now well established in both the peripheral and CNS responses to neuronal injury. Although CCL2 is almost absent in the intact CNS, it is upregulated in several pathological states such as multiple sclerosis and spinal cord damage (McTigue et al., 1998; De Groot and Woodroofe, 2001). Interestingly, CCL2 is upregulated exclusively in neurons of the DRG following peripheral nerve injury (Abbadie et al., 2003; Tanaka et al., 2004; White et al., 2005; Zhang and Koninck, 2006), while it is expressed by neurons and microglia in the spinal cord (Abbadie et al., 2003; Zhang and Koninck, 2006). A spatial and temporal relationship between CCL2 expression and spinal glial activation following nerve injury is evident (Zhang and Koninck, 2006), suggesting that neuronal CCL2 may serve as a trigger for spinal microglia activation. Here we set out to address this possibility directly.

CCL2 binds with high affinity to the chemokine receptor CCR2, G-protein coupled receptor (Bacon et al., 2002). Conflicting results

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exist about the spinal cord cell types which express CCR2, with predominantly microglial (Abbadie et al., 2003), and exclusively neuronal (Gosselin et al., 2005) expression both reported. Importantly, CCR2 knockout mice fail to develop tactile allodynia following nerve injury (Abbadie et al., 2003), and both peripheral and intrathecal administration of CCL2 have been shown to induce mechanical allodynia (Abbadie et al., 2003; Thacker et al., 2003; Tanaka et al., 2004; Thacker et al., 2005).

Despite this body of evidence, several pertinent questions remain unanswered such as the relationship between DRG and spinal expression of CCL2 and CCR2. Furthermore, this study assesses the ability of CCL2 to activate spinal microglia and its neuromodulatory functions in order to validate CCL2 as a crucial factor in neuropathic pain.

2. Materials and methods

2.1. Induction of mononeuropathy

Unilateral peripheral neuropathy was induced in Male Wistar rats (170–200 g; Harlan, UK), according to the method previously described by Bennett and Xie (1988), or Kim and Chung (1992). Briefly, rats were anaesthetized with an intraperitoneal injection of medetomidine (0.25 mg/kg) and ketamine (60 mg/kg). In chronic constriction injury (CCI) animals, four chromic gut ligatures were tied loosely (with approximately 1 mm spacing) around the left common sciatic nerve. The nerve was constricted to a barely discernible degree, so that circulation through the epineurial vasculature was impeded but not totally interrupted. In spinal nerve ligation (SNL) animals, the L5 spinal nerve was isolated, tightly ligated and transected on the peripheral side of the ligation. For investigation of CCL2 axonal transport, SNL animals also received a tight ligation of the L5 dorsal root.

2.2. Intrathecal and intraspinal drug administration

Rats were injected intrathecally with Recombinant Rat CCL2/JE/MCP-1 ([10 μl at 300 ng/ml]; R&D systems, UK), CCL2 neutralizing antibody ([10 μl at 100 ng/ml]; goat anti-mouse CCL2/MCP-1/JE neutralizing antibody which also recognized rat CCL2 R&D systems, UK) or the appropriate vehicle (either 10 μl of 0.9% saline or IgG [10 μl at 300 ng/ml, R&D systems, UK]). Intrathecal injections were performed by direct lumbar puncture between the L5 and L6 vertebrae of the spine using a 26G 3/8″ needle (Mestre et al., 1994), under isofluorane/O_2 anaesthesia. For intraspinal injection of CCL2 (2 μl at 300 ng/ml; R&D systems, UK), or vehicle (2 μl of 0.9% saline), rats were anesthetized with medetomidine (0.25 mg/kg) and ketamine (60 mg/kg). A small (4 mm) laminectomy was performed at the spinal cord L5 level and an incision in the dura mater carefully made. Intraspinal injections were performed using a Hamilton syringe with a beveled glass tip attached. Microinjection was performed into the left dorsal horn at the L4–L5 level (depth 500 μm).

2.3. Immunohistochemistry

Rats were perfused under pentobarbitone anaesthesia with heparinised 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The DRG and lumbar spinal cord were excised, post-fixed for 4 h in the perfusion fixative, cryoprotected and then frozen in optimum cutting temperature embedding medium (VWR, UK). Twenty micrometre transverse sections were cryostat cut and thaw-mounted onto glass slides. Sections were incubated with primary antibodies for CCL2 (10 μg/ml; goat anti-mouse CCL2; R&D Systems, UK), $P2X_3$(1:500, rabbit anti-$P2X_3$, marker for small purinergic fibres; Neuromics, USA), CGRP (1:4000, rabbit anti-CGRP, marker for small peptidergic fibres; Bachem, UK), NF200 (1:400, mouse anti-neurofilament 200, marker for large myelinated fibres; Chemicon, UK), or OX42 (1:200; mouse anti-rat Cd11b; Serotec, UK). Sections were then incubated with corresponding secondary antibody solutions (IgG-conjugated Alexa Fluor™ 488 or 546, 1:1000, Molecular Probes, USA). Slides were washed, cover-slipped with Vectashield mounting medium (Vector Laboratories, CA, USA) and visualized under a Zeiss Axioplan 2 fluorescent microscope.

Quantitative assessment of microglial activation was carried out by determining the number of positive cells within four boxes of 10^4 μm^2 placed over areas of the lateral, central and medial dorsal horn on ipsilateral and contralateral dorsal horns of the spinal cord, respectively. The cell numbers within these areas were counted by an experimenter blind of treatment.

2.4. Real time reverse transcription (RT) polymerase chain reaction (PCR)

Expression levels of *CCL2* and *CCR2* mRNA in adult rat DRGs and lumbar spinal cord were examined by real time RT-PCR (Rotorgene 3000, Corbett Instruments, Australia) relative to *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). For each time point (day 1 and day 4) tissue from 4 sham and 4 CCI rats were used. Rats were terminally anaesthetised with pentobarbitone and ipsilateral and contralateral L5 DRGs and L4–L6 spinal cord segments were quickly dissected and frozen on dry ice. Total RNA was obtained from single DRGs using a column-based method (RNeasy, Qiagen). Total RNA was obtained from L4–L6 spinal cord segments by homogenizing in Trizol (Invitrogen) and treating with DNase on-column (RNeasy, Qiagen). Tissue from different rats was not pooled. DNase treatment was performed because rat *CCR2* and GAPDH are predicted to have a single exon; spanning design was therefore not possible. Concentrations of nucleic acid were established using spectrophotometry (Nanodrop, Ambion) and quality confirmed by capillary gel electrophoresis (Bioanalyzer, Agilent). Reverse transcription was performed using 200 ng RNA from DRG or 750 ng RNA from spinal cord using random hexamers (250 ng/reaction) according to manufacturer's instructions (SuperScript II, Invitrogen). Standard curves were generated using reference cDNA obtained by reverse transcribing total RNA from adult rat lumbar DRGs and spinal cord three days after sciatic nerve transection. Primer sequences were designed using Primer3 and BLAST to discriminate *CCL2* and *CCR2* from others family members (e.g. CCR5). CCL2 primers (forward: ATGCAGTTAATGCCCCACTC, reverse: TTCCTTATTGGGGTCAGCAC) yielded a 167 basepair (bp) product. CCR2 primers (forward: GACCGAGTGAGCTCAACATTT, reverse AACCCAACTGAGACTTCTTGC) yielded a 59 bp product. GAPDH primers (forward: CCTGCACCACCAACTGCTTAGC, reverse GCCAGTGAGCTTCCCGTTCAGC) yielded a 239 base pair product. PCR reaction volumes consisted of 12.5 μl SyBr Green PCR 2X master mix (Applied Biosystems), 1 μl mix of forward and reverse primers (25 ng/μl each) and 11.5 μl water containing cDNA (20 ng). Gain was calibrated automatically after denaturing at 95 °C for 10 min. Each of 40 cycles consisted of denaturing at 95 °C for 15 s, annealing/extending at 60 °C for 60 s, and acquiring fluorescence signals. Specificity of product and lack of primer dimers was confirmed by agarose gel electrophoresis and by performing melt analysis from 60 to 95 °C.

2.5. Nociceptive behavioural testing

2.5.1. Thermal hyperalgesia

Thermal response latencies were tested using the method described by (Hargreaves et al., 1988). Briefly, each animal was

placed in a clear acrylic cubicle (22 × 16.5 × 14 cm) on top of a glass floor in a temperature controlled room (~22 °C) and allowed to acclimatize for 15 min before testing. Paw withdrawal latencies were averaged over three consecutive tests, at least 5 min apart, in response to the thermal challenge from a calibrated (output of 190 mW/cm^2) radiant light source. A cut-off of 20 s was imposed to prevent tissue damage.

2.5.2. Mechanical allodynia

Mechanical withdrawal thresholds (PWT) were tested using a Dynamic Plantar Aesthesiometer (Ugo Basile, Italy) as previously described (Clark et al., 2007b). Briefly, each animal was placed in a clear acrylic cubicle (22 × 16.5 × 14 cm) on top of a metal grid in a temperature controlled room (~22 °C) and allowed to acclimatize for 15 min before testing. The stimulus was applied via an actuator filament (0.5 mm diameter) which under computer control applied a linear ramp of 2.5 g/s to the plantar surface of the paw. Paw withdrawal stops the stimulation and records the threshold. The withdrawal threshold is calculated as the average of three consecutive tests with at least 5 min between each test. A cut-off of 50 g was imposed to prevent tissue damage.

2.6. Isolated dorsal horn-with dorsal roots attached preparation

Horizontal dorsal horn slices (400 μm thick) with dorsal roots attached were obtained from the lumbar spinal cord of adult male rats 4 days post-SNL surgery as described previously (Malcangio and Bowery, 1996; Malcangio et al., 2000; Clark et al., 2006). Briefly, lumbo-sacral spinal cord was excised and longitudinally hemisected producing a horizontal slice with L4 and L5 dorsal roots attached. One slice was obtained from each rat, mounted in the central compartment of a three compartment chamber and continuously superfused (1 ml/min) with oxygenated (95% O_2 + 5% CO_2) Krebs' solution (in mol/L: NaCl, 118; KCl, 4; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5 and glucose, 11) containing 0.1% bovine serum albumin (BSA) and 20 μg/ml bacitracin to minimize protein degradation. The dorsal roots were placed in the lateral compartments and immersed in mineral oil to avoid dehydration. Experimental protocol was carried out as previously described (Lever et al., 2001). Before, during and after dorsal root stimulation 8 ml fractions of superfusate were collected from the central compartment in ice cooled glass tubes to minimize CCL2 loss. Three 8 ml-fractions were collected before stimulation to measure basal levels of the chemokine. These values were then pooled and expressed as "Basal". High intensity (20 V, 0.5 ms, 10 Hz) stimulation of the dorsal roots was applied for 8 min in order to recruit both A- and C-fibre afferents. Four fractions were collected after electrical stimulation to assess recovery to basal levels of CCL2 (R1-4).

2.7. CCL2 ELISA

To quantify CCL2 content collected superfusate samples were desalted and concentrated by Ultrafree-15 5K centrifugal device (Millipore, UK). Retentates were then lyophilised, reconstituted in 150 μl sample buffer (R&D Systems, UK) and assayed for CCL2 content by ELISA. 96-Well colorimetric 'Sandwich' ELISA plates (R&D Systems, CCL2/JE/MCP-1 Quantikine® ELISA Kit) were used. Rat recombinant CCL2 standards (50 μl of 0–1000 pg/ml; sensitivity 2 pg/ml) and 50 μl of unknown samples were run in duplicate following the manufacturer instructions. The optical density of each well was determined at a wavelength of 450 nm. Samples were considered CCL2 positive when the signal was higher than background signal (modified Krebs' solution) and within the range of the standard curve. Data are expressed as percentage of CCL2 content in the basal fractions.

3. Results

3.1. Expression changes following peripheral nerve injury

We examined the expression of CCL2 protein following injury using immunohistochemistry. Following CCI, *de novo* expression of CCL2 protein was observed in adult rat DRG cells as early as 1 day post-injury (Fig. 1). For example, CCL2 protein was observed in 26.0 ± 5.0% of the ipsilateral L5 DRG cells 7 days after CCI (Fig. 1D) compared with 7.0 ± 1.2% in sham (Fig. 1D) and 3.0 ± 0.4% in naive (Fig. 1D) DRG. The increased expression of CCL2 following peripheral nerve injury persisted until the last time-point studied (Fig. 1). Quantitative analysis revealed that this increase was statistically significant at all time points examined ($P < 0.001$, two way ANOVA post-hoc Tukey).

Next the populations of DRG neurons displaying CCL2 immunoreactivity (IR) were examined at several time points following CCI (Fig. 2A and B). A small number of CCL2-IR neurons displayed IR for either NF200 or CGRP (24% and 22% of total cell profiles at 7 days post-CCI, respectively; Fig. 2A and B). However, CCL2 was most commonly expressed in $P2X_3$ positive cells (54% of total cell profiles at 7 days post-CCI; Fig. 2A and B). Therefore following injury CCL2 is mostly expressed by non-peptidergic, purinergic C-fibres. Furthermore, CCL2 expression was observed mainly in small diameter neurons (73% < 35 μm diameter cells at 7 days post-CCI; Fig. 2C) suggesting expression mainly in C-fibers.

We further investigated changes in CCL2 protein using the SNL model, in order to examine the expression of CCL2 in both injured and un-injured neurons. Following SNL a significant increase in the number of DRG cells expressing CCL2 protein, in both the injured L5 and the uninjured L4 ganglia were observed (Fig. 3A–C). In injured L5 ganglia 26.7 ± 2.6% of DRG cells expressed CCL2 7 days following injury (Fig. 3B–C). CCL2 expression was also observed in 18.6 ± 3.1% of L4 (undamaged) DRG neurons 7 days following L5 SNL (Fig. 3A and C). Double staining with activating transcription factor 3 (ATF3) showed, for example, that 7 days post-SNL 68% of L5 cells expressing CCL2 were injured (Fig. 3B and D), suggesting that the majority of CCL2 expressing cells are injured/stressed, whilst only 24% of ATF3 positive cells co-expressed CCL2 (Fig. 3B and E) which indicates that only a minority of damaged/stressed cells express CCL2.

Quantitative real-time RT-PCR was performed in DRG and spinal cord 1 and 4 days following CCI (Table 1) in order to investigate where in the pain-processing pathway CCL2 and CCR2 is synthesised. We observed a significant increase of CCL2 mRNA in L5 DRG 1 day, but not 4 days, post-injury compared to both the contralateral L5 DRG and sham ipsilateral L5 DRG (2.35 ± 0.23 and 1.65 ± 0.16-fold increase, respectively). Interestingly, we found a significant up-regulation of CCL2 mRNA in the ipsilateral dorsal horn of the spinal cord 4 days post-injury compared to both the contralateral, and sham ipsilateral dorsal horn (4.66 ± 1.28 and 4.58 ± 1 fold increase, respectively; Table 1). In contrast, we did not observe any significant increase in CCR2 mRNA levels either in DRG or spinal cord, although there was a slight increase in CCR2 mRNA expression in the L5 DRG 4 days post-injury (Table 1).

3.2. Anterograde transport and release of CCL2 by primary afferent fibres

In order to assess the hypothesis that CCL2 protein may be anterogradely transported from the DRG cell bodies to the central terminals following injury we tightly ligated the L5 spinal nerve, as well as the L5 dorsal root, and examined the axonal transport of CCL2 by immunohistochemistry (Fig. 4A). As previously observed, increased CCL2 expression was evident in the L5 DRG (Fig. 4A,

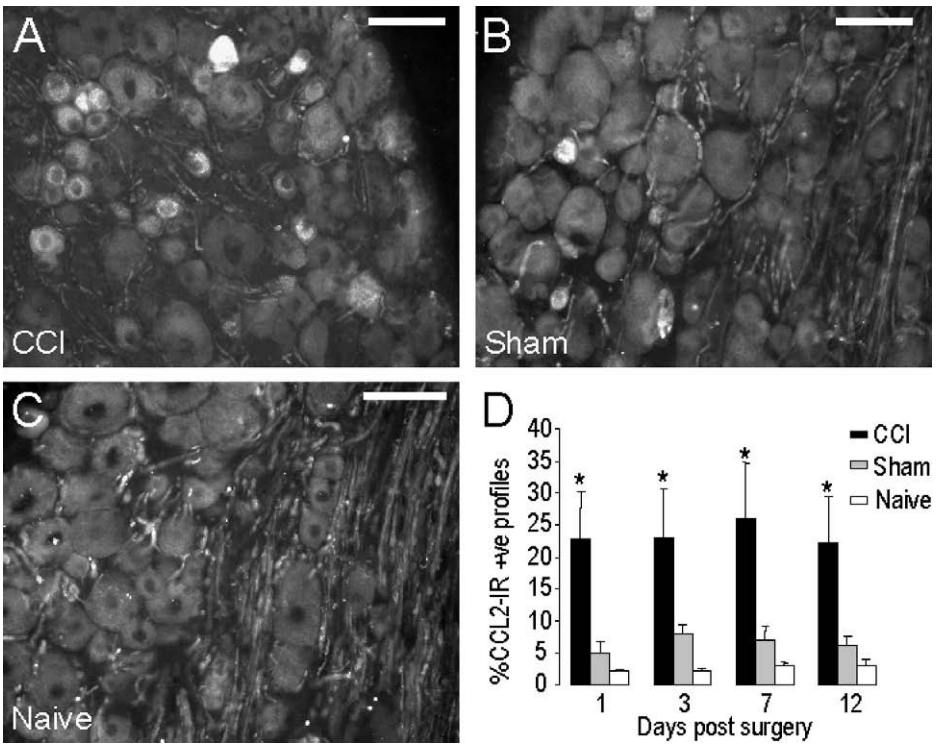

Fig. 1. CCL2 expression in the DRG is enhanced following nerve injury: (A) CCL2-IR in the ipsilateral L5 DRG 7 days following CCI, (B) CCL2-IR in the L5 DRG 7 days following sham surgery, (C) CCL2-IR in the naïve L5 DRG and (D), number of CCL2 positive profiles in the DRG is significantly increased 1 to 12 days following nerve injury. Scale bars = 50 μm. $^{*}P < 0.05$ compared to sham, two-way ANOVA, post-hoc Tukey.

panel 2) but interestingly, also in the ventral root following injury (Fig. 4A, panel 4). Importantly, accumulation of CCL2 was observed proximal to the spinal nerve ligature and distal to the dorsal root ligature (Fig. 4A, panels 1 and 3) demonstrating injury induced transport of CCL2 anterogradely towards both the periphery and the dorsal horn of the spinal cord.

We therefore examined the possibility that CCL2 may function as a primary afferent neuromodulator. We measured the levels of this chemokine in the superfusate of an *ex vivo* isolated dorsal horn-with dorsal roots attached preparation after stimulation of primary afferent neurons (Malcangio et al., 2000). Basal levels of CCL2 in spinal cord superfusates from sham and neuropathic animals were not significantly different (0.85 ± 0.23 pg and 0.42 ± 0.09 pg per 8 ml fraction, respectively). Supramaximal electrical stimulation of the dorsal roots (20 V, 0.5 ms, 10 Hz, for 8 min) evoked rapid release of CCL2 from neuropathic, but not sham preparations (Fig. 4B). Under neuropathic conditions, CCL2 levels in superfusates were significantly increased to 358 ± 91% of basal values ($P < 0.05$, one way ANOVA post-hoc Tukey). In contrast, no-significant release of CCL2 occurred following stimulation of sham preparations (Fig. 4B). Thus, following a peripheral nerve injury, CCL2 is released in an activity dependent manner from the central terminals of primary afferent fibers in the dorsal horn and may function as a neuromodulator.

3.3. Mechanical allodynia and microglial activation following intraspinal or intrathecal CCL2

Following peripheral nerve injury, spinal microglia activation is reported in regions where the damaged primary afferent fibres project. To test the hypothesis that CCL2 released from sensory neurons may contribute to the injury-induced microglia response we investigated the pain behavioral response of spinally administered CCL2. Intrathecal injection of CCL2 (10 μl at 300 ng/ml) induced significant mechanical allodynia 24 h following administration (Fig. 5A), compared to controls ($P < 0.05$, two way ANOVA with repeated measures post-hoc Tukeys). CCL2 induced mechanical hypersensitivity was prevented by prior administration of a CCL2 neutralizing antibody (10 μl at 100 ng/ml; Fig. 5A). Neutralizing antibody alone did not alter mechanical withdrawal thresholds (Fig. 5A).

We also examined the behavioral and biological effects of CCL2 microinjection (2 μl at 300 ng/ml) into the L5 spinal segment of naive rats. This induced a significant, segmentally appropriate, mechanical allodynia in CCL2-treated animals compared to vehicle-treated (0.9% saline) animals (Fig. 5B), apparent from the first day of testing (day 1) and persisting for at least 4 days ($P < 0.05$, two way ANOVA with repeated measures post-hoc Tukeys). Mechanical paw withdrawal thresholds (PWT) decreased from 39.3 ± 1.6 g at baseline to 21.8 ± 2.6 g at day 4 (Fig. 5B). However, there was no significant change in thermal PWT following CCL2 treatment (Fig. 5C). Furthermore, intraspinal CCL2 also led to activation of spinal microglia as seen by enhanced OX42 IR compared to vehicle (Fig. 5D–E). The maximal effect is observed at the site of injection (average 152 ± 20 cells per 2500 μm^2 in the CCL2 treated animals vs 50 ± 14 cells per 2500 μm^2 in vehicle treated animals) and extends to at least 400 μm laterally and 800 μm rostro-caudally from the injection site (Fig. 5F–G). After CCL2 treatment, microglial cells exhibit a hypertrophied type illustrated by rod-like, devoid of branching processes cells compared to vehicle treated animals.

3.4. Endogenous CCL2 contributes to mechanical allodynia and microglial activation induced by peripheral nerve injury

Finally, to determine the functional importance of endogenous CCL2 in neuropathic pain, we tested the effect of intrathecal administration of a CCL2 neutralizing antibody (anti-CCL2)

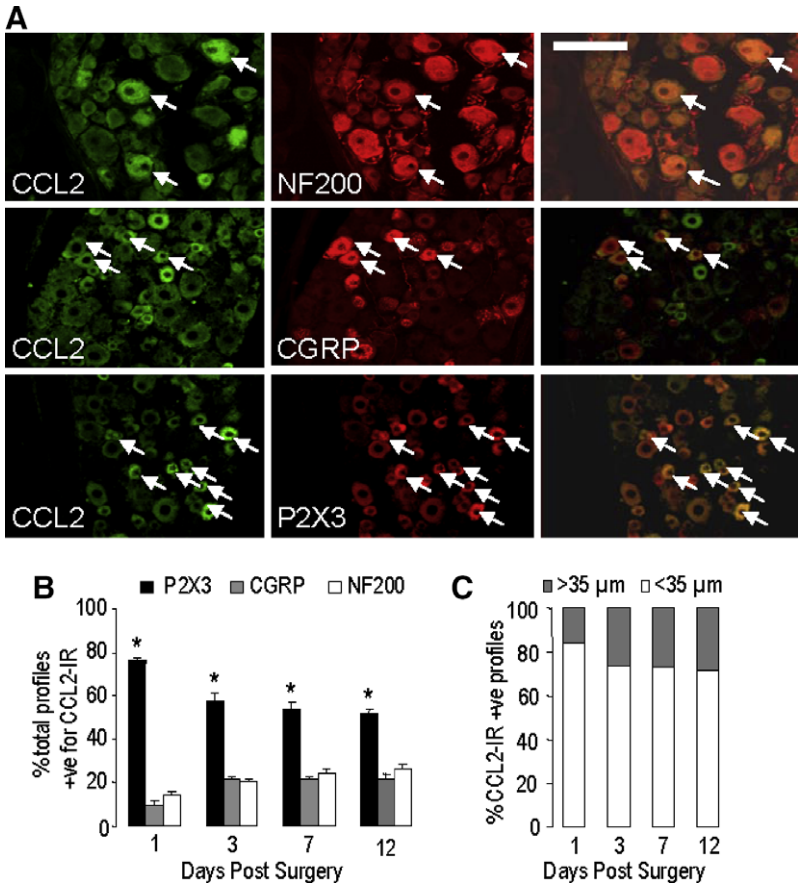

Fig. 2. CCL2 expression predominates in small diameter $P2X_3$ positive DRG neurons: (A) CCL2-IR (green) in populations of DRG neurons expressing NF200 (red; top), CGRP (red; middle), and $P2X_3$ (red; bottom). Scale bars = 100 µm. (B) CCL2-IR is most commonly expressed in $P2X_3$-IR cell profiles compared to other phenotypes. $^{*}P < 0.05$ compared to sham, two-way ANOVA, post-hoc Tukeys. (C) The majority of CCL2-IR profiles have cell body diameters of less than 35 µm.

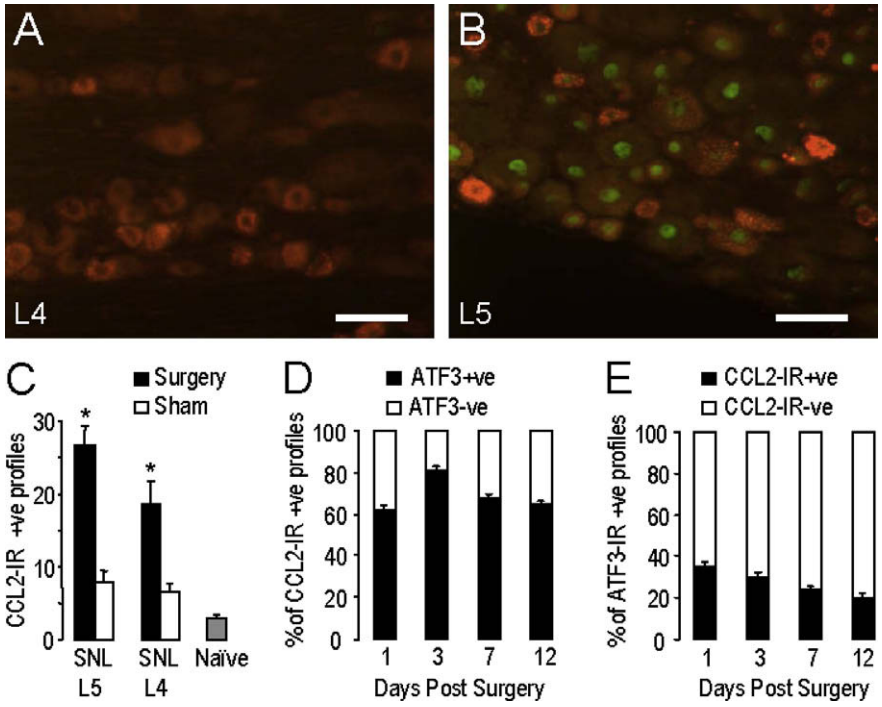

Fig. 3. CCL2 is expressed by both damaged and undamaged DRG neurons. (A) CCL2-IR in un-injured (ATF3 negative) L4 DRG neurons 7 days following L5 SNL. (B) CCL2-IR (red) in injured L5 DRG neurons (co-expressing ATF3, green) 7 days following L5 SNL. Scale bars = 100 µm. (C) Number of CCL2 positive profiles is significantly increased 7 days following SNL in both L4 and L5 DRG. $^{*}P < 0.05$ compared to sham, one-way ANOVA, post-hoc Tukey. (D) Percentage of CCL2-IR L5 DRG neurons expressing ATF3 following injury. (E) Percentage of ATF3-IR L5 DRG neurons expressing CCL2 following injury.

Table 1
Fold change (with standard errors) of CCL2 and CCR2 mRNA in ipsilateral L5 DRG and ipsilateral dorsal horn following CCI

	Tissue	D1 vs contra	D4 vs contra	D1 vs sham ipsi	D4 vs sham ipsi
CCL2	DRG	2.35 ± 0.23*	1.79 ± 0.31	1.65 ± 0.16*	2.48 ± 0.42
	Dorsal horn	2.60 ± 0.60	4.66 ± 1.28*	2.51 ± 0.57	4.58 ± 1.00*
CCR2	DRG	1.33 ± 0.25	1.92 ± 0.36	1.01 ± 0.19	1.23 ± 0.23
	Dorsal horn	1.17 ± 0.34	1.91 ± 0.44	1.09 ± 0.32	0.68 ± 0.16

* $P < 0.05$ (paired t-tests for comparisons with contra; unpaired t-tests for comparisons with sham).

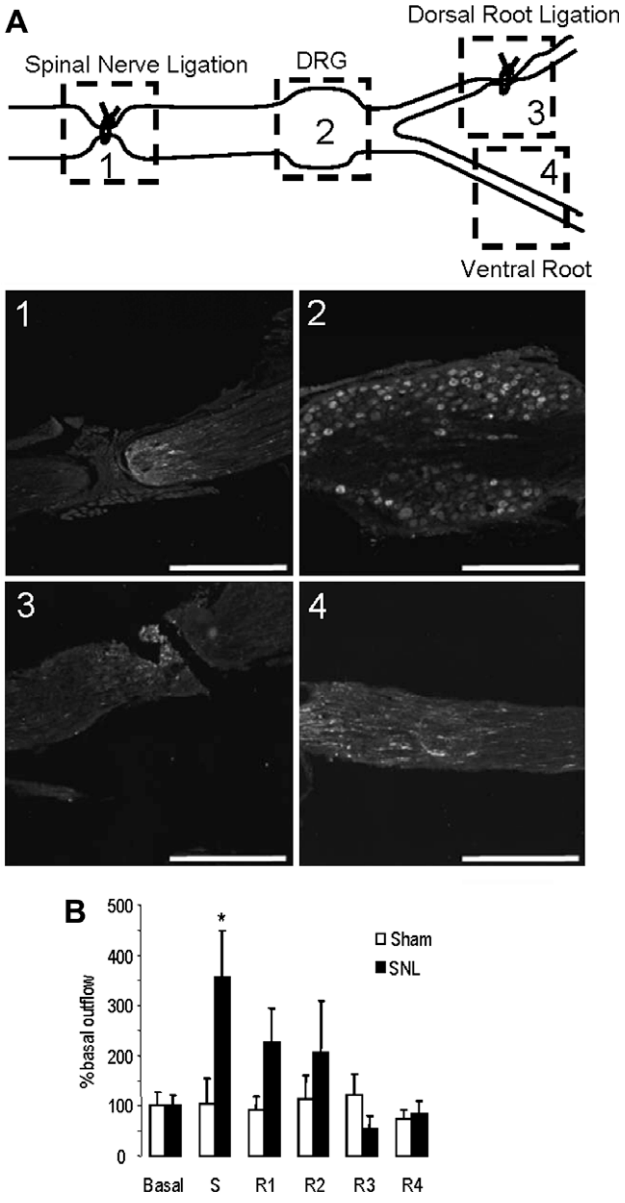

Fig. 4. CCL2-IR is transported from the DRG to the injury site and is released from central terminals of primary afferent fibres in the dorsal horn following nerve injury. (A) CCL2-IR accumulates proximal to the spinal nerve ligature and distal to the dorsal root ligature. Panels 1–4 represent high power photomicrographs representing the areas identified in schematic, CCL2-IR at the spinal nerve ligation (1), DRG (2), dorsal root ligation (3), and ventral root (4). Scale bars = 1 mm. (B) High intensity stimulation of dorsal roots evokes significant CCL2 release from neuropathic (SNL; $n = 6$), but not sham ($n = 7$), dorsal horn slices, 4 days following injury. S = stimulated fraction, R1-4 = recovery fractions following stimulation. *$P < 0.05$, vs basal outflow, one way-ANOVA, Tukey post-hoc.

administered 24 h before and after the induction of neuropathy. Anti-CCL2 treatment resulted in a significant attenuation of ipsilateral neuropathic allodynia following CCI (Fig. 6A). PWT in the IgG treated animals were 27.5 ± 1 g before the injury and dropped by about a third to 19.8 ± 1 g, 21.2 ± 1.3 g, 17.8 ± 0.7 g, 21.1 ± 0.9 g and 17.5 ± 2 g, at days 1, 2, 3, 4 and 7 after CCI, respectively. When anti-CCL2 was administered, PWT were 27.6 ± 0.7 g before injury and 25.7 ± 1.3 g, 27.4 ± 1.5 g, 24.9 ± 1.1 g, 25.3 ± 2 g and 24.2 ± 2.7 g at the same time points. The PWT of anti-CCL2 treated animals are significantly different from IgG treated CCI animals at all time points ($P < 0.05$ compared to CCI rats treated with IgG, Repeated measures ANOVA, post-hoc Tukeys), illustrating a key role for CCL2 in the development of tactile allodynia. The anti-CCL2 treatment does not alter contralateral PWT at any time point (Fig. 6B). Anti-CCL2 treatment also significantly attenuated microglial activation after CCI, as assessed by OX42 IR (Fig. 6C–E). At day 4 following CCI, microglial cell numbers in the dorsal horn were 18.65 ± 0.65 per $10^4\ \mu m^2$ in IgG treated animals and 6.75 ± 0.38 per $10^4\ \mu m^2$ in anti-CCL2 treated animals (Fig. 6E). Anti-CCL2 treatment resulted in a 50% reduction in the numbers of activated microglia cells in the ipsilateral dorsal horn ($P < 0.05$, one way ANOVA, post-hoc Tukeys) (Fig. 6E).

4. Discussion

In this study we provide biochemical and behavioral evidence to suggest that neuronal expression of the chemokine CCL2 is a mechanism by which neuroimmune interactions can arise. We demonstrate that peripheral nerve injury enhances CCL2 expression in small diameter nociceptive DRG neurons, is anterogradely transported from the DRG and released in the dorsal horn with activity. Secondly, we demonstrate that spinal CCL2 induces both mechanical allodynia and microglial activation in naïve rats and the inhibition of endogenous CCL2 following nerve injury attenuates these two phenomena.

We observed *de novo* expression of CCL2 protein in DRG cells as early as 1 day following two types of peripheral nerve injury, which persist for several weeks following injury. These data confirm previous reports demonstrating an increase of CCL2 protein expression in the DRG at 1 day following a partial nerve ligation (PNL) (Tanaka et al., 2004). Furthermore, CCL2 protein was observed mainly in non-peptidergic, purinergic (i.e. $P2X_3$-IR) C-fibres following injury confirming the findings of White and colleagues (White et al., 2005) and suggests that there is preferential expression of CCL2 by nociceptive neurons. Using real time RT-PCR we observed a significant increase of CCL2 mRNA in ipsilateral L5 DRG 1 day, but not 4 days, post-CCI. The increased CCL2 mRNA in DRG 1 day post-injury, in contrast to increased protein for several weeks suggests that an alteration in CCL2 translation occurs in DRG cells early after nerve injury (Richardson and Lu, 1994).

Interestingly, we found a significant up-regulation of CCL2 mRNA in the ipsilateral dorsal horn of the spinal cord at 4 days post-injury. The delay in up-regulation between DRG and the dorsal horn of the spinal cord suggest that CCL2 mRNA and/or protein may be transported to the sensory terminals. An increase of CCL2 protein expression in the superficial layers of the spinal cord from day 3 following injury has been reported (Zhang and Koninck,

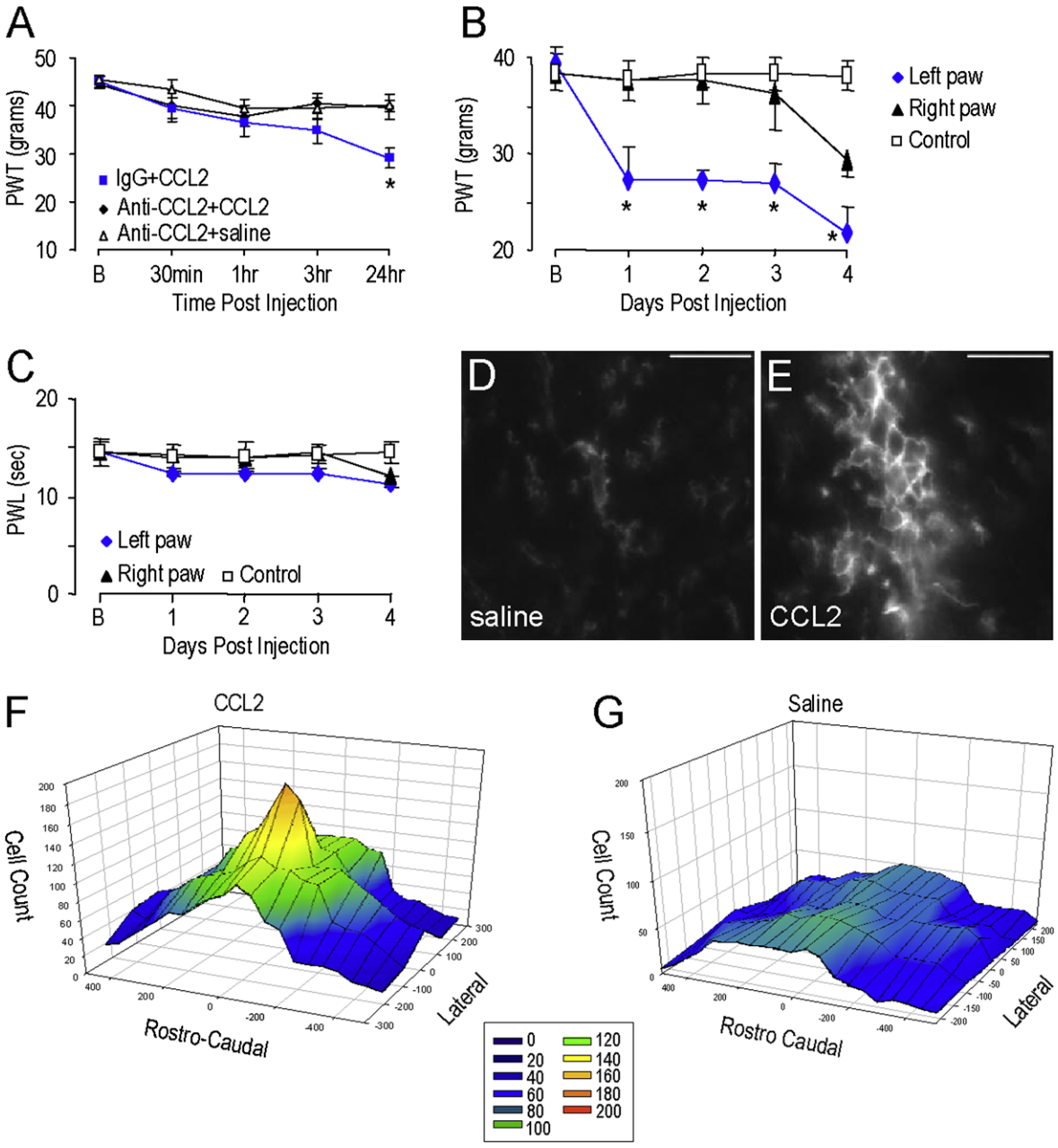

Fig. 5. Spinal injection of CCL2 induces mechanical allodynia and spinal microglia activation. (A) Intrathecal CCL2 induces significant mechanical allodynia which is prevented by prior administration of intrathecal CCL2 neutralising antibody. (B) CCL2 induces significant mechanical allodynia ipsilateral to injection. $^{*}P < 0.05$ compared to neutralising antibody (A) or saline (B) treated groups, two-way ANOVA with repeated measures, post-hoc Tukeys. $n = 8$ per group. (C) CCL2 does not result in thermal hyperalgesia. (D) Minimal OX42-IR following administration of saline. (E) Extensive OX42-IR following CCL2 administration. Scale bar = 25 μm. (F)–(G) Quantification of OX42 positive profiles following spinal administration of CCL2 (F) and saline (G).

2006). Axonal transport of CCL2 containing vesicles and their release from the neurites of cultured DRG neurons was recently observed (Jung et al., 2007). This suggests that CCL2 protein may be anterogradely transported from the DRG cell bodies to the central terminals. Indeed, we directly observed accumulation of CCL2 proximal to the spinal nerve and distal to the dorsal root ligature demonstrating injury induced transport of CCL2 towards both the periphery and the dorsal horn of the spinal cord. Therefore, it is tempting to speculate that CCL2 mRNA may also be transported to the central terminals. However, the evidence for translation here is still very contentious. Alternatively, a local increase of CCL2 mRNA transcription may occur in neurons of the superficial lamina of the dorsal horn and thereafter the protein released locally.

In contrast, we did not observe any significant increase in CCR2 mRNA levels either in DRG or spinal cord. These findings differ from previous work reporting alterations in CCR2 expression in the DRG following peripheral nerve injury (Abbadie et al., 2003; White et al., 2005). White and colleagues utilized a direct DRG compression model (White et al., 2005). This paradigm may lead to a greater activation of cytokines and other mediators upstream to CCL2/CCR2 than in the CCI model, and may explain this discrepancy. However, Abbadie and colleagues were also unable to detect significant increases in CCR2 mRNA in murine spinal cord (Abbadie et al., 2003). To our knowledge only one study, employing a model of bone cancer induced pain, has demonstrated an increase of CCR2 protein in the dorsal horn of the spinal cord (Vit et al., 2006). Nev-

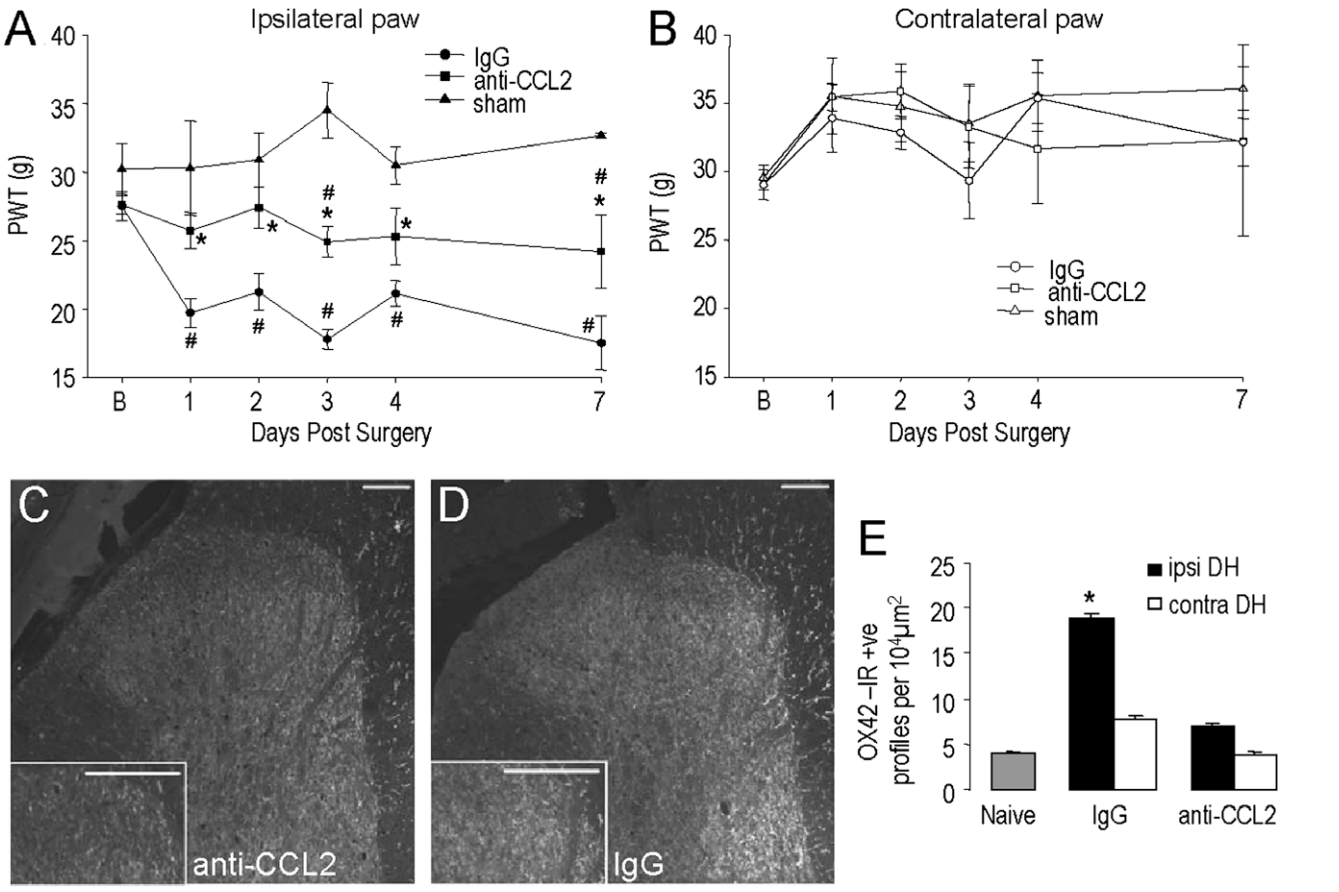

Fig. 6. Intrathecal administration of CCL2 neutralising antibody reverses mechanical allodynia and microglia activation following CCI. (A) Anti-CCL2 significantly attenuates ipsilateral mechanical allodynia following CCI. #$P < 0.05$ compared to sham animals, $^{*}P < 0.05$ compared to CCI rats treated with IgG, two-way ANOVA with repeated measures, post-hoc Tukeys. $n = 8$ per group. (B) Anti-CCL2 does not alter contralateral PWT. (C) OX42-IR in the ipsilateral dorsal horn of anti-CCL2 treated rat 4 days following CCI. (D) OX42-IR in the ipsilateral dorsal horn of IgG treated rat 4 days following CCI. Scale bars = 50 µm. (E) Quantification of OX42 positive profiles. Intrathecal anti-CCL2 significantly attenuates microglia activation following CCI. $^{*}P < 0.05$ compared to IgG treated animals, one-way ANOVA, post-hoc Tukeys.

ertheless, the CNS expression profile of CCR2 is heavily debated. Some studies demonstrate an almost exclusive expression of CCR2 in neurons of the CNS (Gosselin et al., 2005; Banisadr et al., 2005). However, CCR2 expression is detected only in microglia following PNL (Abbadie et al., 2003).

Our real-time RT-PCR and immunohistochemistry results suggest that CCL2 is anterogradely transported from DRG cell bodies to the central terminals of primary afferent fibres where it may be translated and/or directly released. We tested this hypothesis using *ex vivo* isolated dorsal horn preparation. Activity evoked release of CCL2 in the dorsal horn was not observed in sham preparations while its increase in SNL preparations indicates that CCL2 may act as a neuromodulators only under neuropathic conditions.

Following peripheral nerve injury, spinal microglia activation is reported in regions where the damaged primary afferent fibres project (Watkins and Maier, 2003; Marchand et al., 2005; Clark et al., 2007a; Clark et al., 2007b). To test the hypothesis that CCL2 released from sensory neurons may contribute to the injury-induced microglia response, we examined the behavioral and biological effects of spinal CCL2. Intrathecal administration of CCL2 induces mechanical allodynia 24 h following administration and was prevented by pre-treatment with a CCL2 neutralizing antibody. In addition, intraspinal CCL2 injection led to mechanical allodynia but not thermal hyperalgesia. The lack of effect of CCL2 on thermal hyperalgesia is unexpected, however the majority of studies examining CCL2 have not tested thermal hyperalgesia, only investigating mechanical allodynia (Tanaka et al., 2004; Zhang et al., 2007). However, Abbadie and colleagues failed to observe any change in thermal pain behaviour in CCR2 KO mice compared to wild type animal following intraplantar CFA administration (Abbadie et al., 2003). Nonetheless, these data support previous work demonstrating that intrathecal CCL2 induces neuropathic pain-like behaviors (Tanaka et al., 2004). However, in contrast to this study using a dose of 100 ng/rat, we only administered 3 ng/rat which could explain the relative delay observed on the effect of intrathecal CCL2 here. Intraspinal CCL2 also induced extensive microglial activation in the ipsilateral dorsal horn, suggesting that endogenous CCL2 may represent a mediator capable of initiating microglia activation.

The presence of endogenous CCL2 appears to be vital for the full expression of neuropathic pain following a peripheral nerve injury. CCL2 neutralizing antibody effectively prevented neuropathic pain behaviors following CCI. These results support previous work demonstrating a reduction in neuropathic pain behaviors in CCR2 null mice (Abbadie et al., 2003). We also observed a significant reduction in injury induced spinal microglial activation following anti-CCL2 treatment. Together these data suggest that spinal CCL2/CCR2 signaling is critical for spinal microglial activation and the development of neuropathic pain after peripheral nerve damage.

Together, our data establish that neuronal expression of the chemokine CCL2 is a mechanism by which neuroimmune interactions can arise. There are multiple reports that the activation of spinal microglia is a critical event in the generation of neuropathic pain behaviors but the initiators of this activation remain disputed.

Here, we clearly show that CCL2 released from the central terminals of primary sensory neurons in neuropathic pain states may act directly on spinal microglia. Several other candidates have been implicated in microglial activation but the exact mechanism(s) remain elusive. Tsuda and colleagues reported that activation of microglia required $P2X_4$ receptors, which were upregulated and specifically expressed by microglia (Tsuda et al., 2003). However, $P2X_4$ antisense treatment attenuated pain behaviors but failed to alter microglial activation after peripheral nerve injury, indicating that the increase in $P2X_4$ receptor expression in spinal microglia may take place downstream of their activation. More recently, the cysteine protease Cathepsin S has been implicated in microglial mediated neuropathy (Clark et al., 2007b). In contrast to $P2X_4$, inhibition of this enzyme attenuates both established pain behaviors and microglia activation following PNL (Clark et al., 2007b). Fractalkine has also been proposed as a key mediator of neuronal-glial communications in chronic pain models. Fractalkine is expressed by both spinal and DRG neurons (Verge et al., 2004; Lindia et al., 2005) whereas its receptor (CX3CR1) is expressed by microglia, and is upregulated in two models of neuropathic pain (Verge et al., 2004; Lindia et al., 2005). Intrathecal treatment with neutralizing antibodies against both fractalkine and CX3CR1 can attenuate neuropathic pain behaviors (Milligan et al., 2004; Clark et al., 2007b). However, conflicting reports demonstrate both neuroprotective and neurotoxic effects of microglial CX3CR1 signaling in other CNS injury paradigms. Intrathecal antisense directed towards the Toll-like receptor 4 (TLR4), expressed exclusively by microglia, decreased glial activation as well as pain behavior in neuropathic rats (Tanga et al., 2005). Furthermore, TLR4 deficient mice did not exhibit pain behavior after peripheral nerve injury. However, this receptor is activated by several exogenous and endogenous ligands and this study did not provide an identified activator of TLR4 in neuropathic conditions.

Our study, strongly demonstrates that CCL2 may be a key factor in spinal neuro-microglial interactions following peripheral nerve injury. However, CCL2 via CCR2 may also act indirectly by increasing vascular permeability and promoting the recruitment of blood borne monocytes/macrophages, which in turn may promote microglial activation and/or even differentiate into fully functional microglia (Zhang et al., 2007). The literature is still controversial on the infiltration of blood borne monocytes into the spinal cord after nerve injury, with opposing results being reported (Zhang et al., 2007; Ajami et al., 2007) therefore, we cannot conclude of an exclusive effect of CCL2 on existing microglia or the recruitment of blood borne monocytes/macrophages. Finally, a recent study reported that CCL2 may have a direct effect on GABAergic neurons (Gosselin et al., 2005). It is now well established, that chemokines are able to excite cultured DRG neurons (Oh et al., 2001; Sun et al., 2006), but the direct influence of CCL2 on spinal neurons requires further study.

In conclusion, our study provides evidence that CCL2, induced mainly in damaged neurons after peripheral nerve injury, is released centrally by nerve terminals, contributing to microglial activation in the spinal cord. In turn, microglia may release a number of mediators that contribute to the initiation of neuropathic pain. This study suggests that glial cells form an interdependent network that is now considered a functional unit within the CNS. Despite the involvement of distinct cell types, they converge to create a state of hyperexcitability. In this network, we believe that CCL2 is a key player in the neuroimmune communications underlying peripheral neuropathic pain states.

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