CCL2 has similar excitatory effects to TNF-α in a subgroup of inflamed C-fiber axons

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ABSTRACT

Peripheral nerve inflammation can cause neuronal excitability changes that have been implicated in the pathogenesis of chronic pain. Although the neuro-immune interactions that lead to such physiological changes are unclear, in vitro studies suggest that the chemokine CCL2 may be involved. This in vivo study examines the effects of CCL2 on untreated and inflamed neurons and compares its effects to those of TNF-α. Extracellular recordings were performed in the anesthetized rat on isolated neurons with C-fiber axons. On untreated neurons, CCL2 as well as TNF-α had negligible effects. Following neuritis, both cytokines transiently caused the firing of action potentials in 27-30% of neurons that were either silent or had background (ongoing) activity. The neurons with ongoing activity that responded to either cytokine had significantly slower baseline firing rates (median = 3.0 spikes/min (IQR 3.0)) compared to the non-responders (median = 24.4 spikes/min (IQR 24.6); p<0.001). In an additional group, 26-27% of neurons that were sensitized due to repeated noxious mechanical stimulation of the periphery also responded to the effects of both cytokines. Neither cytokine caused axons to become mechanically sensitive. Immunohistochemistry confirmed that the cognate CCL2 receptor, CCR2, is mainly expressed on glia and is therefore not likely to be an axonal target for CCL2 following inflammation. In contrast, the cognate TNF-α receptor, TNFR1, was present on untreated and inflamed neurons. In summary, CCL2 can excite inflamed C-fiber neurons with similar effects to TNF-α, although the underlying mechanisms may be different. The modulatory effects of both cytokines are limited to a subgroup of neurons that may be subtly inflamed.

KEYWORDS

Ongoing activity, cytokines, electrophysiology, chronic pain, nociceptors
INTRODUCTION

Following nerve injury, primary sensory neurons develop signs of increased excitability, whereby cut tips of regenerating axons develop ongoing activity and mechanical sensitivity (Chen and Devor 1998, Howe et al. 1977, Michaelis et al. 1995, Scadding 1981, Tal & Eliav, 1996). Such increases in excitability from regenerating axons may contribute to symptoms in patients with chronic pain where there is a detectable nerve injury. In many patients however, a nerve injury is not always apparent. Examples of such conditions include complex regional pain syndrome, compressive neuropathies and diffuse limb pain (Janig and Baron 2003; Dilley et al. 2011). Evidence from a model of localised peripheral nerve inflammation (the neuritis model) suggests that in these cases, painful symptoms may be in part generated from inflamed neural tissue in the absence of gross pathological changes such as axonal degeneration or demyelination. In the neuritis model, animals show signs of pain hypersensitivity (e.g. mechanical allodynia and heat hyperalgesia) (Chacur et al. 2001, Eliav et al 1999) and intact C-fiber axons develop ongoing activity and axonal mechanical sensitivity (AMS; Bove et al. 2003; Bove 2009; Bove & Dilley 2010; Dilley et al. 2005; Eliav et al. 2001). Signs of increased axonal excitability following neuritis (i.e. ongoing activity and AMS) are consistent with the symptoms of spontaneous pain as well as painful responses to limb movements that stretch nerve trunks.

The neuro-immune interactions that lead to the development of inflammation-induced axonal hyperexcitability are unclear. Such interactions are likely to be complex and involve a host of different inflammatory mediators. Several lines of evidence suggest that chemokines, a group of chemotactic cytokines, may play an important role in such mechanisms (Oh et al. 2001; Thacker et al. 2009). Of particular interest is the chemokine CCL2 and its cognate receptor CCR2. CCL2 is traditionally known for its role as a monocyte chemoattractant, although more recently it has been reported to act within the peripheral nervous system in inflammatory pain pathways. The physiological effects of CCL2 on peripheral neurons are apparent following nerve injury, where it can directly excite injured dorsal root ganglion (DRG) neurons (Sun et al. 2006; Wang et al. 2010; White et al. 2005). Its role in nerve injury mechanisms is further supported by the upregulation of CCR2 on injured DRG neurons (Bhangoo et al. 2007; Jung et al. 2009; Xia et al. 2010). Behavioral studies also suggest that CCL2 may be involved in the development of pain hypersensitivity in inflammatory pain models (Abbadie et al. 2003; Qin et al. 2005; Tanaka et al. 2004).

Despite a body of evidence that infers a role for CCL2 in chronic pain, there are limited in vivo studies into the effects of CCL2 on inflamed nociceptive axons where there is no frank nerve injury. Therefore, the present study expands on previous work by testing the electrophysiological effects of CCL2 on inflamed C-fiber axons in the neuritis model and following acute inflammation at their peripheral terminals. It also compares the effects of CCL2 to those of the pro-inflammatory cytokine, tumor necrosis factor α (TNF-α), to determine whether common excitatory effects exist between cytokines. The role of TNF-α in pain mechanisms has been investigated extensively. For example, the direct exposure of uninjured DRG neurons to TNF-α can lead to the development of ongoing activity (Leem and Bove 2002; Liu et al. 2002; Sorkin et al. 1997; Zhang et al. 2002), although there is substantial controversy over the extent of this effect (Leem and Bove 2002). Following nerve injury however, it is in agreement that TNF-α can further sensitize neurons and increase the rate of firing from those neurons that are already ongoing (Liu et al. 2002; Schafers et al.
Behavioral studies have also reported the development of pain hypersensitivity following the administration of TNF-α (Opree and Kress 2000; Schafers et al. 2003; Wagner and Myers 1996; Zelenka et al. 2005). In particular, TNF-α has been implicated in the development of pain hypersensitivity following neuritis (Gazda et al., 2001). The physiological effects of TNF-α are reported to be mediated via its cognate receptor, TNFR1 (George et al. 2005; Schafers et al. 2008; Shubayev and Myers 2000). In order to gain an insight into the mechanisms by which both CCL2 and TNF-α may exert their physiological effects, the neuronal expression of the cognate receptors for both CCL2 and TNF-α (i.e. CCR2 and TNFR1) were also investigated along untreated and inflamed (neuritis) axons.
MATERIALS AND METHODS

Experiments were carried out in strict accordance with the UK Animals (Scientific Procedures) Act (1986). A total of 66 adult male Sprague Dawley rats (240-520 g, average 370 g) were used in this study.

Two experimental paradigms were carried out to assess the effects of CCL2 and TNF-α on neurons that were either untreated or inflamed mid-axonally in the neuritis model (paradigm 1), or acutely inflamed at the peripheral terminals following repeated noxious mechanical stimulation of the periphery (paradigm 2).

Surgery

Thirty animals underwent neuritis surgery prior to electrophysiological recordings/immunohistochemistry. Animals were anesthetized and maintained on isoflurane (1.75%) in oxygen. The left sciatic nerve was exposed at the mid-thigh by blunt dissection through the biceps femoris muscle, and a 7-8 mm length carefully separated from adjacent connective tissue. The nerve was loosely wrapped in a strip (approximately 5 mm x 10 mm) of sterile Gelfoam (Spongostan™; Ferrosan, Denmark) saturated with approximately 150 µl Complete Freund’s adjuvant (Sigma, Dorset, UK; diluted 1:1 using sterile saline). The muscle and skin were closed using 4/0 monofilament sutures (Vicryl; Ethicon, West Lothian, UK) and the animals were allowed to recover.

Electrophysiology: Paradigm 1

Single unit electrophysiological recordings from C-fiber axons were carried out on untreated animals (n=34 neurons from 9 animals) as well as at 3-6 days post-neuritis (n=66 neurons from 26 animals), which coincides with the peak physiological changes associated with the lesion (Dilley and Bove 2008b). Animals were anaesthetized with 1.5 g/kg 25% w/v urethane i.p. Smaller doses of urethane were given i.p. as required to maintain absence of pinch withdrawal and corneal reflexes. Body temperature was maintained at physiological levels (37°C) by using a rectal thermal probe linked through a feedback loop to a heating pad (Harvard Apparatus, Kent, UK). A lumbar laminectomy was performed from L2 to L5 to expose the spinal canal. The surrounding skin was sutured to a metal ring to form a mineral oil pool. The dura mater was opened and the L5 dorsal root was cut close to the dorsal root entry zone. The cut end of the dorsal root was placed onto a glass platform (9 mm x 5 mm).

The sciatic nerve was exposed in the mid-thigh and cleared from surrounding connective tissue for 8-10 mm. In neuritis animals, the Gelfoam surrounding the nerve was removed. The treatment site or equivalent was positioned through a small notched plastic well (5 x 8 x 4 mm) to allow suspension of test agents around the nerve (Fig. 1A). The notched edges of the chamber were sealed using petroleum jelly to prevent leakage of test agents. The skin flaps were sutured to a metal ring to produce a mineral oil pool that was necessary for electrical stimulation. Since noxious stimulation of the skin during receptive field identification can sensitize neurons (Bove and Dilley 2010), receptive fields were not identified. Instead, bipolar stimulating electrodes were positioned under the sciatic nerve.
distal to the well to ensure that neurons were conducting through the lesion site (Fig. 1A). Due to movement artefacts caused by electrical stimulation of the sciatic nerve, the three terminal branches were crushed distally.

Individual fine filaments (6 -10 µm diameter) were teased from the dorsal root using finely sharpened forceps and placed over a gold bipolar recording electrode. Filaments were teased until single action potentials could be evoked using electrical stimulation of the sciatic nerve. Only filaments with clearly identifiable waveforms were studied. Electrical stimulation (square wave pulses, 0.5 millisecond duration, 10 to 20 V amplitude) was applied using a constant voltage isolated stimulator (Digitimer, Hertforshire, UK) to identify each neuron and determine conduction latencies. Action potentials were amplified (1 K to 5 K), band-pass filtered (10 to 5000 Hz), and monitored with an oscilloscope. Neuronal activity was digitized and recorded with Spike 2 software (Cambridge Electronic Designs, Cambridge, UK) for off-line analysis. Neurons were classified as having C-fiber axons if their conduction velocity was <1.5 m/s. Recordings were carried out from neurons which were either silent, or which were firing ectopically (ongoing) during an initial baseline period. One to eight neurons were assessed for cytokine effects in each animal. Recordings were also made from larger multi-unit filaments to assess the proportion of ongoing activity.

Axonal mechanical sensitivity (AMS) was tested manually using a soft silicone tapered probe, which delivers forces up to 20 cN (Dilley and Bove 2008a). The mechanical stimulus was applied successively along the length of sciatic nerve that was supported by the well (i.e. at the neuritis lesion site or equivalent in untreated animals). The duration of each mechanical stimulus was 1–2 seconds. If one or more action potentials were initiated during this period, the axon was considered to be mechanically sensitive (Fig. 1C). Using the probe in this manner does not interrupt the conduction of action potentials, which was confirmed by electrical stimulation of the sciatic nerve after testing for AMS. Axonal mechanical sensitivity testing dislodged the petroleum jelly seals of the well, and therefore testing was only performed at the end of the test agent exposure. Due to this factor, it was not possible to test every neuritis neuron for the development of AMS.

Electrophysiology: Paradigm 2

In a separate set of experiments, single unit electrophysiological recordings from C-fiber axons were carried out on a group of untreated animals with intact receptive fields (n=60 neurons in 20 animals). The electrophysiological procedures were similar to those described for paradigm 1, except that the sciatic nerve in the mid-thigh was positioned on a small plastic platform (10 mm x 5 mm) that was notched to support the nerve (Fig. 1B). In these experiments, neurons were identified electrically using bipolar stimulating electrodes placed under the L5 dorsal root (square wave pulses, 0.5 millisecond duration, 2 to 10 V amplitude). The three terminal branches of the sciatic nerve were not crushed so as to allow identification of receptive fields. Once a neuron had been identified by electrical stimulation, receptive fields were searched for below the knee by squeezing the periphery, using either fingers or blunt forceps. The loose properties of the skin were exploited to carefully discriminate cutaneous versus deep fields (Leem and Bove 2002). After a receptive field was located, the neuron was electrically “collided” to ensure that that the electrical and mechanically evoked responses were from the same neuron. Collision involves stimulating
the neuron mechanically at its receptive field whilst simultaneously stimulating it electrically at the dorsal root. If the electrical stimulus occurred during the relative refractory period of the neuron following the mechanical stimulation, the action potential was delayed or not initiated (Fig. 1D). Identification of the receptive fields of high threshold mechanoreceptors in deep structures requires noxious mechanical stimulation of the periphery. Over the duration of an experiment, repeated noxious mechanical stimulation leads to acute inflammation, which sensitizes neurons and causes ongoing activity (Bove and Dilley 2010). One to seven neurons were assessed for cytokine effects in each animal.

Axonal mechanical sensitivity was tested as per paradigm 1 except that mechanical stimulus was applied successively along the length of sciatic nerve that was supported by the platform. Axonal mechanical sensitivity was tested prior to and following the application of the test agent. Through conduction was confirmed following the second AMS test by re-stimulation of the distal receptive field.

Test solutions and procedure

Recombinant human CCL2 (Peprotech, London, UK) was diluted in 0.1% BSA saline to a final concentration of 500 ng/ml (pH 6.5-7.0). Recombinant human TNF-α (Peprotech, London, UK) was diluted in a vehicle (0.1% bovine serum albumin (BSA) in 0.9% w/v saline) to give aliquots of 100 ng/ml that were stored at -20°C. Aliquots were diluted to a final concentration of 0.05 ng/ml in 0.1% BSA saline (pH 6.5-7.0). Aliquots were stored at -20°C prior to use.

Doses were determined following preliminary experiments. Ascending concentrations of CCL2 (250, 500, 1000 ng/ml) and TNF-α (0.025, 0.05, 0.1 ng/ml; 10 min exposure at each concentration) were assessed in five untreated neurons (in two animals) and four inflamed neurons following neuritis (in one animal). In untreated animals the cytokines were shown to have no effect, whereas following neuritis one ongoing neuron responded to each cytokine. Peak responses were observed at 500 ng/ml for CCL2 (increase in firing rate from baseline at 250 ng/ml = 81%, 500 ng/ml = 184%, and 1000 ng/ml = 98%) and 0.05 ng/ml for TNF-α (increase in firing rate from baseline at 0.025 ng/ml = 43%, 0.05 ng/ml = 52%, and 0.1 ng/ml = 42%). The most efficacious doses were consistent with previous reports (Oh et al. 2001; Sun et al. 2006; Wang et al. 2010; White et al. 2005, Leem and Bove 2002; Schafers et al. 2003; Sorkin et al. 1997).

For paradigm 1, the vehicle (0.1% bovine serum albumin (BSA saline); 200 μl) was applied to the well and the baseline activity of each neuron was recorded for 15 minutes. At the end of the baseline period, the BSA saline was removed and the test agent was applied for 15 minutes (either BSA saline (control), TNF-α or CCL2; 200 μl). At the end of the test period, the test agent was removed and the sciatic nerve was washed copiously. Note that all effects were transient (see Results). Further neurons were sought but were not recorded from for at least 20 minutes after the test agent had been washed off. Each animal was only tested for the effect of one test agent.

For paradigm 2, test agents (200-500 μl) were delivered to the nerve using absorbent cotton (5 x 5 x 5 mm), which was positioned on and around the sciatic nerve so as to ensure maximum exposure. The notched platform prevented leakage of the test solution into the
surrounding soft tissue. Both delivery approaches (well and absorbent cotton) have successfully been used in previous studies (Leem and Bove 2002; Sorkin et al. 1997). The duration of exposures was the same as for paradigm 1.

**Immunohistochemistry**

Three untreated and 5 neuritis-treated animals were sacrificed with an overdose of sodium pentobarbital. The ipsilateral L5 dorsal root ganglia (DRG) and neuritis treatment site, or equivalent region of untreated sciatic nerves, were removed and snap frozen in isopentane on dry ice. Sections were cut at 8 μm using a cryostat (Leica, Germany), mounted onto gelatin-coated slides and fixed for 7 minutes with 4% paraformaldehyde. DRG sections were sequentially mounted on seven consecutive slides, so that the eighth section was on the same slide as the first section. A maximum of six sections were mounted on each slide.

Sciatic nerves were stained using the avidin-biotin detection system (Vectastain Elite Kit, Vector Laboratories, Cambridgeshire, UK). Endogenous peroxidase was initially blocked for 30 minutes using 0.3% H₂O₂ in absolute methanol. Non-specific binding sites were blocked for 30 minutes with either 2% normal goat serum (CCR2) or 2% normal rabbit serum (TNFR1). Tissue sections were incubated overnight at 4°C with anti-rat CCR2 (1:200 in 2% goat serum; Abcam, Cambridge, UK) or anti-human TNFR1 (1:50 in 2% rabbit serum; R&D Systems, Oxfordshire, UK). This was followed by incubation for 1 hour at room temperature with either biotinylated goat anti-rabbit antibody (1:200, Vector) for CCR2-probed sciatic nerves or biotinylated rabbit anti-goat antibody (1:200, Vector) for TNFR1-probed sciatic nerves. 0.05% Tween-PBS rinses (three times, 5 minutes each) were performed following primary and secondary antibody and ABC treatments. Sciatic nerve sections were developed with diaminobenzidine (DAB) plus nickel chloride (Vector) and were then dehydrated, cleared with xylene (Fisher Scientific, Leicestershire, UK), and coverslipped using Histomount (Fisher). Sections were viewed under a light microscope (Leica Microsystems, Wetzlar, Germany) and photographed.

DRGs were labelled using immunofluorescence. Non-specific sites were blocked for 30 minutes with either 2% normal goat serum (CCR2) or 2% normal rabbit serum (TNFR1) (Vector). Tissue sections were incubated overnight at 4°C with anti-rat CCR2 (1:200 in 2% goat serum; Abcam) or anti-human TNFR1 (1:50 in 2% rabbit serum; R&D Systems). This was followed by incubation for 1 hour at room temperature with Alexa Fluor 488 goat anti-rabbit fluorescent antibody (1:200, Invitrogen) for CCR2-probed DRGs or Alexa Fluor 488 rabbit anti-goat fluorescent antibody (1:200, Invitrogen, Glasgow, UK) for TNFR1-probed DRGs. PBS rinses (three times, 5 minutes each) were performed following the primary and secondary antibody. DRGs were double-labelled with DAPI (4′,6-diamidino-2-phenylindole; 1:5000, Invitrogen) then coverslipped using glycerol/PBS mounting medium (Citifluor, London, UK). Sections were viewed under a fluorescence microscope (Leica Microsystems, Wetzlar, Germany) at 488 and 350 nm excitation and photographed at each wavelength.

**Data analysis**
A neuron was classified as silent if it did not fire during the 5 minute epoch prior to cytokine application (baseline period), whereas a neuron was classified as having background (ongoing) activity if it fired at least once during the baseline period. Silent neurons were considered responsive to cytokines if they fired at least once following application. Ongoing neurons were considered responsive to cytokine application if their firing rate increased by >50% compared to the baseline rate (50% represents the mean standard deviation in firing rate during the entire baseline recording period (3 x 5 min epochs) for all ongoing neurons). The firing rates presented following cytokine application represent the peak response, which could represent either the first or second 5 minute epoch post application.

For immunohistochemistry, the number of cytokine receptor and DAPI positive neuronal profiles were counted in each section, and the percentage of receptor positive cells determined. To avoid repeated counting of cells, the sections examined were at 56 μm intervals. All cell counting was performed blind without knowledge of the condition. Non-specific staining with secondary antibody was not found in the absence of primary antibody in any case.

All data was assessed for normality using Shapiro-Wilk tests to determine appropriate statistical analysis. All comparisons of proportions were made using two-tailed Fisher Exact tests (when expected frequencies were less than 5) or Chi-square tests with Yates correction. Comparisons of conduction velocities, ongoing rates, time from experimental setup and diameters of DRG cell bodies were performed between groups using two-tailed Mann-Whitney tests. Comparisons of ongoing rates pre- and post- treatment were made using two-tailed Wilcoxon Signed Rank tests (one-tailed in the case of the responder groups). All data is given as the median and the interquartile range (IQR) unless stated.
RESULTS

Paradigm 1: Untreated group

CCL2 and TNF-α do not excite untreated neurons

Recordings from multiunit filaments revealed an ongoing activity incidence of <7% in untreated neurons (7/102). Thirty four neurons were examined for the effects of BSA saline, CCL2 or TNF-α, none of which were ongoing prior to application of the test agents. The median conduction velocity of these neurons was 0.89 m/sec (IQR 0.28). None of the neurons tested responded to BSA saline application (n=12 neurons) (Fig. 2). Similarly, none of the neurons tested responded to TNF-α (n=12 neurons). Following CCL2 application, one neuron responded (1/10 neurons; p=0.45 compared to BSA saline, Fisher Exact test), firing at a rate of 0.2 spikes/min. None of the neurons in the untreated group developed AMS following the application of BSA saline (n=12 neurons), CCL2 (n=10 neurons) or TNF-α (n=12 neurons).

Paradigm 1: Neuritis group

Neuritis alters the physiology of C-fiber axons

Recordings from multiunit filaments revealed a significantly higher incidence of ongoing activity in the neuritis group (38% (n=8/21 neurons)) compared to the untreated group (7% (n=7/102 neurons); p=0.0005, Fisher Exact test). During the last recording of the experiment where an ongoing neuron was present, the sciatic nerve was transected (first distal and then just proximal to the neuritis lesion site) to determine the origin of the ongoing activity. In all such neurons (n=6), ongoing activity persisted after the distal transection, but ceased following the proximal transection. The median baseline firing rate of characterised ongoing neurons was 16.8 spikes/min (IQR 31.95; n=36 neurons).

The median conduction velocity in the neuritis group was 0.71 m/sec (IQR 0.33), which was significantly slower than that of the untreated group (p=0.0003, Mann-Whitney test). This slowing was mainly due to the slowed conduction velocity of the ongoing neurons (median = 0.56 m/sec (IQR 0.37) compared to 0.84 m/sec (IQR 0.31) for the silent neurons; p=0.0002, Mann-Whitney test). There was a negative correlation between ongoing activity rate and conduction velocity (i.e. as the ongoing activity rate increased, the conduction velocity decreased; r=0.47, p=0.004, analysis of variance).

CCL2 and TNF-α excite a subgroup of inflamed neurons

Table 1 summarises the baseline firing rates of responsive and non-responsive ongoing neurons, and their rates post-cytokine application.

None of the neurons tested post-neuritis (n=0/21 neurons (11 silent, 10 ongoing)) responded to BSA saline application (Fig. 2).

Twenty seven percent of neurons (n=6/22 neurons) responded to CCL2 application (p=0.02 compared to BSA saline, Fisher Exact test; Fig. 2), which included 20% (2/10) of silent and...
33% (4/12) of ongoing neurons. The firing rates of the two responsive silent neurons were 0.2 and 1.2 spikes/min post-CCL2 (Fig. 3A). The ongoing neurons that responded to CCL2 had significantly slower baseline firing rates compared to the ongoing non-responders (p=0.02, Mann-Whitney Test, Table 1, Fig. 3B). All CCL2 responsive neurons (6/6) increased their activity 0-5 minutes (i.e. during the first 5 minute epoch) after CCL2 application. The effect was transient, and began to recover towards baseline after 10 minutes of exposure (Fig. 3E).

Thirty percent of neurons (n=7/23 neurons) responded to TNF-α application (p=0.009 compared to BSA saline, Fisher Exact test, Fig. 2), which included 22% (2/9) of silent and 36% (5/14) of ongoing neurons. The firing rates of the two responsive silent neurons were 0.4 and 0.6 spikes/min post-TNF-α (Fig. 3C). Similar to CCL2, the ongoing neurons that responded had significantly slower baseline firing rates compared to the ongoing non-responders (p=0.01, Mann-Whitney test, Table 1, Fig. 3D). All TNF-α responsive neurons increased their activity within 10 minutes after application. Similar to CCL2, the effect was transient, and began to recover towards baseline after 10 minutes of exposure.

CCL2 and TNF-α affect inflamed neurons with unaltered conduction velocities

The conduction velocities for each treatment group are summarized in Table 2. The median conduction velocity of the neurons that responded to CCL2 and TNF-α was 39% faster compared to the non-responders (p=0.006, Mann-Whitney test). The proportion of responsive neurons was thus higher at faster conduction velocities (Fig. 4). The conduction velocity of the responders was comparable to the conduction velocity of the untreated group (p=0.90, Mann-Whitney test).

CCL2 and TNF-α do not acutely cause AMS

Due to the experimental design (see Methods), it was not possible to test for AMS prior to cytokine or BSA saline application in the neuritis group. Axonal mechanical sensitivity was present in 14% (6/43) of neurons tested. There was no difference in the proportions of neurons with AMS following either BSA saline (n=1/11 neurons), CCL2 (n=3/22 neurons) or TNF-α (n=2/10) treatment (p=0.86, 2x3 Fisher Exact test).

Paradigm 2: Acute inflammation at the peripheral terminals

In this group, the lower limb was repeatedly noxiously stimulated whilst searching for receptive fields. This process resulted in visible swelling of the lower limb and foot, which increased over time.

Slow ongoing activity developed during the course of the experiment. Ongoing neurons were identified significantly later from the time of experimental setup (median time at which 50% of ongoing neurons were identified = 151 min (IQR 64)) compared to the neuritis group (median time = 118 min (IQR 59); p=0.02, Mann-Whitney test; Fig. 5A). The median baseline
Firing rate of ongoing neurons was 2.8 spikes/min (IQR 10.50; n=30), which was significantly slower than that of the neuritis group (p<0.001, Mann-Whitney test).

The majority of receptive fields were located in deep (49/60 neurons) rather than cutaneous structures.

CCL2 and TNF-α excite a subgroup of sensitized neurons

Table 3 summarizes the baseline firing rates of responsive and non-responsive ongoing neurons, and their rates post-cytokine application.

None of the neurons tested (n=0/19 neurons (9 silent, 10 ongoing)) responded to BSA saline application.

Twenty seven percent of neurons (n=6/22 neurons) responded to CCL2 application (p=0.02 compared to BSA saline, Fisher Exact test), which included 33% (4/12) of silent and 20% (2/10) of ongoing neurons. Five of the six responsive neurons were recorded late in the experiment (i.e. after 151 min, Fig. 5B). The firing rate post-CCL2 application of the responsive silent neurons was 0.2 spikes/min (IQR 0.00). The baseline rate of the ongoing responders was similar to the baseline rate of the ongoing non-responders (Table 3). Four of the six CCL2 responsive neurons increased their activity 0-5 minutes (i.e. during the first 5 minute epoch) after CCL2 application. The effect was transient, and began to recover towards baseline after 10 minutes of exposure.

Twenty six percent of neurons (n=5/19 neurons) responded to TNF-α application (p=0.046 compared to BSA saline, Fisher Exact test), which included 11% (1/9) of silent and 40% (4/10) of ongoing neurons. All responsive neurons were recorded late in the experiment (i.e. after 151 min, Fig. 5B). The firing rate post-TNF-α application of the responsive silent neuron was 0.2 spikes/min. The baseline rate of the ongoing responders was similar to that of the ongoing non-responders (p=0.48, Mann-Whitney Test, Table 3). All TNF-α responsive neurons increased their activity within 10 minutes after application. Similar to CCL2, the effect was transient, and began to recover towards baseline after 10 minutes of exposure.

Receptive field location had no bearing on whether acutely inflamed neurons were responsive to cytokine treatment (3/6 cutaneous vs. 8/35 deep; p=0.32, Fisher Exact test).

None of the axons in the sensitization group developed AMS following the application of BSA saline (n=19 neurons), CCL2 (n=22 neurons) or TNF-α (n=19 neurons).

Immunohistochemistry

CCR2 is expressed at negligible levels in DRG neuronal cell bodies

CCR2 was expressed at extremely low levels within the ipsilateral L5 DRG cell bodies in both the untreated (2.1%; 17/825 cell bodies, n=3 animals) and neuritis groups (0.6%; 7/1198 cell bodies, n=5 animals), although the difference between groups was significant (p=0.005, Chi-square test; Figs. 6A, 6B and 6E).
There was no significant difference in TNFR1 expression within the ipsilateral L5 DRG cell bodies between the untreated (mean=3.2% ± 2.7, total 25/487 cell bodies, n=3 animals) and neuritis groups (mean=6.7% ± 3.3, total 97/1525 cell bodies, n=5 animals; p=0.41, Chi-square test; Figs. 6C, 6D and 6E). The median diameter of the cell bodies that expressed TNFR1 was significantly smaller in the neuritis (32.4 µm) compared to untreated group (41.0 µm; p=0.03, Mann-Whitney test).

**CCR2 and TNFR1 are differentially expressed in sciatic nerve**

Immunolabelling for CCR2 in untreated sciatic nerve showed low-level staining of endothelial structures, such as Schwann cells and myelin, but no obvious axonal staining (Fig. 7A). Following neuritis, the magnitude of Schwann cell and myelin staining increased substantially (Fig. 7B), although axonal CCR2 staining remained unchanged. There was also a lack of CCR2 staining of endothelial cells and monocytes within the Gelfoam.

Immunolabelling for TNFR1 in the untreated sciatic nerve showed clear axonal staining, as well as labelling of the endothelial cells and perineurium (Fig. 7C, D). Following neuritis, there was also notable axonal staining (Fig. 7E).
DISCUSSION

The present study examined the physiological effects of CCL2 on inflamed neurons and compared its actions to those of TNF-α. The effects of CCL2 on neuronal excitability are less well explored than TNF-α, and previous CCL2 studies have focused on excised/dissociated DRG (Sun et al. 2006; Wang et al. 2010; White et al. 2005). In these studies, CCL2 had limited effects on untreated neurons but caused depolarisation of chronically compressed isolated DRG. Our study has extended these previous findings to show that in vivo, CCL2, as well as TNF-α, can excite inflamed neurons, whereas both cytokines had negligible effects on untreated neurons. The proportion of inflamed neurons that responded to CCL2 and TNF-α were similar (26-30%), yet the effects of CCL2 occurred at a concentration that was ten thousand times more concentrated compared to TNF-α. The lack of effect by TNF-α on untreated neurons contrasts from previously published findings (Sorkin et al. 1997; Leem & Bove 2002) where a comparable dose of TNF-α excited C-fiber neurons that were considered uninjured. However, our study suggests that the “uninjured” neurons in these previous studies may have been acutely inflamed due to repeated mechanical stimulation of the periphery during characterization of receptive fields. Schafers and colleagues (2003) also reported an excitation of untreated C-fiber axons by TNF-α. However, this study was carried out in vitro and thus the complete excision of tissue from its native environment could in itself affect axonal physiology, increasing the susceptibility of the neurons to cytokine effects. It is however in agreement that TNF-α is considerably less potent on untreated compared to injured neurons (Liu et al. 2002; Schafers et al. 2003).

Neuritis model

CCL2 and TNF-α had analogous excitatory effects on inflamed axons in the neuritis model and following acute inflammation of the peripheral terminals. Since the neuritis model does not result in substantial degeneration or demyelination (Eliav et al. 1999; Bove et al. 2003; Dilley et al. 2005), it can be concluded that inflammation alone is sufficient to increase the susceptibility of neurons to the effects of inflammatory mediators. In both models, cytokines excited axons that were already ongoing as well as those that were silent.

In the neuritis model, 38% of neurons developed baseline ongoing activity, accompanied by a slowing of conduction velocity. Interestingly, the ongoing neurons that were responsive to CCL2 and TNF-α had substantially slower firing rates (median = 3.0 spikes/min) compared to the ongoing neurons that did not respond (median = 24.4 spikes/min). These responsive neurons also had conduction velocities that were within normal range (i.e. comparable to the untreated group) even though many were already ongoing. It is therefore likely that the responsive neurons that either had slow rates of ongoing activity and relatively normal conduction velocities, or were silent, may be those that were only subtly altered by the neuritis. These observations suggest that both cytokines were only effective on inflamed neurons that had undergone sub-maximal physiological changes.

In both untreated and neuritis groups (i.e. paradigm 1), the three terminal branches of the sciatic nerve were crushed during electrophysiological procedures to prevent movement artefacts during electrical stimulation. Consistent with previous studies on acute crush (Grossmann et al. 2009; Michaelis et al. 1995), recordings from the untreated group
confirmed that the crush did not significantly affect the physiology of C-fiber axons during the few hours of the experiment, i.e. the level of ongoing activity was negligible (<7%), and conduction velocities were not slowed. The lack of ongoing neurons in the untreated group meant that it was only feasible to examine the effects of cytokines on silent neurons in this group.

**Acute inflammation of the peripheral terminals**

CCL2 and TNF-α were also tested on C-fiber neurons whose receptive fields had been characterized by mechanical stimulation of the periphery (paradigm 2). Due to the nociceptive properties of many of these neurons, and the presence of receptive fields in deep tissue (i.e. muscle), repeated noxious mechanical stimulation was required to stimulate the terminals. Towards the end of each experiment there was observable swelling of the periphery. Ongoing activity, which occurred later in these experiments compared to the neuritis, was therefore probably the result of sensitization of C-fiber neurons triggered by acute inflammation of the terminals (Bessou and Perl 1969; Perl et al. 1976). The development of slow ongoing activity (median = 2.8 spikes/min) following noxious mechanical stimulation of the periphery has previously been reported (Bove and Dilley 2010), and contrasts from the faster pattern of ongoing activity that is typically observed in the neuritis model. When peripheral fields are not searched, the level of ongoing activity from C-fiber neurons is extremely low (Bove and Dilley 2010).

The majority of neurons that were excited by CCL2 as well as TNF-α (both silent and ongoing) were recorded late from the start of the experiment, which suggests that those neurons were acutely inflamed. The initial slow firing rate of the responsive ongoing neurons was comparable to the rate of those that responded to the cytokines in the neuritis group. A similar slow baseline firing rate was also reported in the studies on TNF-α by Sorkin et al. (1997) and Leem and Bove (2002), suggesting that a number of their responsive neurons may have been acutely inflamed due to repeated noxious mechanical stimulation of the periphery.

**Axonal mechanical sensitivity**

The development of AMS in the neuritis model is well established (Bove et al. 2003; Dilley et al. 2005; Eliav et al. 2001), although the underlying mechanisms are only partly understood (Dilley and Bove 2008a; Eliav et al. 2009). In this study, AMS was tested after the application of CCL2 as well as TNF-α. The lack of effect in the untreated group suggests that these cytokines cannot acutely induce AMS. Similarly, in the neuritis group the proportion of neurons with AMS was comparable to previous reports (Dilley and Bove 2008b; Dilley et al. 2005). However, the role of CCL2 and TNF-α in the chronic development of AMS cannot be ruled out.
Mechanisms

Part of this study was aimed at understanding the neuronal mechanisms that underlie the physiological actions of CCL2 as well as TNF-α. The most likely targets for both cytokines are their principle receptors, namely CCR2 and TNFR1. Differences in expression patterns of these receptors might explain why inflamed neurons are more susceptible to the effects of these cytokines. For example, CCR2 is induced on primary sensory neurons following frank nerve injury (Bhangoo et al. 2007; Jung et al. 2009; Xia et al. 2010). Therefore, in injured axons, the physiological actions of CCL2 may be mediated via direct axonal interactions with its receptor. In the present study, the notable expression of CCR2 on glia and low level DRG expression following neuritis suggests that CCL2 may act via an indirect pathway that involves glial cells. Alternatively, a direct axonal mechanism might exist through other CCL2 receptors (e.g. CCR4 (Yang et al. 2007)) that may be upregulated along inflamed axons.

It is well established that the binding of CCL2 to its receptor can result in a G-protein coupled modulation of neuronal ion channels. For example, CCL2 has been shown to activate transient receptor potential receptors TRPV1 and TRPA1 (Jung et al. 2008) as well as inhibit voltage-gated potassium channels (Sun et al. 2006; Wang et al. 2010). Interactions with such channels on inflamed axons may lead to an increase in axonal excitability and possibly the development of ongoing activity. However, since CCR2 is mainly located on non-neuronal cells following neuritis, the binding of CCL2 to CCR2 would require an additional step in this pathway. For example, the activation of CCR2 on glial cells may trigger the release of other mediators (e.g. nerve growth factor) that are able to activate neuronal ion channels. There is also evidence that CCL2 can directly bind to and modulate the activity of ion channels independent of CCR2. It has been shown that CCL2 can directly modulate voltage-gated calcium channels (You et al. 2010), although such interactions result in an inhibition of these currents and therefore a likely decrease in excitability.

The results for TNFR1 expression suggest that TNF-α may act directly on C-fiber axons via this receptor. In both untreated and neuritis groups, TNFR1 was expressed along small numbers of axons. This finding is in agreement with previous studies showing TNFR1 expression along peripheral axons following nerve injury (George et al. 2005; Shubayev and Myers 2000). The neuronal expression of TNFR1 was also confirmed in L5 DRG, although levels were fairly low in both the untreated and neuritis groups. Despite the lack of a significant increase in TNFR1 following neuritis, there was a significant shift towards smaller cell bodies expressing the receptor. This shift indicates a move towards greater TNFR1 expression in inflamed C-fiber neurons. In addition, TNF-α may also function via TNFR2, which is considered to play a prominent role following chronic nerve injury (Schafers et al. 2008).

Binding of TNF-α to TNFR1 may increase axonal excitability sufficient to cause ongoing activity by activating the voltage-gated sodium channel Na$\alpha$1.8 (Czeschik et al. 2008) or inhibiting potassium channels (Liu et al. 2008). The modulation of Na$\alpha$1.8 by TNF-α is reported to occur via a p38 MAP kinase pathway (Jin and Gereau 2006). Surprisingly, TNFR1 is expressed on both untreated and inflamed axons yet TNF-α is only effective on inflamed neurons. This difference in physiological effect may reflect the shift in TNFR1 expression on C-fiber axons following neuritis or it may implicate a role for other TNF-α receptors.
It is well established that nerve injury causes changes in the expression patterns of ion channels along peripheral axons (Black et al. 1999). In the neuritis model, it is known that the inflammation is sufficient to cause transcription changes in the dorsal root ganglia (Dilley et al., 2005). Therefore, synthesis of new ion channels may account for the development of ongoing activity in 38% of neurons following neuritis and could also provide new targets for both CCL2 and TNF-α on inflamed axons. Of particular interest in this mechanism are Na,1.8 and hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels, which are upregulated along DRG neurons following nerve injury (Gold 2003; Jiang et al. 2008).

Changes in ion channel kinetics may also be a contributing factor in the development of ongoing activity (Black et al. 2004). Such kinetic changes may render ion channels more susceptible to modulation by cytokines.

The upregulation and insertion of specific receptors or ion channels on C-fiber axons might explain why CCL2 as well as TNF-α are effective on inflamed neurons following neuritis. However, such transcriptional changes are unlikely to explain why neurons recorded late (within hours) following noxious mechanical stimulation of the periphery (paradigm 2) also responded to these cytokines. The actions of CCL2 and TNF-α on what were likely to be acutely inflamed axons, suggests that intracellular mechanisms may play an important role.

For example, the activation of certain intracellular pathways by acute peripheral inflammation may lead to the modification of native ion channels, not only at the site of inflammation but also at remote axonal sites.

Clinical application

There is substantial evidence to indicate a role for inflammation in chronic pain conditions in the absence of a frank nerve injury (Bove 2009;Coderre and Bennett 2010; de Mos et al. 2009; Dilley et al. 2005; Elliott et al. 2008). The present study infers a specific role for both CCL2 and TNF-α in the maintenance of pain in such conditions. For example, CCL2 or TNF-α-mediated increases in ongoing activity from nociceptors may trigger or exacerbate the sensations of spontaneous pain reported by patients. It is also well established that ongoing input from the periphery is necessary for the generation and maintenance of central sensitization mechanisms within the spinal cord (Gracely et al. 1992). Therefore, both cytokines may play a role in the early development or maintenance of these central pain mechanisms. In addition, this study could provide a novel insight into the mechanisms involved in the "double-crush" phenomena, whereby a preceding injury to the peripheral nervous system may predispose the nervous system to a second injury (Upton and McComas 1973). In these patients, the release of inflammatory mediators onto an already inflamed nerve, remote from the original injury, may further sensitize neurons, leading to an exacerbation or development of new symptoms.

In the present study, the axonal responses to both CCL2 and TNF-α were transient and occurred within minutes following cytokine application. In contrast, spontaneous pain in patients can be persistent. However, similarities in the physiological effects of CCL2 and TNF-α suggest that other cytokines may also be excitatory, and thus when a nerve is exposed to a milieu of such mediators, pain sensations may be prolonged.
In summary, both CCL2 and TNF-α can modulate axonal firing in a subgroup of inflamed neurons. This subgroup may represent a window during the inflammatory response when a neuron is undergoing physiological changes and is more vulnerable to the effects of certain cytokines. Moreover, CCL2 and TNF-α have negligible effects on uninjured neurons but instead these cytokines functioned only to augment excitability in neurons that were already undergoing change. Similarities between the physiological function of CCL2 and TNF-α suggest that there are possibly many cytokines that can excite nociceptive neurons that have been subtly altered during an inflammatory response.

ACKNOWLEDGEMENTS

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GRANTS

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DISCLOSURES

The authors would like to state that there are no conflicts of interest regarding this work.


Fig. 1. Schematic diagram of the experimental set up for (A) Paradigm 1 and (B) Paradigm 2. Each diagram shows the sciatic nerve in the thigh with the trifurcation behind the knee. The recording site at the L5 dorsal root is also shown (Rec). A: For Paradigm 1, a well was used to apply treatments to the lesion site or equivalent. To confirm that recorded axons passed through the treatment site, the nerve was stimulated distally (Stim). The three terminal branches were crushed to prevent movement artefacts (Crush). B: For Paradigm 2, the sciatic nerve was positioned on a plastic platform notched to support the nerve. Treatments were applied using absorbable cotton positioned around the nerve on the platform (grayed area). The nerve was electrically stimulated at the L5 dorsal root (Stim). C: A typical C-fiber axon with AMS recorded from the neuritis group. The short horizontal lines above the trace represents the duration of the mechanical stimulation of the nerve. D: Electrical collision of an action potential. Five consecutive traces were triggered by electrical stimulation. In trace 3, an action potential was also elicited by mechanically stimulating the neuron’s receptive field (marked *). In this trace, the electrically stimulated action potential (marked **) was delayed because it fell within the relative refractory period of the previous action potential. DRG = dorsal root ganglion.

Fig. 2. Percentages of neurons responding to BSA saline, CCL2, and TNF-α application in the untreated and neuritis groups. Higher proportions of neurons responded to the cytokines in the neuritis group vs. BSA saline (*p<0.05, Fisher Exact test). The total number of neurons sampled and the number that are responsive are shown.

Fig. 3. A-D: Typical responses of neuritis-treated C-fiber axons to CCL2 and TNF-α application (A: CCL2 silent, B: CCL2 ongoing, C: TNF-α silent, D: TNF-α ongoing). Horizontal lines above traces represent the exposure period to BSA saline and CCL2/TNF-α. E: Interspike-interval plot for a C-fiber axon with baseline ongoing activity that shows the transient response to CCL2 application.

Fig. 4. Percentages of neurons responding to cytokine treatment (CCL2 and TNF-α combined) in the neuritis group based on conduction velocity. As conduction velocity increases, the proportion of responders increases. The total number of neurons sampled and the number that are responsive are shown.

Fig. 5. A: Cumulative plot showing the time from start of experiment at which neurons with baseline ongoing activity were identified in the untreated group with peripheral field searches (Paradigm 2; n=30 neurons) and neuritis group (Paradigm 1; n=36 neurons). Time points represent 30 min bins. Dotted line represents the time from set up that 50% of ongoing neurons were identified, which was significantly earlier in the neuritis (50% neurons identified by 118 min) group compared to the untreated group (50% of neurons identified by 151 min),
*p<0.05 (Mann-Whitney test). B: Percentage of neurons (silent and ongoing) recorded early (<151 min post-setup) and late (>%151 min) in the untreated group with peripheral field searches (Paradigm 2) that responded to cytokine treatment (CCL2 and TNF-α combined). Significantly more late neurons responded compared to early neurons (*p<0.05, Fisher Exact test). The total number of neurons sampled and the number that are responsive are shown.

Fig. 6. A-D: CCR2 and TNFR1 immunofluorescent labelling of DRG cell bodies in the untreated and neuritis groups. A: CCR2 untreated, B: CCR2 neuritis, C: TNFR1 untreated, D: TNFR1 neuritis. The nuclei of DAPI positive neuronal cells appear as large circular pale blue structures compared to the smaller elongated brighter blue nuclei of the satellite cells. The CCR2 and TNFR1 positive cells appear bright green. Scale bar = 50 µm. E: Summary of CCR2 and TNFR1 expression in DRG cell bodies. There was a significant decrease in CCR2 expression in the neuritis compared to untreated group (**p<0.01, Chi-square test). Error bars represent S.E.M.

Fig. 7. CCR2 and TNFR1 immunolabelling of the sciatic nerve in the untreated and neuritis group. Sciatic nerve from an untreated (A) and neuritis animal (B), showing CCR2 staining of Schwann cells and associated myelin (arrows in A and B). Note increase in staining following neuritis. Sciatic nerve from an untreated (C, D) and neuritis animal (E), showing TNFR1 labelling of axons (arrows in D and E), endothelial cells and the perineurium (arrow in C). Scale bar = 20 µm for A, B, C and E, and 10 µm for D. (A-C, E = transverse sections; D = longitudinal section).

### TABLES

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<th>Rate of ongoing neurons (spikes/min) (IQR)</th>
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Table 1. Rates of neuritis ongoing neurons pre- and post-cytokine application. Baseline rates of ongoing responders are significantly slower than baseline rates of non-responders for both cytokines (*p<0.05, Mann-Whitney test). Post-cytokine responder rates were significantly increased compared to baseline (†p<0.05, Wilcoxon Signed Rank test). Values are given as the median firing rate and IQR.

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Table 2. Conduction velocities in the neuritis group. Neurons which responded to cytokine treatment had significantly faster conduction velocities than those that did not respond (*p<0.05; †p<0.01, Mann-Whitney tests). Values are given as the median conduction velocity and IQR.

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Table 3. Rates of ongoing neurons pre- and post-cytokine application in Paradigm 2. Post-TNF-α responder rates were significantly increased compared to baseline (*p<0.05, Wilcoxon Signed Rank test). Values are given as the median firing rate and IQR.