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

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Submitted 2 March 2011; accepted in final form 19 August 2011

Richards N, Batty T, Dilley A. CCL2 has similar excitatory effects to TNF-α in a subgroup of inflamed C-fiber axons. J Neurophysiol 106: 2838–2848, 2011. First published August 24, 2011; doi:10.1152/jn.00183.2011.—Peripheral nerve inflammation can cause neuronal excitability changes that have been implicated in the pathogenesis of chronic pain. Although the neuroimmune 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 with 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, which were either silent or had background (ongoing) activity. The neurons with ongoing activity, which responded to either cytokine, had significantly slower baseline firing rates [median = 3.0 spikes/min [interquartile range (IQR) 3.0]] compared with the nonresponders [median = 24.4 spikes/min (IQR 24.6)]; P < 0.001. In an additional group, 26–27% of neurons, which 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 (TNFR), 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, which may be subtly inflamed.

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 and 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 (Dilley et al. in press; Janig and Baron 2003). Evidence from a model of localized 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 2009; Bove and Dilley 2010; Bove et al. 2003; 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 neuroimmune 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 with those of the proinflammatory cytokine 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
and maintained on isoflurane (1.75%) in oxygen. The left sciatic nerve was marked, saturated with petroleum jelly surrounding the nerve, and was closed using 4/0 monofilament sutures (Vicryl, Ethicon, West Dorset, UK; diluted 1:1 using sterile saline). The muscle and skin surrounding the nerve was sutured to a metal ring to form a mineral oil seal to surround the nerve. 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 × 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 (5 × 8 × 4 mm).fi 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-ms duration, 10–20 V amplitude) was applied using a constant voltage-isolated stimulator (Digitimer, Hertfordshire, UK) to identify each neuron and determine conduction latencies. Action potentials were amplified (1–5 K), band-pass filtered (10–5,000 Hz), and monitored with an oscilloscope. Neuronal activity was digitized and recorded with Spike 2 software (Cambridge Electronic Designs, Cambridge, UK) for offline 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 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 multiunit filament recordings to assess the proportion of ongoing activity.

AMS was tested manually using a soft silicone-latered probe, which delivers forces up to 20 cN (Dilley and Bove 2008a). The mechanical stimulus was applied successively along the length of the sciatic nerve, which 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 s. If no or one more action potentials were initiated during this period, the axon was considered to be mechanically sensitive (Fig. 1C). With the use of the probe in this manner, the conduction of action potentials is not interrupted, which was confirmed by electrical stimulation of the sciatic nerve after testing for AMS. AMS 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.

**Materials and Methods**

Experiments were carried out in strict accordance with the UK Animals (Scientific Procedures) Act (1986). Experimental protocols were approved by the University of Sussex Ethical Review Committee for Animal Research and UK Home Office. A total of 66 adult male Sprague Dawley rats (240–520 g; average 370 g) was used in this study.

Two experimental paradigms were carried out to assess the effects of CCL2 and TNF-α on neurons, which were either untreated or inflamed midaxonally in the neuritis model (paradigm 1) or acutely inflamed at the peripheral terminals following repeated nociceptive 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- to 8-mm length carefully separated from adjacent connective tissue. The nerve was loosely wrapped in a strip (~5 mm × 10 mm) of sterile gelfoam (Spongostan, Ferrosan, Denmark), saturated with ~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 nine animals), as well as at 3–6 days postneuritis (n = 66 neurons from 26 animals), which coincides with the peak physiological changes associated with the lesion (Dilley and Bove 2008b). Animals were anesthetized with 1.5 g/kg 25% w/v urethane intraperitoneally. Smaller doses of urethane were given intraperitoneally, as required to maintain the test agents with 1.5 g/kg 25% w/v urethane intraperitoneally. Smaller doses of urethane were given intraperitoneally, as required to maintain the rate of firing from those neurons that are already ongoing (Liu et al. 2002; Schafer et al. 2003). Behavioral studies have also reported the development of pain hypersensitivity following the administration of TNF-α (Opree and Kress 2000; Schafer 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 (TNFR), TNFR1 (George et al. 2005; Schafers et al. 2008; Shubayev and Myers 2000). To gain 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.

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 × 5 mm), which 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-ms duration, 2–10 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 below the knee by squeezing the periphery, using either fingers or blunt forceps. The loose properties of the skin were exploited to carefully discriminate cuta-
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) was diluted in a vehicle (0.1% BSA in 0.9% w/v saline) to give aliquots of 100 ng/ml, which 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, 1,000 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 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 1,000 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 (Leem and Bove 2002; Oh et al. 2001; Schafers et al. 2003; Sorkin et al. 1997; Sun et al. 2006; Wang et al. 2010; White et al. 2005). For paradigm 1, the vehicle (0.1% BSA saline; 200 μl) was applied to the well, and the baseline activity of each neuron was recorded for 15 min. At the end of the baseline period, the BSA saline was removed, and the test agent was applied for 15 min [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 for at least 20 min 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 × 5 × 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 been used successfully 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 five neuritis-treated animals were killed with an overdose of sodium pentobarbital. The ipsilateral L5 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 Microsystems, Wetzlar, Germany), mounted onto gelatin-coated slides, and fixed for 7 min with 4% paraformaldehyde. DRG sections were mounted sequentially on seven consecutive slides so that the eighth section was on the same slide as

![Image of schematic diagram of the experimental setup 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 lumbar (L)5 dorsal root ganglion (DRG) 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 3 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 (gray area). The nerve was electrically stimulated at the L5 DRG (Stim). C: a typical C-fiber axon with axonal mechanical sensitivity recorded from the neuritis group. The short horizontal lines above the trace represent 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.](http://jn.physiology.org/)

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the first section. A maximum of six sections was mounted on each slide.

Sciatic nerves were stained using the avidin-biotin detection system [Vectastain Elite Avidin Biotinylated Enzyme Complex (ABC) Kit, Vector Laboratories, Cambridgeshire, UK]. Endogenous peroxidase was blocked initially for 30 min using 3% H2O2 in absolute methanol. Non-specific binding sites were blocked for 30 min with either 2% normal goat serum (CCD2) or 2% normal rabbit serum (TNR1). 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 h at room temperature with either biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories) for CCR2-probed sciatic nerves or biotinylated rabbit anti-goat antibody (1:200, Vector Laboratories) for TNFR1-probed sciatic nerves. Tween-PBS rinses (0.05%; three times, 5 min each) were performed following primary and secondary antibody and ABC treatments. Sciatic nerve sections were developed with diaminobenzidin plus nickel chloride (Vector Laboratories) and were then dehydrated, cleared with xylene (Fisher Scientific, UK), and coverslipped using Histomount (Fisher Scientific). Sections were viewed under a light microscope (Leica Microsystems) and photographed.

DRGs were labeled using immunofluorescence. Non-specific sites were blocked for 30 min with either 2% normal goat serum (CCRD2) or 2% normal rabbit serum (TNFR1; Vector Laboratories). 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 h at room temperature with Alexa Fluor 488 goat anti-rabbit fluorescent antibody (1:200; Invitrogen, Glasgow, UK) for CCR2-probed DRGs or Alexa Fluor 488 rabbit anti-goat fluorescent antibody (1:200; Invitrogen) for TNFR1-probed DRGs. PBS rinses (three times, 5 min each) were performed following the primary and secondary antibody. DRGs were double-labeled with 4’,6-diamidino-2-phenylindole (DAPI; 1:5,000; Invitrogen) and then coverslipped using glycerol/PBS mounting medium (Citifluor, London, UK). Sections were viewed under a fluorescence microscope (Leica Microsystems) 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-min 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 with the baseline rate [50% represents the mean SD in firing rate during the entire baseline recording period (3 × 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-min epoch postapplication.

For immunohistochemistry, the number of cytokine receptor and DAPI-positive neuronal profiles was 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 were assessed for normality using Shapiro-Wilk tests to determine appropriate statistical analysis. All comparisons of proportions were made using two-tailed Fisher’s exact tests (when expected frequencies were <5) or χ² tests with Yates’ correction. Comparisons of conduction velocities, ongoing rates, time from experimental setup, and diameters of DRG cell bodies were performed among 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 are given as the median and the interquartile range (IQR) unless stated otherwise.

**RESULTS**

**Paradigm 1: Untreated Group**

CCL2 and TNF-α do not excite untreated neurons. Recordings from multiunit filaments revealed a significantly higher incidence of <7% in untreated neurons (seven of 102). Thirty-four neurons were examined for the effects of BSA saline, CCL2, or TNF-α. none of which was ongoing prior to application of the test agents. The median conduction velocity of these neurons was 0.89 m/s (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 (one of 10 neurons; P = 0.45 compared with BSA saline, Fisher’s exact test), firing at a rate of 0.2 spike/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 with the untreated group (7%; n = 7/102 neurons; P = 0.0005, Fisher’s 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 characterized 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/s (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

![](http://jn.physiology.org/2841/2011/106/12/403/fig2.jpg)

**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’s exact test). The total number of neurons sampled and the number that is responsive are shown.
the ongoing neurons [median = 0.56 m/s (IQR 0.37) compared with 0.84 m/s (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; $r = 0.47; P = 0.004$, ANOVA).

**CCL2 and TNF-α excite a subgroup of inflamed neurons.**

Table 1 summarizes the baseline firing rates of responsive and nonresponsive ongoing neurons and their rates postcytokine application.

None of the neurons tested postneuritis ($n = 0/21$ 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 with BSA saline, Fisher’s exact test; Fig. 2), which included 20% (two of 10) of silent and 33% (four of 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 with the ongoing nonresponders ($P = 0.02$, Mann-Whitney test; Table 1 and Fig. 3B). All CCL2 responsive neurons (six of six) increased their activity 0–5 min (i.e., during the first 5-min epoch) after CCL2 application. The effect was transient and began to recover toward baseline after 10 min of exposure (Fig. 3E).

Thirty percent of neurons ($n = 7/23$ neurons) responded to TNF-α application ($P = 0.009$ compared with BSA saline, Fisher’s exact test; Fig. 2), which included 22% (two of nine) of silent and 36% (five of 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 with the ongoing nonresponders ($P = 0.01$, Mann-Whitney test; Table 1 and Fig. 3D). All TNF-α responsive neurons increased their activity within 10 min after application. Similar to CCL2, the effect was transient and began to recover toward baseline after 10 min 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 with the nonresponders ($P = 0.006$, 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 with 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 MATERIALS AND METHODS, Electrophysiology: paradigm 1), it was not possible to test for AMS prior to cytokine or BSA saline application in the neuritis group.

**Table 1.** Rates of neuritis ongoing neurons pre- and postcytokine application

<table>
<thead>
<tr>
<th></th>
<th>Baseline:</th>
<th>Baseline:</th>
<th>Postcytokine:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonresponders</td>
<td>Responders</td>
<td>Responders</td>
</tr>
<tr>
<td>BSA saline</td>
<td>28.0 (24.6)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>CCL2</td>
<td>16.3 (36.0)</td>
<td>1.7 (2.7)*</td>
<td>11.2 (12.8)†</td>
</tr>
<tr>
<td>TNF-α</td>
<td>26.8 (33.6)</td>
<td>3.4 (4.6)*</td>
<td>10.0 (12.4)†</td>
</tr>
<tr>
<td>CCL2 and TNF-α</td>
<td>24.6 (24.4)</td>
<td>3.0 (3.0)*</td>
<td>10.0 (15.0)†</td>
</tr>
</tbody>
</table>

Baseline rates of ongoing responders are significantly slower than baseline rates of nonresponders for both cytokines (*$P < 0.05$, Mann-Whitney test). Postcytokine responder rates were increased significantly compared with baseline (†$P < 0.05$, Wilcoxon signed-rank test). Values are given as the median firing rate and interquartile range (IQR). n/a, Not applicable.
AMS was present in 14% (six of 43) of neurons tested. There was no difference in the proportions of neurons with AMS following BSA saline (n/11005, 1/11 neurons), CCL2 (n/11005, 3/22 neurons), or TNF-α (n/11005, 2/10) treatment (P/11005 0.86, 2×3 Fisher’s exact test).

Paradigm 2: Acute Inflammation at the Peripheral Terminals

In this group, the lower limb was repeatedly, noxiously stimulated while 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 was identified = 151 min (IQR 64)] compared with 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 was 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 nonresponsive ongoing neurons and their rates postcytokine application.

None of the neurons tested (n = 0/19 neurons; nine 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 with BSA saline, Fisher’s exact test), which included 33% (four of 12) of Table 3.

Table 2. Conduction velocities in the neuritis group

<table>
<thead>
<tr>
<th>Conduction Velocity (m/s; IQR)</th>
<th>All</th>
<th>Responders</th>
<th>Nonresponders</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA saline</td>
<td>0.73 (0.43)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>CCL2</td>
<td>0.75 (0.31)</td>
<td>0.94 (0.49)</td>
<td>0.70 (0.18)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.55 (0.26)</td>
<td>0.85 (0.37)*</td>
<td>0.46 (0.22)</td>
</tr>
<tr>
<td>CCL2 and TNF-α</td>
<td>0.68 (0.20)</td>
<td>0.85 (0.35)†</td>
<td>0.61 (0.16)</td>
</tr>
</tbody>
</table>

Values are given as the median conduction velocity and IQR.

Conduction velocities in the neuritis group

<table>
<thead>
<tr>
<th>Rate of Ongoing Neurons (Spikes/min; IQR)</th>
<th>Baseline: Nonresponders</th>
<th>Baseline: Responders</th>
<th>Postcytokine: Responders</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA saline</td>
<td>4.8 (10.5)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>CCL2</td>
<td>6.8 (11.2)</td>
<td>5.3 (6.6)</td>
<td>9.5 (10.9)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.2 (1.7)</td>
<td>1.8 (3.2)</td>
<td>4.8 (7.8)*</td>
</tr>
</tbody>
</table>

Post-TNF-α responder rates were increased significantly compared with baseline (P < 0.05, Wilcoxon signed-rank test). Values are given as the median firing rate and IQR.

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 is responsive are shown.

Fig. 5. A: cumulative plot showing the time from start of the experiment, at which neurons with baseline ongoing activity were identified in the untreated group with peripheral field searches (paradigm 2; n = 30 neurons) and the neuritis group (paradigm 1; n = 36 neurons). Time points represent 30-min bins. Dotted lines represent the time from setup, at which 50% of ongoing neurons was identified, which was significantly earlier in the neuritis (50% of neurons identified by 118 min) group compared with 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 postsetup) 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 with early neurons (P < 0.05, Fisher’s exact test). The total number of neurons sampled and the number that is responsive are shown.

Paradigm 2: Acute Inflammation at the Peripheral Terminals

In this group, the lower limb was repeatedly, noxiously stimulated while 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 was identified = 151 min (IQR 64)] compared with 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 was 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 nonresponsive ongoing neurons and their rates postcytokine application.

None of the neurons tested (n = 0/19 neurons; nine 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 with BSA saline, Fisher’s exact test), which included 33% (four of 12) of Table 3.

Table 3. Rates of ongoing neurons pre- and postcytokine application in paradigm 2

<table>
<thead>
<tr>
<th>Rate of Ongoing Neurons (Spikes/min; IQR)</th>
<th>Baseline: Nonresponders</th>
<th>Baseline: Responders</th>
<th>Postcytokine: Responders</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA saline</td>
<td>4.8 (10.5)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>CCL2</td>
<td>6.8 (11.2)</td>
<td>5.3 (6.6)</td>
<td>9.5 (10.9)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.2 (1.7)</td>
<td>1.8 (3.2)</td>
<td>4.8 (7.8)*</td>
</tr>
</tbody>
</table>

Post-TNF-α responder rates were increased significantly compared with baseline (P < 0.05, Wilcoxon signed-rank test). Values are given as the median firing rate and IQR.
silent and 20% (two of 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 spike/min (IQR 0.00). The baseline rate of the ongoing responders was similar to the baseline rate of the ongoing nonresponders (Table 3). Four of the six CCL2 responsive neurons increased their activity 0–5 min (i.e., during the first 5-min epoch) after CCL2 application. The effect was transient and began to recover toward baseline after 10 min of exposure.

Twenty-six percent of neurons (n = 5/19 neurons) responded to TNF-α application (P = 0.046 compared with BSA saline, Fisher’s exact test), which included 11% (one of nine) of silent and 40% (four of 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 spike/min. The baseline rate of the ongoing responders was similar to that of the ongoing nonresponders (P = 0.48, Mann-Whitney test; Table 3). All TNF-α responsive neurons increased their activity within 10 min after application. Similar to CCL2, the effect was transient and began to recover toward baseline after 10 min of exposure.

Receptive field location had no bearing on whether acutely inflamed neurons were responsive to cytokine treatment (three of six cutaneous vs. eight of 35 deep; P = 0.32, Fisher’s 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%; seven of 1,198 cell bodies; n = 5 animals), although the difference between groups was significant (P = 0.005, χ² test; Fig. 6, A, B, and E).

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 97/1,525 cell bodies; n = 5 animals; P = 0.41, χ² test; Fig. 6, C–E). The median diameter of the cell bodies that expressed TNFR1 was significantly smaller in the neuritis (32.4 μm) compared with the untreated group (41.0 μm; P = 0.03, Mann-Whitney test).

**CCR2 and TNFR1 are differentially expressed in sciatic nerve.** Immunolabeling 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. Immunolabeling for TNFRI in the untreated sciatic nerve showed clear axonal staining, as well as labeling of the endothelial cells and perineurium (Fig. 7, C and 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 with 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 depolarization 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 was similar (26–30%), yet the effects of CCL2 occurred at a concentration that was 10,000 times more concentrated compared with TNF-α. The lack of effect by TNF-α on untreated neurons contrasts from previously published findings (Leem & Bove 2002; Sorkin et al. 1997), 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 with 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 (Bove et al. 2003; Dilley et al. 2005; Eliav et al. 1999), 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 with 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 with the untreated group), although 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 altered subtly 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. Toward the end of each experiment, there was observable swelling of the periphery. Ongoing activity, which occurred later in these experiments compared with 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 been reported previously (Bove and Dilley 2010) and contrasts from the faster pattern of ongoing activity, which 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) was 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 with 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 Leem and Bove (2002) and Sorkin et al. (1997), suggesting that a number of their responsive neurons may have been acutely inflamed due to repeated noxious mechanical stimulation of the periphery.

AMS

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 with 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)], which 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 (TRP) 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 toward smaller cell bodies expressing the receptor. This shift indicates a move toward greater TNFR1 expression in inflamed C-fiber neurons. In addition, TNF-α may 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 Na1.8 (Czeschik et al. 2008) or inhibiting potassium channels (Liu et al. 2008). The modulation of Na1.8 by TNF-α is reported to occur via a p38 MAPK 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 TNFRs.

It is well established that nerve injury causes changes in the expression patterns of ion channels along peripheral axons (Black et al. 2004). In the neuritis model, it is known that the inflammation is sufficient to cause transcription changes in the DRG (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. In this mechanism, Na\(_{\text{v}1.8}\) and hyperpolarization-activated, cyclic nucleotide-gated channels, which are upregulated along DRG neurons following nerve injury, are of particular interest (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, suggest 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.

Summary

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.

ACKNOWLEDGMENTS

The authors thank Professor Bruce Lynn, Dr. Geoffrey Bove, and Dr. Jane Greening for their helpful comments on the manuscript.

GRANTS

Partial support for this study was through a grant from the Pain Relief Foundation (Registered Charity No: 277732) and a Wellcome Trust summer studentship, which supported T. Batty.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.R. and A.D. conception and design of research; N.R., T.B., and A.D. performed experiments; N.R. and A.D. analyzed data; N.R., T.B., and A.D. prepared figures; N.R., T.B., and A.D. drafted manuscript; N.R., T.B., and A.D. edited and revised manuscript; N.R., T.B., and A.D. approved final version of manuscript.

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