Inhibition of Microglial Activation Attenuates the Development but Not Existing Hypersensitivity in a Rat Model of Neuropathy

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ABSTRACT

Microglia, the intrinsic macrophages of the central nervous system, have previously been shown to be activated in the spinal cord in several rat mononeuropathy models. Activation of microglia and subsequent release of proinflammatory cytokines are known to play a role in inducing a behavioral hypersensitive state (hyperalgesia and allodynia) in these animals. The present study was undertaken to determine whether minocycline, an inhibitor of microglial activation, could attenuate both the development and existing mechanical allodynia and hyperalgesia in an L5 spinal nerve transection model of neuropathic pain. In a preventive paradigm (to study the effect on the development of hypersensitive behaviors), minocycline (10, 20, or 40 mg/kg intraperitoneally) was administered daily, beginning 1 h before nerve transection. This regimen produced a decrease in mechanical hyperalgesia and allodynia, with a maximum inhibitory effect observed at the dose of 20 and 40 mg/kg. The attenuation of the development of hyperalgesia and allodynia by minocycline was associated with an inhibitory action on microglial activation and suppression of proinflammatory cytokines at the L5 lumbar spinal cord of the nerve-injured animals. The effect of minocycline on existing allodynia was examined after its intraperitoneal administration initiated on day 5 post-L5 nerve transection. Although the postinjury administration of minocycline significantly inhibited microglial activation in neuropathic rats, it failed to attenuate existing hyperalgesia and allodynia. These data demonstrate that inhibition of microglial activation attenuated the development of behavioral hypersensitivity in a rat model of neuropathic pain but had no effect on the treatment of existing mechanical allodynia and hyperalgesia.

Chronic pain can occur after peripheral nerve injury, infection, or inflammation. To elucidate pathogenic factors, and thus to develop therapeutic strategies aimed at halting its progression, our laboratory investigates the neuropathology of this syndrome in search of putative culprits. Aside from the neuronal component, it seems that the non-neuronal cells of the CNS, such as glial cells, also have a role in the initiation and maintenance of persistent pain states (DeLeo and Yezierski, 2001; Watkins et al., 2001a,b; Raghavendra and DeLeo, 2003). Robust glial (both microglia and astrocytes) activation was observed at the lumbar spinal cord in various rodent models of chronic pain. These procedures include spinal nerve injury (Sweitzer et al., 2001; Raghavendra et al., 2002), peripheral inflammation (Watkins et al., 1997; Bao et al., 2001), peripheral nerve inflammation (Milligan et al., 2003), and chronic opioid treatment (Raghavendra et al., 2002). To a certain extent, glial activation is triggered secondarily to injury, but the triggering mechanism does not need to be lethal because minor alterations in ionic homeostasis or strong depolarization can lead to micro- and astrogliosis (Yrjanheikki et al., 1998; Raghavendra and DeLeo, 2003).

Although gliosis may sometimes be associated with beneficial effects, often gliosis seems to be deleterious (Vila et al., 2001). For instance, microglial cells, the resident macrophages in the brain, have the ability to react promptly in response to insults of various nature, quickly proliferate, become hypertrophic, and increase or express de novo a plethora of surface markers (Banati et al., 1993; Kreutzberg, 1996). Astrocytic activation usually follows the microglial response and maintains the state of synaptic differentiation initially associated with microglia (Kreutzberg, 1996). The multifunctional nature of activated microglia encompasses the up-regulation of cell surface markers such as the macrophage antigen complex-1 (Mac-1), phagocytosis, and the production of cytotoxic molecules, including reactive oxygen spe-
cies, nitric oxide, prostaglandins, and a variety of proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) (Hopkins and Rothwell, 1995). Many of these substances such as nitric oxide, prostaglandins, and proinflammatory cytokines released after microglial activation, play a role in development of chronic pain states (DeLeo and Colburn, 1999; Raghavendra and DeLeo, 2003).

Minocycline, a semisynthetic second-generation tetracycline, is an antibiotic that possesses superior penetration into the CNS via the brain-blood barrier (Aronson, 1980). Minocycline has emerged as a potent inhibitor of microglial activation and has no direct action on astroglia or neurons (Amin et al., 1996; Tikka and Koistinaho, 2001; Tikka et al., 2001a,b). Its anti-inflammatory property is completely separate from its antimicrobial action. It is an effective neuroprotective agent in experimental brain ischemia (Yrjanheikki et al., 1998), in the R6/2 mouse model of Huntington's disease (Chen et al., 2000), in traumatic brain injury (Sanchez Mejia et al., 1998), in the R6/2 mouse model of Huntington’s disease (1999). Briefly, rats were anesthetized with halothane in an O2 atmosphere for the Study of Pain guidelines (Covino et al., 1980). Approval all procedures in this study. Efforts were made to limit the number of animals necessary to achieve statistical significance as set forth by the International Society for the Study of Pain guidelines (Covino et al., 1980).

Surgery. The unilateral peripheral mononeuropathy was produced according to the method described earlier by Colburn et al. (1999). Briefly, rats were anesthetized with halothane in an O2 carrier (induction 4%, maintenance 2%). A small incision to the skin overlying L5-S1 was made followed by retraction of the paravertebral muscle from the vertebral transverse processes. The L6 transverse process was partially removed exposing the L4 and L5 spinal nerves. The L5 spinal nerve was identified, lifted slightly, and transected. The wound was irrigated with saline and closed in two layers with 3-0 polyester suture and surgical skin staples. Sham surgeries were identical except for the transection of the L5 nerve.

Behavioral Tests and Experimental Design. Mechanical hyperalgesia (decreased threshold to noxious stimuli) and mechanical allodynia (heightened response to normally non-noxious stimuli) were evaluated in sham and L5-spinal nerve-transected rats. Mechanical nociceptive thresholds were evaluated using an Analgesy-Meter (Ugo Basile, Comerio, Italy) as explained by Stein et al. (1990). Rats were gently held and incremental pressure (maximum 250 g) was applied onto the dorsal surface of the ipsilateral hind paw. Development of mechanical hyperalgesia was expressed as a relative decrease in nociceptive threshold to mechanical stimuli. Mechanical sensitivity to non-noxious stimuli was measured by applying 2- and 12-g von Frey filaments (Stoelting, Wood Dale, IL) on the plantar surface of the ipsilateral hind paw, as described previously (Colburn et al., 1999). The number of paw withdrawals in three sets of 10 stimulations/each set to this normally non-noxious stimulus determined mechanical allodynia. All the behavior was recorded before surgery, and on postnerve transection days 1, 3, 5, 7, and 10.

Prevention of Peripheral Nerve Injury-Induced Mechanical Hyperalgesia and Allodynia. In a systemic preventive paradigm 10, 20, or 40 mg/kg minocycline (Sigma-Aldrich, St. Louis, MO) or saline vehicle (n = 8/treatment) was administered by the i.p. route. Treatment was initiated 1 h before surgery and continued daily to day 10 post-transection. Mechanical hyperalgesia and allodynia were tested in the morning at 15 h post-treatment. The selection of minocycline doses and the rationale for the dosing regime is within the range at which it was reported to be a neuroprotectant in rodents (Yrjanheikki et al., 1999; Wu et al., 2002; Zhu et al., 2002; Zhang et al., 2003).

Attenuation of Established Peripheral Nerve Injury-Induced Mechanical Hyperalgesia and Allodynia. Minocycline (10, 20, or 40 mg/kg) was administered i.p. in an existing hyperalgesia/allodynia strategy (n = 8/treatment). Daily i.p. drug administration was initiated on day 5 post-transection and continued for the duration of the study. All injections were completed 15 h before behavioral testing.

Microglial Activation in Neuropathic Pain

Materials and Methods

Animals. Male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 175 to 200 g at the start of surgery, were used. The animals were allowed to habituate to the housing facilities for at least 1 week before the experiments were begun. Behavioral studies were carried out in a quiet room between the hours of 9:00 AM and 11:00 AM. The Institutional Animal Care and Use Committee at Dartmouth College approved all procedures in this study. Efforts were made to limit distress and to use the minimum number of animals necessary to achieve statistical significance as set forth by the International Society for the Study of Pain guidelines (Covino et al., 1980).

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Tissue Collection for Real-Time Reverse Transcription-Polymerase Chain Reaction, RNA Protection Assay, Western Blot, and ELISA Analysis. To quantify mRNA (for glial fibrillary acidic protein (GFAP), Mac-1, Toll-like receptor-4 (TLR4), and cytokine mRNA), and GFAP and cytokine protein levels, a separate group of rats was used. After behavioral testing on day 11 postsurgery, rats were euthanized by CO2 asphyxiation followed by immediate decapitation. An 18-gauge needle was inserted into the caudal end of the vertebral column and the spinal cord was expelled with ice-cold phosphate-buffered saline. The spinal cord was frozen immediately on dry ice and stored at −80°C until homogenization. The L5 lumbar spinal cord was isolated from the intact frozen cord at the time of mRNA and protein quantification. Total RNA was isolated from the L5 lumbar spinal cord by the TRIzol extraction method (Invitrogen, Carlsbad, CA). Total RNA was treated with DNase and the concentration of the total RNA was measured spectrophotometrically. Gene-specific primers and probe were designed and the selected sets of primers/probe were blasted against the GenBank to confirm their specie and gene specificity. The primers and probes selected for this experiment met the G+C content requirement and had a melting temperature of 60 and 70°C, respectively, and are shown in Table 1. The reverse transcription (RT) reaction was carried out in a 100-µl total reaction volume containing 10 µl of 10× RT buffer, 4 µl of 25× dNTPs, 5 µl of Multiscribe reverse transcriptase (50 U/µl), 31 µl of RNAase-free water, and 10 µg of DNase-treated total RNA in a 50-µl volume. The RT reaction was carried out at 25°C for 10 min, 37°C for 120 min, and 95°C for 5 min in the Mastercycler Gradient Eppendorf (Brinkmann Instruments, Westbury, NY). The iCycler IQ Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA) continually monitors the increase in fluorescence, which is directly proportional to the PCR product. The Real-Time PCR reactions were carried out in a total reaction volume of 25 µl containing the final concentration of 1.5 U of Platinum TaqDNA polymerase; 20 mM Tris HCl (pH 8.4); 50 mM KCl; 3 mM MgCl2; 200 µM dGTP, dCTP, and dATP; 400 µM dUTP; and 1 U of uracil DNA glycosylase; 900 nM forward and
Mechanical allodynia is reported as the average number of 2-g (top) and 12-g (bottom) von Frey filament-induced mechanical allodynia compared with saline treatment (Figs. 1 and 2) as assessed using an analgesymeter. Preemptive treatment with minocycline resulted in an overall statistically significant (p < 0.01 for 20 and 40 mg/kg) decrease in both 2-g (top) and 12-g (bottom) von Frey filament-induced mechanical allodynia compared with saline treatment. Preemptive treatment with minocycline resulted in an overall statistically significant (p < 0.05 for 10 mg/kg and p < 0.01 for 20 and 40 mg/kg) decrease in mechanical hyperalgesia compared with saline treatment. Mechanical hyperalgesia is reported as relative decrease in baseline threshold (in grams) ± S.E.M. (n = 8/treatment). Paw pressure threshold measured before surgery represents baseline responses. Horizontal line indicates the time frame of minocycline administration (i.e., day 0 to day 10).

### Table 1

<table>
<thead>
<tr>
<th>Genes</th>
<th>Accession No.</th>
<th>Primers/Probes</th>
<th>Primers and Probes&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>GAPDH</td>
<td>NM_01008</td>
<td>Forward primer</td>
<td>5'-CCCCCAAGTATCCCTTTGAC-3'</td>
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<td></td>
<td></td>
<td>Reverse primer</td>
<td>5'-TGACCCAGCTACTCCTTTGAC-3'</td>
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<td>Mac-1</td>
<td>U59801</td>
<td>5'-GCACCAAGTACATGCAC-3'</td>
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<tr>
<td>TLR4</td>
<td>NM_0191178</td>
<td>Taqman probe</td>
<td>5'-CCCGAGACAGCGCAGAGCA-3'</td>
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<td>GFAP</td>
<td>NM_017009</td>
<td>Taqman probe</td>
<td>5'-CCCGAGACAGCGCAGAGCA-3'</td>
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<sup>a</sup> The Taqman probe has a reporter fluorescent dye, FAM (6-carboxyfluorescein) at the 5' end and fluorescence dye quencher, TAMRA (6-carboxytetramethyl-rhodamine) at the 3' end.

**RNase Protection Assay.** Assessment of the temporal cytokine mRNA expression in the L5 lumbar spinal cord was performed using a ribonuclease protection assay technique. A MultiProbe RNase protection assay kit was used (BD Pharmingen, San Diego, CA). Total RNA (15 μg) was hybridized to 32P-labeled antisense RNA probes transcribed using the rat cytokine-1 multiprobe template set (including IL-1α/β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, TNF-α/β, and interferon-γ, L32, glyceraldehyde-3-phosphate dehydrogenase), resulting in double-stranded target RNA. After RNase digestion, protected RNA, and probe were resolved on a denaturing polyacrylamide gel and visualized by exposing the gel to a Phosphor screen (Amersham Biosciences Inc., Sunnyvale, CA). The Phosphor screen was scanned and fluorescence monitored. Because SYBR Green I only binds double-stranded DNA, the fluorescent signal decreases as the melting temperature is reached. Analysis of the melting curve allows the confirmation of specific PCR products.
using the Phosphorimager Typhoon 9410 (Amersham Biosciences Inc.). Bands were quantified using ImageQuant software. The value for the normalized quantity for each band was obtained by dividing with the L32 house keeping gene control. Individual mRNA concentration in terms of fold activation was calculated as the ratio of their expression compared with sham-operated animals, in which normal values were 1.

**Western Blot Analysis of GFAP.** Tissue homogenates of L5 lumbar spinal cord (prepared as explained under protein estimation by ELISA), and standard protein markers were subjected to SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose filters. Nonspecific binding was blocked by incubation with 1% bovine serum albumin. Primary antibody rabbit anti-rat GFAP (Dako, Carpinteria, CA) was diluted in a buffer containing 0.05% Tween 20. Blots were visualized using 3,3-diaminobenzidine and peroxidase-conjugated goat anti-rabbit immunoglobulin. Protein determinations were performed using BCA protein assay kit (Pierce Chemical, Rockford, IL).

**Cytokine Protein Estimation by ELISA.** Quantitative determination of IL-1β, IL-6, and TNF-α protein was performed on the L5 spinal cord harvested on day 11 postsurgery. Tissue homogenization was prepared as described previously (Raghavendra et al., 2002). In brief, weighed sections of L5 spinal cord were homogenized in homogenization buffer consisting of a protease inhibitor (Roche Diagnostics, Mannheim, Germany) using Power Gen 125 tissue tearer (Fisher Scientific Co., Suwanee, GA). Samples were spun at 20,000g for 30 min at 4°C. Supernatant was aliquoted and stored at −80°C for future protein quantification. IL-1β, TNF-α (R & D Systems, Minneapolis, MN), and IL-6 (BioSource International, Camarillo, CA) protein concentrations were determined using the quantitative sandwich enzyme immunoassay according to the manufacturer’s protocol. IL-1β, IL-6, and TNF-α protein quantification was determined by comparing samples to the standard curve generated from the respective kits.

**Statistical Analysis.** Values are expressed as means ± S.E.M. Comparisons between groups were performed using analysis of variance for repeated measurements followed by Tukey-Kramer multiple comparisons test using InStat (GraphPad Software Inc., San Diego, CA). P < 0.05 was considered significant.

**Results**

**Effect of Preemptive and Postsurgery Treatment of Minocycline on L5 Spinal Nerve Transaction-Induced Mechanical Allodynia and Hyperalgesia.** Before L5 spinal nerve transection, all groups exhibited comparable baseline thresholds to noxious mechanical stimuli (p > 0.5) (145 ± 14 g). Non-noxious mechanical stimuli (2 and 12 g of von Frey filament) did not produce any paw withdrawal response (allodynia) in animals before L5 nerve transection. As in our previous study (Raghavendra et al., 2002), L5 spinal nerve transection produced mechanical allodynia and hyperalgesia, whereas sham surgery produced no significant behavioral hypersensitivity. Minocycline (10, 20, or 40 mg/kg i.p.) administration initiated 1 h before surgery (preemptive) attenuated the development of mechanical allodynia and hyperalgesia in L5 spinal nerve-transected rats. An overall (across the entire study period) statistically significant reduction in both mechanical allodynia and hyperalgesia (p < 0.05 for 10 mg/kg and p < 0.01 for 20 and 40 mg/kg) was observed with the minocycline-treated group compared with saline-treated (control) animals (Figs. 1 and 2). Preemptive treatment with 20 or 40 mg/kg minocycline showed a similar magnitude of behavioral hypersensitivity attenuation. On the contrary, initiation of minocycline treatment (10–40 mg/kg i.p.) to rats after day 5 of nerve injury did not show any significant difference in the mechanical allodynia (p > 0.5) and hyperalgesia (p > 0.5) compared with vehicle-treated nerve-injured rats (Figs. 3 and 4).

**Minocycline Treatment Inhibited the Microglial Activation in L5 Nerve-Transected Rats.** After L5 nerve transection, mRNA for Mac-1, TLR4, and GFAP increased significantly (p < 0.01) compared with sham-operated animals. Initiation of minocycline (40 mg/kg i.p.) administration preemptively or day 5 after postinjury inhibited (p < 0.01) the nerve-induced expression of Mac-1 and TLR4. Conversely, only preemptive treatment of minocycline (40 mg/kg i.p.) suppressed GFAP (p < 0.05), whereas postinjury administration failed to effect the GFAP expression at L5 lumbar spinal cord after nerve injury (Table 2). Similarly, quantification of GFAP protein by Western blot analysis also revealed that only preemptive minocycline treatment suppressed (p < 0.01) the astrogliosis at the lumbar spinal cord of L5 nerve-transected rats (Fig. 5).

**Minocycline Treatment Suppressed Proinflammatory Cytokines Expression after L5 Nerve Transaction.** Minocycline attenuation of behavioral hyperalgesia and allodynia after nerve injury could be due to its inhibitory effect...
Fig. 4. Effect of minocycline administration on the existing mechanical hyperalgesia in nerve-injured rats. Minocycline (10, 20, or 40 mg/kg i.p.) or saline administration was initiated on day 5 post-L5 spinal nerve transection. Treatment with minocycline (10, 20, and 40 mg/kg i.p.) to L5 nerve-transected rats did not reverse the existing mechanical hyperalgesia. Mechanical hyperalgesia is reported as relative decrease in baseline threshold (in grams) ± S.E.M. (n = 8/treatment). Paw pressure threshold measured before surgery represents baseline responses. Horizontal line indicates the time frame of minocycline administration (i.e., postinjury day 5 to day 10).

Fig. 5. GFAP protein levels in L5 lumbar spinal cord from sham-operated (A), saline-treated L5 spinal nerve-transected (B), and preemptive (C) and postinjury (D) minocycline-treated (40 mg/kg) L5 spinal nerve-transected rats. Immunoreactive bands (50 kDa) detected by Western blot analysis (top) were evaluated with densitometry. GFAP levels were expressed as percentage of the sham surgery group. Values are mean ± S.E.M. (n = 4). *p < 0.01 versus sham; †, p < 0.01 versus saline (B)-treated rats.

Table 2
Relative expression of mRNA of Mac-1, TLR-4 (markers for microglia), and GFAP (marker for astrogliosis) in the sham-operated and L5 lumbar spinal cord of L5 nerve-transected rats (saline or minocycline treated)
The mRNA levels were normalized to the corresponding GAPDH (housekeeping gene) level for each sample to adjust for uncontrolled variability between samples. Values are mean ± S.E.M. (n = 4).

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Saline</th>
<th>Minocycline (40 mg/kg) Treatment</th>
<th>Preemptive</th>
<th>Postinjury</th>
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<tr>
<td>Mac-1</td>
<td>2.9 ± 0.3</td>
<td>12.6 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.9 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>TLR-4</td>
<td>0.4 ± 0.02</td>
<td>1.4 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.6 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.7 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>GFAP</td>
<td>111.2 ± 3.7</td>
<td>290.6 ± 12.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>147 ± 7.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>278.5 ± 14.1&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup>p < 0.01 vs. sham; <sup>b</sup>p < 0.01; and <sup>c</sup>p < 0.05 vs. saline-treated group.

on microglia and subsequent production of proinflammatory cytokines. To test this hypothesis, we studied the level of cytokines in the L5 lumbar spinal cord of minocycline (40 mg/kg i.p.) treated rats. Both mRNA (by RNA protection assay) and protein analysis (by ELISA) revealed that a preemptive and postsurgery treatment strategy of minocycline inhibited cytokines at various degrees. Both treatment schedules attenuated (p < 0.01 for preemptive and p < 0.05 for postsurgery treatment) the production of IL-1β and TNF-α compared with vehicle-treated animals. However, the degree of inhibition of IL-1β and TNF-α by postsurgery minocycline treatment was less than the preemptive treatment. Also, preemptive minocycline treatment attenuated IL-6 expression (p < 0.01) compared with the vehicle-treated group, but the postsurgery treatment had no effect (Tables 3 and 4; Fig. 6).

Discussion
This study used minocycline, a microglial activation inhibitor, to selectively assess the role of microglial activation in the etiology of peripheral nerve injury-induced mechanical hypersensitivity in rats. Minocycline was effective in reducing mechanical allodynia and hyperalgesia in a preventive strategy but not on existing behavioral hypersensitivity paradigms. Reversal of this behavioral hypersensitivity associated with nerve injury by minocycline was associated with its
ability to suppress microglial activation and inflammatory immune responses at the lumbar spinal cord.

In animal models of neuropathic pain, including the L5 spinal nerve transection model, both astrocytes and microglia are activated, which may contribute to the development of chronic pain states (DeLeo and Yezierski, 2001; Watkins et al., 2001a,b; Raghavendra and DeLeo, 2003). Although the modulators or inhibitors of microglia and astroglia are shown to reduce the severity of pain in animal models of chronic pain (Meller et al., 1994; Sweitzer et al., 2001; Raghavendra et al., 2003), it is not clear which cell type has a major role in the initiation and maintenance of chronic pain state. In vivo and in vitro studies showed that minocycline provides neuroprotection by inhibiting microglial activation under various pathological conditions without affecting neurons, astroglia, or oligodendroglial progenitors (Amin et al., 1996; Yrjanheikki et al., 1998; Du et al., 2001; Tikka and Koistinaho, 2001; Tikka et al., 2001a,b; Wu et al., 2002; Zhang et al., 2003). In the present study, when minocycline administration was started at the time of L5 nerve transection (preemptive treatment) it reversed allosthenia and hyperalgesia, which is associated with its ability to suppress microgliosis. Alternatively, initiation of minocycline treatment at day 5 after nerve transection (treatment of existing hypersensitivity) failed to attenuate the behavioral hyperalgesia and allodynia, although it inhibited microglial activation. Minocycline may have actions on recruited monocytes or lymphocytes (Kloppenburg et al., 1996). However, in our previous studies we showed that such leukocyte trafficking was observed beginning at day 3 after L5 nerve transection (Sweitzer et al., 2002). In the present study, preemptive minocycline attenuated L5 nerve injury-induced allosthenia and hyperalgesia from day 1 after injury. This suggests that the antiallodynic and antihyperalgesic action of minocycline at this point was mediated by direct inhibitory effect on glial cells.

The following assumptions can be drawn by the above-mentioned observation. First, activated microglia contribute to the initiation of pathological pain responses after L5 nerve injury. Microglia is the early responding glial cells in the CNS after injury, and products released from activated microglia lead to astroglial activation which in turn maintains long-term pathological state (Svensson et al., 1993; Kreutzberg, 1996; Popovich et al., 1997). In the recently concluded study using the identical animal model of neuropathic pain, we observed that messenger levels for Mac-1, TLR4, and CD14 (all microglial markers) were up-regulated as early as 4 h after nerve injury, which is also associated with development of sensory hypersensitivity. Conversely, significant up-regulation of mRNA for GFAP was observed only after post-transection day 4 (F. Y. Tanga, V. Raghavendra, and J. A. DeLeo, manuscript submitted for publication). Similar observations were reported after injury to spinal cord or peripheral nerves (Svensson et al., 1993; Kreutzberg, 1996; Eriksson et al., 1997; Popovich et al., 1997; Gilmore and Kane, 1998), where microglia but not astrocytes proliferate, become hypertrophic, and express several marker molecules and mediators that in turn activate astrocytes. This suggests preemptive minocycline treatment induced-inhibition of hyperalgesia/allodynia and astrogliosis is the consequence of early microglial activation inhibition.

Second, astroglial activation contributes to the maintenance of pathological pain states. Reactive astrocytes maintain the state of synaptic differentiation initially associated with the microglial response. After nerve injury, the process of hypertrophic astrocytes takes over the perineuronal position and replaces the microglia (Kreutzberg, 1996; Eriksson et al., 1997; Popovich et al., 1997). Postoperative administration of inhibitors or modulators of astrocytes attenuate chronic pain states, induced by nerve injury or inflammogens (Meller et al., 1994; Watkins et al., 1997; Sweitzer et al., 2001; Raghavendra et al., 2003). Because minocycline does not inhibit activated astrocytes, it fails to reverse the existing hyperalgesia and allosthenia in nerve-injured rats.

The release of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α from activated glia in the CNS contributes to the development of central sensitization associated with peripheral nerve injury or inflammation (DeLeo and Yezierski, 2001). Both microglia and astroglia have the ability to release proinflammatory cytokines in response to nerve injury and inflammation (Aloisi, 2001; Dong and Benveniste, 2001). In the present study, apart from suppression of glial activation, preemptive treatment of minocycline also suppressed inflammatory immune responses at L5 lumbar spinal cord in nerve-injured rats. This suggests anti-allodynic and antihyperalgesic actions of minocycline are attributed to their ability to suppress central proinflammatory immune responses. However, administration of minocycline postsurgery did decrease IL-1β and TNF-α level, but failed to suppress IL-6 level. Also, the magnitude of inhibition of IL-1β
ators such as IL-1
neuroprotective effect of minocycline is presumably due to
chronic brain insults (Yrjanheikki et al., 1999; Tikka and
microglial activation and subsequent neuroprotective effect
kines suggests minocycline may act at a transcriptional level
astroglial response, because these cells also contribute to the
postoperative minocycline treatment to inhibit an activated
was significantly less than its preemptive treatment. This
peripheral nerve transection.

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References
Amin AR, Aturr MG, Thakker GD, Patel RN, Patel IR, and
Assoc 176:1061–1068.
microglia. Glia 7:111–118.
Adjuvant-induced arthritis: IL-1β, IL-6 and TNF-α are up-regulated in the spinal
Chen M, Ona VO, Li M, Ferrante RJ, Fink KB, Zhu S, Bian J, Guo L, Farrell LA,
Hersch SM, et al. (2000) Minocycline inhibits caspase-1 and caspase-3 expression
and delays mortality in a transgenic mouse model of Huntington disease. Nat Med
6:797–801.
Colburn RW, Rickman AJ, and DeLeo JA (1999) The effect of site and type of
nerve injury on spinal glial activation and neuropathic pain behavior. Exp Neurol
157:289–304.
Covace BG, Duhner R, Gybels J, Losterlitz HW, Lieskind JC, Sternbach RA,
Vlyvick L, Yamamura H, and Zimmerman M (1980) Ethical standards for investi-
gations of experimental pain in animals. Pain 9:141–143.
DeLeo JA and Yenizersky RP (2001) The role of neuroinflammation and neuroimmune
Du Y, Ma Z, Lin S, Dodel RC, Gao F, Bales TR, Tripathou LC, Czerny E, Perry KW,
Nelson DL, et al. (2001) Minocycline prevents nigrostriatal dopaminergic neuro-
degeneration in the MPTP model of Parkinson’s disease. Proc Natl Acad Sci USA
98:14689–14674.
analysis of the glial cell reaction in primary sensory termination areas following
sciatric nerve injury and treatment with nerve growth factor in the adult rat. Exp
Brain Res 114:393–404.
Gilmore SA and Kane CJM (1998) Microglia, but not astrocytes, react to sciatic nerve
Kloogerman B, Brinkman RM, Cui ZW, Hillen MH, Miltenburg AMM, Daha MR,
Breedveld FC, Dijkman BA, and Verweij CL (1996) The tetracycline derivative
minocycline differentially affects cytokine production by monocytes and T lym-
Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. Trends
Meller ST, Dysktra C, Graybycki D, Murphy S, and Gehbhart GF (1994) The possible
role of IL-6 in nociceptive processing and hyperalgesia in the spinal cord of the rat.
Neuropharmacology 33:1471–1478.
Milligan ED, O’Connor KA, Nguyen KT, Armstrong CB, Twining C, Gaykema RPA,
glycoprotein gp 120 induces enhanced pain states mediated by spinal cord
Milligan ED, Twining C, Chaucer M, Biedenkapp J, O’Connor KA, Poole S, Tracey K,
Raghavendra V and DeLeo JA (2003) The role of astrocytes and microglia in persistent
Raghavendra V, Rutkowski MD, and DeLeo JA (2002) The role of spinal neuroimmu-
none activation in morphine tolerance/hyperalgesia in neuropathic and sham
Raghavendra V, Tanga F, Rutkowski MD, and DeLeo JA (2003) Anti-hyperalgesic and
morphine sparing actions of propofol/fenefollow following nerve injury in rats:
mechanisms of suppression of spinal proinflammatory cytokines. Pain, in press.
Sanchez Mejia RO, Ona VO, Li M, and Friedlander RM (2001) Minocycline reduces
traumatic brain injury-mediated caspase-1 activation, tissue damage and neuro-
Stein C, Gramsch C, and Herr A (1990) Intrinsic mechanisms of antinociception in
1298.
36:499–506.
peripheral nerve injury induces leukocyte trafficking into the central nervous
Swietzer SM, Schubert P, and DeLeo JA (2001) Propofol/fenefollow, a glial modulating
agent, exhibits anti-allodynic properties in a rat model of neuropathic pain. J
Pharmacol Exp Ther 297:1210–1217.
cline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhib-
Tikka TM and Koistinaho JE (2001) Minocycline provides neuroprotection against
N-methyl-D-aspartate neurotoxicity by inhibiting microglia. J Immunol 166:7527–
7533.
Tikka T, Usenius T, Tenhunen M, Keinanen R, and Koistinaho J (2001b) Tetracy-
lcline derivatives and ceftizoxime, a cephalosporin antibiotic, protect neurons
Vila M, Jackson Lewis V, Guegan C, Wu DC, Teismann P, Chiu DK, Tieu K, and
Neurotol 14:483–489.
involvement of spinal cord glia in subcutaneous formalin induced hyperalgesia in
Watkins LR, Milligan RD, and Maier SF (2001a) Spinal cord glia: new players in
Watkins LR, Milligan RD, and Maier SF (2001b) Glial activation: a driving force for
Wu DC, Jackson-Levis V, Vila M, Tieu K, Teismann P, Vadecht C, Chiu D, Ischiro-
poulos H, and Przedborski S (2001) Blockade of microglial activation is neuropro-
tective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of
cyclines inhibits microglial activation and are neuroprotective in global brain isch-
Zhang SC, Goets JD, and Duncan ID (2003) Suppression of activated microglia
promotes survival and function of transplanted oligodendroglial progenitors. Glia
Zhu X and Stavrevskaia I, Druda Z, Kim BYS, Ona V, Li M, Sarang S, Liu AS,

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