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

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Abbreviations: IL: interleukin; TNF: tumor necrosis factor; GFAP: glial fibrillary acidic protein; ICC: immunocytochemistry; RPA: RNA protection assay; GAPDH: glyceraldehydes-3-phosphate; Mac-1: macrophage antigen complex-1; TLR-4: Toll like receptor 4

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Abstract

Microglia, the intrinsic macrophages of the CNS, 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 hr prior to 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 post-injury 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 appears 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 and 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 (Bao et al., 2001; Watkins et al., 1997), 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 since 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 appears 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 natures, 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 species, nitric oxide (NO), prostaglandins (PGs) and a variety of proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor- α (TNF- α) (Hopkins and Rothwell, 1995). Many of these substances such as NO, PGs and proinflammatory cytokines released following microglial activation, play a role in development of chronic pain states (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 and 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., 2001), and in the 6-hydroxydopamine and 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Wu et al., 2002). In the present study, we report that in a rat model of neuropathic pain induced by L5 spinal nerve transection, systemic administration of minocycline: 1) Reversed the development of mechanical allodynia and hyperalgesia when the treatment started at the time of nerve transection, but not when the treatment started day 5 after nerve injury; (2) Prevented microglial and astroglial activation in the pre-emptive treatment, but prevented only microglial activation when the treatment started day 5 after nerve injury; and (3) Reversed hyperalgesia and allodynia which was associated with minocycline's ability to inhibit the production of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α in the L5 lumbar spinal cord.

Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing 175g - 200g 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 0900 to 1100. 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).

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 O_2 carrier (induction 4%, maintenance 2%). A small incision to the skin overlying L5-S1 was made followed by retraction of the paravertebral musculature 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 250g) 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 gm 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 3 sets of 10 stimulations/each set to this normally non-noxious stimulus determined mechanical allodynia. All the behavior was recorded before surgery, and on post- nerve 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, MO, USA) or saline vehicle (n=8/treatment) was administered by the i.p. route. Treatment was initiated 1 hr prior to 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; Zhu et al., 2002; Wu et al., 2002; Zhang et al., 2003).

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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 prior to behavioral testing.

Tissue Collection for Real Time RT-PCR, RPA, Western Blot and ELISA Analysis

To quantify mRNA (for Glial Fibrillary Acidic Protein (*GFAP*), macrophage antigen complex-1 (*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 post-surgery, rats were euthanized by CO_2 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 -80^0C 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 Corp. Carlsbad, CA, USA).

Real Time RT-PCR

The DNA sequence of rat Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), GFAP, Mac-1 and TLR4 were obtained from the GeneBank. Only the open reading frame or CDS, coding for proteins were chosen from these sequences. Gene specific primers and probe were designed and the selected sets of primers/probe

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were blasted against the GeneBank 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 (T_m) of 60°C 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 10X RT buffer, 4 µl of 25X dNTPs, 5 µl of Multiscribe reverse transcriptase (50 U/µL), 31 µl of RNAse free water and 10 µg of DNase treated total RNA in a 50µl 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 Instrument Inc. NY, USA). The iCycler IQ™ Multicolor Real-Time PCR detection system (Bio-Rad, CA, USA) 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 1.5U of Platinum Taq DNA polymerase, 20 mM Tris HCl (PH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 µM dGTP, dCTP, and dATP, 400 µM of dUTP and 1 U of UDG (Uracyl DNA glycosylase), 900 nM of forward and reverse primers, 300 nM of Taqman probe, 5 µL of a 10-fold dilution of cDNA (50 ng) from the RT step. Relative standard curves were generated by plotting the threshold value (C_T) versus the log of the amount of total cDNA added to the reaction (1 Pg to 10000 Pg) according to the protocol described in Bulletin #2 (Applied Biosystems, CA) and used to compare the relative amount of target genes from control to sham groups and L5 nerve transected animals. Calculation of C_T, standard curve preparation and quantification of mRNA in the samples were performed by the software provided with the iCycler system. The melting curve analysis was used to determine the specificity of each primer set. The reaction temperature was raised slowly to the melting temperature of the duplex DNA product and fluorescence monitored. Since 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.

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 (RPAse) kit was used (PharMingen, San Diego, CA). Total RNA (15 μg) was hybridized to ^{32}P-labeled anti-sense RNA probes transcribed using the rat cytokine-1 (rCK-1) multi-probe template set (including IL-1α/β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, TNF-α/β and IFN-γ, L32, GAPDH) 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 (Molecular Dynamics, Sunnyvale, CA, USA). The Phosphor screen was scanned using the Phosphor Imager Typhoon 9410 (Molecular Dynamics, Sunnyvale, CA, USA). 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-

PAGE. Separated proteins were transferred to nitro-cellulose filters. Non-specific binding was blocked by incubation with 1% bovine serum albumin. Primary antibody rabbit anti-rat GFAP (Dako Corp., Carpinteria, CA) was diluted in a buffer containing 0.05% Tween-20. Blots were visualized using 3, 5 - diaminobenzidine and peroxidase conjugated goat anti-rabbit immunoglobin. Protein determinations were performed using BCA^{TM} Protein Assay Kit (Pierce, 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 post surgery. Tissue homogenization was prepared as previously described (Raghavendra et al., 2002). In brief, weighed sections of L5 spinal cord were homogenized in homogenization buffer consisting of a protease inhibitor (Boehringer Mannheim, Germany) using Power Gen 125 tissue tearer (Fisher Scientific, Suwanee, GA, USA). Samples were spun at 20,000 g for 30 min at 4^0C. Supernatant was aliquoted and stored at -80^0C for future protein quantification. IL-1β, TNF-α (R&D systems, Minneapolis, MN, USA) and IL-6 (Biosource, Camarillo, CA, USA) protein concentrations were determined utilizing 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 (ANOVA) for repeated measurements followed

by Tukey-Kramer multiple comparisons test using InStat (GraphPad software, Inc, CA, USA). $P < 0.05$ were considered significant.

Results

Effect of pre-emptive and post-surgery 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 gm). Non noxious mechanical stimuli (2 and 12 gm of van-Frey filament) did not produce any paw withdrawal response (allodynia) in animals prior to L5 nerve transection. As in our previous study (Raghavendra et al., 2002), L5-spinal nerve transection produced mechanical allodynia and hyperalgesia, where as sham surgery produced no significant behavioral hypersensitivity. Minocycline (10, 20 or 40 mg/kg, i.p.) administration initiated 1 hr prior to surgery (pre-emptive) 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 (Fig 1 and Fig 3). Pre-emptive treatment with 20 or 40 mg/kg of minocycline showed a similar magnitude of behavioral hypersensitivity attenuation. On the contrary, initiation of minocycline treatment (10 to 40 mg/kg, ip) 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 to vehicle treated nerve injured rats (Fig 2 and Fig 4).

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Minocycline treatment inhibited the microglial activation in L5-nerve transected rats.

Following L5 nerve transection, mRNA for *Mac-1*, *TLR-4* and *GFAP* increased significantly ($p<0.01$) compared to sham operated animals. Initiation of minocycline (40 mg/kg, i.p.) administration pre-emptively or day 5 after post-injury inhibited ($p<0.01$) the nerve-induced expression of *Mac-1* and *TLR-4*. Conversely, only pre-emptive treatment of minocycline (40 mg/kg, i.p.,) suppressed *GFAP* ($p<0.05$), whereas post-injury administration failed to effect the *GFAP* expression at L5 lumbar spinal cord following nerve injury (Table 2). Similarly, quantification of GFAP protein by western blot analysis also revealed that only pre-emptive 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 following L5 nerve transaction.

Minocycline attenuation of behavioral hyperalgesia and allodynia following nerve injury could be due to its inhibitory effect 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 RPA) and protein analysis (by ELISA) revealed that a pre-emptive and post-surgery treatment strategy of minocycline inhibited cytokines at various degrees. Both treatment schedules attenuated ($p<0.01$ for pre-emptive, and $p<0.05$ for post-injury treatment) the production of IL-1β and TNF-α compared to vehicle treated animals. However, the degree of inhibition of IL-1β and TNF-α by post-surgery

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minocycline treatment was less than the pre-emptive treatment. Also, pre-emptive minocycline treatment attenuated IL-6 expression ($p<0.01$) compared to the vehicle treated group, but the post-surgery treatment had no effect (Table 3 and 4, and 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 and 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 and 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 (pre-emptive treatment) it reversed allodynia 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 earlier studies we showed that such leukocyte trafficking was observed beginning at day 3 following L5 nerve transection (Sweitzer et al., 2002). In the present study, pre-emptive minocycline attenuated L5 nerve injury-induced allodynia and hyperalgesia from day 1 after injury. This suggests that the anti-allodynic and anti-hyperalgesic action of minocycline at this point was mediated by direct inhibitory effect on glial cells.

The following assumptions can be drawn by the above observation:

1) *Activated microglia contribute to the initiation of pathological pain responses following L5 nerve injury*. Microglia is the early responding glial cells in the CNS following 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 following 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 (submitted). Similar observations was 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 which in turn activate astrocytes. This suggests pre-emptive minocycline treatment induced-inhibition of hyperalgesia/allodynia and astrogliosis is the consequence of early microglial activation inhibition.

2) *Astroglial activation contributes to the maintenance of pathological pain states.* Reactive astrocytes maintain the state of synaptic differentiation initially associated with the microglial response. Following 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). Post-operative administration of inhibitors or modulators of astrocytes attenuate chronic pain states, induced by nerve injury or inflammogens (Meller et al., 1994; Sweitzer et al., 2001; Watkins et al., 1997; Raghavendra et al., 2003a). Since minocycline does not inhibit activated astrocytes, it fails to reverse the existing hyperalgesia and allodynia 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, pre-emptive treatment of minocycline also suppressed inflammatory immune responses at L5 lumbar spinal cord in nerve injured rats. This suggests anti-allodynic and anti-hyperalgesic actions of minocycline are attributed to their ability to suppress central proinflammatory immune responses. However, administration of minocycline post-surgery did decrease IL-1β and TNF-α level, but failed to suppress IL-6 level. Also, the magnitude of inhibition of IL-1β and TNF-α by post-operative administration of minocycline was significantly less than its pre-emptive treatment. This varying effect of minocycline might reflect the inability of post-operative minocycline treatment to

inhibit an activated astroglial response, as these cells also contribute to the production of proinflammatory cytokines following nerve injury.

Inhibition of mRNA expression for proinflammatory cytokines suggests minocycline may act at a transcriptional level to inhibit proinflammatory cytokines release. Inhibition of microglial activation and subsequent neuroprotective effect of minocycline has also been demonstrated in *in vitro* (Tikka et al., 2001b), and in other experimental models of acute and chronic brain insults (Yrjanheikki et al., 1999; Tikka and Koistinaho, 2001; Tikka et al., 2001a). These studies reported neuro-protective effect of minocycline is presumably due to the blockade of p38 mitogen-activated protein kinase (MAPK), caspase-1, and caspase-3 in microglia (Tikka and Koistinaho, 2001; Zhu et al., 2002). Activation of these cellular events are known to enhance the production of proinflammatory mediators such as IL-1β, IL-6 and TNF-α.

Minocycline is, to our knowledge, the first non-toxic drug with proven human safety record shown to selectively inhibit microglial activation in the CNS. As demonstrated in this study, minocycline attenuates the development of hyperalgesia and allodynia in the rat model of neuropathic pain. Given its safety in chronic disease, its oral bioavailability and its ability to cross the blood-brain barrier, minocycline could be evaluated for its effectiveness in human trials for the prevention of neuropathic pain in diabetes, human immunodeficiency virus (HIV) infection and traumatic nerve injury. Overall, this study not only demonstrates the effectiveness of minocycline in preventing nerve injury-induced neuropathic pain, but also showed the distinct role played by microglia in regulating the induction of a chronic pain state induced by peripheral nerve transection.

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Figure Legend

Fig. 1. Effect of pre-emptive administration of minocycline on the development of nerve injury-induced mechanical allodynia. Minocycline (10, 20 or 40 mg/kg, i.p.) or saline administration was initiated 1 hr prior to the nerve transection. Development of mechanical allodynia was recorded from post injury day 1 to day 10. Pre-emptive 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 both 2g (upper panel) and 12g (lower panel) von Frey filament-induced mechanical allodynia compared with saline treatment. Mechanical allodynia is reported as the average number of paw withdrawals out of 30 ± S.E.M. (n = 8/treatment). Horizontal line indicates the time frame of minocycline administration (i.e., day 0 to day 10). Day 0 mechanical allodynia represents baseline pre injury responses.

Fig. 2. Effect of post-injury administration of minocycline on the existing mechanical allodynia 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 decrease 2g (upper panel) and 12g (lower panel) von Frey filament-induced mechanical allodynia compared with saline treatment. Mechanical allodynia is reported as the average number of paw withdrawals out of 30 ± S.E.M. (n = 8/treatment). Horizontal line indicates the time frame of minocycline administration (i.e., post-injury day 5 to day 10). Day 0 mechanical allodynia represents baseline pre-transection responses.

Fig. 3. Effect of pre-emptive administration of minocycline on the development of nerve injury-induced mechanical hyperalgesia. Minocycline (10, 20 or 40 mg/kg, i.p.) or saline administration was initiated 1 h prior to the nerve transection. Development of mechanical hyperalgesia was recorded from post injury day 1 to day 10 using an analgesymeter. Pre-emptive 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 prior to surgery represents baseline responses. Horizontal line indicates the time frame of minocycline administration (i.e., day 0 to day 10).

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 prior to surgery represents baseline responses. Horizontal line indicates the time frame of minocycline administration (i.e., post-injury 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 pre-emptive (C) and post-injury (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). *$p<0.01$ vs. sham, and $^{\dagger}p<0.01$ vs. saline (B) treated rats.

Fig. 6. Representative RNase protection assay demonstrating cytokine mRNA expression in L5 lumbar spinal cord of sham operated (A), saline treated L5 spinal nerve transected (B), and pre-emptive (C) and post-injury (D) minocycline treated (40 mg/kg) L5 spinal nerve transected rats. (n = 4/group).

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Table 1. Primers and Taqman probe sequence for the rat genes characterized in this experiment.

Genes	Accession #	Primers/Probes	Primers and Probes*
GAPDH	NM_01008	forward primer	5' CCCCCAATGTATCCGTTGTG 3'
		reverse Primer	5' TAGCCCAGGATGCCCTTTAGT 3'
		taqman Probe	5' TGCCGCCTGGAGAAACCTGCC 3'
Mac-1	U59801	forward primer	5' CTGCCTCAGGGATCCGTAAAG 3'
		reverse Primer	5 'CCTCTGCCTCAGGAATGACATC 3'
		taqman Probe	5' CCCGGGACAATGCCGCGAA 3'
TLR4	NM_0191178	forward primer	5' GATTGCTCAGACATGGCAGTTTC 3'
		reverse Primer	5' CACTCGAGGTAGGTGTTTCTGCTAA 3'
		taqman Probe	5' TCCTTGCTGAGGCAGCAGGTCGAAT 3'
GFAP	NM_017009	forward primer	5' TGGCCACCAGTAACATGCAA 3'
		reverse Primer	5' CAGTTGGCGGCGATAGTCAT 3'
		taqman Probe	5' CAGACGTTGCTTCCCGCAACGC 3'

*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.

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Table 2. Relative expression of mRNA of *Mac-1*, *TLR-4* (markers for microgliosis) and *GFAP* (marker for astrogliosis) in the sham operated and L5 lumbar spinal cord of L5 nerve transected rats (saline or minocycline treated).

	Sham	Saline	Minocycline (40 mg/kg) treatment	
			Preemptive	Post-injury
Mac-1	2.9 ± 0.3	12.6 ± 0.5^{a}	$3.6 \pm 0.6^{a,b}$	$3.9 \pm 0.5^{a,b}$
TLR-4	0.4 ± 0.02	1.4 ± 0.05^{a}	$0.6 \pm 0.03^{a,b}$	$0.7 \pm 0.04^{a,b}$
GFAP	111.2 ± 3.7	290.6 ± 12.8^{a}	$147 \pm 7.9^{a,c}$	278.5 ± 14.1^{a}

The mRNA levels were normalized to the corresponding *GAPDH* (house keeping gene) level for each sample to adjust for uncontrolled variability between samples. Values are mean ± S.E.M. (n = 4). $^{a}p<0.01$ vs sham, $^{b}p<0.01$ and $^{c}p<0.05$ vs saline treated group.

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Table 3. Effect of pre-emptive and post-injury administration of minocycline on the induction of mRNA for cytokines in the L5 lumbar spinal cord of the L5 spinal nerve transected rats.

	Saline	Minocycline (40 mg/kg) treatment	
		Preemptive	Post-injury
IL-1β	4.3 ± 0.4^{a}	$1.8 \pm 0.2^{a,b}$	$3.1 \pm 0.3^{a,c}$
IL-6	5.1 ± 0.6^{a}	$2.4 \pm 0.8^{a,b}$	4.7 ± 0.4^{a}
TNF-α	3.6 ± 0.3^{a}	$1.6 \pm 0.3^{a,b}$	$2.5 \pm 0.2^{a,c}$

A multiprobe ribonuclease assay was performed using total mRNA from lumbar spinal cord of different groups of rats. The value for the normalized quantity for each band was obtained by dividing with the *L32* housekeeping 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. Values are mean ± S.E.M. (n= 4). $^{a}p<0.01$ vs sham, $^{b}p<0.01$ and $^{c}p<0.05$ vs saline treated group.

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Table 4. Effect of pre-emptive and post-injury administration of minocycline (40 mg/kg) on the cytokines levels in the L5 lumbar spinal cord of the L5 spinal nerve transected rats.

	Sham	Saline	Minocycline (40 mg/kg) treatment	
			Preemptive	Post-injury
IL-1β	39.5 ± 4.8	208 ± 18.4[a]	88.5 ± 12.3[a,b]	143.5 ± 18.5[a,c]
IL-6	201 ± 19	698 ± 61[a]	306 ± 28[a,b]	654 ± 43[a]
TNF-α	22.5 ± 3.1	98.5 ± 13[a]	36 ± 11.8[a,b]	54.5 ± 15.2[a,c]

Values are pg/mg total protein ± S.E.M. (n =4). [a]$p<0.01$ vs sham, [b]$p<0.01$ and [c]$p<0.05$ vs saline treated group.

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Fig. 1.

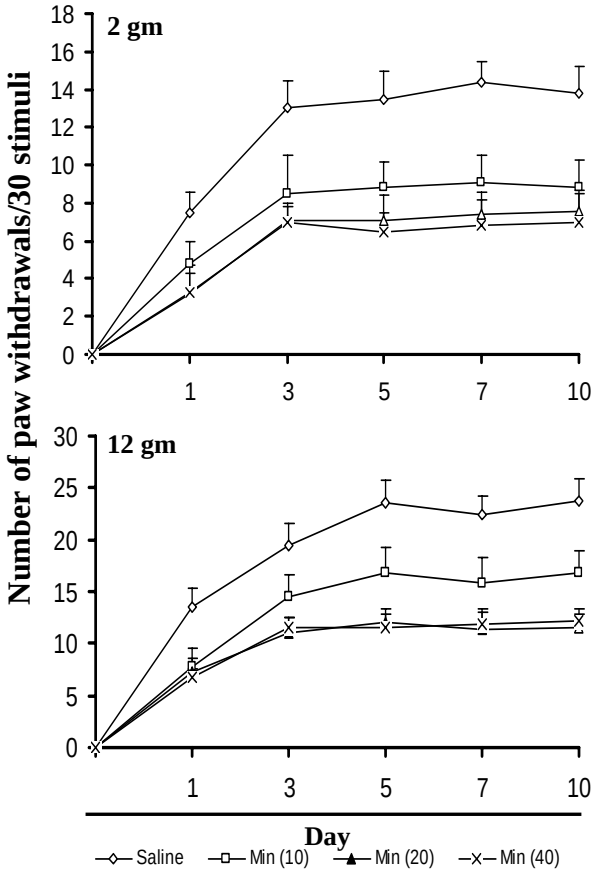

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Fig. 2.

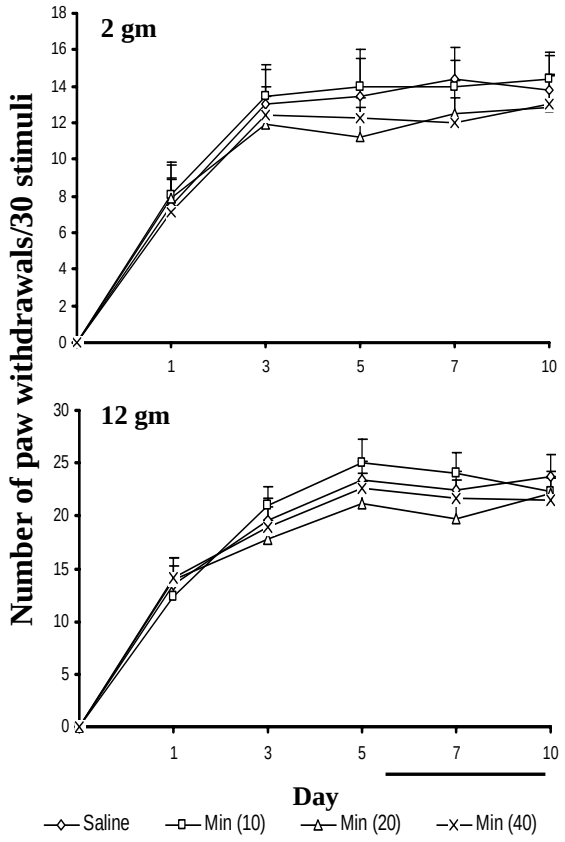

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Fig. 3.

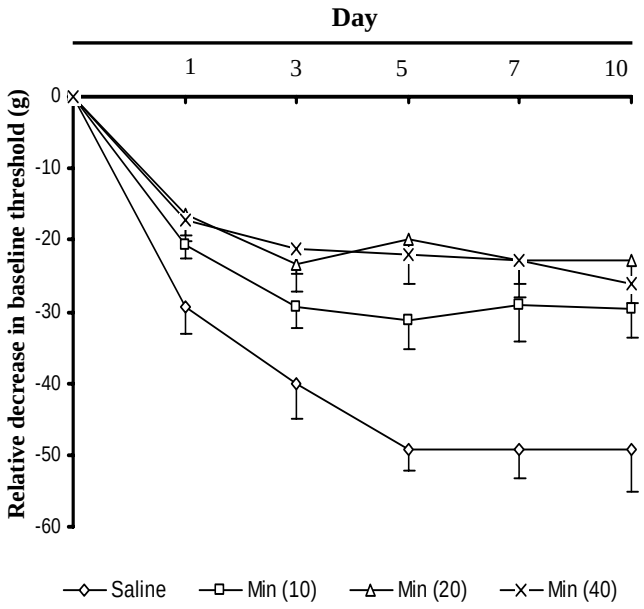

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Fig. 4.

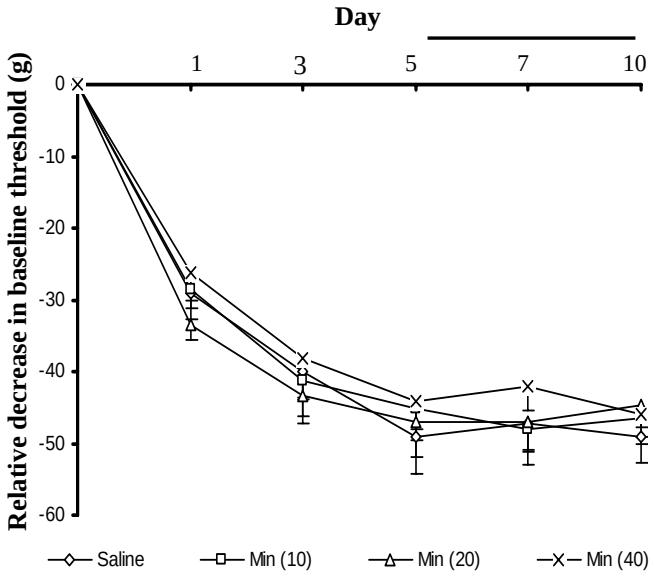

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Fig. 5.

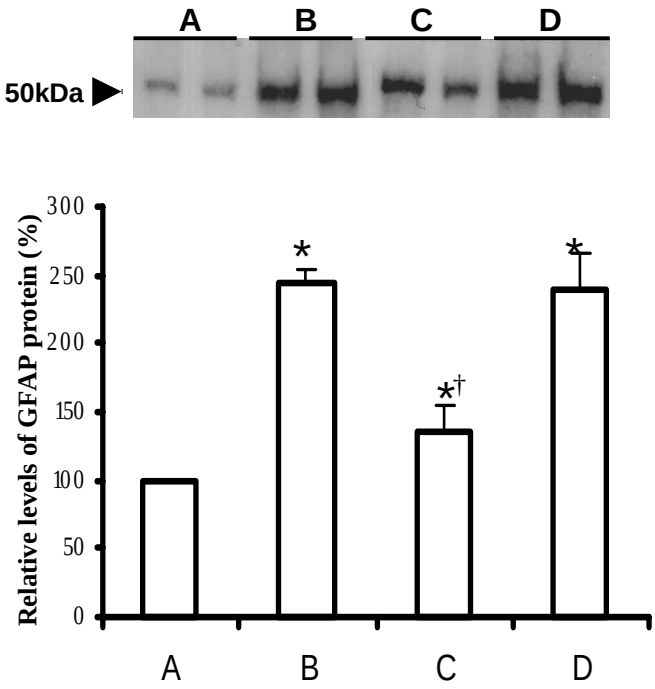

Fig. 6.