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Spinal Microglial Proliferation is Evident in a Rat Model of Painful Disc Herniation Both in the Presence of Behavioral Hypersensitivity and Following Minocycline Treatment Sufficient To Attenuate Allodynia

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Although spinal glia acquire a reactive profile in radiculopathy, glial cell proliferation remains largely unstudied. This study investigated spinal glial proliferation in a model simulating painful disc herniation; the C7 nerve root underwent compression and chromic gut suture exposure or sham procedures. A subset of injured rats received minocycline injections prior to injury. Allodynia was assessed and bromodeoxyuridine (BrdU) was injected 2 hr before tissue harvest on day 1 or 3. Spinal cell proliferation and phenotype identification were assayed by fluorescent colabeling with antibodies to BrdU and either glial fibrillary acidic protein (astrocytes) or Iba1 (microglia). At day 1, ipsilateral allodynia was significantly increased ($P < 0.001$) for injury over sham. Minocycline treatment significantly decreased ipsilateral allodynia to sham levels at day 1 ($P < 0.001$). At day 3, ipsilateral allodynia remained and contralateral allodynia was also present for injury ($P < 0.003$) over sham. The number of BrdU-positive cells in the ipsilateral spinal dorsal horn at day 1 after injury was significantly elevated ($P < 0.001$) over sham. Approximately 70% of BrdU-positive cells labeled positively for Iba1; dividing microglia were significantly increased ($P < 0.004$) in the ipsilateral dorsal horn at day 1 following injury compared with sham. Spinal cellular proliferation after injury was not changed by minocycline injection. By day 3, the number of BrdU-positive cells had returned to sham levels bilaterally. Data indicate that spinal microglia proliferate after injury but that proliferation is not abolished by minocycline treatment that attenuates allodynia, indicating that spinal microglial proliferation may be related to injury and may not be linked to changes in sensory perception.

Key words: astrocytes; microglia; BrdU; Iba1; pain

Glial cells in the central nervous system (CNS) support the function of healthy neurons and respond rapidly to injury. In the normal, nonpathological CNS, microglia are active, performing constant surveillance of the surrounding parenchyma, and astrocytes maintain homeostasis at neuronal synapses (Kreutzberg, 1995, 1996; DeLeo et al., 2006; Hanisch and Kettenmann, 2007). With the intermediate filament glial fibrillary acidic protein (GFAP) and the cell surface marker OX42 as phenotypic markers of a reactive profile, spinal astrocytes and microglia have been reported to become alert following nerve or nerve root injury (Colburn et al., 1999; Hashizume et al., 2000; Hubbard and Winkelstein, 2005; Clark et al., 2007). Pharmacologic agents that block the induction of a reactive profile in either microglia alone or both microglia and astrocytes can reduce behavioral hypersensitivity, further implicating reactive glia as having a role in pain (Raghavendra et al., 2003; Gwak et al., 2008; Mika, 2008). Although studies have assessed glial reactivity by using expression of GFAP and OX42 in models of pain, those cellular markers provide limited insight into the extent or functional consequences of cellular activation.

Astrocytic and microglial reactivity following trauma to the CNS includes cell proliferation, changes in morphology, and release of inflammatory mediators such as nitric oxide, reactive oxygen intermediates, and proinflammatory cytokines (Lendhal et al., 1990; Hill-Felberg et al., 1999; Zai et al., 2005). When astrocytes detect a change in homeostasis, they also up-regulate the

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intermediate filaments vimentin and GFAP and reexpress the embryonic protein nestin; reactive microglia transform to a phagocytic state and increase expression of cell surface markers (Frisen et al., 1995; Lin et al., 1995; Duggal et al., 1997; Shibuya et al., 2002; Eskes et al., 2003; Pekny and Lane, 2007). Markers of the glial response to changes in homeostasis have been described following direct trauma to the CNS, but few studies have examined these indicators of reactivity in the spinal cord following injury to peripheral neural tissues. Although spinal microglial proliferation has been reported following lumbar dorsal root transection and lumbar nerve transection (Liu et al., 1998, 2000), no study has evaluated such proliferation following dorsal root injury when the neural tissues that are injured remain intact following their injury.

Radicular pain can result from varied transient tissue insults, including root compression from vertebral motions, foraminal occlusion, or disc herniation (Kelly et al., 2000; Nuckley et al., 2002). We previously characterized a model of cervical nerve root injury in the rat that combines mechanical compression with an inflammatory insult to simulate cervical disc herniation that produces nerve root impingement (Rothman and Winkelstein, 2007). That injury produces significant increases in allodynia and further exacerbates a host of spinal inflammatory responses compared with responses from either a mechanical or a chemical injury applied to the root alone (Rothman and Winkelstein, 2007), corroborating reports of different behavioral hypersensitivity patterns for different types of root injuries (Colburn et al., 1999; Winkelstein et al., 2001; Rothman et al., 2005). However, it remains to be seen whether a transient nerve root injury such as that from disc herniation is sufficient to induce proliferation of cells in the spinal cord and whether that cellular response has a direct relationship with behavioral hypersensitivity.

Although glial reactivity is widely studied in models of pain, existing studies do not provide a complete description of the reactive profile of such cells. No study has quantified glial proliferation in painful cervical nerve root injury or evaluated whether proliferation has a causal connection with behavioral hypersensitivity. Therefore, this study quantifies spinal cell proliferation in a model of nerve root injury-mediated pain, determines whether the dividing cells are glia, and also examines whether blocking glial responses to changes in homeostasis can affect behavioral hypersensitivity or cell proliferation in the spinal cord.

MATERIALS AND METHODS

Experiments were performed using male Holtzman rats (Harlan Sprague-Dawley; Indianapolis, ID) weighing 250–350 g at the start of the study, with a 12–12-hr light–dark cycle and free access to food and water. All experimental procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and were carried out according to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

Bromodeoxyuridine (BrdU) was used to test for the presence of dividing cells at three different time points in the spinal cord following cervical nerve root injury. Rats underwent one of two procedures: transient compression of the right C7 root with additional chromic suture irritation to simulate a disc herniation with nerve root compression (n = 15) or sham (n = 4). All rats were tested for mechanical allodynia before injury and on each day on which tissue was harvested. The C7 spinal cord was harvested for immunohistochemical analysis of BrdU at day 1 (n = 6 injury, n = 4 sham) and day 3 (n = 6 injury), in separate groups. In addition, a pilot study was performed to assess spinal responses at day 7 following injury (n = 3); no BrdU-positive cells were detected in the spinal cord at day 7 following injury. Accordingly, no additional studies were performed at the day 7 time point for this study. A sham group was included only for day 1 assessment, to provide the most liberal estimate of behavioral outcomes and cell proliferation in the acute period following surgery.

Surgical Procedures

All procedures were performed with rats under inhalation anesthesia (4% isoflurane for induction, 2% for maintenance). A microvascular clip and chromic gut suture were used to model transient compression and chemical irritation, respectively (Rothman and Winkelstein, 2007). Briefly, rats were placed in a prone position, and an incision was made in the skin from the base of the skull to the second thoracic vertebra. A C6/C7 hemilaminectomy and partial facetectomy were performed on the right side to expose the C7 dorsal root. Procedures involved transient compression of the C7 dorsal nerve root for 15 min with a 10 gf microvascular clip (World Precision Instruments, Sarasota, FL) with additional placement of four pieces of 3-0 chromic gut suture on the right C7 dorsal nerve root proximal to the dorsal root ganglion (DRG). After injury, chromic suture pieces were left in place for the duration of the study. Procedures for sham involved the same protocol but included only exposure of the C7 dorsal root. All wounds were closed after surgery with 3-0 polyester suture and surgical staples. Rats were allowed to recovered in room air and monitored continuously.

Minocycline Injections

To assess relationships among mechanical allodynia, cell proliferation, and microglial reactivity, an additional set of injured rats (n = 4) also received injections of minocycline, a compound that suppresses the function of microglia, but not astrocytes or neurons, in vitro (Sigma, St. Louis, MO); it is commonly accepted that minocycline has the same effect in vivo (Raghavendra et al., 2003; Hua et al., 2005; Ledeboer et al., 2005; Mika et al., 2009). Intraperitoneal injections (45 mg/kg) were given 12 hr prior to injury, 1 hr prior to injury, and again 6 hr after injury, with dosing chosen based on previous studies of lumbar nerve root injury (Raghavendra et al., 2003; Mika et al., 2009). All rats in the minocycline study were tested for mechanical allodynia at day 1, and spinal cord tissue was harvested for immunohistochemical analysis at day 1.

Behavioral Assessment

Each rat was evaluated for bilateral forepaw mechanical allodynia before surgical procedures to determine baseline values and again on the day of tissue harvest. A single tester performed all testing and was blinded to the surgical procedures. Allodynia was measured for each unoperated rat before the surgical procedure to provide an assessment of baseline values. For each testing session, after 20 min of acclimation, rats were stimulated on the plantar surface of each forepaw using three von Frey filaments (1.4, 2, 4 g; Stoelting). Each testing session had three rounds of 10 stimulations, separated by 10 min. Total withdrawals were recorded for each forepaw of each rat and averaged for each group.

Immunohistochemistry Procedures

To assess bilateral glial cell proliferation in the spinal dorsal horn, rats were injected with BrdU, a thymidine analog that is incorporated into the DNA of dividing cells. Tissue was harvested from the C7 cervical spinal cord on days 1 and 3 following injury. Spinal cord from the C7 cervical level was assayed by using fluorescent colabeling with an antibody to BrdU and either to GFAP to label astrocytes or to ionized calcium-binding adapter molecule 1 (Iba1) to label microglia.

BrdU (Sigma) was delivered via intraperitoneal injection at 2 hr prior to perfusion (50 mg/kg) to label dividing cells. Rats were then deeply anesthetized, followed by transcardiac perfusion with 200 ml phosphate-buffered saline (PBS) and 300 ml of 4% paraformaldehyde in PBS (pH 7.4). After perfusion, the cervical spinal cord was exposed by laminectomy, the C7 segment of the cervical cord was harvested, and tissue was postfixed in the 4% paraformaldehyde solution for 20 min. Tissue was transferred to 30% sucrose/PBS and stored for 3 days at 4°C. Samples were freeze-mounted with OCT medium (Triangle Biomedical Sciences; Durham, NC) for axial cryosectioning.

Four serial C7 spinal cord sections (20 μm) from each rat were prepared for free-floating immunohistochemical staining. Slices were incubated in 2 N HCl for 60 min at 37°C, followed by two washes in 10 mM sodium borate to neutralize the acid solution. Sections were then blocked with 5% normal goat serum (Invitrogen, Carlsbad, CA) for 60 min, followed by incubation in a primary antibody solution containing mouse anti-BrdU (1:500; Invitrogen) and rabbit anti-GFAP (1:20,000; Dako, Carpintera, CA) or mouse anti-BrdU and rabbit anti-Iba1 (1:1,000; Wako, Richmond, VA) overnight at 4°C. Sections were then treated with an Alexa594-conjugated goat anti-rabbit secondary antibody (1:250; Invitrogen) and an Alexa488-conjugated goat anti-mouse secondary antibody (1:250; Invitrogen) for 60 min. Previous studies were performed to determine optimal antibody dilutions. Spinal cord tissue from the C7 level of naïve rats (n = 2) was also processed for comparison. A negative control with no primary antibody staining was always included for verification of specificity of immunohistochemical techniques.

For each glial marker, both the ipsilateral and the contralateral dorsal horns from representative cord sections from each rat for each colabeling paradigm (BrdU/GFAP and BrdU/Iba1) were photographed at ×100 with a digital camera and stereomicroscope system and Axiovision software (Zeiss, Thornwood, NY). Images were uniformly adjusted to maximize contrast and cropped (1,000 × 400 pixels) to include only the superficial laminae I–III; manual cell counts were used to determine the number of BrdU-positive cells in the dorsal horn, the number of BrdU-positive cells colabeled with Iba1, and the number of BrdU-positive cells colabeled with GFAP, as previously reported (Zai et al., 2005). Values were then averaged and expressed as the number of cells per square millimeter of tissue. A subset of samples was also imaged with a Radiance 2000 Confocal System to verify fluorescent colabeling with z-stacked images.

Statistical Analysis

Significant differences in allodynia between groups were detected by a one-way ANOVA at each time point, separately for each of the ipsilateral and contralateral forepaws. For BrdU, BrdU/Iba1, and BrdU/GFAP cell counts, a one-way ANOVA compared differences between groups at day 1. Significance was defined at $P < 0.05$ (Systat, Richmond, CA).

RESULTS

Behavioral Assessment

Sham procedures did not significantly increase the frequency of paw withdrawals elicited by von Frey stimulation compared with baseline values for either forepaw. In contrast, the number of ipsilateral paw withdrawals elicited by stimulation following injury was elevated and was significantly greater than that of sham at both day 1 ($P < 0.001$) and day 3 ($P < 0.001$; Fig. 1A). Treatment with minocycline significantly decreased the number of paw withdrawals in the ipsilateral forepaw compared with that of untreated rats at day 1 ($P < 0.001$) and reduced those ipsilateral paw withdrawals to sham levels (Fig. 1A). Results for testing the ipsilateral forepaw using the 1.4 g and 2 g filaments were consistent with those from the 4 g filament studies (data not shown).

In the contralateral forepaw, the number of paw withdrawals after injury was not increased over sham values at day 1 for stimulation by the 1.4 g and 4 g filament but was significantly increased over sham for testing with the 2 g filament only ($P = 0.002$; data not shown). At day 3 after injury, the number of paw withdrawals was significantly elevated over sham values ($P = 0.001$) for testing with all filaments (Fig. 1B). Treatment with minocycline significantly reduced contralateral paw withdrawal frequency compared with untreated rats at day 1 for testing with the 2 g filament ($P = 0.014$; data not shown).

BrdU Immunohistochemistry

BrdU-positive cells were detected in nearly all samples probed in this study (Figs. 2, 3). At day 1 after sham procedures, small, nonsignificant numbers of proliferating cells were detected in the ipsilateral dorsal horn (Figs. 2A, 4A), and almost no proliferating cells were detected in the contralateral dorsal horn (Figs. 2B, 4B). At day 1

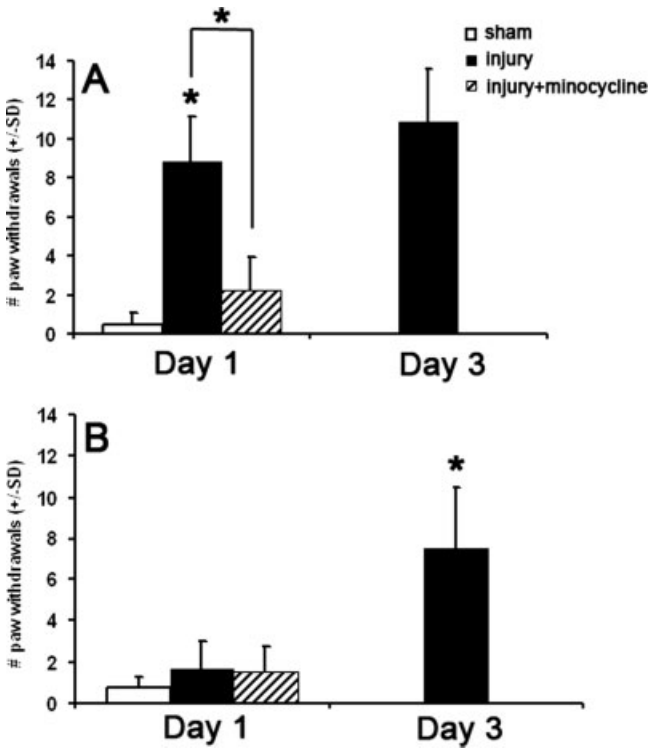

Fig. 1. Average mechanical allodynia in the ipsilateral (**A**) and contralateral (**B**) forepaws for sham, injury, and injury treated with minocycline (injury + minocycline), for testing with the 4 g von Frey filament. A: Ipsilateral allodynia was significantly increased over sham at both days after injury (*$P < 0.001$). Ipsilateral allodynia was significantly decreased after injury + minocycline compared with untreated rats (*$P < 0.001$). B: In the contralateral forepaw, injury did not significantly increase allodynia over sham at day 1. By day 3, injury produced allodynia in the contralateral forepaw that was significantly increased over sham (*$P < 0.002$).

after injury, the number of proliferating cells was significantly increased in the ipsilateral dorsal horn compared with sham (Figs. 2, 4A; $P < 0.001$). This increase in BrdU-positive cells was also present after treatment with minocycline; at day 1 after injury and minocycline treatment, the number of proliferating cells was significantly increased over sham ($P = 0.049$; Figs. 2E, 4A). However, by day 3 after injury, the number of proliferating cells had returned to sham levels (Figs. 2G, 4A). Although some proliferating cells were counted in the contralateral dorsal horn at day 3 after injury (Fig. 2H), there was no significant increase in the number of BrdU-positive cells in the contralateral dorsal horn after injury compared with sham for either day 1 or day 3, for either minocycline or untreated rats (Figs. 2, 4B).

Fluorescent Colabeling

At both of the time points probed in this study (days 1 and 3), the BrdU-positive cells were predominantly colabeled with Iba1 (Fig. 3); fluorescent colabeling demonstrated colocalization of BrdU and Iba1 (Fig. 3C), suggesting that these dividing cells were microglia. At day 1 after injury, the number of dividing microglia in the ipsilateral dorsal horn was significantly higher than sham ($P = 0.001$; Fig. 5A). After injury and minocycline treatment, the number of dividing microglia was still significantly elevated over sham ($P = 0.004$). By day 3, the number of dividing microglia had decreased to sham levels (Fig. 5A). Trends were similar but nonsignificant for the contralateral dorsal horn (Fig. 5B). Colocalization of BrdU and Iba1 was verified in a subset of samples by confocal microscopy (Fig. 3).

Fluorescent colabeling demonstrated a distinct absence of colocalization of BrdU with GFAP (Fig. 3F). At day 1 following injury, the number of BrdU-positive cells that also stained positively for GFAP in the ipsilateral dorsal horn was nearly zero. Among the six samples probed in this study, no BrdU-positive cells that were also positive for GFAP were detected in five of the samples. Although one sample did demonstrate colocalization of BrdU and GFAP, this sample displayed only one such cell per section. This finding was unchanged after minocycline injection. By day 3 after injury, this trend of lack of colabeling was similarly low, with only one sample of the six probed at day 3 displaying BrdU-positive cells that were also GFAP positive. The contralateral side of the dorsal horn exhibited no colocalization of BrdU and GFAP for any time point in any of the groups probed.

DISCUSSION

This study demonstrates spinal microglial proliferation in a model of radicular pain resulting from combined chemical and mechanical insults, as can occur from disc herniation. Our results demonstrate a robust and significant increase in ipsilateral mechanical allodynia at days 1 and 3 after injury that is in agreement with previous findings showing these increases at day 1, with allodynia persisting as late as day 7 following injury (Rothman and Winkelstein, 2007). Minocycline treatment abolished ipsilateral mechanical allodynia at day 1 after injury (Fig. 1). However, cellular proliferation in the spinal cord at day 1 after injury was robust and significantly higher than that observed in the sham group, regardless of whether minocycline was given (Figs. 2, 4). By day 3, proliferation in the spinal cord after injury returned to sham responses, and no proliferating cells were identified by day 7 (data not shown). In general, at both day 1 and day 3, approximately 70% of those cells identified as dividing also labeled positively for Iba1 (microglia), although nearly none of them were positive for GFAP (astrocytes; Fig. 3).

Although this study is the first to show cellular proliferation in the spinal cord in a model simulating disc herniation, the immediate increase in spinal microglial proliferation noted here (Figs. 3, 5) is consistent with reports of glial proliferation in other models of painful lumbar nerve or nerve root injury. For example, glial proliferation has been reported in the dorsal horn of the spinal cord at 1 day after lumbar nerve compression,

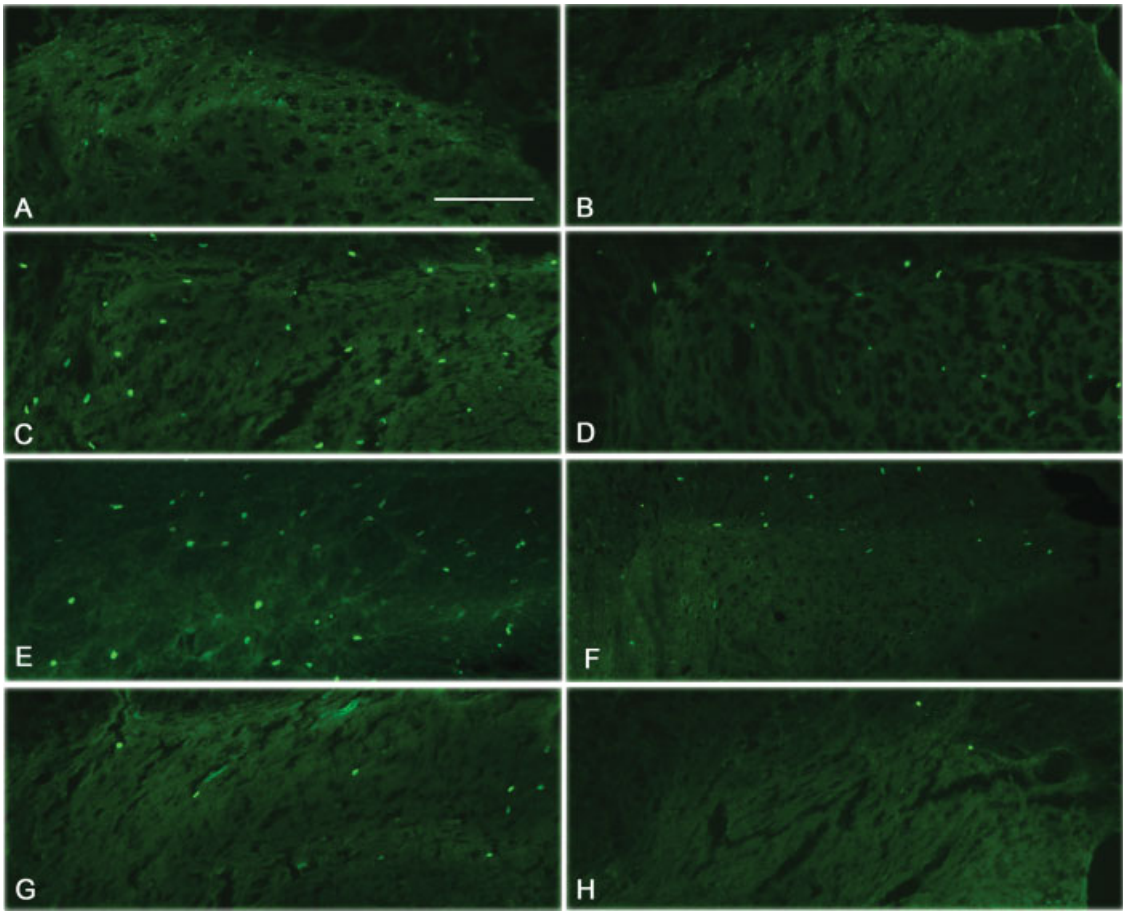

Fig. 2. Representative ipsilateral (**A,C,E,G**) and contralateral (**B,D,F,H**) C7 spinal cord sections showing the dorsal horn stained against BrdU after sham (A,B), injury (C,D,G,H), or injury + minocycline (E,F) on day 1 (A–F) and day 3 (G,H). Scale bar = 100 μm.

nerve transection, or dorsal rhizotomy (Liu et al., 1998, 2000; Echeverry et al., 2008). Most of the dividing cells in those models were also identified as microglia. An increase in the number of microglia in the spinal cord was also detected by stereological analysis at day 3 after peripheral nerve injury (Beggs and Salter, 2007); that finding agrees with the evidence of early increases in OX42 expression in the spinal cord associated with neuropathic pain (Inoue, 2005; Clark et al., 2007; Cao and Zhang, 2008). Spinal microglial reactivity, as measured by OX42 immunoreactivity, was greater for nerve root ligation by chromic suture compared with that produced by a silk ligation, suggesting that spinal microglia may be responsive to chemical irritation (Hashizume et al., 2000). Our current data show an early and significant increase in the number of dividing microglia in the ipsilateral dorsal horn following injury (Figs. 3, 5), implying not only that spinal microglia react quickly following root injury but also that they have a have a multifaceted response to injury.

Most studies of spinal microglia in pain models utilize the cell surface marker OX42; however, Iba1 is a sensitive and specific marker of the induction of a reactive profile in microglia (Imai et al., 1996; Imai and Kohsaka, 2002; Romero-Sandoval et al., 2008; Scholz et al., 2008; Tozaki-Saitoh et al., 2008). Iba1 has a functional role in intracellular calcium signaling, which is stimulated when microglia react to a change in homeostasis (Ito et al., 1998; Okere and Kaba, 2000; Imai and Kohsaka, 2002; Yamada et al., 2006). In contrast, OX42 is involved in adhesion and migration of microglia and is apparent on the membrane of microglia (Ross, 2002). Both OX42 and Iba1 are used to assess microglial reactivity, and both are present in the naïve spinal cord (Moss et al., 2007; Romero-Sandoval et al., 2008). However, Iba1 is involved in intracellular signaling, whereas OX42 is involved in extracellular signaling (Imai and Kohsaka, 2002; Ross, 2002), implying that staining patterns for the two proteins will likely be different. We can hypothesize that increases in OX42 after injury will peak earlier than Iba1, because signaling molecules bind to microglia on the surface of the cell and then induce intracellular calcium changes. Descriptions of changes in both of these proteins in the spinal cord

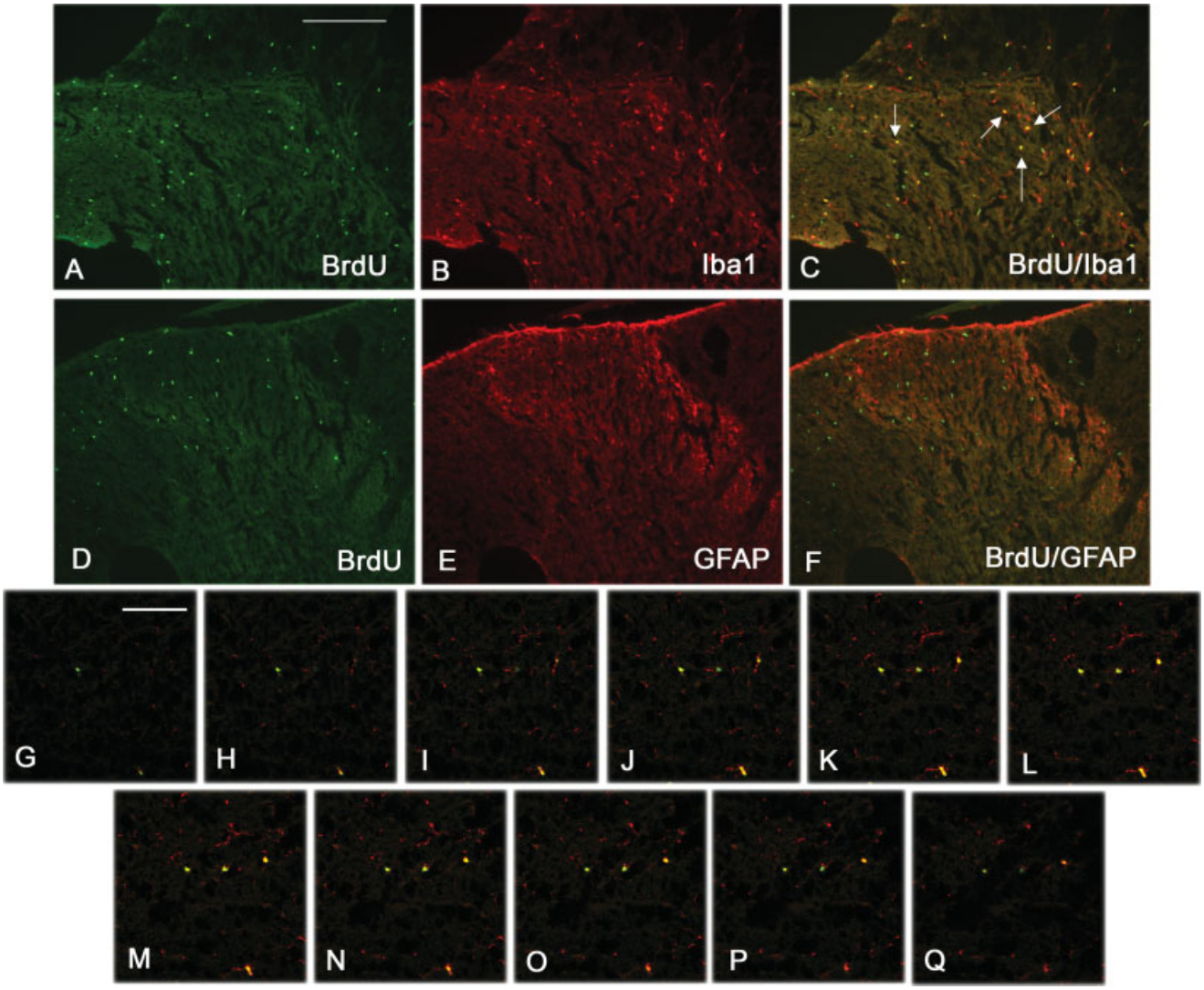

Fig. 3. Representative spinal cord sections taken from day 1 after injury showing BrdU (**A,D**), Iba1 (**B**), GFAP (**E**), and fluorescently colabeled (**C,F**) staining. Arrows in C indicate BrdU-positive and Iba1-positive cells. Confocal microscopy in the z-plane verified fluorescent colabeling (**G–Q**). Scale bars = 100 μm in A (applies to A–F); 20 μm in Q (applies to G–Q).

are likely required to outline fully the time course of microglial reactivity after injury. Furthermore, a description of the changes in activation of mitogen-activated protein kinase (p38) may also provide insight regarding the time course of the spinal microglial response to changes in homeostasis, since p38 is phosphorylated in spinal microglia in models of pain, and its inhibition can reduce allodynia (Hua et al., 2005; Wen et al., 2009). Although the work described here does not explicitly compare Iba1 staining between injury and sham, our data suggest that Iba1 may be useful to define differences in spinal microglial reactivity in this model, since the number of proliferating microglia is significantly increased in injury (Fig. 5).

Microglial proliferation was present at day 1 regardless of whether minocycline was administered (Fig. 5) and despite minocycline treatment effectively reducing allodynia (Fig. 1). The reduction in mechanical allodynia reported here is in agreement with previously published work with models of low back pain and further supports the hypothesis that reactive microglial might directly mediate behavioral hypersensitivity (Raghavendra et al., 2003; Ledeboer et al., 2005; Narita et al., 2006; Mika, 2008). However, the fact that cellular proliferation in the spinal cord persists in the absence of mechanical allodynia (Figs. 1, 4) implies that spinal microglial proliferation may actually be a response to tissue damage and not linked to changes in sensory

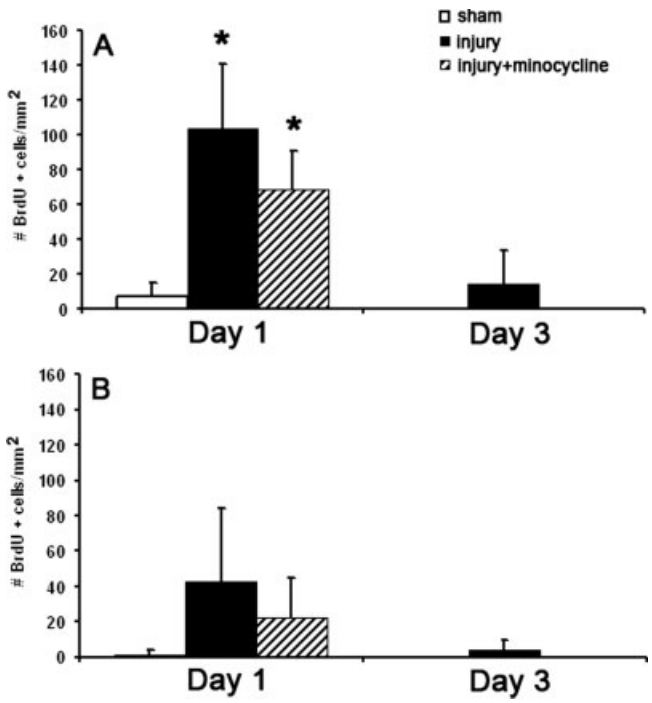

Fig. 4. Quantification of BrdU-positive cells in the ipsilateral (**A**) and contralateral (**B**) dorsal horns at days 1 and 3 after injury. Cell numbers are expressed as the number of positive cells per square millimeter. A: At day 1 after injury either with (injury + minocycline) or without (injury) minocycline treatment, the number of BrdU-positive cells significantly increased in the ipsilateral dorsal horn compared with sham ($*P < 0.001$). A,B: By day 3, there were no significant difference between any groups in either the ipsilateral or the contralateral side.

perception. This finding regarding proliferation is in agreement with similar reports on microglial reactivity in the absence of pain. In particular, in a study of pain induced by formalin injection in the hindpaw, bupivacaine blocked the induction of mechanical allodynia but did not prevent increases in OX42 expression in the spinal cord (Fu et al., 2000). As immune cells, microglia are heavily involved in healing activities such as phagocytosis and release of inflammatory mediators after injury, and our findings further support the idea that these immune activities may persist as a respone to tissue damage or inflammation and may actually be unaffiliated with sensory changes.

However, in the current study, it is possible that an aspect of microglial reactivity other than proliferation is responsible for behavioral hypersensitivity. It is likely that, in this model, minocycline inhibits the *functions* of microglia rather than the further proliferation of these cells. In context with previous work showing that soluble tumor necrosis factor receptor-1 treatment reduced both mechanical allodynia and spinal GFAP expression in this and other nerve root models (Winkelstein et al., 2001; Rothman et al., 2009), we hypothesize that minocycline may mediate behavioral hypersensitivity for this nerve root injury via the reduction of inflammatory cytokine production by spinal microglia. Other possibilities for the modification of behavior by administration of minocycline that was observed in our study could be via the inhibition of migration of microglial cells, since in vitro studies of stimulated microglia demonstrate that minocycline inhibits microglial migration (Nutile-McMenemy et al., 2007). It is also possible that minocycline is inhibiting the activation of p38 mitogen-activated protein kinase; after a painful paw incision, p38 has been show to be phorphorylated in the dorsal horn of the spinal cord, and this effect and the associated hypersensitivity are reduced by minocycline (Wen et al., 2009).

Data presented here provide evidence that spinal astrocytic proliferation does not occur after this cervical nerve root injury (Fig. 3). This is contradictory to reports of GFAP colocalization with BrdU in the spinal dorsal horn as early as 48 hr following dorsal rhizotomy and as late as 14 days after peripheral nerve injury (Liu et al., 2000; Kozlova, 2003). Although it is possible that spinal astrocytes are dividing at a time point not studied here, previous work shows an increase in GFAP reactivity at day 7 after injury (Rothman and Winkelstein,

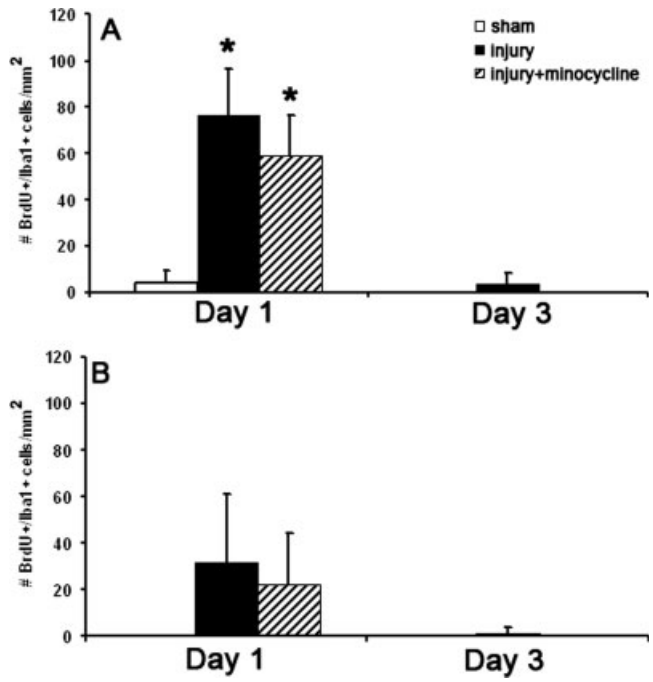

Fig. 5. Quantification of dividing microglia in the ipsilateral (**A**) and contralateral (**B**) dorsal horn of the spinal cord at days 1 and 3 after injury. Cell numbers are expressed as number of positive cells per square millimeter. A: At day 1 after injury, the number of dividing microglia significantly increased in the ipsilateral dorsal horn compared with sham ($*P < 0.001$). This increase in the number of dividing microglia was also significant after injury and treatment with minocycline compared with sham ($*P = 0.004$). B: By day 3, there were no significant differences between groups on either the ipsilateral or the contralateral side.

2007), implying that astrocytic proliferation, if present at all, would be evident before or at day 7. Taking this into account along with the BrdU results presented here (Figs. 2, 3), we hypothesize that the increases in GFAP observed at day 7 previously might reflect an up-regulation of the intermediate filament, rather than an actual increase in the number of astrocytes. It is likely that, although a transient mechanical injury, even with a longer lasting chemical insult, induces an up-regulation of GFAP, these insults might not be sufficient to induce proliferation of spinal astrocytes. At least one study of sciatic nerve transection (Echeverry et al., 2008) also did not observe dividing astrocytes in the spinal cord, further supporting variation in spinal astrocytic responses for different injury paradigms. The BrdU immunohistochemistry results presented here (Fig. 3) also support the hypothesis that measuring GFAP reactivity only might not be sufficient as an indicator of astrocytic activation. Other markers of astrocytic reactivity, such as proliferation, reexpression of nestin, production of proinflammatory cytokines, and expression of growth factors must also be studied to define fully the complete astrocytic response to injury, particularly in models of pain in which spinal astrocytic responses may be more subtle because the spinal cord is remote from the actual injury site and is not directly traumatized.

Although these data support the hypothesis that spinal microglial reactivity plays a role in radicular pain, further studies are needed to establish a direct link between behavioral hypersensitivity and the collection of spinal microglial responses. Current work is based on a single bolus injection of BrdU at each time point. It is, therefore, a conservative estimate of the amount of cell proliferation that may be occurring in the spinal cord. Furthermore, because the time difference between injection and harvest was only 2 hr, this study was not able to probe migration of newly generated cells; newly generated cells in the white matter have been shown to migrate to the gray matter following dorsal rhizotomy (Kozlova, 2003), and this may affect the resulting hypersensitivity. In addition, Nutile-McMenemy et al. (2007) determined that minocycline may prevent microglial migration in vitro, emphasizing that the time between BrdU injection and tissue harvest might also affect the results of BrdU-positive spinal microglial detected in the in vivo minocycline studies reported here. Although approximately 70% of the dividing cells measured in this study were identified as microglia (Figs. 3, 5), the phenotype of the remaining 30% remains unknown. It is hypothesized that these cells may be oligodendrocyte precursors based on literature showing colocalization of BrdU with the condroitin sulfate proteoglycan NG2, an oligodendrocyte marker, in the spinal cord following dorsal rhizotomy (Echeverry et al., 2008).

Results presented here demonstrate spinal microglial proliferation in a model of painful radiculopathy simulating cervical disc herniation. Data suggest a role for spinal microglia in mediating robust increases in behavioral hypersensitivity for this injury in association with spinal cord cellular reactivity. However, although previous work in this model demonstrates increased spinal GFAP at these and other time points, very few proliferating astrocytes were observed in the current study. This finding suggests that, although astrocytes in the spinal cord respond to nerve root injury, their response might not necessarily include all of the same hallmarks of astrocytic pathology as when the spinal cord is itself directly injured. Consequently, there may be intermediate states between ''resting'' and ''activated'' for spinal astrocytes, during which some markers of activation are present and others are not. Further studies into other markers of astrocytic activation are necessary to outline the pathology of astrocytes in the spinal cord in pain.

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REFERENCES

Beggs S, Salter MW. 2007. Stereological and somatotopic analysis of the spinal microglial response to peripheral nerve injury. Brain Behav Immun 21:624–633.

Cao H, Zhang YQ. 2008. Spinal glial activation contributes to pathological pain states. Neurosci Biobehav Rev 32:972–983.

Clark AK, Gentry C, Bradbury EJ, McMahon SB, Malcangio M. 2007. Role of spinal microglia in rat models of peripheral nerve injury and inflammation. Eur J Pain 11:223–230.

Colburn RW, Rickman AJ, 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.

DeLeo JA, Tawfik VL, LaCroix-Fralish ML. 2006. The tetrapartite synapse: path to CNS sensitization and chronic pain. Pain 122:17–21.

Duggal N, Schmidt-Kastner R, Hakim AM. 1997. Nestin expression in reactive astrocytes following focal cerebral ischemia. Brain Res 768:1–9.

Echeverry S, Shi X, Zhang J. 2008. Characterization of cell proliferation in rat spinal cord following peripheral nerve injury and the relationship with neuropathic pain. Pain 135:37–47.

Eskes C, Juillerat-Jeanneret L, Leuba G, Honegger P, Monnet-Tschudi F. 2003. Involvement of microglia-neuron interactions in the tumor necrosis factor-alpha release, microglial activation, and neurodegeneration induced by trimethyltin. J Neurosci Res 71:583–590.

Frisen J, Johansson CB, Torok C, Risling M, Lendahl U. 1995. Rapid, widespread, and longlasting induction of nestin contributes to the generation of glial scar tissue after CNS injury. J Cell Biol 131:453–464.

Fu XY, Light AR, Maixner W. 2000. Relationship between nociceptor activity, peripheral edema, spinal microglial activation and long-term hyperalgesia induced by formalin. Neuroscience 101:1127–1135.

Gwak YS, Crown ED, Unabia GC, Hulsebosch CE. 2008. Propentofylline attenuates allodynia, glial activation and modulates GABAergic tone after spinal cord injury in the rat. Pain 138:410–422.

Hanisch UK, Kettenmann H. 2007. Microglia: active sensor and versatile effector cells in the normal and pathological brain. Nat Neurosci 10:1387–1394.

Hashizume H, DeLeo JA, Colburn RW, Weinstein JN. 2000. Spinal glial activation and cytokine expression after lumbar root injury in the rat. Spine 25:1206–1217.

Hill-Felberg SJ, McIntosh TK, Oliver DL, Raghupathi R, Barbarese E. 1999. Concurrent loss and proliferation of astrocytes following lateral fluid percussion brain injury in the adult rat. J Neurosci Res 57:271–279.

Hua XY, Svensson CI, Matsui T, Fitzsimmons B, Yaksh TL, Webb M. 2005. Intrathecal minocycline attenuates peripheral inflammation-induced hyperalgesia by inhibiting p38 MAPK in spinal microglia. Eur J Neurosci 22:2431–2440.

Hubbard RD, Winkelstein BA. 2005. Transient cervical nerve root compression in the rat induces bilateral forepaw allodynia and spinal glial activation: Mechanical factors in painful neck injuries. Spine 30:1924–1932.

Imai Y, Kohsaka S. 2002. Intracellular signaling in M-CSF-induced microglia activation: role of Iba1. Glia 40:164–147.

Imai Y, Ibata I, Ito D, Ohsawa K, Kohsaka S. 1996. A novel gene iba1 in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. Biochem Biophys Res Commun 224:855–862.

Inoue K. 2005. The function of microglia through purinergic receptors: Neuropathic pain and cytokine release. Pharmacol Ther 109:210–226.

Ito D, Imai Y, Ohsawa K, Nakajima K, Fukuuchi Y, Kohsaka S. 1998. Microglia-specific localisation of a novel calcium binding protein, Iba1. Brain Res Mol Brain Res 57:1–9.

Kelly JD, Aliquo D, Sitler MR, Odgers C, Moyer RA. 2000. Association of burners with cervical canal and foraminal stenosis. Am J Sports Med 28:214–217.

Kozlova EN. 2003. Differentiation and migration of astrocytes in the spinal cord following dorsal root injury in the adult rat. Eur J Neurosci 17:782–790.

Kreutzberg GW. 1995. Microglia, the first line of defence in brain pathologies. Arzneimittelforschung 45:357–360.

Kreutzberg GW. 1996. Microglia: a sensor for pathlogical events in the CNS. Trends Neurosci 19:312–318.

Ledeboer A, Sloane EM, Milligan ED, Frank MG, Mahony JH, Maier SF, Watkins LR. 2005. Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. Pain 115:71–83.

Lendhal U, Zimmerman LB, McKay RD. 1990. CNS stem cells express a new class of intermediate filament protein. Cell 60:585–595.

Lin RCS, Matesic DF, Marvin M, McKay RDG, Brustle O. 1995. Re-expression of the intermediate filament nestin in reactive astrocytes. Neurobiol Dis 2:79–85.

Liu L, Persson JK, Svensson M, Aldskogius H. 1998. Glial cell responses, complement, and clusterin in the central nervous system following dorsal root transaction. Glia 23:221–238.

Liu L, Rudin M, Kozlova EN. 2000. Glial cell proliferation in the spinal cord after dorsal rhizotomy or sciatic nerve transection in the adult rat. Exp Brain Res 131:64–73.

Mika J. 2008. Modulation of microglia can attenuate neuropathic pain symptoms and enhance morphine effectiveness. Pharmacol Rep 60:297–307.

Mika J, Wawrzczak-Bargiela A, Osikowicz M, Makuch W, Przewlocka B. 2009. Attenuation of morphine tolerance by minocycline and pentoxifylline in naive and neuropathic mice. Brain Behav Immun 23:75–84.

Moss A, Beggs S, Vega-Avelaira D, Costigan M, Hathway GJ, Salter MW, Fitzgerald M. 2007. Spinal microglia and neuropathic pain in young rats. Pain 128:215–224.

Narita M, Yoshida T, Nakajima M, Narita M, Miyatake M, Takagi T, Yajima Y, Suzuki T. 2006. Direct evidence for spinal cord microglia in the development of a neuropathic pain-like state in mice. J Neurochem 97:1337–1348.

Nuckley D, Konodi M, Raynak G, Ching R, Mirza S. 2002. Neural space integrity of the lower cervical spine. Effect of normal range of motion. Spine 27:587–595.

Nutile-McMenemy N, Elfenbein A, DeLeo JA. 2007. Minocycline decreases in vitro microglial motility, β1-integrin, and Kv1.3 channel expression. J Neurochem 103:2035–2046.

Okere CO, Kaba H. 2000. Heterogenous immunohistochemical expression of microglia-specific ionized calcium binding adaptor protein (Iba1) in the mouse olfactory bulb. Brain Res 877:85–90.

Pekny M, Lane EB. 2007. Intermediate filaments and stress. Exp Cell Res 313:2244–2254.

Raghavendra V, Tanga F, DeLeo JA. 2003. Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. J Pharmacol Exp Ther 306:624–630.

Romero-Sandoval A, Chai N, Nutile-McMenemy N, Deleo JA. 2008. A comparison of spinal Iba1 and GFAP expression in rodent models of acute and chronic pain. Brain Res 1219:116–1126.

Ross GD. 2002. Role of the lectin domain of Mac-1/CR3 (CD11b/CD18) in regulating intercellular adhesion. Immunol Res 25:219–227.

Rothman SM, Winkelstein BA. 2007. Chemical and mechanical nerve root insults induce differential behavioral sensitivity and glial activation that are enhanced in combination. Brain Res 1181:30–43.

Rothman SM, Kreider RA, Winkelstein BA. 2005. Spinal neuropeptide responses in persistent and transient pain following cervical nerve root injury. Spine 30:2491–2496.

Rothman SM, Huang Z, Lee KE, Weisshaar CL, Winkelstein BA. 2009. Cytokine mRNA expression in painful radiculopathy. J Pain 10:90–99.

Scholz J, Abele A, Marian C, Häussler A, Herbert TA, Woolf CJ, Tegeder I. 2008. Low-dose methotrexate reduces peripheral nerve injury-evoked spinal microglial activation and neuropathic pain behavior in rats. Pain 138:130–142.

Shibuya S, Miyamoto O, Auer RN, Itano T, Mori S, Norimatsu H. 2002. Embryonic intermediate filament, nestin, expression following traumatic spinal cord injury in adult rats. Neuroscience 114:905–916.

Tozaki-Saitoh H, Tsuda M, Miyata H, Ueda K, Kohsaka S, Inoue K. 2008. P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. J Neurosci 28:4949–4956.

Wen YR, Suter MR, Ji RR, Yeh GC, Wu YS, Wang KC, Kohno T, Sun WZ, Wang CC. 2009. Activation of p38 mitogen-activated protein kinase in spinal microglia contributes to incision-induced mechanical allodynia. Anesthesiology 110:155–165.

Winkelstein BA, Rutkowski M, Sweitzer SM, Pahl JL, DeLeo JA. 2001. Nerve injury proximal or distal to the DRG induces similar spinal glial activation and selective cytokine expression but differential behavioral responses to pharmacologic treatment. J Comp Neurol 439:127–139.

Yamada M, Ohsawa K, Imai Y, Kohsaka S, Kamitori S. 2006. X-ray structures of the microglia/macrophage-specific protein Iba1 from human and mouse demonstrate novel molecular conformation change induced by calcium binding. J Mol Biol 364:449–457.

Zai LJ, Yoo S, Wrathall JR. 2005. Increased growth factor expression and cell proliferation after contusive spinal cord injury. Brain Res 1052:147–155.