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Pain. Author manuscript; available in PMC 2009 August 15.

Published in final edited form as:
Pain. 2008 August 15; 138(1): 130–142. doi:10.1016/j.pain.2007.11.019.

Low-dose methotrexate reduces peripheral nerve injury-evoked spinal microglial activation and neuropathic pain behavior in rats

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Abstract

Peripheral nerve injuries that provoke neuropathic pain are associated with microglial activation in the spinal cord. We have investigated the characteristics of spinal microglial activation in three distinct models of peripheral neuropathic pain: spared nerve injury (SNI), chronic constriction injury, and spinal nerve ligation. In all models, dense clusters of cells immunoreactive for the microglial marker CD11b formed in the ipsilateral dorsal horn 7 days after injury. Microglial expression of ionized calcium binding adapter molecule 1 (Iba1) increased by up to 40% and phosphorylation of p38 mitogen-activated protein kinase, a marker of microglial activity, by 45%. Expression of the lysosomal ED1-antigen indicated phagocytic activity of the cells. Unlike the peripheral nerve lesions, rhizotomy produced only a weak microglial reaction within the spinal gray matter but a strong activation of microglia and phagocytes in the dorsal funiculus at lumbar and thoracic spinal cord levels. This suggests that although degeneration of central terminals is sufficient to elicit microglial activation, it does not account for the inflammatory response in the dorsal horn after peripheral nerve injury. Early intrathecal treatment with low-dose methotrexate, beginning at the time of injury, decreased microglial activation, reduced p38 phosphorylation, and attenuated pain-like behavior after SNI. In contrast, systemic or intrathecal delivery of the glucocorticoid dexamethasone did not inhibit the activation of microglia or reduce pain-like behavior. We confirm that microglial activation is crucial for the development of pain after nerve injury, and demonstrate that suppression of this cellular immune response is a promising approach for preventing neuropathic pain.

1. Introduction

Peripheral nerve injury elicits a marked immune response distal to the axonal lesion site, in the dorsal root ganglion (DRG), and in the spinal cord. Macrophages clear myelin debris from peripheral nerve fibers during Wallerian degeneration and eliminate the remains of the cell bodies of degenerating injured sensory neurons (Hu and McLachlan, 2003). A similar role has been proposed for spinal microglial cells, as scavengers that remove the central terminals of injured primary afferents (Aldskogius et al., 1999). Recently it has become evident though,

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that microglial activation in the dorsal horn of the spinal cord actively contributes to the development of neuropathic pain (DeLeo and Yezierski, 2001; Watkins and Maier, 2003; Marchand et al., 2005; Tsuda et al., 2005; Scholz and Woolf, 2007). Following a peripheral nerve lesion, spinal microglia adopts a state of heightened activity that is associated with the phosphorylation of p38 mitogen-activated protein kinase (Mapk) (Jin et al., 2003; Tsuda et al., 2004), an acquisition of phagocytic properties (Liu et al., 1998), and the secretion of neurotrophic factors (Coull et al., 2005) and cytokines (DeLeo and Yezierski, 2001). Mice deficient in genes that contribute to the recruitment or activation of spinal microglia, such as *chemotactic cytokine receptor 2* (Abbadie et al., 2003), *Toll-like receptor 2* (Kim et al., 2007) *or 4* (Tanga et al., 2005), and the purinergic receptors *P2rx4* (Tsuda et al., 2003) and *P2rx7* (Chessell et al., 2005) show reduced pain-like behavior after nerve injury.

Pharmacological suppression of the spinal microglial response to nerve injury may therefore constitute a reasonable strategy for preventing and managing neuropathic pain. The antibiotic minocycline (Ledeboer et al., 2005), the methylxanthine propentofylline (Raghavendra et al., 2003), and fluorocitrate, an inhibitor of the citric acid cycle (Clark et al., 2007), all reduce microglial activation and attenuate pain-like behavior in animal models of nerve injury, but they have not been tested clinically. Immunosuppression as a supplement to analgesic therapy has only been studied in postherpetic neuralgia based on the assumption that a peripheral immune response to the reactivation of herpes zoster virus in the DRG contributes to persistent pain in this condition (Kotani et al., 2000; van Wijck et al., 2006). One way forward would be to utilize clinically available drugs and test in animal models the extent to which modulation of the spinal immune response may contribute to the management of neuropathic pain in patients. We have now investigated the effects of low-dose methotrexate and dexamethasone, two immunosuppressive strategies commonly used in patients, on microglial activation in the spinal cord and pain-like behavior in rats after spared nerve injury (SNI), a model of persistent neuropathic pain.

2. Methods

2.1. Nerve injury models

We used adult male Sprague Dawley rats (160–200 g; Charles River Laboratories) for all experiments. Surgery was carried out under 3% isoflurane anesthesia. For spared nerve injury (SNI), two of the three peripheral branches of the sciatic nerve, the common peroneal and the tibial nerves, were ligated with silk (5-0) and distally transected, leaving the third branch, the sural nerve, intact (Decosterd and Woolf, 2000). For chronic constriction injury (CCI), we loosely tied four chromic gut ligatures (4-0) around the proximal sciatic nerve (Bennett and Xie, 1988). Spinal nerve ligation (SNL) consisted of a tight ligation of the L5 spinal nerve (Kim and Chung, 1992). For rhizotomy, we transected the L4 and L5 dorsal roots (Fig. 1). For sham surgery, we exposed the sciatic nerve, the L5 spinal nerve, or the lumbar dorsal roots without injury.

All experiments adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP). The Subcommittee on Research Animal Care (SRAC) of Massachusetts General Hospital, which serves as the institutional animal care and use committee as required by the Public Health Service (PHS), approved the experimental protocol.

2.2. Drug treatment

Methotrexate (Sigma-Aldrich; MTX) stock solutions were prepared in 0.1 mol/L NaOH at 0.5 mg/ml and diluted 1:10 in 0.1 mol/L phosphate buffered saline (PBS) before use; the pH of the solution was adjusted to 7.4–7.6. Dexamethasone (4 mg/ml, Dexa for injection by ct; DEX)

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was diluted in 0.1 mol/L PBS (pH 7.4). For systemic treatment, we injected 0.2 mg MTX or 2.5 mg DEX per kg body weight intraperitoneally once a day, starting 3 days after injury. Behavioral tests were carried out 3 hours after the injections. For continuous intrathecal infusion, MTX and DEX were diluted in artificial cerebrospinal fluid and filled into osmotic pumps (Alzet). The pumps were implanted subcutaneously and connected to 32 G spinal catheters (ReCathCo) immediately after nerve injury or with a delay of 7 days, as indicated. MTX was delivered intrathecally for 14 days at a flow rate of 0.1 mg/kg/day; DEX was infused at a rate of 0.02 mg/kg/day. Control animals received the appropriate vehicle solutions.

2.3. Immunostaining

We perfused terminally anesthetized rats transcardially with 4% paraformaldehyde in phosphate buffer (0.1 mol/L; pH 7.4). The L4 and L5 spinal cord segments were dissected, postfixed for 2 h before being transferred into phosphate-buffered sucrose (20%) for overnight cryoprotection at 4°C. We embedded the spinal cords in Tissue-Tek (Sakura Finetek) and cut transverse sections (10 μm) using a cryotome. Nonspecific binding sites were blocked with 10% normal serum or a mixture of blocking reagent (Roche) and bovine albumin (Sigma-Aldrich) in PBS containing 0.1% Triton X-100 (Sigma-Aldrich). We incubated the sections overnight at 4°C with primary antibodies directed against CD11b (1:1000, Serotec), ionised calcium binding adapter molecule 1 (Iba1) (1:300; Wako), or ED1 antibody (1:300; Serotec). Antibody binding sites were visualized with species-specific secondary antibodies (Molecular Probes). We incubated the stained sections in 70% methanol containing 0.01% Sudan black (Sigma-Aldrich) for 5–10 min at room temperature to reduce lipofuscin-like autofluorescence. For the immunostaining of p38 Mapk phosphorylated at residues threonine 180 and tyrosine 182 (phospho-p38), we heated the slides in a microwave oven for 20 s while covered with sodium citrate buffer (pH 6) to improve antigen retrieval. We incubated the sections over 2 nights at 4°C with a rabbit antibody specific for phospho-p38 (1:100; Cell Signaling) and visualized the binding sites using either a VECTASTAIN Elite ABC Kit (Vector Laboratories) or a secondary antibody conjugated with horseradish peroxidase (1:100; Jackson ImmunoResearch) and a tyramide signal amplification kit (Molecular Probes). We show representative images for the immunostaining results obtained in the spinal cords of 5–6 animals per group. Sections were imaged by confocal microscopy using a LSM 510 or a LSM 5 Pascal system (Zeiss) for laser scanning microscopy.

2.4. Western blot

For immunoblotting, tissue samples were homogenized by sonication in a protein extraction buffer containing 50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA Na_2 dihydrate, 2% sodium dodecyl sulfate, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethyl sulfonyl fluoride, the protease inhibitor cocktail Complete Mini (Roche), and Phosphatase Inhibitor Cocktails 1 and 2 (Sigma-Aldrich). Tissue extracts were subjected to denaturing NuPAGE Novex 4–12% Bis-Tris gel electrophoresis (Invitrogen) and transferred to polyvinylidene fluoride membranes (Bio-Rad). After blocking nonspecific binding sites with 5% nonfat milk in Tris buffered saline (TBS) containing 0.1% Tween 20 for 1 h at room temperature, we incubated the membranes with the primary antibody overnight at 4°C. Antibody-protein complexes were labeled with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, followed by incubation with a chemiluminescent substrate for 1–3 min (SuperSignal West Femto Maximum Sensitivity; Pierce). Chemiluminiscent bands were detected using Hyperfilm ECL (Amersham). We used primary antibodies directed against Iba1 (1:500; Wako), phospho-p38 (1:1000; Cell Signaling), total p38 (1:1000; Cell Signaling), p38β (1:1000; Invitrogen), and glyceraldehyde-3-phosphate dehydrogenase (Gapdh, 1:10,000; Santa Cruz Biotechnology). Before reblotting the membranes with a different antibody, we stripped them using a Re-Blot Plus Western blot recycling kit (Millipore). We normalized the signal intensity of Iba1-immunoreactive bands relative to Gapdh and that of phospho-p38-immunoreactive bands

relative to total p38 Mapk. Dorsal horn samples from 5–6 animals per group were processed and analyzed separately.

2.5. Enzyme immunoassay

To quantify p38 Mapk phosphorylation by EIA, we employed a TiterZyme Enzyme Immunometric Assay Kit (AssayDesigns). The EIA uses a monoclonal antibody against p38 immobilized on a microtiter plate to bind total p38 Mapk. Captured phospho-p38 is detected with a rabbit polyclonal antibody, followed by incubation with a horseradish peroxidase-conjugated antibody directed against rabbit IgG and visualization of the immune complex by addition of the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine. The optical density of the reaction product is directly proportional to the quantity of phospho-p38. Tissue samples from 6 animals per group were assayed and analyzed separately.

2.6. Behavioral tests

All tests were performed by a blinded investigator. After habituation, we determined the threshold for paw withdrawal by graded-strength von Frey monofilaments (0.02, 0.03, 0.05, 0.08, 0.13, 0.22, 0.36, 0.60, 1.00, 1.66, 2.75, 4.57, 7.58, 12.6 and 20.9 g) to assess mechanical allodynia. To measure cold allodynia, we applied a drop of acetone to the plantar hindpaw and measured the time that the animal spent licking, shaking, or lifting the paw during the following 2 min.

2.7. Data analysis

Data are presented as mean ± SEM. We employed a Student's *t*-test to compare the signal intensities of immunoreactive bands in the Western blot analyses and optical densities of the EIA reaction products. We analyzed the effect of MTX and DEX on neuropathic pain-like behavior by calculating the areas under the effect-versus-time curves (AUCs) based on a linear trapezoidal rule and comparing them to the AUCs of vehicle-treated controls using a Student's *t*-test.

3. Results

3.1. Patterns of spinal microglial activation after peripheral nerve and dorsal root injuries

Upon activation, resting microglia upregulates complement receptor type 3 (Streit et al., 1999). Using immunostaining for the αM subunit of this receptor (cluster determinant 11b; CD11b), we found a consistent temporal pattern of microglial proliferation in the SNI, CCI, and SNL models of peripheral nerve injury. Microglial activation was most prominent in the L4 spinal cord segment after SNI and CCI, and in the L5 segment after SNL (Fig. 2). In all models, CD11b immunoreactivity in the ipsilateral dorsal and ventral horns began to increase 3 days after injury, was maximal after 7 days, and decreased after 14 days. After SNI and CCI dense clusters of intensely stained microglia formed particularly in the medial part of the ipsilateral dorsal horn (Fig. 2A and B). Equally dense collections of active microglia with thickened cellular processes ensheathed the cell bodies of motor neurons in the ventral horn (Fig. 2A). At 21 days after SNI microglial activation was still marked, although the cells were more diffusely spread across the medial two thirds of the dorsal horn and the lateral part of the ventral horn. Over the following 3 weeks the microglial activation slowly decreased (Fig. 2C). Compared to SNI and CCI, active microglia was distributed more evenly across the ipsilateral L5 dorsal horn at all times after SNL (Fig. 2B). The morphology and scattered distribution of microglia in the contralateral spinal cord and in sham-operated controls resembled that of resting cells (shown for SNI; Fig. 2A).

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Several other parameters in addition to the increased immunoreactivity for CD11b confirmed a heightened level of microglial activity after nerve injury. Microglial cells in the dorsal horn became intensely immunoreactive for Iba1 (Fig. 3A), a protein involved in membrane ruffling and phagocytosis (Kanazawa et al., 2002). Seven days after SNI, at the peak of microglial activation, Iba1 expression in the ipsilateral dorsal horn increased to 140 ± 9% compared to naïve controls ($p < 0.01$); the Iba1 expression level in the contralateral dorsal horn remained unchanged (Fig. 3B). Similar increases in Iba1 expression occurred after CCI and SNL (Fig. 3C). We furthermore found a large number of cell profiles expressing the rat homologue of human CD68, a lysosome membrane glycoprotein of 110 kDa labelled by the mouse monoclonal antibody ED1 (Fig. 3D). ED1-immunoreactivity is a marker of phagocytic activity (Damoiseaux et al., 1994). ED1-immunoreactive profiles in the dorsal horn occurred within the same distribution as microglia. However, the morphology of these profiles differed from CD11b- and Iba1-positive microglia. ED1-immunoreactive profiles were larger, had an amoeboid shape, and lacked the fine ramifications of resting microglia or the thick cell processes of activated microglia (Fig. 3D), suggesting that these profiles belonged to microglial cells that had transformed into phagocytes (Streit et al., 1999), infiltrating perivascular cells, or circulating macrophages invading the spinal cord across the blood-brain barrier (Sweitzer et al., 2002). The immune response in the dorsal horn was also associated with an increase in cell profiles immunoreactive for phospho-p38 (Fig. 3E), indicating activation of the p38 Mapk signalling pathway. We detected phospho-p38 only in profiles of microglial cells, not neurons (Fig. 3F).

The immune response to transection of the L4 and L5 dorsal roots differed considerably from that observed after peripheral nerve lesions. Following dorsal rhizotomy, we found microglial activation and phagocytes predominantly in the ipsilateral dorsal funiculus of the lumbar and lower thoracic spinal cord, along the path of the central axons of primary sensory neurons (Fig. 4). Active microglia and phagocytes were also present in Clarke's nucleus (Fig. 4), a relay site ventral to the gracile and cuneate columns at spinal levels T1 through L2 where the central projections of proprioceptive primary afferents of the hindlimb terminate. This particular immune response pattern presumably reflects the involvement of microglia and phagocytes in the removal of central axons that undergo Wallerian degeneration (Liu et al., 1998).

3.2. Methotrexate, but not dexamethasone, suppresses the microglial response to peripheral nerve injury

MTX and the glucocorticoid DEX are two drugs commonly employed for immunosuppressive treatment. MTX is an inhibitor of dihydrofolate reductase. MTX blocks the *de novo* synthesis of purine and thymidylate and reduces cell-mediated immune reactions. Multiple mechanisms contribute to the immunosuppressive effects of glucocorticoids. These include inhibition of prostaglandin and leukotriene synthesis, and blockade of cytokine production and release by macrophages and T lymphocytes. We tested the effect of MTX and DEX on nerve injury-induced microglial activation in the SNI rodent model of persistent peripheral neuropathic pain (Decosterd and Woolf, 2000).

Distribution of systemically administered MTX into the central nervous system is limited by a clearance process involving organic anion transporters that produce a brain-to-blood efflux (Kool et al., 1999). Therefore, we delivered MTX directly to the spinal cord using continuous intrathecal infusion, beginning on the day of the nerve injury. We selected a dose of MTX (0.1 mg per kg body weight and day) that was well below the cytotoxic doses employed in rodent models of cerebral tumors (Kooistra et al., 1989). Seven days after SNI, we found very few activated microglial cells in the dorsal horn of animals treated with intrathecal MTX (Fig. 5A). Continuous infusion of MTX caused striking morphological changes in the microglia, with an approximately three-fold increase in size compared to vehicle-treated animals and processes

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that were greatly enlarged and thickened, but less ramified (Fig. 5A). We used the total level of Iba1 expression in the dorsal horn, determined by Western blot, as an indicator of microglial activation, representing a combination of the number of activated microglial cells and the degree of activation in individual cells. Continuous intrathecal administration of MTX for 7 days following SNI decreased Iba1 protein levels in the dorsal horn to 68 ± 8%, compared to vehicle controls ($p < 0.05$; Fig. 5B); after 14 days of treatment, Iba1 expression was reduced to 51 ± 3% ($p < 0.05$). This effect was only observed when the treatment was started immediately after the nerve injury. If the treatment was delayed by 7 days, so that the continuous intrathecal infusion of MTX for 7 days began after the injury-induced microglial activation had reached its maximum, we found no change in CD11b immunostaining in the dorsal horn, the morphology of microglial cells, or Iba1 expression (data not shown). Systemic treatment with daily intraperitoneal injections of 0.2 mg/kg MTX had no effect on the microglial activation in the ipsilateral dorsal horn 7 days after SNI, as determined by CD11b immunostaining and Western blot for Iba1 (data not shown).

Although DEX readily passes the blood brain barrier and distributes within the central nervous system, daily intraperitoneal injections of 2.5 mg/kg DEX did not lead to changes in CD11b immunostaining intensity, the distribution or morphology of active microglial cells (Fig. 5C), or the total Iba1 expression level in the ipsilateral dorsal horn 7 days after SNI (Fig. 5D).

3.3. Methotrexate reduces the nerve injury-induced phosphorylation of p38 Mapk

Two isoforms of p38 Mapk are expressed in the spinal cord. Whereas p38α is found in neurons, microglia primarily expresses p38β (Svensson et al., 2005). Phosphorylation of microglial p38 is involved in the development of pain after peripheral nerve injury (Jin et al., 2003; Svensson et al., 2003; Tsuda et al., 2004; Piao et al., 2006) and contributes to hyperalgesia in animal models of central sensitization (Svensson et al., 2005).

Intrathecal infusion of MTX, beginning at the time of injury, reduced the number of profiles immunoreactive for phospho-p38 (Fig. 6A), reflecting the overall decrease in the density of microglial cells (immunolabeled for CD11b) that we observed in MTX-treated rats after SNI (Fig. 5A). We quantified p38 phosphorylation in the ipsilateral L4 dorsal horn using Western blot and an EIA. In vehicle-treated animals, both total p38 and isoform p38β expression were substantially increased 7 days after SNI, at the peak of microglial activation (Fig. 6B). Phosphorylation levels of p38 were 45% higher compared to uninjured (naïve) rats, as determined by EIA (Fig. 6B). MTX markedly reduced p38 phosphorylation. Seven days after SNI, the phospho-p38 level in rats treated with continuous intrathecal MTX infusion was comparable to that in naïve animals (Fig. 6B).

3.4. Methotrexate attenuates neuropathic pain-like behavior

Microglial activation after SNI was temporally associated with the onset of pain-like behavior. We therefore examined if immunosuppression with MTX prevents or reduces the nerve injury-induced increase in sensitivity to punctate mechanical stimuli (mechanical allodynia) and cold (thermal allodynia).

Continuous intrathecal delivery of MTX, beginning at the time of nerve injury, attenuated both mechanical and cold allodynia ($p < 0.05$; Fig. 7A). The antinociceptive effect became evident 7 days after SNI, when microglial activation was most pronounced, and persisted until the end of the treatment (14 days after injury). Continuous intrathecal MTX infusion that was delayed by 7 days still reduced mechanical allodynia after SNI ($p < 0.01$), but its effect on cold allodynia was not significant (Fig. 7B). To investigate if systemically delivered MTX may contribute to a decrease in neuropathic pain-like behavior by suppressing the cellular immune response to nerve injury in the peripheral nervous system, we treated an independent group of animals with

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daily intraperitoneal injections of MTX (0.2 mg/kg), a dose that prevents joint destruction in models of arthritis (Magari et al., 2003). Systemic treatment with MTX reduced cold allodynia ($p < 0.05$) but had no effect on heightened mechanical sensitivity after SNI (Fig. 7C), indicating that a central site of action is most likely responsible for the attenuation of mechanical allodynia produced by intrathecal MTX, whereas the reduction in cold allodynia may result from immunomodulation in the DRG.

We also tested if DEX reduces neuropathic pain-like behavior and found that daily intraperitoneal injections of 2.5 mg/kg DEX had no effect on mechanical or cold allodynia after SNI (Fig. 8A), although lower doses of DEX (0.1 and 0.5 mg) are sufficient in the rat to suppress joint destruction in chronic arthritis (Cuzzocrea et al., 2005) or zymosan-induced paw inflammation (Tegeder et al., 2001). Continuous intrathecal infusion of 0.02 mg/kg/day DEX for 7 days, beginning on the day of nerve injury, even led to an increase in pain sensitivity (Fig. 8B), probably because the rats failed to tolerate the drug. Weight gain over the treatment period was reduced in rats receiving DEX through either intraperitoneal injection or intrathecal infusion, whereas MTX-treated animals showed normal weight gain.

4. Discussion

SNI and CCI produce partial lesions of the distal sciatic nerve, whereas SNL constitutes a proximal injury that includes nearly all afferents terminating in the L5 dorsal horn. The chromic gut sutures used for CCI generate an inflammatory reaction, which is absent in SNI. Despite these differences, the temporal pattern of microglial activation in the three models of peripheral neuropathic pain was remarkably consistent. In each model, microglial activation was most prominent 7 days after injury. At that time numerous amoeboid cells with reduced ramification expressed a lysosomal antigen immunolabeled by ED1, indicating phagocytic activity. After rhizotomy, activated microglia and phagocytes occurred, but predominantly in the ipsilateral dorsal column and Clarke's nucleus, most likely as part of the immune response to Wallerian degeneration of the lesioned central axons of large caliber primary sensory neurons (Liu et al., 1998; Colburn et al., 1999). In contrast, the clusters of activated microglia and phagocytes in the dorsal horn after SNI, CCI, or SNL cannot be explained by a need to remove detritus from the central terminals of sensory neurons, because primary sensory neurons die late (several weeks) after severing of the distal axon, with a substantial preponderance of small over large cells (Tandrup et al., 2000).

What triggers microglial activation in the spinal cord after nerve injury is unknown. Reduced activation is observed in mice lacking Toll-like receptor 2 (Kim et al., 2007) or 4 (Tanga et al., 2005), chemotactic cytokine receptor 2 (Abbadie et al., 2003), or the ligand-gated ion channel P2rx4 (Tsuda et al., 2003), suggesting involvement of chemokine (C-C motif) ligand 2 (Zhang and De Koninck, 2006) and adenosine triphosphate (ATP). Tonic P2rx4 activation by ATP in spinal microglia leads to release of brain-derived neurotrophic factor, which provokes a depolarizing shift in the anion reversal potential of a subpopulation of dorsal horn lamina I neurons. This causes a paradoxical excitatory effect of γ-aminobutyric acid (GABA) that contributes to nerve injury-induced mechanical allodynia (Coull et al., 2005). Mice deficient of another purine receptor, P2rx7, show no increase in sensitivity to mechanical or thermal stimuli in models of neuropathic or inflammatory pain, presumably because the P2rx7-mediated release of inflammatory cytokines is blocked (Chessell et al., 2005). Other candidates for early activating signals include macrophage colony stimulating factor, interleukin 6, CD200, and the neuronal chemokine fractalkine (Tsuda et al., 2005). Fractalkine-mediated signaling between neurons and microglia appears to enhance pain after CCI or inflammation of the sciatic nerve (Milligan et al., 2005; Zhuang et al., 2007).

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The time course of microglial activation and the occurrence of phagocytes after SNI, CCI, or SNL coincide with the manifestation of nerve injury-induced transsynaptic apoptosis of dorsal horn neurons. Within four weeks after SNI, more than 20% of superficial dorsal horn neurons are lost (Scholz et al., 2005), which might explain increased phagocytic activity. On the other hand, it is unlikely that the degeneration of dorsal horn neurons is responsible for the bulk of microglial activation because clearance of apoptotic cells is usually not accompanied by an inflammatory reaction (Savill and Fadok, 2000).

Although the initial signal that provokes activation of spinal microglia after peripheral nerve injury remains unclear, the contribution of active microglia to the development of pain-like behavior is well established (DeLeo and Yezierski, 2001; Watkins and Maier, 2003; Marchand et al., 2005; Tsuda et al., 2005; Scholz and Woolf, 2007). We demonstrate in the SNI model of persistent neuropathic pain that MTX, a drug commonly used for long-term immunosuppression, inhibits microglial activation in the dorsal horn and provides an analgesic effect. Continuous intrathecal treatment with MTX at a dose that is 50-fold lower than the threshold dose for central nervous system toxicity (Kooistra et al., 1989) substantially reduced the number of activated microglial cells in the ipsilateral dorsal horn after nerve injury. The remaining cells were greatly enlarged, suggesting that MTX, in addition to blocking mitosis, provoked metabolic changes similar to those described in glial cell cultures (Serrano and Schimke, 1990). In order to achieve this marked immunosuppressive effect, the intrathecal infusion of MTX needed to begin early after nerve injury, before microglial activation reached its peak.

Phosphorylation of p38 Mapk is an important component of the spinal microglial response to both nerve injury (Jin et al., 2003; Tsuda et al., 2004) and peripheral inflammation (Svensson et al., 2003; Boyle et al., 2006). Mapk signaling pathways regulate the transcription and mRNA stability of proinflammatory genes such as *tumor necrosis factor*, *interleukin 6*, and *cyclooxygenase 2* (Chang and Karin, 2001). Intrathecal administration of a p38 inhibitor reduces pain-like behavior after CCI and SNL (Jin et al., 2003; Garry et al., 2005), indicating the importance of the p38 Mapk pathway in neuropathic pain. Intrathecal treatment with MTX, beginning at the time of nerve injury, reduced p38 phosphorylation in the dorsal horn of animals after SNI to almost pre-injury levels and decreased both mechanical and cold allodynia, two major features of clinical neuropathic pain. Delayed intrathecal infusion of MTX, beginning 7 days after SNI, at a time when microglial activation was already maximal, still attenuated mechanical allodynia. In contrast, systemic administration of MTX at a dose that reduces joint inflammation in rodents (Magari et al., 2003) had no effect on mechanical allodynia although it diminished the nerve injury-induced hypersensitivity to innocuous cold. Since systemic MTX acts primarily in the periphery (Sweitzer et al., 2002), this suggests that the immune response to injury in the peripheral nerve or the DRG (Hu and McLachlan, 2003; Schafers et al., 2003) is MTX-sensitive and contributes to the development of cold allodynia, whereas heightened sensitivity to mechanical stimuli after nerve injury is mediated by a central mechanism that involves microglial activation in the spinal cord and p38 Mapk phosphorylation (Tsuda et al., 2005).

A reduction of mechanical allodynia after repeated injections (3 per week) of MTX (1 mg/kg) intrathecally and at the nerve lesion site has been reported in a study employing loose ligation of the L5 spinal nerve with chromic gut (Hashizume et al., 2000). Density and morphology of microglial cells in the dorsal horn did not change with this treatment schedule, although the single doses injected were 10-fold higher than the MTX dose delivered continuously over 24 hours in the present study. Sustained intrathecal treatment may therefore suppress microglial activation much more efficiently than intermittent high-dose bolus injections.

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Systemic or intrathecal treatment with the glucocorticoid DEX failed to prevent or reduce the overall activation of microglia or pain-like behavior after SNI. Previous studies on the analgesic potential of glucocorticoids in animal models of neuropathic pain have produced mixed results. Diminished hyperalgesia and allodynia were described in a model of perineural inflammation induced by sciatic nerve ligatures soaked in complete Freund's adjuvant (Clatworthy et al., 1995), and after SNL or spinal nerve transection (Takeda et al., 2004; Xie et al., 2005), two surgical procedures that produce a lesion accompanied by an inflammatory reaction close to the DRG. On the other hand, glucocorticoids failed to attenuate allodynia after sciatic nerve transection (Kingery et al., 1999) or brachial plexus avulsion (Rodrigues-Filho et al., 2004). The efficacy of glucocorticoids in neuropathic pain models therefore appears to be determined by the degree of peripheral inflammation rather than the central immune response, which, we find, is similar after SNL and sciatic nerve injury.

Spinal microglial activation has recently emerged as an intriguing mechanism in the pathogenesis of neuropathic pain. Its consistent occurrence in different models of peripheral nerve injury underscores the potential for immunosuppressive therapy in neuropathic pain management. Our results suggest that glucocorticoids are unlikely to have much utility, except in those conditions of nerve injury that are associated with a sizeable component of peripheral inflammation. A recent clinical study demonstrated, however, neither a substantial nor persistent benefit of epidural steroid injections in patients with sciatica, which involves a mixed neuropathic and inflammatory etiology (Arden et al., 2005). We demonstrate here that intrathecal infusion of MTX, unlike DEX, does have an immunosuppressive and analgesic effect in the SNI model of peripheral neuropathic pain. Intrathecal MTX, given early after nerve injury at a dose that does not cause neurotoxic side effects, substantially reduced microglial activation in the spinal cord. Early intrathecal treatment with MTX reduced the development of neuropathic pain-like behavior while treatment with a later onset decreased established mechanical allodynia. Intermittent systemic treatment with low-dose MTX is a standard therapy for progressive rheumatoid arthritis. However, because of the particular pharmacokinetics of MTX, intrathecal administration would be required to suppress microglial activation in the spinal cord and produce, in this way, an analgesic effect in early neuropathic pain. A less invasive treatment regimen that limits the cellular immune response in the spinal cord would provide a potentially exciting opportunity to inhibit the development of neuropathic pain in the acute phase.

Acknowledgements

Supported by the Deutsche Forschungsgemeinschaft (IT), GlaxoSmithKline (CJW), and the National Institutes of Health (JS, CJW).

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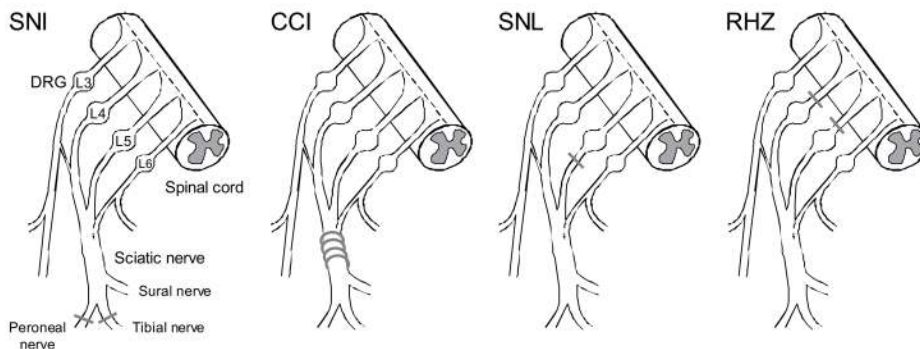

Fig. 1.
Peripheral nerve and dorsal root injuries. Spared nerve injury (SNI) consists of ligating and transecting the peroneal and the tibial nerves, leaving the third peripheral branch of the sciatic nerve, the sural nerve, intact. For chronic constriction injury (CCI), four chromic gut sutures were loosely ligated around the sciatic nerve. For spinal nerve ligation (SNL), the L5 spinal nerve was tightly ligated. Rhizotomy (RHZ) consists of cutting the L4 and L5 dorsal roots proximal to the dorsal root ganglion (DRG).

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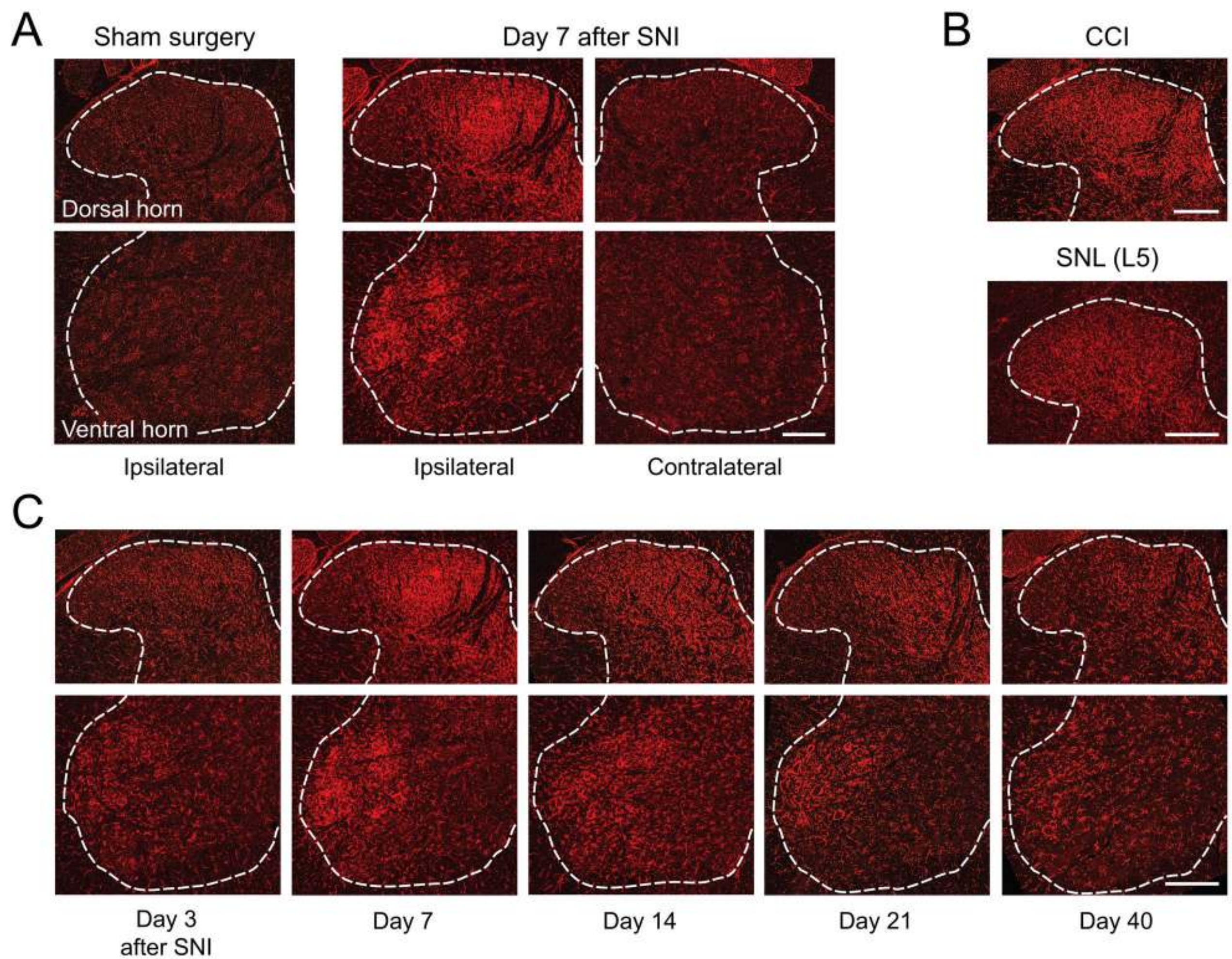

Fig. 2.

Patterns of spinal microglial activation after partial peripheral nerve injury. (A) Activation of microglia (immunolabeled for CD11b) after spared nerve injury (SNI) was most prominent in the ipsilateral dorsal and ventral horns of the L4 spinal cord and peaked 7 days after injury. (B) We also found clusters of active microglia in the medial two thirds of the L4 dorsal horn 7 days after chronic constriction injury (CCI). In comparison, microglial activation was more diffusely spread across the L5 dorsal horn 7 days after spinal nerve ligation (SNL). (C) Microglial activation was sustained for 3 weeks after SNI. Scale bars, 200 μm.

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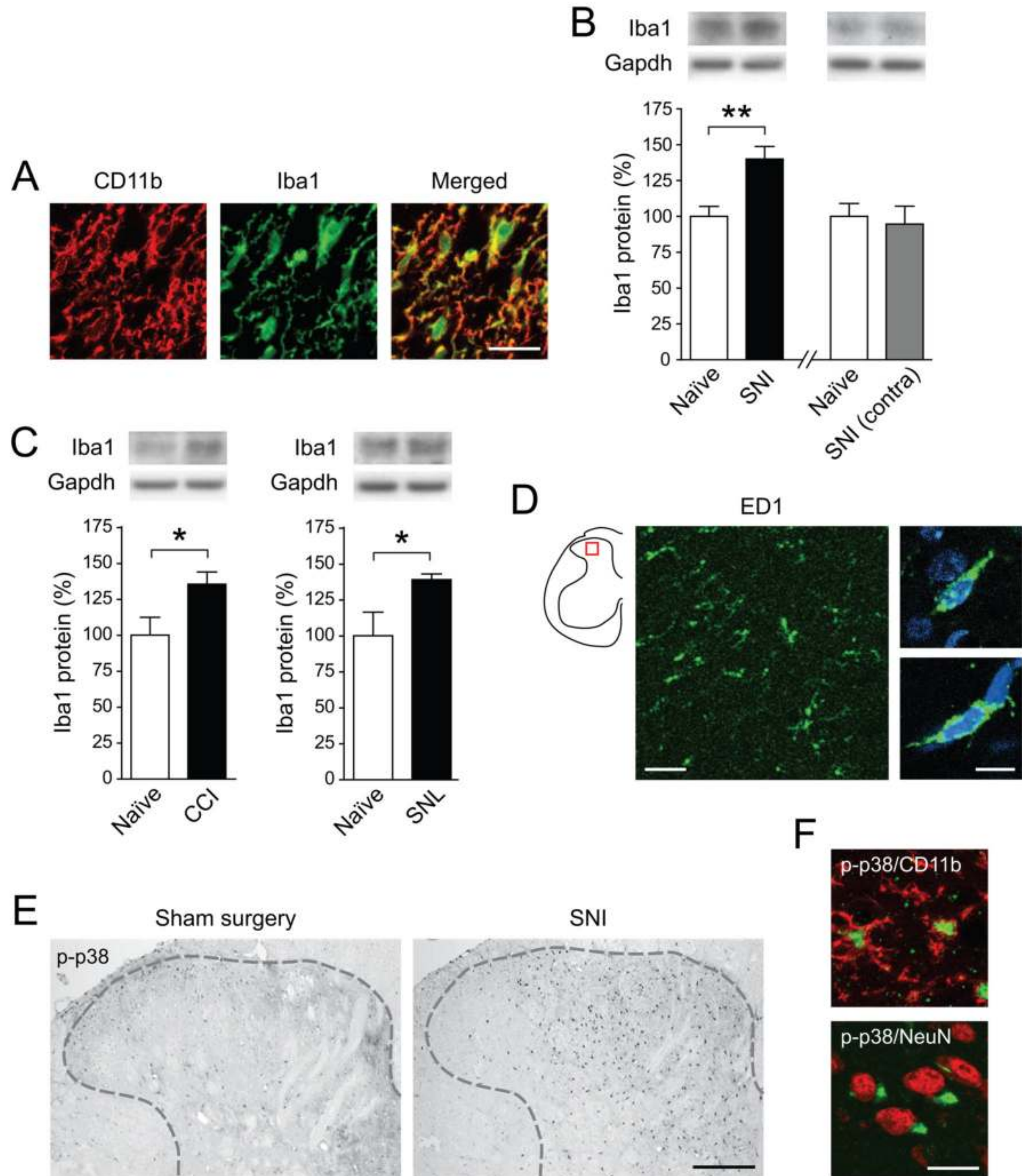

Fig. 3.

Indicators of microglial cell activation in the L4 dorsal horn 7 days after spared nerve injury (SNI). (A) Microglial cells (CD11b) in the dorsal horn expressed ionised binding calcium adapter molecule 1 (Iba1). (B) Seven days after SNI, the overall expression level of Iba1 in the ipsilateral dorsal horn increased by 40% compared to naïve controls. Iba1 expression in the contralateral dorsal horn was unchanged (n = 5–6). (C) We found a similar increase in Iba1 expression in the ipsilateral dorsal horn 7 days after CCI and SNL (n = 5–6). (D) Amoeboid phagocytes immunoreactive for the rat homologue of lysosomal CD68 (immunolabeled by an ED1 antibody; green) occurred within the areas of microglial activation. Nuclear chromatin counterstained with bisbenzimide (blue). (E) Numerous phospho-p38-immunoreactive profiles

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were found across the medial part of the ipsilateral L4 dorsal horn 7 days after SNI. (F) Phospho-p38 (green) was detected in activated microglia (CD11b; red), but not neurons (neuronal nuclei protein, NeuN; red). Gapdh, glyceraldehyde-3-phosphate dehydrogenase. * $p < 0.05$, ** $p < 0.01$. Images in A and E represent projections of z-stacks consisting of optical slices of 0.5-μm and 1-μm thickness. Scale bars indicate 20 μm (A, large panel in D, and F), 10 μm (small panels in D), or 200 μm (E).

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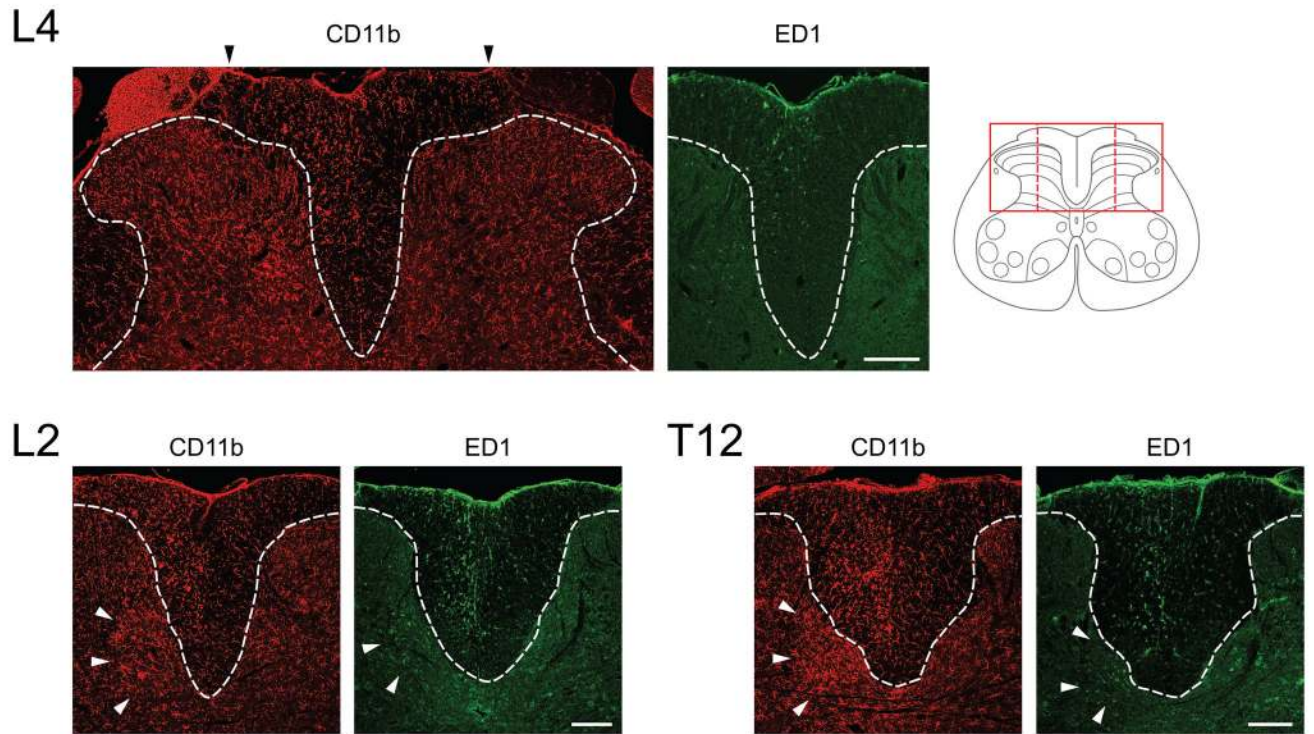

Fig. 4.

Immune response after rhizotomy. Seven days after a transection of the L4 and L5 dorsal roots, active microglia (CD11b) and phagocytes (ED1) occurred predominantly in the ipsilateral dorsal funiculus of the L4 and L5 spinal segments; compared to the peripheral nerve injuries, microglial activation was less pronounced within the dorsal horn gray matter. Microglial and phagocyte activation in the dorsal funiculus extended into the L2 and T12 spinal levels. Here, we also found active microglia and phagocytes in Clarke's nucleus (arrowheads), a relay site for proprioceptive afferents of the hindlimb. The left panel in the depiction of the immune response in the L4 spinal cord is a montage of 3 confocal images (image boundaries indicated by arrows; see scheme). Scale bars, 200 μm.

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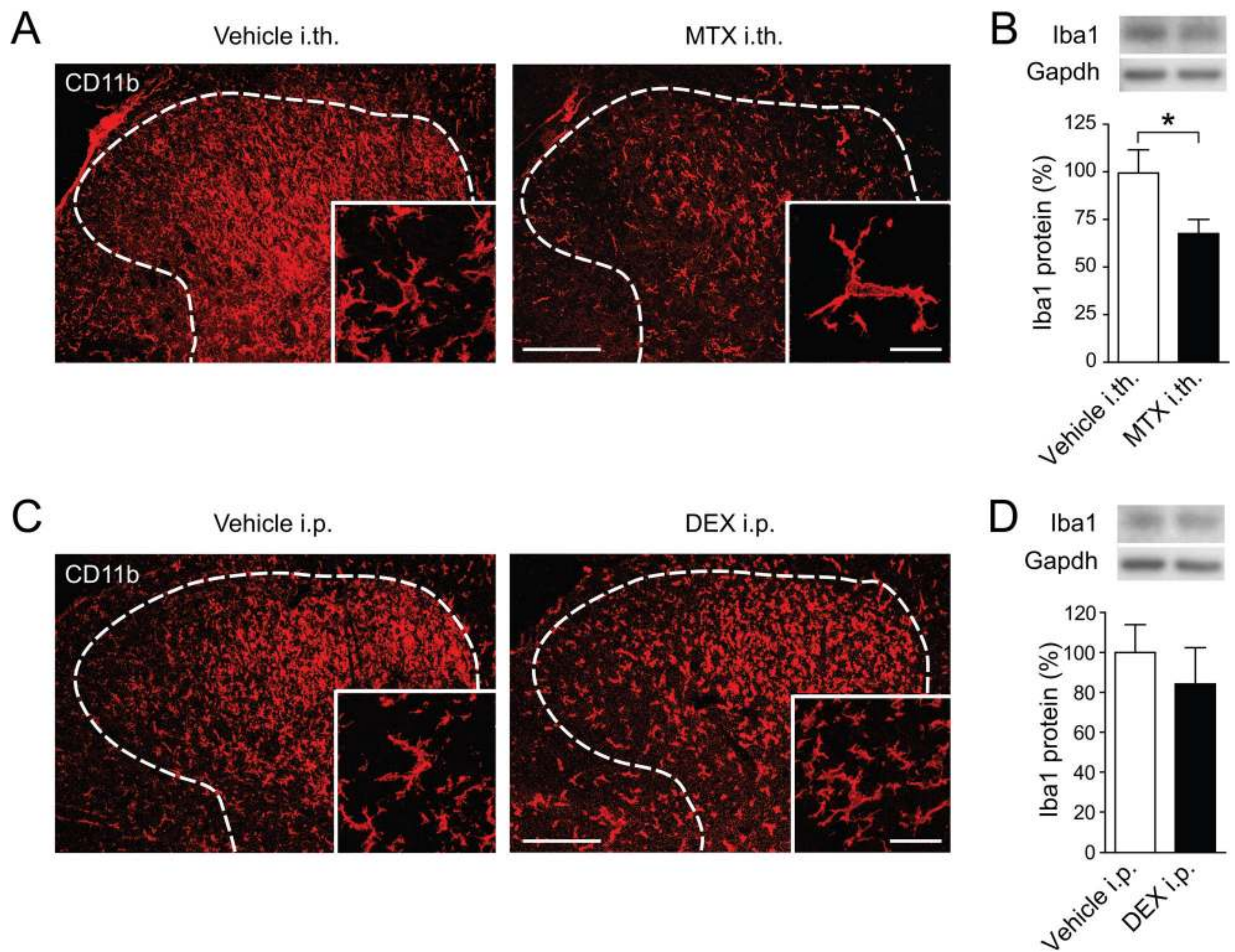

Fig. 5.

Low-dose methotrexate (MTX), but not dexamethasone (DEX), inhibited microglial activation in the ipsilateral L4 dorsal horn 7 days after spared nerve injury (SNI). (A) Continuous intrathecal administration of MTX (0.1 mg/kg/day), beginning at the time of nerve injury, led to a marked decrease in microglial cells (CD11b). Microglial cells in animals treated with MTX were approximately three-fold bigger compared to vehicle-treated controls and had greatly enlarged and thickened processes. (B) Continuous intrathecal treatment with MTX reduced Iba1 expression in the ipsilateral dorsal horn by more than 30% compared to vehicle-treated controls (n = 5–6). (C) The distribution and morphology of activated microglia (CD11b) were unchanged in animals receiving daily intraperitoneal injections of DEX (2.5 mg/kg/day). (D) Systemic treatment with DEX had no effect on Iba1 expression in the ipsilateral dorsal horn (n = 5–6). Gapdh, glyceraldehyde-3-phosphate dehydrogenase. * $p < 0.05$. Inserts in A and C are projections of z-stacks obtained at 100 × magnification (slice thickness, 1 μm). Scale bars in A and C indicate 200 μm (large panels) or 20 μm (inserts).

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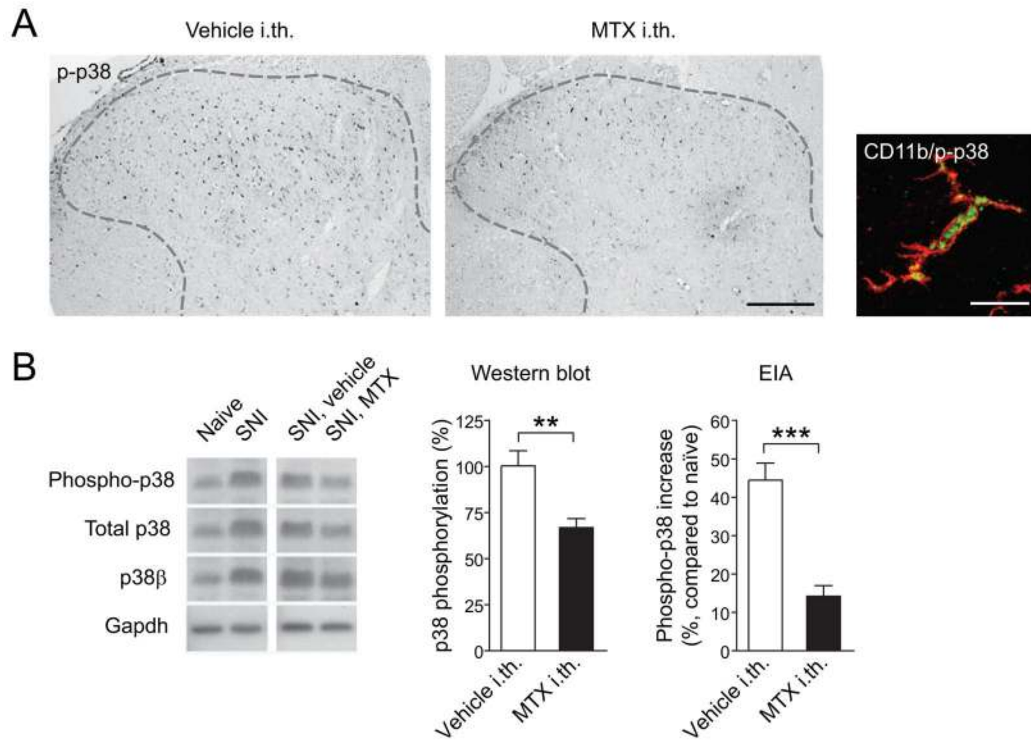

Fig. 6.

Continuous intrathecal infusion of methotrexate (MTX) for 7 days, beginning at the time of spared nerve injury (SNI), decreased microglial p38 Mapk phosphorylation. (A) Microglial cells (CD11b; red) in animals treated with intrathecal MTX were still active, as indicated by the presence of phospho-p38 (green). However, MTX largely reduced the number of phospho-p38-immunoreactive profiles in the ipsilateral dorsal horn. (B) Total expression of microglial p38β and p38 phosphorylation were increased in the ipsilateral dorsal horn 7 days after SNI. MTX substantially diminished the nerve injury-induced increase in p38 phosphorylation, as demonstrated by Western blot analysis ($n = 5–6$) and enzyme immunoassay (EIA) ($n = 6$). Gapdh, glyceraldehyde-3-phosphate dehydrogenase. ** $p < 0.01$; *** $p < 0.001$. The small panel in A shows a projection of a z-stack obtained at 100 × magnification (slice thickness, 1 μm). Scale bars indicate 200 μm (large panels in A), or 20 μm (small panel).

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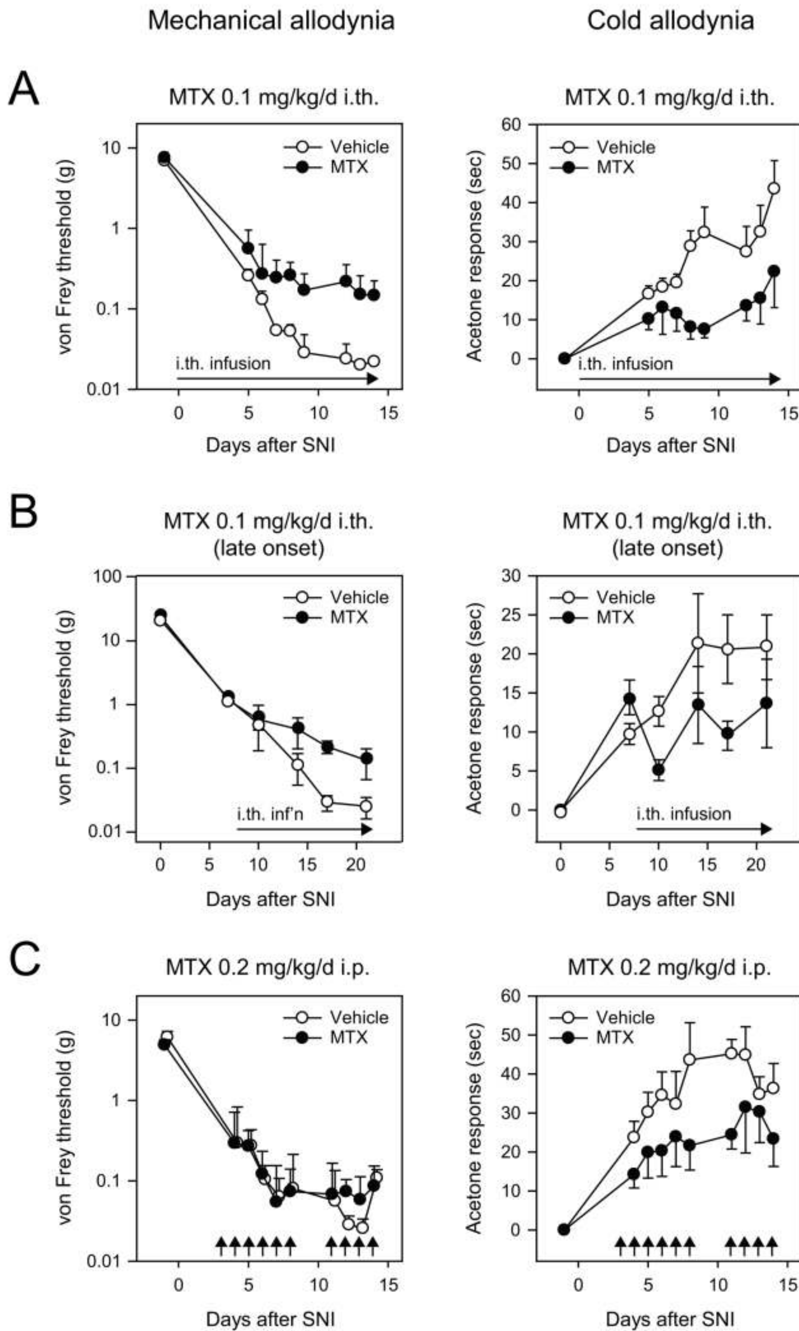

Fig. 7.

Immunosuppression with methotrexate (MTX) attenuated neuropathic pain-like behavior. (A) Continuous intrathecal delivery of MTX (0.1 mg/kg/day), beginning at the day of nerve injury, decreased both punctate mechanical allodynia (tested with von Frey filaments) and cold allodynia (acetone) after spared nerve injury (SNI) ($p < 0.05$). (B) Later intrathecal infusion of MTX, beginning after microglial activation had reached its peak at 7 days following SNI, reduced existing mechanical allodynia ($p < 0.01$). The decrease in cold allodynia was not significant. (C) In contrast, daily intraperitoneal injections of MTX (0.2 mg/kg/day; arrows) attenuated cold allodynia after SNI ($p < 0.05$) but did not diminish the nerve injury-induced increase in sensitivity to punctate mechanical stimuli. n = 7–9 for all behavioral tests.

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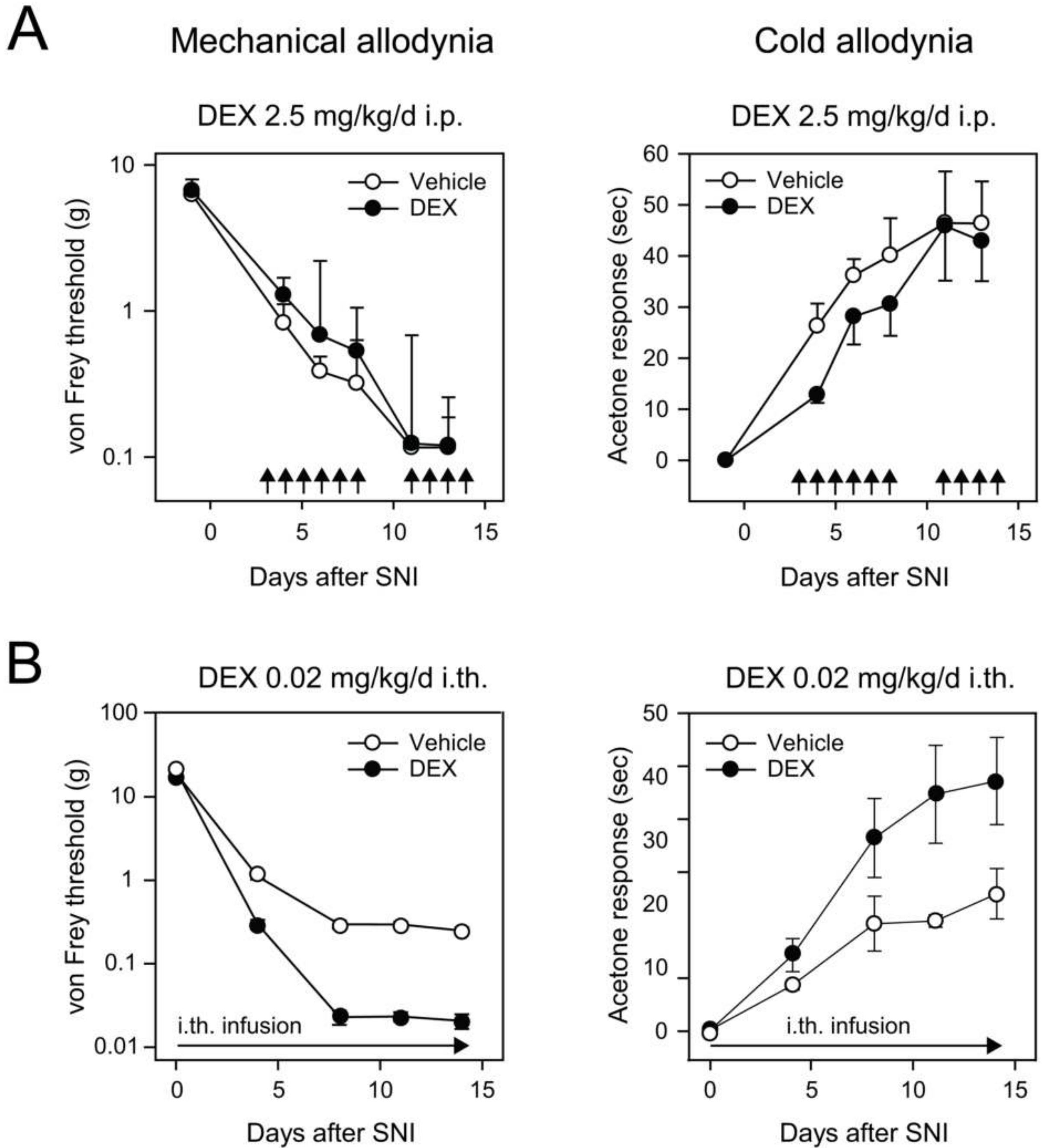

Fig. 8.

Treatment with dexamethasone (DEX) had no effect on pain-like behavior after spared nerve injury (SNI). (A) Daily intraperitoneal injections of DEX (2.5 mg/kg/day; arrows) did not reduce mechanical or cold allodynia. (B) Pain-like behavior after SNI increased in rats treated with continuous intrathecal infusion of DEX (0.02 mg/kg/day), most likely reflecting a heightened general irritability of the animals which failed to tolerate DEX. $n = 8–9$.