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Early Blockade of Injured Primary Sensory Afferents Reduces Glial Cell Activation in Two Rat Neuropathic Pain Models

Wenrui Xie, Judith A. Strong, and Jun-Ming Zhang

Pain Research Center, Department of Anesthesiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0531, USA

Abstract

Satellite glial cells in the dorsal root ganglion (DRG), like the better-studied glia cells in the spinal cord, react to peripheral nerve injury or inflammation by activation, proliferation, and release of messengers that contribute importantly to pathological pain. It is not known how information about nerve injury or peripheral inflammation is conveyed to the satellite glial cells. Abnormal spontaneous activity of sensory neurons, observed in the very early phase of many pain models, is one plausible mechanism by which injured sensory neurons could activate neighboring satellite glial cells. We tested effects of locally inhibiting sensory neuron activity with sodium channel blockers on satellite glial cell activation in a rat spinal nerve ligation (SNL) model. SNL caused extensive satellite glial cell activation (as defined by GFAP immunoreactivity) which peaked on day 1 and was still observed on day 10. Perfusion of the axotomized DRG with the Na channel blocker tetrodotoxin (TTX) significantly reduced this activation at all time points. Similar findings were made with a more distal injury (spared nerve injury model), using a different sodium channel blocker (bupivacaine depot) at the injury site. Local DRG perfusion with TTX also reduced levels of nerve growth factor (NGF) in the SNL model on day 3 (when activated glia are an important source of NGF), without affecting the initial drop of NGF on day 1 (which has been attributed to loss of transport from target tissues). Local perfusion in the SNL model also significantly reduced microglia activation (OX-42 immunoreactivity) on day 3 and astrocyte activation (GFAP immunoreactivity) on day 10 in the corresponding dorsal spinal cord. The results indicate that early spontaneous activity in injured sensory neurons may play important roles in glia activation and pathological pain.

Keywords

satellite glial cells; spontaneous activity; GFAP; spinal nerve ligation; spared nerve injury; nerve growth factor

Recent research has illuminated the complex, two-way communication between glia and neurons. In several pain models, peripheral nerve injury or chronic inflammation has been shown to cause activation of microglia and astrocytes at the corresponding level of the spinal cord. Activated glia proliferate, undergo morphological changes, and show increased expression of the markers glial fibrillary acidic protein (GFAP; astrocytes) or OX-42 (microglia) as well as increased levels of activated MAP-kinases and various molecules thought to play direct roles in pain processing, including prostaglandins, proinflammatory cytokines, and neurotrophins. The importance of these glia responses to pathological pain states has been demonstrated by numerous studies. For example, intrathecal injection of glia-specific

inhibitors can prevent development of neuropathic pain, and injection of activated glia into the spinal cord can cause pathological pain behaviors in uninjured animals (for review, see (DeLeo et al., 2004, Ji et al., 2006, Watkins et al., 2007)).

In the sensory ganglia, the main type of glial cell in the cellular regions is the satellite glial cell (SGC). The SGC, which have some characteristics of both astrocytes and microglia, normally form a single layer wrapped around each sensory neuron soma, a distinctive structure not seen in the central nervous system (Hanani, 2005). The SGCs respond to sensory neuron damage or inflammation by proliferating and expressing GFAP, similar to glia responses in spinal cord and brain. They may also begin expressing neurotrophins, activated MAP kinases, and other molecules that may contribute to the abnormal pain sensitivity. They demonstrate increased gap junction coupling with each other and with neurons, which also contributes to enhanced pain responses (Woodham et al., 1989a, Stephenson and Byers, 1995, Elson et al., 2004, Watkins et al., 2007, Zhang et al., 2007). However, perhaps due to the greater difficulty in applying reagents to the DRG *in vivo*, there is much less known about the possible causal role of SGC in pathological pain states.

The mechanism by which peripheral nerve injury or inflammation causes activation of glia is not well understood. One possibility is abnormal spontaneous activity in the sensory neurons. Spontaneous activity is a common feature of chronic pain models, and generally begins early after injury or inflammation, well before other changes such as spinal glia activation are observed (Govrin-Lippmann and Devor, 1978, Wall and Devor, 1983, Study and Kral, 1996, Hu and Xing, 1998, Liu et al., 2000, Xie et al., 2006). Experiments using a variety of pain models and blockade methods demonstrate that blocking this spontaneous activity is a highly effective way to prevent development of pathological pain behaviors (Xiao and Bennett, 1995, Yoon et al., 1996, Boucher et al., 2000, Lyu et al., 2000, Lai et al., 2002, Chaplan et al., 2003, Xie et al., 2005). Early blockade of spontaneous activity is also effective at reducing other pathological changes, such as sympathetic sprouting into the DRG (Zhang et al., 2004, Xie et al., 2007). There are a number of known mechanisms by which excess neuronal activity might plausibly affect activity of nearby glia. These include activity-induced neuronal release of substances such as fractalkine, substance P, ATP, nitric oxide, and glutamate, that are known regulators of glia cells (Hanani, 2005).

In fact, a recent study showed that in the spared nerve injury (SNI) model of neuropathic pain, blockade of the sciatic nerve (to prevent spontaneous activity at the injury site from reaching the DRG) reduced microglia activation in spinal cord (Wen et al., 2007). In the present study, we were interested in determining whether blocking spontaneous activity could also block activation of the glial cells in the DRG and spinal cord in two established rat models of neuropathic pain.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague-Dawley rats weighing 200–250 g were used for all experiments. Rats were housed one or two per cage under a controlled diurnal cycle of 12 h light and 12 h dark with free access to water and food. The ambient environment was maintained at constant temperature ($22 \pm 0.5^\circ\text{C}$) and relative humidity (60–70%). All the surgical procedures and the experimental protocol were approved by the institutional animal care and use committee of the University of Cincinnati (Cincinnati, OH).

Procedures for spared nerve injury, spinal nerve ligation, and local nerve blockade
Spared nerve injury (SNI)—Rats were anesthetized with isoflurane. The sciatic nerve and its three terminal branches, the sural, common peroneal and tibial nerves, were

exposed at low-thigh level. The common peroneal and tibial nerves were tightly ligated with 6–0 silk. The nerve distal to the ligature was sectioned and 2–4 mm of the nerve stump was removed. Great care was taken to avoid any contact with or stretching of the intact sural nerve. For suppressing spontaneous afferent activity in injured sciatic nerve, both severed nerve branches and the more proximal area before the sciatic nerve divides were completely and evenly covered with 200 mg bupivacaine OH powder immediately after the sciatic nerve was injured. This procedure has previously been demonstrated to reduce transmission of sensory signals for 3–4 days (Xie et al., 2005). In experiments to control for possible systemic effects of the bupivacaine OH powder, the same procedure was used to make the SNI model, but the bupivacaine OH powder was deposited between the gluteus superficialis and biceps femoris muscles, which are located at the right lateral surface of the rat hind leg, and have no contact with the sciatic nerve.

Spinal nerve ligation (SNL)—Rats were anesthetized with isoflurane. An incision was made on the back between L2 and S1. The L4 spinal nerve was exposed and tightly ligated with 6-0 silk and cut about 5 mm distal to the ligature. L4 was chosen for ligation instead of L5 because it was anatomically more accessible for local perfusion. An osmotic pump filled with TTX (250 µg/ml, flow rate: 1 µl/hr for 7 days) or artificial cerebrospinal fluid (ACSF, vehicle) was implanted to perfuse the axotomized DRG through a fine tubing inserted into the sectioned spinal nerve. In experiments to control for possible systemic effects of TTX, the same procedure was used to make the SNL model and implant the osmotic pump filled with TTX, but no tubing was attached to the pump.

In control experiments to determine the likelihood of the TTX diffusing into the spinal cord from the perfused DRG, TTX was replaced with fluorescein (Sigma), which has a molecular weight similar to that of TTX (332 vs. 319 gram/mole). For these experiments, fluorescein in DRG and dorsal root was examined with a whole mount DRG preparation (Xie et al., 2007) and spinal cord sections were prepared from fresh tissue without fixation to avoid possible washing out of fluorescein.

Immunohistochemical staining of GFAP and NGF in sectioned DRG and spinal cord tissue, and of OX-42 in spinal cord tissue

On postoperative 6 hours, 24 hours, day 3, 6, 10 or 14, as indicated, rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.). For OX-42 staining, rats were fixed by perfusing 200–300 ml of periodate-lysine-paraformaldehyde fixative (4% paraformaldehyde in lysine (75 mM)–0.1 M phosphate buffer, and 10 mM sodium periodate, pH=7.4) through the left ventricle of the heart. For GFAP and NGF staining, rats were fixed by perfusing 200–300 ml of Zamboni's fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH=7.4) through the left ventricle of the heart.

Ipsilateral DRGs (L4 for the SNL model, L4 and L5 for the SNI model) were removed. In experiments in which spinal cord sections were examined, sections were taken at the L4 level. Tissue was post-fixed in the perfusion fixative for 30 minutes (OX-42) or 2 hours (NGF and GFAP) at room temperature. The ganglia and spinal cord were horizontally sectioned with a Cryostat at thicknesses of 8 µm and 30 µm, respectively. DRG sections were incubated in antibody to GFAP (Immunostar, Hudson, WI) at a dilution of 1:100 and Alexa Fluor 488 conjugated mouse anti neuronal nuclei monoclonal antibody (Neu-N; 1:100) (Millipore, Billerica, MA), or antibody to NGF (Millipore) at a dilution of 1:500 overnight at 4°C, followed by reaction with secondary antibody conjugated to Alexa Fluor 594 (1:1000, Invitrogen, Carlsbad, CA) for 1 hour at room temperature. Free floating spinal cord sections were incubated in antibody to GFAP at a dilution of 1:200 overnight at 4°C, followed by reaction with secondary antibody conjugated to Alexa Fluor 594 (1:1000, Invitrogen), or in mouse anti-rat

CD11B monoclonal antibody (Millipore) at a dilution of 1:500 overnight at 4°C, followed by reaction with goat anti-mouse secondary antibody conjugated to Alexa Fluor 594 (1:1000, Invitrogen). After drying, the sections were mounted on coverslips with Vector Hard Set mounting medium (Vector Laboratories Inc., Burlingame, CA, USA).

Slides from control and experimental groups were labeled with numbers so that the person performing the image analysis was blinded as to the experimental group. In addition, all images were captured and analyzed by an investigator other than the one who performed immunohistostaining to avoid possible bias. Images from ~10 sections of each DRG and ~20 to 30 sections of each spinal cord sample were captured under a confocal microscope using Slidebook 4.1 imaging acquisition software (Intelligent Imaging Innovation, Denver, CO). In the DRG sections, to measure the density of satellite glia activation, number of neurons encircled by GFAP-positive glia was counted and then normalized by the total number of neurons in the analyzed image area to give a percentage of neurons surrounded by activated SGC. To measure the expression of NGF in the DRG neurons, the summed intensities of NGF signal were measured and normalized by the cellular area in each analyzed section to give an intensity ratio. In the spinal cord, the summed intensity of GFAP or OX-42 signal in the dorsal horn was also measured and normalized by the analyzed image area to give an intensity ratio, which was compared between different experimental groups. In all immunohistochemical experiments, data from at least three animals were included to control for interanimal variability.

Data analysis—Analysis of the effects of nerve blockade at different time points on quantitative measures derived from immunohistochemical data were conducted using one-way analysis of variance with the Bonferonni's Multiple Comparison post-test to determine which points differed significantly from control and on which days the nerve blockade effect was significant. For these analyses multiple measurements from one animal were used to calculate the average value for that animal, and the number of animals was used as the N value for statistical tests.

RESULTS

Effects of nerve blockade on satellite glia activation in the DRG

In SNL model, the activation of SGC (as measured by the percent of DRG neurons surrounded by GFAP-positive glia) in the axotomized L4 ganglion was elevated more rapidly than in the SNL model. Six hours after SNL, 0.07% of neuronal profiles were surrounded by GFAP-positive glia which was not significantly different from that seen in control animals (1.04%). However, marked glia activation was observed as early as the first 24 hours after spinal nerve ligation (Fig 1). Glia activation in this model peaked on POD 1, then gradually declined but was still significantly elevated above control on POD 10 (Fig. 1, Fig. 2). Local perfusion infusion of the DRG with the neuronal activity blocker TTX for the first 7 days after spinal nerve injury significantly inhibited the satellite glia activation. On POD 10, i.e. 3 days after the pump was emptied (Fig. 1 and Fig. 2A), satellite glia activation in the DRG treated with TTX was not significantly elevated above control. In order to control for systemic effects of TTX, additional experiments were done in which the TTX perfusion pump was implanted at the time of SNL but the pump was allowed to discharge under the skin instead of being directed into the DRG with fine tubing. When the TTX was applied systemically in this way, rather than directly into the DRG, the reduction in satellite glia activation by TTX was no longer observed. Instead, when examined on POD 6, systemically applied TTX actually increased the percentage of neurons surrounded by activated glia, from 53.4 ± 5.4 (observed on POD 6 in untreated animals) to $95.1 \pm 1.3\%$, $p < 0.0001$.

An additional experiment was performed to estimate whether TTX perfused into the L5 DRG was likely to significantly increase TTX levels in the corresponding level of the spinal cord. In this experiment, fluorescein (250 µg/ml) instead of TTX was put into the osmotic pump. Fluorescein was chosen as a molecule with molecular weight quite close to that of TTX. Microscopic observation of the DRG in whole-mount showed that fluorescein was readily observed on POD 3 in the perfused L4 DRG and its dorsal root, but not in the adjacent L3 or L5 DRG, or in spinal cord sections (30 µm) to which these DRG project.

In order to determine how general this effect of neuronal blockade on SGC activation, might be, we conducted a similar experiment using the SNI model. In this case, the local nerve blockade was done with a depot form of bupivacaine placed at the injury site, which is much further away from the DRG than the spinal nerve ligation site (see Methods). In the SNI model, satellite glia activation was slower than in the SNL model. It was not significant at POD 1, peaked on POD 3, then began to gradually decline. Nerve blockade at the injury site significantly reduced the amount of glia activation (Fig. 2). In experiments to control for possible systemic effects of the bupivacaine, a similar amount of the drug was deposited between the gluteus superficialis and biceps femoris muscles, which are located at the right lateral surface of the rat hind leg, and have no contact with the sciatic nerve. Glia activation was evaluated on POD 3. In these experiments, the reduction in glia activation by bupivacaine was no longer observed; glia activation with off-target bupivacaine was not significantly different than seen in the SNI model with no bupivacaine present ($67.6 \pm 5.4\%$ vs. $54 \pm 4.6\%$, $p = 0.17$).

Effects of nerve blockade on NGF levels in DRG

One proposed role for activated glia is synthesis of NGF after axotomized cells lose the supply from target tissues. We therefore also examined the effects of early nerve blockade on NGF immunoreactivity in the DRG (Fig. 3 and 4). As previously observed by others (see Discussion), the NGF immunoreactivity in the DRG was significantly altered after spinal nerve ligation. NGF immunoreactivity in the cellular regions of the DRG was dramatically reduced within 24 hours after spinal nerve injury. However, NGF levels recovered back to normal levels on POD 3, followed by decreases at POD 7 and POD 10. The early decrease in NGF on POD1 was not affected by early neuronal blockade with TTX. However, the subsequent recovery of NGF levels on POD3 was significantly less in DRG perfused with TTX for the first 7 days after spinal nerve ligation.

Effects of SNL and nerve blockade on microglia activation in spinal cord

Previous studies (see Discussion) have shown that SNL causes a significant activation of microglia in the spinal cord which is largest around POD 3 – 7, a finding we confirmed in preliminary experiments. It is plausible that abnormal activity in the injured DRG is one important signal for this microglia activation, so we also examined the effect of nerve blockade in the axotomized DRG on microglia activation in the corresponding level of the spinal cord. SNL caused a significant increase in OX-42 staining in the ipsilateral dorsal spinal cord on POD3 (Fig 5, 6). The microglia activation on POD 3 in the ipsilateral dorsal spinal cord was significantly increased (4.7 fold) from control; there was a tendency for increased activation (1.7 fold) on the contralateral side as well though this was not significant after correction for multiple testing. Nerve blockade via perfusion of the axotomized DRG with TTX starting at the time of injury led to a significant reduction of OX-42 staining on POD 3 compared to vehicle treated animals, although microglia activation in the TTX-treated animals was still significantly greater than in uninjured controls.

Effects of nerve blockade on astrocyte activation in spinal cord

Preliminary experiments confirmed previously published reports that astrocyte activation in spinal cord (as measured by GFAP staining) was much slower than SGC and spinal microglia activation, and was significant on POD 10 but not at earlier time points (POD 3, 7). Hence POD 10 was chosen for testing the effects of early nerve blockade (Fig. 6, 7). The increase in GFAP immunoreactivity in the dorsal horn on POD 10 was significantly greater ipsilateral to the injury (3.0-fold vs. 1.7 fold); the trend for increased GFAP intensity on the contralateral side did not reach significance compared to normal animals. Nerve blockade via perfusion of the injured site with TTX for the first 7 POD led to a significant reduction of SNL-induced ipsilateral GFAP staining on POD 10.

DISCUSSION

In this study, we demonstrate that local perfusion of the DRG with the sodium channel blocker TTX markedly reduced SGC activation in the axotomized DRG after spinal nerve ligation. Control experiments indicated that TTX was unlikely to be acting systemically or at the spinal cord level. The generality of the result was confirmed by using another model, the spared nerve injury model, along with a different local blockade method, a depot form of bupivacaine placed at the injury site, which is relatively far from the DRG. The observed time courses of satellite glia activation in these two models were also consistent with a causal role for spontaneous activity. Satellite glia activation after spinal nerve ligation was very rapid, showing a peak on POD 1. Spontaneous activity in this model is also evident very soon after the ligation, showing a peak in both average firing frequencies and percentage of spontaneously active fibers on POD1 (Sun et al., 2005). In contrast, we observed that the peak of glia activation after SNI did not occur until day 3. To our knowledge no one has detailed the time course of spontaneous activity after SNI, but in the sciatic nerve transection model, in which the entire sciatic nerve rather than just 2 branches is transected at a similar anatomical level, spontaneous activity from the injury site is not observed at 24 hours, but begins to rise sharply on POD 3 (Govrin-Lippmann and Devor, 1978). A time course of SGC activation similar to that we observed in the SNI model (low on day 1, significant by day 3) was observed in trigeminal ganglia following a peripheral tooth injury (Stephenson and Byers, 1995).

Abnormal spontaneous activity is observed in models of both neuropathic and inflammatory pain (Zhang et al., 1997, Devor, 1999, Xie et al., 2006). The importance of this ectopic activity, which often precedes other pathological changes, is demonstrated by experiments showing that early blockade of this activity is highly effective in blocking development of pathological pain. Such early blockade can also interrupt other pathological changes, including maintenance of spontaneous activity at later time points, and sprouting of sympathetic fibers into the DRG (Xie et al., 2007). Our study suggests that satellite glia activation, another pathological change observed in many pain models (Fenzi et al., 2001, Hanani et al., 2002, Ohtori et al., 2004, Huang et al., 2005, Peters et al., 2005, Dublin and Hanani, 2007), may also be triggered at least in part by abnormal neuronal activity. The literature suggests many plausible mechanisms by which abnormal neuronal activity might be communicated to the neighboring satellite glia cells. A very simple mechanism is K^+ accumulation in the restricted extracellular space between neurons and their SGC sheath. In cultured astrocytes, elevation of K^+ can lead to changes in GFAP expression (Canady et al., 1990). Active neurons might also influence nearby SGC by releasing neuropeptides, ATP, other transmitters, or cytokines (Fields and Stevens, 2000, Nakatsuka et al., 2001, Rydh-Rinder et al., 2001, Sanada et al., 2002, Skoff et al., 2003, DeLeo et al., 2004); for which satellite glia cells express receptors (Hanani, 2005, Burnstock, 2007, Li et al., 2008). DRG neurons can release such messengers from their soma as well as from their terminals, in an activity-dependent fashion (Harding et al., 1999, Sanada et al., 2002, Zhang et al., 2007); somatically released molecules would be very close to surrounding satellite

glia cells. Such a mechanism has been proposed based on studies of neuronal calcitonin gene related peptide and its receptor on adjacent SGC in the trigeminal ganglion (Li et al., 2008). Electrical recordings of single glial cells and neurons suggests that neuronal activity is more intricately linked with that of glial cells than previously thought (Murphy et al., 1993, Schmidt et al., 1999, Gunzel and Schlue, 2000). For example, depolarization and hyperpolarization of glial membranes has been measured in response to similar electrical activity in adjacent neurons (Lohr and Deitmer, 1999).

Although many mechanisms might plausibly account for activation of neighboring glia by spontaneously active sensory neurons, this raises the question of why normal neuronal activity does not cause some glia activation. In normal DRG, unlike normal CNS, there is virtually no expression of GFAP, the commonly used activation marker for both satellite glia and CNS astrocytes. In the CNS, it is thought that neuronal activity can release local factors that may either inhibit or stimulate microglial activation (Biber et al., 2007), so it is plausible that in the DRG neurons as well that increasing activity could lead to a qualitative shift in neuronal effects on glia. One possibility to be considered is that bursting activity, a subset of observed forms of spontaneous activity which is quite rare in normal sensory neurons, is particularly effective at activating nearby glia cells. It is known in other systems that the pattern of activity can strongly affect what transmitters are released. Another, not mutually exclusive, possibility is that normal neuronal activity is not strong enough to lead to glia activation. Activated glial cells are known to release substances that in turn enhance the excitability of the neighboring neurons. For example, stimulation of DRG neurons induces somatic release of ATP, which can activate receptors on the satellite glia cells and potentiate their release of tumor necrosis factor- α , which in turn enhances the excitability of the neuron (Zhang et al., 2007). The existence of such positive feedback loops means that the relationship between neuronal activity and glia activation could be highly nonlinear. Hence, we do not view our study as evidence that abnormal neuronal activity is the sole cause of satellite glia activation – indeed, neuronal blockade was only partially effective at blocking satellite glia activation – but rather, we propose that abnormal neuronal activity is an important part of a complex two-way communication process between neuron and glial cell that initiates many features of the abnormal chronic pain state.

The above discussion assumes that the local nerve blockade in our experiments acted on neurons rather than directly on the satellite glia cells. Glia cells can also express sodium channels, but these are at much lower density than those found in neurons, insufficient for action potential generation, and are thought to be in a closed state at the normal glia resting potential (Sontheimer et al., 1996, Verkhratsky and Steinhauser, 2000). We cannot completely eliminate the possibility that local perfusion of the DRG with TTX blocked glia activation through direct effects on satellite glia cells; however, this seems unlikely in view of the current literature on glia cells. In addition, we found similar results using the SNI model and a different blocker, bupivacaine, which was applied at the remote injury site where ectopic neuronal activity originates.

Another pathological change that has been studied in some detail in several pain models is altered NGF levels in the sensory ganglia (McMahon et al., 2006). The changes in NGF we observed with immunohistochemistry after spinal nerve ligation are similar to those reported by others using various methods. Spinal nerve ligation (or other forms of axotomy) results in an initial decline in NGF levels, due to disruption of transport from target tissues. NGF levels then rebound, as satellite glial cells begin to synthesize NGF and supply it to the neurons (Lee et al., 1998, Zhou et al., 1999). The effects of local nerve blockade on NGF we observed are quite consistent with the effects we observed on glia activation. The initial decline in NGF, presumably due to disruption of NGF transport from the periphery after axotomy, was unaffected by local nerve blockade. However, the partial recovery on POD 3 was blocked by

local nerve blockade. Since such nerve blockade also blocked satellite glia activation, this differential effect is consistent with the previous work showing that activated glia are the sources of NGF at this later time point. This result also provides a plausible mechanism for the previous finding that early nerve blockade reduces sympathetic sprouting in the DRG in this model (Zhang et al., 2004), insofar as local NGF has been implicated in such sympathetic sprouting (Zhou, 1996, Zhou et al., 1999). Since NGF has excitatory effects on sensory neurons (McMahon et al., 2006), this potentially represents another positive feedback loop between spontaneously active neurons and neighboring activated satellite glia.

The activation of spinal microglia and subsequently of spinal astrocytes in the SNL model, and contributions of these cells to pain behaviors, have been studied in much more detail than satellite glia in the DRG. Our observations were generally consistent with this literature regarding the time course and localization of microglia and astrocyte activation (Tanga et al., 2004, Romero-Sandoval et al., 2008). A previous study using the SNI model (Wen et al., 2007) also showed that local sodium channel blockade at the injury site (using a somewhat different method) reduced microglia activation in the corresponding level of the spinal cord. In a different model, cryoneurolysis of the spinal nerve, even a short-acting perineural application of bupivacaine just prior to the injury was highly effective in reducing spinal microglia activation (Colburn et al., 1997). These studies along with the results presented here suggest that the abnormal activity in the sensory neurons may play a key role in activation of microglia in the corresponding target region of the spinal cord. A number of studies have suggested that activation of microglia precedes and is required for the later activation of astrocytes (Ji et al., 2006); the reduced activation of astrocytes we observed following local nerve blockade in the DRG might be predicted on the basis of the observed decrease in microglia activation though we cannot rule out additional effects of DRG activity on astrocyte activation.

In this study, we have relied upon GFAP immunoreactivity as the only marker for activation of satellite glia cells in the DRG. In the CNS, GFAP has been a standard tool for many decades as an early, robust marker of astrocyte activation that is elevated in a wide range of pathological conditions and models. More recent work has shown the functional importance of this protein in the astrocyte responses (Eng et al., 2000). Much less is known about the satellite glia in DRG and other sensory ganglia. They are a unique cell type sharing some but not all features with astrocytes in the CNS. One important distinction is that their basal level of GFAP expression in normal animals is quite low, as we also report in this study, making this a particularly useful marker for quantitative and semi-quantitative measurement of SGC activation. Upregulation of GFAP in SGC after nerve injury or inflammation has been reported in several different models. Nerve injury or inflammation has been shown to lead to proliferation and hypertrophy of SGC, and to upregulate various other molecules in the SGC, including neurotrophins, transforming growth factor α , tumor necrosis factor α , IB4, and functional gap junctions (Hanani, 2005). In the present study we did not use these potential additional markers of SGC activation for several reasons: some have been described only in a single model; some are observed only in a subset of the cells that express GFAP or in SGC surrounding a subset of sensory neurons (Ohtori et al., 2004, Miyagi et al., 2006), some appear to develop more slowly than the GFAP signal (e.g. the proliferation response; (Humbertson et al., 1969)) or have not been studied at time points early after nerve injury; and often the association of a particular molecule with SGC activation is essentially defined by using GFAP to measure SGC activation (Takeda et al., 2007). Indeed, the initial interpretation of GFAP upregulation as a sign of SGC activation seems to have depended heavily on comparison to the earlier studies on CNS astrocytes (Woodham et al., 1989b, Stephenson and Byers, 1995). Hence, while GFAP seems to be the most widely used and robust early marker for activation of SGC, it must be emphasized that little is known about the nature of this activation in SGC, or of the functional role GFAP plays in SGC. More generally, it is likely that the term

“activation” as it is applied to glia cells in both CNS and PNS is an oversimplification, implying as it does a cell with only two states rather than what is more likely a cell with a broad range of finely tuned responses.

In conclusion, this study demonstrated that in two different nerve injury models, early blockade of sensory neuron activity, at or near the sites where spontaneous activity originates, was highly effective in reducing the activation of satellite glia cells in the DRG. Such blockade was also effective in reducing glia activation in the spinal cord in the SNL model (this study) as well as in the SNI model (Wen et al., 2007). These findings suggest that abnormal neuronal activity plays important roles in activation of glia cells. A number of plausible mechanisms have been described in the literature by which this neuron-glia interaction could occur, including mechanisms in which positive feed-back processes between the active neuron and the activated glia cell could magnify abnormal neuron activity and glia activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

List of Abbreviations Used

ATP, Adenosine-5'-triphosphate
 CNS, central nervous system
 DRG, dorsal root ganglion
 GFAP, glial fibrillary acidic protein
 MAP kinase, mitogen-activate protein kinase
 NGF, nerve growth factor
 POD, post-operative day
 SGC, Satellite Glial Cells
 SNI, spared nerve injury
 SNL, Spinal Nerve Ligation
 TTX, tetrodotoxin

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Figure 1. Early DRG neuronal blockade with TTX reduced satellite glia activation after spinal nerve ligation

Sections of DRG stained for GFAP (red) and Neu-N (Green) a, b: GFAP was barely detectable in normal rat DRG. Following SNL, the expression of GFAP was dramatically increased on POD 1 (c), and remained very high on POD 3 (e) and POD 6 (g). Applying TTX locally to the axotomized DRG for 7 days starting at the time of nerve injury reduced the nerve-injury induced GFAP expression at all these time points. Scale bar = 10 μ m

Figure 2. Time course of local nerve blockade effects on satellite glia activation in DRG
 Top: Effect of SNL with and without nerve blockade (via perfusion of the axotomized L4 DRG with TTX) on satellite glia activation (percent of neurons surrounded by GFAP-positive satellite glia) in L4 DRG. Bottom: effect of SNI with and without nerve blockade (via bupivacaine depot placed at the nerve injury site) on glia activation in L4 DRG. #, glia activation at these time points was significantly different from control (POD 0). * glia activation significantly different from the same time point with no blockade.

Figure 3. Early DRG neuronal blockade via TTX reduced NGF expression following peripheral nerve injury

Significant expression of NGF can be found in cellular areas of normal DRG (a, b). Following SNL, NGF immunoreactivity in axotomized DRG was markedly decreased on POD 1 (c), however, it recovered back to normal on POD 3 (e), then decreased again on POD 7 (g). Qualitatively similar changes in NGF were found in the axotomized DRG blocked with TTX starting at the time of nerve injury. However, after POD 1 (d) the overall level of NGF immunoreactivity in DRG perfused with TTX (f & h) was lower than in the DRG without nerve blockade. i & j were stained as the negative control. They were processed under the same conditions as the other panels except that no anti-NGF was used. Scale bar = 10 μ m.

Figure 4. Effect of SNL with and without nerve blockade (TTX applied to axotomized DRG) on NGF immunoreactivity in the axotomized L4 DRG

*, group differed significantly from control. #, significant effect of TTX for that POD.

Figure 5. Early DRG blockade via TTX reduced microglia activation caused by spinal nerve ligation in the spinal cord

In the normal spinal cord, a basal level of OX-42 is expressed in microglia (a & b). Spinal nerve ligation caused a significant increase of OX-42 expression on the side of spinal cord ipsilateral to the nerve injury (d), more so than on the contralateral side (c) on POD 3. Early DRG neuronal blockade via TTX perfusion for the first 3 days after SNL reduced spinal OX-42 expression (e & f) after SNL. The panels on the left and the right side show dorsal horn portions of the spinal cord, the sides contra- and ipsi- lateral to nerve injury, respectively. Scale bar = 50 μ m.

Figure 6. Effect of perfusion of the axotomized DRG with TTX on glia activation in spinal cord
 In control animals no differences were observed between contralateral and ipsilateral sides, so data have been combined into one point. Top: Microglia marker OX-42 was examined on POD 3. Bottom: Astrocyte marker GFAP was examined on POD 10. *, significantly different from control. #, significant differences between the indicated pairs of groups.

Figure 7. Early DRG blockade via local TTX perfusion reduced the expression of GFAP in the spinal cord caused by spinal nerve ligation

In the normal spinal cord, a basal level of GFAP is expressed in astrocytes (a & b). Spinal nerve ligation caused significant increase of GFAP expression on the side of spinal cord ipsilateral to the nerve injury (d), more so than on the contralateral side (c) on POD 10. Early DRG neuronal blockade via TTX perfusion for the first 7 days after SNL reduced spinal GFAP expression (e & f) observed on POD10 after SNL. Sections shown in g & h were stained as a negative control, processed in the same way as the preceding panels except that no primary antibody (anti-GFAP) was used. The panels on the left and the right side show spinal cord dorsal horn regions contra- and ipsi- lateral side to nerve injury, respectively. Scale bar = 50 μ m.