Published in final edited form as:

Neuroscience. 2009 July 7; 161(3): 895–903. doi:10.1016/j.neuroscience.2009.03.055.

Remote Astrocytic and Microglial Activation Modulate Neuronal Hyperexcitability and Below-Level Neuropathic Pain after Spinal Injury in Rat

Young Seob Gwak and Claire E. Hulsebosch

Department of Neuroscience & Cell Biology, University of Texas Medical Branch, Galveston, TX 77555-1043

Abstract

In this study, we evaluated whether astrocytic and microglial activation mediates below-level neuropathic pain following spinal cord injury. Male Sprague-Dawley (225–250 g) rats were given low thoracic (T13) spinal transverse hemisection and behavioral, electrophysiological and immunohistochemical methods were used to examine the development and maintenance of below-level neuropathic pain. On post operation day 28, both hindlimbs showed significantly decreased paw withdrawal thresholds and thermal latencies as well as hyperexcitability of lumbar (L4-5) spinal wide dynamic range (WDR) neurons on both sides of spinal dorsal horn compared to sham controls (* $p < 0.05$). Intrathecal treatment with propentofylline (PPF, 10 mM) for 7 consecutive days immediately after spinal injury attenuated the development of mechanical allodynia and thermal hyperalgesia in both hindlimbs in a dose related reduction compared to vehicle treatments (* $p < 0.05$). Intrathecal treatment with single injections of PPF at 28 days after spinal injury, attenuated the existing mechanical allodynia and thermal hyperalgesia in both hindlimbs in a dose related reduction (* $p < 0.05$). In electrophysiological studies, topical treatment of 10 mM PPF onto the spinal surface attenuated the neuronal hyperexcitability in response to mechanical stimuli. In immunohistochemical studies, astrocytes and microglia in rats with spinal hemisection showed significantly increased GFAP and OX-42 expression in both superficial and deep dorsal horns in the lumbar spinal dorsal horn compared to sham controls (* $p < 0.05$) that was prevented in a dose related manner by PPF. In conclusion, our present data support astrocytic and microglial activation that contributes to below-level central neuropathic pain following spinal cord injury.

Keywords

allodynia; hyperexcitability; spinal cord injury; spinal glia; WDR neuron

Introduction

Traumatic spinal cord injury (SCI) induces maladaptive neuronal circuits that result in central neuropathic pain (CNP). CNP is divided into three categories relative to the injury site: above-level, at-level and below-level central neuropathic pain (Siddall et al., 1997).

Send Correspondence and Reprint Requests to: Claire E. Hulsebosch, Ph.D., Department of Neuroscience&Cell Biology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1043, USA, Phone: 409-772-2939, Fax: 409-772-3222, cehulseb@utmb.edu, Young Seob Gwak, Ph.D., Department of Neuroscience&Cell Biology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1043, Phone: 409-772-5194, Fax: 409-772-3222, ysgwak@utmb.edu.

Section Editor :

Pain Mechanisms: Dr. Linda S. Sorkin, Department of Anesthesiology, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0818, USA

Several types of rodent SCI-induced CNP models are used to elucidate the neuronal circuit changes of somatosensory information following spinal cord injury. It is well known that one of the representative features of SCI-induced CNP is abnormal and excessive transmission of somatosensory information from non-noxious as well as noxious stimuli, which results in central sensitization of spinal dorsal horn neurons, characterized by neuronal hyperexcitability to cutaneous stimuli (Christensen and Hulsebosch, 1997; Gwak et al., 2006). If neuronal hyperexcitability exists in somatosensory circuits, normally non-noxious stimuli become noxious (allodynia) and formerly noxious stimuli become more noxious (hyperalgesia) (Gwak et al., 2004a, b).

Spinal systems are composed of both neuronal and non-neuronal cells. Spinal glia, especially astrocytes and microglia, outnumber neurons and play important roles in maintaining homeostasis in the central nervous system (Kuffler et al., 1984). Studies on astrocytic and microglial roles have focused on modulating homeostasis by regulation of neurotransmitters through interaction with CNS neurons (Chesler and Kaila, 1992; Anderson and Swanson, 2000). However, recent studies consistently report that activation of astrocytes and microglia are important in peripheral neuropathic pain induced by spinal nerve ligation or inflammation (Colburn et al., 1999; Watkins et al., 2001a; Raghavendra et al., 2003). Additionally, our earlier report also supports the functional role of activated spinal astrocytes in central neuropathic pain following spinal cord injury (Gwak and Hulsebosch, 2005).

It is well known that propentofylline (PPF), a methylxanthine derivative, inhibits phosphodiesterase activity, adenosine uptake and produces neuroprotection by decreasing the synthesis of pro-inflammatory cytokines (DeLeo, et al., 1987; Raghavendra et al., 2003). Recent animal studies report that PPF inhibits astrocytic and microglial activation in peripheral neuropathic models, which results in the attenuation of mechanical allodynia (Sweitzer et al., 2001; Raghavendra et al., 2003). However, the role of astrocytic and microglial activation in central neuropathic pain, especially below-level pain, is not fully understood.

Previously, our studies have reported that spinal transverse hemisection at T13 results in mechanical allodynia, thermal hyperalgesia and hyperexcitability of spinal dorsal horn neurons (Gwak et al., 2004a, b, 2006). Additionally we have demonstrated that spinal glial activation may modulate postsynaptic circuits via changes in glutamate receptor expression and GABAergic tone following spinal cord surgical injury (Gwak and Hulsebosch, 2005; Gwak et al., 2008). Previously, we reported that treatment with PPF immediately after SCI attenuated the development of mechanical allodynia and tested glial soma hypertrophy but we did not directly test the effect of PPF given at a chronic time point on behavioral responses, indices of glial activation or on dorsal horn neuronal hyperexcitability (Gwak et al., 2008). In this study, we examined whether PPF given immediately or at a chronic time point attenuates mechanical and thermal allodynia, modulates regional spinal astrocytic and microglial activation, and tested whether PPF inhibited increased hyperexcitability of spinal dorsal horn neurons in remote regions (below-level) from the spinal injury.

2. Experimental Procedures

2.1 Animal preparation

A total of 72 male Sprague-Dawley (225–250 g) rats were used in this study. All animals were obtained from Harlan Sprague-Dawley, Inc. (Houston TX), and housed on a light/dark cycle of 12/12h, with a reverse day/night cycle and fed *ad libitum*. We certify that experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animal (NIH Publication No. 80-23). Experimental

procedures were reviewed and formally approved by the UTMB Animal Care and Use Committee and were consistent with the NIH Guide for the Care and Use of Laboratory Animals which ensures minimizing the discomfort and number of animals used and ensures humane treatment.

2.2 Spinal cord injury

Spinal cord injury (60 rats) was produced by spinal transverse hemisection, from dorsal to ventral, of the spinal cord at T13 (Gwak et al., 2004a, b). Under masked-inhalation anesthesia with isoflurane (induction 2% and maintenance 1.5%), the surgical field was shaved and a longitudinal incision was made exposing vertebra T11 and T12, which were determined by locating the last rib which is T13 and counting two vertebral segments rostrally. After laminectomy, the T13 spinal cord was laterally hemisected with a #11 scalpel, followed by confirmation with a 28 gauge needle which was pulled laterally while applying suction without damage to the posterior spinal artery or branches with the aid of a surgical microscope (KAPS, Germany). Muscle and fascia were sutured and skin closed with autoclips, and animals were allowed to recover on a 36.5°C heating pad. Following surgery, animals were maintained under the same preoperative conditions and the general health of the animals was carefully monitored. For age- and weight-matched control groups, sham animals (12 rats) had only the dura cut without lesion to the spinal cord after laminectomy. Less than 5% of rats were excluded from this study due to thoracic level overhemisection, confirmed by histological section, and as determined by BBB rank scores (Basso, et al., 1995) of less than 20 on the contralateral hindlimb on postoperative day 1. At the time of sacrifice of all rats, the lesion site was sectioned and histological analyses confirmed that the transverse dorsal to ventral hemisection was unilateral and included the ipsilateral dorsal column system, lateral funiculus and ventral funiculus and included ipsilateral grey matter to the central canal. On post operation day (POD) 7, early PPF treatments demonstrated improved BBB scores (15.6 ± 0.8) compared to the vehicle treatment (9.2 ± 2), we do not know the mechanistic basis for the improvement in locomotion (see Freund, et al., 2007), which entails further study.

2.3.1 Intrathecal Implantation

Intrathecal implantation to inject drug was done by insertion of polyethylene tubing into the intrathecal space before spinal hemisection. Briefly, under isoflurane anesthesia, a pre-measured length of PE-10 tubing (I.D. 0.28mm and O.D. 0.61mm,) was passed caudally from the T8 to the L3 level of the spinal cord and 2 cm of the free end was left exposed in the upper thoracic region. Saline was injected daily to prevent clogging of the tubing. To protect against slipping or loss of the inserted tubing by the rat, the tube was loosely tied using an over hand knot (0.5 cm diameter), fixed by Glas Ionomer Base Cement (Shofu Inc, Japan) and sutured to the surrounding paravertebral musculature just proximal to the intrathecal entry site. The exposed intrathecal tubing was sealed by sterile stainless steel wire to prevent infection. The wire was removed for saline and/or PPF administration and immediately replaced after injection.

2.3.2 Measurement of mechanical threshold

Rats were individually housed in clear plastic boxes (8 × 8 × 24cm) above a metal mesh (0.5 × 0.5 cm) and acclimated for 15 min to avoid the stress of environmental change. Because rats are nocturnal animals, the animals were housed in a reverse day/night cycle and tested during their “awake” time at the same time in the afternoon. To test the threshold of mechanical withdrawal responses of both hindlimbs, calibrated von Frey filaments were applied to the center of glabrous surface of the hindpaws, not on the keratinized foot pads, in 6 applications with 10 secs between stimuli using the Dixon up/down methods (Dixon, 1980). Beginning with the 4.31 log unit von Frey filament [a series of von Frey filament log

unit; 3.61 (0.45g), 3.84 (0.74g), 4.08(1.26g), 4.31 (2.04g), 4.56 (3.31g), 4.74 (5.50g), 4.93 (8.32g), 5.18 (14.45g)], the final calculation of 50% withdrawal mechanical threshold was determined by the formula, $\log(50\% \text{ threshold}) = X_f + \kappa\delta$. X_f = value of the final von Frey filament (log unit), κ = correction factors (from calibration table), and δ = mean differences of log units between stimuli; the 18 g pressure of 50% threshold measurement (the maximum score according to up-down method) was selected as the cut-off value (Chaplan et al., 1994). Tests in mechanical and thermal sensitivity were not done on animals with ipsilateral BBB scores less than 10, since the first hindpaw plantar placement score is 9 where plantar placement is accompanied by weight support in stance only (Basso et al., 1995). Thermal paw withdrawals was accompanied by supraspinal behaviors (such as avoidance, disengage behavior) consistent with the receipt of noxious stimuli in both sham and hemisection groups.

2.3.3 Thermal stimuli

Rats were placed on a glass plate over a light box, and a radiant heat stimulus (4.7 amps, San Diego Instruments) was applied by aiming a beam of light through a hole in the light box onto the center of the glabrous surface for each paw through the glass plate. The light beam was turned off automatically by a photocell when the rat lifted the limb, allowing the measurement of time between the start of the light beam and the paw withdrawal response, recorded in seconds (Bennett and Xie, 1988; Hargreaves et al., 1988).

2.4 Immunohistochemistry

On post operation day (POD) 28, rats were deeply anesthetized with sodium pentobarbital (80mg/kg, i.p.) and perfused intracardially with heparinized physiological saline followed by 4% cold buffered paraformaldehyde solution. After perfusion, the lumbar L4/5 spinal segments were removed immediately and post-fixed overnight in 4% paraformaldehyde, followed by cryoprotection in 30% sucrose/4% paraformaldehyde for several days. Prior to section, the spinal cords were embedded in OCT compound, and then sectioned at 20 μ m. After washing sections with 0.1M PBS 3 X in 10 min, tissues were blocked with 0.1M PBS + 0.15 % triton X-100 + 5% NGS for 30 min. Primary antibodies for GFAP (for astrocytes staining, Chemicon, 1:500) and OX-42 (for microglia staining, Serotec, 1:200) were diluted in phosphate-buffered saline (PBS) and incubated with 1% NGS for overnight. After PBS wash, sections were incubated with secondary antibody (Alexa 488, 1:200, Molecular Probes). Sections were collected by free-floating methods and mounted on gel-coated slides with mounting media (Vectashield). We randomly collected three sections from each animal, where the mean of the three values were counted as one data point (N= 4 animals in each group) to test the immunoreaction product intensity of specific proteins associated with astrocytes and microglia, respectively. Sampled tissues were collected from superficial (laminae I–II) and deep dorsal horn (laminae III–V) of the lumbar spinal cord (L4/5) to analyze immunoreaction product intensity of GFAP and OX-42, respectively. Although lamina-by-lamina analyses are more rigorous (see Christensen and Hulsebosch, 1997; Polgar et al., 2008; Polgar and Todd, 2008), functionally it is reasonable to examine lamina regions that correlate to functional processing. For example, pain and thermal primary afferents project to superficial laminae (I and II) and mechanical and pressure primary afferents project to laminae III–V. Second order neurons elaborate extensively across laminae (Al-Kahter et al., 2008), so it would be impossible to guess functional outcome on a lamina-by-lamina basis. However, by dividing the dorsal horn into superficial and deep, the functional implications of intensity differences with the regional anatomy yield insight into somatosensory dysfunction after spinal injury, which our data supports. To measure the area intensity of GFAP (astrocytes) or OX-42 (microglial) immunoproducts, we captured images with a confocal microscope and Lasersharpe Imaging software (Bio-Rad). We used the Threshold Image function in the MetaMorph 6.1 software program to set the low and high

thresholds for the measurement of immunofluorescent intensity which was determined to be signal. After setting the low and high thresholds for the wavelength of interest (to exclude DAPI nuclear stain) using the “Region Measurements” function in the MetaMorph 6.1 software program, the region selected was manually traced to measure the intensity of immunoreaction product in the superficial and deep dorsal horn, respectively. The measured intensity and area transferred to Excel sheet automatically, after which analysis can be done, including statistics.

2.5 *in vivo* Electrophysiology

The evoked activity of lumbar (L4-L5) wide dynamic range (WDR) dorsal horn neurons was measured by *in vivo* extracellular single unit recording techniques (Gwak et al., 2006; 2008). Briefly, a laminectomy (T12-L3) exposed the lumbar enlargement in order to record the WDR neuronal activity. The rat was held in place by a stereotaxic apparatus within a grounded Faraday cage. The head was fixed using a rat head adaptor with ear bars and the spinal column was clamped at T12 and L3 vertebrae using rat vertebra clamps (David Kopf, USA). The rats were anesthetized by infusion (Harvard, CA, USA) of sodium pentobarbital (5mg/hr/300g rat) via an indwelling jugular vein cannula during recording. Single-unit recordings using carbon filament-filled glass electrode (0.4–0.8 M Ω , Kation Scientific, MN, USA) were made by exploring the dorsal horn during the application of mechanical stimuli onto the peripheral receptive fields. After isolation of a single WDR dorsal horn neuron, the activity was amplified (DAM 80, WPIM FL, USA) and then connected on an oscilloscope and a spike counter (interface, CED 1401+, Cambridge, England) allowing real-time recording of peristimulus time histograms (PSTH) and waveforms of spikes using spike2 program (Cambridge, UK). The paradigm of recording is as follows: after isolation of single unit, the activity was recorded by background activity (without stimulation, 20 seconds), brushing the skin with a camel hair brush (brush stimuli, 10 seconds), a large clamp (Bulldog Clamps, Tiemann) that produced a sense of firm pressure (pressure stimuli, 10 seconds) and a small clamp (Serrefines, Tiemann) that produced a distinctly painful sensation (pinch stimuli, 10 seconds). Dorsal horn neurons were classified as WDR neurons if they displayed increased responsiveness to brush, pressure and pinch stimuli consistent with the graduated increase in intensity of stimulation (Gwak et al., 2006; 2008). The excitability of WDR spinal dorsal horn neurons in response to evoked stimuli was recorded before PPF application and 10, 30, 60, and 120 min after topical application of PPF.

2.6 Drug application

We used four different animal groups in the experimental design. Group A (n=18) was subjected to behavioral tests after “early” treatment of PPF. Intrathecal injection of 15 μ l volume, flushed by 10 μ l saline, of 1 (4.6 μ g) and 10 (46 μ g) mM propentofylline (PPF, 3,7-dihydro-3-methyl-1-(5-oxohexyl)-7-propyl-1H-purine-2,6-dione, M.W. 306.4, Sigma) or saline vehicle was initiated immediately after spinal transverse hemisection and continued on POD 1, 2, 3, 4, 5, 6 and 7. Intrathecal application was done at the same time in the morning. Group B (n=15) was subjected to behavioral tests on POD 28 before (pre-drug) and after “late” intrathecal treatment of PPF. By POD 28, allodynic behavioral outcomes were at maximum values. Group C (n=16) and Group D (n=23) were subjected to tests of immunohistochemistry and electrophysiology on POD 28, respectively.

2.7 Statistical Analysis

Statistical analysis of behavioral tests was performed using the One-Way (comparison of between pre-injury and post-injury) analysis of variance (ANOVA) with repeated measures on time factor followed by the Student-Newman-Keuls Method for comparisons. Statistical analysis of neuronal activity and morphological changes were performed using student's *t*-test, using the Sigmaplot program (Ver 3.1). An alpha level of significance was set at 0.05

for all statistical tests. Data were combined with ipsilateral and contralateral sides because data showed no significant differences in side to side comparisons and are expressed as means $\pm$ S.E. * $p < 0.05$

3. Results

3.1 Mechanical allodynia and thermal hyperalgesia: Early treatment with PPF

The mean mechanical threshold in sham controls and all spinal hemisection groups (before hemisection) was 17.7 ± 0.4 g and 17.8 ± 0.1 g, respectively. After hemisection, the mechanical threshold of the vehicle group ($n=5$) significantly decreased when compared to thresholds before hemisection (Figure 1 A). On post operation day (POD) 14, however, early intrathecal treatment of 10 mM PPF over the first 7 days after spinal hemisection partially attenuated the change in mechanical threshold (11.4 ± 1 g, $n=5$) whereas the 1 mM PPF group (6.5 ± 0.8 g, $n=5$) did not show significant differences when compared to the vehicle group (7.5 ± 0.5 g, Figure 1A, * $p < 0.05$). The attenuation of mechanical allodynia lasted the entire test period. The sham group ($n=3$) did not show significant differences when compared to values before spinal injury (Figure 1A).

The mean thermal latency before hemisection of all groups was 14.1 ± 0.5 seconds. After spinal transverse hemisection, the thermal latency of the vehicle group significantly decreased when compared to values before hemisection (* $p < 0.05$, Figure 1B). On POD 14, however, early treatment of the 10 mM PPF prevented the decrease of thermal latency (14.1 ± 0.7 seconds) whereas the 1 mM PPF (10.7 ± 0.6 seconds) group did not show significant differences when compared to the vehicle group (11.1 ± 0.4 seconds), respectively (Figure 1B, * $p < 0.05$). This attenuation of thermal hyperalgesia lasted the entire test period. The sham group did not show significant differences when compared to the values measured before spinal injury.

3.2 Mechanical allodynia and thermal hyperalgesia: late treatment with PPF

The mean mechanical threshold before spinal transverse hemisection of all groups was 17.3 ± 0.3 g. On POD 28, the mechanical threshold of rats with hemisection (before the 10 mM PPF application, $n=5$) was 3.3 ± 0.3 g which was significantly decreased when compared to the values before hemisection (* $p < 0.05$, Figure 2A). However, the intrathecal treatment of the 10 mM PPF ($n=5$) significantly increased the mechanical threshold whereas the 1 mM PPF group ($n=5$) did not show any significant differences when compared to the values before PPF treatment. These differences were not significant at 24 hrs after intrathecal application.

The mean thermal latency before spinal transverse hemisection of all groups was 13.7 ± 0.5 seconds. On POD 28, the thermal latency of rats with hemisection (before the 10 mM PPF application) was 9.4 ± 0.4 seconds which was significantly decreased when compared to values before hemisection (* $p < 0.05$). However, intrathecal treatment of the 10 mM PPF significantly increased thermal latency whereas the 1 mM PPF group did not show significant differences when compared to before PPF treatment (Figure 2B, * $p < 0.05$). These differences were not significant at 24 hrs after intrathecal application.

3.3 Increase of GFAP and OX-42 immunoproducts

On POD 28, rats with hemisection ($n=4$) displayed increased immunoproduct of GFAP and OX-42 immunoreaction with soma hypertrophy and thickened branches in both lumbar superficial and deep dorsal horns that were significantly greater on both ipsilateral and contralateral sides when compared to sham controls ($n=4$); however there was no side to side difference in either GFAP or OX-42 expression in the hemisected group on POD 28.

The mean intensity of GFAP in laminae I and II was 41.8 ± 2.1 (superficial) and for laminae III, IV and V was 22.2 ± 0.8 (deep), respectively. However, spinal hemisection produced significantly increased GFAP intensity of 75.4 ± 7.7 (for superficial laminae) and 37.6 ± 3.6 (for deep laminae) compared to sham controls, respectively (* $p < 0.05$). The mean GFAP intensity after 10 mM PPF treatment ($n=4$) was 55.9 ± 5 (superficial laminae) and 31.0 ± 1.8 (deep laminae), respectively and showed significant differences compared to the hemisection group (* $p < 0.05$, Figure 3). The mean intensity of OX-42 was 11.3 ± 0.7 (superficial) and 15.1 ± 1.11 (deep), respectively. However, spinal hemisection produced significantly increased OX-42 intensity to 25.9 ± 1 (superficial) and 27.5 ± 1.2 (deep) compared to sham controls, respectively (* $p < 0.05$). The mean OX-42 intensity after 10 mM PPF treatment was 14.7 ± 0.8 (superficial) and 17.7 ± 0.5 (deep), respectively and showed significant differences compared to hemisection groups (# $p < 0.05$, Figure 4). We interpret these results to indicate astrocytic (GFAP) and microglial (OX-42) activation occurs in segments remote from a spinal injury and that PPF attenuates the activation responses. While OX-42 was also expressed in activated infiltrating macrophages, these cells appear 24 to 48 hours after injury and are not present at time points and regions selected in the present study.

3.4 Attenuation of hyperexcitability by PPF

In sham controls, the average evoked activity of spinal WDR ($n=5$) neurons was 6.5 ± 2.1 spikes/sec (brush), 13.7 ± 2.8 spikes/sec (pressure) and 15.01 ± 3.5 spikes/sec (pinch), respectively. On POD 28 (before PPF application), the evoked activity was 17.8 ± 2.2 spikes/sec (brush), 26.5 ± 4.7 spikes/sec (pressure) and 30.1 ± 4.5 spikes/sec (pinch) which significantly increased when compared to the values before hemisection, respectively (Figure 5, * $p < 0.05$). However, the evoked activity on 2 hrs after topical application of the 10 mM PPF ($n=8$) was 8.03 ± 1.6 spikes/sec (brush), 14.7 ± 3.5 spikes/sec (pressure) and 15.4 ± 3.6 spikes/sec (pinch) and showed significant attenuations whereas the 1 mM PPF ($n=6$) did not show significant differences when compared to vehicle treatment groups ($n=4$), respectively (Figure 5, # $p < 0.05$).

Discussion

The present data demonstrate that bilateral astrocytic and microglial activation (characterized by somatic hypertrophy accompanied by increased immunoreaction product) and hyperexcitability of spinal dorsal horn neurons several segments caudal to a spinal transverse hemisection contribute to below-level central neuropathic pain. As a test of this, we demonstrated that propentofylline (PPF) given immediately as well as given chronically after spinal transverse hemisection can attenuate mechanical allodynia, thermal hyperalgesia and hyperexcitability after SCI. We also demonstrated that PPF prevents hypertrophy of both astrocytes and microglia, a classic measure of activated or “reactive” gliosis (Colburn et al., 1999). Thus, we conclude that bilateral activation of astrocytes and microglia in the spinal lumbar dorsal horn is related to below-level central neuropathic pain following unilateral thoracic spinal injury.

Interestingly, despite a unilateral central spinal lesion, we did not see a side to side difference in either GFAP or OX-42 immunointensity, as was reported in a variety of unilateral peripheral injury models 7 days or less after injury (see Colburn, et al., 1999). There are several differences between our work and Colburn’s work such as injury type, tested region, time since injury etc. Since about 30% of synaptic projections of primary afferents are known to decussate to the contralateral side (Chung et al., 1989), it is not surprising that a peripheral injury on one side will affect the contralateral side and that the ipsilateral side will exhibit more robust glial activation. However, in our spinal injury model, primary afferent collaterals as well as descending and ascending tracts are severed

unilaterally, and all have contralateral projections (Skagerberg and Bjorklund, 1985; Hains et al., 2002) which then degenerate, contributing to glial activation on the contralateral side.

Astrocytes and microglia are known to modulate the environment of the central nervous system in terms of glutamate homeostasis and modulation of inflammatory responses (Giulian, 1987; Walz, 1989). However, it is important to note that activation of spinal astrocytes and microglia are proposed to contribute to neuropathic pain syndromes following peripheral nerve injury (Garrison et al., 1991; Coyle, 1998; Sweitzer et al., 2001). Therefore, we tested the morphological changes consistent with the classical definition of astrocytic and microglial activation (hypertrophy and increased GFAP or OX-42 immunopositive) as well as began to test the functional role of spinal astrocytes and microglia following spinal cord injury. To elaborate, on POD 28, increased GFAP and OX-42 positive cells, in both superficial and deep dorsal horns, displayed increased cell body area and thickened branches compared to sham controls, consistent with astrocytic and microglial activation that occurred on both sides of the lumbar spinal dorsal horn following low thoracic spinal hemisection (Gwak et al., 2008). We tested astrocytes and microglia for morphological changes on POD 28 after spinal transverse hemisection because we hypothesized that astrocytic and microglial activation persists for weeks to months after neural injury (Gould and Goshgarian, 1997; Tanga et al., 2004). However, the role of persistent astrocytic and microglial activation in central neuropathic pain following spinal cord injury is not clear.

It is known that substances are released by activated astrocytes and microglia that are candidates for maintaining central neuropathic pain. For example, substances known to contribute to neuropathic pain such as excitatory amino acids (EAAs), reactive oxygen species (ROS), and proinflammatory cytokines, are released by activated astrocytes and microglia (Piani et al., 1991; Martin, 1992; Tanaka et al., 1994). Specifically, the high concentration of extracellular glutamate after spinal cord injury initiates the activation of neuronal, astrocytic and microglial glutamate receptors (Piani et al., 1992; Gwak and Hulsebosch, 2005) which then mediate membrane depolarization with resulting influx of Na^+ and Ca^{2+} ions into cells (Sontheimer et al., 1988). In turn, elevated Ca^{2+} concentrations in astrocytes and microglia stimulate the release of glutamate, proinflammatory substances, such as prostaglandins (PGs) and cyclooxygenase (COX) as well as both reactive oxygen (ROS) and nitrogen species (NOS) (Shafer and Murphy, 1997; Johnstone et al., 1999). These pain mediating substances released from activated astrocytes and microglia initiate activation of neuronal membrane receptors (such as NMDA receptor, Lee et al., 2007) and ion channels (such as sodium channels, Lampert et al., 2006), which result in persistent hyperexcitability of spinal dorsal horn neurons.

Recent literature demonstrated that astrocytic and microglial activation is involved in the development of neuropathic pain after unilateral peripheral nerve injury. The activation of astrocytes and microglia after unilateral peripheral nerve injury predominantly occurs in the superficial layer of the ipsilateral spinal cord consistent with the somatotopy of nociceptive processing (Colburn et al., 1999; Coyle, 1998). However, our data show activation of astrocytes and microglia several segments remote from a unilateral spinal injury and that the activation occurs bilaterally and in the entire dorsal horn of the spinal cord (laminae I–V) (Gwak et al., 2008). Previously, we demonstrated that spinal astrocytic and microglia activation contributed to upregulation of Group I metabotropic glutamate receptors and loss of GABAergic tone after unilateral spinal cord injury (Gwak and Hulsebosch, 2005; Gwak et al., 2008). In addition, early treatment with PPF prevented the loss of GABAergic tone, attenuated development of mechanical allodynia and inhibited astrocytic and microglial activation in the dorsal horn. However, in the present study we report that activation of astrocytes and microglia actively contribute to the development and maintenance of mechanical allodynia as well as thermal hyperalgesia following unilateral spinal injury.

Following our previous publication (Gwak et al., 2008), we wanted to test the changes in immunoreaction product intensity of the superficial (laminae I and II) vs. deep (laminae III–V) dorsal horn because of well known functional differences in these regions; pain and thermal primary afferents project to superficial laminae and mechanical and pressure primary afferents project to deep laminae. Our previous study focused on the single cell level, and did not reflect regional changes (Gwak et al., 2008). Furthermore, in the present manuscript we report our electrophysiological data with PPF, and demonstrate that activation of astrocytes and microglia actively contributed to neuronal hyperexcitability of spinal WDR dorsal horn neurons in remote regions following unilateral spinal cord injury; whereas, our earlier study focused on GABAergic tone and reported the attenuation of hyperexcitability by GABA (Gwak et al., 2008).

The direct comparison of the contribution of activated spinal glia on neuropathic pain-like outcomes following spinal cord injury versus peripheral nerve injury is very difficult. However, our findings suggest that spinal injury results in a more robust and persistent activation of spinal glia than that which occurs following peripheral nerve injury. In another words, spinal injury would be expected to produce greater alterations in spinal intracellular cascades compared to peripheral nerve injury, such as, increased production of proinflammatory cytokines, ROS/NOS, etc. in both neurons and glia.

The pharmacological properties of propentofylline are thought to regulate the synthesis and release of proinflammatory cytokines such as, IL-1 α and TNF- α (Si et al., 1998). These cytokines, in turn, cause enhanced expression of substance P, inducible nitric oxide synthase (iNOS), and COX-2, which are all candidates for potentiating nociceptive transmission in spinal systems (Watkins et al., 2001b). Proinflammatory cytokines trigger neuronal intracellular activation of mitogen activated proteins kinases (MAPKs) that initiate activation of transcription factors, such as pCREB (Crown et al., 2006). Finally, activation of MAPK and transcription factors modulate expression of neuronal receptors and inflammatory cytokine production, which results in sensitization of dorsal horn neurons at the level of injury (Crown et al., 2006) and several segments remote and below the level of spinal injury (Gwak et al., 2007). Taken together, our findings suggest that persistent release of pain mediators from dysfunctional, activated astrocytes and microglia initiate and maintain the central neuropathic pain following spinal cord injury.

In summary, our data suggest that neuronal-glia interactions at remote regions from the spinal injury are involved in altered dorsal horn circuits, and thus participate in abnormal somatosensory processing after spinal injury, which results in persistent central neuropathic pain. Thus, treatment of dysfunctional non-neuronal cells, such as astrocytes and microglia, could provide a potential therapeutic strategy in the attenuation of central neuropathic pain syndromes.

Acknowledgments

Supported by the Dunn and West Foundations, Mission Connect of TIRR Foundation, and NIH grants NS11255 and NS39161.

References

- Al-Khater KM, Kerr R, Todd AJ. A quantitative study of spinlthalamic neurons in laminae I, III, and IV in lumbar and cervical segments of the rat spinal cord (p spc1). *J Comp Neurol* 2008;511:1–18. [PubMed: 18720412]
- Anderson CM, Swanson RA. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. *Glia* 2000;32:1–14. [PubMed: 10975906]

- Basso M, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 1995;12:1–21. [PubMed: 7783230]
- Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 1988;33:87–107. [PubMed: 2837713]
- Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Meth* 1994;53:55–63.
- Chesler M, Kaila K. Modulation of pH by neuronal activity. *Trends Neurosci* 1992;15:396–402. [PubMed: 1279865]
- Christensen MD, Hulsebosch CE. Spinal cord injury and anti-NGF treatment results in changes in CGRP density and distribution in the dorsal horn in the rat. *Exp Neurol* 1997;147:463–475. [PubMed: 9344570]
- Chung K, McNeil DC, Hulsebosch CE, Coggeshall RE. Changes in dorsal horn synaptic disc numbers following unilateral dorsal rhizotomy. *Comp Neurol* 1989;283:568–577.
- Colburn RW, Rickman AJ, DeLeo JA. The effect of site and type nerve injury on spinal glial activation and neuropathic pain behavior. *Exp Neurol* 1999;157:289–304. [PubMed: 10364441]
- Coyle DE. Partial peripheral nerve injury leads to activation of astroglia and microglia which parallels the development of allodynic behavior. *Glia* 1998;23:75–83. [PubMed: 9562186]
- Crown ED, Ye Z, Johnson KM, McAdoo DJ, Hulsebosch CE. Increases in the activated forms of ERK 1/2, p38 MAPK, and CREB are correlated with the expression of at-level mechanical allodynia following spinal cord injury. *Exp Neurol* 2006;199(2):397–407. [PubMed: 16478624]
- DeLeo JA, Toth L, Schubert P, Rudolph K, Kreutzberg GW. Ischemia-induced neuronal cell death, calcium accumulation, and glial response in the hippocampus of the Mongolian gerbil and protection by propentofylline (HWA 285). *J Cereb Blood Flow Metabol* 1987;7:745–751.
- Dixon WJ. Efficient analysis of experimental observations. *Annual Review Pharmacol and Toxicol* 1980;20:441–462.
- Freund P, Wannier T, Schmidlin E, Bloch J, Mir A, Schwab ME, Rouiller EM. Anti-Nogo A antibody treatment enhances sprouting of corticospinal axons rostral to a unilateral cervical spinal cord lesion in adults macaque monkey. *J Comp Neurol* 2007;502:644–659. [PubMed: 17394135]
- Garrison CJ, Dougherty PM, Kajander KC, Carlton SM. Staining of glial fibrillary acidic protein (GFAP) in lumbar spinal cord increases following a sciatic nerve constriction injury. *Brain Res* 1991;565:1–7. [PubMed: 1723019]
- Giulian D. Ameboid microglia as effectors of inflammation in the central nervous system. *J Neurosci Res* 1987;18:155–171. [PubMed: 3500323]
- Gould DJ, Goshgarian HG. Glial changes in the phrenic nucleus following superimposed cervical spinal cord hemisection and peripheral chronic phrenicotomy injuries in adult rats. *Exp Neurol* 1997;148:1–9. [PubMed: 9398444]
- Gwak YS, Unabia GC, Hulsebosch CE. Propentofylline attenuates allodynia, glial activation and modulates GABAergic tone after spinal cord injury in the rat. *Pain* 2008;138:410–422. [PubMed: 18353556]
- Gwak YS, Unabia GC, Hulsebosch CE. Activation of neuronal, not glial, p-38 MAPK maintains hyperexcitability of dorsal horn neurons at caudal region following spinal cord injury in rat. *Society for Neurosciences*. 2007 2007 Program No. 286.9.
- Gwak YS, Nam TS, Paik KS, Hulsebosch CE, Leem JW. Activation of spinal GABAergic receptors attenuates chronic central neuropathic pain after spinal cord injury. *J Neurotrauma* 2006;23(7):1111–1124. [PubMed: 16866624]
- Gwak YS, Hulsebosch CE. Upregulation of Group I Metabotropic Glutamate Receptors in Neurons and Astrocytes in the Dorsal Horn following Spinal Cord Injury. *Exp Neurol* 2005;195:236–243. [PubMed: 16004983]
- Gwak YS, Hains BC, Johnson KM, Hulsebosch CE. Effect of age at time of spinal cord injury on behavioral outcomes in rat. *J Neurotrauma* 2004a;21:983–993. [PubMed: 15318998]
- Gwak YS, Hains BC, Johnson KM, Hulsebosch CE. Locomotor recovery and mechanical hyperalgesia following spinal cord injury depends on age at time injury in rat. *Neurosci Lett* 2004b;362:232–235. [PubMed: 15158021]

- Hains BC, Everhart AW, Fullwood SD, Hulsebosch CE. Changes in serotonin, serotonin transporter expression and serotonin denervation supersensitivity: involvement in chronic central neuropathic pain after spinal hemisection in the rat. *Exp Neurol* 2002;175:347–362. [PubMed: 12061865]
- Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988;32:77–88. [PubMed: 3340425]
- Johnstone M, Gearing AJ, Miller KM. A central role for astrocytes in the inflammatory response to beta-amyloid; chemokines, cytokines and reactive oxygen species are produced. *J Neuroimmunology* 1999;93:182–193. [PubMed: 10378882]
- Kuffler, SW.; Nicholls, JG.; Martin, AR. From neuron to Bain. Sunderland, MA: Sinauer Associate Inc.; 1984. Physiology of neuroglial cells; p. 323–360.
- Lampert A, Hains BC, Waxman SG. Upregulation of persistent and ramp sodium current in dorsal horn neurons after spinal cord injury. *Exp Brain Res* 2006;174(4):660–666. [PubMed: 16718433]
- Lee CJ, Mannaioni G, Yuan H, Woo DH, Gingrich MB, Traynelis SF. Astrocytic control of synaptic NMDA receptors. *J Physiol* 2007;581:1057–1081. [PubMed: 17412766]
- Martin DD. Synthesis and release of neuroactive substances by glia cells. *Glia* 1992;5:81–94. [PubMed: 1349588]
- Piani D, Frei K, Do KQ, Cuenod M, Fontana A. Murine brain macrophages induce NMDA receptor mediated neurotoxicity in vitro by secreting glutamate. *Neurosci Lett* 1991;133:159–162. [PubMed: 1687755]
- Piani D, Spranger M, Frei K, Schaffner A, Fontana A. Macrophage-induced cytotoxicity of N-methyl-D-aspartate receptor positive neurons involves excitatory amino acids rather than reactive oxygen intermediates and cytokines. *Eur J Immunology* 1992;22:2429–2439. [PubMed: 1355433]
- Pölgar E, Todd AJ. Tactile allodynia can occur in the spared nerve injury model in the rat without selective loss of GABA or GABA(A) receptor from synapses in laminae I–II of the ipsilateral spinal dorsal horn. *Neuroscience* 2008;156(1):193–202. [PubMed: 18675320]
- Pölgar E, Al-Khater KM, Shehab S, Watanabe M, Todd AJ. Large projection neurons in laminae I of the rat spinal cord that lack the neurokinin 1 receptor densely innervated by VGLUT2-containing axons and possess GluR4-containing AMPA receptors. *J Neurosci* 2008;28(49):13150–13160. [PubMed: 19052206]
- Raghavendra V, Tanga F, Rutkowski MD, DeLeo JA. Anti-hyperalgesic and morphine-sparing actions of propentofylline following peripheral nerve injury in rats: mechanistic implications of spinal glia and proinflammatory cytokines. *Pain* 2003;104:655–664. [PubMed: 12927638]
- Skagerberg G, Björklund A. Topographic principles in the spinal projections of serotonergic and non-serotonergic brainstem neurons in the rat. *Neuroscience* 1985;15:445–480. [PubMed: 4022334]
- Shafer RA, Murphy S. Activated astrocytes induce nitric oxide synthase-2 in cerebral endothelium via tumor necrosis factor alpha. *Glia* 1997;21:370–379. [PubMed: 9419012]
- Si Q, Nakamura Y, Ogata T, Kataoka K, Schubert P. Differential regulation of microglial activation by propentofylline via cAMP signaling. *Brain Res* 1998;812:97–104. [PubMed: 9813261]
- Siddall PJ, Taylor DA, Cousins MJ. Classification of pain following spinal cord injury. *Spinal Cord* 1997;35(2):69–75. [PubMed: 9044512]
- Sontheimer H, Kettenmann H, Backus KH, Schachner M. Glutamate opens Na⁺/K⁺ channels in cultured astrocytes. *Glia* 1988;1:328–336. [PubMed: 2906630]
- Sweitzer SM, Schubert P, DeLeo JA. Propentofylline, a glial modulating agent, exhibits antiallodynic properties in a rat model of neuropathic pain. *J Pharmacol Exp Ther* 2001;297:1210–1217.
- Tanaka M, Sotomatsu A, Yoshida T, Hirai S, Nishida A. Detection of superoxide production by activated microglia using a sensitive and specific chemiluminescence assay and microglia-mediated PC12h cell death. *J Neurochem* 1994;63:266–270. [PubMed: 8207432]
- Tanga FY, Raghavendra V, DeLeo JA. Quantitative real-time RT-PCR assessment of spinal microglia and astrocytic activation markers in a rat model of neuropathic pain. *Neurochem Int* 2004;45:397–407. [PubMed: 15145554]
- Walz W. Role of glial cells in the regulation of the brain ion microenvironment. *Prog in Neurobio* 1989;33:309–333.
- Watkins LR, Milligan ED, Maier SF. Spinal cord glia: new players in pain. *Pain* 2001a;93:201–205. [PubMed: 11514078]

Watkins LR, Milligan ED, Maier SF. Glial activation: a driving force for pathological pain. Trends Neurosci 2001b;24:450–455.

Figure 1.

Partial prevention of mechanical allodynia and thermal hyperalgesia by early treatment with PPF. Early intrathecal treatment of 10 mM PPF (square, $n=5$) partially attenuated the development of mechanical allodynia (A) and thermal hyperalgesia (B) when compared to the vehicle treatment (diamond, $n=5$) whereas 1 mM PPF (circle, $n=5$) had no effect ($*p<0.05$). However, the sham group (triangle, $n=3$) did not display significant changes from presurgical values. Data are expressed as means $\pm$ S.E. Arrow : intrathecal injections of PPF.

Figure 2.

Attenuation of mechanical allodynia and thermal hyperalgesia by late intrathecal treatment of PPF on post operative day 28. Intrathecal (i.t.) treatment of 10 mM PPF (circle, n=5) partially attenuated the existing mechanical allodynia (A) and thermal hyperalgesia (B) when compared to the vehicle treatment (triangle, n=5, *p<0.05). However, 1 mM PPF (square, n=5) had no effect. Data are expressed as means $\pm$ S.E. Small arrow : hemisection, big arrow : intrathecal injection of PPF, PS : prior to hemisection, PI : prior to intrathecal injection (28 days after hemisection).

Figure 3.

Morphological changes of spinal astrocytes in the lumbar dorsal horn following spinal transverse hemisection at T13. (A–D) Astrocytes visualized by GFAP immunoreaction product from spinal segments L4/L5 in the dorsal horn. (B) Post operation day (POD) 28 after hemisection (n=4), astrocytes in the entire dorsal horn displayed significantly increased area intensity (low magnification) and hypertrophy (high magnification) when compared to the sham control (A). (C) The intrathecal 1 mM PPF treatment did not display any significant changes when compared to hemisection groups. (D) The intrathecal 10 mM PPF treatment attenuated the increased area intensity and hypertrophy when compared to hemisection groups. (E) Intensity comparisons from immunoreaction products in both superficial and deep dorsal horns of sham controls (n=4), spinal hemisection (n=4), spinal hemisection + 1 mM PPF (Hemi + 1 mM PPF, n=4) and spinal hemisection + 10 mM PPF (Hemi + 10 mM PPF, n=4) for GFAP immunoreaction. Data are expressed as means $\pm$ S.E. *p<0.05-between sham and hemisection; #p<0.05-between hemisection and PPF treatments. Sampled images displayed in the whole dorsal horn (low magnification) and in representative cells from laminae IV–V (high magnification), respectively. Scale bar : 300 μ m.

Figure 4.

Morphological changes of spinal microglia in the lumbar dorsal horn following spinal transverse hemisection at T13. (A–D) microglia visualized by OX-42 immunoreaction from spinal segments L4/L5 in the dorsal horn. (B) Post operation day (POD) 28 after hemisection, microglia in the entire dorsal horn displayed increased area intensity (low magnification) and hypertrophy (high magnification) when compared to the sham control (A). The intrathecal 1 mM PPF treatment (C) attenuated the increased area intensity and hypertrophy only in superficial dorsal horn whereas the intrathecal 10 mM PPF (D) treatment attenuated the increased area intensity and hypertrophy in both superficial and deep dorsal horns when compared to hemisection groups. (E) Intensity comparisons from immunoreaction products in both superficial and deep dorsal horns of sham controls, spinal hemisection, spinal hemisection + 1 mM PPF (Hemi + 1 mM PPF) and spinal hemisection + 10 mM PPF (Hemi + 10 mM PPF) for OX-42 immunoreaction. Data are expressed as means $\pm$ S.E. * $p < 0.05$ -between sham and hemisection; # $p < 0.05$ -between hemisection and PPF treatments. Sampled images displayed whole dorsal horn (low magnification) and representative cells from laminae IV–V (high magnification), respectively. Scale bar : 300 μ m.

A) Comparison of Activity

B) Histograms

Figure 5.

Attenuation of neuronal hyperexcitability by spinal treatment of PPF following spinal transverse hemisection at T13. (A) Spinal WDR dorsal horn neurons displayed significantly increased evoked activity in response to brush, pressure and pinch stimuli (10 seconds, each) after spinal hemisection (SCI-vehicle) compared to sham controls ($n=5$). Two hours after spinal treatment of 10 mM PPF ($n=8$), the activity was significantly attenuated whereas 1mM PPF ($n=6$) had no effect when compared to vehicle treatment groups ($n=4$, $*p<0.05$). (B) Real time peristimulus histograms with raw spike activity below in 10 sec bin widths (scale bar) during single neuron recordings from a spinal hemisected rat (SCI) and

hemisection+10 mM PPF treatment rat (SCI + 10 mM PPF). Data are expressed as means $\pm$ S.E.