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Propentofylline attenuates allodynia, glial activation and modulates GABAergic tone after spinal cord injury in the rat

Young Seob Gwak¹, Eric D. Crown¹, Geda C. Unabia¹, and Claire E. Hulsebosch^{*}

Department of Neuroscience & Cell Biology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1043, USA

Abstract

In this study, we evaluated whether propentofylline, a methylxanthine derivative, modulates spinal glial activation and GABAergic inhibitory tone by modulation of glutamic acid decarboxylase (GAD)₆₅, the GABA synthase enzyme, in the spinal dorsal horn following spinal cord injury (SCI). Sprague–Dawley rats (225–250 g) were given a unilateral spinal transverse injury, from dorsal to ventral, at the T13 spinal segment. Unilateral spinal injured rats developed robust bilateral hindlimb mechanical allodynia and hyperexcitability of spinal wide dynamic range (WDR) neurons in the lumbar enlargement (L4–L5) compared to sham controls, which was attenuated by intrathecal (i.t.) administration of GABA, dose-dependently (0.01, 0.1, 0.5 μ g). Western blotting and immunohistochemical data demonstrated that the expression level of GAD₆₅ protein significantly decreased on both sides of the lumbar dorsal horn (L4/5) after SCI ($p < 0.05$). In addition, astrocytes and microglia showed soma hypertrophy as determined by increased soma area and increased GFAP and CD11b on both sides of the lumbar dorsal horn compared to sham controls, respectively ($p < 0.05$). Intrathecal treatment with propentofylline (PPF 10 mM) significantly attenuated the astrocytic and microglial soma hypertrophy and mechanical allodynia ($p < 0.05$). Additionally, the Western blotting and immunohistochemistry data demonstrated that i.t. treatment of PPF significantly prevented the decrease of GAD₆₅ expression in both sides of the lumbar dorsal horn following SCI ($p < 0.05$). In conclusion, our present data demonstrate that propentofylline modulates glia activation and GABAergic inhibitory tone by modulation of GAD₆₅ protein expression following spinal cord injury.

Keywords

Astrocytes; Central neuropathic pain; Glutamic acid decarboxylase; Microglia; Spinal cord injury

1. Introduction

Spinal cord injuries (SCI) induce maladaptive plasticity in the central nervous system including hyperexcitability of spinal dorsal horn neurons, which results in the development and maintenance of central neuropathic pain (CNP) syndromes [8,21,29]. One of the principal spinal pathophysiological mechanisms for the hyperexcitability of spinal dorsal horn neurons is a disruption in the balance between excitatory and inhibitory input onto somatosensory spinal circuits [24,36].

*Corresponding author. Tel.: +1 409 772 2939; fax: +1 409 772 3222. E-mail addresses: E-mail: ysgwak@utmb.edu (Y.S. Gwak), E-mail: ercrown@utmb.edu (E.D. Crown), E-mail: gcunabia@utmb.edu (G.C. Unabia), cehulseb@utmb.edu (C.E. Hulsebosch).

¹Tel.: +1 409 772 5194; fax: +1 409 772 3222.

It is well known that traumatic neural injury causes decreased GABAergic tone in the spinal cord, which often results in neuropathic pain [24,30]. GABA is a major endogenous inhibitory neurotransmitter that is synthesized by the rate-limiting enzyme glutamic acid decarboxylase (GAD), which exists as two different isoforms, GAD₆₅ and GAD₆₇ [18]. GAD₆₅ primarily synthesizes GABA in the axon terminal whereas GAD₆₇ synthesizes GABA in the cytoplasm of the cell body. Immunohistochemical studies showed that GAD₆₅ and GAD₆₇ are widely distributed in the spinal cord, especially in lamina II [33]. Recently, Liu and colleagues reported that GAD gene therapy for increasing the synthesis of GABA attenuated neuropathic pain-like behaviors in rats, such as mechanical allodynia, after SCI [32]. Taken together, these data suggest that one candidate for decreased GABAergic tone following SCI may be a loss of GABAergic neurons and/or may be a decrease in the levels of GAD protein.

In addition to the modulatory role of GABA in spinal cord synaptic circuits, spinal glia plays an important role in maintaining the balance of excitatory and inhibitory tone in the central nervous system. Recently, the literature demonstrates that activated spinal glia also plays an important role in the development and maintenance of peripheral neuropathic pain states [11, 48]. Additionally, astrocytes are actively involved in GABA uptake, which is released from neurons, to control the extracellular concentrations of GABA [6,41]. GABA is converted to glutamine, which is transported to neurons and then acts as a substrate for glutamate in neurons. Thus, astrocytes are important factors controlling the production/reuptake of GABA.

Propentophylline (PPF) is a methylxanthine derivative and a general anti-inflammatory agent that decreases the synthesis of proinflammatory cytokines after peripheral nerve injury [39], inhibits phosphodiesterase activity and adenosine uptake, and is neuroprotective [14,31]. Additionally, PPF prevents activation of spinal glia, both astrocytes and microglia, with attenuation of neuropathic behavior, such as mechanical allodynia [44,45].

It is known that traumatic neural injury causes abnormal changes in both neuronal and non-neuronal cells; however, little is known about mechanisms underlying decreased GABAergic inhibitory tone. In this study, we examined whether propentofylline may modulate glial activation and GABAergic tone following spinal cord injury. We focused on GAD₆₅ enzyme expression because of its role in modulating fast synaptic transmission at axon terminals.

2. Materials and methods

2.1. Animal preparation

A total of 117 male Sprague–Dawley (225–250 g) rats were used in this study. All animals were obtained from Harlan Sprague–Dawley, Inc., housed with a light/dark cycle of 12/12 h, and fed *ad libitum*. Experimental procedures were reviewed by the UTMB Animal Care and Use Committee and were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animal.

2.2. Spinal cord injury

Unilateral spinal cord injury was done as previously described [21]. Briefly, under masked-inhalation anesthesia with isoflurane (induction 2% and maintenance 1.5%), laminectomy of the T11–T12 vertebral segment was done and followed by a dorsal to ventral unilateral transverse cut of spinal segment T13 with a # 11 scalpel blade. To ensure completeness of the injury, an insulin syringe with a 28 gauge needle was inserted dorsal–ventrally at the midline of the cord and pulled laterally with the aid of a surgical microscope (KAPS, Germany) to avoid overhemisection and spinal root damage. The injury lesion was confirmed by postmortem morphology to be unilateral and included the dorsal column system, Lissauer's tract, lateral funiculus, ventral funiculus, and gray matter. For control groups, sham surgery

was produced by only laminectomy of the T11–T12 vertebra in rats of the corresponding body weight.

2.3. Behavioral measurement

To evaluate the neuropathic pain-like behavioral outcomes, we measured 50% withdrawal mechanical threshold for both the contralateral (uninjured) and the ipsilateral (injured) hindlimbs. Briefly, individual rats were housed in transparent plastic cages ($8 \times 8 \times 24$ cm) and acclimated for 30 min to avoid the stress induced by change of environments. Brisk hindpaw withdrawals were quantified when accompanied by active attention of the rat to the stimulus by head turning, biting attacks on the stimulus, and whole-body postural changes in response to mechanical stimuli [7]. The inclusion of these complex behaviors excludes simple hyperreflexia, which is a segmental response [47,49]. The 50% withdrawal mechanical threshold was determined by a modification of Dixon's up/down method [5,16] in which von Frey filaments (Stoelting, Wood Dale, IL) were sequentially applied (increasing or decreasing) to the glabrous surface of the paw in 6 applications with 10 s interstimulus intervals, beginning with the 4.31 log unit von Frey filament (a series of von Frey filaments log unit; 3.61, 3.84, 4.08, 4.31, 4.56, 4.74, 4.93, 5.18). The final calculation of 50% withdrawal mechanical threshold was determined by the formula, $\log(50\% \text{ threshold}) = X_f + \kappa\delta$. X_f is the value of the final von Frey filament (log unit), κ is the correction factors (from calibration table), and δ is the mean differences of log units between stimuli. The 18 g pressure of 50% threshold selected as the cut-off value [5].

2.4. Intrathecal implantation and drug administration

Intrathecal implantation was done by inserting polyethylene tubing in order to inject the drug directly into the intrathecal space. Briefly, under isoflurane anesthesia, a pre-measured length of PE-10 tubing (I.D. 0.28 mm and O.D. 0.61 mm), 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. GABA (0.01, 0.1, 0.5 μ g, Sigma) or propentofylline (PPF, 1 and 10 mM, 3,7-dihydro-3-methyl-1-(5-oxohexyl)-7-propyl-1*H*-purine-2,6-dione, M.W. 306.4, Sigma) was dissolved in saline and was injected intrathecally with 15 μ l volume and flushed by 10 μ l saline or saline vehicle. The GABA doses were determined based on previous reports by us and by others [24,30], while the propentofylline doses were determined based on the reports of Sweitzer et al. [44]. All behavioral tests were done in the evening. It is critical to test for motor-side effects associated with drug delivery, since pain-related behaviors could be masked. Thus, post-locomotor function was tested by a blind observer following hemisection. We used two motor tests: (1) a test developed by Basso, Beattie and Bresnahan (BBB), the BBB Locomotor Rating Scale (to ensure hindlimb function [2] and (2) a 4 point scale developed by Marion Murray which tests for the presence of tremors during the period of drug efficacy, where 0 is no tremors, 1 is few tremors, 2 is increased frequency of tremors but less than grade 3, and 3 is continuous tremors during the entire observation period [26]. The BBB, is a 21 point ordinal scale originally designed to measure locomotor recovery after SCI, but which can be used for loss of hindlimb function as well, where 0 is no hindlimb movement and 21 is consistent and coordinated gait evident during normal hindlimb function. Briefly, scores from 0 to 7 rank the ability for isolated movements in each of the three hindlimb joints, scores of 8–13 describe paw placement, stepping and forelimb–hindlimb coordination; and scores of 14–21 describe degrees of paw position, toe clearance, trunk stability and tail position. No compound tested in this study showed any significant motor-side effects, as indicated by scores in both tests (all scored no change in BBB rank, and 0 on the Murray tremor scale) during and after the period of drug efficacy. Thus, we suggest that the agents tested produced anti-allodynia effects, that were not due to locomotor deficits, stereotypical alterations in motor activity, hyper-locomotion or balance loss.

2.5. Extracellular recording

Four weeks after spinal cord injury, extracellular single-unit recording of wide dynamic range (WDR) neurons was performed. The rats were anesthetized by injection of sodium pentobarbital (50 mg/kg, i.p.) and then a laminectomy of vertebral segments T12–L3 was performed to expose the lumbar enlargement (L3–L5). Tracheal and jugular vein cannulae were inserted for artificial ventilation and supplemental paralysis with pancuronium bromide (2–4 mg/kg/h), respectively. The animal was held in place by a stereotaxic apparatus and rectal temperature was maintained at 37 °C. Extracellular single-unit recordings were performed using a carbon filament-filled single glass microelectrode, to depths from 150 to 800 µm in the lumbar (L4/5) dorsal horn. The neurons were characterized as WDR neurons if they displayed graded responses to increased intensities of mechanical stimuli [10]. After the unit activity was identified from background activity, using Bracken forceps as a 'light touch' search stimulus, three mechanical stimuli were applied to that unit's peripheral receptive field. These were (1) brush stimulation of the skin with a cotton applicator, (2) pressure stimulation by applying a large arterial clip (ROBOZ, USA) with a weak grip to a fold of the skin (120 g/mm²), and (3) pinch stimulation by applying a small arterial clip with a strong grip to a fold of the skin (600 g/mm²). Background activity was recorded for 20 s, and the three mechanical stimuli were applied successively for 10 s each with an interstimulus interval of 20 s. For an analysis of evoked responses of neurons, the number of impulses generated over the stimulation period was counted and expressed as the mean impulse rate per stimulation.

The unit activity was amplified and filtered at 300–3 kHz (DAM80; World Precision Instruments, Sarasota, FL, USA), fed either directly or via an oscilloscope (World Precision Instruments, Sarasota, FL, USA) into the data acquisition unit (CED-1401; Cambridge Electronic Design, Cambridge, UK), and stored on a Pentium HP computer in order to construct the wave forms or plot the peri-stimulus time histograms (spikes/1 s bin width). The stored data were analyzed with Spike2 software (version 5.03 Cambridge Electronics Design). As a control to ensure that a single and the same WDR unit was held for the duration of the recording experiment, we used the Spike2 program to compare the action potential shape and amplitude. If the action potential had the same reproducible shape and amplitude throughout the recording period, it is very unlikely to have come from two different WDR neurons.

2.6. Immunocytochemistry

To test the levels of GAD₆₅ expression and glial activation following spinal cord injury on post-operation day (POD) 28, rats were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused intracardially with heparinized physiological saline followed by 4% cold buffered paraformaldehyde solution. After perfusion, the lumbar spinal cord (L4/5) was removed immediately and post-fixed overnight in 4% paraformaldehyde, followed by cryoprotection in 30% sucrose/4% paraformaldehyde over the course of several days. Prior to sectioning, spinal cords were embedded in OCT compound, and then sectioned at 20 µm. Primary antibodies for GAD₆₅ (Sigma, 1:2000), glial fibrillary acidic protein (GFAP, Chemicon, stains astrocytes, 1:500), and CD11b (OX-42, Serotec, stains microglia, 1:200) were incubated with 1% NGS overnight at 4 °C. After PBS wash, sections were incubated with goat anti-rabbit secondary antibodies (1:200, Molecular Probes). Sections were collected by free-floating methods and mounted on gel-coated slides with mounting media (Vectashield). Images were captured with a Fluroview confocal microscope (Olympus-Leed) and evaluated by a computer-assisted image analysis program (MetaMorph 6.1). To measure the area of GFAP (astrocytic cells) or OX-42 (microglial) immunopositive somata, we used the Threshold Image function in Measure of MetaMorph 6.1 to set the low and high thresholds for the immunofluorescent intensity which was determined to be a signal. Our image data were collected using the same region and the same size of field within laminae to avoid any variance and differential staining between laminae. After setting thresholds, the Region Measurements

function was selected, the Excel spread sheet was opened, and the cell was traced. Only cells in which the cytoplasm was continuous with soma that contained nuclei were measured. The same thresholds were used to measure cell areas in all experimental groups. The measured areas transferred to Excel automatically, after which analysis can be done, including statistics. MetaMorph 6.1 is calibrated to provide standardization of linear and/or areal measurements. A standardized field area was sampled from regions arbitrarily selected within dorsal horn regions that were randomly selected.

2.7. Western blotting

Five rats from each of the four conditions (sham, SCI + vehicle, SCI + 1 mM PPF, SCI + 10 mM PPF) were sacrificed for Western immunoblotting. To examine changes in GAD₆₅ expression following spinal cord injury by Western blot, all subjects were overdosed with pentobarbital (100 mg/kg) and perfused intracardially with 250 ml cold heparinized (1 ml/1 L) saline (0.9%) and the L4/5 spinal cord was removed and dissected while on dry ice. The dorsal aspect of the spinal cord segments was dissected from the ventral portions of the cord and frozen in dry ice. The collected tissue was mechanically homogenized in ice-cold Tris-buffered saline containing 40 mM Tris-HCl (pH 7.5), 2% SDS, 2 mg/ml aprotinin, 2 mg/ml antipain, 2 mg/ml chymostatin, 2 mg/ml bestatin, 2 mg/ml pepstatin-A, 2 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mM EDTA. Homogenates were centrifuged at 10,000g for 10 min. The supernatant was collected and centrifuged again at 10,000g for 10 min and then stored at -80 °C. Protein concentrations of the homogenate were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). We have shown previously that this extraction method is efficient at collecting both the cytoplasmic and nuclear protein fractions [13].

Prior to electrophoresis, samples were heated for 4 min at 95 °C in an equal volume of sample buffer (100 mM Tris, pH 6.8, and 2% SDS, 2% 2-mercaptoethanol, 0.001% bromophenol blue, 20% glycerol) and then loaded onto a polyacrylamide gel in equal protein amounts (10 µg per lane). Each gel contained equal amounts of protein from each of the four conditions (sham, SCI, SCI + 1 mM PPF, SCI + 10 mM PPF), allowing for comparisons within a given gel. The stacking gel was 4% acrylamide, prepared in 0.13 M Tris, pH 6.8, and 0.1% SDS, and the separating gel was 10% acrylamide, prepared in 0.38 M Tris, pH 8.8, and 0.1% SDS. Samples were separated by electrophoresis in Tris-glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) at 300 V for approximately 30 min. Proteins were transferred overnight (12–14 h) to a PVDF membrane at 30 V in a transfer buffer containing 20% MeOH, 20 mM Tris, 150 mM glycine, pH 8.0. Membranes were incubated for one hour at room temperature in blocking buffer containing 5% non-fat powdered milk in Tris-buffered saline (TBS)-Tween (20 mM Tris, 137 mM NaCl, 0.1% Tween 20), then washed for 10 min in TBS-Tween. Membranes were incubated overnight with primary antibodies to GAD₆₅ (Sigma, 1:2500). To control for equal protein loading, beta actin (1:5000) immunoreactivity was used to verify equal loading of proteins on the PVDF membrane and this method found no significant differences between the 4 groups (all *F*s < 1.0, *p* > 0.05). After washing of the primary antibody, membranes were incubated in horseradish peroxidase-conjugated anti-rabbit IgG diluted 1:20,000 in blocking buffer for 2 h and washed three times in TBS for 30 min. Peroxidase activity was detected using the Pierce Super-Signal West Femto Maximum Sensitivity Substrate kit, images were collected by exposing the membranes (exposure time varied from 30 s to 5 min) on chemiluminescence film (Hyperfilm ECL, Amersham Pharmacia Biotech, England), and integrated density values were calculated using LabWorks software (UVP, Upland, CA).

2.8. Experimental protocols

We divided the rats into four different experiments:

First experiment—To test whether maintained mechanical allodynia is caused by decreased GABAergic tone, different concentrations of GABA (0.01, 0.1, 0.5 μ g) were intrathecally (i.t.) delivered on post-operative day (POD) 28 after SCI and 50% mechanical threshold was measured at 30, 60, 120, 180 min after drug administration ($n = 15$, 5 rats per one of the three doses) and compared to the saline vehicle group ($n = 5$).

Second experiment—To test whether SCI causes the hyperexcitability of spinal WDR neurons, the evoked activity of lumbar WDR dorsal horn neurons was recorded in “real time” using extracellular electrophysiological recording techniques in a whole animal preparation following SCI ($n = 20$) and compared to the sham controls ($n = 5$). To test whether hyperexcitability of spinal lumbar dorsal horn neurons after SCI is mediated by decreased GABAergic tone, GABA (0.1 μ g) was topically delivered on to the spinal surface ($n = 20$, 10 ipsilateral and 10 contralateral) and evoked activity was recorded at 2, 10, 30, and 60 min after drug administration.

Third experiment—To test whether spinal glia activation mediates mechanical allodynia, rats were given intrathecal PPF (1 or 10 mM, for 7 days after SCI) to inhibit glial activation and 50% mechanical threshold was measured on POD 7, 14, 21, and 28. On POD 28 after SCI, each group was sacrificed and measured for changes in cell areas by measuring the area of immunoreaction product of glial fibrillary acid protein (GFAP, marker for astrocytes) and CD11b (OX-42, marker for microglia) in the lumbar dorsal horn of the two PPF treated groups and these data were compared to sham and untreated SCI controls ($n = 4$ per group).

Fourth experiment—To test whether the treatment with PPF (1, 10 mM) prevents the decrease in GAD₆₅ expression ($n = 4$, each group), we measured expression of GAD₆₅ protein in the lumbar dorsal horn (L4/5) by double immunostaining of neurons and GAD₆₅ proteins and Western blot ($n = 5$, each group) on POD 28 of the PPF treated group and these data were compared to the sham and untreated SCI controls.

2.9. Statistical analysis

Statistical analysis of behavioral outcomes was performed using the One-Way (comparison of pre-injury and post-injury) or Two-Way (comparisons between group of rats) analysis of variance (ANOVA) with repeated measures on time factor followed by the Student–Newman–Keuls Method for multiple comparisons, using the Sigmasat program (Ver. 3.1). An alpha level of significance was set at 0.05 for all statistical tests. Data are expressed as means $\pm$ standard error (means $\pm$ SE).

3. Results

3.1. Attenuation of mechanical allodynia by intrathecal administration of GABA

Prior to SCI, the average mechanical threshold of all groups was 16.6 ± 0.2 (contralateral) and 16.4 ± 0.3 (ipsilateral). Rats given SCI displayed significant decreases in mechanical threshold on POD 28 (5 ± 0.6 contralateral and 4.6 ± 0.1 ipsilateral) compared to the values prior to SCI ($p < 0.05$). However, intrathecal administration of GABA significantly attenuated the mechanical threshold (Fig. 1). Specifically, intrathecal administration of 0.1 and 0.5 μ g significantly increased the mechanical thresholds in both hindlimbs (10.5 ± 1.4 , 10.5 ± 1.5 contralateral and 9.1 ± 1.6 , 13.2 ± 1.5 ipsilateral, respectively) when compared to POD 28 values (before GABA application). However, 0.01 μ g GABA and vehicle treatment did not show any significant differences compared to the SCI group. We did not observe any significant motor deficits after intrathecal GABA administration at any dose.

3.2. Attenuation of hyperexcitability of spinal dorsal horn neurons by GABA

In the sham control, the average responsiveness of lumbar WDR dorsal horn neurons was 12.1 ± 1.8 (brush), 14.6 ± 2.1 (pressure), 19.7 ± 2.6 (pinch) spikes/s in response to evoked brush, pressure, and pinch stimuli, respectively (Fig. 2). On POD 28 after SCI, the average responsiveness was significantly increased to 28.1 ± 2.4 (brush), 32 ± 5.8 (pressure), 40.6 ± 3.4 (pinch) spikes/s in the contralateral side (average depth $548 \pm 58 \mu\text{m}$) and 35.1 ± 4.5 (brush), 47.6 ± 5.5 (pressure), 55.7 ± 10.9 (pinch) spikes/s in the ipsilateral side (average depth $527 \pm 47 \mu\text{m}$) compared to the sham controls ($p < 0.05$), respectively. However, the average responsiveness of lumbar WDR dorsal horn neurons to evoked stimuli after topical application of $0.1 \mu\text{g}$ GABA was significantly decreased to 19.3 ± 2.1 (brush), 21.1 ± 3.2 (pressure), 23.8 ± 3.2 (pinch) spikes/s in the contralateral side and 18.1 ± 2.5 (brush), 26.6 ± 6 (pressure), 31.4 ± 8 (pinch) spikes/s in the ipsilateral side compared to the SCI group (before GABA application, $p > 0.05$), respectively.

3.3. Attenuation of mechanical allodynia by propentofylline

The average mechanical threshold before SCI for all groups was 16 ± 0.9 g. On POD 7 after SCI, mechanical thresholds of the SCI group (vehicle, 1 and 10 mM PPF treatment) were significantly decreased when compared to threshold values after SCI (Fig. 3, $p < 0.05$). On POD 14, however, early treatment with 10 mM PPF significantly increased the mechanical threshold (9.8 ± 0.5 g) whereas 1 mM PPF (5.8 ± 0.4 g) did not show significant differences when compared to the vehicle group (5.9 ± 0.5 g). Additionally, 10 mM PPF treatment significantly blocked the mechanical allodynia compared to the 1 mM PPF treatment group ($p < 0.05$). This attenuation of mechanical allodynia was also observed on POD 21 and 28. The sham group did not show significant differences when compared to the values measured before spinal injury.

3.4. Inhibition of spinal glia activation

After normalization of the sham group to 100%, the soma areas of astrocytes and microglia were significantly increased following SCI (Figs. 4 and 5). In astrocytes, the soma area of the SCI group were $148.4 \pm 23\%$ (contralateral) and $138.3 \pm 11.8\%$ (ipsilateral) and displayed significant increases compared to sham controls ($p < 0.05$). Early inhibition of spinal astrocytic activation by 10 mM PPF prevented astrocytic soma hypertrophy ($87.7 \pm 9.6\%$ and $106.6 \pm 8.1\%$). One mM PPF, however, did not show any significant changes compared to the SCI group (Fig. 4). In microglia, the soma area of the SCI group was $125.1 \pm 6.5\%$ (contralateral) and $125 \pm 7.7\%$ (ipsilateral) and displayed significant increases compared to sham controls ($p < 0.05$). Early inhibition of spinal microglia activation by 10 mM PPF prevented microglial soma hypertrophy ($106.6 \pm 6.6\%$ and $103.5 \pm 4.7\%$). One mM PPF, however, did not show any significant changes compared to the SCI group (Fig. 5).

3.5. Decreased expression of GAD₆₅ protein by glia activation

For immunohistochemistry, the expression levels of GAD₆₅ in the SCI group were significantly decreased ($63.9 \pm 4.5\%$) in lamina II of lumbar dorsal horn compared to the sham controls ($p < 0.05$, after normalization of sham at 100%). Inhibition of spinal glia activation by 10 mM PPF treatment prevented the decrease of GAD₆₅ expression ($97.3 \pm 4.4\%$, $p < 0.05$) whereas 1 mM PPF did not show any significant changes ($67.8 \pm 3.2\%$) compared to the SCI group (Fig. 6). For Western blot analysis, the expression levels of GAD₆₅ protein in the SCI group were significantly decreased ($43 \pm 14\%$) compared to the sham controls ($p < 0.05$, after normalization of sham as 100%). Inhibition of spinal glia activation by 10 mM PPF treatment prevented the decrease of GAD₆₅ protein level ($90.9 \pm 19.6\%$) whereas 1 mM PPF did not show any significant changes ($74.6 \pm 20.1\%$) compared to the SCI group (Fig. 7).

4. Discussion

These data demonstrate glial activation and loss of GAD₆₅ bilaterally after a unilateral spinal cord injury (SCI) persists for months after SCI and contributes to the development and persistence of bilateral mechanical allodynia. Early administration after SCI of propentophylline (PPF), a methylxanthine derivative, reduced astrocytic and microglia activation, prevented the loss of GABAergic inhibitory tone, as measured by preserved levels of the GABA synthase enzyme, GAD₆₅, and attenuated bilateral hindlimb mechanical allodynia weeks after injury. Specifically, PPF resulted in attenuation of astrocytic and microglia activation, as measured by decreased hypertrophy measured by decreased GFAP and OX-42, respectively, and also prevented downregulation of neuronal GAD₆₅. We interpret these data to indicate a tight coupling of glial/neuronal interactions in which activated glial cells contribute to loss of GABAergic inhibitory tone. Furthermore, the coupling can be disrupted by inhibiting glial activation, thus preserving GABAergic inhibitory tone, if agents preventing glial activation are given early following spinal cord injury. Surprisingly, we report that the effects of glial inhibition, including attenuation of mechanical allodynia, persist for weeks after PPF treatment.

SCI induces a loss of endogenous spinal GABAergic inhibition both near the level injury [17] and several segments below [24]. Unilateral spinal hemisection induces bilateral hindlimb mechanical allodynia and hyperexcitability of lumbar spinal dorsal horn neurons [8,21] and activation of spinal GABAergic receptors attenuates bilateral mechanical allodynia and hyperexcitability of spinal dorsal horn neurons [24]. However, the mechanisms that lead to decreased spinal GABAergic inhibitory tone after SCI are not clear. The present data suggest that spinal astrocytic and microglia activation may induce a decrease in spinal GABAergic inhibitory tone by downregulation of neuronal GAD₆₅ expression in the spinal dorsal horn following SCI. More interestingly, unilateral SCI induces bilateral activation of spinal glia and decreases in GAD₆₅ expression levels.

Glia modulate physiological homeostasis in the nervous system. However, apart from their role in the control of physiological homeostasis, the literature demonstrates that pathophysiological states induce activation in both astrocytes and microglia (characterized by proliferation, hypertrophy, and hyperalgesia) and also play an important role in the maintenance of neuropathic pain [34,48]. SCI induces astrocytic activation and inhibition of astrocytic activation attenuates mechanical allodynia in chronic central neuropathic pain in regions remote to a spinal cord injury [22]. Another physiological role of glia is to modulate extracellular GABA concentrations by uptake via GABA transporters [3]. Following GABA uptake, GABA is converted to glutamate by GABA transaminase, glutamate is converted by glutamine synthase to glutamine, which freely passes to neurons and serves as a substrate for the production of either glutamate or GABA, if neurons contain GAD [42]. Massive release of GABA from neurons into the extracellular space, and reuptake of extracellular GABA by activated glia is one mechanism (the “glutamate–glutamine cycle”) for the control of GABA synthesis by modulation of GAD via concentration-dependent feedback mechanisms [4,43].

How is the expression of GAD modulated following spinal cord injury? One hypothesis is that SCI-induced glial activation results in death of GABA containing neurons by apoptosis and/or necrosis [40,50]. Activated glia release neurotoxic substances such as proinflammatory cytokines, reactive oxygen species (ROS), and excitatory amino acids (EAAs) [15]. Proinflammatory cytokines induce release of tumor necrosis factor α , interleukin-1 (IL-1), interleukin-6 (IL-6), all of which affect and enhance pain sensitivity and activate cellular cascades leading to apoptosis. Another hypothesis involves the modulation of molecular mechanisms as a result of SCI. Spinal cord injury initiates the upregulation of immediate early genes, transcription factors, mitogen activated-protein kinases, extracellular signal-regulated

kinases (ERK, p38) in neurons as well as glia [52]. For example, SCI induces persistent upregulation of the phosphorylated form of cyclic AMP response element-binding protein (pCREB) in the spinal cord in chronic states after SCI [13]. This factor initiates transcription of a number of early genes [37] thought to play a role in central sensitization after SCI.

While mechanisms of astrocytic and microglia activation that contribute to changes in neuronal gene expression in the chronic states following spinal cord injury are unclear, we propose the novel hypothesis that activation of different pathways in different glial cell types contribute to increased neuronal hyperexcitability by glial production of proinflammatory factors that are known to sensitize CNS neurons [37]. For example, following sciatic nerve ligation, sequential activation of ERK in neurons then microglia and astrocytes [52] was reported, while others reported increased activation of p38 and p42/44 MAP kinases where p38 was reduced by TNF α inhibitors and by PPF [19]. Others demonstrate that IL-1 β driven wind-up is inhibited by PPF suggesting glial involvement in C-fiber mediated effects on dorsal horn neurons [1]. More recently, several segments below an SCI, pERK activation was demonstrated in microglial but not neurons or astrocytes, leading to increased microglial PGE₂ release that contributed to neuronal hyperexcitability and mechanical allodynia after SCI [51]. By comparison, several segments below a unilateral thoracic SCI, activated p38 is expressed in microglia and neurons but not in astrocytes [25]. Pharmacologically blocking phosphorylation (activation) of p38 results in reduced neuronal electrophysiological hyperexcitability and antiallodynic behavior [25]. Taken together, spinal cord injury produces differential activation of astrocytic and microglial pathways leading to increased extracellular glial cytokine production, specifically via p-p38 and pERK to pCREB pathways in the spinal dorsal horn [12], that render dorsal horn neurons hyperexcitable.

The time frame and role of glial attenuation of neuropathic pain is not well known. Reports in peripheral neuropathic models suggest that activated microglia are involved in the early phase of neuropathic pain whereas activated astrocytes are involved in the late phase of neuropathic pain [52]. However, activated microglia are reported to be involved in the late phase of central neuropathic pain several segments below a contusion spinal cord injury [27]. Our data demonstrate that both astrocytes and microglia are still activated in the chronic phase (i.e., maintained persistent pain) several segments below a unilateral spinal cord injury. Additionally, our present data show a different time frame for attenuation of pain behavior after PPF treatment in our SCI model compared to peripheral neuropathy models. For example, one report found that attenuation of mechanical allodynia in a peripheral neuropathy model is observed within days after intrathecal pretreatment of PPF [44]. However, the present data show that following SCI, early PPF post-injury treatment demonstrates attenuation of mechanical allodynia that is evident 1 week following administration and persists for weeks.

While we can only speculate regarding the latency of attenuation of mechanical allodynia between the peripheral neuropathic and the present central neuropathic model, there are many possible reasons. The simplest explanation is that pre-injury treatment with PPF is more effective in inhibiting glia activation, whereas the present study is post-injury treatment. Another possibility is that the proposed glial inactivation produced by PPF in the peripheral neuropathic model occurs within the same spinal segments of the nerve lesion. In the central neuropathic model of the present study, the hind-limb behavior is several segments away from the central lesion, and there appears to be a threshold effect such that glial inactivation must occur over a greater time course. This is certainly true for the development of central neuropathic pain syndromes which take several weeks to develop in both people and in rodent models, but once developed, persist for life [9,20,24]. It should be remembered that within days to weeks following SCI, there are significant levels of inflammatory cytokines [38], excitatory amino acid receptor overexpression [23,35], changes in neurotransmitters and transporters [26,46] and other processes that contribute to alterations in somatosensory

processing (see [28,29] for reviews). Thus, any intervention may have a delayed response, particularly if the events are cumulative and multi-factorial; i.e., glial to neuronal to intracellular signaling to alterations in transmitter/receptor/transporter expression.

Nevertheless, the current data demonstrate that the propentofylline treatment inhibits spinal astrocytic and microglia activation, prevents GAD₆₅ downregulation, and attenuates mechanical allodynia following SCI. While it is unclear if glial inactivation is causally related to downregulation of GAD₆₅ following spinal cord injury; the hypothesis that glial activation results in loss of GABA neurons or alterations of pathways are consistent with the expanding glial/neuronal literature. Thus, selective inhibition of astrocytic and/or microglia activation or molecular approaches to GAD gene regulation are possible early therapeutic intervention strategies that may alleviate persistent central neuropathic pain in patients following spinal cord injury.

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(A) Contralateral**(B) Ipsilateral** **Fig. 1.**

Intrathecal spinal administration of GABA immediately after SCI attenuates mechanical allodynia measured 28 days later. Unilateral SCI ($n = 5$) results in bilateral mechanical allodynia on the contralateral (uninjured side) and the ipsilateral (injured side) hindlimbs compared to pre-injury values before spinal hemisection (BH). On post-operation day 28 (B i.t. before intrathecal administration), intrathecal 0.1 (#) and 0.5 (*) μg GABA administration significantly affects the mechanical allodynia on both hindlimbs compared to before intrathecal administration ($p < 0.05$). Arrow reflects the time point of intrathecal administration.

Fig. 2.

Topical administration of GABA attenuates evoked hyperexcitability of lumbar WDR dorsal horn neurons. (A) Displays the typical peri-stimulus histogram of lumbar WDR dorsal horn neurons induced by GABA administration. Four weeks after spinal cord injury (after SCI), the lumbar WDR dorsal horn neurons showed hyperexcitability (compared to uninjured, before SCI). Topical administration of GABA (arrow, 0.1 μ g) significantly attenuated the hyperexcitability. (B) Displays the significant changes in evoked activity in the lumbar WDR dorsal horn neurons among three different groups ($p < 0.05$). The data reflect evoked activity at 30 min post-injection.

Fig. 3.

Inhibition of glial activation attenuates the development of mechanical allodynia when administered immediately after SCI. All spinal cord injured rats developed mechanical allodynia compared to before SCI and the sham controls. Intrathecal treatment of 10 mM PPF (diamond, $n = 4$) significantly attenuated the mechanical allodynia on POD 14 and lasted until POD 28 (* $p < 0.05$) whereas 1 mM PPF (square, $n = 4$) did not show any significant changes compared to the SCI alone group (triangle, $n = 4$).

Fig. 4.

Inhibition of astrocytic activation by intrathecal treatment of PPF. (A) Astrocytes show thin branches and small soma areas in the sham controls. (B) Four weeks after spinal cord injury, astrocytes show thickened branches and enlarged soma areas (hypertrophy). (C) Treatment with 1 mM PPF showed no significant changes compared to the SCI alone group. (D) Treatment with 10 mM PPF inhibited astrocytic hypertrophy. Scale bar: 20 μ m. (E) Astrocytic morphology as demonstrated with GFAP immunoreaction product displays significant changes in astrocytic areas among the three different groups after normalization to the sham group at 100%.

Fig. 5.

Inhibition of microglial activation by intrathecal treatment of PPF. (A) Microglia show thin branches and small soma areas in the sham controls. (B) Four weeks after spinal cord injury, microglia show thickened branches and enlarged soma areas (hypertrophy). (C) Treatment with 1 mM PPF showed no significant changes compared to the SCI alone group. (D) Treatment with 10 mM PPF inhibited astrocytic hypertrophy. Scale bar: 10 μ m. (E) Microglia morphology as demonstrated with OX-42 immunoreaction product displays significant changes in microglial areas among the three different groups after normalization to the sham group at 100%.

Fig. 6. Propentofylline treatment prevents the downregulation of glutamic acid decarboxylase (GAD) 65 levels. Top: low magnification shows GAD₆₅ expression in the entire dorsal horn and high magnification (scale bar: 20 μm) shows the expression of GAD₆₅ within neurons (green) and GAD₆₅ (red) around neurons in lamina II. Bottom: statistical analysis among the four different groups after normalization to the sham group at 100%. Intrathecal treatment with 10 mM PPF significantly attenuated the downregulation of GAD₆₅ expression whereas 1 mM PPF did not produce significant changes compared to the SCI alone group ($p < 0.05$).

Fig. 7.

Propentofylline treatment prevents decreases of GAD₆₅ mRNA expression. (A) Unilateral spinal cord injury caused a decrease of GAD₆₅ mRNA expression level on both sides of the spinal cord compared to the sham controls. Intrathecal treatment with 10 mM PPF significantly prevented the decrease of GAD₆₅ mRNA expression levels ($p < 0.05$) whereas 1 mM PPF did not show any significant changes compared to the SCI alone group. (B) Statistical analysis among the four different groups after normalization to the sham group at 100%.