

Published in final edited form as:

Respir Physiol Neurobiol. 2012 January 15; 180(1): 61–68. doi:10.1016/j.resp.2011.10.011.

Glial activation in the spinal ventral horn caudal to cervical injury

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Abstract

Microglia and astrocytes play complex roles following spinal cord injury (SCI), contributing to inflammatory processes that both exacerbate injury and promote functional recovery by supporting neuro-protection and neuroplasticity. The crossed phrenic phenomenon (CPP) is an example of respiratory plasticity in which C₂ cervical hemisection (C₂HS) strengthens crossed-spinal synaptic pathways to phrenic motor neurons ipsilateral to injury. We hypothesized that microglia and astrocytes are activated in the phrenic motor nucleus caudal and ipsilateral to C₂HS, suggesting their potential for involvement in the CPP. To test this hypothesis, an incomplete cervical spinal hemisection (C₂ lateral injury; C₂LI) was performed, and rats were allowed to recover for 1, 3, 14 or 28 days before collecting perfused spinal tissues. Microglia (via OX42) and astrocytes (via GFAP) were visualized with immunofluorescence microscopy in the C₄-C₅ ventral horn, the region encompassing most of the phrenic motor nucleus. OX42-occupied fractional area ipsilateral to injury increased with C₂LI (vs. sham) at 1 (12.5 +/- 1.8%, p<0.001), 3 (29.0 +/- 1.9%, p<0.001), 14 (26.1 +/- 3.1%, p<0.001) and 28 (19.2 +/- 2.0%, p<0.001) days post-C₂LI. GFAP-occupied fractional area also increased with C₂LI at 3 (24.4 +/- 3.2%, p<0.001) and 14 (16.8 +/- 8.3%, p=0.012) days, but not at 1 (6.2 +/- 3.9%, p=0.262) or 28 (10.6 +/- 3.9%, p=0.059) days post-C₂LI. Thus, microglia and astrocytes are activated in the phrenic motor nucleus caudal to C₂LI, suggesting that they play a role in functional deficits and/or recovery following spinal injury.

Keywords

astrocyte; microglia; phrenic; inflammation; crossed-phrenic

1. Introduction

Strengthening surviving neural pathways following spinal cord injury (SCI) via spontaneous or induced neuroplasticity is an important therapeutic strategy (Winslow and Rozovsky, 2003). However, factors that enable or disable plasticity-induced recovery from SCI must

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Author Disclosure Statement:

No competing financial interests exist.

be understood before we can maximize success in restoring function. One important factor that may modulate plasticity is widespread and long-lasting spinal inflammation associated with spinal injuries (Ankeny and Popovich, 2008; Fleming et al., 2006; Popovich et al., 1997; Sroga et al., 2003; Wu et al., 2005). Microglia, the resident CNS immune cells, are activated following spinal injury and are thought to induce neuropathic pain (Coull et al., 2005; Hulsebosch, 2008). On the other hand, microglia may also promote functional recovery (Rabchevsky and Streit, 1997; Rapalino et al., 1998; Schwartz and Yoles, 2006). For example, microglia release trophic substances, such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF) and tumor necrosis factor α (TNF α), that often promote plasticity (Bessis et al., 2007; Coull et al., 2005; Di Filippo et al., 2008; Skold et al., 2000; Yan et al., 2001).

Astrocytes are also involved in inflammatory processes, and may enhance or suppress functional recovery following SCI. For instance, inhibition of Nf κ B-mediated transcription in the astrocytes of spinally injured mice increases the density of translesion neural projections and improves behavioral recovery (Brambilla et al., 2005; Brambilla et al., 2009). Spinal hemisection at T₁₃ activates astrocytes in lumbar dorsal horn for at least 28 days post-surgery in rats (Gwak and Hulsebosch, 2009). Further, treatments that suppress astrocyte activation improve recovery from SCI and decrease signs of neuropathic pain.

Little is known concerning the role of glial activation in the recovery of respiratory motor function following cervical injury. High cervical spinal hemisection at spinal segment C₂ (C₂HS) strengthens latent bulbospinal pathways that cross the spinal midline to phrenic motor neurons below the injury (Porter, 1895). This spontaneous functional recovery is known as the crossed phrenic phenomenon (CPP; Goshgarian, 2003). Plasticity in bulbospinal and propriospinal pathways underlies the CPP (Lane et al., 2008; Moreno et al., 1992; Vinit et al., 2008), and the CPP underlies partial recovery of diaphragmatic function ipsilateral to C₂HS (Goshgarian, 2003; Vinit and Kastner, 2009). Although phrenicotomy causes long-lasting activation of microglia and astrocytes in the phrenic motor nucleus, and this activation is exacerbated by C₂HS (Gould and Goshgarian, 1997), no previous studies describe specific reactions of microglia and astrocytes to C₂HS alone in this critical region for respiratory motor control.

An understanding of spatial and temporal patterns of glial activation following C₂HS is necessary before we will appreciate the potential roles of these cells in enhancing or diminishing plasticity that is vital for functional recovery of breathing capacity. Since the histological changes underlying the CPP likely occur at the level of the phrenic motor nucleus, we chose to examine microglia and astrocyte activation in the C₄-C₅ ventral horn. Glia may suppress or enhance spontaneous compensatory plasticity necessary for full recovery of breathing capacity via production of pro-inflammatory/toxic and/or pro-survival/pro-plasticity molecules. However, this can only occur if the cells are in close proximity to respiratory-related motor neurons.

In this study, we hypothesized that microglia and astrocytes are activated below a C₂ lateral injury (C₂LI) in the phrenic motor nucleus. To test this hypothesis, we examined temporal profiles of neurochemical/morphological activation of microglia and astrocytes after C₂ lateral injury in adult rats. Increased OX42 (microglia) and GFAP labeling (astrocytes) is observed bilaterally in the C₄ ventral horn 1-28 days post-C₂LI. Thus, glia are activated in temporal and spatial domains appropriate to play a role in compensatory respiratory plasticity following cervical spinal injury.

2. Materials and Methods

2.1 Rats

Adult (aged 3-5 months) male Lewis rats (n = 41) were obtained from Harlan (300-350g) and housed individually with free access to food and water and access to one enrichment item (a small PVC tube standard to all rats housed at facility). The University of Wisconsin, School of Veterinary Medicine Animal Care and Use Committee approved all procedures.

2.2 Surgery

Rats were administered buprenorphine (0.1mg/kg), carprofen (4mg/kg), enrofloxacin (5mg/kg) and medetomidine (500ug/kg) prior to delivery of isoflurane by orotracheal intubation. Rats were ventilated with 1.5-2% isoflurane (balance oxygen) for the duration of surgery. After surgery, rats were administered atipamezole (500µg/kg). All drugs were delivered subcutaneously. The spinal cord was approached dorsally for lateral injury surgery (C₂LI). A 2-3 cm incision was made in the skin from the rostral side of the C₁ spinous process in the caudal direction. All underlying muscle layers and connective tissues over the C₂ spinous process were retracted from the C₂ vertebra. A complete dorsal C₂ laminectomy was performed and the left-side C₂ dorsal root was visualized. A 1-mm gap was created in the spinal cord just caudal to the C₂ dorsal root with a blunt-tipped 25-gauge needle connected to a suction pump after cutting the dura over C₂ with microdissection scissors. The surgery created a lesion that extended from the spinal midline to the ventral edge of the spinal cord. Performing all procedures except for C₂ durotomy and lateral injury created sham subjects. Following surgery, deep muscles of the back were not sutured, but both intermediate and superficial muscle layers were sutured together in a simple continuous pattern using 3-0 absorbable sutures. The incised skin was held together with wound clips for 14 days following surgery before removal. Rats were treated, twice daily, with buprenorphine (0.1mg/kg), carprofen (4mg/kg) and enrofloxacin (5mg/kg) for 3 days following surgery.

2.3 Immunohistochemistry

Rats were perfused transcardially, under isoflurane, with 4% formaldehyde after surgery and recovery. Spinal segments C₁-C₃ and C₄-C₆ were collected and sectioned to 40µm using a sliding frozen microtome. Sections from the site of injury were visualized with Cresyl Violet to confirm the extent of injury (Figure 1). For immunohistochemistry, 4 sections from the spinal cord immediately caudal to the site of injury (C₂) and 4 sections taken from spinal segments C₄-C₅ were stained from each animal. Sections were labeled with antibodies raised against glial fibrillary acidic protein (GFAP, Millipore), a protein expressed by reactive astrocytes (Eng et al., 2000), and CD11b (OX42, Santa Cruz Biotechnology), a protein expressed by microglia and invading macrophages in the central nervous system (Robinson et al., 1986). It was not possible to differentiate between resident microglia and invading macrophages by morphology or CD11b expression, therefore the cells present at the site of injury may represent a mixture of both populations. However, the cells present in the area of the phrenic motor nucleus are most likely composed of resident microglia (David and Kroner, 2011). A fluorescent Nissl counterstain (Neurotrace 435/455, Invitrogen) was applied to sections for 20 minutes to locate presumptive phrenic motor neurons in the C₄-C₅ ventral horn (Bagley et al., 2007; Windelborn and Lipton, 2008). Sections were mounted on slides with ProLong Gold Antifade Mounting Medium (Invitrogen) and imaged using a Nikon wide-field epifluorescent system with MetaMorph 7 imaging software.

2.4 Image Analysis

Raw images were analyzed using Image J (NIH, Bethesda, MD). Analysis was performed using images taken at 400x magnification of fields surrounding putative phrenic motor

neurons at spinal segment C₄-C₅ (i.e. phrenic motor nucleus) or in the vicinity of large Neurotrace-positive neurons in the area immediately caudal to the C₂ ventral horn. Location of putative phrenic motor neurons was based on previous studies using retrograde tracers from the diaphragm (Guenther et al., 2010). The full microscopic field of each image was analyzed, except in cases that bubbles or dust were eliminated from the analysis. The fractional area occupied by GFAP or OX42 label in raw image files was computed by Image J (as the percentage of the total field occupied by label-positive pixels) and interpreted as the fractional area of reactive astrocyte or microglia, respectively. Increased fractional area of OX42 staining indicates greater microglial activity (Ling et al., 1990; Ling and Wong, 1993; Robinson et al., 1986; Roy et al., 2006). This analysis does not allow a distinction between greater cell sizes versus greater cell number. Background staining was subtracted from each image using Image J's "set threshold from background" plug-in, choosing a cell-free area from each image for selection of a background region of interest. Images were then processed for publication using Photoshop CS3 (Adobe). Processing consisted of adjustments to an image's brightness and contrast and was applied equally across all images.

2.5 Statistics

Analyses were performed using ANOVAs indicated in figure legends; individual differences were detected with Fisher's LSD *post hoc* test.

3. Results

3.1 Sham surgery elicits microglial and astrocytic activation

Baseline measurements of the fractional areas of microglial (OX42) and astrocytic (GFAP) fluorescence were made in spinal cord sections from sham-treated rats. Microglial activation was estimated based on the fractional area of a microscopic field occupied by OX42 staining in the C₂ or C₄ ventral horn. Because a visual trend appeared in sham-operated tissues, analyses were performed on microglial and astrocytic staining across recovery times in both spinal segments C₂ and C₄. The fractional area of sham C₂ ventral horn occupied by OX42 fluorescence was significantly increased at 3, 14 and 28 days post-surgery vs. the fractional area at 1-day post-surgery (Figure 2A, black bars). OX42 fractional area peaked at 14-days post-surgery; values at 14-days were significantly greater than those at either 1-day or 3-days post-surgery.

The fractional area of sham C₄ ventral horn occupied by OX42 fluorescence was also significantly increased at 3, 14 and 28 days post-surgery vs. fractional area at 1-day post-surgery (Figure 2A, grey bars). OX42 fractional area peaked at 14-days post-surgery, when values were significantly greater than those at either 1-day or 3-days recovery. Representative images for OX42 are shown from spinal segments C₂ (Figure 2C) and C₄ (Figure 2D).

In contrast to OX-42, no significant differences were measured across post-surgical times for GFAP fractional area in sham rats at either spinal segment (Figure 2B). Representative images for GFAP are shown from spinal segments C₂ (Figure 2C) and C₄ (Figure 2D).

Negative controls were created for non-specific fluorescence by omitting primary antibody or by omitting primary and secondary antibodies from the immunohistochemical procedures. Detectable fluorescence in these cases was minimal (data not shown).

3.2 Increased microglial and astrocytic markers at injury site (C₂)

Following C₂LI, ventral horn regions ipsilateral and contralateral to injury were examined for changes in microglia and astrocyte activation. The fractional area of ipsilateral C₂ ventral

horn occupied by OX42 fluorescence was significantly increased in injured over sham animals at 1-day (23.5 +/- 6.7% vs. 5.4 +/- 1.0%, $p < 0.001$), 3-days (41.0 +/- 3.4% vs. 13.1 +/- 0.9%, $p < 0.001$), 14-days (41.1 +/- 2.8% vs. 20.5 +/- 0.8%, $p < 0.001$) and 28-days (41.2 +/- 3.4% vs. 16.4 +/- 2.1%, $p < 0.001$) post-surgery (Figures 3A, black bars, and 3C, red). Contralateral to injury, the fractional area of C₂ ventral horn occupied by OX42 fluorescence was not significantly different from sham at 1 (10.4 +/- 2.6%, $p = 0.245$), 14 (21.6 +/- 3.1%, $p = 0.826$) or 28 (24.1 +/- 3.4%, $p = 0.075$) days post-C₂LI (Figures 3A, grey bars, and 3D, red). At 3 days post-C₂LI, contralateral OX42 fractional area was significantly greater than sham (21.6 +/- 3.1%, $p = 0.049$). Comparisons between ipsilateral and contralateral tissues were made by two-way ANOVA based on fractional areas normalized to time-matched shams (Figure 3) because of the shifting baseline fraction areas in sham tissues (Figure 2). The normalized OX42 fluorescence in ipsilateral C₂ ventral horn was significantly greater than contralateral C₂ ventral horn at 1 ($p = 0.017$), 3 ($p < 0.001$), 14 ($p = 0.002$), and 28 ($p = 0.001$) days post-C₂LI. Thus, microglial activation ipsilateral to injury was already significant at 1-day, and reached a peak by 3-days but then remained elevated for at least 28-days post-C₂LI.

The fractional area occupied by GFAP fluorescence was used as a measure of astrocyte activation. The fractional area of ipsilateral C₂ ventral horn occupied by GFAP fluorescence was significantly increased in injured over sham animals at 1-day (22.4 +/- 3.2% vs. 9.8 +/- 1.9%, $p = 0.012$), 3-days (50.3 +/- 6.7% vs. 8.8 +/- 1.4%, $p < 0.001$), and 28-days (42.2 +/- 4.3% vs. 17.8 +/- 3.9%, $p < 0.001$) post-surgery (Figures 3B, black bars, and 3C, green). GFAP fractional area 14 days post-surgery was not significantly different from sham (40.7 +/- 8.1% vs. 26.5 +/- 7.2%, $p = 0.056$). Contralateral to injury, the fractional area of C₂ ventral horn occupied by GFAP fluorescence was also significantly different from sham at 1 (25.9 +/- 2.3%, $p = 0.047$), 3 (37.1 +/- 3.5%, $p < 0.001$) and 28 (36.8 +/- 3.3%, $p = 0.003$) days post-C₂LI (Figures 3B, grey bars, and 3D, green). At 14 days post-C₂LI, contralateral GFAP fractional area was not significantly greater than sham (36.8 +/- 6.4%, $p = 0.116$). The normalized GFAP fluorescence in ipsilateral C₂ ventral horn was significantly greater than contralateral C₂ ventral horn at 3 ($p = 0.03$) days post-C₂LI, but did not differ from ipsilateral fraction area at 1 ($p = 0.591$), 14 ($p = 0.725$) or 28 ($p = 0.368$) days. Thus, the timecourse and side-to-injury characteristics of astrocyte activation differed from microglia activation following C₂LS.

3.3 Increased microglial and astrocytic markers caudal to injury

Microglia and astrocyte activation were also estimated caudal to injury in the C₄-C₅ ventral horn, segments that contain most of the phrenic motor nucleus. Image fields were chosen based on the presence of presumptive phrenic motor neurons (large Neurotrace-positive cells often localized in a centralized cluster of the ventral horn; Guenther et al., 2010).

The fractional area occupied by OX42 fluorescence in the ipsilateral C₄ ventral horn significantly increased in injured vs. sham rats at 1-day (17.6 +/- 1.8% vs. 5.0 +/- 0.7%, $p < 0.001$), 3-days (38.6 +/- 1.9% vs. 9.7 +/- 0.5%, $p < 0.001$), 14-days (41.7 +/- 3.1% vs. 15.6 +/- 2.9%, $p < 0.001$) and 28-days (32.9 +/- 2.0% vs. 13.7 +/- 1.5%, $p < 0.001$) post-C₂LI (Figures 4A, black bars, and 4C, red). OX42-positive cells appeared thick and condensed in injured rats. This morphological state is indicative of activated microglia, providing further evidence that individual microglia were activated in the phrenic motor nucleus (i.e. caudal to injury) at all times tested. The large number of glial cells and overlapping processes made it difficult to differentiate one cell from another, so cell numbers were not determined. Contralateral to injury, the fractional area of C₄ ventral horn occupied by OX42 fluorescence was significantly greater than sham at 1 (10.1 +/- 0.9%, $p = 0.032$), 3 (22.0 +/- 1.5%, $p < 0.001$) and 28 (18.4 +/- 1.5%, $p = 0.048$) days post-C₂LI (Figures 4A, grey bars, and 4D, red). OX42 fractional area did not differ significantly from sham 14 days post-C₂LI

(20.9 +/- 0.9%, $p=0.054$). Sham-normalized ipsilateral C₄ OX42 fractional area was significantly greater than the contralateral side at 1 ($p=0.007$), 3 ($p<0.001$), 14 ($p<0.001$) and 28 ($p<0.001$) days post-C₂LI.

The fractional area occupied by GFAP fluorescence in the ipsilateral C₄ ventral horn was increased in injured vs. sham rats at 3-days (34.4 +/- 3.2% vs. 10.0 +/- 2.5%, $p<0.001$) and 14-days (32.2 +/- 8.3% vs. 15.4 +/- 2.7%, $p=0.012$) post-C₂LI (Figures 4B, black bars, and 4C, green). GFAP-positive cells appeared hypertrophied in spinally injured *versus* sham rats, indicating a more reactive state following C₂LI. GFAP fractional area did not differ significantly from sham on 1 (17.1 +/- 3.9% vs. 10.9 +/- 1.4%, $p=0.262$) or 28 (27.2 +/- 3.9% vs. 16.6 +/- 2.0%, $p=0.059$) days post-C₂LI. Contralateral to injury, the fractional area of C₄ ventral horn occupied by GFAP was significantly greater than sham at 3 (36.0 +/- 4.5%, $p<0.001$), 14 (32.4 +/- 8.0%, $p=0.011$) and 28 (30.9 +/- 3.5%, $p=0.012$) days post-C₂LI (Figures 4B, grey bars, and 4D, green). At 1-day post-C₂LI, contralateral GFAP did not differ significantly from sham fractional area (19.7 +/- 2.9%, $p=0.117$). When normalized to time-matched shams, ipsilateral GFAP fractional areas did not differ from contralateral at any time post-C₂LI ($p>0.5$). These results indicate a rapid and persistent inflammatory response at sites remote from spinal injury, and that this response occurs in areas critically involved in the control of inspiration. However, unlike microglia, astrocytes were activated without significant differences between ipsilateral and contralateral GFAP following C₂LI.

4. Discussion

4.1 Significance of findings

We demonstrated that glial activation occurs at sites caudal to cervical spinal injury, including the phrenic motor nucleus, a site critical for respiratory motor control. Previous reports have demonstrated rapid inflammatory responses at the site of spinal injury, as well as at distant sites caudal to injury (Byrnes et al., 2006; Carlson et al., 1998; Fleming et al., 2006; Gwak and Hulsebosch, 2009; Popovich et al., 1997). We extend those findings here by focusing on the inflammatory response in or near the phrenic motor nucleus. C₂ hemisection is frequently studied as a model of respiratory insufficiency and functional recovery following cervical spinal injury. C₂ hemisection eliminates 90% of bulbospinal pathways to phrenic motor neurons, and induces spontaneous respiratory plasticity and partial functional recovery below the site of injury (Goshgarian, 2003; Vinit and Kastner, 2009). This is the first report of chronic glial activation (and possible inflammation) in the phrenic motor nucleus following C₂ lateral injury.

The injury performed in this manuscript would have previously been grouped into the broad category of C₂ hemisection. However, a recent report described several variations of the C₂ hemisection, and the surgery performed most closely matches what was termed 'C₂ hemilesion with moderate ventromedial sparing' (Fuller et al., 2009). In that report, the term 'C₂ hemisection' is reserved for injuries in which a complete ablation of one-half of the spinal cord is performed at spinal segment C₂.

4.2 Sham surgery

Increased OX42 staining was measured over time following sham surgeries. This finding suggests that some damage was done to the spinal cord by laminectomy and/or durotomy. On the other hand, measurements of GFAP over time remained stable following sham surgery. Unfortunately, naïve rats were not run at the time of these experiments. Thus, additional statistical analyses were necessary including the comparison of both raw fraction areas and fraction areas normalized to sham values.

4.3 Sidedness of activation

C₂ hemisections cause lateralized effects on the spinal cord and on animal physiology. For instance, expression of long-term facilitation is observed in the ipsilateral (Golder et al., 2005), but not contralateral, phrenic nerve of rats 1-2 months post-surgery (Doperalski and Fuller, 2006). It was suggested that this differential response is a result of a compensatory increase of respiratory drive to contralateral phrenic motor neurons that limits their ability to express LTF. Identifying mechanisms by which ipsilateral respiratory drive can be heightened is, thus, an important step towards inducing functional recovery via mechanisms of plasticity. Thus, will be important to identify if the glial activation described here is pro-plasticity versus pro-inflammatory since inflammation may be a formidable barrier to full expression of spinal respiratory plasticity (Huxtable et al., 2011).

Like physiological measures of recovery, at least some cell types involved in lateralized effects are expected to exhibit some degree of sidedness following C₂ lateral injury. Microglial activation was distinct ipsilateral to injury, making it a candidate for a cell that contributes to the lateralized effects of C₂ lateral injury. Measurements of contralateral OX42 were, for the most part, greater than sham, but they were significantly less than ipsilateral measurements at all times investigated. In contrast, astrocytic GFAP staining did not show sidedness following C₂ lateral injury at any time studied, suggesting that microglia may play a more important role in this respect.

4.4 Timecourse vs. physiological literature

A previous report indicated that neither microglia nor astrocyte fractional areas are increased in the C₄ ventral horn within 24 hours post-C₂ lateral injury (Gould and Goshgarian, 1997). Our results were only partially consistent with those findings. Microglial staining in the area of the phrenic motor nucleus was significantly increased ipsilateral to injury after 1 day. Staining peaked at 3-days post-C₂LI and remained elevated for at least 28 days. The pattern contralateral to injury was similar, but OX42 staining was considerably less than the ipsilateral levels response. Although astrocytic staining in the area of the phrenic motor nucleus was not significantly greater than sham at 1 day post-C₂LI, it was significantly increased by 3-days post-C₂LI, and remained elevated for at least 28 days. It is possible that the difference between results in this study and those from the earlier study are due to the gender of rats used. Gould and Goshgarian (1997) used female rats, while we used males. Lower estrogen levels in male rats may allow a greater inflammatory response following spinal injury (Sribnick et al., 2005), potentially explaining earlier activation of microglia in the phrenic motor nucleus of our male rats.

Neurochemical responses related to inflammation wane by 2-4 weeks post-surgery in other models of spinal injury (e.g. TNF α and p75 neurotrophin receptor expression), despite the fact that morphological signs of glial activation persist (Gwak and Hulsebosch, 2009; Popovich et al., 1997). Thus, our results are consistent with earlier reports from other spinal regions using different models of spinal injury since cervical (C₃₋₅) microglia and astrocytes remained morphologically activated 28 days post-C₂LI. The potential role of these cells in functional recovery at extended time points is an interesting topic for further investigation. However, since microglia and astrocytes produce a variety of growth factors following spinal injury (Brown et al., 2004; Dougherty et al., 2000; Skold et al., 2000), these cells may affect respiratory motor plasticity in either positive or negative ways by producing pro-inflammatory/toxic and pro-plasticity/pro-survival molecules.

Phrenic motor plasticity induced by intermittent hypoxia is suppressed by lipopolysaccharide (LPS) induced inflammation (Vinit et al., 2011). Similarly, injury-induced inflammation may at least transiently impair plasticity following cervical spinal

injury. For example, intermittent hypoxia-induced phrenic motor facilitation is minimal below a C₂ hemisection at 2-weeks post-surgery, is marginal at one month (depending on the rat strain), but is fully expressed by two months post-surgery (Golder and Mitchell, 2005). Although an argument was made that these dynamics reflect the loss of and then partial recovery of serotonergic innervation below the injury, the present data suggest an unrecognized factor with the potential to modulate the capacity for intermittent hypoxia induced plasticity following spinal injury, namely time-dependent changes in the inflammatory activities of microglia and astrocytes near phrenic motor neurons. Further experiments assessing production of pro-plasticity and pro-inflammatory factors by glia in the phrenic motor nucleus at various times after injury would help to test this possibility.

4.5 Conclusions

Microglia and astrocytes release many factors in the vicinity of phrenic motor neurons that could enhance or suppress respiratory motor plasticity. In the early stages post-injury, inflammatory molecules (e.g. cytokines and prostaglandins) may undermine the capacity for plasticity (Byrnes et al., 2006; Pineau and Lacroix, 2007; Yang et al., 2005; Zhao et al., 2007), whereas production of pro-plasticity molecules (e.g. BDNF and VEGF) may become more prominent with time post-injury (Krenz and Weaver, 2000; Peng et al., 2006; Sieck and Mantilla, 2009). Each of these growth/trophic factors has the potential to induce and/or potentiate plasticity. The net effects of glial activation will presumably depend on their balance of pro-plasticity vs. pro-inflammatory molecules.

Here we show that glial activation is rapid and persistent, suggesting that these cells likely participate in complex time-dependent responses following C₂ hemisection. Future investigations must explore the timing and identity of glial protein expression. Such information will provide a better understanding of the cellular and molecular environment in which any new/potential treatments for SCI must operate and may help define times of greatest efficacy.

Acknowledgments

Supported by National Institutes of Health R37 HL69064. J.A. Windelborn was supported by NIH T32HL007654.

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Highlights

> We examined microglia and astrocyte activation in the phrenic motor nucleus after C2 hemisection. > Microglia activation was measured by OX42 immunofluorescence. > Astrocytic activation was measured by GFAP immunofluorescence. > Microglial activation increased at 1-28 days after injury. > Astrocytic activation increased at 1-28 days after injury.

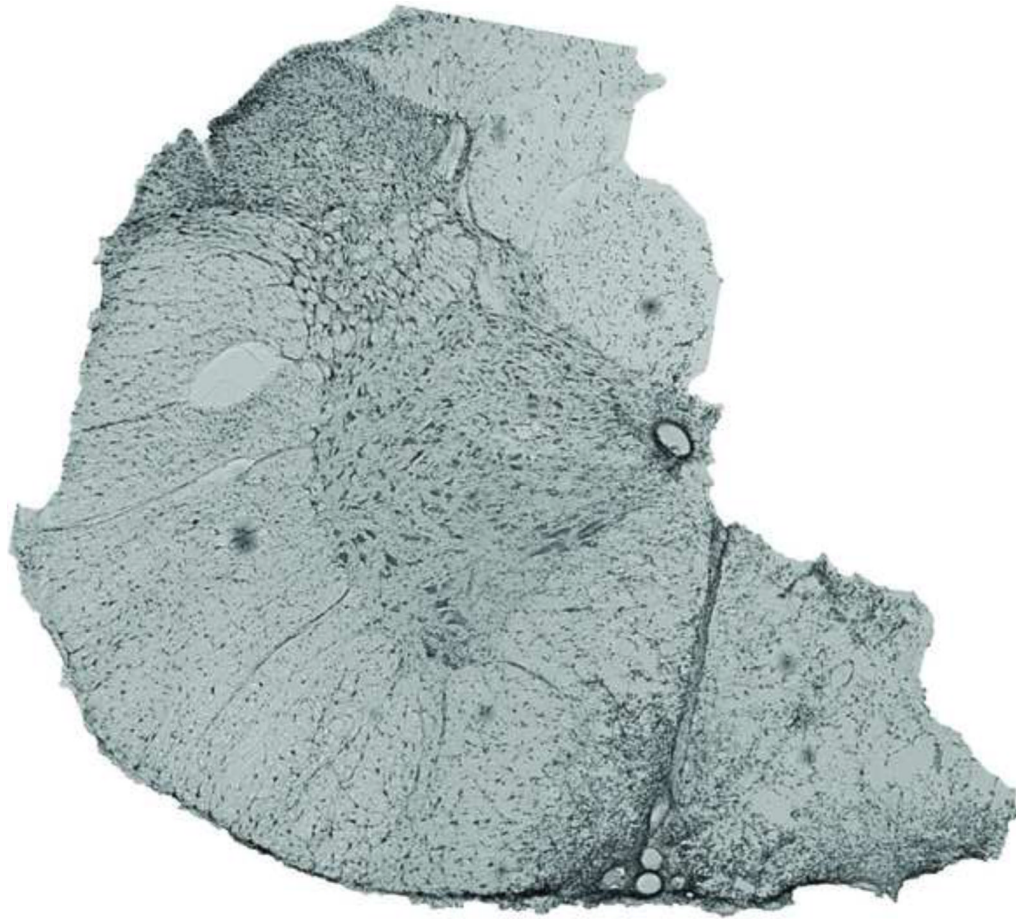


Figure 1.

A coronal section taken from the site of C₂ lateral section shows a typical extent of injury. C₂ lateral section was incomplete in these studies, with the ventromedial funiculus remaining intact. These images are similar in many respects to many previous studies concerning spontaneous recovery of respiratory motor function following cervical spinal hemisection (Fuller et al., 2009; Goshgarian, 1981). The micrograph shows a full coronal section and was obtained using phase-contrast microscopy.

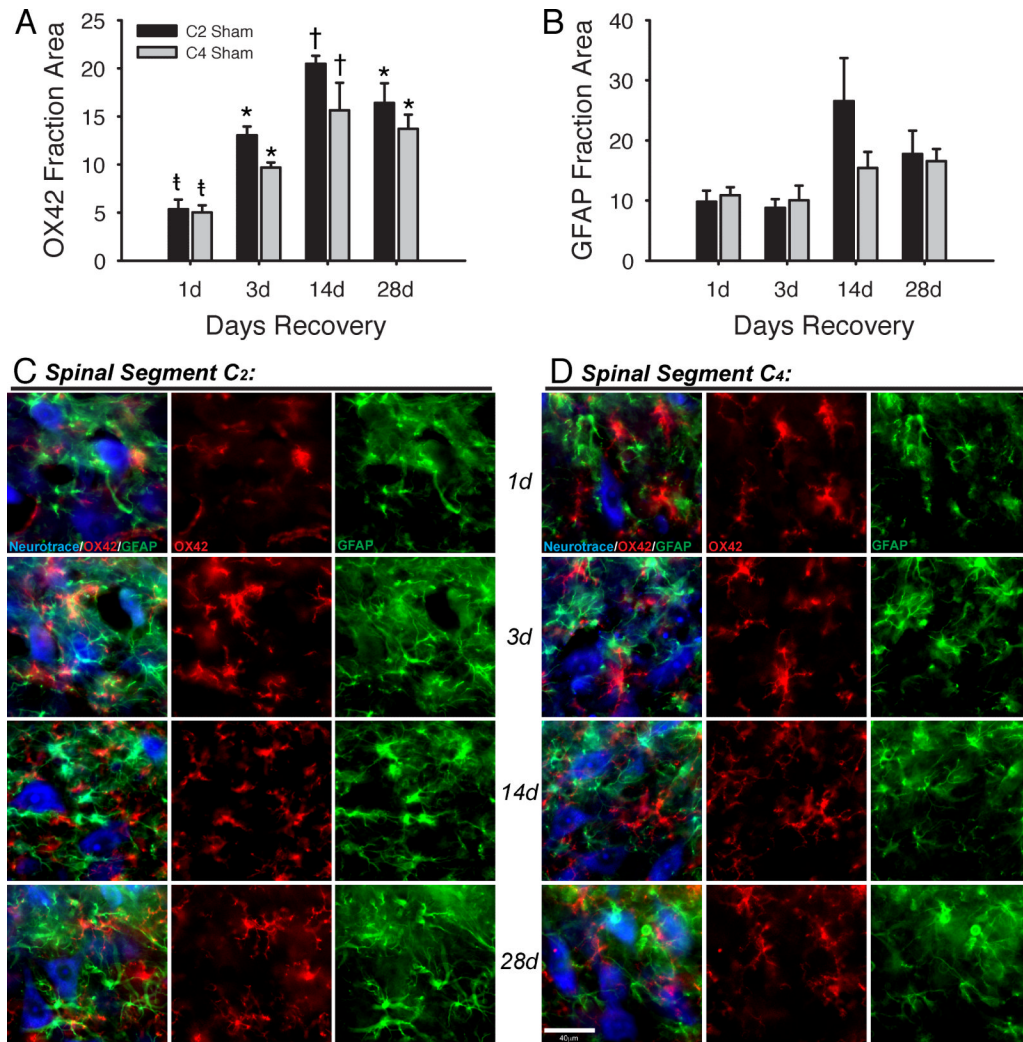


Figure 2.

Immunohistochemistry revealed significant differences between sham tissues after different post-surgical durations. The fraction of C₂ and C₄ ventral horn occupied by OX42 (A) was significantly different between days. The fraction of C₂ and C₄ ventral horn occupied by GFAP (B) did not differ significantly between days. A representative image from each day post-surgery is provided in rows for both C₂ (C) and C₄ (D). All images were taken from sham tissues. The first column in each panel is a merged image showing Neurotrace (blue), OX42 (red) and GFAP (green). OX42 and GFAP are shown individually in panels to the right of the merged image. One-Way ANOVA run for each antigen separately within each spinal segment; $p < 0.05$: *vs. 1d, †vs. 1d and 3d, ‡vs. 3d. Scale bar is 40 μ m.

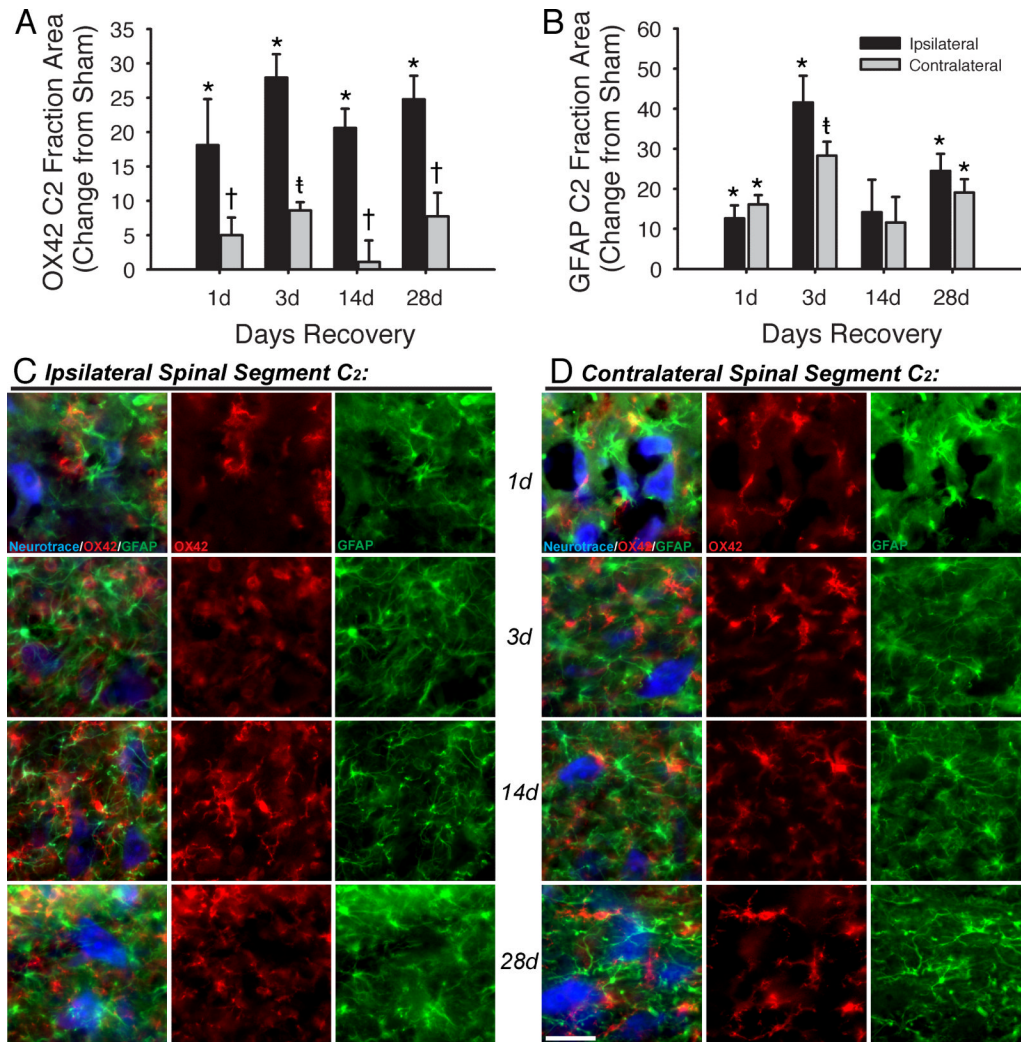


Figure 3. Microglial and astrocytic staining is increased in the ventral horn immediately caudal to the site of C₂ lateral section. OX42 staining (A) ipsilateral to injury is increased vs. sham at all times post-injury. Contralateral to injury, increases are observed at only 3 days post-injury. Additionally, increases contralateral to injury are significantly lower than increases measured ipsilateral to injury. GFAP staining (B) was increased vs. sham at 1, 3, and 28 days post-injury. In contrast to OX42 staining, no difference was observed between ipsilateral and contralateral GFAP except 3 days after injury. Images taken from the C₂-C₃ ventral horn of spinally injured rats are shown for sites ipsilateral (C) and contralateral (D) to injury, with each row containing a field from 1, 3, 14, or 28 days after injury. The left-hand column in each panel shows a merged image, with Neurotrace in blue, OX42 in red and GFAP in green; the central column shows only OX42; the right-hand column shows only GFAP. Scale bar is 40 μ m. Three-way ANOVA tested differences between sham, contralateral and ipsilateral area fractions across all recovery times. Two-way ANOVA tested normalized differences between contralateral and ipsilateral changes from sham across all recovery times. * $p < 0.05$ vs. sham; † $p < 0.05$ vs. ipsilateral; ‡ $p < 0.05$ vs. sham and ipsilateral.

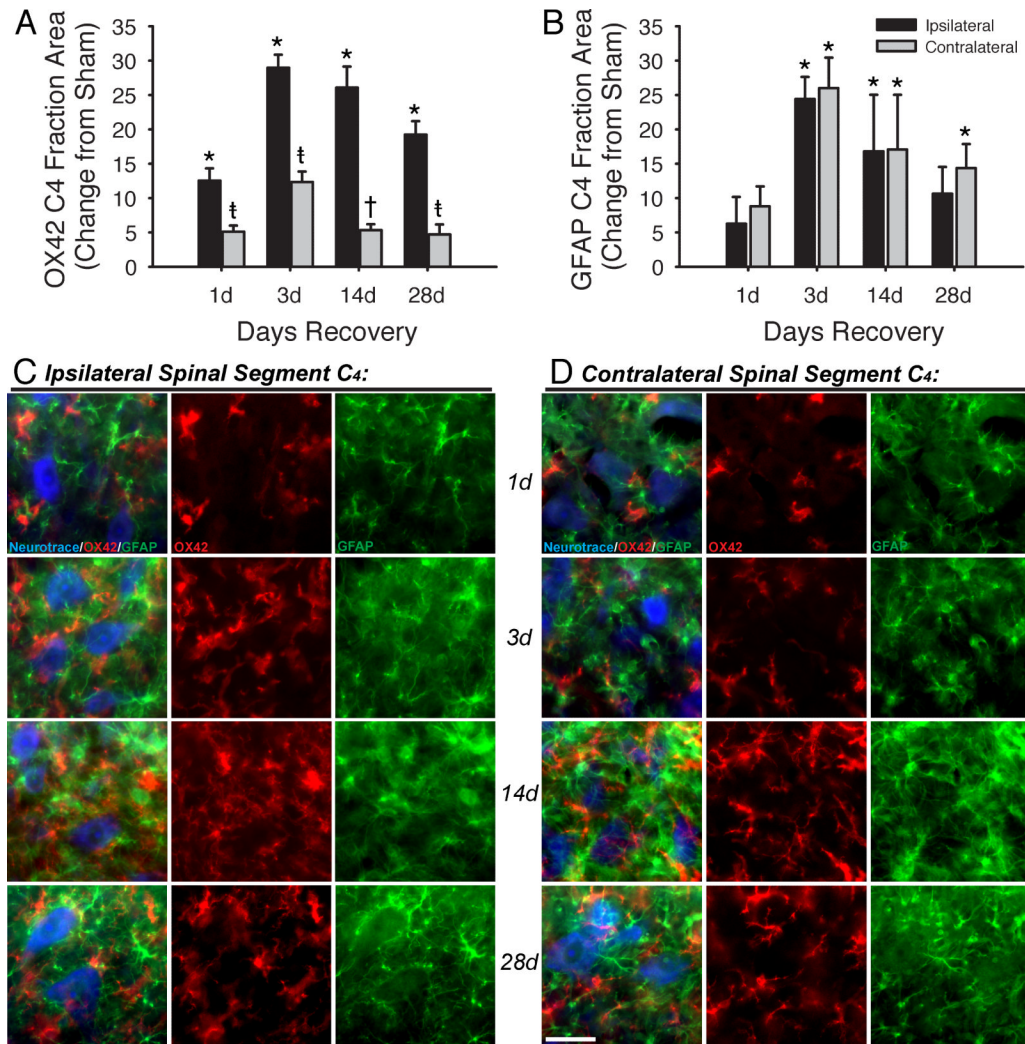


Figure 4.

Microglial and astrocytic staining is increased in the region of the phrenic motor nucleus after spinal injury. OX42 staining (A) ipsilateral to injury is increased vs. sham at all times post-injury. Contralateral to injury, increases are observed at 1, 3 and 28 days post-injury. Additionally, increases contralateral to injury are significantly lower than increases measured ipsilateral to injury. GFAP staining (B) did not increase vs. sham at 1 day post-injury, but was significantly increased vs. sham at 3, 14 and 28 days post-injury. In contrast to OX42 staining, no difference was observed between ipsilateral and contralateral GFAP after injury. Images taken from the C₄ ventral horn of spinally injured rats are shown for sites ipsilateral (C) and contralateral (D) to injury, with each row containing a field from 1, 3, 14, or 28 days after injury. The left-hand column in each panel shows a merged image, with Neurotrace in blue, OX42 in red and GFAP in green; the central column shows only OX42; the right-hand column shows only GFAP. Scale bar is 40 μ m. Three-way ANOVA tested differences between sham, contralateral and ipsilateral area fractions across all recovery times. Two-way ANOVA tested normalized differences between contralateral and ipsilateral changes from sham across all recovery times. * $p < 0.05$ vs. sham; † $p < 0.05$ vs. ipsilateral; ‡ $p < 0.05$ vs. sham and ipsilateral.