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Transient changes in spinal cord glial cells following transection of preganglionic sympathetic axons

Aminata P. Coulibaly and Lori G. Isaacson*

Center for Neuroscience and Behavior, Department of Zoology, Miami University, Oxford, OH 45056

Abstract

Following peripheral nerve injury, retrograde signals originating from the injury site may activate intrinsic factors in the injured neurons, possibly leading to regenerative growth. Retrograde influences from peripheral injury sites may lead to the activation of glial cells in the vicinity of the centrally located cell bodies of the injured neurons. Few studies have examined changes in the spinal cord intermediolateral cell column (IML), which houses sympathetic preganglionic cell bodies, following injury to distal axons in the cervical sympathetic trunk (CST). The goal of the present study was to determine if transection of the CST results in plasticity in glial cells in the IML. At 1 day following injury, changes in the expression of microglial marker Iba1 were observed and the typical oligodendrocyte-neuronal relationship was altered. By 7 days, astrogliosis, microglial aggregation, and increased numbers of oligodendrocytes, as well as enhanced glial-glial and glial-neuronal relationships were present. The majority of cases were similar to controls at 3 weeks following injury and no changes were observed in any cases at 10 weeks following the injury. These results revealed changes in astrocytes, microglia, oligodendrocytes in the spinal cord following transection of preganglionic axons comprising the CST, indicating their ability to respond to distal axonal injury.

Keywords

peripheral nerve injury; central response; oligodendrocyte response; cervical sympathetic trunk; intermediolateral cell column (IML); astrogliosis; microglial aggregation

INTRODUCTION

The underlying mechanisms leading to regeneration of peripheral neurons following injury are poorly understood. Following peripheral nerve injury, positive signals as well as removal of negative signals from the injury site can activate intrinsic factors in the injured neurons, typically leading to regenerative growth (reviewed in Abe and Cavalli, 2008). As a consequence the injured neurons are thought to secrete factors into the local environment that can activate non-neuronal cells following injury. Such retrograde influences may lead to the activation of glial cells in the vicinity of the cell bodies of the injured neurons. The activation of central glia has been reported near the motor neurons of the hypoglossal

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*Corresponding author Lori G. Isaacson, PhD, Center for Neuroscience and Behavior, Department of Zoology, 280 Pearson Hall, Phone: 513-529-3142, FAX: 513-529-6900, isaacslg@muohio.edu.

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nucleus (Svensson et al., 1993; Yamada et al., 2011), the facial nucleus (Rohlmann et al., 1994; Kalla et al., 2007), and the ventral horn of the spinal cord following distal injury (Gilmore et al., 1990; Coyle, 1998). Whether this phenomenon occurs following injury to autonomic neurons is unknown.

Few studies have examined the specific changes in the spinal cord intermediolateral cell column (IML), which houses sympathetic preganglionic cell bodies, following injury to distal axons in the cervical sympathetic trunk (CST). Tang and Brimijoin (2002) reported a 50% neuronal loss in the IML by 10–12 weeks following CST immunolesion or transection. While these studies demonstrated changes in IML neurons, the possibility of plasticity in glial cells in the IML following CST injury was not explored. Further, though central glial cells such as astrocytes and microglial cells have been shown to respond in other models, there are no investigations of whether oligodendrocytes respond following peripheral axon injury.

The goal of the present study was to determine if transection of the CST results in plasticity in astrocytes, microglia, and/or oligodendrocytes in the IML. Transection was chosen as the means of injury in order to compare with similar models that utilized transection to injure peripheral axons associated with facial (Rohlmann et al., 1994) and hypoglossal (Svensson et al., 1993; Yamada et al., 2011) nerves. Changes in glial cells in the IML following transection of the CST were investigated at short term (1 day, 7 days) and long term (3 weeks and 10 weeks) survival times. The short term time points were chosen to parallel previous studies of the facial (Rohlmann et al., 1994) and hypoglossal (Svensson et al., 1993) nuclei, where glial cell activation was observed in response to peripheral nerve transection as early as 12 hrs and 2 days following injury, respectively. Further, examination at the longer survival times allowed for an assessment of changes in the IML during the period when pupillary function reportedly occurred in rabbits (approximately 40 days after CST crush; Butson, 1950) and cats (15–30 days after CST crush and approximately 60 days following CST transection; Langley, 1895). Our results revealed changes in IML astrocytes and microglial cells, as reported in other models, as well as in oligodendrocytes, following transection of the CST, with the most obvious changes occurring at the 7 day time point.

METHODS

Animals

Young adult (3 months of age) female Sprague Dawley rats (Harlan Labs, Indianapolis, IN) were housed in the Miami University Animal Facilities in a 12:12 light:dark environment at regulated temperature (~21°C). The cervical sympathetic trunk (CST) was bilaterally transected and animals survived for 1 day (n=4), 7 days (n=4), 3 weeks (n=10) or 10 weeks (n=5) following the injury. Data were compared with respective age-matched controls that either received sham surgery or underwent no surgical procedures, and that corresponded to the 1 day and 7 day (n=4), 3 week (n=10), and 10 week (n=4) survival time points. Because no changes were observed in any of the sham cases, data collected from shams and unoperated control groups were pooled. All methods used in this study were approved by the Miami University Institutional Animal Care and Use Committee (IACUC) and efforts were taken to minimize discomfort and pain to the animals and to minimize the number of animals used in the study.

Transection of the cervical sympathetic trunk

Rats were anesthetized either with Ketamine (80 mg/kg):Rompun (14mg/kg) cocktail or via the inhalant 2.5% isoflurane. A 3 cm ventral incision was made on the neck region of the animal. The CST was exposed and gently separated from surrounding tissue and transected

approximately 2 mm from the entry into the SCG (Nagata et al., 1987; Sun and Zigmond, 1996). Sham animals underwent the same procedure but the CST was not transected. As an additional control for the surgery and to show that the response by cells in the IML was specific to injury of the CST, additional levels of the spinal cord (upper cervical and lower thoracic) were examined from the same animals in which a response in the IML was observed.

Tissue preparation and immunohistochemistry

Animals were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The entire spinal cord was removed and spinal cord segments C8-T3 were blocked from the cord using the dorsal roots as a guide (Rando et al., 1981) and stored individually in 0.1M PB in 1 dram glass vials. The segments were individually embedded in optimal cutting temperature (O.C.T.) medium (Ted Pella Inc.) and cut in the coronal plane at 18µm thickness. Sections were collected in series onto 15 microscope slides with the first section on slide one and the second section on slide 2, etc. so that representative rostral-caudal sections of the spinal cord segment were mounted on one slide, with each section on the slide approximately 270 µm apart.

Sections were mounted on Superfrost microscope slides. Antibodies were directed against: 1) the astrocyte marker glial fibrillary acidic protein (GFAP; Abcam) which resulted in a distinct 50kDa band in western analysis; 2) the microglial markers OX42 (Serotec), a clone of the cluster determinant (CD) 11b molecule, and ionized calcium binding adaptor molecule (Iba) 1, as described in Romero-Sandoval et al. (2008); 3) the oligodendrocyte marker, adenomatous polyposis coli gene clone (APC-CC1; Abcam) and the full length TrkB receptor (MW: 145 kDa; sc-12; Santa Cruz Biotech) which has been characterized in previous studies to label oligodendrocytes (Frisen et al., 1993; Skup et al., 2002; Macias et al., 2005; McCartney et al., 2008) as well as preganglionic neurons (McCartney et al., 2008) and which resulted in a distinct 145kDa and 90kDa band in TrkB western analysis of CNS tissues (Wu et al. 1996; Ohira et al. 1999). These non-neuronal cell markers were used in conjunction with antibodies directed against the neuronal marker choline acetyltransferase (ChAT; Millipore), a common marker for preganglionic neurons in the IML (reviewed in Woolf, 1991), and activating transcription factor-3 (ATF-3; Santa Cruz Biotech), a marker for injury (Tsujino et al., 2000; Kataoka et al., 2007), to identify the boundaries of the IML (Fig.1) and to examine any relationships of glial cells with injured (ATF-3 immunopositive) neurons. In addition, incubation with no primary antibody served as a control for each of the antibodies examined.

Sections were incubated overnight in 0.1M PBS-0.2% Triton-X solution, blocked with normal donkey serum, and then incubated for 48 hrs at 4°C in primary antibody: chicken anti-GFAP (1:500), rabbit anti-TrkB (1:200), mouse anti-APC-CC1 (1:1000), mouse anti-rat OX42 (1:100), goat anti-Iba1 (1:200, Abcam), goat anti-ChAT (1:200), or rabbit anti-ATF-3 (1:200) diluted in 0.1M PBS. Following a series of rinses, sections were incubated for 2 hours in AlexaFluor conjugated antibodies (made in donkey; 1:200) directed toward the primary host antibody. Sections then were rinsed and coverslipped using fluorescent mounting medium with DAPI (Vectashield). Images were captured with either an Olympus FV500 or a Zeiss 720 laser scanning confocal microscope.

Analysis of glial cells in the IML

Because the neurons that project axons into the CST are located throughout the upper thoracic spinal cord (Rando et al., 1981; Strack et al., 1988), a qualitative analysis of cord levels C8-T3 was carried out to assess the rostral-caudal extent of the glial response to CST

transection. Sectioning in series, as described above, allowed for qualitative assessment of the entire segment and then the quantification of select sections in the series.

Reactive astrocytes were characterized by an increase in cell body size, shortening and thickening of processes and an apparent increase in GFAP protein expression (reviewed in Cao and Zhang, 2008). Activated microglia were identified by enlarged cell bodies and short and thick extensions (Ayoub and Salm, 2003; reviewed in Romero-Sandoval et al., 2008). Only a qualitative assessment of astrocyte activation was carried out due to the complex pattern of astrocyte staining.

Quantitative analysis of microglia was carried out from level T1 of the spinal cord as this level showed the most obvious response to CST transection. For quantification of microglia, cell counts were obtained from images that were acquired at 200X magnification and then enlarged to 500X using Olympus FV Viewer software. The microglial markers OX42 and Iba1 were examined in the IML. Though microglial aggregation following injury was observed at 7 days following injury using both markers, the Iba1-immunoreactive (-ir) cells, which showed 99% colocalization with OX42 (Fig.3), appeared more defined and allowed for more accurate quantification. Therefore the Iba1 marker was used to quantify the microglial response and to document microglial relationships with IML neurons and astrocytes. The number of Iba1-ir cells present within a rectangular area ($0.45\text{mm} \times 0.55\text{mm}$) of the IML from four images were averaged to obtain a mean value for a single spinal cord (a single case). The use of the nuclear marker DAPI was used to confirm the cellular nature of each cell that was counted.

Quantitative analysis of oligodendrocytes was carried out from level T1. For quantification of oligodendrocytes, cell counts were obtained from images that were acquired at 200X magnification and then enlarged to 500X using Olympus FV Viewer software. The total number of TrkB-ir oligodendrocytes in a field of view that included the IML, and that encompassed an area of approximately $0.63\text{ mm} \times 0.63\text{ mm}$, was recorded from four confocal images per case. The use of the nuclear marker DAPI was used to confirm the cellular nature of each cell that was counted. Counts typically were obtained from tissues that were double labeled with ChAT. Any TrkB/ChAT-ir profiles, which presumably represented neurons (McCartney et al., 2008), were not included in the analysis.

In order to study the relationship of astrocytes and microglia cells with injured neurons, Z-stacked confocal images (8 scans acquired at $3\text{ }\mu\text{m}$ intervals) were analyzed following triple immunolabeling of the spinal cord with GFAP, Iba1 and ATF-3. A confocal Z-stack was used to generate the orthogonal view depicted in Figure 6.

Statistical analysis

Three independent experiments were carried out where the analysis of the number of microglia, oligodendrocytes and ChAT neurons with TrkB abutting cells was carried examined. In the first experiment, changes at 1 day and 7 days following transection were assessed. A second experiment was carried out to examine the changes at 21 days following injury while the third experiment assessed long term changes at 10 weeks following the injury. Therefore data were analyzed using a one way ANOVA (for comparisons with 1 day and 7 day survivals with one age-matched control group) or student's t-test (comparisons of 3 week and 10 week survivals with respective age-matched controls). Prior to statistical analysis, a mean was obtained for each set of controls and each case from that time point (both controls and treatments) was expressed as the percent control. These data were square root transformed prior to statistical analysis. The square root transformed data are shown in the graphs in Figures 4 and 7. All significance is reported at $p < 0.05$ and in all graphs the error bars represent standard error of the mean.

RESULTS

Astrogliosis in the IML following CST transection

The IML, which houses the ChAT-ir cell bodies of preganglionic sympathetic neurons, is situated between the dorsal and ventral horns at a lateral position in the gray matter (Fig. 1) primarily at spinal cord levels C8-T3 (Rando et al., 1981; Strack et al., 1988). The area encircled in Figure 1 shows the specific location of ChAT-ir cell bodies in the IML and the specific area analyzed in this study. ChAT-ir neurons also were located in the ventral horn (VH), which represent somatic motor neurons.

ATF-3, which was used as an injury marker to assist in identifying cell bodies having transected axons (Tsujino et al., 2000; Kataoka et al., 2007), was absent in the spinal cord at 1 day following axotomy except for two ATF-3-ir neurons in T1 in one of the four cases that was examined at this time point. GFAP-ir astrocytes appeared similar to the control cases, exhibiting multiple thin processes emanating from a small cell body (Fig. 2). However, at 7 days following transection, ATF-3-ir neurons were commonly observed in the IML and occasionally seen in the intercalated region and central autonomic area (Rando et al., 1981). The presence of ATF-3 was paralleled by a change in astrocyte morphology from non-reactive to active or reactive (Fig. 2). Compared to the control group where delicate 'knob-like' GFAP-ir astrocyte processes made contacts with neurons in the IML (Fig. 2), at 7 days following CST transection, brightly fluorescent reactive GFAP-ir profiles extended long filamentous processes in close proximity to ATF-3-ir neuronal cell bodies (Fig. 2) and occasionally ATF-3 negative neurons. At the 7 day time point, the activated state was observed only at cord levels C8-T1 and no obvious astrocyte activation was observed at any other spinal cord levels examined (T2-T3 or upper cervical or lower thoracic). At the 3 week time point, ATF-3-ir neurons were observed in T1 in a proportion (3 of 10) of the cases and, in these same cases, astrocyte activation also was observed. Representative activated (3 wk, upper panel) and non-activated (3 wk, lower panel) cases in T1 at the 3 week time point are shown in Figure 2A. No ATF-3-ir neurons were observed at any other cord levels at this time point. At 10 weeks post-injury, no ATF-3-ir neurons were observed and in all cases, astrocytes in the IML appeared similar to those in the controls (Fig. 2A).

Alterations in microglia in the IML following CST transection

Iba1-ir cells were observed throughout the IML in control tissues (Fig. 3,4). No obvious changes in the appearance of Iba1-ir cells were observed in the IML at any levels examined at the 1 day time point (Fig. 4), yet quantitative analysis of Iba1-ir cells at T1 revealed a significant decrease compared with the age-matched control group (Fig. 4C). At 7 days following injury, characteristics of microglial activation were observed in the IML at cord levels C8-T1 (Fig. 4). The activated microglial cells tended to aggregate in the IML and this obvious clustering of activated microglia was detectable even at low magnification (Fig. 4A). The number of Iba1-ir cells in the IML in T1 at the 7 day time point was increased by 51% compared with the age-matched control group (Fig. 4C). At this time point the activated Iba1-ir profiles were in close proximity to ATF-3-ir neurons, showing thickened processes adjacent to ATF-3-ir (Fig. 4A,B) as well as ATF-3 immunonegative neuronal cell bodies (Fig. 4B, inset). No aggregation of microglia was observed in any spinal cord level in any of the 3 week cases studied. Yet, in the same 3 week cases in which astrocyte activation occurred and ATF-3 neurons were present, microglia were observed in close proximity to the injured neurons (Fig. 4, upper 3wk panel). No activated microglia were observed and the number of Iba1-ir cells was similar to the controls in the remaining 3 week cases and at 10 weeks following injury (Fig. 4). No microglial clustering was observed in the dorsal or ventral horns, and no evidence for microglia activation was observed at levels T2 or T3 of

the injured cases at any time point, in the controls, or at levels above and below the IML at any time point examined.

Glial-neuronal relationships in the IML following CST transection

Triple immunolabeling allowed for the visualization of microglia-astrocyte-neuronal interactions. The use of orthogonal views of confocal Z-stacks obtained from cryostat sections of the IML at T1 following 7 day CST transection yielded several examples of astrocyte processes in close proximity to activated microglia, which in turn were adjacent to ATF-3-ir neurons within the astrocyte sheath (Fig. 5). Such interactions were not observed in the IML at any other time points or in the control tissues.

Alterations in oligodendrocytes in the IML following CST transection

The TrkB antibody used in this study was directed against the full length TrkB receptor and labeled small profiles that co-localized the oligodendrocyte marker, APC-CC1 (Fig. 6), confirming previous studies of their oligodendrocyte nature (Skup et al., 2002; McCartney et al., 2008). In addition, lightly labeled preganglionic neurons that co-localized ChAT (McCartney et al. 2008) were observed in the IML (Fig. 7). In the control tissues TrkB-ir oligodendrocytes were distributed throughout the spinal gray matter and, as shown in Figure 7A, and frequently were in close apposition with ChAT-ir cell bodies. The number of TrkB-ir oligodendrocytes was unchanged at 1 day following injury (Fig. 7B), yet the number of ChAT-ir neurons abutted by TrkB-ir was significantly decreased by 43% at this time point (Fig. 7C). At 7 days following injury, a significant 29% increase in TrkB-ir cells in the IML was observed (Fig. 7B) and the number of ChAT-ir neurons with abutted TrkB-ir cells remained significantly decreased by 42% (Fig. 7C). At 3 weeks following injury, the number of TrkB-ir cells in the IML was similar to age-matched controls (Fig. 7B), yet the number of ChAT neurons with abutting TrkB oligodendrocytes remained significantly decreased by 33% (Fig. 7C). By 10 weeks following injury, the number of TrkB-ir cells as well as the ChAT neurons with abutting TrkB-ir oligodendrocytes was similar to controls (Fig. 7C).

DISCUSSION

Summary of the present study

In the present study, the transection of preganglionic axons in the periphery resulted in alterations in central glial cell populations near the injured cell bodies located in the IML. These changes were most obvious at 7 days following the injury and appeared to subside by the 10 week time point. In addition to changes in astrocytes and microglial cells, which have been shown in other injury models, oligodendrocytes were increased in the IML at 7 days following CST transection and loss of the typical juxtaposition of oligodendrocytes to IML neurons was observed at several time points following the injury. This transient response suggests the possibility that signals arising from the distal injury can either directly or indirectly activate glial cells near the IML cell bodies of the injured axons.

Glial activation in the IML

Glial cell activation is a phenomenon that has been widely documented in many injury models and has been proposed to play a variety of roles in the central and peripheral nervous systems (reviewed in Cao and Zhang, 2008; reviewed in Renault-Mihara et al., 2008; Romero-Sandoval et al., 2008). In the CNS, astrocyte activation can lead to scar formation, which in turn inhibits axonal regeneration (Fawcett and Asher, 1999; Silver and Miler, 2004; Yiu and He, 2006). Yet the inhibition of astrocyte activation following spinal cord injury led to increased bleeding at the lesion site, suggesting a possible beneficial role (Pekny et al.,

1999). Glial cell activation in the PNS following peripheral nerve injury may play a protective role. The main consensus is that Schwann cell activation at the distal peripheral nerve injury site leads to the removal of axonal debris (Stoll et al., 1989; Reichert et al., 1994), a result of Wallerian degeneration (Stoll et al., 1989), and the production of neurotrophic factors in order to optimize the conditions for axonal regeneration (reviewed in Verdu et al., 2000).

The gliosis observed in the present study is different from the many studies of CNS and PNS injury that reveal activated glial cells at the site of injury, since the activated glial cells were observed in the vicinity of injured cell bodies in the IML following a distal injury in the periphery. We propose that injury signals emanating from these injured neurons, which were identified by the presence of nuclear ATF-3 immunoreactivity (Tsujino et al., 2000; Kataoka et al., 2007), were responsible for the observed changes. Indeed, Armstrong and colleagues (2008) showed an enhanced neuroinflammatory response in mice lacking pituitary adenylyl cyclase activating peptide (PACAP). While not showing direct evidence, this study does implicate that local neuronal release of PACAP, which is upregulated in facial motor neurons following axotomy (Zhou et al., 1999), might modulate the response by microglial cells.

Activated astrocytes, which can result from migration (Okada et al., 2006) as well as proliferation (Faulkner et al., 2004), also have been reported in the facial (Rohlmann et al., 1994; Hao et al., 2007) and the hypoglossal (Svensson et al., 1993; Yamada et al., 2011) nuclei following axotomy of respective peripheral axons, and in the spinal cord following sciatic nerve injury (Coyle, 1998). Similarly, at 7 days following CST injury, we observed a transformation in the morphology of astrocyte processes in the IML from short and delicate with 'knob-like' endings making occasional contacts with IML neurons to long, thin processes in close proximity to the injured neuronal cell bodies. A similar relationship was observed in the facial motor nucleus following axotomy (Kalla et al., 2001) as well as in the ventral (Svensson et al., 1993; Hu et al., 2004) and dorsal (Coyle, 1998; Hu et al., 2004) horns following sciatic nerve injury.

We observed an increase in the number of microglial cells at 7 days following injury, which has been attributed to proliferation (Graeber et al., 1988). Increased microglia appears to be a characteristic of injury, as observed in other CNS nuclei following transection of peripheral axons, such as the facial (Jones et al., 2005; Blinzinger and Kreutzberg, 1968) and hypoglossal (Svensson et al., 1993; Yamada et al., 2011) nuclei following axotomy of respective peripheral axons, and in the spinal cord following sciatic nerve injury (Coyle, 1998) as in the dorsal horn following spinal nerve transection in a model of mechanical allodynia (Romero-Sandoval et al., 2008), a partial nerve transection model of chronic pain (Coyle, 1998), and in the sciatic inflammatory model (Ledeboer et al., 2005). Both protective and detrimental roles have been attributed to the presence of microglia following injury. For example, though activated microglia have been implicated in the onset of neuropathic pain (Ledeboer et al. 2005), the implantation of activated microglial cells into the DRG following injury led to the regeneration of sensory neurons (Prewitt et al., 1997). Jones and colleagues (2005) proposed that activated microglia in the mouse facial nucleus following axotomy served as antigen presenting cells to infiltrating T lymphocytes, which in turn stimulate nearby glial cells to release growth factors to facilitate neuronal survival. Our preliminary findings revealed a similar CD4 T cell infiltration into the IML following CST transection (unpublished observation), supporting a potential neuroprotective function in our model. Yet Yamada et al. (2011) proposed that microglia in the mouse hypoglossal nucleus following axonal transection might have detrimental effects on neurons.

In addition to obvious changes in the number and activation state of microglial cells observed at 7 days post injury, the expression of the Iba1 marker, a cytosolic ionized calcium binding adaptor molecule which may be involved in microglial motility (Imai et al. 1996), was dramatically decreased in the IML at 1 day following injury. It is not clear why the expression of the Iba1 marker would decrease prior to aggregation or clustering. The down regulation of Iba1 may represent an early step in the series of changes that take place following the injury.

In our model, microglial cells were observed in close proximity to both ATF-3 immunoreactive and non-immunoreactive neurons. Indeed the sympathetic model used in this study allowed the opportunity to examine the interaction of glial cells and non-injured neurons, since a small proportion of IML neurons send axons to the stellate ganglion (Pilowsky et al. 1992) and would not have been injured by the CST transection. Though speculative, it is possible that the activated microglia might somehow influence non-injured neurons, possibly to mediate the production of survival factors as proposed by Jones and colleagues (2005). Alternatively, it is possible that not all injured preganglionic neurons showed increased ATF3 immunoreactivity.

Close proximity of microglial and astrocyte processes to the injured neurons may contribute to the stripping of presynaptic inputs, a phenomenon observed in various injury paradigms and first described by Blinzinger and Kreutzberg (1968). For example, microglial cells were shown to participate in the synaptic stripping in the facial (Blinzinger and Kreutzberg, 1968; Kalla et al., 2001) and hypoglossal (Yamada et al., 2011) nucleus following axonal transection, though it was recently reported that microglia may play only a minimal role in this phenomenon in the hypoglossal nucleus (Perry and O'Connor 2010). Astrocytes also have been implicated in synaptic stripping in the spinal cord (Svensson et al., 1993; Krassioukov and Weaver, 1996) following distal axon injury. It is not known whether synaptic stripping, which may aid in neuronal recovery by decreasing neuronal overstimulation (Kalla et al., 2001), occurs in the IML following CST injury and/or whether there exists a relationship between the activated microglial and astrocytes and any loss of presynaptic inputs.

We observed Iba1- and GFAP-ir cells in close proximity in the IML at the 7 day time point, suggesting that the CST transection resulted in enhanced interactions between astrocytes and microglia following injury. Both microglial and astrocyte processes surrounded the cell bodies of the injured neurons. Interestingly, an analysis of Z-stacked confocal images showed that the microglial sheath was inside the astrocyte envelope. Similarly, Hu et al. (2007) observed both microglial and astrocyte processes interacting with the injured neurons, such that the microglial envelope was within the astrocyte sheath. However, Svensson and colleagues (1993) used electron microscopy to show astrocyte processes within the microglial envelope in the hypoglossal nucleus following injury. It is possible that astrocyte processes, which could not be resolved at the light microscopic level, also are in contact with the injured neurons in our model.

Previous studies suggest a potential interaction between astrocytes and microglial following injury. For example, blocking microglia proliferation with the anti-mitotic drug cytosine-arabinoside led to decreased GFAP expression after axotomy of the hypoglossal nerve (Svensson et al., 1993). Also a positive correlation between GFAP and OX42 was shown following both sciatic nerve injury and hypoglossal nerve axotomy (Olsson et al., 1988; Gilmore et al., 1990; Svensson et al., 1993), leading to the hypothesis that reactive astrocytes release cytokines into the extracellular space to activate and recruit additional microglia. Activated microglia and astrocyte interactions following injury were thought to contribute to the development of neuropathic pain (Romero-Sandoval et al., 2008), implying

that the disruption of this activation may be a therapeutic approach to block pain hypersensitive behaviors (Milligan et al., 2003). Indeed, it was shown that blocking microglial and/or astrocyte activation led to the inhibition of mechanical allodynia (Milligan et al., 2003; Raghavendra et al., 2003; reviewed in O'Callahan and Miller, 2010).

The main consensus in the literature is that microglial activation is an acute response to injury that precedes astrocyte activation, which in turn is maintained for longer time periods following the injury (Colburn et al., 1997; Kalla et al., 2001; Romero-Sandoval et al., 2008). Our results support this idea to some extent. For example, alterations in microglia were observed at one day following injury, prior to the detection of any obvious changes in astrocytes. In addition, in the 3 week cases that showed signs of astrogliosis, we observed no signs of microglial aggregation revealing that astrocyte activation, though subsided at 10 weeks, may have lasted longer than the activation of microglia.

To our knowledge, we are the first to report changes in oligodendrocytes following peripheral axon injury. Two types of changes in the oligodendrocytes were observed – an increase in number and a change in the typical close apposition that was observed in control cases although the functional consequences of these changes are unknown. These cells typically are involved in the myelination of axons (Delcomyn, 1998), and their presence near neuronal cell bodies has an unknown function, particularly since the majority of preganglionic fibers in the rat appear to be unmyelinated (Hedger and Webber, 1976). Yet their close proximity to IML neurons suggests a supportive relationship between these two cell types. The loss of this neuronal-oligodendrocyte relationship was observed at 1 day following injury, prior to the increase in the number of the oligodendrocytes and the activation of astrocytes and microglia observed at 7 days. Though the number of cells was similar to controls by 3 weeks, their relationship with the IML neurons remained altered. It is possible that the activated microglia and astrocytes, which become closely associated with IML neurons following injury, contribute to the altered relationship between the oligodendrocytes and injured IML neurons, much like the phenomenon of synaptic stripping. Interestingly, the changes in oligodendrocytes observed at 1 day following injury parallel the reduction in Iba1 protein expression observed at this same time point. The changes in these cells observed at 1 day following injury represent a more subtle response to the injury that occurs prior to the observation of overt astrogliosis, microglial activation, and the increased number of oligodendrocytes.

The expression of the full-length TrkB receptor on glial cells identified as general marker for oligodendrocytes has been previously documented (Skup et al., 2002; McCartney et al., 2008), and suggests that these cells respond to neurotrophins such as brain derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4). Previous studies in our lab (McCartney et al., 2008) as well as others (Skup et al., 2002) have shown co-localization of full length TrkB with RIP, a marker that recognizes 2',3'-cyclic nucleotide 3'-phosphodiesterase (Watanabe et al. 2006) in both mature and immature oligodendrocytes (Daston and Ratner, 1994). Similarly, we show here the co-localization of full length TrkB with APC-CC1, a general marker for all oligodendrocyte activity states. Therefore, it is not known whether the increase at 7 days results from an increase in the number of immature oligodendrocytes and thus from a proliferative response.

What role the increased number of TrkB-ir oligodendrocytes plays in this response and the potential mechanisms underlying their regulation by neurotrophins remain to be determined. BDNF has promyelinating effects on CNS axons by directly activating full length TrkB receptors that are expressed by oligodendrocytes (Xiao et al, 2010). Following injury, oligodendrocytes have been shown to degenerate at the injury site following spinal cord injury (McTigue et al., 2001; reviewed in Chen and Xie, 2009) which may lead to myelin

degeneration, and subsequent disturbances in axonal function (Chen and Xie, 2009). Yet it has been shown that oligodendrocyte progenitor cells express neurotrophic factors following injury which may serve to facilitate neuronal survival (Casha et al., 2001; Zhang et al., 2006). The implantation of these progenitor cells between 7 days and 10 weeks after spinal cord injury was shown to enhance remyelination and lead to a substantial increase in recovery (Zhang et al., 2006).

Though the exact role of the glial changes is unknown, it is possible that the activated glial cells may serve a neuroprotective function at early time points following the injury. Indeed, cell death is not thought to be the primary cause of glial activation following injury (Yamada et al., 2011). Consistent with this idea, no signs of DNA 'nicking' (with TUNEL staining) were observed in the IML at the 7 day time point (Tang and Brimijoin, 2002; Isaacson, unpublished data), when changes in glial cells were most obvious, suggesting little neuronal death at this time point. In addition, we observed that, though the number of ChAT-ir neurons in the IML appeared reduced at T1 level of the spinal cord at 7 days following CST transection, the neurons were still present and typically showed ATF-3 immunoreactivity. Others have reported that the loss of ChAT expression in centrally located neurons following peripheral axon injury is a transient event and does not involve cell death (Lams et al., 1988; Wang et al., 1997; Morcuende et al., 2011). However, though they found no evidence of TUNEL staining at 1 week or 8 weeks following CST transection, Tang and Brimijoin (2002) reported a significant decrease in the number of ChAT neurons at the 10 week survival time point following CST transection, suggesting that a loss of neurons in the IML occurred between 1 week and 10 week survival time point.

The reinnervation of the SCG following CST transection can provide one measure of the regenerative potential of the IML neurons. In our preliminary observations, we observe little ChAT protein (using western and immunohistochemical analyses) in the SCG at 10 weeks following CST transection. This is in agreement with Tang and Brimijoin (2002) who observed relatively little ChAT activity in the SCG at this time point. Further, Raisman et al. (1974) observed little recovery of synaptic loss in the SCG and low ChAT activity at 6 months following CST transection though regeneration was observed following crush or freezing of the CST. We observed a slight increase in ChAT protein in the SCG (using western analysis) between 10 weeks and 16 weeks following CST transection (unpublished observation), suggesting that some reinnervation of the SCG may be taking place at longer survival term time points. In the guinea pig, Nja and Purves (1977) observed full reinnervation of the SCG after 3–4 months following CST transection. Interestingly, ChAT re-expression in motor cell bodies can proceed without target reinnervation (Matsuura et al., 1997; Wang et al., 1997), indicating that ChAT reinnervation of the SCG may not necessarily coincide with the return of ChAT expression in the IML. We conclude that SCG reinnervation following CST transection is a slow process and that, though the glial alterations have subsided, the IML neurons may remain focused on recovery at long survival times.

Extent and time course of the IML response

Previous retrograde labeling studies have reported that preganglionic neurons projecting to the SCG are located primarily at levels C8- T2, with additional projections from levels T3 to T5 (Rando et al., 1986; Strack et al., 1988; Hosoya et al., 1991; Poulat et al., 1992). Yet, in the present study, the obvious activation of astrocytes, microglial cells, and oligodendrocytes was restricted to spinal cord levels C8 and T1. If the response is dependent on retrograde signals from the injury site arriving at the IML, it is possible that 7 days following the injury was too early to detect activation at more caudal spinal cord levels. However, if this were the case, we would expect some signs of glial activation as well as ATF-3 immunoreactivity in more caudal levels (T2-T5) at the 3 week survival time point.

Perhaps the transient glial activation occurred in more caudal spinal cord levels between the 7 day and 3 week time points examined in this study.

Tsujino et al. (2000) observed weak ATF-3 mRNA expression in the spinal cord at 1 day following sciatic nerve transection. This correlates well with our findings that few neurons expressed ATF-3 protein in the IML at this same time point. Also consistent with our results, after peaking at 3–7 days, ATF-3 expression in the spinal cord following sciatic nerve injury gradually decreased and was diminished at 3 weeks and was absent by 6 weeks following the injury (Tsujino et al., 2000). Similarly, though ATF3-ir neurons and astrocyte activation were present in 30% of the cases in the present study, the majority of cases at the 3 week survival time point showed no ATF-3 and glial morphology similar to that observed in the controls, suggesting that recovery may be getting underway at this time point in some individuals. This is in contrast to results observed at 84 days after sciatic nerve ligation, where astrocyte morphology was not back to normal in the dorsal and ventral horns (Coyle, 1998). It may be that the persistence of astrogliosis in that model occurred because the ligation was left in the animal, leading to a persistent gliosis state.

CONCLUSION

Similar to that observed in other models, the glial response in the IML following CST transection was robust at the 7 day survival time point. In addition to astrocytes and microglia in the IML, oligodendrocytes also showed a response to peripheral nerve injury. Activated astrocytes were observed at 1 day following injury in the facial (Rohlmann et al., 1994) and hypoglossal (Svensson et al., 1993) nucleus, yet no obvious astrocyte activation was observed in our model. It is possible that this difference is a reflection of the cell body distance from the injury site. Though no glial activation was observed, less obvious changes were detected in the glial cells in the IML at 1 day following CST transection, such as a decreased number of microglial cells and loss of the typical apposition of oligodendrocytes with IML neuronal cell bodies. Similar to other models, the glial response was diminished at long term time points and had subsided in the IML by 10 weeks following the injury. The transient nature of the glial activation in the spinal cord following distal axonal injury suggests that this component of the response is an acute reaction to the injury. Whether these changes lead to neuroprotection is a topic for future investigations.

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Figure 1.

Location of the IML in the rat spinal cord. Confocal micrograph of a spinal cord hemisection at level T1 showing the location of the intermediolateral cell column (IML, circled area) between the dorsal horn (DH) and ventral horn (VH). The IML is comprised of ChAT-immunoreactive (-ir) preganglionic neurons (green). Note the presence of ChAT-ir motor neurons in the VH. The central canal (cc; white arrow) is located on the midline of the cord. Scale bar = 100 μ m.

Figure 2.

Time course of changes in astrocytes in the IML at level T1 following CST transection. **A.** GFAP-ir astrocytes (green, arrow) exhibited typical characteristics in the control (cont) and at 1 day following injury (1d, arrow). At 7 days (7d) following injury, reactive astrocytes were observed in the IML (arrow) and in 30% of the spinal cords examined at the 3 week (3wk) time point (arrow, upper 3 wk panel). Changes in astrocyte morphology coincided with the presence of ATF-3-ir neuronal cell bodies (red, right column). Astrocytes appeared similar to controls in 70% of the 3wk cords (arrow, lower 3 wk panel) and by 10 weeks following transection, astrocytes (arrow) appeared similar to controls and ATF-3 was absent. Scale bar = 50 μ m. **B.** Changes in astrocytes in the IML at higher magnification. In control

tissues (cont), astrocytes exhibited small cell bodies with short processes forming “knob-like” contacts (arrows) with neuronal cell bodies. At 7d following CST transection, astrocytes showed enlarged cell bodies with processes (white arrows) in close proximity to ATF-3-ir neurons (yellow arrows). DAPI (blue) shows cellular nature of the stained profiles. Scale bar = 25 μ m.

Figure 3.

Analysis of OX42 and Iba1 co-localization in the IML at cord level T1. OX42 (A., green) and Iba1 (B., red) were localized in small cells in the IML from controls and following 1 day (1d) and 7 day (7d) CST transection. C. The merged image with DAPI (blue) shows numerous profiles co-localizing these two markers. Scale bar = 100 μ m. Inset: Boxed area in C. shown at higher magnification reveals details of double labeled profiles (arrows). Scale bar = 50 μ m.

Figure 4.

Changes in Iba1-ir cells in the IML at level T1 following CST transection. **A.** In the controls and at 1 day (1d) following injury, microglial cells (arrows) were evenly distributed in the IML. However, at 7 days (7d) following injury, aggregates of microglia were observed in the IML and these clusters were in close proximity to ATF-3-ir neurons (arrows). Though ATF-3-ir neurons and reactive astrocytes were present in 30% of cases at the 3 week (3wk) time point (See Fig. 2), there was no evidence of microglial clustering (3wk, upper panel) in these cases, yet microglia were in close proximity to ATF-3-ir neurons (arrows). In the remaining 3wk cases (3wk, lower panel), microglia appeared similar to controls and 10wk cases. Scale bar = 50 μ m. **B.** Characteristics of Iba1-ir microglia at higher magnification. In

controls (cont), thin processes emanated from microglial profiles (arrow). At 7d following injury, the Iba1-ir cell bodies (white arrows) were enlarged with processes in close proximity to neurons that showed signs of injury (ATF-3-ir, yellow arrow). Iba1-ir processes also were observed enveloping non-immunoreactive neurons (inset, arrow). DAPI (blue) shows cellular nature of the stained profiles. Scale bars = 25 μ m. **C.** Quantitative analysis revealed a significant decrease in Iba1-ir cells at 1d following injury. By 7d the number of Iba1-ir cells was significantly increased. The number of microglia returned to control values by the 3wk time point. As described in the Methods, the data were square root transformed prior to statistical analysis and the transformed data were plotted for demonstration in this graph. Sq rt, square root. Error bars represent the standard error of the mean. Significance reported at $p < 0.05$.

Figure 5.

Composite image created from a Z-stacked confocal series (8 images, 3 μm intervals) shows the association of astrocytes (green), microglia (red) and injured neurons (white) in the IML at 7d following CST transection. Details of three representative glial-neuronal relationships are shown in the orthogonal views (orange arrows) as well as at higher magnification (insets). In each instance, astrocyte processes (white arrows) are in close proximity to microglial cells (yellow arrows), which in turn appear to envelope the injured neuronal cell body. DAPI (blue) shows cellular nature of the stained profiles. Scale bar = 100 μm , Inset = 20 μm .

Figure 6.

Co-localization of TrkB-ir profiles in the IML with the oligodendrocyte marker, APC-CC1. Numerous APC-CC1-ir (A., green) and TrkB-ir (B., red) profiles were observed in the gray matter of the spinal cord. The merged image (C.) shows almost complete co-localization of these two markers. Insets: At higher magnification, the profiles (arrows) and the co-localization of these oligodendrocyte markers are evident (arrows). DAPI (blue) shows the cellular nature of the staining. A.–C., Scale bar = 100 μm, Insets = 20 μm.

Figure 7.

Changes in TrkB-ir oligodendrocytes in the IML following injury. **A.** In this confocal micrograph taken from T1 level of the spinal cord, small TrkB-ir cells (red, yellow arrows) showed a close association with ChAT-ir neurons (green, white arrows) in the controls (cont). Note the light TrkB immunoreactivity in some of the ChAT-ir neurons. At 7 days (7d) following transection, the number of TrkB-ir profiles (yellow arrows) appeared increased and the typical juxtaposition of these cells with ChAT-ir neurons (white arrows) was not apparent. The number of TrkB cells appeared similar to controls at 3 week (3wk) yet few ChAT-ir neurons showed abutting TrkB-ir cells. A 10 weeks (10wk; yellow arrows) the number of TrkB-ir cells and the number of ChAT-ir neurons with abutting TrkB-ir cells

appeared similar to controls. DAPI was used to show the cellular nature of the positive profiles. Scale bar = 50 μ m. **B.** Quantitative analysis of TrkB-ir cells in the IML revealed a significant increase at 7d following injury. As described in the Methods, the data were square root transformed prior to statistical analysis and the transformed data were plotted for use in this graph. Significance reported at $p < 0.05$. Error bars represent the standard error of mean. **C.** The number of ChAT-ir neurons with abutting TrkB-ir cells was significantly decreased at 1d, 7d, and 3 wk following CST transection but was similar to controls at the 10 week survival time point. Data were square root transformed prior to statistical analysis and the transformed data are shown here. Sq rt, square root. Significance reported at $p < 0.05$. Error bars represent the standard error of the mean.