OLIGODENDROGENESIS FOLLOWING EXPERIMENTAL SPINAL CORD INJURY

DISSERTATION

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the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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ABSTRACT

Trauma to the spinal cord causes oligodendrocyte (OL) cell death and axonal demyelination. Spontaneous recovery following spinal cord injury (SCI) is quite limited due to severe axonal damage and very poor axonal regeneration. Thus the conductivity of the spared but demyelinated axons is of paramount importance. Since OLs are the myelinating cells of the CNS the first step in addressing this issue would be to understand OL loss and replacement. The mature central nervous system (CNS) contains oligodendrocyte progenitor cells (OPCs) which make the replacement of lost OLs possible. Multiple investigators have focused on transplantation of oligodendrocyte precursors to improve SCI outcome but few studies have looked at an endogenous potential for OL replacement. Thus our goals were fourfold: study SCI induced changes in endogenous OPCs in different regions of the spinal cord over time; evaluate the inherent capability of the injured cord to generate new OLs; determine if any noted oligogenesis affects OL population dynamics and finally determine which endogenous factors contributing to OPC proliferation and differentiation.

In the CNS, OPCs are identified by NG2 and PDGFαR (platelet derived growth factor alpha receptor) expression. Upregulated NG2 expression has been observed in several CNS injury models. Therefore, we were interested in overall expression patterns
of NG2 after SCI. Using a rat spinal contusion model we examined NG2 proteoglycan
distribution up to 10 weeks following SCI along an 8mm length of the injured spinal
cord. NG2 immunoreactivity occupies about 6% of total naïve spinal cord cross-
sectional area. Following SCI no changes in the overall NG2 protein expression were
seen at the epicenter 3 days post injury (dpi) but between 7-70dpi, NG2 expression
increased significantly to occupy almost 50% of the cross-sectional area. Even 4mm
distal to the injury site, NG2 immunoreactivity was still elevated 2-fold over naïve
between 7-70dpi. Although NG2 expression increased in all of the tissue, early
upregulation of NG2 was especially prominent along the lesion border.

In addition to assessing the overall changes in the NG2 proteoglycan distribution,
we also examined the temporo-spatial changes in numbers of proliferating NG2+ OPCs.
Multiple BrdU pulse paradigms revealed that NG2+ OPCs proliferated throughout week 1
and accumulated along the lesion border. By 14dpi NG2/BrdU cells at the white matter
(WM) lesion border were significantly higher than spared white matter (SWM), and gray
matter (GM) border contained more NG2/BrdU cells than spared gray matter (SGM).
Interestingly the GM border also contained more proliferating OPCs than WM border at
14dpi.

To determine if the proliferating OPCs gave rise to new OLs, we examined
whether new OLs (BrdU+) were present in regions of high NG2 cell proliferation. Mature
OLs are post-mitotic and cannot re-enter the cell cycle hence a BrdU+ OL must have
differentiated from a cycling progenitor. At all days examined during the first 2 weeks
post injury, significantly higher oligodendrogenesis occurred in all regions examined
compared to naïve, with the greatest increases occurring in the gray matter and in regions
around the lesion border. The enhanced oligogenesis normalized OL numbers in the spared white matter by 7dpi, and along the lesion border at 7d and 14dpi OL numbers were more than 2-fold greater than in normal WM.

In addition to being an area of active oligogenesis early after SCI, the lesion border is also the region of the presumptive glial scar and is associated with active changes in astrocytes. Astrocytes produce multiple growth factors important for OPC proliferation and differentiation. We hypothesized that astrocyte derived growth factors contribute to enhanced oligodendrogenesis in the perilesion area. We chose to examine ciliary neurotrophic factor (CNTF) since it promotes the rate of OPC proliferation and maturation as well as enhances OL survival. Using western blot we determined that CNTF protein expression continually rises between 5d and 28dpi. We next used immunocytochemistry to determine the potential cellular sources of CNTF and the regional distribution. Between 3 and 28dpi CNTF expression increased significantly ±2mm around the epicenter both in SGM and SWM. Following SCI astrocytes were the major source of CNTF, although we also noted CNTF immunoreactivity on OLs and Schwann cells. From this it was clear that CNTF immunoreactivity was present throughout the injured tissue, with most immunoreactivity localized to lesion borders, which, as stated above, were the regions showing the greatest level of oligodendrogenesis.

Another potential factor that may contribute to oligogenesis after SCI is fibroblast growth factor-2 (FGF-2). FGF-2 promotes NG2 cell proliferation and migration and studies have shown that CNTF enhances FGF-2 mediated effects. Therefore, we also
examined FGF-2 expression at 7dpi after SCI. Significantly higher numbers of FGF-2 expressing cells were present in the WM bordering the lesion compared to the SWM. SGM and GM borders both contained equally high numbers of FGF-2+ cells, which were increased significantly over naïve GM. Thus we detected increased expression of FGF-2 following SCI especially along WM border and GM regions where we had previously noted maximum oligogenesis.

Hence collectively, our work shows that subsequent to marked NG2 cell proliferation, there is a previously unrecognized robust oligogenic response during the first 2 weeks following SCI. This oligogenesis was particularly prominent along the lesion border and resulted in replacement and normalization of OL numbers in spared tissue. This marked increase in OLs may be due, at least in part, to SCI-induced up regulation of CNTF and FGF-2. Thus the mature CNS has a remarkable self-reparative potential. Our understanding of in-vivo mechanisms underlying this significant oligogenesis in such severe conditions will further our strategies aimed at better outcomes following cell transplantation or enhancing the endogenous repair mechanisms not only following SCI but additional disorders targeting OL loss.
DEDICATION

Dedicated to my mother
ACKNOWLEDGMENTS

The journey of my PhD is full of the guidance, assistance and love from many people. The first and foremost is my guide and mentor Dr Dana M McTigue. Often people tell me that the relationship between the boss and their first graduate student is very special. To me it surely is. Dana you have been a scientific and a life coach, I have loved to work with and for you; you will be my guide for many years to come. You are “super-awesome”.

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My friends Megan and Myron deserve a special mention here. Megan, I cannot think of my years as a NGSP student without you. All the meetings in which we were roommates and especially SFN 2004 which was too much fun and all the gab-fests we have indulged in. Myron, thank you for being a friend, when I had very few friends.
Many new people became a part of daily scientific adventures when we moved into a new space with the Popovich Lab. Thank you every one in the Popovich and McTigue labs for all the extra fun, food & pounds; all the advice on experiments and the great environment.

The contribution of my family to who I am as a person and as a scientist is immeasureable. If you have wondered where my high energy personality comes from look no further than the Tripathi family, it runs in our blood. My mom has inspired me to be ambitious and unrelenting in my professional pusuits. Not even I could be as proud of my PhD as my mom would have been. I love you and I miss you too much. My siblings have been my best friends; Ritu, Swati and Skand you will be close to my heart always. My work ethic comes from my dad who taught me work is worship; I wish I could work as hard as he does. On the other hand I married a man who believes life is a romance; enjoy everything as it passes by. Love you Pravin and thank you for marrying me because god alone knows, few can handle my craziness.

Overall my life at Ohio State has been a very eventful one personally and professionally. Thank you Dana for making it such a memorable journey!
VITA

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<td>α</td>
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<tr>
<td>β</td>
<td>beta</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
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<td>CNTFRα</td>
<td>Ciliary neurotrophic factor alpha</td>
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<td>CSPG</td>
<td>Chondrotin sulphate proteoglycan</td>
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<td>FGF-2</td>
<td>Fibroblast growth factor-2</td>
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<td>PDGF-αR</td>
<td>Platelet derived growth factor alpha receptor.</td>
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<td>SCI</td>
<td>Spinal cord injury</td>
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<tr>
<td>SWM</td>
<td>Spared white matter</td>
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<td>SGM</td>
<td>Spared gray matter</td>
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<td>OPC</td>
<td>Oligodendrocyte progenitor cells</td>
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<td>OL</td>
<td>Oligodendrocyte</td>
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CHAPTER 1

Introduction

Rio Hortega once wrote” those who invent no techniques rarely succeed in making discoveries of any importance”, and so he did. In the 1920s, primarily due the innovation and efforts of Rio Hortega in developing new staining techniques, oligodendrocytes were discovered as distinct glial cells in the central nervous system (CNS). He initially described them as glia of scarce radiations with sedentary attributes and probably myelinogenic properties (Pasik and Pasik, 2004). Four decades later undeniable evidence linking oligodendrocytes to the myelin membranes of the CNS was published (Bunge et al., 1962).

The mammalian CNS is a complex and highly specialized network of neural cells containing neurons, astrocytes, oligodendrocytes and microglia. Injury to this system unleashes a cascade of damaging and reparative events (Kakulas, 1984; Schwab and Bartholdi, 1996; Hagg and Oudega, 2006). Unfortunately the balance of these events more often than not leans towards poorer recovery and negative outcomes, especially in humans (Bunge et al., 1993).
Oligodendrocyte cell death following SCI

Among the various pathological events occurring following SCI, oligodendrocytes (OLs) perish very quickly. As early as 15 mins after spinal trauma 50% of OLs close to the injury site (epicenter± 1mm) are lost (Grossman et al., 2001). Apart from the immediate OL death, slow apoptotic death of OLs continues to occur in spinal cord tissue several segments away (Crowe et al., 1997). Early oligodendrocyte death is most likely mediated via excitotoxic mechanisms since blocking of glutamatergic receptors (AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate) on OLs using NBQX (2,3-dihydroxy-6-nitro-7-sulphamoyl-benzoquinoxaline) prevents substantial OL loss (Rosenberg et al., 1999). Delayed and ongoing OL death takes place due to the presence of a host other molecules present in the injured CNS. Many proinflammatory cytokines (IL-1β, TNFα and IFNγ) produced by activated macrophages post SCI have
been shown to kill OLs (Streit et al., 1998; Merrill and Scolding, 1999; Pineau and Lacroix, 2007). Also, activation of the death receptors (p75$^{NTR}$, p55TNFR, FAS) via their specific ligands leads to the activation of capases and programmed cell death (Casaccia-Bonnefil, 2000). These receptors along with their ligands (proNGF and NGF, TNF and FAS-L, respectively) have all been shown to be upregulated following SCI (Streit et al., 1998; Casha et al., 2001; Beattie et al., 2002). Strategies used to manipulate these death receptor mediated events have ameliorated OL loss following rodent SCI (Stirling et al., 2004; Ackery et al., 2006; Yune et al., 2007). The most potent effect of OL cell death is demyelination as the primary function of the OL in the CNS is to myelinate axons. Since a single OL is responsible for myelinating multiple internodes on various axons even a partial loss of OLs would have wide-spread effects (Bunge, 1968; Sternberger et al., 1978).

**Demyelination an important issue after SCI:**

Acute and chronic demyelination has been shown in multiple animal models of SCI (Bresnahan et al., 1976; Totoiu and Keirstead, 2005). More importantly, several degrees of demyelination ranging from minimal to extensive have been reported in post-mortem human SCI tissues (Norenberg et al., 2004; Guest et al., 2005). Demyelinated segments persist up to several years following SCI. Axonal myelination is indispensable for efficient saltatory conduction. Loss of myelin along the axons leads to redistribution of sodium channels and also exposes fast acting K+ channels which are normally ‘hidden’ below the myelin (Bostock et al., 1981; Chiu and Ritchie, 1982; Wang et al., 1995) causing disruption of action potential conduction. Hence loss of even a few
myelinated internodes can leave an axon dysfunctional or lead to a complete conduction block (for review, see (Nashmi and Fehlings, 2001)).

Additional evidence of dysfunctional axonal performance probably due to demyelination comes from studies using 4-amino pyridine (4-AP) in animal models and humans. 4-AP is a fast acting K+ channel blocker. Myelinated axons are unresponsive to 4-AP since there is no fast acting K+ channel activity (Sherratt et al., 1980). Following demyelination, exposure and activation of fast acting voltage gated K+ channels leading to a block in action potential propagation has been demonstrated (Blight, 1983a); whereas application of 4-AP to demyelinated nerve preparations restored conduction (Blight, 1989). Preliminary clinical studies using 4-AP (or Fampridine) in human SCI patients have demonstrated improved somatosensory and motor evoked potentials indicating improved synaptic transmission and axonal conduction (Hayes et al., 1994). Additionally, 4-AP treatment improved strength and sensation in the legs, reduced spasticity and pain, and positively impacted the quality of life in spinally injured patients (Hayes et al., 1993; Hayes et al., 1994; Potter et al., 1998a; Potter et al., 1998b). These results are evidence that there is scope for functional improvement by optimizing conduction of axon preserved after injury.

Many spinal cord injuries in humans are anatomically incomplete, i.e there is a central core lesion with a residual rim of spared tissue (Bunge et al., 1993; Kakulas, 1999). There is evidence to suggest that in animal models of SCI even a preservation of 5-10% white matter discernibly improves injury outcomes (Blight, 1983b; Fehlings and Tator, 1995). In the human condition, however, the location of tissue sparing and the quality of spared tissue appear to be of greater importance than just the amount of spared...
tissue (Kakulas, 1999). Hence preserving and enhancing function of axons in the spared rim of tissue is key to improving spontaneous functional recovery following SCI. Thus, if remyelination of all demyelinated axons could be successfully achieved we can, at the minimum, optimize functionality of the spared axons. Since oligodendrocytes are post-mitotic cells and remyelination cannot occur if generation of new oligodendrocytes is suppressed (Keirstead and Blakemore, 1997), new oligodendrocytes are needed to achieve remyelination of denuded axons.

Circle of life: replacing dead oligodendrocytes with new ones:

Since dramatic oligodendrocyte loss occurs very early after SCI (within minutes), oligoprotective interventions, although possible, are probably of limited therapeutic utility. Also, because myelinating OLs are post-mitotic and are incapable of replenishing endogenous OL populations (Keirstead and Blakemore, 1997), therefore, the best strategy would be to harness the potential of progenitor cells capable of differentiating into OLs. This is a real possibility since the mammalian CNS, remarkably, has a pool of endogenous progenitor cells that can generate oligodendrocytes even in adulthood (Nishiyama et al., 1999). These progenitors are commonly referred to as oligodendrocyte progenitor cells (OPCs). OPCs were discovered as CNS derived progenitor cells capable of differentiating into oligodendrocytes or type 2 astrocytes in vitro, hence are also referred to as O2A cells (Raff et al., 1983). Subsequently multiple reports identifying OPCs in human have been published (Chang et al., 2000; Maeda et al., 2001). OPCs in the CNS tissue are mainly identified by their NG2 and/or PDGFrαR expression (Nishiyama et al., 1996a). Until very recently there was only circumstantial evidence to
indicate that NG2+ cells (presumably the OPCs) could generate OLs in vivo but work from Akiko Nishiyama’s lab has proved that NG2+ cells indeed give rise to myelinating oligodendrocytes and protoplasmic astrocytes during development (Zhu et al., 2008). After CNS development is complete, a portion of these NG2+ progenitors are believed to persist in a quiescent state in the adult CNS. OPCs within the adult CNS may be a result of natural “maturation” of non-differentiated perinatal OPCs (Miller, 2002). Following a CNS challenge, these NG2+ OPCs can shed their latent state and enter a reactive phase during which they hypertrophy and upregulate their NG2 expression. They also proliferate and migrate towards regions of damaged parenchyma. NG2+ cells have been shown to proliferate and are believed to be the cells that differentiate into OLs in the adult CNS following a “demand” for myelinating cells or purely in response to pathological conditions (Watanabe et al., 2002; Nielsen et al., 2006). Specifically, following SCI NG2+ OPCs undergo protracted proliferation and a modest increase in OL numbers has been noted (McTigue et al., 2001).

Immense amount of research efforts have been focused towards transplanting stem cells or glial restricted precursors into the injured spinal cord to obtain remyelination (Myckatyn et al., 2004). Many of these studies have achieved significant remyelination and have also shown a partial locomotor recovery in injured rodent spinal cords (McDonald et al., 1999; Cao et al., 2005; Keirstead et al., 2005). This suggest that demyelination is a significant limiting factor in recovery from SCI. However, what has been explored to a lesser extent is why despite significant endogenous OPC proliferation following SCI does complete remyelination fail.
Hence our goals were to study, the overall NG2 distribution, temporo-spatial distribution of proliferating NG2 cells, formation of new oligodendrocytes and overall oligodendrocyte population dynamics following spinal contusion injury. In addition, we also examined what factors could be potentially involved in these events.
CHAPTER 2

General methodology

Spinal cord injury (All chapters)

Female Sprague Dawley rats (225-250g) received a moderate to severe spinal contusion using the electromagnetic OSU injury device (Stokes et al., 1992) and were randomly assigned to survive 3, 5, 7, 14, 28, 70 or 112dpi depending on the study. Using ketamine (80mg/kg)/xylazine (10mg/kg) anesthesia (i.p.), a laminectomy at vertebral level T8 was performed under aseptic conditions. Rats were stabilized in a spinal frame and a computer controlled impact probe was swiftly driven into the spinal cord a distance of 0.75-1.1mm (for different studies) over 23 msecs. After spinal contusion, the overlying muscles were sutured and the skin closed with wound clips. The animals were given saline and placed in warm recovery cages. Prophylactic antibiotic (Gentocin 1mg/kg s.c) was given for 5 days. The animals received manual bladder expression until self-voiding returned, usually within 10-14 days. Naïve animals were included as non-injury controls. All procedures were in accordance with the Ohio State University Institutional Animal Care and Use Committee (IACUC) guidelines.
Deeply anesthetized animals were perfused intra-cardially with cold 0.1M PBS followed by 4% paraformaldehyde/0.1M PBS (n=5-6/group). Spinal cords were removed and post-fixed with 4% paraformaldehyde/0.1M PBS for 2 h, then transferred to 0.2M phosphate buffer at 4°C overnight. The tissue was subsequently cryoprotected with 30% sucrose for 2-3 d and spinal cord around the epicenter was dissected, frozen on dry ice and embedded in OCT (Electron Microscopy Sciences, Hatfield, PA). Spinal cords were sectioned at 10µm on a cryostat and serially mounted on pre-cleaned glass slides (ColorFrost/plus, Fischer Scientific).

To identify the lesion epicenter, sections were double-labeled for neurofilament (NF; RT97 clone, Developmental Studies Hybridoma bank, Iowa City, IA) and eriochrome cyanine (EC; Sigma, St Louis, MO). Briefly, sections were pre-incubated with BP+ (4% BSA/PBS/0.1% Triton) and then with 1:2000 mouse anti-NF overnight at 4°C, followed by secondary antibody. The sections were treated with 6% hydrogen peroxide in methanol followed by Elite Avidin Biotin Conjugate (ABC; Vector Lab Inc., Burlingame, CA) and visualized with 3, 3’-Diaminobenzidine substrate (DAB; Vector). Slides were rinsed, treated with acetone (5 min), rinsed and immersed in EC solution for 30 min. The sections were differentiated in 5% Iron Alum and completely differentiated in borax ferricyanide. Slides were dehydrated, cleared using Histoclear (National Diagnostics, Atlanta GA) and coverslipped.
Staining for CC1/GFAP/P0 (Chapters 4&5):

To visualize oligodendrocyte cell bodies, the CC1 antibody (Chapter 5: Oncogene Research Products, Cambridge, MA; Chapter 6: Abcam,) was used. We have previously noted that a subset of Schwann cells and astrocytes become labeled with the CC1 antibody when using the DAB process (see McTigue et al., 2001); thus, a triple-label protocol of CC1, GFAP (glial fibrillary acidic protein) and P0 (Schwann cell myelin) was used to eliminate these cells from OL counts. CC1 (1:500), P0 (1:6000, Dr Juan Archelos, Society to Support Research, Diagnosis and Treatment of Neurological Disorders, Austria) and GFAP immunohistochemistry (1:4000, Sigma) was performed and sections were counterstained with methyl green.

Staining for Olig2/cell markers (Chapter 3, 4 &5)

To verify that NG2 and CC1 cells belong to the OL lineage, a subset of sections was also triple-labeled for Olig2, BrdU and CC1 or NG2. The Olig2 antibody (R&D systems) was applied at 1:2000 (1:500 for chapter 5) followed by 1:1000 donkey anti-goat Alexa Fluor 546; CC1 or NG2 antibodies were followed by 1:1000 goat anti-mouse Alexa Fluor 633 (for triple label) or Alexa Fluor 488 (in case of double-label) and finally if needed the BrdU antibody was applied 1:100 for 2 h followed by 1:1000 goat anti-mouse Alexa Fluor 488.
Cell Counts (Chapter 3&4)

Figure 2.1: Diagram of a spinal cord section 1.0mm rostral to lesion epicenter demonstrating sample box placement. Spared gray matter is denoted with gray and spared white matter with white; lesioned tissue is stippled. Sample boxes include 6 in the outlying spared white matter (ivory), 2 in outlying spared gray matter (grey), and 9 along the lesion border. Of the lesion border samples, 3 are in the gray matter / lesion border (black); the remaining 6 are in the white matter / lesion border (orange).

Cell counts were performed at 0.5 mm intervals throughout a 5mm segment of tissue centered on the lesion epicenter. For each distance, adjacent cross-sections stained for NG2/BrdU, CC1/GFAP and CC1/BrdU were analyzed. For NG2/BrdU and CC1/GFAP cell counts, sections were viewed at 40X using a light microscope (Zeiss Axioskop Plus 2) fitted with a counting reticule. A counting box (0.125 x 0.125mm) was used to sample regions of spared white matter (WM), spared gray matter (when present) and the lesion border, and the number of identifiable cells within each box was counted. For spared WM measurements (SWM), sample boxes were placed just inside the pial border in the dorsolateral, lateral and ventral funiculi (see Fig 2.1). Boxes were placed in the same general location on all sections. Only those regions in which the sample box was at least 0.125 mm away from the lesion border were included in spared WM measurements; in this way, tissue sampled in spared WM never consisted of lesion border
tissue. For spared gray matter (GM), boxes were placed centrally in dorsal horns and ventral horns at a minimum distance of 0.125mm from the lesion border (Fig. 2). When this was not possible, the samples were not collected from the area. Because GM was completely destroyed near the lesion epicenter, samples were only collected in sections ≥1.0mm rostral or caudal to epicenter. For lesion border measurements, boxes were placed such that the innermost edge was located at the lesion / spared tissue interface with the remainder of the box directed toward the meninges (Fig 2.1). Sample box location was selected on low power print-outs of each tissue section. Since cellular details could not be discerned on the printouts (e.g., the number of OLs that would fall within each box), bias in box placement was avoided. A box was placed in the dorsal-most aspect of the lesion border and then non-overlapping boxes were evenly placed around the lesion circumference. A minimum of three boxes were placed along the edge of the lesion poles (≥1.5mm from epicenter) and typically 6-10 boxes were used in sections containing larger lesion sites, which allowed maximum sampling of the lesion border. Data from lesion borders falling within white matter and gray matter were collected separately. The number of cells per box per region (e.g., spared WM) was averaged for each section and then divided by the box area (0.0156mm²) to determine the number of profiles/mm² for each area. Measurements from 3-5 animals per region were averaged and counts expressed as profiles/mm² at each distance or as an overall mean by averaging data from all distances throughout the 5mm of tissue. For comparisons with normal spinal cords, sample boxes were placed in equivalent regions of WM and GM in cross-sections from uninjured spinal cords and the average number of cells/mm² determined.
Protein extraction (chapters 5 & 6)

Rats were anaesthetized and very briefly perfused with cold 0.1M phosphate buffered saline (PBS) (n=3/time point). Spinal cord blocks (4mm) centered on epicenter were rapidly dissected and frozen using liquid nitrogen. The tissue was stored at -70°C until protein extraction. Proteins were extracted from spinal cords by homogenization with 400µl of T-PERS (Pierce, Rockford, IL), 1X EDTA (Pierce) and 1X protease inhibitor cocktail (Pierce). The homogenates were centrifuged at 10,000 rpm for 5 min and the supernatants were collected. Total protein content was determined using the Bradford assay and aliquots of supernatant samples were stored at -70°C.

Electrophoresis/western blots (Chapters 5 & 6)

Protein samples containing 1X Nupage LDS (Invitrogen, Carlsbad, CA) and 1X Nupage reducing agent (Invitrogen) were heated for 5 min at 95°C. 10µg (for CNTF and CNTFRα) or 12.5µg (for FGF-2) of total protein was loaded per lane and separated on 4-12% Bis-Tris Nupage gel (Invitrogen) in 1X Nupage MES running buffer (Invitrogen) at 200V for 40 min. Subsequently, proteins were transferred onto a nitrocellulose membrane at 30V for 60min. The membrane was blocked for 1 h with 5% non-fat dry milk in 0.5% Tween-20/0.1M PBS and incubated with the primary antibody of interest overnight at 4°C. The membrane was washed with 0.5% Tween/0.1M PBS multiple times and probed with the appropriate HRP (Horseradish peroxidase)-conjugated secondary antibody for 1 h at room temp. Chemiluminescence was imparted to the primary-secondary antibody complex by incubating with solution from Super Signal West Pico kit (Pierce) which detects HRP. The signal was detected by exposing the membrane to autoradiographic...
film (ISC Bioexpress) for 10sec – 1min (depending on the signal intensity) and
developed using 100plus automatic X-ray film processor (All-ProImaging, NY). A
chemiluminescent protein ladder (Cell Signaling Technology, MA) was used to
determine molecular weights and α-tubulin was used as loading control. Final
concentrations of primary antibodies used for western blotting were as follows: anti-
CNTF, 1µg/ml (R&D systems, Minneapolis, MA), anti-CNTFRα, 1µg/ml (BD
Pharmingen, San Diego, CA), anti-FGF2, 1:500 (Upstate, Charlottesville, VA) and anti-
α-tubulin, 1:2500 (Abcam, Cambridge, MA).

*Western blot analysis (Chapters 5 & 6)*

The developed western blot films were scanned and saved as gray scale tiff
images. Bands on the images were analyzed using the MCID Elite 7.0 software (Imaging
Research Inc., Ontario, Canada). After lanes of interest were identified, MCID generated
an optical density profile. Density measurements of the bands (seen as peaks) were
displayed in relative optical density (ROD) units. Band profiles of sharp peaks could be
seen riding on a variable background baseline. Therefore, to obtain accurate density
measurements baseline density components were subtracted from band density peaks.
The individual peaks (minus the background) were then defined, sampled and quantified.
The profile measure of each individual band/peak is reported as mean density value of the
sampled peak multiplied by area of the sampled peak and is an estimate of signal
intensity. The ROD of CNTF or CNTFRα bands was normalized to the respective ROD
of α-tubulin bands to obtain the ratios which were then graphically represented and
analyzed.
Statistics (All chapters)

GraphPad Prism 5 software was used for statistical data analyses. Depending on the data set, either a one-way ANOVA followed by Tukey post-hoc tests or a two-way repeated measures ANOVA followed by Bonferroni post-hoc tests was performed. For two-way ANOVA analysis, group means were used to fill in missing values (in case of torn/unavailable sections). A p value <0.05 was considered significant.

Image montages (All chapters)

Anatomical plates containing light or confocal microscopic images were assembled using Adobe Photoshop software (San Jose, CA). When deemed necessary, brightness and contrast were enhanced to allow reproduction of the image as originally viewed in the microscope.
CHAPTER 3

NG2 distribution: extracellular and cellular NG2 following SCI

Introduction

The NG2 molecule belongs to the chondroitin sulfate proteoglycan (CSPG) family. Being a transmembrane protein, NG2 is expressed on the cell surface but it can be proteolytically cleaved and released as an extracellular matrix molecule (Nishiyama et al., 1995). In the normal CNS NG2 expression is noted mainly on oligodendrocyte progenitor cells (OPCs) and blood vessels and hence NG2 is widely recognized as a marker for OPCs in the adult CNS. Apart from being a marker for OPCs, NG2 promotes cell migration and growth factor activities of PDGF and FGF-2 (Nishiyama et al., 1996b; Burg et al., 1997; Stallcup, 2002). Following SCI, NG2+ cells undergo prolonged proliferation lasting many weeks (McTigue et al., 2001). Recently, it has been shown that the NG2 cell population undergoing proliferation after SCI is indeed an adult OPC population and can give rise to oligodendrocytes in vitro (Yoo and Wrathall, 2007).

Despite its acceptance as an OPC marker in normal adult CNS, the use of NG2 as an antigenic fingerprint for OPCs following CNS trauma has been controversial. Macrophages, Schwann cells, meningeal cells and endothelial cells that invade the CNS
parenchyma also express NG2 and contribute to the increased NG2 immunoreactivity after SCI (Jones et al., 2002; McTigue et al., 2006).

In addition to its beneficial roles, NG2 proteoglycan has been shown to inhibit axonal regeneration following SCI. NG2 by itself or as a part of a host of CSPGs has been shown to inhibit neurite outgrowth and axonal regeneration (Chen et al., 2002). Degradation of CSPGs (including NG2) has been shown to improve axonal regeneration and behavioral recovery after SCI (Bradbury et al., 2002).

Hence NG2 proteoglycan appears to be involved in several injury induced processes some of which may supress recovery by inhibiting axon regeneration while others may promote repair by helping OPCs migrate, proliferate and differentiate into OLs. Thus the NG2 molecule is of great interest in many ways after spinal trauma. Therefore, we first wanted to study the total NG2 immunoreactivity in the contused spinal cord and next determine the temporo-spatial distribution of NG2+OPCs. Previously, McTigue et al. (2001) have shown protracted NG2+ OPC proliferation following SCI. However, in that study NG2/BrdU cell counts were collapsed across entire cross-sections hence information regarding which sub-regions of the injured spinal cord show the highest NG2 proliferation and distribution is lacking. The Wrathall group has examined NG2 cell division after SCI but their analysis focused only in the ventral white matter (Zai and Wrathall, 2005). Since it is likely that tissue dynamics vary greatly depending on proximity to the lesion and perhaps between gray and white matter, here we compared NG2 cell proliferation in WM and GM bordering the lesion with that in outlying spared WM and GM using a clinically relevant rodent model of spinal contusion.
Materials and methods

Spinal cord injury: Different injury severities were used for different experiments discussed in this chapter. For evaluation of overall NG2 expression a displacement of 1.1mm was used. Displacements for NG2/BrdU cell counts and proliferating NG2 cell migration counts were 0.75mm and 0.8mm respectively.

NG2 immunohistochemistry: Mouse anti-NG2 antibody (Gift from Dr William Stallcup) was used for overall NG2 immunoreactivity assessment and NG2/BrdU counts following SCI. Mouse anti-NG2 antibody (US Biological, Swampscott, MA) was used for IHC in NG2/BrdU counts for examining their migration.

NG2 immunoreactivity quantitation: Sections labeled for NG2 spanning 8 mm of spinal cord centered on the epicenter were digitized using the MCID Elite image analysis system (InterFocus Imaging Ltd., Cambridge, UK). Digitized images were manually outlined, taking care to exclude NG2+ meninges on section edges which avoided artificial inflation of NG2 levels. Cavities within the sections were excluded from analysis, because the goal was to determine the proportion of tissue expressing NG2. Next, the segmentation range was set such that only positively labeled NG2 was selected. The cross-section area was determined and the amount of NG2 within that area was quantified as target area, which was divided by cross-sectional area to determine proportional area, or the percent of tissue expressing NG2. Data obtained at each time point were averaged and expressed as mean ± standard error of mean. Results were
compared with data collected from naïve noninjured spinal cords using 2-way analysis of variance followed by Bonferroni multiple comparisons test.

*For NG2/BrdU cell counts:* See chapter 2 General methodology for the counting method.

*For Proliferating NG2 cell migration estimation:* To determine if the NG2/BrdU cells along the lesion border were due to local proliferation or due to NG2 cell migration from spared tissue we counted NG2/BrdU cells using stereological technique. Using a computerized/motorized microscope stage, spinal cord cross-sections were digitized, the region of interest was identified and a 33% sampling criterion was set. The software then randomly chose counting fields (totally 33% of the region of interest) and placed sample boxes. The microscope stage moved from one sample box to the next automatically completely eliminating personnel bias regarding counting frame placement in a section. The NG2/BrdU counts were still done manually with observer discrimination.

**Results**

**Chronically elevated NG2 expression following SCI**

At 3 dpi, tissue pathology was evident in sections 4 mm distal to the epicenter (Fig. 3.1 A). Over time, NG2 levels rose within lesions and at the lesion-spared tissue border (Fig. 3.1 B, C). Elevated NG2 was especially prevalent on tissue bridges throughout the cavities (Fig. 3.1 B-F). As shown in Figure 3.1, the total amount of tissue present decreases over time as ongoing cavitation replaces tissue damaged by the initial
trauma. Consequently, NG2-expressing cells occupy a larger proportion of the remaining tissue over time.

NG2 immunoreactivity was quantified in the epicenter and sections 2 and 4mm rostral/caudal (Fig. 3.1 G). Data are expressed as the proportion of tissue displaying NG2 immunoreactivity and are compared with uninjured thoracic spinal cords, in which NG2 immunoreactivity comprised 6% to 7% of the cross-sectional area. In sections 2 and 4mm rostral to epicenter, NG2 increased more than 2-fold by 7 dpi, rose again at 28 dpi, and persisted through at least 70 dpi. In the epicenter region, NG2 levels rose significantly between 3 and 7 dpi and continued to rise through 70 dpi, at which time >50% of tissue was positive for NG2. This level was maintained for at least 112 dpi. Caudally, NG2 expression was similar to that seen rostrally, i.e. NG2 continually increased so that by 70 dpi, levels were 4- to 5-fold greater than controls. Thus, at least 8 mm of spinal cord tissue displayed chronically elevated NG2 levels.
Figure 3.1: NG2 expression after spinal cord injury in cross-sections from 2 to 4 mm rostral (R) and caudal (C) and lesion epicenter (Epi). (A) Three days postinjury (dpi) tissue pathology is evident in all sections. (B) At 28 dpi, NG2 is increased within and around the lesions and NG2 + tissue bridges are present throughout the cavities. (C) At 70 dpi, cavitation has progressed, especially 2 mm from epicenter. At 4 mm R and C, tissue in the dorsal funiculus has collapsed and displays high NG2 levels. (D) High-power view of epicenter box in C showing NG2 cells and processes. (E) High-power view of dorsal funiculus box in 4-mm caudal section from (C); tissue in this region has collapsed and displays high NG2 levels. (F) High-power view of region indicated by arrow in 4-mm caudal section in C. In this region, NG2 expression is near normal levels. Scale bar = 200 µm (A-C), 20 µm (D-F). (G) Proportional area of NG2 immunoreactivity over time postinjury (3 days, 14 days, 70 days, 112 days: n = 4; 7 days, 28 days: n = 5). Dashed line indicates NG2 level in uninjured thoracic sections (approximately 6-7%). *, p < 0.05; **, p < 0.01; ***, p < 0.001. Bars are mean ± standard error of mean.
Overall, the continual rise in NG2 proportional area was the result of increasing NG2 cell numbers and increased NG2 deposition in the extracellular matrix. This is in line with our and other previous data demonstrating significant NG2 cell proliferation for at least 28 days and NG2 deposition after contusion injury (McTigue et al., 2001). Decreasing total cross-sectional area due to cavitation and tissue loss also contributes to this increase in the relative proportion of NG2 immunoreactive area after injury. Collectively, these data reveal that NG2 levels increased early after SCI and remained chronically elevated especially along the lesion borders.

**Anatomical characteristics of the lesion border**

In the experiments described above we noted increased NG2 immunoreactivity around the lesion perimeter after contusion SCI; other investigators too have reported increased NG2 expression at the interface of lesion and spared tissue in a different SCI model. Interestingly, Frei et al (2000) have noted increased mRNA expression of Proteolipid protein (PLP), a major component of CNS myelin, around spinal lesions. Additionally, the lesion border is also home to reactive and hypertrophied astrocytes which form the glial scar. Hence, we became interested in this NG2 rich area surrounding spinal lesions and its gliogenic potential.

To better understand injury induced changes in NG2 expression along lesion perimeter, we first began with examining the characteristics of the lesion border. In order to have sufficient residual tissue after SCI, to be able to differentially examine spared tissue from lesion borders, we chose a less severe injury than in the previous experiment. Using a lesser injury severity compared to previous work shown in Fig 3.1 (0.75mm vs.
1.1mm displacement), we again observed elevated NG2 expression at 7 and 14dpi in tissue bordering the lesion compared to spared GM or WM near the meninges (Fig. 3.2 A,B). We also noted on adjacent slides that this NG2-rich border typically contained a number of axons lacking myelin based on the absence of EC stain (Fig. 3.2 C,D). The presence of bare axons along the lesion border was confirmed by examining comparable regions of semi-thin epon embedded sections (Fig. 3.2 E-G). Hence, increased NG2 expression along the lesion borders was accompanied with the presence of bare axons.

We next examined how this lesion border containing NG2 expression changed over time.
**Figure 3.2: Bare axons and augmented NG2 expression were present along lesion borders.** (A) NG2/BrdU immunoreactivity was increased at the gray matter / lesion cavity (**) border 1mm caudal to epicenter at 14dpi. (B) Spared white matter from same section in A displaying comparatively lower NG2 than the lesion edge (seen in A). (C,D) 7dpi section taken 2mm caudal to epicenter labeled for myelin (EC, blue) and axons (NF, brown). D is a higher magnification of lesion in C. Axons lacking myelin line the lesion border (**). (E-G) 1µm transverse (E,F) or longitudinal (G) sections from 7dpi epicenter sections stained with Toluidine blue. Lesion cavities (**) are located in upper left corners. Several axons lacking myelin (arrowheads) are present. A node of Ranvier is located along a bare axon in G (arrow). Oligodendrocytes in E are denoted by asterisks. **Insert:** High power view of boxed area in E. Note the appearance of processes extending from the oligodendrocyte toward nearby unmyelinated axons. Scale = 20µm A-D; 10µm E-G.
Figure 3.2
**Evolution of the lesion border area**

In order to determine how the region of high NG2 expression and demyelinated axons changed over distance and time after SCI, we mapped the area that fit these criteria. In general, this NG2 reactive zone followed the contours of the lesion (Fig 3.3A-F), and varied in size over time along the 5mm length of injured tissue. At 3dpi it was non-existent i.e no significant increase in NG2 reactivity was specifically noted along the lesion borders. At 7dpi, the NG2 reactive area (containing bare axons) ranged from 0.4-0.7mm² and decreased to 0.2-0.3mm² by 14dpi (Fig 3.3G). Thus the lesion border is a dynamic region in terms of the spread of NG2 reactivity.

We next wanted to determine if changes in NG2 cell proliferation and accumulation were responsible for the changes in NG2 immunoreactivity along the lesion borders and how that changed over time. From the above observations it became obvious that the NG2 reactive lesion border was distinguishable from spared and lesioned areas; therefore we examined the lesion borders, spared white matter and spared gray matter separately for NG2 cell analyses. Also, since the area of the NG2 reactive lesion border is dynamic (Fig 3.3G), to maintain objectivity in our subsequent data analysis involving the lesion borders we chose a distance of 0.125mm from the lesion into the residual parenchyma to serve as the lesion border. The 0.125mm criteria was chosen since this a region that was within the NG2 reactive area at 7d and 14dpi in all sections examined (Fig 3.3G).
Figure 3.3: Diagrammatic representation and quantification of the NG2 reactive zone. A-F: The dynamic shape of the NG2 reactive zone (indicated in black) is illustrated through the rostral (R) – caudal (C) extent of the lesion 14dpi. The reactive zone followed the contours of the lesion, though it was often thickest near grey matter areas (hashed regions). G: Reactive zone area was measured on digitized images. A decrease in the reactive zone area was observed between 7 and 14dpi.
NG2 cells proliferate and accumulate in the perilesion reactive zone by 7dpi

NG2 cell proliferation in WM and GM bordering the lesion was compared with that in outlying spared WM and GM in animals receiving BrdU at different intervals after SCI (see Fig. 3.4). For baseline data, NG2/BrdU cells were counted in spinal cords of naïve animals that received BrdU for 7d prior to sacrifice. This revealed that NG2 cell division in normal GM was twice that of WM (19.5 cells/mm² vs. 9.5 cells/mm²; Fig 3.5).

**Figure 3.4: BrdU administration I.** Time course of BrdU injections, denoted by a solid line; time of sacrifice is indicated by “x”. Rats were randomly divided into groups receiving BrdU on days 1-3 post-injury (“3d”), days 4-7 post-injury (“7d”) or days 1-7 post-injury (“14d”). Rats received one BrdU injection (50mg/kg) per day.

**NG2 cell division during 1-3dpi:** To examine acute NG2 cell proliferation, rats received BrdU on 1-3dpi. During this time, the number of NG2/BrdU cells in outlying WM and in WM adjacent to the lesion doubled (25 cells/mm² vs. 9.5 cells/mm²; Fig. 3.5B), although this did not reach significance. In contrast, NG2/BrdU cells in outer spared GM and in GM bordering the lesions were significantly elevated compared to naïve GM (Fig 3.5B). Spared GM also contained more NG2/BrdU cells than spared WM (Fig. 3.5B). Since GM is absent near the epicenter, most acute NG2 cell proliferation took place in GM near the lesion extensions ≥1mm distal to epicenter (Fig. 3.5E).
Figure 3.5: NG2/BrdU cells increased after SCI, particularly at the lesion perimeter. The timing of BrdU injections is shown above each graph. Fig. 2.1 diagram is repeated to facilitate comparisons of graphical data with sample regions (boxes are not to scale). (A) Average of NG2/BrdU cells in white or gray matter from naive spinal cord cross-sections. Gray matter contained significantly more cells. (B) At 3dpi, NG2/BrdU cell numbers were higher in spared gray matter (SGM) compared to nearby spared white matter (SWM) and naive GM. GM lesion borders also contained more NG2/BrdU cells than naïve GM. (C) At 7dpi in rats given BrdU on 4-7dpi, NG2/BrdU cells were increased in WM and GM lesion borders compared to naïve and spared WM/GM, respectively. GM borders also contained more cells than WM borders. (D) At 14dpi in rats given BrdU on 1-7dpi, NG2/BrdU cells were elevated in WM lesion borders compared to SWM and naïve WM. SGM contained more double-labeled cells than naïve GM; GM lesion borders contained more NG2/BrdU cells than naïve and WM borders. (E) In the lesion border, most NG2/BrdU cells were distributed ≥1.0 mm distal to epicenter at 3d and 7dpi (* vs. naïve at same distance). *p<0.05 vs. naïve; ^p<0.05 border vs. spared tissue; +p<0.05 WM vs. GM. All data represent mean ± SEM.
**NG2 cell division during 4-7dpi:** NG2 cell division during the latter half of week one post-injury was investigated in rats given BrdU for 4-7dpi. During this time, NG2 cell proliferation fell to naive levels in spared WM and GM (Fig. 3.5 C). In contrast, NG2 cells in WM and GM lesion borders continued to proliferate at levels significantly greater than in naïve WM/GM or spared WM/GM within the same sections (Fig 3.5C). Interestingly, NG2 cell proliferation in GM lesion borders was greater than that in nearby WM lesion borders (Fig. 3.5C). Similar to above, sections ≥1.5mm distal to epicenter contained most NG2/BrdU cells (Fig. 3.5E).

**NG2/BrdU cells remaining at 14dpi:** To evaluate if NG2 cells dividing during the first week post-injury were retained through 14dpi, a group of rats received BrdU on 1-7dpi and survived to 14dpi (Fig. 3.4). In spared WM, the number of NG2/BrdU cells was not different from naïve levels. In WM lesion borders, however, NG2/BrdU cell numbers were similar to 7dpi values and were elevated compared to naïve and spared WM (Fig. 3.5D). Likewise, GM lesion borders and GM closer to meninges maintained elevated numbers of NG2/BrdU cells compared to naïve spinal cords and WM lesion borders (Fig. 3.5D). Thus, many NG2 cells that proliferate during the first week post-injury are maintained through 14dpi in spared GM and along lesion borders. It should be noted, however, that NG2 cells dividing during 1-3dpi and 4-7dpi are unlikely to be the same cells based on the BrdU injection regimen. Thus, if these numbers were considered additive, it would appear that ~half of the NG2/BrdU cells were no longer present at 14dpi.
NG2 cell migration

In order to determine if the increased NG2/BrdU cells at the lesion border were due to local proliferation at the border or due to migration of proliferating NG2 cells from spared tissue, spinally injured rats received BrdU on 3dpi, half of these animals were sacrificed later on 3dpi itself and the remaining were sacrificed 7dpi (Fig 3.6). By perfusing half the animals two hours after BrdU administration, the labeled cells would not have had the time to migrate, thereby, allowing us to determine where they were located when they divided; the 7d group allowed us to determine if the dividing cells migrated away from their original location between 3d and 7dpi.

Figure 3.6: BrdU administration II. Injured rats (n=9) received 2 injections of BrdU (50mg/kg) separated by 2h on 3dpi. Four rats were sacrificed 2 hours after the last BrdU injection on 3dpi; the remaining rats were sacrificed 7dpi.

We examined distances 2mm rostral and caudal to the epicenter since it was here that we saw the maximum NG2/BrdU cell accumulation along lesion border (see Fig 3.5E). We counted NG2/BrdU cells at the lesion border and in the spared tissue using stereological techniques (see methods).
Figure 3.7: NG2 cells proliferating on 3dpi are mainly located in the lesion border by 7dpi. A) Overall, by 7dpi NG2 cells undergoing division on 3dpi had reduced in the SWM but increased in WM border. B) In the GM, proliferating NG2 cells at 3d remained unchanged by 7d in the SGM but had increased in the GM borders. n=4-5/group. The dotted lines represent the average number of NG2/BrdU cells in naïve white matter or gray matter (n=3). p<0.05 was considered significant. For 2mm rostral data in white matter p values are indicated on top of the bars.

**White matter:** At 3dpi, 2mm caudal to the epicenter, NG2/BrdU cells were equally distributed between SWM and WM border (Fig 3.7A). In the SWM, NG2 cells dividing on 3dpi had reduced significantly by 7dpi. In contrast, at the WM border, NG2 cell proliferating on 3dpi had risen significantly by 7dpi compared to 3d counts.

Changes in NG2/BrdU cell counts 2mm rostral to the epicenter revealed a trend very similar to 2mm caudal data; the data here approached but did not meet p<0.05 criteria to be considered statistically significant.

**Gray matter:** Similar to WM, at 3dpi 2mm caudal to epicenter, the numbers of NG2/BrdU cells were equal in SGM and GM border (Fig 3.7B). There was no change in the cell numbers between 3d and 7dpi in the SGM, but NG2/BrdU cells in GM border
had tripled by 7dpi. A similar trend in the GM regions 2mm rostral to the epicenter was also observed.

**NG2 cells after SCI express Olig2:** In order to confirm the lineage identity of the proliferating NG2 cells after SCI, we used double-label immunofluorescence for NG2 and Olig2. Olig2 expression by NG2 cells was also examined since we have noted previously that NG2+ cells inside contusion lesions express markers not typically associated with OPCs (McTigue et al., 2006). Olig2 is a transcription factor believed to be exclusively expressed in cells of the OL lineage (Zhou et al., 2000; Kitada and Rowitch, 2006). Most NG2 cells bordering the lesions also expressed Olig2, and a subset of NG2 cells were labeled with Olig2 and BrdU (Fig 3.8). This indicates that although not all NG2 cells may function as OPCs in the adult CNS, a large portion of the proliferating NG2 cells that accumulate along lesion borders likely function as OL lineage cells.

![Figure 3.8: NG2 cells after SCI express Olig2](image)

**Figure 3.8: NG2 cells after SCI express Olig2.** (A,B) Confocal images revealed that many NG2+ cells following SCI were also labeled with Olig2 confirming their oligodendrocyte lineage. A subset of proliferating NG2 cells were also Olig2+ (C,D). Scale bar=20µm.
Discussion

The purpose of the experiments in this chapter was to better understand the distribution of the NG2 proteoglycan and to determine the temporo-spatial response of NG2 cells following SCI. In the present study, total NG2 immunoreactivity rose >2-fold by 7 dpi compared with naive spinal cords. This is in line with previous work showing maximal NG2 cell proliferation occurs during the first week postinjury (McTigue et al., 2001; Zai and Wrathall, 2005). Interestingly, NG2 expression continued to rise throughout the lesions over time, resulting in a significant elevation as late as 112 dpi. Spinal parenchyma at the lesion/border interface also contained reactive cells displaying high levels of NG2. Thus, throughout the entire rostrocaudal extent of tissue pathology (>8 mm), NG2 proteoglycan was elevated within and around the lesions. These results contrast with data by others using dorsal spinal knife cuts in which NG2 rose within 1 mm of the lesion and peaked 1 to 2 weeks postinjury (Jones et al., 2002; Tang et al., 2003). The protracted rise in NG2 in our model likely is the result of the greater pathology caused by contusion injury and the accompanying progressive cavitation. This model may induce a larger number of NG2 cells to proliferate, which would contribute to higher and prolonged NG2 levels. Indeed, (McTigue et al., 2006) have shown that NG2 cell numbers continually rise over time inside the lesions, which may be the result of chronic proliferation and infiltration from the surrounding tissue.

In addition to increased overall NG2 immunoreactivity we also showed increased NG2 cell proliferation. NG2 cells dividing during the first 3 days after injury resulted in higher NG2/BrdU numbers in GM regions at 3dpi. NG2 cells dividing during the latter part of week 1 resulted in higher NG2/BrdU along the lesion borders of WM and GM.
And overall NG2 cells dividing throughout week 1 were maintained in GM and lesion borders up to 14dpi. It is also possible that the absolute number of NG2 cells in the lesions borders did not change over time, but that the area available for them to occupy was continually reduced by ongoing cavitation. Hence, they would be forced to coalesce into the remaining tissue (For more explanation regarding this possibility, refer to data and discussion in chapter 4). Nevertheless, the density of proliferating NG2 cells was the highest in the lesion borders. NG2 cells have been shown previously to proliferate and migrate towards and into damaged tissue (Levine and Reynolds, 1999; Chari and Blakemore, 2002; Watanabe et al., 2002), which suggests that lesioned environments emit tropic signals for these cells. Also, in other disease models like stroke and focal demyelination increased oligodendrocyte formation has been noted in the tissue surrounding the lesions (Watanabe et al., 2002; Talbott et al., 2005). Our data supports the fact that following SCI, the region adjacent to lesioned tissue contains cues that promote the migration and accumulation of proliferating NG2 cells and hence has a high potential for oligodendrogenesis.

To determine if the increased NG2/BrdU cells in the lesion border were due to local proliferation and/or migration of cells from spared tissue to the lesion border, we examined NG2/BrdU cell counts at 3dpi and 7dpi after BrdU administration on 3dpi. NG2 cells were dividing (BrdU+) in SWM and WM border at 3dpi. Over the next 4 days their numbers decreased in the SWM but increased in the WM along the lesion border. The decrease in SWM may be due to cell migration, differentiation or cell death, all of which are plausible events. However based on our data in chapter 4 it is clear that at least a portion of the cells differentiated into oligodendrocytes. The increased NG2/BrdU cells
at 7dpi along the WM lesion border is likely, at least in part, due to local proliferation, since a) we know that NG2 cells were dividing at the lesion border on 3dpi and b) NG2 cells continue to divide between 4d-7dpi following SCI. However, we cannot rule out that migration of NG2/BrdU cells from SWM dividing on 3dpi could have contributed to increased cell numbers.

In contrast to WM, the only GM region in which noticeable changes in proliferating NG2 cells were seen between 3d-7dpi was the GM lesion border. Since no other region showed an increase in the NG2/BrdU cell population, local proliferation is the most likely explanation for increased numbers along GM lesion borders. It is important to note here that since OPCs are capable of self-renewal and asymmetric division, proliferating NG2 cells could easily have migrated from spared regions to the lesion border without any decrease in the NG2/BrdU cells in the spared regions (Wren et al., 1992; Levine and Reynolds, 1999). Hence, although we may not have completely answered whether cells from spared tissue migrate to the lesion border we have shown definitively that NG2 cells do proliferate locally along the lesion border in response to injury. More experiments are needed to examine the issue of contribution of local proliferation versus that of migration from other regions. Therefore, local NG2 cell proliferation and/or NG2 cell migration both may have lead to the increased presence of NG2/BrdU cells along the lesion border.

A potential negative consequence of increased NG2 in the perilesion zone is reducing axon regeneration. Several studies have shown that NG2 can prevent neurite growth in vitro (Dou and Levine, 1994; Fidler et al., 1999; Chen et al., 2002; Ughrin et al., 2003). Because NG2 and other CSPGs accumulate along lesion borders after SCI,
several experiments have been designed to promote axon regeneration by removing CSPGs from the glial scar. Given evidence showing NG2 cells are associated with potential OL replacement, promoting axon growth with such strategies may come at the expense of losing cells needed to support and myelinate those axons. Interestingly, studies are emerging which suggest that cellular expression of NG2 in vivo may not be as growth inhibitory as previously believed and may even promote growth of certain fiber populations (de, Jr., Tajrishi et al., 2005; McTigue et al., 2006; Yang et al., 2006; Hossain-Ibrahim et al., 2007). The ultimate fate and function of NG2+ glia after CNS injury is not completely understood. Although adult OPCs express NG2, not all NG2 cells necessarily function as OPCs. Indeed, work by others has suggested that OPCs may comprise only a small subset of adult NG2 cells (Dawson et al., 2000). Furthermore, a previous study showed that many NG2 cells migrating into spinal contusion cavities do not express the typical OPC markers (McTigue et al., 2006). This would imply that at least a portion of NG2 cells present after SCI may not be oligodendrocyte progenitors. However, most NG2 cells along the lesion borders co-expressed Olig2, which is accepted as an indicator of OL lineage cells indicating that a lot of NG2 cells are indeed OPCs.

Thus in conclusion, we showed an overall increase in NG2 expression after SCI which persists chronically. This increased NG2 expression was prominent along lesion borders between 7-14dpi. In addition to the increased NG2 immunoreactivity along lesion borders, significantly higher proliferating NG2 cells were also located in the SGM and the lesion borders, a portion of which were OPCs. Interestingly, we have also noted some bare axons along the lesion borders, this is important because OPCs may be proliferating and migrating to the lesion borders in response to signals from demyelinated axons that
need myelination. Overall, our data indicates that the lesion border contains a high gliogenic potential and subsequent studies will examine if concomitant increase in oligodendrocytes is noted in the same regions (shown in chapter 4).
CHAPTER 4

Oligodendrocyte genesis in the adult contused spinal cord.

Introduction

Oligodendrocytes (OLs) are vulnerable to injury and disease conditions, and significant OL loss occurs after spinal cord injury (SCI) (Crowe et al., 1997; Grossman et al., 2001; McTigue et al., 2001). Accordingly, demyelinated axons have been detected in the injured spinal cords of rodents, cats, monkeys and humans (Bresnahan et al., 1976; Blight, 1983a; Totoiu and Keirstead, 2005). Hence, there have been numerous attempts to restore myelination after SCI by transplanting exogenous progenitor/stem cells that can generate OLs (McDonald et al., 1999; Cao et al., 2005; Keirstead et al., 2005; Cummings et al., 2005). Surprisingly, however, the possibility of stimulating endogenous oligogenesis after traumatic injury has received relatively less attention (Zai and Wrathall, 2005; Yang et al., 2006).

Myelinating OLs are post-mitotic and cannot contribute to cell replacement when neighboring cells are lost. However, differentiation of surviving OPCs after SCI may represent a viable endogenous mechanism for OL replacement. To generate new oligodendrocytes, NG2+ progenitors must first proliferate since oligogenesis and CNS remyelination can be prevented by x-irradiation, which kills all dividing cells within the irradiated region (Keirstead and Blakemore, 1997). After traumatic SCI, NG2 cells within
and around the lesions undergo protracted proliferation, which is associated with a modest increase in OLs at 14 days post-injury (dpi) (McTigue et al., 2001). More recently, Zai and Wrathall (Zai and Wrathall, 2005) showed that NG2 cell proliferation at 3d after spinal contusion was associated with new OL formation in residual ventral white matter; a portion of these new OLs persisted for at least 6 weeks post-injury. A separate study recently affirmed that cells proliferating at 24h after dorsal hemisection, which were mostly NG2+, gave rise to oligodendrocytes in spared tissue by 3 weeks post-injury (Horky et al., 2006). Thus, NG2 cell division between 1-3dpi is correlated with new OL formation.

Understanding oligodendrocyte replacement in spared white matter is significant, since this region contains apoptotic OLs after SCI (Li et al., 1996; Crowe et al., 1997; Liu et al., 1997; Warden et al., 2001). However, less attention has been paid to OL loss or generation in spared gray matter. Indeed, gray matter contains a large number of OLs which myelinate axons traversing across the midline and/or toward local targeted synapses. Since experimental and clinical spinal contusion lesions often extend rostrally and caudally in close proximity to spared gray matter, the issue of OL loss and replacement in gray matter is important. Additionally, OL replacement in tissue at the lesion epicenter has received sparse attention. The functional capacity of this tissue is highly relevant to the overall outcome from SCI, because this region contains the sole surviving axons that pass through the injury site and maintain connections between the brain and spinal cord. Therefore, it is important to understand what, if any, endogenous repair processes occur in this fragile tissue.
In the previous chapter we noted a marked increase in proliferating NG2 cells around contusion lesions (McTigue et al., 2006). This occurred in the region characterized as the glial scar, where reactive astrocytes accumulate after SCI (Reier et al., 1983; Windle et al., 1952). Since proliferating NG2 cells in this region include oligodendrocyte progenitor cells, this perilesion area may be an important region of gliogenesis. Indeed, a recent report using a primate hemisection model showed that OLs formed early after injury were maintained chronically in tissue near the lesion site (Yang et al., 2006). Thus, the region in which NG2 cells accumulate after SCI, i.e., the glial scar, may be an important area of oligogenesis.

Here, we compared OL genesis in WM and GM bordering the lesion with that in outlying spared WM and GM using a clinically relevant rodent model of spinal contusion. To determine if augmented OL genesis was reflected in altered OL numbers, the total number of OLs was quantified in the same regions. Our results revealed that ongoing OL genesis throughout the first two weeks post-injury resulted in a normalization of OL numbers in spared white matter, despite concurrent OL apoptosis reported to occur during this time (Li et al., 1996; Crowe et al., 1997; Liu et al., 1997; Warden et al., 2001). The most striking result of this study was the marked and protracted formation of new OLs in tissue adjacent to the lesions. Oligogenesis occurred throughout the first and second weeks post-injury and resulted in a significant elevation in OLs, such that the numbers were markedly greater than in normal white or gray matter. A lower number of newly formed astrocytes also were present along the lesion border and, to a lesser extent, the surrounding gray matter. Thus, tissue adjacent to the lesion cavity,
typically classified as the glial scar, is a dynamic zone of glial proliferation and gliogenesis after SCI.

**Materials and Methods**

BrdU administration: Bromo-deoxyuridine (BrdU; Roche Diagnostics, Indianapolis, IN) was injected once daily (50mg/kg, i.p.). Rats were randomly divided into groups that received one of four different BrdU injection regimens (Fig. 4.1). The different groups were used to examine OL formation during 1-3dpi ("3d"), 4-7dpi ("7d") or 8-14dpi ("14d(2)"); because adult OPC proliferation and differentiation may require more than 3-4d, a fourth group received BrdU on 1-7dpi and survived to 14dpi ("14d"). In addition, three naïve rats received BrdU for 7d and then were sacrificed.

**Figure 4.1: BrdU administration III.** Time course of BrdU injections, denoted by a solid line; time of sacrifice is indicated by "x". Rats were randomly divided into groups receiving BrdU on days 1-3 post-injury ("3d"), days 4-7 post-injury ("7d"), days 1-7 post-injury ("14d") or days 8-14 post-injury ("14d(2)"). Rats received one BrdU injection per day.

For new OL counts, sections double-labeled for CC1 and BrdU were viewed using a Zeiss 510 Meta Laser Scanning confocal microscope. Optical sections (<1μm) were captured and saved as single and merged channels. Non-overlapping sample boxes
(0.05\text{mm}^2; 4-8 in spared WM or lesion border, 2-4 in gray matter) were placed throughout each region under investigation. The number of single-labeled CC1 cells and double-labeled CC1/BrdU cells was counted in each sample box. The number of double-labeled cells per box per region was averaged for each section and then divided by the box area to determine the average number of profiles/mm$^2$ for each area. Data were also expressed as the percent of CC1 cells counted in each box that were double-labeled with BrdU. Group means were calculated at each distance and as the overall average for the 5mm of tissue.

Sections from the 14d group (BrdU given at 1-7dpi) were also double-labeled for GFAP and BrdU to determine if new astrocytes arose from cells dividing during the first week post-injury. For this analysis, sections from epicenter, 1.5 and 2.5mm rostral and caudal were examined with confocal microscopy and sample boxes used as above. Lastly, the total number of cells in each region in naïve and 14dpi sections was estimated using sample boxes to count Draq5+ nuclei in sections from the epicenter and 1mm and 2mm rostral to the epicenter.

To estimate the total number of cells located in different sub-regions of the sections (see above) in naïve or 14dpi spinal cords, cell nuclei were labeled with Draq5 (1:3000 in PBS, Biostatus Limited, UK), which is visualized by excitation at 633nm in the high red range.

To examine new astrocyte formation, sections were double-labeled with GFAP and BrdU antibodies. Rabbit anti-GFAP (1:10,000, Dako Corporation, Carpinteria CA)
and mouse anti-BrdU (1:400) were applied together following 2N HCl treatment at 37°C for 20 min. Secondary antibodies were applied for 30min.

Results

New oligodendrocytes are formed in the reactive lesion border after injury

Since proliferating NG2 cells can give rise to OLs, we hypothesized that new OLs would be generated along lesion borders and, to a lesser extent, in spared tissue. Thus, sections were examined for the presence of CC1 cells containing BrdU+ nuclei, which would indicate they were new OLs that arose from cells proliferating after SCI (Fig 4.2); to determine if new OLs were generated in naïve adult spinal tissue, sections from uninjured spinal cords were also examined for BrdU+ CC1 cells. The average number of CC1/BrdU cells in uninjured spinal cords was 2.3 cells/mm², which equates to 0.98% of all OLs sampled. This is consistent with work by Horner et al. (Horner, Power et al., 2000), who reported that 0.82% of OLs were new in adult naïve rat spinal cords.
Figure 4.2: Enhanced oligodendrogenesis in perilesion tissue after SCI. (A-C): Merged (A) and single channel (B,C) confocal images (<1µm) from 14dpi tissue 2.5 mm rostral to epicenter. Multiple oligodendrocytes (CC1+, red) with BrdU+ nuclei (green) are present in the perilesion zone (arrows). Single-labeled OLs (arrowhead) and BrdU+ cells are also present. Lesion border is delineated by dotted white line; * denotes lesion. Insert: Higher magnification of box in A showing single-labeled and double-labeled OLs. (D-F) Multiple single-labeled OLs (arrowhead) and occasional double-labeled OLs were noted in spared white matter. Images were taken 1.5 mm caudal to epicenter. Scale bar = 20µm for A-F.
New OL formation between 1-3dpi: During the first 3dpi, increased oligogenesis occurred in spared WM and GM, which led to more new OLs compared to naïve WM (3.5 cells/mm²) and naïve GM (0.4 cells/mm²), respectively (Fig. 4.3A). In tissue adjacent to lesion cavities, the average number of new OLs was 16.9 cells/mm², which was significantly greater than in naïve tissue (indicated by dotted line in Fig. 4.3A). These new OLs were located in WM and GM lesion borders.

New OL formation between 4-7dpi: Few new OLs were detected in spared WM and GM of rats given BrdU on 4-7dpi (Fig 4.3A). However, OL genesis had continued during this time in tissue along the lesion border, where significantly more CC1/BrdU cells were present compared to naïve and surrounding spared WM/GM (Fig 4.3A). Thus, enhanced oligogenesis along the lesion border is not restricted to 1-3dpi as in spared tissue, but continues throughout the first week post-injury.
Figure 4.3: Prominent OL genesis occurred after SCI. (A) BrdU injection times are denoted above graph. Number of CC1/BrdU cells in naïve cords is indicated by dashed line. Data from WM and GM lesion borders was combined. Between 1-3dpi, CC1/BrdU cells in spared white matter (SWM), spared gray matter (SGM) and lesion border had increased compared to naïve. Between 4-7dpi, OL genesis continued along lesion borders. By 14dpi in rats given BrdU on 1-7dpi, new OLs drastically increased in SGM and lesion borders compared to earlier times. Progenitors dividing during week one post-injury likely continue dividing and differentiating during week two, which would explain the rise in new OLs between 7-14dpi in GM. OL genesis continued during 8-14dpi along lesion borders. (B) In rats given BrdU at 1-7dpi, more new OLs were observed at 14dpi in lesion borders compared to SWM at most distances examined. All data represent mean ± SEM. *p<0.05 vs. naïve; ^p<0.05 border vs. spared tissue; +p<0.05 WM vs. GM.
New OLs formed by 14dpi from progenitors proliferating on 1-7dpi: Because adult OPCs have a slower proliferation and differentiation rate than neonatal progenitors (Wolswijk and Noble, 1989), it is possible that >3-4d would be required for development of some new OLs. Thus, a group of animals received BrdU on 1-7 dpi and survived to 14dpi. In spared WM, where NG2 cell division was elevated between 1-3dpi but not 4-7dpi, the number of CC1/BrdU cells was almost twice compared to 3dpi (Fig 4.3A), suggesting that progenitor cells dividing on 1-3dpi differentiate during the following 11 days. An even larger increase was noted in spared GM, where the average was >40 cells/mm² (Fig 4.3A). This was significantly greater than naïve GM and nearby spared WM, which is in accordance with greater NG2 cell proliferation occurring in GM in our model (Chapter 3). The largest increase in new OLs was detected along lesion borders where >50 cells/mm² were present (Fig. 4.3A). Based on the total number of fluorescent-labeled CC1 cells sampled at least 17.3% of OLs present along lesion borders at 14dpi were newly generated from progenitors dividing during 1-7dpi. Within the 5mm of spinal tissue examined, most new OLs were in sections ≥1mm distal to epicenter (Fig 4.3B), which also is in accordance with NG2 cell proliferation and with the greater amount of GM sparing in those sections (see Chapter 3).

New OLs formed between 8-14dpi: Our previous work revealed that NG2 cells proliferate during the second week post-injury, raising the possibility that OL genesis also continues during that time. Therefore, a group of rats was given BrdU on 8-14 dpi. New OLs were generated in these spinal cords along the lesion border, where an average of 14 CC1/BrdU cells/mm² (or 5.2% of OLs sampled) was detected (Fig 4.3A). Thus,
new OL formation from progenitor cells dividing during week two post-injury occurs but is restricted to tissue in the vicinity of the lesion border. Based on the above data, it is possible that oligogenesis continues at times beyond 14dpi.

CC1/BrdU cells within the lesion border express Olig2: The CC1 antibody has been used in numerous studies to identify OLs. To verify in our hands that CC1 cells in lesion borders belonged to the OL lineage, sections were triple-labeled for CC1, BrdU and Olig2, which is a transcription factor expressed by cells of the OL lineage. After SCI, the majority of CC1 cells within lesion borders contained Olig2+ nuclei. In addition, multiple cells triple-labeled for CC1, Olig2 and BrdU were detected (Fig 4.4). This and the fact that very few CC1 cells co-expressed NG2 (0.6% of CC1 cells sampled, Table1); strongly suggests that CC1/BrdU cells formed after SCI are oligodendrocytes.
Figure 4.4: CC1/BrdU cells along lesion border express Olig2. Single label confocal images of sections triple-labeled for Olig2 (red), BrdU (green) and CC1 (blue) in gray matter (GM; A-C) and white matter (WM; E-G) lesion borders are shown here at 7dpi. Merged images are shown in D,H. Multiple triple-labeled Olig2/CC1/BrdU cells (arrows) were observed. Olig2/CC1 double-labeled cells were also detected (arrowhead).

<table>
<thead>
<tr>
<th></th>
<th># of CC1 cells</th>
<th># of CC1/NG2 cells</th>
<th>% double-labeled</th>
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<tbody>
<tr>
<td>3dpi</td>
<td>458</td>
<td>5</td>
<td>1.09%</td>
</tr>
<tr>
<td>7dpi</td>
<td>485</td>
<td>2</td>
<td>0.41%</td>
</tr>
<tr>
<td>14dpi</td>
<td>760</td>
<td>2</td>
<td>0.26%</td>
</tr>
<tr>
<td>1703</td>
<td>1703</td>
<td>9</td>
<td>0.53%</td>
</tr>
</tbody>
</table>

Table 1: Less than 1% of CC1+ cells co-labeled with NG2. Images were collected on a confocal microscope and single and double-labeled cells were manually counted. Sections 1.5mm caudal and 2mm caudal to the epicenter were used for counting. n=3-4/group.
Changes in oligodendrocyte number over the first two weeks post-injury

Oligodendrocyte numbers at 3dpi: To determine if OL genesis after SCI altered overall OL numbers, sections were labeled with CC1 using DAB immunohistochemistry. At 3dpi, OL numbers in WM along the meningeal border were significantly reduced near the lesion epicenter (Fig. 4.5A). Interestingly, OL numbers in WM bordering lesion cavities were not reduced; instead, OL levels were comparable to uninjured WM (Fig. 4.5B,C). In GM near the meninges and along the lesion border, OL numbers were not different from naive and were significantly greater than in neighboring WM (Fig. 4.5C). Thus, only outlying WM near the epicenter displayed marked OL loss at 3dpi.
Figure 4.5: Changes in OL numbers after SCI. (A) By 3dpi, CC1+ OLs had decreased in spared white matter (WM) near the epicenter compared to equivalent regions of naïve WM. By 7dpi, OL numbers had risen slightly and by 14dpi OLs had further increased such that all distances were similar to naïve WM and several distances were greater than 3dpi levels. (B) In WM lesion borders at 3dpi, CC1 cell number was not different from naïve WM. By 7 and 14dpi, more OLs were present in WM lesion borders compared to naïve WM and 3dpi data. (C) OL numbers in each region were averaged over the 5mm of tissue examined. At 3dpi, outlying GM and GM borders contained more OLs than SWM and WM borders, respectively. By 7dpi, OLs in WM lesion borders were greater than naïve and SWM. GM lesion borders now contained more OLs than naïve GM, WM borders and spared GM. OL numbers were similar at 14dpi in all areas. (D) Total cell numbers at 14dpi were compared in sections stained with Draq5. Naïve GM contained more cells than naïve WM. After SCI, spared WM and WM borders had a comparable increase in cell number. Spared GM cell number was not different after SCI; GM lesion borders contained more cells than naïve GM, spared GM and WM lesion borders. A,B: *p<0.05 vs. naïve; ^p<0.05 vs. 3dpi; +p<0.05 vs. 7dpi. C,D: *p<0.05 vs. naïve; ^p<0.05 border vs. spared; +p<0.05 WM vs. GM. All data represent mean ± SEM.
**Oligodendrocyte numbers at 7dpi:** Between 3-7dpi, OL numbers had risen in every region examined. In spared WM, OL counts were no longer less than naive (Fig. 4.5A). WM lesion borders contained more OLs than comparable regions in naïve and 3dpi spinal cords and also had more than in nearby spared WM (Fig. 4.5B,C). While spared GM displayed OL numbers comparable to 3dpi, in GM lesion borders OL numbers had continued to rise to a level that was greater than in outlying spared GM, spared WM and naïve GM (Fig. 4.5C).

**Oligodendrocyte numbers at 14dpi:** By 14dpi in spared WM, OL numbers had increased again such that levels near the epicenter were equal to normal values and were considerably greater than at 3dpi (Fig. 4.5A). The same trend occurred in WM lesion borders, where OL numbers were greater than 7dpi values in some rostral sections (Fig. 4.5B,C). OL numbers in spared GM and GM lesion borders were similar to 7dpi levels (Fig. 4.5C, Fig 4.6).

**Total cell counts:** Spinal contusion markedly alters tissue morphology due to edema and tissue contraction over time. These phenomena could passively affect cell numbers within tissue compartments by changing the available space in which cells reside. To examine how overall cell numbers in WM and GM changed after SCI, the total number of nuclei was quantified at 14dpi in the regions examined above. It was noted that, not surprisingly, normal uninjured GM contains more cell nuclei than WM (Fig. 4.4D). After SCI, the number of cells in outlying spared WM and WM along the lesion borders increased to a similar level (Fig. 4.4D). Interestingly, cell numbers in spared GM
were not different after SCI, but were significantly greater in GM along lesion borders (Fig. 4.4D). Thus, overall changes in cell density along the lesion border are not altered equally between WM and GM, which would indicate that tissue contraction and/or expansion was unlikely to have mediated the changes in OLs noted along the lesion borders, especially since a significant rise in OLs was noted in spared GM, which did not display an overall difference in cell nuclei at 14dpi.

**Figure 4.6: Increased CC1+ OLs at the lesion/spared tissue interface.** Sections from 14dpi at 1.0mm caudal are labeled for CC1 (brown) and GFAP (black) and counterstained with methyl green. Only single-labeled CC1 cells were counted in Fig 4.5. (A) Border between lesion (*) and grey matter; note the presence of many OLs along the lesion border. (B). Outlying spared white matter near pial border in the same section contained fewer OLs. Scale = 20µm A,B.

**A low number of new astrocytes are formed after SCI**

Because astrocytes also accumulate along lesion borders over the first two weeks post-injury, and may be derived from proliferating NG2 cells (Alonso, 2005), sections from the 14dpi group were double-labeled for GFAP and BrdU to examine whether new astrocytes were generated after SCI. A limited number of BrdU+ astrocytes indeed were
present (Fig. 4.7), the majority of which were located along the lesion border where on average 20.4 cells/mm² were GFAP+/BrdU+ (table 2). The adjacent gray matter contained 12.6 cells/mm² new astrocytes, while nearby spared white matter contained very few double-labeled cells (2.4 cells/mm²). In every location sampled at 14dpi, the number of BrdU+ astrocytes was markedly less than BrdU+ OLs (Table 2).

![Figure 4.7: A limited number of new astrocytes were formed after SCI, especially along lesion borders. Merged (A) and single channel confocal images (B,C) from 14dpi lesion border, 1.5 mm rostral to epicenter, showing two astrocytes (green; arrows) with BrdU+ nuclei (red). Lesion cavity is denoted by asterisk. Scale bar = 20µm.](image)

<table>
<thead>
<tr>
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<th>CC1/BrdU Cells/mm²</th>
<th>GFAP/BrdU Cells/mm²</th>
</tr>
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<tbody>
<tr>
<td>SWM</td>
<td>11.5 ± 1.9</td>
<td>2.38 ± 1.63</td>
</tr>
<tr>
<td>SGM</td>
<td>40.9 ± 6.6</td>
<td>12.58 ± 4.39</td>
</tr>
<tr>
<td>Lesion border</td>
<td>49.3 ± 7.3</td>
<td>20.39 ± 5.83</td>
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**Table 2: Comparison between new oligodendrocyte and new astrocyte formation.** In all regions of the injured spinal cord CC1/BrdU (new OL) numbers are higher than GFAP/BrdU (new astrocyte) numbers at 14dpi. Confocal images were used to count the double-labeled cells. Sections 2.5mm rostral-2.5mm caudal were used for comparison. n=3-5/group.
A portion of newly generated OLs remyelinate axons post SCI

To determine if the OLs generated following SCI remyelinated axons, we examined plastic embedded sections stained with Toluidine blue. At 28dpi, we observed axons which had maintained their original pre-injury OL myelin, recognized by their thick myelin layers (Fig 4.8 B). We also observed axons that had been remyelinated by Schwann cells (Fig 4.8 B); this was evident by the presence of the characteristic thick myelin sheath and Schwann cell body next to the myelinated axon. More importantly, around the injury core we noted clusters of thinly myelinated axons indicating OL remyelination (Fig 4.8 B). Since mature OLs in the CNS are incapable of remyelinating bare axons, the OL remyelination seen here is evidence that the newly generated OLs remyelinated spinal axons. Thus, at least a portion of the new OLs around lesion borders contribute to anatomical repair by remyelinating denuded axons.
Figure 4.8: Oligodendrocyte remyelination along lesion borders 28dpi. A resin-embedded (1µm) section stained with Toluidine blue 2mm caudal to the epicenter shows the lesion core (*), blue dotted lines demarcate the gray matter. The red arrow head shows the same region in A and B. B Axons displaying preserved OL myelin were noted (yellow arrows) following SCI. Clusters of OL remyelinated axons were seen at the lesion border (denoted by yellow dotted lines). Schwann cell mediated remyelination was also seen as indicated by green arrows.

Discussion

Traumatic SCI results in central cystic cavitation surrounded by a rim of surviving tissue. Within this rim of tissue, however, considerable cell death and axon degeneration occur. Therefore, the amount of tissue preserved or replaced in this region will considerably influence functional outcomes after SCI. Indeed, studies have shown that preserving as little as 10% of tissue at the impact site can have a significant impact on locomotor recovery (Blight, 1983).

Numerous interventions have been tested in attempt to improve recovery from SCI; these studies are clearly needed as complete regeneration of the adult spinal cord does not occur. However, the extent of endogenous repair that does occur is still
somewhat of a mystery. Most studies that focus on repair phenomena have focused on axon regeneration. An issue receiving less attention is the extent to which spinal cord cell populations are spontaneously replenished. For instance, most studies exploring OL fate after SCI have focused on the timing and distribution of OL loss (Crowe et al., 1997; Grossman et al., 2001; McTigue et al., 2001). In this report, we characterized for the first time the substantial formation of new oligodendrocytes during the first 14dpi in tissue directly adjacent to spinal lesions. This work complements and extends work by Zai and Wrathall (2005) who documented new OL formation in spared ventral white matter. We examined the lesion border area since it contains upregulated NG2 expression after SCI (McTigue et al., 2006), and proliferating NG2 cells preferentially accumulated in this region (Chapter 3). This perilesion zone is typically thought of as the glial scar, containing upregulated chondroitin sulfate proteoglycans (CSPGs) and reactive astrocytes that collectively prevent axon growth (for reviews, see Fawcett and Asher, 1999; Silver and Miller, 2004). Here we have determined that this dynamic area also exhibits a substantial and protracted gliogenic potential. Indeed, by 7dpi, the lesion border contained OL numbers exceeding twice those in non-injured spinal cords. This extensive OL genesis occurred in regions of elevated NG2 cell division, which is pertinent since proliferating NG2 cells are generally accepted as the source of new OLs in the adult CNS.

By 14dpi, at least 17% of OLs sampled along the lesion border were generated from cells dividing during week one post-injury, and another 5% were from cells dividing during the second week. The actual number of new OLs is likely even higher than the combined percentage of 22% due to the high metabolism of BrdU (Kriss and Revesz, 1962) and the conservative injection regimen currently used, in which peak BrdU
bioavailability would be ~2 h after injection. Progenitor cells dividing outside that time would not be labeled with BrdU, and therefore neither would their progeny. Thus, it is likely that a larger percentage of OLs in the lesion/spared tissue interface were newly generated after SCI, especially given the large increase in overall OL numbers.

A potential alternate explanation for increased cell numbers is that tissue compression and/or shrinkage after SCI led to elevated OL numbers. For instance, we have shown previously that epicenter cross-section area decreases continually for at least 5 months post-injury in rats (McTigue et al., 2001). Hence similar numbers of cells in a smaller tissue area would appear as an increase. If tissue loss was the sole cause for rising OL numbers, the implication would be that surviving and new OLs were preserved despite ongoing loss of other cells. Given their usual vulnerability to CNS injuries, this would be an interesting phenomenon. However, our total cell counts do not support shrinkage as the main explanation for elevated OLs. We noted that outlying spared WM and the WM lesion border exhibited similar increases in total cell number but not in new OL number. Furthermore, spared GM displayed a dramatic rise in BrdU+ OLs at 14dpi despite having no change in overall cell number. Thus, changes in total cell numbers are not consistently reflected by changes in specific glial populations, nor are they distributed evenly as might be expected if passive tissue shrinkage was the cause.

The presence of numerous proliferating NG2 cells and OLs within the glial scar region has multiple potential consequences. For instance, the OLs may be critical for subsequent remyelination of spared or growing axons in this region. In our study we noted intact axons lacking myelin in the border zone throughout the first two weeks post-injury. A similar distribution of bare axons was detected in human SCI tissue, where
demyelinated axons were observed in tissue next to lesion cavities in a subset of samples (Guest, Hiester, and Bunge, 2005). We did observe OL remyelinated axons along the lesion borders a month following SCI. Due to the technical inability of performing immunocytochemistry on resin embedded tissue we were unable to determine directly if the OL remyelination we saw was definitively from a newly generated OL. Other studies in the past have also shown OL remyelination 3-5 weeks following SCI close to the perilesion tissue (Gledhill et al., 1973; Griffiths and McCulloch, 1983). Remyelination by new OLs in this perilesion zone was addressed recently by Yang et al. (Yang et al., 2006). Using a primate spinal hemisection model, they found evidence that new OLs arising during the first 5dpi remyelinated nearby axons by 2 months post-injury, suggesting that these cells have the functional capacity of mature OLs. The large number of OLs in GM and along the lesion border also may support neuron functions. For instance, OLs produce growth factors that promote neuronal survival (Sortwell et al., 2000), and OL-axon contact influences axon caliber and ion channel distribution (Waxman and Ritchie, 1985; Sanchez et al., 1996; Kaplan et al., 1997; Edgar et al., 2004). Thus, the large population of OLs formed in the gliogenic lesion border may serve multiple reparative functions.

In contrast to the perilesion zone, OL numbers did not change appreciably between 7 – 14dpi in spared white and gray matter closer to the meningeal border, despite concurrent oligogenesis therein. This may represent a balance between OL loss and replacement, since OL apoptosis has been detected distal to epicenter during the first two weeks after SCI (Li et al., 1996; Crowe et al., 1997; Liu et al., 1997; Warden et al., 2001). Similarly, in a perforant path axonal lesion paradigm, oligogenesis was detected
without a concomitant rise in OL numbers which again was hypothesized to be due to a balance between OL death and replacement (Nielsen et al., 2006).

In moderate to severe SCI, the lesion epicenter is surrounded by white matter. Distally the lesion extensions typically localize to the dorsal funiculus and are often bordered by gray matter, which appears to have a substantial oligogenic capacity. In comparing WM and GM in naive and SCI tissue, we consistently detected approximately twice as many NG2/BrdU cells and OLs in gray matter. The number of OLs in GM bordering lesions was often striking, reaching levels >1000 OLs/mm². The oligogenic nature of GM may be due to the higher proportion of progenitor cells, different growth factor expression and/or differential inflammatory infiltration. For instance, greater T cell influx and microglial activation occur in GM compared to WM in spinal cords of rats with experimental autoimmune encephalomyelitis (Popovich et al., 1997). Furthermore, neurons can synthesize Sonic hedgehog and platelet-derived growth factor, two important molecules critical for oligogenesis during development (Ellison et al., 1996; Traiffort et al., 1999). Others have also noted differences in NG2 cell and OL formation or function in gray matter versus white matter (Baracskay et al., 2002). Our results are in contrast, however, to those of Dawson et al. (2003), who noted more abundant NG2 cells and OLs in white matter compared to gray matter. This may be due to differences in antibody use (CC1 vs. CNPase) or sampling techniques.

In our data, the largest increase in OLs was detected in the same regions containing the highest NG2/BrdU cell numbers. Both cell types displayed a biphasic spatial pattern which was lower at the lesion epicenter and higher distally; these distal sections contained more spared tissue, especially GM, and therefore a larger pool of
surviving OPCs from which to draw. Collectively, these data suggest that a sizable portion of NG2 cells along the lesion border functioned as OL progenitors. Additionally, we noted rare occurrences of CC1 cells that co-expressed NG2. Their presence suggests that NG2 cells can transition into CC1+ cells, a phenomenon reported previously (Zai and Wrathall, 2005).

Although the CC1 antibody labels a low number of astrocytes and Schwann cells when used with DAB immunohistochemistry (McTigue et al., 2001), our dilution protocol for immunofluorescence eliminated all but the OL labeling. However, because of decreased sensitivity, the fluorescent protocol may not detect all OLs. Therefore, the number of CC1/BrdU fluorescent cells should not be directly compared with the number of DAB labeled CC1 cells. The fact that astrocyte/Schwann cell labeling was eliminated with immunofluorescence allows confidence, though, that cells counted as CC1/BrdU+ cells were indeed oligodendrocytes. This is further supported by co-expression of Olig2 by most CC1 and CC1/BrdU cells. It is important to note that not all CC1 cells in the adult spinal cord are Olig2 positive since expression of Olig2 following differentiation is down-regulated (Zhou et al., 2000; Wegner, 2001); this would suggest that Olig2-negative CC1 cells in our study were further differentiated than the “newer” Olig2+ cells. Collectively, the observations that CC1 cells often expressed Olig2 but rarely NG2 strongly suggests that these cells were newly generated oligodendrocytes. To further verify that CC1 cells are mature OLs, it would be useful to co-label with myelin protein markers. In the current study, multiple combination protocols of CC1 plus MBP, PLP, MOG, MAG or CNPase were attempted with unsatisfactory results.
Based on the distribution of new OLs, our results suggest a gradient effect in which tissue contacting the lesion had the highest oligogenic capacity, which receded outward toward the pial border. Other studies have also shown that OL numbers rise outside a variety of CNS lesions (Frei et al., 2000; Gard and Pfeiffer, 1990; Mandai et al., 1997). Thus, NG2 cell accumulation and oligodendrogenesis may be a general response to factors released by dying cells, exposed axons, reactive astrocytes and/or infiltrating inflammatory cells. For instance, reactive astrocytes are a source of growth factors during development and after SCI (Raff et al., 1988; Noble et al., 1988; Follesa et al., 1994). Activated microglia and macrophages, which are abundant in the SCI lesion cavity and border, also secrete numerous molecules which can affect OL formation (Raivich and Banati, 2004), such as transforming growth factor-β (TGFβ) which can promote OL differentiation (McKinnon et al., 1993). Of note, our group has previously detected a marked elevation in TGFβ and TGFβ receptor expression after SCI, the distribution and timing of which correlates with oligogenesis observed currently (McTigue et al., 2000). Future studies are needed to clarify the multiple interactions between NG2 cells and lesion constituents that orchestrate the timing and location of OL formation after SCI.

In summary, spinal trauma induces OL death, but also elicits significant OL regeneration, especially in the gliogenic zone bordering the lesion cavity. Ongoing studies in the laboratory are working toward revealing the underlying mechanisms controlling this gliogenesis. The remarkable capacity of the adult CNS to endogenously replace OLs provides hope that by understanding the cellular interactions involved in this process, better strategies can be devised to treat SCI and other disorders in which oligodendrocyte loss and demyelination are major contributors to dysfunction.
CHAPTER 5

Increased growth factor expression in regions of elevated oligogenesis

Introduction

Traumatic injury to the spinal cord results in a rapid and significant loss of oligodendrocytes (OLs) at and near the lesion epicenter. Fortunately, replacement of dying OLs is possible due to an endogenous pool of NG2+ cells typically referred to as oligodendrocyte progenitor cells (OPCs) or polydendrocytes (Nishiyama et al., 1996a; Baracskay et al., 2007), which are present in the naïve and injured adult spinal cord (McTigue et al., 2001; Yoo and Wrathall, 2007). OPCs are stimulated to proliferate for several weeks following spinal cord injury (SCI), which is accompanied by the formation of new oligodendrocytes (Zai and Wrathall, 2005; Tripathi and McTigue, 2007); at least a portion of the new glial cells persist chronically after SCI (Zai and Wrathall, 2005; Yang et al., 2006).

In recent work, we showed that accumulation of proliferating NG2+ cells and generation of new OLs is especially robust along the lesion penumbra during the first two weeks after SCI (Tripathi and McTigue, 2007). In addition to being an area of active oligogenesis, the lesion border is well known to display prominent astrocyte hypertrophy.
and accumulation over time after SCI. Astrocytes can produce multiple growth factors which potently regulate OPC proliferation and differentiation into OLs as well as promote OPC and OL survival (McMorris and McKinnon, 1996; Park et al., 2001). Thus, astrocyte-derived factors, if present at the right time and place, may play an active role in post-SCI gliogenesis. One such potential astrocytic factor is ciliary neurotrophic factor (CNTF), which is a pleiotropic cytokine belonging to the interleukin-6 (IL-6) family of molecules. In addition to its potent neuroprotective effects, CNTF enhances the rate of OPC generation and maturation, protects OLs against tumor necrosis factor-α (TNFα) induced toxicity and promotes myelinogenesis and myelin protein formation (Barres et al., 1993; Mayer et al., 1994; Barres et al., 1996; Marmur et al., 1998; Talbott et al., 2007). Notably, the absence of CNTF reduces OPC proliferation and exacerbates OL pathology in experimental autoimmune encephalomyelitis (EAE) (Linker et al., 2002). Since CNTF null mice develop normally and display only modest abnormalities later in life (Masu et al., 1993) but have worse outcomes following CNS insults, CNTF has been postulated to play its most important role in an injured environment. Following neurotrauma, CNTF mRNA and protein have been shown to increase transiently close to the injury site (Oyesiku et al., 1997). Hence, CNTF is an attractive candidate for potentially influencing post-traumatic oligodendrocyte survival and formation.

In addition to its direct actions, CNTF can potentiate the effects of other growth factors such as insulin-like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2) and platelet-derived growth factor (PDGF), all of which play key roles at various stages of oligogenesis (Engel and Wolswijk, 1996; McMorris and McKinnon, 1996). CNTF
enhances their effects by acting synergistically on downstream targets (as with PDGF) (Dell'Albani et al., 1998), by upregulating their production (FGF-2, IGF-1) (Albrecht et al., 2002) and by increasing expression of their receptors, i.e., FGF-1R and IGF-IR (Jiang et al., 1999). Thus, the potential mechanisms for CNTF-induced effects on oligodendrocyte lineage cells are many-fold.

Previous studies have examined CNTF and CNTF receptor α (CNTFRα) mRNA expression and immunohistochemical localization in intact brain and spinal cord and following spinal hemisection (Ip et al., 1993; Oyesiku et al., 1997; Lee et al., 1998). However, comprehensive information on CNTF and CNTFRα protein levels following contusion injury is lacking. Therefore we systematically characterized CNTF protein expression in a rat spinal contusion model at 3d - 28d post-injury (dpi) in spared white and gray matter and the lesion border (where gliogenesis appears highest). We also examined overall post-injury expression of CNTFRα protein, which has activity both as a bound and soluble receptor (Davis et al., 1993). As stated above, CNTF can induce FGF-2 production; thus, we evaluated the distribution and number of FGF-2+ cells after SCI. Lastly, to get a potential indicator of CNTF/CNTFRα signaling activity, we examined the expression of phosphorylated STAT3 (signal transducer and activator of transcription 3, pSTAT3) in naïve and injured tissue, since phosphorylation of this intracellular signaling mediator is initiated by CNTF binding to its receptor (Dell'Albani et al., 1998).

Collectively, the results reveal that elevated CNTF was detected throughout the spared tissue early after SCI but was restricted to the lesion border at more chronic times. pSTAT3 levels were also increased, especially in tissue adjacent to the lesion, and were maintained at an elevated level for at least 28 dpi. Lastly, FGF-2 expression closely
mirrored that of CNTF; it was elevated early after SCI and chronically along the lesion border. Thus, these two factors are viable candidates for playing a role in oligodendrocyte survival and generation after SCI.

**Materials and Methods**

To assess temporo-spatial CNTF expression, CNTF labeling was achieved using an immunoperoxidase method. Sections were pre-incubated with BP+ and then incubated overnight in 1:800 goat anti-CNTF antibody (0.5mg/ml stock, R&D systems, Minneapolis, MN). The next day, secondary antibody (1:2000, horse anti-goat) was applied for 1 h at room temp; sections were then treated with 6% hydrogen peroxide in methanol followed by Elite ABC and DAB substrate. IHC was performed on all sections at the same time to eliminate variability. To verify the specificity of the CNTF antibody, 10µg of CNTF antibody was incubated with 100ng of recombinant rat CNTF (R&D systems) for 1hr at 37°C (as per manufacturer’s recommendation) and the above procedure was repeated. No positive signal for CNTF was detected under these conditions.

In order to identify the cellular sources of CNTF, immunofluorescent double-labeling of CNTF with cell specific markers was used. The CNTF antibody was applied at 1:100 overnight followed by 1:1000 anti-goat Alexa Fluor 546 (Molecular Probes, Eugene, OR) for 30min; rabbit anti-GFAP antibody (1:4000, Dako Cytomation, Carpinteria, CA), mouse anti-CC1/APC antibody (1:400, Abcam, Cambridge, MA) or rabbit anti-p75 (1:250, Promega, Madison, WI) followed by 1:1000 Alexa Fluor 488 (Molecular Probes). Cell nuclei were labeled with DRAQ5 (1:3000, Biostatus Limited,
UK), which is visualized by excitation at 633 nm in the high red range. Sections were viewed with a Zeiss 510 Meta Laser Scanning Confocal microscope. To confirm the oligodendrocytic identity of CC1/CNTF+ cells, we performed double labeling for Olig2 and CNTF. Olig2 is a transcription factor expressed almost exclusively in nuclei of cells belonging to oligodendrocyte lineage (Kitada and Rowitch, 2006). Olig2 antibody (R&D systems) was applied at 1:500 overnight followed by 1:1000 anti-goat Alexa Fluor 546 followed by the CNTF antibody (1:100) and Alexa Fluor 488 for 30mins.

To examine OL numbers along lesion borders at 28dpi, oligodendrocyte cell bodies were visualized using the CC1 antibody (see general methodology chapter 2).

To determine if phosphorylated STAT3 (pSTAT3) was elevated following SCI, we performed single-labeling or double-labeling of pSTAT3 with cell specific markers. Spinal cord sections were subjected to antigen retrieval using Antigen unmasking solution (1:100, Vector) for 5min at 96°C. The pSTAT3 antibody against STAT3 phosphorylated at Tyr705 (1:100, Cell signaling technologies, Danver, MA) was applied followed by 1:500 anti-rabbit Alexa Fluor 546. The astrocyte and OL marker antibodies were used as above and DRAQ5 was used as nuclear label. For pSTAT3 and NG2 co-localization immunoperoxidase method was used (antigen retrieval was not necessary here). pSTAT3 antibody was applied at 1:200 overnight at 4°C, followed by secondary antibody. Elite ABC and DAB substrate was used to obtain the pSTAT3 IHC. Sections were then incubated with mouse anti-NG2 antibody (1:100, US Biologicals,
Swampscott, MA) followed by secondary antibody and then elite ABC and SG substrate (Vector). The sections were counterstained with methyl green.

For FGF-2 IHC, FGF-2 antibody was applied (1:1000, Upstate, Charlottesville, VA) overnight, followed by 1:800 horse anti-mouse antibody for 1h at room temperature. Sections were treated with 6% hydrogen peroxide in methanol followed by Elite ABC and DAB substrate to visualize FGF-2 labeling.

All IHC protocols included controls in which the primary or secondary antibodies were omitted. These controls consistently revealed an absence of labeling.

**CNTF IHC quantification:** To compare CNTF expression in residual white matter and gray matter following injury, MCID software was used to digitize cross-sections of injured spinal cords. Images were collected at 1mm intervals throughout a 4mm segment of tissue centered on the lesion epicenter. The sections were manually delineated into white matter or gray matter and segmentation range was set to select only profiles positively labeled for CNTF within each region. Total scan area and target area for each region was measured and used to calculate proportional area stained for CNTF.

To specifically compare CNTF expression in white matter border vs. outlying spared white matter and gray matter border vs. outlying spared gray matter, sample boxes (0.12mm x 0.12mm) were placed within each sub-region as performed previously in chapter 3 and 4 (see general methodology). A minimum of three boxes were placed along the edge of the lesion in distal sections (2mm from epicenter) and typically 6–10 boxes were used in sections containing larger lesion sites; this method allows maximum
sampling of the lesion border. Data from lesion borders located within white matter and gray matter were collected separately.

Measurements from 5-6 animals throughout the 4 mm of tissue per region per distance were averaged to obtain an overall mean ± SEM (standard error of the mean). For comparisons with normal spinal cords, data was collected from sample boxes placed in equivalent regions of white matter and gray matter in cross-sections from uninjured spinal cords.

FGF-2 counts: FGF-2 labeled sections were digitized at 20X magnification as stated above and the segmentation range was set to identify positively labeled FGF-2+ nuclei. An average size for FGF-2+ profiles was determined by sampling multiple cells in different sections. The mean diameter of FGF-2+ profiles was entered in the MCID Elite7.0 software; FGF-2 labeled profiles <30% of the mean size were excluded from counts. Sample boxes were placed in the white matter and gray matter regions as described above and the image analysis software was set to automatically count FGF-2+ cells in the boxes, based on the positive segmentation and pre-set mean size and exclusion criteria. Measurements from 5-6 animals throughout the 4 mm of tissue per region per distance were averaged as an overall mean ±SEM.
Results

**CNTF protein expression increases following SCI**

Using western blots, a continual rise in CNTF protein was observed between 5d and 28dpi in 4mm of tissue centered on the contusion epicenter (Fig. 5.1A). A single band at 22kDa was detected for CNTF. Densitometric analysis of the CNTF bands normalized to α-tubulin loading controls revealed that CNTF protein had increased 1.5 fold by 5dpi and had tripled by 7dpi, although the data were not significant (Fig. 5.1B). By 14dpi, CNTF protein had significantly increased compared to naïve tissue and then continued to rise such that at 28dpi, it was significantly higher than that in naïve and 5dpi spinal cord tissue (Fig. 5.1B).

![Figure 5.1: Increased CNTF expression 5-28 days post-injury (dpi).](image)

(A) Western blot analysis of 4mm of SCI tissue centered on lesion epicenter revealed a single CNTF band (22kDa) which increased over time after injury; α-tubulin was used as loading control. 10µg of total protein was loaded/lane. (B) Ratio of the relative optical density (ROD) of CNTF bands normalized to α-tubulin showed significantly higher CNTF expression at 14d and 28dpi. One way-ANOVA followed by Tukey post-hoc tests was used for statistical analysis and p<0.05 was considered significant; n=3/group.
Immunohistochemistry (IHC) was used to compare the spatio-temporal distribution of CNTF after SCI in residual gray matter and white matter to that in comparable regions of uninjured spinal cord sections. As expected, a low level of CNTF immunoreactivity was present in uninjured controls (Fig. 5.2A,B); in these sections, less than 1% of white matter or gray matter was positively labeled for CNTF (Fig. 5.3A,B).

Figure 5.2: Increased CNTF immunoreactivity was noted in white matter (WM) and gray matter (GM) regions of the injured spinal cord. (A-B) As expected, a low level of CNTF expression was observed in normal GM (A) and WM (B). Dotted line in A delineates dorsal horn (DH) gray matter and adjacent white matter. Note the high CNTF expression in the dorsal root in naïve tissue. (C) 14dpi white matter immunolabeled with CNTF antibody that had been pre-incubated with recombinant rat CNTF; no positive signal was detected. Markedly increased CNTF expression was observed after SCI in GM (D; 7dpi) and WM (E,F; 28dpi). CNTF expression was especially high in white matter bordering the lesion. Images were taken at the following locations: (D) 1.2mm rostral, (C) 1.5mm rostral, (E) 1mm rostral, (F) 2.5mm caudal to epicenter. Lesion is indicated by *; arrows indicate CNTF labeling. Scale=20µm for A-F.
**Gray matter:** As gray matter is destroyed at the lesion epicenter, all gray matter analyses were performed on sections 1 - 2mm rostral and caudal to epicenter. At 3dpi, CNTF immunoreactivity was not different from that in naïve gray matter (Fig. 5.3A). By 7dpi, CNTF was significantly higher in sections 1mm rostral to epicenter compared to naïve and 3dpi levels (Fig. 5.2A,D; Fig. 5.3A). CNTF immunoreactivity continued to rise such that by 14dpi, it was markedly higher both rostral and caudal to epicenter compared to naïve and previous time points. Between 14d and 28dpi, CNTF immunoreactivity was either maintained or further increased in residual gray matter, with the highest levels occurring 1mm rostral to epicenter (Fig. 5.3A). By 70dpi, CNTF levels in gray matter approximated naïve levels (data not shown). Hence CNTF immunoreactivity increased in gray matter between 3d and 28dpi and returned to baseline by 10 weeks post injury.

**White matter:** Similar to gray matter, significantly elevated CNTF immunoreactivity was not detected until 7dpi, at which time it was elevated compared to naïve in all distances examined (Fig. 5.3B). By 14dpi, CNTF had increased further at the epicenter, and by 28dpi, CNTF immunoreactivity was even higher than 14dpi levels in sections rostral and caudal to epicenter (Fig. 5.3B). Hence, in spared white matter after spinal contusion, CNTF levels increased continuously through 28dpi. Between 28d and 70dpi, CNTF expression distal to the injury site decreased and was indistinguishable from that at 3dpi; epicenter CNTF levels remained comparable to that at 7dpi (data not shown).
Figure 5.3: CNTF expression is significantly elevated after SCI, especially at lesion borders. (A) Quantification of CNTF immunoreactivity after SCI revealed a significant increase in gray matter by 7d post-injury (dpi) in sections 1-2mm rostral to epicenter (0 on x-axis). Between 7d - 28dpi, CNTF expression continued to increase at most distances compared to naïve gray matter and previous time points. (B) CNTF increased modestly in white matter at 3dpi. By 7dpi, CNTF was significantly higher in white matter at all distances examined compared to naïve. By 28dpi, CNTF expression in white matter was greater than naïve, 7d and 14dpi at most distances examined. (C) Diagram of an injured spinal cord section depicting sample box placement used to generate data in D. Sample box area = 0.0144 mm². White boxes represent spared white matter samples, gray boxes show gray matter samples and hatched boxes show samples along lesion borders. Boxes are not to scale. (D) At 7d and 28dpi, CNTF expression was significantly higher in spared white matter, white matter border and gray matter border compared to naïve; at 28dpi, CNTF along white matter and gray matter lesion borders was significantly elevated compared to the respective outlying spared tissue. Data spanning 4mm of spinal tissue are collapsed across distance. For A & B, analysis included two-way repeated measures ANOVA followed by Bonferroni post-hoc analyses; for D, one-way ANOVA followed by Tukey post-hoc tests was used. For all analyses, p<0.05 was considered significant (n=4-6/group). For A and B, sections were manually delineated into white matter or gray matter and segmentation range was set to select only profiles positively labeled for CNTF. The proportional area labeled for CNTF was calculated using target area/total scan area. For D, the proportional area labeled for CNTF was similarly calculated for each sample box.
Lesion border versus meningeal border: In performing these analyses, it became apparent that CNTF immunoreactivity was especially prominent along lesion cavities, which is the region previously shown to contain more proliferating NG2+ progenitors and new OLs compared to outlying spared tissue along the meningeal border (Tripathi and McTigue, 2007). To determine if quantifiable differences in CNTF between lesion border and outer regions could be detected, we used a sample box technique to distinguish CNTF expression in white matter and gray matter bordering the lesion from that in white matter/gray matter near the meninges at 7d and 28dpi (Fig. 5.3C). At 7dpi, CNTF was upregulated to a comparable level in white matter bordering the lesion and the meninges (Fig. 5.3D). In gray matter, however, CNTF had only increased significantly in the lesion border tissue (Fig. 5.3D).

By 28dpi, CNTF expression in outer white matter had declined slightly compared to 7dpi levels. In contrast, CNTF had further increased in white matter bordering lesion cavities; thus, lesion border white matter now contained more CNTF than outlying white matter (Fig. 5.2E-F; Fig. 5.3D). In gray matter, the difference between peri-lesion and outlying CNTF expression was amplified at 28dpi, as CNTF levels had risen further along lesion borders (Fig. 5.3D). Thus, during the first month post-injury, CNTF expression was elevated throughout white matter but was restricted to lesion borders in gray matter.
Figure 5.4: Increased oligodendrocyte numbers were present along the lesion border at 28dpi. Our previous work revealed that oligodendrocyte numbers are elevated along lesion borders through at least 14d post-injury. Here, sections from 28d post-injury double-labeled for oligodendrocytes (CC1; brown) and astrocytes (GFAP, black) reveal that elevated oligodendrocyte numbers are maintained along lesion borders (B) compared to outlying white matter (WM; A) through at least 28dpi. Arrows indicate CC1+ oligodendrocytes; arrow heads denote GFAP+ astrocytes. Asterisk denotes lesion. Both the images were taken from the same section 1 mm caudal to epicenter. Scale bar = 20µm.

In the previous study (chapter 4), we showed that increased oligodendrogenesis and elevated oligodendrocyte numbers were maintained in tissue adjacent to spinal lesions throughout the first 14d after SCI (Tripathi and McTigue, 2007). Since our current data reveal a sustained elevation of CNTF through 28dpi, we asked whether the elevation in OLs numbers in the lesion penumbra existed through 28dpi as well. Using immunohistochemistry, a clear elevation in OL numbers along lesion borders was still detectable at 28dpi compared to spared tissue adjacent to meningeal borders (Fig 5.4)

Astrocytes and OLs display CNTF immunoreactivity

To identify potential cellular sources for CNTF after SCI, tissue sections were double-labeled with glial markers and examined using confocal microscopy. As expected,
CNTF co-localization with GFAP+ astrocytes was detected in naive and injured spinal cord cross-sections (Fig. 5.5A-C). CNTF+ astrocytes were especially prominent along the lesion border. CNTF immunoreactivity was also observed on a subset of CC1+ oligodendrocytes (Fig. 5.5D-F), as has been noted previously (Dobrea et al., 1992). We confirmed CNTF immunoreactivity on oligodendrocyte lineage cells by co-labeling with Olig2 (Fig. 5.5G-I). CNTF immunoreactivity was not detected on NG2+ cells (glial progenitor cells which are also Olig2+) further indicating that the round Olig2+/CNTF+ profiles were oligodendrocytes.

Because Schwann cells can express CNTF and enter the spinal cord following injury, we double-labeled sections with CNTF and P0 (expressed by myelinating Schwann cells) or p75, which is expressed by non-myelinating Schwann cells. No double-labeling was detected with CNTF and P0+ profiles (data not shown). In contrast, a small subset of p75+ cells did express CNTF immunoreactivity (5.5J-L). Most p75+ cells displayed a bipolar morphology, suggestive of migrating Schwann cells; however p75 can also be expressed by oligodendrocytes and other cells indicating that the p75/CNTF cells may not be exclusively Schwann cells. CNTF immunoreactivity was not present on OX42+ microglia and macrophages (data not shown).
Figure 5.5: Astrocytes, oligodendrocytes and Schwann cells display CNTF immunoreactivity after SCI. (A-C) Single channel (A,B) and merged (C) confocal images (≤1µm) reveal many GFAP+ astrocytes (green) were co-labeled with CNTF (red). (D-F) CNTF immunoreactivity (red) was also observed on CC1+ oligodendrocytes (green); merged image shown in F. (G-I) The oligodendrocyte lineage identity was confirmed by co-localization of Olig2 (H) with CNTF (G). The single-labeled Olig2 cells in H are most likely OPCs; note astrocyte-like CNTF+ cells in I do not show Olig2 labeling. (J-M) A subset of p75+ Schwann cells expressed CNTF. Images G-I were taken 14dpi 2.5mm rostral to epicenter, all other images were taken from 28 dpi sections: (A-C) 1.2mm caudal, (D-F) 0.75mm caudal, (J-K) 0.45mm caudal & (L-M) 1.5mm caudal to epicenter. All sections were counterstained with DRAQ5 and the high red signal converted to blue. Arrows indicate double-labeled cells; arrowheads indicate single-labeled cells. Asterisk denotes lesion in D-F. Scale bar=20µm.
Figure 5.5
CNTF receptor α expression following SCI

Since the direct effects of CNTF are mediated by binding the CNTFRα, overall changes in protein levels for this receptor were examined in SCI tissue using western blot. This method allowed optimal detection of the membrane bound and soluble form of the receptor. A prominent band at 60kDa was detected for CNTFRα at all days post-injury examined (3d – 28dpi); a second weaker 55kDa band was also detected at 3dpi and, to a lesser extent, at 5dpi (Fig. 5.6A). The 55kDa protein may be a breakdown product of the receptor or a differentially glycosylated form of CNTFRα (Zvonic et al., 2003). Quantitation of the 60kDa band using densitometry revealed that at 3dpi, CNTFRα expression was substantially decreased compared to naïve spinal cords (Fig. 5.6B). Although a modest increase occurred between 3d – 14dpi, CNTFRα expression remained significantly lower than naïve levels until 28 dpi.
Figure 5.6: CNTF receptor α (CNTFRα) expression initially declines then rises after SCI. (A) CNTFRα was detected as a prominent 60kDa band in spinal cord samples using western blot; an additional lighter 55kDa band was also noted at 3d and 5dpi. α-tubulin was used as loading control. 10µg of total protein was loaded/lane. (B) Densitometric analysis of the 60kDa band showed a dramatic and significant decrease in CNTFRα expression as early as 3dpi which rebounded to naive levels by 28dpi. One-way ANOVA followed by Tukey post-hoc test was performed and p<0.05 was considered significant (n=3/group).
FGF-2 expression following SCI

A known action of CNTF within the CNS is upregulation of FGF-2 expression, another astrocyte-derived growth factor (Albrecht, Dahl, Stoltzfus, Levenson, and Levison, 2002). Hence we examined FGF-2 immunoreactivity following SCI as above for CNTF. Using western blots on 4mm of spinal tissue, we observed an FGF-2 band at 22kDa in uninjured spinal cords. At 3dpi, there was an additional band ~24kDa which was absent on all subsequent days post-injury (Fig. 5.7). Between 5d – 28dpi, FGF-2 protein increased as seen by the increasing intensity of the 22kDa and the 20kDa bands (Fig. 5.7).

Figure 5.7: FGF-2 protein levels increase 3d-28d post-injury. In naïve spinal cords, FGF-2 was detected as a faint band at 22kDa. At 3dpi, this band and a 24kDa band was detected. Between 5d-28dpi, the 22kDa FGF-2 band intensified indicating increased FGF-2; a 20kDa band also appeared which intensified over time. α-tubulin signal reveals comparable loading in all lanes. A total of 12.5µg protein was loaded /lane.

Using immunohistochemistry, FGF-2+ cells were quantified in 7d and 28 dpi tissue and compared to naïve values. In contrast to CNTF which is expressed by cell bodies and processes, FGF-2+ immunoreactivity appears as punctate labeling of cell nuclei thereby facilitating automated counts. In naïve spinal cords, gray matter had thrice the number of FGF-2+ cells compared to white matter (420.1 ± 84.9 vs. 145.3 ± 23.7) (Fig. 5.8A,D; Fig. 5.9). At 7dpi, FGF-2+ cell numbers were significantly increased in
outer spared gray matter and gray matter lesion borders compared to naïve gray matter (Fig. 5.8, 5.9). In white matter, however, FGF2+ cell numbers were only increased in tissue adjacent to lesion cavities; indeed, the numbers were greater than those in outlying spared white matter and naive white matter (Fig. 5.8A-C; Fig. 5.9). Between 7d and 28dpi, FGF-2+ cell number continued to rise in gray matter lesion borders while it remained relatively unchanged in outlying gray matter (Fig. 5.8D-F; Fig. 5.9). In white matter at 28dpi, elevated FGF-2+ cell numbers were maintained along the lesion border. Thus, in contrast to CNTF, gray matter appears to contain a more robust FGF-2 response compared to white matter after SCI.

Figure 5.8: FGF-2+ cell numbers increase after SCI. (A-C) At 7dpi, elevated numbers of FGF-2+ cells were present at the white matter (WM) lesion border (C) compared to outer spared WM (SWM) near meninges (B) and naive WM (A). (D-F) Many FGF-2+ cells were observed 28dpi at the gray matter (GM) borders (F) compared to spared GM (SGM; E) and naive GM (D). B-C: 1mm rostral; E-F: 2mm caudal to epicenter. Scale bar=20µm.
Figure 5.9: FGF-2+ cell numbers increase at the lesion borders post-SCI. FGF2+ cells were counted every 1mm in 4mm of spinal tissue spanning the lesion using sample boxes (refer to Fig. 3C). Naive white matter had fewer FGF-2+ cells compared to naive gray matter. At 7dpi, significantly more FGF-2+ cells were present in white matter border, spared gray matter (SGM) and gray matter border compared to naive tissue. Notably, white matter border also had significantly more FGF-2+ cells than spared white matter (SWM). At 28dpi, elevated numbers of FGF-2+ cells were only detected in white matter and gray matter lesion borders compared to their respective spared regions as well as naïve tissue. One-way ANOVA was used to compare white matter and gray matter regions over time with their respective controls. p<0.05 was considered significant (n=4-6/group).
pSTAT3 expression and localization following SCI

Expression of pSTAT3 in naïve spinal cord was very low with few positively labeled cells. Following contusion, pSTAT3 expression was elevated in injured spinal tissue as early as 1dpi (data not shown). At 1d and 3dpi, the pSTAT3 distribution pattern was scattered throughout the residual tissue following SCI and was also present in cells within the lesion cavity. pSTAT3 expression remained elevated at 7d, 14d and 28dpi, at which times it was mainly concentrated along the lesion borders and was present in NG2+ cells, OLs and astrocytes (Fig. 5.10, 5.11).

Figure 5.10: NG2 cells with phosphorylated STAT3+ (pSTAT3) nuclei were common following SCI. NG2+ cells (grayish-black) with pSTAT3+ nuclei (brown) were seen in gray matter (A; 3dpi, 1.8mm rostral to epicenter) and white matter (B; 7dpi; 1.5mm rostral to epicenter) close to lesion cavity. Arrows indicate double-labeled cells; * indicates lesion. Insets show larger magnification of double-labeled cells in A and B. Sections were counterstained with methyl green. Scale bar=10µm.
Figure 5.11: Oligodendrocytes and astrocytes along lesion borders display pSTAT3+ nuclei following SCI. (A-C) Using confocal microscopy, many oligodendrocytes (CC1+, green; A) with pSTAT3+ nuclei (red; B) were detected along lesion borders. Higher magnification inset in B shows nuclear localization of pSTAT3. (D-F) Astrocytes (GFAP, green; A) also contained pSTAT3+ nuclei (red; E,F). Nuclear labeling is shown in blue (DRAQ5). Arrows indicate double-labeled cells; arrowheads indicate single-labeled pSTAT3 cells. * denotes lesion and the dotted line indicates lesion border. Images were taken from 14dpi sections at 1.5 mm rostral (A-C) and 0.5 mm rostral (D-F) to the epicenter. Scale = 20µm.
Discussion

In this study, we show a continual rise in CNTF and CNTFRα protein for at least 28d after spinal contusion injury, a model which mimics the majority of spinal injuries sustained by humans (Bunge et al., 1993; Norenberg et al., 2004). This work extends previous studies which examined CNTF after SCI but mainly focused on acute post-injury times, mRNA expression and/or used spinal hemisection injuries. For instance, CNTF mRNA was significantly elevated 3d after spinal contusion in rats (Zai et al., 2005) and was shown to increase between 1-3dpi and then decrease by 10-14dpi after hemisection (Oyesiku et al., 1997; Nakamura and Bregman, 2001). Additionally, Lee et al (Lee, Kim, Shin, Moon, and Chun, 1998) showed that following spinal hemisection, CNTF peaked at 7dpi and then declined to baseline by 14dpi. In contrast, we observed a continual rise in CNTF (and CNTFRα) over the first four weeks post-injury. Since hemisection is a more focal injury compared to contusion, which involves a larger area of tissue damage up to several mm rostral-caudal (Siegenthaler et al., 2007), the changes in CNTF after hemisection may have been accordingly shorter-lived. In addition to elevated CNTF expression, increased FGF-2+ cell numbers were observed, which may have been at least partially mediated through CNTF signaling (see below). Lastly, we examined pSTAT3 expression, as this molecule is a major part of the CNTF signaling cascade. Increased pSTAT3 cell numbers were noted as early as 1dpi and remained elevated, at least along lesion border, up to 28dpi. Thus, collectively, our data indicate that the necessary components for CNTF signaling are indeed upregulated after SCI and are particularly prominent in the lesion penumbra.
We were especially interested to examine CNTF expression in tissue bordering lesion cavities since our previous work described reduced OL loss and increased new OL generation there compared to white matter adjacent to the meninges during the first two weeks post-injury (Tripathi and McTigue, 2007). To shed light on mechanisms that may contribute to these effects, we initiated investigations to determine potential signaling molecules that are expressed in the correct location and time after SCI. Since astrocyte proliferation and hypertrophy are known to occur along lesion borders after injury, and CNTF is a prominent astrocyte-derived growth factor, investigating the spatio-temporal expression of CNTF after SCI was a logical choice. CNTF is well known for its effects on OL lineage cells, such as enhancing survival, inducing progenitor cell differentiation and promoting myelin formation (Barres et al., 1996; Marmur et al., 1998; Stankoff et al., 2002). In addition, a role for CNTF in adult myelinogenesis has been suggested, since it is upregulated within and around remyelinating lesions (Albrecht et al., 2003). Our results suggest that CNTF-induced effects in white matter after SCI will not be restricted to the lesion border since elevated CNTF levels were detected throughout post-injury white matter. Therefore, CNTF may play a global role in white matter OL survival and generation after SCI. For instance, we previously noted that OL numbers were reduced in white matter at 3dpi but returned to naïve levels by 7dpi (Tripathi and McTigue, 2007); this increase over the first week post-injury may be due in part to the action of CNTF. In contrast to white matter, CNTF was only upregulated in gray matter bordering the lesions, suggesting that it may influence the marked OL genesis and rise in OL numbers that occur in gray matter lesion borders after SCI (Tripathi and McTigue, 2007). This
also suggests that signals for inducing CNTF expression may be different and/or more widespread in white matter compared to gray matter.

The initial increase in CNTF after SCI is likely due to acute upregulation of molecules known to evoke CNTF production, such as interleukin-1β and TNF-α (Kamiguchi et al., 1995; Herx et al., 2000). mRNAs for both cytokines rise almost immediately after spinal contusion and then decline rapidly by 24h (Streit et al., 1998), although there is a smaller secondary wave of IL-1β and TNFα produced at 14dpi (Pineau and Lacroix, 2007). The prolonged elevation of CNTF detected after spinal contusion may also be due to an autocrine-paracrine effect of CNTF on astrocytes. Astrocytes express CNTF receptor and CNTF, thus an initial burst of CNTF may act locally on the same or nearby astrocytes to promote further CNTF secretion (Monville, Couplier et al., 2001). Although the ability of astrocytes to secrete CNTF has been questioned due to its lack of a typical secretory signaling sequence, in vitro experiments have shown that CNTF is indeed secreted and can be detected in astrocyte conditioned media if re-uptake by the CNTF receptor is blocked (Kamiguchi et al., 1995).

Astrocytes bind and respond to CNTF with hypertrophy and increased GFAP expression (Winter et al., 1995; Levison et al., 1998; Albrecht et al., 2002; Albrecht et al., 2003). Therefore, immunohistochemical detection of CNTF on GFAP+ astrocytes after SCI was not surprising. Interestingly, we also observed CNTF immunoreactivity on a subset of OLs. Although astrocytes are typically thought to be the sole source of CNTF in the adult CNS, one other report has shown CNTF immunoreactivity in the naïve spinal cord on GFAP-negative cells with an OL-like morphology (Dobrea et al., 1992). Whether OLs actually produce CNTF or become labeled due to CNTF binding to cell surface
receptors remains to be determined. In addition to astrocytes, another potential source for CNTF after SCI is non-myelinating Schwann cells (Dobrea et al., 1992). These cells infiltrate spinal cords during the first week post-injury and reside there chronically, where some eventually myelinate axons within the lesion cavity as well as throughout the rim of spared white matter (Blight and Young, 1989; McTigue et al., 2006). The observation of CNTF immunoreactivity on bipolar p75+ suggests that migratory non-myelinating Schwann cells contribute to the chronic elevation in CNTF after spinal contusion. Their level of contribution, however, is lower relative to the astrocytic component.

The effects of CNTF on CNS parenchyma are mediated by a tripartite receptor complex consisting of CNTFRα, which is the CNTF-signal recognition component, and the two signal-transducing subunits LIFRβ and gp130 (for review, see Sleeman, Anderson et al., 2000). CNTF binding to CNTFRα causes the transmembrane LIFRβ and gp130 subunits to dimerize, leading to activation of the JAK-STAT pathway (Sleeman et al., 2000; Horvath, 2000; Kisseleva et al., 2002). In the naïve adult spinal cord, CNTFRα mRNA expression and immunoreactivity has been demonstrated exclusively on neurons (Ip et al., 1993; MacLennan et al., 1996). Following injury, increased CNTFRα mRNA has been detected in certain neuronal populations and in astrocytes close to injury sites (Ip et al., 1993; Rudge et al., 1994). An additional report on spinal hemisections showed an early increase in CNTFRα mRNA caudal to the injury which declined by 10dpi (Oyesiku et al., 1997). In contrast, we detected a substantial decline in total CNTFRα protein expression early after SCI, which likely reflects the complete loss of neurons at and close to the injury epicenter. The protracted rise of the CNTFRα between 3d and 28d
in our study is likely due to upregulation by reactive astrocytes and, perhaps, OLs (Miotke et al., 2007). It is also possible that cleaved and soluble CNTFRα infiltrated the lesion areas from distal spinal segments. Indeed, Davis et al. (1993) showed that following peripheral nerve injury, the GPI link anchoring the CNTFRα to cell surfaces is cleaved thereby releasing a soluble form of the receptor from the skeletal muscles. This is important as the combination of CNTF and soluble CNTFRα imparts CNTF sensitivity to cells lacking CNTFRα but expressing LIFRβ and gp130 subunits, such as macrophages and oligodendrocytes (Davis et al., 1993; Kobayashi and Mizisin, 2000; Butzkueven et al., 2002).

CNTF can influence the function of wide-ranging cell types and therefore may indirectly affect OL lineage cells through interactions with other cellular constituents, the most obvious of which is astrocytes. Astrocytes are highly responsive to CNTF, which induces their proliferation, hypertrophy and upregulation of GFAP – all of which are noted along the lesion border or “glial scar” after SCI. CNTF also stimulates astrocytes to release an as yet unknown trophic factor that promotes OL progenitor survival and proliferation (Albrecht et al., 2007). Another well characterized effect of CNTF is the induction of FGF-2 by astrocytes (Albrecht et al., 2002; Albrecht et al., 2003). Under normal circumstances, astrocytes produce a low molecular weight isoform of FGF-2; however, following injury, higher molecular weight isoforms are prevalent (Li et al., 2006; Yu et al., 2007). Increased FGF-2 mRNA after SCI has been reported at 1d and 7dpi, and higher molecular weight isoforms of FGF-2 protein were detected at 4d and 7d after SCI (Follesa et al., 1994; Mocchetti et al., 1996). Our data confirm and expand upon these in that we detected an overall increase in FGF-2 up to 28dpi, which included
upregulation of three different isoforms of FGF-2. Characterization of FGF-2+ cell
distribution revealed that, similar to CNTF, FGF-2 cell numbers increased by 7dpi in
white matter and gray matter lesion borders. In contrast to CNTF, however, FGF-2 was
increased in outer spared gray matter but not outlying white matter. Our data are in
agreement with Koshinaga et al. (Koshinaga et al., 1993) who noted increased FGF-2+
cells at 5d and 12dpi near the edge of photo-chemically induced spinal lesions. Therefore,
FGF-2 and CNTF upregulation may be interrelated in tissue along lesion borders but
independent signals for FGF-2 expression may be present in spared gray matter.

FGF-2 has well known protective effects on OLs and OL progenitors; therefore, if
its upregulation is mediated at least in part by CNTF, this would be potential indirect
mechanism for CNTF to affect OL lineage cells along SCI lesion borders. FGF-2 in turn
may accentuate CNTF-induced effects, since it has been shown that FGF-2 regulates
CNTF-mediated gene expression and differentiation (Song and Ghosh, 2004).

Interestingly, while FGF-2 promotes OPC proliferation and migration, it can also prevent
OL differentiation and hence may hinder oligogenesis (Goddard et al., 2001; Armstrong
et al., 2002). In our model, it is possible that the acute rise in FGF-2 along lesion borders
functioned to promote NG2 cell proliferation and migration to the lesion border (Simpson
and Armstrong, 1999), which would be a necessary prerequisite for oligogenesis. Once
there, the cells may receive a greater influence from the rising levels of CNTF, which is a
pro-differentiation factor, leading to oligogenesis. On the other hand, it is also possible
that beyond a certain expression level, CNTF effects overcome those of FGF-2. Indeed,
work by Mayer et al. (1993) has shown that the inhibition of OL differentiation by FGF-2
can be overridden by astrocyte-conditioned medium, which likely contained CNTF.
Further studies are needed to determine for certain the link between CNTF and FGF-2 expression after SCI and their individual and combined roles.

An indirect effect on OL lineage cells by CNTF and CNTFRα may also occur through altered macrophage function after SCI. For instance, CNTF promotes macrophage chemotaxis (Kobayashi and Mizisin, 2000; Cen et al., 2007) and thus may recruit macrophages from within and around the lesion toward the spared tissue. Macrophages are a complex cell population with a multitude of actions in the injured CNS, one of which may be promoting OL replacement and remyelination (Kotter et al., 2001; Kotter et al., 2005). CNTF also induces mRNA expression for the anti-inflammatory molecule SOCS-3 (suppressor of cytokine signaling) (Bjorbaek et al., 1999). Thus, elevation of CNTF in the injured spinal cord likely affects multiple cell populations, including astrocytes, OLs, progenitor cells, microglia/macrophages and neurons surviving in gray matter near the lesions.

It is well accepted that CNTF-induced actions are mediated by activation of CNTFRα which ultimately leads to phosphorylation of STAT3. In our tissue, we noted an increase in pSTAT3 expression, particularly along the lesion border at times when CNTF (and FGF-2) were upregulated. pSTAT3 plays diverse roles in cellular functions that are highly relevant to SCI, such as promoting cell proliferation, migration, differentiation and protection against apoptosis (Catlett-Falcone et al., 1999; Wu and Bradshaw, 2000; Zhang et al., 2005; Yoshimatsu et al., 2006; Okada et al., 2006). The detection of pSTAT3 in NG2+ progenitors after SCI indicates that these cells were probable targets of CNTF, which may promote their proliferation, survival and differentiation into OLs close to the lesion border. Phosphorylation of STAT3 after SCI
may not be exclusively induced by CNTFRα activation since other growth factors (e.g., PDGF, IGF-1, FGF-2) and cytokines (IL-6 family) can induce STAT3 phosphorylation. Indeed, IL-6 and LIF are potent inducers of STAT3 phosphorylation and are upregulated early after SCI (Streit et al., 1998; Pineau and Lacroix, 2007). Hence, their signaling may account for the early post-injury expression of pSTAT3 that occurred during a time of relatively low CNTF and CNTFRα expression. The more prolonged expression of pSTAT3 along the lesion borders is likely due at least in part to CNTF (and to a lesser extent FGF-2) expression.

Thus, all elements appear to be in place to mediate active participation by CNTF in post-injury events. Indeed, two recent in vivo studies suggest that increasing CNTF levels promotes neural repair in the adult CNS. First, CNTF infusion after SCI enhanced tissue sparing, including a preservation of rubrospinal tract axons and improved locomotor recovery (Ye et al., 2004). In the other, CNTF administration improved pathological outcomes in a multiple sclerosis mouse model (Kuhlmann et al., 2006). Furthermore, CNTF, CNTFRα and pSTAT3 are all upregulated in human MS brains (Dutta et al., 2007), suggesting a potential neuroprotective role in this disease. Thus, in vitro and in vivo experiments strongly support the beneficial effects of CNTF on OL lineage cells and overall tissue pathology after CNS injury. However, it has also been argued that increased astrocytic hypertrophy induced by CNTF may be an impediment to neuronal regeneration following SCI. For instance, Ishii et al. (Ishii et al., 2006) showed that neutralization of CNTF following SCI led to better regeneration of host corticospinal tract axons. They also reported a more favorable environment for distribution and differentiation of transplanted stem cells. It is difficult to interpret these results in terms
of endogenous oligogenesis since the study examined glial differentiation of transplanted cells. The enhanced host regeneration may have been related to decreased astrocyte hypertrophy rather than a potential reduction in endogenous oligogenesis. The fact that most transplanted cells became oligodendrocytes suggests that enhanced oligogenesis did not reduce the regenerative capacity of host axons. Furthermore, Talbott et al. (Talbott et al., 2007) recently showed that although CNTF has positive effects on OPC survival and maturation in vitro, increasing its levels in vivo did not promote earlier remyelination in a chemically-induced model of demyelination. As the demyelination model in that study undergoes spontaneous remyelination, one possible explanation is that the lesion was already saturated with endogenous growth factors and additional CNTF was therefore unable to accelerate the remyelination process. However, this does not rule out a possible role for endogenous CNTF in recovery processes after CNS injury.

Collectively, our results demonstrate significant and prolonged expression of CNTF, FGF-2 and pSTAT3 in gray matter and white matter after SCI and reveal that chronic expression of these factors corresponds to regions of elevated OLs, proliferating OL progenitors and new OL genesis noted previously shown in chapters 3 and 4 (Tripathi and McTigue, 2007). Thus, these factors are viable candidates for playing roles in endogenous repair processes initiated after spinal injury. Ongoing and future studies will seek to determine in which aspects of OL replacement, if any, these two factors play a direct role after SCI.
CHAPTER 6

Targeting CNTF expression following SCI

Introduction

CNTF was initially identified as a growth factor that promotes neuronal survival but subsequent work has uncovered its role in oligodendrocyte progenitor cell proliferation, maturation and myelogenesis. We have shown that there is increased CNTF expression following SCI, especially in regions with enhanced oligogenesis (Chapter 4 and 5). In order to unequivocally demonstrate the role of CNTF in the formation of new oligodendrocytes following trauma, it is pertinent to study oligodendrocyte population dynamics in the absence of CNTF from the injured CNS milieu. Since, transgenic animal technology is not readily accessible in rats, to achieve efficient down regulation of CNTF we turned to RNA interference (RNAi). RNAi has revolutionized the study of effects of several gene products and proteins on various cellular functions without having to manipulate the genetic make-up of the cells/animals at the germline level.

*What is RNAi and how does it work?:* RNAi is a process of post-transcriptional gene silencing by which double stranded RNA (dsRNA) cause sequence-specific degradation of homologous mRNA sequences in a cell. It was discovered in 1998 by Andrew Fire and Craig Mello in Caenorhabditis elegans (Fire et al., 1998). RNAi can be
naturally occurring or can be induced by introduction of dsRNA in a cell. When a dsRNA is present in a cell it is processed by the RNase III enzyme Dicer in an ATP-dependent reaction. Dicer cleaves dsRNAs into 21-23 nucleotide short interfering RNA (siRNA) with 2-nucleotide 3' overhangs. siRNA can also be synthesized outside the cell and then be introduced into a cell. siRNAs are incorporated into the RNA-inducing silencing complex (RISC) which consists of an Argonaute (Ago) protein as one of its main components. Ago cleaves and discards the sense strand of the siRNA duplex leading to activation of the RISC. The antisense strand of the siRNA then guides RISC to its homologous mRNA, resulting in the endonucleolytic cleavage of the target mRNA eventually leading to down-regulation of the target protein (Rand et al., 2005; Matranga et al., 2005).

The purpose of the studies here was to determine the most efficient and effective way to down-regulate the CNTF protein in vivo using siRNA. To that effect three different strategies were employed. First we attempted to evaluate the effects of naked CNTF-siRNA administration into the injury site following SCI. Next, we used a 25kDa cationic polymer, polyethyleneimine (PEI) in conjunction with the CNTF-siRNA. PEI readily complexes with siRNA and protects it from RNase degradation enhancing its half-life (Urban-Klein et al., 2005; Werth et al., 2006). Also, complexation of siRNA with PEI improves the cellular uptake of the siRNA (Aigner, 2005); 25kDa PEI is considered among the better non-viral gene delivery systems. Lastly, we made a lentivirus expressing shRNA (short hairpin RNA) against rat CNTF for use in-vitro and in-vivo. A lentiviral vector was chosen over other viral vector systems because of its ability to primarily infect non-dividing cells (Naldini et al., 1996b), i.e astrocytes in our
studies, since astrocytes were found to be the cell type with maximum contribution to the CNTF expression post-SCI. Also the use of lentivirus enables a stable and long term expression of shRNA in a cell for many months since it integrates with the host DNA (Naldini et al., 1996a).

**Materials and Methods**

For methods regarding spinal cord injury, protein extraction, electrophoresis and western blot please refer to the sections under general methodology in chapter 2. An injury severity of 0.8mm displacement was used for all the studies in this chapter.

An open-field locomotor test, Basso, Beattie and Bresnahan (BBB) scale, was used to assess locomotor recovery of rats following SCI. The BBB scale is specifically designed to evaluate the hind limb movements of rats following SCI (Basso et al., 1995).

**siRNA design and delivery**

*Un-bound or naked siRNA:* Sequences of dsRNA against rat CNTF mRNA were carefully designed using online siRNA design tools from Dharmacon (Dharmacon Inc, Lafayette, CO). Two different siRNA constructs (21 nucleotide duplexes) against the rat CNTF were combined and used (see below).

**CNTF siRNA #1** sense sequence CCUGACUCUCUUAUGGAAUU
antisense sequence UUCCAUAAGAGCAGUGU.

**CNTF siRNA #2** sense sequence ACUGAUCGUUGGAGAGGAAUU
antisense sequence UCUCACUAAGACUGU.

Two different doses of siRNA (low dose 5µg/µl or high dose 10µg/µl) were used for invivo administration. On the day of siRNA administration (2d, 3d and 4dpi), the
injury site was re-exposed and using a Hamilton syringe, 5µl of CNTF siRNA or 1X PBS was injected into the injury site (n=3/group). Hence the animals received either 25µg or 50µg of siRNA/injection. The syringe was rinsed in 3 changes of RNase free water between animals. The animal were surgically closed and treated as they were on the day of their injury. All procedures were performed aseptically. The animals were sacrificed at 5dpi and their spinal cords harvested (epicenter ± 2mm).

**Complexation of the siRNA with Polyethylenimine (PEI):** In order to minimize RNA degradation and improve its cellular uptake, a 25 kDa polymer polyethylenimine (PEI, Sigma, St. Louis, MO) was complexed with the siRNA. The same two siRNA sequences mentioned above were used here too. siRNA was diluted to 50µg/µl in RNase free PBS with 25µg of each siRNA. Its conjugation to PEI was carried out by modification of the work by Tan et al. (Tan, Yang et al., 2005). Briefly, 100mM PEI solution was prepared in 10ml of 5% dextrose/ 1X PBS (pH=7.4). For experimental controls a proprietary sequence of scrambled siRNA (Ambion, Austin, TX) was also complexed with the polymer in the exact same way as the siRNAs for CNTF. PEI alone was not used as a control since it is highly cytotoxic in its unbound form.

PEI-siRNA conjugation was done by addition of 2µl siRNA (50µg/µl) to 18µl of 100mM PEI solution and gently vortexing. PEI/RNA ratio was 6 equivalents of PEI nitrogen per phosphate of RNA. PEI-siRNA complex was allowed to form for at least 20minutes at room temperature. A final conc. of 5µg/µl of the CNTF siRNA (2.5µg/siRNA) was used for injections.
On the day of siRNA administration the injury site was re-exposed and 5µl of PEI-siRNA or 1X PBS was injected into the injury site using a Hamilton syringe. The syringe was rinsed in 3 changes of RNase free water between animals. Animals were injected either on 1 and 3dpi or 2, 3 and 4dpi or 1, 3 and 5dpi and were sacrificed on 7dpi. Spinal cord tissue (epicenter ± 2mm) was dissected and processed for western blot analysis or immunohistochemistry.

Subcloning of shRNA: Two sets of distinct hairpin DNA oligonucleotides were designed to silence rat CNTF. The oligonucleotide sequences were designed to contain Xho1 sites at the ends for subsequent subcloning into the pSICOR (Addgene, Cambridge, MA) lentiviral vector and are designated as CNTF 1 and CNTF 2. The sequences are as follows

CNTF1: TCGAGCCTGACTGCTTTTATGGAATTTTAGTAAGCCACAGATGTATTTCATAAAGGACAGTCAGGTTC and CGGACTGACGAGAATACCTTAAATCACTTCGGTGTCTACATAAGGTAT TCTCGTCAGTCCAAAGGCT

CNTF2: TCGAGACTGATCGTTGAGTGAGATTTAGTGAAGCCACAGATGTATCTCACTCAACGAGAATACCTCAGTCCAAAGGCT

Generation of lentivirus: The pSICOR plasmid containing the sequences to silence CNTF expression was packaged into lentiviral particles pseudo-typed with vesicular stomatis virus glycoprotein (VSVG) and produced by quadruple transfection in HEK-293 cells, as previously described (Kafri et al., 1997). Briefly, Gag, Pol and Rev DNAs were co-transfected with the pSICOR vector utilizing the calcium phosphate
procedure. Media containing the virus was first collected 48 hours after initial transfection and then every 24 hours for 4 days. The media was filtered and centrifuged at 90,000g for 90min at 4°C. The resulting pellet was dissolved in 10% glycerol/1X PBS. The viral titer was determined by infection of 293 cells utilizing the GFP tag contained within the pSicoR plasmid. Also, the presence of loxP sites flanking the Xho1 site makes it possible to excise and shut down shRNA expression, in presence of Cre, allowing CNTF expression in the cells to be re-established if needed (Fig 6.1).

![Image](198x308 to 417x527)

Figure 6.1 pSicoR map adapted from the addgene website. The boxes show the loxP sites and the Xho1 site where the shRNAs were inserted.

**Astrocyte culture:** Astrocyte cultures were prepared using modified methods from McCarthy and de Vellis (1980). All chemicals are from Sigma unless otherwise noted, and all procedures were performed under sterile conditions. Brains from 3d rat pups were dissected and meninges removed. Cortices were placed in a solution of 0.9mg/ml Bovine Serum Albumin (BSA) and 0.135mM MgSO₄ in Krebs Ringers Buffer (KRB) (Solution 101
1). Cortexes were dissociated by triturating with a 25ml serological pipette and centrifuged for 5 min at 300 relative centrifugal force (rcf). Next, the supernatant was removed and 0.28mg/ml trypsin in Solution 1 was added. This solution was shaken for 30 min (200 rotations per minute (rpm), 37°C). Next, dilute triturating solution (12.8μg/ml DNA-ase, 83.2μg/ml trypsin inhibitor and 0.25mM MgSO₄ in Solution 1) was added, and the final volume was mixed and centrifuged for 5 min at 2000 rpm. The supernatant was removed and full-concentration triturating solution (0.08mg/ml DNA-ase, 0.52mg/ml trypsin inhibitor, and 1.5mM MgSO₄ in Solution 1) was added. This solution was triturated 25x, then 1.2mM Mg and 0.096mM Ca in Solution 1 added, mixed, and centrifuged for 7 min at 300 rcf. The pellet was resuspended in enough astrocyte media (0.11mg/ml sodium pyruvate (Invitrogen), 0.584mg/ml L-Glutamine, 10% Fetal Calf Serum (Invitrogen), and 40μg/ml gentamicin (Invitrogen) in DMEM (Invitrogen)) to allow for plating of 2-3 brains per T75 flask. Isolated cells were given fresh media at 24 hours then as needed until the shakeoff procedure, and stored in a 37°C, 5% CO₂ incubator.

For the shakeoff, flasks were shaken on an orbital shaker for 1h at 250 rpm and 37°C to remove microglia. The media was replaced with fresh media and allowed to equilibrate for 2 hours, then the cells were shaken ≥18 hours at 200 rpm and 37°C. The astrocytes remain attached while other cells are lifted into the media. The astrocytes were trypsized and replated on glass cover slips or directly on 24 well plates for experiments.
Results

*CNTF expression following administration of naked siRNA:* We did not observe any changes in CNTF protein expression 5dpi as examined by western blot technique in the animals that received 5µg/µl of CNTF-siRNA (Fig 6.2). A dose of 10µg/µl of CNTF-siRNA did reduce CNTF expression by 44%, albeit insignificantly (Fig 6.2). No differences in locomotor recovery of these animals were noted based on the administration of CNTF siRNA (data not shown).

![Bar graph showing CNTF expression](image)

**Figure 6.2:** No change in CNTF expression, after naked CNTF siRNA administration, 5dpi. Protein extracted from epicenter ± 2mm was examined for CNTF expression 5dpi using western blot. No change in CNTF was noted in the animals that received 5µg/µl of siRNA; 44% reduction in CNTF protein expression was noted in the high dose group, although it was not statistically significant. n=3/group.

*Administration of PEI-siRNA (CNTF) I:* In order to improve the uptake of siRNAs into the cells and to protect them from degradation, we decided to complex the siRNAs with PEI. Also, in the previous experiment the animals survived only up to 5dpi which was perhaps insufficient time to observe the complete down-regulation of CNTF protein; therefore, here we let our animals survive to 7dpi.
Administration of PEI-siRNA (CNTF) into the injury site on 1d and 3 dpi significantly reduced CNTF expression by ~70% on 7dpi compared to PBS injected controls (Fig 6.3). The delivery of PEI-siRNA (CNTF) on 2d, 3d and 4dpi also significantly reduced CNTF expression on 7dpi by ~53% compared to PBS injections (Fig 6.3). We also examined CNTF expression on 7dpi after administering PEI-siRNA on 1d, 3d and 5dpi; although noticeable down regulation of CNTF was seen (Fig 6.4) it was not as robust as the other PEI-siRNA injection paradigms when assessed with western blotting (data not shown). Hence it appeared that PEI-siRNA delivery on 1d and 3dpi post injury was the most successful in reducing the CNTF expression by 7dpi.

Figure 6.3: Significant down regulation of CNTF protein following PEI-siRNA (CNTF) administration following SCI. A 70% reduction in CNTF was seen on 7dpi after siRNA administration on 1d and 3dpi. Also siRNA injections 2d, 3d and 4dpi reduced CNTF expression by 53% on 7dpi.
Figure 6.4: Reduced CNTF expression after PEI-siRNA (CNTF) administration to injured animals. (A) Increased CNTF expression 7dpi at the lesion epicenter compared to un-injured naïve animals (C). (B) Increased CNTF expression persists at 1mmR to the epicenter. (D) 7dpi CNTF expression is dramatically reduced at the epicenter of a rat receiving siRNA on 1, 3 & 5dpi. (E-F) Decreased CNTF expression is also seen 1mmR to the epicenter. Scale bar= 100µm.

We also assessed locomotor recovery of the injured animals and interestingly saw that the group of animals which received PEI-siRNA (CNTF) on 1d and 3dpi had significantly lower BBB locomotor scores (avg=0.89) than PBS injected (avg=8.17) and the ones that received PEI-siRNA (CNTF) on 2d, 3d and 4dpi (avg=8.17) (Fig 6.5). This meant that an early reduction in endogenous CNTF expression following SCI lead to poorer locomotor recovery in rats.
Figure 6.5: Poorer locomotor recovery in animals receiving PEI-siRNA (CNTF) on 1d and 3dpi. No difference in BBB scores were noted in PBS injected controls from uninjected controls (data not shown). Also, PEI-siRNA (CNTF) administration on 2, 3 and 4dpi did not change BBB scores compared to PBS injected animals, (n=3/group).

Administration of PEI-siRNA (CNTF) II: After ascertaining that PEI-siRNA (CNTF) injections are most effective in down regulating CNTF expression when given 1d and 3dpi, we repeated the experiment with other experimental controls. In order to eliminate the factor that injecting the spinal cord multiple times by itself may be increasing CNTF and confounding the results, we included surgery controls, which got surgically re-opened but no intraspinal injections. We also included animals which received PEI-siRNA (scrambled) on 1d and 3dpi. Again the CNTF expression was examined at 7dpi. The scrambled siRNA sequence ideally should not downregulate any proteins. We obtained a 50% down-regulation in CNTF expression using the PEI-siRNA (CNTF); surprisingly we also got a very similar suppression of CNTF in the animals that received PEI-siRNA (scrambled) (Fig 6.6). Also, this time we did not observe any significant differences in the locomotor recovery of injured rats among the three experimental groups (Fig 6.7).
Figure 6.7: A 50% decrease in CNTF expression was seen on 7dpi after PEI-siRNA administration. Both groups of animals receiving PEI-siRNA irrespective of the siRNA sequence showed half the CNTF protein expression on 7dpi compared to non-injected injured rats. This reduction was not statistically significant. The dotted line indicates 50% of control value. n=3/group.

Figure 6.7: No change in locomotor recovery of spinally injured rats following PEI-siRNA administration. An initial decline in overall BBB scores was noted in all rats following SCI. The spontaneous recovery of animals in all the 3 groups over week 1 post injury was comparable. n=11 for control and n=13 for PEI-siRNA groups.
Since we saw a down-regulation of CNTF protein even with scrambled siRNA when complexed with PEI we decided to use a viral vector for delivery of siRNA into the cells. We successfully subcloned a short hairpin RNA (shRNA) against CNTF into a plasmid with GFP expression and the ability to conditionally shut down the expression of shRNA. We noted multiple astrocytes infected with the virus in vitro as seen with GFP expression (Fig 6.8). Due to a low virus titre we did not probe the astrocyte cultures for CNTF expression via western blot. Efforts are ongoing to improve lenti-virus titres to achieve >90% transfection efficiency.

Figure 6.8: Astrocytes infected with GFP expressing lentivirus carrying shRNA against CNTF. Confocal image shows GFAP+ (red) astrocytes with GFP (green) immunoreactivity in vitro. Image was taken 5 days following infection the virus. Scale bar=20 µm.

Discussion

The aim of conducting studies which can effectively down regulate CNTF expression following spinal trauma was to mainly understand the role of CNTF in post-traumatic oligogenesis. In our initial experiments, naked siRNA against CNTF was unable to achieve significant reduction in CNTF expression following SCI. The high siRNA doses required to even detect CNTF suppression in naked siRNA experiments are
likely due to the rapid degradation of siRNA invivo since the half life of unprotected naked siRNA in presence of RNases is estimated to be 2-10 min (Morrissey et al., 2005; Sato et al., 2007). Also, since reduction in CNTF mRNA may take some time to reflect as CNTF protein changes it is possible that examining CNTF protein expression at 5dpi was premature. Nevertheless, we did achieve 50% suppression of CNTF expression in our high dose paradigm.

In the next set of experiments using PEI-siRNA after SCI, we did note a significant decrease in CNTF expression following PEI-siRNA (CNTF) administration under all the injection paradigms compared to PBS injections. It was intriguing that 2 injections of PEI-siRNA (CNTF) were more efficient at reducing CNTF expression than 3 injections. Probably the act of re-injecting the spinal cord, as it is beginning to heal, causes re-activation of CNTF production. On the other hand it could also be due to RNAi rebound owing to a higher dose of siRNA in the system. It has been shown that target genes can rebound after being suppressed by siRNA for a period of time; the higher the dose of siRNA the quicker rebound of the target mRNA (Hong et al., 2005).

In addition to a decrease in CNTF protein we also noted changes in locomotor behavior. We do not think the loss of spontaneous locomotor recovery in animals which had a 70% reduction in CNTF was merely due to an injection on day 1 since we have injected PBS and naked siRNAs previously on 1dpi and subsequent days without any detectable effects on locomotor recovery of injured rats. We also don’t think it is the result of including the PEI along with the siRNA, since animals that got PEI-siRNA complex on 2d, 3d and 4dpi had locomotor scores comparable to PBS injected rats. It is possible that the behavioral effects seen may be due to specifically a combination of PEI-
siRNA being injected on 1dpi. We think that a dramatic reduction (70%) in CNTF protein following siRNA administration may be the responsible for the poorer locomotor recovery of rats following SCI.

When we repeated the PEI-siRNA experiment with non-injected controls and PEI-siRNA (scrambled) we found that PEI-siRNA (CNTF) and PEI-siRNA (scrambled) both reduced CNTF expression by half compared to un-injected controls. It is possible that the scrambled siRNA sequence we used in our study has an off target effect due to partial sequence complementarity to the mRNA of interest (CNTF in our case)(Svoboda, 2007). Additionally it is possible that our delivery polymer PEI itself may be down regulating certain genes (including CNTF) via non-specific effects. Studies have shown that other complexation agents like lipofectamine, even when not complexed with RNA or DNA, can down regulate certain genes non-specifically (Akhtar and Benter, 2007). Another possibility is the oversaturation of the RNA machinery in the cell, by the use of high siRNA doses, causing cell dysfunction and death.

To circumvent some of these issues we are working on a lentiviral vector as an in-vivo delivery tool for CNTF shRNA expression into cells following SCI. In the present experiments we could not consistently and specifically down regulate CNTF expression but we have certainly made progress and gained insight into this technique. We hope that experiments using the lenti-viral vector will overcome the shortcomings of our previous endeavors and address the question of CNTF’s role in post-traumatic gliogenesis. In addition to studying the effects of CNTF on oligodendrocyte genesis, manipulating
CNTF levels in vivo also provides us with a tool to study the relationship between CNTF and other injury related events such as astrocyte hypertrophy, neuron survival, axon regeneration, microglial activation and macrophage invasion. CNTF is known to affect these processes following CNS insults (Kobayashi and Mizisin, 2000; Albrecht et al., 2002; Ye et al., 2004; Ishii et al., 2006). Also since CNTF has been shown to upregulate the astrocytic expression of FGF-2, reducing CNTF levels using RNAi techniques will help us to examine the contribution of upregulated CNTF to the increase in FGF-2 levels after injury. Hence future experiments in the lab will explore these and other possibilities.
GENERAL DISCUSSION

Neurons have long enjoyed “star-status” in the CNS and deservedly so. It is the neurons with their axonal projections that communicate between CNS and the rest of the body making multiple life sustaining and enabling events possible. Following spinal cord injury there is extensive loss of neurons at the injury site and severe axonal damage functionally disconnecting the higher levels of the CNS from the rest of the body below the level of the lesion (Hayes and Kakulas, 1997; Kakulas, 1999). Therefore research efforts directed at solving the spinal cord injury problem have often focused on generating new neurons and regenerating axons.

While the neuron has its place as the leading man/lady, the supporting cast of glia play important roles in the CNS and in almost all neuron mediated events. Glial cells nourish, protect and facilitate the neurons, enabling their critical function in all CNS activities. Oligodendrocytes perform all of these functions and are indispensable to the CNS (Bunge et al., 1962; Du and Dreyfus, 2002; Yin et al., 2006). Hence, enhancing and achieving axonal regeneration in the injured spinal cord without appropriate strategies to remyelinate those axons may be eventually of limited functional significance. Indeed, genetic mutations leading to dysfunctional myelin production by oligodendrocytes is the
cause of devastating illnesses in humans, some of which are Pelizaeus-Merzbacher
disease, Krabbe’s disease, Alexander’s disease and multiple leukodystrophies (Suzuki,
2003; Mignot et al., 2004; Schiffmann and van der Knaap, 2004; Garbern, 2007). Other
conditions with OL/myelin pathology and complex etiologies are multiple sclerosis and
Schizophrenia (Franklin, 2002b; Uranova et al., 2007). Oligodendrocyte death and
myelin destruction also occur as a result of SCI and understanding the underlying
mechanisms has been an active area of research (Blight, 1985; Grossman et al., 2001).

The discovery of NG2+ OPCs, capable of differentiating into OLs in vitro and in
vivo has opened whole new vista for oligodendrocyte replacement therapy. The NG2
proteoglycan has received significant research interest not only because it is a marker for
OPCs and promotes its migration but also because of its role in inhibiting axonal
regeneration following SCI. We characterized overall NG2 distribution following a
contusion injury, which is a clinically relevant model of human SCI and found that NG2
immunoreactivity continued to rise in the residual tissue up to 2 months post-injury
(chapter 3). We also determined that spinal cord injury lesion borders contain a higher
number of proliferating NG2 cells.

Since the lesion border is also the site of the glial scar, believed to be an
impediment to axonal re-growth, is the enhanced presence of NG2 proteoglycan and the
higher numbers of NG2+ cells adding brick and mortar to this wall? Recent research
shows that cellular expression of NG2 in vivo may not be growth inhibitory as previously
thought (Jones et al., 2003; Yang et al., 2006; McTigue et al., 2006). Additionally,
evidence from NG2 deficient mice reveals that NG2 is not a major contributor to axon regeneration failure following CNS injuries (Hossain-Ibrahim et al., 2007). These data emphasize that studies which examine components of the extracellular matrix out of context i.e. in vitro should be viewed with some degree of reservation. Apart from it role in generating OLs and its debatable role in inhibiting axon regeneration in an injured environment the function of the NG2+ cells in the naïve CNS still remains to be explored. Hence, much thought must be given in any therapeutic application enhancing or reducing its levels in vivo (i.e via proteases or inhibitory peptides) and a balance between the multiple aspects of NG2 proteoglycan activity must be considered.

The exciting finding in our studies was the increased presence of proliferating NG2+ cells along the lesion borders accompanied by enhanced oligodendrocyte formation. Although new oligodendrocyte formation has been shown previously in the injured spinal cord, the extent and robust nature of oligodendrogenesis was not previously appreciated (Zai and Wrathall, 2005; Yang et al., 2006). These results are significant since they reveal the endogenous potential for genesis of large numbers of OLs in the adult spinal cord. Some may argue that this enhanced oligodendrogenesis is pathological and/or ectopic. And although it is possible that an increased OL presence along lesion borders may be “pathological”, i.e prevent axonal regeneration due to presence of myelin proteins; it is important to remember that they also can also myelinate bare axons following SCI. Whether the prominent oligogenesis along the lesion perimeter is ectopic is debatable. Since OLs are normally present in the white matter and gray matter, although different factors may influence their genesis, the oligogenesis seen in
our study cannot be described ectopic just based on the fact that it is more robust in the gray matter regions (Pastor et al., 1998; Baracskay et al., 2002; Dawson et al., 2003; Tripathi and McTigue, 2007). Also, oligodendrocytes during development are generated in much larger numbers than needed and then “matched-up” with the axons that need myelin; the remaining oligodendrocytes that are unable to partner with an axon perish (Barres et al., 1992; Trapp et al., 1997; Barres and Raff, 1999). We do not yet know how long these OLs that are generated along the lesion border last and what is their myelinating potential. However, the increased OL presence and OL remyelination we observed at 28dpi along lesion borders indicates that at least a portion of the OLs survive and go on to remyelinate spinal axons since pre-existing OLs would not have been able to remyleinate. Ongoing studies in the McTigue lab are addressing these issues in detail.

The result of enhanced oligogenesis along the lesion borders motivated us to explore endogenous mechanisms responsible for it following spinal trauma. We examined astrocyte derived CNTF due to the dense astrocyte presence along the lesion borders and the role of CNTF in promoting many stages along the OL lineage. Our findings revealed that CNTF and FGF-2 are both upregulated in regions of oligogenesis. The distribution pattern of these growth factors revealed they may be acting differently in the gray matter compared to white matter to promote oligodendrocyte formation. It is unlikely that any of these growth factors is acting alone, since both CNTF and FGF-2 have been shown to be linked to each other expression and activity it is highly possible that they are acting synergistically. To further examine the causal relationship of CNTF in injury induced OL formation, we used RNAi. The magnitude and influence of RNAi to
the field of biological sciences is evident in the fact that the Nobel Prize for Physiology in 2006 was awarded to Andrew Fire and Craig Mello “for their discovery on RNA interference: gene silencing by double stranded RNA”. RNAi is now being seriously considered as therapeutic option in humans for various diseases (Gewirtz, 2007; Grimm and Kay, 2007). Unfortunately, we were not successful in establishing a stable down-regulation of the CNTF protein in vivo. Hence ongoing studies are examining the potential of siRNA delivery using a lentiviral vector. Compared to other viral vectors, lentiviral vector has been shown to be the most effective gene delivery vehicle following SCI (Abdellatif et al., 2006) and I am sure Dr McTigue’s laboratory will continue to pursue this direction.

What may be the purpose of studying this endogenous oligodendrogenesis phenomenon? We already know that OPCs undergo a protracted period of proliferation following SCI; also demyelinated axons persist chronically following SCI in animals and humans. We also know that transplanting cells capable of differentiating into OLs and remyelinating clearly demonstrate improvements in recovery following rodent SCI. All these evidence reveal that there is demyelination after SCI which does not get repaired completely via endogenous processes. More importantly that this demyelination is amenable to manipulation pharmacologically (via 4-AP) and can be augmented by cells capable of becoming OLs and remyelinating and improve functional recovery following SCI. The question then is why despite significant endogenous OPC proliferation does complete remyelination fail. Is it because these OPCs do not differentiate into OLs or the resulting OLs do not remyelinate? Also, are the OPCs proliferating at the wrong times in
the wrong places? Hence to understand this problem we examined the location of proliferating OPCs in the injured spinal cord and if there was oligodendrogenesis in the regions that showed increased OPC proliferation. We have established that following SCI proliferating OPCs are located in regions showing enhanced oligogenesis especially in the gray matter and the lesion borders. If we know what is “calling” and/or accumulating OPCs in a specific region, what is helping them metamorphose into OLs and eventually myelinate, we can then understand why they may not be doing so in regions which remain demyelinated. Understanding the underlying mechanisms behind this oligogenic niche will be critical to elucidating and solving the failures of endogenous remyelination.

It is important to point out that OPCs are not the only available answer to the remyelination conundrum. Another option to achieve remyelination the non-OPC way is to use Schwann cells, which are the myelinating cell of the peripheral nervous system. Indeed, Schwann cells do invade the CNS parenchyma following SCI and remyelinate CNS axons (Blakemore, 1983; Blight and Young, 1989). Many investigators have shown enhanced remyelination can be achieved via Schwann cell transplants and this remyelination does result in enhanced conduction of action potentials (Honmou et al., 1996; Pearse et al., 2007). It must be noted here that Schwann cells may influence SCI outcome not just by remyelination but also by promoting axonal regeneration and providing growth factors (Xu et al., 1997; Oudega and Xu, 2006). Surprisingly though, very few studies have evaluated the long term effects of peripheral myelin on central axons (Felts and Smith, 1992; Black et al., 2006). One study suggests that oligodendrocyte myelin has neuroprotective effects and replacing PLP (major component
of CNS myelin) with P0 (major component of peripheral myelin) in the CNS causes axonal degeneration and major locomotor dysfunction (Yin et al., 2006). Although replacing PLP with P0 is not the same as replacing OLs with Schwann cells, it draws attention to the absence of beneficial effects of central myelin in the event of peripheral myelination. Hence, studies evaluating effects of peripheral Schwann cell transplantation to achieve remyelination in the injured CNS must carefully examine long term effects of such experiments. Also, the relationship of Schwann cells with astrocytes and microglia (the other glia of the CNS) must be studied thoroughly to achieve successful results following Schwann cell transplants.

In addition to Schwann cells other cells like olfactory ensheathing cells, bone marrow cells and embryonic stem cells have also been explored as remyelinating therapy in the CNS (Kato et al., 2000; Liu et al., 2000; Akiyama et al., 2002; Franklin, 2002a). Hence our repertoire of potential ways to tackle CNS demyelination is constantly growing.

Even as we learn more and more about endogenous processes occurring in disease conditions, we as a scientific community are entering a new phase of modern medicine in which stem cell transplants are a viable option for treating CNS disorders. Indeed, OPCs have arrived on the clinical trial scene as therapeutic intervention for SCI with Geron Corporation gearing up to go from bench-side to bed-side.

laboratories around the world will help understand the basic science behind cell transplant studies and ongoing endogenous processes and help prevent any premature abortion of these and any subsequent clinical trials in SCI. From the multitude of laboratory studies and clinical trials on human SCI subjects it is becoming clear that there may not be a single magic-bullet which will fix all the issues and combinatorial therapies will be needed. Since SCI is not just a problem of demyelination it is unlikely that OPC transplants will solve it, therapies targeting neuron loss, axonal regeneration and balancing immunological issues will be as critical as promoting and protecting surviving tissue function. In addition to surgical and pharmacological treatments, physical therapy approaches will also be beneficial towards overall improvements following SCI.

Our data demonstrate that biochemical and pathophysiological changes following SCI are not just acute and transient; many events continue occurring chronically reminding us of the protracted evolving nature of the injury. Our understanding of the oligodendrocyte has come a long way since the early 1900s when they were described as the “third element” by Ramón y Cajal (García-Marin et al., 2007). Hopefully this work will add to the existing knowledge about oligodendrocyte and its progenitor cell response following SCI and will further our understanding towards solving the demyelination issues not only following SCI but also in multiple disease conditions targeting OLs and myelin.
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