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Chronically Increased Ciliary Neurotrophic Factor and Fibroblast Growth Factor-2 Expression After Spinal Contusion in Rats

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Abstract

Demyelination and oligodendrocyte loss following spinal cord injury (SCI) are well documented. Recently, we showed oligodendrocyte progenitor cell (OPC) accumulation and robust oligodendrocyte genesis occurring along SCI lesion borders. We have since begun investigating potential mechanisms for this endogenous repair response. Here, we examined ciliary neurotrophic factor (CNTF) and fibroblast growth factor-2 (FGF-2) expression, because both factors alter progenitor proliferation and differentiation and are increased in several CNS disorders. We hypothesized that CNTF and FGF-2 would increase after SCI, especially in regions of enhanced oligogenesis. First, CNTF protein was quantified using Western blots, which revealed that CNTF protein continually rose through 28 days post injury (dpi). Next, by using immunohistochemistry, we examined the spatiotemporal expression of CNTF in cross-sections spanning the injury site. CNTF immunoreactivity was observed on astrocytes and oligodendrocytes in naïve and contused spinal cords. Significantly increased CNTF was detected in spared white and gray matter between 5 and 28 dpi compared with uninjured controls. By 28 dpi, CNTF expression was significantly higher along lesion borders compared with outlying spared tissue; a similar distribution of phosphorylated STAT3, a transcription factor up-regulated by CNTF and to a lesser extent FGF-2, was also detected. Because CNTF can potentiate FGF-2 expression, we examined the distribution of FGF-2⁺ cells. Significantly more FGF-2⁺ cells were noted along lesion borders at 7 and 28 dpi. Thus, both CNTF and FGF-2 are present in regions of elevated OPC proliferation and oligodendrocyte generation after SCI and therefore may play a role in injury-induced gliogenesis.

Indexing terms

CNTF receptor; astrocyte; spinal cord injury; progenitor; STAT3; oligodendrocytes; FGF-2; NG2

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 because of an endogenous pool of NG2⁺ cells typically referred to as *oligodendrocyte progenitor cells* (OPCs) or *polydendrocytes* (Nishiyama et al., 1996; Baracskey 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

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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 persists 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 are especially robust along the lesion penumbra during the first 2 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 (TNF α)-induced toxicity, and promotes myelinogenesis and myelin protein formation (Barres et al., 1993, 1996; Mayer et al., 1994; 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). Because 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. After 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 posttraumatic 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 up-regulating their production (FGF-2, IGF-1; Albrecht et al., 2002), and by increasing expression of their receptors, i.e., FGF-1R and IGF-1R (Jiang et al., 1999). Thus, the potential mechanisms for CNTF-induced effects on oligodendrocyte lineage cells are manifold.

Previous studies have examined CNTF and CNTF receptor- α (CNTFR α) 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 CNTFR α protein levels following contusion injury is lacking. Therefore, we systematically characterized CNTF protein expression in a rat spinal contusion model at 3 days to 28 days post injury (dpi) in spared white and gray matter and the lesion border (where gliogenesis is highest). We also examined overall post injury expression of CNTFR α protein, which has activity both as a bound and as a 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. Finally, as a potential

indicator of CNTF/CNTFR α signaling activity, we examined the expression of phosphorylated STAT3 (signal transducer and activator of transcription 3, pSTAT3) in naïve and injured tissue, because 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 bordering 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

Spinal cord injury

Female Sprague Dawley rats (225–250 g) received a moderate spinal contusion using the electromagnetic OSU injury device and were randomly assigned to survive for 3, 5, 7, 14, or 28 dpi. With ketamine (80 mg/kg)/xylazine (10 mg/kg) anesthesia, 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 0.8 mm over 23 msec. After spinal contusion, the overlying muscles were sutured and the skin was closed with wound clips. The animals were given saline and placed in warm recovery cages. Prophylactic antibiotic (Gentocin 1 mg/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 noninjury controls. All procedures were carried out in accordance with The Ohio State University Institutional Animal Care and Use Committee (IACUC) guidelines.

Protein extraction

Rats were anesthetized and very briefly perfused with cold 0.1 M phosphate-buffered saline (PBS; pH 7.4; n = 3/time point). Spinal cord blocks (4 mm) centered on epicenter were rapidly dissected and frozen using liquid nitrogen. The tissue was stored at -70°C until protein extraction. Proteins were extracted by homogenization of spinal cords with 400 μl T-PERS (pH 7.6; Pierce, Rockford, IL), 1 $\times$ EDTA (Pierce), and 1 $\times$ protease inhibitor cocktail (Pierce; catalog No. 78410). The homogenates were centrifuged at 10,000 rpm for 5 minutes, and the supernatants were collected. Total protein content was determined by using the Bradford assay, and aliquots of supernatant samples were stored at -70°C .

Electrophoresis/Western blot

Protein samples containing 1 $\times$ Nupage LDS (Invitrogen, Carlsbad, CA) and 1 $\times$ Nupage reducing agent (Invitrogen) were heated for 5 minutes at 95°C . Then, 10 μg (for CNTF and CNTFR α) or 12.5 μg (for FGF-2) of total protein was loaded per lane and separated on 4–12% Bis-Tris Nupage gel (Invitrogen) in 1 $\times$ Nupage MES running buffer (Invitrogen; pH 7) at 200 V for 40 minutes. Subsequently, proteins were transferred onto a nitrocellulose membrane at 30 V for 60 min. The membrane was blocked for 1 hour with 5% nonfat dry milk in 0.5% Tween-20/0.1 M PBS and incubated with the primary antibody of interest

overnight at 4°C. The membrane was washed with 0.5% Tween/0.1 M PBS multiple times and probed with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. Chemiluminescence was imparted to the primary–secondary antibody complex by incubating with solution from the Super Signal West Pico kit (Pierce) that detects HRP. The signal was detected by exposing the membrane to autoradiographic film (ISC BioExpress) for 10 seconds to 1 minute (depending on the signal intensity) and developed with a 100plus automatic X-ray film processor (All-ProImaging). A chemiluminescent protein ladder (Cell Signaling Technology, Beverly, 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, 1 μ g/ml; anti-CNTFR α , 1 μ g/ml; anti-FGF2, 1:500; and anti- α -tubulin, 1:2,500. For detailed information on antibodies see Table 1.

Western blot analysis

The developed Western blot films were scanned and saved as gray-scale tiff images. Bands on the images were analyzed in MCID Elite 7.0 software (Imaging Research Inc., St. Catherine's, Ontario, Canada). After lanes of interest were identified, MCID generated a density profile based on the darkness of the bands. 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 quantitated. 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 CNTFR α bands was normalized to the respective ROD of α -tubulin bands to obtain the ratios, which were then graphically represented and analyzed.

Tissue processing and immunohistochemistry

Deeply anesthetized animals were perfused intracardially with cold 0.1 M PBS followed by 4% paraformaldehyde/ 0.1 M PBS (n = 5–6/group). Spinal cords were removed and postfixed with 4% paraformaldehyde/0.1 M PBS for 2 hours, then transferred to 0.2 M phosphate buffer at 4°C overnight. The tissue was subsequently cryoprotected with 30% sucrose for 3 days, and 6 mm of 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 10 μ m on a cryostat and serially mounted on precleaned glass slides (ColorFrost/plus, Fischer Scientific; catalog No. 12-550-17). For information on antibodies used for immunohistochemistry (IHC) see Table 1.

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 preincubated with BP⁺ (4% BSA/PBS/ 0.1% Triton) and then with 1:2,000 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, Burlingame, CA) and visualized with 3,3'-

diaminobenzidine substrate (DAB; Vector). Slides were rinsed, treated with acetone (5 minutes), rinsed, and immersed in EC solution for 30 minutes. 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.

To assess temporal and spatial patterns of CNTF expression, CNTF labeling was achieved using an immunoperoxidase method. Sections were preincubated with BP⁺ and then incubated overnight in 1:800 goat anti-CNTF antibody (0.5 mg/ml stock). On the next day, secondary antibody (1:2,000 horse anti-goat) was applied for 1 hour 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, 10 µg of CNTF antibody was incubated with 100 ng recombinant rat CNTF (R&D Systems, Minneapolis, MN) for 1 hour at 37°C (per manufacturer's recommendation), and the above-described procedure was repeated. No positive signal for CNTF was detected under these conditions.

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 1:1,000 anti-goat Alexa Fluor 546 (Molecular Probes, Eugene, OR) for 30 minutes or rabbit anti-GFAP antibody (1:4,000; Dako Cytomation, Carpinteria, CA) or mouse anti-CC1/APC antibody (1:400) followed by 1:1,000 Alexa Fluor 488 (Molecular Probes). Cell nuclei were labeled with DRAQ5 (1:3,000; Biostatus Limited), 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 the oligodendrocyte lineage (Kitada and Rowitch, 2006). Olig2 antibody was applied at 1:500 overnight followed by 1:1,000 anti-goat Alexa Fluor 546 followed by the CNTF antibody (1:100) and Alexa Fluor 488 for 30 minutes.

To examine OL numbers along lesion borders at 28 dpi, oligodendrocyte cell bodies were visualized with the CC1 antibody. 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-labeling protocol of CC1, GFAP, and P0 (Schwann cell myelin) was used to differentiate these cells. CC1 (1:500), P0 (1:6,000; Dr. Juan Archelos, Society to Support Research, Diagnosis and Treatment of Neurological Disorders, Austria), and GFAP immunohistochemistry (1:4,000; Sigma) was performed using standard immunoperoxidase methods, and sections were counterstained with methyl green.

To determine whether 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 5 minutes at 96°C. The pSTAT3 antibody against STAT3 phosphorylated at Tyr705 (1:100) was applied, followed by 1:500 anti-rabbit Alexa Fluor 546. The astrocyte and OL marker antibodies were used as described above, and DRAQ5 was used as nuclear

label. For pSTAT3 and NG2 colocalization, the 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), 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 (1:1,000) overnight, followed by 1:800 horse anti-mouse antibody for 1 hour 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.

Antibody specificity

CNTF—The CNTF antibody specificity for spinal cord was confirmed by Western blot; it recognized a single band at the predicted 22 kDa (data shown in Results). Antibody specificity was further confirmed by performing IHC after preadsorption with recombinant rat CNTF, which revealed no positive staining (data shown in Results).

FGF-2—This antibody recognized three bands in rat spinal cord tissue at ~20, ~22, and ~24 kDa (data shown in Results). All of these bands have been previously reported in the literature following rat SCI (Mocchetti et al., 1996). The staining pattern of the FGF-2 antibody was consistent with that shown previously following rat SCI in which cell nuclei around the lesion site were labeled (Zai et al., 2005).

CNTFR α —As expected, in our study this antibody recognized a band at 60 kDa and an additional band at 55 kDa in certain injured spinal cord samples. The 55-kDa band is most likely a differentially glycosylated form. In comparison with other studies, these results are consistent with results showing CNTFR α -positive bands at 60 kDa and 55 kDa using rat retina and mouse cardiomyocytes (Beltran et al., 2005; Raju et al., 2006).

α -Tubulin—This antibody was used as a loading control for Western blots. It recognized a single and predicted band at 53 kDa in our rat SCI tissue (data shown), which is consistent with previous work in which α -tubulin was detected as a 53 kDa band in Western blots using rat spinal cord tissue (Mutti et al., 2007).

CC1 clone of the APC antibody—The APC antibody was originally described as recognizing CNS cells with a morphology and spatial distribution consistent with OLs; it was also shown to recognize a single band at 300 kDa from rat brains (predicted mol. wt. of APC is 312 kDa; Bhat et al., 1996). This antibody is considered an OL marker because it double-labels cells expressing the OL markers such as Olig2, Nogo-A, and GST-pi (Linares et al., 2006; Tripathi and McTigue, 2007; Kuhlmann et al., 2007). In addition, IHC of CNS tissue from mice expressing GFP under the PLP promoter (which is expressed only in mature OLs) showed complete overlap of GFP and CC1 cells; this study also revealed

double-labeling of GFP cells with CNPase and PLP but not GFAP and determined that the GFP⁺ cells displayed electrophysiological properties of mature OLs, further confirming that the labeled cells were mature OLs (Fuss et al., 2000).

P0—This antibody (P07 clone) was developed and extensively characterized by Archelos et al. (1993) by using Western blot, IHC, and competition ELISA. In their study, Western blots with rat sciatic tissue demonstrated a single band at 29 kDa, as expected. The authors also demonstrated that IHC on rat and human sciatic nerve tissue revealed exclusive localization of staining to peripheral myelin; preabsorption of the antibody with extracellular domain of P0 completely eliminated all staining.

GFAP—This antibody revealed an astrocyte staining pattern consistent with many published reports using naïve and injured rat CNS showing specific labeling of astrocyte cell bodies and processes (Lee et al., 1998; Ishii et al., 2006; Talbott et al., 2007). Also, Western blots with this antibody in our laboratory yielded results very similar to published work with injured rat spinal cord, with a distinct band at ~50 kDa and multiple bands between 50 and 70 kDa (Nesic et al., 2005).

Olig2—Antibody specificity was verified by the manufacturer by ELISA and Western blots with recombinant Olig2 and with IHC with mouse spinal cord tissue. In addition, we have directly compared labeling in rat spinal cord by this antibody and that of the Olig2 DF308 antibody (Ligon et al., 2004), which has been shown to be specific for Olig2 in Olig2^{-/-} mice (Ligon et al., 2006). The labeling pattern with the manufactured Olig2 antibody was identical to that of DF308 and revealed nuclear staining specifically in OL-lineage cells.

pSTAT3—We detected nuclear labeling with pSTAT3 antibody within multiple cells following SCI, which is consistent with the nuclear staining pattern in rat SCI tissue detected by Yamauchi et al. (2006) with the same antibody. This antibody also recognized pSTAT3 α of expected molecular weight (~86 kDa) in Western blot performed on the injured mouse spinal cord (Yamauchi et al., 2006).

NG2—Antibody specificity was proved by the manufacturer in Western blots of rat brain and recombinant protein, which showed identical patterns (manufacturer's technical information). Furthermore, we have detected identical labeling patterns of rat spinal cord tissue between this antibody and antibodies generated by William Stallcup that have been shown to be specific by Western blots and IHC in CNS tissue from NG2 knockout mice (Grako et al., 1999; McTigue et al., 2006).

p75—Specificity of this antibody for rodent p75 was shown in previous studies with Western blots on tissue from rat spinal cord (Mutti et al., 2007) or mouse brain (Paul et al., 2004); the results showed a single band at 75 kDa, which was absent in mice lacking expression of the full-length p75 protein (Paul et al., 2004).

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 1-mm intervals throughout a 4-mm 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 were measured and used to calculate proportional area stained for CNTF.

To compare CNTF expression in specifically white matter border vs. outlying spared white matter and gray matter border vs. outlying spared gray matter, sample boxes (0.12 mm × 0.12 mm) were placed within each subregion, as performed previously (Tripathi and McTigue, 2007). For spared tissue measurements, only regions in which the sample box was at least 0.12 mm away from the lesion border were included; in this way, tissue sampled in spared regions never consisted of lesion border tissue. When this was not possible, samples were not collected. Spared gray matter samples were collected only in sections 1–2 mm rostral/caudal to the epicenter, because the lesion epicenter sections contained no measurable gray matter. 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 (an example is depicted in Fig. 3C). Sample box location was selected on low-power printouts of NF/EC-stained tissue of each section. Because cellular or IHC details could not be discerned on the printouts, bias in box placement was avoided. A box was placed in the dorsalmost aspect of the lesion border, and then nonoverlapping boxes were evenly placed around the lesion circumference. At minimum three boxes were placed along the edge of the lesion in distal sections (2 mm from epicenter), and typically six to ten 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 five or six animals throughout the 4 mm of tissue per region per distance were averaged to obtain an overall mean $\pm$ SEM. For comparisons with normal spinal cords, data were 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 20× 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 count FGF-2⁺ cells automatically in the boxes, based on the positive segmentation and preset mean size and exclusion criteria. Measurements from five or six animals throughout the 4 mm of tissue per region per distance were averaged as an overall mean $\pm$ SEM.

Statistical analysis

GraphPad Prism 5 software was used for statistical data analyses. Depending on the data set, either a oneway 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). $P < 0.05$ was considered significant.

Anatomical plates containing light or confocal microscopic images were assembled in Adobe Photoshop 7.01 (Adobe, San Jose, CA). When deemed necessary, brightness and contrast were enhanced to allow reproduction of the image as originally viewed in the microscope. The red Draq5 signal was converted to blue in the confocal software.

RESULTS

CNTF protein expression increases following SCI

On Western blots, a continual rise in CNTF protein was observed between 5 and 28 dpi in 4 mm of tissue centered on the contusion epicenter (Fig. 1A). A single band at 22 kDa 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 5 dpi and had tripled by 7 dpi, although the data were not significant (Fig. 1B). By 14 dpi, CNTF protein had significantly increased compared with naïve tissue and then continued to rise such that, at 28 dpi, it was significantly higher than that in naïve and 5-dpi spinal cord tissue (Fig. 1B).

IHC was used to compare the spatiotemporal distribution of CNTF after SCI in residual gray matter and white matter with that in comparable regions of uninjured spinal cord sections. As expected, a low level of CNTF immunoreactivity was present in uninjured controls (Fig. 2A,B); in these sections, less than 1% of white matter or gray matter was positively labeled for CNTF (Fig. 3A,B).

Gray matter—Because gray matter is destroyed at the lesion epicenter, all gray matter analyses were performed on sections 1–2 mm rostral and caudal to epicenter. At 3 dpi, CNTF immunoreactivity was not different from that in naïve gray matter (Fig. 3A). By 7 dpi, CNTF was significantly higher in sections 1 mm rostral to the epicenter compared with naïve and 3-dpi levels (Figs. 2A,D, 3A). CNTF immunoreactivity continued to rise such that, by 14 dpi, it was markedly higher both rostral and caudal to epicenter compared with naïve and previous time points. Between 14 and 28 dpi, CNTF immunoreactivity was either maintained or further increased in residual gray matter, with the highest levels occurring 1 mm rostral to epicenter (Fig. 3A). By 70 dpi, CNTF levels in gray matter approximated naïve levels (data not shown). Hence, CNTF immunoreactivity increased in gray matter between 3 and 28 dpi and returned to baseline by 10 weeks post injury.

White matter—Similar to the case in gray matter, significantly elevated CNTF immunoreactivity was not detected until 7 dpi, at which time it was elevated compared with naïve at all distances examined (Fig. 3B). By 14 dpi, CNTF had increased further at the epicenter, and, by 28 dpi, CNTF immunoreactivity was even higher than 14-dpi levels in sections rostral and caudal to epicenter (Fig. 3B). Hence, in spared white matter after spinal

contusion, CNTF levels increased continuously through 28 dpi. Between 28 and 70 dpi, CNTF expression distal to the injury site decreased and was indistinguishable from that at 3 dpi; epicenter CNTF levels remained comparable to the level at 7 dpi (data not shown).

Lesion border vs. 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 elevated NG2 levels, more proliferating NG2⁺ progenitors, and new OLs compared with outlying spared tissue along the meningeal border (McTigue et al., 2006; Tripathi and McTigue, 2007). To determine whether 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 7 and 28 dpi (Fig. 3C). At 7 dpi, CNTF was up-regulated to a comparable level in white matter bordering the lesion and the meninges (Fig. 3D). In gray matter, however, CNTF had increased significantly only in the lesion border tissue (Fig. 3D).

By 28 dpi, CNTF expression in outer white matter had declined slightly compared with 7 dpi 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 (Figs. 2E,F, 3D). In gray matter, the difference between perilesion and outlying CNTF expression was amplified at 28 dpi, insofar as CNTF levels had risen further along lesion borders (Fig. 3D). Thus, during the first month post injury, CNTF expression was elevated throughout white matter but was restricted to lesion borders in gray matter.

In a previous study, we showed that increased oligodendrogenesis and elevated oligodendrocyte numbers were maintained in tissue adjacent to spinal lesions throughout the first 14 days after SCI (Tripathi and McTigue, 2007). Because our current data reveal a sustained elevation of CNTF through 28 dpi, we asked whether the elevation in OLs numbers in the lesion penumbra existed through 28 dpi as well. By using IHC, a clear elevation in OL numbers along lesion borders was still detectable at 28 dpi compared with spared tissue adjacent to meningeal borders (Fig. 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 via confocal microscopy. As expected, CNTF colocalization with GFAP⁺ astrocytes was detected in naïve and injured spinal cord cross-sections (Fig. 5A–C). CNTF⁺ astrocytes were especially prominent along the lesion border. CNTF immunoreactivity was also observed on a subset of CC1⁺ oligodendrocytes (Fig. 5D – F), as has been noted previously (Dobrea et al., 1992). We confirmed CNTF immunoreactivity on oligodendrocyte lineage cells by colabeling with Olig2 (Fig. 5G – I). CNTF immunoreactivity was not detected on NG2⁺ cells (glial progenitor cells that 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 nonmyelinating 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 (Fig. 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 might not be exclusively Schwann cells. CNTF immunoreactivity was not present on OX42⁺ microglia or macrophages (data not shown).

CNTFR α expression following SCI

Because the direct effects of CNTF are mediated by binding the CNTFR α , overall changes in protein levels for this receptor were examined in SCI tissue by Western blot. This method allowed optimal detection of the membrane-bound and soluble form of the receptor. A prominent band at 60 kDa was detected for CNTFR α at all days post injury examined (3–28 dpi); a second, weaker, 55-kDa band was also detected at 3 dpi and, to a lesser extent, at 5 dpi (Fig. 6A). The 55-kDa protein may be a breakdown product of the receptor or a differentially glycosylated form of CNTFR α (Zvonic et al., 2003). Quantitation of the 60-kDa band using densitometry revealed that, at 3 dpi, CNTFR α expression was substantially decreased compared with naïve spinal cords (Fig. 6B). Although a modest increase occurred between 3 and 14 dpi, CNTFR α expression remained significantly lower than naïve levels until 28 dpi.

FGF-2 expression following SCI

A known action of CNTF within the CNS is up-regulation of FGF-2 expression, another astrocyte-derived growth factor (Albrecht et al., 2002). Hence, we examined FGF-2 immunoreactivity following SCI as described above for CNTF. By using Western blots on 4 mm of spinal tissue, we observed an FGF-2 band at 22 kDa in uninjured spinal cords. At 3 dpi, there was an additional band ~24 kDa, which was absent on all subsequent days post injury (Fig. 7). Between 5 and 28 dpi, FGF-2 protein increased, as shown by the increasing intensity of the 22-kDa and 20-kDa bands (Fig. 7). With IHC, FGF-2⁺ cells were quantified in 7- and 28-dpi tissue and compared with 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 with white matter (420.1 ± 84.9 vs. 145.3 ± 23.7 ; Figs. 8A,D, 9). At 7 dpi, FGF-2⁺ cell numbers were significantly increased in outer spared gray matter and gray matter lesion borders compared with naïve gray matter (Figs. 8, Fig. 9). In white matter, however, FGF2⁺ cell numbers were increased only in tissue adjacent to lesion cavities; indeed, the numbers were greater than those in outlying spared white matter and naïve white matter (Figs. 8A–C, 9). Between 7 and 28 dpi, FGF-2⁺ cell number continued to rise in gray matter lesion borders, although it remained relatively unchanged in outlying gray matter (Fig. 8D–F, 9). In white matter at 28 dpi, 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 with white matter after SCI.

pSTAT3 expression and localization following SCI

Expression of pSTAT3 in naïve spinal cord was very low, with few positively labeled cells present. After contusion, pSTAT3 expression was elevated in injured spinal tissue as early as 1 dpi (data not shown). At 1 and 3 dpi, 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 7, 14, and 28 dpi, at which times it was mainly concentrated along the lesion borders and was present in NG2⁺ cells, OLs, and astrocytes (Figs. 10, 11).

DISCUSSION

In this study, we show a continual rise in CNTF and CNTFR α protein for at least 28 days after spinal contusion injury, a model that mimics the majority of spinal injuries sustained by humans (Bunge et al., 1993; Norenberg et al., 2004). This work extends previous studies that examined CNTF after SCI but focused mainly on acute post injury times, mRNA expression, and/or used spinal hemisection injuries. For instance, CNTF mRNA was significantly elevated 3 days after spinal contusion in rats (Zai et al., 2005) and was shown to increase between 1 and 3 dpi and then decrease by 10–14 dpi after hemisection (Oyesiku et al., 1997; Nakamura and Bregman, 2001). Additionally, Lee et al. (1998) showed that, after spinal hemisection, CNTF peaked at 7 dpi and then declined to baseline by 14 dpi. In contrast, we observed a continual rise in CNTF (and CNTFR α) over the first 4 weeks post injury. Because hemisection is a more focal injury compared with contusion, which involves a larger area of tissue damage up to several millimeters rostrally-caudally (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 might have been partially mediated through CNTF signaling (see below). Finally, we examined pSTAT3 expression, because this molecule is a major part of the CNTF signaling cascade. Increased pSTAT3 cell numbers were noted as early as 1 dpi and remained elevated, at least along the lesion border, up to 28 dpi. Thus, collectively, our data indicate that the necessary components for CNTF signaling are up-regulated after SCI and are particularly prominent in the lesion penumbra.

We were especially interested to examine CNTF expression in tissue bordering lesion cavities, because our previous work described reduced OL loss and increased OL generation there compared with white matter adjacent to the meninges during the first 2 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 at the correct location and time after SCI. Because astrocyte proliferation and hypertrophy occur along lesion borders after injury, and CNTF is a prominent astrocyte-derived growth factor, investigating the spatiotemporal expression of CNTF after SCI was a logical choice. CNTF is well known for enhancing OL 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, because it is up-regulated within and around remyelinating lesions (Albrecht et al., 2003). Our results indicate that CNTF-induced effects in white matter after SCI will not

be restricted to the lesion border; 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 3 dpi but returned to naïve levels by 7 dpi (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 up-regulated only in gray matter bordering the lesions, suggesting that it may influence the marked OL genesis and rise in OL numbers occurring 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 with gray matter.

The initial increase in CNTF after SCI likely is due to acute up-regulation of molecules known to evoke CNTF production, such as interleukin-1 β and TNF α (Kamiguchi et al., 1995; Herx et al., 2000). mRNAs for both cytokines rise almost immediately after SCI and then decline rapidly by 24 hours (Streit et al., 1998), although there is a smaller, secondary wave of IL-1 β and TNF α at 14 dpi (Pineau and Lacroix, 2007). The prolonged elevation of CNTF after spinal contusion might also be due to an autocrine-paracrine CNTF effect on astrocytes. Astrocytes express CNTFR α and CNTF, thus an initial burst of CNTF may act locally on the same or nearby astrocytes to promote further CNTF secretion (Monville et al., 2001).

It is known that astrocytes express CNTF (Winter et al., 1995; Levison et al., 1998; Albrecht et al., 2002, 2003), so detection of CNTF on GFAP⁺ astrocytes after SCI was not surprising. Interestingly, we also observed CNTF immuno-reactivity on a subset of OLs. Although astrocytes are typically thought of as the source for CNTF in the adult CNS, one 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 as a result of CNTF binding to surface receptors remains to be determined. Another potential source for CNTF after SCI is nonmyelinating Schwann cells (Dobrea et al., 1992). These cells infiltrate spinal cords early after injury and reside there chronically. The observation of CNTF immunoreactivity on bipolar p75⁺ cells suggests that migratory nonmyelinating Schwann cells contribute to the chronic elevation in CNTF after spinal contusion. Their contribution, however, is lower relative to the astrocytic component.

The effects of CNTF on CNS parenchyma are mediated by a tripartite receptor complex including CNTFR α (Sleeman et al., 2000; Horvath, 2000; Kisseleva et al., 2002). In the naïve adult spinal cord, CNTFR α mRNA expression and immunoreactivity have been demonstrated exclusively on neurons (Ip et al., 1993; MacLennan et al., 1996). After injury, increased CNTFR α mRNA has been detected in certain neuronal populations and in astrocytes close to injury sites (Ip et al., 1993; Rudge et al., 1994). For instance, a study with spinal hemisection showed an early increase in CNTFR α mRNA caudal to the injury, which declined by 10 dpi (Oyesiku et al., 1997). In contrast, we detected a substantial decline in total CNTFR α 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 CNTFR α between 3 and 28 dpi in our study likely is due to up-regulation by reactive astrocytes and, perhaps, OLs (Miotke et al., 2007). It is also possible that cleaved and soluble CNTFR α infiltrated

the lesion areas from distal spinal segments. Indeed, Davis et al. (1993) showed that the GPI link anchoring the CNTFR α to cell surfaces can be cleaved, thereby releasing a soluble form of the receptor.

CNTF may indirectly affect OL-lineage cells through interactions with other cells, the most obvious of which are astrocytes. CNTF 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, 2003). FGF-2 in turn may accentuate CNTF-induced effects by regulating CNTF-mediated gene expression and differentiation (Song and Ghosh, 2004). Astrocytes normally produce a low-molecular-weight isoform of FGF-2; following injury, however, higher-molecular-weight isoforms are prevalent (Li et al., 2006; Yu et al., 2007). Increased FGF-2 mRNA early after SCI has been reported, and higher-molecular-weight isoforms of FGF-2 protein were detected at 4 and 7 days after SCI (Follesa et al., 1994; Mocchetti et al., 1996). Our data confirm and expand upon these data, in that we detected an overall increase in FGF-2 up to 28 dpi, which included up-regulation of three different isoforms. Characterization of FGF-2⁺ cell distribution revealed that, similar to CNTF, FGF-2 cell numbers increased by 7 dpi 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 those of Koshinaga et al. (1993), who noted increased FGF-2⁺ cells near the edge of photochemically induced spinal lesions. Therefore, FGF-2 and CNTF up-regulation may be interrelated in tissue along lesion borders, but independent signals for FGF-2 expression may be present in spared gray matter.

Although 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 prerequisite for oligogenesis. Once there, the cells may receive a greater influence from the rising levels of CNTF, which is a prodifferentiation factor, leading to oligogenesis. Also, it is possible that, beyond a certain expression level, CNTF effects may overcome those of FGF-2. Indeed, work by Mayer et al. (1993) showed 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.

It is well accepted that CNTF-induced actions are mediated by activation of CNTFR α , which ultimately leads to phosphorylation of STAT3 (Horvath, 2000; Kisseleva et al., 2002). With our tissue, we noted an increase in pSTAT3 expression, particularly along the lesion border at times when CNTF (and FGF-2) were up-regulated. 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. It should be noted that phosphorylation of STAT3 after SCI might not be induced exclusively by CNTFR α activation, insofar as other growth factors (e.g., PDGF, IGF-1, FGF-2) and cytokines (IL-6 family) can also induce STAT3 phosphorylation.

Two recent in vivo studies suggest that increasing CNTF levels in the adult CNS promotes neural repair. 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). 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. (2006) showed that neutralization of CNTF following SCI led to better regeneration of corticospinal tract axons and a more favorable environment for differentiation of transplanted stem cells. It is difficult to interpret these results in terms of endogenous oligogenesis, insofar as that study examined glial differentiation of transplanted cells. Moreso, the fact that most transplanted cells became oligodendrocytes suggests that enhanced oligogenesis per se did not reduce the regenerative capacity of host axons. Furthermore, 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. The demyelination model in that study undergoes spontaneous remyelination, so 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 an integral 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 the regions of elevated OLs, proliferating OL progenitors, and new OL genesis noted previously (Tripathi and McTigue, 2007). Thus, these factors are viable candidates for contributing to endogenous repair processes initiated after SCI. Ongoing and future studies will determine in which aspects of OL replacement, if any, these two factors play a direct role after SCI.

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Fig. 1.

Increased CNTF expression 5–28 days post injury (dpi). **A:** Western blot analysis of 4 mm of SCI tissue centered on the lesion epicenter revealed a single CNTF band (22 kDa), which increased over time after injury; α -tubulin was used as loading control, and 10 μ g of total protein was loaded per lane. **B:** Ratio of the relative optical density (ROD) of CNTF bands normalized to α -tubulin showed significantly higher CNTF expression at 14 and 28 dpi. One-way ANOVA followed by Tukey post hoc tests was used for statistical analysis and $P < 0.05$ was considered significant; $n = 3/\text{group}$.

Fig. 2.

Increased CNTF immunoreactivity was detected 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). Dashed 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:** WM at 14 dpi immunolabeled with CNTF antibody that had been preincubated with recombinant rat CNTF; no positive signal was detected. Markedly increased CNTF expression was observed after SCI in GM (**D**; 7 dpi) and WM (**E,F**; 28 dpi). CNTF expression was especially high in white matter bordering the lesion. Images were taken at the following locations: D, 1.2 mm rostral; C, 1.5 mm rostral; E, 1 mm rostral; F, 2.5 mm caudal to epicenter. Lesion is indicated by the asterisk; arrows indicate CNTF labeling. Scale bar = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 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 7 days post injury (dpi) in sections 1–2 mm rostral to epicenter (0 on x-axis). Between 7 and 28 dpi, CNTF expression continued to increase at most distances compared with naïve gray matter and previous time points. **B:** CNTF increased modestly in white matter at 3 dpi. By 7 dpi, CNTF was significantly higher in white matter at all distances examined compared with naïve. By 28 dpi, CNTF expression in white matter was greater than naïve and 7 and 14 dpi 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; hatched boxes show samples along lesion borders. Boxes are not to scale. **D:** At 7 and 28 dpi, CNTF expression was significantly higher in spared white matter, white matter border, and gray matter border compared with naïve; at 28 dpi, CNTF along white matter and gray matter lesion borders was significantly elevated compared with the respective outlying spared tissue. Data spanning 4 mm of spinal tissue are collapsed across distance. For A and 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.

Fig. 4.

Increased oligodendrocyte numbers were present along the lesion border at 28 dpi. For color figures, see the article online. Our previous work revealed that oligodendrocyte numbers are elevated along lesion borders through at least 14 dpi. Here, sections from 28 dpi double-labeled for oligodendrocytes (CC1; brown) and astrocytes (GFAP, black) reveal that elevated oligodendrocyte numbers are maintained along lesion borders (**B**) compared with outlying white matter (WM; **A**) through at least 28 dpi. Arrows indicate CC1⁺ oligodendrocytes; arrowheads denote GFAP⁺ astrocytes. Asterisk denotes lesion. Both the images were taken from the same section 1 mm caudal to epicenter. Scale bar = 20 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 5. Astrocytes, oligodendrocytes, and Schwann cells display CNTF immunoreactivity after SCI. For color figures, see the article online. **A–C:** Single-channel (A,B) and merged (C) confocal images ($\times 1 \mu\text{m}$) reveal that many GFAP⁺ astrocytes (green) were colabeled with CNTF (red). **D–F:** CNTF immunoreactivity (red) was also observed on CC1⁺ oligodendrocytes (green); a merged image shown is in F. **G–I:** The oligodendrocyte-lineage identity was confirmed by colocalization of Olig2 (H) with CNTF (G). The single-labeled Olig2 cells in H are most likely OPCs; note that 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 at 14 dpi 2.5 mm rostral to epicenter; all other images were taken from 28 dpi sections: A–C, 1.2 mm caudal; D–F, 0.75 mm caudal; J,K, 0.45 mm caudal; and L–M, 1.5 mm caudal to epicenter. All sections were counterstained with DRAQ5, and the high red signal was converted to blue. Arrows indicate double-labeled cells; arrowheads indicate single-labeled cells. Asterisk denotes lesion in D–F. Scale bars = 20 μ m in F (applies to A–F); 20 μ m in I (applies to G–I); 20 μ m in M (applies to J–M). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 6.

CNTF receptor α (CNTFR α) expression initially declines then rises after SCI. **A:** CNTFR α was detected as a prominent 60-kDa band in spinal cord samples using Western blot; an additional, lighter, 55-kDa band was also noted at 3 and 5 dpi. α -Tubulin was used as loading control, and 10 μ g of total protein was loaded per lane. **B:** Densitometric analysis of the 60-kDa band showed a dramatic and significant decrease in CNTFR α expression as early as 3 dpi, which rebounded to naïve levels by 28 dpi. One-way ANOVA followed by Tukey post hoc test was performed, and $P < 0.05$ was considered significant ($n = 3/\text{group}$).

Fig. 7.

FGF-2 protein levels increase from 3 to 28 dpi. In naïve spinal cords, FGF-2 was detected as a faint band at 22 kDa. At 3 dpi, this band and a 24-kDa band was detected. Between 5 and 28 dpi, the 22-kDa FGF-2 band intensified, indicating increased FGF-2; a 20-kDa band also appeared, which intensified over time. α -Tubulin signal reveals comparable loading in all lanes. In total 12.5 μ g protein was loaded per lane.

Fig. 8.

FGF-2⁺ cell numbers increase after SCI. **A–C:** At 7 dpi, elevated numbers of FGF-2⁺ cells were present at the white matter (WM) lesion border (C) compared with outer spared WM (SWM) near meninges (B) and naïve WM (A). **D–F:** Many FGF-2⁺ cells were observed 28 dpi at the gray matter (GM) borders (F) compared with spared GM (SGM; E) and naïve GM (D). B,C, 1 mm rostral; E,F, 2 mm caudal to epicenter. Scale bar = 20 μ m.

FGF-2: Spared vs Lesion Border

Fig. 9.

FGF-2⁺ cell numbers increase at the lesion borders post-SCI. FGF2⁺ cells were counted every 1 mm in 4 mm of spinal tissue spanning the lesion using sample boxes (refer to Fig. 3C). naïve white matter had fewer FGF-2⁺ cells compared with naïve gray matter. At 7 dpi, significantly more FGF-2⁺ cells were present in white matter border, spared gray matter (SGM), and gray matter border compared with naïve tissue. Notably, white matter border also had significantly more FGF-2⁺ cells than spared white matter (SWM). At 28 dpi, elevated numbers of FGF-2⁺ cells were detected only in white matter and gray matter lesion borders compared with 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 ($n = 4-6/\text{group}$).

Fig. 10. NG2 cells with phosphorylated STAT3⁺ (pSTAT3) nuclei were common following SCI. For color figures, see the article online. NG2⁺ cells (grayish-black) with pSTAT3⁺ nuclei (brown) were seen in gray matter (**A**; 3 dpi, 1.8 mm rostral to epicenter) and white matter (**B**; 7 dpi, 1.5 mm rostral to epicenter) close to lesion cavity. Arrows indicate double-labeled cells; asterisk indicates lesion. **Insets** show higher magnification of double-labeled cells in A and B. Sections were counterstained with methyl green. Scale bar = 10 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 11. Oligodendrocytes and astrocytes along lesion borders display pSTAT3⁺ nuclei following SCI. For color figures, see the article online. **A–C:** By 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; D) 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. Asterisk denotes lesion, and the dotted line indicates lesion border. Images were taken from 14 dpi sections at 1.5 mm rostral (A–C) and 0.5 mm rostral (D–F) to the epicenter. Scale bar = 20 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE 1

Information on Antibodies Used in This Study

Antibody	Company	Catalog no.	Immunogen	Species
CNTF	R&D Systems, Minneapolis, MN	AF-557-NA	Recombinant rat CNTF	Goat
CNTFR α	BD Pharmingen, San Diego, CA	558891	Human CNTFR α aa residues 1–372	Mouse
FGF-2	Upstate Charlottesville, VA	05–118	Bovine brain FGF-2	Mouse
pSTAT3 (tyr705)	Cell Signaling Technologies, Danvers, MA	9145	Peptide sequence ADPGSAAPyLTKFIC (y denotes phosphorylated tyrosine residue)	Rabbit
GFAP	Dako, Carpinteria, CA	Z0334	GFAP from bovine spinal cord	Rabbit
CC1/APC	Abcam, Cambridge, MA	ab16794	Recombinant human APC, aa residues 1–226	Mouse
NG2	US Biological, Swampscott, MA	C57067-70F	Cell line expressing truncated rat NG2	Mouse
α -Tubulin	Abcam, Cambridge, MA	ab7291	Full length α -tubulin from chicken brain	Mouse
p75	Promega, Madison, WI	G3231	Recombinant human p75 (cytoplasmic domain)	Rabbit
Olig2	R&D Systems, Minneapolis, MN	AF2418	Recombinant human Olig2	Goat
P0	Provided by Dr. Juan Archelos, Austria	—	Extracellular domain of P0 protein, aa residues 1–124	Mouse
Neurofilament (clone RT97)	Developmental Studies Hybridoma Bank, Iowa City, IA	RT97	Semipurified neurofilament from Wistar rat brains	Mouse