Published in final edited form as:

Auton Neurosci. 2013 December ; 179(0): . doi:10.1016/j.autneu.2013.07.002.

Transection of Preganglionic Axons Leads to CNS Neuronal Plasticity Followed by Survival and Target Reinnervation

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Abstract

The goals of the present study were to investigate the changes in sympathetic preganglionic neurons following transection of distal axons in the cervical sympathetic trunk (CST) that innervate the superior cervical ganglion (SCG) and to assess changes in the protein expression of brain derived neurotrophic factor (BDNF) and its receptor TrkB in the thoracic spinal cord. . At 1 week, a significant decrease in soma volume and reduced soma expression of choline acetyltransferase (ChAT) in the intermediolateral cell column (IML) of T1 spinal cord were observed, with both ChAT-ir and non-immunoreactive neurons expressing the injury marker activating transcription factor 3. . These changes were transient, and at later time points, ChAT expression and soma volume returned to control values and the number of ATF3 neurons declined. No evidence for cell loss or neuronal apoptosis was detected at any time point. Protein levels of BDNF and/or full length TrkB in the spinal cord were increased throughout the survival period. In the SCG, both ChAT-ir axons and ChAT protein remained decreased at 16 weeks, but were increased compared to the 10 week time point. These results suggest that though IML neurons show reduced ChAT expression and cell volume at 1 week following CST transection, at later time points, the neurons recovered and exhibited no significant signs of neurodegeneration. The alterations in BDNF and/or TrkB may have contributed to the survival of the IML neurons and the recovery of ChAT expression, as well as to the reinnervation of the SCG.

Keywords

CNS response to peripheral injury; retrograde signaling following injury; neurotrophin regulation; brain derived neurotrophic factor (BDNF); full length TrkB (TrkB. FL); truncated TrkB (TrkB. T1); intermediolateral cell column (IML)

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INTRODUCTION

axon injury, it is thought that retrograde signals originating from the injury site can activate the parent cell bodies, possibly leading to survival and regenerative growth (Abe and Cavalli, 2008).

In a previous study, a significant loss of IML neurons at spinal cord level T1 was reported at 10 weeks following CST transection. This loss was based on a reduced number of choline acetyltransferase (ChAT) immunoreactive (-ir) neurons in the IML that also contained the retrograde tracer Fast Blue, which was applied to the SCG at the time of injury (Tang and Brimijoin, 2002). Yet, in this same study, a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, which was carried out weekly throughout the survival period, failed to reveal any evidence of apoptosis in the IML (Tang and Brimijoin, 2002). Similarly little evidence of neuronal cell death following transection of peripheral axons has been reported in similar models. For example, the loss of ChAT expression, reduced soma size, and increased expression of activating transcription factor 3 (ATF3), a nuclear injury marker expressed by all injured neurons (Tsujino et al., 2000), was reported at the sacral level of the spinal cord following pelvic nerve transection (Peddie and Keast, 2011), but there was no evidence of cell death and these responses to injury subsided over time. In the nucleus ambiguus (Chang et al., 2004) and hypoglossal nucleus (Lams et al., 1988; Armstrong et al., 1991), loss of ChAT expression without significant cell death was observed after axonal transection, suggesting that, though ChAT immunoreactivity was reduced, the neurons were not lost. A slight neuronal loss in the hypoglossal nucleus following axonal transection was reported in a different study (Chang et al., 2004), yet there is general agreement that, following injury, an initial loss and then substantial recovery of ChAT expression occurs over time following axonal transection. In an attempt to understand whether similar changes occur in the IML following CST, we documented changes in ChAT expression as well as in the soma size of IML neurons at short term and long term survival points, and also determined whether the injured neurons in the IML expressed ATF3.

Because brain derived neurotrophic factor (BDNF) mRNA in the SCG is thought to play a role in the regulation of the innervating IML neurons (Causing et al., 1997), which in turn express the high affinity full length TrkB receptor (TrkB.FL; Skup et al., 2002; McCartney et al., 2008; Coulibaly and Isaacson, 2012), and alterations in BDNF and TrkB have been observed in the spinal cord following injury (Dougherty et al., 2000; Morcuende et al., 2011; Gomes et al., 2012), we investigated whether BDNF and/or TrkB in the spinal cord were affected by the distal preganglionic axon transection. In the present study, the protein expression of BDNF, as well as TrkB.FL, which elicits signal transduction in the expressing cells (For review see Barbacid, 1995), and truncated TrkB receptor 1 (TrkB.T1), which lacks the tyrosine kinase domain and is thought to play a role in BDNF sequestration (Biffo et al., 1995; Lu et al., 2005), was compared across treatments.

The third objective of this study was to examine the SCG in order to determine whether changes in IML neuronal cell bodies following CST transection were accompanied by alterations in ChAT protein and tyrosine hydroxylase (TH), the rate limiting enzyme in the production of norepinephrine. Because the regeneration of peripheral axons is estimated at ~1–3mm/day (Gordon et al., 2007), the reinnervation of the SCG following transection of the CST would be expected to occur quickly. Following CST transection, Nja and Purves (1977) noted full reinnervation of the guinea pig SCG at 3 months and the return of function in the cat SCG was observed as early as 11.4 days following CST transection (Butson, 1950). Yet, in other models, though ChAT protein expression in the cell bodies of injured neurons returned, the ChAT reinnervation of their peripheral targets remained altered (Wang et al., 1997). Indeed, ChAT activity in the SCG remained reduced at 10 weeks following

CST transection (Tang and Brimijoin, 2002) and, at 6 months, though tyrosine hydroxylase (TH) activity appeared to return to control values, Raisman et al. (1974) observed little ChAT activity and few synapses in the rat SCG, suggesting that the reinnervation of the rat SCG following CST transection proceeded slowly. Here we used western analysis and immunohistochemistry to monitor the changes in ChAT and TH protein expression in the SCG at short term and long term survival times following CST transection. Portions of this study were published in abstract form (Coulibaly et al., 2009; Coulibaly et al., 2011).

METHODS

Animals

Young adult (3 months of age) female Sprague Dawley rats (Harlan Labs, Indianapolis, IN) were housed in the Miami University Animal Facilities in a 12:12 light:dark environment at regulated temperature. The CST was bilaterally transected and animals survived for 1 day (n=8), 1 week (n=8), 3 weeks (n=13), 10 weeks (n=8), or 16 weeks (n=6). Data from these animals were compared with findings from respective age-matched controls that either received sham surgery or underwent no surgical procedures. Because no changes were observed in any of the sham cases when compared to control, data collected from shams and unoperated control groups were pooled to obtain a control groups that corresponded to 1 day-1 week (n=13), 3 week (n=13), 10–16 week (n=12) survival time points. All methods used in this study were approved by the Miami University Institutional Animal Care and Use Committee and efforts were taken to minimize discomfort and pain to the animals and to minimize the number of animals used in the study. Though previous retrograde labeling studies showed that preganglionic neurons projecting to the SCG were located primarily at levels C8- T2, with additional projections from levels T3 to T5 (Rando et al., 1981; Strack et al., 1988; Hosoya et al., 1991; Poulat et al., 1992), robust glial plasticity following CST transection in a previous study was reported primarily at T1 of the spinal cord (Coulibaly and Isaacson, 2012). Therefore all analyses shown in this study were carried out at the T1 level of the spinal cord. T1 level of the spinal cord was identified by counting the nerve roots extending from the cord and the T1 level was verified by noting its location immediately caudal to the cervical enlargement.

Transection of the cervical sympathetic trunk

Rats were anesthetized either with Ketamine (80 mg/kg):Rompun (14mg/kg) cocktail or via the inhalant 2.5% isoflurane. A 3 cm ventral incision was made on the neck region of the animal. The CST was exposed and gently separated from surrounding tissue and transected approximately 2 mm from the entry into the SCG (Sun and Zigmond, 1996). The proximal stump was placed carefully back into original position after the cut in close proximity to the distal stump. The procedure was repeated on the other side. The incision was closed using sutures and tissue glue (Nexaband, Phx, AZ). The CST was exposed, but not transected, in sham animals.

Success of the surgery was assessed by the extent of ptosis following the surgery and post-surgical examination of the transection sites. The level of ptosis was documented bilaterally in each case and was ranked from 0 (no evidence) to 5 (most severe). Ptosis rankings were obtained immediately following the surgery, and on a daily basis for the first week. For longer survival time points, ptosis was documented weekly until just prior to sacrifice, when a final ptosis ranking was obtained.

Tissue collection and immunofluorescence studies

Animals were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Spinal cord and SCG tissues were removed, and stored in

0.1M PB, cryoprotected by infiltrating with 30% sucrose in 0.1M PB, embedded in optimal cutting temperature medium (Ted Pella Inc.), and cut using a MICROM HM 550 series cryostat. Coronal sections of spinal cord (18 μ m) and longitudinal sections of the SCG (12 μ m) were mounted on Superfrost microscope slides, incubated overnight in 0.1M PBS-0.2% Triton-X solution, blocked with normal donkey serum, and then incubated for 48 hrs at 4°C in primary antibody. Spinal cord sections were incubated in a cocktail of goat anti-ChAT (1:200, Millipore) and rabbit anti-ATF3 (1:200; Santa Cruz Biotech), or in rabbit anti-caspase 3 (casp3; 1:200, Cell Signaling) followed by Neurotrace (1:200; Molecular Probes), a Nissl stain conjugated to Alexa 500/525. SCG sections were incubated in goat anti-ChAT (1:200). Following a series of rinses, sections were incubated for 2 hours in AlexaFluor conjugated antibodies (1:200; Molecular Probes) directed against the primary antibody host. Sections then were coverslipped using fluorescent mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vectashield). Images were captured with either an Olympus FV500 or a Zeiss 720 laser scanning confocal microscope.

For casp3 controls, positive and negative control slides were obtained from Cell Signaling Technology (#8104). The prepared paraffin slides of Jurkat cells that were pre-treated with etoposide (positive controls) or received no treatment (negative controls) were de-paraffinized processed for casp3 immunohistochemistry as described above.

TUNEL assay

Spinal cord sections from each survival time point were tested for apoptotic cell death using a TUNEL assay (Roche). Sections were processed according to protocol instructions, and then processed for ATF3, ChAT, or Iba1 (1:500; Abcam) immunolabeling as described above. To validate the protocol, both negative and positive controls were carried out using uninjured spinal cord tissue. The positive control was exposed to 6M HCl for 4 minutes to initiate DNA fragmentation, while the negative controls were processed without terminal deoxynucleotidyl transferase followed by the normal protocol.

Analysis of neurons in the IML

Cell counts were obtained from confocal images that were acquired at 200X magnification using an Olympus FV500 laser scanning confocal microscope and then enlarged to 500X for viewing on a computer monitor using Image Pro software. For the total number of neurons (ChAT only +ATF3 only +ChAT/ATF3) and for the number of ChAT-ir neurons (ChAT only +ChAT/ATF3) in the IML, the number of cells in each category was recorded from the left and right IML from two different spinal cord sections per animal and then averaged to obtain a value for the spinal cord for each animal. In order to provide information regarding the extent of variability within the control sets, a mean was obtained for each set of controls and then the values from each cord in the data set (both controls and treatments) were expressed as the proportion of control and then subjected to statistical analysis. This results in a mean that might be slightly more or less than 100% and allows for a calculated SEM for the control group. This is a more realistic demonstration of the controls rather than merely setting the control group at 100% and comparing treatments to this value. Therefore, a mean was obtained for each set of controls and then the values from each cord in the data set (both controls and treatments) were expressed as the proportion of control and then subjected to statistical analysis. In the first experiment, changes at 1 day and 1 week following transection were analyzed using a one way ANOVA followed by the Fisher post hoc comparison test to compare to one group of age-matched sham controls that survived from 1 day to 1 week following sham surgery. In the second experiment, data obtained from animals at 3 weeks following transection were analyzed using a Student's test compared to age-matched sham controls, while the third experiment assessed long term changes using a one way ANOVA followed by the Fisher post hoc comparison test to compared data

obtained at 10 and 16 weeks following the injury to age-matched sham controls that survived from 12–14 weeks following sham surgery. Because ATF3-ir cells were absent in the control cases, the number of ATF3 cells present at 1 week was compared with the number present at 3 weeks to determine whether any changes occurred between these 2 time points. Significance was reported at $p < 0.05$. All values were reported as mean $\pm$ SEM.

For the determination of cell volume, data were collected using the method previously described in Flak et al. (2009). In brief, two Z-stacks per spinal cord (one stack from the IML on each side) were obtained using images that were acquired at 0.5 μ m intervals (30–40 images) with the 40X oil objective. The volume of at least 4 neurons from each stacked image (and thus at least 8 neurons per cord) was determined by obtaining a mean radius measurement for each neuron and then using the following equation for the volume of the sphere: $V = 4/3 \pi r^3$. The mean radius for each neuron was obtained by measuring the distance from the center of the nucleus to the edge of each cell at 4 different angles: 0, 90, 180 and 270 degrees. Each neuron was analyzed using this approach in 4 different images in the stack to yield 16 radius measurements, which then were averaged for use in the volume equation to obtain a final volume measurement for the neuron. Volumes from individual neurons from the left and right IML were averaged to obtain a value for each spinal cord. Values from each cord in the data set (both controls and treatments) were expressed as the percent control and then subjected to statistical analysis. Because few ChAT-ir neurons were present at 1 week following transection, soma volume was obtained from neuronal profiles in the IML that were stained with Neurotrace (1:200; Molecular Probes) and then compared with the volume of Neurotrace profiles from the age-matched controls. At later time points (3, 10, and 16 weeks) the neuronal volume was measured in ChAT-ir neurons. The values obtained from 1 week and 3 week treatments were compared to respective age-matched sham controls using Student's *t* test. Data from 10 and 16 week treatments were compared to one group of age-matched sham controls that survived 12–14 weeks following sham surgery using one way ANOVA. Significance was reported at $p < 0.05$. All values were reported as mean $\pm$ SEM.

Semi-quantitative western analysis

Protein from hemisections of T1 level of the spinal cord (Macias et al., 2007) and whole SCGs was extracted by sonicating tissue in 0.01M Tris-HCl buffer (pH 7.4) containing 1% sodium dodecyl phosphate (SDS) and 1% protease inhibitor cocktail. Protein concentration of entire SCG or hemisection of the spinal cord at T1 was determined from the supernatant by BCA protein assay and sample preparation was performed in accordance with the Laemmli method (Laemmli, 1970). SDS-PAGE (5% stacking/10% resolving) was used to separate proteins, and a Precision Plus protein standard was used to confirm molecular mass. Following the transfer of 2400mA at 4°C in transfer buffer (25mM Tris, 192 mM glycine, 10% v/v methanol) to PVDF membrane, blots were submerged in methanol, allowed to dry, cut so that the standard was processed separately, and rehydrated in methanol. For the western analysis of TH (SCG), BDNF (cord), and TrkB (cord), the membrane containing protein samples was blocked for three hours in 4% nonfat dry milk in Tris-buffered saline with Tween20 (TBST) and incubated overnight at 4°C in primary antibody (*SCG*: mouse anti-TH, BD Biosciences, 1:200,000; *cord*: rabbit anti-BDNF, N-20, Santa Cruz Biotech, 1:750; rabbit anti-TrkB.FL, 794, Santa Cruz Biotech, 1:500; rabbit anti-TrkB.T1, c13, Santa Cruz Biotech, 1:500) and the membrane containing the standard was incubated in TBST alone. Membranes were rinsed and incubated in appropriate secondary antibody (goat anti-mouse HRP IgG, 1:120,000; goat anti-rabbit HRP IgG, 1:10,000; rabbit anti-goat HRP IgG, 1:10,000; Chemicon) for 2 hours or in streptactin-HRP (for the standard; 1:500,000, Bio-Rad Labs), then rinsed and submerged in Supersignal West Pico Chemiluminescent Substrate for five minutes. Protein was visualized with X-ray film. For western analysis of

ChAT (SCG), membranes were incubated in 4% BSA at overnight at 4°C followed by incubation (2.5 hours) with goat anti-ChAT (1:500, Millipore) and then processed as described above. Blots were then stripped at room temperature, blocked, and re-probed for GAPDH (1:100,000; Fitzgerald Industries International) followed by goat anti-mouse HRP IgG (1:100,000; Chemicon). Films were scanned and densitometry measurements were obtained using ImageQuant 5.2. A ratio of the protein of interest to GAPDH was determined for each case. Prior to statistical analysis, a mean was obtained for each set of controls and each case (both controls and treatments) was expressed as the percent control. These proportional data were then square root transformed for statistical analysis and for presentation in the figures.

For the western analysis of BDNF and TrkB in the spinal cord, changes at one week and 3 weeks following transection were compared to respective age-matched sham controls using Student's t-test. For long term survivals, comparisons of 10 week and 16 week survivals were made to one age-matched control group (sacrificed approximately 12–14 weeks following sham surgery).

For the western analysis of ChAT and TH in the SCG, proportional data obtained from comparisons to age-matched controls at each survival time point were compared across treatments using one way ANOVA followed by the Fisher post-hoc comparison test. As shown in Figure 7, significant differences of each injury time point with the control group as well as any differences between the 10 week and the 16 week survival time point were reported. Significance was reported at $p < 0.05$.

Analysis of ChAT-ir profiles in the SCG

ChAT-ir profiles in the SCG were analyzed from images that were acquired at 200X magnification and then enlarged to 500X for viewing on a computer monitor using Image Pro software. The number of fluorescent profiles in 3–4 different regions within each SCG was determined using Image Pro software and averaged to generate the overall axonal density value for each SCG. A mean density was obtained for the control group and then all values (controls and treatments) were expressed as the percent control. The proportional data obtained from the comparisons to age-matched controls at each survival time point were compared across treatments using one way ANOVA followed by the Fisher post-hoc comparison test. Significant differences with the control group as well as any difference between the 10 week and the 16 week survival time point were reported. Significance was reported at $p < 0.05$. All values were reported as mean $\pm$ SEM.

RESULTS

Analysis of ptosis

The severity of ptosis following CST transection was ranked over the survival period from 0 (no ptosis) to 5 (most severe ptosis). Ptosis typically ranged from 4.5–5.0 at 1 day following transection and remained relatively unchanged (rank of ~4.5) at one week following transection. By 3 weeks ptosis generally was moderately severe with a rank of ~3.5–4.0. Ptosis decreased to a rank of 2 by 10 weeks following transection. Little recovery of ptosis was observed between 10 weeks and 16 weeks following transection with the animals typically exhibiting mild ptosis with a rank of approximately 2 at the time of sacrifice.

Changes in the IML following CST transection

ChAT-ir neurons were prevalent in the IML at cord level T1 taken from the control cases (Fig. 1, 2) with a mean of 7.8 ± 0.8 cells per IML. At 1 day following injury, the number of ChAT-ir neurons was similar to controls and ATF3 neurons were rare, with only two

ATF3-ir neurons present on one side of one of the four cases examined (not shown). At 1 week following transection, a significant 67% decrease in the number of ChAT-ir neurons in the IML was observed (compared to controls) and ATF3 immunoreactivity was prevalent in both ChAT-ir and ChAT non-immunoreactive neurons. Of the total number of ATF3 neurons present at this time point, 72% did not co-localize ChAT, while the remaining 28% of the ATF3-ir neurons also exhibited ChAT immunoreactivity. Though ChAT-ir neurons were significantly reduced at the one week survival time point, no significant change was observed when the total number of IML neurons (ChAT only + ChAT/ATF3 + ATF only) was compared with that obtained from age-matched controls (Fig. 2). The changes observed at one week appeared to be restricted to spinal cord level T1, as no ATF3-ir neurons and no significant changes in ChAT-ir neurons were observed in cord levels T2 (control: 7.8 ± 0.85 vs 1 week transection 6.8 ± 0.97 ; or T3 (control 6.0 ± 0.71 vs 6.8 ± 0.12). Values are provided as mean $\pm$ SEM. or T3.). Values are provided as mean $\pm$ SEM. No ATF3 immunoreactivity was observed in any glial cell type).

At 3 weeks following transection, occasional ATF3-ir neurons were present in the IML in 6 of the 9 injured cases, but there was no evidence of ATF3 in the remaining 3 cases (Figs.1, 2). When compared with the one week time point, a 79% decrease in ATF3-ir neurons was observed (Fig. 2), suggesting that the injury response was subsiding. Although 57% of the ATF3-ir neurons that were present did not co-localize ChAT, the number of ChAT-ir neurons at this survival time point was not significantly different from age-matched controls. Subsequently, the total number of neurons in the IML (ChAT only + ChAT/ATF3 + ATF only) also was not different from that observed in the controls (Figs.1, 2). Similar to that observed in the 1 week animals, the changes observed at 3 weeks appeared to be restricted to spinal cord level T1, as no ATF3-ir neurons and no significant changes in ChAT-ir neurons were observed in cord levels T2 (control: 7.8 ± 0.85 vs 3 weeks transection 6.6 ± 1.02 –) or T3 (control 6.0 ± 0.71 vs 7.3 ± 1.33). Values are provided as mean $\pm$ SEM.

At 10 and 16 weeks following the injury, no ATF3-ir cells were present in the IML. Analysis of the number of ChAT-ir neurons revealed no significant differences compared to age-matched controls (Figs.1, 2).

The neurons in the IML at one week following injury appeared to be smaller in size compared to the controls, similar to that reported in a similar injury model (Peddie and Keast, 2011). In order to assess alterations in cell volume in the IML due to injury and to determine whether any size alterations were sustained at longer survival time points, the cell volume of IML neurons was quantified and compared to age-matched controls. The typical cell volume for preganglionic neurons in the each of the control groups averaged approximately $1,100 \mu\text{m}^3$. At 1 week following injury the volume of IML neurons was significantly decreased by 50% (Fig. 3). The volume of ChAT-ir neurons in the IML at later survival time points was similar to that of their age-matched controls (Fig. 3).

Because previous reports have indicated the possibility of cell death in the IML following CST transection (Tang and Brimijoin, 2002), a TUNEL assay was carried out on tissues taken from animals surviving 1 week, 3 week, and 10 week and 16 weeks following injury. TUNEL positive neurons were observed in the positive control (HCl treated) tissues (Fig. 4). However, in the treatment cases, TUNEL positive neurons were essentially absent. At 1 week post injury, occasional small profiles showed positive staining for TUNEL and these typically co-localized Iba1 (Fig. 4C insets), a marker for microglia in the CNS (Imai et al., 1996). No TUNEL positive neuronal profiles were observed. At 3 weeks following transection, no TUNEL positive staining was observed in the IML (Fig. 4). At the 10 week survival time point, one neuron, which co-localized ChAT (not shown), was positive for TUNEL in the five cases studied and of the 25 neurons that were examined at this time

point. No evidence for cell death was observed at the 16 week time point (Fig. 4) and no TUNEL staining was observed in any ATF3-ir neurons (not shown).

The localization of casp3, an early and definite sign of apoptotic cell death (Deshmukh and Johnson, 1997; Radović et al., 2008), was assessed in the IML to confirm the lack of cell death in the injured cases. The general neuronal cell body marker, Neurotrace, a fluorescent Nissl stain, was used to identify the IML neurons. Qualitative analysis showed that Neurotrace labeled most of the neurons in the IML as well as a few glial cells. Casp3-ir cells were observed in the positive controls (Fig. 5A), yet no casp3-ir neurons were found in IML neurons at any time points following injury (Fig. 5), supporting the idea that no neuronal death occurred in the IML following distal axonal transection. At the 10 week and 16 week time points, small casp3-ir cells, which did not co-localize Neurotrace, were present in both the controls and the treatment cases (Figs. 5C, G, H) and were likely glial in nature.

Changes in spinal cord BDNF and/or TrkB following CST transection

The effects of CST transection on BDNF in the spinal cord were evaluated using western analysis. Though unchanged at 1 week, the mature BDNF isoform (mBDNF; 15 kDa) was generally increased in the spinal cord over the survival period. Mature BDNF was significantly increased by 34% in the spinal cord at 3 weeks following injury, and though the increase in mBDNF at 10 weeks did not reach significance, a significant 80% increase in mBDNF was observed at the 16 week survival time point. The proBDNF isoform (32–37 kDa) was unchanged at 1 week following injury and, following an increase at the 3 week survival time point, returned to control values at 10 weeks. However, by 16 weeks, at a time point when mBDNF reached its highest levels, proBDNF protein was significantly decreased by 59% compared with age-matched controls (Fig. 6).

The changes in BDNF observed in the spinal cord following CST transection were accompanied by changes in the TrkB receptor. TrkB.FL was significantly increased by 40% at 1 week following injury (Fig. 6), at a time when ATF3-ir expression in the IML was prevalent, the number of ChAT-ir neurons was significantly decreased, and the glial cells were shown in a previous study to be increased (Coulibaly and Isaacson, 2012) (Coulibaly and Isaacson, 2012). At the same survival time point, a significant 64% decrease was observed in protein levels of TrkB.T1. No changes in TrkB.FL or TrkB.T1 were observed at 3 weeks. While TrkB.FL protein was unchanged at 10 weeks and 16 weeks following injury, a significant decrease in TrkB.T1 was observed at 10 weeks, though by 16 weeks, the levels of TrkB.T1 protein expression rebounded and were similar to control values (Fig. 6).

Changes in the SCG following CST transection

ChAT-ir profiles in the SCG from control cases were obvious with dense immunopositive fibers coursing through the ganglion and targeting neuronal cell bodies (Fig. 7). Following CST transection, ChAT-ir fibers in the SCG were significantly decreased at every time point examined. As expected, a depletion of ChAT-ir fibers was observed at 1 day and 1 week following CST transection. At later time points, a slight recovery in the number of ChAT-ir fibers was observed in the SCG (Fig. 7), and by 16 weeks, though still decreased compared with the controls, the density of ChAT-ir fibers was significantly increased compared to the 10 week time point (Fig. 7). Semi-quantitative western blot analysis of ChAT protein in the SCG showed similar trends, with little ChAT detected in the SCG at 1 day, 1 week, 3 weeks and 10 weeks following injury. However, though significantly reduced compared to controls, ChAT protein in the SCG at 16 weeks was significantly increased compared to the 10 week time point, indicating that some recovery may be taking place. TH western analysis in the SCG revealed a significant decrease in TH in the SCG at early time points, but by 16 weeks, the levels of TH protein were similar to controls (Fig. 7).

DISCUSSION

The effects of distal CST transection on preganglionic neurons in the IML were documented at short and long term survival time points. The most obvious changes in preganglionic neurons in the IML occurred at one week post injury, as demonstrated by decreased cell soma volume, decreased number of ChAT-ir neurons, and increased number of neurons that expressed the injury marker ATF3, majority of which did not co-localize ChAT. When total number of ChAT-ir and ATF3-ir neurons was considered, no significant cell loss was observed. These changes were transient, and at later time points, ChAT expression and soma volume returned to control values and the number of ATF3 neurons declined. Because no evidence for neuronal loss or apoptosis was detected at any survival time point examined in this study, we conclude that no significant neuronal death occurred in the IML at short term or long term time points following CST transection. The morphological changes at early survival time points were accompanied by alterations in BDNF and TrkB protein in the spinal cord and, though no obvious morphological changes were observed at long term time survivals, BDNF and/or TrkB were altered at the longer survival times. The CST transection initially resulted in complete denervation of the SCG, and the SCG was not yet fully innervated by ChAT-ir fibers at the 16 week survival time point. Though significantly decreased at early time points, TH protein levels in the SCG were similar to controls at 16 weeks following the CST transection.

Cell survival following peripheral injury

There was little evidence for cell death in the IML following CST transection at any of the time points examined in this study. These findings are similar to that observed in other models. For example, though the number of ChAT-ir neurons was reduced in the hypoglossal and vagal nuclei at 4 weeks following axotomy, there was no loss of neurons when evaluated using Nissl stain (Lams et al., 1988), suggesting that, though ChAT immunoreactivity was lost, the neurons were present. Results in a subsequent study of the hypoglossal nucleus following peripheral axon transection paralleled these findings (Armstrong et al., 1991), although a slight reduction in ChAT-ir neurons in the hypoglossal was reported by Chang et al. (2004). Loss of ChAT immunoreactivity without cell death also was observed in the nucleus ambiguus after axonal transection (Chang et al., 2004). Similarly, the loss of ChAT expression without cell death was reported at the sacral level of the spinal cord following pelvic nerve transection (Peddie and Keast, 2011). In that study, a reduction in cell soma size, a reduction in ChAT expression, and an increase in ATF3 were observed at one week. Yet by 4 weeks following the lesion, the neurons appeared similar to controls and no neuronal loss was observed. We also observed the most robust changes at the 1 week survival time point, with the return of ChAT expression and cell volume to control values occurring by 3 weeks following the injury.

Yet, unlike our results in the IML and previous findings in other models, Tang and Brimijoin (2002) reported a significant loss of sympathetic preganglionic neurons in the IML at 10 weeks following transection of the CST. In that study, the fluorescent retrograde label True Blue was injected into the SCG at the time of injury and analysis of cell loss in the IML involved counting retrogradely labeled cellular profiles. In addition, when the number of ChAT-ir neurons in the IML was considered, a significant decrease at the T1 level of the cord was observed at 10 weeks. No significant loss was reported at T2 or T3. Though we observed a trend for a decrease in ChAT-ir neurons in the IML in T1 at 10 weeks following CST transection, the decrease did not reach significance, and by 16 weeks following the injury, the number of neurons in the IML was relatively similar to control values. Thus, it appears that most neurons survived the injury, and by 16 weeks following the injury were able to begin restoring normal preganglionic innervation of the SCG. Similar to our results, in which a TUNEL assay yielded little evidence for neuronal cell death in the

IML following CST transection, a TUNEL assay carried out by Tang and Brimijoin (2002) yielded no apoptotic neurons over the 10 week survival period. In addition, though we observed small casp 3-ir profiles that were not positive for Neurotrace at 10 weeks and 16 weeks in both controls and treatments, no neuronal profiles were detected. Overall, these findings lead to the conclusion that no significant loss of preganglionic neurons occurred in the IML over the 16 week survival following CST transection.

Role of ATF3 following injury

Following injury, the neuronal cell body undergoes a variety of changes known as neuronal reaction and chromatolysis (Navarro et al., 2007). Some of the changes include synthesis of new proteins, such as the transcription factor ATF3, a protein that has been proposed as a marker for injured neurons (Tsujino et al., 2002; Kataoka et al., 2007). It is thought that ATF3, a member of the CREB/ATF family, and an immediate early gene (Tarn et al., 1999; Hai and Herman, 2001), is activated by retrograde signals from the injury site (Abe and Cavalli, 2008). As with many of the proteins involved in gene transcription following injury (Abe and Cavalli, 2008), the role played by ATF3 in the survival of the injured neuron remains unclear. However there is evidence suggesting that ATF3 expression in neurons following injury has a neuroprotective effect, and that a relationship exists between ATF3 expression and axonal regeneration and neuroprotection (For review, see Hunt et al., 2012). Therefore we hypothesize that ATF3 did not play a detrimental role in the IML following transection of the CST, but may have served a neuroprotective role to promote neuronal survival and regeneration.

Similar to other models, the expression of ATF3 was transient in nature with a significant reduction in the number of ATF3-ir neurons in the IML at 3 weeks following the CST transection. Peddie and Keast (2011) observed that ATF3 protein peaked in the sacral spinal cord at 1 week following pelvic nerve injury, and then decreased by 4 weeks following the injury. Tsujino and colleagues (2002) found that ATF3 gene expression was relatively weak in the spinal cord at 1 day following sciatic nerve transection, peaked from 3–7 days, and gradually decreased so that it was diminished at 3 weeks, and then “faded away” by 6 weeks.

It has been proposed that ATF3 expression decreased once the regenerative process was underway in the DRG (Seijffers et al., 2007). However, in our model, a decrease in ATF3 expression was observed at 3 weeks post-injury, yet ChAT reinnervation in the SCG was not complete even at 16 weeks post-injury, suggesting that the *initiation* of the regenerative process rather than the reinnervation of the target can lead to a decrease in ATF-3 expression. The slow reinnervation of the SCG was reflected by the ptosis, which was evident at 16 weeks following injury. The hypothesis that ATF-3 expression decreases following initiation of regeneration rather than target reinnervation is supported by other models (Matsuura et al., 1997; Wang et al., 1997).

Regulatory influences on spinal cord neurons: effects of injury

In the present study BDNF protein in the spinal cord was increased following CST transection and may have contributed to the neuronal response that took place following injury. Neurotrophin signaling appears to play an important role in the neuronal response to injury

injury (Sofroniew et al., 1993; Yan et al., 1994; Yin et al., 2001; Spalding et al., 2005; Sahenk et al., 2008) by affecting cell survival (Hammond et al., 1999; Ichim et al., 2012), axonal regeneration (Oudega and Hagg, 1999; Yin et al., 2001) and target reinnervation (Michalski et al., 2008) and it is known that BDNF plays an important role in the

maintenance of motoneurons in the adult spinal cord (Koliatsos et al., 1993) and in the survival of motor (Koliatsos et al., 1993; Boyd and Gordon, 2001; Garraway et al., 2011) and sensory neurons (Song et al., 2008) following spinal cord injury. Therefore we postulate that BDNF and/or TrkB served a neuroprotective role to prevent neuronal death in the IML following the injury.

Sympathetic preganglionic neurons in the IML co-localize TrkB.FL (Skup et al., 2002; McCartney et al., 2008; Coulibaly and Isaacson, 2012) indicating their responsiveness to BDNF. BDNF mRNA is expressed in the SCG (Causing et al., 1997), and thus BDNF protein originating from the SCG may regulate sympathetic preganglionic neurons in the IML (Causing et al., 1997). The transection of the CST would interrupt any retrograde signals derived from the SCG and therefore affect BDNF protein levels in the spinal cord. Yet BDNF levels were increased in the spinal cord, even though reconnection with the SCG was incomplete. Other models have demonstrated that BDNF production is induced in glial cells, including microglia, astrocytes and CD4 cells in the central nervous system following peripheral injury (Dougherty et al., 2000; Jones et al., 2005). It is therefore possible that the activated glial cells which were documented in previously in this injury model (Coulibaly and Isaacson, 2012) increased the extracellular availability of BDNF, thereby supplying trophic support to the injured IML neurons.

Changes in Trk receptor expression can influence neurotrophin activities. In the course of this study, changes were observed in both full length and truncated BDNF receptors. An increase in TrkB.FL occurred at one week following injury, at a time many other responses in the IML were taking place. Others have shown that TrkB expression is altered following injury (Morcuende et al., 2011; Gomes et al., 2012), suggesting that it is important for the neuronal response following injury, possibly to facilitate regeneration (Hollis et al., 2009). A significant 64% decrease was observed in TrkB.T1 at the one week survival time point. Though TrkB.FL and TrkB.T1 isoforms, which were abundant in the brain and spinal cord, show 100% homology in their extracellular domains (Barbacid, 1995), TrkB.T1 possesses an intracellular domain that lacks tyrosine kinase activity. The increase in TrkB.FL may have contributed to neuronal survival (Koliatsos et al., 1993; McTigue et al., 1998), while decreased TrkB.T1 may reflect a reduced role in ligand sequestration (Biffo et al., 1995) to enhance BDNF availability for neuroprotective purposes.

The alterations in BDNF and TrkB may have played a role in restoring ChAT expression in the IML following CST transection. There is evidence that BDNF availability regulates the expression of ChAT (Sofroniew et al., 1993; Garcia et al., 2010).

Reinnervation of the SCG following CST transection

The reinnervation of the SCG following CST transection can provide one measure of the regenerative potential of the IML neurons. Functional studies showed recovery (i.e. pupil size) in both the rabbit and cat by two weeks after transection of the CST (Langley, 1895; Butson, 1950). In addition, following CST transection, Nja and Purves (1977) noted full reinnervation of the guinea pig SCG at 3 months and return of function of the cat SCG was observed as early as 11.4 days (Butson, 1950). Therefore we expected full reinnervation at the 3 week post injury time point. Indeed, based on regenerative growth rate of 1–3 mm/day (Olson, 1969; Kirpekar et al., 1970; Gordon et al., 2007) and that the distance from the CST stump to the SCG is only a few millimeters, we were surprised to discover that, even at 4 months following transection, the number of ChAT-ir fibers and levels of ChAT protein in the SCG remained significantly reduced.

It is curious that ChAT reinnervation remained significantly decreased at the 10 and 16 week time points, even when ChAT in the IML appeared similar to controls and no ATF3

neurons were present yet this lack of reinnervation may explain the presence of moderate ptosis even at long term survival time points. Tang and Brimijoin (2002) also observed reduced ChAT activity in the SCG at 10 weeks following CST transection, and Raisman et al. (1974) observed little ChAT activity and few synapses in the rat SCG at long survival time points following CST transection. In fact, following compression injury of the CST revealed that connections within the SCG were still pathological after one year (Serebryakova, 2008). Others have shown in similar models that little target reinnervation took place even though ChAT re-expression tended to occur (Wang et al., 1997). These studies, which are consistent with our findings, suggest that the reinnervation of the rat SCG following CST transection proceeded very slowly. Though ChAT protein levels remained depressed, TH protein appeared similar to controls at the 16 week time point. This is consistent with Raisman et al. (1974), who reported little ChAT activity and few synapses in the rat SCG at 6 months following CST injury while TH activity appeared to return to control values. We conclude that SCG reinnervation following CST transection was a slow process, and that, though overt changes in IML neurons had subsided, the IML neurons remained focused on recovery for long periods following the injury. Yet the relatively slow reinnervation of the SCG, however, did not affect the recovery of TH protein expression by the SCG neurons.

CONCLUSIONS

In addition to the robust glial response in the vicinity of the parent cell bodies in the IML following CST transection (Coulibaly and Isaacson, 2012), The neuronal cell bodies of transected axons in the IML showed transient changes in ChAT and ATF3 expression as well as soma volume that were paralleled by significant changes in BDNF and TrkB expression in the spinal cord. The most robust neuronal changes were observed at one week following the injury, at a time glial plasticity also was robust. TrkB.FL protein was increased at one week, suggesting that BDNF/TrkB signaling may play a role in both the neuronal and glial responses to distal axon injury. Though the morphological changes tended to subside at longer survival time points, BDNF remained increased throughout the survival period as the reinnervation of the SCG proceeded. We conclude that BDNF and/or TrkB may have played a role in the recovery of ChAT expression and neuroprotection in the IML at short term time points, and may have served to facilitate the reinnervation of the SCG, which proceeded slowly over the 16 week survival period.

Acknowledgments

This study was supported by NIH NS051206 awarded to LGI and NSF DBI-0821211 awarded to the Center for Advanced Microscopic Imaging, Miami University. We thank Matt Duley and Richard Edelmann for their assistance with confocal microscopy and image analysis, Zoe Hesp and Kara Francis for their help with the quantitative analysis of the SCG, and James Oris, Miami University for his help with the statistical analysis.

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Figure 1.

Transient changes in ChAT and ATF3 following CST transection. All images depicted are coronal sections of T1 spinal cord. **A.-C.** ChAT-ir neuronal cell bodies (green, yellow arrows) were observed in the control IML, shown here at cord level T1. No ATF3 profiles were present (red). **D.-F.** At one week following injury, ChAT immunoreactivity was reduced and few ChAT-ir neurons (green, yellow arrows) were present. White arrows mark the location of IML neurons expressing the nuclear injury factor ATF3 (red), most of which lacked ChAT immunoreactivity. One neuron (asterisk) showed immunoreactivity for both ChAT and ATF3. **G.-I.** ChAT-ir neurons (green, yellow arrows) appeared similar to controls

by 3 weeks following the injury. Few neurons expressed ATF3 immunoreactivity (red, white arrows). The ChAT-ir neurons in the IML at 10 weeks (**J.-L.**) and 16 weeks (**M.-O.**) appeared similar to controls. In the right column, merged images (ChAT + ATF3) were combined with DAPI to indicate the presence of cellular profiles. All images, scale bar = 50 μm .

Figure 2.

The total number of preganglionic neurons remained unchanged following CST transection.

A. The number of ChAT-ir was significantly decreased at 1 week following CST transection but was similar to age-matched sham controls at all other time points. **B.** Though ChAT-ir neurons were decreased, the preganglionic neurons were still present and expressed the nuclear injury marker ATF3. ATF3-ir neurons were observed at 1 day, 1 week, and 3 weeks following the injury, were most abundant at the 1 week survival time point, and significantly increased compared with the 1 day time point. ATF3-ir was diminished at 3 weeks (compared to 1 week). No ATF3-ir profiles were observed in any of the controls or at 10

weeks or 16 weeks following injury. C. Analysis of the *total* number neurons (ChAT + ATF3) revealed no significant differences across the treatments following CST transection. Error bars represent the standard error of mean. n=3–7 cases per group. *, significantly different from controls at $p < 0.05$; np, not present.

Figure 3.

Reduced soma volume observed at one week was reversed at the long term survival time points. A significant decrease in the volume of ChAT-ir neurons was observed at 1 week following the injury. However volumes were similar to controls at later time points. Error bars represent the standard error of mean. n=3–6 cases per group. *, significantly different from controls at $p < 0.05$.

Figure 4.

No TUNEL positive neurons were observed in the IML following CST transection. **A.** TUNEL staining in the positive control (HCL treated) showed numerous positive nuclear profiles. DAPI in **A'** shows the cellular nature of the positive staining. **B.-B'**. No TUNEL staining was observed in the negative control tissues. **C.-C'**. At one week following injury, small TUNEL/DAPI profiles were frequently observed and were found to co-localize Iba1 (inset), indicating their microglial phenotype. **D.-F.** No TUNEL positive staining was observed at longer survival time points. n=3–5 cases per group. A.-F.', scale bar = 50 μ m. Inset, scale bar = 10 μ m.

Figure 5.

No caspase 3 (casp3)-ir neurons were observed in the IML following CST transection. **A.** Casp3-ir staining in the positive controls showed numerous immunopositive profiles. DAPI in **A'** shows the cellular nature of the positive profiles. **B.-B'**. No casp-ir profiles were observed in the negative control tissues. **C.** Small casp3 immunoreactive profiles (inset) were observed in the 16 week sham control cases. These profiles, which were present in both controls and the 16 week injury animals (see **H.**), did not co-localize Neurotrace (Neuro) (see **H'**. which shows lack of Neurotrace staining with casp3 profiles at 16 week survival time point in **H.**) and were likely glial in nature. **D.-H.** No evidence of casp3-ir neurons was observed at any time points examined. n=3–5 cases per control group and treatment. A.-H.', scale bar = 50 μ m. Inset, scale bar = 10 μ m.

Figure 6.

Increased mature BDNF (mBDNF) and full length TrkB (TrkB.FL) in the spinal cord following CST transection. Representative bands from time points in which significance was observed are shown along with respective age-matched control taken from the same membrane. **A.** Western blots analysis of mBDNF revealed no changes were at one week following injury, yet mBDNF levels generally were increased over longer survival time points. mBDNF levels were significantly increased at 3 weeks and 16 weeks following injury. Protein expression of proBDNF was increased at 3 weeks, but the levels decreased over time and was significantly decreased at 16 weeks. **B.** At one week following injury,

increased TrkB.FL was paralleled by a decrease in TrkB.T1. TrkB.T1 was decreased at 10 weeks following injury but was similar to controls by 16 weeks. n=3–5 cases per group. Error bars represent the standard error of mean. *, significantly different from controls at $p < 0.05$.

Figure 7.

ChAT and TH in the SCG following loss of afferent input. **A.** ChAT-ir fibers can be observed coursing through the SCG from controls (cont). At 1 day (1d) and 1 week (1 wk) following CST transection only a few ChAT-ir fibers were present. Frequent ChAT-ir fibers could be seen in the SCG at later time points, yet the numbers appeared much less than controls. Scale bar = 50 μ m. **B.** Quantitative analysis revealed that the number of ChAT-ir fibers was significantly decreased at each time point examined. However ChAT-ir fibers were increased at 16 weeks compared with the 10 week time point, indicating a potential recovery taking place. **C.** ChAT protein in the SCG as determined by western analysis yielded similar results to the immunohistochemical analysis, with significant decreases in ChAT at each time point examined, but an increase at 16 weeks compared with the 10 week time point. **D.** TH protein in the SCG was significantly decreased early, but was similar to controls by 16 weeks following the injury. $n=3-5$ cases per group. Error bars represent the standard error of mean. *, significantly different from controls at $p < 0.05$; #, significantly different from 10 week survival time point.