ABSTRACT

PLASTICITY IN THE INTERMEDIOLATERAL CELL COLUMN OF THE SPINAL CORD FOLLOWING INJURY TO SYMPATHETIC POSTGANGLIONIC AXONS

by

Sean Michael Gannon

The effects of transection of sympathetic postganglionic axons on uninjured, preganglionic neurons arising from the spinal cord were examined in this study. Protein levels of choline acetyltransferase (ChAT) were decreased in the superior cervical ganglion after axotomy at short term and long term survival time points, suggesting a response by the uninjured ChAT preganglionic axons. Yet no parallel changes were observed in ChAT protein expression or in the morphology of preganglionic somata in the spinal cord intermediolateral cell column. However, at long term survival time points following the axotomy, a decrease in the number of synaptic boutons making contact with the somata of IML neurons was observed, suggesting that injury to peripheral axons can result in chronic retrograde transsynaptic changes and providing evidence that the effects of peripheral injury can cascade into the central nervous system.
PLASTICITY IN THE INTERMEDIOLATERAL CELL COLUMN OF THE SPINAL CORD FOLLOWING INJURY TO SYMPATHETIC POSTGANGLIONIC AXONS

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<tr>
<td>ACA</td>
<td>Anterior cerebral artery</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<td>ChAT</td>
<td>Choline acetyltransferase</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CRF</td>
<td>Corticotropin-releasing factor</td>
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<tr>
<td>CRF1</td>
<td>Corticotropin-releasing factor receptor 1</td>
</tr>
<tr>
<td>CRF2</td>
<td>Corticotropin-releasing factor receptor 2</td>
</tr>
<tr>
<td>CST</td>
<td>Cervical sympathetic trunk</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>IML</td>
<td>Intermediolateral cell column</td>
</tr>
<tr>
<td>IOD</td>
<td>Integrated Optical Density</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NT-4</td>
<td>Neurotrophin 4</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
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<tr>
<td>RVM</td>
<td>Rostral ventromedial medulla</td>
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<tr>
<td>SCG</td>
<td>Superior cervical ganglion</td>
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<tr>
<td>SG</td>
<td>Stellate ganglion</td>
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<tr>
<td>SGC</td>
<td>Satellite glial cells</td>
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<tr>
<td>SPN</td>
<td>Sympathetic preganglionic neuron</td>
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<tr>
<td>syn</td>
<td>Synaptophysin</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween20</td>
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<tr>
<td>TrkB.FL</td>
<td>full-length tyrosine receptor kinase B</td>
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1. **PROJECT SUMMARY**

The goal of this project was to determine whether injury to peripheral axons results in retrograde transneuronal effects on undamaged neurons in the central nervous system (CNS). Sympathetic postganglionic axons that arise from neuronal somata in the superior cervical ganglion (SCG) were transected (axotomy) and the uninjured sympathetic preganglionic neurons (SPNs) that innervate the injured SCG neurons were examined for potential retrograde effects. Because previous studies have shown that preganglionic axonal endings in the SCG were remodeled following axotomy of postganglionic SCG axons, and that alterations in target-derived growth factors may play a role in this plasticity, we hypothesized that preganglionic neuronal somata in the spinal cord would be affected by the injury to postganglionic axons. The effects of peripheral injury to SPNs were assessed by examining the number and morphology of choline acetyltransferase (ChAT) expressing neurons in the spinal cord intermediolateral cell column (IML), and assessing any changes in synaptic input to IML neuronal somata. We found that postganglionic axotomy resulted in reduced ChAT protein expression in the SCG, but no discernible effects on the ChAT-immunoreactive (-ir) SPNs in the IML were observed. However, at long term time points, the number of synaptic boutons contacting IML somata was decreased, and corticotropin-releasing factor (CRF) fibers in the IML were increased. These findings suggest that injury to peripheral axons can result in chronic retrograde transneuronal changes in the CNS, and provide evidence that the effects of peripheral injury can cascade into the CNS.
2. **INTRODUCTION**

2.1 *The injury model*

The objective of this study was to characterize the effects of peripheral nerve injury on the CNS. Specifically, the experiments tested the hypothesis that transection (or axotomy) of the axons of postganglionic neurons of the superior cervical ganglion (SCG) will lead to retrograde transneuronal changes in the intermediolateral cell column (IML) of the spinal cord. We conducted a detailed analysis of the sympathetic preganglionic neurons (SPNs) in the IML of the rat spinal cord following axotomy of postganglionic axons arising from the SCG, the most rostral of the sympathetic chain ganglia (Purves et al., 2012; See Figure 1).

The majority of retrograde tracing studies have shown that the IML neurons span from the eighth cervical (C8) to fifth thoracic (T5) spinal cord segments (Rando et al., 1981; Appel and Elde, 1988; Strack et al., 1988; Strack et al., 1989; Pyner and Coote, 1994a; 1994b). The primary innervation to the SCG is provided by IML neurons located between segments T1 and T3 (Rando et al., 1981; Appel and Elde, 1988; Strack et al., 1988; Pyner and Coote, 1994a; 1994b). The transection of preganglionic axons showed the greatest effects at the T1 cord segment with decreased ChAT expression (Tang and Brimijoin, 2002; Coulibaly et al., 2013) and gliosis (Coulibaly and Isaacson, 2012), suggesting that T1 is a primary source of input to the SCG.

The majority of preganglionic neurons are located in the IML and the nearby lateral funiculus, but they have also been observed in the intercalated nucleus and the central autonomic nucleus of the spinal cord (Rando et al., 1981; Barber et al., 1984; Strack et al., 1988; Hosoya et al., 1991). The preganglionic axons enter the ganglion as the cervical sympathetic trunk (CST) to innervate the SCG neurons. In turn, the postganglionic axons arising from the SCG innervate multiple targets in the head that include cerebral blood vessels (Arbab et al., 1986; Hesp et al., 2012), irides, submandibular glands, and the pineal gland (Luebke and Wright, 1992).

2.2 *Transneuronal effects of neuronal injury: role of target-derived neurotrophin*

The experiments in this study tested the hypothesis that uninjured SPNs can be affected by injury to postganglionic axons. Several previous studies have described changes in preganglionic axons innervating the SCG following injury to postganglionic axons that arise from the SCG. The axotomy or crush of SCG axons resulted in a decrease in preganglionic
synapses onto SCG neurons (Matthews and Nelson, 1975; Purves, 1975; Nja and Purves, 1978; Smolen, 1983; Paggi et al., 2006). The preganglionic innervation of small intensely fluorescent cells in the SCG increased nearly 4.5 times after postganglionic axotomy and remained increased (compared to controls) at 128 days after injury (Case and Matthews, 1986). The decreased synaptic input may be mediated by a decrease in the expression of subunits of the nicotinic acetylcholine receptor (nAChR) by the SCG neurons (Zhou et al., 1998; Yeh et al., 2001; Zhou et al., 2001; Paggi et al., 2006).

There is evidence that a reduction or alteration in target-derived neurotrophin in the SCG following axotomy can play a role in the transneuronal effects observed following axotomy. It is well known that target-derived neurotrophins such as nerve growth factor (NGF) are necessary for the normal maintenance and survival of innervating neurons (reviewed in Chowdary et al., 2012) and that the transection of an axon results in the disconnection of the soma with its target and subsequently prevents the retrograde transport of neurotrophins to the soma. It has been shown previously that the transection of SCG postganglionic axons disrupts the retrograde transport of NGF from peripheral targets (Hendry et al., 1974; Shoemaker et al., 2006; Walker et al., 2009), suggesting that the loss of target-derived NGF may play a role in any effects observed following axotomy. Further, Nja and Purves (1978) observed that, in addition to injury to postganglionic axons, injection of NGF antibody which reduces NGF levels in the SCG was sufficient to decrease the number of preganglionic synapses in the SCG.

The finding that NGF antiserum can alter preganglionic innervation of postganglionic neurons suggests that NGF plays a role in the maintenance of preganglionic synapses in the SCG and that the loss of NGF in the SCG that occurs following axotomy may lead to the observed remodeling of preganglionic input in the SCG. Indeed, the altered expression of nAChRs, which is implicated in the reduction in synaptic input observed in the SCG (Zhou et al., 1998; Yeh et al., 2001; Zhou et al., 2001; Paggi et al., 2006) may result from loss of NGF, since the application of NGF after axotomy partially rescued nAChR expression in the SCG (Zhou et al., 1998; Yeh et al., 2001).

There is evidence suggesting that transneuronal effects of injury can occur in other models. Johnson and Cowey (2000) observed a loss of retinal ganglion cells in the macaque following removal of the portion of a striate cortex corresponding to the macular retina, suggesting a transneuronal retrograde effect on the uninjured retina originating from the injury.
site. In addition, Leong and colleagues (2011) observed a 23% decrease in the number of neurons in the ipsilateral rostral ventromedial medulla (RVM) following ligation of the fifth lumbar spinal nerve of rats. Compared to controls, the number of serotonergic neurons in the ipsilateral RVM was reduced and serotonergic varicosities in the ipsilateral substantia gelatinosa were also significantly reduced. Nguyen and colleagues (2011) reported extensive remodeling of the preganglionic cholinergic innervation to the stellate ganglion (SG) after an induced heart attack in rabbits. In conclusion, these studies demonstrate that CNS neurons can be affected by a downstream injury.

2.3 Regulation of preganglionic input to the SCG

While it is well-characterized that NGF regulates postganglionic neurons in the SCG, the neurotrophin regulation of the innervating SPNs is not well understood. Because the loss of NGF can affect preganglionic synapses in the SCG, it was initially thought that NGF may regulate SPNs as well as postganglionic neurons. Yet the findings of Schwab and Thoenen (1977) suggested an indirect role for NGF in regulating preganglionic synaptic input. These investigators compared the rate of NGF and textanus toxin transport to the rat SCG after injection into the eye and found that NGF did not pass beyond the dendrites and somata of SCG neurons, while tetanus toxin continued to the innervating synaptic terminals. This finding provides evidence that NGF may regulate the production of a different neurotrophin in the SCG, which in turn regulates the SPNs.

There is evidence to suggest that BDNF and/or NT-4 may regulate preganglionic input to the SCG. Neurons of the IML express the full-length tyrosine receptor kinase B (TrkB.FL; McCartney et al., 2008; Coulibaly and Isaacson, 2012), which is the receptor for the neurotrophins brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT-4). In addition, SCG neurons have been found to express mRNA for BDNF (Causing et al., 1997; Roosen et al., 2001) and BDNF protein (Wetmore and Olson, 1995; Hawk, 2013) and also express NT-4 protein (Roosen et al., 2001). Causing and colleagues (1997) proposed that BDNF protein derived from the SCG was likely to be retrogradely transported by SPNs to the IML to regulate preganglionic neurons. While Roosen and colleagues (2001) found that mice bred to express little or no NT-4 showed a 23% decrease in the number of SPNs in segments T1 to T5, the IML neurons that specifically project to the SCG were not as affected as other neurons.
(Roosen et al., 2001), suggesting that NT4 may not play an important role in the regulation of the SPNs that project to the SCG.

Previous studies in our lab suggest that BDNF is a likely candidate for the normal regulation of SPNs that project to the SCG. Axotomy of the SCG resulted in a significant decrease in the amount of proBDNF in the SCG at 7 days after injury (Hawk, 2013). The decrease in SCG proBDNF, which is cleaved into mature BDNF before being taken up by TrkB.FL (Lu et al., 2005), is mirrored by a decrease in mature and proBDNF in the IML at 7 days (Hawk, 2013). This finding suggests that a decrease in BDNF in the SCG may lead to a decrease in BDNF in the IML and that postganglionic axotomy has significant effects on the neurotrophin expression by uninjured neurons in the IML.

2.4 Effects of SCG axotomy beyond the preganglionic neurons

The overall objective of this study was to understand whether peripheral axon injury might affect neurons in the CNS. One specific aim was to determine whether injury to postganglionic axons might affect not only the SPNs, but also influence the neurons that provide input to the preganglionic cells bodies in the IML. Synaptic input to the SPNs may be affected by a phenomenon known as synaptic stripping that is associated with the soma of an injured axon (Blinzinger and Kreutzberg, 1968; Yamada et al., 2011). The removal of synaptic input to the soma in response to axon injury is thought to involve microglia and astrocytes, which tend to envelope the neuronal somata and, therefore, displace afferent synaptic input to the neuron (Blinzinger and Kreutzberg, 1968; Yamada et al., 2011). The phenomenon may have a neuroprotective effect on the injured neurons (Yamada et al., 2011).

The IML neurons receive projections from the paraventricular nucleus (PVN) of the hypothalamus which utilize corticotropin releasing factor (CRF) as a neurotransmitter (Jansen et al., 1995), as well as the A5 cell group, the rostral ventrolateral medulla, ventromedial medulla, caudal raphe nuclei, mesencephalic central gray matter, and the lateral hypothalamic area (Strack et al., 1989). Numerous studies have focused on the connection between the PVN and the SPNs (Hosoya et al., 1991; Ranson et al., 1998; Pyner and Coote, 1984b) and found direct apposition of anterogradely labeled fibers from the PVN onto both ipsilateral and contralateral SPNs that project to the SCG (Ranson et al., 1998). In addition, single neurons in the PVN can provide dual innervation to SPNs and innervate those that project to the SCG as well as neurons that
project to the SG (Pyner and Coote, 1984b; Jansen et al., 1995). CRF fibers, thought to arise from the PVN, have been observed densely aggregating through lamina VII of the spinal cord, especially around the IML of the cat (Krukoff, 1986), mouse (Korosi et al., 2007) and rat (Merchenthaler et al., 1983; Merchenthaler, 1984). Korosi and colleagues (2007) found that SPNs in the IML of the mouse expressed the mRNA for CRF receptor 1 (CRF1) and CRF receptor 2(CRF2), both of which bind CRF, and also express CRF receptor protein, which is a protein that is homologous region for both the CRF1 and CRF2.

Because the PVN contains a large population of CRF immunoreactive (-ir) neurons, and provides a likely source for the CRF fibers found in the spinal cord (Jansen et al., 1995), we chose to examine whether input from CRF labeled axons onto IML neurons was altered following axotomy of postganglionic axons. Any changes in the synaptic input onto the SPNs that innervate the SCG following postganglionic axotomy would suggest that peripheral neuronal injury could cascade to neurons located entirely in the CNS.

2.5 Changes in neuronal somata following axonal injury

All SPNs express the synthesizing enzyme choline acetyltransferase (ChAT; Barber et al., 1984), which catalyzes the production of acetylcholine. In the present study, the number of neurons in the IML expressing ChAT and as well as the area of ChAT-ir neurons in the IML were characterized following axotomy of the SCG. These two measures were chosen because these parameters have been examined previously following direct injury to IML neurons (preganglionic axonal transection) (Coulibaly et al., 2013) where the loss of ChAT expression and reduced volume of in SPNs were observed at 7 days following preganglionic transection. We compared the findings of the present study with the results of Coulibaly et al. (2013) in order to determine whether postganglionic injury can similarly affect preganglionic neurons in the IML and to understand whether the changes in soma size are a direct effect of injury or an indirect response, possibly due to loss of target derived neurotrophins.

Interestingly, following preganglionic transection, ChAT-ir neurons in the IML lose ChAT expression at early time points, but the neurons were still present and by 3 weeks following the injury, the neurons appeared similar to the sham controls. Lams and colleagues (1988) observed a similar response, where at 7 days after axotomy of vagal and hypoglossal axons, both ChAT and acetylcholinesterase expression were significantly decreased, yet no
significant decrease in neuronal counts using Nissl staining and retrograde tracing was observed. The neurons in that model also recovered at long term survival time points.

Neuronal atrophy, the shrinkage of neuronal somata, is another morphological change that has been observed following direct neuronal injury and that was assessed in the present study. Giehl and Tetzlaff (1996) observed a 49% decrease in the cross-sectional area of corticospinal neurons at 7 days following transection. At the same survival time point, Peddie and Keast (2011) observed a decrease in the soma size of sacral SPNs after axotomy of the pelvic nerves. In addition, Coulibaly and colleagues (2013) reported a significant decrease in the soma volume of ChAT-ir neurons in the IML at 7 days after CST transection.

3. **SIGNIFICANCE**

Numerous studies have indicated that direct injury to a neuron has numerous deleterious effects on normal function and phenotype (Lams et al., 1988; Giehl and Tetzlaff, 1996; Zhou et al., 1998; Yeh et al., 2001; Zhou et al., 2001; Paggi et al., 2006; Peddie and Keast, 2011; Hesp et al., 2012; Coulibaly et al., 2013). The effects that such injuries have on uninjured neurons are unclear, but there is evidence that uninjured neurons can be negatively affected by injury to a target neuron (Matthews and Nelson, 1975; Purves, 1975; Nja and Purves, 1978; Smolen, 1983; Johnson and Cowey, 2000; Paggi et al., 2006; Leong et al., 2011). Therefore these studies seek to determine whether retrograde influences of peripheral injury occur and whether the peripheral injury affects neurons in the spinal cord. Alterations in retrograde signaling (Millecamps and Julien, 2013), particularly communications involving neurotrophins, are implicated in the pathology of neurodegenerative diseases such as Alzheimer’s and Huntington's (Gauthier et al., 2004; Salehi et al., 2006). Therefore it is important to understand how neurons communicate to ensure the proper survival of neurons in the peripheral and central nervous systems.

4. **RATIONALE AND HYPOTHESES**

Studies of peripheral neuronal injury have generally only examined the injured neuron and surrounding cells. There are several studies that have examined alterations in synaptic input to the SCG following axotomy of postganglionic axons (Matthews and Nelson, 1975; Purves, 1975; Nja and Purves, 1978; Smolen, 1983; Paggi et al., 2006), yet no studies have examined the changes in the somata in the spinal cord. The studies of the SCG suggest that the effects of
peripheral injury could extend retrogradely into the CNS and so the experiments in this study are
designed to assess whether any changes in the spinal cord can be detected following axotomy of
the postganglionic axons arising from the SCG.

Other studies in our lab have characterized the response of preganglionic axons to direct
injury (preganglionic transection), but it was not known whether these effects resulted from
factors arising from the injury site or possibly from the loss of retrogradely transported
neurotrophin derived from the peripheral targets. Therefore, we compared the CNS effects of
preganglionic transection with that observed following postganglionic axotomy and took
advantage of the hierarchical chain of sympathetic neurons, where neurons in the brain innervate
the SPNs in the IML, which in turn innervate the postganglionic neurons in the SCG, neurons
that are well known for their dependence on target-derived neurotrophin for survival. Changes
in uninjured neurons in the IML were examined following transection of the postganglionic
axons arising from the SCG at short term (1 day, 7 days) and long term (8 weeks, 12 weeks)
survival time points. These survival times were chosen to allow for comparison to previous
studies (Walker et al., 2009; Hesp et al., 2012).

5. SPECIFIC AIMS OF THIS STUDY
Aim 1. The first aim of the project was to conduct a detailed analysis of SPNs in the IML at T1
level of the spinal cord at short term (1 day, 7 days) and long term (8 weeks, and 12 weeks)
survival times. Changes in the number, soma area, and soma volume of neurons expressing
ChAT were quantified using an antibody directed against ChAT to label SPN somata and 4’, 6-
diamidino-2-phenylindole (DAPI) as a nuclear marker. Because we had preliminary data
showing that ChAT protein in the SCG was significantly reduced following axotomy at all
survival time points examined, and the only source for ChAT in the SCG would be the SPNs, we
hypothesized that SPN somata in the spinal cord would show a response to the axotomy.

Aim 2. The second aim of this project was to determine whether the synaptic input to IML
neurons was altered following axotomy of the SCG. Changes in synaptic input onto the SPNs
were determined using an antibody directed against synaptophysin as a marker for presynaptic
terminals and anti-CRF as a well characterized input to the IML region. The input was analyzed
using methods described by Flak and colleagues (2009). We hypothesized that, because others
have observed retrograde transneuronal effects in the CNS following injury, synaptophysin and CRF in the IML would show changes at both short term and long term survival time points following axotomy of the SCG.

6. METHODS
6.1 Animals
Young adult 3 month old 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 Miami University Institutional Animal Care and Use Committee (IACUC) approved the methods used in this study and all efforts were taken to minimize pain and discomfort to the animals.

6.2 Surgery
Young adult female rats were anesthetized with the inhalant anesthetic isoflurane (2%). A 3 cm ventral incision was made in the neck of each animal. The postganglionic axons of the SCG on both sides of the neck were uncovered and gently removed from the surrounding tissues. The axons were transected 2 mm from their origin in the SCG (Nagata et al., 1987; Sun and Zigmond, 1996). The incision was then closed with the use of discontinuous surgical Look™ nylon sutures (Angiotech, Vancouver, BC) and Nexaband tissue glue (World Precision Instruments, Sarasota, FL). Sham animals underwent the same surgery, but the axons were not transected. The surgeries were considered successful when the rats exhibited ptosis or eyelid droopiness. The animals were sacrificed at the time points of 1 day, 7 days, 8 weeks, and 12 weeks after the surgery. A total of 33 animals were perfused for immunohistochemistry and 18 animals were decapitated for western analysis. A detailed listing of the animals used at each survival time point is provided below.

6.3 Tissue preparation for immunohistochemistry and western analysis
For immunohistochemistry, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (80mg/kg) and perfused with 50 ml of 0.9% saline followed by 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The spinal cords were removed and
stored in 0.1 M PB at 4°C. For western blot analysis, animals were decapitated using a Harvard guillotine, and the tissue was removed and snap-frozen with liquid nitrogen, and stored at -80°C.

6.4 Immunohistochemistry procedure

The first thoracic segment of the spinal cord corresponding to the first thoracic vertebrae was removed from each spinal cord. The segments were embedded in optimal cutting temperature medium (Ted Pella, Inc.) and cut into 18 μm thick sections using a MICROM HM 550 series cryostat. Sections were mounted on Superfrost Plus microscope slides (Fisher Scientific). The sections were desiccated for 6-12 hours and then incubated overnight in a 0.1 M phosphate buffer saline (PBS) containing 0.6% Triton X, and 0.1% normal donkey serum (Jackson Research Laboratories). The sections were then incubated for two days in primary antibody diluted in a 0.1 M PBS, 0.6% Triton X, and 0.7% normal donkey serum solution. The primary antibodies used were mouse anti-synaptophysin (1:500; Millipore, Billerica, MA; marker for presynaptic boutons), rabbit anti-CRF (1:10,000; gift from Dr. Wylie Vale; marker for PVN axons), and goat anti-ChAT (1:200; Millipore; marker for SPNs in the IML). The sections were then rinsed in PBS four times for 5 minutes each and then incubated for 2 hours in the appropriate secondary antibodies. Each secondary was AlexaFluor conjugated and directed toward the host of the primary antibody. The sections were rinsed in PBS three times for 5 minutes each and then in a final 5 minute rinse with PB before being mounted on microscope slides and coverslipped with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA) with DAPI. Images were captured with a Zeiss 710 Laser Scanning Confocal System and Zen 2009 Light Edition software. Because we were using the CRF antibody for the first time, a series of sections was processed with all steps of the immunohistochemical labeling except for the primary antibody. Comparisons were made of the no primary antibody sections with sections that received primary antibody in the same experiment. The results of this experiment are shown in Figure 7.

6.5 Analysis of ChAT-ir neuron numbers and area

The spinal cords from animals surviving for 1 day (n=8), 7 days (n=8), 8 weeks (n=10) and 12 weeks (n=10) following injury were analyzed to determine the number of ChAT-ir neurons in the IML and analysis of cell area. The group of controls sacrificed at 7 days
following sham surgery (n=7) was referred to as ‘short term controls’. Similarly, the group of controls that survived for 8-12 weeks after sham surgery (n=12) was referred to as ‘long term controls’.

For the analysis of the number of ChAT-ir neurons, 2-4 individual images of the left and of the right sides of the spinal cord were acquired using the 20X objective. The number of ChAT-ir neurons exhibiting an obvious nucleus labeled with DAPI was counted. The left and right IMLs from each animal were considered separate cases since the peripheral axons on each side of the animals were transected and the response to peripheral transection in the spinal cord has been determined to be unilateral. The data were square root transformed for analysis to account for data that are not normally distributed and/or have heterogeneous variance. Because there were no significant differences between the short term and long term control groups, the square root transformed data for the short and long term controls were combined for comparison to each survival time point. Combining of the square root transformed data for the short and long term controls was used for all the other analyses taken from confocal micrographs, since there was no significant difference between the two control groups for any analysis. The mean number of ChAT-ir neurons from each survival time point was compared with the counts obtained from to the combined square root transformed data for the short and long term control. Data were analyzed using a one way ANOVA followed by a Fisher’s post hoc comparison test. Significance was reported at p<0.05. Square root transformed data are depicted in the graphs shown in Figure 5A.

Analysis of cell soma area was conducted using the same images that were used for neuronal counts. For soma area measurements, Image Pro Plus V6.3 software was used to trace a line around individual neuronal somata and to measure the area of each ChAT-ir neuron in the IML as described in Peddie and Keast (2011). Data were square root transformed and the area for ChAT-ir neurons from each treatment group was compared to the combined square root transformed data for the short and long term controls. The values obtained from 1 day, 7 day, 8 week, and 12 week treatments were compared to the combined square root transformed data for the short and long term controls using a one way ANOVA followed by a Fisher’s post hoc comparison test. Significance reported at p<0.05. Square root transformed data are depicted in the graphs shown in Figure 5C.
6.6 Analysis of ChAT-ir neuronal volume

The volume of ChAT-ir neurons was analyzed using Z-stacks of confocal images that were taken with a 40X oil objective at 0.5 µm intervals, typically obtaining approximately 36 scans per section. These methods were previously used in our lab as described by Coulibaly et al. (2013), which in turn were modified from Flak et al. (2009). Z-stacks were obtained from both right and left IMLs and each side was considered as a separate case. Animals from the survival time points of 1 day (n=8), 7 days (n=8), 8 weeks (n=10) and 12 weeks (n=10) were analyzed and compared to combined ‘short term controls’ (n=7) and ‘long term controls’ (n=12).

Within each Z-stack, a range of two to six ChAT-ir neurons that exhibited a visible nucleolus were chosen for volume measurements. Approximately 4–5 neurons per case were analyzed. A neuron was chosen if it was present through at least 9 consecutive scans and had distinguishable boundaries throughout each of the scans. The mean radius was obtained by collecting four measurements in each of the four images, each 90 degrees apart, taken from the center of the nucleolus to the edge of the neuron (Flak et al., 2009). The resulting sixteen radii measurements were then averaged to get a mean radius per neuron. The mean radius of each neuron was then inserted into the formula for the volume of a sphere: \( v = \frac{4}{3}\pi r^3 \) (Flak et al., 2009) to obtain the volume for each neuron. The volume measurements of cells from each case were averaged to obtain a mean neuronal volume. Data then were square root transformed for analysis and for presentation in Figure 5B. The values obtained from 1 day, 7 day, 8 week, and 12 week treatments were compared to the combined square root transformed data for the short and long term controls (n=19) using a one way ANOVA followed by a Fisher’s post hoc comparison test. Significance reported at p<0.05.

6.6.1 Analysis of boutons contacting IML neuronal somata

The survival time point of 1 day after axotomy was not included in bouton analyses and immunoreactivity analyses, to be described in 6.7, due to poor labeling of syn-ir and CRF-ir puncta in the majority of cases. The poor labeling may have been the result of poor fixation or degradation of antigen in these tissues.

The innervation of IML neurons was analyzed from confocal Z-stacks that were acquired using a 40X oil objective at 1.0 µm intervals, typically obtaining approximately 20 scans per section. For the analysis of syn-ir inputs, data were collected from animals at 7 days (n=7), 8
weeks (n=8) and 12 weeks (n=6) following axotomy and short (n=3) and long term (n=6) controls. Data then were square root transformed for analysis. The values obtained from 7 day, 8 week, and 12 week treatments were compared to the combined square root transformed data for the short (n=3) and long term (n=6) controls using a one way ANOVA followed by a Fisher’s post hoc comparison test. Significance reported at p<0.05. For determining CRF/syn colocalization, animals from survival time points of 7 days (n=7), 8 weeks (n=4) and 12 weeks (n=3) were compared to the combined square root transformed data for the short (n=3) and long term (n=4) controls using a one way ANOVA followed by a Fisher’s post hoc comparison test. Significance reported at p<0.05. The number of animals for CRF/syn colocalization differs from the ones used for syn-ir boutons because experimenter error resulted in CRF images being taken with incorrect parameters for some cases and these were eliminated from the study.

For quantification of synaptic input, the sections were triple-immunolabeled with ChAT, CRF, and syn and with DAPI which was used as a nuclear marker. Three scans were analyzed: 1) a scan taken from middle of each stack; 2) a scan above the middle scan and 3) a scan below the middle scan. The number of CRF and synaptophysin boutons in apposition to the soma of a ChAT-ir neuron was counted for all neurons with distinguishable boundaries in each of the three scans. Boutons were considered in apposition to the soma if there was no visible space between the bouton and the neuronal membrane (Figure 2). The average number of boutons on a neuron from all three z-scans was averaged to obtain an average number of boutons per neuron. The number of syn-ir boutons and boutons that colocalized both CRF and syn were counted for each case. The data were then square root transformed for analysis and for presentation in Figures 7C and 7D. The values obtained from 7 day, 8 week, and 12 week treatments were compared to the combined square root transformed data for the short and long term controls using a one way ANOVA followed by a Fisher’s post hoc comparison test. Significance reported at p<0.05.

6.7 Analysis of immunoreactivity of CRF and synaptophysin after axotomy

The area of coverage of the entire field of view and the IOD of the section quantified the number of profiles of CRF and syn immunoreactivity in the entire field of view, rather than just the innervation of neuronal somata. The same z-stacks described in section 6.6.1 above were used for area of coverage and IOD. For this determination, three collapsed subsets, made up of three scans each, were taken from the middle nine scans of each full z-stack. The three collapsed
subsets from each case were quantified in ImagePro Plus V6.3 after determining the minimum threshold for detection of fluorescence. The area of coverage and the IOD of the 3 collapsed subsets were averaged to obtain a mean value for each animal. Data then were square root transformed for analysis and for presentation in Figures 7A and 7B for syn and Figures 8A and 8B for CRF. The values obtained from 7 day, 8 week, and 12 week treatments were compared to the combined square root transformed data for the short and long term controls using a one way ANOVA followed by a Fisher’s post hoc comparison test. Significance reported at p<0.05.

6.8 Semi-quantitative western analysis

Protein from the SCG tissues was isolated by sonicating tissue in 0.01M Tris-HCl buffer (pH 7.4) with 1% sodium dodecyl phosphate and 1% protease inhibitor. Protein concentration was determined by BCA protein assay (Pierce) and sample preparation was performed with the Laemmli method (Laemmli, 1970). SDS-PAGE (5% stacking/10% resolving) was used to separate proteins, along with a Precision Plus protein standard (Bio-Rad Laboratories). Following transfer of 2400mA at 4°C in transfer buffer (25mM Tris, 192 mM glycine, 10% v/v methanol) to PVDF (Millipore; Billerica, MA; 0.45 µm pore size) membrane, blots were submersed in methanol, allowed to dry, cut so that the standard was processed separately, and rehydrated in methanol. Blots were blocked overnight with 4% bovine serum albumin (BSA) in Tris-buffered saline with Tween20 (TBST) overnight, incubated for 2 hours in goat anti-ChAT (1:500, Millipore; ab144P) or TBST alone for the standard. Membranes were rinsed in TBST, incubated in donkey anti-goat HRP IgG (1:50,000, Santa Cruz Biotechnology; streptactin-HRP 1:500,000, Bio-Rad Labs for the standard) for one hour, rinsed with TBST, and submersed in Supersignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA) for five minutes. Protein was visualized with CL-X Posure™ x-ray film (Thermo Fisher Scientific, Waltham, MA). Blots were then stripped in IgG elution (stripping) buffer (Thermo Fisher Scientific, Waltham, MA) for one hour at room temperature and then blocked in 4% milk in Tris-buffered saline with Tween20 (TBST) for three hours. Blots were then incubated overnight with mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH 1:100,000, Fitzgerald Industries International, Acton, MA) for use as an internal control or with TBST alone for the standard. Membranes were washed four times for five minutes each in TBST, incubated in secondary antibody (goat anti-mouse HRP IgG 1:100,000, Millipore) for two hours for the
blot and the standard, washed four times for five minutes each in TBST, and submerged in Supersignal West Pico Chemiluminescent Substrate for five minutes. Protein was visualized with CL-X Posure™ x-ray film (Thermo Fisher Scientific, Waltham, MA). Films were scanned and analyzed for densitometry relative to GAPDH with ImageQuant 5.2. Protein from animals surviving for 1 day (n=4), 7 days (n=3), and 12 weeks (n=4) from axotomy and from 1 day sham controls (n=3); 7 day sham controls (n=3); 12 week sham controls (n=3) was analyzed. The data were then square root transformed for analysis and for presentation in Figure 3. The values obtained from 1 day, 7 day, and 12 week treatments were compared to the combined square root transformed data for 1 day, 7 day, and 12 week sham controls using a one way ANOVA followed by a Fisher’s post hoc comparison test. Significance reported at p<0.05.

7. RESULTS
7.1 Decreased ChAT protein in the SCG following axotomy

Western analysis of the SCG for ChAT revealed a prominent band at 68 kDa. Analysis across treatments revealed a significant decrease in ChAT protein at 1 day, 7 days, and 12 weeks after axotomy when compared to the controls ($F(3,19) = 14.09$, $p = 0.001$; Figure 3).

7.2 ChAT expression and cellular morphology unaffected by SCG axotomy

There were no significant differences in the number or appearance of ChAT-ir neurons at 1 day, 7 days, 8 weeks, or 12 weeks following axotomy when compared to the control group ($F(4, 50) = 1.3$, $p = 0.28$; Figures 4, 5A).

The cellular morphology of the SPNs was examined. Qualitative analysis of the ChAT-ir neurons showed no obvious morphological differences between the control and treatment cases. There were no significant differences in the average soma volume of ChAT-ir neurons at 1 day, 7 days, 8 weeks, or 12 weeks following axotomy when compared to the sham controls ($F(4, 50) = 1.4$, $p = 0.26$; Figure 5B).

There were no significant differences in the areas of ChAT-ir neurons in the IML for 1 day, 7 days, 8 weeks, or 12 weeks cases when compared to the sham controls ($F(4, 50) = 0.69$, $p = 0.6$; Figure 5C).
7.3 Synaptic input to preganglionic neurons affected by axotomy at long term survival time points

The general pattern of syn and CRF input to the IML is shown in Figure 6. There were no significant differences in the syn IOD (Figure 7A; \(F(3, 26) = 1.00, p = 0.41\)) or syn area of coverage (Figure 7B; \(F(3, 26) = 0.31, p = 0.82\)) in the IML at any time point following axotomy. In addition, there were no significant differences in the number of syn-ir boutons apposing neurons at 7 days when compared with combined controls, but the number of syn-ir boutons apposing neurons in the IML was significantly decreased by 17% at 8 weeks and 16% at 12 weeks following axotomy \((F(3, 26) = 0.09, p = 0.001; \text{Figure 7C})\). However when the number of syn-ir boutons that expressed CRF was assessed, no significant differences were observed at 7 days, 8 weeks, or 12 weeks when compared to the controls \((F(3, 17) = 1.3, p = 0.31; \text{Figure 7D})\).

Comparisons were made of sections processed using anti-CRF with those in which the primary antibody was omitted. Analysis of the no antibody sections revealed frequent nonspecific puncta associated with the soma of the ChAT-ir neurons (Figure 8). The long term controls and axotomy cases showed more abundant non-specific puncta than the short term controls and axotomy cases (Figure 8), suggesting that the increased background may be an effect of aging. In addition to the nonspecific labeling, numerous specific CRF-ir puncta were observed throughout the IML. Because the non-specific labeling appeared to be similar between the controls and the treatment groups, it was assumed that the quantification of the CRF puncta would elucidate only changes in specific labeling patterns. Quantification of the overall CRF immunoreactivity in the IML sections revealed a significant increase in CRF area of coverage in the section \((F(3, 27) = 19.43, p = 0.001)\) and IOD \((F(3, 27) = 20.58, p = 0.001)\) at 8 and 12 weeks, but no change at 7 days when compared to the merged control group (Figure 9). Similarly the analysis of CRF area and IOD revealed that the 12 week time point was significantly increased compared to 8 week axotomy time point.
The injury model

PVN of hypothalamus

Spinal cord (Intermediolateral cell column)

Superior Cervical Ganglion

Site of postganglionic axotomy

Peripheral targets (cerebral blood vessels, submandibular gland, pineal gland, irides, etc.)

Figure 1
Figure 1: The injury model used in this study involving the axotomy of the postganglionic axons of the superior cervical ganglion (SCG). The preganglionic axons of the intermediolateral cell column (IML) of the spinal cord (green neuron) project axons to innervate neurons in the SCG (purple neuron). The injury site involves the postganglionic axons that project from the SCG to peripheral targets in the head. The IML receives input from multiple regions of the brain; one of the more prominent regions is the paraventricular nucleus of the hypothalamus (PVN; red neuron). The gray curve distinguishes between the central and peripheral nervous systems.
Analysis of syn-ir boutons within a confocal Z-stack
Figure 2: Analysis of syn-ir boutons within a Z-stack of confocal images. Three scans from a z-stack of confocal images taken through the IML, each separated by 3 µm, from a control animal to show how the changes in syn-ir boutons and ChAT-ir preganglionic neurons in the IML were analyzed. Neurons depicted with the same color asterisk can be followed through the z-stack. Syn-ir boutons pointed out with similar color arrows can be followed throughout the z-stack. Scale for all images, 50µm.
Western analysis of ChAT in the SCG following axotomy

A)

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B)

![Graph showing percent control (sqrt) for different time points](* indicates significant difference)

Figure 3
**Figure 3:** Western analysis of ChAT in the SCG following axotomy.  A. Analysis of ChAT protein (68 kDa) in the SCG at 1 day, 7 days, and 12 weeks following axotomy of the SCG. GAPDH (36 kDa) served as a loading control.  25 µg protein loaded in the 1 day and 7 day lanes; 20 µg protein loaded in the 12 week lane. B. Semi-quantitative analysis revealed a significant decrease at all of the time points that were examined.  n=3-4 per treatment; *, significantly different from control, p<0.05.  Error bars represent the standard error of mean.
ChAT-ir neurons in the IML following axotomy of the SCG

Figure 4
Figure 4: ChAT-ir neurons in the IML showed no obvious changes following axotomy of the SCG. Confocal orthogonal Z-stack micrographs of ChAT-ir preganglionic neurons in the IML at 1 day, 7 days, 8 weeks, and 12 weeks after axotomy. Arrows denote a typical ChAT-ir neuron that would have been analyzed to obtain the cell counts, area, and volume data. The bars on the side of the micrograph show the depth of the tissue. DAPI was used to show the cellular nature of labeled profiles. Scale for all images, 50 µm.
Analysis of ChAT-ir neurons in the IML following axotomy

Figure 5

A) IML cell counts

B) Volume of IML neurons

C) Area of IML neurons
**Figure 5:** No changes in the number or morphology of ChAT-ir neurons in the IML were observed following SCG axotomy. A. Quantitative analysis of the number of IML neurons at 1 day, 7 days, 8 weeks, and 12 weeks showed no significant differences compared to age-matched controls. B. Quantitative analysis of preganglionic soma volume at 1 day, 7 days, 8 weeks, and 12 weeks showed no significant differences compared to controls. C. Quantitative analysis of preganglionic soma area at 1 day, 7 days, 8 weeks, and 12 weeks showed no significant differences compared to controls. Error bars represent the standard error of mean.
Synaptic boutons in the IML following axotomy of the SCG

Figure 6
Figure 6: Confocal micrographs showing the synaptic boutons in the IML in controls and at 7 days, 8 weeks, and 12 weeks after axotomy. The overall synaptic input is immunolabeled with anti-synaptophysin. Anti-CRF was used as a marker for input specifically from the PVN. Scale for all images, 50 µm.
Analysis of syn-ir boutons in the IML following axotomy of the SCG

Figure 7
Figure 7: Boutons in association with neuron somata in the IML were decreased at long term survival time points following axotomy. A. Analysis of the area of coverage by synaptophysin in the IML at 7 days, 8 weeks, and 12 weeks following axotomy showed no significant differences compared to controls. B. Analysis of the integrated optical density (IOD) of synaptophysin in IML sections at 7 days, 8 weeks, and 12 weeks following axotomy showed no significant differences compared to controls. C. Quantitative analysis of the number of boutons per neuron at 7 days, 8 weeks, and 12 weeks following axotomy revealed a significant decrease at 8 and 12 weeks following axotomy. D. The number of double labeled syn/CRF-ir boutons showed no significant differences compared to controls at any time point. Error bars represent the standard error of mean; *, significantly different from control, p<0.05.
Control experiment for CRF immunolabeling studies

Figure 8
Figure 8: Control experiment for the specificity of CRF immunolabeling. Confocal micrographs showing the comparison of CRF no antibody controls with sections that received primary antibody. In the 7 day axotomy tissues, there were few non-specific puncta (yellow arrows) in the no CRF antibody control tissues (A; A’) when compared with tissues that received CRF antibody (B; B’). Boxed areas are shown at high magnification. Specific immunoreactivity due to incubation with the anti-CRF antibody is shown in white and also is denoted by the white arrows. C.-D. Tissues taken from the 12 week axotomy animals showed non-specific puncta (yellow arrows) that appears to be within the ChAT-ir somata (red) (C; C’) which are detected also in the tissues that were processed with anti-CRF (D; D’). Scale for all images, 50µm, insets = 20µm
Increased CRF in the IML following SCG axotomy

Figure 9

A) CRF Area of Coverage

B) CRF IOD
Figure 9: CRF immunoreactivity in the IML was increased following SCG axotomy. A. Analysis of the area of coverage of CRF-ir boutons revealed a significant increase at 8 and 12 weeks when compared to age-matched controls. B. Analysis of the integrated optical density (IOD) of CRF-ir boutons showed a significant increase at 8 and 12 weeks when compared to controls. The IOD at the 12 week time point was significantly increased compared to the 8 week time point. Error bars represent the standard error of mean; *, significantly different from control, p<0.05; #, significantly different from 8 week ax.
8. DISCUSSION

8.1 Effects of axotomy on the CNS

The most notable change in the spinal cord following axotomy of SCG axons involved the synaptic input to the SPN cell bodies in the IML, where a decrease in syn-ir boutons contacting IML neurons was observed at long term survival time points. This decrease was not the result of a loss of CRF boutons since the number of syn/CRF double labeled boutons was unchanged. These findings suggest that synaptic input to the IML arising from sources other than CRF axons may have been affected by the injury. Overall, these results suggest that injury to postganglionic axons can result in transneuronal retrograde effects in the CNS to affect uninjured neurons in the sympathetic outflow pathway.

ChAT protein in the SCG was significantly decreased following axotomy of postganglionic axons, suggesting that uninjured preganglionic neurons that innervate the SCG are affected by the injury. However, no obvious changes in the number or morphology of ChAT preganglionic cell bodies in the spinal cord were detected. No changes in the number of ChAT-ir SPNs or the area and volume of ChAT-ir somata were found at any time points. This is in contrast to studies of transection of preganglionic axons where a decrease in the number of ChAT-ir SPNs (Tang and Brimijoin, 2002; Coulibaly et al., 2013) and cell volume (Coulibaly et al., 2013) were observed. These findings lead to the conclusion that direct injury to the axon, rather than possibly the loss of connection with the peripheral targets, is necessary to induce the dramatic changes in SPNs observed following the transection of preganglionic neurons.

The lack of any obvious changes in ChAT-ir SPNs together with substantial decreases in ChAT protein in the SCG at short term and long term time points suggest that the effects on the SPN somata may be more subtle than a complete loss of ChAT expression. Indeed, the finding that the synaptic input to the IML SPNs is affected suggests that the SPNs do respond to the injury. However, the changes in the neurons themselves appear to be subtle and could not be detected using the approaches in this study.

The transection of a peripheral axon prevents the retrograde transport of factors such as neurotrophins that are supplied by the target tissues (reviewed in Ben-Yaakov and Fainzilber, 2009). This loss of retrogradely transported neurotrophins after axonal injury is referred to as ‘negative injury signaling’ (Ben-Yaakov and Fainzilber, 2009) and can have widespread effects
on the injured neurons (Nja and Purves, 1978; Hyatt Sachs et al., 2007). In our model, it is possible that the loss of target-derived factors, such as neurotrophins, resulting from the disconnection of the postganglionic axons with their peripheral targets may have accounted for the changes in synaptic input to the IML neurons. If this is true, then it might be concluded that neurotrophin derived from peripheral targets plays an important role in the regulation of synapses in the CNS, particularly in the chain of neurons involved in sympathetic outflow. However, it is possible that a continued source of neurotrophin (i.e. BDNF or NT-4), possibly provided by other sources, and was sufficient to maintain the number, neurotransmitter expression, and morphology of the ChAT-ir SPNs.

8.2 Role of neurotrophin in the effects of axotomy on the spinal cord

Western analysis revealed a significant decrease in BDNF in the T1 spinal cord and SCG at 7 days following postganglionic axotomy (Hawk, 2013). However BDNF levels in the cord and IML returned to control levels by 8 weeks following the injury (Hawk, 2013). The maintained supply of BDNF to the preganglionic neurons suggests that the observed decrease in syn-ir boutons at 8 and 12 weeks may be due to an alteration in the supply of another target derived factor such as NT-4.

The rebound of the levels of BDNF in the SCG and spinal cord may be due to satellite glial cells in the SCG that extend processes between SCG neurons and preganglionic axons after postganglionic axotomy (Matthews and Nelson, 1975; Paggi et al., 2006). These cells have been found to express BDNF mRNA in dorsal root ganglia (DRG; Zhou et al., 1999) and BDNF protein in DRG and the SCG (Wetmore and Olson, 1995), which suggests that they may be capable of producing BDNF and supplying it to the preganglionic axons. In the SCG of axotomized adult guinea pigs, the satellite glial population was found to be increased at 1 to 3 weeks after axotomy and remained significantly above control values at 12 weeks (Purves, 1975). This continued supply of BDNF may have been sufficient to compensate for the loss of target derived factors and maintain the morphology and phenotype of the SPNs. However, it did not appear to be sufficient to maintain synaptic input to the SPNs.

Another possibility for the lack of an obvious effect on SPNs may be due to the fact that a number of SPNs project collaterals to both the SCG and the SG (Pyner and Coote, 1984b; Jansen et al., 1995). The SG expresses BDNF mRNA as well as NT-4 mRNA and protein (Roosen et
al., 2001), thus making it an additional source of trophic support for SPNs that project to the SCG. The ChAT immunoreactivity of axotomized facial nerve motoneurons was partially rescued by BDNF (Yan et al., 1994), which suggests that the lack of changes in SPNs may result from the fact that the BDNF supply was not affected to the same degree as if the preganglionic axons themselves were transected.

The lack of observable changes in SPNs in our model is comparable to the chemical ablation of SCG neurons by guanethidine. Long-term guanethidine treatment resulted in the cell death of 98% of the SCG neurons at 6 weeks (Burnstock et al., 1971; Heath and Burnstock, 1977) or 8 weeks (Tang et al., 1998) post treatment, but the loss of SCG neurons did not affect the number of SPNs at 8 (Tang et al., 1998) or 10 weeks (Tang and Brimijoin, 2002) after the treatment. It is possible that the satellite glial cells survived and provided a source of BDNF for the SPNs after guanethidine treatment.

8.3 Decrease in synaptophysin boutons at long term survival time points

The number of synaptophysin boutons per SPN was significantly decreased at 8 weeks and 12 weeks following axotomy of the SCG. In addition to the potential alterations in neurotrophin, the long term effects of axotomy may be due to the recent finding that the SCG neurons themselves have not yet recovered at 12 weeks following the injury (Hesp et al., 2012). Western analysis of the SCG revealed that TH protein levels remain decreased by 67% at 12 weeks after axotomy (Hesp et al., 2012) and that the SCG has not fully reinnervated many of its targets at this same time point (Hesp et al., 2012).

The decrease in syn-ir boutons in the IML at 8 weeks and 12 weeks following injury occurs at the same time that cell death in the SCG has been reported following axotomy. No cell death was observed at 7 days following axotomy (Walker et al., 2009), while at 8 weeks, the number of SCG neurons was 46% that of controls in rats (Smolen, 1983) and in a different study the number of SCG neurons was 54% of controls at 12 weeks following the injury in guinea pigs (Purves, 1975). These studies suggest that neuronal death in the SCG occurs at a time point after 7 days and suggests that the death of SCG neurons may lead to a cascade of events that involves decreased innervation of the SPN neurons.

The exact mechanism to explain how syn-ir boutons decrease at 8 and 12 weeks cannot be elucidated by this study. Possible explanations include a decrease in the expression of syn in
individual boutons synapsing onto SPN somata and/or the removal of boutons by synaptic stripping (Blinzinger and Kreutzberg, 1968; Yamada et al., 2011). Evidence for synaptic stripping will require future research to determine if astrocyte and microglia gliosis occurs around SPNs in a similar manner as 7 days after CST transection (Coulibaly and Isaacson, 2012). We have preliminary data to suggest that no astrogliosis or microgliosis occurs in the IML at 7 days following SCG axotomy (Hawk, unpublished data), but the later time points have not been examined for gliosis.

8.4. CRF in the IML following axotomy

No changes in the number of syn-ir boutons colocalizing with CRF were observed at any time point even though syn-ir boutons were decreased at 8 weeks and 12 weeks following the injury. This finding suggests that synaptic inputs expressing other neurotransmitters may account for the decrease in boutons. Alternatively, it appears that not all CRF terminals colocalize with syn. CRF is a neuropeptide that is transported in large dense core vesicles which express syn in lower levels than small clear vesicles (Winkler, 1997). Sámano and colleagues (2009) found that the majority of vesicles containing only neuropeptides in the SPNs do not also express syn. This leaves open the possibility some of the background puncta near or against the somata of SPNs that did not colocalize with syn may actually be CRF boutons. In addition it may be that other inputs are affected by the injury and were not detected using our experimental approach.

The increased expression of CRF in the IML at long term time points could be a compensatory mechanism to promote the production and release of BDNF by surrounding glia. Astrocytes and microglia have been shown to express the mRNA for CRFR1 and CRFR2 (Steven et al., 2003). CRF receptor protein was found in cultured astrocytes (Kapcala and Dicke, 1992) and the addition of CRF to cultured astrocytes led to an influx of calcium by the cells (Takuma et al., 1994). The addition of CRF to cultured microglia has been shown to promote the release of BDNF (Yuan et al., 2010). Therefore, it is possible that the increased CRF in the IML following injury leads to the release of BDNF from nearby glia. In turn, the BDNF release from the surrounding glial cells may exert neuroprotective effects and reverse the reduced BDNF levels that were observed at the 7 day survival time point.
The increased CRF in the IML may arise from descending input and/or enhanced expression of CRF by neurons in the IML (Merchenthaler, 1984; Krukoff, 1986; Korosi et al., 2007). CRF-ir neurons have been found in thoracic and lumbar IMLs in Sprague-Dawley rats (Merchenthaler, 1984), cats (Krukoff, 1986), and mice (Korosi et al., 2007), and it is possible that some of the CRF fibers observed in the IML arise from neurons in the spinal cord rather than the PVN of the hypothalamus. Regardless, it appears that either the descending axons or the spinal cord neurons that express CRF are affected by the peripheral injury, possibly to increase BDNF levels in the spinal cord at long term survival time points.

9. CONCLUSION

The overall goal of this project was to examine the effects of postganglionic axotomy on uninjured SPNs in the spinal cord. Though ChAT protein expression was decreased in the SCG at both short term and long term survival time points following the postganglionic axotomy, no discernible effects on SPNs in the IML were detected. The lack of any changes on the neuron somata in the spinal cord may be the result of continued supply of BDNF and/or NT-4 from sources in the spinal cord or from satellite cells in the SCG. Though no changes were observed in the somata, a decrease in syn-ir boutons making contact with the neuronal somata was observed at 8 and 12 weeks following the injury and CRF puncta in the IML were increased at these same survival time points. Since no long term alterations in BDNF were observed in the SCG or spinal cord, it is possible that changes in some other factor resulted in the alterations in the synaptic input to the IML somata. Further, it is possible that the increase in CRF may have served to promote the release of BDNF from nearby glial cells to prevent any obvious alterations in the SPNs. Overall these findings suggest that retrograde influences from axonal injury in the periphery can result in long term subtle changes in the CNS.
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