The overall goal of this project was to understand the changes in peripheral neurons and their vascular targets following axotomy (transection) of sympathetic postganglionic axons arising from the superior cervical ganglion (SCG). Changes were documented at short- and long-term survival time points following the injury. At seven days following axotomy, the extracerebral blood vessels were denervated of sympathetic axons, yet sensory and parasympathetic perivascular axons were increased and decreased, respectively. Protein expression of vascular endothelial growth factor (VEGF) in the vasculature was unchanged, but VEGF was increased in the SCG as well as the trigeminal ganglion, which houses the parent cell bodies of the sensory perivascular axons. At 12 weeks, the sympathetic innervation showed some recovery, yet sensory and parasympathetic innervation remained altered, as did VEGF in the SCG and trigeminal ganglion. These results reveal the presence of long term plasticity in peripheral neurons following axotomy of the SCG.
Plasticity of Peripheral Neurons Following Axotomy of the Superior Cervical Ganglion

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# TABLE OF CONTENTS

1. Introduction
   - Sympathetic model and overall goal of this study 1
   - The extracerebral blood vessels: innervation by... 1
   - Neurotrophin regulation of peripheral neurons 3
   - VEGF as a regulator of sympathetic neurons 5
   - Changes in peripheral neurons following injury 7

2. Rationale and Hypotheses 9

3. Specific Aims 9

4. Methods
   - Animals and tissue processing 11
   - Surgical procedures and axotomy of the SCG 11
   - Immunofluorescence procedures 11
   - Analysis of axons of extracerebral blood vessels 12
   - Western blot analysis and semi-quantitative analysis 13

5. Results
   - Changes in sympathetic perivascular axons associated following axotomy 14
   - Changes in sensory and parasympathetic perivascular axons following axotomy 15
   - Changes in VEGF following axotomy of the SCG 17

6. Discussion
   - Plasticity of sympathetic innervations of... 49
   - Increased CGRP sensory innervations following aectomy of the SCG 50
   - Decreased VACChT parasympathetic innervation following aectomy of the SCG 51
   - Changes in VEGF changes and the role of... 52

7. Conclusions 55

8. References 58
LIST OF TABLES

Table 1. Summary of changes in perivascular axonal populations… 42
LIST OF FIGURES

Figure 1. The extracerebral blood vessels at the base of the brain 18
Figure 2. TH perivascular axons associated with the ACA following axotomy 20
Figure 3. Quantitative analysis of the density of … 22
Figure 4. CGRP-ir and VACHT-ir perivascular axons show no colocalization 24
Figure 5. CGRP-ir perivascular axons associated with the ACA following axotomy 26
Figure 6. Quantitative analysis of the density of … 28
Figure 7. CGRP-ir perivascular axons associated with the MCA following axotomy 30
Figure 8. Quantitative analysis of the density of… 32
Figure 9. VACHT associated with the ACA following axotomy 34
Figure 10. Quantitative analysis of the density of… 36
Figure 11. VACHT-ir axons associated with the MCA following axotomy 38
Figure 12. Quantitative analysis of the density of… 40
Figure 13. VEGF western analysis of the extracerebral blood vessels 43
Figure 14. VEGF western analysis of the superior cervical ganglion (SCG) 45
Figure 15. VEGF western analysis of the trigeminal ganglion 47
1. INTRODUCTION

Sympathetic model and overall goal of this study.

The sympathetic nervous system is part of the peripheral nervous system (PNS), which connects the brain and spinal cord to the limbs and target organs. The PNS can be divided into the somatic nervous system and the autonomic nervous system. One function of the somatic nervous system is to process sensory information and the neuronal cell bodies are located in sensory ganglia located just outside of the brain and spinal cord. The somatic nervous system also consists of cell bodies in brain and spinal cord that have axons that connect to skeletal muscles to regulate movement (Purves et al., 2008). The autonomic nervous system is comprised of the sympathetic nervous system and parasympathetic nervous systems. Primary functions of sympathetic and parasympathetic neurons are to regulate pupil dilation, heart rate and contraction, and urinary output (Wallin and Charkoudian, 2007; Charkoudian and Rabbitts, 2009).

The cell bodies of autonomic neurons are located in ganglia in the periphery. The superior cervical ganglion (SCG) is the most rostral sympathetic ganglion and is located at the bifurcation of the common carotid artery and is comprised of a cluster of postganglionic neuronal cell bodies and glial cells. The cell bodies in the SCG project postganglionic axons through the external and internal carotid nerves to innervate the head and neck targets such as the irides, the submandibular gland, the pineal gland, and the extracerebral blood vessels, which are located at the base of the brain (Fig.1). The postganglionic axons that innervate the extracerebral blood vessels regulate lumen diameter and consequently monitor blood flow into the brain. The overall goal of the present study is to understand the regulation of these sympathetic neurons and their innervation of peripheral targets such as the extracerebral blood vessels.

The extracerebral blood vessels: innervation by three different neuronal populations.

The extracerebral blood vessels, located within the subarachnoid space at the base of the brain (Fig.1), provide arterial blood supply to the brain and originate from the vertebral and the internal carotid arteries. The internal carotid artery arises from the common carotid, passes through the neck to enter the cranial cavity, and then branches
into the anterior cerebral (ACA) and the middle cerebral (MCA) arteries (Fig.1) at the base of the brain. The representative extracerebral blood vessels for examination in this study are the MCA and ACA.

The anterior extracerebral blood vessels, such as the MCA and ACA receive innervation from sympathetic axons that arise primarily from the SCG (Kajikawa 1968; Sato et al., 1980; Handa et al., 1990; Hamel, 2006), while the more caudal vessels such as the caudal basilar and vertebral, receive sympathetic innervation from stellate ganglion, located at the level of the first two thoracic vertebrae (Kajikawa, 1968; Hernández-Pérez and Stone, 1974). The main neurotransmitters released by sympathetic perivascular axons are norepinephrine and neuropeptide Y, which function to maintain vascular tone (Hernández-Pérez et al., 1975) and serve as vasoconstrictors (Kajikawa, 1968; Hernández-Pérez et al., 1975) to regulate blood flow into the brain. The sympathetic perivascular axons can be visualized using an antibody to tyrosine hydroxylase (TH), which serves as the rate-limiting enzyme in the biosynthesis of norepinephrine (noradrenaline) and epinephrine (adrenaline) (Nagatsu et al., 1964).

In addition to sympathetic innervation, the extracerebral blood vessels also are innervated by two other neuronal populations. The sensory perivascular axons arise from the trigeminal ganglion (Ruskell and Simons, 1987; Saito and Moskowitz, 1989), which is located in the cranial vault. These sensory perivascular axons show robust immunoreactivity for calcitonin gene related peptide (CGRP), which is released from activated trigeminal sensory nerves to function as vasodilators of the extracerebral blood vessels (Hamel, 2006). The trigeminovascular pathway plays a protective role to restore vessel tone after vasocontractile stimuli (McCulloch et al., 1986). As a vasodilator, infusion of CGRP can also provoke migraine, a neurovascular disorder, and blockade of CGRP receptors may inhibit migraine (Villalón and Olesen, 2009).

It is not known whether sensory perivascular axons are altered when sympathetic innervation is lost following axotomy. Documenting the changes in sensory innervation associated with the extracerebral blood vessels during sympathetic denervation and reinnervation following axotomy of the SCG is one goal of the present study. Increased CGRP levels were observed in rat parotid gland after axotomy of the SCG (Ekström and Ekman, 2005), which were thought to be the result of increased sensory innervation.
following loss of sympathetic axons. In addition, CGRP was shown to be synthesized within damaged sensory axons following injury to the sensory axons of the sural nerve in rats and was an important neuropeptide for the interactions between axons and Schwann cells during peripheral nerve regrowth (Toth et al., 2009). Therefore, we hypothesize that sensory perivascular axons will increase following axotomy of the SCG and loss of sympathetic innervation.

Parasympathetic axons also innervate the extracerebral blood vessels and arise mainly from the sphenopalatine and otic ganglia (Walters et al., 1986; Hardebo et al., 1991). The parasympathetic nerves release the neurotransmitter acetylcholine (ACh). Upon stimulation, the parasympathetic system works as a potent dilator of the extracerebral blood vessels (Hamel, 2006) and can increase cerebral blood flow (Seylaz et al., 1988). After axotomy of the SCG, other parasympathetic markers, such as vasointestinal peptide, were increased (Ekström and Ekman, 2005). Yet a different study showed loss of parasympathetic markers associated with perivascular axons when sympathetic innervation was lost (Hasan and Smith, 2009).

Because parasympathetic perivascular axons release ACh, a useful marker for these axons is vesicular acetylcholine transporter (VAChT), a protein that is required for the storage and release of ACh (Eiden et al., 2004). VAChT has been used as a marker for cholinergic nerves in many studies (Giordano et al., 2004; Schütz et al., 2008; Murabayashi et al., 2009). We will use VAChT to determine whether the parasympathetic innervation of the extracerebral blood vessels is altered following axotomy of the SCG. We expect to see a decrease in parasympathetic axons following loss of sympathetic innervation.

**Neurotrophin regulation of peripheral neurons**

The activities of peripheral neurons are regulated by target-derived proteins called neurotrophins. Neurotrophins are synthesized by the target tissues, internalized by the innervating neurons, and retrogradely transported to the cell body (Fahnestock 1991; Campenot and MacInnis, 2004) to carry out survival activities. All members of the neurotrophin family activate intracellular signaling pathways via a dual receptor system.
utilizing trk, a tyrosine kinase receptor, and p75, a member of the tumor necrosis factor receptor super-family (Teng and Hempstead, 2004).

While neurotrophins are critical to the survival of uninjured sympathetic neurons, they also play a survival role after injury of sympathetic neurons. One well-studied neurotrophin is nerve growth factor (NGF), a 13kDa protein, which binds the trkA receptor to promote survival effects (Reichardt, 2006) and which has been show to be altered in the SCG following axotomy. For example, Shoemaker et al. (2006) observed a decrease of NGF protein levels in the SCG following axotomy and this was believed to be the result of disconnection of the SCG with its peripheral targets. Similarly, the 22-24kDa NGF species in the SCG was significantly reduced by 99% following 7 day axotomy (Walker et al., 2009). There is evidence that NGF might play a neurotrophic role after injury. Transection of sciatic nerve led to an increased expression of NGF by Schwann cells in the sciatic nerve (Heumann et al., 1987). In addition, NGF was also found to promote the neural survival and neurite outgrowth of SCG neurons in vitro (Ma et al., 2009).

Another neurotrophin found in the periphery is brain derived neurotrophic factor (BDNF). The function of BDNF is mediated by TrkB receptor (Reichardt, 2006). BDNF/TrkB-signaling stimulated intracellular pathways that are important for neuronal survival, morphogenesis, and plasticity. Following axotomy of sciatic nerve, BDNF expression was found increased in large TrkB cells in dorsal root ganglion (Michael et al., 1999), suggesting a role for BDNF following injury to sensory neurons. In another study, BDNF was applied to regenerating tibial nerve and found to be a positive neurotrophic factor for regeneration and axonal growth (Gordon, 2010).

Neurotrophin-4 (NT-4), also mediated by TrkB receptor (Barbacid, 1994), has been shown to regulate the survival of peripheral neurons. In NT-4 null mice, a loss of sensory mechanoreceptors in the sensory neurons was observed starting at 3 weeks after birth (Stucky et al., 1998). NT-4 was also found in distal muscle targets after sciatic nerve axotomy and this target-derived NT-4 was thought to play a role in axonal regeneration (Funakoshi et al., 1993). NT-4 was also found to promote sympathetic (Reis et al., 2002) as well as sensory (Stucky et al., 1998) neuronal survival.
Neurotrophin-3 (NT-3) is a neurotrophin that is also important for the regulation of peripheral neurons. NT-3 is thought to be produced by target tissues, internalized and retrogradely transported back to the cell body in the SCG (Zhou and Rush, 1996). Neuron loss in SCG was found in NT-3 deficient mice, and axonal outgrowth was also affected in vitro (Francis et al., 1999). NGF and NT-3 may work together to promote neuron survival in the SCG as administration of NT-3 in NGF-antiserum treated newborn rats inhibited neuronal loss in SCG (Tafreshi et al., 1998).

**VEGF as a regulator of sympathetic neurons**

Other factors regulate the survival as well as the regenerative properties of peripheral neurons. One factor is vascular endothelial growth factor (VEGF), a growth factor that was first found to be an angiogenic factor that regulates blood vessel formation (Ferrara et al., 1996), but more recently has been shown to have neuroprotective and neurotrophic roles in the nervous system (Sköld and Kanje, 2008; Ruiz de Almodovar et al., 2009). The human VEGF gene has eight exons, which are separated by seven introns, and different splicing sites result in different isoforms of VEGF (Tischer et al., 1991). VEGF-A was found to be expressed in all vascularized parts of the body during development, especially in heart, intestine and parts of the brain (Sköld and Kanje, 2008). VEGF-B is expressed in brain vessels and found to be important for homeostasis (Nag et al., 2002). The amino acid of VEGF-B167 is 44% identical to VEGF165, however, VEGF-B knockout mice are found to be healthy and fertile, indicating its redundancy in healthy conditions (Ruiz de Almodovar et al., 2009). VEGF-C and VEGF-D are found in the heart (Achen et al., 1998). VEGF-C is expressed in heart, placenta, and ovary during development but not in the CNS. And it was found to be of importance for lymphangiogenesis (Sköld and Kanje, 2008).

VEGF-A promoted neurite outgrowth in postnatal rodent retinal neurons (Böcker-Meffert et al., 2002) and was also demonstrated to have neurotrophic effects by increasing survival and decreasing apoptotic proteins in cultured cortical neurons (Sanchez et al., 2010). In another experiment, Jin et al. (2002) showed that VEGF-A can stimulate neurogenesis both in vitro and in vivo. Following injury such as stroke, VEGF-A can increase the permeability of blood vessels to ensure sufficient blood
supply and may also work as a neuroprotective factor or an axon guidance molecule (Sköld and Kanje, 2008). VEGF-A injection into the lateral ventricle of rat brains after a transient middle cerebral artery occlusion reduced infarct volume and promoted cortical newborn neurons (Wang et al., 2009).

VEGF-A may also serve to regulate sympathetic neurons, particularly those innervating vascular targets (Sköld and Kanje, 2008; Marko and Damon, 2008; Long et al., 2009). In vitro and in vivo experiments revealed that VEGF-A can promote sympathetic axon growth at sympathetic neurovascular junctions (Böcker-Meffert et al., 2002; Marko and Damon, 2008). Addition of VEGF to explanted cell cultures of sympathetic and sensory neurons promoted axonal outgrowth as well as Schwann cell survival and proliferation, which was inhibited when the mitogen-activated protein kinase (MAPK) pathway was blocked (Sondell et al., 1999b). In another experiment, Sondell et al. (2000) found that VEGF accumulated distal to the ligature of the sciatic nerve, which suggests that VEGF was produced at the targets and retrogradely transported back to the neurons. Other studies have concluded that VEGF is produced by vascular targets (Sondell et al., 1999b; Marko and Damon, 2008) and plays a role in sympathetic reinnervation of peripheral blood vessels following injury (Marko and Damon, 2008; Long et al., 2009).

The function of VEGF in affecting sympathetic neuron function is mediated by three receptors, VEGF receptor 1 (VEGF1), VEGF receptor 2 (VEGFR2), and neuropilin1 (NRP1; Sköld and Kanje, 2008; Ruiz de Almodovar et al., 2009). VEGFR1 was the first VEGF receptor to be identified, though its exact role has not been elucidated (De Vries et al., 1992). VEGFR1 has been shown to have weak tyrosine kinase activity but high affinity for VEGF (Ruiz de Almodovar et al., 2009). One study has shown that mice lacking the extracellular part of VEGFR1 has an increased number of endothelial progenitors which results in vascular disorganization (Fong et al., 1999), indicating VEGFR1 is not important for angiogenesis and rather it works as a regulator to prevent VEGF binding to VEGFR2 (Park et al., 1993). It has been suggested that VEGFR-1 may act as a mediator to prevent excessive VEGFR-2 activation (Olsson et al., 2006).
It has been proposed that VEGF stimulates axon outgrowth via the activation of VEGFR-1 (Marko and Damon, 2008). Yet Marko and Damon (2008) found that VEGF affects sympathetic growth cones by binding to VEGFR1. Other studies showed that VEGFR2 plays important roles in vascular permeability, endothelial cell survival, proliferation and migration (Ferrara et al., 2003). The mechanism for VEGF to stimulate axonal outgrowth of sympathetic and sensory neurons was thought to be mediated through VEGFR2 (Sondell et al., 1999b) and it has been shown that VEGFR2 is very important for angiogenesis since VEGFR2 knockout mice die at E8.5 because of disorganized blood vessels (Shalaby et al., 1995). VEGFR2 also mediated regeneration of motor neurons in mouse models for amyotrophic lateral sclerosis (ALS) (Lunn et al., 2009). Thus it is possible that the reinnervation is affected by both of the two VEGF receptors.

The three VEGF receptors are all tyrosine kinases receptors (Sköld and Kanje, 2008). Among all the three receptors, VEGFR-2 is considered as the major mediator. It induces vascular permeability and angiogenesis. It also stimulates migration, proliferation, and survival of neural cells in the nervous system (Ruiz de Almodovar et al., 2009). Based on different phosphorylation sites, VEGFR can activate different downstream kinases. Phosphorylation of VEGFR-2 at Tyr1175 mediates activation of the mitogen-activated protein kinase (MAPK) and result in proliferation of endothelial cells; phosphorylation of Tyr951 regulates endothelial-cell migration. Also, phosphorylation at Tyr1175 (VEGFR-2) can also mediate endothelial cell survival (Olsson et al., 2006). Studies by Sondell et al. (2000) suggest that VEGF induces axon outgrowth via VEGFR-2. VEGFR-2 was found at the nerve cell bodies of SCG, and VEGF-induced axonal outgrowth was blocked by the VEGFR-2 inhibitor (Sondell et al., 1999a; Sondell et al., 2000).

**Changes in peripheral neurons following injury**

Our understanding of nerve regeneration in the PNS has increased significantly during the past several decades as a result of advances in cellular and molecular
biology. The peripheral neurons, such as those of the SCG, are different from neurons in the CNS in that they have the capacity of regeneration after injury (Langley, 1895; Ramon y Cajal, 1928; Butson, 1950; Purves and Thompson, 1979). The factors that contribute to this regeneration are uncertain and are currently under investigation.

One means of promoting regeneration involves the production of retrograde signals at the injury site that are transported to the injured cell bodies where they activate transcription factors, adhesion molecules, growth-associated proteins and structural components that are needed for regeneration (Navarro et al., 2007). Proteins such as dyneins are necessary for this retrograde process to occur (Perry and Fainzilber, 2009). Axotomy of the post-ganglionic axons of SCG likely results in the activation of such retrograde signals, which can result in many changes in the SCG. Following axotomy, the injured sympathetic neurons of SCG showed decreased mRNA (Koo et al., 1988; Sun and Zigmond, 1996) and protein levels of TH (Walker et al., 2009). Some have postulated that the majority of neurons in the SCG survive after axotomy and cell death is rare in adult peripheral neurons (Boeshore et al., 2004), but others have shown extensive cell death following crush injury (Purves, 1975). In one study by Boeshore et al. (2004), DNA microarray technology and real-time RT-PCR analysis were used to examine the gene changes after axotomy of SCG. The arg I gene was found to increase after the injury, which has anti-apoptotic effects in vitro (Esch et al., 1998).

Following peripheral axon injury, nerve fibers distal to the lesion site undergo a process known as Wallerian degeneration, in which the axons undergo degeneration and removal (Fawcett and Keynes, 1990). Schwann cells and macrophages are thought to play a positive role in removing the degenerating debris. They can eliminate the degenerative debris and provide injured neurons a healthy environment for recovery and prepare the environment for regeneration (Fawcett and Keynes, 1990). Due to this elimination of fibers distal to the injury site, we expected that the extracerebral blood vessel targets of the SCG would be denervated following axotomy of the SCG. Yet the degree of reinnervation and the changes in other perivascular populations have not been studied and are one focus of this project.
2. RATIONALE AND HYPOTHESES

Though numerous studies have addressed the changes in the SCG following axotomy, few studies have related these changes observed in the parent cell bodies with the changes in the peripheral targets following loss of sympathetic innervation, leading to the goals outlined in the present study. Work in our lab has focused on the innervation of the extracerebral blood vessels following transection (axotomy) of the sympathetic axons that arise from the SCG. We have documented that the sympathetic axons associated with the middle cerebral artery (MCA) are decreased after axotomy, and remain significantly decreased at 12 weeks (Zhu et al., 2010). However, changes in sympathetic innervation and the time course of sympathetic reinnervation of the ACA, a more anterior vessel in the arterial tree (Fig.1), were unknown. In Aim 1 the sympathetic innervation of the ACA following short term and long term axotomy was examined in order to understand the time course of sympathetic denervation and reinnervation of this vessel and to compare these processes with that associated with the MCA, which has already been documented in our laboratory.

How the changes in sympathetic axons during the denervation and reinnervation processes affect the other, uninjured, perivascular axons is unknown. In Aim 2 of this study, we documented the response of the sensory and parasympathetic perivascular axons associated with the MCA and ACA after axotomy of the SCG and related these changes to the sympathetic denervation and reinnervation.

The regulatory influences on cerebrovascular axons following injury were the focus of Aim 3. VEGF has been shown to promote the sympathetic reinnervation of the femoral artery (Marko and Damon, 2008), yet its role in regulating the cerebrovascular innervation following injury is unknown. Because VEGF appears to regulate both sympathetic and sensory neurons (Sondell et al., 2000), the third objective of this study is to examine the pattern of VEGF protein expression in sympathetic and sensory neurons as well as the blood vessel targets following axotomy of the SCG and during the sympathetic denervation and reinnervation processes.

3. SPECIFIC AIMS
**Aim 1:** To examine the time course of denervation and reinnervation of the ACA following short term (1 day, 7 days) and long term (8 weeks and 12 weeks) axotomy of the SCG.

- TH immunohistochemical analysis of the ACA was carried out to assess changes in sympathetic innervation and to compare with previous analysis of the MCA as well as to relate to the changes in uninjured populations observed in Aim 2 of this study.

**Aim 2:** To document any changes in the sensory and parasympathetic perivascular axons associated with the extracerebral blood vessels following short term and long term axotomy of the SCG.

- Immunohistochemical analysis of the extracerebral blood vessels (ACA and MCA) to document changes in CGRP (sensory) and VACHT (parasympathetic) immunoreactive axons associated with these vessels was carried out. I hypothesized that the sensory innervation will increase while parasympathetic innervation will decrease following sympathetic denervation. Whether alterations in these populations are sustained at long term survival time points during the sympathetic reinnervation process was unknown.

**Aim 3:** To assess any changes in VEGF regulation following axotomy of the SCG.

- The pattern of VEGF protein expression in the extracerebral blood vessel targets as well as the SCG and trigeminal ganglion at short term and long term survival time points was examined. Because others have shown that VEGF plays a role in sympathetic reinnervation following injury, I hypothesized that VEGF would be altered in the blood vessels or the SCG. In addition, because uninjured sensory perivascular axons are expected to show plasticity following injury to the sympathetic population (via axotomy of the SCG), VEGF western analysis of the trigeminal ganglion was carried out to determine any changes in the parent sensory neurons.

4. **METHODS**
Animals and tissue processing.

Young adult (3 months) female Sprague Dawley (Harlan) rats were housed in the Miami University Animal Facilities in a 12:12 light:dark environment at regulated temperature. Treatment groups for the experiment were: cont: received no treatment; sham: SCG was exposed but no transection was performed; axotomy: transection of SCG axons with a 1 day, 7 day, 8 week or 12 week survival period. All procedures were approved by the Miami University Institutional Animal Care and Use Committee.

Surgical procedures and axotomy of the SCG.

Young adult female Sprague Dawley rats (200-225 gm; Harlan Labs) were anesthetized with the inhalant 2.5% isoflurane. A ventral incision approximately 3 cm in length was made in the neck region. For axotomy, the axons of the SCG were exposed and both the external carotid nerve and internal carotid nerve were gently separated from surrounding tissues. Both nerves were transected approximately 2 mm from their origin in the SCG with microdissecting scissors (Nagata et al., 1987; Sun et al., 1996). The procedure was repeated on the other side. Animals survived for 24 hours (control: n=6; axotomy: n=6), 7 days (control: n=6; axotomy: n=6), 8 weeks (control: n=6; axotomy: n=6), or 12 weeks (control: n=6; axotomy: n=6). The incision was closed using sutures and tissue glue (Nexaband, Phx, AZ). Success of the procedure was assessed by the extent of ptosis (eyelid droopiness), as well as post-surgical examination of the surgery sites.

Immunofluorescence procedures.

For immunohistochemistry, animals were sacrificed via transcardial perfusion with 4% paraformaldehyde in 0.1M phosphate buffer (PB). Brains (with attached vessels) were stored in 0.1M PB at 4°C. Vessels were clipped from the base of the brain and incubated for 24 hours at 4°C in a blocking solution of 0.3% Triton-X and 0.1% normal donkey serum (Jackson Research Labs) in 0.1M phosphate buffer saline (PBS). For assessing uninjured axonal populations, vessels were then incubated for 48 hours at 4°C in rabbit TH (1:200; Millipore; catalog number AB152; for sympathetic axons), rabbit anti-CGRP (1:500; Peninsula Laboratories; catalog number T-4032; for sensory
perivascular axons), goat anti-VACHT (1:200; Abcam; catalog number ab43875; for parasympathetic axons). The vessels were then be rinsed 4 times for 5 minutes each with 0.1M PBS and then placed in secondary antibody (1:200; donkey anti-goat-488; catalog number A-11055; or 1:200 donkey anti-rabbit-594; catalog number A-21207; Molecular Probes) diluted in 0.1M PBS for 2 hours at room temperature. Following incubation, vessels were rinsed with 0.1M PBS 3 times for 5 minutes each followed by another 5 minute rinse in 0.1M PB. Vessels were mounted on glass microscope slides, and coverslipped with Vectashield mounting medium.

**Analysis of axons of extracerebral blood vessels.**

Vessels were viewed using a Zeiss 710 confocal laser scanning microscope (Center for Advanced Microscopy and Imaging) and analyzed using Image pro 6.0 software. Images were captured at 200X magnification and saved as TIFF files. For each blood vessel, six images (two from each region: proximal, middle, distal) were analyzed. To quantify the density of fluorescent perivascular axons, the vessel image was displayed on the computer monitor at 200X magnification and two 6 inch X12 inch rectangular grids were placed over the images on the computer monitor. The total number of axonal intersections with gridlines in the horizontal and vertical planes in each grid was recorded and a mean from the counts of the two rectangular grids was obtained as the axonal density in that image. A mean then was obtained for the two images of each region and that value was used in the statistical analyses. The total density for each vessel was obtained by adding the density values from each of the three regions. Because no significant differences were found between the controls from the different age groups, data from the control vessels were pooled to form one control group (n=12). Data were subjected to ANOVA with the Fisher’s post-hoc comparison test. Significance was reported at p<0.05.

For simplicity, the results described below focused on two types of statistical comparisons. First, any changes in each of the survival time points compared with the control group were described. Second, any changes between the 7 day time point, in which almost total sympathetic denervation was observed, and the 12 week time point,
in which some recovery was noted, were described in order to understand how the changes in each of the different axonal populations might be related.

**Western blot analysis and semi-quantitative analysis**

For western analysis, animals were sacrificed using a Harvard guillotine apparatus. SCGs were removed and snap-frozen in liquid nitrogen and stored at -80°C until further processing. Total protein was extracted by sonicating tissue in 0.01M Tris-HCl buffer (pH 7.4) containing 1% sodium dodecyl phosphate and 1% protease inhibitor cocktail (Sigma). Protein concentration was determined from the supernatant by BCA protein assay (Pierce) and sample preparation was performed in accordance with the Laemmli method (Laemmli 1970). SDS-PAGE (5% stacking/12% resolving) were used to separate proteins, along with a Precision Plus protein standard, which was used to confirm molecular mass. Following 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. The membrane containing samples were 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 rabbit anti-VEGF-A (1:2000; SC-507; Santa Cruz Biotech) and the membrane containing the standard was incubated in TBST alone. Membranes were then washed four times for five minutes each in TBST, incubated in secondary antibody (goat anti-rabbit HRP IgG 1:100,000; Chemicon; catalog number AP132P; streptactin-HRP 1:500,000; Bio-Rad Labs for the standard; catalog number 161-0380) for two hours at room temperature. Membranes were washed four times for five minutes each in TBST, and submerged in Supersignal West Pico Chemiluminescent Substrate for five minutes. After placing membranes in an autoradiography cassette, protein was visualized with X-ray film. Blots were then stripped in IgG Elution Buffer, which eluted previously applied primary antibodies from the membrane, for one hour at room temperature, blocked, and re-probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by incubating overnight in mouse anti-GAPDH (1:80,000 for SCG; 1:50,000 for blood vessel; Fitzgerald Industries
International; catalog number 10R-G109A) washing four times for five minutes each in TBST, incubating in secondary antibody goat anti-mouse HRP IgG (1:100,000 for SCG; 1:50,000 for blood vessel, Chemicon; catalog number AP124P) for two hours at room temperature, washing four times for five minutes each in TBST, submerging in Supersignal West Pico Chemiluminescent Substrate for five minutes, and developing as normal.

The optical density of VEGF was normalized to GAPDH for each sample to produce a ratio. A mean of the ratios were obtained for the control cases and comparisons were then made to the ratios obtained from each of the treatment samples. Ratios for each tissue were collected from at least 3 animals per treatment and data were subjected to analysis using the Student’s t-test with statistical significance reported at p<0.05.

5. RESULTS

Changes in sympathetic perivascular axons associated with the ACA.

Using TH as a marker for sympathetic axons, the sympathetic perivascular axons associated with the ACA were examined following axotomy of the SCG. The sympathetic innervation in the controls was robust with axons passing in both the longitudinal and circumferential planes (Fig. 2). The total sympathetic innervation of the ACA was significantly decreased (compared with controls) following axotomy of the SCG at all of the time points examined. As shown in Figures 2 and 3, the sympathetic innervation was decreased at 1 day and vessel was almost completely denervated by 7 days following axotomy of the SCG. By 12 weeks, the total sympathetic innervation of the ACA, though significantly decreased compared with the controls, was increased compared with the 7 day treatment group, indicating a potential recovery of the sympathetic innervation over time. Regional analysis of the ACA revealed a reduction in sympathetic innervation across all three regions of the vessel at every time point examined. By 12 weeks however, the regional sympathetic innervation of the ACA, though significantly decreased compared with the controls, was increased compared
with the 7 day treatment group and indicated a potential recovery. These results are similar to that observed in the MCA in a previous study (Zhu et al., 2010).

Changes in sensory and parasympathetic perivascular axons following axotomy.

Changes in the sensory and parasympathetic populations were examined in the ACA and MCA following axotomy of the SCG at time points when the vessels were almost completely denervated of sympathetic axons (1 day and 7 days) and when sympathetic innervation appeared to be returning (8 weeks and 12 weeks). The distribution of sensory perivascular axons associated with the extracerebral blood vessels, which arise from the trigeminal ganglion (Wanaka et al., 1986; Tsai et al., 1988; Edvinsson, 1987; Suzuki et al., 1989), was observed by immunofluorescence for calcitonin gene related peptide (CGRP) (Ruskell and Simons, 1987; Saito and Moskowitz, 1989). The extracerebral blood vessels also receive parasympathetic innervation from the sphenopalatine and otic ganglia (Walters et al., 1986; Hardebo et al., 1991) and these parasympathetic axons can be labeled with an antibody directed toward VACht (Eiden et al., 2004).

First, co-localization studies were carried out to ensure that the markers used for sensory (CGRP) and parasympathetic (VACht) axons did not co-localize. In Figure 4, a control MCA and one from a 12 week axotomy case are shown and no colocalization of these two markers was observed. Similarly, though not shown here, previous studies showed no colocalization of CGRP and TH and no colocalization of VACht and TH in the perivascular axonal population in the controls or following axotomy of the SCG.

Changes in sensory innervation. The normal appearance and distribution of CGRP-ir perivascular axons associated with the ACA and MCA are shown in Figures 5-8. As shown in Figures 5 and 7, the perivascular sensory axons appeared thinner and finer than the sympathetic axons and their overall density appeared less than the sympathetics, yet sensory axons showed a longitudinal and circumferential distribution pattern that was somewhat similar to sympathetic axons. The changes in sensory innervation of the ACA and MCA followed similar trends and generally increased following sympathetic denervation and reinnervation. While at 1 day following axotomy the density of sensory axons associated with both vessels was similar to controls, by 7
days, the density of sensory axons in both vessels was significantly increased compared to controls. A trend for an increase was observed at 8 weeks, and at 12 weeks following axotomy, the density of CGRP-ir axons was significantly increased and similar to the 7 day time point, indicating a sustained increase in sensory innervation at this time point. Regional analysis of the ACA (Fig. 6) indicated that the increase (compared with controls) was observed consistently in the middle and distal segments of the vessel at the 7 day and 12 week time points and that these time points generally were similar. However in the MCA (Fig. 8), the sensory innervation in the distal segment was significantly elevated compared with the 7 day time point.

**Changes in parasympathetic innervation.** The typical appearance and distribution of the parasympathetic perivascular axons are shown in Figures 9-12. As shown in Figures 9 and 11, the parasympathetic innervation of the ACA and MCA in all cases showed primarily a circumferential pattern. Following axotomy, the parasympathetic innervation of the two vessels followed similar trends across treatments and generally was decreased during the sympathetic denervation and reinnervation periods. In the control cases, VACHT-ir parasympathetic axons typically showed a circumferential distribution pattern with occasional axons in a longitudinal orientation (Fig. 9, 11). The total number of parasympathetic axons associated with the ACA was decreased at 1 day, 7 days and 8 weeks, but at 12 weeks following axotomy was similar to controls (Figs. 9, 10), revealing a potential recovery in the parasympathetic axons, similar to that observed with regard to the sympathetic axons. However, though not different from the control group, the parasympathetic density at 12 weeks was similar to the 7 day time point. The regional analysis of the ACA revealed decreases in parasympathetic innervation at both short and long term time points (Fig. 10), yet the 12 week time point was not significantly different from controls, even though in each region of the ACA, the 12 week cases were similar to the 7 day time point (Figs. 9,10). With respect to the MCA, similar decreases in the total number of parasympathetic axons were observed (Fig. 12). The decrease was not observed at 1 day, but at all other time points, including 12 weeks, a decrease in perivascular density compared to controls was observed (Figs. 11, 12). Regional analysis of the MCA
revealed decreases in some segments at both short and long term time points (Fig. 12). Again, the 7 day and 12 week cases were statistically similar in each regional segment.

**Changes in VEGF following axotomy of the SCG.**

Western analysis was used to assess the changes in VEGF in the extracerebral blood vessels, the SCG, and the trigeminal ganglion following following axotomy of the SCG. The 42 kDa isoform was the primary form observed in the extracerebral blood vessels, and this isoform was similar to controls at each time point examined (Fig. 13). In SCG, a 42kDa and 80 kDa isoform were observed. Though unchanged at 1 day, the 42kDa VEGF isoform was significantly increased to 273% of controls at 7 days following axotomy (Fig. 14) and was increased to 340% and 353% of control values at 8 weeks and 12 weeks axotomy, respectively. In contrast the 80kDa isoform was generally decreased and was 23%, 13%, and 2% of the control values at 7 days, 8 weeks, and 12 weeks, respectively (Fig. 14). VEGF protein also was altered in the trigeminal ganglion (Fig. 15). Though the 42kDa isoform was significantly decreased to 73% and 49% of control values at 1 day and 8 weeks following axotomy, this isoform was increased to 161% of controls at 12 weeks following axotomy. The 80kDa VEGF was significantly increased to 198% and 147% of control values at 7 days and 12 weeks, but was significantly decreased to 46% of control values at 8 weeks following axotomy (Fig. 15).
The extracerebral blood vessels at the base of the brain

Figure 1
**Figure 1.** Diagram of the extracerebral blood vessels located at the base of the brain. Western analyses were carried out on all vessels from one side that comprise the Circle of Willis. Immunohistochemical analysis was performed on the anterior cerebral artery (ACA) and the middle cerebral artery (MCA) as individual samples. Identification the proximal, middle, and distal portions of the ACA and MCA were based on the proximity of the vessels to the Circle, with the proximal segment located closest to the Circle and the distal segments farthest from the Circle. (Based on [http://www.stroke-recovery-advocate.com/brain-blood-supply.html](http://www.stroke-recovery-advocate.com/brain-blood-supply.html))
TH perivascular axons associated with the ACA following axotomy

Figure 2
Figure 2. TH immunohistochemistry of the ACA following axotomy. ACA from control cases exhibited robust TH immunoreactivity (red). Axons passed in both the longitudinal and circumferential planes. TH immunoreactivity appeared reduced following a 1 day (1d) axotomy, especially at the middle and distal portion. TH immunoreactivity was virtually absent at all three portions of the ACA by 7 days (7d) after the axotomy. TH immunoreactivity remained decreased at 8 weeks (8wk) compared with controls, some longitudinal axons appeared in the ACA. By 12 weeks (12wk) following axotomy, the density appeared to increase compared with earlier time points. Scale bar, 100µm.
A. Total number of TH perivascular axons: ACA

B. TH perivascular axons: regional analysis

Figure 3
Figure 3. Quantitative analysis of the density of TH-immunoreactive (-ir) sympathetic axons associated with the ACA. **A.** Total sympathetic innervation was significantly decreased (compared with controls) following axotomy of the SCG at all of the time points examined. Sympathetic innervation was decreased at 1 day (1d) and the vessel was almost completely denervated by 7 days (7d) following axotomy of the SCG. By 12 weeks (12wk), the total sympathetic innervation of the ACA, though significantly decreased compared with the controls, was increased compared with the 7 day treatment group, indicating a potential recovery of the sympathetic innervation over time. **B.** Regional analysis revealed a reduction in sympathetic innervation across all three regions of the vessel at every time point examined. By 12 weeks the regional innervation, though significantly decreased compared with the controls, was increased compared with the 7 day treatment group and indicated a potential recovery. *, significantly different from control, p<0.05; #, significantly different from 7 day treatment, p<0.05. Number of samples examined: cont (n=12), 1d (n=3), 7d (n=3), 8wk (n=3), 12wk (n=3).
CGRP-ir and VACHT-ir perivascular axons show no colocalization

Figure 4
**Figure 4.** Immunohistochemical analysis of CGRP (red arrows, left panels) and VACHT (green arrows, middle panels) associated with the MCA from a 12 week control (upper) and from a rat receiving a 12 week axotomy (lower). At the 12 week time point after axotomy, CGRP-immunoreactive (-ir) axons were increased while VACHT-ir axons were decreased. Note in the merged images (right panels) the complete segregation of the two axonal populations and no colocalization of the two markers. Scale bar, 100µm.
CGRP-ir perivascular axons associated with the ACA following axotomy

Figure 5
Figure 5. CGRP immunoreactive (-ir) axons associated with the ACA following axotomy of the SCG. The perivascular sensory axons appeared thinner and finer than the sympathetic axons, yet a longitudinal and circumferential distribution pattern was present. By 7 days (7d) following axotomy, the density of sensory axons appeared increased at proximal, middle and distal portions of the ACA compared to controls. At 12 weeks (12wk) following axotomy, the density of CGRP-ir axons was significantly increased compared with controls and statistically similar to the 7 day time point, indicating a sustained increase in sensory innervation at this time point. Scale bar, 100µm.
A. Total number of CGRP perivascular axons: ACA

B. CGRP perivascular axons: regional analysis

Figure 6
Figure 6. Quantitative analysis of the density of CGRP-immunoreactive (-ir) sensory axons associated with the ACA.  

**A.** The density of CGRP-ir axons was increased at 7 days (7d) following axotomy, and at 12 weeks (12wk), the density was significantly increased compared with controls but statistically similar to the 7 day time point, indicating a sustained increase in sensory innervation.  

**B.** Regional analysis of the ACA revealed a significant increase (compared with controls) in the middle and distal segments of the vessel at the 7 day (7d) and 12 week (12 wk) time points and that these time points generally were similar. *, significantly different from control, p<0.05. Number of samples examined: cont (n=12), 1d (n=3), 7d (n=3), 8wk (n=3), 12wk (n=3).
CGRP-ir perivascular axons associated with the MCA following axotomy

Figure 7
**Figure 7.** CGRP-immunoreactive(-ir) perivascular axons associated with the MCA following axotomy of the SCG. The MCA from control cases exhibited a typical distribution of longitudinal and circumferential CGRP-ir sensory axons. By 7 days (7d) following axotomy, the density of sensory axons appeared increased compared to controls, the increase was more prominent at proximal portion of the MCA. The increase continued at 12 weeks (12wk) following axotomy. Scale bar, 100μm.
A. Total number of CGRP perivascular axons: MCA

B. CGRP perivascular axons: MCA regional analysis

Figure 8
**Figure 8.** Quantitative analysis of the density of CGRP-immunoreactive(-ir) sensory axons associated with the MCA.  

**A.** Analysis of the total density of the CGRP-ir axons on the MCA revealed a significant increase in CGRP-ir axons at 7 days (7d) and 12 weeks (12wk) following axotomy of the SCG. The density in the 12 week treatment was similar to the 7 day cases.  

**B.** Regional analysis of the density of CGRP-ir axons associated with the MCA revealed significant increase at 7 days after axotomy at the proximal portion of the MCA. By 12 weeks following axotomy, a significant increase in CGRP-ir axons was observed at all three regions of the MCA. *, significantly different from control, p<0.05. Number of samples examined: cont (n=12), 1d (n=3), 7d (n=3), 8wk (n=3), 12wk (n=3).
VAChT associated with the ACA following axotomy

Figure 9
Figure 9. VACHT-immunoreactive(-ir) perivascular axons associated with the ACA following axotomy. VACHT-ir axons showing a circumferential pattern were typically observed in the control cases. Abundant circumferential VACHT-ir axons were observed in the proximal, middle, and distal portion of the ACA in the control group. These axons appeared reduced in the 1 day (1d), especially at middle and distal portion. The decrease was also observed at 7 day (7d) and 8 week (8wk) cases, which dramatic decrease was observed at all three portions of the ACA. By 12-week axotomy, the density of VACHT-ir parasympathetic axons appeared to return to control values. Scale bar, 100µm.
A. Total number of VACHT perivascular axons: ACA

![Bar chart showing density of perivascular axons over time (cont, 1d, 7d, 8wk, 12wk).](image)

B. VACHT perivascular axons: regional analysis

![Bar chart showing density of perivascular axons for proximal, middle, and distal regions.](image)

Figure 10
Figure 10. Quantitative analysis of the density of VACHT-immunoreactive(-ir) parasympathetic axons associated with the ACA. A. The total number of parasympathetic axons associated with the ACA was decreased at 1 day, 7 days and 8 weeks following axotomy, but at 12 weeks was similar to controls, revealing a potential recovery over time in these axons. However, though not different from the control group, the parasympathetic density at 12 weeks was similar to the 7 day time point. B. The regional analysis of the ACA revealed decreases in parasympathetic innervation at both short and long term time points, yet the 12 week time point was not significantly different from controls although in each region of the ACA, the 12 week cases were similar to the 7 day time point. *, significantly different from controls, p<0.05. Number of samples examined: cont (n=12), 1d (n=3), 7d (n=3), 8wk (n=3), 12wk (n=3).
VACChT-ir axons associated with the MCA following axotomy

Proximal | Middle | Distal
---|---|---
cont | | 1d
| 7d | 8wk | 12wk

Figure 11
Figure 11. VACHT-immunoreactive(-ir) perivascular axons associated with the MCA following axotomy. VACHT-ir axons showing a circumferential pattern were typically observed in the control cases. These axons appeared to be reduced in the 7 day (7d) and 8 week (8wk) and 12 week (12wk) survival time points following axotomy. Scale bar, 100µm.
A. Total number of VACHT perivascular axons: MCA

B. VACHT perivascular axons: regional analysis

Figure 12
Figure 12. Quantitative analysis of the density of VAChT-immunoreactive(-ir) parasympathetic axons associated with the MCA. **A.** No change was observed at 1 day (1d) following axotomy, but at all other time points, including 12 weeks (12wk), a decrease in VAChT-ir perivascular density compared to controls was observed. **B.** Regional analysis of the MCA revealed decreases in some segments at both short and long term time points. Again, the 7 day and 12 week cases were statistically similar in each regional segment. Number of samples examined: cont (n=12), 1d (n=3), 7d (n=3), 8wk (n=3), 12wk (n=3).
Table 1. Summary of changes in perivascular axonal populations associated with the ACA and MCA.

<table>
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<tr>
<td>axons</td>
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VEGF in the extracerebral blood vessels following axotomy

A.

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</table>

B.

VEGF in the extracerebral blood vessels

![Bar chart showing VEGF levels at different times after axotomy.]

Figure 13
**Figure 13.** VEGF western analysis of the extracerebral blood vessels. **A.** Western analysis of VEGF associated with the extracerebral blood vessels revealed a prominent 42kDa band. The presence of GAPDH served as a loading control. Each lane represents a different vessel. 20 µg protein loaded. **B.** Semiquantitative analysis revealed that VEGF protein expression was unchanged following axotomy when compared to corresponding age-matched controls.
VEGF in the SCG following axotomy

A.

<table>
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</table>

B.

VEGF in the SCG

![Graph](image9.png)

Figure 14
Figure 14. VEGF western analysis of the superior cervical ganglion (SCG). A. Western analysis of VEGF associated with the SCG revealed prominent bands at 42kDa and 80kDa. Each lane represents a single superior cervical ganglion. The presence of GAPDH served as a loading control. 25 µg protein loaded. B. Semiquantitative analysis revealed changes in both isoforms of VEGF protein (42kDa and 80kDa) following axotomy of the SCG. Though unchanged at 1 day, the 42kDa VEGF isoform was significantly increased to 273% of controls at 7 days following axotomy and was increased to 340% and 353% of control values at 8 weeks and 12 weeks axotomy, respectively. In contrast the 80kDa isoform was generally decreased and was 77% of controls at 7 days and decreased to 87% and 98% of the control values at 8 weeks and 12 weeks respectively. *, different from control, p<0.05.
A. VEGF in trigeminal ganglion following axotomy

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<tr>
<td>12wk</td>
<td>80 kDa</td>
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</tr>
</tbody>
</table>

B. VEGF in the trigeminal

- 42kD
  - 1d
  - 7d
  - 8wk
- 80kD
  - 1d
  - 7d
  - 8wk

Figure 15
Figure 15. VEGF western analysis of the trigeminal ganglion. Each lane represents a single trigeminal ganglion. **A.** Western analysis of VEGF associated with the trigeminal ganglion revealed prominent bands at 42kDa and 80kDa. Each lane represents a trigeminal sample. The presence of GAPDH served as a loading control. 25 µg protein loaded. **B.** Semiquantitative analysis revealed changes in both isoforms of VEGF protein (42kDa and 80kDa) following axotomy of the SCG. Though the 42kDa isoform was significantly decreased at 1 day and 8 weeks following axotomy, this isoform was increased to 161% of controls at 12 weeks. The 80kDa VEGF was significantly increased at 7 days and 12 weeks, but was decreased at 8 weeks following axotomy. *, different from control, p<0.05.
6. DISCUSSION

Plasticity of sympathetic perivascular innervation following axotomy of the SCG.

In order to assess the regenerative growth potential of the SCG neurons following axotomy, we examined the TH reinnervation of the extracerebral blood vessels, which serve as primary targets of the SCG, following axotomy. The typical sympathetic innervation pattern of extracerebral blood vessels by postganglionic axons arising from the SCG has been examined previously (Kajikawa, 1968; Sato et al., 1980; Handa et al., 1990; Hamel, 2006), yet no studies have documented the denervation and reinnervation of the extracerebral blood vessels over short-term and long-term survival time points following axotomy of the SCG. The results of our study reveal that sympathetic TH innervation of the ACA and MCA following axotomy of the SCG generally did not fully recover over the 12 week survival period. Whether TH innervation returns to control values at longer survival time points is unknown. These findings reveal that peripheral nerve regeneration typically is a slow process and regenerative potential of these axons is not as robust as the previously reported growth rate of 3mm/day (Gordon et al., 2007) for regenerative growth of peripheral axons.

We have examined the sympathetic innervation of other vascular and peripheral targets in our laboratory following axotomy of the SCG and have found somewhat variable results. For example, similar to the relatively incomplete reinnervation of the ACA and MCA, sympathetic innervation of the basilar artery (Fig. 1) also did not recover by 12 weeks following axotomy. However, at this same time point, the sympathetic innervation was similar to control values in the internal carotid artery (Fig. 1). In the pineal gland, a small gland located on the dorsal surface of the brain between the cerebral hemispheres, the TH innervation remained significantly decreased at 12 weeks following axotomy (Zhu et al., 2010). Yet, the innervation of the submandibular glands (smg), located in the neck region, was similar to control values by 12 weeks following axotomy (Zhu et al., 2010). Perhaps the distance needed by the regenerating axons to traverse to reinnervate their original target has an impact on the time course of reinnervation. Indeed, the targets that are farthest away and are innervated by the internal carotid nerve take longer to reinnervate than more proximal targets (smg). Yet, western analysis of the SCG revealed that TH protein in the SCG, which was
significantly reduced relative to controls at 1 day and 7 days, remained decreased at 12 weeks following axotomy (Morris et al., 2010), indicating that recovery is far from complete at this survival time point. Sun and Zigmond (1996) showed that TH mRNA in SCG was similar to controls at 12 weeks following axotomy. These results suggest that TH mRNA levels in the SCG return to normal for quite some time prior to the normal expression of TH protein.

**Increased CGRP sensory innervation following axotomy of the SCG.**

The origin and distribution pattern of sensory innervation in vascular tissues have been described by others (Tsai et al., 1988; Cherruau et al., 2003; Ekstrom and Ekman, 2005), yet documenting the distribution of sensory innervation of the extracerebral blood vessels following short-term and long-term axotomy of the SCG has not been reported previously. In the present study, we analyzed the density of the CGRP-ir sensory axons associated with the ACA and MCA at time points when sympathetic denervation and reinnervation are occurring. In the control tissues, the fine, varicose sensory perivascular axons passed along the vessels in both the longitudinal and circumferential planes in a pattern similar to that described by Tsai and colleagues (Tsai et al., 1988). By 7 days following axotomy, we observed a significant increase in sensory axons associated with both the ACA and MCA and the increase persisted at the 12 week time point. Similarly, Kessler et al. (1983) observed a significant increase in sensory axons in the iris following axotomy of the SCG. The increase in sensory innervation may be the result of loss of competition by the sympathetic axons. Indeed, Kessler et al. (1983) postulated that the increase in sensory innervation of the iris was likely the result of increased availability of target-derived growth factors such as NGF provided by the target in the absence of sympathetic input. In support of this idea, though NGF was not examined in this study, we have observed an increase in both NGF and NT-3 in the extracerebral blood vessels following long term removal of the SCG (Randolph et al., 2007).

Because the increase in sensory perivascular axons persisted even when recovery of the sympathetic innervation appears to be ongoing, other factors may also be involved in mediating the changes in sensory neurons during the time of
compensatory sprouting. It has also been postulated that CGRP may play an important role in axonal outgrowth and regeneration (Toth et al., 2009). Following sural sensory axon injury, CGRP was increased in regenerating sensory neurons and it was thought to enhance the regenerative properties of the sensory axons. Though speculative, it is possible CGRP released by the sensory perivascular axons might somehow contribute to the sympathetic reinnervation process.

**Decreased VACHT parasympathetic innervation following axotomy of the SCG.**

Previous studies have been conducted to determine the origin and distribution of the parasympathetic axons of the extracerebral blood vessels (Hardebo et al., 1991; Bleys et al., 2000). By using retrograde axonal tracers, Hardebo and co-workers (1991) found that parasympathetic axons innervating the extracerebral blood vessels in the monkey originated from the sphenopalatine ganglion and the internal carotid ganglion. In rat, parasympathetic axons that innervate the extracerebral blood vessels arise mainly from the sphenopalatine and otic ganglia (Walters et al., 1986; Hardebo et al., 1991) as well as the cavernous sinus ganglia (Bleys et al., 2000).

Following axotomy of the SCG and loss of sympathetic input to the extracerebral blood vessels, we observed a general reduction in VACHT-ir perivascular axons and these axons remained significantly decreased at 12 weeks following axotomy. Consistent with these findings, decreased parasympathetic innervation, using nitric oxide synthase as a parasympathetic marker, associated with the internal carotid artery was observed at 6 weeks following sympathectomy (Warn et al., 1997). These authors proposed that target derived factors released following the removal of sympathetic axons may have suppressed NOS expression by the parasympathetic axons (Warn et al., 1997). In addition, Kemplay (1980) observed a loss in cholinesterase activities of the submandibular gland at 7 and 14 days following axotomy but cholinesterase activity returned to control values at 8 and 12 months. Neurons in the cardiac ganglion have been shown to be heterogeneous including both cholinergic and adrenergic neurons (Weihe et al., 2005). It has been proposed by Hasan and Smith (2009) that cardiac ganglion is innervated by both extrinsic sympathetic innervation and intrinsic noradrenergic neurons. Similarly, Hasan and Smith (2009) observed a reduced number
of parasympathetic neurons in the cardiac ganglion following loss of sympathetic input and proposed that sympathetic innervation is important for modulating the cholinergic properties of this ganglion.

These studies suggest that the parasympathetic axons were still present, but that their typical expression of parasympathetic neurotransmitters was reduced by the loss of sympathetic input, leading to the overall conclusion in our study that the protein expression of VACHT, but not the presence of the perivascular axons, was reduced following axotomy of the SCG, and that sympathetic axons somehow modulate the expression of VACHT in the parasympathetic nervous system. In our study, we did not use a general axonal marker to determine whether the parasympathetic axons were still present. This is an experiment that will help with the interpretation of our results and will determine whether the axons are still present. However, in contrast to these findings, an increase in vasoactive intestinal peptide, a peptide believed to be associated with parasympathetic axons, was observed in the parotid and submandibular glands using radioimmunoassay techniques at 28 days following removal of the SCG (Ekström and Ekman, 2005). The authors of this study proposed that the increased NGF in the glands resulting from loss of sympathetic input may have increased parasympathetic activity.

**Changes in VEGF and the role of VEGF following axotomy of the SCG.**

In addition to a role in angiogenesis (Ferrara, 1996; Autiero et al., 2005), VEGF can play an important role in the development of the nervous system (Ruiz de Almodovar et al., 2009), neurodegenerative diseases (Bogaert et al., 2006), and peripheral neuropathies (Bogaert et al., 2006; Skold and Kanje, 2008; Ruiz de Almodovar et al., 2009). VEGF also reportedly plays a role in regulation of the sensory and sympathetic neurons (Sondell et al., 1999b; Marko and Damon, 2008; Long et al., 2009).

It is believed that VEGF is produced by peripheral vascular targets such as vascular cells associated with arteries such as the femoral artery (Marko and Damon, 2008) and then retrogradely transported to parent cell bodies to carry out its regulatory activities (Sondell et al., 2000) with VEGF receptors located on sympathetic and sensory neurons (Sondell et al., 2000). Sondell and co-workers (1999b) detected both
VEGF and its receptor VEGFR2 in the SCG and sensory neurons of the DRG. VEGF in the peripheral nervous system functions to stimulate axonal outgrowth (Sondell et al., 2000), and enhance cell survival and proliferation (Sondell et al., 1999a).

In their studies of the femoral artery, Marko and Damon (2008) detected a 42kDa VEGF isoform associated with this vascular target. This is similar to our analysis of the extracerebral blood vessels. We also observed the 42 kDa VEGF isoform in the SCG and the trigeminal. This is somewhat similar to a report of a 37kDa VEGF isoform in the SCG and dorsal root ganglion by Sondell and colleagues (1999b). In addition to the 42kDa VEGF isoform, an 80kDa VEGF isoform was prominent in both the SCG and the trigeminal ganglion. Though the data sheet for the antibody that we used in this study indicates no cross reactivity with VEGFC, it is interesting to note that the typical size of VEGFC is indeed 80 kDa, as we observed in our immunoblots.

Following severing the femoral nerve to remove sympathetic input to the femoral artery, VEGF was found to coordinate the sympathetic reinnervation of femoral arteries (Marko and Damon, 2008), leading to the experiments carried out in the present study. Here we used western analysis to document VEGF protein in the extracerebral blood vessels following the sympathetic denervation and reinnervation that occurs after axotomy of the SCG. However unlike Marko and Damon (2008) we found no differences in VEGF protein associated with the vessels during the denervation and reinnervation period.

Yet we did find significant changes in the SCG and trigeminal ganglia following axotomy of the SCG. No studies have examined changes in VEGF expression in a sympathetic ganglion following axotomy. Interestingly, even though VEGF protein expression was not changed in the vascular targets, which are thought to synthesize VEGF, the expression of this protein was dramatically altered by 1 week, and remained altered at 8 weeks and 12 weeks following axotomy of the SCG. The two VEGF isoforms were differentially affected, with the 42kDa isoform increasing and the 80kDa isoform decreasing over time.

There are at least two possible explanations for the VEGF changes in the SCG following axotomy. It is possible that VEGF that was produced by the vascular targets was then transported to the SCG by regenerating sympathetic axons. Retrograde
transport of VEGF has been shown in sensory neurons in vitro (Sondell et al., 2000), supporting this possibility. However, changes due to retrograde transport would not be possible at the early time points since the peripheral targets are denervated following axotomy and no perivascular axons would be present and/or available for transport. This possibility could, however, explain the changes at later time points, once regeneration of sympathetic axons was underway. A second possibility is that neurons and/or glial cells have the capability to biosynthesize VEGF and that VEGF production by SCG cells was altered following the injury. This possibility is more likely, particularly at early time points. Indeed, VEGF-ir neuronal cell bodies in the SCG were observed in a previous study (Sondell et al., 1999b). However it was not clear whether this VEGF protein was produced by the neurons or had been transported from peripheral vascular targets. VEGF receptor 2 protein and mRNA (Sondell et al., 1999b) were observed in neurons in the SCG but the potential for VEGF mRNA expression in the SCG has not been examined.

VEGF protein also was altered in the trigeminal ganglion following axotomy of the SCG, at time points when sympathetic denervation and reinnervation was occurring and when sensory perivascular populations were showing enhanced CGRP immunoreactivity. It is important to note that, rather than regeneration, the sensory perivascular axons were undergoing compensatory sprouting in response to the axotomy of the SCG, suggesting that VEGF plays a role not only in the regeneration of peripheral neurons, but in the plasticity in the sensory nervous system that takes place following loss of sympathetic input. Similar to the sympathetic system, the changes in VEGF could be the result of retrograde transport from the vascular targets to the trigeminal neurons (Sondell et al., 2000) or might result from changes in VEGF biosynthesis that, following axotomy, resulted from retrograde signals produced at the injury site and that were transported to the cell bodies in the trigeminal ganglion. At 8 weeks following axotomy, a decreasing trend of CGRP-ir sensory axons was observed in both ACA and MCA. It is interesting to note that at the same time point, a significant decrease of both VEGF isoforms (42kDa and 80kDa) was observed in the trigeminal ganglion. This parallel between sensory innervation and VEGF protein expression in the trigeminal ganglion suggests a possible regulatory influence of VEGF on CGRP
expression by the trigeminal. Indeed, VEGF mRNA and protein was observed in the trigeminal ganglion (Yu et al., 2008) and was shown to play a role in the regeneration of sensory axons in the cornea following wounding of the cornea with a scalpel blade.

Given the dual role for VEGF regulation of sensory and sympathetic neurons, it is possible at earlier time points (24 hours, 1 day, and 8 weeks), sensory axons utilized VEGF from the extracerebral blood vessels. While at long term (12 weeks), sensory axons received VEGF from neurons in the trigeminal ganglion. At 8 weeks, because the sensory axons are switching its VEGF source to trigeminal ganglion, the sprouting of sensory axons might compromised at this time point. The sympathetic axons received VEGF from the SCG, so I am thinking the recovery of the sympathetic axonal population on extracerebral blood vessel might be related with the reinnervation of the sympathetic axons after axotomy. The density of sympathetic axons showed a potential recovery at 8 week and 12 weeks. This trend is similar to the increased level of VEGF observed in SCG. And some other growth factor, for example NGF, could also involved in the process.

In contrast to the SCG, both isoforms in the trigeminal ganglion showed increases following axotomy at the 12 week time point when sympathetic reinnervation was occurring. The VEGF alterations paralleled the sustained increase in sensory perivascular axons, leading to the conclusion that the sensory nervous system shows long term changes following axotomy of the SCG, indicating a close relationship between the two neuronal populations in their innervation of peripheral targets.

7. CONCLUSIONS

Our goal in this project was to examine the changes in the peripheral neurons and their vascular targets following axotomy of the SCG. We found that, similar to the other extracerebral blood vessels, the sympathetic innervation of the ACA at 7 days following axotomy was significantly decreased following axotomy of the SCG. By 12 weeks, the sympathetic innervation of the ACA, though significantly decreased compared with controls, was increased compared with the 7 day treatment group, suggesting a potential recovery of the sympathetic innervation. This is similar to the results observed with the MCA, which was conducted in a previous study (Zhu et al.,
The reinnervation process was much slower than anticipated, since regeneration of peripheral neurons has been estimated to take place at approximately 3mm/day (Gordon et al., 2007) and these axons must traverse approximately 40-50 mm to reach these vessels. Yet there were signs that the sympathetic innervation was increasing over the longer survival time points.

At the 7 day survival time point, when the ACA and MCA were denervated of sympathetic axons, the density of sensory perivascular axons associated with these vessels was significantly increased, suggesting a compensatory sprouting response by the sensory axons. Interestingly, though sympathetic reinnervation of the vessels was taking place at 12 weeks following the injury, the sensory innervation remained enhanced. When the sensory axons return to normal is unknown, but there were few signs that the sensory innervation was returning to control values at the 12 week time point. Because sympathetic innervation of the extracerebral blood vessels is known to function in vasoconstriction (Kajikawa, 1968; Hernández-Pérez et al., 1975), and sensory perivascular axons are known vasodilators (Hamel, 2006), a potential vasodilation of the vessel may results from these long term changes which in turn could alter cerebral blood flow.

In contrast to the response by sensory axons, the parasympathetic innervation of the ACA and MCA showed a general decrease following axotomy of the SCG and loss of sympathetic innervation. Whether the axons were present but showing a decrease in the expression of parasympathetic neurotransmitter or whether an actual reduction in the number of parasympathetic fibers occurred is unknown. At 12 weeks following the injury, the parasympathetic and sympathetic innervation associated with the ACA followed similar trends and, similar to sympathetic axons, the parasympathetic axons appeared to be recovering. However, the total perivascular density of parasympathetic axons associated with the MCA remained decreased even though the sympathetic axons were increasing. It appears that the regulatory influences associated with the ACA and MCA are different, as reflected by the differences in the innervating populations. Interestingly, parasympathetic axons also serve as vasodilators (Hamal, 2006) and therefore it is important to note that a reduction in the activity of these axons is countered by the increase in sensory vasodilators.
Assessment of VEGF protein revealed VEGF isoforms that were prominent in the extracerebral blood vessels as well as the SCG and the trigeminal ganglion. Though the 42kDa isoform was unchanged in the blood vessels following injury, it showed a robust increase in the SCG as well as the trigeminal ganglion, particularly at 12 weeks following the injury. An 80kDa VEGF isoform also was prominent in the SCG and trigeminal ganglion. Though the 80kDa isoform was differentially affected in the SCG, in the trigeminal ganglion, it was increased at both short term and long term survival time points.

In conclusion, though sympathetic nerve regeneration associated with the extracerebral blood vessels appeared to be progressing at 12 weeks following axotomy of the SCG, other changes also were occurring in parallel to the reinnervation process. The other perivascular populations (sensory and parasympathetic) continued to be impacted by the injury, even at long term time points. At least some of these changes may be mediated by the alterations in VEGF in sympathetic axons, which were undergoing regeneration, as well as in the sensory population, which was undergoing compensatory sprouting. Future studies will need to address the site of VEGF biosynthesis in order to fully understand how this regulatory protein contributes to the plasticity that is observed following axotomy of the SCG.
8. REFERENCES


