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Sympathetic reinnervation of peripheral targets following bilateral axotomy of the adult superior cervical ganglion

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Abstract

The ability of adult injured postganglionic axons to reinnervate cerebrovascular targets is unknown, yet these axons can influence cerebral blood flow, particularly during REM sleep. The objective of the present study was to assess quantitatively the sympathetic reinnervation of vascular as well as non-vascular targets following bilateral axotomy of the superior cervical ganglion (SCG) at short term (1 day, 7 days) and long term (8 weeks, 12 weeks) survival time points. The sympathetic innervation of representative extracerebral blood vessels [internal carotid artery (ICA), basilar artery (BA), middle cerebral artery (MCA)], the submandibular gland (SMG), and pineal gland was quantified following injury using an antibody to tyrosine hydroxylase (TH). Changes in TH innervation were related to TH protein content in the SCG. At 7 days following bilateral SCG axotomy, all targets were significantly depleted of TH innervation, and the exact site on the BA where SCG input was lost could be discerned. Complete sympathetic reinnervation of the ICA was observed at long term survival times, yet TH innervation of other vascular targets showed significant decreases even at 12 weeks following axotomy. The SMG was fully reinnervated by 12 weeks, yet TH innervation of the pineal gland remained significantly decreased. TH protein in the SCG was significantly decreased at both short term and long term time points and showed little evidence of recovery. Our data demonstrate a slow reinnervation of most vascular targets following axotomy of the SCG with only minimal recovery of TH protein in the SCG at 12 weeks following injury.

Keywords

extracerebral blood vessels; perivascular axons; tyrosine hydroxylase innervation density

1. Introduction

The sympathetic postganglionic neurons located in the superior cervical ganglion, which innervate vascular targets such as the extracerebral blood vessels, serve to influence cerebral blood flow, particularly during REM sleep (Cassaglia et al., 2009). In response to axotomy (transection of postganglionic axons) these neurons reportedly ‘shift’ priorities from neurotransmission to regeneration (Boeshore et al., 2004; Sun and Zigmond, 1996). Indeed, some have suggested a potential recovery in the SCG by 8 weeks following axotomy (Klimaschewski et al., 1994; Klimaschewski et al., 1996; Sun and Zigmond, 1996), leading to the conclusion that SCG axons have successfully regenerated and subsequently

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reinnervated their peripheral targets. Yet extensive cell death in the SCG has been reported following injury to postganglionic axons (Hendry, 1975b; Purves, 1975), leading to confusion in the literature regarding if and when axotomized SCG axons reinnervate their peripheral targets (see Purves and Thompson, 1979) and/or whether recovery of the SCG neurons indeed occurs. In addition, no quantitative assessment of the innervation density of vascular targets of the SCG following axotomy has been carried out to determine the extent of sympathetic reinnervation.

In the current study, the sympathetic denervation and reinnervation of several peripheral (vascular and non-vascular) targets of the SCG were assessed quantitatively following axotomy of the SCG. Using TH as a marker for sympathetic axons, the sympathetic innervation of representative extracerebral blood vessels [internal carotid artery (ICA), basilar artery (BA), middle cerebral artery (MCA)], as well as the submandibular gland (SMG) and pineal gland was quantified following bilateral axotomy at short term (1 day, 7 day) and long term (8 week and 12 week) survival time points. We also analyzed the presence of TH protein in the SCG at these same time points in order to relate changes in the innervation of the peripheral targets with TH protein content in SCG neurons. Our data demonstrate a relatively slow reinnervation process of the vascular targets of SCG neurons at 12 weeks following axotomy with only minimal recovery of TH protein in the SCG.

2. Results

Analysis of ptosis

The severity of ptosis following axotomy of the SCG was ranked over the survival period from 0 (no ptosis) to 5 (most severe ptosis). Ptosis typically ranged from 3.5-4.5 at 1 day following injury and remained relatively unchanged (rank of ~4) at 7 days following axotomy (Hendry, 1975a). By 8 weeks ptosis generally was reversed in most of the cases with 6 of the 8 cases examined at this survival time point showing a ptosis that ranked 0-1. In the two remaining cases, one showed ptosis that ranked 1.5 (right) and 2.0 (left) and the ptosis ranked 2.5 (right) and 3.0 (left) in the second case. At 12 weeks following injury, little ptosis was observed in any of the axotomized cases and the rank average was approximately 0-1.

Analysis of peripheral targets

The ICA from control cases showed distinctive TH immunoreactive (-ir) axons passing in the longitudinal and circumferential planes and as described in previous studies (Handa et al., 1990; Hernandez-Perez and Stone, 1974; Kajikawa, 1968; Marfurt et al., 1986; Fig. 1). At 1 day following axotomy, the total TH innervation density of the ICA was reduced, and by 7 days the vessel appeared completely denervated. Yet by 8 weeks TH-ir axons were present and the vessel showed robust TH innervation (Fig.1). Quantitative analysis of the TH innervation density associated with the ICA revealed a significant decrease at 1 day and 7 days following axotomy. However, the TH innervation density of the ICA was similar to controls at both 8 week and 12 weeks and showed a significant increase compared to the 7 day survival time point (Fig. 1), suggesting complete reinnervation by 8 weeks following axotomy.

Three regions or segments of the BA (caudal, middle, rostral) were analyzed for changes in TH innervation density following bilateral axotomy of the SCG. The total TH perivascular density associated with the caudal segment, the region closest to the vertebral artery, remained consistently unchanged at all survival time points and seemed to be unaffected by the axotomy (Fig. 2). At 1 day following axotomy of the SCG, the middle and rostral BA segments showed a significant loss of TH-ir axons, and the exact site on the middle BA

where SCG input was lost could be discerned (Fig. 2). At 7 days, longitudinal axons occasionally were observed in the middle and rostral segments (Fig. 2), yet few circumferential fibers were present and the total innervation density was significantly decreased when compared with the control group. By 8 weeks, frequent TH-ir axons were associated with the middle and rostral BA, mostly passing in the longitudinal plane, yet the total TH innervation density remained significantly decreased (compared to controls) in both segments, and the density of both segments remained statistically similar to the 7 day treatment, suggesting little recovery. Both longitudinal and circumferential TH perivascular axons were present in these segments at 12 weeks following the injury, yet the axons exhibited a somewhat disorganized pattern compared to the controls (Fig. 2). Quantitative analysis revealed that the density of the middle segment at 12 weeks was similar to controls, suggesting a potential recovery of innervation, yet the innervation was also similar to the 7 day treatment, indicating that the innervation had not progressed significantly compared with the 7 day time point. At this same 12 week time point, the total density of the rostral BA remained significantly decreased compared with the controls and, similar to the 8 week time point, was unchanged compared to the 7 day treatment, suggesting that little recovery had taken place (Fig. 2).

The sympathetic innervation of the MCA from control cases consisted of a distinct perivascular plexus passing in both longitudinal and circumferential orientations (Fig. 3). At 7 days following bilateral axotomy, the entire length of the MCA showed almost complete denervation of sympathetic perivascular axons. The reappearance of TH-ir axons that occurred over time took place in the proximal-distal direction. At 8 weeks following axotomy the proximal MCA showed robust innervation and the total TH density was significantly increased compared with the 7 day time point, suggesting that some reinnervation had occurred. Yet the plexus appeared disorganized with no distinct pattern of circumferential and longitudinal branching, and the density remained significantly decreased compared to controls. At 12 weeks following axotomy this segment appeared more organized with distinct circumferential and longitudinal axons present (Fig.3), and though the total perivascular density remained significantly reduced compared to the controls, it was increased compared to the 7 day time point (Fig.3), suggesting that reinnervation was progressing. The TH density of the middle and distal MCA segments showed little indication of reinnervation at 8 weeks following axotomy, with the total perivascular density showing a significant decrease compared to the control group and showing no change compared to the 7 day treatment (Fig. 3). At the 12 week time point, the total TH perivascular density of both the middle and distal segments remained decreased compared to controls, yet showed a significant increase compared to the 7 day time point, suggesting that some reinnervation may be occurring.

In addition to the analysis of total TH axon density, the density of TH-ir circumferential and longitudinal axons for each vessel was analyzed separately during the reinnervation process. The longitudinal and circumferential TH axons associated with the ICA and BA revealed similar reinnervation patterns to that reported above for total TH density. However two changes in circumferential or longitudinal axons associated with the MCA were observed that were somewhat different from that observed for total perivascular density. First, though the total axon density as well as the longitudinal axon density of the proximal segment of the MCA remained decreased compared to controls at the 12 week time point, the density and pattern of circumferential axons was similar to control values. Second, in the middle portion of the MCA, similar to the total perivascular density, the longitudinal axons were significantly increased at the 12 week time point compared to 7 days, yet the density of circumferential axons remained decreased and was similar to the 7 day time point.

The SMG and pineal gland from control cases exhibited robust TH immunoreactivity (Fig.4) in the form of distinct TH-ir puncta distributed throughout the tissues. At 1 day and 7 days following axotomy, TH-ir puncta were absent in both tissues (Fig. 4), indicating a substantial loss of TH innervation. The reinnervation patterns, however, differed between these two nonvascular targets. In the SMG, though the density was significantly decreased compared to controls, frequent TH-ir axons were present at 8 weeks following axotomy, and by 12 weeks the TH innervation density was approximately 60% of the control cases, was similar to controls, and showed a significant increase compared to the 7 day time point (Fig. 4). However, in the pineal gland, few puncta were present at 8 weeks following axotomy, and, though frequent TH-ir profiles were present at 12 weeks after axotomy, the TH innervation density remained significantly decreased compared to controls and similar to the 7 day time point, suggesting no significant reinnervation had taken place by 12 weeks (Fig. 4).

TH protein analysis of the SCG

At 1 day and 7 days following axotomy, TH was significantly reduced compared to controls as previously reported (Fig. 5; Walker et al., 2009). Semi-quantitative western analysis revealed that TH protein remained significantly decreased at both 8 weeks and 12 weeks (Fig. 5), and there were no significant differences between these long term time points and the 7 day time point, indicating no increase in TH at the longer time points.

3. Discussion

Previous studies have indicated that SCG neurons recover by 8 weeks following axotomy (Klimaschewski et al., 1994; Klimaschewski et al., 1996; Sun and Zigmond, 1996), suggesting the possibility that peripheral targets of the SCG have become successfully reinnervated. For example, Klimaschewski reported that, after 60 days, TH was colocalized in neurons in the SCG that also expressed galanin (Klimaschewski et al., 1994), a peptide that is upregulated in SCG neurons following injury (Zhang et al., 1994), leading to the conclusion that regeneration was underway. A subsequent study concluded, based on the reversal of ptosis, the loss of galanin, and the presence of retrogradely labeled neurons in the SCG following Fast Blue administration into the SMG, that regeneration had occurred at 60 days following crush axotomy (Klimaschewski et al., 1996). Further, Zigmond reported a return of TH mRNA levels in the rat SCG to control levels by 2 months after injury (Sun and Zigmond, 1996), suggesting a potential recovery of TH expression in the SCG following axotomy. Because of these findings and the idea that peripheral neurons have the capacity to regenerate (Navarro, 2007) at a rate of approximately 3 mm/day (Gordon et al., 2007), we expected robust reinnervation of the vascular targets and recovery of the SCG by 12 weeks following injury and took a quantitative approach to determine the innervation density of peripheral targets following axotomy. Indeed, the ICA showed full sympathetic reinnervation following axotomy. However, the TH innervation of the MCA and BA remained significantly reduced at the 12 week survival time point. These decreases in TH innervation were accompanied by a significant reduction of TH protein in the SCG, which showed little evidence for recovery between the 8 week and 12 week survival time points.

Long term alterations in the SCG following axotomy have been reported in previous studies. For example, substantial cell death was reported in the adult guinea pig SCG following crush injury to postganglionic axons (Purves, 1975; Bowers et al., 1984) with an estimated 50% cell loss at 1-3 months following the injury (Purves, 1975). Apoptosis also was reported in the rat SCG just days following axotomy (Hou et al., 1998). Similarly, though ptosis was reversed by 6-8 weeks in the adult, Hendry (1975a) observed that TH activity in the SCG remained decreased. Further, long lasting decreases in TH activity in the rat SCG were observed when crush axotomy was performed at 6 days postnatally, with activity only

4% of control values at 70 days following the injury (Hendry, 1975b). Though age may have played a role in that study, we observed a similar lack of recovery in the SCG when axotomy was performed in the adult.

However, others have reported that TH gene expression in the SCG appeared to be fully recovered at 8 weeks following axotomy (Sun and Zigmond, 1996). It may be that the select targets receiving TH innervation by 8-12 weeks (i.e. proximal MCA, ICA, SMG) provide sufficient neurotrophic support to promote TH gene expression in the SCG, while the loss of target-derived factors resulting from the reduced TH innervation of most targets had a negative effect on the expression of TH protein. Reduced TH protein in SCG cell bodies could result from a number of mechanisms, such as decreased protein translation and/or increased protein degradation in the cell body, or possibly altered (i.e. enhanced) TH transport to recovered terminals associated with the proximal MCA, ICA, or SMG.

Several targets showed little improvement in innervation between the 8 week and 12 week time points, suggesting that regeneration proceeds slowly and/or complete reinnervation may never take place in these targets. Similar to our findings, pineal innervation remained significantly reduced at 3 months and showed no improvement between 2 months and 3 months following bilateral crush axotomy of the SCG (Bowers et al., 1984). Though the pupil appeared normal, no return of function was observed at 105 or 172 days following crush injury of postganglionic axons in the rabbit (Langley, 1895). Although, consistent with our findings, projection neurons to the SMG appeared normal (Klimaschewski et al., 1996), only 12% of neurons that normally projected to the iris remained following axotomy of the SCG. Indeed it was reported previously that only a small portion of axotomized SCG neurons successfully reinnervated their original targets (Hendry et al., 1986). Further, regeneration studies following SCG axotomy in the guinea pig revealed that postganglionic axons had little ability to follow their original path following axotomy and that regrowth of postganglionic axons was likely to be nonspecific (Purves and Thompson, 1979).

Though some targets showed no improvement between 8 and 12 weeks survival time points, our data revealed that TH reinnervation of select vascular segments was occurring. Remodeling of perivascular axons associated with the proximal MCA was evident between the 8 week and 12 week time points and, by the 12 week survival time point, the disorganized plexus observed at 8 weeks appeared to have rearranged into an organized pattern of circumferential and longitudinal perivascular axons similar to the control cases.

The separate analyses of longitudinal and circumferential TH axons revealed additional information regarding the reinnervation process of the extracerebral blood vessels following SCG axotomy. Most notable in this study were the patterns observed in longitudinal and circumferential axons associated with the MCA. For example, even though the longitudinal axonal density, similar to the total axon density, remained decreased compared with controls at the 12 week time point, as mentioned above, the density and innervation pattern of the circumferential axons associated with the proximal MCA were similar to controls and appeared to have recovered from the injury, revealing that at least a portion of the axonal population had been restored. Further, the longitudinal axons associated with the middle portion of the MCA were significantly increased at 12 weeks compared with 7 days, while the circumferential axons, as well as the total perivascular density, remained similar to the 7 day time point. This result suggests that the longitudinal axons may precede the appearance of the circumferential population and is consistent with the sympathetic innervation pattern of extracerebral blood vessels during development in which sympathetic axons first extend in longitudinal bundles along the Circle of Willis, and circumferential axons subsequently proliferate to form a standard perivascular plexus (Tsai et al., 1989).

The perivascular axons reinnervating the proximal MCA may have originated from regenerating SCG axons which traversed the ICA to innervate the proximal MCA and then coursed proximal to distal on the MCA (Handa et al., 1990). The denervated BA appeared to be recovering, though somewhat slowly, in a caudal to rostral direction. Indeed, the regenerating SCG axons could have taken a caudal approach, and traversed the vertebral artery and caudal BA to approach the middle and rostral segments of the BA (Handa et al., 1990).

Another source of TH reinnervation, particularly with regard to the middle and rostral BA, might involve compensatory sprouting of stellate axons that innervated more caudal regions of the artery (Arbab et al., 1986). Compensatory sprouting of contralateral postganglionic axons has been shown following unilateral SCG removal (Handa et al., 1991). If this was indeed the source of reinnervation, the axonal growth axons appeared to be proceeding very slowly from the caudal BA to reach the middle and rostral BA. It is interesting to note that the density of the middle BA at 12 weeks, while similar to control values, was not significantly different from the 7 day time point, indicating that reinnervation of this segment had not progressed compared with early denervation time points. If compensatory sprouting was responsible for the reinnervation of the BA, it seems that 12 weeks would be a sufficient recovery time for sprouting to occur. It is possible that the denervated segments of the BA never become fully reinnervated. Handa and colleagues (1991) noted that, while proximal vessels in the circle of Willis were reinnervated by compensatory sprouting of postganglionic axons following unilateral ganglionectomy, other components of the circle, such as distal branches, showed no reinnervation even by 16 weeks.

Whether any functional capabilities have been reestablished is unknown. A recent study revealed that function of the tail artery was restored even when sympathetic reinnervation was sparse (Tripovic et al., 2011). Indeed there is some debate regarding the actual function of sympathetic innervation of the extracerebral blood vessels (See Strandgarrd and Sigurdsson, 2008; Levine et al., 2008), but in general it is thought that sympathetic innervation of the extracerebral vasculature plays a role in vascular tone (Hamel, 2006) and the regulation of cerebral blood flow (Edvinsson et al., 1976; Levine et al., 2008), particularly in conditions of extreme increases or decreases in blood pressure, and, as recently shown, during REM sleep (Cassaglia et al., 2009).

The data presented here represent a quantitative approach to determining the relative contribution of the SCG to the innervation of the rat basilar artery, which receives bilateral innervation from both the SCG (Handa et al., 1990) and stellate ganglia (Arbab et al., 1986). Previous reports concerning the contribution of the SCG to innervation of the BA have been somewhat unclear. The removal of the SCG in the macaque resulted in variable reductions in sympathetic innervation of the BA (Hernandez-Perez and Stone, 1974), while studies by Kajikawa (1968) suggested that the SCG contributes very little to innervation of the BA in the rat, with a depletion of catecholaminergic fibers associated with the BA following removal of the stellate ganglion, but no change following SCG removal. The use of anterograde tracers into the SCG suggested that the SCG provides some innervation to the rat BA (Arbab et al., 1986; Handa et al., 1990; Tamaki and Nojyo, 1987). While Arbab and colleagues (1986) showed only sparse innervation of the BA following WGA-HRP application to the SCG, Tamaki and Nojyo (1987) and Handa et al. (1990) reported a relatively dense plexus along the total length of the BA using a similar experimental approach. Because the axotomy in the present study was carried out bilaterally, we were able to visualize a clear delineation on the BA at which SCG innervation transitioned to stellate ganglion innervation, such that the TH innervation density of the region rostral to this site showed a dramatic decrease following axotomy of the SCG, while the density caudal to this point was not affected by the procedure. Our findings suggest that the caudal

region of the BA receives only a minor contribution from the SCG, while the middle and rostral BA receive almost exclusively from the SCG.

Our results revealed that TH innervation density of the pineal was significantly decreased at the 12 week survival time point, while the SMG showed complete reinnervation. The reasons for the discrepancy between these two peripheral targets are unknown. The pineal gland, which receives dense innervation by the SCG (Marfurt et al., 1986; Tamaki and Nojyo, 1987), showed a complete loss of innervation, based on norepinephrine uptake studies, following bilateral axotomy of the SCG (Bowers et al., 1984), and the innervation remained significantly decreased at 3 months following the injury (Bowers et al., 1984). The number of SCG neurons that innervate the pineal is relatively small (approximately 3%; Bowers et al., 1984) and these neurons are thought to be vulnerable to the injury procedure. The SMG receives a more robust innervation from the external nerve, which seems to recover more readily. The SMG tissues may provide a more significant trophic source for regenerating SCG neurons and thus facilitate regeneration and/or survival. Different patterns of regeneration were observed in cutaneous nerves compared with those innervating muscle following sciatic nerve crush injury (Jenq and Coggeshall, 1985) where cutaneous sensory reinnervation lagged the motor reinnervation by axons reinnervating muscle. Conversely, comparison of the reinnervation patterns of afferents and efferents of the gut revealed a much slower reinnervation by preganglionic motor neurons compared with sensory afferents (Phillips et al., 2003). These variations may reflect different trophic influences by target tissues.

Our data showing sustained reductions in TH in the SCG as well lack of reinnervation of some peripheral targets at long survival time points following injury are more consistent with studies that revealed a lack of recovery in the SCG and reduced innervation of SCG peripheral targets. We conclude that previous studies, which suggested recovery of the SCG and regeneration of SCG neurons by 8 weeks following axotomy, may have overestimated the extent of recovery taking place in the injured neurons and did not consider the sympathetic innervation of the vascular targets. While a recovery of sympathetic vascular target innervation may occur, the process proceeds relatively slowly and some vascular segments may never reach the full innervation pattern exhibited prior to the injury.

4. Experimental Procedure

Axotomy of the SCG

Young adult female Sprague Dawley rats (200-225 gm; Harlan Labs) were anesthetized with inhaled isoflurane gas (2%). A ventral incision approximately 3 cm in length was made in the neck region. The axons of the SCG were exposed and the external carotid and internal carotid nerves 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 and Zigmond, 1996). The procedure was repeated on the other side. The incision was closed using sutures and tissue glue (Nexaband, Phx, AZ).

Success of the surgery was assessed by the extent of ptosis following the surgery and post-surgical examination of the transection sites. The level of ptosis was documented bilaterally in each case and was ranked from 0 (no evidence) to 5 (most severe). Ptosis rankings were obtained immediately following the surgery, and on a daily basis for the first week. For longer survival time points, ptosis was documented weekly until just prior to sacrifice, when a final ptosis ranking was obtained. In all cases examined in this study, ptosis was obvious at early time points (ranging from 3.5-4.5) and all treatment data shown here were obtained from successful transections. Because no statistical differences in TH innervation were

observed between the sham and unoperated controls, data from these two groups were pooled to form one control group.

Tissue collection and immunofluorescence

Animals were sacrificed via transcardial perfusion with 4% paraformaldehyde in 0.1M phosphate buffer (PB). The intradural segment of the ICA, the MCA, and BA were clipped from the base of the brain and processed as whole mounts. The SMG and pineal glands were infiltrated with 30% sucrose in 0.1M PB, embedded in OCT freezing medium, and sectioned at 12 μ m on a cryostat microtome. Tissues were incubated for 24 hrs 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) and then incubated for 48 hrs at 4°C in rabbit anti-TH (1:500; Millipore). Tissues were rinsed 4 times (5 min each) with 0.1M PBS and placed in donkey anti-rabbit-594 (1:200; Molecular Probes) diluted in 0.1M PBS for 2 hrs at room temperature, then rinsed 4 times (5 min each) with 0.1M PBS followed by a final 5 min rinse in 0.1M PB. Vessels were mounted on glass microscope slides. All tissues were mounted with Vectashield (Vector Labs) mounting medium.

Protein isolation and semi-quantitative western blot analysis of the SCG

Total SCG protein was obtained by sonicating tissues in 0.01M Tris-HCl buffer (pH 7.4) containing 1% SDS and 1% protease inhibitor cocktail (Sigma-Aldrich) and protein concentration was determined using a BCA assay (Pierce Biotechnology, Inc.). Samples were prepared as described by Laemmli (1970). SDS-PAGE (5% stacking/10% resolving) was used to separate proteins and the Precision Plus protein standard. Proteins were transferred onto PVDF membrane overnight at 4°C and a total of 2,400 mAmps in transfer buffer (25mM Tris, 192mM glycine, 10% (v/v) methanol). The membrane containing protein was incubated in 4% non-fat dry milk diluted in TBST for 4 h at RT, incubated overnight at 4°C in mouse anti-tyrosine hydroxylase (1:200,000, BD biosciences), rinsed with TBST, and incubated 2 hr at RT in goat anti-mouse HRP IgG (1:100,000; Millipore). Membranes were rinsed with TBST, covered with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc.) for 5 min, and then placed in an autoradiography cassette. Membrane containing protein was stripped and reprobed for GAPDH (1:80,000; Fitzgerald Industries International) and goat anti-mouse HRP IgG (1:100,000; Millipore). Films were scanned into ImageQuant 5.2 (Amersham Biosciences) and densitometry readings for all bands were obtained with the local background subtracted. The densitometry of TH was normalized to GAPDH for each sample to produce a ratio. Ratios for each tissue were collected and data were subjected to analysis using one way ANOVA and the Fischer's post hoc comparison test. Values are reported as mean $\pm$ SEM. Significance was reported at $p < 0.05$.

Analysis of TH-ir axons innervating peripheral targets

Tissues were viewed using a Zeiss 710 or an Olympus FV500 confocal laser scanning microscope (Center for Advanced Imaging, Miami University) and analyzed using Zen or Fluoview v4.3 software. For blood vessel quantification, images were captured at 200x (ICA) or 400x (MCA, BA) magnification, and saved as TIFF files. The MCA was quantified at three regions: a) proximal - region closest to the ICA; b) middle - mid portion; c) distal - region farthest from the ICA. Likewise, the BA was quantified in three regions: a) rostral - region closest to the Circle of Willis, b) middle - mid portion, c) caudal - farthest from Circle of Willis. Quantification was carried out manually on confocal images displayed on a computer monitor. A rectangular grid (200 μ m $\times$ 100 μ m) was divided into 72 equal squares and was placed over two different regions on each the upper and lower planes of the vessel for a total of four areas analyzed on each segment of the vessel. A measurement was obtained for longitudinal and circumferential perivascular density by counting the number of

horizontal and vertical grid line crosses, respectively. A value for total innervation density (longitudinal + circumferential) then was calculated for each vessel region and compared across treatments. For SMG and pineal analyses, images were captured at 400x magnification, saved as TIFF files, and the number of fluorescent profiles in two different images per case was determined using ImagePro 6.0 software and a mean for each case was obtained. Values from each treatment were compared with the values obtained from the control group and subjected to statistical analysis using ANOVA followed by Fisher's post hoc comparison test. Comparisons were made between each treatment and the control group, and, in order to assess any significant reinnervation, the 8 week and 12 week treatment groups were compared to the 7 day treatment for each tissue examined. Significance was reported at $p < 0.05$. All values were reported as mean $\pm$ SEM.

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Highlights

Reinnervation of targets following axotomy of the superior cervical ganglion (SCG) was examined.

Significant decreases in TH innervation of targets were observed at 12 weeks following SCG axotomy.

TH protein in the SCG also remained decreased at 12 weeks following bilateral axotomy of the SCG.

TH innervation of blood vessel targets and TH protein in the SCG recover slowly following SCG axotomy.

Figure 1.

Sympathetic denervation and reinnervation of the internal carotid artery (ICA) following axotomy of the SCG. **A.** The ICA from control cases showed distinctive circumferential and longitudinal axons (arrows) along the vessel. At 1 day after axotomy, the density of TH immunoreactive axons appeared reduced (arrows), and by 7 days the ICA was completely denervated. The ICA appeared to be fully reinnervated and the innervation was similar to control levels by 8 weeks following the injury (arrows). Scale = 100 μ m. **B.** Quantitative analysis of TH associated with the ICA revealed a significant decrease in the density of perivascular axons at 1 day and 7 days following axotomy. The TH density was similar to controls at 8 weeks and 12 weeks following axotomy. At both 8 weeks and 12 weeks, TH density was significantly increased compared with the 7 day time point. Significance was reported at $p < 0.05$. Error bars represent the standard error of the mean. *, significantly different from control group; #, significantly different from 7 day time point. cont, $n = 7$; 1d, $n = 3$; 7d, $n = 4$; 8wk, $n = 3$; 12 wk, $n = 3$.

Figure 2.

Changes in TH innervation of the basilar artery (BA) following axotomy of the SCG. **A.** At 1 day following axotomy, the point of denervation resulting from the loss of SCG innervation can be easily discerned along the middle BA (arrow). Caudal to this denervation point, the BA showed no change in perivascular density and remained unchanged at all survival time points while the density of the segment rostral to this point was decreased. At 7 days following injury, the density remained decreased in this middle segment with a few longitudinal axons that could be seen passing along the vessel middle and rostral segments, but few circumferential perivascular axons were present. The density of the middle and

rostral segments appeared decreased even at long term survival time points. Scale = 100 μ m.

B. Quantitative analysis revealed no change in caudal BA at any time point examined. The middle BA was significantly decreased compared with controls at 1d, 7d, and 8 wks following axotomy. At 12 weeks following axotomy, TH density associated with this segment was not different from controls, but also remained similar to the 7 day time point. The TH density of the rostral BA was significantly decreased at each survival time point examined. The middle and rostral BA segments showed no increase compared with the 7 day time point even at long survival times. Error bars represent the standard error of the mean. Significance was reported at $p < 0.05$. *, significantly different from control group. cont, n=8; 1d, n=4; 7d, n=4; 8wk, n=4; 12 wk, n=3.

Figure 3.

Changes in TH innervation of the middle cerebral artery (MCA) following axotomy of the SCG. **A.** The density of TH perivascular axons associated with the MCA was analyzed at proximal (closest to the ICA), middle (mid), and distal (farthest from the ICA) segments of the vessel. At 7 days following injury, all segments of the MCA showed almost complete denervation. At 8 weeks, the proximal portion showed significant reinnervation, but with a disorganized plexus (arrows), while few axons were present at the middle segment (arrows). The distal segment remained denervated at 8 weeks. At 12 weeks, a more orderly plexus was present on the proximal segment and axons on the middle and distal segments were observed. Scale = 200 μ m. **B.** Quantitative analysis of TH innervation density associated

with the MCA revealed a significant decrease compared with controls in each segment at short term and long term survival time points. Yet, by 12 weeks, the TH density was significantly increased compared with the 7 day time point in each of the segments examined. Error bars represent the standard error of the mean. Significance was reported at $p < 0.05$. *, significantly different from control group; #, significantly different from 7 day time point. cont, n=8; 7d, n=3; 8wk, n=5; 12 wk, n=3.

Figure 4.

TH reinnervation of the submandibular gland (SMG) and pineal gland following axotomy. TH innervation density in the SMG from control cases was abundant (arrows) but was significantly reduced at 7 days following axotomy. Frequent TH-ir axons were present at 8 weeks following axotomy, and by 12 weeks the TH axonal density (arrows) was approximately 60% of the control cases. Scale=100 μ m, all micrographs. TH innervation of the pineal gland was robust in the controls (arrows). Following 7 day axotomy, TH density was virtually absent, and, though TH was present at 8 weeks and 12 weeks after axotomy (arrows), the TH innervation density remained decreased compared with controls. Scale=100 μ m, all micrographs. **B.** Quantitative analysis revealed that TH density in the SMG was similar to control values by 12 weeks following axotomy and was significantly increased compared with the 7 day time point. However, TH in the pineal gland remained significantly decreased at 12 weeks following axotomy compared with controls, with no evidence of recovery compared to the 7 day treatment. Error bars represent the standard error of the mean. Significance was reported at $p < 0.05$. *, significantly different from control group; #, significantly different from 7 day time point. cont, $n=4$; 7d, $n=3$; 8wk, $n=5$; 12 wk, $n=3$.

Figure 5.

TH western analysis of the SCG. **A.** TH protein expression in the SCG was reduced following axotomy at every time point examined. Each lane represents a different SCG. 10 μ g protein was loaded. GAPDH served as the loading control. **B.** Semi-quantitative analysis of TH in the SCG revealed a significant decrease in TH at each time point examined. TH remained significantly decreased by 77% at 12 weeks following axotomy and was not significantly different from the 7 day treatment. Error bars represent the standard error of the mean. Significance was reported at $p < 0.05$. *, significantly different from control group. cont, $n=4$; 7d, $n=3$; 8wk, $n=5$; 12 wk, $n=3$.