Structural neuroplasticity following T5 spinal cord transection: increased cardiac sympathetic innervation density and SPN arborization

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Lujan HL, Palani G, DiCarlo SE. Structural neuroplasticity following T5 spinal cord transection: increased cardiac sympathetic innervation density and SPN arborization. Am J Physiol Regul Integr Comp Physiol 299: R985–R995, 2010. First published July 28, 2010; doi:10.1152/ajpregu.00329.2010.—When the spinal cord is injured at or below thoracic level 5 (T5), cardiovascular control is markedly unbalanced as the heart and blood vessels innervated by upper thoracic segments remain under brain stem control, whereas the vasculature of the lower body is affected by unregulated spinal reflexes. Importantly, the regulation of heart rate and cardiac function is abnormal after spinal cord injury (SCI) at T5 because sympathetic outflow to the heart is increased. An increase in tonic sympathetic outflow may be attributable to multiple mechanisms, such as increases in cardiac sympathetic innervation density, altered morphology of stellate ganglia neurons, and/or structural neuroplasticity of cardiac sympathetic preganglionic neurons (SPNs). Furthermore, these neuroplastic changes associated with SCI may be mediated by nerve growth factor (NGF). NGF is a neurotrophin that supports the survival and differentiation of sympathetic neurons and enhances target innervation. Therefore, we tested the hypothesis that T5 spinal cord transection (T5X) is associated with an increased left ventricular (LV) NGF content, LV sympathetic innervation density, and cardiac SPN arborization. In intact and paraplegic (9 wk posttransection) rats, LV NGF content (ELISA), LV sympathetic innervation density (tyrosine hydroxylase immunohistochemistry), and cardiac SPN arborization (cholora toxin B immunohistochemistry and Sholl Analysis) were determined. Paraplegia, compared with intact, significantly increased LV NGF content, LV sympathetic innervation density, and cardiac SPN arborization. Thus, altered autonomic behavior following SCI is associated with structural neuroplastic modifications.

Since World War II, there has been a dramatic reduction in the mortality rates during the acute period following a spinal cord injury (SCI) (81). However, mortality rates in the chronic period (>2 yr following injury) have changed very little, and people with SCI have a significantly reduced life expectancy (17, 28, 81). The reduced life expectancy is due, in part, to a higher prevalence and earlier onset of cardiovascular diseases (57). In fact, cardiovascular disease is now a leading cause of death and morbidity for individuals with SCI (17, 28, 81).

Excessive sympathetic activity is responsible for, and/or contributes to, the morbidity and mortality associated with cardiovascular disease. Accordingly, efforts to reduce sympathetic activity are the first-line therapy for most, if not all, cardiovascular diseases. Importantly, sympathetic activity is elevated above the lesion in individuals with midthoracic SCI, and the regulation of heart rate and cardiac function is abnormal because sympathetic outflow to the heart is increased (14). For example, we recently documented that thoracic level 5 (T5) spinal cord transection (T5X) increased cardiac sympathetic innervation density and SPN arborization (14). When the spinal cord is injured at or below thoracic level 5 (T5), cardiovascular control is markedly unbalanced as the heart and blood vessels innervated by upper thoracic segments remain under brain stem control, whereas the vasculature of the lower body is affected by unregulated spinal reflexes. Importantly, the regulation of heart rate and cardiac function is abnormal after spinal cord injury (SCI) at T5 because sympathetic outflow to the heart is increased. An increase in tonic sympathetic outflow may be attributable to multiple mechanisms, such as increases in cardiac sympathetic innervation density, altered morphology of stellate ganglia neurons, and/or structural neuroplasticity of cardiac sympathetic preganglionic neurons (SPNs). Furthermore, these neuroplastic changes associated with SCI may be mediated by nerve growth factor (NGF). NGF is a neurotrophin that supports the survival and differentiation of sympathetic neurons and enhances target innervation. Therefore, we tested the hypothesis that T5X is associated with an increased left ventricular (LV) NGF content, LV sympathetic innervation density, and cardiac SPN arborization. In intact and paraplegic (9 wk posttransection) rats, LV NGF content (ELISA), LV sympathetic innervation density (tyrosine hydroxylase immunohistochemistry), and cardiac SPN arborization (cholora toxin B immunohistochemistry and Sholl Analysis) were determined. Paraplegia, compared with intact, significantly increased LV NGF content, LV sympathetic innervation density, and cardiac SPN arborization. Thus, altered autonomic behavior following SCI is associated with structural neuroplastic modifications.

MATERIALS AND METHODS

Surgical Procedures

Experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wayne State University and complied with The American Physiological Society’s Guiding Principles.
in the Care and Use of Animals. Eighteen adult Sprague-Dawley male rats (n = 7; T5X and n = 11; Intact (sham T5X)) were studied to determine LV NGF content, LV sympathetic innervation density,stellate neuron size and number, and cardiac SPN sparation.

All surgical procedures were performed using aseptic surgical techniques. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip), atropinized (0.05 mg/kg ip), intubated, and prepared for aseptic surgery. Supplemental doses of pentobarbital sodium (10–20 mg/kg ip) were administered if the rat regained the blink reflex or responded during the surgical procedures.

First surgical procedure: spinal cord transection. After anesthesia was induced, rats were intubated and positioned prone over a thoracic roll that slightly flexed the trunk. The fourth thoracic vertebra was exposed via a midline dorsal incision and the spinous process and laminae were removed. Two ligatures (6-0 silk) were tightened around the underlying spinal cord between the fifth and sixth thoracic segments, and the spinal cord was completely transected by cutting between the ligatures with scissors (48, 49). In this way, there was minimal bleeding. Identical procedures were used for the Intact rats; however, the spinal cord was preserved in situ. Sym pathetic innervation to the heart is derived from preganglionic fibers that exit the spinal cord at the first through fourth thoracic levels (80). Transection between the fifth and sixth thoracic levels of the spinal cord preserves supraspinal control of cardiac sympathic activity. The completeness of the transection was confirmed by visual inspection of the lesion site. During the acute recovery period (~10 days), all rats were handled at least six times daily. During these periods, visual inspections and physical manipulations were performed to detect and prevent pressure sores. In addition, the urinary bladder was voided by manual compression, and all animals were weighed. After this acute recovery period, rats required only daily inspection, and the bladders did not require manual compression. The diets of all rats were continuously supplemented with palatable, nutritious, and enrichment treats (Bio-Serv, Frenchtown, NJ). No other dietary interventions were necessary. At day 7 posttransection, the rats received a motor activity score using criteria described previously (84). The motor activity score was assessed by placing the animal on a paper-covered table and observing spontaneous motor activity for 1 min. Motor scores ranged from 0 to 5. A motor score of 5 indicates normal walking, whereas a score of 0 indicates no weight-bearing or spontaneous voluntary movement in the hind limbs. All rats had a motor score of 0, which indicates no weight bearing. All rats were allowed to recover for 9 wk. Upon completion of the studies, the site of the spinal transection was confirmed by autopsy.

Second surgical procedure: stellate ganglia injections. T5X and Intact animals were anesthetized as described above, and the stellate ganglia were approached (one at a time) via a ventral thoracotomy. The entire stellate ganglia from seven T5X and six Intact rats were sectioned at 10-μm intervals, thaw mounted on Superfrost Plus slides (Fisher Scientific), and stained with cresyl violet. The cardiac sympathetic postganglonic cell bodies, on every 10th section, were counted and measured with the aid of a ×20 objective and a MicroBrightField NeuroLucida software interfaced with a BH-2 Olympus microscope (50, 51). Only neurons with distinct, prominent nuclei were counted. Based on a study by Jones (29), no correction for split nuclei is necessary in 10-μm sections.

Spinal cord. The spinal cord blocks (T1–T4) from seven T5X and six Intact rats were sectioned horizontally at 40-μm intervals. Tissue sections were washed in 10 mM Tris, 0.9% NaCl, 0.05% thimerosal in 10 mM phosphate buffer, pH 7.4 (TPBS) containing 0.3% Triton X-100 for 3 × 10 min and then incubated in 10% heat-inactivated normal horse serum (Invtrogen) in TPBS-Triton for at least 1 h. The sections were then incubated in goat anti-CTB antiserum (1:4000 List Biologicals) in TPBS-Triton containing 10% normal horse serum for 3 days at room temperature. After rinsing (in TPBS, ×3 10 min each), sections were incubated with biotinylated donkey anti-goat immunoglobulin (1:500; Jackson Laboratories) in TPBS-Triton with 1% normal horse serum overnight at room temperature. Sections were rinsed again (TPBS, ×3 10 min each) and incubated 4–6 h in 1:1500 ExtrAvidin-Peroxidase in TPBS-Triton (cat. no. E-2886; Sigma). Immunoreactive axons were revealed with the nickel-intensified diaminobenzidine reaction [Fig. 1 (44)].

Tissue Processing, Analysis, and Immunohistochemistry

Stellate ganglia. The entire stellate ganglia from seven T5X and six Intact rats were sectioned at 10-μm intervals, thaw mounted on Superfrost Plus slides (Fisher Scientific), and stained with cresyl violet. The cardiac sympathetic postganglonic cell bodies, on every 10th section, were counted and measured with the aid of a ×20 objective and a MicroBrightField NeuroLucida software interfaced with a BH-2 Olympus microscope (50, 51). Only neurons with distinct, prominent nuclei were counted. Based on a study by Jones (29), no correction for split nuclei is necessary in 10-μm sections.

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Structural Analysis. Morphological features of cardiac SPNs in the Intact and injured spinal cords were analyzed as described by Nelson and colleagues (58–60). Specifically, each neuron was analyzed using the Sholl analysis [Fig. 2, (79)] of dendritic branching, which assumes that dendritic arborization is an indirect measure of available postsynaptic space. A series of concentric rings calibrated at 10-μm intervals was superimposed on each neuron and centered on the cell body. Intersections between dendrites and each concentric ring were then counted. The location and number of intersections were plotted (Fig. 3) and used for statistical comparisons. In each animal, two to three horizontal sections through the intermediateolateral column were examined, and nine to ten neurons located rostral (T2–T4) to the injury were measured using NeuroLucida software (MBF Bioscience, Williston, VT). Each cell’s morphometric features were measured within one 40-μm-thick horizontal section. Only cells with clearly distinguishable perikarya and dendritic trees were assessed. Specifically, to be selected for analysis, CTB-labeled neurons satisfied the following criteria: 1) dark and consistent staining in the entire dendritic tree, 2) lack of truncated dendrites, and 3) relative separation...
remaining LV free wall was removed, cryoprotected, and embedded in blocks in O.C.T. compound. The LV blocks from four T5X and four Intact hearts were cut in transverse cross sections, at 10-μm intervals, serially thaw mounted on 25 Superfrost Plus slides (4 sections/slide), and stored at −20°C until analysis. The sequence of processing was planned so that each adjacent section was separated by 150 microns.

On the day of analysis (66), the slides were air dried, washed 3× 10 min in PBS (10 mM phosphate buffer containing 0.9% NaCl, and 0.1% sodium azide). The slides were then treated with 10 mg/ml sodium borohydride (3× 10 min) to reduce autofouescence followed by one wash of PBS. The slides were incubated for 1.5 h at room temperature in PBS containing 2% BSA-fraction 5 (dilution buffer) followed by rabbit antityrosine hydroxylase (cat. no. AB152, 1:300 in dilution buffer; Millipore) overnight at room temperature. After being rinsed (PBS, 3× 10 min each) slides were incubated with Alexa Fluor 488 goat anti-rabbit immunoglobulin (1:300 in PBS containing 5% goat serum) for 1.5 h at room temperature. After being rinsed, slides were treated with 10 mM CuSO4/50 mM NH4C2H3O2 for 30 min, rinsed, and coverslimped with buffered glycerol.

LV INNERVATION DENSITY ANALYSIS. Identical areas of the heart were analyzed from four Intact and four T5X rats. Photos were obtained, using consistent camera settings, from slides that had been processed together. Subsequently, four sections at least 150 μm apart from each heart were analyzed. Specifically, four regions of each LV free wall was quantified by threshold discrimination with the aid of a ×20 objective using the MicroBrightField Neurolucida software interfaced with a BH-2 Olympus microscope (51).

All photomicrographs were treated in an identical manner. Black and white photos were opened in the Neurolucida software program, and the brightness/contrast tool was used to adjust each image. The threshold tool was then used to identify nerve fibers. The automated function set a beginning threshold level, but this did not reliably include all sympathetic fibers, while excluding all nonneuronal tissue. The threshold was then manually adjusted to ensure that only specific tyrosine hydroxylase staining was identified. Innervation density (i.e., tyrosine hydroxylase-positive fibers) was expressed as the percent area that was above the threshold. Specifically, the area of the tyrosine hydroxylase-positive fibers was divided by the area of the section. The
data were averaged to obtain one data point per animal. Heart tissue obtained from Intact and T5X animals revealed many tyrosine hydroxylase immunopositive sympathetic terminals (Fig. 4).

**ELISA for Left Ventricular NGF Content**

The LV free wall from six Intact and six T5X animals was pulverized into fine powder using a liquid nitrogen-cooled mortar and pestle. Approximately 70 mg of tissue was suspended in lysis buffer [137 mM NaCl, 20 mM Tris·HCl (pH 8.0), 1% NP40, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mM sodium vanadate]. The samples were homogenized with Kontes Pellet Pestle Micro Grinders (~30 s), vortexed, and centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant was diluted 1:20 in DPBS buffer (0.2 g KCl, 8.0 g NaCl, 0.2 g KH2PO4, 1.15 g Na2HPO4, 133 mg CaCl2·2H2O, and 100 mg MgCl2·6H2O per 1 liter ddH2O). All samples were stored at −80°C.

The concentration of NGF was determined using Promega’s NGF Emax ImmunoAssay System (Promega, Madison, WI). Subsequently, the samples and standards were assayed in duplicate according to the manufacturer instructions. MaxiSorp Plates (cat. no. 439454; Nunc) were incubated with carbonate coating buffer containing polyclonal anti-NGF overnight at 4°C. The next day, the plates were washed one time, blocked with one-time block and sample buffer for 1 h at room temperature, and washed again. Next, 100 μl of either sample or standard was added to each well and incubated for 6 h at room temperature. The wells were then incubated with a secondary monoclonal anti-NGF overnight at 4°C. Subsequently, the wells were incubated with anti-rat IgG conjugated to horseradish peroxidase for 2.5 h at room temperature. Between incubations, the plates were washed five times (unless otherwise stated). A TMB One solution was used to develop color in the wells for 10 min at room temperature. The absorbance was read at A450 within 30 min in a Molecular Devices ThermoMax microplate reader with SOFTmax PRO v3.1 software (Sunnyvale, CA).

**Data Analysis**

All data are reported as means ± SE. Using a Student’s unpaired t-test, we compared LV NGF content (Fig. 5), LV sympathetic nerve fiber density and number (Fig. 4, A and B), SPN soma size, maximum dendritic length, and number of intersections/animal (which represents the total dendritic field; Fig. 6, A, B, and C) as well as stellate neuron number and size (Fig. 7, A and B) between Intact and T5 spinal cord transected rats.

The Sholl analysis was evaluated by the use of a two-way repeated-measures ANOVA applied to the dendritic intersections found at each concentric ring.
concentric ring. Specifically, a two-way repeated-measures ANOVA [group (Intact or T5X) × branching order] was applied to the numbers of dendrites according to their order of branching (Fig. 3). Post hoc Tukey analysis was used to document a significant difference between the two groups for every point on Fig. 3A. Significance was set at $P < 0.05$.

RESULTS

Determination of Left Ventricular NGF Content

An ELISA was used on a small, ~1-mm², full-width, cross section of the LV free wall from six Intact and six T5X animals to determine NGF content. Fig. 5 presents LV NGF content for Intact and T5X rats. Left ventricular NGF content was significantly higher in T5X rats (~5.4-fold).

Determination of Left Ventricular Sympathetic Innervation Density

Tyrosine hydroxylase immunohistochemistry was used to determine sympathetic innervation density on the remaining LV free wall of Intact and T5X rats, because tyrosine hydroxylase is the rate-limiting enzyme in norepinephrine synthesis and is routinely used to determine cardiac sympathetic innervation density (20, 47). Innervation density was expressed as the percent area of tyrosine hydroxylase-positive fibers. Specifically, the area of the tyrosine hydroxylase-positive fibers was divided by the area of the section. Four sections, at least 150 microns apart, from each animal were analyzed, and the data were averaged to obtain one data point per animal. Left ventricular sympathetic nerve fiber density (Fig. 4A) and number of LV sympathetic nerve fibers (Fig. 4B) were significantly higher in the SCI group compared with the Intact group. C: these results are consistent with photomicrographs (×20) of TH immunoreactive sympathetic fibers in 1 SCI (right) and 1 Intact rat (left). Many more TH immunoreactive sympathetic fibers are seen in the photomicrograph from the SCI rat. Scale bar = 25 μm; *$P < 0.05$ Intact vs. SCI.
number of LV sympathetic nerve fibers (Fig. 4B) were increased in T5X rats. These results were consistent with Fig. 4C showing photomicrographs of tyrosine hydroxylase immunoreactive sympathetic fibers in one Intact (left) and one T5X rat (right). Left ventricular sympathetic nerve fiber density and number were significantly higher in the T5X group compared with the Intact group.

**Determination of Cardiac Sympathetic Preganglionic Dendritic Intersections: Structural Analysis**

Morphological features of cardiac SPNs in the six Intact and seven T5X spinal cords were analyzed using the Sholl analysis of dendritic branching, which assumes that dendritic arborization is an indirect measure of available postsynaptic space. A series of concentric rings calibrated at 10-μm intervals were superimposed on each neuron and centered on the cell body. Intersections between dendrites and each concentric ring were then counted. To identify SPNs, CTB was injected into the stellate ganglia to retrogradely label SPNs projecting mainly to the heart. This is an important procedure because it is well documented that the majority of cardiac sympathetic axons and nerve terminals originate in the cell bodies of the bilateral stellate ganglia. Specifically, bilateral stellate ganglionectomy in the rat reduces cardiac norepinephrine content by 89–100% (64, 90), suggesting that 90% of cardiac sympathetic innervation arises from the stellate ganglia. In this context, we mainly examined cardiac projecting SPNs. Specifically, Fig. 3 presents the Sholl analysis of the mean number of intersections per CTB-labeled cardiac SPN between each ring in a series of concentric rings. The Sholl analysis was evaluated by the use of a two-way repeated-measures ANOVA applied to the dendritic intersections found at each concentric ring. Specifically, a two-way repeated-measures ANOVA [group (Intact or T5X) × branching order] was applied to the numbers of dendrites according to their order of branching (Fig. 3). Significance was set at $P < 0.05$. The two-way ANOVA revealed a significant group effect, significant branching order effect, and significant group by branching order interaction. Post hoc Tukey analysis documented a significant difference between the two groups for every point on Fig. 3A. These results suggest that SPN arborization was increased in T5X rats.

**Determination of SPN Soma Area, Dendritic Length, and Total Number of Dendritic Intersections**

SPN soma area (Fig. 6A), maximum dendritic length (Fig. 6B), and the total dendritic field (Fig. 6C) were increased in T5X rats. The increased SPN soma size and maximum dendritic length are consistent with the increase in the total number of dendrites (Fig. 6C), since processes within the cell body, such as synthesis of proteins, are responsible for the maintenance of overall cell structure.

**Determination of the Size and Number of Cardiac Projecting Postganglionic Neurons in the Stellate Ganglia**

Nissl staining with cresyl violet was used to determine the size and number of cardiac projecting postganglionic neurons in the entire stellate ganglia. The cardiac sympathetic postganglionic cell bodies, on every 10th section, were counted and measured with the aid of a ×20 objective and MicroBrightField Neurolucida software interfaced with a BH-2 Olympus microscope (50, 51). Only neurons with distinct, prominent nucleoli were counted. There were no changes in stellate neuron number or size (Fig. 7A and B) between Intact and T5X rats.

**DISCUSSION**

In this study, we assessed the potential mechanisms by which spinal cord transection at T5 alters the autonomic nervous system, in particular, the augmentation of tonic sym-

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![Fig. 6. Morphological features of cardiac SPNs in 6 Intact and 7 T5X spinal cords were analyzed using the Neurolucida software. Shown are the soma area (A), maximum dendritic length (B), and number of intersections/animal (C) of SPNs projecting to the stellate ganglia in 2 groups of rats, Intact and T5 SCI. Morphological features showed a significant increase in SPN soma size, dendritic length, and the total dendritic field in the SCI group compared with Intact group. *$P < 0.05$ Intact vs. SCI.](http://ajpregu.physiology.org/)
pathetic outflow. We hypothesize that T5X would be associated with increased LV NGF content, increased LV sympathetic innervation density, and increased cardiac SPN arborization. Sham-operated Intact rats were compared with paraplegic (9 wk post-T5X) rats. Following T5X, there was a significant increase in LV NGF content (Fig. 5), LV sympathetic innervation density (Fig. 4), and cardiac SPN arborization (Figs. 3 and 6). In contrast, T5X did not alter soma number or size of stellate ganglia neurons (Fig. 7). These structural neuroplastic changes in cardiac innervation may contribute to the cardiovascular dysfunction associated with SCI.

In addition to cardiovascular dysfunction, several additional complications associated with SCI (e.g., chronic pain, spasticity, and autonomic dysreflexia) are also mediated, in part, by NGF-induced structural neuroplastic changes within the spinal cord and peripheral tissues. Specifically, SCI produces changes in small-diameter peptidergic afferent fibers, increases the density of lumbosacral propriospinal projections, induces sprouting of the corticospinal tract, causes a reorganization of the neural pathways regulating bladder and urethral sphincter function, and alters vascular responsiveness (3, 5, 16, 22, 24, 34, 36, 46, 61, 62, 77, 89).

Importantly, trophic factors, such as NGF, mediate many of these structural neuroplastic changes. For example, it is well known that increased levels of NGF in the bladder, spinal cord, and dorsal root ganglia are associated with bladder hyperreflexia after SCI. Furthermore, immunoneutralization of NGF in the spinal cord suppressed NGF levels and also suppressed bladder hyperreflexia (77). Similarly, central sprouting of small-diameter primary afferent fibers in the dorsal horn of the spinal cord occurs concurrently with the development of autonomic dysreflexia. Furthermore, blocking intraspinal NGF prevented small-diameter afferent sprouting and decreased (by 43%) the hypertension induced by colon stimulation (36, 38, 55).

Our recent work (13, 14, 48, 49, 70, 71) and the present study extend the concept of structural neuroplastic changes, associated with NGF content, mediating complications associated with SCI. Furthermore, these results are consistent with recent work documenting that individuals and animals with
paraplegia have elevated heart rates (23), increased blood pressure variability (71), episodic bouts of life-threatening hypertension as part of a condition termed autonomic dysreflexia (12, 13), and elevated sympathetic activity above the level of the lesion (14, 31, 48, 70). Paraplegia also alters cardiac electrophysiology and the abundance of Ca2+ regulatory proteins in a manner that increases the susceptibility to electrically-induced as well as ischemia and reperfusion-induced ventricular arrhythmias (14, 48, 49, 70, 71). These findings are important because patients with high-level spinal cord injuries prioritize the recovery of autonomic functions, such as cardiovascular and sexual function and bowel and bladder control, above the ability to walk (1). As stated profoundly by Christopher Reeve, “Spinal cord injury is a ferocious assault on the body that leaves havoc in its wake. Paralysis is certainly part of its legacy, but there are other equally devastating consequences including autonomic dysfunction: compromised cardiovascular, bowel, bladder, and sexual function. Treatments and cures for these losses would greatly improve the quality of life for all of us living with SCI” [September 30, 2004; (69)].

*TSX Increased LV NGF Content*

TSX increased LV NGF content (Fig. 5). These results confirm a recent report (48). NGF is expressed in the heart and other sympathetic targets (33). The production of NGF in target organs determines the density of innervation by the sympathetic nervous system (32). For example, overexpression of NGF within the heart of transgenic mice causes hyperinnervation (21). Accordingly, individuals and animals with paraplegia have elevated heart rates (23). Elevated heart rates are an important consideration because several long-term cohort studies have found resting heart rate to be a risk factor for mortality from coronary heart disease, cardiovascular diseases, cancer, or all causes (19, 86).

*TSX Increased LV Sympathetic Innervation Density*

The increased LV NGF content was associated with an increased LV sympathetic innervation density (Fig. 4). The concept of plasticity is well established with respect to heart muscle; however, the importance of plasticity of cardiac innervation has only recently received attention (83). In this context, investigators have recently provided evidence implicating sympathetic nerve sprouting in ventricular arrhythmogenesis and sudden cardiac death (7, 10, 43). For example, hypercholesterolemia, a change in rate or activation sequence, or enhanced sympathetic nerve activity induces proarrhythmic nerve sprouting and sympathetic hyperinnervation. The hyperinnervation is associated with dispersion of repolarization, changes in calcium currents, and increased susceptibility to ventricular arrhythmias (41, 43, 91). Importantly, myocardial damage and cardiac dyssynchrony, related to calcium overload, begins as early as 15 min after SCI in humans and animals (78). Thus, cardiac dyssynchrony, associated with midthoracic SCI, may increase cardiac NGF content. The increased cardiac NGF content may increase cardiac sympathetic innervation density and increase the susceptibility to ventricular tachyarrhythmias induced by programmed electrical stimulation (70, 71), myocardial reperfusion (49), and myocardial ischemia (48).

*TSX Altered the Morphology of Cardiac Projecting SPNs*

Specifically, TSX increased soma size, maximum dendritic length, and dendritic number of mainly cardiac projecting SPNs rostral to the injury (Figs. 3 and 6). These results are in agreement with a recent report by Kalincik et al. (30) who documented that T4 spinal cord transection increased the soma size of SPNs rostral to the transection, but differ from the results of Krassioukov and Weaver (34, 35), where alterations of soma size were not seen rostrally at 7 or 30 days postinjury. The innovative feature of the present study is that stellate ganglia projecting SPNs were analyzed. In the previous studies (30, 34, 35), heterogenous populations of SPNs with unknown targets were analyzed. In sharp contrast, in the present study, a nearly homogenous population of SPNs projecting to the heart was studied. Specifically, we injected CTB into the stellate ganglia to retrogradely label mainly SPNs projecting to the heart. It is well documented that the majority of cardiac sympathetic axons and nerve terminals originate in the cell bodies of the bilateral stellate ganglia. Bilateral stellate gangliectomy in the rat reduces cardiac norepinephrine content by 89–100% (64, 90) suggesting that > 90% of cardiac sympathetic innervation arises from the stellate ganglia. Therefore, this paper presents the first report examining morphometric features of SPNs mainly projecting to the heart following SCI.

*Potential Mechanisms*

Although the mechanisms mediating the increased LV NGF content and structural neuroplastic changes are unknown, it is important to note that increases in cardiac NGF content due to abnormal cardiac electrical conduction, disease, or injury can produce adverse sympathetic remodeling (7). Importantly, abnormal electrical conduction (71), unbalanced autonomic activity (48), and myocardial damage related to calcium overload begin as early as 15 min after SCI in humans and animals (78). This may be due, in part, to a baroreflex-mediated increased cardiac sympathetic efferent activity and decreased cardiac parasympathetic activity following acute hypotension due to loss of sympathetic vasoconstrictor drive below the site of the lesion. The baroreflex-induced unbalanced autonomic activity may cause myocardial electrical uncoupling, arrhythmias, myocardial damage, and thus stimulate NGF production, which further promotes cardiac sympathetic hyperinnervation. Thus the initial hypotension and baroreflex-mediated unbalanced autonomic activity may cause abnormal cardiac conduction and thus explain, in part, the increased cardiac NGF content and LV sympathetic hyperinnervation. However, it is unclear whether cardiac-produced NGF is transported to the spinal cord. In this context, Krenz and Weaver (37) documented increased NGF levels around the lesion site following T4X spinal cord transection. The mechanisms responsible for the production of NGF, as well as the site of origin, remain unclear. However, these data suggest that the demand for trophic factors may be activity dependent. Specifically, SPNs with intact descending innervation (and thus active) may require trophic factors. This is suggested because it is well known that neurotrophins act as modulators of activity-dependent plasticity (4). For example, the trophic effect of brain-derived neurotrophic factor on dendritic length and branching in retinal ganglion cells is dependent on their activity (11). In
a similar manner, Kalincik et al. (30) documented differential regulation of SPNs above vs. below a T4 spinal cord transection. Finally, activity in locomotor circuits is well known to lead to structural plasticity, changes in electrophysiological properties, and altered function within the spinal cord (6, 88). Thus altered morphology of SPNs could be associated with their hyperactivity and altered cardiac electrophysiology in spinal cord-injured humans and animals (14).

Limitations

It is well known that sympathetic innervation of the heart is directed by chemoattractive and chemorepulsive factors (18, 27). Specifically, the chemoattractive factor, NGF, supports sympathetic neuron survival and promotes cardiac axon outgrowth (15, 18). In contrast, the chemorepulsive factor semaphorin 3a attenuates sympathetic axon extension in the heart (27). Importantly, a gradient of chemoattractive and chemorepulsive factors, and accordingly, heterogeneity of sympathetic innervation density, exists across the ventricle with more sympathetic fibers in the subepicardium than in the subendocardium (27, 68). This is an important consideration because heterogeneous sympathetic innervation density in the left ventricle increases the occurrence of ventricular arrhythmias (7, 8, 27, 47, 72). In the present study, there was no attempt to determine regional sympathetic innervation density, and this is a limitation to the study. However, this is the first report documenting increased LV sympathetic innervation density following midthoracic SCI. Furthermore, hyperinnervation is associated with dispersion of repolarization, changes in calcium currents, and increased susceptibility to ventricular arrhythmias (41, 43, 91).

As stated in the introduction, NGF is produced in the target tissue (heart) and is transported back to the ganglia, where NGF promotes an increase in sympathetic innervation density. Thus, NGF levels should be elevated in the ganglia following T5X. However, we did not determine NGF levels in the stellate ganglia following T5X. Having measurements of stellate ganglia NGF content would have provided important data supporting the conclusions of this study. In addition, what is less clear is whether cardiac NGF is transported back to the spinal cord. As stated above, Krenz and Weaver (37) demonstrated that SCI induces increases in NGF content within certain populations of microglia and astroglia. Furthermore, B and T cells responding to the injury were found to contain NGF. These results suggest that neuronal remodeling of SPN following SCI is not mediated by cardiac NGF, but possibly from NGF released from the site of the injury. This possibility merits further investigation.

We performed bilateral stellate ganglia injections in this study as well as in two recent studies (50, 51) to identify all SPNs with the potential to promote ventricular arrhythmias in animals with spinal cord injuries (48, 49). However, unilateral injections may have provided more specific regional information. Specifically, sympathetic control of heart rate is primarily exerted by the right stellate ganglion (67, 74), while the left stellate ganglion is primarily responsible for ventricular arrhythmic events (74–76). However, the response to ischemia is complex and involves the cardio-cardiac sympathetic reflex with the efferent limb mainly through the right stellate ganglion and the afferent limb mainly through the left stellate ganglion (52–54). Specifically, cardiac sympathetic afferents are excited by ischemia and elicit a cardio-cardiac sympathetic reflex (9, 53) that plays a major role in the genesis of ventricular arrhythmias (73). Since the response to ischemia involves afferent and efferent fibers from both ganglia, we designed the present study to examine both components.

Finally, > 90% of the sympathetic supply to the heart originates from the stellate ganglion (64, 65). However, the stellate ganglia is not a pure cardiac sympathetic ganglion, because a small minority of its neurons innervate other structures (2, 39, 87). Thus, some noncardiac projecting SPNs may have been analyzed. Nevertheless, the approach used in this study (bilateral stellate ganglia injections) was viewed as a significant advantage over nonspecific labeling of all SPNs with unknown targets, since the majority of SPNs labeled project to the heart.

Conclusion

T5X increased LV NGF content, LV sympathetic innervation density, and cardiac SPN arborization. These results provide mechanistic support of previous findings that paraplegia increases cardiac sympathetic tonus (48), alters cardiac electrophysiology (14), and increases the susceptibility to ventricular tachyarrhythmias induced by programmed electrical stimulation (70, 71) as well as myocardial reperfusion (49) and the clinically relevant ischemia-induced sustained ventricular tachycardia (48) in conscious animals.

Cardiovascular diseases are a growing concern for individuals with SCI. In fact, the prevalence of cardiovascular diseases in individuals with paraplegia is much higher than in the general population (82). Furthermore, the morbidity and mortality from cardiovascular disease exceeds that caused by renal and pulmonary complications, the primary cause of mortality in previous decades (56). Excessive sympathetic activity is responsible for, and/or contributes to, the morbidity and mortality associated with cardiovascular disease. Accordingly, the increased susceptibility to cardiovascular diseases may be mediated, in part, by NGF-induced cardiac sympathetic hyperinnervation. This hypothesis merits further investigation.

Perspective and Significance

It is important to note that worldwide efforts are in progress for the regenerative repair of injured spinal cords (25, 42). However, it is unknown whether regenerating or sprouting axons will form functionally appropriate connections and avoid forming aberrant synapses (63). Results from this study and many others (36, 55, 77) document that several complications associated with SCI are mediated, in part, by structural neuroplastic changes within the spinal cord and peripheral tissues. The potential complications of inappropriate autonomic rewiring are a serious concern and merit further investigation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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