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The changes in human spinal sympathetic preganglionic neurons after spinal cord injury

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We have applied conventional histochemical, immunocytochemical and morphometric techniques to study the changes within the human spinal sympathetic preganglionic neurons (SPNs) after spinal cord injury. SPNs are localized within the intermediolateral nucleus (IML) of the lateral horn at the thoraco-lumbar level of the spinal cord and are the major contributors to central cardiovascular control. SPNs in different thoracic segments in the normal spinal cord were similar in soma size. SPNs in the IML were also identified using immunoreactivity to choline acetyltransferase. Soma area of SPNs was 400.7 ± 15 μm^2 and 409.9 ± 22 μm^2 at the upper thoracic (T3) and middle thoracic (T7) segments, respectively. In the spinal cord obtained from a person who survived for 2 weeks following a spinal cord injury at T5, we found a significant decrease in soma area of the SPNs in the segments below the site of injury: soma area of SPNs at T8 was 272.9 ± 11 μm^2. At T1 the soma area was 418 ± 19 μm^2. In the spinal cord obtained from a person who survived 23 years after cord injury at T3, the soma area of SPNs above (T1) and below (T7) the site of injury was similar (416.2 ± 19 and 425.0 ± 20 μm^2 respectively). The findings demonstrate that the SPNs in spinal segments caudal to the level of the lesion undergo a significant decrease of their size 2 weeks after spinal cord injury resulting in complete transection of the spinal cord. The impaired cardiovascular control after spinal cord injury may be accounted for, in part, by the described changes of the SPNs. The SPNs in spinal segments caudal to the injury were of normal size in the case studied 23 years after the injury, suggesting that the atrophy observed at 2 weeks is transient. More studies are necessary to establish the precise time course of these morphological changes in the spinal preganglionic neurons.

Keywords: atrophy; cardiovascular control; spinal cord injury; morphometry

Introduction

Cardiovascular control in the human is significantly impaired after spinal cord injury.[1–3] Clinical observations provide much of our current understanding of the characteristics of this disabling condition;[4–6] however, little is known about the morphological changes that occur within the spinal cord structures which control the cardiovascular system. A substantial portion of the current literature on spinal cord pathology is focused on characterizing the changes at the site of injury. Such work often describes the extent of demyelination or cavitation within the human spinal cord, or defines complete or incomplete injuries of the spinal cord.[7–11] After spinal cord injury, preservation of descending pathways through the area of cord injury or the maintenance of neuronal populations in the gray matter below the spinal cord injury could play a crucial role in determining long term neurological outcome.[11–13]

This study used a novel approach to study changes in spinal sympathetic preganglionic neurons (SPNs) after spinal cord injury in order to understand the mechanisms underlying the impaired cardiovascular control. In this study we examined the effect of partial deafferentation (loss of supraspinal descending pathways) of spinal cord neurons by a complete spinal injury. As the efferent component of the central nervous system (CNS), SPNs send tonic signals to different target organs such as blood vessels, the heart and the adrenal medulla, therefore, SPNs are crucial for central cardiovascular control.[14–16] Previous research in animal models demonstrated that following spinal cord transection, SPNs show signs of atrophy in the acute stage of spinal injury. However, with time in the chronic stage SPNs regain their normal morphology.[17] These morphological changes

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within the SPNs may occur as a result of partial deafferentation, because of a loss of descending projections from medullary neurons. A portion of the descending medullary input to SPNs is thought to synapse directly.[18] Previously, neurons within the lateral horn of the human cord were studied only in cases with multiple system atrophy and autonomic failure.[19–21] In our study, we investigated the changes in humans SPNs following spinal cord injury and compared this with our previous animal findings.[17]

The SPNs within the human spinal cords were identified. The soma area and diameter of SPNs were analyzed in one case with intact spinal cord and in two cases after spinal cord injury. The cases with human spinal cord injury were chosen on the basis of data from previous animal experiments.[17] Therefore, the cases of acute and chronic spinal injury were selected and anatomical completeness was confirmed for comparison of SPNs. SPNs were compared in upper and middle thoracic (T) segments in the control case and above and below the site of injury in cord-injured cases.

Methods

Two spinal cords from persons who had suffered spinal cord injury prior to death were selected from the bank of autopsy materials at the Miami Project to Cure Paralysis of the University of Miami School of Medicine. One specimen of spinal cord from a person without spinal injury was provided by the Department of Pathology from the London Health Sciences Centre, University Campus, The University of Western Ontario.

Spinal cords were removed within 24 h of death in all three cases. The cords were placed immediately in 10% buffered formalin for a period of 2 weeks, and then stored in phosphate buffer (0.1 M, pH 7.2) at 4°C. In the cases with spinal cord injury, the site of injury was examined and the completeness of injury was determined. The level of damage was determined by physical examination of the cord and root identification. The severity of the injury (complete/incomplete) was determined from histological analysis of the site of injury.

Alternative axial sections of the spinal cords were prepared and examined in all three cases. Three sets of 30–40 sections were prepared from each segment of the spinal cords in each case. One set of sections was stained for general histology (haematoxylin-eosin). The second set of sections was stained for neurons and their processes using a silver staining technique (Bielchowsky).[22] Finally, the third set of sections was processed immunocytochemically for choline acetyltransferase (ChAT). Antigen retrieval incorporating high-temperature microwave heating of formalin-fixed, paraffin-embedded tissue sections before immunostaining was used in this case.[23,24] Antibodies to ChAT made in goat (1/1000 dilution; Chemicone, USA) were used in our study. Then, biotinated anti-goat antibody made in donkey (1 : 400 dilution, Biocan Scientific, Canada) and rhodamine lissamine conjudated to streptavidin antibody (1 : 150 dilution Biocan Scientific, Canada) were used for staining.[25] Immunofluorescent neurons were detected using a Leitz microscope equipped with an epifluorescence system containing N2 filter.

First, the sections were examined under a bright field microscope for the gross anatomy. The presence of major artifacts within examined segments was determined. Then examination was focused only on the lateral horns of the spinal cord. This is a clearly identified area of the spinal cord (Figures 1A, 2A and C). The location and morphology of SPNs were analyzed using a Leitz microscope with the Microcomputer Imaging Device imaging system (MCID, St. Catharines, Ontario). The somas were outlined and assessed for area size, median and maximum diameters using MCID software. The majority of measured cells were elongated, not circular. In elongated targets the median diameter is a measure of width, while the maximum diameter is a measure of length.[26] Therefore, both diameters were chosen for a better representation of spatial dimensions of the measured cells. Only preganglionic neurons within the intermediolateral nucleus (IML) of the spinal cord, as a clearly identified area of the spinal cord, were analyzed (Figure 1). Moreover, this area of the cord contained cholinergic neurons identified by the presence of ChAT. In each segment at least 20 SPNs were selected and soma area, median and maximum diameters were measured and compared in upper and middle thoracic segments of the spinal cords. In the two cases with spinal cord injury we were able to compare the SPNs above and below the site of spinal injury. The neurons below the injury represent neurons which lost descending input from supraspinal structures. To eliminate bias of the observer, measurements were made without the knowledge of the segment and case number. The age and sex factor was eliminated by choosing the specimens from men of the same age.

Statistical analysis

The quantitative cell analysis was used cautiously in our study. To determine statistical morphological differences in the SPNs at different levels of the human spinal cord and between the cases, a one-way analysis of variance with repeated measures was used. Tukey's test was used for comparison of mean values.[27] All data in the paper were presented as mean $\pm$ SEM. Differences were considered significant when $P<0.05$.

Results

Case 1 (Case with normal spinal cord)

This 43-year-old male had died from myocardial infarction. Postmortem interval (PMI) in this case was 10 h. The third (T3) and the seventh (T7) thoracic

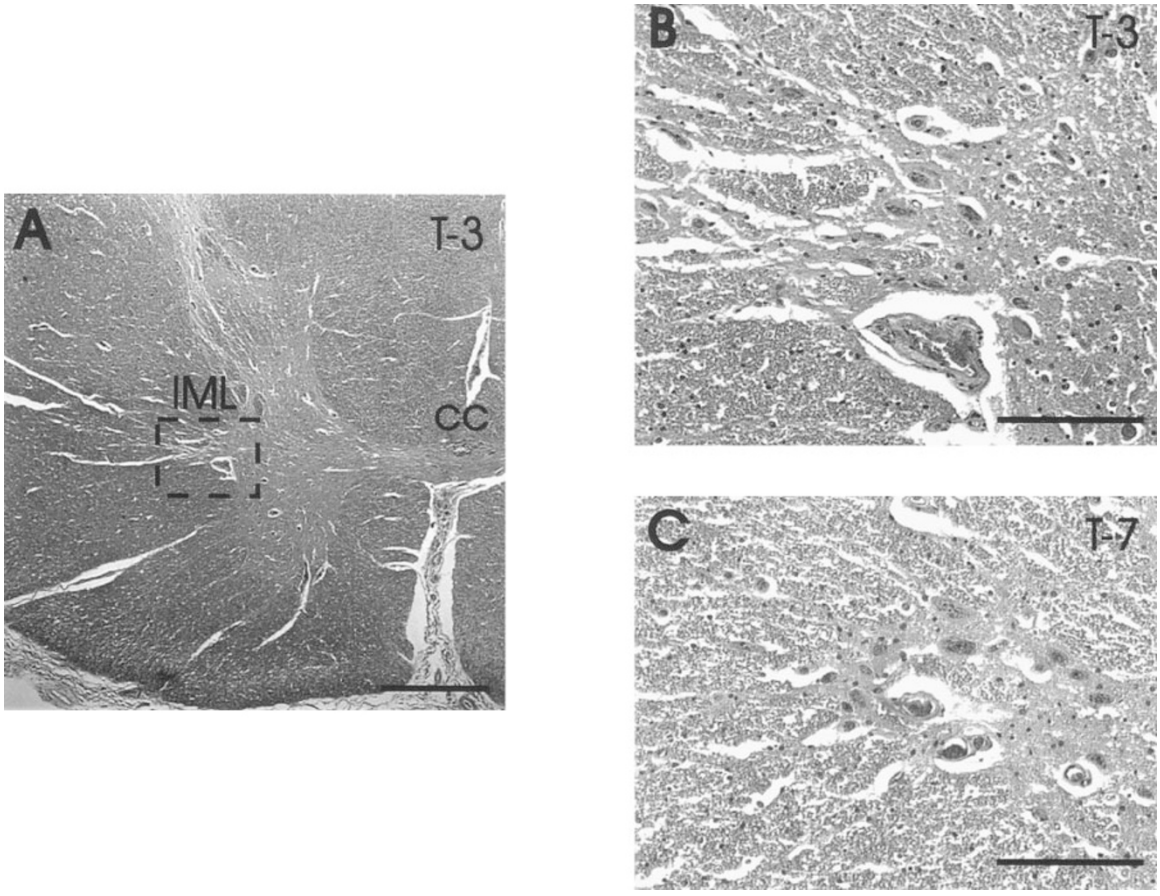

Figure 1 Haematoxylin/eosin stained sections through the third (T3) and seventh (T7) thoracic segments of human cord (Case 1, without cord injury). (**A**) shows a section of the spinal cord at low magnification. (**B**) is a higher power photomicrograph of the area outlined in panel A. (**C**) is a high magnification photomicrograph of the IML from the seventh thoracic segment. Abbreviations: cc - central canal; IML - intermediolateral nucleus (nucleus within the lateral horn of the cord); T - thoracic. Calibrations on (**A**) is 1 mm and on (**B** and **C**) are 100 μm

segments of the spinal cord were examined from this case. The microscopic examination revealed the normal morphology of the spinal cord at both levels with clear symmetrical representation of white and gray matter (Figure 1A). A typical butterfly-shaped gray matter with prominent lateral horn was observed at both levels of the spinal cord. The lateral horn was easily detected as a prominent triangularly-shaped part of the gray matter at the level of the central canal. At both levels the intermediolateral nuclei were clearly represented (Figure 1B and C). The SPNs were clearly detected within this area and had oval or spindle shaped cell bodies, which were elongated in the mediolateral direction. The SPNs in this area were also immunopositive to the cholinergic neurotransmitter enzyme ChAT. Motoneurons within the ventral horn of the spinal cord were also identified by immunoreactivity to the ChAT. The soma area of the SPNs at T3 segment was $400.7 \pm 15\ \mu m^2$ and the median diameter $14.0 \pm 0.5\ \mu m$ (Table 1). At the T7 segment the soma area and median diameter were $409.9 \pm 22\ \mu m^2$ and $14.1 \pm 0.8\ \mu m$ accordingly. No significant differences between the soma areas, median and maximum diameters of SPNs within these two segments were observed in this case.

Case 2 (Acute spinal cord injury case)

This 38-year-old male had died 2 weeks after spinal cord injury (gunshot wound) at the T5 level. The PMI in this case was 20 h. The functional and anatomical completeness of the injury was confirmed by both clinical and post-mortem examinations. The soma area of SPNs at T1 above the site of injury and at the T8 segment below the injury was examined. We found that the general morphology of the spinal cord within the segments chosen for the study was mostly intact (Figure 2A and C). Clear preservation of white and gray matter was observed in both segments. However, a small area of hemorrhage within the dorsal funiculus was present at T8 level (Figure 2C). The lateral horn with intermediolateral nucleus was easily identified in spinal sections at both levels of the spinal cord and did not show any signs of compression or deformation.

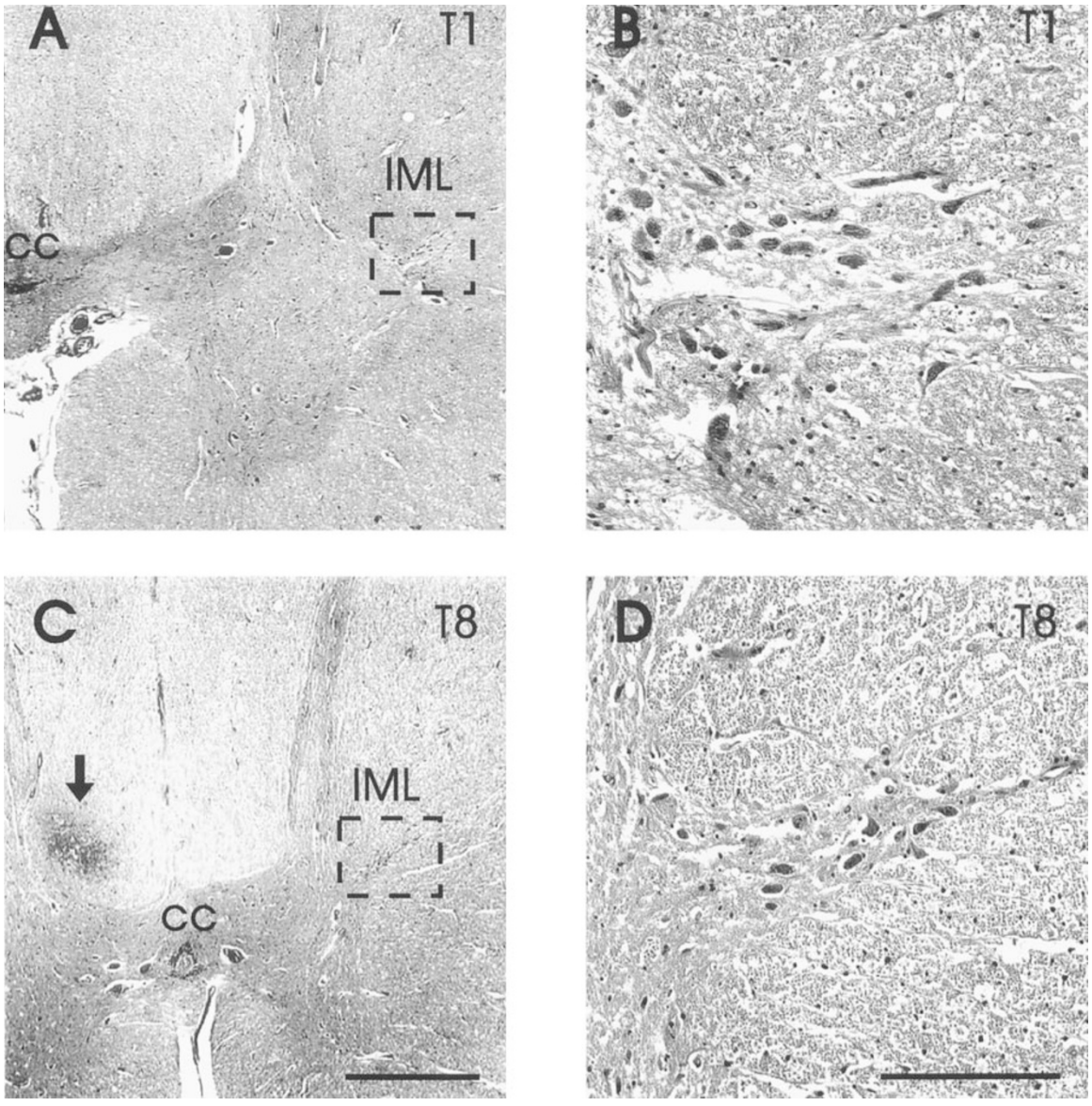

Figure 2 Haematoxylin/eosin stained sections through the first (**A** and **B**) and eighth (**C** and **D**) thoracic segments of human spinal cord (Case 2, 2 weeks after spinal injury). (**B** and **D**) are the areas of IML with sympathetic neurons at higher magnification, that are outlined in (**A** and **C**) respectively. A small contusion haemorrhage within the dorsal funiculus is clearly present at T8 level (indicated by arrow on **C**). Abbreviations as in Figure 1. Calibrations for (**A** and **C**), on (**C**) is 1 mm and for (**B** and **D**), on (**D**) is 200 μm

The SPNs within the T1 segment had clearly identified nuclei and oval or spindle-shaped cell bodies. The SPNs in this area were also immunopositive to the cholinergic neurotransmitter enzyme ChAT. The approximate soma area and the median diameter of SPNs within this segment were 418.7 ± 19 μm^2 and 13.4 ± 0.5 μm respectively (Table 1). The majority of somata of SPNs below the site of injury were shrunken with asymmetric nuclei. The soma area and the median diameter of these neurons were 272.9 ± 11 μm^2 and 11.2 ± 0.4 μm. The soma area of SPNs below the site of injury was significantly smaller ($P < 0.01$) than the SPNs in the upper thoracic segment in this case or than SPNs from the control and chronic cord injury cases.

Case 3 (Chronic spinal cord injury case)
This 42-year-old male had died 23 years after the spinal cord injury (gunshot wound) at T3 level. The PMI in this case was 22 h. This case was classified as functionally complete and anatomically incomplete (a small number of axon/myelin units were preserved at the periphery of the gray matter at the site of injury). The T1 and T7 segments were studied in this case. The

Table 1

		Soma area (μm^2)	*Median diameter* (μm)	*Max. diameter* (μm)
Control case	T3	400.7 ± 15	14.0 ± 0.5	17.7 ± 0.6
(no SCI case)	T7	409.9 ± 22	14.1 ± 0.8	18.3 ± 0.7
Case 34	T1	418.7 ± 19	13.4 ± 0.5	16.9 ± 0.5
(acute case SCI)	T8	272.9 ± 11*#	11.2 ± 0.4*#	14.5 ± 0.4*#
Case 57	T1	416.2 ± 19	13.1 ± 0.7	17.4 ± 0.8
(chronic case SCI)	T7	425.0 ± 20	13.0 ± 0.4	17.0 ± 0.6

*Significantly different from neurons at T1 level. #Significantly different from neurons between the cases

morphology of the T1 segment was generally normal. However, the T7 segment was significantly altered by the degeneration and atrophy of the spinal cord below the site of injury. In spite of this, the lateral horns were easily identified at both levels (data not shown). The soma area and median diameter of SPNs at T1 were approximately 416.2 ± 19 μm^2 and 13.1 ± 0.7 μm, respectively (Table 1). The SPNs at T7 level had a soma area of 425.0 ± 20 μm^2 with a median diameter of 13.0 ± 0.4 μm. There was no significant difference in soma areas of SPNs between the upper and middle thoracic segments in this case. The SPNs within the lateral horn were also immunopositive to the cholinergic neurotransmitter enzyme ChAT.

Discussion

In this study we have shown that partial central deafferentation of spinal neurons by a middle thoracic injury leads to significant atrophy of SPNs at 2 weeks after cord injury. Twenty-three years after injury, no atrophy was present. To our knowledge these are the first observations of changes in human SPNs in the IML after spinal cord injury. This neuronal population is crucial for cardiovascular control.[18,28] Comparison of soma areas of the SPNs in the thoracic segments of the spinal cord of the person who died 2 weeks after the spinal trauma shows a significant decrease in soma area caudal to the site of spinal injury. However, the soma area of the IML neurons within the upper and middle thoracic segments in the person 23 years after spinal injury were similar to the SPNs in spinal cord from a person without cord injury. The study of this distinct neuronal population gives us information about the extent of degenerative changes within the spinal cord after the injury.

The major purpose of this study was to compare previous findings in animals with the human data. The neuronal atrophy and dendrites retraction after deafferentation, a condition characterized as transneuronal degeneration, was previously described in different neuronal populations in animals.[29–34] Also, it has been known that the extent of atrophy is related to the degree of the deafferentation.[35] In some experimental studies the neuronal changes were described after the spinal cord injury at acute and chronic stage of injury.[17,29] In this study the SPNs studied in segments above the injury site will still have intact descending supraspinal connections, will not be affected by partial deafferentation and will be similar to the neurons from the control case. However, SPNs from the segments below the site of injury will represent a centrally deafferented neuronal population with loss of supraspinal connection.

Our morphological findings revealed an interesting correlation with the changes in cardiovascular control that were reported in clinical observations. The atrophy of the SPNs in the acute stage of spinal cord injury in humans, may contribute to the condition observed immediately after the spinal cord injury in animals[36–38] and humans known as 'spinal shock'.[5,39–41] After acute spinal injury there is a flaccid paralysis, with urinary bladder atonia, paralytic bowel and sympathetic underactivity resulting in low systemic arterial pressure. Besides the immediate depression of autonomic functions resulting from a loss of important excitatory input from the brainstem,[16] the atrophy of SPNs can also contribute to the sympathetic atonia. With time after the injury, the sustained excitatory responses develop that cause the autonomic dysreflexia.[2,42,43] Usually, this condition can only be seen during the chronic stage of human spinal cord injury. Autonomic dysreflexia in humans becomes obvious 6 months after the cord injury, particularly in people with cervical or high to midthoracic cord injury.[42,44–47]

Probably at a later stage of cord injury other mechanisms contribute to the impaired cardiovascular control. For example, in animals, after cord injury, dorsal root afferents have been shown to sprout[48] and spinal neurons first lose synaptic inputs and then appear to replace them with synapses from a different source.[49,50] Therefore, new inappropriate afferent inputs from different afferent sources could support the long-term dysreflexia after spinal cord injury that occurs in humans. The return to normal morphological appearance of SPNs at the chronic stage of spinal injury may be a result of these new connections. This possibility requires future investigation.

In this study, we confirmed that in the chronic stage of cord injury in humans, the morphology of SPNs was re-established. To some extent the similar correlation in morphological changes of SPNs were observed in previous animal studies. The re-establish-

ment of dendritic arbour and soma area at chronic stage of spinal cord injury in animal models coincides with stable and intense autonomic dysreflexia.[17] Therefore, it is possible that the functional changes are a result of rearrangement within the spinal cord that occurred after the injury.

The IML contains spinal sympathetic preganglionic neurons and occupies the apical region of the lateral horn in the thoracic and upper lumbar segments.[28,51] We have to acknowledge that the term 'IML' and 'lateral horn' are not the same. The majority of SPNs are located within the IML, however, some of them can also be found in the gray matter medial to the apical region of the lateral horn.[52] Also, some neurons can be found within the white matter of the lateral funiculus adjacent to the lateral horn. The IML is a well defined cell column within lamina VII, that occupies a large portion of the spinal gray matter and extends across the spinal cord bilaterally (Figure 1A). We did not have any difficulty in identifying this area within the spinal cord. Many other neurons are located within this area beside the SPNs that could be easily identified using neurotracing techniques.[53,54] We cannot use retrograde tracers *in vivo* in humans, to study neuronal morphology. However, our recent study in fixed human spinal cord tissue showed that IML neurons retrogradely labeled with the lipophilic tracer DiI[52] have similar morphological characteristics to those of SPNs as presented in this study. In this study, the SPNs were identified by well known anatomical criteria such as their localization within the lateral horn of the spinal gray matter. Also, SPNs in our study were identified by using immunoreactivity to the cholinergic neurotransmitter enzyme ChAT. ChAT containing neurons were identified within the lateral horn (presumably SPNs) of the spinal cord (data not shown). Even though motoneurons were not in the focus of the present study, they were clearly identified within the ventral horn by immunoreactivity for ChAT. SPNs and motoneurons are known to be cholinergic and contain the acetylcholine synthetizing enzyme ChAT.[25,55,56]

One limitation of this study is the small number of cases that we were able to investigate. Although we examined only three human cases, we were able to detect degenerative changes within the specific neuronal population of the spinal cord. We were particularly interested in comparing the SPNs within the same spinal cord located above and below the site of injury. The SPNs above the site of injury had intact supraspinal connections and served as a control for neurons below the injury with interrupted bulbar connections with reaction to central deafferentation. The comparison of soma area of SPNs at different levels in the control case (Case 1, without cord injury) was necessary to be sure that the SPNs have the same features at different levels of intact spinal cord. The examination of the soma area of SPNs in Case 1 showed no significant difference between two segments of the spinal cord. Moreover, the possible difference in size of the SPNs that could arise from difference in the sex or age of the person, were eliminated by choosing cases within the same age group and the same sex. There is well documented evidence for changing neuronal properties with age in animal studies.[57,58] In our study, all spinal cords were obtained from male subjects and the age difference within the group was only 4 years (Cases 1 and 2 – 42-year-old, Case 3 – 38-year-old).

In conclusion, we still do not fully understand the mechanisms of the disrupted autonomic control after spinal cord injury. This study confirmed that the neuronal reactions observed in animals also occurred in humans in response to spinal cord injury. The described morphological changes within the SPNs could lead to better understanding of the CNS reaction to injury and to the designing of possible treatment strategies. Moreover, the comparison of the time course of these changes will allow the prediction of the best time frame for therapies. Also, the interest in studying the SPNs is continuing among scientists and clinicians because many questions have to be asked concerning the function of these neurons and their involvement in other cardiovascular disorders such as hypertension.

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Note added in proof

My acquaintance with Dr Richard Bunge began when we collaborated on a project between The Miami Project and my laboratory at The John P Robarts Research Institute, London, Ontario, Canada. This collaboration began soon after my relocation from the Former Soviet Union and at the beginning of my research career in North America. It was a very difficult and challenging time. Many colleagues were very helpful and offered support with my research initiatives, one of whom was Dr Richard Bunge. After studying sympathetic preganglionic neurons in the animal model of spinal cord injury in the laboratory, I was very interested to see what was happening in a clinical situation with human spinal neurons. Knowing that The Miami Project has an extensive bank of human spinal cord specimens, I discussed this project, for the first time, with Dick during a meeting of the Society of Neuroscience. At that time I knew about his illness. He kept his grace, integrity and his smile despite prolonged and debilitating

treatments. As a result of our preliminary discussions, I was invited to the Miami Project to present my research and discuss my proposal in detail. I was fascinated with the courage and strength Dick portrayed. Dick retained his good spirit and optimism despite having to take frequent breaks during the day because of his chemotherapy treatments. He was excited by future research possibilities as if nothing else was going on in his life. Detailed and careful observations of the biological phenomena, which was always documented in his work, was at the forefront of Dick's work. Similar to many who worked with Dick, I will always remember him as a great scientist, educator and mentor.

Dr AV Krassioukov

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