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Short-Term Changes of NADPH Diaphorase-Exhibiting Neuronal Pools in the Spinal Cord of Rabbit after Repeated Sublethal Ischemia

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Summary

The aim of this study was the histochemical characterization of NADPH diaphorase-positive neuronal pools in the rabbit lumbosacral segments using a model of single, repeated and multiple sublethal spinal cord ischemia. Following a single 8-min sublethal spinal cord ischemia and 1-hour reperfusion, the staining of NADPH diaphorase-exhibiting neurons in the dorsal horn, pericentral region, dorsal gray commissure and sacral parasympathetic nucleus was comparable with the control sections. In contrast to the foregoing sublethal ischemia, a regionally different somatic NADPH diaphorase (NADPHd) staining was found after multiple sublethal spinal cord ischemia. Whereas an almost complete loss of the staining of large NADPHd-exhibiting somata in the pericentral region was detected, the staining of the NADPHd-exhibiting neuronal pools in the deep dorsal horn and sacral parasympathetic nucleus was fully preserved. Concomitantly, a prominent reduction of small NADPH diaphorase -positive neurons was noted in the superficial dorsal horn layers of lower lumbar and sacral segments.

Key words

NADPH diaphorase • Rabbit • Spinal cord • Sublethal ischemia

Introduction

It is generally accepted that NADPHd/NOS-containing and NO synthesizing neurons are remarkably spared from cell death in N-methyl-D-aspartate (NMDA) receptor-induced neurotoxicity, vascular stroke, Huntington's disease and Alzheimer's disease (Thomas and Pearse 1964, Ferrante *et al.* 1985, Beal *et al.* 1986, Koh *et al.* 1986, Koh and Choi 1988, Uemura *et al.* 1990, Hyman *et al.* 1992, Dawson et al. 1993). NO has been implicated in many normal and pathophysiological functions such as neurotransmission (Snyder and Bredt 1991, Dawson *et al.* 1992, O'Dell *et al.* 1994), regulation of vascular tone (Moncada *et al.* 1991, Knowles and Moncada 1992), modulation of the calcium transport systems in the myocardium (Zahradníková and Križanová 1997), NMDA-mediated cytotoxicity (Dawson *et al.* 1991) and platelet aggregation (Radomski *et al.* 1987). It should be noted that in search for a detailed regional distribution of various histochemically detectable NADPHd-exhibiting neurons in the spinal cord of rabbits and dogs (Maršala *et al.* 1998, 1999) a great variety of

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neuronal types has been discovered. Some of them were perhaps a part of spinal cord microcircuits mainly those occurring in the superficial dorsal horn and middle-sized, large NADPHd-exhibiting neurons localized in the pericentral region and deep dorsal horn along the rostrocaudal axis of the spinal cord. The functional significance of these parvo- and magnocellular NADPHd-positive neuronal populations is as yet unknown. However, in a single episode of transient spinal cord ischemia, spinal cord gray matter layers rich in NADPHd-positive neurons were found to be refractory to ischemia-reperfusion-induced injury (Maršala *et al.* 1997).

On the other hand, short episodes of sublethal cerebral ischemia, each brief enough to allow recovery without detectable neuropathological damage at the light microscopic level, can cause prominent selective necrosis if such sublethal ischemia is introduced repeatedly and spaced at shorter, e.g. 1-hour intervals (Hanyu *et al.* 1995, Kato and Kogure 1990, Tomida *et al.* 1987). However, the response and role of NADPHd-exhibiting neuronal pools under special conditions of repeated sublethal ischemia requires further study. The need to clarify a possible region-specific response of NADPHd-exhibiting and/or NOS immunoreactive neurons is strengthened by recent findings pointing to quite obvious phenotypic differences, morphological heterogeneity among spinal cord NADPHd-exhibiting neurons (Lee *et al.* 1993, Wetts and Vaughn 1994), regionally specific effects of nitric oxide donors and cGMP on the electrical activity of neurons in the rat spinal cord (Schmid and Pehl 1996) and regional differences in membrane-bound phospholipids (Lukáčová *et al.* 1998, Lukáčová 1999, Pavel and Lukáčová 1999). In the present work, we tried to determine if short repeated sublethal spinal cord ischemia may have 1) a histochemically detectable influence on NADPHd-exhibiting neurons and 2) if such exposure will result in a lamina-dependent homo- or heterogeneous response of NADPHd-exhibiting neuronal pools.

Methods

This study is based on the induction of the sublethal spinal cord ischemia model in adult rabbit of both sexes (n=9) weighing 2.5-3.5 kg (Zivin *et al.* 1982). The animals were anesthetized with pentobarbital (30 mg/kg, i.v). The right femoral artery was cannulated (Portex cannula 0.5-0.9) to monitor distal blood pressure and heart rate, using a pressure monitor LDP-102 (Tesla, CR). A small subcostal incision on the right side was then performed, providing access to the abdominal aorta at the level of the renal arteries. A snare ligature with a long file was then placed around the aorta, distal to the left renal artery. Blood samples taken from the central ear artery were periodically analyzed for PaO_2, $PaCO_2$, and pH (Automatic Gas Check 995-Hb, Austria). The body temperature was monitored with a rectal probe inserted 4 cm into rectum and maintained between 38 and 39 °C using a heating pad. Before the induction of sublethal spinal cord ischemia, the animals were divided into two groups: 1) sham-operated control (n=3) group with abdominal surgery but without tightening of the ligature and 2) three 8-min occlusions of the abdominal aorta (n=6) spaced at 1-hour intervals. At the end of the sublethal ischemia-reperfusion period, all animals (including those of sham-operated control group) were fixed by transcardial perfusion with saline followed by 4 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 and processed for NADPH diaphorase histochemistry.

After perfusion fixation with saline followed by freshly prepared 4 % paraformaldehyde + 0.1 % glutaraldehyde that was buffered with 1 M sodium phosphate, pH=7.4, the lower thoracic and all lumbosacral segments were dissected out and stored *in toto* in the same fixative for 3-4 h. After postfixation, the spinal cord was divided into lower thoracic (Th8-Th13), lumbar (L1-L7), and sacral (S1-S4) segments. The segments were then cryoprotected in an ascending concentration of sucrose (15-30 %) with the same phosphate buffer, and stored overnight at 4 °C. Frozen transverse sections (40 μm thick) were cut from all the segments studied and processed for NADPHd activity by using a modified histochemical procedure (Scherer-Singler *et al.* 1983).

NADPHd staining occurring in cell bodies is described as somatic; the staining showing the dendritic and axonal processes is described as fiber-like and dense neuropil NADPHd staining, where most of the reaction product, which is dotted in appearance, is described as punctate non-somatic NADPHd positivity. For qualitative analysis of the NADPHd-exhibiting neuronal pool in Th8-S4 segments, 25-35 sections were used from each segment. Control sections were treated in the same solution but without NADPH to enable testing for endogenous reduction were then rinsed in 0.1 M phosphate buffer (pH=7.4), mounted on slides, air-dried overnight and coverslipped with Entellan.

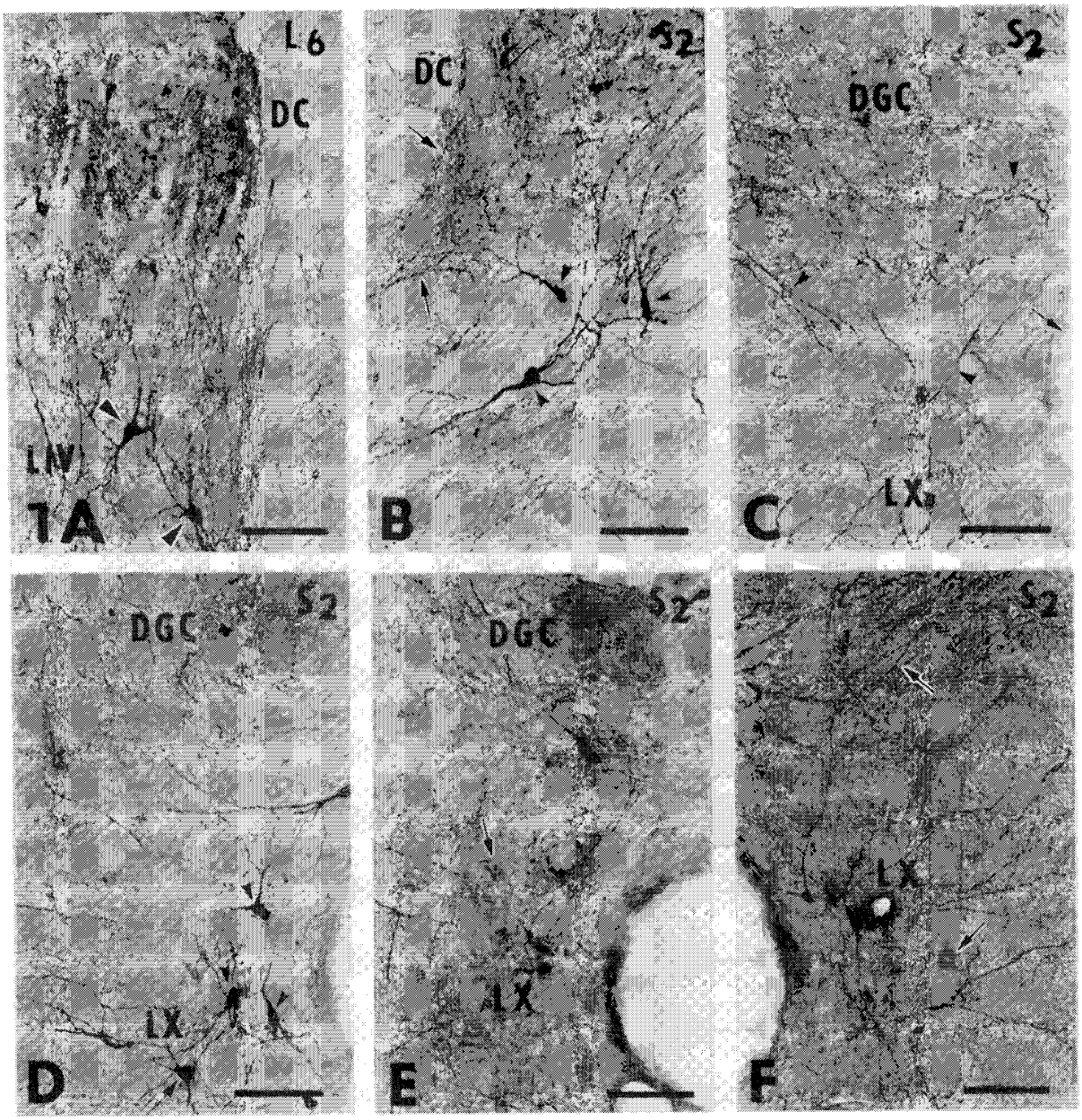

Fig. 1. ***[A]*** *microphotograph showing intensely stained large NADPHd-exhibiting neurons (arrowheads) in the medial portion of lamina IV (LIV); DC – dorsal column; L6 segment (L6).* ***[B]*** *many varicose fibers (arrows) and intensely stained neurons (arrowheads) are seen in the deep dorsal horn; DC – dorsal column; S2 segment (S2).* ***[C]*** *many extremely thin varicose fibers are seen across the pericentral region (arrowheads), close to the central canal (arrow) and in the dorsal gray commissure (DGC); S2 segment (S2).* ***[D]*** *control section cut through pericentral region (LX), depicting large NADPHd-positive neurons (arrowheads); DGC-dorsal gray commissure; S2 segment (S2).* ***[E]*** *large NADPHd-positive neurons are not seen in the pericentral region (LX, arrow); DGC-dorsal gray commissure; S2 segment (S2).* ***[F]*** *occasionally, pale shadows of NADPHd-positive neurons (arrow) are seen; arrow points to a varicose axon in the deep portion of the dorsal gray commissure. All bars represent 100 μm.*

Results

A regionally different somatic NADPHd-staining was noted bilaterally at the L4-S4 segmental level in the dorsal horn, and in the sacral parasympathetic nucleus (SPN), whereas an almost complete loss of the staining of large, multipolar NADPHd-exhibiting somata was found in the pericentral region. While somata of

NADPHd-positive neurons in lamina X known to be highly NADPHd-positive in control sections (Fig. 1D) appeared as completely depleted from NADPHd activity in S1-S3 segments (Figf 1E and 1F), the number of large NADPHd-exhibiting neurons in the deep dorsal horn and large stellate neurons in the SPN appeared to be only slightly reduced. However, some neurons in laminae III-VI were intensely stained (Fig. 1A, 1B). Concurrently with this finding, the number and staining of small elongated bipolar neurons and round, triangular, or oval NADPHd-exhibiting neurons located in the superficial dorsal horn layers was reduced (Figs 2A, 2B and 2C).

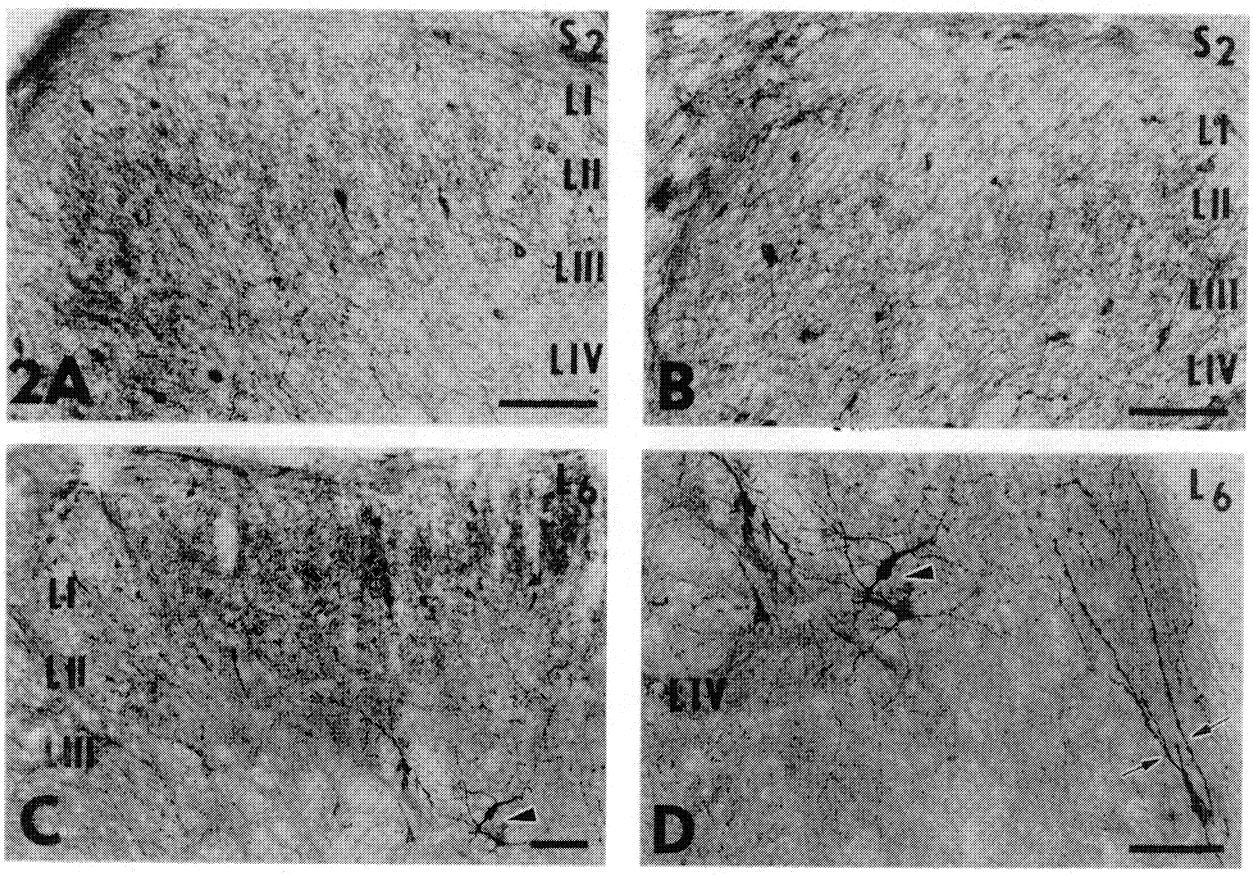

Fig. 2. ***[A]*** *the number of small NADPHd-positive neurons in the superficial layers (LI-LIII) is greatly reduced; in lamina IV (LIV) no large NADPHd-positive neurons are seen; S2 segment (S2).* ***[B]*** *considerably reduced number of small NADPHd-positive neurons was seen across the superficial layers (LI-LIII); in lamina IV (LIV) no large NADPHd-positive neurons are seen; S2 segment (S2).* ***[C]*** *the number of small NADPHd-positive neurons in the superficial layers (LI-LIII) is greatly reduced; arrowhead points to a group of intensely stained neurons in the deep dorsal horn; L6 segment (L6).* ***[D]*** *many intensely-stained NADPHd-exhibiting neurons (arrowhead) are seen in lamina IV (LIV); a large number of varicose fibers (arrows) was found in the medial portion of the dorsal horn; L6 segment (L6). All bars represent 100 μm.*

Simultaneously, abundant and intense fiber-like NADPHd-positive staining was found in laminae III-VI and lamina X (Figs 1C and 2D). With regard to the pericentral region, the course and ramification of the dense meshwork of NADPHd-positive fibers and varicosities was derived from two thin fiber bundles seen at L6-S3 segment level. A more compact medial axonal bundle containing 1-2 μm thick varicose axons was seen along the medial border of the dorsal horn at L6-S3 segment level close to the dorsal funiculus running in the ventromedial direction and penetrating into the ventral half of the dorsal gray commissure in the sacral segments (Fig. 3). A beaded appearance of these axons was quite apparent with some almost regularly spaced large varicosities (3-4 μm in diameter). However, the majority of these medially oriented varicose axons could be followed more ventrally into the pericentral region where a dense punctate non-somatic NADPHd-positive staining

was easily detectable. This is possibly due to terminal arborizations of varicose axons seen in great abundance in the pericentral region. More loosely arranged intensely-stained axonal bundles containing thin axons (0.5-1.5 μm in diameter) rich in densely-packed varicosities were found extending from the ventromedial border of the SPN. They terminated in the lateral portion of lamina X and in the dorsomedial part of the ventral horn. Isolated varicose axons of both components were oriented transversally and, after crossing into the most ventral portion of the dorsal commissure, they were traceable in the medial part of the pericentral region reaching into the subependymal layer, close to the central canal. An apparently increased punctate non-somatic NADPHd staining was noted in the neuropil of Onuf's nucleus in the S1-S3 segments.

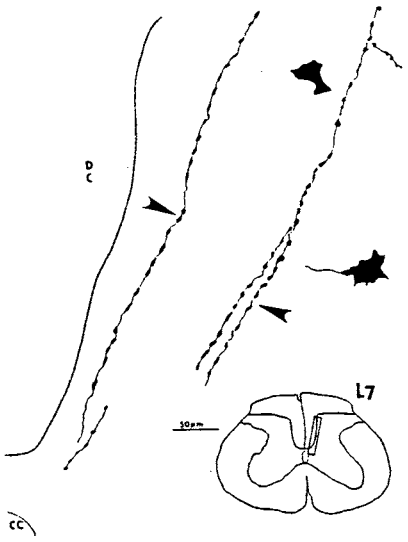

Fig. 3. *Camera lucida drawing demonstrating the location of the medial component of long ventromedially oriented varicose intensely stained NADPHd-positive fibers (arrowheads) along the medial border of the dorsal horn in the L7 segment (L7); DC – dorsal column. Bar represents 50 μm.*

Discussion

The present study demonstrated that the response of NADPHd-exhibiting neurons in the lower lumbar and sacral segments of the rabbit to multiple 8-min sublethal ischemia spaced at 1-hour intervals resulted in a differential segment- and lamina-dependent up-regulation of NADPHd activity. It was repeatedly reported that brief and sublethal cerebral ischemia can selectively injure some vulnerable neurons when such short ischemia is induced at certain intervals (Araki *et al.* 1990, Kato and Kogure 1990, Lukáčová 1999). Several causal factors could influence this cumulative effect of neuronal damage that is most severe when sublethal ischemia is repeated at 1-hour intervals. These factors mainly concern impairment of protein synthesis (Araki *et al.* 1990), postischemic hypoperfusion which is maximal 1-hour after 2-min ischemia (Kato *et al.* 1990), the excitotoxic mechanism (Kato and Kogure 1990, Lin *et al.* 1992), alterations in receptor sensitivity (Kato *et al.* 1991), regional changes in [^{3}H] inositol 1,4,5-triphosphate binding (Kato *et al.* 1994) and changes in the biogenesis of membrane phospholipids (Lukáčová *et al.* 1998, Pavel and Lukáčová 1999). The transition from ischemic neuronal necrosis to infarction in repeated sublethal ischemia suggests that there is a threshold infarction in repeated ischemia, a phenomenon defined as a volume of tissue necrosis, in which all neurons, glial cells, and blood vessels are involved (Hanyu *et al.* 1995).

The principal finding of the present study is that well-defined NADPHd-exhibiting neuronal pools in the lumbosacral segments respond to multiple sublethal spinal cord ischemia spaced at 1-hour intervals in a region-specific manner. This finding relates to NADPHd activity of small neurons in the superficial dorsal horn classified as type 1 and type 2, large neurons in the deep dorsal horn layers classified as type 3 and type 4 (Maršala *et al.* 1998, 1999) and neurons in the sacral parasympathetic nucleus in S1-S3 segments. All of them appeared to be strongly enhanced considering the intensity of somatic NADPHd staining. However, large profusely-branched and intensely-stained neurons occurring in control sections of the pericentral region (lamina X) were almost completely lacking. It was repeatedly confirmed that a dramatic NOS up-regulation assessed by NADPHd histochemistry or NOS immunocytochemistry may occur in many central neurons despite the fact that these neurons normally lack the enzyme, or may express it only very weakly (Chen and Aston-Jones 1994, Herdegen *et al.* 1993, Saxon and Beitz 1994, Yu 1997). In contrast, NADPHd staining and NOS immunopositivity were proved to persist for a long period in the central neurons, e.g. in the mammillary body after mammillothalamic tract transection (Chen and Aston-Jones 1994). Similarly, some brainstem neurons projecting to the cerebellum and Purkinje cells can

survive and remain NOS immunoreactive for a long period of time after cerebellar lesion (Saxon and Beitz 1994).

More importantly, the present study demonstrates that a region-specific response, including the down-regulation of NADPHd activity in different neuronal NADPHd-exhibiting pools, does exist, at least in the paradigm of multiple sublethal spinal cord ischemia. The loss of NADPHd staining of neurons around the central canal in lamina X and adjacent medial lamina VII may have serious implications, because several anatomical studies have demonstrated that primary nociceptive afferent fibers project to the area surrounding the central canal (Light and Perl 1979, Miller and Seybold 1987) and lamina X neurons have been implicated in the transmission of nociceptive information (Honda and Perl 1985). Approximately 10 % of NOS positive neurons in lamina X are spinothalamic tract neurons (Lee *et al.* 1993). Moreover, neurons in the pericentral region have a diversity of neurotransmitters and/or neuromodulators such as neurotensin, dynorphin A, met-enkephalin, somatostatin, γ-aminobutyric acid, leu-enkephalin, CCK and serotonin (Glazer and Basbaum 1981, Sasek *et al.* 1984, Miller and Seybold 1987, Newton *et al.* 1986). Recently, a considerable phenotypic diversity of the spinal cord NADPHd-exhibiting neurons was described. By a combination of diaphorase histochemistry with choline acetyltransferase (ChAT) immunocytochemistry, it was revealed that 71 % of the cholinergic neurons were diaphorase-positive around the central canal (lamina X), whereas neurons in lamina II were diaphorase-positive only (Wetts and Vaughn 1994).

Finally, comparative NADPHd histochemical studies detected a close association between NADPHd-exhibiting somata and their dendrites located in the pericentral region (lamina X) and the spinal cord vasculature (Maršala *et al.* 1998, 1999). At the light microscopic level, NADPHd-exhibiting somata were observed to have processes that form an intricate network of NADPHd-positive fibers closely associated with longitudinally running spinal cord vessels that are located in lamina X and somewhat laterally from the subependymal layer. Interestingly, however, the loss of somatic NADPHd staining in the pericentral region and the adjacent medial part of the intermediate zone in L6-S3 segments following multiple sublethal spinal cord ischemia had no appreciable influence on fiber NADPHd staining in both regions. In the transverse and sagittal sections, many extremely thin axons were seen to enter and enrich the intricate network of intensely-stained NADPHd-positive fibers. Among them the fibers with beaded varicosities (ranging 1.5-4.5 μm in diameter) clearly prevailed giving rise to extensive arborizations in the pericentral gray matter including the subependymal layer.

The present experiments have revealed that many pericentrally persisting NADPHd-exhibiting fibers in the L6-S3 segments share some of the characteristics known for vasoactive intestinal polypeptide (VIP) occurring in the visceral afferent pathway (Vizzard *et al.* 1994).

Several pathological mechanisms may be considered as causal factors leading to an unusual loss of NADPHd staining of lamina X neurons in L6-S3 segments after multiple sublethal ischemic insults. In the central and peripheral nervous system, there is a remarkable topographic overlapping between NADPHd-exhibiting and NOS immunoreactive neurons (Bredt *et al.* 1991, Dawson *et al.* 1991, Hope *et al.* 1991, Kummer *et al.* 1992). However, there are some areas of the central nervous system where the number of NADPHd-exhibiting neurons is higher than the number of NOS immunoreactive somata (Grozdanovic *et al.* 1995, Lee *et al.* 1993, Lukáčová *et al.* 1999, Vizzard *et al.* 1994).

Phenotypic differences exist among NADPHd-exhibiting neurons characterizing the majority of the pericentral neurons (lamina X) as cholinergic and NADPHd-positive neurons (i.e. excitatory acting elements), whereas neurons in laminae I-II neurons are NADPHd-positive only (perhaps acting as mostly inhibitory elements). These differences may have some relevance with regard to the down-regulation of NADPHd activity of lamina X neurons resulting in a complete loss of NADPHd staining following multiple sublethal ischemic insults. This assumption is strengthened by the finding that, while the majority of neurons increased their electrical activity during superfusion with the NO-donor sodium nitroprusside in lamina X, neurons in laminae I-II were mainly inhibited by sodium nitroprusside in the slice preparation of the spinal cord (Schmid and Pehl 1996). A causal analysis of the rich network of NADPHd-exhibiting fibers in the pericentral region and in the core of the ventral horn in L6-S3 segments and of the loss of somatic NADPHd staining in the same regions supports the existence of a second distinct NADPHd-exhibiting afferent pathway. This pathway contains dorsal root afferents from S1-S3 small dorsal root ganglia neurons (Kawatani *et al.* 1986),

the fine unmyelinated fibers express VIP that is histochemically detectable through NADPHd staining. The existence of a NOS co-expression in VIP-containing neurons in this afferent pathway is possible, because NO was found to participate as a neuronal co-mediator of VIP/cholinergic perivascular vasodilatory acting fibers (Kummer *et al.* 1992).

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