Neuronal Nitric Oxide Synthase in the Rabbit Spinal Cord Visualised by Histochemical NADPH-diaphorase and Immunohistochemical NOS Methods

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Abstract. The NADPH-diaphorase (NADPH-d) staining method is widely used in the investigation of both the central and peripheral nervous systems. Neuronal nitric oxide synthase (nNOS) has previously been shown to be responsible for the NADPH-d activity in neurons. However, NADPH-d activity does not always fully represent the enzyme nNOS. We investigated the distribution of NADPH-d activity and nNOS protein in the rabbit spinal cord for all groups of neurons and Rexed's laminae.

In most laminae the distribution of NADPH-d activity was identical to nNOS immunoreactivity. Both were present in the dorsal horn and in pericentral areas of the spinal cord, but some differences existed. The superficial part of the dorsal horn (laminae I–III) stained more intensely for NADPH-d than for nNOS. However, the most prominent difference was seen in the lateral part of the dorsal horn – the lateral collateral pathway (LCP). The LCP stained strongly for NADPH-d activity, while nNOS staining was absent.

Although there is an excellent correlation between NADPH-d staining and nNOS immunohistochemical staining in the spinal cord in general, the presence of staining differences necessitates the use of immunohistochemistry for some specialized applications.

Key words: NADPH-diaphorase — Histochemistry — NOS immunohistochemistry — Rabbit — Spinal cord

Introduction

Nitric oxide (NO) as an unconventional neurotransmitter is involved in many physiological and pathological processes. It is produced from L-arginine by the enzyme nitric oxide synthase (NOS). NO subserves a variety of functions: in addition to its property of a neurotransmitter, in vascular tissue it has been recognized to be a feasible candidate of endothelium-derived relaxing factor, and in the immune sys-

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tem it is a mediator of cytotoxicity (Dawson et al. 1992; Kluchová 1999). Although its localization was already described in various tissues (Grozdanovic et al. 1992; Gabbot and Bacon 1993; Munoz et al. 2000), its biological importance is still not completely clear. As NO is a gas with half-life of 5 s, its direct measurement and localisation in the tissue is difficult (Bredt and Snyder 1992). Therefore, a number of investigators attempted to purify NOS to produce antibodies for precise localization of the site of NO synthesis.

Several isoforms of NOS have been described and are distinguished by their tissue distribution and the mode of regulation (Nathan and Xie 1994; Norris et al. 1995). Isoform I is constitutively expressed, and the enzymatic activity is regulated by Ca^{2+} and calmodulin. It is referred to as neuronal NO synthase – nNOS (alternatively ncNOS, bNOS). Isoform II of NO synthase is usually not constitutively expressed, but it can be induced in macrophages and many other cells. This enzyme is Ca^{2+} independent and also referred to as inducible – iNOS (macNOS). Isoform III is expressed primarily in endothelial cells. In the literature, the enzyme is also referred to as -eNOS (ecNOS). Like isoform I, this isoform is also constitutively expressed and regulated by Ca^{2+} and calmodulin (Pollock et al. 1995). Although the product of these isoforms of NOS is the same – NO, their roles in physiological and pathological conditions are different. The production of isoform-specific antibodies to NOS has allowed investigators to identify which isoform is present in a specific cell or tissue. Antibody probes can be targeted by use of synthetic peptides as immunogens to recognize selectively any of the three isoforms of NOS.

Prior to development of specific antibodies for immunohistochemical localization, the presence of NOS was inferred by the histochemical demonstration of dehydrogenase: reduced nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d). Enzyme histochemical procedures for the demonstration of various dehydrogenases is based on the ability of reduced cofactors formed in the dehydrogenase reaction to reduce subsequently a dye, e.g. tetrazolium salt to a visible reaction product. This appears to take place either non-enzymatically or *via* second enzyme which can utilize the reduced cofactor to reduce the dye and is therefore termed a diaphorase. NADPH-diaphorase histochemistry is a procedure based on diaphorase activity that selectively stains various discrete populations of neurons. The technique provides a Golgi-like image of these selected neurons, delineating cell bodies, dendritic trees and axonal networks (Scherer-Singler et al. 1983).

NADPH-d activity has been detected in neural structures at various sites both in the mammalian and non-mammalian nervous system (Hope and Vincent 1989; Druga and Syka 1993; Spessert and Claassen 1998; Kluchová et al. 1999, 2001; Munoz et al. 2000). At many of these sites, NADPH-d activity is a marker for neuronal nitric oxide synthase, so that nNOS activity can be detected as the activity of NADPH-diaphorase (Hope et al. 1991).

Since histochemical NADPH-d method is widely used for the visualisation of NOS-containing nerve structures, the present study was proposed to examine the correlation between NADPH-d activity and neuronal NOS immunoreactivity in the rabbit spinal cord.

Materials and Methods

Animal treatment and tissue preparation

Five adult rabbits of both sexes were used in this study. Experimental animals were anesthesized with pentobarbital (30 mg/kg, i.v.) and immediately sacrified *via* intracardiac perfusion with saline followed by 4% paraformaldehyde +0,1% glutaraldehyde buffered with 0,1 mol/l sodium phosphate, pH = 7.4. The fixatives were freshly made up immediately prior to perfusion.

Following the perfusion the lumbosacral part of the spinal cord was carefully dissected out and stored in the same fixative for 3–4 hours. Then it was placed in ascending concentrations of sucrose (15–30%) in the same phosphate buffer for cryoprotection and stored overnight at 4℃. Spinal cord segments were sectioned transversally in a freezing microtome to the thickness of 45 μm.

Histochemical procedure

NADPH-d histochemical detection was performed as reported in our previous studies (Maršala et al. 1997, Kluchová et al. 2000). Sections were incubated for an hour at 37℃ in the solution of 1,5 mmol/l nitroblue tetrazolium (NBT, Sigma Chemicals, N-6876), 1.0 mmol/l ß-nicotinamide adenine dinucleotide phosphate (NADPH, Sigma Chemicals, N-1630), 0.5% Triton X-100 dissolved in 0.1 mol/l phosphate buffer (pH 7.4), 10.0 mmol/l monosodium malate (Malic acid, Sigma Chemicals, M-1125).

Control sections were treated in the same way but without NADPH in the reaction medium. This was to test for endogenous reduction activity in the corresponding blue formazan product (Hope and Vincent 1989).

Following the reaction, sections were rinsed in 0.1 mol/l phosphate buffer (pH 7.4), mounted on slides, air-dried overnight and coverslipped with mounting medium Entellan.

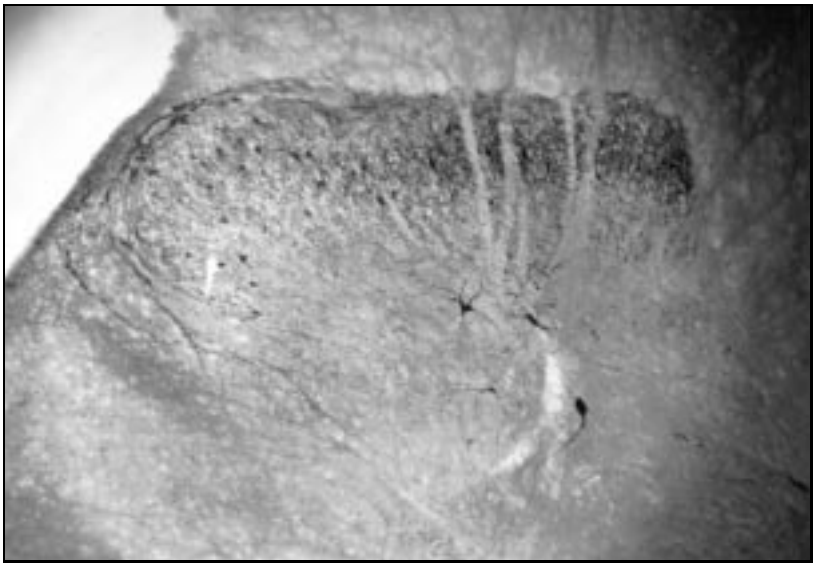
nNOS-immunohistochemistry

Spinal cord sections were treated for nNOS using modified method according to Bredt et al. (1990). They were processed using the free-floating method. After removing them from cryoprotective solution, sections were washed several times in PBS, then pretreated with 0.3% H_2O_2, 20% methylalcohol in PBS for 30 min. Then, sections were washed again and blocked with 5% bovine serum albumin.

For processing nNOS-immunoreactivity, sections were incubated in the Monoclonal Anti-Nitric Oxide Synthase-Brain antiserum (bNOS Sigma), 1 : 1,500 dilution with 1% bovine serum albumin and 0.3% Triton X-100 in PBS overnight at room temperature. After several washes in PBS the sections were incubated with biotinylated anti-mouse secondary antibody (1 : 90, Biogenex) for 1.5 h. Then they were rinsed with PBS and incubated in streptavidin peroxidase solution (1 : 90, Biogenex) for 1 h at room temperature.

Following incubation, sections were washed several times in PBS. Finally, they were developed in diaminobenzidine – H_2O_2 (DAB) as the chromogen, washed

1a



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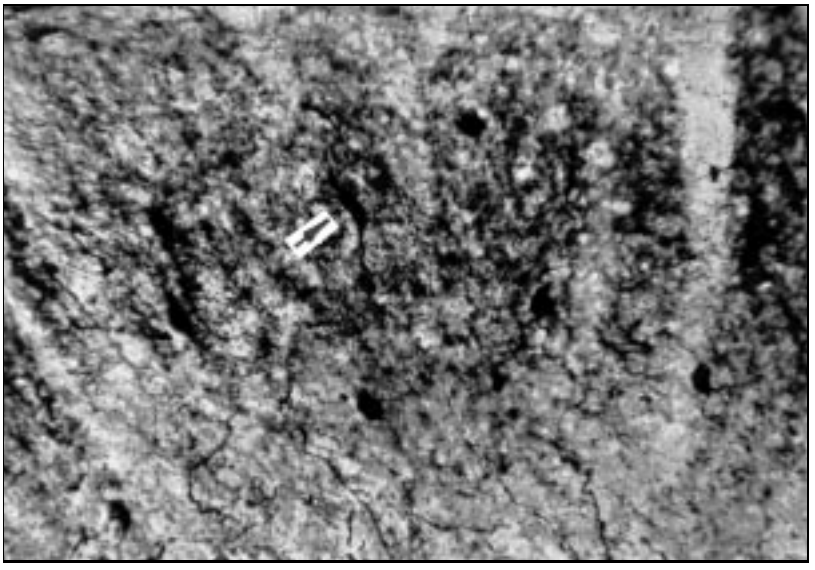
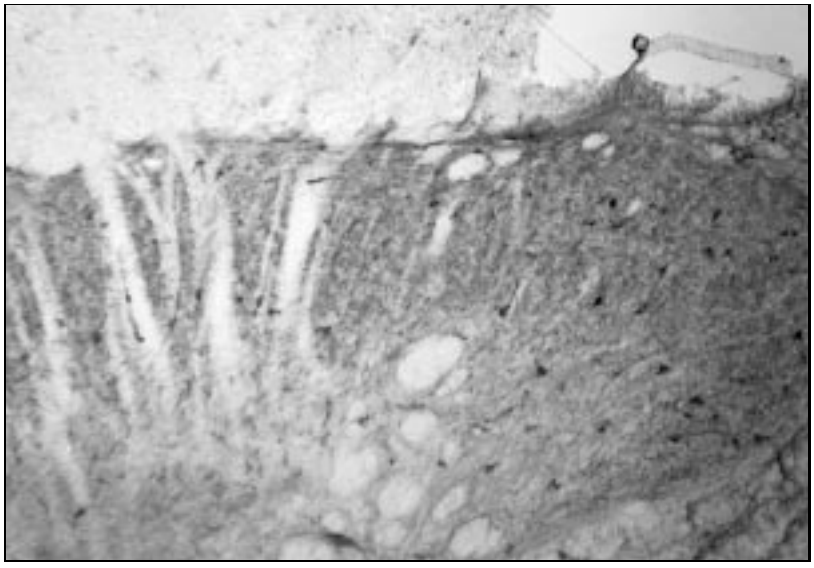


Figure 1a. View of NADPH-d positive neural structures in lamina I–III of the rabbit spinal cord. In its medial part (on the right side), the staining is interrupted by entering afferent fibers (× 63).
Figure 1b. More detailed investigation of superficial layers (lamina I–III) of the dorsal horn shows higher density of NADPH-d stained neuropil. Small spindle-shaped neurons (arrow) are hardly seen due to intensively stained background (× 160).

1c



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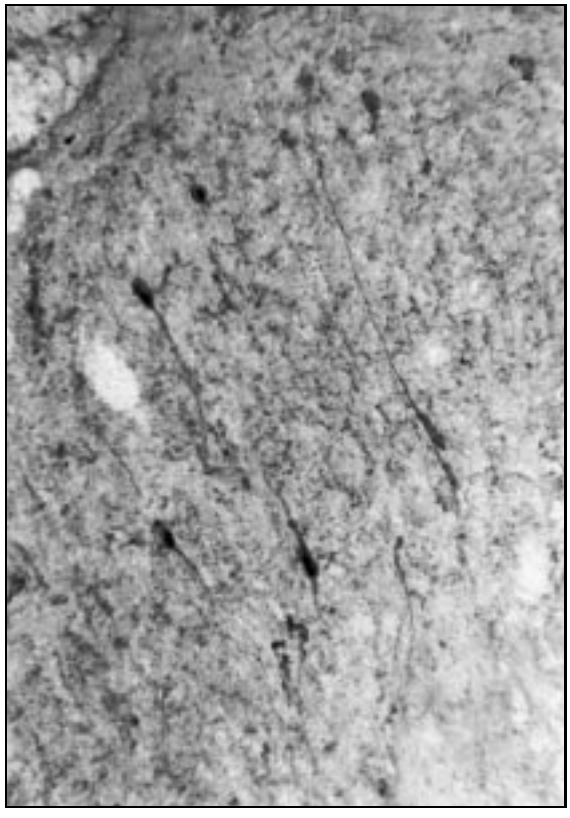


Figure 1c. Superficial dorsal horn of the rabbit lumbosacral spinal cord stained by using nNOS immunohistochemistry (× 63).
Figure 1d. Lighter background (neuropil) of lamina II enables better recognition of nNOS positive small spindle-shaped neurons in higher magnification (× 160).

in distilled water and mounted on the Colorfrost/Plus Microscope slides (Fisher Scientific, USA), air-dried and coverslipped with Entellan.

On few sections the primary antibody bNOS was omitted from the staining procedures, so that no nNOS positive cells were detected.

Results

The examination of nNOS and NADPH-d positive neurons in transverse sections of lumbosacral spinal cord revealed differences both in their laminar and segmental distribution and in morphology of their bodies and processes. Groups of neurons were evaluated according to their morphological and functional properties as well as their location in transversely sectioned tissue (Rexed 1954).

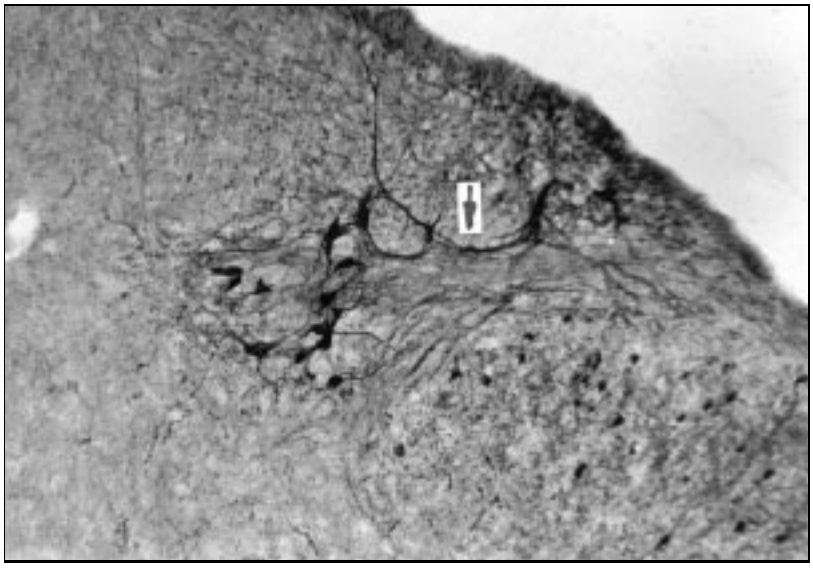
In the dorsal horn, its superficial part (laminae I–III) was clearly stained both for NADPH-d and for nNOS. Its lumbosacral part (L5-S1) showed dark blue NADPH-d staining which was interrupted mostly in the medial part of the dorsal horn (Fig. 1a). Detailed investigation revealed a high accumulation of punctate nonsomatic NADPH-d positivity and fiberlike NADPH-d staining (Fig. 1b). Small round or bipolar cells were intensely stained and their location was more frequently seen in the lateral portion of the dorsal horn. The distribution of nNOS immunoreactivity in this region was similar (Fig. 1c), colour of the reactive product was yellow. Detailed inspection of this region showed much lighter nNOS positive reaction of neuropil so that neurons were seen more in contrast (Fig. 1d). Their bodies were mostly of bipolar morphology with clearly seen processes oriented ventrodorsally.

Laminae I–III in sacral segments of the spinal cord (S2-S4) were similar in the distribution when investigated both by the NADPH-d histochemistry (Fig. 2a) and nNOS immunohistochemistry (Fig. 2b). The most prominent difference was seen in the staining of the fibre bundle extending ventrally from lamina I along the lateral edge of the dorsal horn into the sacral parasympathetic nucleus (SPN). These fibers of lateral collateral pathway (LCP) were clearly detected by NADPH-d histochemistry (Fig. 2a) whereas nNOS immunohistochemistry showed no staining of this region (Fig. 2b).

Around the central canal (lamina X), there were located groupings of 4–8 neurons. Their processes could be clearly identified, they were extending mostly towards the intermediate zone and the anterior horn. Both NADPH-d activity (Fig. 3a) and nNOS immunoreactivity (Fig. 3b) were positive without any difference in the distribution of the nerve structures. It should be stressed that in case of presence of some vessels in the investigated section, nNOS immunohistochemistry showed only contours of a vessel while coloured nNOS staining was absent (Fig. 3b).

In lamina VII of the sacral spinal cord, there was a loose aggregation of NADPH-d (Fig. 2a) and nNOS positive cells (Fig. 2b) located at the gray-white margin of this lamina, well known as the SPN. Cells were mainly triangular in shape, loosely arranged in the net of positively stained fibers.

2a



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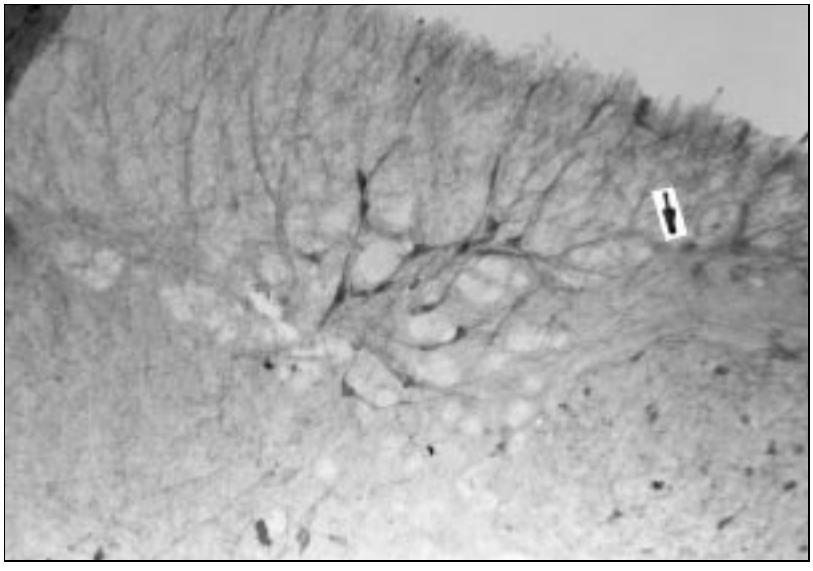
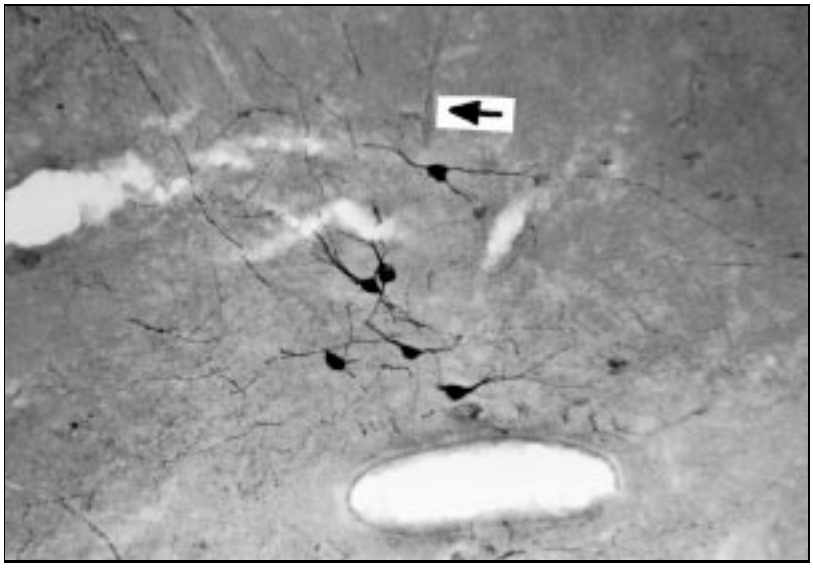


Figure 2a. Neurons and fibers of the SPN were clearly visible after using NADPH-d histochemical method. In comparison with nNOS staining the difference was seen in the fibre bundle (arrow) extending ventrally from lamina I along the lateral edge of the dorsal horn into the SPN. Fibers are known as lateral collateral pathway (LCP) (× 63).
Figure 2b. In sacral segments of the spinal cord (S2-S4) nNOS positive triangular cells of the sacral parasympathetic nucleus (SPN) are visible. However, connection between them and the superficial laminae of the dorsal horn – LCP, can not be seen (arrow) (× 63).

3a



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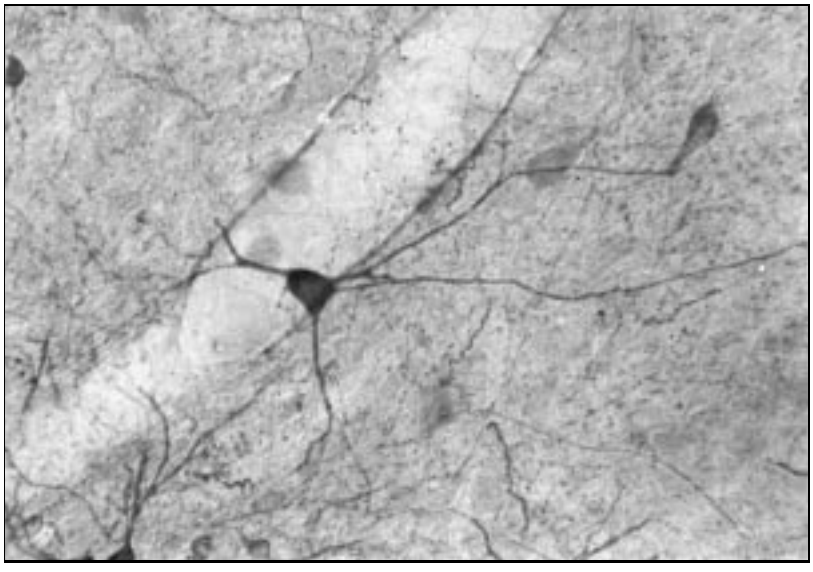

Figure 3a. NADPH-d positive nerve cells and vessels (arrow) in the intermediate zone (× 63).
Figure 3b. nNOS positive nerve cell in the intermediate zone in contrast with nNOS negative vessel behind it confirms the specifity of nNOS antibody (× 160).

The region of ventral horn remained unstained after using both NADPH-d histochemical and nNOS immunohistochemical methods.

Discussion

The aim of the present study was to compare and describe the distribution of NADPH-d activity and nNOS immunoreactivity in the rabbit spinal cord. Experiments have revealed similar distributions in many regions of gray and white matter in lumbosacral part of the spinal cord. However, some differences have been found.

There was a prominent NADPH-d positive staining in laminae I–III. This region demonstrated in addition to intensively stained small, spindle-shaped neurons also the most dense accumulation of the reaction product which had a punctate non-somatic appearance, presumed to be of synaptic origin and representing axonal terminals and dendritic spines (Saito et al. 1994). The examination of nNOS immunoreactivity showed surprisingly lighter staining of this neuropil although neurons of the same morphology were clearly seen. According to Vizzard et al. (1994), primary afferent cells in the neuropil may make a considerable contribution to the dense plexus of nNOS/NADPH-d fibers in the superficial dorsal horn.

A major difference between NADPH-d and nNOS staining was observed in the fibers of the LCP on the lateral edge of the dorsal horn in the sacral spinal cord. This location and its selective segmental distribution was similar to the central projections of visceral afferents in the pelvic nerve labelled in previous experiments (Morgan et al. 1981) by axonal transport of horseradish peroxidase (HRP) and which designed the lateral collateral pathway – LCP. The presence of NADPH-d positivity and the absence of nNOS immunoreactivity in these fibers was observed in guinea pig (Doone et al. 1999) as well as in cat (Vizzard et al. 1994) and dog (Vizzard et al. 1997). This difference can be related to several factors some of which could be technical, such as (1) NOS protein in the afferent axons might be an isoform other than nNOS so that it could not be detected by this specific antibody (Dawson et al. 1998) or (2) the thresholds for detection of NADPH-d and nNOS staining are different (Doone et al. 1999). With regard to this possibility it should be noted that Dun et al. (1993) have described nNOS positive fibers in the lower lumbar spinal cord of the cat projecting along the lateral edge of the dorsal horn. In other experiments (Vizzard et al. 1994), a different batch of nNOS antibody was used than in the studies by Dun et al. (1993), and the former have not detected nNOS immunoreactivity in this region of the spinal cord. However, differences in processing or tissue preparation may have led to variations in the detection of neuronal elements with low levels of nNOS.

The mismatch between two histochemical markers (NADPH-d and nNOS) might also be related to a real biochemical difference. Other examinated regions both in the gray and white matter of the rabbit spinal cord showed the correlation between NADPH-d activity and nNOS immunoreactivity. Also catalytic nNOS activity was similar to nNOS immunoreactivity in the lateral edge of the dorsal horn (Lukáčová and Pavel 2000).

The detection of NOS using the NADPH-d technique was studied by many investigators (Matsumoto et al. 1993; Tracey et al. 1993; Norris et al. 1995; Spessert and Claassen 1998). In the central nervous system, nNOS and eNOS will reduce nitroblue tetrazolium to provide a rapid and convenient histochemical method to visualize NOS. NADPH-d staining is independent of NO formation by NOS as the formalin fixation of the tissue used prior to diaphorase staining blocks the NO-forming part of the enzyme activity, but leaves the ability to transfer electrons from NADPH to reduce nitroblue tetrazolium. In the central nervous system (CNS) there is a good correlation between sites of NADPH-d activity and nNOS, but only after the exposure of the tissue to a fixative (Matsumoto et al. 1993; Spessert and Layes 1994).

A number of enzymes other than NOS, including various haemoproteins and cytochrome oxidases, can also reduce tetrazoliums (Hope and Vincent 1989). Colocalization studies of Norris et al. (1994) suggest that diaphorase activity in fixed rat CNS tissue cannot be accounted for by such an enzyme activity. It was confirmed by identical pattern obtained from NADPH-d histochemistry and nNOS immunohistochemistry by the examination of the frog spinal cord (Munoz et al. 2000). Outside the CNS, diaphorase staining is a much less reliable marker for NOS due to the ability of many other enzymes to reduce nitroblue tetrazolium (Norris et al. 1995). In these circumstances the specifici of the antibody probe is required.

Acknowledgements. The experimental work has been supported by the VEGA Grant No. 2/7222/20. The authors wish to thank Miss Ivana Perunska and Mrs. Ruzena Weiserova for their expert technical assistance.

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Final version accepted: October 25, 2001