Densitometric patterns of NADPH diaphorase staining in the spinal cord of dog

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Segmental and laminar distribution of NADPHd activity was studied in the normal spinal cord of the dog and basic densitometric patterns of somatic, fiber-like and punctuate, non-somatic NADPHd staining were described in the gray and white matter. Prominent NADPHd activity was noted in the superficial and deep dorsal horn, pericentral region, intermediolateral cell column, Lissauer’s tract and in the vertical and horizontal limbs of the medial longitudinal bundle of the ventral column in the cervical and upper thoracic segments.

Key words: densitometry, NADPH diaphorase, spinal cord, dog.

Introduction

The use of nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) histochemistry alone or combined with the nitric oxide synthase immunoreactivity (NOS-IR) allowed for a morphologically distinct and topographically precise localization of small neuronal pools synthesizing, releasing and transporting NOS, an enzyme responsible for nitric oxide (NO) synthesis. The discrete loci, nuclei or solitary NOS-IR neurons have been identified not only in the cortex, brain stem and spinal cord, but also in the peripheral nervous system (Vincent & Johanson, 1983; Vincent et al., 1983a, 1983b; Aimi et al., 1991; Dawson et al., 1991; Hope et al., 1991; Valschanoff et al., 1992; Vincent & Kimura, 1992; Vizzard et al., 1993, 1994; Saito et al., 1994; Maršala et al., 1997, 1998, 1999; Lukáčová et al., 1999; Maršala & Jalč, 2000). At many sites the NADPHd staining and/or NOS immunoreactivity can achieve three different staining patterns, somatic, fiber-like and punctuate, non-somatic. NADPHd staining and NOS-IR occurring with various intensity in the cell bodies is described as somatic, whereas the staining showing the dendritic and axonal processes including collaterals is described as fiber-like. More or less dense punctuate, non-somatic neuropil NADPHd and NOS-IR staining, where most of the reaction product

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is dotted in appearance is described as punctuate, non-somatic NADPHd or NOS-IR positivity. However, varying ratio of somatic, fiber-like and punctuate NADPHd staining and NOS-IR can be seen in different regions of the brain stem and spinal cord (Marsala et al., 1998, 1999).

Immunocytochemistry of the neuronal nitric oxide synthase (nNOS) showed that the occurrence of this enzyme is almost completely homotopic with the localization of neurons stained for NADPHd (Dawson et al., 1991; Hope et al., 1991). Therefore, NADPHd histochemistry have been repeatedly used to demonstrate neuronal changes of NO synthesizing neurons under various experimental conditions (Sandell et al., 1986; Solodkin et al., 1992; Morton et al., 1993; Rebeck et al., 1993; Vizzard et al., 1993; Zhang et al., 1993; Kuo et al., 1994; Traub et al., 1994; Choi et al., 1996; Xu et al., 1998; Marsala & Jalic, 2000; Xu et al., 2000). Although NADPHd histochemistry is a relatively simple histochemical method, the results of somatic, fiber-like and punctuate, non-somatic NADPHd staining appear to be very sensitive to many experimental conditions, such as deafferentation, fixation of the material, incubation time (Sandell et al., 1986; Morton et al., 1993; Kuo et al., 1994), and temperature. More importantly, examining the NADPHd histochemical staining and NOS-IR and, more specifically, considering the sensitivity of somatic and neuropil staining considerable differences were disclosed in some regions, e.g., in the striatum. It was found that increasing the incubation time only increased the optical density of NADPHd staining in the neuropil and, in contrast, the number of NADPHd-exhibiting cells was relatively consistent across incubation times (Kuo et al., 1994).

In the present study an attempt was made to specify the differences of somatic, fiber-like, and punctuate NADPHd staining in the gray and white matter of the spinal cord in the normal dog, including different segments and layers using the densitometric analysis. Densitometric patterns of NADPHd positivity in the undamaged spinal cord may be helpful in experimental studies aimed at a causal interpretation of changes affecting the NOS-containing neuronal pools in various experimental and pathologic conditions.

Material and methods

Tissue sampling, sectioning, examination of sections and the performance of the densitometric analysis

Adult dogs (n = 6) of both sexes weighing 12-18 kg were used in this study. The animals were deeply anesthetized with pentobarbital (50 mg/kg, i.v.) and perfused transcardially with saline followed by freshly prepared 4% paraformaldehyde +0.1% glutaraldehyde buffered with 1M sodium phosphate, pH = 7.4. Following perfusion fixation, the spinal cords were carefully dissected out and stored in toto in the same fixative for 3-4 hours. After post fixation, the spinal cord was divided into cervical, thoracic, lumbar, sacral and coccygeal segments, and each segment was then secondarily divided into three small blocks comprising the upper, middle, and lower segmental levels, respectively. Specimens 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 (50 µm thick) were cut from all segments studied and processed for NADPH-d activity by using a modified histochemical procedure (Scherer-Singler et al., 1983) as follows: (1) The sections were incubated for 1 h at 37°C in a solution containing 1mg/mL of Nitroblue Tetrazolium (NBT, Sigma Chemicals, N-8760), 0.5mg/mL of β-nicotinamide adenine dinucleotide phosphate (NADPH, Sigma Chemicals, N-6300), 0.8% Triton X-100 dissolved in 0.1M phosphate buffer (pH = 8.0), and 1.25mg/mL monosodium malate (Malic acid, Sigma, M-1125). (2) Control sections were treated in the same solution but without NADPH, thus testing for endogenous reduction activity in the corresponding blue formazan product. (3) The sections were then rinsed in 0.1 M phosphate buffer (pH = 7.4), mounted on slides, air-dried overnight and coverslipped with Entellan. (4) Some sections were stained by the Nissl method to specify in more detail the laminar division of the spinal cord gray matter. A micrometer grid was used to determine the cell diameter of NADPH-d-exhibiting neurons.

The densitometric analysis was performed using transverse sections stained for NADPHd histochemistry. Precise loci identified in the gray and white matter on transverse sections were used for the assessment of the densitometric patterns in both compartments of the spinal cord (Fig. 1).

How the densitometric patterns depicting the NADPH diaphorase positivity should be read and interpreted?

Regional and laminar densitometric analysis of somatic, fiber-like and punctuate NADPHd staining was performed on a PC using the UTHSCSA Image Tool program allowing for a reliable detection of subtle changes in the differences of the optical density of NADPHd staining in various spinal cord segments and regions under physiological conditions. Basically, the densitogram displays the distribution of the gray values in the area-of-interest, e.g., the superficial dorsal horn, pericentral region of the spinal cord or some other spinal cord gray matter nuclei where NADPHd positive cell bodies, fibers and puncta may occur. Each densitogram is displayed as a two-dimensional graph. The values along the vertical y-axis represent the relative number of pixels as the smallest elements of an image that can be individually displayed, represented as 8-bit unsigned integers in monochrome (i.e. single value) palettes, ranging in values from 0 to 255. In
Fig. 1. Schematic drawing of a transverse section demonstrating the loci in the gray and white matter regions (open squares 1–20) used for the densitometric analysis. 1 – medial dorsal horn, laminae I-II; 2 – lateral dorsal horn, laminae I-II; 3 – medial dorsal horn, laminae III-IV; 4 – lateral dorsal horn, laminae III-IV; 5 – medial lamina VII; 6 – lateral lamina VII; 7 – medial ventral horn, lamina VIII; 8 – lateral ventral horn, lamina IX; 9 – pericentral region, lamina X; 10 – deep dorsal horn, laminae V-VI; intermediolateral cell column at Th7 segment level; 12 – intercalated nucleus at Th7 segment level; 13 – ventral column close to the ventral horn; 14 – lateral column; 15 – cuneate fascicle; 16 – superficial gracile fascicle; 17 – deep gracile fascicle; 18 – Lissauer’s tract; 19 – vertical limb of the medial longitudinal bundle; 20 – horizontal limb of the medial longitudinal bundle.

the gray scale palette a pixel value of 0 is displayed as black and 255 as white. Black, dark-blue and light-blue colour characteristics for neuronal NADPHd staining and several closely related intermediaries are transformed into a gray scale corresponding to black, or dark-gray, medium-gray and light-gray, respectively. In terms of the gray scale system which corresponds with the pixel depth, black is less than white and on most systems, the numeric value of pixels which are black is represented using a number which is less than that for white. Densitometric patterns resulting after NADPHd histochemical procedure of a dense accumulation of small intensely-stained neuronal cell bodies or large, multipolar NADPHd-exhibiting neurons monitored as large black areas are highly characteristic and, are commonly located close to 0 value or in the left third of the densitogram on the horizontal x-axis displaying a variable height of the pixel spikes depending on different extent of dark or dark-gray areas under study. Contrary to this, thick, short NADPHd positive dendritic shafts occurring solitary or as aggregated dark profiles can be found usually in the left third of the densitogram as tall, often repeated pixel spikes reaching up to the lower third of the y-axis. Densely packed punctuate, non-somatic NADPHd positivity appears in the gray scale palette of the densitogram in the left half with many spikes in the ascending branch of the curve, which is sloping down abruptly thus pointing to a massive reduction of medium and dark-gray puncta characteristic for NADPHd staining of axonal terminals and dendritic spines.

Results

Densitometric patterns of NADPH diaphorase activity in the gray matter of the spinal cord

Considerable laminar differences were found in regard to the densitometric patterns of NADPH diaphorase activity prepared from different layers (laminae I–VI and VII–X) of the spinal cord gray matter at L7 segment level. Large extent of the densitograms on both horizontal and vertical axes taken from medial (Fig. 2A), and lateral (Fig. 2B) portion of laminae I–II and medial portion (Fig. 2C) and, to a lesser extent of the lateral portion (Fig. 2D) of laminae III–IV point to a high NADPH diaphorase activity in the superficial portion of the dorsal horn. Contrary to this, greatly reduced NADPH diaphorase activity was noted in the intermediate zone (lamina VII) and, moreover, demonstrating noticeable differences between medial and lateral part of lamina VII. With regard to the latter, the main peak of NADPHd activity depicted in the midfield of the densitogram (Fig. 2F, LAT-arrowhead) is preceded in the former by a burst of short repeated spikes (Fig. 2E, MED-arrow) considered to be a densitometric expression of thick fibres, perhaps dendrites highly positive for NADPHd staining currently seen in the medial portion of the intermediate zone. A slightly broadened base of the densitogram taken from lamina VIII of the ventral horn may reflect the occurrence of NADPHd-exhibiting somata seen in this layer all along the lumbar and cervical enlargement (Fig. 2G).

Considering the shape and extent of the densitogram taken from motoneuronal pool in the ventral horn (lamina IX) NADPH diaphorase activity seems to be low and comparable with that found in certain regions of the spinal cord white matter (Fig. 2H). However, some loci in the ventral horn having a close relation with large motoneurons and occurring in the ventrolateral motor group appeared to be more intensely stained for NADPHd; their identity should be tested using an experimental approach, e.g., after dorsal and ventral rhizotomy. A comparatively high NADPH diaphorase activity was detected in the pericentral region (lamina X) where, except small and large NADPHd-exhibiting cell bodies many NADPHd positive fibers and numer-
Fig. 2. Densitometric patterns of NADPHd staining in the medial (A), lateral (B) superficial dorsal horn (laminae I-II) and in the medial (C) and lateral (D) parts of laminae III-IV. In the densitogram from the medial portion of lamina VII (E) arrow points to short spikes corresponding with highly positive NADPHd staining and high spikes (arrowhead) in the lateral part (F) of lamina VII corresponds with NADPHd-exhibiting somata. A low level of NADPHd staining was noted in the medial ventral horn-lamina VIII (G) and in the lamina IX (H). In the pericentral region in lamina X (I) many NADPHd positive dendrites (arrowhead) considerably influence NADPHd positivity of this field.

ous NADPHd stained puncta seen in the neuropil could be found. Both these components, i.e. the fibers and puncta are enclosed within the left half of the densitogram (Fig. 2I – arrowhead).

Large, multipolar intensely-stained, but more
or less loosely arranged NADPHd-exhibiting neurons together with sparsely disseminated highly NADPHd-positive puncta in the neuropil of laminae V-VI clearly modulate the extent of the base and a stepwise appearance of the ascending branch of the densitogram (Fig. 3A-arrowhead). Similarly, a dense accumulation of NADPHd-positive puncta and dendritic fragments occurring in the neuropil of the intermediolateral (Fig. 3B-arrowhead) and intercalate (Fig. 3C-arrowhead) nuclei, both forming a part of the intraspinal thoraco-lumbar sympathetic chain, strongly elongate the base and influence the densitometric pattern.

Densitometric patterns of NADPH diaphorase activity in the white matter of the spinal cord

Densitometric patterns of NADPH diaphorase activity prepared from the white matter of the dorsal, lateral and ventral columns and taken at a precisely identified segment level demonstrated an almost homogenous appearance with a comparatively low or mildly expressed NADPHd positivity which was strictly bound to the light microscopically verified axonal profiles (Fig. 4A, B, C, D and E). However, considerably modified densitometric patterns were obtained from those portions of the dorsal and dorsolateral regions of the lateral column packed with many thin NADPHd positive axons occurring in the extent of the lumbosacral and cervical enlargement, a finding broadening the base and extent of the densitograms taken from the Lissauer’s tract (Fig. 4F). Similarly, a high NADPHd positivity can be deduced from densitograms taken from the ventral column at the cervical and upper thoracic level and depicting the position of the vertical limb of the medial longitudinal bundle at C4 segment level (Fig. 5A MLB, VL) and horizontal limb of the medial longitudinal bundle at C6 segment level (Fig. 5B MLB, HL). Both limbs contained a high concentration of thick NADPHd-exhibiting axons.

Discussion

It is generally accepted that the histochemical staining for NADPH diaphorase can be used to visualize a distinct population of central and peripheral neurons (Vincent et al., 1983a, 1983b; Vincent & Kimura 1992). Recently, it has been demonstrated that NADPHd staining corresponds almost completely with the immunocytochemical staining of neurons containing neuronal NOS (Dawson et al., 1991; Hope et al., 1991; Lukácsová et al., 1999) and NOS is the synthetic enzyme which converts arginine to nitric oxide (NO). Since the time NADPHd histochemistry was introduced into the neurobiology and neuromorphology an enormous interest has been noted examining the alterations of NADPHd staining under various experimental conditions with different neuronal populations in both central and peripheral nervous system (Bredt & Snyder 1990; Snyder 1991), including neuronal pathways participating in the pain stimuli processing and their termination in the spinal cord (Sprke et al., 1993; Zhang et al., 1993; Steel et al., 1994; Choi et al., 1996; Orendáčová et al., 2000, 2001). Moreover, the feasibility of NADPHd histochemistry as
Fig. 4. Densitometric patterns of NADPHd activity found in the white matter of the ventral (A) and lateral (B) column at L7 level, cuneate fascicle (C) at C7 level, superficial gracile fascicle (D) at C7 level, deep gracile fascicle (E) at C7 level and in the Lissauer's tract (F) at S1 level.

A useful technique for examining different neuronal populations provides an unusually simple and precise method to localize NOS-containing neurons and their possible changes in various experimental paradigms (Mařála et al., 1997, 1998, 1999; Mařála & Jalč, 2000).

Basically, three different types of images related with NADPHd neuronal positivity can appear after an accurately performed NADPHd histochemical staining. First, somatic NADPHd positivity usually seen as a dark, dark-blue or light-blue cytoplasmic staining leaving a centrally localized light ring of unstained nucleus thus allowing for a characterization of the cell body size and shape. Using these criteria NADPHd-exhibiting neurons can be categorized into regionally different types (Valschanoff et al., 1992; Mařála et al., 1999). Second, a fiber-like NADPHd positivity depicting variously shaped short and long, or thin and thick dendrites and axons and, here again, a tremendous mixture of NADPHd dendritic and axonal staining can be identified. Third, a punctuate, non-somatic NADPHd staining occurring in the neuropil of various gray matter regions appears mostly as a densely-packed accumulation of small, intensely-stained particles, 0.5–1.5 μm in diameter, sometimes obscuring the outlines of the neuronal cell bodies and fibers.

While all three components of NADPHd staining are easily distinguishable by light microscope no reliable method as yet exists allowing for detection the ratio between somatic, fiber-like and punctuate NADPHd positivity in general, and, for specifying the quantitative changes of NAD-
PHd histochemistry under different experimental conditions, in particular. In an attempt to detect a possible correlation between neuron counts and optical density of NADPH diaphorase histochemistry a quantitative study was undertaken using an automated densitometry (RAS system, Amersham) and the influence of incubation time, fixation time, and temperature to further characterize striatal NADPHd histochemistry with regard to the neuron counts and optical density was studied (Kuo et al., 1994). The term “optical density” was used in this study to express an overall NADPHd staining of the neuropil comprising both fiber-like and punctuate, non-somatic NADPHd positivity as specified before. It should be noted that the quantification based on the above mentioned approach is an indirect one, using the optical density of the optic tract as a reference value. Since neither neurons nor fibers in the optic tract stained with NADPHd, the optical densities of the optic tract were measured and used to calibrate the staining intensities. The calibrated striatal densities were calculated as the optical densities of the striatum divided by that of the optic tract (Kuo et al., 1994).

The results of the densitometric analyses of NADPHd staining demonstrated in our study are easily applicable to similar NOS immunocytochemical visualization using the UTHSCSA Image Tool program and confirmed a high degree of exactness seen in the quantitative assessment of the punctuate, non-somatic NADPHd staining occurring in the neuropil. In connection with this finding a short comment seems to be needed. While the somatic NADPHd staining makes the neuronal cell bodies readily identifiable and quantifiable in each gray matter region (Aimi et al., 1991; Vizzard et al., 1994; Marsala et al., 1997), an extremely high density of NADPHd-exhibiting punctuate, non-somatic NADPHd staining occurring in the neuropil of some brain regions is hardly to be quantitatively assessed at light microscopic level. Densitometric quantitative assessment of punctuate, non-somatic NADPHd positivity as performed in the present study may be used as a valuable tool for studying, e.g., the dynamic changes of terminal portions and presynaptic boutons of C-polymodal afferent fibers in the superficial dorsal horn i.e., in the region where the dorsal and ventral rhizotomies failed to demonstrate any significant changes in the density of punctuate NADPHd staining or, as seen in other experimental paradigms, only quite inconclusive results could be detected (Vizzard et al., 1993; Zhang et al., 1993). Similarly, the densitometric analysis as described above can be applied in the tract-tracing studies characterizing the density of somatic NADPHd staining or NOS immunopositivity of retrogradely reacting NOS-containing cell bodies after axotomy performed proximally or distally from the cell body (Xu et al., 2000). Both modalities of a purposeful application of the UTHSCSA Image Tool program are the object of a detailed experimental study in our laboratory aimed at explaining the basic characteristics of NOS-containing neurons participating in the formation of short neuronal microcircuits in the spinal cord dorsal horn or forming a part of the long projection ascending spinal cord connections.
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References


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