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Catalytic Nitric Oxide Synthase Activity in the White and Gray Matter Regions of the Spinal Cord of Rabbits

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Summary

The latest research reveals that nitric oxide as a gas messenger may diffuse into the surrounding extracellular fluid and act locally upon neighboring target cells. However, several observations raise the possibility that nitric oxide may also be released at a greater distance from the neuronal cell body. The catalytic nitric oxide synthase (cNOS) activity was therefore studied in the cervicothoracic and lumbosacral segments of the spinal cord of rabbits, including the white matter of dorsal columns (DC), lateral columns (LC) and ventral columns (VC), as well as the gray matter of dorsal horns (DH), intermediate zone (IZ) and ventral horns (VH). Lower cNOS activity was found in the white matter of both cervicothoracic (47 %) and lumbosacral (30 %) regions, whereas that detected in the gray matter of the lumbosacral part of the spinal cord was considerably higher (70 %). Enzyme activity varied from 43.4 to 77.2 dpm/μg protein in the cervicothoracic segments of the gray matter in the descending order: DH>VH>IZ. Similar cNOS activity was found in the white matter of the cervicothoracic segments (42.1-62.8 dpm/μg protein). When the activity of cNOS was compared in the lumbosacral segments, the highest enzyme activity was found in DH of the gray matter (198.7 dpm/μg protein) and the lowest cNOS in DC (45.8 dpm/μg protein) of the white matter. It was concluded that the white matter of the spinal cord contains similar cNOS activity in comparison to the gray matter.

Key words
cNOS activity • Spinal cord segments • White and gray matter regions • Rabbit

Introduction

The latest research on the multifarious actions of nitric oxide synthase (NOS)-containing neurons and on the role of nitric oxide (NO) in neuronal transmission has revealed that according to morphological and functional properties of NOS-containing neurons, NO as a product of these cells and as a gas messenger acting on the principle of a unicellular paracrine gland may diffuse into the surrounding extracellular fluid and act locally upon neighboring target cells, e.g. on smooth muscle cells in the vessels wall, or on neighboring neurons, thus modifying the activity of neuronal microcircuits (Iadecola *et al.* 1993). However, several observations have raised the possibility that NO may also be released at a greater distance from the neuronal cell body (Lee *et al.* 1993, Miyazaki *et al.* 1996) and therefore, anterograde axoplasmic transport of NOS along with other synaptically acting enzymes and neurotransmitters may serve as a mechanism allowing for remote synaptic activity of NO.

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In this connection, anterograde tracing combined with immunocytochemistry and immunoelectron microscopy disclosed that long vagal afferents to the nucleus tractus solitarii contain brain nitric oxide synthase (Lin *et al.* 1998). Therefore, the existence of NOS-containing presynaptic boutons and release of NO implicates that the rate of anterograde axoplasmic transport in NOS-containing long ascending and descending nervous pathways in the brain stem and spinal cord may be of primary importance. While a detailed distribution of NADPHd/NOS-containing neurons in the gray matter of the spinal cord, brain stem and cortex including some comparative and developmental aspects has been elaborated (Bredt *et al.* 1991, Saito *et al.* 1994, Dun *et al.* 1993, Valtschanoff *et al.* 1992, Vincent and Kimura 1992, Egberongbe *et al.* 1994, Burnett *et al.* 1995, Vizzard *et al.* 1994, Vincent 1994, Maršala *et al.* 1997, 1998, 1999, Lukáčová *et al.* 1999), studies describing the distribution of NADPHd/NOS positivity in the white matter in general and the occurrence of axonal NADPHd/NOS positivity in well-characterized compartments of the white matter, e.g. capsula interna, corpus callosum, crura cerebri, pyramis medullae oblongatae and white columns of the spinal cord are scanty and inconclusive. With regard to the latter, occasional NADPHd-exhibiting neurons with rostrocaudally elongated dendrites traceable for several millimeters were found in the dorsal funiculus at all spinal cord levels and a few NADPHd-exhibiting neurons with similar characteristics were found in the lateral and ventral funiculi as well (Valtschanoff *et al.* 1992). However, how these neurons contribute to the axonal NADPHd staining seen in the white matter in all planes is as yet not settled. Similarly, the origin of a more dense axonal NADPHd positivity localized in the ventral part of the dorsal funiculus and sparcer in the lateral and ventral funiculi (Valtschanoff *et al.* 1992) and NOS-fibers forming strand-like tracts traced in the dorsal, ventral and lateral spinal cord white matter and apparently terminating in adjacent gray matter regions (Dun *et al.* 1993) needs a more detailed scrutiny.

Considerable region-dependent differences in the NOS activity were encountered in the gray matter of the rabbit spinal cord, demonstrating a high level of NOS activity in lower lumbar and sacral segments compared with lower cervical and upper thoracic segments (Lukáčová *et al.* 1999) under normal conditions. However, it remains to be clarified whether similar region- or segment-dependent differences in the NOS activity occur in the lateral, ventral and dorsal funiculi and, if so, whether such differences might imply considering the deleterious effect of NO on white versus gray matter during ischemia and reperfusion. It is well established that white matter of the CNS, although more resistant than gray matter, is also irreversibly damaged by anoxia/ischemia and, as has recently been shown by *in vitro* experiments using optic nerve as a representative central white matter tract, the activation of Na^+ channels plays a critical role in anoxia/ischemia-induced axonal damage (Stys 1996).

In the present work, we have used cervicothoracic (C6-Th2) and lumbosacral (L5-S3) segments of the rabbit spinal cord in an attempt to quantify the NOS activity under physiological conditions in three white matter regions, and to compare this enzyme activity with that in different gray matter regions.

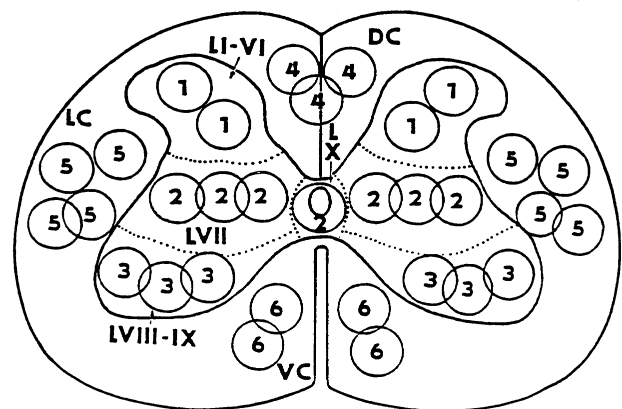

Fig. 1. *The specimens from 600 μm thick transverse sections of the cervicothoracic (C6-Th2) and lumbosacral (L5-S3) segments were punched by needles (id 0.6 or 0.8 mm) on a plate cooled in liquid nitrogen (–15 °C). The punches were done separately from the laminae I-VI (dorsal horns) – (no. 1), laminae VII and X (intermediate zone) – (no. 2), laminae VIII-IX (ventral horns) – (no. 3), dorsal columns (DC) – (no. 4), lateral columns (LC) – (no. 5) and ventral columns (VC) – (no. 6). Punches numbered 1-6 were taken separately from spinal cord segments, pooled into a single sample and used for biochemical analysis.*

Methods

Five male rabbits, weighing 2.5-3.5 kg were used in the experiment. The animals were anesthetized with thiopental (30 mg/kg, i.v.), the backbone was excised and the spinal cord was quickly excised *in toto*

and put into ice-cold isotonic saline. The spinal cord was cleaned from its envelopes, carefully frozen in liquid nitrogen and then cut into C6-Th2 and L5-S3 segments. For regional distribution of cNOS activity, the spinal cord segments were cut on a cryostat at –12 °C into 600 μm slices and gray matter regions: laminae I-VI (dorsal horns), laminae VII and X (intermediate zone), laminae VIII-IX (ventral horns), and white matter regions: dorsal, lateral and ventral columns (Fig. 1) were punched by needles (id 0.6 or 0.8 mm) on a plate cooled in liquid nitrogen (–15 °C) from spinal cord segments.

NOS radioassay

Catalytic NOS activity was determined by the conversion of [^{3}H] arginine to [^{3}H] citrulline according to the method of Bredt and Snyder (1990) with slight modification by Strosznajder and Chalimoniuk (1996). Frozen spinal cord samples were homogenized in 100-150 μl of an ice-cold Tris-HCl buffer (10 mM, pH=7.4). Aliquots of the homogenates (200 μg/ml) were incubated for 45 min (37 °C) with 10 μM [^{3}H] L-arginine (1 μCi), 1 mM NADPH, 1 μM calmodulin in HEPES buffer (50 mM, pH=7.4) containing 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 100 μM flavin mononucleotide (FMN), 100 μM flavin adenine dinucleotide (FAD), 2 mM $CaCl_2$, 15 μM tetrahydrobiopterin (H_4B) in a final volume of 300 μl. The reaction was stopped by an addition of 1 ml of ice-cold Hepes buffer (100 mM, pH =5.5) containing 10 mM EDTA. Samples were applied to a Dowex AG 50W-X8 cationic-exchange column (Na^+ form) to remove the [^{3}H]-L-arginine. Columns were washed with 2 ml of deionized water to elute the [^{3}H]-citrulline. Samples were centrifuged at 1 000 xg for 5 min and aliquots (0.5 ml) of supernatant fractions were mixed with 5 ml of Bray's fluid into scintillation vials and then counted in the Beckman LS-3801 spectrometer. Cpms were converted to dpms using [^{3}H]-quenched standards. Levels of [^{3}H]-citrulline were computed after subtracting the blank which represented nonspecific radioactivity in the absence of enzyme activity. Protein determination was done using a Bradford assay (1976). Results from analyses were expressed as dpm/μg protein.

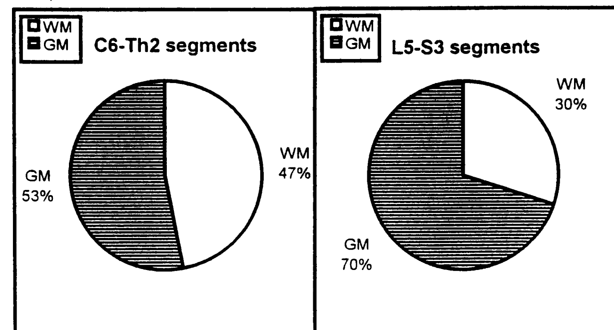

Fig. 2. *The percentage distribution of cNOS activity in the gray matter and white matter of the cervicothoracic (C6-Th2) and lumbosacral (L5-S3) segments of the rabbit spinal cord. The values of cNOS activity in the gray matter regions (dorsal horns, intermediate zone and ventral horns), and in the white matter regions (dorsal, lateral and ventral columns), which were summed up separately in the cervicothoracic and lumbosacral part of the spinal cord, were taken as 100 %. Data are means of 5 experiments ± S.E.M. The results are expressed as dpm/μg protein.*

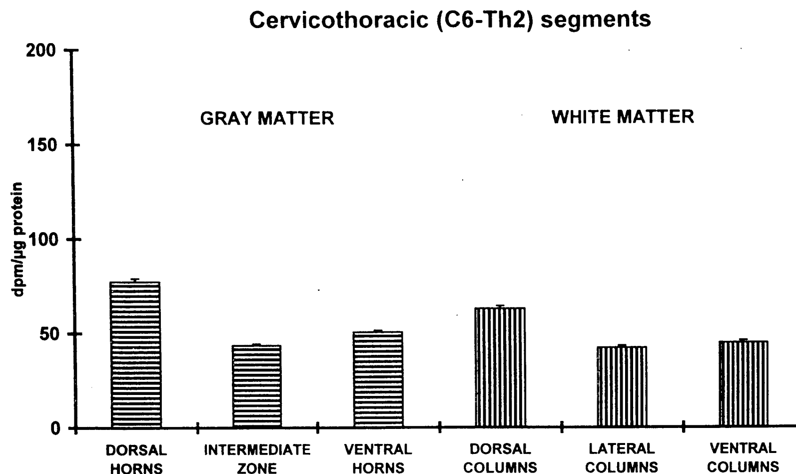

Fig. 3. *The cNOS activity in the cervicothoracic (C6-Th2) segments of the rabbit spinal cord divided into three gray matter regions (dorsal horns, intermediate zone, ventral horns) and into three white matter regions (dorsal, lateral and ventral columns). Data are means of 5 experiments ± S.E.M. The results are expressed as dpm/μg protein.*

Results

The cNOS activity studied in the gray and white matter regions of cervicothoracic and lumbosacral segments of the rabbits spinal cord point to considerable quantitative differences (Fig. 2). Lower cNOS activity was found in the white matter of both cervicothoracic (47 %) and lumbosacral (30 %) segments. In the white matter of cervicothoracic segments, the activity of cNOS closely resembled that detected in the gray matter, whereas that detected in the gray matter of lumbosacral segments was considerably higher (70 %).

Enzyme activity varied from 43.4 to 77.2 dpm/μg protein in the cervicothoracic segments of the gray matter in the descending order: DH>VH>IZ (Fig. 3). Similar cNOS activity was found in the white matter (42.1 to 62.8 dpm/μg protein). It is known that a long-term inhibition of NOS activity causes spinal cord infarction at the cervicothoracic level followed by forelimb motor dysfunctions (Blot *et al.* 1994). It seems possible, that low NOS activity in these spinal cord regions was the primary site in which the effect of NOS inhibition was apparent.

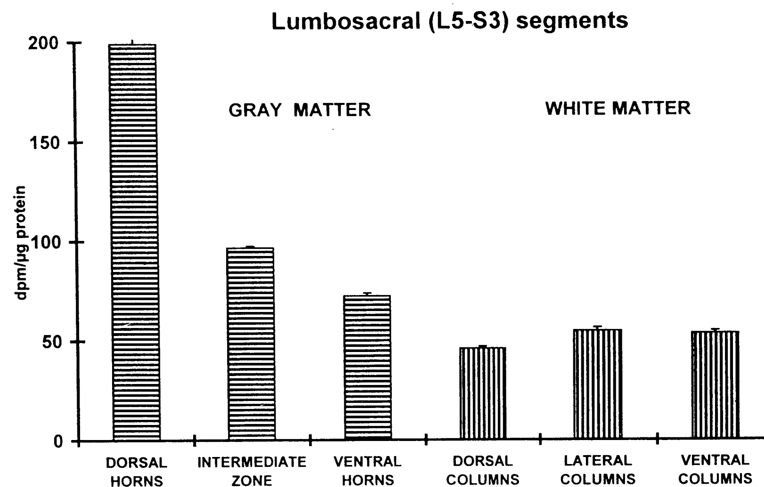

Fig. 4 . *The cNOS activity in the lumbosacral (L5-S3) segments of the rabbit spinal cord divided into three gray matter regions (dorsal horns, intermediate zone, ventral horns) and into three white matter regions (dorsal, lateral and ventral columns). Data are means of 5 experiments ± S.E.M. The results are expressed as dpm/μg protein.*

When comparing the activity of cNOS in the lumbosacral regions of the spinal cord, the highest conversion rate of radioactive [^{3}H] citrulline from [^{3}H]-arginine was found in DH of the gray matter (198.7 dpm/μg protein) and in LC of the white matter (54.6 dpm/μg protein) (Fig. 4). In contrast, the lowest cNOS activity was shown in VH (72.3 dpm/μg protein) and in DC (45.8 dpm/μg protein) of the lumbosacral gray and white matter.

It was concluded that enzyme activity, detected by radioassay, clearly prevails in the lumbosacral segments, and, that un equal distribution in enzyme activity within a gray and white matter regions does really exist. Moreover, our study clearly shows that three of the above mentioned white matter columns contain comparatively high levels of cNOS activity as compared with the gray matter regions.

Discussion

In the present study, we have demonstrated that the level of the catalytic activity of NOS under physiological conditions, assessed separately in the white and gray matter in the lower lumbar and sacral segments, significantly differs from that found in the lower cervical and upper thoracic segments. With regard to the latter, the cNOS activity in the gray matter, taken as a whole, closely resembles that detected in the three white matter columns. On the other hand, the cNOS activity assessed in the gray matter versus white matter of L5-S3 segments clearly prevails and strongly indicates a high quantitative distribution of NO synthesizing NADPHd-exhibiting and/or NOS immunoreactive neurons detected in the lower lumbar and sacral segments (Maršala *et al.* 1999, Lukáčová *et al.* 1999). It should be noted, however, that

intrinsic spinal NADPHd-exhibiting and/or NOS immunoreactive perikarya and fibers are the primary source supplementing to catalytic NOS activity. Furthermore, an extrinsic source coming *via* NADPHd/NOS positive dorsal root afferents and terminating in the dorsal horn in lower lumbar and sacral segments may provide a second potential mechanism by which the dorsal root ganglia neurons could contribute to a higher catalytic NOS activity in the dorsal horns of lumbosacral segments. This assumption is strongly supported by experiments examining the effect of unilateral transection of the L6-S1 dorsal and ventral roots resulting in a complete loss of NADPHd staining in the Lissauer tract and lateral collateral pathway in these segments (Vizzard *et al.* 1993). The above findings confirm the contention that cNOS activity in the spinal cord not only has a differential segmental distribution but also exhibits considerable regional differences of NOS activity in the white matter columns across various spinal cord regions. This observation concerns the cervical and lumbar enlargements, thus broadening the scope of the regional differences in cerebral cNOS activity, a phenomenon which may, in part, be responsible for the differences seen in the degree of ischemia-induced vulnerability and subsequent nervous tissue injury (Ashwal *et al.* 1998, Bredt 1995,Kuppusamy *et al.* 1995, Salter *et al.* 1995, Lukáčová *et al.* 1998).

It is widely accepted that the nitric oxide which is continuously synthesized and released from endothelial cells may act as a mediator providing a basal vasodilator tone (Faraci 1990), influencing the calcium transport systems in the myocardium (Zahradníková and Križanová 1997). However, it seems possible that the NO influencing basal tone in cerebral and spinal cord vessels is not only of endothelial origin but may also be derived from neurons and the glia (Toda *et al.* 1993). The close relation of NADPHd-exhibiting cell bodies and fibers with the capillary network and the vessel wall of the brain and spinal cord vasculature supports this view (Iadecola *et al.* 1993, Maršala *et al.* 1998, 1999). Finally, this assumption is supported by several studies using staining for NADPH diaphorase, suggesting that nNOS is present in the axon terminals and dendrites closely associated with the wall of microvessels in the brain parenchyma (DeFelipe 1993, Estrada *et al.* 1993, Regidor *et al.* 1993). A recent quantitative assessment of NOS catalytic activity (Lukáčová *et al.* 1999), supported by a qualitative histochemical NADPH diaphorase and immunocytochemical study comprising the gray matter of several spinal cord segments in the rabbit, and further confirmed by a radioassay analysis of the white matter of both spinal cord enlargements in present study, may help to understand the pathophysiological mechanisms leading to a highly characteristic damage at lower cervical and upper thoracic spinal cord levels appearing after a long-term inhibition of NOS activity.

Since it is known that a long-term systemic inhibition of NOS activity causes highly prominent spinal cord infarcts at the cervical and cervicothoracic level followed by forelimb motor dysfunctions (Blot *et al.* 1994), it seems possible that the spinal cord regions (which are poorly endowed with NO synthesizing neurons enriched by a comparatively high number of NOS transporting axons and in which a comparatively low cNOS activity takes place), may be the primary site in which the effects of NOS inhibition is apparent.

The principal finding of the present study is that three spinal cord white matter columns studied at lumbosacral and more prominently at the cervicothoracic level contain a high level of NOS catalytic activity. This finding is notable because the spinal cord white matter is known to be poor in NADPHd-exhibiting and/or NOS immunoreactive cell bodies, and, therefore, the catalytic activity of the white matter may be almost completely derived from short and long axons emerging from NADPHd-exhibiting perikarya belonging to long ascending or descending neuronal pathways (Lee *et al.* 1993). The participation of the propriospinal NADPHd-positive fibers adding to the NOS activity is highly probable (Maršala, personal communication).

Sporadic notes describing the white matter NADPHd positivity and NOS fiber immunoreactivity have appeared in different animal species (Valtschanoff *et al.* 1992, Dun *et al.* 1993). Some of them include the description of NADPHd positive fibers, perhaps axons in all planes of sectioning, traceable in longitudinal planes along the whole length of the section. The NADPHd-positive fibers were found to be dense in the ventral part of the dorsal funiculus and sparser in lateral and ventral funiculi (Valtschanoff *et al.* 1992). Similarly, NOS immunoreactive fibers forming strand-like tracts could be traced in the dorsal, ventral and lateral white matter. They were most numerous in the thoracic and lower lumbar/sacral segments, where they seemed to terminate in adjacent gray matter regions (Dun *et al.* 1993). It should be noted that these descriptions are based purely on histochemical and immunocytochemical analyses. In summary, our present study clearly defines NOS activity in the white matter of both spinal cord enlargements.

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Reprint requests

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