Ultrastructural features and synaptic connections of NADPH-Diaphorase positive neurones in the rat spinal dorsal horn and central grey matter

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SUMMARY

Nitric oxide (NO) is a recently identified neuronal messenger within the central and peripheral nervous system. NO is synthesized by specific populations of spinal neurones, and is implicated in nociception and hyperalgesia. We have studied here the ultrastructural features and synaptic connections of NADPH-diaphorase positive neurones in the superficial dorsal horn and lamina X, which are the main sites of processing for somatic and visceral nociceptive information relayed to the cord. Labelled neurones in the dorsal horn were islet cells with myelinated axons, and thus likely corresponded to projection neurones. Their dendrites were usually post-synaptic to unlabelled axons or, at times, appeared as peripheral elements in type II glomeruli. In lamina X, labelled neurones were of the multipolar type. NADPH-diaphorase positive processes included myelinated and small unmyelinated axons and dendrites of varying size. Therefore lamina X receives a direct axonal input from local and/or supraspinal neurones producing NO. These results provide morphological evidence that the synaptology of NO producing neurones varies in different areas of the spinal cord involved in pain processing.

Key words: Spinal cord - Nitric oxide - Pain - Ultrastructure

INTRODUCTION

The superficial dorsal horn (laminae I-II) and the central grey region (lamina X) of the spinal cord are involved in somatosensation, particularly nociception (Honda , 1985; Honda and Perl, 1985; Light, 1992; Meller and Gebhart, 1994; Nahin et al., 1983, 1984; Willis and Coggeshall, 1991).

Lamina II (the substantia gelatinosa of Rolando) is the major site of integration of nociceptive afferent input to the dorsal horn. Primary afferent fibers involved in nociception contain and likely release a number of biologically active small molecules among which are the transmitter amino acids L-glutamate and L-aspartate (De Biasi and Rustioni, 1988; Maxwell et al., 1990; Merighi et al., 1991; Ueda et al., 1994; Valtschanoff et al., 1994) and numerous peptides, including the calcitonin gene-related peptide (CGRP) and substance P (Cuello et al., 1993; De Biasi and Rustioni, 1988; Merighi et al., 1991; Ribeiro-Da-Silva, 1995). Many of these substances together with several other transmitters/modulators of nociception and/or analgesia have also been immunohistochemically localized in axons and terminals of lamina X (Barber et al., 1979; Honda and Lee, 1985; La Motte et al., 1982). More direct implication of this area in pain perception has been provided by the demonstration that thinly myelinated primary afferent fibers from both cutaneous and subcutaneous nociceptors terminate in the grey matter surrounding and immediately dorsal to the central canal (Light and Perl, 1979; Mense et al., 1981).

The free radical gas nitric oxide (NO) is a recently identified intracellular messenger molecule which appears to be involved in a wide range of physiological and pathological processes (Bredt and Snyder, 1994; Moncada et al., 1991; Moncada and Higgs, 1993). In the superficial dorsal horn, NO synthesis linked to n-methyl-D-aspartate (NMDA) receptor activation has been implicated in the maintainance of hyperalgesia in several models of persistent pain

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(Meller and Gebhart, 1993, 1994). NO is synthesized in neural tissue from L-arginine by the enzyme nitric oxide synthase (NOS), which is present throughout the central nervous system (CNS) (Anggard, 1994; Bredt and Snyder, 1990; Vincent and Hope, 1992). In fixed tissue, NOS corresponds to nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-diaphorase) (Buwalda et al., 1995; Dawson et al., 1991; Hope et al., 1991; Matsumoto et al., 1993). Therefore, the histochemical method for NADPH-diaphorase has been widely used to investigate the distribution of neurones containing NOS.

At the light level, NOS immunoreactivity and/or NADPH-diaphorase positivity have been detected in both the superficial dorsal horn and the central grey region of the spinal cord of several mammals (for review see Vincent, 1995). We studied here the electron microscopic distribution of NADPH-diaphorase-positive neurones in these areas. We wanted to obtain detailed information on the connectivity and synaptology of these neurones in order to shed more light on their possible role in pain processing.

Materials and Methods

Studies were performed on four male Wistar rats (240-260 g body weight). All experiments were carried out in strict accordance with the Italian and UE Regulations and were authorized by the Italian Ministry of Health (ref. 600.8/82.20/AG1826). Under deep pentobarbital anesthesia (60 mg/100 g), animals were injected intracardially with 1 ml heparin (5000 U/ml), and perfused through the descending aorta with Sörensen buffer 0.1 M, pH 7.4 containing 0.8% NaCl, 0.025% KCl, 0.05% $NaHCO_3$ and saturated with a mixture of O_2:CO_2 (95:5), followed by cold fixative solution. The latter consisted of 2% glutaraldehyde and 1% paraformaldehyde in Sörensen buffer 0.1 M, pH 7.4. After perfusion, the spinal cord was removed, cut in 4-5 mm thick blocks and postfixed for 2-4 hours. Coronal and horizontal slices from different levels of the cord were cut on a Vibratome (Oxford, UK) at a thickness of 50-100 μm. Sections were then preincubated in PBS containing 0.25% Triton X-100 for 10 minutes at room temperature and processed for the histochemical visualization of NADPH-diaphorase. To do so, they were transferred to a freshly prepared buffer-Triton solution containing 1 mg/ml β-NADPH (Sigma, UK) and 0.2 mg/ml nitro blue tetrazolium (NBT) (Sigma, UK) for 2-4 hours at 37°C. The reaction was monitored under the microscope and stopped by transferring sections in PBS. As an alternative to the use of NBT, which is not osmiophilic, some sections were incubated with 0.1 M PBS containing 1 mg/ml β-NADPH (Sigma, UK) and 0.6 mg/ml 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl) tetrazolium chloride (BSPT, Sigma, UK)(Wolf et al. 1992). BSPT was initially dissolved in 3-4 drops of dimethylformamide.

NBT- and BSPT-labelled sections were then postfixed in osmium ferrocyanide for 1 hour at 4° C, stained with 1% uranyl acetate in maleate buffer for 1 hour at 4° C, dehydrated in increasing concentrations of ethanol and flat-embedded in Araldite. Ultrathin sections were counterstained further with uranyl acetate and lead citrate before observation with a Siemens Elmiskop 102 or a Philips CM10 electron microscope.

Results

NADPH-diaphorase activity can be easily detected in aldehyde-fixed nervous tissue by means of a tetrazolium salt technique, based on the reduction of tetrazolium salts to insoluble and coloured formazan deposits. To study the distribution of positive nerve cells in the spinal cord at the light microscopic level and to directly correlate it with ultrastructural observations, we used NBT as a chromogen. NADPH-diaphorase-stained neurones are deep purple/blue and are histochemically labelled with a Golgi-like appearance. Therefore, it is very easy to follow their processes up to the finest axons and/or dendritic tips. As already reported by others (Dun et al., 1993; Laing et al., 1994; Morris et al., 1994; Valtschanoff et al., 1992; Vizzard et al., 1994a,b), labelled neurones were found in laminae I-II and X and the intermediolateral cell column of the thoracic spinal cord. Cells in laminae I and II were small, round or oval with dendritic trees showing a bipolar shape and oriented along a rostrocaudal axis. Positive neurones in lamina X were either oval or fusiform with three to five thin cell processes extending towards the dorsal horn or central canal.

For electron microscope analysis, in addition to the NBT staining we marked positive neurones with BSPT. This latter chromogen is a non-osmiophilic tetrazolium salt that, unlike NBT, yields an osmiophilic formazan. The BSPT formazan was found to depict very precisely the subcellular localization of enzyme activity, appearing as a very fine granular material of high electron density. By such an approach, the formazan deposists were mainly observed on intracellular membranes of the rough endoplasmic reticulum and the outer nuclear envelope (Fig.1A and insert). A positive reaction was also detected at the levels of the Golgi apparatus and the outer mitochondrial membranes. In NBT-stained preparations, the reaction end product had a more floccular aspect, without a precise subcellular localization (Fig. 1 B-C-D and Fig. 2), so

that it was impossible to ascribe with certainty the enzyme activity to specific cell organelles. Nevertheless, to study the connectivity of NADPH-diaphorase positive nerve cells in the spinal cord, we decided to use this latter approach because the resulting formazan could be easily detected also in very fine cell processes, including very small axons.

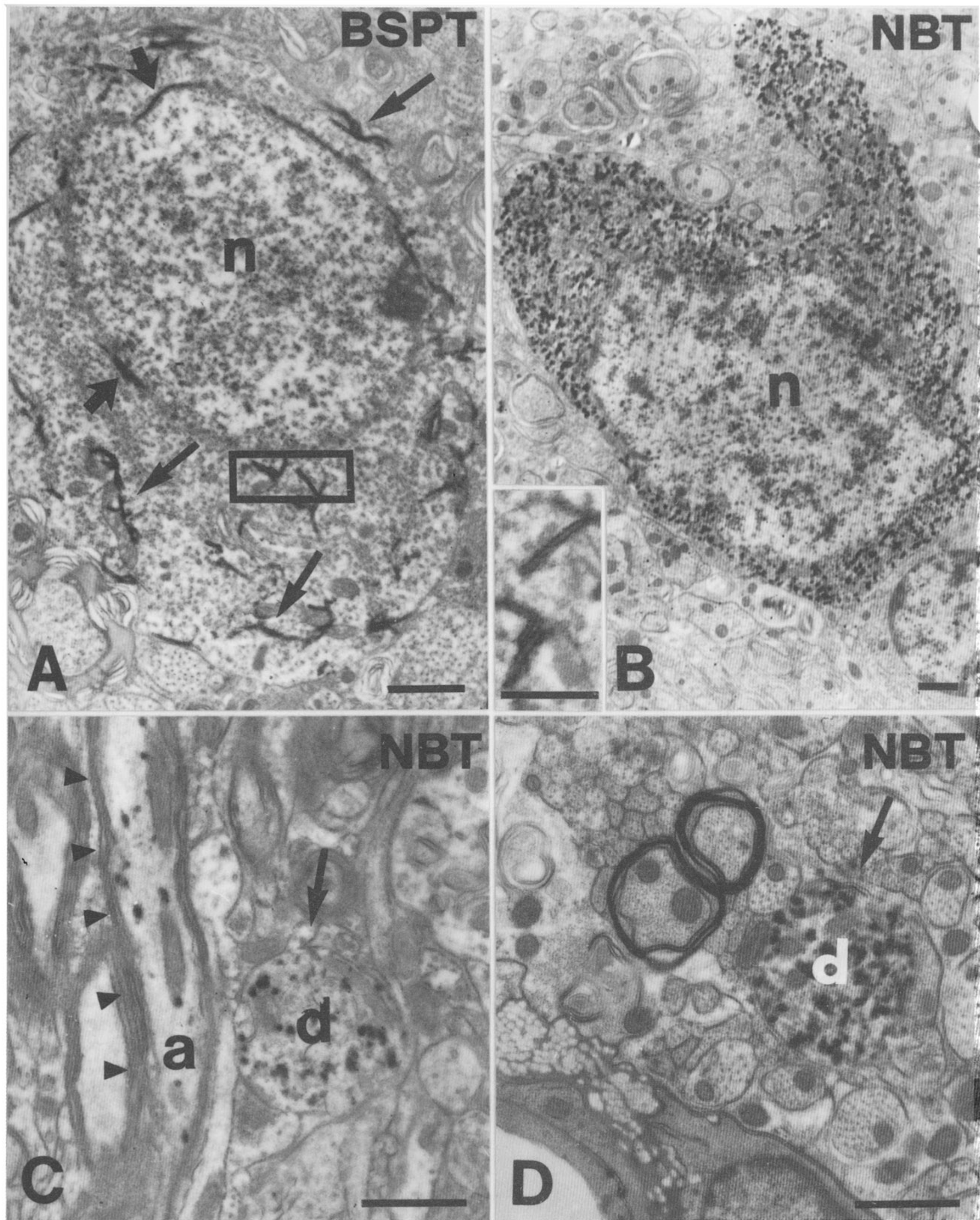

Fig.1.– Ultrastructural visualization of NADPH-diaphorase-positive neurones and processes in the dorsal horn. Positive neuronal cell bodies in lamina II show a different subcellular localization of formazan deposits depending on the chromogen used to reveal diaphorase activity. When using BSPT (**A**), staining is apparent at the level of the rough endoplasmic reticulum (**long arrows**) and nuclear membrane (**short arrows**). At higher magnification (**insert**), the electrondense BSPT label is localized to membranes of the reticulum cisternae. NBT-positive reaction (**B-C-D**) appears in the form of coarser deposits which fill completely almost the neuronal cell body and processes without any clear association with specific cell organelles. Note in **C** positivity within a small myelinated axon (**arrow heads**). **a** = axon; **d** = dendrite; **n** = nucleus. Scale bars = 1μm

NADPH-diaphorase-positive cell bodies and dendrites were observed in all laminae of the dorsal horn but were particularly abundant in the inner part of the lamina II (lamina II_i). Labelled cell bodies were of small to medium size and showed a characteristically indented nucleus. They often received synapses from unlabelled axons. NADPH-diaphorase-positive

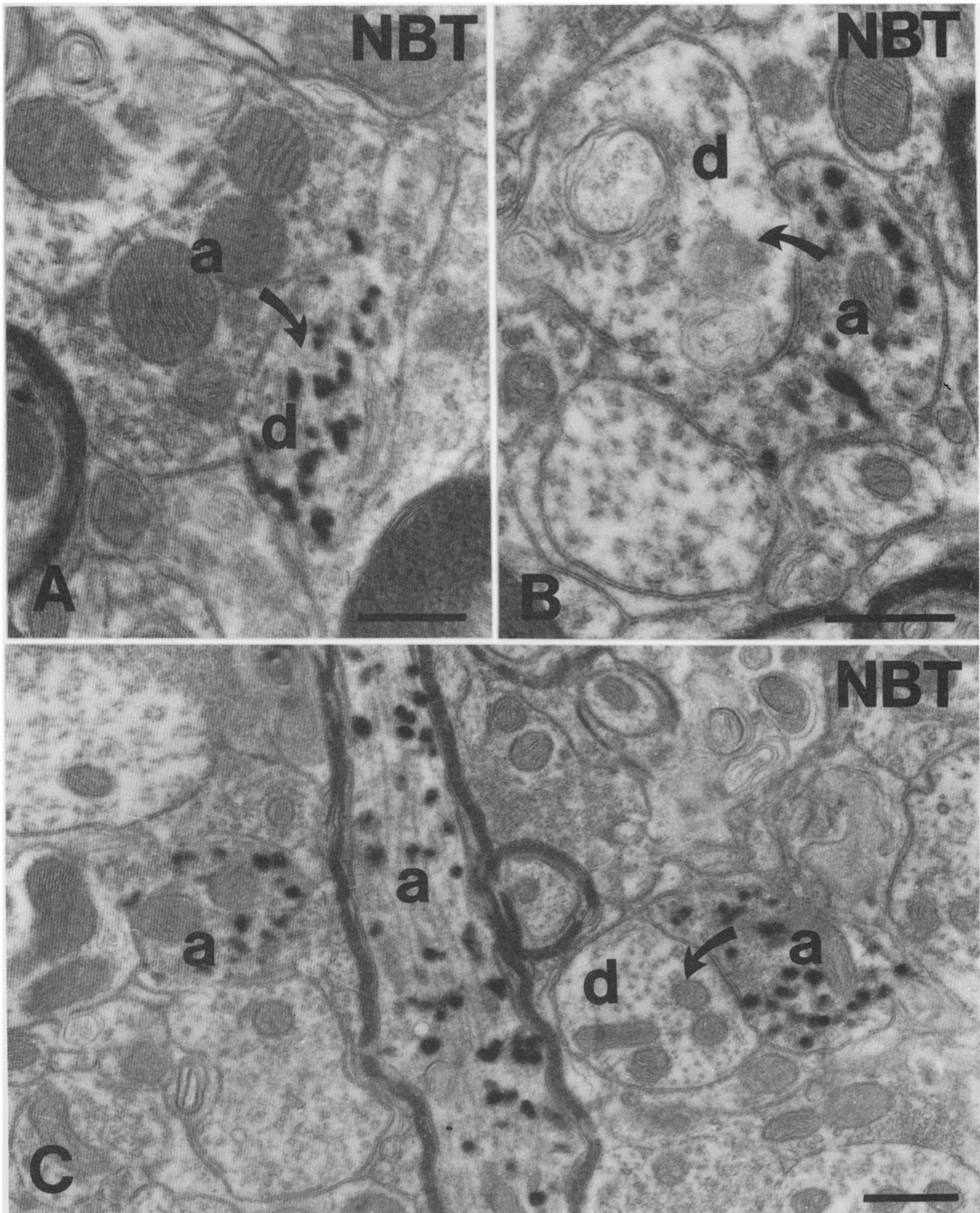

Fig.2.– Distribution and connectivity of NADPH-diaphorase positive nerve processes in lamina X.
At least two different synaptic configurations were observed in this region: negative axon terminals presynaptic to NADPH-diaphorase-positive dendrites (**A**) and NADPH-diaphorase positive axon terminals presynaptic to NADPH-diaphorase-negative dendrites (**B-C**). Small positive myelinated axons (**C**) were also detected in lamina X. **Curved arrows** indicate the direction of synaptic input. **a** = axon; **d** = dendrite. Scale bars = 0.5 µm.

dendrites usually received synapses of the asymmetric type from unlabelled axonal endings, which were generally free of dense core vesicles, and from unlabelled dendrites of varying size. Some positive dendrites were at times observed at the periphery of type II glomeruli. Only a single example was found of a NADPH-diaphorase positive peripheral dendrite that was part of a type I glomerulus after examination of several hundreds of labelled profiles. NADPH-diaphorase positive myelinated axons were found in Lissauer's tract and laminae I-II (Fig. 1C, D). Some very small neuronal processes were filled by the formazan deposits and scattered throughout these laminae, but it was impossible to identify their dendritic or axonal nature simply on the basis of their complement of organelles and/or vesicles.

In lamina X, NADPH-diaphorase-positive neurones originated both myelinated (Fig. 2C) and small unmyelinated axons (Fig. 2B, C). The latter were often observed to form axo-dendritic or axo-somatic contacts of the asymmetric type (Fig. 2B, C). We also frequently found negative terminals containing a number of clear synaptic vesicles, rounded mitochondria and very rarely a few large dense core vesicles, which were presynaptic to NADPH-diaphorase-positive dendrites (Fig. 2A).

Discussion

Subcellular localization of NADPH-diaphorase activity

In several areas of the brain a one-to-one correspondence between neurones expressing NOS mRNA or NOS immunoreactivity and those showing NADPH-diaphorase positivity was found (Bredt et al. 1991; Dawson et al. 1991), and it is now widely recognized that NOS corresponds to NADPH-diaphorase in aldehyde-fixed tissue. Moreover, the recent demonstration that after knocking out the NOS gene there is a complete loss of NADPH-diaphorase activity in the nervous system provided definitive evidence for the correspondence of NADPH-diaphorase and NOS activities (Huang et al. 1993).

We decided to use the histochemical staining to label NO producing neurones in the spinal cord because: *i.* the intensity of the enzyme reaction was not quenched by fixation with high percentage glutaraldehye, thus rendering it possible to obtain satisfactory ultrastructural preservation of our samples; *ii.* addition of small amounts of a detergent to the incubation medium allowed the staining of positive nerve cells through the whole thickness of Vibratome sections up to 100 μm, without having a detrimental effect on ultrastructure.

Our results demonstrate that the use of different tetrazolium salts for the histochemical localization of NADPH-diaphorase yields different subcellular localization of positive reaction sites. NBT formazan deposits were scattered throughout the neuronal cell body and processes without any preferential association with cell organelles. On the other hand, by using BSPT enzyme activity was localized to the rough endoplasmic reticulum, mitochondria and nuclear membranes, as previously observed in some other areas of the brain (Darius et al. 1995; Tang et al., 1995; Wolf et al. 1992). Biochemical studies indicate that brain NOS is a cytosolic enzyme and those who have used NOS immunocytochemistry have not been able to observe a specific association with any subcellular organelle or membrane (Bredt and Snyder, 1990; Llewellyn-Smith et al. 1992). However, highest enzyme activity was detected in the microsomal fraction from the rat brain by others (Kuonen et al. 1988). Therefore, considering the difficulties in obtaining a reliable subcellular localization by a pre-embedding approach (Ribeiro-Da-Silva et al. 1993) and the results of the present work, it seems reasonable to assume that both a membrane-bound and a cytosolic fraction of the enzyme exist in neurones.

NADPH-diaphorase positive neurones in the superficial dorsal horn

The distribution of NADPH-diaphorase positive neurones in the rat dorsal horn as described here is in substantial agreement with previous reports at the light level (Aimi et al. 1991; Dun et al. 1993; Valtschanoff et al. 1992; Vincent, 1995; Vizzard et al. 1994a, b).

In lamina I, NADPH-diaphorase-stained neurones had dendritic trees oriented in the rostrocaudal plane and flattened mediolaterally. Some of these cells morphologically resembled certain types of marginal neurones (Gobel, 1979). In lamina II, most NADPH-diaphorase-positive neurones morphologically corresponded to the islet cells described by Gobel (1979), one of the two most represented neuronal types within the gelatinosa. They had perikarya with diameters ranging from 8 to 18 μm and dendrites predominantly travelling along a rostro-caudal axis. In addition, positive neurones often received dendro-dendritic synapses. Thus, they clearly did not correspond to stalked cells, the second major neuronal type of the gelatinosa, which have fan-like dendritic arbors and different synaptic configurations (Gobel et al., 1980).

In the substantia gelatinosa, NADPH-diaphorase-positive neurones originated small myelinated axons. Therefore, they likely corresponded to projection neurones and/or propriospinal neurones. The possibility that at least some of

the positive cells in the substantia gelatinosa are propriospinal neurones is in agreement with studies on Golgi-stained preparations (Abdel-Maguid and Bowsher, 1984). As to the possibility that some of the NADPH-diaphorase-positive neurones are projection neurones, this is strengthened by their close morphological resemblance with type A spinotectal neurones (Lima and Coimbra, 1989), group I or type C spinoreticular cells (Lima et al. 1991), spinolateroreticular type I cells (Lima and Coimbra, 1990), spinothalamic type 2 neurones (Lima and Coimbra, 1988), type III spinothalamic cells (Abdel-Maguid and Bowsher, 1984), and to some physiologically identified wide dynamic range (WDR) gelatinosa neurones, many of which send their axons to the spino-thalamic tract (Treede et al. 1992). In addition, NOS immunoreactivity was described in certain rat spinothalamic cells (Lee et al. 1993).

Positive neurones in deeper laminae, as previously observed by Valtschanoff et al. (1992), have a morphology that closely resembles that of intracellularly filled spinal neurones projecting to the dorsal column nuclei or spinocervical tract cells.

NADPH-diaphorase positive neurones in lamina X

Light microscopic level studies of lamina X showed that the majority of the NADPH-diaphorase-positive nerve cells were located dorsal and lateral to the central canal (Tang et al., 1995). At electron microscopic level, by using NBT we detected a positive reaction in cell bodies, dendrites of varying size, small unmyelinated and myelinated axons. The cytological features and synaptic connectivity of these cells, as observed here, are in substantial agreement with a previous report on the distribution of NADPH-diaphorase in the rat lamina X using BSPT as a chromogen, except for the failure to demonstrate labelling in myelinated axons in that study (Tang et al., 1995). This might be explained considering the already discussed differences in subcellular localization of diaphorase activity after use of BSPT or NBT formazans.

The neuropil of the central grey region displays three main types of terminals, which have been classified based upon their vesicular constituents (Miller and Seybold, 1987). These three terminal types respectively contained: *i.* small round clear vesicles; *ii.* small flattened clear vesicles; and *iii.* small round clear vesicles and large dense core vesicles (LGVs). The NADPH-diaphorase-positive terminals that we observed in lamina X were generally filled with round agranular vesicles only. For this reason, and considering that it is now widely accepted that LGVs represent the subcellular site of storage for biologically active neuropeptides (De Biasi and Rustioni, 1988; Merighi et al., 1991, 1992), we believe that the NO input to lamina X comes from sources other than the peptidergic afferents, which are widely represented in this lamina (LaMotte, 1988; LaMotte and Shapiro, 1991). Alternatively, NADPH-diaphorase-positive axon terminals in lamina X may arise from supraspinal regions. In keeping, neurones of the hypotalamic paraventricular nucleus, the parabrachial nucleus, the nucleus tractus solitarius, the ventrolateral medulla and raphe nuclei, known to project to this region, are all NADPH-diaphorase-positive (Mizukawa et al., 1989; Vincent and Kimura,1992). Another possibility is that positive terminals arise from local neurones producing NO. This is supported by the finding that physiologically identified cells around the central canal issue a great number of axon collaterals that ramify within a short distance from their cell bodies (Honda and Perl, 1981).

The presence of NADPH-diaphorase-positive terminals in lamina X, but not in laminae I-II indicates that the synaptology of NO-producing neurones varies in the different areas of the spinal cord involved in nociception, and might be of physiological relevance, although, at present, an axonal release of NO has only been demonstrated with certainty in the peripheral nervous system (Bult et al., 1990).

In the CNS, there is a wide body of evidence indicating that NO released at dendrites is able to influence a series of very important synaptic events and has a key role in the synchronization of synaptic activity. As to the role of this molecule in pain perception, a much clearer picture is coming out in the superficial dorsal horn, where severals groups have demonstrated functional links between NMDA receptor activation, production of NO and release of sensory neuropeptides. In keeping, we have recently demonstrated that NO produced at the level of islet cell dendrites in lamina II_i stimulates release of substance P from primary afferents in lamina II_o, in the absence of direct synaptic contacts (Aimar et al., 1998). This indicates that in the dorsal horn NO acts simply by transmembrane diffusion as suggested after *in vitro* experiments (Kelm et al., 1988).

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