

The Distribution of Nitric Oxide Synthase in the Rat Spinal Cord

Mümtaz NAZLI, Richard MORRIS

Department of Veterinary Preclinical Sciences, University of Liverpool, Liverpool, UK.

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Abstract: Since the proposal that nitric oxide acts as an intercellular signalling molecule in the nervous system, a large number of studies have been carried out to determine its functions in neural tissue. In this study, neuronal nitric oxide synthase using the highly specific polyclonal nature of an antibody is observed mainly in laminae I-II, lamina X and in the intermediolateral cell column of the thoraco-lumbar spinal cord. The distribution of neuronal nitric oxide synthase was confirmed and extended the work done in previous studies. Distinct concentrations of neuronal nitric oxide synthase expressing small to medium sized neurones were seen in two bands: one in lamina I and the second in lamina II. It is suggested that these neurones are probably Islet type interneurons and could be inhibitory interneurons. Therefore, based on the observations of nitric oxide synthase containing neurones in laminae I-II, laminae IV-V and lamina X, it suggests that nitric oxide is involved in nociception.

Key Words: Hyperalgesia, nitric oxide, nitric oxide synthase, nociception.

Ratların Omuriliğinde Nitrik Oksit Enziminin Dağılımı

Özet: Nitrik oksit'in sinir sisteminde hücrelerarası uyarıları iletiminde rol alan molekül olduğu kabulünden itibaren, bunun sinirsel dokulardaki fonksiyonlarını tesbit etmek amacıyla çok sayıda çalışmalar yapılmıştır. Bu çalışmada, poliklonal doğal antikorlara oldukça yüksek oranda duyarlılık gösteren antikorun kullanımıyla, nöronal nitrik oksit sentez bölgelerinin esas olarak, thoraco-lumbar omuriliğin lamina I, II, X ve intermediolateral kornuda lokalize olduğu gözlenmiştir. Bu sonuçlar daha önce yapılan çalışmalarla paralellik gösterdiği gibi, bazı ilave yeni bilgiler de sağlamıştır. Nöronal nitrik oksit sentez bölgelerinin küçükten orta hacime kadar değişen büyüklükte ve belirgin bir şekilde olan nöronları lamina I ve internal lamina II' de iki ayrı bant şeklinde gözlenmiştir. Bu nöronların Islet veya inhibitör tipte internöronlar olabileceği düşünülmektedir. Böylece, lamina I-II, IV-V ve lamina X'deki lokalize olan nitrik oksit sentez bölgelerinin bulunduğu nöronlara göre nitrik oksitin ağrı duyusunun iletiminde rol oynadığı sanılmaktadır.

Anahtar Sözcükler: Hiperalji, nitrik oksit, nitrik oksit enzimi, ağrı olgusu.

Introduction

Nitric oxide (NO) represents a new class of neuronal messenger and acts as a signalling molecule in the mammalian central nervous system (CNS) (1,2). NO is produced from L-arginine by the enzyme nitric oxide synthase (NOS), an enzyme requiring several co-factors (3). The predominant NOS isoform in the brain is the calcium-calmodulin-dependent, neuronal form (type 1) (nNOS), which we used in our study (4).

NO may act within the neurone or act more like a classical neurotransmitter (5). Several studies suggested that NOS is a neuromediator of nociceptive regulation in the CNS (6). NO has been implicated in both the sensitization of peripheral nerve terminals and the alteration in spinal neurones which result from peripheral tissue injury (6,7). Hyperalgesia could be mediated by an

enhanced release of NO from primary afferents and spinal neurones which express NOS (8). Nerve injury or axotomy caused upregulation of NOS (8). Hence, if as in LTP, NO acted to cause synaptic facilitation it could be a major contribution to hyperalgesia. If NO is involved in secondary hyperalgesia it could act by facilitating the actions of L-glutamate on N-methyl-D-aspartate (NMDA) receptors. The L-glutamate released from primary afferents can act at either NMDA or non-NMDA receptors (6).

Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) is now known to be a co-factor for NOS and hence earlier studies using the NADPH-d method revealed the distribution of NOS prior to this enzyme being known (9). NOS has been demonstrated in three fairly well-defined regions: the intermediolateral

cell column (IML), LX and LI-II (10-15). The high concentration of NOS in the IML (11,12) has been shown by retrograde labelling to be in sympathetic preganglionic neurones (SPNs) (10,15,16). These findings in the rat IML have been confirmed and extended by ultrastructural examination (17).

In view of the recent studies implicating NO in nociception, detailed localization and morphology of the nNOS expression was performed in the thoraco-lumbar spinal cord using the immunocytochemical method.

Materials and Methods

Twenty adult Wistar rats, weighing approximately 250 g, of either sex, were used in this study. The rats were deeply anaesthetized and perfused with a fixative containing 4% paraformaldehyde in 0.1M phosphate buffer saline (PBS). The thoracic and lumbar parts of the spinal cord were removed, postfixed in fixative (4-6 h) and then cryoprotected with 30% sucrose in 0.1M PBS overnight at 4 °C. Tissue blocks were cut on a freeze knife microtome into 40µm transverse sections and processed free-floating. The endogenous peroxidase and non-specific binding sites for antibodies were suppressed by treating sections with 1% hydrogen peroxide for 30 minutes and 10% normal donkey serum for an hour at room temperature respectively. Furthermore, sections were processed for standard immunocytochemistry by the avidin-biotin-peroxidase complex (ABC) method (18).

Antibodies to nNOS were raised against the whole nNOS molecule created by the use of an insect expression

system. The specificity of the antiserum was confirmed by its lack of endothelial cell staining. Also it has been shown to produce no staining in macrophages stimulated to produce the inducible isoform of NOS (Dr. T. Thippeswamy, personal communication). The sections were incubated in primary antiserum to nNOS receptor diluted 1:5000, in PBS containing normal donkey serum (2.5%) and TritonX-100 (2%), overnight at 4 °C. Subsequently, the binding of primary antisera was detected using biotinylated anti-sheep antisera (1:1000) (Jackson) and streptavidin-conjugated horseradish peroxidase (1:1000) (Amersham). Finally, the chromogen protocol of Shu et al. (19) was used to reveal the distribution of bound peroxidase. In two sample sections, NOS punctuate profiles seen as dots were counted in LI-II_i per dorsal horn by light microscope (x40).

Results

Immunocytochemical staining with this novel nNOS antibody produced intense labelling of many neurones in the spinal cord (Figs. 1, 2). The reaction product labelled the cytoplasm of the soma and the dendrites. Many stained axons with varicosities were also found throughout the grey matter of the spinal cord. NOS-immunoreactive (-IR) neurones were found throughout the spinal cord, with many in LI-II_i, and others were scattered in deeper laminae, LX and in the IML.

Lamina I-II_i: Neurones in LI and LI_i of the lumbar spinal cord are shown in Fig. 3. Stained neurones were small, round or oval and typically bipolar (soma diameter: 12-13µm, mean intersoma distance: 17-18µm). Their

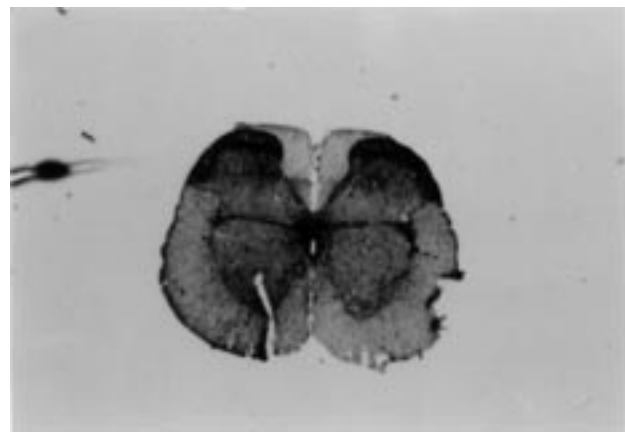
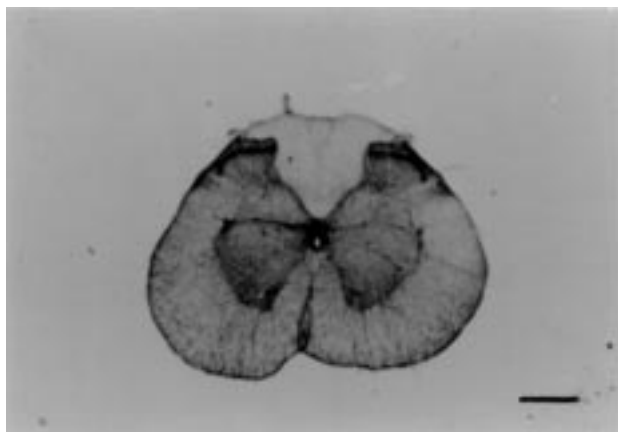


Figure 1 and 2. Low power photomicrographs of the thoracic and lumbar sections to illustrate NOS-IR. Bar: 500µm.

dendrites (two dendrites, mean stained dendritic length: 23-24 μ m) were typically orientated dorsally, ventrally and extended to LIII. Staining in LI-II_i appeared to show a density variation such that LIIIo contained fewer punctate structures and soma. The soma were most numerous in LII_i (Fig. 3). The total number of NOS punctate profiles in LI-II_i per dorsal horn was approximately 1573 (2 sample sections counted). The neurones in LI also showed a dorsal-ventral orientation to their dendrites. This is surprising as many neurones in this lamina have a mediolateral and rostra-caudal organization to their dendrites.

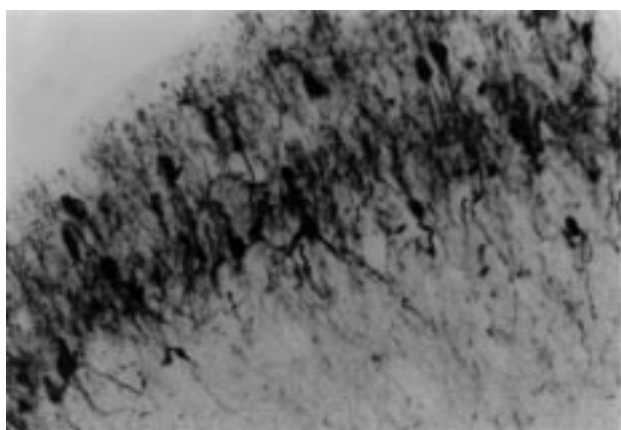


Figure 3. Higher power photomicrographs of the dorsal horn to illustrate NOS staining in the lumbar part of the spinal cord. In LI and LII_i, NOS was present in both small cells of the lumbar spinal cord and in smaller punctate structures which consist of axons and dendritic profiles. The staining tended to form two bands, one in LI and a second in LII_i, where a distinct band of neurones and a dense neuropil are present spreading into LIII.

Lamina III-VI: Bundles of stained processes ran along the edge of the dorsal funiculus with occasional processes entering this tract (Fig. 4). Many of the neurones in the neck of the dorsal horn gave rise to processes which ran towards the dorsal commissure (Fig. 4). Some bipolar cells are also seen on the edge of the dorsal funiculus (Fig.5). Neurones, most of which were triangular shaped, were scattered in the intermediate portion of the grey matter in LIII-VI (Fig. 6). In the deep dorsal horn, NOS-IR neurones (soma diameter: 12-13 μ m, mean intersoma distance: 48-49 μ m) had usually three dendrites (mean stained dendritic length: 12-13 μ m).

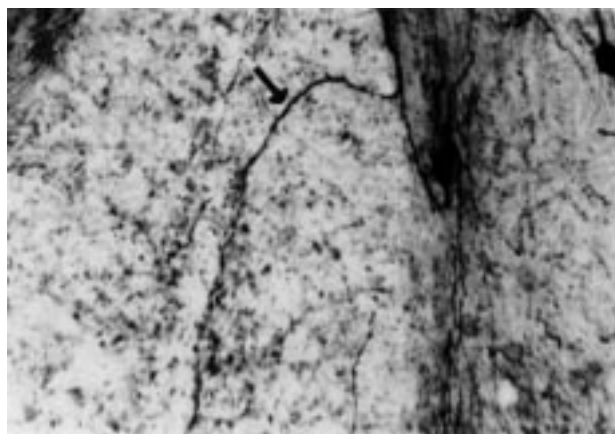


Figure 4. NOS neurones were seen occasionally on the edge of the dorsal funiculus with some processes entering the dorsal column (small arrow).

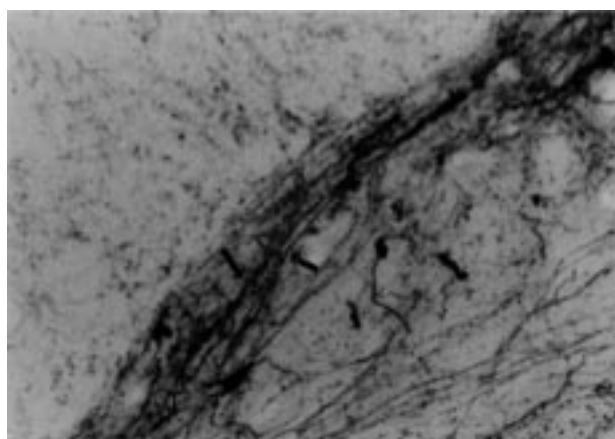


Figure 5. Densely labelled NOS neurones gave rise to processes which were seen running close to the edge of the dorsal funiculus towards the dorsal commissure, LX and dorsally towards superficial laminae (small arrows).

Lamina X: Many NOS-IR neurones were located in LX, with a higher density in the dorsal commissure region (Fig. 7). There were heavily stained punctate neuropils and triangular or multipolar cell bodies (soma diameter: 13-14 μ m, mean inter soma distance: 26-27 μ m). Typically these neurones give rise to 4 principal dendrites (distance to first branch: 10-11 μ m) in the section plane.

Intermediolateral horn-Sympathetic preganglionic neurones: Heavily labelled NOS positive neurones were found in the IML (Fig. 8). Neuronal cell bodies (soma diameter: 25-26 μ m, mean intersoma distance: 29-

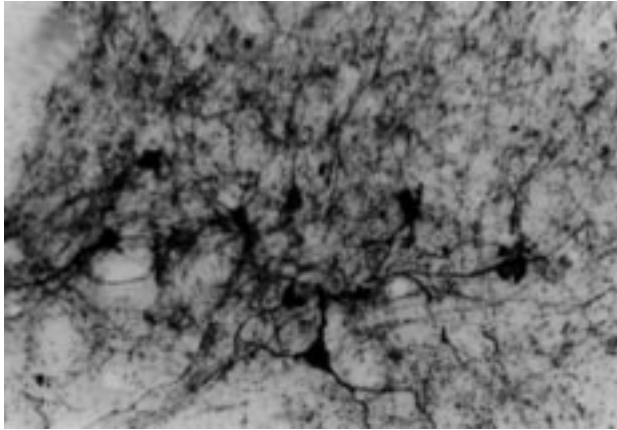


Figure 6. NOS expressing neurons in the neck of the dorsal horn. NOS neurons were sparsely present and had processes extended towards LX and superficial laminae.

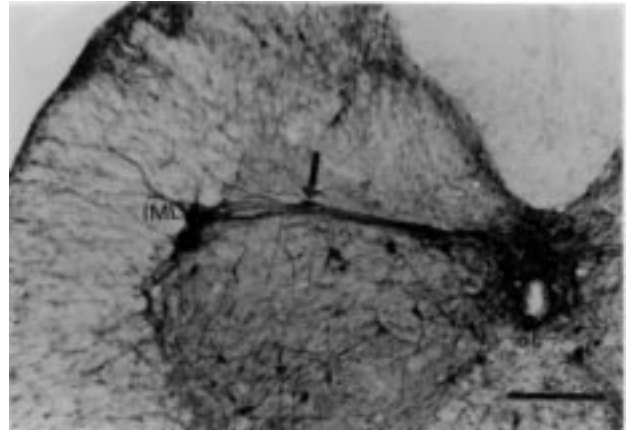


Figure 8. NOS-IR in the IML and LX. A system of characteristic dendrites and axons runs between these regions and laterally into the white matter. There is a NOS expressing neurone in intercalated nucleus (small arrow). Bar: 200µm.

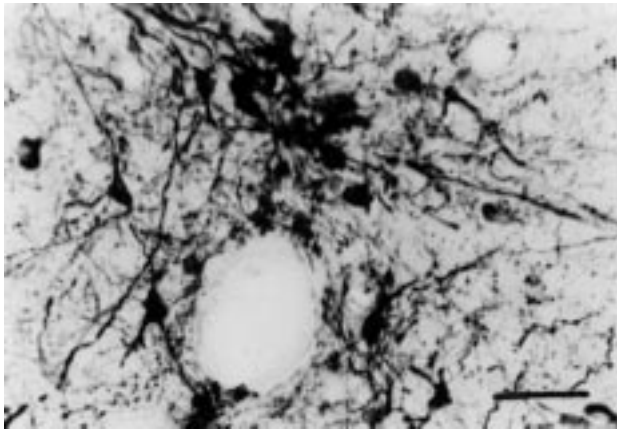


Figure 7. NOS in LX. Many heavily labelled NOS-IR neurones, usually with triangular or multipolar soma were seen, particularly in the region of the dorsal commissure. LX contained a dense network of dendrites and axon varicosities. Bar: Figs 3-7: 50µm.

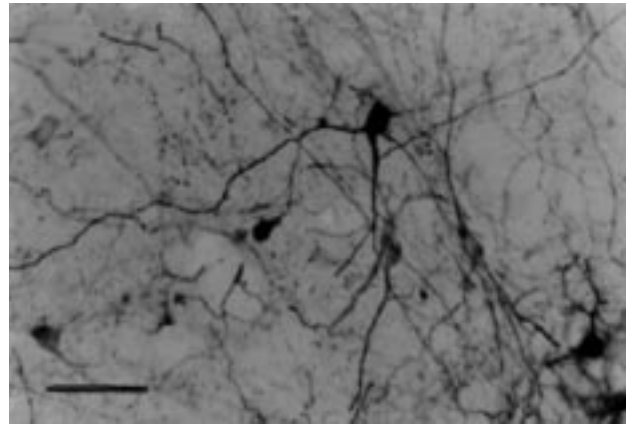


Figure 9. NOS in the ventral horn. Moderately labelled multipolar NOS neurones with their dendrites directed to the LX, ventral commissure and deeper laminae. Bar: 50µm.

30µm) and processes of the principal IML nucleus, a well-defined neuronal population located in the lateral horn at the border line of the white/grey matter, were found to be stained intensely for NOS activity. NOS-IR processes (distance to first branch: 21-22µm) from cells in the IML extended medially towards to LX and laterally into the lateral funiculus (Fig. 8). These neurones were also observed in the region between the IML and the LX (intercalated nucleus) which contained SPNs.

Ventral horn and lateral spinal nucleus: NOS positive neurones were occasionally seen in LVII and LVIII of the ventral horn. The cell bodies were either bipolar or

triangular shaped and lightly stained. Dendrites from these NOS positive neurones projected throughout the ventral horn (Fig. 9). Moderately labelled NOS neurones occasionally were seen in the region of the lateral spinal nucleus (LSN).

General neuropil: Throughout the grey matter large numbers of punctuate stained structures were observed as well as occasional axons with varicosities. In the ventral horn, some of these could be seen directly over the soma of motoneurones with which they possibly make synaptic contact. This staining almost certainly represents nNOS in synaptic structures and hence, NO could be formed in many synapses in the spinal cord. This enzyme

is also extensively distributed in the soma and dendrites of stained neurones. If all this enzyme is available for NO synthesis it suggests that NO could be released from many functionally different regions of a neurone.

Discussion

The antiserum used in this study was highly sensitive and, at the concentration used, was probably highly specific for nNOS (type 1). It gave a similar pattern of staining to another anti-nNOS antiserum raised in a different species (20), but the staining of dendrites and axons was much more extensive and intense. Using nNOS antiserum in this study, labelled neurones and cell processes were found throughout the spinal cord. The present study confirmed previous reports (11,12,15).

Primary afferents: The NOS-IR fibres in the superficial dorsal horn may originate from small diameter primary afferents. Although small numbers (less than 5%) of dorsal root ganglion (DRG) neurones express NOS in adult rats, it is possibly transported towards the terminals of these axons, where it synthesises NO, which may have a role in synaptic transmission. NOS staining is found in axon profiles in the DRG and in axons in the dorsal roots. However, in an ultrastructural study, it was found that primary afferent fibres are unlikely to contribute significantly to NOS fibre staining in the superficial dorsal horn (13). This is because the synapses containing NOS were also labelled for g-amino butyric acid (GABA), which is not found in primary afferents. Furthermore, the NOS positive synaptic profiles were not glomerular in structure and few contained dense core vesicles. This suggests that the majority of the small punctuate structures observed in LI and LII_i are synapses from intrinsic neurones. Zhang et al. (8) have demonstrated that after peripheral injury, NOS production increased in the DRG and spinal cord neurones, many of which contained substance P (SP) and calcitonin gene-related peptide (CGRP). It has been suggested that NO might serve as a transmitter for nociception in LI and LII (11,12).

Laminae I-III: NOS is expressed in large numbers of small neurones which have dendrites running in a mainly dorsal/ventral direction in transverse sections. Their dendritic trees appear limited and they have the overall shape of small interneurones probably of the Islet type (21). The studies of both Laing et al. (14) and

Valtschanoff et al. (13) indicate that these cells also express GABA and hence would be inhibitory interneurones. As Islet cells have local axon arborizations with few long projection axons to other laminae, it would appear as though these cells are local inhibitory interneurones. Furthermore, the small interneurones in LI-III can be subdivided into several groups. Glutamate is contained in substantial numbers of these small interneurones and is not co-localized with either GABA or NOS but is co-localized with calbindin. On this basis it seems as though LII contains both excitatory and inhibitory interneurones (14).

LI-III is the termination site of the majority of small diameter cutaneous afferents implicated in the processing of noxious intensities of stimuli. Hence, the observation of a concentration of NOS containing neurones in this region led to the suggestion that NO was involved in nociception (6). Intrathecal application of NO-blocking drugs has been shown to reduce thermal hyperalgesia and this is mediated via c-GMP. The co-localization of NOS with GABA suggests that in some way NO acted synergistically with GABA. At a simplistic level this may be thought to lead to reduced excitation in spinal circuits and it is difficult to see how this would lead to the increased activity implicated in hyperalgesia. Activation of spinal NMDA receptors results in thermal hyperalgesia and facilitation of nociceptive reflexes (22). In the spinal cord slices, it was demonstrated that the selective glutamate receptor agonist NMDA could lead to the synthesis of cGMP via LII, via the mediation of NO (23). Overall, these data suggest that activation of NMDA receptors by glutamate can lead to an increased synthesis of NO. This in turn leads to synaptic modulation, which results in hyperalgesia. The precise synapse at which NO has this effect remains to be identified and whether NO leads to increased release of an excitatory or an inhibitory transmitter also needs to be established. Overall, it appears as though the spinal actions of NO show strong parallels with the role of NO in synaptic plasticity in regions such as the hippocampus.

However, recently it has been shown that NMDA receptor activation was not necessary for an action of NO on spinal cord neurones. Also, NO had a predominantly inhibitory effect on LI-II neurones (24).

Deep dorsal horn: More scattered NOS positive neurones were seen throughout the deeper laminae of the dorsal horn LIV-VI. These had a triangular and

multipolar structure with more extensive dendrites. Valtchanoff et al. (12) have also shown sparse NADPH-d positive neurones in the deeper dorsal horn, which were multipolar in shape with long radiating dendrites. In the present study, some of the NOS neurones which were bipolar were seen running parallel to the medial border of the dorsal horn. Their dendrites were dorso-ventrally oriented and largely confined to the transverse plane.

Lamina X: A number of NOS expressing neurones were found in LX, which is consistent with previous studies (11,12,15,16). Some of the NOS positive neurones in LX may project to the dorsal reticular nucleus or to the hypothalamus and also some of these cells may be sympathetic preganglionic neurones at the thoracic level (12,16).

Intermediolateral horn-Sympathetic preganglionic neurones: It has been shown that, in the rat, many SPNs (10,11) contain NADPH-d and NOS-immunoreactivities. In the IML, NOS neurones with their bundles of dendrites and axons travelled medially towards LX in our study. Neurochemical heterogeneity has been demonstrated amongst the cell bodies of spinal autonomic neurones. In rat IML, the cell bodies of NOS containing SPN are also non-uniformly distributed along the thoracic cord (10). A retrograde labelling study has shown that nearly half of the choline acetyl transferase-IR IML neurones also contain NOS (16). The thoracic IML seems to have two distinct subpopulations of SPN. Subpopulations of SPNs probably innervate distinct peripheral target neurones, such as the main peripheral ganglia or the adrenal gland (25).

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Conclusion

Many small neurones of LI-LII₁ contain a high concentration of NOS. These neurones are probably Islet type interneurones (21). GABA has been shown to be co-localized in these neurones and in NOS containing synaptic terminals and hence, they could be inhibitory interneurones (13,14). LI and LII of the lumbar spinal cord receives many C-fibre afferent inputs which respond to noxious stimulation, and some of these have been shown to directly innervate NOS expressing neurones (13). Therefore, based on the observation of NOS containing neurones in LI and LII, it has been suggested that NO is involved in nociception. Intrathecal application of NOS blockers indicates that NO seems to have a relatively specific role in the facilitation of thermal responses seen in neuropathic and inflammatory pain models. At present it is not possible to explain the specific contribution made by NO to nociception and thermal hyperalgesia.

NOS is also present in many neurones in LX. It has also been shown that many LX neurones have nociceptive properties (26), further reinforcing the view that NO has a role in nociception. The presence of NOS in many preganglionic and parasympathetic neurones also suggests an important role for NO in these neurones.

Acknowledgement

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