Turk J Vet Anim Sci
24 (2000) 453-458

Evidence that the Neurokinin 1 Receptor is Expressed in spinal Neurones Which Do Not Synthesize Nitric Oxide in the Rat

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Received: 25.10.1999

Abstract : The co-localization of the distribution of the neurokinin 1 receptor with nitric oxide was investigated in laminae I-II, III-IV, X and in the intermediolateral horn of the spinal cord in thirteen adult rats. Silver-intensified gold and standard immunocytochemical labelling or standard double immunocytochemical labelling were employed in 40µm-thick sections of the spinal cord. There were many NK1 receptor-expressing neurones in the dorsal horn of the spinal cord, but none of these expressed NOS. Although neurones expressing the NK1 receptor and NOS were in close proximity in LX and IML, co-localization of the NK1 receptor and NOS in single neurones was extremely rare in these regions.

Key Words: Neurokinin, nitric oxide, nociception, spinal cord, substance P.

Sıçanlarda Nörokinin 1 Reseptörünün Lokalize Olduğu Omurilik Sinir Hücrelerinde Nitrik Oksit'in Bulunmayışının Kanıtlanması

Özet : Onüç (13) adet ergin sıçanların omuriliğinin lamina I-II, III-IV, X ve intermedio lateral kornularında nörokinin 1 ile nitrik oksit sentezinin dağılımlarının birlikte lokalizasyonunun varlığı araştırıldı. Omurilikten alınan 40 mikron kalınlığındaki kesitlere Gümüşle güçlendirilmiş Altın ile birlikte standart immunositokimyasal boyama metodu veya iki aşamalı standart immunositokimyasal boyama metodu kullanıldı. Omuriliğin dorsal kornularında Nörokinin 1 reseptörünü eksprese eden birçok nöron mevcut olmasına karşılık bunların hiçbiri nitrik oksit sentezi eksprese etmedi. İntermediyolateral kornu ve lamina X' de nörokinin 1 reseptörü ve nitrik oksit sentezi eksprese eden nöronlar birbirlerine çok yakın ilişki içinde olmasına rağmen bunların bu bölgelerde birlikte aynı nöronda lokalizasyonları çok nadir olarak gözlendi.

Anahtar Sözcükler: Nörokinin, nitrik oksit, ağrı, omurilik ve substans P.

Introduction

The neurokinin 1 (NK1) receptor has the highest affinity for substance P(SP) of the known neurokinin receptors, and hence is a logical target for this peptide (1). Recently, with the cloning of the NK1 receptor (2,3), it has become possible to map the cellular distribution of this receptor throughout the central nervous system (CNS) using antibodies raised against peptide sequences forming components of the receptor (4-7). In the spinal cord, the NK1 receptor has been shown to be expressed in several specific neuronal groups, including neurones in laminae (L) I, LIII-V, LX and in the intermediolateral horn (IML) (8-12). In laminae I and III-IV, the NK1 receptor bearing neurones receives inputs from SP-containing synapses, most of which would be predicted to be of primary afferent origin. As SP is released in the spinal cord following noxious peripheral stimulation, it is generally accepted that the release of SP from primary afferents is involved in the signalling of noxious events (13,14). Primary afferents containing SP also contain small round clear vesicles which are immunoreactive to antibodies raised against L-glutamate (15,16). Several other lines of evidence strongly support the view that SP-containing primary afferents co-release L-glutamate. The L-glutamate released from these afferents can act at either N-methyl-D-aspartate (NMDA) or non-NMDA receptors. Several studies have proposed mechanisms for secondary hyperalgesia, which involves the modification of NMDA receptor conductance as a result of the release of SP (17).

Recently it has been shown that nitric oxide (NO) acts as an intercellular signalling molecule in the CNS (18,19).

The enzyme which synthesizes NO and nitric oxide synthase (NOS) in neurones has been cloned (20,21) and its antiserum [a-neuronal (n)NOS-type 1] has been raised in rabbit and sheep respectively (22,23). In the spinal cord, nNOS is found in several groups of neurones, a few in LI, and many in the inner part of LII, around the central canal and in the IML. Both around the central canal and in the IML the neurones look very similar to those expressing NK1 receptors (24,25,26). NO is involved in secondary hyperalgesia and acts by facilitating the actions of L-glutamate on NMDA receptors. In view of these observations it was considered of value to study more closely whether co-localization of the NK1 receptor and NOS occurs.

Materials and Methods

Fifteen adult Wistar rats of both sexes weighing approximately 250 g were deeply anaesthetized with pentobarbitone sodium (40 mg/kg). Rats were perfused by vascular perfusion with oxygenated Krebs solution followed by fixation with 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS). The thoraco-lumbar part of the spinal cord was removed, postfixed in the same fixative for 4-6 hours at 4°C and cryoprotected with 30% sucrose in PBS at 4°C overnight and then sectioned on a freeze knife microtome into 40µm sections. Sections were collected into tubes containing PBS and processed free floating. After washing with PBS, the sections were treated with 1% H_2O_2 (30 min) and 10% donkey serum (30 min), both in PBS, to suppress endogenous peroxidase and non-specific binding of antibodies, respectively. Polyclonal anti-NK1 receptor antibody used as a primary antibody, raised in rabbit, kindly supplied by Dr Vigna (7), was diluted at 1:20,000 and incubated at room temperature for 72 hours. Antibody used to localize nNOS, raised in sheep and kindly supplied to us by Dr P. Emson (23), was diluted at 1:5000 and incubated at 4°C overnight. The results obtained with this nNOS antibody were very similar to those obtained with both NADPH-diaphorase staining and with another nNOS antibody raised in rabbit (22). nNOS antibody (23) can be employed at a much lower dilution (1:10,000) and gives much more intense specific staining of dendrites and axons. After incubating of the primary antibodies, the sections were washed with TBS (Tris buffer saline, adjusted to pH 8.2) for 15 m intervals. These antibodies and subsequent antibodies used were diluted with PBS containing 2% Triton X-100, 2.5% bovine serum albumin (2.5% normal donkey serum for nNOS) and 0.25% sodium azide. Secondary antibodies were either gold labelled anti-species or unconjugated, biotinylated antibodies appropriate to the first antibody, and the subsequent steps used were either silver intensification or streptavidin bridge protocols. A range of chromagen methods was employed to reveal the distribution of horse-radish peroxidase. The primary antibodies were tested with sequential application or co-incubation. Controls involving omission of one or both primary antibodies were also undertaken routinely. Several protocols gave acceptable results. The most ideal form of theoretical perspective involved the use of a gold labelled secondary antibody followed by silver intensification to detect the first primary antibody bound to one antigen. The second antigen was detected with the second primary antibody followed by the appropriate biotinylated antispecies antibody, streptavidin-biotin HRP and Vector VIP. This resulted in granular black silver staining and a contrasting more amorphous red-purple Vector VIP stain. This double staining system does not have any potential for false positives due to non-specific binding of antibodies or chromagen (27).

Results

In all regions of the spinal cord investigated, co-localization of NK1 receptor and NOS in a single neurone was extremely rare. While an NK1 receptor stained with silver intensified gold had a dotted appearance, NOS, which is a enzyme, stained lightly with Vector VIP. There were heavy NK1 receptor- and NOS-immunoreactivities in LI (Figures. 1 and 2). Many neurones in LI and LII_i contained NOS, but none of these expressed NK1 receptors. An atypically large NK1 receptor-expressing neurone was found in LII (Figure 1). NK1 receptor-expressing neurones of LIII-IV, which have dendrites traversing LII, were also NOS negative (Figures 1 and 2). Surprisingly, despite their very similar size, shape and reasonably high densities, none of the neurones in LX which contain NOS or bear an NK1 receptor appear to express both. In the IML, both the NK1 receptor and NOS were rarely expressed together by the same neurone (Figure 3). There were both NK1 receptor-and NOS-expressing neurones and dendrites in the intercalated nucleus (between LX and IML). Their dendrites run laterally into the IML and medially into the LX, but there

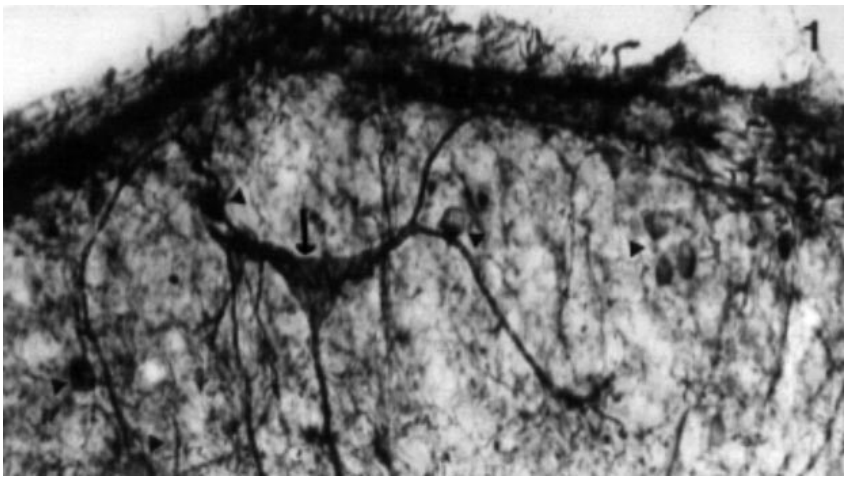

Figure 1. Immunoreactivity was dense for the NK1 receptor in LI and LIII and for NOS in LI and LII_i of the lumbar spinal cord. An atypically large NK1 receptor-expressing neurone was found in LII (small arrows). Many NOS cells are seen in LI-LII and scattered in LIII (arrowheads).

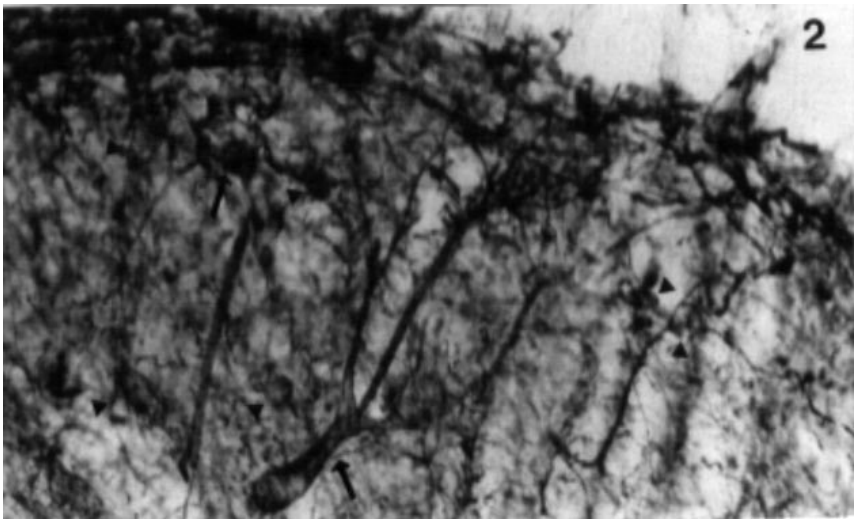

Figure 2. An example of NK1 receptor-expressing neurones in LIII (small arrows). These neurones typically have large soma and prominent dorsally directed dendrites. Many NOS expressing neurones are also present in LI and LII_i (arrowheads).

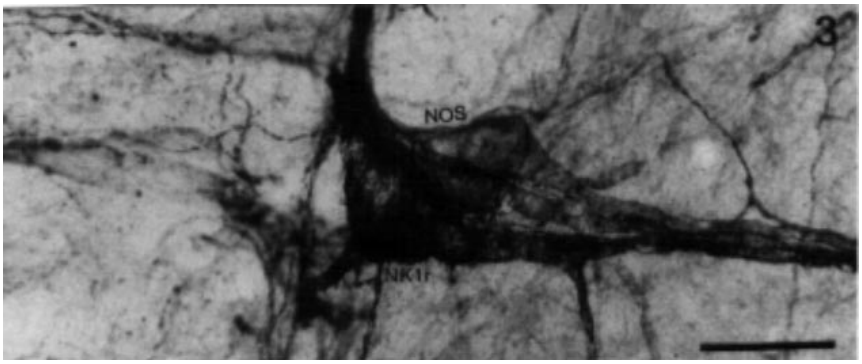

Figure 3. Co-expression of NK1 receptor and NOS in the IML. Sympathetic preganglionic neurones rarely express both the NK1 receptor (arrow NK1r) and NOS (arrow NOS).

was no co-localization of the NK1 receptor and NOS in the same neurone (Figure 4).

On the basis of detailed studies in the cat, principally using the Nissle staining method, Rexed distinguished nine layers (laminae) in the grey matter of the spinal cord, together with a region around the central canal (LX) (Figure 5) (28). This system of laminae was used in the present study.

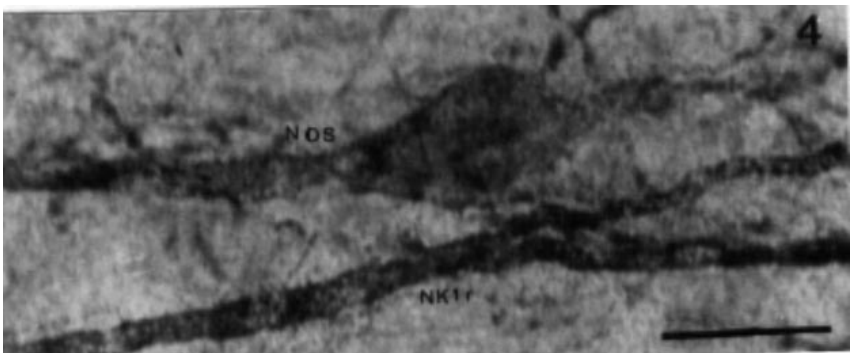

Figure 4. Immunoreactive for both NK1 receptor (arrow NK1r) and NOS (arrow NOS) expressing neurones and dendrites are seen separately in the intercalated nucleus (between LX and IML).

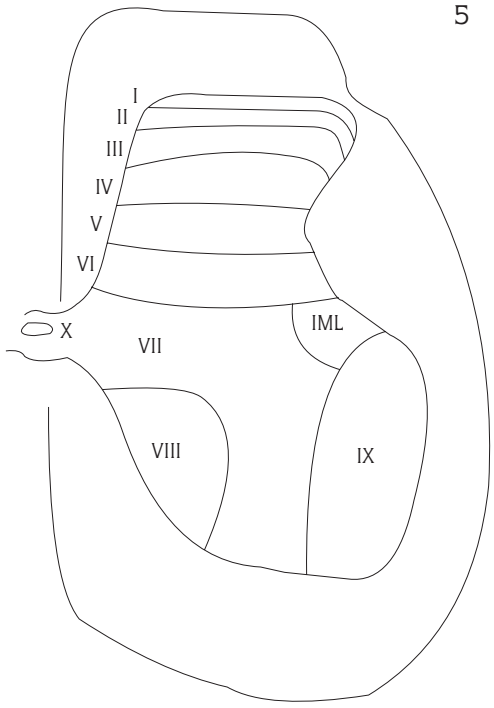

Figure 5. Diagrammatic representation of the cytoarchitectonic scheme of Rexed for the cat's lumbar spinal cord. Laminae I-VI are considered the dorsal horn (From Rexed 1952). Scale bars: Figures 1, 2, and 3: 50µm (x40); Figure 4: 25µm (x100).

Discussion

In a number of regions of the spinal cord, nNOS is found in neurones of similar size and location to those expressing the NK1 receptor. This is particularly apparent in the IML and LX. Overall, the present data demonstrate that the vast majority of spinal neurones in the adult rat which express either the NK1 receptor or nNOS do not express both proteins. These observations have a number of surprising implications. Probably the most surprising is that it suggests that at least two separate populations of sympathetic preganglionic neurones exist in the IML. This will be returned to later in this discussion.

In the outer dorsal horn, nNOS is concentrated in two bands of small neurones with dorsally and ventrally directed dendrites. This distribution is obviously different to that of the NK1 receptor, and co-localization was not seen. These small nNOS-positive dorsal horn neurones are found to contain γ-amino butyric acid (GABA) (24,25,29) and hence would be presumed to be inhibitory interneurones. It is unlikely that NK1 receptor-expressing neurones in LI are inhibitory interneurones as many have axons projecting to the thalamus. Large LIII-IV antenna neurones which express the NK1 receptor also have a different morphology to any of the neurones which contain GABA.

It has been suggested that the release of SP in the spinal cord may activate NOS (24) and this could be involved in the role of nNOS in thermal hyperalgesia. The present observations would suggest that this is unlikely. NO has a predominantly inhibitory effect on neurones in LI and LII (30). It would be interesting to establish whether NO influences NK1 receptor-expressing neurones.

It was surprising that neurones surrounding the central canal in the LX showed no co-localization of these two proteins. The densities of both of these proteins in the LX is high and it would be interesting to establish what proportion of the total neurone population in this region contains one of these markers. They certainly seem to divide the population into two groups. It would be interesting to see if this difference in neurochemical identity correlates with other aspects of their anatomy in terms of synaptic input and axonal targets. Many neurones in the LX of the lumbar spinal cord are known

to belong to the central autonomic nucleus (31,32) and therefore might serve afferent sympathetic functions (33).

In the IML, a few neurones contained both an NK1 receptor and nNOS, but the majority only expressed one protein. Retrograde labelling studies confirmed earlier findings that the IML neurones expressing NOS have axons projecting out of the spinal cord (34) and on the basis of their morphology and location will be sympathetic preganglionic neurones. Similarly, some IML neurones staining for the NK1 receptor had axons projecting out of the CNS. This was not so readily demonstrated as the NK1 antigen was more difficult to demonstrate with fluorescent dyes and long incubation periods that were required tended to reduce the Fluoro-Gold fluorescence. Their morphology and subsequent retrograde labelling studies identify many of these IML NK1 receptor-expressing neurones as sympathetic preganglionic neurones (35-37). However, less than one-third (29.9%) of the total number of sympathetic preganglionic neurones in the IML of the adult rat express the NK1 receptor (36). This suggests that sympathetic preganglionic neurones do not form a homogeneous population and that they could have diverse functions. Studies of NOS expressing neurones in the IML clearly reinforce this view of several distinct populations of sympathetic preganglionic neurones.

Acknowledgement

This study was supported by the University of Kafkas, Kars, Turkey.

References

1. Regoli, D., Drapeau, G., Dion, S. and Couture, R.: New selective agonists for neurokinin receptors: pharmacological tools for receptor characterization. Trends Pharmacol. 1988; 9: 290-295.
2. Hershey, A.D. and Krause, J.E.: Molecular characterization of a functional cDNA encoding the rat substance P receptor. Science. 1990; 247: 958-961.
3. Yokota Y, Sasai Y, Tanaki K, Fugiwara T, Tsuchida K, Shigemoto R, Kakizuka A, Ohkubo H. and Nakanishi, S.: Molecular characterization of a functional cDNA for rat substance P receptor. J. Biol. Chem. 1989; 264 (30): 17649-17652.
4. Moussaoui, S.M., Hermans, E., Mathieu, A.M., Bonici, B., Clerc, F., Guinet, F., Garret, C. and Laduron, P.M.: Polyclonal antibodies against the rat NK1 receptor: characterization and localization in the spinal cord. NeuroReport. 1992; 3: 1073-1076.
5. Shigemoto, R., Nakaya, Y., Nomura, S., Ogawa-Meguro, R., Ohishi, H., Kaneko, T., Nakanishi, S. and Mizuno, N.: Immunocytochemical localization of rat substance P receptor in the striatum. Neurosci Lett. 1993; 153: 157-160.
6. Bret-Dibat, J.L., Zouaoui, D., Dery, O., Zerari, F., Grassi, J., Maillet, S., Conrath, M. and Couraud, J.Y: Antipeptide polyclonal antibodies that recognize a substance P-binding site in mammalian tissues: a biochemical and immunocytochemical study. J. Neurochem. 1994; 63: 333-343.
7. Vigna, S.R., Bowden, J.J., McDonald, D.M., Fisher, J., Okamoto, A., McVey, D.C., Payan, D.G. and Bunnett, N.W.: Characterization of antibodies to the rat substance P (NK1) receptor and to a chimeric substance P receptor expressed in mammalian cells. J. Neurosci. 1994; 14 (2): 834-845.
8. Bleazard, L., Hill, R.G. and Morris, R.: The correlation between the distribution of NK1 receptor and the action of tachykinin agonist in the dorsal horn of the rat indicates that substance P does not have functional role on substantia gelatinosa (lamina II) neurones. J. Neurosci. 1994; 14: 7655-7664.
9. Nakaya, Y., Kaneko, T., Shigemoto, R., Nakanishi, S. and Mizuno, N.: Immunohistochemical localisation of substance P receptor in the central nervous system of the adult rat. J. Comp. Neurol. 1994; 347: 249-274.
10. Brown, J.L., Liu, H., Maggio, J.E., Vigna, S.R., Mantyh, P.W. and Basbaum, A.I: Morphological characterization of substance P receptor-immunoreactive neurones in the rat spinal cord and trigeminal nucleus caudalis. J. Comp. Neurol. 1995; 356: 327-344.
11. Littlewood, N.K., Todd, A.J., Spike, R.C., Watt, C. and Shehab, S.A.S.: The types of neurones in spinal dorsal horn which possess neurokinin-1 receptors. Neuroscience. 1995; 66: 597-608.
12. Marshall, G.E., Shehab, S.A.S., Spike, R.C. and Todd, A.J.: Neurokinin 1 receptors on lumbar spinothalamic neurones in the rat. Neuroscience. 1996; 72: 255-263.
13. McCarson, K.E. and Goldstein, B.D.: Release of substance P into the superficial dorsal horn following nociceptive activation of the hindpaw of the rat. Brain Res. 1991; 568: 109-115.
14. McCarson, K.E. and Krause, J.E.: NK-1 and NK-3 type tachykinin receptor mRNA expression in the rat spinal cord dorsal horn is increased during adjuvant or formalin-induced nociception. J. Neurosci. 1994; 14 (2): 712-720.
15. De Biasi, S. and Rustioni, A: Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of the spinal cord. PNAS. U.S.A. 1988; 85: 780-782.

16. Merighi, A., Polak, J.M. and Theodosis, T.: Ultrastructural visualization of glutamate and aspartate immunoreactivities in the rat dorsal horn, with special reference to the co-localization of glutamate, substance P and calcitonin gene related peptide. Neuroscience. 1991; 40: 67-80.

17. Dubner, R. and Basbaum, A.I.: In The Textbook of Pain (Wall, P.D. and Melzack, R. Eds.). Churchill-Livingstone, London, 225-241, 1994.

18. Garthwaite, J.: Glutamate, nitric oxide and cell-cell signalling in the nervous system. TINS. 1991; 14: 60-67.

19. Garthwaite, J. and Boulton, C.L.: Nitric oxide signaling in the central nervous system. Annu. Rev. Physiol. 1995; 57: 683-706.

20. Bredt, D.S., Hwang, P.M., Glatt, C.E., Lowenstein, C., Reed, R.R. and Synder, S.H: Cloned and expressed nitric oxide synthase structurally resembled cytochrome P-450 reductase. Nature (London). 1991; 351: 714-718.

21. Huang, P.L., Dawson, T.M., Bredt, D.S., Snyder, S.H., Fishman, M.C.: Targeted disruption of the neuronal nitric oxide synthase gene. Cell. 1993; 75: 1273-1286.

22. Schmidt, H.H.H.W., Gagne, G.D., Nakane, M., Pollock, J.S., Miller, M.F. and Murad, F.: Mapping of neural nitric oxide synthase in the rat suggests frequent co-localization with NADPH-diaphorase but not soluble guanyly cyclase, and novel paraneural functions for nitrinergic signal transduction. J. Histochem. and cytochemistry. 1992; 40 (10): 1439-1456.

23. Herbison, A.E., Simonian, S.X., Norris, P. and Emson, P.C.: Relationship of neuronal nitric oxide synthase immunoreactivity to GnRH neurones in the ovariectomized and intact female rat. J. Neuroendocrinol. 1996; 8: 73-82.

24. Valtschanoff, J.G., Weinberg, R.J. and Rustioni, A.: NADPH Diaphorase in the spinal cord of rats. J. Comp. Neurol. 1992a; 321: 209-222.

25. Laing, I., Todd, A.C., Heizmann, C.W. and Schmidt, H.H.H.W.: Subpopulation of GABAergic neurones in laminae I-II of rat spinal dorsal horn defined by coexistence with classical transmitters, peptides, nitric oxide synthase or parvalbumin. Neuroscience. 1994; 61: 123-132.

26. Saito, S., Kidd, G.J., Trapp, B.D., Dawson, T.M., Bredt, D.S., Wilson, D.A., Traystman, R.J., Synder, S.H. and Hanley, D.F. Rat spinal cord neurons contain nitric oxide synthase. Neuroscience, 1994; 59: 447-456.

27. Shu, S., Ju, G., Fan, L.: The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. Neurosci. Lett. 1988; 85: 169-171.

28. Rexed, B.: The cytoarchitectonic organization of the spinal cord of the cat. J. Comp. Neurol. 1952; 96: 415-495.

29. Valtschanoff, J.G., Weinberg, R.J., Rustioni, A. and Schmidt, H.H.H.W.: Nitric oxide synthase and GABA colocalise in lamina II of rat spinal cord. Neurosci. Lett. 1992b; 148: 6-10.

30. Pehl, U. and Schmidt, H.A.: Electrophysiological responses of neurones in the rat spinal cord to nitric oxide. Neuroscience. 1997; 77 (2): 563-573.

31. Hancock, M.B. and Peveto, C.A.: A preganglionic autonomic nucleus in the dorsal grey commissure of the lumbar spinal cord of the rat. J. Comp. Neurol. 1979; 183: 65-72.

32. Strack, A.M., Sawyer, W.B., Marubio, L.M. and Loewy, A.D.: Spinal origin of sympathetic preganglionic neurones in the rat. Brain Res. 1988; 455: 187-1.

33. Hakim, M.A., Hirooka, Y., Coleman, M.J., Bennett, M.R. and Dampney, R.A.L.: Evidence for a critical role of nitric oxide in the tonic excitation of rabbit renal sympathetic preganglionic neurones. J.Physiol. 1995; 482 (2): 401-407.

34. Bredt, D.S., Hwang, P.M. and Snyder, S.H.: Localisation of nitric oxide synthase indicating a neural role for nitric oxide. Nature (London). 1990; 347: 768-770.

35. Llewellyn-Smith, I.J., Pilowsky, P., Minson, J.B. and Chalmers, J.: Synapses on axons of sympathetic preganglionic neurons in rat and rabbit thoracic spinal cord. J. Comp. Neurol.1995; 354: 193-208.

36. Grkovic, I. and Anderson, C.R.: Distribution of immunoreactivity for the NK1 receptor on different subpopulations of sympathetic preganglionic neurons in the rat. J. Comp. Neurol. 1996; 374: 376-386.

37. Llewellyn-Smith, I., Martin, C.L., Minson, J.B., Pilowsky, P.M., Arnolda, L.F., Basbaum, A.I. and Chalmers, J.P.: Neurokinin 1 receptor-immunoreactive sympathetic preganglionic neurons: target, specificity and ultrastructure. Neuroscience. 1997; 77: 1137-1149.