IMMUNOHISTOCHEMICAL DISTRIBUTION OF NEUROPEPTIDE Y AND NEUROPEPTIDE Y Y1 RECEPTOR IN THE RAT LUMBAR SPINAL CORD

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A N - terminal sequence of the rat neuropeptide Y (NPY) Y1 receptor was used as a novel antigen to raise polyclonal antisera in rabbits. Immunocytochemical staining was used to compare the distribution of NPY and NPY Y1 receptors in the rat's lumbal spinal cord. The NPY Y1 receptor antibody stained a narrow band in lamina (L) II of the superficial dorsal horn. The pattern of staining was more widespread being particularly clear in deeper laminae (LIII-IV). By comparison with the localization of the receptor, neuropeptide Y was more widely distributed throughout the dorsal horn. Normally, primary afferent fibres do not contain neuropeptide Y. Several neuropeptide Y receptors have been identified and our results suggest that neuropeptide Y released in the dorsal horn acts as neuropeptide Y receptorss in addition to Y1.

Key words: neuropeptide Y, neuropeptide Y Y1 receptor, pain, spinal cord

INTRODUCTION

Neuropeptide Y (NPY) is a small peptide of 36 amino acids which was first isolated from mammalian brain tissue (Allen *et al*., 1983). The first clone for an NPY receptor was isolated from a rat's forebrain cDNA library (Eva *et al*., 1990). At least five different NPY receptor subtypes have now been described, Y1, Y2, Y3, Y4 and Y5 (Wahlestedt *et al*., 1992; Bard *et al*., 1995; Gerald *et al*., 1996; Weinberg *et al*., 1996; Yan *et al*., 1996).

Immunocytochemical studies have demonstrated that NPY occurs not only in the brain but also in the spinal cord (Ch'ung *et al*., 1985; Llewellyn-Smith *et al*., 1990). Examination of the spinal cord of various species, particularly rat (Sasek and Elde, 1985; De Quidt and Emson, 1986), has revealed that NPY-immunoreactive axons and terminals are found throughout the grey matter at all segmental levels of the cord. Within the dorsal horn a heavy concentration of NPY-immunoreactive axons and varicosities is found within the superficial laminae (Gibson *et al*., 1984; Sasek and Elde, 1985; De Quidt and Emson, 1986; Doyle and Maxwell, 1993; Rowan *et al*., 1993), with the highest concentration in LI and the outer part of LII (LII_0) (Hunt *et al.*, 1981; Gibson *et al*., 1984; Sasek and Elde, 1985;

396   Acta Veterinaria (Beograd), Vol. 55. No. 5-6, 395-401, 2005.
                                              Nazli M: Immunohistochemical distribution of neuropeptide
                                                Y and neuropeptide Y Y1 receptor in the rat lumbar spinal cord

Doyle and Maxwell, 1993; Rowan *et al*., 1993). This distribution of NPY terminals is similar to that of NPY binding mapped by autoradiography (Kar and Quirion, 1992). In animals, in which prior sciatic nerve section has been carried out the NPY Y1 receptor distribution showed no change whilst NPY was apparently found at greater density in LII of the spinal dorsal horn and in the gracile nucleus (Nazli and Morris, 2000).

In view of the successful generation of a number of antibodies to other G-protein coupled receptors, we decided to raise one to the NPY Y1 receptor. The N-terminal was chosen as the target for raising antibodies as it is on the outside of the cell and as such could be potentially valuable in studies on living cells.

The aim of the present study was to raise the NPY Y1 receptor antibody against the N-terminal peptide sequence. The distribution of NPY Y1 receptor in the lumbar spinal cord has been compared with that of NPY.

MATERIAL AND METHODS

Production of antiserum: An N-terminal peptide sequence of the NPY Y1 receptor (VSENSPFLAFENDDCHLPL, 18-37) was synthesized by automated solid-phase peptide synthesis and purified by reversed-phase high-pressure liquid chromatography and its correct structure was confirmed by mass spectrometry. The peptide was conjugated to Keyhole Limpet Haemocyanin. Three New Zealand rabbits were used to produce NPY Y1 receptor antibodies. Pre-immune bleeds were taken from the rabbits and serum was prepared and centrifuged to remove any remaining solid material. Serum with added sodium (Merck) was aliquoted and stored at -20°C. Sera were obtained after repeated immunizations with the antigen solution. The rabbits were bled (6 times) by the ear artery fourteen days after each immunization (5 times).

Immunocytochemistry: Eighteen adult Wistar rats of both sexes, weighing approximately 250 g, were deeply anaesthetised with a lethal dose of pentobarbitone sodium (40 mg/kg). The blood was washed from the circulation by transcardial perfusion with oxygenated Krebs solution followed by fixation with 4 % paraformaldehyde in 0.1M phosphate buffered saline (PBS). The lumbar spinal cord was 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 40ìm transverse sections and processed free-floating. The endogenous peroxidase and non specific binding sites for antibodies were suppressed by treating sections at room temperature with 1% hydrogen peroxide for 30 minutes and 10% normal donkey serum for an hour respectively. Further, the antisera were tested for anti-NPY Y1 receptor$_{18-37}$ on lumbar spinal cord sections using immunocytochemistry (Hsu *et al*., 1981). The sections were incubated with NPY Y1 receptor$_{18-37}$ antisera at various dilutions (1:100, 1:250, 1:500, 1:1000, 1:2000, 1:3000 and final optimum dilution: 1:1000) and NPY antibody (Peninsula Lab) at 1:1000, overnight at 4°C. Antibody omission and absorption controls were carried out for anti-NPY Y1 receptor$_{18-37}$ sera. Absorption controls were undertaken with 50% vol. of serum/mixed with 50% vol. of antigen (NPY Y1$_{18-37}$). The sections were incubated subsequently with a

Acta Veterinaria (Beograd), Vol. 55. No. 5-6, 395-401, 2005.   397
Nazli M: Immunohistochemical distribution of neuropeptide
Y and neuropeptide Y Y1 receptor in the rat lumbar spinal cord

biotinylated anti-rabbit IgG antibody (Jackson) and then with horse-radish peroxidase-conjugated streptavidin (Amersham) at room temperature for an hour respectively. Finally the chromogen protocol of Shu *et al*. (1988) was used to reveal the distribution of bound peroxidase.

RESULTS

Results of antibody production study: No immunoreactivity was seen in the lumbar spinal cord following application of preimmune sera. One of the rabbits had started to produce the antibodies against the NPY Y1 antigen by the 4th blood sampling and a similar pattern of immunoreactivity was observed in the fifth and final bleeds tested. NPY Y1 receptor-immunoreactivity was observed with the cell bodies and nerve processes as a band in LII of the lumbar spinal cord (Fig. 1), but two rabbits sera gave relatively weak staining.
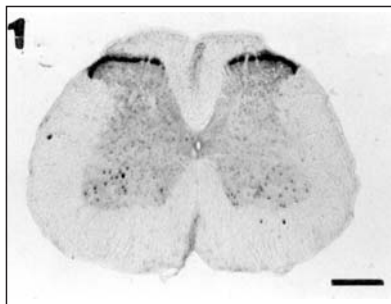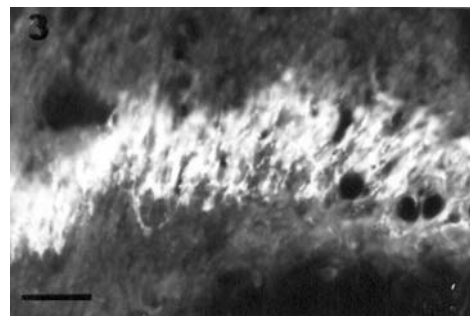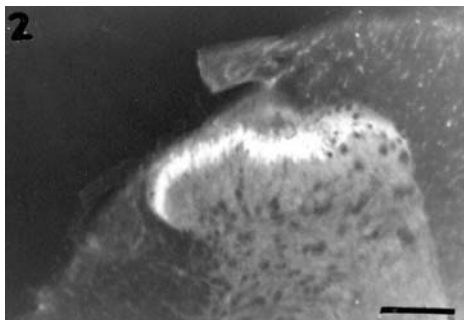

Figure 1. Low power photomicrograph shows NPY Y1 receptor-LI in LII (sera test - rabbit 1). Bar: 500 μm

Distribution of NPY and NPY Y1 receptor in the spinal cord: Antibodies against NPY Y1 receptor produce selective staining as a dense band in LII of the lumbar spinal dorsal horn (Fig 1). Immunolabelling was also seen in motoneurones of the ventral horn (Fig 1). There were several neurones expressing heavily stained NPY Y1 receptors , as well as in processes LII of the superficial dorsal horn (Fig 2 and 3).

NPY immunoreactive fibres were seen throughout the lumbar spinal dorsal horn (LI-VI) (Fig 4). NPY-immunoreactive axons and varicosities were found in LI-VI, but within each laminae differences were noted with respect to their appearance and density.

Figures 2 and 3. High power photomicrographs showing NPY Y1 receptor-LI in a distinct band of positive nerve processes and cell bodies in LII. Bars: Fig. 2; 200 μm, Fig. 3; 50 μm

398   **Acta Veterinaria (Beograd), Vol. 55. No. 5-6, 395-401, 2005.**
Nazli M: Immunohistochemical distribution of neuropeptide
Y and neuropeptide Y Y1 receptor in the rat lumbar spinal cord

NPY-immunoreactivity was particularly dense in LI and LII of the dorsal horn with heavily stained immunoreactive fibres and axons (Fig 5). There were moderate numbers of NPY fibres in LIII-IV with occasional scattered fibres along the dorso-medial border of the dorsal horn in LV-VI running across the midline (Fig 6).
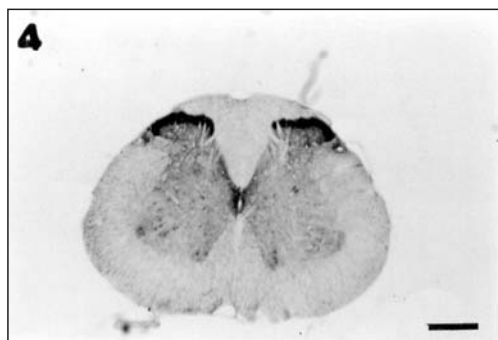

Figure 4. Low power photomicro- graph of NPY-IR fibres in the lumbal spinal cord. Bar: 500 μm
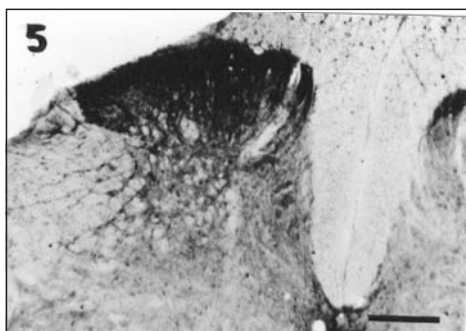

Figure 5. High power photomicrograph shows NPY-IR in a large numbers of axons and varicosities in the dorsal horn. NPY-IR terminals are found occasionally in contact with neuronal cell bodies in the superficial and deep dorsal horn. Bar: 200 μm
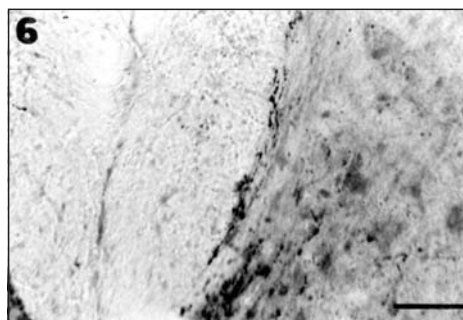
Figure 6. NPY-immunoreactive fibres and axons are seen along the dorsomedial border of the dorsal horn in LV-VI and travelling across the midline. These fibres are also found in contact with neuronal cell bodies in this region. Bar: 50 μm

DISCUSSION

Using a novel antigen from the N-terminal sequence of the rat NPY Y1 receptor, an antiserum was raised which gives staining comparable to a recently

Acta Veterinaria (Beograd), Vol. 55. No. 5-6, 395-401, 2005.   399
Nazli M: Immunohistochemical distribution of neuropeptide
Y and neuropeptide Y Y1 receptor in the rat lumbar spinal cord

described antibody to the C-terminal region (Zhang *et al.*, 1994). This pattern of staining and absorption tests with the antigen suggests that this polyclonal antibody specifically recognises the NPY Y1 receptor.

The staining given by this antibody was highly localized to a narrow band in LII of the dorsal horn. By comparison with the distribution of NPY, NPY Y1 receptor was much less widely distributed. Several NPY receptors have now been identified and this suggests that NPY released in the spinal cord is probably acting at more than one receptor type. Furthermore, it suggests that the NPY Y1 receptor has a highly specific function in a small subpopulation of spinal neurones. This reinforces the view that specificity of action of neuropeptides in the dorsal horn is probably determined by the distribution of target receptors. Indeed, if volume transmission is an important process targeting specificity could only be achieved through the highly localized distribution of receptors. In this respect, these systems resemble the action of hormones where the specificity of action is determined by the distribution of target receptors and not the localization of release sites.

Whilst NPY is distributed quite widely in the cord, it is present in a higher density in LI and II. This confirms other studies which have shown NPY to be most dense in LI (Hunt *et al.*, 1981; Sasek and Elde, 1985; Doyle and Maxwell, 1994). NPY is present in both neurones soma and processes in LII.

Rowan *et al*. (1993) have shown that all NPY neurones contain γ-amino butyric acid (GABA) in LI-III of rat spinal dorsal horn. In view of the fact that NPY in the dorsal horn originates mainly from intrinsic spinal neurones, it is probable that all NPY-containing neurones in the superficial dorsal horn are inhibitory interneurones. However, not all GABA-immunoreactive neurones showed NPY-immunoreactivity. This suggests that the superficial dorsal horn contains several different populations of inhibitory interneurones only some of which contain NPY. Large Islet cells in LII and similar cells in LIII are usually GABA-immunoreactive, while Stalked and small Islet cells in LII and neurones with dorso-ventrally orientated dendrites in LIII are not GABA-immunoreactive (Powell and Todd, 1992; Todd and McKenzie, 1989). Hunt *et al.* (1981) also suggested that NPY is present in Islet cells, which are also GABA- immunoreactive. Hence, in LII, it would appear that both NPY and the NPY Y1 receptor are probably expressed in intrinsic neurones. Whether the NPY Y1 receptors are auto-receptors on NPY containing neurones in LII, or are on a separate population of neurones remains to be determined.

It has been shown that the highest levels of basal-release of immunoreactive NPY were detected in the mid-dorsal horns of intact animals (cat and rat) and that this was unaltered by peripheral noxious thermal or mechanical stimuli (Mark *et al*., 1997). Since NPY is virtually absent in primary afferents it is probable that spontaneous release within the spinal cord comes from NPY-containing intrinsic spinal neurones. By a process of exclusion, it appears that spontaneous firing of intrinsic spinal neurones was responsible for the large ongoing release of immunoreactive NPY. This finding is in contrast to the other studied peptides in the spinal cord of the rat and cat, such as SP (Duggan *et al*., 1988) and galanin (Hope *et al*., 1994). This leads to a rather curious conclusion that NPY is present in

400

Acta Veterinaria (Beograd), Vol. 55. No. 5-6, 395-401, 2005.
Nazli M: Immunohistochemical distribution of neuropeptide
Y and neuropeptide Y Y1 receptor in the rat lumbar spinal cord

intrinsic inhibitory neurones of LII and is being released continuously. Noxious stimuli which would be expected to activate C-fibres terminating in LII do not however lead to an increased release of NPY. It would be interesting to explore this further to establish the changes that occur in NPY release in inflammation or neuropathic pain models.

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Acta Veterinaria (Beograd), Vol. 55. No. 5-6, 395-401, 2005.
Nazli M: Immunohistochemical distribution of neuropeptide
Y and neuropeptide Y Y1 receptor in the rat lumbar spinal cord

401

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IMUNOHEMIJSKA DISTRIBUCIJA NEUROPEPTIDA Y I NJEGOVOG RECEPTORA Y1 U LUMBALNOM DELU KIČMENE MOŽDINE PACOVA

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SADRŽAJ

Imunizacijom kunića N-terminalnom sekvencom Y1 receptora pacova za neuropeptid Y (NPY) dobijen je poliklonski antiserum koji je korišćen za imunohemijsko bojenje radi izučavanja distribucije NPY i NPY Y1 receptora u lumbalnom delu kičmene moždine pacova. Antitela za NPY Y1 receptor su se vezivala u obliku uske trake u L II segmentu površnog dela dorzalnih rogova. Ovo bojenje je bilo posebno jasno u dubljim segmentima L III i L IV. Upoređivanjem lokalizacije receptora zapažena je distribucija NPY pretežno u dorzalnim rogovima. Primarna aferentna vlakna nisu sadržavala NPY. Identifikovano je nekoliko receptora za NPY i ovi rezultati ukazuju da se NPY, oslobođen u dorzalnim rogovima, vezuje i za druge receptore osim Y1.