Kafkas Univ. Vet. Fak. Derg.
2003, 9(1): 69-74

IMMUNOCYTOCHEMICAL LOCALISATIONS OF THE OREXIN-A WITH CALBINDIN D AND NITRIC OXIDE SYNTHASE IN THE RAT SPINAL CORD

Mümtaz NAZLI*

Geliş Tarihi : 16.05.2003

Summary: Orexin (hypocretin) is synthesized by neurones in the lateral hypothalamus and is reported to regulate the food intake, energy metabolism and endocrine control. The aim of this study was to investigate and compare the distributions of orexin-A- with calbindin D- and neuronal nitric oxide synthase-containing neurones in the spinal cord of adult Wistar rats using immunocytochemistry. The orexin-A immunoreactive fibres and terminals are observed in the superficial dorsal horn. Calbindin D and neuronal nitric oxide synthase positive cells and processes are also distributed in the superficial dorsal horn. Although calbindin D and neuronal nitric oxide synthase cell populations with the orexin-A fibres are concentrated in the superficial dorsal horn, there was rarely co-localization or direct anatomic contact between orexin-A and other two markers in this region. These findings may suggest that the actions of the orexins with calbindin D and nitric oxide on the food intake, neuroendocrine functions and pain modulation are mediated via independent mechanisms. Thus nitric oxide is a diffusible molecule and could potentially affect the activity of orexin neurones via a non-synaptic mechanism. Calbindin D expressing neurons that were separate populations of small Islet cells in the superficial dorsal horn, are probably excitatory interneurons.

Key Words: Orexin; nitric oxide; calbindin D; hypothalamus; feeding; pain; spinal cord.

Ratların omuriliğinde oreksin-A ile kalbindin D ve nöronal nitrik oksit sentetazın immunositokimyasal lokalizasyonları

Özet: Oreksin (hipokretin) hipotalamusun lateralindeki sinir hücrelerinde sentezlenen, endokrin kontrol, enerji metabolizması ve besin maddesi alımını düzenleyen bir nöropeptitdir. Bu çalışmanın amacı Wistar rat'ın omuriliğinde oreksin-A ile kalbindin D ve nöronal nitrik oksit sentetazın dağılımlarını immunositokimyasal metotla araştırmak ve mukayese etmektir. Orexin-A'nin immunoreaktif fibril ve sonlanmaları dorsal kornunun yüzeysel kısımlarında gözlendi. Kalbindin D ve nöronal nitrik oksit sentetaza ait sinir hücre ve uzantıları da aynı bölgede görüldü. Kalbindin D ve nöronal nitrik oksit sentetaza ait dorsal kornudaki hücre grupları oreksin-A fibrilleriyle dorsal kornunun yüzeysel kısımlarında konsantre olmalarına rağmen, oreksin-A ile diğer iki sinir işaretleyicilerin birbirleriyle aynı sinir hücresinde (ko-ekspres) veya anatomik olarak herhangi bir bağlantının nadiren olduğu belirlendi. Bu bulgular oreksinler ile kalbindin D ve nitrik oksitin besin maddesi alımı, nöroendokrin fonksiyonlar ve ağrı modulasyonu üzerine olan işlevlerinin bağımsız birtakım olaylar aracılığıyla yapıldığını gösterir. Bununla birlikte, nitrik oksit diffuze olabilen bir molekül olup, sinaptik olmayan mekanizmalar aracılığıyla oreksin nöronlarının aktivitelerini etkileyebilir. Dorsal kornunun yüzeysel kısımlarında küçük islet hücreleri halinde bulunan ve kalbindin D'yi ifade eden sinir hücreleri farklı bir grup olup, muhtemelen eksitatör ara nöronlardır.

Anahtar Sözcükler: Oreksin; nitric oksit; kalbindin D; hipotalamus; beslenme, ağrı, omurilik.

INTRODUCTION

Orexin-A (Orx-A) and orexin-B (Orx-B) (also known as hypocretin-1 and hypocretin-2, respectively), are recently discovered neuropeptides that are synthesized in the lateral hypothalamic neurones. Orx-A and orx-B are derived from a single prepro-peptide and activate two closely related G-protein-coupled receptors[1]. Several morphological studies have shown that orexins-like immunoreactivity is highly specifically present in varies regions of the hypothalamus, particularly lateral hypothalamus (LHA)[1-4]. In contrast to the localization of orexins-containing cell bodies in the hypothalamus, the orexins-containing fibres and terminals are widely distributed in the hypothalamus, cerebral cortex, thalamus, brainstem and spinal cord[5-7], suggesting that orexinergic neurones have widespread connections with other regions of the brain[3,6] including a strong distribution to the lamina (L) I-II of the spinal cord[5,6,8]. This provides morphological evidence for a wide spectrum of the physiological roles of orexins-containing neurones. Additionally, orexins are suggested to be associated with the regulation of food intake, energy balance, neuroendocrine functions, sleep-wake cycle and glucose homeostasis[1,9]. There have been many studies on the distribution of orexins-containing perikarya and fibres in many mammals. However, little is known about the morphological characteristics of orexinergic neurones in the superficial dorsal horn of the spinal cord.

Calbindin-D28k (CaB), a calcium binding protein is isolated from the brain[10]. In neurohistological studies, the immunocytochemical detection of these proteins provides an excellent tool to study the histology and neurochemical character of specific subsets of neurones in the dorsal horn of the spinal cord[11-15]. The superficial dorsal horn, which contains many peptidergic neurones, mainly receives nociceptive

* Kafkas Üniversitesi Veteriner Fakültesi Histoloji-Embriyoloji Anabilim Dalı, Kars-TÜRKİYE

information. Nitric oxide (NO) is a highly reactive free radical gas and is predicted to act as a second messenger in the control of important biological functions, like cell-mediated immunological response and synaptic transmission[16]. NO is produced from L-arginine by the enzyme NO synthase (NOS), which appears in distinct isoforms. NOS isoform in the brain is called neuronal form (type-1) (nNOS) and is calcium-calmodulin dependent[17]. NO participates in great variety of neuronal functions including the modulation of food intake, wakefulness, endocrine system and pain perception[18,19]. NO production may affect certain hypothalamo-hypophyseal peptidergic systems. The generation of oxytocin and vasopressin increases in hypothalamic magnocellular neurones, which is accompanied by an up-regulation of the intracellular NOS activity in these neurones[20].

The similar physiological roles and overlapping localization between orx-A and CaB, or nNOS in superficial dorsal horn raise the possibility that the orexin system may interact with the NO system in functional activities. Different morphological types of neurones have been identified in LI of the rat[21]. This has lead to the suggestion that superficial laminae are heavily involved in the processing of primary afferent nociceptive and thermoreceptive activity[22]. The four LI neurone types have been identified in the superficial dorsal horn of the rat, i.e. fusiform, flattened, pyramidal and multipolar[21,23,24]. Variety of its constituent synaptic mediators and this data[25] indicate that the superficial dorsal horn represents a complex neural system. Thus, the present study was undertaken to investigate if CaB, or nNOS, is co-localized in the orexin neurones in the superficial dorsal horn of the Wistar rat. In the present study, double-labeling immunofluorescent immunocytochemistry for orexin and CaB or nNOS was used.

MATERIALS and METHODS

Ten adult Wistar rats, weighing approximately 250 g, were used in this study. Wistar rats were group housed in standard cages and maintained on a 12 h light/dark cycle, 55% humidity, with food and water ad libitum. Primary antisera for immunocytochemical experiments used were polyclonal anti-orexin-A and anti-calbindin D antisera. All antisera were diluted in a solution of 0.1 M phosphate buffered saline (PBS; pH 7.2), 2.5% bovine serum albumin, 0,25% sodium azide and 2% tritonX-100. Antisera specificity was determined in control experiments in which the primary antiserum was omitted or pre-absorbed with an excess of anti-orx-A with control peptide orx-A (1:100).

The rats were deeply anaesthetised with sodium pentobarbitone (150 mg/kg, i.p; Rhône Mérieux Harlow, UK) and perfused transcardially with oxygenated Kreb's solution and fixative containing 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS). All subsequent steps were performed at room temperature unless indicated otherwise. Spinal cord was removed, post-fixed in fixative (4-6 h) and then cryoprotected with 30% sucrose in 0.1 M PBS overnight at 4 °C. Transversal (40$_{\mu}$m) and parallel to the dorsal surface (10 $_{\mu}$m) of the spinal cord sections were cut into 10 $_{\mu}$m in a sample order on a cryostat.

Immunofluorescent method as described by Dun et al.[26], was used to visualize orx-A with CaB and nNOS immunoreactivities. Free-floating sections were washed five times with PBS (15 min per wash), incubated with 10% donkey serum in PBS (1 h) and washed once with PBS prior to incubation with anti-orx-A (1:100) (Peninsula, U.S.A.) antiserum overnight at 4 °C. Sections were washed five times with PBS before incubation (1 h) with either biotinylated rabbit anti-species IgG as appropriate (1:500) then fluorescein (DTAF)-conjugated streptavidin (1:400) (both from Jackson Immuno Research Laboratories, U.S.A.). After the sections were washed with PBS, half of the all sections were incubated with anti-CaB (1:500) (Sigma, U.K.) and the rest of the sections were incubated with anti-nNOS (1:5000) overnight at 4 °C. Subsequently, sections were employed in anti-rabbit CY3 (1:150) (Jackson). Finally, sections were mounted on chrome alum gelatin-coated microscope slides and coverslipped with Vectashield (Vector, U.K.). Sections were examined by conventional fluorescence microscopy (596 nm excitation, 615 nm emission).

RESULTS

No immunoreactive staining was observed when primary antibodies were omitted. Preabsorption of orexin-A antisera abolished immunopositive staining. Immunoreactivity for CaB was found in all lamina of the dorsal horn of the transversal and parallel sections of the dorsal surface of the spinal cord and detected in the cytoplasm of neurones, but not in the nucleus (Figs. 1,2). Most of the neurones expressing positive immunoreaction for CaB were observed in LII (Fig. 2), while they were moderately distributed in LI, LIII and LIV (Fig. 1). Double-immunocytochemistry showed that orexin-A and CaB peptides had rarely synaptic contacts within the neurones of the superficial dorsal horn. In LII, many positive cell bodies darkly stained with punctate profiles were

Kafkas Univ. Vet. Fak. Derg.
2003, 9(1): 69-74

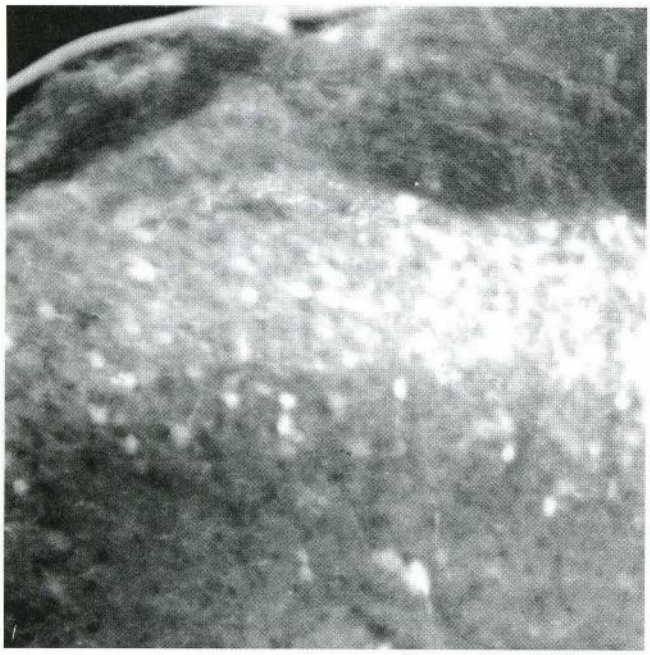

Figure 1. Photomicrograph of the spinal cord showing CaB-immunoreactive neurones and their dendrites in LI-II of dorsal horn, transversal section. **Bar:** 100$_{\mu}$m.
Resim 1. Omuriliğin transversal kesitinde kalbindin D'nin immunreaktif nöron ve dentritlerinin LI-II'deki görünümü. **Bar:** 100$_{\mu}$m.

usually fusiform or occasionally flattened and had long dendrites (Fig. 2). Immunoreactive neurones had usually two dendrites that oriented from dorsal to the ventral in the parallel sections of the dorsal surface (Fig. 2), and had two short dendrites that rostrocaudally oriented in the transversal sections (Fig. 1). Immunoreactive orexin-A fibres, which are also directed ventrally, had many boutons and terminals (Fig. 2). Few immunoreactive fibres for orexin-A made synaptic contact with the dendrites of CaB cells (Fig. 2).

nNOS-immunoreactivity was dense in LI and inner part of the LII (LII_i) of the superficial dorsal horn (Figs. 3,4). Cytoplasm of the cell bodies and dendrites were stained with nNOS antibody. Typical positive bipolar neurones for nNOS were fusiform or occasionally pyramidal and had usually two dendrites (Figs. 3,4). The dendrites of these cells had long and were dorsally to ventrally directed in LI- LII_i at the parallel sections of the dorsal surface. Orexin-A immunoreactive fibres that also dorsally to ventrally oriented had many terminals and boutons. Orexin-A immunoreactive fibres also hardly made synaptic contact with the immunoreactive cell bodies (soma) and dendrites of nNOS in the superficial dorsal horn (Figs. 3,4), although both orexin-A and nNOS immunoreactive processes directed ventrally.

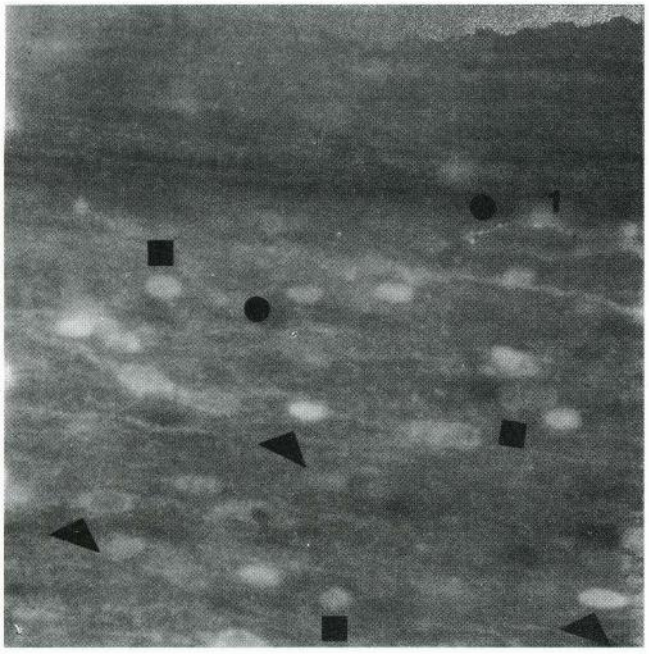

Figure 2. Photomicrograph showing Orx-A- (roundheads) and CaB-immunoreaktivities in LI-II. Rarely seen synaptic contacts between Orx-A fibres and CaB cells and dentrites (number 1). Typically fusiform (arrowheads) and occasionally flattened cells (small square) for CaB. **Bar:** 50 $_{\mu}$m.
Resim 2. Oreksin-A (yuvarlak başlar) ile kalbindin D'nin LI-II deki immunreaktiviteleri. Oreksin-A fibrilleri ile kalbindin D'ye ait hücre ve dentritler arasında çok seyrek olarak görülen sinaptik ilişki (no 1). Kalbindin D'nin çoğunlukla yassı (ok başları) ve seyrek olarak ovalimsi-yassı tipteki hücrelerin (küçük kareler) görünümü. **Bar** 50 $_{\mu}$m.

Figure 3. Photomicrograph showing double labelling of Orx-A and nNOS is in LI-II of the dorsal horn. Typically fusiform (number 1) or occasionally pyramidal type cells for nNOS and their dentrites (arrowheads). Orx-A- immunoreactive fibres and their terminals (large arrowheads). **Bar:** 100$_{\mu}$m.
Resim 3. Dorsal kornunun LI-II'sinde oreksin-A ile nNOS (nöronal nitrik oksit sentataz)'in immunreaktivitelerinin görünümü. nNOS'e ait tipik fusiform (no 1) veya seyrek olarak piramidal tipteki sinir hücreleri (ok başları) ve dentritlerin görünümü. İmmunreaktif oreksin-A fibril ve sonlanmaları (büyük ok başları). **Bar:** 100$_{\mu}$m

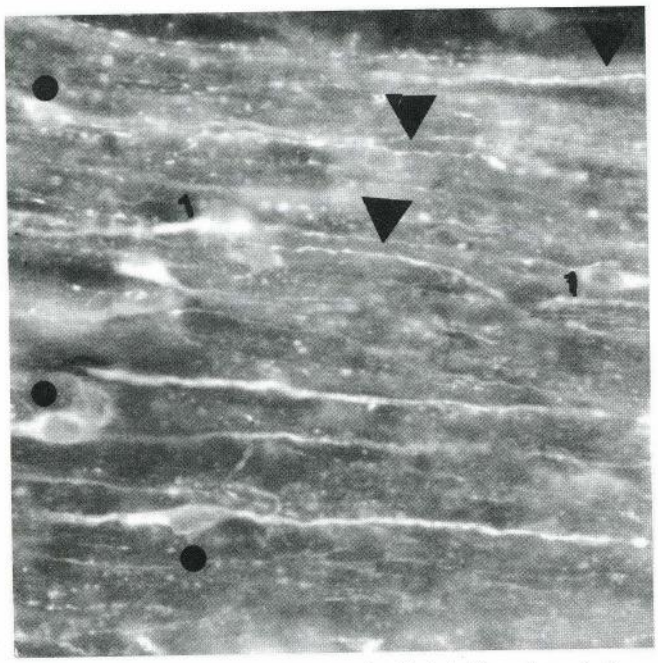

Figure 4. Photomicrograph showing double labelling of orx-A (large arrowheads) and nNOS-immunoreactivities (roundheads) in LI-II. Rarely seen synaptic contacts between Orx-A fibres and nNOS cells and dendrites (number 1). **Bar:** 50µm
Resim 4. Omuriliğin LI-II'sinde oreksin-A (büyük ok başları) ile nNOS (yuvarlak başlar)in immunreaktivitelerinin görünümü. Oreksin-A fibrilleri ile nNOS sinir hücre ve dentritleri arasındaki çok seyrek olan sinaptik ilişki (no 1). **Bar:** 50µm.

DISCUSSION

In the Wistar rat, many immunoreactive orexin-A fibres and terminals were localized particularly in LI and moderately in LII of the dorsal horn, agreeing with other studies[5-8]. NOS and CaB were also examined, as these substances have previously been found in dorsal horn neurones with similar morphology[7,12,15,27-29], and have been suggested as useful markers for subpopulations of inhibitory[30] and excitatory neurones[13,15]. All these findings may provide some morphological information for the distribution patterns of orexin, nNOS and CaB in the superficial dorsal horn, since orexins and NO are regulators of food intake and energy homeostasis.

Orexins were initially suggested to be involved in the regulation of energy balance and food intake based on the observations that centrally or locally administration of the orexins resulted in increased food intake and that food deprivation up-regulated the prepro-orexin mRNA level in the hypothalamus[1,9]. Voltage-clamp recordings and calcium digital imaging experiments using cultured LHA cells revealed that both orexin-1 and -2 induced enhancement of neuronal activity occurred as early as synaptic activity was detected, thus suggesting that orexin might contribute to the development of arousal, sleep regulation, feeding and endocrine control[31]. Intrathecal injection orexin-A suppressed the expression of Fos-like immunoreactivity, induced by formalin injection into the paw in LI-II of LIV-V of the spinal cord, suggesting that spinal orexin-1 receptor was involved in the nociceptive transmission[7]. Date et al[5]. showed that orexin fibres were concentrated in LI of the dorsal horn. This area directly receives pain and thermal stimuli[32] and relay on nociceptive information to the LHA, brainstem and thalamus. The LHA is a site that attenuates nociceptive transmission[33]. Orexin pathway from the LHA to the spinal cord may be involved in the nociceptive modulation system.

NO is known to be modulator in the regulation of feeding and energy balance. Previous studies shown that administration of nNOS inhibitor induced a significant decrease in food intake and body weight in normal and genetically obese rat and mice, while peripheral or central treatment with NO donors increased food intake[34,35]. These findings suggest that NO may also act as a modulator promoting food intake as orexin does. NOS-immunoreactive neurones with a fusiform appearance were described in LI[27], and NOS positive cells resembling the flattened type were described in transverse sections[36]. In the present study, NOS positive cell types were usually fusiform type and occasionally pyramidal type (Figs. 3,4). NO is generated postsynaptically in dorsal horn neurones in response to nociceptive C-fibre discharge and activation of nNOS[37] and is thought to act as a retrograde messenger[38]. CaB may be a marker for spinal projection neurones that process different types of information from those containing parvalbumin, reflecting different physiological properties[11]. While large numbers of calbindin-immunoreactive neurones have been reported in LI, along with present observations, their morphological characteristics suggested a mixed population of fusiform or flattened cells[11,12]. In the current study, CaB positive cells in LI appeared similar to fusiform cells described in this region by Lima and Coimbra[21,23], suggesting that CaB neurones were possibly are excitatory neurones.

In conclusion, no evidence of the co-expressions of nNOS and CaB in a population of the orexin-containing neurones suggests that NO and CaB may play separate role in the mechanisms by which the orexinergic neurones regulate the food intake and energy balance. NO may also interact with orexin in modulation of feeding via non-synaptic route, since some nNOS stained processes were hardly in close proximity to orexinergic neurones. Clarification and further understanding of the processes involved in orexin actions would depend upon a better knowledge of the circuitry of the superficial dorsal horn and fuller

appreciation of the functional part played by the various cell types. At the present level of understanding, it is possible to affirm that the descending connections from the hypothalamus of orx-A-containing neurones has significant functional consequences in terms of regulation of excitability of a substantial number of neurones in part of the superficial dorsal horn. These effects can be rationalized in terms of orexin's enhancement of arousal and support of mechanisms associated with active feeding.

REFERENCES

1 **Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JRS, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M:** Orexins and orexin receptors: A family of hypothalamic neuropeptides and G-protein-coupled receptor that regulate feeding behavior. *Cell*, 92: 573-585, 1998.

2 **DeLecea L, Kilduff TS, Peyron C, Gao XB, Foye PE, Danielson PE, Fukuhara C, Battenberg ELF, Gautvick VT, Bartlett FS, Frankel WN, Van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG:** The hypocretins: hypothalamus -specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci*, USA 95: 322-327, 1998.

3 **Peyron C, Tighe DK, van den Pol AN, DeLecea L, Heller HC, Sutcliffe JG, Kilduff TS:** Neurones containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci*, 18: 9996-10015, 1998.

4 **Horvath TL, Diano S, Van den Pol AN:** Synaptic interaction between hypocretin (orexin) and Neuropeptide Y cells in the rodent and primate hypothalamus: a novel circuit implicated in metabolic and endocrine regulations. *J Neurosci*, 19(3): 1072-1087, 1999.

5 **Date Y, Mondal MS, Matsukura S, Nakazato M:** Distribution of orexin-A and orexin-B (hypocretins) in tha rat spinal cord. *Neurosci Lett*, 288: 87-90, 2000.

6 **Nazli M:** Distribution of orexin-A- and orexin-B-containing neurones and fibres in the rat brain and spinal cord, *Isr J Vet Med*, 56 (4): 143-146, 2001.

7 **Yamamato T, Nozaki-Taguchi N, Chiba T:** Analgesic effect of intrathecally administered orexin-A in the rat hot plate test. *Br J Pharmacol*, 137: 170-176, 2002.

8 **Van den Pol AN:** Narcolepsy: a neurodegenerative disease of the hypocretin system? *Neuron*, 3: 415-418, 2000.

9 **Ida T, Nakahara K, Murakami T, Hanada R, Nakazato M, Murakami N:** Possible involvement of orexin in the stress reaction in rats. *Biochem Biophys Res Common*, 270: 318-323, 2000.

10 **Baimbridge KG, Miller JJ, Parkes CO:** Calcium-binding protein distribution in the rat brain. *Brain Res*, 245: 223-229, 1982.

11 **Yamamoto T, Carr PA, Baimbridge KG, Nagy JI:** Parvabumin- and calbindin D28k-immunoreactive neurons in the superficial layers of the spinal cord dorsal horn of rat. *Brain Res Bull*, 23: 493-508, 1989.

12 **Antal M, Freund TF, Polgar E:** Calcium-binding proteins, parvalbumin- and calbindin-D28k-immunoreactive neurones in the rat spinal cord and dorsal root ganglia: a light and electron microscopic study. *J Comp Neurol*, 295: 467-483, 1990.

13 **Antal M, Polgar E, Chalmers J, Minson JB, Llewellyn-Smith I, Heizmann CW, Somogyi P:** Different populations of parvalbumin- and calbindin-D28k-immunoreactive neurones contain GABA and accumulate ^{3}H-D-aspartate in the dorsal horn of the rat spinal cord. *J Comp Neurol*, 314: 114-124, 1991.

14 **Goodchild AK, Llewellyn-Smith IJ, Sun, OJ, Chalmers J, Cunningham AM, Pilowsky PM:** Calbindin-immunoreactive neurones in the reticular formation of the rat brainstem: catecholamine content and spinal projections. *J Comp Neurol*, 424: 547-562, 2000.

15 **Nazlı M, Morris R:** Comparison of Localisation of the Neurokinin 1 Receptor and Nitric Oxide Synthase with Calbindin D Labelling in the Rat Spinal Cord. *Anatomia Histologia Embryologia*, 29 (3): 141-143, 2000.

16 **Garthwaite J, Boulton CL:** Nitric oxide signaling in the central nervous system. *Annu Rev Physiol*, 57: 683-706, 1995.

17 **Huang PL, Dawson TM, Bredt DS, Snyder SH, Fishman MC:** Targeted disruption of the neuronal nitric oxide synthase gene. *Cell*, 75: 1273-1286, 1993

18 **Stricker-Krongrad A, Beck B, Burlet C:** Nitric oxide mediates hyperphagie of obese Zucker rats: relation to specific changes in microstructure of feeding behavior. *Life Sci*, 58: 9-15, 1996.

19 **Morley JE, Alshaher MM, Farr SA, Flood JF, Kumar VB:** Leptin and neuropeptide Y (NPY) modulate nitric oxide synthase: further evidence for a role of nitric oxide in feeding. *Peptides*, 20: 595-600, 1999.

20 **Calka J, Wolf G, Brosz M:** Ultrastructural demonstration of NADPH-diaphorase histochemical activity in the supraoptic nucleus of normal and dehydrated rats. *Brain Res Bull*, 34: 301-308, 1994.

21 **Lima D, Coimbra A:** A golgi study of the neuronal population of the marginal zone (lamina I) of the rat spinal cord. *J Comp Neurol*, 244: 53-71, 1986.

22 **Han ZS, Zhang ET, Craig AD:** Nociceptive and thermoreceptive lamina I neurons are anatomically distinct. *Nature Neurosci*, 1: 218-225, 1998.

23 **Lima D, Coimbra A:** Morphological types of spinomesencephalic neurons in the marginal zone (lamina I) of the rat spinal cord, as shown after retrograde labelling with cholera toxin subunit B. *J Comp Neurol*, 279: 327-339, 1989.

24 **Lima D, Mendes-Ribeiro JA, Coimbra A:** The spino-latero reticular system of the rat: projections from the superficial dorsal horn and structural characterization of marginal neurons involved. *Neuroscience*, 45: 137-152, 1991.

25 **Todd AJ, Spike RC:** The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn. *Prog Neurobiol*, 41: 609-646, 1993.

26 **Dun NJ, Dun SL, Wu SY, Förstermann U, Schmidt HHHW, Tseng LF:** Nitric oxide synthase immunoreactivity in the rat, mouse, cat and squirrel monkey spinal cord. *Neuroscience*, 54: 845-857, 1993.

27 **Saito S, Kidd GJ, Trapp BD, Dawson TM, Bredt DS, Wilson DA, Traystman RJ, Synder SH, Hanley DF:** Rat spinal cord neurons contain nitric oxide synthase. Neuroscience, 59: 447-456, 1994.

28 **Wu J, Lin Q, Lu Y, Willis WD, Westlund KN:** Changes in nitric oxide synthase isoforms in the spinal cord of rat following induction of chronic arthritis. *Exp Brain Res*, 118: 457-465, 1998.

29 **Nazlı M, Morris R:** The distribution of Nitric Oxide Synthase in the Rat Spinal Cord. *Turkish Journal of Veterinary and Animal Sciences*, 25 (2): 209-215, 2001.

30 **Laing I, Todd AC, Heizmann CW, Schmidt HHHW:** Subpopulation of gabaergic neurons in laminae I-II of rat spinal dorsal horn defined by coexistence with classical transmitters, peptides, nitric oxide synthase or parvalbumin.

Neuroscience, 61: 123-132, 1994.

31 **Van den Pol AN, Patrylo PR, Ghosh PK, Gao XB:** Lateral hypothalamus: early developmental expression and response to hypocretin (orexin). *J Comp Neurol*, 433: 349-363, 2001.

32 **Sugiura Y, Lee CL, Perl ER:** Central projections of identified, unmyelinated (C) afferent fibers innervating mammalian skin. *Science*, 234: 358-361, 1986.

33 **Dafny N, Dong WQ, Prieto-Gomez C, Reyes-Vazquez C, Stanford J, Qiao JT:** Lateral hypothalamus: site involved in pain modulation. *Neuroscience*, 70: 449-460, 1996.

34 **Morley JE, Flood JF:** Effect of competetive antagonism of NO synthase on weight and food intake in obese and diabetic mice. *Am J Physiol*, 266: 164-168, 1994.

35 **Squadrito F, Calapai G, Cucinotto G, Altavilla D, Zingarelli B, Ioculano M, Urna G, Sardella A, Campo GM, Caputi AP:** Anorectic activity of NG-nitro-L-arginine, an inhibitor of brain nitric oxide synthase, in obese Zucker rats. *Eur J Pharmacol*, 230: 125-128, 1993.

36 **Lee JH, Price RH, Williams FG, Mayer B, Beitz AJ:** Nitric oxide synthase is found in some spinothalamic neurons and in neuronal processes that appose spinal neurons that express Fos induced by noxious stimulation. *Brain Res*, 608: 324-333, 1993.

37 **Meller ST, Gebhart GF:** Nitric oxide (NO) and nociceptive processing in the spinal cord. *Pain*, 52: 127-136, 1993

38 **Bredt DS, Synder SH:** Nitric oxide, a novel neuronal messenger. *Neuron*, 8: 3-11, 1992.