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LOCALISATIONS OF THE NEUROPHYSIN AND β-ENDORPHIN WITH OREXIN-A LABELLING IN THE RAT SPINAL CORD

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Summary: The novel neuropeptides called hypocretins (orexins) have recently been described in neurones of the lateral hypothalamus and shown to increase feeding and regulate the endocrine system. In this study, immunohistochemical method was used to map the localization of neurones and fibres containing-orexin-A with -neurophysin and -β-endorphin in the spinal cord of the ten adult Wistar rats. Orexin-A positive cells with their fibres and terminals that oriented towards varies directions were seen throughout the superficial lamina of the spinal cord. Neurophysin and β-endorphin cells and their fibres and terminals were observed in the superficial dorsal horn of the spinal cord.

The present study indicates that orexin-containing neurones may have an important role in modulating of a number of interrelated system functions. Additionally, orexin possibly regulates the neuroendocrine functions, feeding and pain modulation in the superficial lamina of the spinal cord

Key Words: Feeding, Hypothalamus, neuroendocrine, Orexin, pain, spinal cord.

Ratların Omuriliğinde Oreksin-A ile Nörofizin ve β-endorfinin İmmunohistokimyasal Yöntem ile Lokalizasyonu

Özet: Yeni bir nöropeptit olan hipokretin (oreksin) son yıllarda lateral hipotalamusun sinir hücrelerinde ortaya çıkarılmış olup, besin maddesi alımının artmasında ve endokrin sistemin düzenlenmesinde rolü olduğu düşünülmektedir. Bu çalışmada, on adet Wistar ratın omuriliğinde, oreksin-A ile nörofizin ve β-endorfin içeren sinir hücre ve fibrillerinin lokalizasyonunu ortaya çıkarmak amacıyla immunohistokimyasal yöntem kullanıldı. Omuriliğin tüm dorsal kornularının yüzeyinde oreksin-A pozitif hücreleri ile birçok tarafa yönelmiş fibril ve terminasyonları görüldü. Nörofizin ve β-endorfin hücreleri ile bunlara ait fibril ve terminasyonları da aynı bölgede bulundu.

Bu çalışma oreksin içeren sinir hücrelerinin birbirleriyle alakalı birçok sistemdeki fonksiyonların düzenlenmesinde önemli bir role sahip olabileceğini ortaya çıkarmıştır. Ayrıca, omuriliğin yüzeysel kısımlarında oreksinin muhtemelen ağrı modülasyonu, beslenme ve nöroendokrin fonksiyonların düzenlenmesinde rolü olabileceğini düşündürmüştür.

Anahtar Sözcükler: Ağrı, beslenme, hipotalamus, nöroendokrin, omurilik, Oreksin .

INTRODUCTION

Regulation of food intake and energy balance is ultimately controlled by complex homeostatic mechanisms involving various regions of the central nervous system[1-4]. In recent years, a number of neuropeptides including neuropeptide Y, galanin, bombesin, leptin etc. have been implicated in feeding behaviour either as a positive or negative regulator of food consumption[1,5,6]. Injections of orexin into the brain caused rats to increase their food intake and also, food restriction caused an increase in peptide synthesis[1,7]. It suggests a role for the peptide in energy metabolism. An electrophysiological study has showed that the peptide might be involved in the regulation of the neuroendocrine system[8]. The observation of the hypocretin innervation of arcuate nucleus neuropeptide Y cells may provide a signalling modality via which hypocretin could regulate endocrine systems[9]. Immunocytochemical studies have showed that orexin-positive neurones are located at all levels of the spinal cord[10]. Orexin-containing neurones have been the highest density in the lateral hypothalamus[10,11], suggesting control mechanism of feeding, arousal and autonomic control relating to both sympathetic and parasympathetic systems[10]. Since hypocretin-immunoreactive axons project to many regions of the brain, hypocretin are likely to have a regulation of the autonomic nervous system[12].

The presences of the vazopressin and neurophysin were demonstrated in the different brain regions in humans, other mammals and other species[13-16]. The distribution of vazopressin- and neurophysin-immunoreactive cells appeared to be comparable among many mammals including humans[13,15]. General topographic distribution of neurophysin-immunoreactive cells and fibres was seen in human and other mammal's brain[17]. There are some projections from parvocellular neurones of the paraventricular nucleus that contains oxytocin, vazopressin and their associated neurophysins[18-20]. The diverse efferent projections of the various vazopressin, oxytocin, or neurophysin cell groups include hypothalamo-hypophyseal portal system as well as neuronal targets within many regions of the

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central nervous system ranging from portions of the limbic system to the brainstem and spinal cord in many mammalian species, including humans[13,15,16]. Yan et al.[21] have demonstrated the existence of β-endorphin-containing proteins in the human pituitary gland. An immunocytochemical study has shown that the network of paraventricular β-endorphin-immunopositive cell bodies and their processes was present in the brain[22].

Since neurophysin and β-endorphin are hypothalamo-hypophyseal peptides[13,15,21], it would be useful to study these peptides as a marker with the orexin-A that synthesize in hypothalamic neurones. The present study was undertaken to characterize and compare the pattern of the distribution of the neurophysin and β-endorphin with orexin-A-immunoreactivities in the rat spinal cord using immunohistochemistry.

MATERIALS and METHODS

Ten adult Wistar rats, weighing approximately 250g, 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 immunohistochemical experiments were used polyclonal anti-orexin-A, anti-neurophysin and anti-β-endorphin 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-orexin-A with control peptide orexin-A (1:100).

The rats were deeply anaesthetised with sodium pentobarbitone (150 mg/kg, i.p; Rhône Mérieux Harlow, UK) and perfused transcardially via the left ventricle with Kreb's solution followed by fixative containing 4% paraformaldehyde in 0.1 M 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. Parallel to the dorsal surface (10_{μ}m) and transversal (40_{μ}m) of the spinal cord sections were cut in a sample order on a cryostat.

Immunofluorescent method as described by Dun et al.,[23] was used to visualize orexin with neurophysin and β-endorphin 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-orexin-A (1:100) 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, U.S.A.). After the sections were washed with PBS five times, the half of the all sections was incubated with anti-neurophysin (1:200) and the other half of sections was incubated with anti-β-endorphin (1:200) 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

Orexin-A-immunoreactivity was dense in cell bodies and their processes throughout the spinal dorsal horn (Figs. 1,2). Neurophysin- and β-endorphin- immunoreactive processes were detected within the neuronal cytoplasm at the dorsal surface of the spinal cord (Figs. 3,4). No immunoreactivity was observed when primary antibodies were omitted. Preabsorption of orexin-A antisera abolished immunopositive staining.

Orexin-A immunoreactivity was detected in the cytoplasm of neurones in the transversal sections of the dorsal horn (Fig. 2). Throughout the spinal cord, within the dorsal horn, laminae (L) I received immunoreactive fibres. There were many fibres which had numerous boutons and terminals characterized by asymmetrical beading on the axon (Fig. 1). These fibres in LI run to the LII and LIII of the dorsal horn.

Neurophysin-immunoreactive cell bodies and fibres were observed throughout the dorsal horn (Fig. 3). Neurones in LI had elliptical cell bodies that were oriented parallel with the surface of the cord. Fibres had many terminals running varies directions, usually parallel to the superficial dorsal horn (Fig. 3). β-endorphin-immunoreactivity was moderate in the superficial dorsal horn. The immunoreactive fibres of β-endorphin had terminals oriented parallel to the dorsal surface of the cord (Fig. 4).

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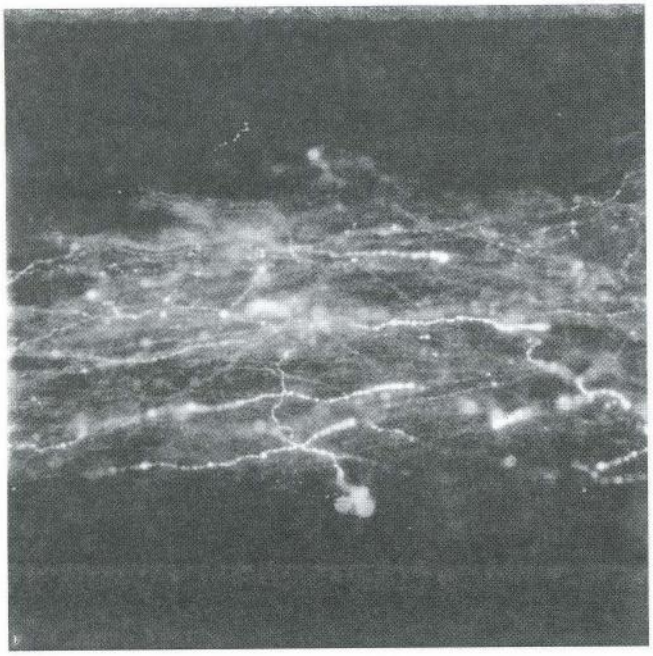

Figure 1. Photomicrograph of a parallel section of dorsal surface of the spinal cord showing orexin-A-immunoreactivity. Several fibres and terminals in LI-II.
Resim 1. Omuriliğin dorsal yüzeyine paralel olarak alınan kesitteki oreksin-A immunoreaktivitesinin görünümü. LI-II'deki oreksin-A'ya ait çok sayıda fibril ve terminasyon.

Figure 3. High power photomicrograph showing neurophysin-immunoreactivity in the marginal layer of the spinal cord.
Resim 3. Büyük büyütme (X40) ile çekilen fotografta omuriliğin en üst laminasında nörofizin'in immunohistokimyasal görünümü.

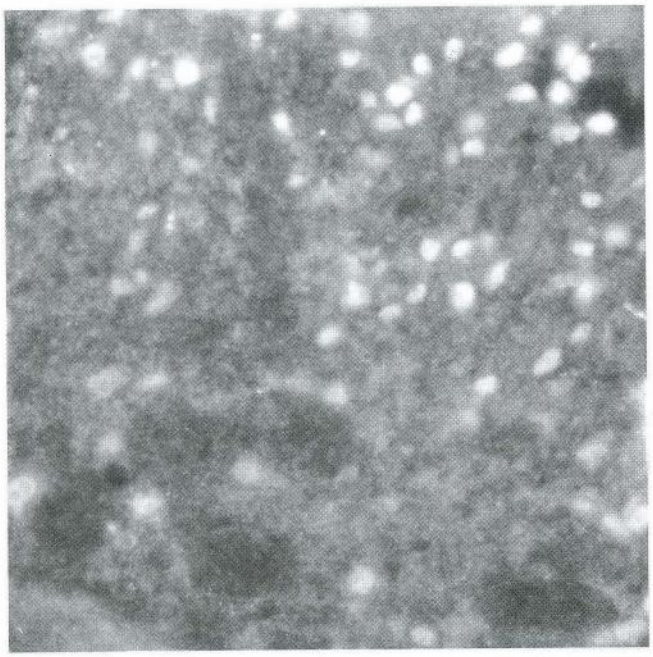

Figure 2. Photomicrograph showing the distribution of fluorescein-labelled orexin-A immunoreactive cells in LI-II of the spinal cord.
Resim 2. İmmun-fluoresan teknik kullanılarak boyanmış omuriliğin LI-II'de immunoreaktif hücrelerdeki oreksin-A'nın dağılımının görünümü.

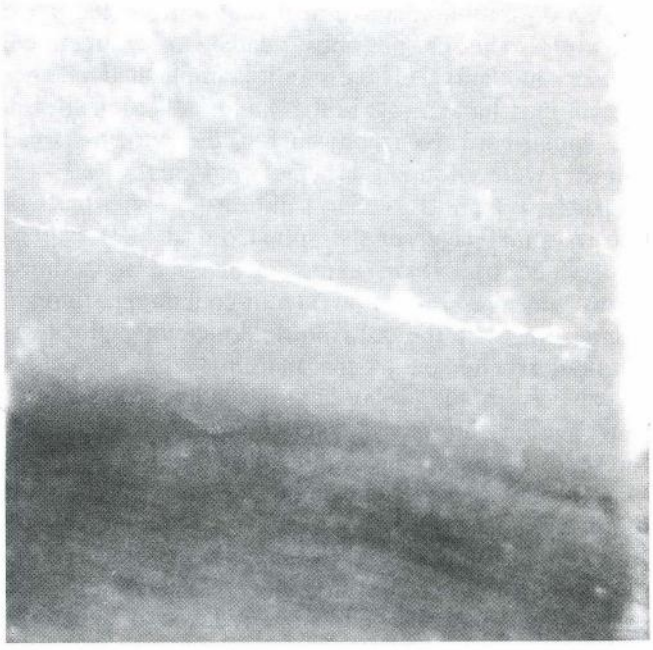

Figure 4. Photomicrograph of higher magnification sections in LI-II of the dorsal horn showing β-endorphin-immunoreactivity. **Bar:** Figures 1-4. 50 $_{u}$m.
Resim 4. Büyük büyütme (X40) ile çekilen fotograf. dorsal kornu'nun paralel kesitlerinde β-endorphin'in immunohistokimyasal görünümü. **Bar:** Resimler 1-4. 50 $_{u}$m.

DISCUSSION

The present study demonstrates that most of the fibres from the lateral hypothalamic area where the orexin-immunoreactive cells are present descend to the superficial dorsal horn of the spinal cord. These findings were in agreement generally with recent studies on the localization in the rat spinal cord of neurones containing the orexin peptides[10]. The physiological role of the orexin in the spinal cord may be addressed by examining the role of the lateral hypothalamus. It has been suggested that orexin might be involved in the regulation of feeding[1,12,24]. When centrally administered to rats orexin-A stimulate food consumption[1]. It is also thought that orexin may play a role in endocrine regulations[8-10,12].

Orexin positive neurones and their dendrites and terminals in this study were observed throughout all superficial lamina of the spinal cord. Immunoreactive orexin neurones were concentrated in the marginal layer of the cord, suggesting that hypocretin may have important roles in the modulation of pain and thermal sensation[25]. Physiological studies have shown three types of cells in LI that respond to mechanical or thermal nociception, and respond to both noxious stimuli and non-pain-related temperature changes[26,27]. The present study demonstrated that orexin-A-immunoreactive terminals surrounded some LI cells. This is possibly related to a functional sensory modality[28]. Therefore, it is implicated whether orexin may have a role in the pain modulation sites in the spinal cord. Neurophysin I- and oxytocin-stained fibres were found in the marginal zone of hypertensive and Egyptian sand rats[20]. Some parts of the marginal layer of the spinal dorsal horn regions innervated by orexin axons receive an innervation from vasopressin- or oxytocin-containing fibres[20], suggesting that the paraventriculo-spinal pathway is particularly related to the marginal zone, which is involved in the relay of ascending nociceptive information through the spinothalamic tract. In the present study, β-endorphin-immunoreactive fibres were seen in the superficial dorsal horn. It was demonstrated that the number of β-endorphin-immunoreactive arcuate neurones and a less dense endorphinergic innervation of para-ventricular neurones were reduced in wild-type mutant mice[22]. This finding suggests that the alterations in the endorphinergic hypothalamic system are a posttranslational event. It is imaginable that β-endorphin plays, along with many other regulatory functions in the hypothalamo-hypophyseal system, an important role as a direct inhibitory neurotransmitter/neuromodulator on nitric oxide synthase-immunoreactive paraventricular neurones[22,29,30].

Orexin-containing neurones and their axons have been demonstrated to selectively innervate variety of brain regions and all level of the spinal cord that could possibly play a role in setting a general tone for brain activation or inactivation[7,12] and in modulation of sensory input[10]. The paraventriculo-spinal pathway is specially related to the marginal layer. In conclusion, the results of the present study suggest that orexin-containing neurones may have a widespread role in regulation of the activities of large number of interrelated systems, such as the feeding and endocrine functions, pain modulation, sensory information in the marginal layer of the dorsal horn.

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