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IMMUNOHISTOCHEMICAL LOCALISATIONS OF OREXIN-A AND THE NEUROKININ 1 RECEPTOR IN THE RAT SPINAL CORD

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Orexins (hypocretins) are a recently described set of hypothalamic peptides that have been implicated in feeding, neuroendocrine regulation and sleep-wakefulness. The aim of this study was to investigate and compare the distribution of orexin-A- and neurokinin 1 receptor-expressing neurones in the spinal cord of adult Wistar rats using immunohistochemistry. Orexin-A immunoreactive fibres and their terminals were observed in the superficial dorsal horn. Neurokinin 1 receptor positive cells and their processes were also observed in lamina I of the dorsal horn. Although neurokinin 1 cells with orexin-A fibres were concentrated in the marginal layer of the dorsal horn, there was occasional direct anatomic contact between orexin-A and neurokinin 1 receptors in this region. The results of the present study suggest that the functions of the orexins and the neurokinin 1 receptor related to feeding, nociception, sensory information and neuroendocrine functions are possibly mediated via dependent mechanisms. Thus neurokinin 1 containing neurones could potentially receive synaptic inputs from inhibitory and excitatory interneurones in the marginal layer.

Key words: feeding, hypothalamus, neurokinin 1 receptor, orexin, pain, spinal cord

INTRODUCTION

Orexin-A (orx-A) / hypocretin-1 and orexin-B (orx-B) / hypocretin-2 are recently identified hypothalamic peptides that are derived from a single prepro-peptide and activate two closely related G-protein-coupled receptors (DeLecea *et al*., 1998; Sakurai *et al*., 1998). There have been many morphological studies showing that orexin-like immunoreactivities are highly specifically present in various regions of the hypothalamus, particularly the lateral hypothalamus (LHA) of the rat (DeLecea *et al*., 1998; Peyron *et al*., 1998; Sakurai *et al*., 1998; Van den Pol *et al*., 1998). Orexin-containing fibres and terminals are widely localized in the cerebral cortex, thalamus, brainstem and spinal cord (Date *et al*., 2000; Van den Pol, 1999; Nazli, 2001; Yamamoto *et al*., 2002; Nazli, 2003a, 2003b), suggesting that orexinergic neurones have widespread connections with other regions of the brain (DeLecea *et al*., 1998; Peyron *et al*., 1998; Nazli, 2001).

Neurones that synthesize the orexins are limited to the LHA, perifornical region; however, they send axonal projections to many regions of the central nervous system (CNS) (Peyron *et al*., 1998) including a strong distribution to the superficial dorsal horn of the spinal cord (Van den Pol, 1999; Date *et al*., 2000). The superficial dorsal horn is heavily involved in the processing of primary afferent nociceptive and thermoreceptive activity (Light, 1992; Han *et al*., 1998). These studies provide morphological evidence for a wide spectrum of physiological roles for orexin-containing neurones. Furthermore, orexins may be involved in the regulation of feeding, neuroendocrine functions, energy metabolism, water balance and the sleep-wake cycle (Peyron *et al*., 1998; Sakurai *et al*., 1998; Van den Pol, 1999; Ida *et al*., 2000). Although, several studies have been undertaken on the distribution of orexin-containing cell bodies and fibres in many species, little is known about the morphological patterns of orexinergic neurones in the superficial dorsal horn of the spinal cord.

The neuropeptide substance P (SP), is released from primary afferent nociceptors and acts via the neurokinin (NK) 1 receptor. SP is present in primary afferent axons that terminate in the superficial laminae of the spinal cord (Hökfelt *et al*., 1975; Hökfelt *et al*., 1975), and also in interneurones within the dorsal horn (Hunt *et al*., 1981). NK1 receptor-immunoreactivity was found to be the densest in lamina (L) I and moderate in LIII-VI, the central canal and intermediolateral horn (Littlewood *et al*., 1995; Nazli, 2000; Todd, 2002; Todd *et al*., 2002). In neurohistological studies, immunohistochemical detection of the NK1 receptor provides an excellent tool to study the histology and neurochemical character of specific subsets of neurones in the dorsal horn of the spinal cord (Goodchild *et al*., 2000).

The overlapping localization between orx-A and NK1 receptors in superficial laminae of the dorsal horn raises the possibility that the orexin system may interact with the NK1 receptor system in functional activities. Different morphological types of neurones have been identified in LI of the rat (Lima and Coimbra, 1986). The dorsal horn plays an important role in the transmission of pain-related information from nociceptive primary afferent neurones to the brain. However, our knowledge of its neuronal and synaptic organisation is still limited (Han *et al*., 1998). The four LI neurone types have been identified in the superficial dorsal horn of the rat, i.e. fusiform, flattened, pyramidal and multipolar (Lima and Coimbra, 1986, 1989). The variety of its constituent synaptic mediators and these data (Lima and Coimbra, 1989) indicate that the superficial dorsal horn is a complex neural system. Thus, the present study was undertaken to investigate if the NK1 receptor was co-localized in the orexin neurones in the superficial dorsal horn of the Wistar rat. Double-labeling immunofluorescent immunocytochemistry for orexin and NK1 receptors was employed.

MATERIAL AND METHODS

Eight adult Wistar rats, weighing approximately 250 g, were used in this study. The rats were group housed in standard cages and maintained in a 12 h light/dark cycle at 55% humidity, with food and water ad libitum. The primary

antisera for immunocytochemical experiments were polyclonal anti-orexin-A and anti-neurokinin 1 receptor antisera. All antisera were diluted in 0.1 M phosphate buffered saline (PBS; pH 7.2), containing 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 PBS. All subsequent steps were performed at room temperature unless indicated otherwise. The spinal cord was removed, post-fixed in fixative containing 4% paraformaldehyde in 0.1M PBS (4-6 h) and then cryoprotected with 30% sucrose in 0.1 M PBS overnight at 4 ^{o}C. Parallel to the dorsal surface of the spinal cord (10 μm), sections were cut in order on a cryostat.

The immunofluorescent method described by Dun *et al*. (1993) was used to visualize orx-A and NK1receptor localization. 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 ^{o}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 had been washed with PBS, all sections were incubated with anti-NK1receptor (1:10000) (Gift from Dr S. Vigna, Duke University Medical Center, Durham, USA) for 3 days at room temperature. Subsequently, sections were placed in anti-rabbit CY3 (1:150) (Jackson). Finally, the 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

Immunoreactive staining was not observed when primary antibodies were omitted. Preabsorption of orexin-A antisera abolished immunopositive staining.

Orexin-A immunoreactivity was seen in LI in the transversal section of the spinal cord (Fig 1) Orexin-A immunoreactive fibres had various orientations and many terminals in LI in parallel sections of the dorsal surface (Figs. 2, 3). NK1 receptor-immunoreactivity was dense in the marginal layer of the dorsal horn (Figs. 2, 3). Many heavily labelled LI neurones were surrounded by fibres and varicosities with intense NK1 receptor immunoreactivity that formed a dense network throughout LI. NK1 expressing neurones that usually had four-five dendrites were of the pyramidal type (Figs. 2, 3). The dendrites of these neurones were long and projected in various directions in LI. Double-immunocytochemistry showed that orexin-A immunoreactive fibres also occasionally made synaptic contact with the immunoreactive cell bodies (soma) and dendrites of NK1 in the marginal layer of the dorsal horn (Figs. 2, 3).

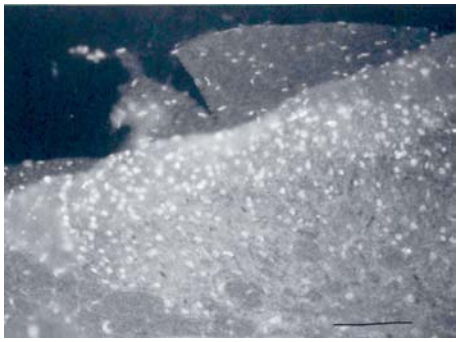

Figure 1. Photomicrograph of the spinal cord showing orexin-A-immunoreactive fibres and their terminals in the marginal layer of the dorsal horn, transversal section. Bar: 100 μm

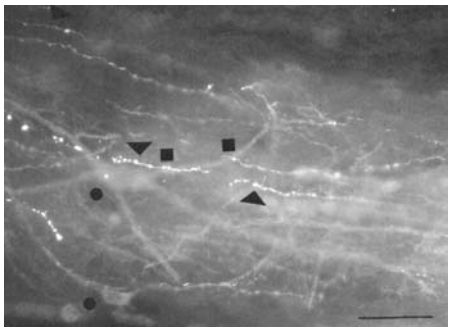

Figure 2. Photomicrograph showing Orx-A- (arrowheads) and NK1-immunoreactivities (small roundheads) in LI. There were occasional synaptic contacts between Orx-A fibres and NK1 cells and dendrites (small squares). Pyramidal type cells for NK1 receptor and their dendrites. Bar: 50 μm

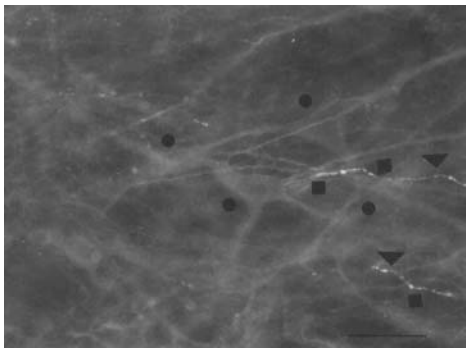

Figure 3. Photomicrograph showing double labelling of Orx-A and NK1 immunoreactivities (small roundheads) in LI of the dorsal horn. Orx-A-immunoreactive fibres and their terminals (arrowheads). Occasionally seen synaptic contacts between Orx-A fibres and NK1 cells and their dendrites (small squares). Bar: 50 μm

DISCUSSION

By the use of immunohistochemistry, the present study provides direct evidence of synaptic contact between the orexin-A and NK1 receptor-producing neural systems of the Wistar rat spinal cord. Many immunoreactive orx-A fibres and terminals were seen particularly in LI and moderately in LII of the dorsal horn, which confirms other studies (Date *et al*., 2000; Van den Pol, 1999; Nazli, 2001; Grudt *et al*., 2002; Yamamoto *et al*., 2002; Nazli, 2003a, 2003b). Thus, the NK1 receptor was previously found in dorsal horn neurones (Nazli, 2000; Yamamoto *et al*., 2002), and was suggested as a useful marker for subpopulations of excitatory neurones (Hunt *et al*., 1981; Marshall *et al*., 1996).

Orexins were initially implicated in the control of feeding behavior, regulation of the neuroendocrine system and energy balance based on the observation that intracerebroventricular injection of orexins stimulates food intake (Sakurai *et al*., 1998). Further, studies reported that orexin might play a role in endocrine regulation (Van den Pol *et al*., 1998; Van den Pol, 1999; Peyron *et al*., 1998). An electrophysiological experiment using cultured LHA cells demonstrated that both orx-A and-B induced enhancement of neuronal activity, which occurred as soon as synaptic activity was detected, suggesting that orexin might contribute to the development of arousal, sleep regulation, feeding and endocrine control (Van den Pol, 2001). The large number of orexin terminals in synaptic contact with other neurones of the LHA suggests that orexin neurones are in an excellent position to modulate the excitability of other neurones with these sensing capabilities.

Spinal cord and other extrahypothalamic projections from the hypothalamus have been described previously, especially a projection from the parvocellular neurones of the paraventricular nucleus that contains oxytocin and vasopressin (Swanson, 1977; Swanson and McKeller, 1979; Van den Pol and Collins, 1994). These axons innervate LI and LX, supporting our finding of a partial overlap with the projection from orexin fibres. Inferences about orexin function can be made on the basis of the laminae in the spinal cord that orexin preferentially innervates. The innervation of the marginal layer, LI, of the cord suggests that orexin may be involved in modulation of pain and thermal sensation. Intrathecal administration of orx-A suppressed the induction of Fos-like immunoreactivity induced by paw formalin injection in LI-II of the spinal cord, suggesting that the orx-A receptor was involved in nociceptive transmission in the spinal cord (Yamamoto *et al*., 2002). Date *et al*. (2000) demonstrated that orexin fibres were concentrated in LI of the dorsal horn. This region directly receives pain and thermal stimuli (Sugiura *et al*., 1986) and relays nociceptive information to the LHA, brainstem and thalamus. The LHA is an area that attenuates nociceptive transmission (Dafny *et al*., 1996). The orexin pathway from the LHA to the spinal cord may be involved in the nociceptive modulator system. This is consistent with observations that stimulation of the LHA in the region of orexin neurones produces pain analgesia (Franco and Prado, 1996), which in turn raises the question of whether or not orexin might possess antinociceptive properties in the cord.

NK1 expressing neurones and their dendrites were localized in LI of the dorsal horn. These neurones resemble the pyramidal types. The population of NK1 cells in LI, which is nociceptive, ascends to the thalamus (Marshall *et al*., 1996). SP-containing primary afferents terminate mainly in LI and LII (Willis and Coggeshall, 1991) and SP released from these fibres is therefore likely to act on neurones which possess NK1 receptors in these laminae. Some NK1 neurones in LI of the rat spinal cord are thought to be inhibitory interneurones which use GABA as a transmitter, while it is likely that most or all of the remaining neurones in these laminae are excitatory cells, which release glutamate (Antal *et al*., 1991; Todd *et al*., 1994). LI neurones could potentially receive a wide variety of synaptic inputs from primary afferents of various modalities, as well as from inhibitory and excitatory interneurones in these laminae (Todd and Spike, 1993).

In conclusion, orx-A immunoreactive fibres and terminals were moderately localized in LI-LII of the dorsal horn. Double immunolabeling results demonstrated that orx-A immunoreactive fibres occasionally made synaptic contact with the NK1 expressing cell bodies in LI of the dorsal horn. The unique neuronal distribution of orexins and their functional activation of neural circuits suggest specific complex roles for the peptides in neuroendocrine control. These results indicate that the NK1 receptor may play a coordinated role in the food intake, modulation of pain and thermal sensation-regulating activity of orx-A cell processes. Clarification of the pathways involved in orexin actions will depend upon better knowledge of the circuitry of the marginal layer of the dorsal horn.

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IMUNOHISTOHEMIJSKA LOKALIZACIJA RECEPTORA ZA OREKSIN-A I NEUROKININ 1 U KIČMENOJ MOŽDINI PACOVA

NAZLI M

SADRŽAJ

Oreksini (hipokretini) su nedavno opisani set hipotalamusnih peptida, koji učestvuju u ishrani, neuroendokrinoj regulaciji i stanju budnosti i sna. Cilj ovih istraživanja je bio imunohistohemijsko ispitivanje i upoređivanje distribucije oreksin-A i neurokinin 1 receptora ekspresornih neurona u kičmenoj moždini odraslih Vistar pacova. U površnim delovima dorzalnih rogova su utvrđena nervna vlakna i završeci imunoreaktivni na oreksin-A. Neurokinin 1 receptor pozitivne ćelije i njihovi nastavci su takođe utvrđeni u lamina I dorzalnih rogova. I ako su neurokinin 1 ćelije i oreksin -A vlakna skoncentrisana u marginalnom sloju dorzalnih rogova, povremeno se u ovoj oblasti javlja direktna anatomska veza između receptora za oreksin- A i neurokinin 1. Rezultati ove studije sugerišu na to da se uloga oreksina, sa neurokinin receptorima u ishrani, nociocepciji, senzornoj infomaciji i neuroendokrinoj funkciji verovatno obavlja preko zavisnih mehanizama. Na ovaj način, neuroni, koji sadrže neurokinin 1 mogu potencijalno primiti ulazni sinaptički impuls od inhibitornih i ekscitatornih interneurona u marginalnom sloju.