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Research Report

# Localization of orexin-A-immunoreactive fibers in the mesencephalic trigeminal nucleus of the rat

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**Abstract**

Orexin A is a neuropeptide located exclusively in neurons in the hypothalamic nuclei involved in the central regulation of many brain functions, related to motor activity and state-dependent processes. Orexins modulate behavioral state via actions across multiple terminal fields. In order to determine whether the mesencephalic trigeminal neurons may receive a direct hypothalamic orexinergic input, the distribution of orexin A immunoreactivity was examined in the rat mesencephalic trigeminal nucleus (MTN), using orexin A immunohistochemistry. Orexin-A-immunostained nerve fibers and terminals were found in a close apposition to the perikarya of primary afferent neurons in the MTN with a marked rostrocaudal gradient in their density. In the caudal pontine MTN, only scattered orexin-A-immunoreactive fibers were found, while more rostrally in the pons, and in the midbrain–pontine junction part of the nucleus, orexin-A-immunopositive varicosities were relatively more abundant, located in close proximity to or often surrounding the neuronal profiles. At the level of the inferior or superior colliculi, a large number of orexin-A-containing neuronal processes and terminal arborizations were observed traveling toward and contacting mesencephalic trigeminal neurons, some of which were multipolar. The results of this study show that MTN neurons receive orexin A hypothalamic innervation with a somatotopic arrangement of the projections in the nucleus. The central orexinergic system may exert direct influence upon jaw movements at the level of the MTN and thus to participate in the control of feeding behavior.


*Theme:* Neural basis of behavior
*Topic:* Neuropeptides and behavior

*Keywords:* Orexin A; Immunohistochemistry; Feeding behavior; Mesencephalic trigeminal nucleus; Rat

## 1. Introduction

Orexin A and B, also known as hypocretins, are recently discovered hypothalamic peptides, which are specific ligands for two different receptors belonging to the G-protein-coupled receptor family [29]. Orexins are located exclusively in a relatively small population of neurons in the posterior and lateral hypothalamic perifornical region and the dorsomedial hypothalamic nucleus [7,25]. These are areas which are classically established to be involved in the central regulation of behavioral state and state-dependent processes [6,30]. Orexin circuits have also been implicated in the facilitation of mastication and feeding behavior [5,14,29]. In fact, orexinergic neurons give rise to an extensive projection system, part of which innervate the groups of neurons associated with the genesis of the masticatory pattern and thus may contribute to the final masticatory motor output (reviewed in [18]).

Despite the restricted location of orexinergic neuronal somata, their fibers and terminals are widely distributed throughout the brain. Orexinergic efferent projections are found to reach the brainstem nuclei involved in the control of feeding, including the trigeminal sensory and motor nuclei [4,25,28,33]. A previous study has also reported that both the orexin receptor-1 and -2 are expressed at protein and mRNA levels in the mesencephalic trigeminal nucleus (MTN) of the rat. On the other hand, orexin A has a high but equal affinity

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for the two receptors, while orexin B has a 10-fold higher affinity for orexin receptor-2 than for orexin receptor-1 [29]. However, except for a recent report on the innervation of the rat MTN neurons from hypothalamic orexin B-immunoreactive fibers [32], little is known as to whether orexin-A-containing neurons send descending projections to this nucleus. More recently, Zhang et al. [33] revealed the presence of orexin-A- and orexin-B-containing axonal projections to the feline MTN, albeit with a different staining density of the orexinergic fibers in the nucleus. Nevertheless, data about the distribution and functioning of the orexinergic system in the MTN in rats are far from complete.

MTN contains primary sensory neurons innervating jaw-closing muscle spindles and mechanoreceptors associated with the teeth [1,2,13]. In their turn, MTN neurons transfer proprioceptive information to the muscle of mastication through their monosynaptic connections with trigeminal motoneurons for controlling jaw movements ([21], reviewed in [16,17]). In addition, jaw-closing motoneurons receive inputs from jaw-muscle spindle afferents through premotoneurons, located in the area surrounding the trigeminal motor nucleus (see [23], and references therein). Furthermore, it has been reported that the proprioceptive afferent feedback to trigeminal motoneurons is crucial for modulating and coordinating the activity of the masticatory muscles during oral motor behaviors [20]. Hence, MTN neurons constitute a neuronal circuit of the mastication control, an important part of feeding behavior.

Therefore, the aim of the present study was to determine whether the primary trigeminal afferent neurons in the rat MTN may receive a direct input from hypothalamic orexin-A-containing efferents. We examined the distribution of orexin A reactivity by immunohistochemistry at the light microscopic level with a subtype-specific antibody.

## 2. Materials and methods

### 2.1. Animals and tissue preparation

Eight adult male Sprague–Dawley rats (250–350 g b.w.) were used in this study. All housing facilities and procedures used were supervised and approved by the Animal Care and Use Committee of the Thracian University and were consonant with the guidelines established by the NIH. The animals were deeply anesthetized with Ketanest (50 mg/kg i.p., Pfizer, New York, USA) and transcardially perfused, first with 100 ml heparinized cold 0.9% NaCl (1 U heparin/ml saline) and followed by 500 ml 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). After perfusion, the brains were extracted, blocked, and postfixed in the same fixative solution for 5–6 h at 4 °C, and then cryoprotected in 20% sucrose in PBS overnight at 4 °C. The brains were embedded in TissueTek OCT compound (Miles Inc., Elkhart, NI, USA), frozen, and 20 μm thick sections were cut in a cryostat at −20 °C. The sections were separated into five series, according to the method proposed by Guillery and Herrup [10]. After rinsing in 0.1 M PBS, each complete series of one-in-five frontal sections, stretching through the entire rostrocaudal dimension of the MTN, were processed for orexin-A immunohistochemistry.

### 2.2. Immunohistochemistry

The immunohistochemical staining procedure was performed on free-floating sections according to the ABC (avidin–biotin–horseradish peroxidase) method [11]. Briefly, specimens were treated with hydrogen peroxide (0.3% in absolute methanol; 30 min) to inactivate endogenous peroxidase, and the background was blocked with 5% normal goat serum. The primary antibody, polyclonal rabbit anti-orexin A (Oncogene, Cambridge, MA, USA, Cat.# PC345), was applied for 24 h at room temperature. It was diluted 1:1000 in a solution that blocks non-specific antibody binding and promotes penetration of the antibody into the tissue, containing 1 ml 0.05% of the preservative thimerosal (Fluka, Buchs, Switzerland), 1 ml 0.1% bovine serum albumin, 1 ml 10% normal goat serum, 1 ml 0.01% sodium azide, and 6 ml 0.1 M PBS. After rinsing in PBS, sections were incubated with the secondary antibody, biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA), and diluted 1:160 in PBS containing 1% normal goat serum and 0.1% Triton X-100, for 6 h at room temperature. After washing the sections, the ABC complex (Vector, 6.25 μl/ml of each compound in PBS) was applied. Following rinsing, peroxidase activity was visualized using 2.4% SG substrate kit for peroxidase (Vector) in PBS for 5 min at room temperature. To reveal the precise location of MTN neurons and labeled fibers in the brainstem, we counterstained them with 0.5% Neutral Red (Sigma, St. Louis, MO, USA). Finally, the sections were dehydrated in a graded series of alcohols, cleared in xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany).

Negative controls included sections that were incubated in the absence of the primary antibody or in the presence of non-immune normal serum in the same dilution as the primary antibody, as well as antigen–antibody preabsorption experiments with the native antigen orexin A ($10^{-6}$ M, Oncogene, Cambridge, MA, USA, Cat.# PC345-100 UG) at 4 °C for 24 h.

In this study, the presence of orexin-A-immunoreactive fibers and their distributional pattern in the MTN was determined semi quantitatively from very sparse (±), sparse (+), and moderate (++) to dense (+++). The relative expression levels were subjectively assessed by visual comparison, and the results of the analysis are summarized in Table 1.

### 2.3. Data analysis and photomicrograph production

After immunostaining, the sections were photographed with AxioCam MRC digital camera linked to a Zeiss