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Research report

Genioglossal hypoglossal muscle motoneurons are contacted by nerve terminals containing delta opioid receptor but not mu opioid receptor-like immunoreactivity in the cat: a dual labeling electron microscopic study

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Abstract

This study has investigated (1) the distribution of delta opioid receptor (DOR) or mu opioid receptor (MOR) containing elements in the hypoglossal nucleus of the adult cat; and (2) the association of these processes with retrogradely labeled genioglossus muscle motoneurons. Cholera toxin B conjugated to horseradish peroxidase (CTB-HRP) was injected into the genioglossus muscle on the right side of four isoflurane-anesthetized cats. Forty-four to 52 h later, the animals were sacrificed. Motoneurons containing HRP were labeled with a histochemical reaction utilizing tetramethylbenzidine (TMB) as the chromogen. The tissues were then processed for immunocytochemistry, using an antiserum raised against DOR or MOR using diaminobenzidine (DAB) as the chromogen. At the light microscopic level, retrogradely labeled cells were observed primarily ipsilaterally in ventral and ventrolateral subdivisions of the hypoglossal nucleus. The majority of these labeled cells were observed immediately caudal to obex. DOR-like immunoreactive processes were apparent at the light microscopic level in the hypoglossal nucleus, but MOR-like immunoreactive processes were not. Both DOR and MOR-like immunoreactive processes were observed in other brainstem areas such as the spinal trigeminal nucleus. At the electron microscopic level, DOR-like immunoreactive nerve terminals formed synaptic contacts with retrogradely labeled genioglossus muscle motoneuronal dendrites and perikarya in the hypoglossal nucleus. Nineteen (19) percent of the DOR terminals contacted retrogradely labeled genioglossus muscle motoneurons. DOR-immunoreactive terminals also synapsed on unlabeled dendrites and somata. Few MOR-like immunoreactive terminals were found at the EM level in the hypoglossal nucleus, and none of these terminals contacted retrogradely labeled neuronal profiles from the GG muscle. These are the first ultrastructural studies demonstrating synaptic interactions between functionally identified hypoglossal motoneurons and DOR terminals, and that enkephalins most likely act presynaptically to modulate the release of other neurotransmitters that affect GG motoneuron activity. These studies demonstrate that hypoglossal motoneurons which innervate the major protruder muscle of the tongue, the genioglossus muscle, are modulated by terminals containing DOR, and that enkephalins acting on DOR but not MOR in the hypoglossal nucleus may play a role in the control of tongue protrusion.
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1. Introduction

Tongue muscles are under the direct control of the hypoglossal nerve (the twelfth cranial nerve). The hypoglossal nerve originates from the hypoglossal nucleus. It has been shown in several species that hypoglossal motoneurons are myotopically arranged [5,12,14,18,19,21].

Nerve terminals containing enkephalins have been described in the hypoglossal nucleus [1,9]. All regions of the hypoglossal nucleus contained both leu- and met-enkephalin; however, density was greatest in the ventral

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subcompartments where protrusor muscle motoneurons are located. It is not known which opiate receptor subtype(s) the enkephalins act upon in the hypoglossal nucleus, nor is it known whether the enkephalins act pre- or postsynaptically. There are five major subtypes of opiate receptors: the mu receptor, delta receptor, kappa receptor, epsilon receptor, and sigma receptor. Opiates have been long known as respiratory depressants in most species. Systemic administration of opiates such as morphine produces a profound decrease in hypoglossal nerve activity in the cat [6]. In the present study, we have used a modified double-labeling electron microscopic method [13] to retrogradely label hypoglossal motoneurons that selectively innervate the genioglossus muscle while simultaneously utilizing immunocytochemistry to visualize central nervous system afferents containing either the delta opioid receptor (DOR) or the mu opioid receptor (MOR). With this technique, one could also ascertain whether the receptors are found in postsynaptic elements as well. The focus of the present study is to determine the location of DOR and MOR within the hypoglossal nucleus and determine the relationship between these processes with genioglossus muscle motoneurons. A preliminary report of these findings has been previously published [16].

2. Materials and methods

Principles of animal care (NIH publication no. 86-23, revised 1985) were followed. All procedures involving animals were approved by the Animal Care and Use Committee of Howard University. Four adult mongrel cats of either sex were sedated with a ketamine/acepromazine (10 mg/kg, i.m.) solution. The animal was intubated and anesthetized with isoflurane. Ten (10) microliters of the beta unit of cholera toxin conjugated to horseradish peroxidase [CTB-HRP (1% in 50% dimethylsulfoxide) List Biochemicals, Campbell, CA] was injected into the right half of the genioglossus muscle from a submental approach. In most animals, there were three injections made into the right half of the genioglossus muscle of approximately 3 μl each. The lateral branch of the right hypoglossal nerve was cut to prevent any nonselective transport of the tracer from retrusor muscles in the area. Two days later, the animals were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused sequentially through the aorta with: (1) 1 l of phosphate buffered saline (PBS), pH 7.4 containing 10,000 U of heparin which was bubbled with 95% oxygen/5% carbon dioxide for 30 min prior to use; (2) 4 l of a solution of 0.5% paraformaldehyde and 2.0% acrolein in phosphate buffer. The brainstem was then removed, blocked, and washed in PBS. Approximately 40-μm-thick sections of brain were cut through n.XII, using a Vibratome and collected in PBS. Every seventh section was processed for light microscopy. For light microscopy, tissues were mounted on glass slides, dehydrated in alcohol, cleared in xylene, coverslipped with Permount, and examined in a Nikon FXA photomicroscope equipped with differential interference contrast optics.

The histochemical method used is that that of Llewellyn-Smith et al. [13]. Sections are preincubated for 20 min in a solution containing 100 ml of 0.1 M sodium phosphate buffer, pH 6.0, 5.0 ml of 1% ammonium paratungstate, 1.0 ml of 0.4% NH_4Cl, 1.0 ml of 20% D-glucose, and 1.25 ml of 0.2% tetramethylbenzidine (TMB) free base in ethanol. Subsequently, the sections are incubated in another 100 ml of the same solution to which is added 100 μl of glucose oxidase. The development of the blue crystalline reaction product, which is formed by the glucose oxidase reaction, is monitored under a dissecting microscope and is usually completed within 30 min. The sections are washed three times in fresh 0.1 M phosphate buffer, pH 6.0 for 10 min each, and then the reaction product is stabilized and enhanced with a cobalt chloride–diaminobenzidine (DAB)–glucose oxidase reaction. Briefly, tissues are incubated for 7 min in a solution containing 100 ml 0.1 M sodium phosphate buffer, pH 6.0, 100 mg diaminobenzidine, 1.0 ml of 0.4% NH_4Cl, 1.0 ml of 20% D-glucose, 2 ml of 1% $CoCl_2$ in water, and 100 μl of glucose oxidase. This second glucose oxidase reaction is terminated by washing in excess sodium phosphate buffer.

The immunocytochemical procedure utilized is an avidin–biotin based method utilizing the Vectastain Elite ABC kit. Briefly, sections are processed through the following incubations: (1) 18–24 h in primary antisera against DOR or MOR (Immunostar, Minnesota) generated in the rabbit diluted 1:1000; (2) 60 min in a 1:200 dilution of biotinylated goat antirabbit secondary IgG (Vector Labs); (3) 60 min in avidin–biotin–HRP complex. All dilutions and washes separating each incubation are in 0.1 M PB/saline/0.1% bovine serum albumin. HRP reaction product is visualized by the glucose oxidase reaction described above using diaminobenzidine as the chromogen in order to obtain an amorphous reaction product at the level of the electron microscope. The duration of this second glucose oxidase reaction is determined by observation through a dissecting microscope and varies between 4 and 10 min. Controls were performed by eliminating the primary antibody and processing the tissue with normal serum instead.

Tissues to be processed for electron microscopy are washed, osmicated, dehydrated, and flat embedded in Epon 812. Silver–gold ultramicrotome sections taken from the surface of blocks of embedded tissue are collected on grids, counterstained with 5% uranyl acetate and Reynolds lead citrate, and examined in a JEOL JEM-1210 transmission electron microscope at 50 kV in the high contrast mode. At least 10 grids containing 2–5 thin sections each, obtained from two plastic embedded sections from each of four cats, were examined. All DOR-labeled terminals were quantified and were described as (a) apposing unlabeled (no retrogradely labeled) dendrites and soma; (b) those contacting unlabeled dendrites and soma; (c) those apposing labeled

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