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Research report

Nitrergic innervation of trigeminal and hypoglossal motoneurons in the cat

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Abstract

The present study was undertaken to determine the location of trigeminal and hypoglossal premotor neurons that express neuronal nitric oxide synthase (nNOS) in the cat. Cholera toxin subunit b (CTb) was injected into the trigeminal (mV) or the hypoglossal (mXII) motor nuclei in order to label the corresponding premotor neurons. CTb immunocytochemistry was combined with NADPH-d histochemistry or nNOS immunocytochemistry to identify premotor nitrergic (NADPH-d$^+$/CTb$^+$ or nNOS$^+$/ CTb$^+$ double-labeled) neurons. Premotor trigeminal as well as premotor hypoglossal neurons were located in the ventro-medial medullary reticular formation in a region corresponding to the nucleus magnocellularis (Mc) and the ventral aspect of the nucleus reticularis gigantocellularis (NRGc). Following the injection of CTb into the mV, this region was found to contain a total of 60 ± 15 double-labeled neurons on the ipsilateral side and 33 ± 14 on the contralateral side. CTb injections into the mXII resulted in 40 ± 17 double-labeled neurons in this region on the ipsilateral side and 16 ± 5 on the contralateral side. Thus, we conclude that premotor trigeminal and premotor hypoglossal nitrergic cells coexist in the same medullary region. They are colocalized with a larger population of nitrergic cells (7200 ± 23). Premotor neurons in other locations did not express nNOS. The present data demonstrate that a population of neurons within the Mc and the NRGc are the source of the nitrergic innervation of trigeminal and hypoglossal motoneurons. Based on the characteristics of nitric oxide actions and its diffusibility, we postulate that these neurons may serve to synchronize the activity of mV and mXII motoneurons.

Theme: Motor systems and sensory motor integration
Topic: Spinal cord and brainstem

Keywords: Nitric oxide; Motor control; Medulla; Premotor interneurons; Neuromodulation; Reticular formation

1. Introduction

Somatic motoneurons are perhaps the most completely studied cells in the central nervous system. There is a wealth of data dealing with their structure, function, electrophysiological properties, as well as their synaptic processes, including the neurotransmitters and neuromodulators that control their activity. In a comprehensive review, numerous substances including amino acids, biogenic amines, and peptides that act as neurotransmitters and neuromodulators are described in the synaptic processes that impinge on these cells [40].

In recent years, nitric oxide (NO) has joined the list of neuromodulatory substances that act on neurons in the central nervous system [6,19]. We have previously reported that synaptic processes, containing the neuronal isoform of nitric oxide synthase (nNOS), are present in close apposition to the dendrites and cell bodies of trigeminal motoneurons in the guinea pig and that nitric oxide exerts an excitatory effect on these cells [1].

Trigeminal motoneurons participate in jaw movements associated with numerous behaviors such as mastication, deglutition, and vocalization [27,28]. During these and related motor acts, a precise coordination takes place between the jaw musculature innervated by trigeminal motoneurons and the tongue musculature that is innervated by hypoglossal motoneurons. It has been suggested that

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this coordination depends on circuits that involve overlapping or shared structures controlling both motor pools [2,13,14,24,25,47].

The present study was designed to determine the location of nitrergic premotor neurons that project to the trigeminal and hypoglossal motoneurons in the cat. An examination of the origins of the innervation of trigeminal motoneurons, prompted by our previous work [1], was combined with a study of the innervation of hypoglossal motoneurons. By means of retrograde and nNOS labeling techniques, we traced the origin of the nitrergic innervation of these brainstem motor nuclei to a population of cells within the ventro-medial medullary reticular formation. Accordingly, a novel population of premotor neurons was discovered in the present work. A portion of these data has been previously reported [32].

2. Methods

Thirteen adult cats of both sexes, weighing between 3.0 and 3.5 kg, were utilized. Eight cats were employed for studies of the trigeminal nucleus and five for experiments on the hypoglossal nucleus.

2.1. Surgical procedures

All experimental procedures were conducted in accord with the *Guide for the Care and Use of Laboratory Animals* (7th edition, National Academy Press, Washington, D.C. 1996) and approved by the Chancellor's Animal Research Committee (ARC) of the UCLA Office for the Protection of Research Subjects (OPRS). Before anesthesia, the animals were premedicated with atropine (0.04 mg/kg, i.m.) and Xylazine® (2 mg/kg, i.m.). Anesthesia was first induced with Ketamine® (15 mg/kg, i.m.) and maintained with a gas mixture of isoflurane in oxygen (2–3%). The head of the cat was positioned in a heavy-duty stereotaxic frame and the calvarium was exposed. A 3.0- to 4.0-mm-diameter hole was drilled in the calvarium overlying the cerebellar cortex; after surgery, the hole was covered with bone wax. This hole provided access to the trigeminal or the hypoglossal nuclei for injection of CTb, as described below. During recovery from surgery, an analgesic (Buprenex® 0.01 mg/kg, i.m.) was administered and an antibiotic (Cephazolin®) was given parenterally for 4 days. All wound margins were regularly cleaned and covered with an antibiotic ointment (Fougera®).

2.2. Micropipette and microelectrode assembly for the injection of cholera toxin subunit b (CTb) and for recording antidromic field potentials

In order to inject CTb, a three-barreled micropipette assembly, consisting of a carbon fiber recording microelectrode and two side barrels containing CTb (List Biological Laboratories, Campbell, CA), was lowered into the trigeminal (stereotaxic coordinates: P4.6, L3.5, H−4; [3]) or the hypoglossal motor nucleus (stereotaxic coordinates: P12.7, L1.2, H−6.5; [3]). The exact location of these motor nuclei in each animal was resolved by recording the antidromic field potential evoked by electrical stimulation of the masseter and hypoglosssal nerves, respectively, as described in previous communications [7,18]. CTb was injected by iontophoresis (2 μA positive current pulses, 7 s on, 7 s off, for 20 min in each barrel) at a site where the antidromic field potential was largest (4 to 5 mV, [7,18,31]).

2.3. Perfusion and immunohistochemical procedures

Ten to fourteen days after the injection of CTb, the animals were sacrificed with an overdose of sodium pentobarbital and perfused with heparinized saline, followed by a solution of 4% paraformaldehyde, 15% saturated picric acid, and 0.25% glutaraldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. The brainstem was removed and immersed for a 24-h post-fixation period in a solution consisting of 2% paraformaldehyde and 15% saturated picric acid in 0.1 M PB at pH 7.4. Following post-fixation, the tissue was kept in a solution of sucrose (25%) in 0.1 M PB at pH 7.4 for 2 days. The brainstem was frozen and cut into 15-μm-thick coronal sections using a cryostat. Each section was placed in one well of a 36-well tray containing a buffered solution of 0.1 M PBS, 0.3% Triton X-100 (PBST), and 0.1% sodium azide. The first section obtained was placed in the first well of the tray and consecutive sections were placed in the remaining wells in serial order. Section number 37 was placed in well 1 and the procedure was repeated until the entire brainstem was sectioned. Therefore, each well contained a sample of the entire brainstem consisting in a set of sections separated by 540 μm from one another (i.e., 15 μm × 36). Using this procedure, neighboring wells contained adjacent sections.

Free-floating sections were processed first for CTb immunocytochemistry and then for either nNOS immunocytochemistry or NADPH-d histochemistry. For CTb immunocytochemical detection, sections were first incubated in goat antiserum against CTb (List Biological Laboratories, CA.) at a dilution of 1:40,000 in PBS–0.25% Triton–0.1% Na Azide (PBST-AZ), pH 7.4 at 4 °C with gentle agitation for 72 h or at 1:20,000 overnight at room temperature. The sections were rinsed over a 30-min period and placed for 90 min at room temperature in biotinylated donkey anti-goat serum (Jackson Immuno Research Laboratories, West Grove, PA) diluted 1:2000 with PBST. After rinsing for 30 min, the sections were incubated in a standard ABC complex (Vector Standard Elite kit, Vector Laboratories, Burlingame, CA) for 90 min at room temperature at a dilution of 1:400. The tissues were then rinsed for a total of 30 min and then processed with the Nickel Ammonium Sulfate enhanced DAB method consisting of immersion in 0.6% Nickel Ammonium Sulfate,