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Differential pontomedullary catecholaminergic projections to hypoglossal motor nucleus and viscerosensory nucleus of the solitary tract

Irma Rukhadze *, Leszek Kubin

Department of Animal Biology, School of Veterinary Medicine, and Center for Sleep and Respiratory Neurobiology, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA 19104-6046, USA

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Abstract

In individuals with a narrow or collapsible upper airway, sleep-related hypotonia of upper airway muscles leads to recurrent airway obstructions. Brainstem noradrenergic neurons reduce their activity during slow-wave sleep and become silent during rapid eye movement sleep; this may cause state-dependent changes in the motor output and reflexes. The loss of noradrenergic excitation is a major cause of sleep-related depression of activity in upper airway muscles innervated by the hypoglossal nerve. Our goal was to identify and compare the pontomedullary sources of catecholaminergic (CA) projections to the hypoglossal motor nucleus (Mo12) and the adjacent viscerosensory nucleus of the solitary tract (NTS). In 10 Sprague–Dawley rats, retrograde tracers, Fluoro-Gold or B sub-unit of cholera toxin, were microinjected (5–20 nl) into the Mo12, NTS, or both nuclei. Tyrosine hydroxylase (TH) was used as a marker for CA neurons. Following tracer injections into the Mo12, retrogradely labeled and TH-positive neurons were found in the A1/C1 (18.5%), A5 (43.5%), A7 (15.0%), and sub-coeruleus (21.0%) regions, and locus coeruleus (1.7%). In contrast, following injections into the NTS, these proportions were: 48.0, 46.5, 0.2, 0.9, and 4.3%, respectively. The projections to both nuclei were bilateral, with a 3:2 ipsilateral predominance. In four animals with one tracer injected into the Mo12 and the other in NTS, TH-positive cells containing both tracers were found only in the A5 region. Thus, the pontomedullary sources of CA projections to the Mo12 and NTS differ, with only A1/C1 and A5 groups having significant projections to these two functionally distinct targets.

Keywords: Apnea; Atonia; Locus coeruleus; Norepinephrine; Sleep; Upper airway

1. Introduction

Hypoglossal motoneurons innervate the genioglossus muscle, an important upper airway dilator. In individuals with a narrow or collapsible upper airway, sleep-related decreases in upper airway muscle activity play a major role in the pathogenesis of obstructive sleep apnea (Remmers et al., 1978; reviewed by Kubin and Davies, 2002). Both norepinephrine and serotonin excite orofacial motoneurons, including those that innervate upper airway muscles (McCall and Aghajanian, 1979; Kubin et al., 1992; Larkman and Kelly, 1992; Funk et al., 1994; Parkis et al., 1995), and both noradrenergic (NE) and serotonergic brainstem neurons reduce their activity during slow-wave sleep and stop firing during rapid eye movement (REM) sleep (Aston-Jones and Bloom, 1981; Reiner, 1986; Jacobs and Azmitia, 1992). These data suggested that sleep-related withdrawal of aminergic excitation may cause decrements of motoneuronal activity, thereby contributing to sleep-related upper airway hypotonia (Kubin et al., 1998). Recently, withdrawal of excitation mediated by α_1-adrenergic receptors was identified as a major cause of sleep-related suppression of activity in hypoglossal motoneurons (Fenik et al., 2005; Chan et al., in press).

Abbreviations: Amb, nucleus ambiguous; AP, area postrema; DMV, dorsal motor nucleus of the vagus; Gr, nucleus gracilis; IRt, intermediate reticular region; Mo5; Mo7; Mo12, motor trigeminal, facial, hypoglossal nuclei; KF, Kölliker-Fuse nucleus; LC, locus coeruleus; LPGi, lateral paragigantocellular region; LRt, lateral reticular nucleus; LSO, lateral superior olive; NTS, nucleus of the solitary tract; scp, superior cerebellar peduncle; sol, solitary tract; Sp5, spinal trigeminal nucleus; SubC (D/V), sub-coeruleus region (dorsal/ventral portions)

* Corresponding author at: Department of Animal Biology 209E/VET, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA 19104-6046, USA. Tel.: +1 215 898 6489; fax: +1 215 573 5186.
E-mail address: rukhadze@vet.upenn.edu (I. Rukhadze).

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The information about the origins of NE innervation of different orofacial motor nuclei is conflicting. It appears that NE cells of the A5, A7 and sub-coeruleus (SubC) regions project to the trigeminal, facial and hypoglossal motor nuclei (Mo5, Mo7 and Mo12) (Grzanna et al., 1987; Aldes, 1990; Aldes et al., 1992), as well as the adjacent viscerosensory nucleus of the solitary tract (NTS) (Moore and Bloom, 1979; Loewy et al., 1986; Byrum and Guyenet, 1987), suggesting anatomically widespread and divergent projections from different groups of NE neurons to functionally diverse brainstem targets. On the other hand, data also suggest that NE projections within the brainstem show substantial target-specificity. For example, most NE neurons innervating the rat Mo5 are located in the ipsilateral A7 region, Mo7 receives most of its NE innervation from the A5 group, whereas, the spinal trigeminal sensory nucleus receives crossed and uncrossed afferents from all pontine NE groups, including the SubC region and locus coeruleus (LC) (Grzanna et al., 1987). In contrast, NE projections to the Mo12 were reported to be bilateral and originate primarily in the SubC region (69%), with lesser projections from the A5 and A7 groups (10% and 21%, respectively) (Aldes et al., 1992). These conflicting data, and the new evidence for an important role of NE withdrawal in sleep-related upper airway hypotonia (Fenik et al., 2005; Chan et al., in press), prompted us to re-examine the sources of NE projections to the Mo12. To verify that our results selectively represented NE projections to the Mo12, we compared them to those targeting an anatomically proximal but functionally different site, the viscerosensory NTS.

Preliminary results have been published (Rukhadze et al., 2005).

2. Materials and methods

The experiments were performed on 10 adult, male Sprague–Dawley rats (body weight: 376 ± 11 g (S.E.)) obtained from Charles River Laboratories. All animal handling procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.1. Tracer injections

The animals were anesthetized with isoflurane (3%) followed by Nembutal (60 mg/kg, i.p.). The head was placed in a stereotaxic holder and the skin and muscles overlying the atlanto-occipital membrane and the membranes overlying the 4th ventricle were cut along the midline and retracted laterally. A glass pipette (A-M Systems, tip diameter 20–25 μm) filled with Fluoro-Gold (FG; Fluorochrome, LLC) or cholera toxin B sub-unit (CTb, List Biological Lab) was inserted into the target site. The injections into the Mo12 were aimed at 0.1 mm rostral to the caudal end of the calamus scriptorius, 0.3 mm lateral to the midline, and 1.1 mm below the medullary surface. The injections into the NTS were placed 0.2 mm rostral to the caudal end of the calamus scriptorius, 0.4 mm lateral, and 0.5 mm below the medullary surface. Three animals received unilateral FG injections into the Mo12 (1%, 20 nl or 4%, 5 nl), three had unilateral FG (1%, 20 nl) injections into the NTS, three had FG (1%, 20 nl) injected into the Mo12 on one side and CTb (1%, 5 nl) into the NTS on the opposite side, and the remaining one received CTb (1%, 5 nl) injection into the Mo12 and FG (1%, 20 nl) injection into the NTS. We used two different concentrations and volumes of FG that delivered the same total amount of the tracer in an attempt to reduce necrosis caused by this tracer at the center of the injection site. The tracers were injected over 10–20 s by applying pressure to the fluid in the pipette while monitoring the movement of meniscus with a calibrated microscope. The pipette was left in place for 5–10 min and then slowly withdrawn. The muscles and skin overlying the operated area were sutured in layers.

2.2. Immunohistochemical procedures

Seven days after tracer injections, the rats were deeply anesthetized with Nembutal (80 mg/kg, i.p.) and transcardially perfused with phosphate-buffered saline (PBS, pH 7.4, with 5 USP units/ml of heparin and 0.004% lidocaine), followed by 4% paraformaldehyde in PBS. The brains were removed, post-fixed in the same solution for 48 h at 4 °C, cryoprotected in 30% sucrose-PBS and then the brain stems were cut on a cryostat (CM 1850, Leica) into five series of 35 μm coronal sections.

In experiments with FG injections only, one series was mounted on gelatin-coated glass slides, dried, dehydrated and coverslipped, and another was subjected to immunohistochemistry to visualize tyrosine hydroxylase (TH). In experiments with CTb injections, the second series was first processed to visualize CTb and then subjected to TH immunohistochemistry.

For CTb immunohistochemistry, the sections were incubated for 48 h at 4 °C in goat CTb antiserum (1:20,000; Lot #7032A2, List) in PBS containing 0.2% Triton X and 5% donkey serum. Subsequently, they were incubated for 2 h in Cy3-labeled, donkey anti-goat antibodies (1:200; Jackson). For TH immunohistochemistry, sections were incubated for 48 h in mouse anti-TH antibodies (1:20,000; Lot #41K4829; Sigma) in PBS containing 0.3% Triton X and 5% horse serum. This was followed by incubation in biotinylated anti-mouse, rat-adsorbed antibodies (1:200; Vector) for 1 h at room temperature, and then for 1.5 h in fluorescein (FITC)-conjugated egg white-avidin (1:1000; Jackson).

2.3. Data analysis

For comparisons of the placement and spread of the tracer among different animals, all injections were represented as the area enclosed within 75% of the distance from the center of the injection site to the furthest points containing visible extracellular deposit of the tracer. Even though the 75% figure was an arbitrary estimate of the effective tracer spread, it offered an objective way with which to compare the injection sites in different animals. Every 5th section from the levels −14.3 mm to −8.72 mm caudal to bregma (Paxinos and Watson, 1997) was examined for the presence of cells containing one or the other tracer and TH immunoreactivity using a Leica DMLB microscope and appropriate filter sets (FG, Cy3, FITC). Cells were regarded retrogradely labeled if they contained FG or CTb grains within a clearly identifiable cell body, the nucleus was visible, and the grains were not visible under filters other than the one appropriate for that tracer. TH-positive cells were identified by uniform distribution of immunofluorescence (FITC) within the cell body and proximal dendrites.

The locations of TH-positive cells, with or without the tracer, were mapped onto closest standard cross sections from a rat brain atlas (Paxinos and Watson, 1997). Photographs were taken with a digital camera (DMC-2, Polaroid) and then enhanced using Photoshop software (Adobe). Image processing was limited to adjustments of the color balance and contrast in order to best represent the image seen under direct microscopic observation.

The variability of the means is characterized by the standard error (S.E.). Statistical comparisons were conducted using Student's unpaired *t*-test (Sigma Plot, Jandel), and the differences were considered significant when $p < 0.05$.

3. Results

3.1. Tracer injection sites and distribution of retrogradely labeled neurons

In 10 rats included in this study, FG or CTb injections were placed in the centers of the target nuclei (Mo12 or NTS) at the level of the caudal end of the AP. Fig. 1A shows a typical example of FG injection into the Mo12, and Fig. 1B the