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Mesopontine cholinergic projections to the hypoglossal motor nucleus

Irma Rukhadze *, Leszek Kubin

Department of Animal Biology 209E/VET, 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

Mesopontine cholinergic (ACh) neurons have increased discharge during wakefulness, rapid eye movement (REM) sleep, or both. Hypoglossal (12) motoneurons, which play an important role in the control of upper airway patency, are postsynaptically excited by stimulation of nicotinic receptors, whereas muscarinic receptors presynaptically inhibit inputs to 12 motoneurons. These data suggest that ACh contributes to sleep/wake-related changes in the activity of 12 motoneurons by acting within the hypoglossal motor nucleus (Mo12), but the origins of ACh projections to Mo12 are not well established. We used retrograde tracers to assess the projections of ACh neurons of the mesopontine pedinculopontine tegmental (PPT) and laterodorsal tegmental (LDT) nuclei to the Mo12. In six Sprague–Dawley rats, Fluorogold or B subunit of cholera toxin, were pressure injected (5–20 nl) into the Mo12. Retrogradely labeled neurons, identified as ACh using nitric oxide synthase (NOS) immunohistochemistry, were found bilaterally in discrete subregions of both PPT and LDT nuclei. Most retrogradely labeled PPT cells (96%) were located in the PPT *pars compacta* region adjacent to the ventrolateral tip of the superior cerebellar peduncle. In the LDT, retrogradely labeled neurons were located exclusively in its *pars alpha* region. Over twice as many ACh neurons projecting to the Mo12 were located in the PPT than LDT. The results demonstrate direct mesopontine ACh projections to the Mo12. These projections may contribute to the characteristic of wakefulness and REM sleep increases, as well as REM sleep-related decrements, of 12 motoneuronal activity.
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Cholinergic (ACh) neurons of the laterodorsal and pedinculopontine tegmental (LDT and PPT) nuclei have increased discharge during wakefulness and/or rapid eye movement (REM) sleep [9,14,27,31]. Some of these neurons have axonal projections to the brainstem regions containing respiratory neurons. They may also project directly to the cranial motor nuclei, including the hypoglossal motor nucleus (Mo12) that innervates the genioglossus, an important upper airway dilator ([13,36]; reviewed in ref. [17]). By acting on different receptors within the Mo12, acetylcholine may increase or decrease the activity of 12 motoneurons [2,4,19]. Nicotinic receptors may mediate wake- and/or REM sleep-related excitation, and decreased ACh cell activity during slow-wave sleep (SWS) may contribute to the characteristic of the state decrements of Mo12 activity. Muscarinic (m2) receptors are also present in the Mo12 [21], and in vitro studies show that they presynaptically suppress glutamatergic inputs to 12 motoneurons [2]. However, interpretation of these findings in the context of sleep/wake-dependent control is difficult because little is known about the sources of ACh projections to the Mo12. In order to define the location of PPT and LDT neurons that may contribute to state-dependent modulation of 12 motoneurons, we studied the topography of mesopontine ACh projections to the Mo12.

Six male Sprague–Dawley rats (body weight: 396 ± 11 g (S.E.), Charles River, MA) were used. 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.

The animals were anesthetized with isoflurane (3%) followed by Nembutal (60 mg/kg i.p.), placed in a stereotaxic head holder and the dorsal medullary surface exposed. Retrograde tracers, Fluorogold (FG; Fluorochrome, LLC; 1%/20 nl in two rats or 4%/5 nl in three rats) or cholera toxin B subunit (CTb; List Biological Lab.; 1%/5 nl in one rat) were pressure injected using glass pipettes (A-M Systems, tip diameters 20–25 μm), with the injection volume monitored by observing the movement of the meniscus in the pipette through a calibrated microscope. The

* Corresponding author. Tel.: +1 215 898 6489; fax: +1 215 573 5186.
E-mail address: rukhadze@vet.upenn.edu (I. Rukhadze).

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pipette was inserted into the Mo12 at the level of the caudal end of the area postrema, aiming at AP −13.68 mm relative to bregma [24], 0.3 mm lateral to midline and 1.1 mm below the medullary surface. Seven days after the 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 heparin and 0.004% lidocaine) and then with 4% paraformaldehyde in PBS. The brains were post-fixed for 48 h at 4 °C, cryoprotected in 30% sucrose and cut into five series of 35 μm coronal sections. One series was subjected to immunohistochemical procedures to visualize nitric oxide synthase (NOS) as a reliable and unique marker for ACh neurons within the mesopontine tegmentum [8,33] and the remaining series were used to provide cytoarchitectonic landmarks or for other procedures. To visualize NOS, sections were incubated for 48 h in mouse anti-NOS antibodies (1:4000; Lot #22K4838; Sigma), in PBS containing 0.3% Triton X and 5% horse serum, then for 1 h at room temperature in biotinylated anti-mouse antibodies (1:200; Vector), and then for 1.5 h in Fluorescein (FITC)-conjugated egg white-avidin (1:1,000; Jackson). The sections from the experiment with CTb were first immunohistochemically processed to visualize CTb and then subjected to NOS immunohistochemistry. They 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).

Every fifth section from AP levels −9.30 to −7.04 mm caudal to bregma [24] was examined for the presence of cells containing the retrograde tracer and NOS-immunoreactivity using a Leica DML microscope and appropriate filter sets (FG, Cy3 and FITC; Chroma Technologies). Cells were regarded retrogradely labeled when they contained FG or CTb grains within a clearly identifiable cell body, the cell nucleus was present, and the grains were not visible under filters other than the one appropriate for a given fluorophore. NOS cells were identified on the basis of a uniform distribution of immunofluorescence (FITC) within the cell body and proximal dendrites. Photographs were taken with a Polaroid digital camera (DMC-2) and then enhanced using Photoshop software (Adobe). Image processing was limited to adjustments of the color balance and contrast in order to most faithfully represent the image seen under direct microscopic observation. The locations of double-labeled cells were mapped onto closest standard cross-sections from a rat brain atlas [24]. Statistical comparisons were conducted using Students's paired *t*-test (Sigma Plot, Jandel) and the variability of the means characterized by the standard error (S.E.).

In all six rats, tracer injections were placed in the center of the Mo12 (Fig. 1A–C). In each animal, some tracer spread was visible in the surrounding areas, such as the dorsal motor nucleus of vagus (DMV) or the reticular formation located lateral or ventral to the Mo12, but with the volumes used, the amount and intensity of such a spread represented a small fraction of that deposited within the Mo12. The pontomedullary distribution of retrogradely labeled neurons was consistent with previous reports (e.g. [5,7,10,32]), and so was the distribution of ACh neurons [1,8,13,28,29,33]. As previously defined in rats, the PPT nucleus was divided into two subdivisions: PPT *pars compacta* (PPT*c*) and PPT *pars dissipata* (PPT*d*) [26,30]. PPT neurons were clustered dorsal and ventrolateral to the superior cerebellar peduncle (scp) at AP −8.8 to −7.04 from bregma (PPT*c* region) and also scattered ventral and medial to the scp (PPT*d* region). LDT neurons were located at AP levels −9.16 to −8.3 either as a dense cluster embedded in the central gray (CG), corresponding to the core of the nucleus, or as diffusely scattered cells distributed dorsal and ventral to the border between the CG and the adjacent dorsomedial reticular formation (the LDT *pars alpha* region—LDTα).

Fig. 1 shows examples of tracer injections and NOS-positive neurons that were retrogradely labeled from the Mo12. In individual animals with FG injection, we found three to seven double-labeled neurons in the PPT, and one to four in the LDT, nucleus (in every fifth 35 μm section), and two cells were found in the animal with CTb injection. Nearly all double-labeled neurons were located in the same discrete subregions of the PPT and LDT nuclei. Of the 24 cells found in all six rats in the PPT nucleus, 23 were located in a subregion of the PPT*c* adjacent to the ventrolateral tip of the scp and only 1 in the PPT*d* region (Fig. 2). In the LDT nucleus, all 10 double-labeled cells found in the 6 rats were located in the LDTα region (Fig. 2). The ratios of ipsi- to contralaterally located double-labeled neurons were 11:13 for the PPT, and 5:5, for the LDT nucleus. Relative to the total number of retrogradely labeled and NOS-positive cells found bilaterally in both nuclei, the mean percentage of those located in the PPT nucleus was 69.8 ± 4.9%; this was significantly more than in the LDT nucleus ($p < 0.02$, $t_5 = 3.9$).

In order to assess the proportions of PPT and LDT cells projecting to the Mo12 relative to all mesopontine ACh cells, we counted NOS-positive neurons in the PPT and LDT nuclei. The number of NOS-positive neurons found bilaterally in different animals (counts obtained from every fifth section and then multiplied by 5) ranged from 1410 to 4250 for the PPT (mean: 2970 ± 500), and from 1465 to 4370 (mean: 2880 ± 380) for the LDT nucleus. These numbers were similar to those reported earlier for the PPT nucleus and slightly lower than in other studies for the LDT nucleus. For example, the numbers of ACh neurons counted bilaterally in the PPT were 2070 [13], 3655 [26] or 3252 [6]. In the LDT nucleus, previously reported bilateral counts of ACh cells were 3304 [3], 4227 [13] or 5526 [11]. Relative to our counts, the percentages of NOS cells retrogradely labeled from the Mo12 were 0.8 ± 0.2% for the PPT and significantly less, 0.30 ± 0.08%, for the LDT nucleus ($p < 0.03$, $t_5 = 3.3$). Thus, a limited number of mesopontine ACh neurons located in discrete portions of the PPT and LDT nuclei have bilateral projections to the Mo12. Such neurons may directly modulate 12 motoneuronal activity in a state-dependent manner.

The information about descending pontine ACh projections to the orofacial motor nuclei has been scanty and conflicting. In one study, ACh projections to the trigeminal (Mo5), facial (Mo7) or Mo12 were reported to be 5:1 ipsilateral and originate mainly in the PPT nucleus [36]. While the main location within the PPT complex of our double-labeled cells is consistent with that study, we also found a similar number of double-labeled cells in the contralateral PPT nucleus and less numerous but