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INHIBITION OF PONTINE NORADRENERGIC A7 CELLS REDUCES HYPOGLOSSAL NERVE ACTIVITY IN RATS

V. B. FENIK,* I. RUKHADZE AND L. 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

Abstract—Noradrenergic (NE) excitatory drive maintains activity of hypoglossal (XII) motoneurons during wakefulness. In predisposed persons, sleep-related decrements of NE cell activity may contribute to hypotonia of upper airway muscles innervated by XII motoneurons. The goal of this study was to determine whether NE neurons of the pontine A7 group, an anatomically identified source of NE projections to the XII nucleus, provide significant, endogenous NE excitatory drive to XII motoneurons. In anesthetized rats, we microinjected clonidine (0.75 mM, 20–40 nl), an α_2-adrenergic receptor agonist that inhibits pontine NE cells, aiming at the A7 region. Nine injections were placed within 0.4 mm from the A7 group identified using tyrosine hydroxylase immunohistochemistry: they reduced XII nerve activity by 31.3±2.8% (standard error) and decreased the central respiratory rate by 6%. Another 21 injections, including eight placed near NE cells of the sub-coeruleus region, were made at distances over 0.5 mm from the A7 group and they did not alter either XII nerve activity or respiratory rate. In control experiments, clonidine injections into the A7 group preceded by injections of an α_2-receptor antagonist, RS-79948, did not change XII nerve activity. Four experiments with unilateral clonidine injections into the A7 region and with Fos immunohistochemistry used as a marker of cell activity revealed that the percentage of Fos-positive A7 cells was significantly reduced on the injected side. There was also a significant positive correlation between Fos expression in A7 cells and XII nerve activity. Thus, decrements of NE excitatory drive from the A7 group may significantly reduce XII nerve activity. In obstructive sleep apnea patients, in whom the muscles innervated by XII motoneurons act as upper airway dilators, this may contribute to sleep-related respiratory disorders. © 2008 Published by Elsevier Ltd on behalf of IBRO.

Key words: locus coeruleus, norepinephrine, pons, sleep, upper airway, obstructive sleep apnea.

In obstructive sleep apnea syndrome patients, activity of the genioglossus, an important airway dilator is higher during wakefulness than in healthy individuals to compensate for their anatomically compromised upper airway patency (Suratt et al., 1988; Mezzanotte et al., 1992; Hendricks et al., 1993; Katz and White, 2004). This activity decreases during non-rapid eye movement (REM) sleep and is often abolished during REM sleep, thus facilitating the occurrence of sleep-related obstructions of the upper airway (Remmers et al., 1978; Sauerland and Harper, 1976; Okabe et al., 1994; Mezzanotte et al., 1996; Ikeda et al., 2001; Katz and White, 2004). Recent studies in rats suggest that sleep-related suppression of genioglossal muscle activity is mainly caused by sleep-related withdrawal of noradrenergic (NE) activation of hypoglossal (XII) motoneurons that innervate the muscles of the tongue (Fenik et al., 2005b; Chan et al., 2006).

Norepinephrine excites XII motoneurons (Funk et al., 1994; Parkis et al., 1995; Al-Zubaidy et al., 1996). Pontine NE neurons have highest levels of activity during wakefulness, reduce activity during non-REM sleep and cease firing during REM sleep (Aston-Jones and Bloom, 1981; Reiner, 1986; Kubin, 2001; Fenik et al., 2002). Since antagonism of NE excitatory receptors (α_1-adrenergic) located in XII nucleus region causes a profound decrease of XII motoneuronal activity (Fenik et al., 2005b; Chan et al., 2006), sleep-related decrements of NE cell activity probably also contribute to decrements of upper airway motor tone.

NE cells of the pontine A7, subcoeruleus (SubC) and A5 groups, as well as the medullary A1/C1 group, have axonal projections to the XII nucleus (Aldes et al., 1992; Rukhadze and Kubin, 2007), but the relative contribution of silencing of cells in different NE nuclei to the decrements of XII motoneuronal activity is unknown. The numbers of NE neurons projecting to the XII nucleus from each NE group cannot be used to infer about the impact of the loss of activity in that cell group on the activity of XII motoneurons because cells of different groups may have different densities and strengths of synaptic connections. In our earlier study (Fenik et al., 2002), we observed that silencing of cells in the ventrolateral pontine A5 group did not significantly alter the activity of XII motoneurons despite anatomical evidence for NE projections from this group to the XII nucleus (Aldes et al., 1992; Rukhadze and Kubin, 2007). Data showing that the dorsolateral pontine NE group designated as A7 has the highest percentage of cells with axonal projections to the XII nucleus (Rukhadze and Kubin, 2007) and that activity of these cells is reduced in a pharmacological model of REM sleep (Rukhadze et al., 2008), prompted us to test whether NE cells located in the pontine A7 group are a source of significant endogenous NE excitatory drive to XII motoneurons. We used local microinjections of clonidine, an α_2-adrenergic receptor agonist that powerfully inhibits activity of pontine NE neurons

*Corresponding author. Tel: +1-215-898-6489; fax: +1-215-573-5186.
E-mail address: vfenik@vet.upenn.edu (V. B. Fenik).

Abbreviations: AP, antero-posterior; B, bregma; DAB-HRP, diaminobenzidine–horseradish peroxidase; EEG, electroencephalogram; Fos+, Fos-immunopositive; NE, noradrenergic; REM, rapid eye movement; SubC, subcoeruleus; TH, tyrosine hydroxylase; TH+, tyrosine hydroxylase–immunopositive; XII, hypoglossal.

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(Reiner, 1985; Huangfu and Guyenet, 1997), into and around the A7 group and measured the resulting changes of XII nerve activity. We found that unilateral clonidine injections into the A7 group significantly reduced XII nerve activity whereas those made at a distance from the A7 region had no effect. A preliminary report has been published (Fenik et al., 2007).

EXPERIMENTAL PROCEDURES

Animal preparation

Experiments were performed on 20 adult male Sprague–Dawley rats (body weight: 325–470 g, mean: 393±9.5 g (standard error (S.E.)) obtained from Charles River Laboratories (Wilmington, MA, USA). All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and followed the guidelines established by the National Institutes of Health. The number of animals used was the lowest necessary to achieve satisfactory power of statistical analysis. Anesthesia was carefully monitored and maintained at adequate level at all time to avoid animal suffering.

The animals were pre-anesthetized with isoflurane (2%) and then anesthetized with urethane (1 g/kg, i.p., supplemented by 50 mg i.v. injections, as needed). The trachea was intubated and a femoral artery and vein were catheterized for arterial blood pressure monitoring and fluid injections, respectively. The right XII nerve was cut and its central end placed inside a cuff electrode for recording (Fenik et al., 2001). The cervical vagi were cut to enhance XII nerve activity and eliminate its reflex modulation by pulmonary afferents. The head of the animal was placed in a stereotaxic holder, two screws were attached to the skull to record the electroencephalogram (EEG) and a bipolar recording electrode was inserted into the hippocampus, as described previously (Fenik et al., 2005b; Lu et al., 2007). Openings were made bilaterally in the interparietal bone and the dura removed for inserting a drug-containing pipette into the pontine A7 region.

The animals were paralyzed with pancuronium bromide (2 mg/kg i.v., supplemented with 1 mg/kg injections, as needed) and artificially ventilated with a mixture of air and oxygen (30–60% O_2) at a rate 50–70 lung inflations/min. After paralysis, the level of anesthesia was assessed by intermittently applying a pinch to the hind limb while recording the arterial blood pressure, EEG, hippocampal and XII nerve activity. Absence of pinch-induced changes in respiratory rate or XII nerve activity and only transient changes in blood pressure, EEG and hippocampal signals, similar to those before paralysis, indicated adequate level of anesthesia. Rectal temperature was maintained at 35.5–36.5 °C with a heating pad. End-expiratory CO_2 (Columbus Instruments capnograph, Columbus, OH, USA) was adjusted at the beginning of the experiment to obtain a steady respiratory modulation of XII nerve activity and then kept constant. The mean systolic blood pressure was 90.4±8.4 mm Hg.

Electrophysiological recordings

EEG (bandwidth 0.5–100 Hz), hippocampal activity (2–20 Hz) and XII nerve activity (30–2500 Hz) were amplified with AC amplifiers (N101, Neurolog System; Digitimer, Hertfordshire, UK). The signals were continuously monitored on an eight-channel chart recorder (TA-11; Gould Instruments, Valley View, OH, USA) and recorded on a 16-channel digital tape recorder (C-DAT; Cygnus Technology, Delaware Water Gap, PA, USA) together with tracheal pressure, end-expiratory CO_2, arterial blood pressure and an event marker. XII nerve activity was fed into a moving average circuit with a time constant of 100 ms (MA-821 RSP; CWE, Inc., Ardmore, PA, USA).

Experimental and histological procedures

The glass pipettes used for the injections (A-M Systems, Carlsborg, WA, USA) had tip diameters of 25–30 μm and were filled with 0.75 mM clonidine hydrochloride, an α_2-adrenoceptor agonist (Sigma-Aldrich, St. Louis, MO, USA) in 0.9% NaCl. Pontamine Sky Blue dye (2%, ICN Biomedicals Inc., Aurora, OH, USA) was included in clonidine solution to mark the injection sites. Once the animal was prepared for recording, one unilateral, or up to two bilateral clonidine injections were made at 10–20 min intervals aiming at the A7 group(s). The injections (20 or 40 nl) were made over 30–60 s by applying pressure to the fluid in the pipette. In two control experiments, 80 nl of RS-79948 hydrochloride (2 mM in 0.9% NaCl; Tocris, Ellisville, MO, USA), an α_2-adrenoceptor antagonist, were injected at the same site 13–19 min prior to clonidine injections. The injected volume was monitored with 2 nl resolution by observing movement of the meniscus through a calibrated microscope.

The experiments were terminated 40 min after the last clonidine injection with additional i.v. injections of urethane (1 g/kg) and heparin (100 units). The animal was intra-arterially perfused with cold (4–6 °C) phosphate-buffered saline (PBS, pH 7.4, with 5 USP units/ml of heparin and 0.004% lidocaine) followed by 4% phosphate-buffered formalin. The pons was removed, post-fixed in 4% phosphate-buffered formalin, cryoprotected in 30% sucrose and cut into three series of 35 μm coronal sections. One series was stained with Neutral Red and used for localization of the injection site and the other two were used for immunohistochemistry. Sections from the animals with unilateral clonidine injections were immunohistochemically processed for Fos, the protein product of the immediate early gene c-fos used as a marker of cell activity, and then for tyrosine hydroxylase (TH), a marker of catecholaminergic neurons, as previously described (Lu et al., 2007). Sections from the animals that received two or more clonidine injections were processed for TH only. The double-labeling procedures were conducted successively, first using Fos antibodies (1:100,000; catalog number PC38; lot D14211; Oncogene, Temecula, CA, USA) and then TH antibodies (1:35,000; catalog number T-1299; lot 41K4829; Sigma-Aldrich). Incubations with primary antibodies were followed by appropriate biotinylated secondary antibodies, avidin-biotin reaction (Vector, Burlingame, CA, USA), and diaminobenzidine–horseradish peroxidase (DAB-HRP) reaction. The DAB-HRP step was heavy metal-intensified for Fos, resulting in black staining of neuronal nuclei, but conducted without the intensification for TH, resulting in golden-brown staining of catecholaminergic cells. Photomicrographs were taken using an upright microscope (Leica DML, Wetzlar, Germany) and a digital camera (DMC Ie, Polaroid, Cambridge, MA, USA). Image processing was limited to brightness, contrast and color adjustments to most faithfully represent the appearance of the specimen under direct microscopic observation (Photoshop CS software, Adobe, San Jose, CA, USA).

Data analysis

The amplitude of the moving average of XII nerve activity and central respiratory rate were measured during a 30–60 s baseline period preceding the injection and during 30–60 s 4.4–15 min following each clonidine injection. This delay was empirically selected because clonidine injections often produced short-lasting changes of arterial blood pressure that were occasionally accompanied by transient changes of XII nerve activity after which the activity stabilized.

Tyrosine hydroxylase–immunopositive (TH+) A7 cells were present in brain cross-sections corresponding to the antero-posterior (AP) levels from −8.72 mm to −9.30 mm from bregma (B) according to a rat brain atlas (Paxinos and Watson, 1997). From the caudal to rostral levels, A7 cell initially appeared as isolated TH+ neurons, then, near the AP level of B-8.90, they became