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REM sleep-like atonia of XII motoneurons is caused by loss of noradrenergic and serotonergic inputs

Victor B. FENIK, Richard O. DAVIES and Leszek KUBIN

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

Correspondence to:

Leszek Kubin, Ph.D.
Department of Animal Biology 209E/VET
School of Veterinary Medicine
University of Pennsylvania
3800 Spruce Street
Philadelphia, PA 19104-6046, USA
Tel. 215-898-1893
Fax. 215-573-5186
E-mail: lkubin@vet.upenn.edu

On line data supplement for the article by Victor B. FENIK, Richard O. DAVIES and Leszek KUBIN: ***REM sleep-like atonia of XII motoneurons is caused by loss of noradrenergic and serotonergic inputs.***

Methods in detail

Animal preparation and monitoring

Experiments were performed on 18 adult male Sprague-Dawley rats weighing 384 g ± 5.4 (SE). The procedures for anesthesia, surgery and recording followed the guidelines of the Institute for Laboratory Animal Research, and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

The animals were anesthetized with isoflurane (2%) followed by urethane. The latter was initially given at a dose of 1 $g \bullet kg^{-1}$ (i.p.) and then supplemented with 50 mg i.v. injections administered at ~1 h intervals to ensure that the cortical and hippocampal signals exhibited no spontaneous activation for at least 15 min prior to each test with carbachol. The trachea was intubated, and a femoral artery and vein were cannulated for arterial blood pressure monitoring and fluid injections, respectively. To record the hypoglossal (XII) nerve electroneurogram, the right XII nerve was dissected and placed in a cuff-type recording electrode (E1). Both cervical vagi were cut to enhance spontaneous XII nerve activity and make it independent of lung volume feedback. The characteristic enhancement of XII nerve activity following vagotomy helped maintain a satisfactory level of spontaneous XII nerve activity throughout these experiments despite microinjections into the XII nucleus of the antagonists whose main effect was to reduce

that activity. Vagotomy also helped avoid total silencing of XII nerve activity at the time when it was suppressed following pontine carbachol injections. The depressant effect of pontine carbachol can occur in both vagotomized and non-vagotomized animals (E2, E3).

As described previously (E4), the animal was placed in a stereotaxic head holder, two openings made in the right parietal bone, and the dura removed for inserting a carbachol-containing pipette and hippocampal recording electrode. For monitoring cortical activity, two screws were attached to the skull, and for hippocampal activity, two insulated platinum wires with tips separated by 0.8 mm were inserted into the hippocampus. The dorsal surface of the caudal medulla was exposed to insert a microinjection pipette into the XII nucleus.

The animals were paralyzed (pancuronium bromide, 2 $mg \bullet kg^{-1}$ i.v., supplemented with 1 $mg \bullet kg^{-1}$ injections at ~2 h intervals) and artificially ventilated with an air-oxygen mixture (O_2 concentration 30-60%) at 50-70 lung inflations/min. Ventilation with a hyperoxic mixture may facilitate the ability of pontine carbachol to elicit REM sleep-like changes (E5). A regular central respiratory rhythm, steady inspiratory bursts in the XII nerve electroneurogram, stable blood pressure, and only transient activations of cortical and hippocampal activities in response to tail or hindlimb pinch indicated that the animal was adequately anesthetized. The mean systolic blood pressure was above 80 mmHg, rectal temperature was maintained at 36-37^{o}C, and the end-expiratory CO_2 was continuously measured (Columbus Instruments capnograph) and in each experiment maintained constant (mean: 5.6% ± 0.1 (SE); range 4.8-6.6%).

Hippocampal (bandwidth 3-8 Hz), cortical (1-100 Hz), and XII nerve (30-2500 Hz) activities were recorded with AC amplifiers (N101, NeuroLog). XII nerve activity was full-wave rectified and a moving average derived (time constant 100 ms; MA-821 RSP; CWE, Inc.). All neural signals, event markers, blood pressure, tracheal pressure and end-expiratory CO_2 were

monitored on a chart recorder (TA-11; Gould Instruments), and recorded on a 16-channel digital tape recorder (C-DAT; Cygnus Technology).

Drug solutions and microinjections

Methysergide maleate salt (5-HT receptor antagonist) and carbamylcholine chloride (carbachol, cholinergic receptor agonist) were obtained from Sigma (St. Louis, MI), and 1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl) piperazine hydrochloride (prazosin, α_1-adrenoceptor antagonist) from RBI (Natick, MA). The stock solution of prazosin was made in distilled water and all other drugs in 0.9% NaCl. Final solutions were prepared in 0.9% NaCl about 1 h before injections and contained either 0.2 mM prazosin and 1.0 mM methysergide mixed together or only one of the drugs. At these concentrations, the antagonists injected together blocked the excitatory effect of the corresponding agonists injected into the XII nucleus in doses sufficient to at least double the XII nerve activity (10 nl of 2 mM phenylephrine or 5 mM 5-HT; data not shown, but see refs. E6, E7).

Pontine injections of carbachol were made using glass pipettes (tip diameter 20-30 μm), filled with 10 mM carbachol and 2% Pontamine sky blue dye (ICN) in 0.9% saline, inserted into the the region of the dorsomedial pontine reticular formation identified as most effective in our earlier studies (E8-E10). Ten nl of carbachol (18.3 ng) were injected over 10-20 s by applying pressure to the fluid in the pipette while monitoring the movement of the meniscus with a microscope calibrated in 1 nl increments.

For injections into the XII nucleus, a glass pipette filled with one or both antagonists, and with the tip beveled to an external diameter of 22-28 μm, was inserted into the right XII nucleus 0.3 mm lateral to the midline and 1.15 mm below the dorsal medullary surface at three locations:

0.5 mm caudal, 0.15 mm rostral and 0.8 mm rostral to the calamus scriptorius. Three successive injections of the antagonist mix, 40 nl each, were placed in the XII nucleus over 5.8 min ± 0.1 (SE). The injection sites and volumes were selected based on the estimate that the extracellular volume that can be filled with the injected solution is ~21% of the total volume (E11). For this available extracellular volume fraction, each 40 nl injection would initially fill a sphere of tissue having a diameter of 0.7 mm, close to the coronal diameter of the XII nucleus in the rat. With the 0.65 mm rostrocaudal spacing of successive injections, the three injections delivered the drugs to the XII nucleus along its entire rostrocaudal extent (~2.5 mm) (see calculations in ref. E12).

Experimental protocol and data analysis

At least two pontine carbachol injections (10 nl each) were made at the beginning of each experiment to verify that the response had an appropriate pattern and was reproducible. Once the control response was established, the pipette filled with antagonist(s) was inserted successively at the three rostrocaudal locations in the XII nucleus described above and injections made. After the last antagonist injection, carbachol was injected into the pons to elicit the first post-antagonist response. Additional carbachol injections were then made during 3 h after the antagonists to observe the time-course of the effects of the antagonists on the effects of pontine carbachol on XII motoneuronal activity. The minimum interval between two successive carbachol injections was 30 min; at this repetition rate, the responses occur without adaptation (E4, E9, E12, E13).

Changes in XII nerve activity were assessed from the moving average of the signal by measuring the difference between its peak during the inspiratory phase of the central respiratory

cycle and an expiratory period when no activity was present. The central respiratory rate was determined from the record of XII nerve activity. The latencies and durations of the responses were measured from the onset of the carbachol injection to the start and end of the changes in hippocampal activity. The time of occurrence of the maximal response was identified from the changes in XII nerve activity and respiratory rate. The average measures for each response were obtained from 30 s segments of records prior to, at the time of the maximal effect, and after the recovery from the effect of carbachol. The level of XII nerve activity during the course of each experiment was expressed as the percentage of the peak inspiratory activity present prior to the last pre-antagonist carbachol injection. This normalization allowed us to average the levels of XII nerve activity across all animals despite the use of arbitrary units for the corresponding measurements within individual animals.

After verification that the data were normally distributed, repeated measures ANOVA or one-way ANOVA with Bonferroni's correction, or paired or unpaired two-tailed Student's t-tests were used for statistical analyses (SigmaStat, Jandel). The repeated measures ANOVA was used to test whether the antagonist(s) altered the response of a given parameter to pontine carbachol injections. For example, the respiratory rates before and during the responses to pontine carbachol elicited prior to the antagonist(s) were compared to the respiratory rates before and during carbachol responses elicited in the same group of animals at certain time after the antagonist(s). Differences were considered significant when $p<0.05$. The variability of the means is characterized by the standard error (SE) throughout the report, and p values refer to paired t-tests unless otherwise noted.

Histology

At the end of the experiments the animals were given urethane (2 g•kg^{-1} i.v.) and decapitated. The brainstem was removed, fixed in formalin and cut into 100 μm sections in the coronal plane for the pons, and sagittal or coronal plane for the medulla. Sections containing the dye were serially mounted and stained with Neutral red. The carbachol injection sites were then plotted on standard brain cross-sections derived from an atlas (E14).

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Editorial

Are Biogenic Amines Involved in Controlling Upper Airway Patency during REM Sleep?

Obstructive sleep apnea (OSA) occurs in at least 9 to 15% of middle-aged adults, and it is likely that its prevalence will rise with the increasing incidence of obesity (1). OSA is associated with daytime somnolence, hypertension, heart failure, and cardiac arrhythmias. The pathophysiology of OSA is characterized by repetitive occlusions of the posterior pharynx during sleep due, in part, to decreased tone in the genioglossus muscle (2, 3). Nasal application of continuous positive airway pressure (CPAP) is the standard form of therapy for treating OSA. Although effective, many patients are unable to, or unwilling, to comply with the use of CPAP and thus there is intensive interest in developing effective pharmacologic therapies (4). However, such advances in therapy will necessitate a clear understanding of the neurochemical control of upper airway motoneurons during sleep–wake states. Two articles in this issue of the *AJRCCM* (pp. 1322–1330 and pp. 1338–1347) examine the state-dependent involvement of bioamines in the control of hypoglossal (XII) motoneuron excitability in rodent models, and provide important insights into potential pharmacologic interventions that may be useful in OSA.

Fenik and colleagues (5) tested the hypothesis that suppression of upper airway motor tone during REM sleep is due to reduced serotonergic (5-HT) and noradrenergic (NE) drive to XII motoneurons. Nerve recordings (XII nerve) were made from anesthetized rats to evaluate the effect of microinjection of antagonists to α_1-noradrenergic (prazosin) and/or 5-HT (methysergide) receptors into the XII nuclei. The vagi were cut to accentuate baseline XII motor activity and REM-like sleep episodes were induced by injecting carbachol into the pontine reticular formation (6). The cocktail of NE and 5-HT receptor antagonists suppressed the baseline amplitude of XII motor discharge during anesthesia before administration of carbachol. This combination also blocked the suppression of XII motor discharge amplitude normally observed during the pharmacologically induced REM sleep–like state. The authors propose that a combined withdrawal of NE and 5-HT effects from XII motoneurons is the main factor underlying their reduced activity during REM sleep.

The novel demonstration of endogenous excitatory drive to XII motoneurons by NE extends past data that showed an excitatory effect of exogenously applied NE (7–9). Evidence of endogenous 5-HT release and excitation of XII motoneurons by applied 5-HT has been previously demonstrated by several studies using *in vitro* and *in vivo* models (8–12). It should also be noted that, in addition to 5-HT, raphe neurons contain the neurotransmitters glutamate, thyrotropin-releasing hormone, and substance P, all of which have excitatory actions on XII motoneurons (9). Thus, the modulation of these transmitters may contribute to changes in pharyngeal motor tone across sleep–wake states.

Fenik and colleagues' emphasis on the combinatorial actions of neuromodulators in controlling XII motoneuron excitability is particularly important. There is an increasing body of work demonstrating that XII motoneuronal excitability is dynamically modulated by neuromodulators that control multiple protein kinases and phosphatases (13). Indeed, targeting intracellular signal transduction cascades that regulate neuronal excitability downstream from the receptor may prove to be an effective pharmacologic strategy for OSA.

The second study reported in this issue, by Sood and coworkers (14), also examines the role of 5-HT in state-dependent control of XII motoneuron excitability. The chronically instrumented rat model developed in Dr. Horner's laboratory was used to measure genioglossus motor activity across natural sleep–wake states. The 5-HT receptor antagonist mianserin or MDL100907 was administered into portions of the XII motoneuron pool via microdialysis. In contrast to what was expected from past studies using more reduced preparations, Sood and colleagues demonstrate that the endogenous 5-HT drive affecting genioglossus activity is normally weak and minimally modulated with sleep state. This apparent contradiction is clarified by their demonstration that vagotomy, which is typical of reduced preparations including the carbachol model of REM sleep, significantly enhances 5-HT modulation of genioglossus activity. Thus, it appears that the potential role of 5-HT in modulating pharyngeal muscle activity might be overestimated in animal experiments using reduced, vagotomized preparations.

Sood and colleagues were careful to temper their conclusions regarding a lack of 5-HT–mediated events in OSA. They point out that 5-HT–mediated reflex compensations in certain patients with OSA, as well as the bulldog and Zucker rat models, could increase airway tone through 5-HT–mediated mechanisms. Indeed, there is evidence for 5-HT–induced plasticity of XII motoneuron activity in response to repeated bouts of intermittent hypoxia (13, 15) and decreased excitatory actions of 5-HT on XII motoneurons after long-term, intermittent hypoxia (16). It would be of interest to expand on the chronically instrumented rat model to examine the neurochemical control of XII motoneuron excitability after exposure to pathophysiologic aspects of OSA, such as airway obstruction and hypoxia.

Further basic neurophysiologic studies will be required to fully ascertain the role of biogenic amines and other neuromodulators controlling XII motoneuron excitability during sleep–wake states. These two studies demonstrate the necessity to consider converging, as well as interacting, neuromodulatory inputs and potential plasticity within the respiratory system, and also underline the need to consider data from multiple preparations before drawing conclusions about regulatory mechanisms.

Conflict of Interest Statement: J.J.G. does not have a financial relationship with a commercial entity that has an interest in the subject matter of the manuscript.

John J. Greer, Ph.D.
University of Alberta
Edmonton, Alberta, Canada

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