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Quantitative Analysis of the Excitability of Hypoglossal Motoneurons during Natural Sleep in the Rat

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Abstract

We describe a novel approach to assess the excitability of hypoglossal motoneurons in rats during naturally-occurring states of sleep and wakefulness. Adult rats were surgically prepared with permanently-placed electrodes to record the EEG, EOG and neck EMG. A stimulating/recording miniature tripolar cuff electrode was implanted around the intact hypoglossal nerve and a head-restraining device was bonded to the calvarium. After a period of adaptation to head-restraint, the animals did not exhibit any sign of discomfort and readily transitioned between the states of wakefulness, NREM and REM sleep. There was no spontaneous respiratory or tonic activity present in the hypoglossal nerve during sleep or wakefulness. Hypoglossal motoneurons were activated by electrical stimulation of the hypoglossal nerve (antidromically) or by microstimulation directly applied to the hypoglossal nucleus. Microstimulation of hypoglossal motoneurons evoked compound action potentials in the ipsilateral hypoglossal nerve. The magnitude of their integrals tended to be higher during wakefulness ($112.6\% \pm 15$; standard deviation) and were strongly depressed during REM sleep ($24.7\% \pm 3.4$), compared to the integral magnitude during NREM sleep. Lidocaine, which was delivered using pressure microinjection to the microstimulation site, verified that the responses evoked in hypoglossal nerve can be affected pharmacologically. We conclude that this animal model can be utilized to study the neurotransmitter mechanisms that control the excitability of hypoglossal motoneurons during naturally-occurring states of sleep and wakefulness.

Keywords

REM sleep; Obstructive Sleep Apnea; cuff electrode; head-restrained rat

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CONFLICT OF INTEREST

None.

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1. INTRODUCTION

The activity of hypoglossal and other upper airway motoneurons is critical in maintaining airway patency during wakefulness (Remmers et al., 1978; Eckert et al., 2009). During sleep, and especially during rapid eye movement (REM) sleep, the activity of hypoglossal motoneurons decreases, which results in a reduction in the tone of genioglossus and other upper airway muscles which results in airway obstruction in Obstructive Sleep Apnea (OSA) patients (ibid). Therefore, understanding the neurochemical mechanisms that underlie the depression of hypoglossal motoneurons during REM sleep is an important goal of basic and clinical neuroscience. However, despite decades of research, there is a significant controversy regarding the mechanisms that are responsible for the REM sleep-related depression of hypoglossal motoneuron activity (RD-HM) (Brooks and Peever, 2008; Lydic, 2008; Kubin, 2008; Berger, 2008; Funk, 2008; Soja, 2008; Chase, 2008). Consequently, new models and approaches are required to establish a foundation for novel studies to advance our understanding of the mechanisms responsible for RD-HM.

Recently, two models have been employed to study the neurotransmitter mechanisms that are involved in RD-HM: 1) the carbachol model of REM sleep using anesthetized rats in which drugs are delivered to the hypoglossal nucleus using the microinjection technique (e.g., Fenik et al., 2005); and 2) chronically-instrumented naturally-sleeping behaving rats with drugs delivered to hypoglossal motoneurons using the reversed microdialysis technique (e.g., Morrison et al., 2003). There is a significant advantage in using chronic animals because behavioral processes can be studied during naturally-occurring sleep and wakefulness. On the other hand, acute preparations provide for the use of variety of electrophysiological techniques, for a better control of the amount of delivered drugs and for more precise localization of target sites.

The technique of using head-restrained, undrugged animals has advantages of both of the preceding models and was successfully implemented for use in rats (e.g., Soulière et al., 2000) and mice (Bryant et al., 2009). It had been also employed to record intracellularly from identified hypoglossal and other motoneurons in head-restrained cats during sleep and wakefulness (Chase et al., 1980; Yamuy et al., 1999; Fung et al., 2000). Here, we describe a novel approach to study the mechanisms of RD-HM using head-restrained rats which includes the recording/stimulation of hypoglossal nerve and hypoglossal motoneurons, as well as precise drug delivery into the hypoglossal nucleus during natural sleep and wakefulness. A preliminary report has been published (Fenik et al., 2010).

2. MATERIAL AND METHODS

Data were obtained from 11 adult Sprague-Dawley male rats (body weight: 300–460 g). The animals were housed individually in the Greater Los Angeles VA Healthcare System vivarium under a 12/12 h light/dark cycle with standard rodent food and water available, *ad lib*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Greater Los Angeles VA Healthcare System and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.1. Surgery procedures

Animals were initially anesthetized with isoflurane (2%) followed by a mixture of ketamine/xylazine (60/7.5 mg/kg i.m., supplemented with 30 mg/kg ketamine as needed). The animals were premedicated with an analgesic Buprenorphine HCl (0.05 mg/kg s.c.; Hospira, Inc., Lake Forest, IL) and an antibiotic, Baytril (5 mg/kg s.c.; Bayer HealthCare LLC, Shawnee Mission, KS). All surgical procedures were conducted under sterile conditions. An

appropriate level of anesthesia was determined by the absence of a withdrawal reflex in response to a pinch applied to a hindlimb. At the completion of the surgical procedures, 25 ml/kg of 0.9% solution of NaCl was injected subcutaneously to assist in restoring the animal's fluid balance.

A teflon coated silver wire was used in the construction of the implanted electrodes (bare/coated diameters: 0.075 mm/0.140 mm; A-M Systems, Inc., Sequim, WA). This wire was employed because of its small diameter and great flexibility, which minimized any potential animal discomfort and reduced the impact on the hypoglossal nerve created by animal movements.

A tripolar cuff electrode was implanted around the intact hypoglossal nerve. Wires from the electrode were tunneled under the skin to the calvarium. The rat was then placed in a stereotaxic instrument in the prone position and the skin overlying the calvarium was reflected to permit the insertion of four stainless steel screws (#0–80, 1/8", Small Parts, Inc., Seattle, WA) to record the electroencephalogram (EEG). The screws were positioned bilaterally at 2 mm from the midline: two screws were placed 2 mm rostral and another two, 3 mm caudal, to bregma. Two additional screws were placed in the parietal bone to strengthen the acrylic attachment to the skull and to provide for electrical grounding. Bregma and lambda points on the skull were positioned horizontally. Two wires, one on each side, were placed in the dorsal neck musculature to monitor the neck muscle electromyogram (EMG). Another pair of wires was implanted subcutaneously close to the external canthus of each eye to record the electrooculogram (EOG). The bare ends of the EMG and EOG electrodes were tipped with silver balls to prevent tissue puncture during the animal's movements. Each wire was soldered to a gold-plated male miniature pin connector (AM Systems) which was affixed to the calvarium with acrylic cement (Co-Oral-It Dental MFG.CO., Diamond Springs, CA). A portion of the caudal part of the parietal bone was removed, the underlying dura mater cut and an acrylic plate was placed on the cerebellum surface. The plate contained two holes to permit the subsequent insertion of a metal electrode for recording or stimulation and a glass pipette for drug injection. Stainless steel screws (hex socket drive, #4–40, 1/8", Small Parts, Inc.) were placed in the preceding holes to protect the brain surface between experimental sessions. The acrylic head plug contained four laterally directed openings (two on each side) which were used to restrain the animal's head movements during experimental sessions.

2.2. Cuff electrode

The nerve cuff electrode was constructed of silicon tubing (inside diameter 1 mm) which was cut to a length of 4 mm (Fig. 1). The tubing also had a longitudinal cut to encase the intact hypoglossal nerve, which resided within the tube. The external tube wall was trimmed, as shown in Figure 1, to accommodate the bare ends of silver wires which went through the wall and formed loops inside the tube in order to make contact with the nerve. One wire was used to make two peripheral contacts which served as the anode during electrical stimulation, whereas a wire that made a central contact was used as the cathode. The bare silver wires were covered with silicone glue (not shown) to insulate them from the surrounding tissue.

Using a cuff electrode that is placed on the hypoglossal nerve has a number of advantages, especially for our approach, compared to recording genioglossus (GG) muscle activity. Despite potential signal shunting within the cuff electrode, it provides access to, and recording from, all axons in the hypoglossal nerve, unlike EMG wires, which sample only a portion of muscle activity. Similarly, single-pulse electrical stimulation applied to the hypoglossal nerve using cuff electrode activates all motoneurons in the ipsilateral hypoglossal nucleus. Also, central stimulation of hypoglossal motoneurons evokes potentials

that are recorded by the cuff electrode regardless of the stimulation site within the hypoglossal nucleus. In addition, using the described cuff electrode allowed us to employ minimally disturbing soft silver wire, which is beneficial for the animal's comfort compared to multithread stainless steel EMG wires that are routinely used to withstand rigorous tongue movements.

2.3. Postoperative procedures and training of the rats

Postoperative care, which was provided for two postsurgical days, included twice-a-day observation of the animal's behavior and injections of Buprenorphine and Baytril to alleviate possible post-surgical discomfort and to prevent infection, respectively. During adaptation and experimental sessions, rats were placed in a small elongated box to restrict their movements. Each rat was adapted for several days beginning with head-free habituation to the box and then, under head-restrained conditions with a gradual increase in the time spent in the head-restraining device. The duration of these sessions increased depending on the rat's behavior. The typical time-course of adaptation and experimental sessions is shown in Table 1. During the initial short-lasting adaptation sessions with the head immobilized, the animals appeared to be very anxious due to the novel situation and did not sleep. However, after a few training sessions, as the rats became familiar with the environment and the condition of head-restraint, they began to spend more time asleep. With subsequent adaptation, sleep latency, which was defined as the time between placing an animal in the head-restraining device and the first episode of non-REM (NREM) sleep, decreased. In addition, the duration and the frequency of REM sleep episodes gradually increased to values similar to those recorded in freely behaving rats (Vetrivelan et al., 2009). Thus, following an adaptation period of 4–6 days, the animals went to sleep within minutes after being placed in the head-restraining device; they did not exhibit any sign of discomfort during experimental sessions (of approximately 5 hours) and they readily transitioned between the states of wakefulness, NREM and REM sleep.

2.4. Experimental procedures

Experimental sessions began with immobilizing the animal's head by inserting one end of each of the four holding bars into the openings in the head plug and attaching the other end to the stereotaxic frame (semi-chronic headholder-880; David Kopf Instruments, Tujunga, CA). A connection was made between the male connectors in the head plug and the recording equipment in order to monitor the EEG, neck EMG, and EOG activity via the permanently-implanted electrodes. The protective screws were removed under aseptic conditions and the cerebellar surface in the openings was treated with a local anesthetic Lidocaine 2% (Sparhawk Laboratories, Inc., Lenexa, KS) to prevent the animal's discomfort during electrode penetration. A metal electrode was positioned on the cerebellum surface at angles in both caudal and medial directions (16–22 degrees and 0–6 degrees from vertical, respectively). The electrode was then advanced through the cerebellum into the hypoglossal nucleus with a hydraulic Micropositioner 650 (David Kopf Instruments) using the field potential evoked by stimulation of hypoglossal nerve as a guide. In order to position a glass pipette tip close to the tip of the metal electrode, its angles and depth were calculated using the coordinates of the metal electrode. At the end of each experimental session, the electrodes were removed, the protective screws were replaced and the rat was returned to its home cage.

2.5. Signal recording and processing

Recorded signals were amplified (Differential AC Amplifier 1700; A-M Systems) and filtered with a bandwidth of 0.1 Hz – 500 Hz for the EEG, 10 Hz – 1 kHz for the EOG and 30 Hz – 5 kHz for the neck EMG and hypoglossal nerve activity. Extracellular neuronal activity was recorded using thin metal electrodes (A-M Systems) connected to an AxoClamp

2B amplifier (Axon Instruments, Inc., Union City, CA) amplifier. During experiments, all signals were monitored oscilloscopically and on a Macintosh computer (Axiograph software; Axon Instruments) which was attached to an A/D converter (Digidata 1322A; Axon Instruments). Records were saved using a digital tape recorder system (Vetter Digital 3000A; A.R. Vetter Co., Inc., Rebersburg, PA) for subsequent analysis. Off-line data analyses were performed using the Axiograph program with a sampling rate of 20 kHz.

2.6. State identification

The states of sleep and wakefulness were identified visually on the basis of the EEG, neck EMG and EOG (Ursin and Serman, 1981). Wakefulness was characterized by a “desynchronized” low amplitude EEG and by a high level of the neck EMG. During NREM sleep, large amplitude spindles appeared in the EEG whereas the neck EMG was relatively stable and of intermediate amplitude. REM sleep was distinguished by a prominent theta rhythm in the EEG and an absence of neck EMG activity (muscle atonia). Eye movements were present only during wakefulness and REM sleep. REM sleep was always preceded by NREM sleep and it was terminated by wakefulness (in most cases) or by a transition to NREM sleep.

2.7. Electrical stimulation and drug injections

Single pulse electrical stimulation (Grass S88; Grass Technologies, West Warwick, RI) was used to activate the hypoglossal nerve (pulse duration 0.2 ms, amplitude 0–300 μ A and frequency 0.2 s⁻¹). Electrical microstimulation (0.2 ms, 0–90 μ A, 0.2 s⁻¹) was also employed to centrally stimulate hypoglossal motoneurons by applying negative current to the metal electrode that was used for recording. In order to mark the stimulation/recording sites, a low-intensity (10 μ A) positive current was passed through the metal electrode for 10–20 s to deposit iron ions.

For drug microinjection, glass pipettes (A-M Systems) were sharpened (tip 25–30 μ) and filled with a drug solution prepared in 0.9% NaCl. The tip of a filled pipette was positioned by a micromanipulator within 50 μ of the tip of the metal electrode. Microinjections were made by applying pressure to the drug solution while monitoring movements of the meniscus with a resolution of 1 nl using a portable digital microscope (Aoli Technology, Ltd., Hong Kong) attached to a computer. To mark the injection sites, 2% Pontamine Sky Blue (Sigma-Aldrich, St. Louis, MO) was added to the injection solution.

2.7. Morphological procedure

For the morphological procedures, the rats were deeply anesthetized with isoflurane (5%) and perfused transcardially with the ice-cold phosphate buffer solution (PBS, pH 7.4) followed by 4% phosphate buffered paraformaldehyde. The medulla was removed and postfixed overnight in fresh fixative at 4°C, cryoprotected with 30% sucrose and cut into 40 μ m sections in the coronal plane. The sections were serially mounted on glass slides and stained with Neutral Red and the Prussian blue reaction (ferric hexacyanoferrate; Sigma-Aldrich, St. Louis, MO) to verify the location of the stimulation and injection sites using a light microscope.

2.8. Monitoring of the cuff electrode integrity

The integrity of the cuff electrode was monitored daily and confirmed by the presence of: 1) bursts of activity in the hypoglossal nerve when the animal moved its tongue during grooming, swallowing, etc.; 2) tongue twitches during electrical stimulation of the hypoglossal nerve; or 3) inspiratory activity in the hypoglossal nerve that appeared under 2% isoflurane anesthesia. In addition, the perfused animals were dissected to verify that

there was no damage to the cuffed hypoglossal nerve and to visually confirm the integrity of the cuff electrode.

2.9. Statistical analysis

The two-tailed paired Student's t-test was used for statistical comparisons. The null hypothesis was rejected at the level of $p < 0.05$. Variability of the mean was expressed as a standard deviation (SD) throughout the text, unless the use of a standard error (SE) was indicated.

3. RESULTS AND DISCUSSION

3.1. Spontaneous activity of the hypoglossal nerve

Figure 2 shows a typical transition from NREM sleep to REM sleep to wakefulness in a well adapted head-restrained rat. In this and all other rats used in this study, there was no spontaneous respiratory or tonic activity present in the hypoglossal nerve during either sleep or wakefulness. However, periods of strong phasic activity were always associated with the animal's tongue movements, e.g., when rats groomed their faces with their forelimbs (see Fig. 2, arrow). Also, high amplitude phasic activation was observed in the hypoglossal nerve during REM sleep that was associated with muscle twitches (Fig. 2, diamonds).

The absence of spontaneous respiratory activity in the GG muscle has been also documented in other studies using behaving rats (Jelev et al., 2001; Lu et al., 2005). However, some studies indicate that tonic and/or respiratory activity in the GG muscle is present during wakefulness and NREM sleep in chronically-instrumented rats (Megirian et al., 1985; Morrison et al., 2003; Sood et al., 2005; Fraigne and Orem, 2011). These different findings may be explained by diverse experimental conditions. Hypoglossal motoneuron activity is well-known to be enhanced by a number of factors including hypercapnia and hypoxia (Hwang et al., 1983; Liu et al., 2003), vagotomy (Glerant et al., 2005), as well as vestibular drives due to a head-tilt position (Rossiter and Yates, 1996). Therefore, using chambers or cages during recording sessions in behaving rats may also favor the accumulation of CO_2 thereby stimulating GG muscle activity. The rat's sleep posture may also have an effect on the GG muscle activity which is minimal or absent when rat's head is positioned horizontally and maximal when the rat's posture is most curved (Megirian et al., 1985). This suggest that neither tonic nor respiratory activity are present in the hypoglossal nerve or GG muscle in rats under isocapnic conditions during naturally occurring sleep; however, such activity may appear in response to specific activating stimuli.

3.2. Antidromic field potentials recorded centrally from hypoglossal motoneurons

Systematic mapping studies began after the animals were fully adapted to head-restraint and readily transitioned between states of sleep and wakefulness. During experimental sessions, a stimulus to the ipsilateral hypoglossal nerve was delivered via the cuff electrode while a metal electrode was advanced into the medulla to record the antidromic field potential (FP). Figure 3A shows an example of a FP profile at different depths measured from the surface of the cerebellar cortex. In this case, the FPs exhibited maximal amplitude at a depth of 7.60 mm which corresponds to the center of the hypoglossal nucleus.

Figure 3B illustrates changes in the shape of FPs when recorded at the same depth but at different medio-lateral coordinates. At the most lateral position of the electrode (0.6 mm lateral to the site of the maximal response), the FP was relatively small and had a positive polarity. According to Rall's mathematical model of multipolar neurons, which also applies to hypoglossal motoneurons, these positive potentials correspond to recordings from the distal dendrites of hypoglossal motoneurons which act as a source of extracellular current

that flows radially into the active soma membrane (Rall, 1962; Nelson and Frank, 1964). Closer to the hypoglossal nucleus (0.3 mm lateral, Fig. 3B), the amplitude of the dendrite potential increased. When the tip of the electrode was positioned within the hypoglossal nucleus (Fig. 3B), a large negative wave appeared in the FPs, which most likely originated from the somas of hypoglossal motoneurons and masked the positive dendrite potential. When recording at more medial locations (0.3 mm medial, Fig. 3B), the negative soma potentials were of smaller amplitude and there were no positive dendrite potentials due to the increased distance from the center of hypoglossal nucleus and the sparse dendritic arbor of hypoglossal motoneurons medially to the hypoglossal nucleus (Altschuler et al., 1994). Figure 3C shows histological verification of the position of the tip of metal electrode which, in this example, was situated within the hypoglossal nucleus.

The latencies of the antidromic FPs ranged from 1.2 ms to 1.48 ms with a mean of $1.29 \text{ ms} \pm 0.11$ ($n=6$ rats), as measured from the beginning of the stimulus artifact to the peak of the negative wave of the FP. Within a given dorso-ventral track, the FP latency remained constant (Fig. 3A). The amplitude of the antidromic FPs recorded from the center of hypoglossal nucleus decreased during REM sleep (Fig. 4). The average FP amplitude during REM sleep was $65.6\% \pm 24$ of the value recorded during NREM sleep ($n=5$ sites in 3 rats, $p < 0.05$; range 32.6% – 89.1%); it tended to increase during wakefulness ($106\% \pm 11$ of the NREM value) (Fig. 4C). The REM sleep-related decrease in the FP amplitude was comparable to that previously reported in cats (Yamuy et al., 1999).

3.3. Orthodromic compound action potentials recorded in hypoglossal nerve

Central single-pulse monopolar electrical microstimulation of hypoglossal motoneurons evoked compound action potentials (CAPs) in the hypoglossal nerve. They were complex potentials that consisted of many superimposed orthodromic action potentials recorded from the axons of motoneurons. Stimulation neither aroused the animals nor caused any sign of discomfort. Figure 5A illustrates the normal transitions between states of sleep and wakefulness during continuous central microstimulation with a current of $83 \mu\text{A}$ that was applied adjacent to the hypoglossal nucleus. The evoked hypoglossal nerve CAPs were suppressed during REM sleep compared to NREM sleep and wakefulness (Fig. 5B).

Figure 6 are examples of CAPs elicited from the center of the hypoglossal nucleus during different states of sleep and wakefulness. At least three prominent negative waves of different latencies were present in these CAPs. The first two relatively small waves had latencies of approximately 1 and 2 ms, respectively (Fig. 6A, arrows 1 and 2). Since these latencies were comparable to the latency of the FPs that were recorded within the hypoglossal nucleus (see above), they likely occurred as a result of direct stimulation of motoneuron axons and somas, respectively. The third wave had the longest latency of 3–4 ms and the largest amplitude (Fig. 6A, arrow 3). It was most likely produced by the excitation of pre-synaptic terminals and/or interneurons which are abundant within the hypoglossal nucleus (Boone and Aldes, 1984; Takasu and Hashimoto, 1988). The “synaptic” wave was strongly depressed during REM sleep compared to the first two waves (Fig. 6).

Since the amplitude of the synaptic component of CAPs varied due to spontaneous minimal changes in the latency of action potentials (see Fig. 6A, NREM sleep traces), CAPs integral was evaluated as the less variable measure of the response. Figure 6B illustrates the time-course of the integral of the synaptic wave of the CAPs that are shown in Fig. 6A during consecutive periods of NREM sleep, REM sleep and wakefulness. On average, the integral of the synaptic component of the CAPs was depressed during REM sleep to $24.7\% \pm 3.4$ relative to NREM sleep ($n=3$ rats, $p < 0.001$; range 21.0% – 27.7%) and it tended to increase during wakefulness relative to the NREM sleep ($113\% \pm 15$ of the NREM value) (Fig. 6C).

Although the rat preparation used in this study did not exhibit any spontaneous tonic or respiratory hypoglossal nerve activity during either NREM sleep or wakefulness, the mechanisms of the RD-HM remained intact and were quite powerful, as evidenced by the strong suppression of the CAPs during REM sleep. This suggests that the neuronal mechanisms of RD-HM are independent of the presence of spontaneous activity in the hypoglossal nerve. This conclusion is also supported by the following findings. 1) The inspiratory activity of medullary premotor neurons that provide an inspiratory drive to hypoglossal motoneurons is only modestly depressed during carbachol-induced REM sleep compared to the depression of the inspiratory component of hypoglossal nerve activity. Therefore, non-respiratory mechanisms must be responsible for the depression of inspiratory activity in hypoglossal motoneurons during REM sleep (Woch et al., 2000). 2) In previous studies, we observed that the magnitude of depression of hypoglossal nerve activity during carbachol-induced REM sleep was virtually independent of the level of spontaneous inspiratory activity in the hypoglossal nerve (Fenik et al., 2004). Under relatively low end-expiratory CO₂ concentration, when a very low level of spontaneous inspiratory activity was present in the hypoglossal nerve, pontine injections of carbachol powerfully abolished that activity. In order to observe some inspiratory activity in the hypoglossal nerve during the REM sleep-like episodes, we had to increase the level of end-expiratory CO₂ which also resulted in increase of spontaneous inspiratory activity in the hypoglossal nerve. At this elevated level of CO₂, we were able to measure the magnitude of RD-HM during the characteristic REM sleep-like episodes (Fenik et al., 2004; 2005). Interestingly, additional increase in the CO₂ level, which further increased spontaneous inspiratory nerve activity, tended to decrease the magnitude of RD-HM. Thus, observations in anesthetized rats also suggest that the mechanism of the RD-HM is independent of the presence of spontaneous inspiratory activity in the hypoglossal nerve. In addition, the tendency of a relatively high CO₂ level to decrease the magnitude of the RD-HM indicates that the magnitude of the RD-HM is maximal under normal isocapnic conditions.

The measurement of the CAPs evoked by direct microstimulation of hypoglossal motoneurons has several advantages compared to the measurement of spontaneous inspiratory activity that was employed by ourselves and others to study the mechanisms of the RD-HM in rats. Since spontaneous inspiratory activity in the hypoglossal nerve or in the GG muscle in rats appears under activated conditions, e.g., hypercapnia or vagotomy, these conditions may recruit additional neuronal pools or mechanisms and result in findings and misleading conclusions that are idiosyncratic to these particular experimental conditions (see Horner, 2007 for review). Thus, the measurement of the CAPs integral allows one to study the mechanisms of RD-HM under normal isocapnic conditions. In addition, since glutamate is the major neurotransmitter that mediates the inspiratory discharge of hypoglossal motoneurons (Steenland et al., 2008; Zuperku et al., 2008), electrical microstimulation is beneficial in studying glutamatergic mechanisms of RD-HM using glutamatergic drugs which would otherwise interfere with the spontaneous inspiratory activity of hypoglossal motoneurons.

Our present data suggest that the synaptic component of the CAPs is more depressed during REM sleep than are the other components of the CAPs. The FPs, which reflect only potentials generated by motoneuronal axons and somas that are evoked by stimulation of the hypoglossal nerve, were also less depressed during REM sleep. These findings emphasize the importance of pre-synaptic mechanisms in mediating the RD-HM (Bellingham and Berger, 1996).

3.4. Effect of microinjections of lidocaine on the CAPs

In two rats, we also demonstrated that it is possible to microinject drugs near the tip of a metal electrode in order to pharmacologically affect the evoked activity of hypoglossal

motoneurons. Glass pipettes, filled with Lidocaine (2%), were stereotactically inserted into the hypoglossal nucleus using precalculated angles and depths to place their tips close to the tip of the metal electrode. Figure 7 shows an example of the effect of Lidocaine that was injected at a distance of 50 μ from the tip of a metal electrode within the hypoglossal nucleus. FPs, which were elicited by stimulation of the hypoglossal nerve and which were recorded by the metal electrode, decreased by approximately 25% after injection of 20 nl of the Lidocaine solution (Fig. 7A and B). CAPs were evoked in the hypoglossal nerve by electrical microstimulation (36 μ A) of the same site within the hypoglossal nucleus using the same metal electrode. Lidocaine microinjection (20 nl) abolished the synaptic component of the CAPs in both rats. These findings indicate that the pharmacology of changes in both FPs and CAPs can be studied using relatively small volumes of drugs during natural states of sleep and wakefulness in this preparation.

4. CONCLUSIONS

We described a novel approach to quantify changes in the excitability of hypoglossal motoneurons during natural states of sleep and wakefulness using rats that are chronically-instrumented to record the EEG, neck EMG and the EOG. In addition, we have developed a specially designed cuff electrode suitable for chronic hypoglossal nerve (or other nerves) recording and stimulation. The conditions of head-restraint in this preparation allowed us to acutely insert electrodes into the brainstem for recording and stimulation and to inject drugs in nanoliter volumes.

Using microstimulation of hypoglossal motoneurons, we determined that the excitability of hypoglossal motoneurons decreases during NREM sleep compared to wakefulness, and that it is strongly suppressed during REM sleep despite the absence of spontaneous tonic or respiratory activity in the hypoglossal nerve. This suggests that the neurochemical mechanisms of RD-HM are independent of the presence of spontaneous activity in hypoglossal motoneurons.

We conclude that the present preparation has significant advantages for studying the neuronal network and neurochemical mechanisms that are responsible for changes in the excitability of hypoglossal motoneurons during natural states of sleep and wakefulness.

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ABBREVIATIONS

CAPs	compound action potentials
EEG	electroencephalogram
EMG	electromyogram
EOG	electrooculogram
FP	field potential
GG	genioglossus
NREM	non-REM
OSA	Obstructive Sleep Apnea
REM	rapid eye movement

RD-HM	REM sleep-related depression of hypoglossal motoneuron activity
SD	standard deviation
SE	standard error

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RESEARCH HIGHLIGHTS

- We describe a new approach to measure the excitability of hypoglossal motoneurons during natural sleep-wake cycles.
- We describe a tripolar cuff electrode that can be chronically implanted around the hypoglossal or other peripheral nerves.
- Not stimulated rats have no spontaneous tonic or respiratory modulated activity in the hypoglossal nerve.
- The integral of elicited compound action potentials in the hypoglossal nerve reflect excitability of hypoglossal neurons.
- The excitability of hypoglossal motoneurons is strongly suppressed during REM sleep compared to NREM sleep and wakefulness.

Figure 1.

Schematic of the cuff electrode which was implanted around the intact hypoglossal nerve for recording and/or stimulation. Note that silver wires, which pass through the silicon tubing (dashed line), are in direct contact with the nerve inside the tubing. The bare silver wires outside the tubing are insulated with silicon glue (not shown).

Figure 2.

Example of recorded signals during consecutive states of NREM sleep, REM sleep and wakefulness. NREM sleep was characterized by large EEG spindle waves. During REM sleep, a characteristic hippocampal theta rhythm appeared in the EEG; the tonic activity of the dorsal neck muscles, which was recorded during NREM sleep, was abolished and typical REM sleep phasic twitching activity appeared in the neck EMG and hypoglossal nerve. Wakefulness typically succeeded REM sleep; it was characterized by a desynchronized EEG and EMG activity that was greater than that during NREM state. Phasic eye movements (EOG) were recorded during REM sleep and wakefulness. There was no spontaneous tonic or respiratory activity in the hypoglossal nerve; however, phasic activity was recorded during REM sleep (diamonds) and during tongue movements during wakefulness (arrow). Closed circles indicate movement artifacts.

Figure 3.

Mapping of field potentials (FPs) evoked by electrical stimulation of the hypoglossal nerve which were recorded within or near the hypoglossal nucleus. A: Examples of FPs that were recorded at different depths (three traces were superimposed for each depth which is shown as numbers on the left) by a metal electrode that protruded through the center of the hypoglossal nucleus (depth of 7.60 mm). B: Examples of FPs recorded within the hypoglossal nucleus and at different medio-lateral distances from the nucleus (each trace is the average of 20 sweeps). C: An example of the histological verification of a stimulation site within the hypoglossal nucleus, which was marked by depositing iron ions from the tip of the metal electrode and revealed by Neutral Red staining and the Prussian Blue reaction.

Figure 4.

Examples of FPs recorded within the hypoglossal nucleus during consecutive periods of NREM sleep, REM sleep and wakefulness. A: Three traces of FPs recorded during each state were superimposed. B: Amplitudes of FPs measured during different states. C: Mean values and SD of the amplitude of FPs during different states. The amplitude was normalized based on its value during NREM sleep.

Figure 5.

Example of a continuous, non-arousing central electrical stimulation applied adjacent to the hypoglossal nucleus. A: The stimulation (pulse duration 0.2 ms, amplitude 83 μA , frequency 0.2 s^{-1}) did not elicit any sign of discomfort and the animal transitioned readily between states of sleep and wakefulness. The large amplitude periodic potentials that were recorded in all three traces are stimulus artifacts. B: The stimulation elicited compound action potentials in the ipsilateral hypoglossal nerve which were typically depressed during REM sleep.

Figure 6.

Electrical stimulation applied within the hypoglossal nucleus elicited compound action potentials (CAPs) in the ipsilateral hypoglossal nerve, which were depressed during REM sleep. A: Examples of five superimposed traces of the CAPs are shown for different states of sleep and wakefulness. Numbers with arrows indicate three components of the CAPs. B: Example of the time-course of the integral of the CAPs during sequential transitions between states. C: Mean integral of the CAPs during different states.

Figure 7.

Effect of lidocaine (20 nl) which was ejected adjacent to the tip of a metal electrode which was positioned within the hypoglossal nucleus. A and B: FPs were evoked by stimulation of the ipsilateral hypoglossal nerve and recorded by a metal electrode. C and D: CAPs were evoked by electric stimuli that were applied to the metal electrode and recorded from the ipsilateral hypoglossal nerve. Arrows with corresponding numbers indicate the three components of the CAPs. See text for details.

Table 1

Time-course of a successful adaptation of a rat to head-restraint.

Day	Procedure	Session duration [hours]	Sleep occurrence and sleep latency [min]	Mean REM sleep duration [min $\pm$ SE] (number of episodes per session)
1	Surgery	-	-	-
2	Post-surgical care	-	-	-
3	Post-surgical care	-	-	-
5	Habituation in the box	3	Rat sleeps	Not recorded
6	Habituation in head- restraint	0.5	No sleep	Not recorded
7	Habituation in head- restraint	1.3	No sleep	N/A
8	Recording/stimulation in head-restraint	3.3	68	1.06 $\pm$ 0.42 (4)
9	Recording/stimulation in head-restraint	5.2	33	2.02 $\pm$ 0.41 (5)
12	Recording/stimulation in head-restraint	2.9	36	1.85 $\pm$ 0.35 (4)
13	Recording/stimulation in head-restraint	4.4	3	2.00 $\pm$ 0.23 (9)
14	Recording/stimulation in head-restraint	4.0	4	2.06 $\pm$ 0.30 (9)
15	Recording/stimulation in head-restraint	4.7	8	1.36 $\pm$ 0.30 (7)