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Hypoglossal motoneurons are endogenously activated by serotonin during the active period of circadian cycle

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Abstract

In obstructive sleep apnea patients, contraction of lingual muscles protects the pharyngeal airway from collapse. Hypoglossal (XII) motoneurons innervate the muscles of the tongue and are themselves under wake-related excitatory drives, including that mediated by serotonin (5-HT). Estimates of endogenous 5-HT activation vary among different studies. We tested whether endogenous drive mediated by 5-HT is present in rat XII motoneurons when measured during the active period of the circadian cycle. We monitored sleep-wake states and lingual and nuchal electromyograms (EMGs) while perfusing the XII nucleus with a vehicle or a 5-HT_2 receptor antagonist (mianserin, 0.2 mM) at the active period onset. EMG levels were measured during each behavioral state and normalized by the mean EMG activity during wakefulness at 4–7am. Wake-related lingual EMG was significantly lower during mianserin perfusion than with the vehicle (53.0 ±9.7% vs. 84.5 ±8.7%; p=0.002). Mianserin had no effect on nuchal EMG or sleep-wake behavior. Thus, rat XII motoneurons receive endogenous serotonergic activation during wakefulness when measured during the dark period. This indicates that XII motoneuronal activity is enhanced by 5-HT output during the active period of the circadian cycle.

Keywords

mianserin; serotonin receptors; sleep; sleep apnea; tongue; upper airway

1. Introduction

Hypoglossal (XII) motoneurons innervate the muscles of the tongue. In obstructive sleep apnea patients, activation of lingual muscles protects the pharyngeal airway from collapse and is required for effective breathing [Remmers *et al.*, 1978; Jordan *et al.*, 2010; White & Younes, 2012; Kubin, 2016]. XII motoneurons receive serotonergic innervation and express the excitatory type 2 receptors for serotonin (5-HT_2) [Manaker & Tischler, 1993; Volgin *et al.*, 2003; Rukhadze *et al.*, 2010]. Systemic or local administration of 5-HT_2 receptor

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antagonists revealed that XII motoneurons receive endogenous excitatory serotonergic drive in intact dogs [Veasey *et al.*, 1996], vagotomized cats [Kubin *et al.*, 1992], and anesthetized and vagotomized rats [Fenik *et al.*, 2005]. However, in chronically instrumented, intact rats, microperfusion of a 5-HT_2 receptor antagonist into the XII nucleus region conducted during the rest period of the circadian cycle revealed only a minimal endogenous serotonergic activation of XII motoneurons [Sood *et al.*, 2005]. This result could be due to generally low level of XII motoneuronal activation during quiet wakefulness in rats, which may have precluded detection of endogenous serotonergic activation for technical reasons. If this were the case, similar measurements conducted during the active period of the circadian cycle (night in rats) could offer more favorable conditions for the detection of endogenous serotonergic drive because rats use their tongues considerably more intensely during the night than during the day. In addition, we recently determined that both 5-HT_{2A} receptor mRNA and protein occur in the XII nucleus at higher levels at the active period onset than at the rest period onset [Volgin *et al.*, 2013]. Accordingly, serotonergic activation of XII motoneurone would be expected to be enhanced during the active period of the circadian cycle.

5-HT release in the brain is maximal during wakefulness, reduced during slow-wave sleep (SWS) and minimal or absent during rapid eye movement sleep (REMS) [Jacobs & Azmitia, 1992]. 5-HT has been proposed to act as an important state-dependent modulator of the level of motoneuronal activity, especially in XII motoneurons [Kubin *et al.*, 1992; Fenik *et al.*, 2005; see Kubin, 2016 for a review]. The important role of the tongue as an airway-protecting muscle in obstructive sleep apnea patients makes the mechanisms of its state-dependent control clinically important, as well as an attractive model with which to investigate the fundamental mechanisms that regulate execution of motor commands in relation to the rest-activity cycle.

Our goal here was to determine whether endogenous 5-HT drives XII motoneuronal activity during the active period of the circadian cycle by means of recording from lingual muscles in chronically instrumented, behaving rats while microperfusing the XII nucleus with a 5-HT_2 receptor antagonist. Preliminary data have been published [Kubin & Mann, 2013].

2. Materials and methods

2.1. Animals

We used adult male Sprague-Dawley rats whose body weight was 330–445 g at the time of instrumentation. Of the 10 rats entered into the study, five animals yielded complete results presented in this report; data from the remaining five rats could not be subjected to within-subject analysis required by our protocol because not all planned recording sessions were successful, and these five animals were excluded from the study. All experimental procedures followed the National Institutes of Health (USA) *Guide for the Care and Use of Laboratory Animals* and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (protocol number: 804875).

Under surgical anesthesia initially induced with ketamine/xylazine (70 mg/kg and 10 mg/kg, i.m., respectively) and subsequently maintained with isoflurane (1.0–1.5%), the rats were

instrumented under aseptic conditions for chronic wired monitoring of lingual EMG, nuchal EMG and cortical EEG, as described previously [Lu *et al.*, 2005; Lu & Kubin, 2009]. Additionally, they had a guide cannula implanted unilaterally above the XII nucleus for subsequent insertion of a probe for reverse microdialysis (CMA-11, 1 mm-long active tip, BAS, West Lafayette, IN). Starting on day 6–8 after instrumentation, each animal was accustomed to handling and experimental conditions through a series of habituation sessions. These included at least one day when the animal was placed in the recording chamber for at least 4 h without being connected to the recording and perfusion devices, followed by at least one day-time recording session conducted without insertion of the dialysis probe, and then at least two overnight recording sessions during which the dialysis probe was inserted and perfused in a random order with artificial cerebrospinal fluid (ACSF) or with ACSF replaced for 2 h with the 5-HT_2 receptor antagonist (mianserin HCl, 0.2 mM; Sigma-RBI, St. Louis, MO) dissolved in ACSF. These initial habituation sessions were completed 16–26 days after instrumentation and were also used to set the optimal gains for EMG and EEG recordings and verify the day-to-day stability of the signals. The composition of ACSF was (in mM): 119 NaCl, 26.2 $NaHCO_3$, 2.5 KCl, 1 NaH_2PO_4, 1.3 $MgCl_2$, 2.5 $CaCl_2$; the solution was bubbled with 5% CO_2/95% O_2 for 10–15 min and then filtered prior to use.

2.2. Experimental protocol

Following habituation, the XII nucleus was perfused with ACSF or mianserin during two separate recording sessions lasting from 3 pm to 10 am on the following day. During one of these sessions, the microdialysis probe was continuously perfused with ACSF only and during the other session the perfusate was switched to mianserin starting at 7:00–7:30 pm (*i.e.*, just after the lights-off/active period onset) and then, after 2 h, switched back to ACSF at 9:00–9:30 pm for the reminder of the recording session. The prefusion rate was 0.8 μl/min which, depending on the length of the perfusion tubing, resulted in a delay of 20–30 min between the time of solution switch at the input and the time of drug delivery to the tip of the perfusion probe.

In two rats, additional recording and microperfusion sessions were conducted during the daytime during which 5-HT (1 mM, serotonin creatinine sulfate; Sigma-RBI) dissolved in ACSF was perfused for 1 h to verify that the characteristic excitatory effect on lingual EMG occurred with a clear onset in rats previously subjected to microdialysis probe insertions into the XII nucleus region, and that the activation selectively affected lingual EMG without altering nuchal EMG or cortical EEG.

All signals were amplified and filtered at 5–3000 Hz for the EMGs and at 1–100 Hz for EEG (Model 8–10B amplifiers, Grass, Warwick, RI) and then digitally acquired using Power-1401 and Spike-2 v.7 data acquisition hardware and software (CED, Cambridge, England). The A/D conversion rate was 2 kHz for the EMGs and 100 Hz for EEG.

At the conclusion of all planned recording sessions, the animals were deeply anesthetized with pentobarbital (100 mg/kg, i.p.), microdialysis probe was inserted into the guide, and the animal was transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. To verify the placement of the perfusion cannula within the XII

nucleus, the brainstems were extracted, post-fixed, cryoprotected in 30% sucrose, sectioned at 50 mμ intervals in parasagittal plane on a cryostat, and the sections were serially mounted, counterstained with Neutral red, dehydrated, coverslipped and photographed using a dark field feature of a microscope (DML, Leica, Wetzlar, Germany) equipped with a digital camera (ProgRes^{CFscan}, Jenoptik, Jena, Germany) because this imaging mode provided an optimal visualization of both the anatomical structures and location of the microdialysis probe track within the tissue (Fig. 1A–B).

To determine the locations and integrity of the recording wires implanted into the base of the tongue, the entire head with the wires implanted in the tongue and connected to the head connector was kept intact while the tongue was gradually sliced in parasagittal plane with a scalpel blade under microscopic control until the bare tips of the recording wires were uncovered. Once the tips were found, their electrical connection to the leads on the head connector was verified. The tongue recording sites were then re-drawn onto a standard sagittal cross-section of a rat tongue. To ensure unambiguous assignment of each recording site within the tongue to the recorded signal, all lingual EMGs were acquired in monopolar configuration relative to a reference point on the skull, as reported previously [Lu & Kubin, 2009]. Ultimately, one of the two lingual EMGs obtained from each animal was selected for analysis, with the selection based on the quality and stability of the signal across all recording sessions. These recording sites are shown in Fig. 1C.

2.3. Data analysis

The states of wakefulness, non-rapid eye movement (NREM; or slow-wave sleep – SWS) and REMS were scored in successive 10 s epochs with a sleep-scoring software (Somnologica-3, Medcare, Buffalo, NY) enhanced by epoch-to-epoch power spectral analysis, as described previously [Lu *et al.*, 2005; Lu & Kubin, 2009; Rukhadze *et al.*, 2011, 2014]. Subsequently, root mean squares of lingual and nuchal EMG were calculated for each scoring epoch and the epochs were sorted by behavioral state and the time of their occurrence within the recording session. Based on our prior studies revealing that respiratory modulation of lingual EMG is rare in rats and that other forms of phasic activation are mainly related to intermittently occurring alimentary or grooming behaviors [Stettner *et al.*, 2013], we measured the root mean squares of total EMG magnitude level above the noise level in successive 10 s intervals without distinguishing between the tonic and phasic forms of activity [Lu *et al.*, 2005; Lu & Kubin, 2009]. The mean EMG levels were then measured separately for each state within the successive 3 h-long intervals starting at 4 pm (*i.e.*, 1 h after the animal was connected to the recording equipment) and ending at 10 am on the next day. To allow for comparison of EMG levels across different recording sessions and animals, mean EMG levels within the successive 3 h-long intervals of each recording session were normalized by the mean calculated during wakefulness at 4–7 am, *i.e.*, when rat EMG is typically maximal among all the 3 h-long intervals in which we conducted our analysis.

Statistical analysis included testing all data sets for normality and equal variance. This was followed by two-way repeated measures analysis of variance (ANOVA), with the two factors being the treatment applied during the recording session (ACSF only, or mianserin for 2 h during the first 3 h-long interval after the active period onset) and the sequential number of

the 3 h-long interval within each recording session. The interaction between the two factors was also tested, and all statistical significance levels derived from ANOVA were corrected for multiple comparisons using the Holm-Sidak method (SigmaPlot v. 12.5, Systat Software, San Jose, CA). When warranted, ANOVA was followed by paired Student's t-tests, with the two-tailed p levels of 0.05 regarded statistically significant. The mean within-subject difference between lingual EMG levels obtained from the corresponding 3-h long intervals in the recording session with microperfusion with ACSF only and the session with mianserin was taken as a measure of the endogenous serotonergic excitatory drive affecting XII motoneurons.

3. Results

3.1. Location of microperfusion probes and lingual recording sites

As in the example shown in Fig. 1B, in all five rats of the present study, the tips of microperfusion probes penetrated dorso-ventrally through the XII nucleus at the antero-posterior levels near the middle of the rostro-caudal extent of the nucleus. The recording sites from which lingual EMG was derived in these five rats were located within the posterior part of the tongue where muscle fibers of the genioglossus (GG) fan out from the tight GG bundle towards the more posterior and dorsal regions of the base of the tongue (Fig. 1C).

In the two rats in which 5-HT was perfused for 1 h through the XII nucleus during the day, lingual EMG was activated rapidly when the drug reached the tip of the probe and the activation gradually declined after the perfusate was switched back to ACSF (Fig. 2). This provided a functional evidence that the probes were located within the XII nucleus.

3.2. Quantification of lingual muscle activity during the rest and active periods of the circadian cycle

Rats are nocturnal animals and exhibit a strong circadian rhythmicity of locomotor activity and the relative proportions of time spent in sleep and wakefulness [Frank & Heller, 1997; Schwierin *et al.*, 1999]. Consistent with these overt behavioral expressions of the circadian rhythm, EMG activation that characterizes bouts of wakefulness is more intense during the active period than during the rest period of the circadian cycle. This feature is illustrated in the raw record of lingual EMG shown in Fig. 3. The relatively enhanced wake-related lingual activity during the night than during the day is probably driven both behaviorally and through specific circadian mechanisms designed to reinforce behavioral activation during the active period of the circadian cycle. In the present study, we set out to estimate the magnitude of endogenous excitatory drive mediated by 5-HT_2 receptors at the onset of the active period of the circadian cycle, *i.e.*, when the output from XII motoneurons is increased above that during the rest period (Fig. 3), and when the excitatory 5-HT_{2A} receptors are expressed in the XII nucleus at higher levels than at the onset of the rest period [Volgin *et al.*, 2013].

3.3. Lingual EMG is reduced when XII nucleus is microperfused with 5-HT_2 receptor antagonist during the active phase of the circadian cycle

To assess the magnitude of endogenous excitation mediated by 5-HT_2 receptors onto XII motoneurons within the XII nucleus, we compared the levels of lingual EMG recorded during the same phase of the circadian cycle while the XII nucleus was microperfused with either ACSF or the 5-HT_2 receptor antagonist, mianserin. Our comparisons focused on the period of lingual activity recorded during the active period of the circadian cycle because, as explained in the Introduction, we expected that conducting our experiments during the active period should help uncover the presence of serotonergic drive in XII motoneurons.

Figure 4 shows the mean levels of lingual EMG measured separately in different sleep-wake states in successive 3 h-long intervals starting from the last 3 h of the rest period (lights-on), then throughout the active period (lights-off), and into the first 3 h of the rest period (lights-on again). In each graph, one curve represents the mean EMG measurements obtained during continuous perfusion of the XII nucleus with ACSF and the other shows data from the recording sessions in which mianserin was perfused through the XII nucleus for 2 h during the first lights-off interval (7–10 pm).

Application of two-way repeated measures ANOVA to lingual EMG magnitudes measured during wakefulness (Fig. 4A) revealed a highly significant effect of the sequential position of the measurement interval within the recording session (six successive 3 h-long intervals) ($F_{5,\ 1,\ 4}$=12.68, p=0.00001) and a trend towards a significant effect of the treatment (mianserin *vs.* ACSF) when assessed across all six measurement intervals ($F_{1,\ 5,\ 4}$=6.24, p=0.066), whereas the interaction between the measurement interval and perfusion medium was not significant (p=0.2). The highly significant effect of the measurement interval reflected the strong dependence of the magnitude of lingual EMG on the circadian time. Indeed, in individual comparisons, wake-related lingual activity in each 3 h-long interval during the active period was significantly higher than in either the first or the last recording interval, *i.e.*, the two intervals representing the rest periods. Depending on the combination of intervals compared, p values ranged from 0.000008 to 0.03. The borderline significant effect of the treatment, as revealed by the two-way ANOVA, was caused by the profoundly reduced lingual EMG during the first 3 h-long interval of the active period with mianserin perfusion when compared to the same measurement interval with ACSF perfusion. When expressed as the percentage of lingual activity measured at 4–7 am (when lingual activity is typically highest), lingual EMG during the 7–10 pm interval was 84.5 ±8.7% (SE - standard error) with ACSF perfusion and only 53.0±9.7% with mianserin perfusion (p=0.002, paired t-test). Notably, in contrast to the recordings with ACSF perfusion only, the level of lingual EMG measured during 7–10 pm with mianserin perfusion was not different from lingual EMG levels measured during the rest periods (ether 4–7 pm or 7–10 am). Thus, mianserin perfusion reduced the nighttime lingual EMG to the level typical of the rest period of the circadian cycle.

In contrast to the wake-related lingual activity, quantification of the effect of the time of day and mianserin perfusion on lingual EMG measured during either SWS or REMS did not reveal any significant effects (Fig. 4B and C). SWS-related lingual EMG levels were

particularly low (only about 5% of the mean level measured at 4–7 am during wakefulness) and exhibited no time-dependence.

During REMS (Fig. 4C), lingual EMG was slightly higher than during SWS, at 10–12% level with still no evidence of circadian variation. There was also no effect of mianserin perfusion which reflects the well-documented absence of activity in central 5-HT neurons during this stage of sleep [Jacobs & Azmitia, 1992].

In contrast to the effects of mianserin on lingual EMG, mianserin had no effect on either the circadian pattern of sleep-wake states (Fig. 5A–C) or nuchal EMG (Fig. 5D–F). This is consistent with the drug having exerted its effect locally within the XII nucleus region. While mianserin had no effects, we found the expected circadian pattern of the amounts of sleep and wake ($F_{5, 1, 4}$=8.78, p=0.00015; $F_{5, 1, 4}$=8.00, p=0.0003; $F_{5, 1, 4}$=4.45, p=0.007 for two-way repeated measures ANOVA separately applied to wakefulness, SWS and REMS percentages, respectively). Similarly, there was a highly significant circadian variation of nuchal EMG levels when measured during wakefulness ($F_{5, 1, 4}$=14.77, p=0.000004), whereas circadian changes of nuchal EMG measured during either SWS or REMS were not significant.

4. Discussion

Our main finding is that antagonism of 5-HT_2 receptors located within the XII nucleus region results in a significant and profound suppression of wake-related lingual muscle activity in chronically instrumented, behaving rats when measured at the onset of the active period of the circadian cycle. Consistent with our earlier studies showing that rat lingual EMG attains a minimum during SWS with hardly any activity present during most SWS epochs [Lu *et al.*, 2005; Lu & Kubin, 2009; Rukhadze *et al.*, 2011], we found no evidence of a circadian modulation of lingual output during SWS. We also found no effect of local antagonism of 5-HT_2 receptors on lingual EMG when measured during either SWS or REMS. These findings are consistent with the wake-related activity of XII motoneurons being significantly driven by endogenous 5-HT, and especially so during the active period, and with minimal or no endogenous serotonergic drive in XII motoneurons during sleep.

In a prior similar study [Sood *et al.*, 2005], 5-HT_2 receptor antagonist was perfused into the XII nucleus region during the lights-on/rest period when XII motoneurons are, on the average, less active than during the night/active period (Figs. 3 and 4A). That study revealed a modest suppressant effects of mianserin on lingual EMG that was significant only during active wakefulness, whereas the effect was negligible when measured across all wakefulness epochs or during different stages of sleep [Sood *et al.*, 2005]. Based on these findings, the authors concluded that, under the baseline, unstimulated conditions, there is only a minimal endogenous serotonergic excitatory drive to XII motoneurons. However, this interpretation did not consider the evidence that extracellular brain 5-HT levels are increased in rats during the active period of the circadian cycle when compared to the rest period [Rueter & Jacobs, 1996]. Hence, the endogenous serotonergic drive to XII motoneurons may be difficult to detect under the conditions of low motor drive characterizing the rest period of the circadian cycle when central release of 5-HT is relatively low. It is also of note that local antagonism

of 5-HT_2 receptors located in the XII nucleus region was previously found to cause significant depression of activity in XII motoneurons when measurements were conducted in bilaterally vagotomized cats and rats regardless whether the animals were decerebrated and unanesthetized [Kubin *et al.*, 1992] or anesthetized with urethane [Fenik & Veasey, 2003; Sood *et al.*, 2003]. To explain these different results, it has been proposed that vagotomy releases endogenous excitatory serotonergic tone onto XII motoneurons [Sood *et al.*, 2005]. However, in the absence of evidence for a specific facilitatory effect of vagotomy on central serotonergic activity, it is more plausible that any enhancement of XII motoneuronal output by any means may facilitate detection of endogenous serotonergic tone by merely increasing the range of lingual activity above the noise level that can be probed with specific pharmacological manipulations, including microperfusion of 5-HT receptor antagonists into the XII nucleus [Sood *et al.*, 2003] or pharmacological inhibition of medullary serotonergic neurons [Sood *et al.*, 2006].

To maximize our ability to detect serotonergic drive in XII motoneurons, we conducted our measurements during the active period of the circadian cycle when the wake-related lingual EMG is significantly increased when compared to the rest period and when endogenous 5-HT release onto XII motoneurons is increased [Rueter & Jacobs, 1996]. Under these conditions, we observed a profound suppression of lingual EMG when the XII nucleus was perfused with the 5-HT_2 receptor antagonist, mianserin. Since the effect of mianserin was limited to the lingual output (no changes in sleep-wake states or nuchal EMG), our data indicate that spontaneously active XII motoneurons received significant endogenous serotonergic excitatory drive. Based on the comparison of our data to the previous findings obtained also from intact, naturally sleeping rats but in experiments conducted during the rest period of the circadian cycle [Sood *et al.*, 2005], we conclude that the increased during the active period level of wake-related lingual activity, with or without additional specific circadian modulatory factors, contributed to our ability to detect the presence of a strong endogenous serotonergic excitatory drive in XII motoneurons. Our interpretation is consistent with other studies of different inputs impinging on upper airway motoneurons where it has been proposed that the magnitude and pattern of response to one input can be uncovered, or enhanced, by changes in the background activity determined by other inputs [*e.g.*, Bailey & Fregosi, 2005, 2006; John *et al.*, 2005].

While an enhancement of spontaneous lingual muscle activity by any means may offer sufficiently favorable condition for detection of central serotonergic excitatory drive in XII motoneurons, there is also evidence that the availability and/or sensitivity of central serotonergic receptors undergoes distinct circadian modulation [Wesemann & Weiner, 1990]. The patterns of these changes are perhaps best characterized for the 5-HT_{1A} receptors [Lu & Nagayama, 1997; Nagayama & Lu, 1998], including a variation among rodent strains [Wesemann & Weiner, 1990]. Nevertheless, there is also evidence for regulation of 5-HT_2 receptors at both mRNA and receptor protein levels [Wohlpart & Molinoff, 1998]. In particular, measurements of 5-HT_{2A} mRNA and protein levels in micropunches of tissue extracted from the XII nucleus of adult rats revealed that both signals attain higher levels at the onset the lights-off/active period than near the end of this period [Volgin *et al.*, 2013]. This would be consistent with the endogenous serotonergic activation of XII motoneurons being relatively enhanced at the onset of the active period when compared to the rest period

of the circadian cycle. Accordingly, our finding of a strong endogenous activation of XII motoneurons mediated by 5-HT_2 receptors may reflect the presence of an endogenous circadian mechanism by which lingual output is enhanced in a manner that favors the normal, physiologic use of the tongue, which is mainly in alimentary behaviors and vocalization. As such, our data are complementary to the evidence that 5-HT_{2A} receptors mediate the CO_2-dependent component of behavioral arousal [Buchanan *et al.*, 2015].

In humans and a few other species whose airway is prone to obstruction under the centripetal force of negative inspiratory pressure, lingual muscles have an important accessory respiratory function in that their activation stiffens the airway wall and prevents, or resolves, sleep-related airway obstructions (Fuller *et al.*, 1999; Remmers *et al.*, 1978; see Horner, 2012; Kubin, 2016; White & Younes, 2012 for reviews). In this context, our finding that endogenous serotonergic activation of XII motoneurons is relatively enhanced during the active period of the circadian cycle suggests that the central neural control of the lingual motor output is endogenously designed to enhance tongue EMG during the part of the circadian cycle during which lingual behaviors, such as feeding, grooming and vocalization, are most appropriate and physiologically desirable. This enhancement may be, at least in part, mediated by an increased at the appropriate circadian time expression of 5-HT_{2A} receptors [Volgin *et al.*, 2013], as this would help align lingual activity with other aspects of behavior typical of the active period of the circadian cycle. Conversely, the relatively low levels of lingual EMG during the rest period may be at least partially related to the reduced endogenous serotonergic drive during this period. Accordingly, in the rat which is a species with fully patent upper airway, serotonergic activation of upper airway motoneurons is sub-optimally designed for protection of the airway against collapse during sleep. In other species, including humans, the presence of a similar circadian dependence of circadian activation of upper airway motor output may exacerbate the occurrence of upper airway collapses during sleep when the latter occurs during the rest period when compared to the occurrence of sleep-disordered breathing during daytime naps. Indeed, there is evidence for circadian variation of central apneic events in rodents [Fink *et al.*, 2014]. Furthermore, in human sleep apnea patients, the duration of apneic episodes occurring during non-REM sleep increases across the night [Charbonneau *et al.*, 1994; Lavie *et al.*, 1981], and it was recently shown that this process extends into the morning hours, i.e., apnea durations are longer and upper airway critical closing pressures are more positive in the morning than in the evening hours [El-Chami *et al.*, 2015]. It has been suggested that these time-dependent effects are related to circadian variation in metabolic rate, body temperature, ventilator chemosensitivity or changes in arousal threshold [El-Chami *et al.*, 2014, 2015; Montserrat *et al.*, 1996]. Underlying all these potential mechanisms may be an intrinsic circadian process that comprehensively aligns motor activity to ventilation and metabolic rate. Underlying all these potential mechanisms may be an intrinsic circadian process that comprehensively aligns motor activity, ventilation and metabolic rate. It is also of note that, in human sleep apnea patients, any circadian variation of the propensity and severity for sleep-related obstructive apneas and hypopneas may be distorted by an increased central sleep drive typical of this population [Leiter *et al.*, 1985; Persson & Svanborg,1996]. Our present data further highlight the need to better understand how endogenous circadian rhythms may affect the propensity for sleep disordered breathing in human subjects.

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Highlights

- Hypoglossal nerve innervates the tongue and protects the upper airway from collapse.
- Serotonin 2 receptors (5-HT_2) may importantly drive hypoglossal (XII) motoneurons.
- We found a strong, wake-related, 5-HT_2 receptor-mediated drive in rat XII motoneurons.
- This drive is evident during the active period of the circadian cycle (night in rats).

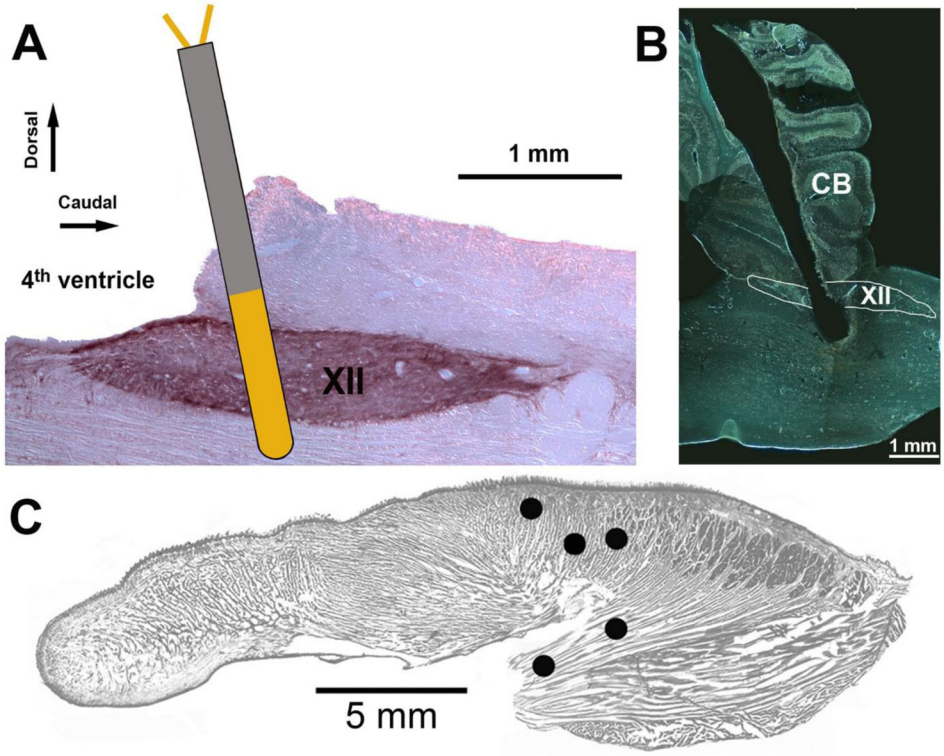

Fig. 1.

A: schematic representation of the placement of microdialysis probe in the hypoglossal (XII) nucleus. The image shows a drawn to scale microdialysis probe superimposed onto a parasagittal section through the dorsomedial medulla. The brain section used as the background was immunostained for 5-HT_{2A} receptor-like protein which delineates the boundaries of the XII nucleus [Fay & Kubin, 2000]. B: parasagittal section through the cerebellum and caudal medulla showing the track of the guide cannula and microdialysis probe inserted into the XII nucleus in one of the rats of the present study. Continuous white line outlines the XII nucleus. CB - posterior cerebellar vermis. C: locations of the lingual EMG recording sites superimposed onto a standard sagittal cross-section of the rat tongue. Lingual recordings were conducted in monopolar configuration relative to a reference electrode on the frontal bone. Circles mark the recording sites in each of the five rats from which data were collected for this study.

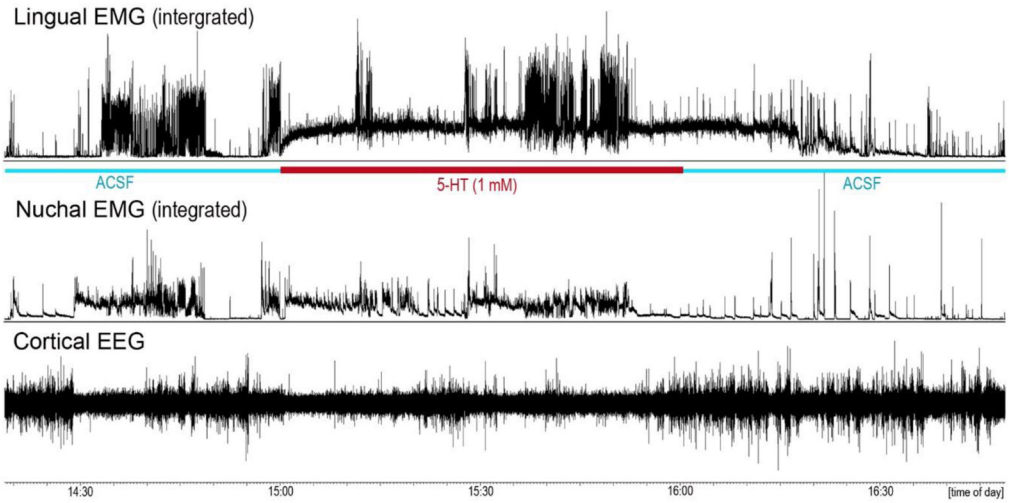

Fig. 2.

Tonic activation of lingual EMG during local microperfusion of 5-HT through the XII nucleus. Records of rectified and integrated lingual and nuchal EMGs collected before, during and after a 1 h-long period of microperfusion of 5-HT (1 mM) through the XII nucleus. The marker line below lingual EMG shows the times when 5-HT reached the XII nucleus and was then switched back to the control medium (ACSF - artificial cerebrospinal fluid). There is a large enhancement of tonic lingual activity during 5-HT perfusion that persists for about 30 min after 5-HT has been switched back to ACSF. Superimposed on this tonic activation are phasic changes in lingual EMG that are similar in pattern to those occurring also prior to 5-HT perfusion. Unlike the tonic activation, the phasic lingual changes coincide with periods of activation present in nuchal EMG and cortical EEG (bottom trace), thus indicating that they are behaviorally driven and unrelated to the local activation of XII motoneurons with 5-HT.

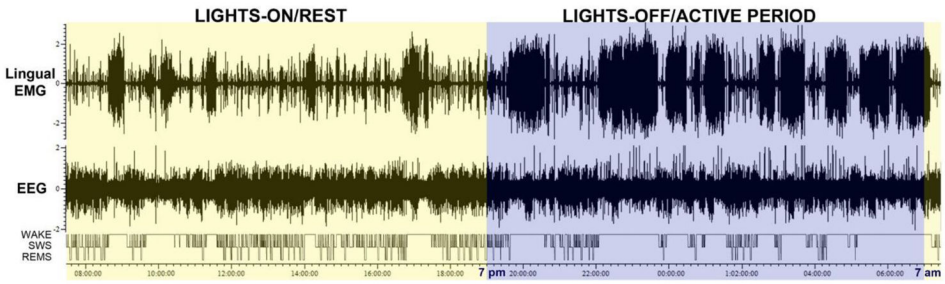

Fig. 3.

Lingual muscle activity is higher during the active period of the circadian cycle (night in rats) than during the rest period. The record shows lingual EMG and cortical EEG collected over a 24 h period. The bottom plot shows the distribution of sleep-wake states (hypnogram) (REMS - rapid eye movement sleep; SWS - slow-wave sleep). It is of note that the wake bout durations, the amount of wakefulness, and the raw magnitude of lingual activity during individual wake bouts are all higher during the lights-off/active period when compared to the lights-on/rest period of the circadian cycle.

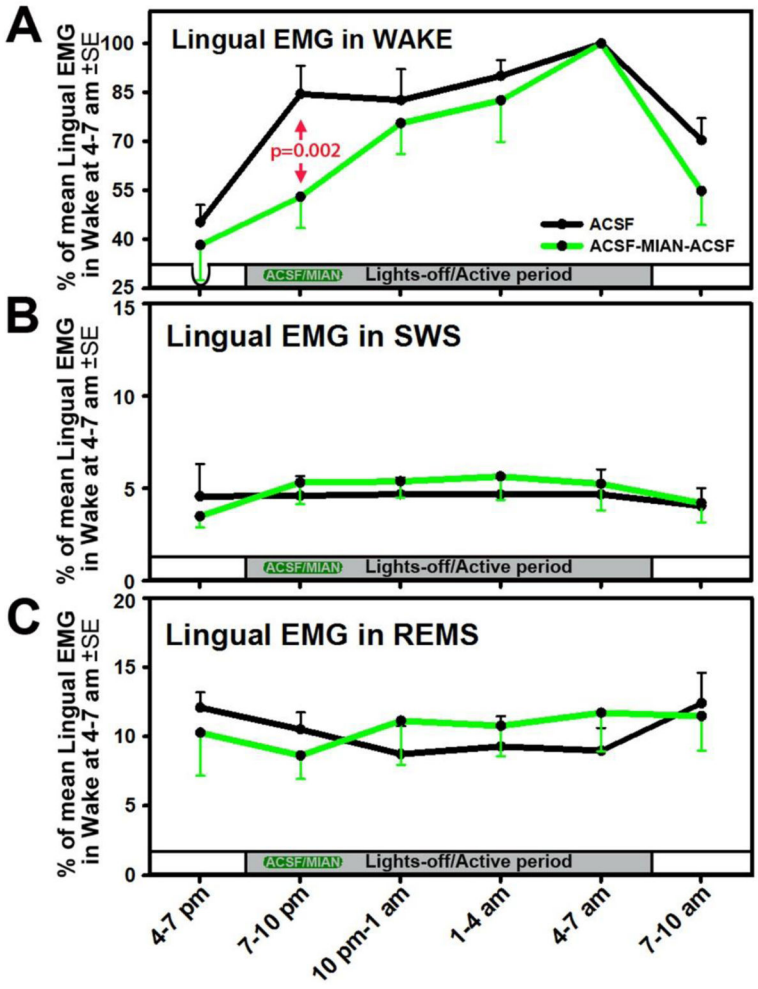

Fig. 4.

Perfusion of the XII nucleus with the 5-HT_2 receptor antagonist, mianserin (MIAN), during the active period of the circadian cycle reduces spontaneous, wake-related lingual activity. The graphs show mean lingual EMG quantified separately during three different behavioral states over successive 3 h-long periods covering the entire lights-off/active period and one preceding and one following 3 h-long lights-on/rest periods. Each graph contains superimposed data from two recording sessions conducted with five rats; in one session, artificial cerebrospinal fluid (ACSF) was continuously perfused through the XII nucleus throughout the recording period, in the other session, ACSF was replaced with mianserin (0.2 mM) during a 2 h period starting just after the lights-off/active period onset. Thus, the comparison of data obtained from the two sessions between 7 pm and 10 pm provides a measure of the effect of mianserin on lingual EMG. When measured during wakefulness, mianserin had a significant suppressant effect on lingual EMG (A). In contrast, mianserin had no effect on lingual EMG during either slow-wave sleep - SWS (B), or rapid eye movement sleep - REMS (C). Lingual EMG measured during the wake epochs was significantly higher during the active periods of the circadian cycle than during the rest

periods, whereas there was no such a difference during either SWS or REMS (see text for details).

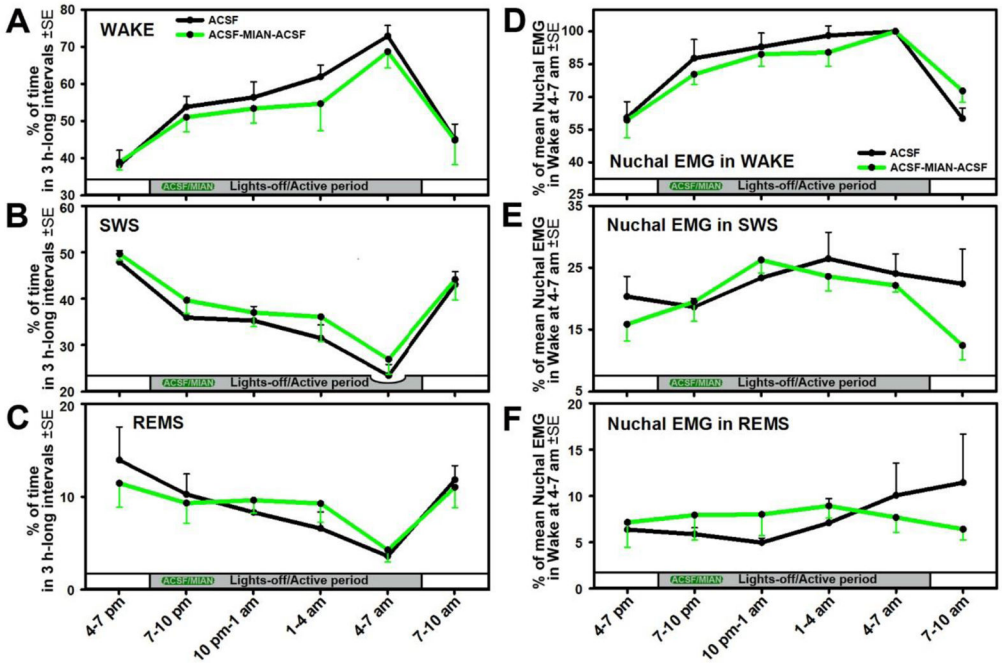

Fig. 5.

Microperfusion of the XII nucleus region with the 5-HT_2 receptor antagonist, mianserin (MIAN) for 2 h at the active period onset did not alter sleep-wake amounts or magnitude of nuchal EMG when compared to perfusion with the vehicle (ACSF - artificial cerebrospinal fluid). A–C: mean data for sleep-wake states from five rats shown in successive 3 h-long quantification intervals staring from the last one prior to lights-off and ending with the first one after lights-on. D–F: mean nuchal EMG levels corresponding to the sleep-wake data shown in A–C. In the mianserin sessions, mianserin was perfused through the XII nucleus for 2 h between 7 pm and 10 pm. The format of these graphs is the same as in Fig. 4. There was a highly statistically significant circadian variation of the amounts of sleep-wake states (A–C), and wake-related nuchal EMG was significantly higher during the lights-off than during the lights-on period (see text for details). However, mianserin perfusion had no effects on either sleep wake states or nuchal output, thus indicating that the effects of mianserin on lingual EMG (Fig. 4) were exerted locally at the level of the XII nucleus.