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Circadian dependence of receptors that mediate wake-related excitatory drive to hypoglossal motoneurons

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Abstract

Serotonin (5-HT), norepinephrine and orexins (ORX) are the three best established mediators of wake-related activation of hypoglossal (XII) motoneurons that innervate the muscles of the tongue. Since the tongue's use is temporarily closely aligned with the rest-activity cycle, we tested whether expression of mRNA for relevant 5-HT, norepinephrine and ORX receptors varies in the XII nucleus with the rest-activity cycle. Adult rats (n=7–9/group) were decapitated at 8–9 am (near rest period onset) or at 6–7 pm (near active period onset). Tissue micropunches were extracted from medullary slices containing the XII motor and sensory external cuneate (ECN) nuclei. 5-HT_{2A}, α_1-adrenergic and ORX type 2 receptor mRNAs were quantified using RT-PCR. Only 5-HT_{2A} receptor mRNA levels differed between the two time points and were higher at the active period onset; no differences were detected in the ECN. Consistent with the mRNA results, 5-HT_{2A} protein levels were also higher in the XII nucleus at the active period onset than at rest onset. Thus, the endogenous serotonergic excitatory drive to XII motoneurons may be enhanced through circadian- or activity-dependent mechanisms that increase the availability of 5-HT_{2A} receptors prior to the active period. Conversely, reduced levels of 5-HT_{2A} receptors during the rest/sleep period may exacerbate the propensity for sleep-disordered breathing in subjects with anatomically compromised upper airway.

Keywords

circadian rhythm; norepinephrine; orexin; serotonin; sleep; tongue; obstructive sleep apnea; upper airway

1. INTRODUCTION

Hypoglossal (XII) motoneurons innervate the muscles of the tongue (genioglossus, hyoglossus, and geniohyoid). Under the normal, healthy conditions, motor functions of the tongue, such as food/fluid intake and phonation, are closely aligned with the rest-activity cycle. However, in subjects with anatomically compromised upper airway, activation of the tongue muscles is also required to maintain sufficient upper airway patency for breathing. In such subjects, upper airway muscle tone, including that of the tongue muscles, is elevated during wakefulness but declines during sleep, which leads to recurrent periods of flow

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limitation or complete upper airway obstructions, episodic hypoxemia and sleep disruption (Sauerland and Harper, 1976; Remmers et al., 1978; Suratt et al., 1988; Mezzanotte et al., 1992; Okabe et al., 1994; Katz and White, 2004; see Kubin and Davies, 2011; Horner, 2012; White and Younes, 2012 for reviews).

Similar to healthy humans (Chokroverty, 1980; Kuna et al., 1994; Katz and White, 2004; Brown et al., 2011), in rats, activity of the tongue muscles and XII motoneurons is high and variable during wakefulness, very low or absent during slow-wave sleep (SWS), and generally absent but punctuated by often large phasic twitches during rapid eye movement sleep (REMS) (Megirian et al., 1978; Lu et al., 2005; Lu and Kubin, 2009; Rukhadze et al., 2011). In obstructive sleep apnea (OSA) patients and rats with experimentally enhanced upper airway muscle tone through the means such as vagotomy, application of stimulants into the XII nucleus or chemical stimulation of breathing, XII motoneurons also exhibit inspiratory modulation (Hwang et al., 1983; Jelev et al., 2001; Morrison et al., 2003; Fenik et al., 2005) which in subjects with anatomically compromised upper airway plays an important role in protecting the airway against obstructions at the time when inspiratory negative pressure exerts a centripetal force on the airway walls. Thus, the accessory respiratory function of XII motoneurons in healthy subjects becomes extremely important in subjects with anatomical conditions that make the upper airway vulnerable to collapse, with both the tonic and inspiratory-modulated activity required to maintain adequate ventilation.

Several distinct neurochemical systems have been identified that collectively mediate the wakefulness-related excitatory effects onto XII motoneurons, thereby facilitating those functions of the tongue that are typically performed during wakefulness, as well as maintaining tongue activity in OSA patients. Of those, the best evidence is currently available for norepinephrine (NE) and serotonin (5-HT) which are released from brainstem cells and for orexins (ORX) which are produced by neurons located in the posterior, lateral hypothalamus (Aston-Jones and Bloom, 1981; Trulson and Trulson, 1982; Estabrooke et al., 2001; Fenik et al., 2002; Lee et al., 2005; Mileykovskiy et al., 2005; Takahash et al., 2008). NE, 5-HT and ORX cells have maximal activity during wakefulness, reduced activity during SWS and minimal or no activity during REMS. Terminals containing NE, 5-HT and ORX are present in the XII nucleus, and XII motoneurons express α_1-adrenergic, 5-HT_2 and ORX type 2 receptors that are all known to mediate excitatory effects (Kubin et al., 1992; Funk et al., 1994; Peyron et al., 1998; Fay and Kubin, 2000; Marcus et al., 2001; Volgin et al., 2001, 2002, 2003; Zhan et al., 2002; Fenik and Veasey, 2003). There is also pharmacological evidence that these modulators mediate major portions of the endogenous excitatory drives that maintain activity of XII motoneurons (Kubin et al., 1992; Fenik et al., 2005; Chan et al., 2006; Stettner and Kubin, 2013).

Under normal conditions, motor activity of the tongue is temporarily closely aligned with the rest-activity cycle. Mechanistically, this may be related to the fact that major excitatory drives to XII motoneurons originate in the premotor circuits that have sleep-wake dependent patterns of activity. However, it is also plausible that the sensitivity of XII motoneurons to these drives is regulated in a circadian manner with a pattern that reinforces the ability of the state-dependent systems to activate XII motoneurons at the time when it is most appropriate, i.e., during the active phase of the circadian cycle. Such a hypothetical mechanism could relay on a circadian- or use-dependent regulation of the availability of excitatory receptors in XII motoneurons. Indeed, 5-HT binding in different regions of the rat brain varies with the circadian time, suggesting that regulation of receptor availability is a potentially important mechanism by which effectiveness of neurotransmitter actions is aligned to the rest-activity cycle (Wesemann et al., 1983; Wesemann and Weiner, 1990; Weiner et al., 1992). Accordingly, our goal was to test whether such mechanisms operate for NE, 5-HT or ORX receptors located in the XII nucleus, as this would add a new dimension to both the normal

regulation of tongue activity across the rest-activity cycle and the ability of central excitatory premotor pathways to maintain upper airway patency in OSA patients.

We found that, of the three receptor systems investigated, 5-HT_{2A} receptor mRNA and protein levels were higher in the XII nucleus at the active period onset than at the rest onset. This may result in a relatively enhanced endogenous serotonergic excitatory drive to XII motoneurons during the active period and a relatively reduced ability of 5-HT to activate XII motoneurons during the rest/sleep period. Preliminary data have been published (Kubin et al., 2010; Volgin et al., 2010).

2. MATHERIALS AND METHODS

Experiments were performed on 18 adult, male Sprague-Dawley rats (300–385g). All animal handling procedures followed the guidelines of the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

2.1. Tissue extraction and mRNA quantification procedures

The animals were housed on a 12:12 light/dark schedule with lights on at 7 am and *ad libitum* access to food and water. They were decapitated under deep isoflurane anesthesia (4%) either between 8 and 9 am (just after the rest period onset) or between 6 and 7 pm (just prior to the active period onset). The brainstems ware rapidly removed and immersed in ice-cold artificial cerebrospinal fluid containing (in mM): 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, and 20 mannitol; pH adjusted with NaOH to 7.4 and osmolarity set at 300±5 mOsm. The medulla was blocked, immersed in the same ice-cold medium, and transverse sections, 500–600 μm thick, were obtained using a tissue slicer (VSLM1; Lafayette, IN). Tissue micropunches, 500 μm in diameter, were extracted bilaterally from the XII nucleus and, for comparison, from the somatosensory external cuneate nucleus (ECN). From each pair of micropunch samples, one was used for total RNA extraction and the other was stored at −80° C for subsequent protein studies. The slices from which the punches were extracted were fixed in formalin, cut into 25 μm sections, mounted and stained with Neutral red to verify the proper placement of the punches (Fig. 1A).

Total RNA was extracted from each micropunch using the RNeasy® Mini Kit (Qiagen, Valencia, CA), re-dissolved in 50 μl of RNase-free water and quantified by densitometry (BioPhotometer, Eppendorf, Germany). One half of the extract was treated with RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) and reverse-transcribed using SuperScript® IIReverse Transcriptase (Life Technologies, Carlsbad, CA)in a total buffer volume of 50 μl. Subsequent PCR reactions were performed using LightCycler® system (Roche Diagnostics, Indianapolis, IN). Fixed aliquots of each cDNA sample (1 μl) were used for polymerase chain reactions (PCRs) with primer sets for the following genes: α_{1A}- and α_{1B}-adrenergic receptors (Volgin et al., 2001), 5-HT_{2A} and 5-HT_{2C} receptors (Volgin et al., 2003), ORX type 2 receptors (Volgin et al., 2002), and α1-tubulin (Volgin and Kubin, 2006) (see Table 1 for accession numbers). PCR amplification was performed in 20 μl of the reaction buffer containing 250 μM of dNTPs, 200 nM of the primers, 2.5 μl of SYBR Green I cDNA-sensitive dye (Sigma-Aldrich, Saint Louis, MO), 1 μl of cDNA sample, and 0.7 μl of Titanium© Taq DNA polymerase (Clontech, Palo Alto, CA). The PCRs comprised 30 s of initial denaturation at 95° C followed by repeated cycles of a 1 s spike at 95° C and 25 s of combined annealing-elongation at 68° C, and were completed with 30 s of final elongation (Fig. 1B). Subsequently, the products were subjected to linear heating (0.2° C/s) to 95° C to assess the quality of each reaction based on the position and shape of the peaks of the melting curves (Fig. 1C). After cooling, the PCR products were displayed on an ethidium bromide-stained 2% agarose gel to confirm that they were of the appropriate size. PCR

reactions were calibrated using external cDNA standards produced in house for each cDNA target, as described previously (Volgin and Kubin, 2006). Ultimately, the target receptor mRNA levels were quantified as the number of cDNA copies per 1,000 copies of the housekeeping gene (tubulin) derived from the same micropunch sample.

2.2. 5-HT_{2A} receptor protein quantification

The amount of protein available from a single tissue micropunch like those collected in this study is, at best, sufficient for quantification of one selected target protein and one reference protein. Since our mRNA data pointed to 5-HT_{2A} receptors as the ones whose levels were likely to vary with circadian time, we used the tissue punches that remained available after the mRNA study to quantify the levels of 5-HT_{2A} receptor-like protein in the XII nucleus and ECN. Each micropunch sample was sonicated in 12 μl of the solubilizing buffer containing 7 M of urea, 2 M of thiourea, 0.25 mM Tris base, 4.0% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate(CHAPS)and 1.0% NP -40. Subsequently, proteins were separated by size on an SDS-PAGE gel and transferred onto nitrocellulose membranes (Mini-Protean-3 system, BioRad, Hercules, CA). Albumin-blocked membranes were incubated overnight with rabbit antibodies against 5-HT_{2A}receptors (1:250, catalog no. 24288, ImmunoStar, Hudson, WI) and β-actin (1:1,000, catalog no. 4970, Cell Signaling, Danvers, MA) at 4 C. Primary antibody binding sites were visualizedby incubation with donkey ECL anti -rabbit, horseradish peroxidase-conjugated IgG (1:10,000, catalog no. NA934V, GE Healthcare, Mickleton, NJ) and the SuperSignal West Dura chemiluminescent substrate (Pierce/Thermo Scientific, Hudson, NH). The chemiluminescent signal was detected using HyBlot CL autoradiography film (Denville Scientific, South Plainfield, NJ). Digital images of the lab eled bands corresponding to 5-HT_{2A} receptor protein and β-actin were quantified using ImageJ software(National Institutes of Health; http://rsb.info.nih.gov/ij/index.html). After subtraction of background staining (Fig. 2B), the amounts of 5-HT_{2A} and β-actin proteins were measured by integration of the intensity of staining within each band obtained from each micropunch sample and the amount of 5-HT_{2A} protein was expressed relative to the density of the band for β-actin in the same tissue sample (Fig. 2C).

2.3. Statistical analysis

All datasets with continuous variables were tested for normality and equal variance (Analyse-It, Leeds, UK). The significance of differences between mRNA or protein levels in tissue samples harvested from each of the two anatomic regions at the two different circadian times was examined using one-way ANOVA followed by post-hoc comparisons for each receptor subtype with Bonferroni's correction for multiple comparisons. If normality criteria were not fulfilled, nonparametric analysis was performed using Kruskal-Wallis ANOVA. The variability of the means is characterized by the standard error (SE), and the differences were considered significant at $p<0.05$.

3. RESULTS

3.1. Receptor mRNA quantification in the XII nucleus and ECN

We compared mRNA levels for selected receptors that mediate wake-related activation of XII motoneurons between two time points separated by about 12 h and selected to represent a period just after the circadian rest phase onset and just before the active phase onset. When quantified relative to mRNA levels for tubulin, 5-HT_{2A} receptor mRNA levels were significantly higher in the XII nucleus at the active period onset than at the rest period onset. For all other receptors studied (5-HT_{2C}, α_{1A}- and α_{1B}-adrenergic, and ORX type 2), the differences between the two time points were not statistically significant (Table 1). In the ECN, the average mRNA levels of 5-HT_{2A}, 5-HT_{2C} and α_{1B} adrenergic receptors were

considerably lower than in the XII nucleus (p=0.002–0.09) and no significant differences were detected between the two time points (Table 1). It may be, however, of note that mRNA levels for 5-HT_{2A} and α_{1B}-adrenergic receptors exhibited relative prominent tendencies towards higher levels at the active period onset than at rest onset (p=0.09 and p=0.06, respectively).

3.2. 5-HT_{2A} receptor protein levels in the XII nucleus and ECN

With the difference in 5-HT_{2A} receptor mRNA levels in the XII nucleus between the rest onset and active period onset of a nearly 3-fold order and a moderate tendency in the same direction in the ECN, we then measured by means of Western blots whether the differences in 5-HT_{2A} receptor protein levels followed the same pattern. We found a significantly higher 5-HT_{2A} receptor to β-actin protein ratio in the XII nucleus samples collected at the active period onset when compared to the rest onset (2.0±0.3 *vs.* 1.0±0.2; p<0.02; Fig. 3, top panel). As with 5-HT_{2A} receptor mRNA, the mean level of 5-HT_{2A} receptor protein was considerably lower in the ECN than in the XII nucleus, and there was only a weak trend in the ECN for the relative level of the 5-HT_{2A} receptor protein to be higher at the active period onset than at the rest onset (p=0.25; Fig. 3, bottom panel).

4. DISCUSSION

Our main finding is that, among the three distinct receptor systems that mediate wake-related activation of XII motoneurons, the levels of at least one receptor subtype, the 5-HT_{2A}, exhibit a distinct and statistically significant difference in the XII nucleus between the time corresponding to the onset of the rest period and the time just preceding the active period when quantified at either the mRNA or protein levels. Importantly the direction of the difference is such that it should favor a stronger activation of XII motoneurons by 5-HT during the early part of the active period when compared to the beginning of the rest period. In contrast to 5-HT_{2A} receptors, no differences between these two time points were suggested by measurements of mRNA levels for 5-HT_{2C} receptor (another excitatory 5-HT receptor subtype present in the XII nucleus), nor for the α_1-adrenergic or ORX type 2 receptors. Furthermore, no significant changes for any of these receptors were detected in ECN, a somatosensory nucleus located in the dorsal medulla relatively close to the XII motor nucleus. These results suggest that the mechanisms driven by the central circadian clock(s) or those secondary to the natural circadian pattern of tongue use can selectively influence the availability of one of several receptor systems that are known to mediate excitatory drive to XII motoneurons and are established mediators of their wake-related activation. As such, our findings unveil a potential mechanism by which the motor output to the tongue muscles is enhanced, or reinforced, during the time most appropriate for the use of the tongue (active period). Conversely, the same mechanism may also be seen as designed to reduce, or disfacilitate, activation of the tongue during the rest period when rats normally sleep and have a reduced need for complex and strong activation of the tongue muscles.

Our present measurements were limited to only two time points separated by approximately 12 h. We used this experimental design because it allows one to grossly assess circadian differences in receptor levels. With this limited approach, we were able to detect a large and statistically significant day-night difference for 5-HT_{2A} receptors that consistently occurred at both the mRNA and protein levels. However, due to the limited temporal density of tissue sampling, our design might have been sub-optimal for capturing the full peak and trough of the process that determines 5-HT_{2A} receptor levels in the XII nucleus, and it is possible that the other receptors for which we did not detect any circadian changes would exhibit circadian variations when tested more frequently or at different phases of the circadian cycle.

Nevertheless, our present positive findings with 5-HT_{2A} receptors are important because they reveal a novel and little investigated mechanisms whose function might be to best align motoneuronal excitability with the active phase of the circadian cycle to achieve optimal motor performance. Additional studies with more frequent tissue sampling may reveal further intricacies of the processes that optimize the central control of the motor output relative to the circadian phases of rest and activity.

The rest-active period difference that we detected may be driven by the central circadian clock, the light-dark cycle, or the closely associated with these variables circadian changes in motoneuronal activity. In mammals, the main circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus but multiple secondary molecular circadian pacemaker circuits are present in other brain regions, as well as in peripheral tissues and organs (Yamazaki et al., 2000; Reppert and Weaver, 2002; Yoo et al., 2004; Herichová et al., 2007; Kaneko et al., 2009; Hughes et al., 2012). Under most conditions, these additional clocks are synchronized by the neural and humoral outputs from the SCN but they are also capable of controlling the functions of different neural circuits and organs in a relatively autonomous manner. For example, in the hypertensive transgenic TGR(mRen-2)27 rats, normal circadian rhythms of blood pressure are inverted, with relatively higher blood pressure during the rest (light) phase than during the active (dark) phase, whereas circadian rhythms of heart rate and locomotor activity are normal (Lemmer et al., 1993; Witte and Lemmer, 1999). These rats have altered profiles of rhythmic expression of clock genes in the SCN and in the medullary regions that control arterial blood pressure (nucleus of the solitary tract and rostral ventrolateral medulla) (Herichová et al., 2007). In fruit flies (*Drosophila)*, circadian rhythms of motor activity are associated with rhythmic circadian changes in the size of synaptic boutons on flight motoneurons that occur independently of synaptic activity or disruption of the rest-activity cycle and persist when motoneurons are disconnected from other major circadian clocks (Mehnert et al., 2007; Mehnert and Cantera, 2008). Thus, the neural circuits important for supporting the most essential functions of the organism appear to be equipped with their own mechanisms that optimize performance relative to the fundamental circadian rhythm of rest and activity both in mammals and evolutionarily lower species. The same is likely to be the case for mammalian motoneurons, and especially those that support alimentary functions. In rats, food intake is significantly higher during the dark (active) period than during the light (sleep) period (e.g., Madrid et al., 1993). Since the tongue plays a key role in alimentary behaviors, it is possible that the circadian change in 5-HT_{2A} receptor expression that we detected is designed to support this function. Alternatively, it may have been driven by synaptic activity related to the circadian changes in the use of the tongue. Considering that the amounts of sleep, including REMS, vary between the dark and light periods, it is possible that certain cellular, humoral or neurochemical mechanisms specific for the generation of the distinct stages of sleep also contribute to the circadian variation of 5-HT_{2A} receptor levels.

Expression of 5-HT receptors also can be regulated by ligand binding (see Sodhi and Sanders-Bush, 2004 for a review). Brain 5-HT levels exhibit circadian variation and, in rats, they are higher during the active phase than during the rest phase (Sanchez et al., 2008), a mechanism that can also contribute to the circadian variability of long-term facilitation of ventilation (c.f., the article by Mateika and Syed in this Special Issue). A strong stimulation of 5-HT_{2A} receptors during the night may lead to receptor internalization resulting in a circadian variation whereby the initially high levels at the onset of the active period gradually decline with prolonged activation. This, in turn, may stimulate synthesis and transport of new receptors to the cell surface with a time constant of the entire process optimized to go along with the circadian period of rest and activity. Thus, there is a potential

that the circadian variation of 5-HT_{2A} receptor levels in the XII nucleus is driven by the circadian variation of 5-HT levels.

Studies conducted in different animal models suggested that the magnitude of endogenous activation of XII motoneurons by 5-HT is species-dependent. It is strong in cats (Kubin et al., 1992; Neuzeret et al., 2009) and dogs (Veasey et al., 1996) but relatively weaker than the effect of NE in rats (Fenik et al., 2005; Sood et al., 2005). However, it is important to note that the studies in rats were conducted during the rest period. Our present data suggest that the excitatory effects of 5-HT on XII motoneurons mediated by 5-HT_{2A} receptors may be limited during the rest period by the receptor availability and that stronger effects could be detected during the active period.

Collectively, our findings suggest that the strength of serotonergic activation of XII motoneurons varies with the circadian cycle and that it is reinforced during the active period by an increased availability of 5-HT_{2A} receptors. Under the normal conditions, this should properly support the main motor functions of the tongue. However, in patients with OSA, reduced levels of 5-HT_{2A} receptors in XII motoneurons during the rest/sleep period would negatively impact the ability of the tongue muscles to protect the upper airway from collapse and to re-open the airway following an obstructive episode. The mechanisms that cause the circadian variation of 5-HT_{2A} receptors in the XII nucleus need additional studies for their clear health relevance for sleep-disordered breathing and their potentially fundamental role in aligning the motor output with the natural periods of rest and activity.

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HIGHLIGHTS

- Activity of hypoglossal (XII) motoneurons helps maintain airflow in the upper airway.
- Serotonin (5-HT) mediates wake-related activation of XII motoneurons.
- 5-HT_{2A} receptor levels are higher in XII nucleus at 6–7 pm than at 8–9 am.
- Receptor changes in motoneurons help align motor performance with circadian rhythm.

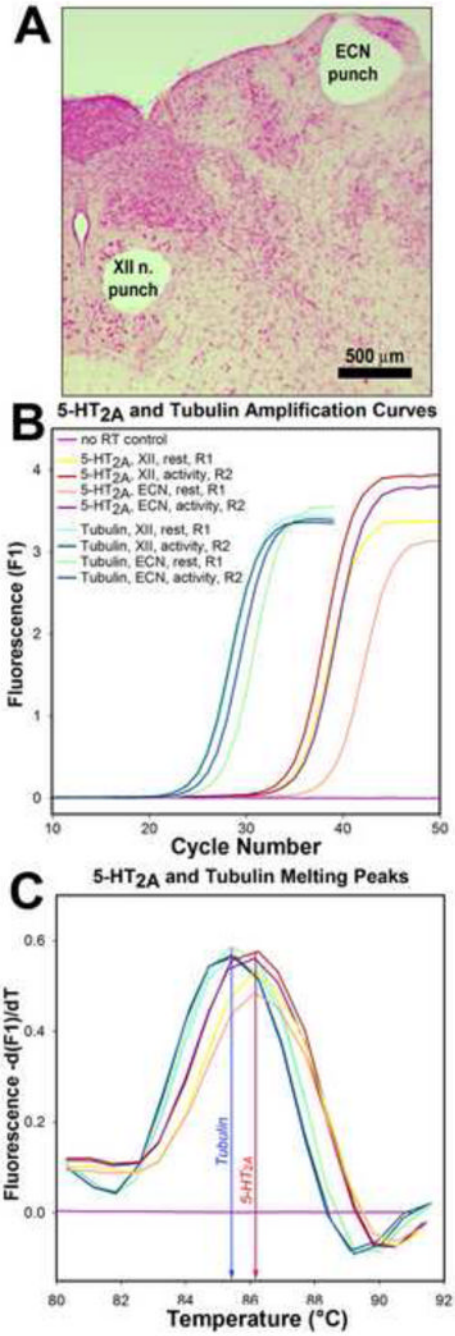

Figure 1.

Example of tissue sampling and of the outputs from RT-PCR reactions. A: location of the tissue micropunches extracted from the XII nucleus and external cuneate nucleus (ECN) visualized in a Neutral red-stained section of a medullary slice. B: PCR amplification curves obtained with a set of cDNA samples from the XII nucleus and ECN from two rats, one at the rest period onset (R1) and the other at the active period onset (R2), and from one control mRNA sample that was not reverse-transcribed. C: melting curves for the set of reactions shown in B demonstrates that the PCR reactions yielded two distinct products that had melting peaks at the temperatures expected for the 5-HT_{2A} receptor and tubulin cDNAs.

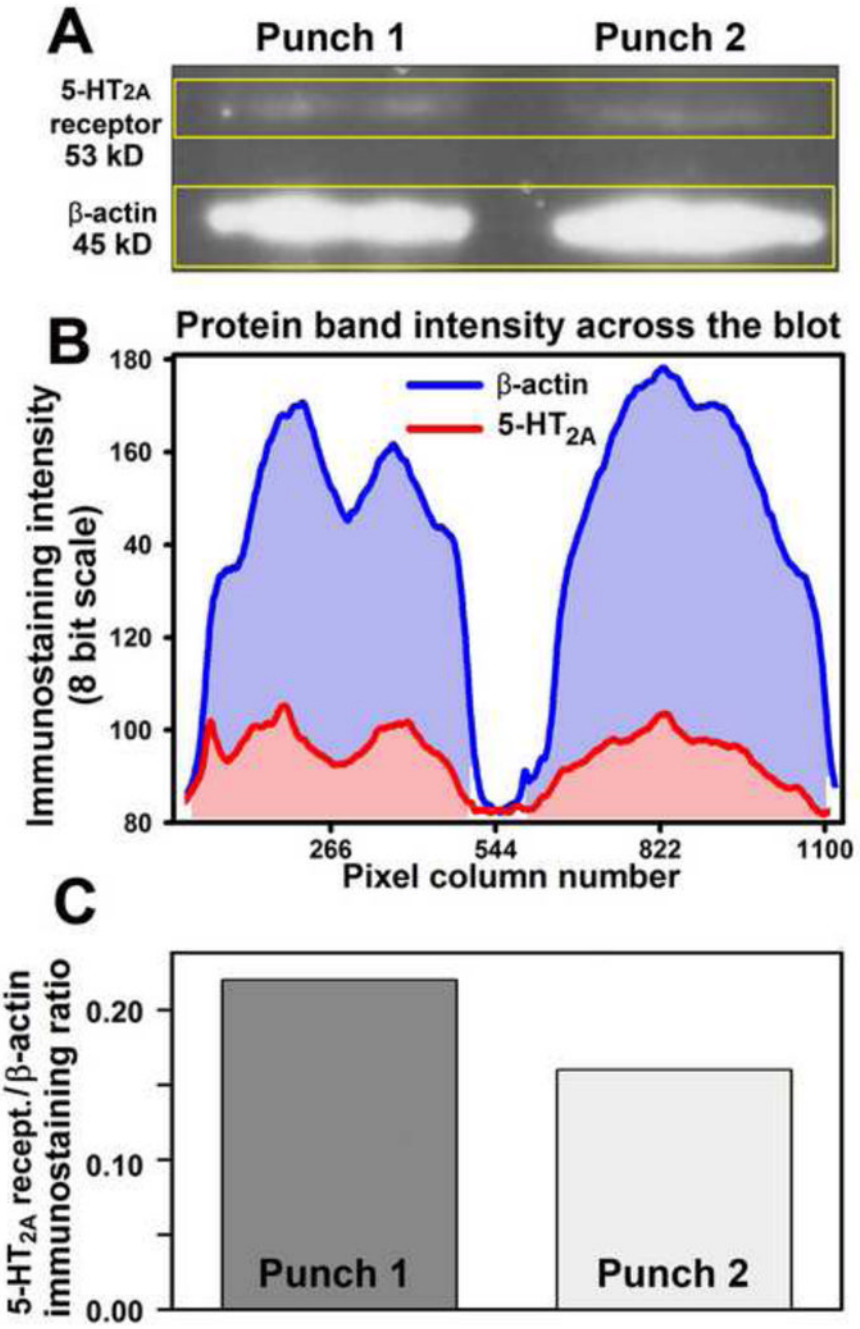

Figure 2.

Quantification of 5-HT_{2A} receptor protein in tissue micropunches by Western blotting. A: image of a blot double-labeled for 5-HT_{2A} receptor protein and β-actin with two protein samples (lanes) obtained from tissue micropunches. The yellow frames enclose the areas scanned for densitometric measurements of protein amounts in each band. B: staining intensity for each of the two bands measured across the gel shown in A obtained after background subtraction. C: bars representing the ratios of 5-HT_{2A} receptor to β-actin protein amounts for the data illustrated in A and B.

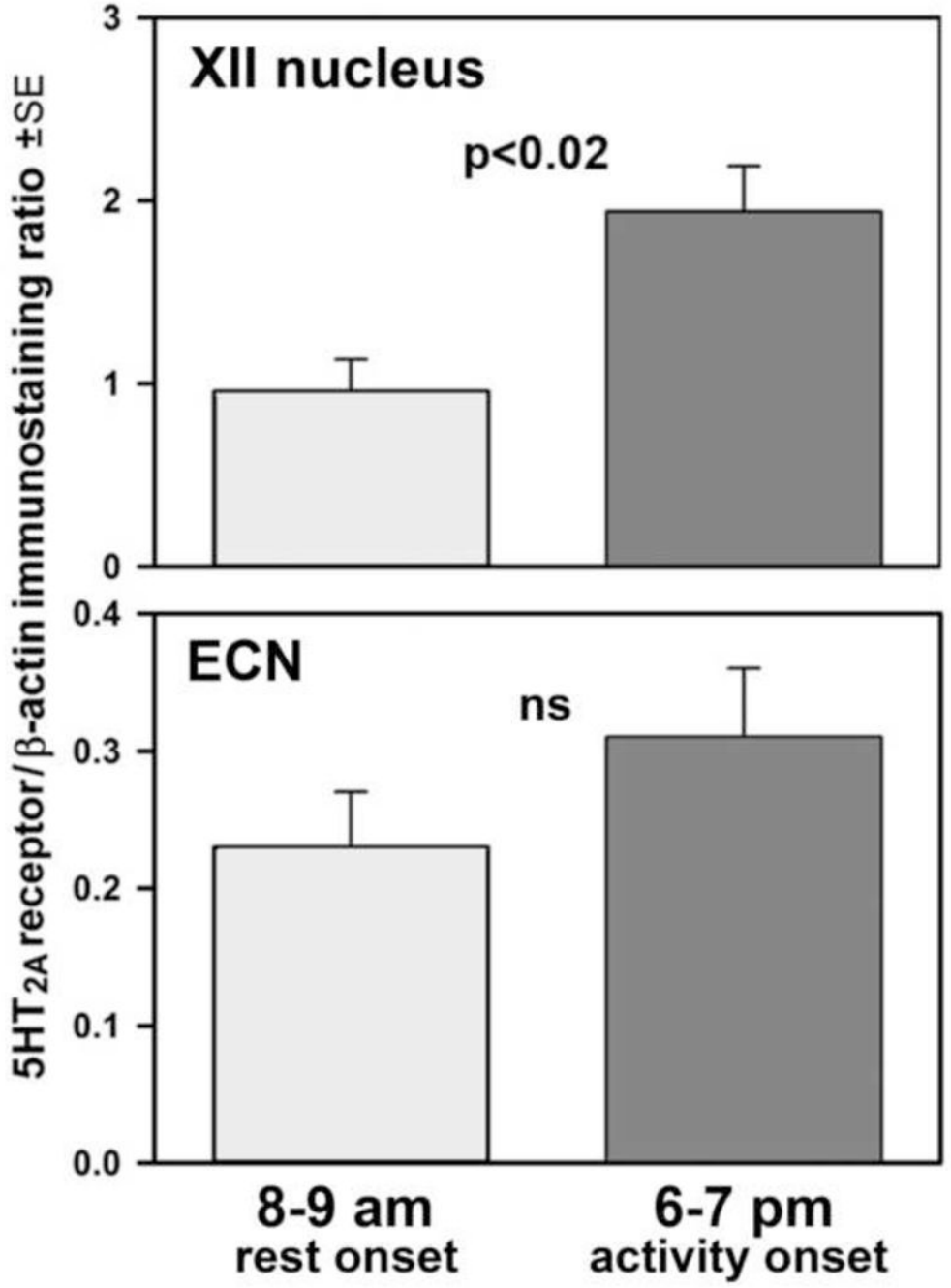

Figure 3.
Mean 5-HT_{2A} receptor protein levels in micropunches extracted from the XII nucleus (top panel) and external cuneate nucleus region (ECN; bottom panel) at rest onset (8–9 am) and active period onset (6–7 pm) normalized by the β-actin contents in the same protein samples (n=6–8 rats per group).

Table 1

Neurotransmitter receptor mRNA levels quantified by RT-PCR near the time of the rest period onset (8–9 am) and near the active period onset (6–7 pm) in the XII nucleus and external cuneate nucleus (ECN).

Transcript and its accession number	Transcript copy numbers* ±SE					
	XII nucleus			ECN		
	8–9 am	6–7 pm	*p* value	8–9 am	6–7 pm	*p* value
5-HT_{2A} M30705	5.2±2.8	14.9±3.7	0.03	0.7±0.3	1.8±0.8	0.09
5-HT_{2C} M21410	49.7±14.0	42.4±10.0	0.82	8.2±3.5	7.3±2.4	0.64
α_{1A}AR U07126	63.5±18.1	44.8±8.1	0.38	41.4±13.6	67.9±17.1	0.12
α_{1B}AR L08609	20.0±2.9	23.1±5.2	0.59	6.5±1.4	13.4±3.0	0.06
ORX 2R AF041246	1291±476	711±175	0.77	807±255	1656±1038	0.85
Tubulin, α1NM_022298 (absolute)	9426±2191	11864±3277	0.54	3767±913	3496±1064	0.77

*Receptor mRNA levels were quantified at each time point and anatomical location as the number of cDNA copies of the target cDNA per 1,000 copies of the housekeeping gene, tubulin, in the same sample. The bottom row shows the mean absolute cDNA copy numbers per sample for tubulin. p values describe for each transcript the statistical significance of the difference between the samples collected at the two different time points from the XII nucleus or the ECN (n=7–9 rats per group).