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Increased tongue use enhances 5-HT$_{2C}$ receptor immunostaining in hypoglossal motor nucleus

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Abstract

Hypoglossal (XII) motoneurons are activated by type 2 receptors for serotonin (5-HT). This activation is especially strong during wakefulness which facilitates diverse motor functions of the tongue, including the maintenance of upper airway patency in obstructive sleep apnea (OSA) patients. We tested whether 5-HT$_2$ receptor levels in the XII nucleus vary with intensity of tongue use. Three groups of rats were housed overnight under conditions of increasing oromotor activity: W-water available *ad lib*; S-sweetened water to stimulate drinking; S+O-sweetened water+oil applied on fur to increase grooming. After the exposures, immunostaining for 5-HT$_{2C}$, but not 5-HT$_{2A}$, receptors was higher in the XII nucleus in S+O than in W rats (65±1.8 (SE) *vs.* 60±2.0 arbitrary units; p=0.008). In the medullary raphé obscurus region, the percentage of c-Fos-positive 5-HT cells was 13% higher (p=0.03) in S+O than in W rats. The positive feedback between tongue use and 5-HT$_{2C}$ receptor immunostaining reveals a novel mechanism potentially relevant for OSA and neuromuscular disorders.

Keywords

drinking; grooming; motor rehabilitation; obstructive sleep apnea; plasticity; serotonin receptors; use-dependence

1. Introduction

The muscles of the tongue play a key role in multiple vital functions, such as food and water intake, vocalization and social interactions [Sawczuk and Mosier, 2001; Travers and Jackson, 1992; Vranish and Bailey 2015]. These functions are predominantly associated with the state of wakefulness and the active phase of circadian cycle. In addition, in persons suffering from obstructive sleep apnea syndrome (OSA), the tonic and inspiration-related activations of tongue muscles have important respiratory roles to prevent upper airway obstructions by protruding the tongue and stiffening the walls of the pharyngeal airway. This

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protective function, while effective during wakefulness, often fails during sleep, leading to recurrent apneic episodes that then terminate with bouts of strong activation of tongue muscles [see Horner, 2012; Kubin, 2016; White and Younes, 2012 for reviews]. Hence, significant efforts have been devoted to better understand the neurophysiology and neurochemistry of the central neural control of hypoglossal (XII) motoneurons which provide motor innervation of the tongue [e.g., Besnard *et al.*, 2009; Fuller *et al.*, 1999; Nasse and Travers, 2014; O'Brien *et al.*, 2004; Selvaratatnam *et al.*, 1998].

Among the many neurotransmitters and neuromodulators that control XII motoneurons, serotonin (5-HT) is one of the most extensively studied [see Kubin, 2016 for a review]. The characteristic pattern of 5-HT cell activity, high during wakefulness, reduced during slow-wave sleep, and silent during rapid eye movement sleep [Jacobs and Azmitia, 1992], is a major reason for the high interest in the role of 5-HT in the regulation of tongue activity with the sleep-wake cycle. Among the 9 known subtypes of 5-HT receptors, 2 are present in the XII motor nucleus and mediate excitatory effects onto motoneurons; they are the 5-HT_{2A} and 5-HT_{2C} subtypes [Fay and Kubin, 2000; Okabe *et al.*, 1997; Volgin *et al.*, 2003; Zhan *et al.*, 2002]. The excitation mediated by these receptors facilitates the maintenance of tongue position and execution of tongue movements during wakefulness [Kubin *et al.*, 1992]. Notably, medullary 5-HT-containing cells are activated during rhythmic and stereotypical oromotor behaviors [Jacobs *et al.*, 2002; Veasey *et al.*, 1995].

Both brain 5-HT and its receptors vary with the circadian cycle but there is only a limited understanding as to how these changes are orchestrated and regulated [Martin, 1991; Miyamoto *et al.*, 2012; Wesemann and Weiner, 1990]. For example, 5-HT_{2A} receptor mRNA and protein had higher levels in the XII nucleus at the beginning, than at the end, of the active period of circadian cycle [Volgin *et al.*, 2013]. In other systems, chronic receptor stimulation with 5-HT_2 agonists suppressed 5-HT_{2A} receptor-mediated behaviors but enhanced a 5-HT_{2C} receptor-mediated behavior [Berendsen and Broekkamp, 1991]. Thus, 5-HT receptors may respond to periodic changes in 5-HT release, with their stimulation leading to receptor internalization, degradation, synthesis and transport to and from the cell surface, as shown for other receptors [*e.g.*, Wierenga *et al.*, 2005; Modirrousta *et al.*, 2007].

An increased availability of 5-HT_{2A} receptors in the XII nucleus coincident with the active period of circadian cycle may have a physiologic role in optimizing the tongue muscle tone and execution of tongue movements [Volgin *et al.*, 2013]. From a clinical perspective, a better understanding of the mechanisms that regulate serotonergic activation of XII motoneurons may help develop novel treatments for OSA. In the present study, we tested the hypothesis that 5-HT_{2A} and/or 5-HT_{2C} receptor immunostaining in the XII nucleus responds to varying intensity of tongue use. Towards this goal, we conducted experiments in rats in which a stereotyped use of the tongue was behaviorally increased in a manner known to be associated with increased activation of medullary 5-HT neurons [Jacobs *et al.*, 2002; Veasey *et al.*, 1995] and, consequently, an increased release of 5-HT onto XII motoneurons. Preliminary results have been published [Das *et al.*, 2016].

2. Materials and Methods

Eighteen adult male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). After delivery, they were housed singly in our animal facility for at least 4 days under a 12 h light (7:00–19:00)/12 h dark cycle and with standard rodent chow and water available *ad lib* to habituate them to the subsequent experimental conditions. All experimental procedures followed the National Institutes of Health (USA) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023) and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (protocol 804875).

2.1 Animal groups and behavioral monitoring

After habituation, three animals at a time were placed for an additional 4 days in three separate cages equipped with infrared beam-based detectors of drinking from a standard water bottle (ENV-251M lickometer; Med Associates, St. Albans, VT) and infrared beam-based monitors of locomotor activity (MicroMax, AccuScan Instruments, Columbus, OH). All animals received standard rat food at all times and tap water for drinking at all times except the last experimental night designed to enhance tongue activity. Specifically, during that night, one rat continued receiving tap water (W rat), another rat received sweetened water containing sucrose (100 mM) and saccharin (6 mM) (S rat) to increase drinking activity with only a moderate increase in sugar intake [Davis and Smith, 1988; Weijnen, 1998], and the third rat also was presented with sweetened drinking water and additionally had peanut oil applied on the fur of the forelimbs and upper body to stimulate grooming (S +O rat). Each such group of three rats is from now on referred to as a “triplet.” In the course of the study, six “triplets” were successively subjected to this protocol.

Both drinking activity and locomotor activity were continuously recorded during the last two nights. Individual “licks” of drinking water were acquired as electric pulses generated by transient interruptions of the lickometer infrared light beam using data acquisition hardware and Spike-2 software (Cambridge Electronic Design, Cambridge, England). Accurate detection of tongue licking movements by the lickometer was validated in one additional rat that was a part of another study. The rat was chronically instrumented for recording of electromyographic activity (EMG) of the tongue, dorsal neck and diaphragm muscles, as well as cortical electroencephalogram, as described elsewhere [Stettner *et al.*, 2013]. Figure 1 shows a typical recording of tongue EMG during drinking from a water bottle attached to a lickometer [cf. Travers and Jackson, 1992]. Locomotor activity of each rat was acquired in successive 10 min intervals as counts of distinct breaks of infrared light beams spaced at 2.5 cm apart. The additional enhancement of tongue use associated with grooming triggered by application of oil to the rat’s fur could not be quantified but we verified by direct observations and video recordings that S+O rats repeatedly generated bouts of intense grooming not seen in either W or O rats both at the beginning and near the end of the experimental night.

At 8 am following the last recording (one hour after lights-on), the rats were anesthetized with pentobarbital (100 mg/kg) and perfused through the aorta with 100–300 ml of phosphate-buffered saline (PBS; pH 7.4, room temperature) containing heparin (5 U/ml) and

lidocaine (0.003%) followed by 200–400 ml of 4% paraformaldehyde in PBS at 4–8°C. The brains were removed, post-fixed in the same fixative overnight, washed in PBS and cryoprotected in 30% sucrose.

2.2 Immunohistochemical procedures and microscopic analysis

Four series of transverse sections, 35 μm thick, of the lower brainstem were cut on a cryostat (Leica, Buffalo, NY). Sections from the three rats of each "triplet" were uniquely marked, combined in one container and submitted together to all immunohistochemical procedures. Free-floating sections were first treated with 1% sodium borohydride in PBS for 15 min and then with 70% methanol and 0.3% H_2O_2 for 15 min. Separate series of sections were then incubated at 4°C for 36–48 h in the blocking solution (4% normal goat serum and 0.2% Triton X-100 in PBS) with one of the following primary antibodies: rabbit anti-5-HT_{2A} receptor (catalog number: 24288, ImmunoStar, Hudson, WI; 1:500), rabbit anti-5-HT_{2C} receptor (catalog number: SAB4501477, Sigma, St. Louis, MO; 1:1000), or rabbit anti-c-Fos (catalog number: SC-52, Santa Cruz Biotechnology, Dallas, TX; 1:400). After washes, sections were incubated for 1 h in a blocking solution containing biotinylated goat anti-rabbit secondary antibodies (catalog number: BA1000, Vector, Burlingame, CA; 1:250) and then for 1 h in avidin-horseradish peroxidase (HRP) complex (ABC kit; Vector). HRP was visualized with diaminobenzidine with nickel ammonium sulfate as the chromogen in the presence of H_2O_2. The sections subjected to c-Fos immunohistochemistry were then additionally incubated at 4°C for 16–20 h with rabbit anti-5-HT antibodies (catalog number: S5545, Sigma, 1:25,000) and 5-HT binding sites were visualized using biotinylated secondary antibodies and the ABC kit as above but with nickel ammonium sulfate omitted, which resulted in a light-brown staining of 5-HT-containing neurons. Thus, 5-HT-positive cells with nuclear c-Fos accumulation could be recognized as brown cells with dark gray-, or black-, stained nuclei. Brain sections from 6 separate rat "triplets" (W, S and S+O rat) were processed for 5-HT_{2A} and 5-HT_{2C} receptor immunohistochemistry, whereas c-Fos/5-HT staining was done with 5 of these 6 "triplets." Negative controls with primary antibodies omitted were previously conducted for 5-HT_{2A} receptor antibodies (Fay and Kubin, 2000), and negative controls with 5-HT_{2C} antibodies were included in this study and were determined to be negative.

Concurrently processed series of sections from all 3 rats of each "triplet" were serially mounted side-by-side, dehydrated, coverslipped, and those designated for analysis were photographed using an upright microscope (Leica DMLB, Wetzlar, Germany) and digital camera (ProgRes CFscan; Jenoptik, Jena, Germany). Images were taken from 4 standard anterior-posterior (A-P) levels covering the caudal half of the XII nucleus (13.68, 13.92, 14.04 and 14.28 mm caudal to bregma according to a rat brain atlas [Paxinos and Watson, 2007]). Each digital image was converted to grayscale and inverted, so that stronger stained regions had higher brightness. Figure 2 shows typical examples of 5-HT_{2A} and 5-HT_{2C} receptor immunostaining, as seen under different magnifications, as well as the corresponding gray scale-converted and then inverted versions of images prepared for quantification of staining intensity. The boundaries of the entire XII nucleus, as well as its dorsal and ventral halves, were outlined and the average density of immunostaining for 5-HT_{2A} and 5-HT_{2C} receptors within each outlined region was measured in arbitrary units

using ImageJ software (National Institutes of Health, Bethesda, MD). Background staining was subtracted by additionally measuring staining intensity in a standard region located in the same brain section just ventral to the XII nucleus where specific immunostaining for 5-HT_{2A} or 5-HT_{2C} receptors was typically low (Fig. 2A and B). The differences between staining intensity within the XII nucleus, or its dorsal and ventral compartments, and the background were calculated for each analyzed brain section and then entered into statistical analysis.

Since sections from the three rats of each 'triplet" had to be mounted on glasses in a standardized manner to facilitate subsequent navigation through this material, the person taking measurements could not be entirely blind to the treatments within each set of brain sections. However, the selection of sections for analysis was based strictly on anatomical criteria unrelated to the treatments, the person conducting quantification of immunostaining was unaware of the hypothesis underlying the study regarding the effect of tongue use on staining intensity, and the measurements of immunostaining were entirely automated. Therefore, there was no room for a subjective bias in data selection or measurements. We previously used this approach to assess the impact of postnatal development, or chronic intermittent hypoxia, on 5-HT_{2A} receptor immunostaining and found it to provide measures consistent with concurrently collected receptor mRNA measures and with functional and immunohistochemical data of others [Rukhadze *et al.*, 2010; Volgin *et al.*, 2003].

Since 5-HT facilitates execution of stereotyped motor patterns, such as drinking and grooming [Jacobs *et al.*, 2002; Veasey *et al.*, 1995] and, at least in some networks, 5-HT regulates 5-HT receptor trafficking [Berendsen and Broekkamp, 1991; Chennaoui *et al.*, 2000], we assessed whether, in our study, the different levels of behavioral stimulation of tongue use were associated with changes in activation of 5-HT neurons. Towards this goal, we determined the proportion of medullary 5-HT neurons with nuclear accumulation of c-Fos, which is an established histochemical method for assessing the level of cellular activation. We focused on caudal medullary 5-HT neurons because they are the source of serotonergic innervation of the XII nucleus [Manaker and Tischler, 1993]. These 5-HT cells aggregate in three anatomically distinct groups referred to as the obscurus, pallidus and parapyramidal raphé regions. Each of these regions contains cells with axonal projections to the XII motor nucleus, with the contributions from the obscurus and pallidus regions (Fig. 4A) being most prominent [Manaker and Tischler, 1993]. To quantify c-Fos expression in medullary 5-HT neurons, we counted all 5-HT neurons found in each of the three distinct raphé regions of interest in brain sections selected to represent four A-P levels appropriate to sample the entire rostro-caudal extent of the caudal medullary raphé: 12.36, 13.08, 13.68 and 14.28 mm caudal to bregma [Paxinos and Watson, 2007]. 5-HT-positive cell bodies were counted when they had at least two primary dendrites, and the average proportion of those containing nuclear accumulation of c-Fos was determined for each rat and each of the three raphé regions.

2.3 Statistical analysis

To minimize the confounding effect of random factors affecting the outcome of immunostaining, brain sections from the 3 rats of each "triplet" were processed, mounted

and analyzed together. All immunostaining data sets were first verified to be normally distributed. They were then subjected to two-way analysis of variance (ANOVA) with interactions and corrections for multiple comparisons (SigmaPlot v. 12.5; Systat Software, Inc., San Jose, CA). Our statistical design included treatment (W, S or S+O) as one factor and the A-P level of each analyzed brain section as the other factor, whereas the data subsets derived from separate "triplets" were entered into analysis as separate "subjects". Accordingly, all receptor immunohistochemistry data sets included 6 "subjects" comprising 3 rats concurrently subjected to one of the three treatments (the "triplet"), and 4 sections selected from each animal representing the four designated AP levels (a total of 72 raw measurements per each data set).

In the analysis of c-Fos expression, the raw numbers of 5-HT-positive cells varied considerably among the brain sections; 57–129 5-HT cells per section for the obscurus region, 21–54 for the pallidus region, and 33–48 for the parapyramidal region. Although this variability had no significant relationship to the treatments, it contributed to variable percentages of 5-HT neurons with c-Fos when calculated on the section-by-section basis. For this reason, the percentages of Fos-positive 5-HT cells were combined across all 4 analyzed brain sections within each of the 3 anatomically distinct raphé regions. We then used 3-way ANOVA with the "triplet" number, treatment, and raphé region as distinct factors. This was followed by paired within each "triplet" Student's t-tests for comparison between treatments.

For the analysis of the impact of experimental conditions on lick counts and locomotor activity, individual licks and locomotor beam breaks were counted over the entire night (7 pm through 7 am), and also within the first and the second half of the night, for the last two nights prior to perfusion. The differences between the first night (all rats presented with plain drinking water - W) and the second night (W or S or S+O for different animals) were assessed using one-way repeated measures ANOVA. When ANOVA revealed a significant effect of the treatment, differences were further explored by direct comparisons between treatment groups using paired t-tests. Results are presented as means ±standard errors (SE).

3. Results

3.1 Licking, locomotor activity, and c-Fos expression in medullary 5-HT neurons following behavioral stimulation of tongue use

Sweetened water, with or without addition of oil on the fur, resulted in significantly increased licking activity when compared to the previous night when the same rats received plain water for drinking. Notably, lick counts were similarly increased when quantified across the entire night (Fig. 3A), and only during the first, or during the second half, of the night (data not shown). This indicated that a satiety, or habituation, did not occur. While direct observations confirmed that application of oil on rat's fur elicited frequent grooming, there was no evidence for competitive interactions among licking, grooming and locomotor activity because there was no difference in lick counts between the S+O and S condition. Furthermore, locomotor activity did not differ among the three treatment groups, indicating that the increased use of the tongue for drinking and grooming did not disrupt the normal locomotor activity of the animal (Fig. 3B).

The percentages of Fos-positive 5-HT neurons significantly varied with all three factors examined, treatment, raphé region, and experimental run, with the 3-way ANOVA-derived p values being 0.007, $4 \cdot 10^{-6}$ and $7 \cdot 10^{-5}$, respectively. In direct comparisons among the treatments, there was a significant difference between S and W rats (t=2.9, p=0.02) and between S+O and W rats (t=3.4, p=0.01), whereas S+O and S rats did not differ. When treatment effect was separately examined within each of the three distinguished raphé regions, a significant difference was detected only within the raphé obscurus and only between W and S+O group (13.5%±2.3 *vs.* 26.7%±6.1, p=0.03; 5 rats per group, with each animal yielding one mean value of Fos expression for each raphé region calculated from 4 brain sections; see Methods) (Fig. 4). In the analysis of the effect of raphé region across all treatments, raphé obscurus had significantly lower percentage of Fos-positive 5-HT cells than either the raphé pallidus or the parapyramidal region (t=6.64, $p<2 \cdot 10^{-5}$ for both).

3.2 Effect of increased use of the tongue on 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor immunoreactivity in the XII nucleus

As described previously [Fay and Kubin, 2000; Volgin *et al.*, 2003], 5-HT$_{2A}$ receptor immunostaining distinctly marks the boundaries of the XII nucleus. In the present study, we found the same to be the case with 5-HT$_{2C}$ receptor immunostaining, although its intensity relative to the dorsally located nucleus of the solitary tract differed less than for 5-HT$_{2A}$ receptors. Nevertheless, we could reliably trace the boundaries of the XII nucleus and separate its dorsal and ventral halves based on the distribution of immunostaining for either receptors (Fig. 2A and B, Fig. 5A and B; cf. Rukhadze *et al.*, 2010). Despite relatively diffuse staining within the XII nucleus (discussed in Fay and Kubin, 2000), individual motoneuronal cell bodies darkly stained for either 5-HT$_{2A}$ or 5-HT$_{2C}$ receptors could be discerned when viewed at a higher magnification (cf. Fig. 2C top and bottom, respectively).

Application of two-way ANOVA with interactions to 5-HT$_{2C}$ receptor immunostaining within the entire XII nucleus revealed a significant effect of both the treatment ($F_{2,5,3}$=5.53, p=0.044, n=72) and experimental run ($F_{2,5,3}$=12.8, p<0.0001, n=72), whereas the effect of the anatomical level of the section was not significant. The experimental run effect was, expectedly, related to large differences in the overall intensity of staining among the different “triplets” of animals belonging to separate experimental runs. However, the interaction between treatment and experimental run was not significant ($F_{2,5,3}$=2.0, p=0.06, n=72), indicating that the inter-group staining differences were of technical nature and independent of the treatment effects. The subsequent pairwise comparisons revealed that immunostaining for 5-HT$_{2C}$ receptors was significantly stronger in S+O than in W rats (65±1.8 *vs.* 60±2.0 arbitrary units; p=0.008), whereas staining intensity for the S group (63±2.4) was intermediate between the W and S+O groups and not significantly different from either one (p=0.17 and p=0.25, respectively; Fig. 5D). The same analysis applied to either the raw immunostaining measured within the background region or the raw immunostaining density within the XII nucleus without background subtraction yielded no significant treatment effects.

In contrast to 5-HT$_{2C}$ immunostaining that increased with intensity of tongue use, 5-HT$_{2A}$ receptor immunostaining measured in the entire XII nucleus exhibited only a minor trend

towards reduced intensity with the increasing use of the tongue (Fig. 5C and D). No statistically significant treatment effect was present for 5-HT_{2A} receptors ($F_{2,3,71}$=1.36, p=0.33).

As was the case with the measurements across the entire XII nucleus, 5-HT_{2C} receptor immunostaining was also significantly stronger in the S+O than in W rats when measured separately within either the dorsal or the ventral compartment of the nucleus (p=0.04 for the dorsal compartment, and p=0.02 for the ventral compartment) (Fig. 6). There was also a consistent difference between 5-HT_{2C} receptor immunostaining in the dorsal and ventral compartments within each treatment group, with a significantly stronger immunoreactivity in the dorsal than in the ventral half (Fig. 6). In contrast, immunostaining for 5-HT_{2A} receptors did not differ between the dorsal and ventral part of the nucleus, consistent with our earlier study [Rukhadze *et al.*, 2010].

4. Discussion

We found that behavioral stimulation of tongue use is associated with an increased 5-HT_{2C} receptor immunostaining in the XII nucleus and an increased c-Fos expression in a subset of 5-HT neurons located in the medullary raphé region most relevant for serotonergic control of XII motoneurons. Based on these findings, we propose that 5-HT_{2C} receptor generation and turnover in the XII motor nucleus is controlled, at least in part, in an activity- and 5-HT-dependent manner. This control can occur within a time frame of 12 hours or less, and its pattern points to a positive relationship between motoneuronal activation and expression of 5-HT_{2C} receptors.

4.1 Different mechanisms and time scales of 5-HT receptor-dependent respiratory plasticity

Various forms of 5-HT receptor-dependent respiratory plasticity have been described for different receptors in studies using different modes and time scales of experimental manipulations. By far the most extensively characterized is the long-term facilitation (LTF) of respiratory motor output that can be elicited in both the phrenic and XII motoneurons in response to a series of short hypoxic episodes applied on the time scale of minutes [see Mahamed and Mitchell, 2007 for a review]. The LTF requires a transient expression of 5-HT_{2A} receptors within the motor nuclei, and is additionally modulated by 5-HT_{2B} and 5-HT_7 receptors [Dale-Nagle *et al.*, 2010; Fuller *et al.*, 2001; Hoffman and Mitchell, 2013; MacFarlane *et al.*, 2011].

Prolonged motor training can increase serotonergic innervation of XII motoneurons [Behan *et al.*, 2012]. In another study, physical training was associated with desensitization of striatal 5-HT_{1B} receptors (inhibitory and mostly presynaptic) [Chennaoui *et al.*, 2000]. However, chronic administration of different 5-HT receptor agonists attenuated behaviors dependent on 5-HT_{1A} and 5-HT_{2A} receptors, but those dependent on 5-HT_{2C} receptors were strengthened or unchanged [Berendsen and Broekkamp, 1991]. Thus, the effects of chronic stimulation of 5-HT receptors might vary among the different control systems and receptor subtypes.

With the regard to XII motoneurons, we previously determined that both mRNA and protein levels for 5-HT_{2A} receptors are lower in the XII nucleus at the end, than at the beginning, of the active period of circadian cycle [Volgin *et al.*, 2013]. This has lead us to hypothesize that 5-HT_2 receptor expression is reduced in response to their increased stimulation. However, in our present study, immunoreactivity for 5-HT_{2A} receptors was not significantly affected by behaviorally elicited increase in tongue use, which suggests that the day-night difference that we detected previously was not driven by the day-night difference in activation of the tongue. Instead, it might have been mediated by the central circadian clock, but this will require separate studies.

In contrast to insignificant effects on 5-HT_{2A} receptor immunoreactivity, 5-HT_{2C} receptors were significantly altered by activation of tongue use and, notably, a combined increase of licking and grooming was associated with an increased 5-HT_{2C} receptor immunostaining in the XII nucleus. Since these effects occurred parallel to an increased activation of a relevant subset of medullary 5-HT neurons, our finding is consistent with an increased turnover of these receptors elicited by their increased stimulation. Whether this change is indicative of a net increase in the effectiveness of XII motoneuronal stimulation mediated by 5-HT_{2C} receptors, as in at least one earlier study [Berendsen and Broekkamp, 1991], or a net loss of functional membrane-bound receptors, cannot be determined based on our present findings. Neurotransmitter receptors can be regulated in a homeostatic manner, such that their increased stimulation results in an accelerated receptor degradation; this helps to maintain approprite balance between excitation and inhibition at the target cell level [Dani *et al.*, 2005; Modirrousta *et al.*, 2007; Volgin *et al.*, 2014]. However, the phenomenon of response potentiation, including that in the respiratory motor output elicited by intermittent hypoxia [Dale-Nagle *et al.*, 2010; MacFarlane *et al.*, 2011], offers an alternative model with which to interpret our present findings. While the cellular and subcellular mechanisms responsible for the activity-dependent increase in 5-HT_{2C} receptor immunostaining that we observed remain to be elucidated, the picture that emerges from our study is that the availability and turnover of the two major excitatory 5-HT receptors present in the XII nucleus are regulated in a different manner. If our result with 5-HT_{2C} receptors is functionally associated with an increased availability of these receptors, it would suggest a new mechanism that is potentially suitable for strengthening of serotonergic activation of XII motoneurons.

We found that the activity-dependent immunostaining increase occurred similarly in the dorsal and ventral halves of the XII nucleus. Such a uniformity may reflect the nature of the behavioral tasks employed in our study, as both licking and grooming require alternating activation of tongue protrussor and retrussor muscles which are, respectively, innervated by in the ventral and dorsal parts of the XII nucleus [Aldes, 1995; Dobbins and Feldman, 1995; McClung and Goldberg, 1999]. Concurrently, we determined that the dorsal half of the nucleus has a slightly but significantly higher level of 5-HT_{2C} receptor immunoreactivity than the ventral half (Fig. 6), a difference that has not been previously reported.

4.2 Limitations of our study

In our interpretation of our findings we emphasize that the two treatments that we used (S and S+O) caused a progressively increasing intensity of tongue use. While this is

mechanistically correct, additional factors may have contributed to our result. In particular, one may consider the increased sugar consumption and the emotional valence of our experimental interventions. Accordingly, data indicate that glucose consumption can increase brain serotonin release [e.g., Smolders *et al.*, 2001]. Furthermore, exposure to sugar is presumably associated with positive emotional effects, whereas exposure to oil applied to fur is presumably a stressor. All these factors could influence our results in a complex manner. However, it is of note that, although only the differences between W and S+O conditions were statistically significant, both our measures of receptor immunostaining and Fos expression in the raphé obscurus region exhibited a gradual increase from W through S to S+O conditions, thus roughly following the intensity of tongue use. Should these outcomes be mostly determined by either the sugar intake or the pleasure-stress balance, we would expect their pattern to deviate from the one following the level of tongue activation across our three experimental conditions. Also, in contrast to the obscurus region, our Fos data for the raphé pallidus and parapyramidal regions had different pattern (Fig. 4B) which could be related to combined effects of emotional stimuli and sweetened water on these nuclei. Indeed, the uniquely significant effect of c-Fos expression in the raphé obscurus region is consistent with functional evidence that this region is particularly involved in activation of the muscles of the tongue [Besnard *et al.*, 2009].

Our methods of receptor quantification did not allow us to differentiate between membrane-bound receptors and those undergoing internalization. Thus, the mechanisms underlying the generation and turnover of 5-HT_2 receptors occurring in our study are unknown. Nevertheless, the increased immunostaining for 5-HT_{2C} receptors after a period of enhanced activation of the tongue and increased 5-HT release was likely driven by an increased generation of new receptors. This interpretation leads us to postulate that there is a positive feedback whereby an increased use of the tongue and the associated increase of 5-HT release lead to elevated turnover of 5-HT_{2C} receptors.

4.3 Conclusions and clinical implications

Our findings suggest that the synthesis of the excitatory 5-HT_{2C} receptors is stimulated by the conditions associated with an increased activation of XII motoneurons. In OSA patients, the pharyngeal portion of the upper airway is anatomically compromised which makes it vulnerable to collapse under the centripetal action of inspiratory pressure [reviewed by Horner, 2012; White and Younes, 2012]. To cope with this condition, OSA patients develop a compensatory increase in upper airway muscle activity, including the muscles of the tongue [Mezzanotte *et al.*, 1992; Suratt *et al.*, 1988]. This activation helps maintain upper airway open, at least during wakefulness, by stiffening and protruding the tongue, but the mechanisms underlying this compensation are not clear [see Kubin, 2016 for a review]. Both the characteristic for OSA chronic occurrence of intermittent hypoxia and the repeated activation of the tongue in association with recurrent terminations of obstructive events have been considered as contributing to this OSA-related compensation. For both modes of stimulation, there is evidence for their beneficial effects on muscle tone in human subjects [Mateika and Syed, 2013; Rousseau *et al.*, 2015; Tamisier *et al.*, 2009; Trumbower *et al.*, 2012]. Our finding of a positive activity-dependence of 5-HT_{2C} receptor expression in the

XII nucleus offers a potential new target for increasing upper airway and postural muscle tone in OSA patients and following neural injury or degeneration.

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Highlights

- Hypoglossal (XII) motoneurons innervate tongue muscles
- Increased tongue use enhances serotonin-2C receptors in the XII nucleus
- This may lead to stronger activation of XII motoneurons
- Strengthening of tongue output is a desired strategy in obstructive sleep apnea

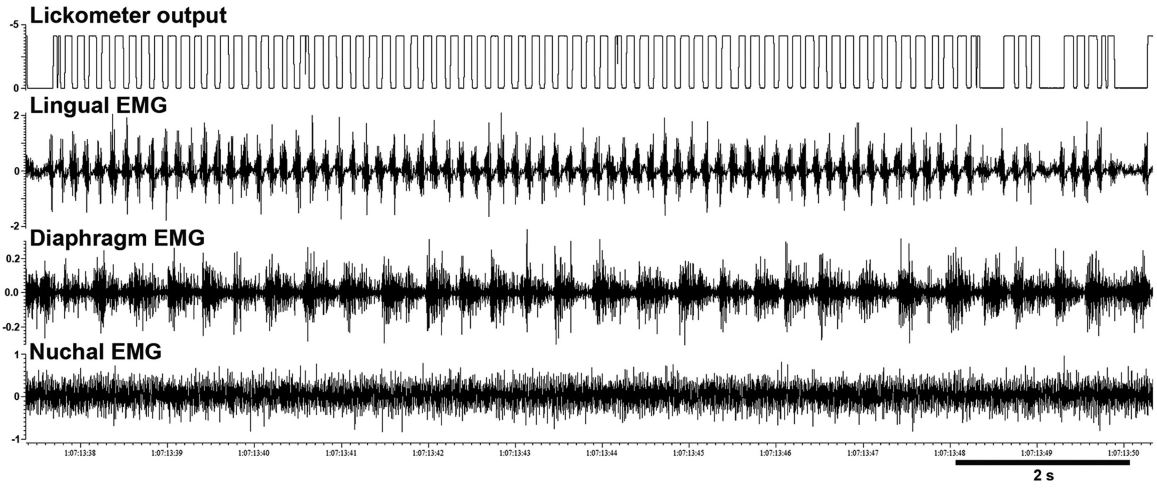

Figure 1:

Lickometer output acquired concurrently with lingual, diaphragm and nuchal electromyograms (EMG) during a bout of drinking. There is a close correspondence between the lickometer pulses and bursts of lingual EMG but no such a correspondence for the respiratory rhythm seen in diaphragm EMG. Lick events were derived from interruptions of an infrared beam positioned in front of the spout of a standard drinking bottle.

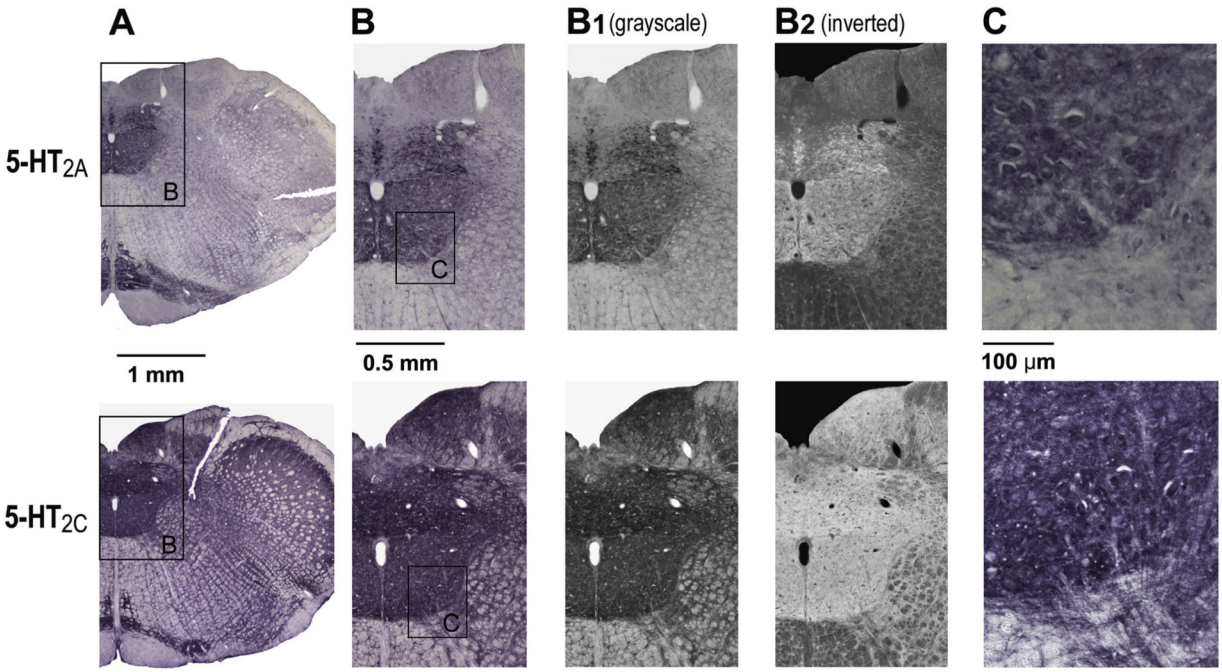

Figure 2:

Examples of 5-HT_2 receptor immunostaining within and around the XII nucleus. The top and bottom rows in A show low-magnification images of brain sections containing the XII nucleus immunostained for 5-HT_{2A} and 5-HT_{2C} receptors, respectively. Panels B show the XII nucleus and its surroundings at a magnification used for quantification of staining intensity. The corresponding panels B1 and B2 have been derived from those in panels B but with color information removed (grayscale only) and then following a black-white inversion conducted prior to measurements of staining intensity (see Methods). At a still higher magnification used in panels C, one can discern individual darkly stained motoneuronal cell bodies in the ventrolateral part of the XII nucleus. The sections shown correspond approximately to the standard A-P level of −14.04 mm from bregma according to a rat brain atlas [Paxinos and Watson, 2007].

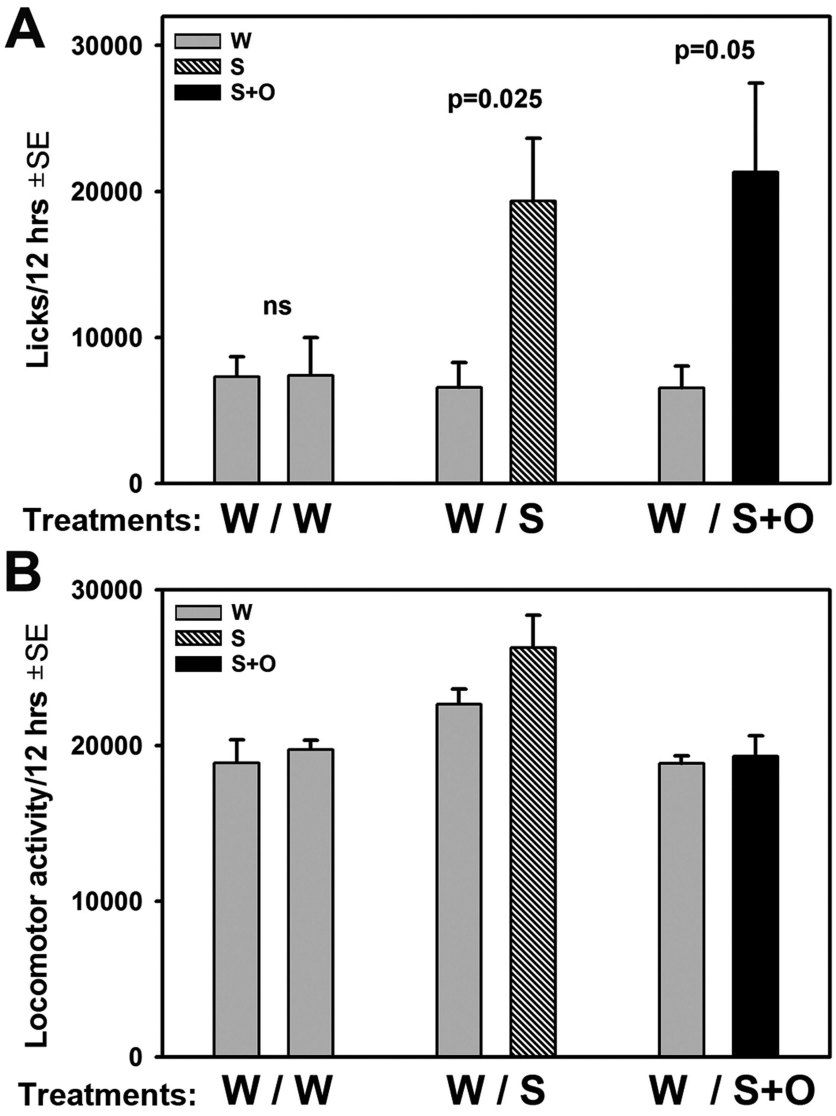

Figure 3:

Mean lick counts (A) and locomotor activity (B) collected during two successive nights of which during the first all three rats were presented with plain drinking water (W) and during the second night one of the rats received again only plain water (W), another had sweetened water (S), and the third one received sweetened water and additionally had oil applied to the fur of the upper body to stimulate tongue use for grooming (S+O). The number of licks counted overnight was nearly tripled when the animals were presented with sweetened water regardless whether oil was also applied to fur or not (A). Locomotor activity did not differ among the three experimental conditions (B). N=6 rats per treatment group.

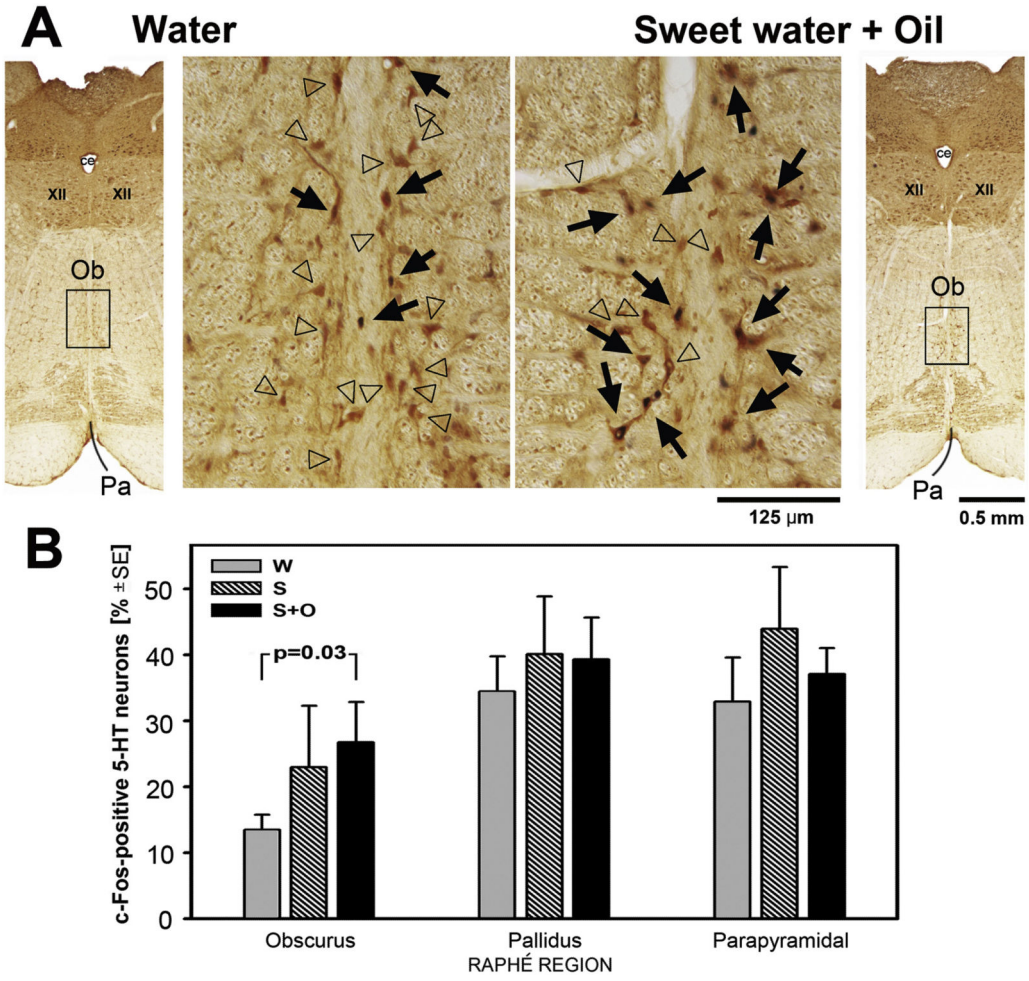

Figure 4:

Stimulation of tongue use for licking and grooming was associated with an increased proportion of 5-HT cells with nuclear accumulation of c-Fos protein in the medullary raphé obscurus region. A: Examples of microscopic images with double-labeling for cell bodies containing 5-HT of which some have nuclear accumulation of c-Fos. The side panels show low-magnification images of the medial medulla, with the raphé obscurus (Ob) regions boxed and the location of the raphé pallidus (Pa) region marked. The XII nucleus and central canal (ce) are also indicated. The central panels show the boxed obscurus regions at a magnification suitable to observe individual 5-HT cells (brown) and that some have c-Fos-positive nuclei (black); open triangles: 5-HT cells without c-Fos; filled arrows: 5-HT cells that are also c-Fos-positive. The proportion of c-Fos-expressing cells is higher in the brain section on the right derived from an animal concurrently exposed to sweet water to enhance drinking and with oil applied to fur to increase grooming than in the brain section on the left from a rat presented with plain water only. B: In all three raphé regions, the proportion of 5-HT cells expressing c-Fos tended to be higher in the rats with enhanced use of the tongue (S or S+O group) when compared to the rats having only plain water for drinking (W). The effect was significant in the raphé obscurus region where the proportion of 5-HT cells with nuclear c-Fos accumulation was significantly higher in the S+O than in W group. N=5 rats

per treatment, with each animal yielding one mean percentage value of c-Fos expression for each raphé region calculated across 4 brain sections).

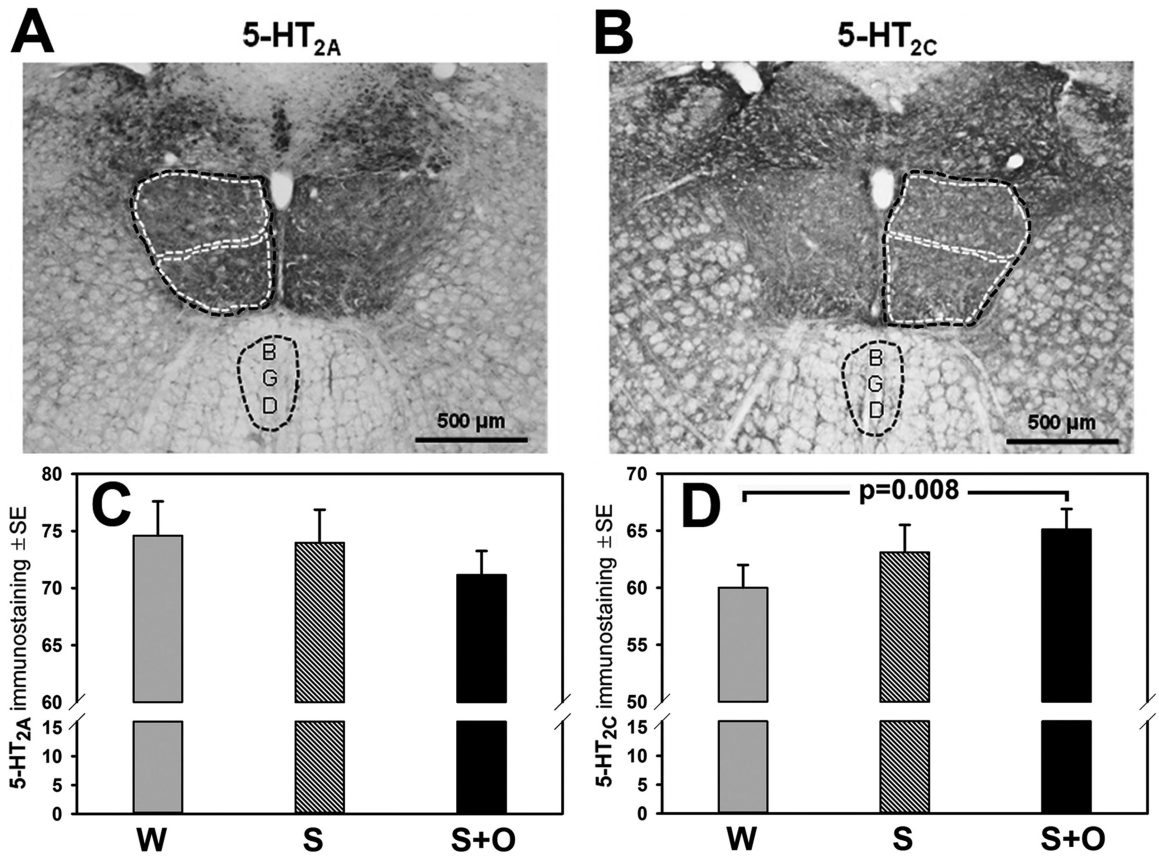

Figure 5:

Images of brain sections immunostained for 5-HT_{2A} (A) and 5-HT_{2C} (B) receptor-like proteins, respectively. The entire XII nucleus and its dorsal and ventral halves are outlined with dashed lines. Intensity of immunostaining was measured within the outlined regions and background staining was subtracted on section-by-section basis (BGD marks the region used for measurement of background staining). C and D: Average specific (background-subtracted) immunostaining for 5-HT_{2A} and 5-HT_{2C} receptors in the entire XII nucleus. For 5-HT_{2A} receptors, there were no differences among the three treatments. For 5-HT_{2C} receptors, immunostaining was significantly higher in the rats with the strongest stimulation of tongue use (S+O group) than in the water (W) group, whereas the S group was intermediate between the other two and not significantly different from either the W or S+O group. N=6 rats per treatment group.

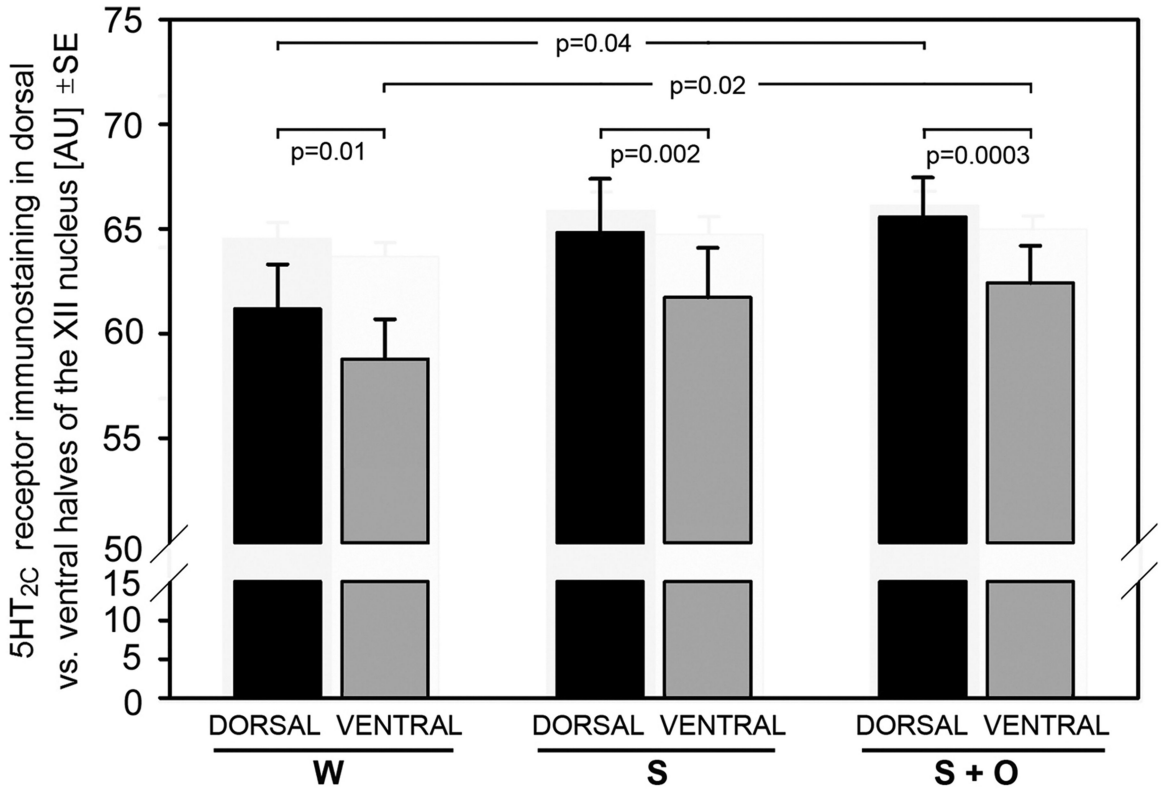

Figure 6:

Comparison of immunostaining for 5-HT_{2C} receptors in the dorsal *vs.* ventral half of the XII nucleus. Within each treatment group, the dorsal part of the nucleus had significantly higher staining intensity than the ventral half. The effect of S+O treatment was significant relative to W treatment within both the dorsal and ventral half of the nucleus, consistent with the finding for the entire XII nucleus (Fig. 5D). Each mean was derived from 6 rats and 4 brain sections per animal.