Chronic Intermittent Hypoxia Alters Density of Aminergic Terminals and Receptors in the Hypoglossal Motor Nucleus

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Rationale: Patients with obstructive sleep apnea (OSA) adapt to the anatomical vulnerability of their upper airway by generating increased activity in upper airway-dilating muscles during wakefulness. Norepinephrine (NE) and serotonin (5-HT) mediate, through α_1 -adrenergic and 5-HT_{2A} receptors, a wake-related excitatory drive to upper airway motoneurons. In patients with OSA, this drive is necessary to maintain their upper airway open. We tested whether chronic intermittent hypoxia (CIH), a major pathogenic factor of OSA, affects aminergic innervation of XII motoneurons that innervate tongue-protruding muscles in a manner that could alter their airway-dilatory action.

Objectives: To determine the impact of CIH on neurochemical markers of NE and 5-HT innervation of the XII nucleus.

Methods: NE and 5-HT terminal varicosities and α_1 -adrenergic and 5-HT_{2A} receptors were immunohistochemically visualized and quantified in the XII nucleus in adult rats exposed to CIH or room air exchanges for 10 h/d for 34 to 40 days.

Measurements and Main Results: CIH-exposed rats had approximately 40% higher density of NE terminals and approximately 20% higher density of 5-HT terminals in the ventromedial quadrant of the XII nucleus, the region that controls tongue protruder muscles, than sham-treated rats. XII motoneurons expressing α_1 -adrenoceptors were also approximately 10% more numerous in CIH rats, whereas 5-HT_{2A} receptor density tended to be lower in CIH rats.

Conclusions: CIH-elicited increase of NE and 5-HT terminal density and increased expression of α_1 -adrenoceptors in the XII nucleus may lead to augmentation of endogenous aminergic excitatory drives to XII motoneurons, thereby contributing to the increased upper airway motor tone in patients with OSA.

Keywords: chronic-intermittent hypoxia; hypoglossal motoneurons; obstructive sleep apnea; norepinephrine; serotonin

Obstructive sleep apnea (OSA) is characterized by recurrent nocturnal episodes of upper airway narrowing or collapse, which lead to reduced ventilation or apnea, blood oxygen (O₂) desaturations, and sleep fragmentation (1, 2). Subjects with OSA adapt to the anatomical vulnerability of their upper airway by generating a higher level of activity in their upper airway-dilating muscles during wakefulness than healthy subjects (3–5). This allows them to maintain airway patency, but the mechanisms underlying this adaptation are unknown.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Patients with obstructive sleep apnea (OSA) have elevated upper airway motor tone during wakefulness and chronically experience nocturnal intermittent hypoxia. Endogenous noradrenergic and serotonergic excitatory drives help maintain upper airway patency.

What This Study Adds to the Field

Animals subjected to chronic intermittent hypoxia have increased noradrenergic and serotonergic innervation of hypoglossal motoneurons; this may cause increased upper airway motor tone during wakefulness.

Hypoglossal (XII) motoneurons innervate the genioglossus and other muscles of the tongue whose active contraction is important for the maintenance of upper airway patency in patients with OSA (2, 6). Sleep-related decrements of lingual muscle activity facilitate the occurrence of sleep-related upper airway obstructions (6–8). Data from healthy animals show that endogenous excitation mediated by norepinephrine (NE) and serotonin (5-HT) is an important contributor to the maintenance of upper airway muscle tone during wakefulness (9–13). This drive is derived from pontomedullary NE and 5-HT cells (14–16) that have state-dependent levels of activity, maximal during wakefulness, moderate during non-REM sleep, and minimal or absent during REM sleep (17–19). The sleep-related withdrawal of aminergic (NE and 5-HT) activation has been identified as a major mechanism underlying sleep-related depression of upper airway muscle activity (9, 12, 13, 20). The excitatory effects of NE and 5-HT on XII motoneurons are mediated by α_1 -adrenergic and 5-HT_{2A} receptors that are abundantly expressed in XII motoneurons (9, 21–23).

Chronic intermittent hypoxia (CIH) is a major component of OSA pathogenesis (24, 25). In rodent models, it elicits arterial hypertension (26), metabolic derangements (27), reduced alertness (28), and cognitive impairments (29), symptoms typical of patients with OSA. However, little is known about the effects of CIH on control of XII motoneurons. We hypothesized that aminergic inputs to XII motoneurons might be altered after exposure to CIH in a way that could contribute to the wake-related upper airway hyperactivity in patients with OSA. Our goal was to assess the neuroanatomical support for this hypothesis, by quantifying the density of NE and 5-HT terminal varicosities and α_1 -adrenergic and 5-HT_{2A} receptors in the XII nucleus in a rat model of CIH. We found that the counts of NE and 5-HT terminals, and XII motoneurons immunoreactive for the α_1 -adrenergic receptor, were higher in rats exposed to CIH than in control animals. Preliminary reports have been published (30, 31).

(Received in original form December 17, 2009; accepted in final form July 8, 2010)

Supported by National Institutes of Health grants HL-047600 and HL-074385 (L.K.).

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 182, pp 1321–1329, 2010

Originally Published in Press as DOI: 10.1164/rccm.200912-1884OC on July 9, 2010

Internet address: www.atsjournals.org

METHODS

An expanded methods section is provided in the online supplement to this article.

Animals and Administration of CIH

The experiments were performed on 22 adult male Sprague-Dawley rats. Eleven rats were subjected to a sine-like pattern of O₂ oscillations with a 3-minute period and a nadir of 6.9% (Figure 1) applied from 7:00 A.M. to 5:00 P.M. daily for 34 to 40 days, and the remaining 11 to identically timed room air exchanges. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Immunohistochemical Procedures

Four to 6 days before the end of CIH/sham treatments, pairs of rats, one CIH-exposed and one sham-treated, were anesthetized with isoflurane, and 10 μ l of a retrograde tracer, tetramethylrhodamine-dextran (rhodamine), was injected into the base of the tongue. Then, 1 day after the last day of CIH/sham exposure, the rats were perfused with phosphate-buffered saline followed by 4% paraformaldehyde. Brainstems were cut into six series of 35- μ m coronal sections. Series of sections from a CIH and a sham-treated rat were then combined and processed immunohistochemically to visualize dopamine β -hydroxylase (DBH, a marker for NE), 5-HT, and α_1 -adrenergic and 5-HT_{2A} receptors. DBH- and 5-HT-labeled sections were also processed to visualize rhodamine in XII motoneurons.

Terminal and Cell Counting Procedures

DBH and 5-HT terminals were counted in 24 pairs of brain sections from CIH/sham-treated rat pairs. The sections covered the caudal half of the XII nucleus at the anteroposterior (A-P) levels from -14.3 mm to -13.68 mm relative to bregma according to a rat brain atlas (32). DBH and 5-HT terminal and *en passant* synaptic varicosities present within a 100×100 μ m counting box positioned in the ventromedial quadrant of the XII nucleus (Figure 2) were counted throughout the depth of each section using a water-immersion objective, with those closely apposed to retrogradely labeled XII motoneurons being distinguished from all others (Figures 2A3–2B3). To assess whether exposure to CIH caused a loss of NE cells, DBH-positive neurons were counted in those pontomedullary cell groups that send axons to the XII nucleus (16). Most sections with DBH terminal staining and all sections with α_1 -adrenergic receptor staining were analyzed by persons not informed of the treatments (K.E.B. and A.P.).

Figure 1. Time course of oxygen level changes in hypoxic chambers during two successive cycles of chronic intermittent hypoxia, as sampled at 13.3-second intervals.

Quantification of α_1 -Adrenergic and 5-HT_{2A} Receptor Expression in the XII Nucleus

α_1 -Adrenergic receptor immunostaining was analyzed by counting all α_1 -adrenoceptor-positive XII motoneurons within the entire cross-section of the XII nucleus on both sides and, separately, within the dorsal and ventral halves of the XII nuclei in 32 pairs of brain sections from eight rat pairs (four sections per rat). For quantification of 5-HT_{2A} receptor staining, digital photographs of one XII nucleus and the reticular formation region ventral to the nucleus were taken under constant illumination, and staining intensity was then densitometrically measured within the entire XII nucleus, its dorsal half, ventral half, and in the reticular region ventral to the XII nucleus (to measure background staining), as described previously (33) (cf. Figure 6A).

Statistical Analysis

For normally distributed variables, repeated-measures analysis of variance, Student *t* test, and linear regression were used, and variability of the means was characterized by the standard error (SE). For nonnormally distributed variables, Mann-Whitney rank sum test was used and variability of the median was characterized by the 25 to 75% interquartile range. Differences were considered significant when *P* was less than 0.05.

RESULTS

Noradrenergic Terminal Density Is Elevated in the XII Nucleus in Rats Subjected to CIH

Figures 2A1 and 2B1 show the XII nucleus in a pair of brain sections matched for A-P level taken from CIH- and sham-treated rats, with the square regions delineating the location of the 100×100 μ m boxes in which we counted DBH-positive terminals. The counting boxes were placed within the ventromedial region of the XII nucleus because most XII motoneurons retrogradely labeled from the base of the tongue were located in this region, with the position of the boxes set to include most labeled motoneurons. All DBH-positive terminals found within the counting box throughout the depth of each analyzed brain section were counted. Figures 2A2 and 2B2 show the counting regions illustrated in Figures 2A1 and 2B1, as seen under $1,000\times$ magnification with a water-immersion objective, and Figures 2A3 and 2B3 show retrogradely labeled XII motoneurons (gray here, brown in the other panels) and all DBH-positive terminal varicosities redrawn from within the counting boxes shown in Figures A1 and B1 (red dots represent those DBH varicosities that were closely apposed to labeled motoneurons).

DBH-positive terminal counts obtained from eight pairs of CIH/sham-exposed rats were higher in most sections from CIH rats (in 20 out of 24 analyzed section pairs) (Figure 3A). The range of DBH-positive terminal counts in individual brain sections was 347 to 1,463 for CIH rats, and 311 to 1,267 for sham-treated rats. The median number of terminals counted in CIH rats was significantly higher than that in sham-treated rats (632 per counting box; 25–75% interval, 497–868 vs. 455 per box; 25–75% interval, 345–671; *P* = 0.021, paired Mann-Whitney rank sum test; Figure 3B).

DBH-positive terminal counts were also compared between CIH- and sham-treated rats at different A-P levels. At the most caudal level (-14.3 mm), DBH terminal counts were significantly higher in CIH- than sham-treated rats (788 ± 89 vs. 529 ± 80 per counting box; *P* = 0.008, paired *t* test, eight pairs of sections). At other A-P levels, the same trend was present but the differences were not statistically significant in the data sets limited to one A-P level only. The density of DBH-positive terminals tended to be higher at the caudal than at rostral levels, but the differences among the distinct A-P levels were not significant within either the CIH or sham group.

DBH axonal varicosities found to be closely apposed to labeled XII motoneurons (gray); black dots show all remaining DBH terminals found throughout the depth of these brain sections.

NE Cell Counts Are Not Different between CIH and Sham-Treated Rats

To assess whether the increased number of NE terminals could be secondary to altered by CIH numbers of brainstem NE neurons that send axonal projections to the XII nucleus (16), in five pairs of CIH/sham-treated rats, we counted DBH-positive cells bilaterally in every sixth brain section. The counts were: 388 ± 16 sham versus 398 ± 20 CIH for the A1 group, 219 ± 14 sham versus 189 ± 32 CIH for the A5 group, 53 ± 6 sham versus 56 ± 7 CIH for the A7 group, and 74 ± 12 sham versus 98 ± 11 CIH for the sub-locus coeruleus region. There were no statistically significant differences between CIH- and sham-treated rats (paired *t* test).

5-HT Terminal Density Is Elevated in the XII Nucleus in Rats Subjected to CIH

Figures 4A and 4B show examples of high-magnification photographs of XII motoneurons retrogradely labeled with rhodamine from the base of the tongue (brown) and 5-HT terminals (black) located in the ventromedial quadrant of the XII nucleus in a matched for A-P level pair of brain section from a CIH-exposed and a sham-treated rat. As illustrated in

Figure 2 for DBH terminals, 5-HT terminals found throughout the depth of each analyzed brain section were redrawn under $1,000\times$ magnification and counted within a $100 \times 100 \mu\text{m}$ counting box placed in the ventromedial region of the XII nucleus. Also as with DBH terminals, 5-HT terminals were counted in 24 pairs of brain sections from eight pairs of CIH/sham-treated rats. The range of total numbers of 5-HT terminals counted was 160 to 911 per counting box in CIH rats and 327 to 846 per counting box in sham-treated rats. The average number of 5-HT terminals was significantly higher in CIH- than sham-treated rats (603 ± 37 vs. 503 ± 31 per counting box; $P = 0.012$, paired *t* test). In most pairs of brain sections (17/24), 5-HT terminal counts were higher in the section from a CIH rat than in the matched for A-P level section from sham-treated rat (Figure 4C).

As with DBH terminals, the rostrocaudal distribution of 5-HT terminals was evaluated. At A-P level -14.08 mm, 5-HT terminal counts were significantly higher in CIH- than in sham-treated rats (636 ± 57 vs. 461 ± 36 per counting box; $P = 0.015$, paired *t* test). At other A-P levels, a similar trend was present but the differences between CIH- and sham-treated rats were not statistically significant. Similarly to DBH-positive terminals, the density of 5-HT terminals tended to be higher at the caudal than rostral levels within both the

identity line). (B) The median number of terminals counted in sections from CIH rats (632 per counting box; horizontal line inside the gray box) was significantly higher than for the sham-treated group (455; $P = 0.021$, paired Mann-Whitney rank sum test; 24 brain sections/group). The gray boxes show 25 to 75% interquartile ranges for the sham (345–671) and CIH rats (497–868); open circles show terminal counts in individual sections.

Figure 2. XII motoneurons retrogradely labeled from the base of the tongue and dopamine β -hydroxylase (DBH)-positive terminals located in the ventromedial quadrant of the XII nucleus in a pair of matched for anteroposterior level brain sections from (A) chronic intermittent hypoxia (CIH)- and (B) sham-exposed rat. (A1, B1) Low-magnification images of the XII nucleus, with rhodamine-labeled XII motoneurons (brown) located mainly in the ventromedial part of the nucleus. A $100 \times 100 \mu\text{m}$ box was placed in this region for counting of DBH-positive terminals (black). (A2, B2) High-magnification images of the counting boxes shown in A1 and B1. Only a small fraction of DBH-positive varicosities contained within the boxes can be seen (those that are within the focal plane of this photograph). (A3, B3) Redrawing of retrogradely labeled XII motoneurons and all DBH-positive terminals contained in the counting boxes shown in panels A2 and B2. Red dots show

Figure 3. Dopamine β -hydroxylase (DBH)-positive terminals in the XII nucleus are more numerous in chronic intermittent hypoxia (CIH)- than sham-treated rats. (A) Relationship between the numbers of DBH-positive terminals counted in CIH- versus sham-treated rats in 24 matched for anteroposterior level pairs of brain sections obtained from eight pairs of CIH/sham-treated rats. For all but four pairs of sections, more terminal varicosities were counted in the section from a CIH rat than in the corresponding section from a sham-treated rat (data points above the identity line). (B) The median number of terminals counted in sections from CIH rats (632 per counting box; horizontal line inside the gray box) was significantly higher than for the sham-treated group (455; $P = 0.021$, paired Mann-Whitney rank sum test; 24 brain sections/group). The gray boxes show 25 to 75% interquartile ranges for the sham (345–671) and CIH rats (497–868); open circles show terminal counts in individual sections.

CIH and sham group, but the differences among the distinct A-P levels were not significant.

DBH and 5-HT Terminals Closely Apposed to Cell Bodies and Proximal Dendrites of XII Motoneurons Are Not Different between CIH- and Sham-treated Rats

DBH and 5-HT terminals closely apposed (with no separating space visible under $1,000\times$ magnification with a water-immersion objective) to cell bodies or proximal dendrites of those retrogradely labeled XII motoneurons that were fully contained within the counting box and had visible nucleus were counted separately from other terminals. Such terminals represented a small fraction (0.5–3.7%) of all terminals contained in each counting box (cf. Figures 2A3 and 2B3).

Thirty-eight retrogradely labeled XII motoneurons with closely apposed DBH terminals were analyzed in CIH rats, and 32 such motoneurons were analyzed in sham-treated rats. The range of DBH terminals closely apposed to cell bodies and proximal dendrites of individual XII motoneurons was 4 to 41 in CIH rats and 3 to 40 in sham-treated animals. The corresponding median numbers were 19 per motoneuron (25–75% interval, 12–26) and 18 per motoneuron (25–75% interval, 12–25) ($P = 0.7$, Mann-Whitney rank sum test).

Twenty retrogradely labeled XII motoneurons from CIH-exposed rats and 21 motoneurons from sham-treated rats were included in the analysis of 5-HT terminals closely apposed to XII motoneurons. The range of closely apposed 5-HT terminals was 6 to 42 per motoneuron in CIH rats and 4 to 40 in sham-treated animals. The corresponding median numbers were 15 per motoneuron (25–75% interval, 13–31) and 15 per motoneuron (25–75% interval, 11–25; $P = 0.7$, Mann-Whitney rank sum test), respectively.

To assess whether exposure to CIH affected the size of XII motoneurons which could, in turn, alter the efficiency of synaptic inputs, we measured the long (a) and short (b) axis of all retrogradely labeled XII motoneurons included in the analysis of DBH and 5-HT terminals and then calculated the estimated motoneuronal cell body area (A) as: $A = 3.14 \cdot a \cdot b / 4$. The median XII motoneuronal area was $271 \mu\text{m}^2$ (25–75% interval, $219\text{--}315 \mu\text{m}^2$) for CIH rats, and $256 \mu\text{m}^2$ (25–75% interval, $223\text{--}323 \mu\text{m}^2$) for sham-treated rats ($P = 0.8$, Mann-Whitney rank sum test). Thus, exposure to CIH was not associated with a significant change of either the number of aminergic synaptic contacts on cell bodies and proximal dendrites or the size of XII motoneurons.

Numbers of XII Motoneurons Positive for α_1 -Adrenoceptor-like Immunoreactivity Are Higher in CIH- than Sham-treated Rats

XII motoneurons with α_1 -adrenoceptor-like immunostaining were counted bilaterally in the entire XII nucleus and, separately, in its dorsal and ventral halves in 32 matched for A-P level pairs of medullary sections from eight pairs of rats (Figures 5A and 5B). The range of α_1 -adrenoceptor-positive XII motoneurons counted in the XII nucleus was 101 to 259 per section for CIH-exposed rats and 35 to 245 per section for sham-treated animals. It was also apparent that, at all A-P levels, more motoneurons were α_1 -adrenoceptor-immunopositive in the ventral than the dorsal half of the nucleus. Two-way, repeated measures analysis of variance revealed a significant effect of the treatment (CIH vs. sham; $F = 4.65$, $P = 0.039$, $df = 1,31$) and location within the nucleus (dorsal vs. ventral; $F = 112.95$, $P = 0.00073$, $df = 1,31$). The average number of α_1 -adrenoceptor-like immunopositive XII motoneurons per brain section was 170 ± 7.5 in CIH rats and 155 ± 8.5 in sham-treated rats

(Figure 5C). Subsequent *post hoc* comparisons within only the dorsal or only the ventral half of the nucleus revealed that the mean number of α_1 -adrenoceptor immunopositive XII motoneurons was significantly higher in the ventral than in the dorsal half of the nucleus in both CIH- and sham-treated rats (99.3 ± 4.4 vs. 71.6 ± 3.9 per section and 93.9 ± 5.0 vs. 61.3 ± 4.0 per section, respectively; $P < 0.0001$ for each comparison).

The number of α_1 -adrenergic receptor-positive XII motoneurons was significantly higher in CIH- than sham-treated rats for the dorsal half of the XII nucleus (71.6 ± 3.9 vs. 61.3 ± 4.0 per section; $P = 0.013$, paired *t* test), whereas the difference was not statistically significant in the ventral half (99.3 ± 4.4 vs. 93.9 ± 5.0 per section; $P = 0.2$, paired *t* test) (Figure 5C).

Similar to the density of DBH-positive terminals, the number of α_1 -adrenoceptor immunopositive XII motoneurons was significantly higher at the most caudal level of XII nucleus (-14.3 mm) in CIH- than sham-treated rats (168 ± 11 vs. 143 ± 17 per section; $P = 0.021$, paired *t* test). At other levels, a similar trend was present but the differences between CIH- and sham-treated rats were not statistically significant.

5-HT_{2A} Receptor-like Immunostaining in the XII Nucleus Is Attenuated in CIH- When Compared with Sham-Treated Rats

Intensity of 5-HT_{2A} receptor-like immunostaining was measured within the entire cross-section of the XII nucleus on one side in 32 pairs of brain sections from eight pairs of CIH/sham-treated rats. With no correction for background staining, the mean intensity of 5-HT_{2A} receptor-like immunostaining did not differ between CIH- and sham-treated rats (181 ± 3.6 vs. 181 ± 3.7 arbitrary units). However, the generally low level of labeling within the reticular formation region located ventral to the XII nucleus (background staining shown in Figure 6A) was significantly more intense in CIH- than sham-treated rats (69.2 ± 4.3 vs. 62.2 ± 3.9 ; $P = 0.009$, paired *t* test) (Figure 6B), suggesting that CIH had an unspecific effect on the overall level of tissue staining for 5-HT_{2A} receptor-like protein. This prompted us to assess whether there was a systematic relationship between labeling intensity within and outside the XII nucleus on section-by-section basis. We found that 5-HT_{2A} receptor-like labeling intensity within the XII nucleus was positively correlated with the intensity of background staining within either sham-treated ($P = 0.001$ for linear regression; $n = 32$ sections) or CIH rats ($P = 0.001$; $n = 32$ sections). One common regression line for all sections is shown in Figure 6C because the slopes of the two regression lines did not differ. Accordingly, to unveil any potential effect of CIH on specific 5-HT_{2A} receptor expression in the XII nucleus, a correction was applied to minimize the effect of CIH on background staining. To achieve this, for each section we calculated the ratio of staining intensity in the XII nucleus to the intensity of background staining. With such a normalization for background labeling, the relative 5-HT_{2A} receptor-like immunostaining within the XII nucleus was significantly lower in CIH- than in sham-treated rats (the ratios were 2.9 ± 0.4 vs. 3.2 ± 0.4 ; $P = 0.015$, paired *t* test) (Figure 6D). This suggested that, if CIH had any effect on 5-HT_{2A} receptor expression in the XII nucleus, it would be a small attenuation of the order of 10%.

5-HT_{2A} receptor-like immunostaining was not significantly different between the dorsal and ventral halves of the XII nucleus in either CIH- or sham-treated rats, and we found no differences between CIH and sham-treated rats when staining intensity was analyzed separately at different A-P levels.

DISCUSSION

Our main finding is that the density of NE and 5-HT terminals, as well as the number of XII motoneurons positive for α_1 -adrenoceptor-like protein, are all increased in the XII nucleus

Figure 4. 5-HT terminal varicosities are more numerous in the XII nucleus in chronic intermittent hypoxia (CIH)- than sham-treated rats. (A, B) High-magnification images showing XII motoneurons retrogradely labeled from the base of the tongue (brown) and terminal fibers and varicosities immunostained for serotonin (5-HT) (black) in a pair of sections matched for anteroposterior (A-P) level from a CIH- and a sham-exposed rat. (C) Relationship between the numbers of 5-HT-positive terminals counted in CIH- versus sham-treated rats for 24 matched for A-P level pairs of brain sections obtained from eight pairs of CIH/sham-treated rats. For most pairs of sections (17/24), more terminal varicosities were counted in the section from a CIH rat than in the corresponding section from a sham-treated rat (data points above the identity line). The average number of 5-HT terminals was significantly higher in CIH- than sham-treated rats (603 ± 37 vs. 503 ± 31 per counting box; $P = 0.012$, paired t test).

in CIH-exposed rats when compared with sham-treated animals. Our data also suggest that 5-HT_{2A} receptor-like immunoreactivity is slightly lower in CIH- than sham-treated rats.

Figure 5. XII motoneurons immunopositive for α_1 -adrenoceptor-like protein are more numerous in chronic intermittent hypoxia (CIH)- than sham-treated rats. (A, B) Examples of α_1 -adrenoceptor immunostaining of XII motoneurons in a CIH- and sham-treated rat, respectively. cc = central canal. (C) In the dorsal half of the nucleus, the mean number of α_1 -adrenoceptor-positive motoneurons was significantly higher in CIH- than in sham-treated rats (paired t test). The mean number of XII motoneurons positive for α_1 -adrenoceptor-like protein also was significantly higher in the ventral than dorsal half of XII nucleus in both CIH- and sham-treated rats (paired t test). In the ventral half of the nucleus, there was only a trend for the mean number of α_1 -adrenoceptor-positive motoneurons to be higher in CIH- than sham-treated rats.

Increased NE and 5-HT Terminal Density in the XII Nucleus after Exposure to CIH

Consistent with an earlier report that the ventral, caudal part of the XII nucleus has particularly high density of NE synaptic varicosities (34), we also noticed that the density of DBH-positive terminals was clearly higher in the ventral than the dorsal half of the nucleus. The XII nucleus can be divided into two functionally different regions based on the distribution of XII motoneurons that innervate different muscles of the tongue. Dorsal motoneurons mainly innervate tongue retractor muscles, whereas ventrocaudal motoneurons have their axons in the medial branch of the XII nerve and innervate tongue protruders

Figure 6. Serotonin (5-HT_{2A}) receptor-like immunostaining is slightly lower in the XII nucleus in chronic intermittent hypoxia (CIH)- than in sham-treated rats. (A) 5-HT_{2A} receptor-like immunostaining in the left XII nucleus and the surrounding regions, as converted to a grayscale digital image before densitometric measurements of staining intensity. The continuous white line outlines the entire XII nucleus, whereas the black line ventral to the XII nucleus encircles the reticular formation region used for measurement of background staining (*bckg*). The white dashed lines encircle the dorsal (XII_d) and ventral (XII_v) halves of the XII nucleus. cc = central canal. (B) With no correction for CIH effect on background staining, the average intensity of 5-HT_{2A} receptor-like staining intensity within the XII nucleus was not different between CIH- and sham-treated rats (32 pairs of brain sections from eight pairs of CIH/sham-treated rats). In contrast, background staining was significantly higher in CIH- than sham-treated rats. (C) 5-HT_{2A} receptor-like immunostaining within the XII nucleus was positively correlated with intensity of background staining when analyzed on section-by-section basis. *R* = correlation coefficient for linear regression. (D) The

ratio of 5-HT_{2A} receptor-like staining intensity within the XII nucleus to background labeling was lower in CIH- than sham-treated rats, suggesting that CIH-treated rats had lower specific expression of 5-HT_{2A} receptor-like protein in the XII nucleus than sham-treated animals ($P = 0.015$, paired *t* test).

(35–37). We also found that most XII motoneurons retrogradely labeled from the base of the tongue were located in the ventromedial region of the caudal XII nucleus. Although one earlier study reported that the dorsal region of the caudal XII nucleus had a higher density of 5-HT terminals than the ventral region (34), this was less evident in our material than the dorsoventral difference in the density of DBH terminals. Therefore, to focus on the effects of CIH on XII motoneurons that innervate tongue protruders, we analyzed both NE and 5-HT terminals in the ventromedial part of the caudal XII nucleus.

We found that the density of both DBH and 5-HT terminals was elevated in CIH when compared with sham-treated rats. One possibility is that such an increase was a result of increased concentration of DBH and 5-HT in the fibers located within the XII nucleus in CIH rats. In support of this, it has been reported that exposure to CIH increases concentration of NE in samples of brainstem tissue (38). However, visual examination of our immunostaining did not support this interpretation because we have not observed in our sections from either CIH- or sham-treated rats any lightly stained DBH or 5-HT fibers and terminals. This would be a necessary prerequisite for interpreting our finding of increased terminal density in CIH rats as resulting from increased levels of DBH or 5-HT in an otherwise unchanged number of terminals. Therefore, we conclude that CIH must have elicited growth of new DBH and 5-HT terminal varicosities (sprouting) in the XII nucleus. It is possible that CIH has a similar effect on these fibers in other areas, but investigation of additional regions was beyond the scope of this project.

It has been reported that, in mice, prolonged exposure to CIH elicits cellular changes characteristic of neuronal damage in NE neurons of the locus coeruleus (39). If this effect also occurred in our CIH-exposed rats and involved those brainstem

NE neurons that have axonal projections to the XII nucleus, a decrease, rather than an increase, of NE terminals would be expected. To clarify this, we counted A1/C1, A5, A7 and sub-locus coeruleus region NE neurons in CIH-exposed and sham-treated rats, as these NE cells project to the XII nucleus (16). We found that NE cell counts in these cell groups were similar to those in untreated rats (16, 19) and did not differ between CIH- and sham-exposed rats of the present study. This is consistent with qualitative observations in mice suggesting that only the locus coeruleus, but not other brainstem NE cell groups, is susceptible to CIH-induced damage (39). The absence of changes in relevant cell numbers in our CIH rats further supports our interpretation that CIH elicited sprouting of NE terminals.

We also evaluated the incidence of DBH and 5-HT terminals closely apposed to cell bodies and proximal dendrites of retrogradely labeled XII motoneurons. In contrast to the total counts of aminergic terminal varicosities in the XII nucleus, we found that the numbers of those closely apposed to XII motoneurons did not differ between CIH- and sham-treated rats. We also found that exposure to CIH was not associated with any systematic changes in the size of XII motoneurons, thus excluding the possibility that the same numbers of aminergic terminals would act on cells of different size in CIH- and sham-treated rats (which could alter the efficiency of synaptic transmission even in the absence of changes in aminergic terminal numbers).

Our negative findings with DBH and 5-HT terminals closely apposed to XII motoneurons can be explained by the observations that most NE and 5-HT terminals target remote dendrites of XII motoneurons, rather than their cell bodies and proximal dendrites (40, 41), or occur as free synaptic endings that are not associated with specific postsynaptic membranes and release their

transmitters into the extracellular space in a mode referred to as “volume transmission” (42). Such a form of chemical communication appears to be particularly appropriate for neurotransmitters and hormones such as NE and 5-HT that affect their targets in relation to the states of vigilance. Based on our results, it appears that CIH preferentially increased the numbers of those NE and 5-HT terminal varicosities that participate in volume transmission.

Increased Expression of α_1 -Adrenergic Receptor-like Protein in XII Motoneurons after Exposure to CIH

We found that the numbers of XII motoneurons immunopositive for α_1 -adrenoceptor-like protein were higher in CIH-exposed than sham-treated rats. The difference was particularly prominent and statistically significant in the dorsal half of the XII nucleus, where α_1 -adrenergic receptor expression was relatively lower than in the ventral half. In contrast to our terminal staining for DBH or 5-HT, our staining for α_1 -adrenoceptor-like protein had variable intensity in different motoneurons (Figure 5). Therefore, our finding of increased numbers of α_1 -adrenoceptor-positive motoneurons in CIH rats can be interpreted as reflective of increased levels of these receptor proteins. The numbers of α_1 -adrenoceptor-positive motoneurons were significantly increased in CIH rats in the dorsal half of the XII nucleus only, probably because our methodology based on counting of XII motoneurons exhibiting any visible level of α_1 -adrenergic receptor expression was not geared toward quantification of changes in α_1 -adrenergic receptor staining intensity in XII motoneurons located in the ventral XII nucleus, of which most exhibited some staining also in sham-treated rats.

Possible Mechanisms Responsible for the Increased NE and 5-HT Terminal Density and α_1 -Adrenergic Receptor Expression in the XII Nucleus in CIH Rats

5-HT and NE terminal densities have been reported previously to increase or decrease under different conditions in various experimental models. For example, 5-HT terminals change in the XII nucleus with aging and are dependent on the level of sex hormones (43), and NE terminal density in the spinal cord increases in response to peripheral nerve injury (44). The latter result is of particular interest because NE fiber sprouting observed in that study was regulated by the brain-derived neurotrophic factor, a factor that also plays an important role in the CIH-induced long-term facilitation of both phrenic and XII motoneuronal activity (45, 46). This respiratory output-enhancing mechanism is also elicited by both NE and 5-HT (47, 48). Thus, although the CIH-induced long-term facilitation has been best characterized in its semiacute form that requires only a few cycles of intermittent hypoxia, data also suggest that CIH lasting several days further enhances the acutely elicited long-term facilitation (46). Collectively, these data suggest that brain-derived neurotrophic factor plays an important role in the sprouting of NE and 5-HT terminals that we found to occur in response to CIH.

The mechanisms by which α_1 -adrenergic receptor expression increases in CIH rats are less clear, but it is possible that this effect occurs in response to an increased release of NE that undoubtedly occurs during exposures to CIH.

Decreased 5-HT_{2A} Receptor-like Immunostaining in the XII Nucleus in CIH-exposed Rats

We found that the intensity of immunostaining for the main excitatory receptor through which 5-HT excites XII motoneurons (5-HT_{2A}) (9, 21, 22) was either unchanged or reduced in CIH when compared with sham-treated rats. The effect was

small, and the suggestion that expression of these receptors was reduced was derived from analysis that required correction for background staining that typically occurs with the antibodies used in the present study. Therefore, our interpretation of the findings with 5-HT_{2A} receptors is tentative. Nevertheless, we note that decreased 5-HT_{2A} receptor levels in the XII nucleus in our rats exposed to CIH are consistent with the observation that XII motoneurons of rats exposed to CIH have reduced excitatory response to exogenous 5-HT (49). We also note that the magnitude of 5-HT_{2A} receptor decrease, as detected in our study, was considerably smaller than the increase in density of 5-HT terminals in the XII nucleus that we found in our CIH-exposed rats. Therefore, it is possible that endogenous 5-HT input may have an enhanced effect on XII motoneurons in CIH-exposed rats despite a slightly decreased expression of 5-HT_{2A} receptors. Nevertheless, our data would suggest that CIH has a relatively lesser effect on serotonergic innervation of the XII nucleus than on its NE inputs.

Functional Implications

In patients with OSA, decrements of upper airway muscle tone during sleep facilitate the occurrence of sleep-related upper airway obstructions (2, 6). In wakefulness, subjects with OSA adapt to the anatomical vulnerability of their upper airway by generating a higher level of activity in their upper airway dilating muscles than healthy subjects (3–5). It has been proposed that this enhancement is due to stronger activation of excitatory reflexes elicited by upper airway negative pressure (50). Our study suggests that CIH exposure increases the magnitude of XII motoneuron excitation mediated by NE and 5-HT afferent. If so, this would represent a novel mechanism by which upper airway motor tone adapts to the conditions imposed by anatomical vulnerability of the upper airway. Facilitatory effects mediated by NE and 5-HT may enhance transmission of reflex effects, and may exert additional central effects that act in concert with reflexes to increase upper airway muscle tone in patients with OSA.

Data from healthy animals show that sleep-related withdrawal of aminergic (NE and 5-HT) activation is a major mechanism responsible for sleep-related depression of upper airway muscle activity (9, 11–13, 20). This wake-related aminergic drive is derived from NE and 5-HT cells of the brainstem (14–16). NE excites XII motoneurons through α_1 -adrenergic receptors (23, 51, 52) and antagonism of these receptors in the rat XII nucleus causes a profound decrease of XII motoneuronal activity (12, 13). Therefore, our finding of an increased density of NE terminals and XII motoneurons expressing α_1 -adrenergic receptors suggests that exposure to CIH may enhance the endogenous NE excitatory input to XII motoneurons.

5-HT, similarly to NE, excites XII motoneurons (9, 20) and 5-HT neurons, similarly to NE neurons, have reduced activity during sleep. 5-HT afferents to the XII nucleus originate in the medullary raphe pallidus and obscurus nuclei and the parapyramidal region (15). Our findings suggest that increased density of 5-HT terminals may also contribute to the increased activity of upper airway muscles in patients with OSA, albeit the effect may be less prominent than in the case of NE input because the increased density of 5-HT terminals may be counterbalanced by reduced expression of the excitatory 5-HT_{2A} receptors, as suggested by our anatomical data and pharmacological experiments (49).

When NE or 5-HT are applied onto XII motoneurons *in vivo*, their dominant effect is excitation mediated by α_1 -adrenergic and 5-HT₂ receptors, respectively (9–13, 20). These effects are exerted postsynaptically on the corresponding receptors expressed in XII motoneurons (21, 23, 33, 51, 52).

However, additional presynaptic excitatory and inhibitory effects of both NE and 5-HT on XII motoneurons also have been described in juvenile and adult rats *in vitro*. 5-HT may enhance activity of XII motoneurons by its presynaptic inhibitory action on transmission of glycinergic inhibition (53), and it may reduce XII motoneuronal activity by its presynaptic inhibitory action on transmission of glutamatergic inputs (54), with both mediated by 5-HT_{1B} receptors. Inhibitory effects mediated by α_2 -adrenoceptors have also been described, but their underlying mechanisms need further studies (55, 56). Thus, although we emphasize the postsynaptic excitatory effects because they are best documented *in vivo* and appear to be most powerful, the net functional effects of the anatomical changes described in our study may be more complex.

The CIH-elicited strengthening of aminergic innervation of the XII nucleus may contribute to the increased upper airway muscle tone that patients with OSA exhibit during wakefulness, which is a major positive adaptation of upper airway control to anatomically compromised upper airway in these patients. Specifically, the increased density of 5-HT and NE terminals and α_1 -adrenergic receptors after exposure to CIH may cause an increased endogenous excitatory aminergic drive to XII motoneurons that is predominantly wake-related. This could directly increase spontaneous activity of upper airway motoneurons and facilitate their responsiveness to other central and reflex excitatory inputs.

Author Disclosure: I.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. V.B.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.E.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.K. received \$1,001–\$5,000 from Galleon Pharmaceuticals and \$1,001–\$5,000 from Sepracor for a one-time consultation.

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