Rats subjected to chronic-intermittent hypoxia have increased density of noradrenergic terminals in the trigeminal sensory and motor nuclei

Pari Mody, Irma Rukhadze, Leszek Kubin*

Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104-6046, USA

**A R T I C L E   I N F O**

Article history:
Received 28 June 2011
Received in revised form 7 September 2011
Accepted 6 October 2011

Key words:
Motoneurons
Noradrenaline
Obstructive sleep apnea
Cranial muscles
Respiratory plasticity

**A B S T R A C T**

Rodents subjected to chronic intermittent hypoxia (CIH) are used to investigate the mechanisms underlying the consequences of the obstructive sleep apnea (OSA) syndrome. Following CIH, rats have an increased density of noradrenergic terminals in the hypoglossal motor nucleus which innervates lingual muscles that protect the upper airway from collapse in OSA patients. Here, we investigated whether such an increase also occurs in other brainstem nuclei. Six pairs of male Sprague-Dawley rats were exposed to CIH or sham treatment for 10 h/day for 35 days, with O₂ level oscillating between 24% and 7% every 3 min. Brainstem sections were immunohistochemically processed for dopamine-β-hydroxylase, a marker for noradrenaline. Noradrenergic terminal varicosities were counted in the center of the trigeminal motor nucleus (Mo5) and the interpolar part of the spinal trigeminal sensory nucleus (Sp5). In the Mo5, noradrenergic varicosities tended to be 9% more numerous in CIH- than sham-treated rats, and in the Sp5 they were 18% more numerous in CIH rats (184 ± 9 vs. 156 ± 8 per 100 × 100 μm counting box; p = 0.03, n = 18 section pairs). These data suggest that CIH elicits sprouting of noradrenergic terminals in multiple motor and sensory regions of the lower brainstem. This may alter motor and cardiorespiratory outputs and the transmission of cardiorespiratory and motor reflexes in CIH rats and, by implication, in OSA patients.

© 2011 Elsevier Ltd. All rights reserved.

Rodents subjected to chronic intermittent hypoxia (CIH) are used to investigate cardiorespiratory, metabolic and other consequences of obstructive sleep apnea (OSA) [3,4]. The hypoglossal motor nucleus (Mo12) innervates the muscles of the tongue that, in OSA patients, are hyperactive and help to maintain airway patency [4,14,20,23]. We recently determined that rats subjected to CIH have an increased density of noradrenergic terminals in the Mo12 [21]. Specifically, noradrenergic terminal varicosities were counted in the ventromedial part of the Mo12 which innervates the genioglossus, the main extrinsic muscle of the tongue. The counts were nearly 40% more numerous in CIH than sham-treated rats.

Like the Mo12, the trigeminal motor nucleus (Mo5) innervates palatal muscles that also contribute to the maintenance of airway patency in OSA patients [13], but it mainly targets the jaw muscles that do not have significant respiratory functions. In contrast to either the Mo5 or Mo12, the spinal trigeminal sensory nucleus (Sp5) has sensory, rather than motor, functions and processes information from both respiratory and non-respiratory sources [8,12]. Thus, our present goal was to determine whether, similarly to the Mo12, noradrenergic innervation is increased following CIH in the Mo5 and Sp5 which have major non-respiratory and some respiratory functions and are located far apart one from the other, in the pons and lateral medulla, respectively. A preliminary report has been published [15].

Twelve adult, male Sprague-Dawley rats were group-housed in standard rat cages placed inside custom-made chambers in which oxygen, nitrogen and room air flows were controlled to obtain the desired profile of changes in oxygen level (OxyCycler, Biosphere, Lacona, NY). At the beginning of the experiment, the mean body weight of CIH rats was 325 ± 9 g (SE), and that of sham-treated rats was 337 ± 16 g. Rats were concurrently exposed in adjacent chambers to CIH or identically timed room air exchanges (sham treatment). CIH was administered for 10 h/day for 35 days, from 7:00 am to 5:00 pm, with oxygen level oscillating between 24% and 7% with a 180 s period, as described by Rukhadze et al. [21]. By the end of the exposure, CIH rats had a lower body weight than sham-treated animals (440 ± 22 g vs. 496 ± 19 g) (c.f. [21]). The material for this study was obtained from three rat pairs that were included in a previous study in which we investigated noradrenergic innervation of the Mo12 [21] and an additional three pairs that were generated specifically for this study. All experiments followed the guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Rat pairs, one CIH-exposed and one sham-treated that were concurrently exposed, were deeply anesthetized and transcardially

* Corresponding author at: Department of Animal Biology 209E/VET, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA 19104-6046, USA. Tel.: +1 215 898 1893; fax: +1 215 573 5186.

E-mail address: ikubin@vet.upenn.edu (L. Kubin).

0304-3940/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.

Fig. 1. DBH⁺ terminal varicosities in the Mo5 in CIH- and sham-treated rats. (A) Typical position of the 100 μm × 100 μm counting box in the Mo5 at the A-P level of −9.8 mm [18]. (B) High-magnification image of DBH⁺ terminals in the counting box shown in (A). (C) DBH⁺ terminal varicosities re-drawn from the image shown in (B). (D) Mean (±SE) numbers of DBH⁺ terminals counted in the Mo5 in sham-treated and CIH-exposed rats. CIH rats tended to have higher numbers of DBH⁺ terminals than sham-treated rats (258 ± 11 vs. 236 ± 10 per counting box; p = 0.07, n = 17 section pairs, paired t-test). Abbreviations: LC, locus coeruleus; Sub CD, dorsal subcoeruleus region.

perfused with 4% paraformaldehyde on the last day of exposure to CIH or sham treatment. Brainstems were cut into six series of 35 μm transverse sections and one series was immunohistochemically processed for dopamine-β-hydroxylase (DBH) (antibody: MAB308, Millipore: 1:500 or 1:2000, depending on the batch of antibodies). Sections from each pair of rats were processed together and then mounted as pairs representing the matching antero-posterior (A-P) levels. DBH-positive (DBH⁺) varicosities were counted in 100 μm × 100 μm counting boxes positioned in the center of Mo5 (A-P levels: −8.8 mm to −9.8 mm, according to a rat brain atlas [18]), and in the interpolar part of Sp5 at the nucleus ambiguous level dorso-ventrally and 100 μm medial to the lateral margin of the Sp5 (A-P levels: −12.3 mm to −13.3 mm). Usually, 4 or 5 sections were available from each rat that belonged to each of the targeted A-P levels. With the additional requirement that the sections from both rats of each pair be matched by their A-P level, three section pairs were available for analysis in each pair, except for one which had only two matched section pairs from the Mo5. To ensure that section selection and matching was done independently of the output measure (noradrenergic terminal density), section pairs were established under low-magnification observation (50×) that was insufficient to assess terminal density. Subsequent re-drawing and counting of terminals was conducted within the counting boxes positioned relative to the anatomic boundaries of the Mo5, Sp5 and other landmarks, as described above, under 1000× magnification with a water-immersion objective. When DBH⁺ terminal counts were normally distributed, they were compared between CIH- and sham-exposed rats within the simultaneously processed CIH/sham rat pairs and section pairs representing the matching A-P levels using paired t-test. One non-normally distributed data set was analyzed using Wilcoxon signed rank test.

In the Mo5, CIH rats tended to have higher numbers of DBH⁺ terminals than sham-treated rats (258 ± 11 (SE) vs. 236 ± 10 per counting box; p = 0.07, n = 17 section pairs, paired t-test) (Fig. 1). DBH⁺ terminal counts varied in individual sections from 194 to 331 in CIH rats, and from 178 to 325 in sham-treated rats. The CIH–sham difference within the pairs of compared sections ranged from 51 less terminals in a section from a CIH rat to 116 more terminals in a section from a CIH rat relative to the corresponding section from the sham-treated rat. When the average terminal counts per section were calculated within each rat and then compared within the six rat pairs, the average counts were higher in the CIH rat in five out of the six pairs, and the statistical trend towards more terminals in CIH rats was maintained (p = 0.058).

In the Sp5, CIH rats had higher numbers of DBH⁺ terminals (184 ± 9 vs. 156 ± 8 per counting box; p = 0.03, n = 18 section pairs; paired t-test) (Fig. 2). DBH⁺ terminal counts varied in individual sections from 154 to 255 in CIH rats, and from 110 to 229 in sham-treated rats. The CIH–sham difference within the pairs of compared sections ranged from 56 less terminals in a section from a CIH rat to 127 more terminals in a section from a CIH rat relative to the corresponding section from the sham-treated rat. When the average terminal counts per section were calculated within each rat, they were higher in the CIH rat for each of the six rat pairs. Since this collapsed data set was not normally distributed, statistical comparison was conducted using Wilcoxon test. The median terminal counts

Fig. 2. DBH⁺ terminal varicosities in the Sp5 in CIH- and sham-treated rats. (A) Typical position of the 100 μm × 100 μm counting box in the Sp5 at A-P level of −12.3 mm [18]. (B) High-magnification image of DBH⁺ terminals in the counting box shown in (A). (C) DBH⁺ terminal varicosities re-drawn from the image shown in (B). (D) Mean (±SE) numbers of DBH⁺ terminals counted in the Sp5 in sham-treated and CIH-exposed rats. CIH rats had higher numbers of DBH⁺ terminals (184 ± 9 vs. 156 ± 8 per counting box; p = 0.03, n = 18 section pairs; paired t-test). Abbreviations: Amb, nucleus ambiguus; A1, noradrenergic cell group of the ventrolateral medulla.

Please cite this article in press as: P. Mody, et al., Rats subjected to chronic-intermittent hypoxia have increased density of noradrenergic terminals in the trigeminal sensory and motor nuclei, Neurosci. Lett. (2011), doi:10.1016/j.neulet.2011.10.015
were 174(25–75% interval: 161–217) for CH rats, and 148 (25–75% interval: 136–170) for sham-treated rats, and the difference was significant at p = 0.031.

Terminal counts were 20 ± 10% higher in CH when compared to sham rats in the Sp5 when calculated for the data sets collapsed to one average DBH⁺ terminal count per rat and nucleus. Owing to the significantly higher average terminal counts in the Mo5 than Sp5, the relative increase was only 9 ± 4% in the Mo5, but the difference between the two nuclei in the relative increase of DBH⁺ terminal density was not statistically significant.

When compared between the Mo5 and Sp5, DBH⁺ varicosities were consistently more numerous in the former, with p < 0.0001 when tested using unpaired t-test separately within CH- or sham-treated groups or for all Mo5 and Sp5 sections combined irrespective of the treatment.

We found that, in the Mo5, noradrenergic terminal density tended to be higher in CH- than sham-treated rats and, in the Sp5, it was significantly higher in CH- than sham-treated rats. In combination with our prior data from the Mo12 [21], this suggests that CH-stimulates noradrenergic terminal sprouting in multiple functionally distinct nuclei in both the medulla and pons.

In both CH- and sham-treated rats, the average density of noradrenergic varicosities was much higher in the Mo5 than Sp5. This may be related, in part, to the different cytoarchitecture of the two nuclei. Whereas the center of the Mo5 is relatively uniformly filled with neuronal cell bodies, the Sp5 contains distinct patches of fiber tracts interspersed among clusters of neurons. Since noradrenergic varicosities do not occur within fiber tracts, their average density within the entire counting box is lower than that within the regions containing neuronal clusters. Nevertheless, the counts in both Mo5 and Sp5 were significantly lower, and less elevated following CH, than in the ventromedial quadrant of the Mo12 (median numbers: 632 vs. 455 in CH- and sham-treated rats, respectively, or a nearly 40% increase) where we have previously counted noradrenergic terminals using the same methodology [21]. Our present results show that CH-induced sprouting of noradrenergic terminals occurs not only in the Mo12 but also in other sensory and motor regions of the lower brainstem. However, the relative magnitude of the increase is considerably higher in the Mo12 than in either Mo5 or Sp5.

Two explanations may be suggested for the different magnitudes of the CH-stimulated increase of noradrenergic innervation among the three nuclei (Mo12 > Sp5 > Mo7): (1) that differences in the magnitude of the effect of CH are secondary to the chronic hypoxic stimulation of ventilation, which probably more strongly activates the central respiratory and reflex inputs to Mo12 and Sp5 neurons than to Mo5 motoneurons; and (2) that these differences are secondary to the different sources of noradrenergic innervation of the three nuclei. With regard to the first possibility, both the central respiratory drive and respiratory reflex inputs from receptors located in the respiratory tract are strongly stimulated during CH. This may result in a re-configuration (plastic changes) in the afferent pathways to Mo12 and Sp5 neurons that involve the noradrenergic system (cf. [16,27]) to a larger degree than the pathways that control Mo5 neurons, which primarily serve alimentary functions. Indeed, the Sp5 region that we investigated receives afferent information from the nasal mucosa through the ethmoidal nerve and from the larynx through the superior laryngeal nerve [5,11,17]; afferent input from these nerves is likely stimulated when breathing is increased during exposure to CH. Within this framework, the specific mechanisms connecting stronger respiratory stimulation to stronger noradrenergic innervation remain to be identified. With regard to different sources of noradrenergic innervation, noradrenergic afferents to the Mo12 are bilateral and originate primarily in the A5, A7, A1 and sub-coeruleus groups of noradrenergic neurons, whereas Mo5 receives predominantly ipsilateral catecholaminergic afferents from A7 and A5 and little or no innervation from the A1 or sub-coeruleus region, and Sp5 is innervated by A1/C, A5, A7, subcoeruleus and also the locus coeruleus proper [46] [19,22]. It is of note that, in mice subjected to long-lasting CH protocols, an oxidative damage occurred in the locus coeruleus but not in other pontomedullary noradrenergic neurons [29]. Thus, different brainstem regions and nuclei may exhibit different patterns of CH-induced changes of their noradrenergic innervation depending on the predominant sources of their noradrenergic innervation.

Norepinephrine provides an important, wake-related excitatory drive to motoneurons that innervate upper airway and other orofacial muscles [26,24], including the genioglossus innervated by the Mo12 and the tensor and levator veli palatini innervated by the Mo5 [13]. Upper airway muscles have increased activity during wakefulness in OSA patients who also experience CH as a consequence of their disorder [14,26]. Accordingly, our results suggest that upper airway muscle hyperactivity in OSA patients is caused, at least in part, by CH. As such, CH may contribute to a positive adaptation in OSA patients that allows them to breathe adequately when they are awake despite the anatomical vulnerability of their upper airway that causes OSA.

OSA patients also develop arterial hypertension as a result of the disorder [19,28], and CH leads to arterial hypertension in rodents [7]. The CH-induced sprouting of noradrenergic innervation in the brainstem may contribute to increased activity in the medullary regions that maintain arterial blood pressure, leading to an elevation of sympathetic output [7,10,25]. As such, CH is also an established cause of some of the major negative consequences of OSA – arterial hypertension. Thus, CH may contribute to both adaptive and maladaptive changes in the central neural control of cardiorespiratory outputs.

Acknowledgments

The study was supported by grant HL-047600 from the National Institutes of Health. The sponsor had no role in the study design, data collection, analysis or interpretation of data, or in the writing and submission of the manuscript.

References

Rats subjected to chronic-intermittent hypoxia have increased density of noradrenergic terminals in the trigeminal sensory and motor nuclei.