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Original Article

Chronic intermittent hypoxia affects endogenous serotonergic inputs and expression of synaptic proteins in rat hypoglossal nucleus

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Abstract: Evidence has shown that hypoxic episodes elicit hypoglossal neuroplasticity which depends on elevated serotonin (5-HT), in contrast to the rationale of obstructive sleep apnea (OSA) that deficient serotonergic input to HMs fails to keep airway patency. Therefore, understanding of the 5-HT dynamic changes at hypoglossal nucleus (HN) during chronic intermittent hypoxia (CIH) will be essential to central pathogenic mechanism and pharmacological therapy of OSA. Moreover, the effect of CIH on BDNF-TrkB signaling proteins was quantified in an attempt to elucidate cellular cascades/synaptic mechanisms following 5-HT alteration. Male rats were randomly exposed to normal air (control), intermittent hypoxia of 3 weeks (IH3) and 5 weeks (IH5) groups. Through electrical stimulation of dorsal raphe nuclei (DRN), we conducted amperometric technique with carbon fiber electrode in vivo to measure the real time release of 5-HT at XII nucleus. 5-HT_{2A} receptors immunostaining measured by intensity and c-Fos quantified visually were both determined by immunohistochemistry. CIH significantly reduced endogenous serotonergic inputs from DRN to XII nucleus, shown as decreased peak value of 5-HT signals both in IH3 and IH5groups, whereas time to peak and half-life period of 5-HT were unaffected. Neither 5-HT_{2A} receptors nor c-Fos expression in HN were significantly altered by CIH. Except for marked increase in phosphorylation of ERK in IH5 rats, BDNF-TrkB signaling and synaptophys consistently demonstrated downregulated levels. These results suggest that the deficiency of 5-HT and BDNF-dependent synaptic proteins in our CIH protocol contribute to the decompensated mechanism of OSA.

Keywords: Obstructive sleep apnea, hypoglossal nucleus, serotonin, chronic intermittent hypoxia

Introduction

Obstructive sleep apnea (OSA) is a prevalent disorder and manifests as the sleep-related narrowed or collapsible upper airway, resulting in nocturnal intermittent hypoxia (IH) and hypercapnic stimulus induced arousal [1]. The root cause of upper airway hypotonia is the suppression of genioglossus (GG) muscle activity which is innervated by hypoglossal nucleus (HN, XII nucleus). Previous studies gained tremendous insight into the diverse neurotransmitter mechanisms involved in state-dependent control of HN. Of note, pretreatment of upper airway dilator motor neurons with serotonin [5-hydroxytryptamine (5-HT)] reduced sleep state-dependent suppression in tonic GG activity, indicating the decrement in serotonin delivery to HN at least in part contributed to upper airway obstruction. Serotonergic neurons concentrated in caudal medullary dorsal raphe nuclei (DRN), which releases serotonin and projects to XII nucleus [2, 3]. The endogenous serotonergic excitatory drives to XII nucleus are mainly mediated by 5-HT_{2A} receptors that are abundantly expressed in hypoglossal motoneurons (HMs) [4]. Based on these observations, modulation of endogenous 5-HT has been attempted as a pharmacological treatment for OSA [5]. However, little beneficial effectiveness of clinical serotonergic drugs such as selective 5-HT reuptake inhibitors [6, 7] was observed in OSA patients. A major characteristic of OSA pathogenesis could not be ignored:

chronic intermittent hypoxia (CIH) itself may modify serotonergic neurochemical input to XII nucleus or 5-HT_{2A} receptor (5-HT_{2A}R) expression. Several neuronal populations have shown CIH exposure altered physiological function and neuronal excitability [8, 9]. These hypoxia-induced alterations will render these pharmacological therapies for sleep apnea more challenging.

Specifically, patients with OSA adapt to the anatomical vulnerability of their upper airway by generating more increased tonic activation of genioglossal and pharyngeal muscles than healthy subjects [10]. This compensatory mechanism is described as respiratory neuroplasticity that allows for airway patency and adequate ventilation. Accumulative evidence suggests long-term facilitation (LTF), as a form of neural plasticity in respiratory motor control following episodes hypoxia, depends on a persistent elevation of serotonin release from caudal raphe neurons and consequent 5-HT receptor activation [11, 12]. That is in contrast to the rationale that deficient serotonergic modulation of HMs fails to keep airway patency during sleep [13]. Therefore, understanding of the 5-HT dynamic changes at XII nucleus during CIH will be essential for central pathogenic mechanism of OSA. For this reason, we conducted amperometric technique with carbon fiber electrode in vivo. Through electrical stimulation of DRN in CIH and sham-treatment rats, we could monitor the real time release of 5-HT at XII nucleus.

5-HT mediated neuroplasticity in hypoglossal motor output also requires de novo synthesis of brain-derived neurotrophic factor (BDNF) [14], and activation of its high affinity receptor tyrosine kinase TrkB [15, 16]. Changes of proteins such as BDNF-TrkB signaling and synaptophysin may suggest this dynamic integration of neurotransmission [17], and probably alter over short- and long-term CIH exposure. In terms of neuroanatomical aspect, we hypothesized that CIH might affect 5-HT_{2A}R expression and hypoglossal motoneurons baseline activity. Thus, 5-HT_{2A} receptors were quantified and c-Fos expression was compared as an indirect way of assessing activities of HMs in rats subjected to CIH or sham treatment. Furthermore, the effect of CIH preconditioning on BDNF-TrkB signaling proteins was investigated in an initial attempt to elucidate cellular cascades/synaptic mechanisms following 5-HT alteration.

Materials and methods

Animals and experimental protocol

Male adult Sprague-Dawley (SD) rats were obtained from the Experimental Animal Centre of Fudan University (Shanghai, China) and kept in the conventional housing unit under standard conditions (two or three per cage, 24 C, 45-65% humidity, 12 h light/dark cycle), with free accessing to food and water. The project was approved by the Medical Experimental Animal Administrative Committee of Shanghai Medical College of Fudan University, accordance with the guidelines implemented by National Institutes of Health Guide regarding the care and use of animals for experimental procedures. All effects were made to minimize animal suffering. The standard CIH protocol was modeled as our previous study described [18]. Rats exposure to intermittent hypoxia (IH) were placed inside custom-made (28.5×30.0×51.5 cm) chambers where flows of oxygen and nitrogen were controlled to obtain the desired profile of changes in oxygen level. CIH was administered for 10 h/day, from 7:00 AM to 5:00 PM, with oxygen level oscillating between 24% and 7% with a period of 60 s (**Figure 1A**). The oxygen concentration was measured automatically using an oxygen analyzer (Corporation, Shanghai, China). Thirty-six adult, male Sprague-Dawley rats (250-350 g) were concurrently exposed to intermittent hypoxia in adjacent chambers (IH group) or identically room air exposure (control sham treatment). Rats belong to IH group (n=24) were randomly divided into the following two experimental groups: the IH group of 3 weeks (7 AM-5 PM daily for 21 days, n=12) and the IH group of 5 weeks (7 AM-5 PM daily for 35 days, n=12). CIH animals did not gain weight during the first 2-3 days of exposure and then gained weight at a slightly slower pace than the control group.

Amperometric detection of serotonin signals with carbon fiber electrode

At the end of modeling for 3 weeks and 5 weeks, six rats from each group were deeply anesthetized with intraperitoneal injection of chloral hydrate (10% in saline, 300 mg/kg) and fixed at the stereotaxic instrument (Life Technology Co. Ltd. of Shenzhen City). A bipolar

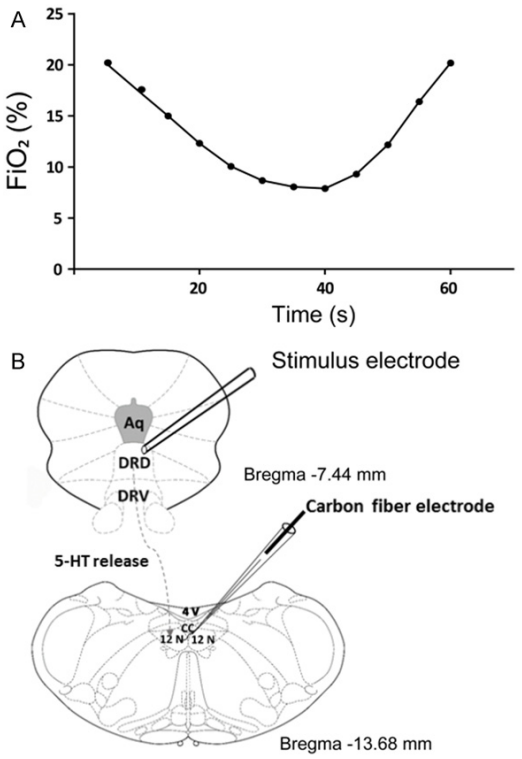

Figure 1. Experimental schema. A. A rat model of CIH resembling sleep apnea. Time course of inspired fraction of oxygen (FiO_2) level changes in CIH chambers during one successive cycles of intermittent hypoxia, as sampled at 5-second intervals. B. Amperometric detection of serotonin 5-HT release evoked by electrical stimulation in DRN and recorded in XII nucleus by carbon fiber electrode *in vivo*. DRN located at bregma -7.44 mm and XII nucleus at bregma -13.68 mm according to the atlas. Aq = aqueduct, DR = dorsal raphe, 4V =4th ventricle, cc = central canal, 12N = XII nucleus.

stainless steel electrode with diameter of 1.0 mm sent electrical stimulation (Isolated Pulse Stimulator model 2100; A-M Systems) into raphe dorsal nuclei (anterior-posterior: -8.16 mm, medio-lateral: 0.2 mm, dorso-ventral: -5.6 mm). The amperometry working electrode was a cylindrical carbon-fiber electrode insulated by a glass capillary. A 7-μm diameter carbon fiber was inserted into a 1.5 mm×10 cm glass capillary, and then the glass capillary was pulled by a vertical puller (05-E, Institute of new technique application, Wuhan, China). Each successfully made CFE had an overall length of 45 mm, with a relatively long sensor tip (100 μm) of naked carbon fiber. In order to improve insulation and reduce noise, the glass capillary of the electrode tip was filled with epoxy and back-filled with 4 M KCl. The detecting carbon fiber electrode was inserted into the XII nuclei (anterior-posterior: -13.08 mm, medio-lateral: ±0.3 mm, dorso-ventral: -7.0 mm), as per the atlas of Paxinos and Watson. The reference electrode was a silver wire coated with AgCl and connected to the neck muscle tissue. A patch-clamp amplifier (PC-2B, INBIO, Wuhan, China) was used under voltage-clamp mode, with the gain of 0.5 mV/pA and a CFE voltage of a constant +700 mV for amperometry. All data were low pass filtered at 20 Hz and acquired by a data acquisition system with a digital interface and software (iPDA-0.1; INBIO, Wuhan, China). Schema illustrating amperometric detection of serotonin release in XII nucleus *in vivo* was shown as **Figure 1B**. 5-HT release signals evoked by electrical stimulation (0.5 mA, 20 Hz, 10 pulses) in dorsal raphe nuclei *in vivo* were analyzed by three indices. The indices include the peak value (maximal amplitude of the secretion signal), the time to peak (time duration from start of the electrical stimulation to the peak amplitude of the secretion signal) and the half-life period (time duration from the peak to half-height of the secretion signal). After this craniocerebral surgery, these rats were euthanized to collect arterial blood samples from the abdominal aortic, and measured partial oxygen tension (PaO_2) and partial pressure of carbon dioxide ($PaCO_2$) with a GEM Premier 3000 blood gas analyser (International Lab, South San Francisco, CA, USA) to confirm the experimental efficiency of CIH protocol. Respiratory rates of rats were counted by a blinded technician. At the end of the experimental, the whole brains were dissected on ice. The raphedorsal nuclei were collected for Nissl staining to verify the extent of electrode lesions and hypoglossal nuclei were extracted for western blots.

Immunohistochemistry

On the last day of 3 or 5 weeks of exposure to CIH or sham treatment, six rats from each group were gently kept awake for 2.5 h and then were deeply anesthetized by chloral hydrate (500 mg/kg) and perfused with 50 ml saline followed by 500 ml 10% formalin through the heart for $5\text{-}HT_{2A}$ and c-Fos immunostaining. The brains were removed, post-fixed for 4 h in 10% formalin, and equilibrated in 20% sucrose in phosphate-buffered saline (PBS) overnight. The brains were frozen and cut on a freezing microtome at 35 μm into transverse sections that collected into four series, which were subjected to $5\text{-}HT_{2A}$ recep-

tors and c-Fos immunohistochemistry. For Nissl staining, sections were mounted on gelatin-coated slides, washed and incubated in 0.25% thionin solution for 2 min, then washed and dehydrated in gradient ethanol, and cleared in xylene before being cover slipped. For 5-HT_{2A} and c-Fos immunostaining, sections were incubated with 0.3% H_2O_2 for 15 min to quench the endogenous peroxidase activity. After washing in 0.1 M phosphate-buffered saline (PBS; pH 7.4), the sections were incubated with primary antibody 5-HT_{2A} (1:500; ab16028; Cambridge, UK) and c-Fos (1:5000, CalBiochem, San Diego, CA, USA)diluted in PBS-T with 0.02% sodium azide for 24 h at room temperature. On the second day, the sections were washed in PBS and incubated in biotinylated secondary antibody (anti-rabbit IgG antibody 5-HT_{2A}, 1:500 and c-Fos 1:1000 in PBST) for 1 h, followed by a 1:1000 dilution of avidin-biotin-peroxidase (Vector Laboratories, Burlingame, CA, USA) for 1 hat room temperature. The peroxidase reaction was visualized with 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma) in PBS and 0.01% H_2O_2. The sections were stained with DAB only and thus c-Fos-positive was labeled brown. The 5-HT_{2A} receptor positive cells were brown in color. After terminating the reaction by PBS-azide, sections from each pair of rats were matched for anteroposterior (A-P) levels, mounted side-by side on glass slides, dehydrated and cover slipped. Sections were examined under bright-field illumination using an Olympus BX51 microscope (Japan).

Imaging, analysis and cell counting

Sections were observed under a photomicroscope (DP72, Olympus, Japan) at 200 magnification and checked at 400 magnification when necessary. C-Fos-positive numbers within entire cross-section of the XII nucleus in each group were initially counted bilaterally. Neurons of XII nuclei were present in both the rostral and the caudal region. Rostro-caudal coordinates in the medulla were calculated relative to the obex: rostral XII nucleus ranged from -12.72 mm to -13.56 mm, and caudal XII nucleus was located between -13.68 mm and -14.76 mm posterior to the bregma.

Ultimately, the c-Fos counts were represented by average counting per section. For quantification of 5-HT_{2A} receptor staining, digital images were converted to a grayscale digital image before densitometric measurements of staining intensity within the dorsal and ventral halves of the XII nuclei separately in 24 representative pairs of brain sections from six rat pairs (four sections per rat), as described previously. Digital images were processed using Photoshop software (Adobe Systems, San Jose, CA).

Tissue collection and western blots

Hypoglossal nuclei were collected on ice from the brains of control and IH groups, and then dissected on ice according to the protocol published by Zhou [19]. Tissues were frozen at -80°C and further homogenized in cold lysis buffer supplemented with protease inhibitors for protein extraction. Proteins (20 μg) were separated using a 10% SDS-PAGE gel. Proteins were transferred to a polyvinylidene fluoride membrane using a wet transfer method. Then the membrane was washed in Tris-buffered salineþTween-20 (TBST; 20 mM Tris, 0.5 M NaCl, 0.1% Tween-20) blocked for 1 h with 5% skim milk made in TBST buffer at room temperature. The membrane was incubated with primary antibodies in TBST overnight at 4°C, followed by incubation in the appropriate secondary antibodies for 1 h atroom temperature. The following antibodies were used: rabbit anti-TrkB (1:1000; sc-8316, Santa Cruz Biotechnology; Dallas, TX), mouse anti-synaptophysin (1:1000; ab8049, Abcam; Cambridge, MA), rabbit anti-BDNF (1:1000; sc-20981, Santa Cruz Biotechnology; Dallas, TX), p-ERK [1/2] (1:1000; No.9101; Cell Signaling Technology; Danvers, MA), donkey anti-rabbit IgG-HRP (1:10000; 711-035-152, Jackson Immuno research; West Grove, PA), donkey anti-mouse IgG-HRP (1:5000; 715-035-151, Jackson Immunoresearch; WestGrove, PA). For detection, the membrane was washed with TBST, followed by distilled water and then detected using a horseradish peroxidase substrate ECL chemiluminescence system (LuminataForte, EMD Millipore; Billerica, MA). Blots were visualized using a VersaDoc imaging system (Bio-Rad Laboratories; Hercules, CA) and analyzed using Image J (version 1.42, National Institutes of Health, USA).

Statistical analysis

Variability of the means was characterized by the SEM. The density of 5-HT_{2A} receptor immunostaining and background were measured using Image J software from digital images

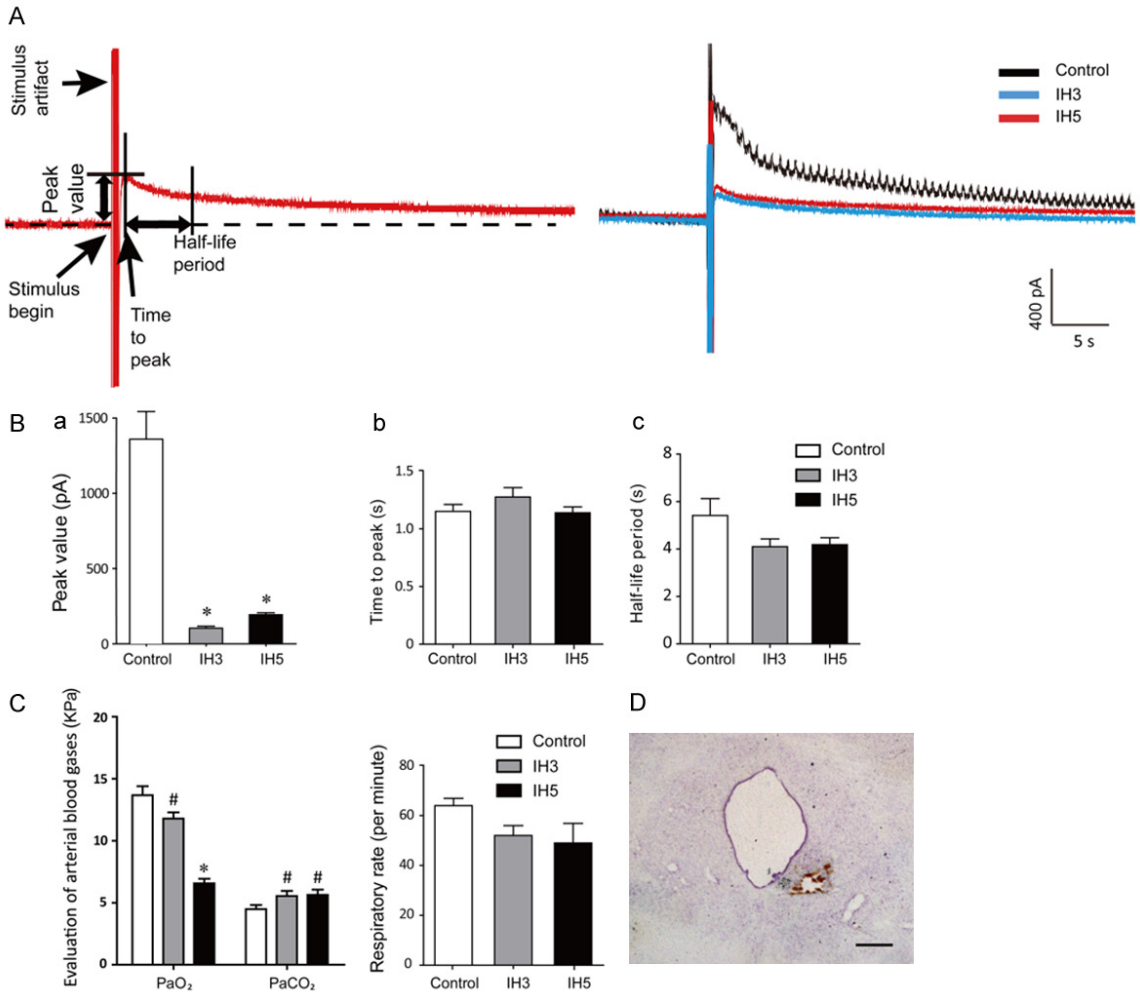

Figure 2. CIH significantly decreased serotonergic inputs from DRN to XII nucleus in vivo. A. Representative traces of typical 5-HT signals with the peak value, time to peak and half-life period separately labeled (left image). Representative raw data of 5-HT signals recorded from control, IH3 and IH5 group rats merged as right image. B. Histogram of the peak value (a), time to peak (b), half-life period (c) of 5-HT release analyzed respectively from control (normal room air), IH3 (exposed to 3 weeks of CIH) and IH5 (exposed to 5 weeks of CIH) group rats. C. Blood gas analysis and respiratory rate of control, IH3 and IH5 group rats. D. Example of a unilateral lesion site made by stimulus electrode in DRN shown as Nissl staining. Scale bar =150 μm. Data were presented as mean ± S.E.M (n=6 rats/group), #P<0.05, *P<0.01 versus control group.

taken under constant illumination and magnification. The density was determined by outlining the nucleus of interest with a cursor and measuring the average grey scale intensity on a pixel-by-pixel basis. The background staining was measured by positioning a copy of the nuclear outline over an area of the same section having the lowest intensity. The differences between these two methods were used to characterize the specific immunostaining, and subjected to statistical analysis using SPSS. C-Fos counts were presented as the total number of marked positive cells per section per rat. Comparisons between groups were made by ANOVA. Bonferroni's test was used for post-hoc comparisons. The differences were regarded as significant when $P<0.05$.

Result

Effects of CIH on 5-HT release in the XII nucleus by electrical stimulation in the DRN

5-HT release signals in the XII nucleus were recorded bilaterally by carbon fiber electrode by electrical stimulating DRN (0.5 mA, 20 Hz, 10 pulses) in three groups under anaesthetized

Table 1. 5-HT release signals recorded in XII nucleus by electrical stimulation in DRN

Parameters	Control group	IH of 3 weeks	IH of 5 weeks	*P*-value
Peak value (pA)	1359.67±552.19	104.22±38.57*	193.21±40.61*	<0.0001
Time to peak (s)	1.15±0.17	1.27±0.25	1.14±0.15	0.488
Half-life period (s)	5.41±2.14	4.09±0.99	4.18±0.89	0.165

Data were expressed as means ± SEM. *P<0.01 vs. control group.

condition. Representative raw data of typical 5-HT signals recorded from three group rats were shown as **Figure 2A**. The peak value, the time to peak and half-life period of 5-HT signal were analyzed respectively. Compared with the control group, CIH significantly decreased peak value of 5-HT signals both in IH3 and in IH5 group rats (**Figure 2Ba**). No significant changes of the time to peak (**Figure 2Bb**) and half-life period (**Figure 2Bc**) of 5-HT signals were recorded in the three groups (n=6/group). In addition, long-term IH for 5 weeks resulted in a smaller increase in 5-HT release from DRN than that for 3 weeks, whereas time to peak and half-life period of 5-HT signals were unaffected. A more detailed description of the parameters of 5-HT release signals detected in XII nucleus following normoxia and hypoxia was described in **Table 1**. There were also no significant differences in the respiratory rate within the three groups (**Figure 2C**). The lesion site made by stimulus electrode in all rats was confirmed by Nissl staining (**Figure 2D**). Accordingly, hypoxia significantly inhibited 5-HT release irrespective of the time of exposure.

Effects of CIH on 5-HT$_{2A}$ receptor expression in the XII nucleus

The dorsal and ventral halves of XII nucleus with were delimited by white triangular shapes shown as **Figure 3A**. Intensity of 5-HT$_{2A}$R immunostaining was measured within the entire cross-section of the XII nucleus in 24 pairs of matched A-P level sections from six pairs of three group rats. With no correction for background staining, the immunostaining of 5-HT$_{2A}$R demonstrated modest intense in IH3 group (dorsal part: 214.5±12.73 and ventral part 225.17±11.14 arbitrary units) than other two groups (*P*=0.88, **Figure 3B**). No significance did exist between control and IH5 group rats (dorsal part: 199.83±9.54 vs. 198.33±12.23 arbitrary units and ventral part: 209.5±11.45 vs. 209±15.98 arbitrary units respectively; *P*=0.96, **Figure 3B**). It was also apparent that, 5-HT$_{2A}$R immunostaining was slightly more intense in the ventral than the dorsal half of the nucleus at all A-P levels, although this intensity was not significantly different in either IH or control rats.

In particular, 5-HT$_{2A}$R intensity within the XII nucleus was found positively correlated with the intensity of background staining within either dorsal (R^2=0.482) or ventral part (R^2=0.755). The slopes of the two linear regression lines were different as shown in **Figure 3D** respectively. Using the same methodology to quantify 5-HT$_{2A}$R [20], we calculated the ratio of staining intensity in XII nucleus to the intensity of background for each section to unveil any potential unspecific effect. After such a normalization for background, the relative 5-HT$_{2A}$R intensity was lower in IH3 group than in control group rats (dorsal part ratios: 4.35±0.79 vs. 6.14±1.31 and ventral part: 4.57±0.86 vs. 6.44±1.38; *P*=0.93, **Figure 3C**). Further, time of exposure to IH seemed to associate with a change of the intensity of 5-HT$_{2A}$R, whereas the difference was not statistically significant in IH5 than in IH3 group rats (dorsal part ratios: 5.8±1.72 and ventral part: 6.11±1.8; *P*=0.94, **Figure 3C**).

Effects of CIH on c-Fos expression in the XII nucleus

In normal condition, the rostral nucleus of the solitarytract (sol) contained very few c-Fos-positive neurons, whereas the marked c-Fos-positive numbers increased to relative high levels in rats subjected to IH (**Figure 4A**). This suggested that, this CIH protocol was successful to have a specific effect on the overall level of c-Fos expression. The range of c-Fos-positive neurons counted in the XII nucleus was 7 to 22 per section for IH-exposed of 3 weeks rats, 5 to 16 per section for IH-exposed of 5 weeks rats and 2 to 9 per section for control group of 24 representative pairs of XII nucleus sections from six rat per group.

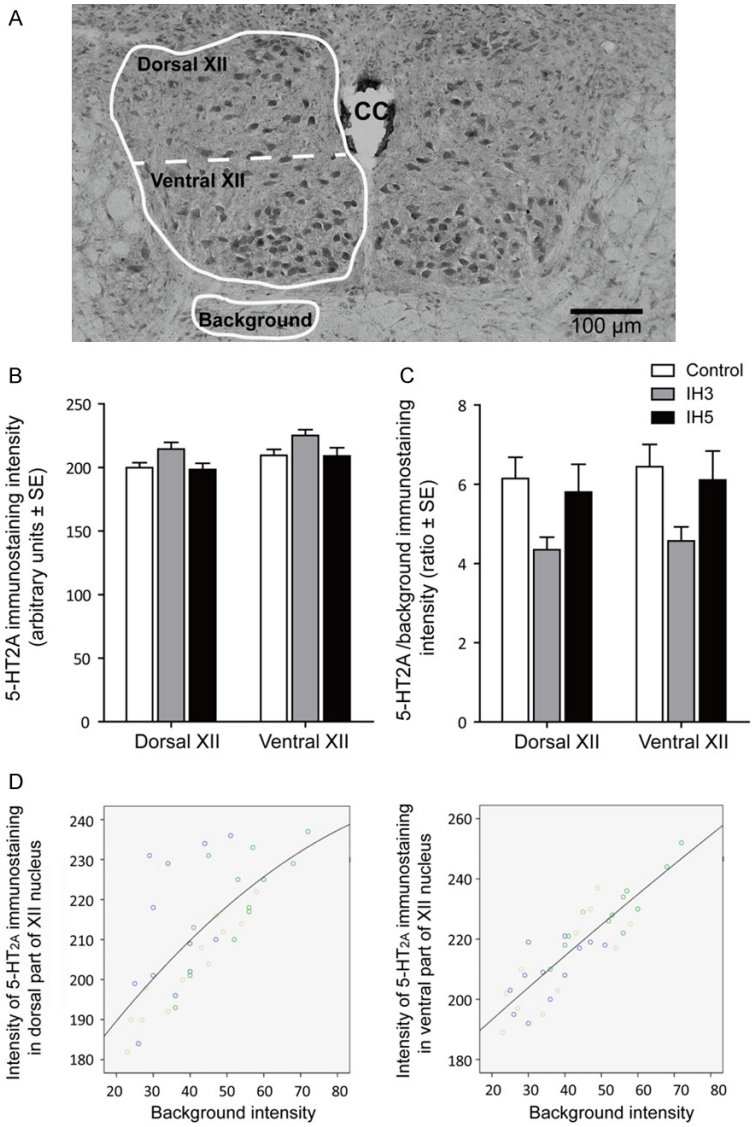

Figure 3. Effect of CIH on 5-HT_{2A} receptor immunostaining in the XII nucleus. A. Representative photomicrograph of 5-HT_{2A} receptor immunostaining with white line delimiting the entire XII nucleus and dotted lines indicating the borders of dorsal and ventral halves of the XII nucleus. The background region encircled used for measurement of ratio of staining intensity. cc = central canal. Scale bar =100 μm. B. The average intensity of 5-HT_{2A} receptor within the dorsal and ventral XII nucleus measured by densitometry from control (normal room air), IH3 (exposed to 3 weeks of CIH) and IH5 (exposed to 5 weeks of CIH) group rats. C. The ratio intensity of 5-HT_{2A} receptor staining within the dorsal and ventra XII nucleus to the background measured by densitometry from control, IH3 and IH5 group rats. D. Intensity of 5-HT_{2A} receptor staining positively correlated with background separately analyzed within the dorsal and ventral XII nucleus. Blue circle = control group; green circle = IH3 group; yellow circle = IH5 group. Data were presented as mean ± S.E.M (n=6 rats/group).

When c-Fos-positive numbers were analyzed, modest differences were found at different A-P levels. Therefore, rostro-caudal coordinates in the entire XII nucleus were separately calculated relative to the atlas. Following hypoxia, c-Fos-positive counts tended to be higher at the caudal than rostral regions in three group rats (**Figure 4A**). At A-P level -14.76 mm that assumed in the caudal part, c-Fos counts were slight higher in IH3 than those in control group rats (18±4 vs. 7±2 per section, n=12; P=0.58, paired t test, **Figure 4B**). After a long-term IH exposure for 5 weeks, there was a slight reduction inc-Fos levels than IH for 3 weeks (13±3 per section, n=12; P>0.05, **Figure 4B**). In the rostral part of the XII nucleus (-13.08 mm), there demonstrated a higher trend of the c-Fos-positive expression in IH3 than control group rats (7±4 vs. 2± 1 per section, n=12; P=0.65, paired t test, **Figure 4B**). Consistent with analysis of c-Fos in caudal XII nucleus, the average number of c-Fos-positive neurons in IH5 group was mildly lower thanIH3 group rats (5±2 per section, n=12; P>0.05, **Figure 4B**). In addition, subsequent comparisons at other matched A-P levels revealed no statistical significance.

Effects of CIH on synaptic proteins expression in the XII nucleus

In contrast to normoxic controls, the expression levels of BDNF in hypoglossal nucleus were significantly inhibited by 3 weeks of CIH exposure (P=0.0016), but subsequently showed a lessened decreasing trend (21% less expression) following 5 weeks of CIH exposure (P=0.0062; **Figure 5A**). Consistent with these changes in BDNF, its receptor TrkB protein expressions in hypoglossal nucleus were decreased by 33% in IH3 group (P=0.0016), while

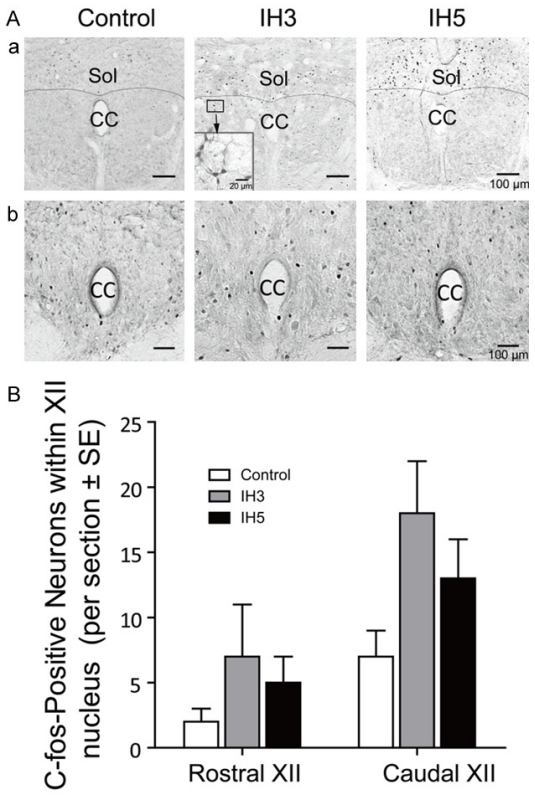

Figure 4. Effect of CIH on c-Fos expression in the XII nucleus. A. Representative photomicrographs for c-Fos expression in the XII nucleus from control (normal room air), IH3 (exposed to 3 weeks of CIH) and IH5 (exposed to 5 weeks of CIH) group rats. Coordinating distance to bregma according to rat brain atlas of *Paxinos and Watson* illustrated as rostral and caudal XII nucleus. Black arrow indicates nuclear c-Fos immunoreactivity and a magnified view (400×) of the area indicated by the inset boxed. B. Numbers of c-Fos-positive neurons in XII nucleus of the three groups. Data were presented as mean ± S.E.M (n=6 rats/group). cc = central canal, Sol = nucleus of the solitary tract. Scale bar =100 μm.

long term of hypoxia in IH5 group only elicited a suppression by 13% compared to normoxic controls (**Figure 5B**). As showed in **Figure 5C**, no compensatory changes but sustained decreases were observed in the presynaptic marker synaptophysin. Engagement of BDNF-TrkB induced by CIH also accompanied a significant decrease of synaptophysin as compared to normal condition (P=0.0006). Specifically, 5 weeks of hypoxia exerted severer inhibitory effect on synaptophysin levels than that of 3 weeks (decreased by 85% & 67% respectively). Since ERK1/2 was phosphorylated by BDNF-dependent TrkB activation and necessary for multiple forms of synaptic plasticity, we measured p-ERK1/2 as attractive candidate molecules in this mechanism. Results demonstrated that expressions of p-ERK were lowly detectable in control and IH3 groups. Conversely, 5 weeks of hypoxia tended to enhance the phosphorylation of ERK1/2 by approximately 73%, consistent with our precious results in kidney tissue.

Discussion

Exposure to chronic hypoxic episodes produces similar patterns as those observed during OSA. Substantially, short term and long term of IH, characterized by different duration and severity, exert different effects. Our novel, most salient finding of the present study is that CIH significantly reduced endogenous serotonergic inputs from DRN to XII nucleus. The decreased peak value of 5-HT signals suggested lower serotonin concentration in XII nucleus in CIH rats. In contrast, neither the time to peak or half-life period of 5-HT was significantly altered by CIH, indicative of no significant changes of 5-HT reuptake at nerve terminal of DRN. Similar to the slightly decreased 5-HT_{2A} receptor activation, BDNF-TrkB signaling and synaptophys consistently demonstrated downregulated levels. Since serotonin is previously thought to have an overall excitatory effect on XII nucleus, the reduced 5-HT levels elicited by CIH at least in part compromise the upper airway tonic in the process of obstructive sleep apnea.

Previous studies have demonstrated hypoxia activated raphe serotonergic neurons activity through increased c-fos labeling [21]; and subsequently released serotonin in the brainstem of anesthetized cats [22]. On the contrary, we found 5-HT at XII nucleus reduced after exposure to CIH. Of note, hypoxia could induce ROS production and affected cytochrome c oxidase activity [23]. Large soma and long axonal projections require high energetic demand and mitochondrial function [24]. Suffering from hypoxia stress, high content of neurofilament protein inevitably leads to misfolding and poor nerve conduction [25]. Therefore, excitatory synaptic transmission such as 5-HT input was easily susceptible to cellular metabolic inflammatory responses [18] and neuronal oxidative damage [26].

Comprehensive studies suggested serotonergic neurons were critical for respiratory respon-

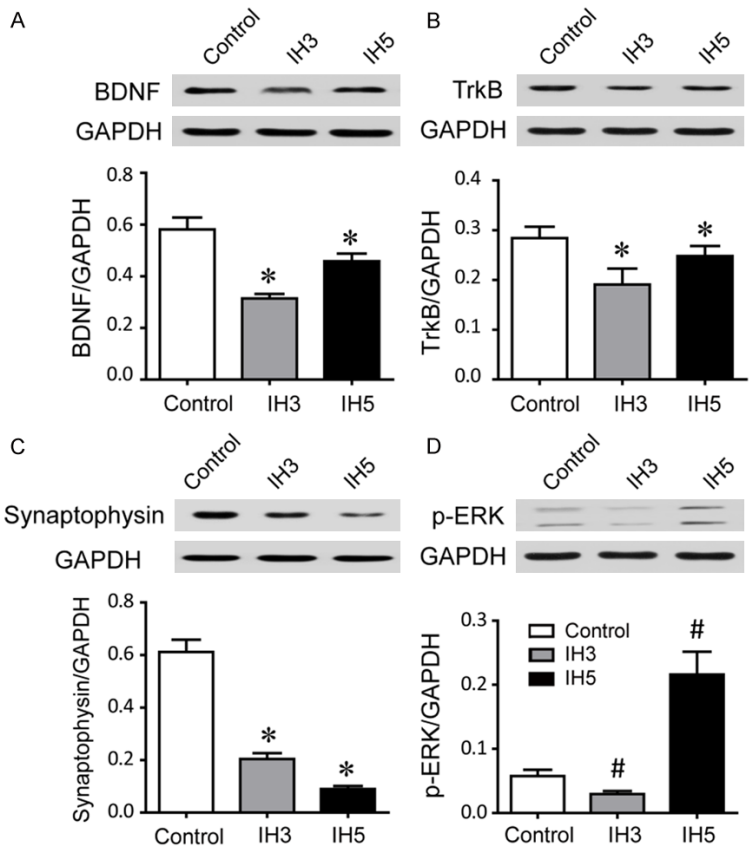

Figure 5. Representative western blots with summary quantifications of BDNF (A), TrkB (B), synaptophysin (C) and phosphorylation of ERK (D) protein expression in XII nucleus. GAPDH was analyzed as a loading control. Data were presented as mean ± S.E.M (n=6 rats/group), #P<0.05, *P<0.01 versus control group.

ses to intermittent hypoxia [27]. It was also acknowledged that specific loss of 5-HT from the brain stem severely compromised the ability to withstand episodes of anoxia [28]. Dysfunction within serotonergic system usually included reduced 5-HT content and inhibited tryptophan hydroxylase-2 (TPH2) activity, and the latter was the enzyme catalyzing the rate-limiting step in central 5-HT biosynthesis [29]. Our data have taken the effect of enzyme into consideration and reflected the ultimately temporal levels of 5-HT. Along with previous evidence, deficient 5-HT levels following episodic hypoxia at least in part support the central pathogenic mechanism of OSA. Analysis of arterial blood gas within three groups following differential CIH exposures was compatible with discrepant severities of OSA. Evidenced by sustained low protein levels of synaptophysin in this study, we speculated that longer term of IH might worsen neural synaptic transmission. Conversely, 5-HT release was elevated in rats subjected to IH exposure for 5 weeks than that exposure for 3 weeks, but still lower than control group. Such an increase may associate with the adaptive responses, and these pathophysiologic changes elicited by CIH were likely to gradually normalize during the prolong exposure period.

As the predominant receptor subtype mediating serotonergic excitatory effects in hypoglossal motoneurons, 5-HT_{2A} receptors tend to undergo transcriptional changes subjected to stress condition [30]. There was a study that measured 5-HT_{2A}R mRNA numbers which were almost unchanged by CIH in a single hypoglossal motoneuron [31]. In agreement with this, we note that the intensity of 5-HT_{2A}R immunostaining was slightly attenuated in IH3 group than in the control group after correction for background staining. Subsequently, the functional downregulated postsynaptic receptors could explain the reduced neural responsiveness, just as a prior observation demonstrated [31], XII motoneurons of rats exposed to CIH had reduced excitatory response to exogenous 5-HT. Meanwhile, cIH might modify a series of intracellular signaling events. Within hypoglossal nucleus, levels of BDNF-TrkB and synaptophysinin IH3 group rats were observed decreased. Erickson demonstrated that the absence of BDNF was associated with severe depression of respiratory control as well as loss of the hypoxic ventilatory drive [32]. Another evidence of the reduced neuroprotection from BDNF/TrkB system was provided in a piglet model [33]. Indeed, several possibilities also exist for interaction between 5-HT and BDNF systems, for example, 5-HT stimulated the expression of BDNF [34]. Previous findings further proposed a schematic cellular/molecular mechanism described as *Q pathway* triggered

by IH [35]. In other words, secreted 5-HT binds to G_q-protein coupled metabotropic receptors such as 5-HT_{2A}R, and then initiates necessary downstream signaling including the activation of protein kinase C, de novo synthesis of BDNF, TrkB and ERK MAP kinase signaling. Although 5-HT_{2A}R was not significantly altered in our study, loss of 5-HT probably failed to initiate BDNF-dependent signalling cascades, shown as downregulated BDNF and TrkB expression. Furthermore, absence of phosphorylation of ERK in IH3 group was the best evidence to explain this, for net phosphorylation state of proteins, may 'tip the scales' towards synaptic strengthening (facilitation) or weakening (depression) [36]. Nevertheless, the precise neuronal mechanism underlying pathophysiological changes elicited by CIH may involve mutual "cross-talk inhibition or augment, and be poorly understood.

We cannot ignore the serotonin-dependent phrenic or hypoglossal LTF after episodic hypoxia [37]. LTF in OSA is postulated as a potential pathophysiological factor, but also a beneficial factor for maintaining respiratory homeostasis during sleep. Regarding the controversial 5-HT levels, one plausible explanation is the diverse hypoxia paradigms: severe protocols of CIH elicit pathologies such as hippocampal apoptosis with learning deficits, while less severe protocols may elicit beneficial (compensatory) plasticity without morbidity. In the present study, it is clear to identify the peripheral marked c-Fos of the nucleus of the solitary tract following hypoxia, indicating that our CIH protocol was efficient to activate carotid chemoreceptors and further affect neurons. Although cholinergic neurons do not always show robust c-Fos staining, c-Fos as a marker of neuronal activity is a simple surrogate measure of spontaneous changing levels. As expected, our results were consistent with the collective studies responding to hypoxia: slightly higher c-fos expression [38].

Contrary to previous expectation, our data appear to be a counterintuitive result in the face of evidence pointing to adaptive compensatory changes of OSA. It has been reported that CIH caused hyperexcitability of XII motoneuron mediated by facilitated NE and 5-HT afferents [39], representing a tonic hyperactivity of upper airway muscles mechanism that stabilizes the anatomical vulnerability of OSA patients. Collective data suggested that endogenous noradrenergic excitatory drive to XII motoneurons was enhanced during CIH. For example, a neuroanatomical study showed the augmented noradrenergic and serotonergic terminals innervation in XII nucleus in rats subjected to CIH [39, 40]. Companied with the increased density of α1-adrenoceptor, XII nerve activity was profoundly reduced by microinjections of α1-adrenergic receptor antagonist into the XII nucleus in CIH-exposed rats [40]. A similar finding with an increased density of noradrenergic terminals in hypoglossal motor nuclei was determined following CIH exposure for 35 days [41]. However, these results seemed to contradict with another line of evidence that lower percentages of central catecholaminergic neurons in the medulla expressed c-Fos in rats subjected to CIH, insufficient to prove an elevated excitatory drive [42]. Indeed, CIH had relatively lesser effect on serotonergic innervation of the XII nucleus than its NE inputs [43]. It is possible that CIH-induced sprouting of noradrenergic innervation may have an enhanced effect on XII motoneurons, despite a decreased endogenous 5-HT input. The ultimate functional consequences depend on all the inputs to XII motoneurons and output factors.

In conclusion, this is the first report of real time monitoring the dynamic release of 5-HT in vivo following normoxia and CIH. As such, our data reflect complex changes in functional and neuroanatomical levels during the different periods of exposure to CIH. From a clinical perspective, understanding of the neural mechanism following 5-HT dynamic change has important implication in efficient pharmacological therapy of OSA. The study provides important information for 5-HT and BDNF-dependent molecular signaling. Except for oxidative propagation, hypoxia itself is a complex process that could result in ion channel alterations, glutamate release and so on. For the reason, further exploration for hypoxia-elicited neurotransmitter alterations should be extended to more precise mechanisms.

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Disclosure of conflict of interest

None.

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References

[1] Remmers JE, deGroot WJ, Sauerland EK and Anch AM. Pathogenesis of upper airway occlusion during sleep. J Appl Physiol Respir Environ Exerc Physiol 1978; 44: 931-938.

[2] Kubin L, Tojima H, Davies RO and Pack AI. Serotonergic excitatory drive to hypoglossal motoneurons in the decerebrate cat. Neurosci Lett 1992; 139: 243-248.

[3] Manaker S and Tischler LJ. Origin of serotoninergic afferents to the hypoglossal nucleus in the rat. J Comp Neurol 1993; 334: 466-476.

[4] Zhan G, Shaheen F, Mackiewicz M, Fenik P and Veasey SC. Single cell laser dissection with molecular beacon polymerase chain reaction identifies 2A as the predominant serotonin receptor subtype in hypoglossal motoneurons. Neuroscience 2002; 113: 145-154.

[5] Smith IE and Quinnell TG. Pharmacotherapies for obstructive sleep apnoea: where are we now? Drugs 2004; 64: 1385-1399.

[6] Veasey SC. Serotonin agonists and antagonists in obstructive sleep apnea: therapeutic potential. Am J Respir Med 2003; 2: 21-29.

[7] Hanzel DA, Proia NG and Hudgel DW. Response of obstructive sleep apnea to fluoxetine and protriptyline. Chest 1991; 100: 416-421.

[8] Pena F and Ramirez JM. Hypoxia-induced changes in neuronal network properties. Mol Neurobiol 2005; 32: 251-283.

[9] Krnjevic K. Early effects of hypoxia on brain cell function. Croat Med J 1999; 40: 375-380.

[10] Suratt PM, McTier RF and Wilhoit SC. Upper airway muscle activation is augmented in patients with obstructive sleep apnea compared with that in normal subjects. Am Rev Respir Dis 1988; 137: 889-894.

[11] Bach KB and Mitchell GS. Hypoxia-induced long-term facilitation of respiratory activity is serotonin dependent. Respir Physiol 1996; 104: 251-260.

[12] Ling L, Fuller DD, Bach KB, Kinkead R, Olson EB Jr and Mitchell GS. Chronic intermittent hypoxia elicits serotonin-dependent plasticity in the central neural control of breathing. J Neurosci 2001; 21: 5381-5388.

[13] Veasey SC, Panckeri KA, Hoffman EA, Pack AI and Hendricks JC. The effects of serotonin antagonists in an animal model of sleep-disordered breathing. Am J Respir Crit Care Med 1996; 153: 776-786.

[14] Baker-Herman TL, Fuller DD, Bavis RW, Zabka AG, Golder FJ, Doperalski NJ, Johnson RA, Watters JJ and Mitchell GS. BDNF is necessary and sufficient for spinal respiratory plasticity following intermittent hypoxia. Nat Neurosci 2004; 7: 48-55.

[15] Schinder AF and Poo M. The neurotrophin hypothesis for synaptic plasticity. Trends Neurosci 2000; 23: 639-645.

[16] Skaper SD. The biology of neurotrophins, signalling pathways, and functional peptide mimetics of neurotrophins and their receptors. CNS Neurol Disord Drug Targets 2008; 7: 46-62.

[17] Moreau JM and Ciriello J. Chronic intermittent hypoxia induces changes in expression of synaptic proteins in the nucleus of the solitary tract. Brain Res 2015; 1622: 300-307.

[18] Wu X, Gu W, Lu H, Liu C, Yu B, Xu H, Tang Y, Li S, Zhou J and Shao C. Soluble receptor for advanced glycation end product ameliorates chronic intermittent hypoxia induced renal injury, inflammation, and apoptosis via P38/JNK signaling pathways. Oxid Med Cell Longev 2016; 2016: 1015390.

[19] Zhou L, Wang ZY, Lian H, Song HY, Zhang YM, Zhang XL, Fan RF, Zheng LF and Zhu JX. Altered expression of dopamine receptors in cholinergic motoneurons of the hypoglossal nucleus in a 6-OHDA-induced Parkinson's disease rat model. Biochem Biophys Res Commun 2014; 452: 560-566.

[20] Liu Q and Wong-Riley MT. Postnatal changes in the expressions of serotonin 1A, 1B, and 2A receptors in ten brain stem nuclei of the rat: implication for a sensitive period. Neuroscience 2010; 165: 61-78.

[21] Erickson JT and Millhorn DE. Hypoxia and electrical stimulation of the carotid sinus nerve induce Fos-like immunoreactivity within catecholaminergic and serotoninergic neurons of the rat brainstem. J Comp Neurol 1994; 348: 161-182.

[22] Richter DW, Schmidt-Garcon P, Pierrefiche O, Bischoff AM and Lalley PM. Neurotransmitters and neuromodulators controlling the hypoxic respiratory response in anaesthetized cats. J Physiol 1999; 514: 567-578.

[23] Wong-Riley MT. Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. Trends Neurosci 1989; 12: 94-101.

[24] Shaw PJ and Eggett CJ. Molecular factors underlying selective vulnerability of motor neurons to neurodegeneration in amyotrophic lateral sclerosis. J Neurol 2000; 247 Suppl 1: I17-27.

[25] Chou SM, Wang HS, Taniguchi A and Bucala R. Advanced glycation endproducts in neurofilament conglomeration of motoneurons in familial and sporadic amyotrophic lateral sclerosis. Mol Med 1998; 4: 324-332.
[26] Xu W, Chi L, Row BW, Xu R, Ke Y, Xu B, Luo C, Kheirandish L, Gozal D and Liu R. Increased oxidative stress is associated with chronic intermittent hypoxia-mediated brain cortical neuronal cell apoptosis in a mouse model of sleep apnea. Neuroscience 2004; 126: 313-323.
[27] Ozawa Y and Okado N. Alteration of serotonergic receptors in the brain stems of human patients with respiratory disorders. Neuropediatrics 2002; 33: 142-149.
[28] Cummings KJ, Hewitt JC, Li A, Daubenspeck JA and Nattie EE. Postnatal loss of brainstem serotonin neurones compromises the ability of neonatal rats to survive episodic severe hypoxia. J Physiol 2011; 589: 5247-5256.
[29] Duncan JR, Paterson DS, Hoffman JM, Mokler DJ, Borenstein NS, Belliveau RA, Krous HF, Haas EA, Stanley C, Nattie EE, Trachtenberg FL and Kinney HC. Brainstem serotonergic deficiency in sudden infant death syndrome. JAMA 2010; 303: 430-437.
[30] Vaidya VA, Terwilliger RM and Duman RS. Role of 5-HT2A receptors in the stress-induced down-regulation of brain-derived neurotrophic factor expression in rat hippocampus. Neurosci Lett 1999; 262: 1-4.
[31] Veasey SC, Zhan G, Fenik P and Pratico D. Long-term intermittent hypoxia: reduced excitatory hypoglossal nerve output. Am J Respir Crit Care Med 2004; 170: 665-672.
[32] Erickson JT, Conover JC, Borday V, Champagnat J, Barbacid M, Yancopoulos G and Katz DM. Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice lacking NT4 and display a severe developmental deficit in control of breathing. J Neurosci 1996; 16: 5361-5371.
[33] Tang S, Machaalani R and Waters KA. Brain-derived neurotrophic factor (BDNF) and TrkB in the piglet brainstem after post-natal nicotine and intermittent hypercapnic hypoxia. Brain Res 2008; 1232: 195-205.
[34] Mattson MP, Maudsley S and Martin B. BDNF and 5-HT: a dynamic duo in age-related neuronal plasticity and neurodegenerative disorders. Trends Neurosci 2004; 27: 589-594.
[35] Gonzalez-Rothi EJ, Lee KZ, Dale EA, Reier PJ, Mitchell GS and Fuller DD. Intermittent hypoxia and neurorehabilitation. J Appl Physiol (1985) 2015; 119: 1455-1465.
[36] Sweatt JD. Mitogen-activated protein kinases in synaptic plasticity and memory. Curr Opin Neurobiol 2004; 14: 311-317.
[37] Mitchell GS and Johnson SM. Neuroplasticity in respiratory motor control. J Appl Physiol (1985) 2003; 94: 358-374.
[38] Lavezzi AM, Ottaviani G and Matturri L. Identification of neurons responding to hypoxia in sudden infant death syndrome. Pathol Int 2003; 53: 769-774.
[39] Rukhadze I, Fenik VB, Benincasa KE, Price A and Kubin L. Chronic intermittent hypoxia alters density of aminergic terminals and receptors in the hypoglossal motor nucleus. Am J Respir Crit Care Med 2010; 182: 1321-1329.
[40] Stettner GM, Fenik VB and Kubin L. Effect of chronic intermittent hypoxia on noradrenergic activation of hypoglossal motoneurons. J Appl Physiol (1985) 2012; 112: 305-312.
[41] Mody P, Rukhadze I and Kubin L. Rats subjected to chronic-intermittent hypoxia have increased density of noradrenergic terminals in the trigeminal sensory and motor nuclei. Neurosci Lett 2011; 505: 176-179.
[42] Herr KB, Stettner GM and Kubin L. Reduced c-Fos expression in medullary catecholaminergic neurons in rats 20 h after exposure to chronic intermittent hypoxia. Am J Physiol Regul Integr Comp Physiol 2013; 304: R514-522.
[43] Chan E, Steenland HW, Liu H and Horner RL. Endogenous excitatory drive modulating respiratory muscle activity across sleep-wake states. Am J Respir Crit Care Med 2006; 174: 1264-1273.