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# Role of 5-hydroxytryptamine expression in cerebellar Purkinje cells in obstructive sleep apnea syndrome☆

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## Abstract

In the present study, electrical stimulation to the rat insular cortex induced apnea or respiratory disturbance, reduced amplitude of genioglossal electromyogram, and decreased electromyogram integrals. In addition, arterial blood gas analysis showed arterial blood acidosis, reduced pH values, increased alkali reserve negative values, decreased peripheral blood 5-hydroxytryptamine content, and increased 5-hydroxytryptamine expression in cerebellar Purkinje cells. Following lidocaine injection to block the habenular nucleus, abnormalities in breath, genioglossal electromyogram, and blood gas values disappeared, and peripheral blood 5-hydroxytryptamine content returned to levels prior to electric stimulation. However, 5-hydroxytryptamine expression in cerebellar Purkinje cells remained high. The results suggested that 5-hydroxytryptamine expression in Purkinje cells did not correlate with ventilation function involving insular cortex and habenular nucleus.

**Key Words:** sleep apnea; obstructive; cerebellum; 5-hydroxytryptamine; insular cortex; habenular nucleus; Purkinje cells

**Abbreviations:** OSA, obstructive sleep apnea; 5-HT, 5-hydroxytryptamine; EMG, electromyogram

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## INTRODUCTION

The pathogenesis of obstructive sleep apnea (OSA) remains poorly understood[1], but increasing evidence demonstrates that some nucleus groups in the central nervous system contribute to OSA[2-3]. Imaging and experimental studies have shown that the insular cortex and habenular nucleus are linked to OSA[4-6]. In addition, OSA patients exhibit abnormal signals in the cerebellum, and hypertension in the presence of OSA can induce cerebellar fastigial nucleus excitation, which influences respiratory movement[7]. A previous study showed that intermittent hypoxia results in cerebellar Purkinje cell and fastigial nucleus neuronal injury in mice, suggesting that cerebellar functions highly correlate with normal and abnormal respiratory movement[8].

The neurotransmitter 5-hydroxytryptamine (5-HT) is involved in OSA[9]. The present study hypothesized that cerebellar 5-HT levels are altered following OSA. 5-HT is released by raphe nuclei neurons and is continuously inhibited by the habenular nucleus[10]. The habenular nucleus is a pathway involved in insular cortical signal transduction. Previous studies have shown that stimulations to the rat insular cortex and habenular nucleus reduce peripheral blood 5-hydroxytryptamine content and genioglossal electromyogram (EMG) values, as well as induce apnea. However, these changes disappear after following inhibition of the habenular nucleus[5-11]. Clinical results have shown that 5-HT-intake inhibitor can effectively treat OSA[12].

To determine the correlation between cerebellar functions and OSA, the present study stimulated the insular cortex prior to and following habenular nucleus blocking to observe 5-HT changes in cerebellar Purkinje cells when respiratory movement was altered.

## RESULTS

### Quantitative analysis of experimental animals

A total of 280 Wistar rats were initially enrolled in the study. Following elimination of rats with infection, bleeding, death, or inaccurate localization of insular cortex and habenular nucleus, 105 were selected and randomly assigned to electric stimulation, electric stimulation + lidocaine, and control groups. A

total of 105 rats were included in the final analysis.

**Effects of insular cortex electrical stimulation and habenular nucleus blocking on animal respiratory movement**

Rat respiratory movement curves were recorded. Following long trains of electric pulse stimulation to the insular cortex, the breathing became shallow and short, followed by 2.5-second apnea. Following deep inspiration, respiratory rhythm was restored, but the breath remained deep and slow. After 10 seconds, respiratory rhythm was normal, but breathing remained shallow. After the habenular nucleus was blocked by lidocaine, breath frequency and depth remained unchanged following electrical stimulation to the insular cortex.

**Effects of insular cortex electrical stimulation and habenular nucleus blocking on genioglossal EMG**

EMG recording showed that following long trains of electric pulse stimulation to the insular cortex, rats immediately exhibited lengthening of the inspiratory phase and significant hypopnea during the deep-inspiration phase, followed by apnea (range 2–10 seconds). In addition, genioglossus cell discharge was significantly inhibited, which manifested as significantly reduced EMG amplitude, nearly baseline. The EMG amplitude decreased even when respiratory-related rhythmic discharge was absent, along with EMG integrals for a period of time following apnea termination. After the habenular nucleus was blocked by lidocaine, the EMG changes disappeared and respiratory movement remained unchanged following electrical stimulation to the insular cortex.

**Effects of insular cortex electrical stimulation and habenular nucleus blocking on arterial blood gas content and pH values**

Blood gas analysis showed that following electric stimulation to the insular cortex, arterial pH values decreased ($P < 0.05$), but negative base excess significantly increased ($P < 0.01$), and blood $K^+$, $Na^+$ and $Cl^-$ concentrations remained unchanged. However, after the habenular nucleus was blocked by lidocaine, those changes were not observed following electric stimulation to the insular cortex (data not shown). Blood gas analysis results were consistent with respiratory movement curves and genioglossal EMG changes.

**Effects of insular cortex electrical stimulation and habenular nucleus blocking on peripheral blood 5-HT**

Following electrical stimulation to the insular cortex, rats developed apnea and peripheral blood 5-HT levels decreased (61.919 ± 63.723 ng/mL *vs*. 288.433 ± 18.105 ng/mL, $P < 0.05$), but 5-HT levels returned to normal after the habenular nucleus was blocked (288.890 ± 37.153 ng/mL). These peripheral blood 5-HT level changes were consistent with respiratory movement curves and genioglossal EMG changes.

**Effects of insular cortex electrical stimulation and habenular nucleus blocking on cerebellar 5-HT expression**

Immunohistochemistry results showed that 5-HT expression changes were not consistent with peripheral blood 5-HT levels or respiratory movement curves and genioglossal EMG changes in response to electrical stimulation before and after habenular nucleus blocking. 5-HT was not expressed in Purkinje cells in the normal cerebellar cortex, but 5-HT expression was detected in the cytoplasm of Purkinje cells following electrical stimulation before and after habenular nucleus blocking (Figure 1).

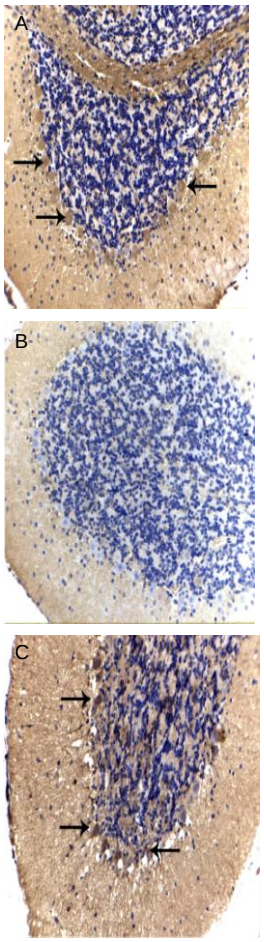


Figure 1 5-hydroxytryptamine (5-HT) expression in cerebellar Purkinje cells (immunohistochemistry, × 200).

Control group (B) does not express 5-HT in cerebellar Purkinje cells, but cerebellar Purkinje cells express 5-HT following stimulation to the insular cortex before (A) and after (C) habenular nucleus blocking with lidocaine. Arrows indicated 5-HT positive cells.

## DISCUSSION

The present study induced apnea *via* electrical stimulation to insular cortex to establish a rat model of OSA[6]. Localization of the insular cortex and habenular nucleus was difficult, and inaccurate localization led to failure in obtaining some experimental results.

The genioglossus is dominated by the hypoglossal nerve, and genioglossus contraction plays an important role in

maintaining upper airway opening during sleeping[13]. 5-HT is the most important neurotransmitter for hypoglossal nucleus[9].

The cerebellum is associated with normal and abnormal respiratory movement, and the cerebellar fastigial nucleus is very sensitive to hypoxia[8]. Therefore, OSA results in cerebellar morphological and functional changes, in addition to structural changes in the insular cortex[3, 14].

Anatomic tracing experiments have shown that the cerebellum receives information from the medulla oblongata or senior motor cortex and participates in dyspnea-induced emotional responses. Abnormal reactions have been detected in the insular cortex and cerebellum following OSA[3].

Purkinje cells do not express 5-HT in a normal cerebellum. However, chronic intermittent hypoxia in OSA patients results in oxidative stress reactions in the cortex, hippocampus, cerebellum, and pons[2]. In the present study, 5-HT was expressed in Purkinje cells following stimulation to the insular cortex before and after habenular nucleus blocking. The effects and role of 5-HT in OSA remain poorly understood. However, electrical stimulation to the insular cortex induced changes in respiratory movement and cerebellar function, because the cerebellum is sensitive to hypoxia and increased $CO_2$, and also receives information from pons and medulla oblongata neurons. Therefore, interaction between the insular cortex and cerebellum could exist. Following hypoxia or acidosis-induced environmental changes *in vivo*, cerebellar 5-HT expression increased, but was not influenced by apnea or blood gas levels. Future studies are needed, however, to determine the role of 5-HT in Purkinje cells.

In addition, hypoxia or acidosis-induced environmental changes *in vivo* result in functional injuries in multiple organs and systems of OSA patients, including abnormalities in the central nervous system[15]. Intravenous 5-HT injection is associated with the cardiovascular system, abnormal blood pressure, and apnea. In addition, neuroimaging has revealed that cerebellar signal changes takes place in cerebellar Purkinje cells following electrophysiological stimulation[9, 16]. Blood pressure abnormalities and neuronal changes in the cerebellar fastigial nucleus suggest that the cerebellum is associated with occurrence and progression of cardiovascular complications in OSA patients, as well as hypotension and bradycardia correction.

In the present study, peripheral blood 5-HT expression significantly decreased following electrical stimulation to the insular cortex, which was accompanied by apnea. 5-HT expression in the blood returned to levels prior to stimulation, and apnea disappeared following electrical stimulation when the habenular nucleus was blocked. These results suggested that 5-HT blood changes were consistent changes in the central nervous system in response to apnea and low blood-oxygen levels. However, these results did not explain why 5-HT expression in Purkinje cells increased after the habenular nucleus was blocked. 5-HT expression increased in Purkinje cells in response to electrical stimulation to the insular cortex, which did not correlate with habenular nucleus activities, and 5-HT expression remained unchanged following habenular nucleus blockage.

Because the present study was an acute animal experiment, rat breath and blood gas values were not dynamically observed. Moreover, the influence of cerebellar 5-HT on emotion, as well as the cerebrovascular system after a long period of hypoxia and acidosis, requires further study to determine the relationship between cerebellar 5-HT changes and OSA.

In conclusion, electrical stimulation to the insular cortex increased 5-HT expression in Purkinje cells, with or without habenular nucleus blockage. However, the correlation between cerebellar 5-HT changes and OSA remains unclear.

## MATERIALS AND METHODS

### Design

A randomized, controlled, animal experiment.

### Time and setting

The experiment was performed at the Key Laboratory of Pathophysiology, the Ministry of Education, Norman Bethune College of Medicine, Jilin University and the Key Laboratory of Physiology, Jilin University, China, from January to December 2006.

### Materials

A total of 280 healthy, adult, Wistar rats, weighing 200–280 g and of either gender, were provided by the Laboratory Animal Department of Jilin University, China (No. SYXK (Ji) 2003-0001). The animals were housed in standard cages with a 12-hour day/night cycle at 22 ± 2°C and were fed food and water twice daily. The experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[17].

### Methods

#### *Preparation prior to electrical stimulation*

The rats were anesthetized by intraperitoneal injection of 20% urethane (6 mL/kg), but the rats remained sensitive to corneal reflex and leg flexion reflex in response to hind limb clipping, and did not exhibit spontaneous movement. During experimentation, the anesthetic was supplemented, if necessary, with 1/5 of the original dose. Rectal temperature was maintained at 36°C, and the experiments were performed under quiet conditions with natural lighting.

#### *Electrical stimulation to the insular cortex*

The rats were placed on a VDT-1 cerebral stereotactic apparatus (Takahashi, Kanagawa, Japan), the scalp was cut open, and the temporal bone was exposed. A concentric circle electrode was inserted into the insular

cortex at the following coordinates: AP: +1.0, L: 5.4–5.5, H: 4.2–4.4[18]. The rats were stimulated by a stimulating electrode with a concentric circle embedded in the insular cortex (external diameter 2.0 mm, 2–5 MΩ) at 0.4 mA, 100 Hz, and 1-ms wave width for 9 seconds[19]. The control group underwent electrical stimulation with identical parameters at four points (up, down, left, and right) that were 1 mm around the insular cortex and habenular nucleus.

***Habenular nucleus blockage with lidocaine***

The coordinates of habenular nucleus were AP: –3.4, L: 0.6 and H: 5.3. The habenular nucleus was blocked by an injection of 0.3 μL lidocaine (2% lidocaine hydrochloride; Seventh Wuxi Pharmaceutical, Wuxi, China) using a microsyringe[10] over a 3-minute time period, followed by electrical stimulation to the insular cortex.

***Recordings of respiratory movement and genioglossal EMG***

The abdominal site displaying maximal amplitude while breathing was connected to a tension-amplifying device, and signals were output to an electrophysiograph (Chengdu Instrument Factory, Sichuan, China) to record respiratory movement curves. For genioglossal EMG recordings, the recording electrode was a Teflon-insulated copper wire (self-made epoxy resin electrode) with 1.0 mm of the electrode tip exposed. When inserted into the muscle, the soft Teflon copper wire was encased in a 27-gauge pinhead tube (clinical syringe, Shanghai Injection Needles, Shanghai, China), which withdrew when inserted. The reference electrode was placed at the left helix. The BL-420E + utilized low-frequency filtering of 60 Hz, with a time constant of 0.3 second.

***Blood gas analysis***

Arterial blood was harvested from the femoral artery when the rats exhibited apnea in response to electrical stimulation to the insular cortex, and apnea disappeared following habenular nucleus blockage. Blood gas analysis was performed using AVL-OPan TI blood gas analyzer (AVL Scientific Corporation, Roswell, NM, USA)[6].

***Determination of peripheral blood 5-HT content***

Venous blood, 1 mL, was collected from the angular vein when the rats exhibited apnea in response to electrical stimulation to the insular cortex. The samples were placed in tubes, 26 μL ethylenediamine tetraacetic acid disodium salt was added (7.5%; Sigma, St. Louis, MO, USA), and the samples were centrifuged at 3 000 r/min for 10 minutes. The supernatant was harvested and stored. The frozen venous blood samples were thawed and 5-HT plasma content was determined using a radioimmunity kit (Serotonin RIA kit; Labor Diagnostika Nord, Nordhorn, Germany) using a HTEC-500 spectrofluorometer (Hitachi, Tokyo, Japan)[20].

***5-HT immunohistochemistry in cerebellar neurons***

Following apnea, the rats were sacrificed by intravenous injection of pentobarbital (100 mg/kg), followed by cardiac perfusion of phosphate buffered saline (PBS) containing 4% paraformaldehyde. The brain and genioglossus tissues were harvested and fixed in PBS solution containing 4% paraformaldehyde for 48 hours. Sagittal paraffin sections of the cerebellum were prepared, dewaxed, washed, incubated in 0.1 M citrate buffer (pH 7.4) at 98°C for 5 minutes for antigen retrieval, incubated in blocking agent (agent A; general type SP kit; Fuzhou Maxim, Fuzhou, China) at room temperature for 15 minutes to block endogenous peroxidase, washed three times with PBS for five minutes each, incubated in rabbit anti-rat 5-HT polyclonal antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight, washed three times in PBS for five minutes each, incubated with biotin-labeled goat anti-rat/rabbit IgG (reagent C) at room temperature for 15 minutes, washed three times with PBS for five minutes each, followed by biotin-labeled streptavidin-peroxidase (reagent D) at room temperature for 15 minutes, and three washes with PBS for five minutes each. The stainings were then visualized with diaminobenzidine and observed with a light microscope (Olympus, Tokyo, Japan), counterstained with hematoxylin, dehydrated, cleared and mounted. The negative control was incubated with PBS instead of primary antibodies. Images were acquired using a Cooled CCD system (Nikon, Tokyo, Japan). Brown yellow particles were regarded as 5-HT-positive expression, and blue staining represented negative expression.

***Histology of insular cortex and habenular nucleus positions***

Pontamine sky blue solution (0.3 μL; Sigma) was injected into the insular cortex and habenular nucleus following experimentation, and the microinjection location was labeled. The animals were then sacrificed and the brain was harvested and fixed in 10% formaldehyde for 1 week, followed by coronal sectioning (30-μm thick sections). The insular cortex and habenular nucleus position was identified according to rat brain stereotaxic coordinates under a microscope[21].

***Statistical analysis***

Data were expressed as mean ± SD, which represented normal distribution. The data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA) with one-way analysis of variance. Intergroup paired differences were compared using the Student-Newman-Keuls-*q* test. $P < 0.05$ was considered statistically significant.

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**Author contributions:** Liping Peng and Min Wang had full access to all data and participated in data analysis. Li Cui, Lihong Zhang, Pan Liu, Jinghua Wang, Shengnan Liu, and Pingping He conducted the animal experiments. Min Huang participated in experiment guidance, data analysis, and interpretation. Shao Wang and Mingxian Li designed the study and contributed to study supervision. Mingxian Li wrote the manuscript.

**Conflict of interest:** None declared.

**Ethical approval:** The project received full ethical approval from the Animal Ethical Committee of the First Affiliated Hospital of Jilin University, China.

## REFERENCES


[1] Buchner NJ, Quack I, Stegbauer J, et al. Treatment of obstructive sleep apnea reduces arterial stiffness. Sleep Breath: In press.

[2] Ramanathan L, Gozal D, Siegel JM. Antioxidant responses to chronic hypoxia in the rat cerebellum and pons. J Neurochem. 2005;93(1):47-52.

[3] Macey PM, Kumar R, Woo MA, et al. Brain structural changes in obstructive sleep apnea. Sleep. 2008;31(7):967-977.

[4] Herigstad M, Hayen A, Wiech K, et al. Dyspnoea and the brain. Respir Med. 2011;105(6):809-817.

[5] Ptak K, Yamanishi T, Aungst J, et al. Raphé neurons stimulate respiratory circuit activity by multiple mechanisms via endogenously released serotonin and substance P. J Neurosci. 2009;29(12):3720-3737.

[6] Sartorius A, Kiening KL, Kirsch P, et al. Remission of major depression under deep brain stimulation of the lateral habenula in a therapy-refractory patient. Biol Psychiatry. 2010;67(2):e9-11.

[7] Rector DM, Richard CA, Harper RM. Cerebellar fastigial nuclei activity during blood pressure challenges. J Appl Physiol. 2006; 101(2):549-555.

[8] Pae EK, Chien P, Harper RM. Intermittent hypoxia damages cerebellar cortex and deep nuclei. Neurosci Lett. 2005;375(2): 123-128.

[9] Barker JR, Thomas CF, Behan M. Serotonergic projections from the caudal raphe nuclei to the hypoglossal nucleus in male and female rats. Respir Physiol Neurobiol. 2009;165(2-3):175-184.

[10] Wang S, Jiang Y, Xiao JS, et al. Spontaneous discharges of habenular nucleus and its inhibitory action on nucleus raphe magnus. Kexue Tongbao. 1980;25:83-88.

[11] Yu L, Huang M, Xu HY, et al. Effects of 5-hydroxytryptamine ascending pathways of dorsal raphe nuclei and habenular nucleus on the respiration and blood pressure of rats. Chin Med J (Engl). 2007;120(24):2308-2310.

[12] Prasad B, Radulovacki M, Olopade C, et al. Prospective trial of efficacy and safety of ondansetron and fluoxetine in patients with obstructive sleep apnea syndrome. Sleep. 2010;33(7):982-989.

[13] Jordan AS, Wellman A, Heinzer RC, et al. Mechanisms used to restore ventilation after partial upper airway collapse during sleep in humans. Thorax. 2007;62(10):861-867.

[14] Joo EY, Tae WS, Lee MJ, et al. Reduced brain gray matter concentration in patients with obstructive sleep apnea syndrome. Sleep. 2010;33(2):235-241.

[15] Lakatosova S, Celec P, Schmidtova E, et al. The impact of serotonergic stimulation on reelin and glutamate decarboxylase gene expression in adult female rats. Bratisl Lek Listy. 2011; 112(2):58-62.

[16] Henderson LA, Yu PL, Frysinger RC, et al. Neural responses to intravenous serotonin revealed by functional magnetic resonance imaging. J Appl Physiol. 2002;92(1):331-342.

[17] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.

[18] Kou ZY, Li MS, Zhang CX, et al. Habenula participates the vasopressor response by stimulation of the insular cortex, central-, lateral amygdaloid nucleus respectively. Zhongguo Yingyong Shenglixue Zazhi. 2003;19(4):334-336.

[19] Wang S, Fu Q, Fu J. Modulation of preoptic area on discharges of parafascicular pain neurons. Kexue Tongbao. 1983;28(3): 401-405.

[20] Cui L, Wang JH, Wang M, et al. Injection of L-glutamate into the insular cortex produces sleep apnea and serotonin reduction in rats. Sleep Breath: In press.

[21] Paxions G, Watson C. The Rat Brain in Stereotaxic Coordinates. 2nd ed. San Diego: Academic Press. 1986.


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