Influence of chronic intermittent hypoxia on growth associated protein 43 expression in the hippocampus of young rats

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Abstract
This study aimed to explore the pathological change to hippocampal neurons and the expression of growth associated protein 43 in 21-day-old young rats following chronic intermittent hypoxia. Hematoxylin-eosin staining results showed varying degrees of degeneration and necrosis in hippocampal neurons depending on the modeling time. Immunohistochemistry revealed that growth associated protein 43 expression in young rats following chronic intermittent hypoxia decreased, but that levels were still higher than those of normal rats at each time point, especially 4 weeks after modeling. During 1-5 weeks after modeling, a slow growth in rat weight was observed. Experimental findings indicate that chronic intermittent hypoxia may induce growth dysfunction and necrosis of hippocampal neurons, as well as increase the expression of growth associated protein 43 in young rats.

Key Words
chronic intermittent hypoxia; brain injury; growth associated protein 43; obstructive sleep apnea hypopnea syndrome; hippocampus; young rats; neural regeneration

INTRODUCTION
Obstructive sleep apnea hypopnea syndrome (OSAHS) has a great impact on multiple systems, especially the nervous system, and can induce cognitive dysfunction1-6. Existing studies regarding OSAHS have mainly focused on adult rat chronic intermittent hypoxia (CIH) models. However, there is a substantial difference between child and adult OSAHS7-10, with child OSAHS classified as an independent clinical syndrome11. Currently, studies on children with OSAHS are few, and many questions remain unanswered. Growth associated protein 43 (GAP-43) mainly exists in differentiated neurons that present with axon outgrowth. GAP-43 content is abundant in the neuronal cytoplasm at the early development stage of the nervous system; the expression of GAP-43 may gradually decrease as the brain becomes mature and sharply increases when the brain is injured12-15. Therefore, GAP-43 is an important marker for studying neural regeneration and repair16-17. At present, various models of anoxic/ischemic brain damage have been produced in adult or neonatal rats to observe changes in GAP-43 expression18-21. However, it is unknown if changes in GAP-43 expression occur after anoxic brain injury in young rats.
The present study aimed to establish a CIH model in 21-day-old young rats to simulate typical pathophysiological characteristics of OSAHS. Body weight gain of CIH young rats at each week was measured, and neuronal pathology in the hippocampus was observed using hematoxylin-eosin staining. In addition, hippocampal GAP-43 expression was determined by immunohistochemistry. We aim to identify (1) the effect of CIH on body weight growth of young rats; (2) GAP-43 expression changes in hippocampal neurons of CIH young rats at the growth and development period; (3) whether expression changes are consistent with that in adult rats.

RESULTS

Quantitative analysis of experimental animals
A total of 60 21-day-old Sprague-Dawley male rats were randomly divided into a control group (normal feeding) and a CIH group (CIH modeling). Ten rats in each group were selected for experiments at 2, 4, and 6 weeks after modeling. In total, 60 rats were involved in the final result analysis.

Body weight gain of young rats
Increased body weight of CIH young rats at each week was significantly decreased compared with the control group during 1–5 weeks after modeling (P < 0.05 or P < 0.01), and there was no significant difference in body mass gain between CIH young rats and control rats at 6 weeks (P > 0.05; Figure 1).

Pathological changes of hippocampal neurons in CIH rats
Hematoxylin-eosin staining and light microscopic observation results showed no degeneration and necrosis of hippocampal neurons in the control group. At 2 and 4 weeks after modeling, hippocampal neurons in the control group were smaller in size, increased in number, had an ordered arrangement, and had unclear intracellular constituents. At 6 weeks, hippocampal neurons in the control group increased in volume, reduced in number, and cell nuclei were visible.

The hippocampal neurons in the CIH group were different at each time point. At 2 weeks after modeling, no apparent degeneration or necrosis of hippocampal neurons was found, and cells had a slightly disordered arrangement. At 4 and 6 weeks, hippocampal neurons showed varying degrees of degeneration and necrosis, which was accompanied by cell swelling, cytoplasmic vacuoles, nuclear shrinkage, and disappearance of intracellular constituents (Figure 2, supplementary Figure 1 online).

Figure 1  Comparison of increased body weight per week in each group for 6 weeks. Data are expressed as mean ± SD of ten rats in each group per week. aP < 0.05, bP < 0.01, vs. control group (independent-samples t-test). Body weight gain = the measured body weight on the tested week – the measured body weight on the previous week.

Figure 2  Histological changes in the hippocampal dentate gyrus in the control group and chronic intermittent hypoxia (CIH) group (hematoxylin-eosin staining, light microscope, × 400).

There was no degeneration or necrosis of neurons in the control group at 2 (A), 4 (B), and 6 weeks (C), and the CIH group at 2 weeks (D). At 4 (E) and 6 weeks (F) in the CIH group, necrotic neurons (arrows) were visible.

Compared with surrounding normal cells, cytoplasmic vacuoles appeared and intracellular components disappeared.
GAP-43 expression in the CIH rat hippocampus

Immunohistochemical staining showed that GAP-43-positive immunoreactive products were brown punctate or granular deposits, which mainly distributed in the cytoplasm of hippocampal neurons (Figure 3). With the modeling time, GAP-43 expression (absorbance) in the CIH group and control group gradually decreased. In the control group, GAP-43 absorbance values were significantly reduced at 4 and 6 weeks compared with 2 weeks ($P < 0.01$), and no significant difference was observed between 4 weeks and 6 weeks ($P > 0.05$). In the CIH group, the absorbance values were similar at 2 and 4 weeks ($P > 0.05$), while they were significantly reduced at 6 weeks ($P < 0.01$). The CIH group showed significantly higher GAP-43 absorbance values than the control group at the same time point ($P < 0.01$; Table 1).

Counting results revealed that the number of GAP-43-positive neurons in the hippocampal CA1 and dentate gyrus region of control rats at 4 and 6 weeks was significantly decreased compared with that at 2 weeks ($P < 0.01$). In the CIH group, there were significantly less positive neurons in the hippocampal CA1, CA3 and dentate gyrus region at 6 weeks, compared with at 2 and 4 weeks ($P < 0.01$). The number of GAP-43-positive neurons in the hippocampal CA1, CA3 and dentate gyrus of CIH young rats was significantly higher than that of control rats at each time point ($P < 0.01$), except the number in the hippocampal CA3 region at 6 weeks ($P > 0.05$; Table 2).

![Figure 3](image_url)

**Figure 3** Expression of growth associated protein 43 (GAP-43) in the hippocampal CA1 region of control rats and chronic intermittent hypoxia (CIH) rats (immunohistochemistry staining, light microscope, ×400).

In the control group (A-C) and CIH group (D-F), GAP-43 was expressed as brown/yellow color in the neuronal cytoplasm. GAP-43 expression in the CIH group was stronger than that in the control group.

At 2 (A, D), 4 (B, E), and 6 weeks (C, F) after modeling, GAP-43-positive neurons in the hippocampal CA1 region gradually decreased (arrows).

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**Table 1** Expression of growth associated protein 43 (absorbance, ×10$^{-2}$) in the rat hippocampus

| Group                                | Time after model establishment (week) |
|--------------------------------------|--------------------------------------|
|                                      | 2                                    | 4                                    | 6                                    |
| Control                              | 6.21±0.88                            | 3.83±0.83$^b$                       | 3.26±0.77$^b$                       |
| Chronic intermittent hypoxia         | 10.16±2.23$^a$                       | 9.09±2.92$^a$                       | 5.54±1.97$^{abc}$                  |

Data are expressed as mean ± SD of ten rats for each time point in each group. $^aP<0.01$, vs. control group (independent-samples t-test). $^bP<0.01$, vs. 2 weeks; $^cP<0.01$, vs. 4 weeks (one-way analysis of variance).

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**Table 2** Comparison of growth associated protein 43-positive cells (n/200 μm × 100 μm) in the rat hippocampus

| Time after model establishment (week) | CA1     | CA3     | Dentate gyrus |
|--------------------------------------|---------|---------|---------------|
|                                      | Control | CIH     | Control       | CIH     | Control | CIH     |
| 2                                    | 28.1±9.6| 57.6±10.9$^a$ | 18.7±8.7      | 36.6±14.3$^a$ | 40.2±12.0 | 93.4±17.4$^a$ |
| 4                                    | 15.8±6.3$^a$ | 60.3±11.1$^a$ | 14.5±5.7      | 35.0±10.2$^a$ | 22.9±5.3$^a$ | 85.5±13.3$^a$ |
| 6                                    | 12.9±7.1$^b$ | 30.5±9.8$^{abc}$ | 15.2±4.5      | 23.1±12.4$^{abc}$ | 19.0±3.7$^b$ | 37.3±13.3$^{abc}$ |

Data are expressed as mean ± SD of ten rats for each time point in each group. $^aP<0.01$, vs. control group (independent-samples t-test); $^bP<0.01$, vs. 2 weeks; $^cP<0.01$, vs. 4 weeks (one-way analysis of variance). CIH: Chronic intermittent hypoxia.
DISCUSSION

OSAHS is a common sleep problem among children, which can cause growth retardation, mental retardation, hypertension and impairment of learning and memory\textsuperscript{[22-23]}. OSAHS can occur in infancy and adolescence, especially in 2–5 years old children\textsuperscript{[2]}. Twenty-two days after birth is the weaning period of rats, which is equal to the childhood period of human cognitive development\textsuperscript{[24]}. Rats aged 3–12 months are generally classified as adults, and those less than 3 months of age are known as early developing rats\textsuperscript{[25]}. The present study adopted 21-day-old Sprague-Dawley rats, to observe the effect of CIH on body weight gain in early developing rats and GAP-43 expression in the hippocampus.

Our results showed an increase in body weight in both the control group and CIH group. However, growth in CIH rats was significantly slower than that in the control group, which was consistent with the studies of Xia et al.\textsuperscript{[26]}. The majority of scholars believe that the reason may be due to the fact that long-term chronic hypoxia affects growth hormone secretion in the pituitary gland, thus leading to growth retardation\textsuperscript{[27-28]}. In addition, the increased weight was not significant at 6 weeks. This could be due to enhanced hypoxia resistance, which reduces the impact on growth. However, the underlying mechanism remains unclear.

GAP-43 plays an important role in nerve fiber growth, development, axonal regeneration and synaptic function maintenance\textsuperscript{[26]}. An increasing number of studies regarding brain damage models have demonstrated that GAP-43 expression was transiently increased after injury, and then decreased to normal levels, which indicates that GAP-43 can promote the regeneration and repair of injured cerebral neurons, as well as protect brains against injury\textsuperscript{[14-15]}. GAP-43 expression in hippocampal neurons of CIH young rats was significantly increased compared with the control group at each time point, and peak expression levels were longer (for 4 weeks). This result was inconsistent with previous findings\textsuperscript{[19]}, which showed a transient increase (3–14 days) of GAP-43 expression in adult rats following brain injury. After brain injury in early developing rats, hippocampal tissue has an active self-repair capacity, suggesting that the nervous system may initiate protective mechanisms and repair damaged neurons immediately after hypoxic brain damage. One of the important repair pathways that promote synaptic growth involves an increase in GAP-43 expression\textsuperscript{[30]}. Increasing GAP-43 expression in the brain of CIH young rats may occur as a result of compensatory mechanisms for hypoxia stimulating non-damaged neurons, thus increasing the synthesis of GAP-43. Also, it is believed to be associated with the release of GAP-43 from the dead nerve cells\textsuperscript{[13]}. In this study, only a small number of hippocampal neurons appeared to degenerate and undergo necrosis, although varying degrees of pathological changes were visible in CIH young rats. This result is evidence that increased GAP-43 expression in the hippocampus of CIH young rats was not completely dependent on the release of necrotic neurons.

Regulation of GAP-43 is a very complex process that is affected by many factors, and mechanisms underlying the increase in GAP-43 in CIH rats remain unclear. In summary, CIH-induced slow body weight gain, different degrees of pathological injury of hippocampal neurons, and enhanced expression of GAP-43 in CIH young rats all support that hypothesis that CIH greatly affects the growth and development of neonatal rats, but has a certain compensatory restorative ability for CIH-induced brain injury in young rats. Further studies are required to conclusively determine the mechanism underlying hypoxic injury stimulation on GAP-43 synthesis and changes in GAP-43 expression.

MATERIALS AND METHODS

Design
A randomized controlled animal experiment.

Time and setting
Experiments were performed from July 2009 to March 2011 in the Physiology and Pathology Laboratory of Luzhou Medical College, China.

Materials
Sixty healthy, male, 21-day-old Sprague-Dawley young rats, weighing 50 ± 5 g, were provided by the Experimental Animal Center of Luzhou Medical College, China (license No. SYXK (Chuan) 2004–065). All experimental procedures adhered to the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China\textsuperscript{[31]}.

Methods
Establishment of CIH models
The CIH rat model was prepared according to the method of Yang et al.\textsuperscript{[8]}. Rats in the CIH group were placed daily in a hypoxic cabin, which was filled with soda lime and silica gel to absorb carbon dioxide and water vapor, the bulkhead was present with small gaps to provide a balance between the cabin pressure and the atmospheric pressure. During experimentation, the cabin was filled with nitrogen at 9:00 a.m. to 5:00 p.m. and discharged with mixing air, for 6 minutes each. The air transport and exhaust unit was programmed and controlled based on monitored oxygen concentrations.
within the cabin, to ensure 8% minimum oxygen concentration within the hypoxia cabin. Hypoxia gas was expelled and fresh air was added to recover oxygen concentrations to 21%. The carbon dioxide concentration in the cabin was maintained at < 3%, and the temperature was 22 ± 2°C. During hypoxia intervention, rats were allowed normal feeding and drinking. Control rats were placed in the same hypoxia cabin, which was filled with fresh air, and other treatments were performed in the same as that of the CIH group.

**Body weight determination**
The body weight of experimental rats was measured at the beginning of the experiment, and then once per week. Body weight gain = body weight measured on the tested week – body weight measured on the previous week.

**Pathological changes of hippocampal neurons detected by hematoxylin-eosin staining**
Ten rats in each group were anesthetized with ether and perfused with 4% (w/v) paraformaldehyde at 2, 4, and 6 weeks after CIH modeling. The brain was harvested and tissues were cut into three pieces at the optic chiasm posterior border and the junction between the cerebral peduncle and the pons, fixed in 4% (w/v) paraformaldehyde for more than 4 hours, followed by routine dehydration, xylene transparency and embedded in paraffin. Sections were cut at 4 μm, stained with hematoxylin-eosin and observed under light microscopy (Nikon, Tokyo, Japan).

**Hippocampal GAP-43 expression detected by immunohistochemical staining**
Hippocampal tissue was cut into slices. Specimens were conventionally dewaxed to water and incubated with 3% (v/v) H2O2 at room temperature for 10 minutes, to block endogenous peroxidase. Sections subjected to heat-induced antigen retrieval, were incubated with reagents using a two-step non-biotin enzyme-conjugated anti-rabbit IgG poly-antibody; Beijing Zhongshan Golden Bridge Biological Technology Co., Ltd, Beijing, China; 50 μL; 1:200) at 4°C overnight. Sections were washed, and incubated with reagents using a two-step non-biotin detection kit (polymer auxiliary agent and horseradish enzyme-conjugated anti-rabbit IgG poly-antibody; Beijing Zhongshan Golden Bridge Biological Technology Co., Ltd, Beijing, China; 50 μL; 1:1). Diaminobenzidine (Beijing Zhongshan Golden Bridge Biological Technology Co., Ltd) was used for coloration for 5–10 minutes. Cells stained brown/yellow in the cytoplasm were considered positive. A series of procedures were performed, including hematoxylin mild counterstain, dehydration, transparency and mounting. A negative control was included, which was treated with PBS instead of the primary antibody. Specimens were viewed by light microscopy.

**Image analysis**
Determination of hippocampal GAP-43 absorbance value: GAP-43 absorbance in hippocampal neurons was measured using the ECLIPSE88i photomicrography system (Nikon, Tokyo, Japan) and Image-Pro Plus 6.0 image analysis software (Media Cybernetics Inc., Silver Spring, MD, USA). Three high-power fields (400 ×) in the hippocampal dentate gyrus, CA1, CA2, CA3 and CA4 were randomly selected from three sections in each rat, to determine the absorbance value of positive staining. The average value was calculated, serving as the absorbance value of the rat hippocampus. At the same time, the absorbance of the optic beam on the same section was taken as the background, GAP-43 absorbance value – background absorbance = corrected absorbance, which is the actual absorbance value of GAP-43 immunoreactive products.

Counting of hippocampal GAP-43 neurons: Three non-overlapping high-power fields (400 ×) in the hippocampal dentate gyrus, CA1 and CA3 were randomly selected from three sections in each rat, to count the number of positive neurons within an area of 200 μm × 100 μm. The average value was considered as the number of GAP-43-positive cells at each time point.

**Statistical analysis**
Measurement data were expressed as mean ± SD and data were statistically analyzed with SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). The data at different time points within the same group were compared with one-way analysis of variance, and data among groups were compared using the independent-samples t-test. A $P < 0.05$ was considered a significant difference.

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**Author contributions:** Yan Chen drafted the manuscript and had full access to the study design, implementation and evaluation. Chunling Zhao was responsible for the study design, implementation and provided technical support. Chunlai Zhang designed the study and provided technical support. Lirong Luo and Guang Yu implemented the study.

**Conflicts of interest:** None declared.

**Ethical approval:** The experiment was approved by the
Experimental Animal Ethics Committee of Luzhou Medical College in China.

Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org.

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