Research Report

Neurogenesis in the adult rat brain after intermittent hypoxia

Ling-ling Zhu, Tong Zhao, Hai-sheng Li, Huiqing Zhao, Li-ying Wu, Ai-shi Ding, Wen-hong Fan, Ming Fan*

Department of Brain Protection and Plasticity, Institute of Basic Medical Science, Beijing 100850, China

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Abstract

Intermittent hypoxia has been found to prevent brain injury and to have a protective role in the CNS. To address the possible causes of this phenomenon, we made investigative effort to find out whether intermittent hypoxia affects neurogenesis in the adult rat brain by examining the newly divided cells in the subventricular zone (SVZ) and dentate gyrus (DG). The adult rats were treated with 3000 and 5000 m high altitude 4 h per day for 2 weeks consecutively. 5-Bromo-2-deoxyuridine-5-monophosphate (BrdU) immunocytochemistry demonstrated that the BrdU-labeled cells in the SVZ and DG increased after 3000 and 5000 m intermittent hypoxia. The number of BrdU-labeled cells in the SVZ returned to normal level 4 weeks following intermittent hypoxia. However, the BrdU-labeled cells in the DG had a twofold increase 4 weeks subsequent to intermittent hypoxia. From these data, we conclude that intermittent hypoxia facilitates the proliferation of neural stem cells in situ, and that the newly divided cells in the SVZ and DG react differently to hypoxia. We are convinced by these findings that the proliferation of neural stem cells in SVZ and DG may contribute to adaptive changes following intermittent hypoxia.

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1. Introduction

Progenitor cells in the SVZ of the lateral ventricle and in the dentate gyrus of the hippocampus can proliferate throughout the life of the rodent [5,15]. Proliferating cells constitute an average of 10% of the cell population in the SVZ in adult mice under physiological conditions [4]. The proliferation, migration, and differentiation of neural stem cells in response to ischemia in CNS have been under extensive investigation, yet scan attention has been given to the data on the proliferation of neural stem cells in the SVZ and the dentate gyrus populations following hypoxia.

Exposure to intermittent hypoxia has been suggested to be beneficial in biological organisms. Several studies have been shown that intermittent hypoxic treatment is capable of preventing brain injury induced by systemic administration of kainic acid in rats [1]. This observation is predicated on the suggestion that hypoxic insults trigger a host of intrinsic adaptive processes designed to promote tissue protection and regeneration [2].

The proliferation of progenitor cells in CNS has been studied by labeling dividing cells with systemic application of the thymidine analog, 5-bromodeoxyuridine [8]. Bromodeoxyuridine (BrdU) is incorporated into the DNA of dividing cells in the S phase of the mitotic cycle and is detectable immunohistochemically. This method has been widely used to study the neurogenesis in the adult brain [4,6,8,12,16,17]. Here, we have attempted to investigate whether intermittent hypoxia activates the proliferation of neural progenitors in the subventricular...
zone (SVZ) and dentate gyrus (DG) in the adult rat brain.

2. Materials and methods

2.1. Animals

Male Wistar rats (160–180 g body weight) were used in the experiments. Animals were housed individually and had free access to food and water under the condition of a 12:12-h light/dark cycle. These animals were maintained according to the guidelines stated in Guide for the Care and Use of Laboratory Animals, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council, China (1985).

2.2. Intermittent hypoxic treatment

Animals were given intermittent hypoxia as described before [1] with minor modification. Male Wistar rats, weighing 160–180 g, were randomly divided into three groups. Two groups were subjected to hypoxia environment, which is equivalent to an altitude of 3000 m or 5000 m high altitude, respectively, in a chamber for 4 h/day for 2 weeks, and the other rats were maintained in the normoxic condition. After intermittent hypoxia, the animals were placed back in their home cages and kept there until perfusion. Rats had free access to food throughout the experiments. The number of rats in each group was 4 to 6.

2.3. BrdU administration

All rats received BrdU injection as previously described [7] with minor modification. BrdU (Sigma), 50 mg/kg in saline i.p., was given once daily on 3 consecutive days after intermittent hypoxia. Two injection paradigms were used. In the first experiment, the rats were sacrificed 24 h after the last BrdU injection. This schedule labels cells undergoing DNA replication over 3-day spans ending 2 weeks after hypoxia. In the second experiment, the rats were killed after 2 or 4 weeks following the last BrdU injection. This schedule is to trace the long-term BrdU-labeled cells.

2.4. Brain preparation

All the rat brain was prepared as described before [22]. The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with heparinized saline (20 U/ml) followed by 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min. Brains were removed and post-fixed in the same fixative solution containing 15% sucrose overnight. They were then transferred to a 30% sucrose solution in 0.1 M phosphate buffer for 1 day. The brains were frozen on dry ice and kept in −80 °C until sectioning. Coronal sections were cut at 30 μm by a freezing microtone and collected into 0.1 M phosphate buffer. Forebrain sections are collected from 1.3 mm to −0.3 mm Bregma, and the CA sections are collected from −2.2 to −4.0 Bregma [23]. The sections were then processed for immunohistochemistry.

2.5. Immunohistochemistry

For detection of BrdU, sections were pretreated with 2 N HCl for denaturing the DNA, and were first incubated with a mouse monoclonal antibody against BrdU (Molecular Probe, U.S.A., and diluted 1:1000) for 48 h at 4 °C. After washing in 0.1 M phosphate buffer, sections were then incubated with biotinylated anti-mouse IgG (Vector Laboratories U.S.A., diluted 1:1000) at 4 °C overnight. BrdU-immunoreactivity was visualized as a black nuclear precipitate using a nickel-intensified 3,3′-diaminobenzidine (DAB) procedure [22].

For detection of BrdU- and NeuN-immunohistochemistry, the BrdU-labeled sections were then incubated with mouse anti-NeuN antibody (Chemicon, 1:5000) for 48 h at 4 °C. After washing in 0.1 M phosphate buffer, sections were then incubated with biotinylated anti-mouse IgG (Vector Laboratories U.S.A., diluted 1:1000) at 4 °C overnight. NeuN-immunoreactivity was visualized as brown precipitate using 3,3′-diaminobenzidine (DAB) procedure.

2.6. Cell counting and statistical analysis

BrdU-positive cells in the SVZ and SGZ were counted blindly in eight to ten DAB-stained, 30-μm coronal sections per animal, spaced 120 μm apart. Cells were counted under high power (20× objective) on Leica microscope with magnifying digital camera, and the image was displayed on a computer monitor. SVZ area includes lateral ventricular and subventricle ependema. Results were expressed as the average number of BrdU-positive cells per section in SVZ and per rat in DG, and reported as the mean ± SEM. Difference in data among groups was analyzed with the Student’s t test, with P < 0.05 considered significant.

3. Results

3.1. Effects of intermittent hypoxia on the body weight and BrdU-labeled cells in the adult rat brain

The body weight of the rat receiving 3000 m (344 ± 25 g, n = 6) and 5000 m (339 ± 28 g, n = 6) high altitude for 2 weeks did not decrease compared with that in the control (350 ± 18 g, n = 4). The rats without injection of BrdU showed no positive BrdU-labeled cells in the whole brain. In the control rats, the distribution of the number of BrdU-labeled cells in the restricted brain regions (subventricular zone, lateral ventricle, and dentate
gyrus) (Figs. 2A and 3C) was consistent with that in the previous reports [10,13,15,19].

3.2. Effects of intermittent hypoxia on the number of BrdU-labeled cells in the SVZ

To determine whether intermittent hypoxia affects BrdU-labeled cells in the adult rat brain, the newly divided cells in the subventricular zone (SVZ) were examined. The adult rats were treated with 3000 and 5000 m high altitude; BrdU-immunohistochemistry demonstrated an increase in the BrdU-labeled cells in the SVZ, and that rats treated with 3000 and 5000 m high altitude had 62 ± 5.8% and 35 ± 3.4% more BrdU-labeled cells in the SVZ following intermittent hypoxia (Figs. 1, 2, and 4 left). These results show that intermittent hypoxia increases the BrdU-labeled cells in the SVZ.

3.3. Effects of intermittent hypoxia on the number of BrdU-labeled cells in DG

With the same method above, we also tried to identify whether intermittent hypoxia affects BrdU-labeled cells in the dentate gyrus (DG) of adult rat brain by examining the number of BrdU-labeled cells in the DG. The number of BrdU-labeled cells in the control rats was insignificant, and there were usually 2–4 BrdU-positive cells per section in the DG. However, the BrdU-labeled cells in the DG increased after 3000 and 5000 m high altitude. Rats treated with 3000 m high altitude had 42 ± 4.2% more BrdU-labeled cells in the DG. But there was no significant difference in the number of BrdU-positive cells between 5000 m and control groups, though the mean number of BrdU-labeled cells in the 5000-m group was quite remarkable (Figs. 3 and 4 right). These data show that 3000 m intermittent hypoxia also increases the BrdU-labeled cells in the DG.

3.4. Effects of intermittent hypoxia on the number of BrdU-labeled cells in SVZ and DG at the 28th day after intermittent hypoxia

We further tracked the number of BrdU-labeled cells 2 and 4 weeks subsequent to intermittent hypoxia. The number of BrdU-labeled cells in the SVZ were 119 ± 23.2 and 126 ± 21 separately in the 3000- and 5000-m groups, and returned to normal level (100.4 ± 18) 4 weeks following intermittent hypoxia. In contrast, the number of BrdU-labeled cells in the DG were 64 ± 3.0 and 69 ± 5.2 separately in the 3000-m and 5000-m groups, and had a two-times increase 4 weeks following intermittent hypoxia, in comparison with control group (29 ± 2.2) (Figs. 5 and 6). Interestingly, in the wake of 2 and 4 weeks, the increased...
cells in the DG were not detected to differentiate into neurons. From these data, we conclude that intermittent hypoxia facilitates the neurogenesis of the adult rat brain, and that the neural precursors in the SVZ and DG response differently to hypoxia.

4. Discussion

In the present study, we have demonstrated that intermittent hypoxia increases BrdU-labeled cells in SVZ and DG, and that hypoxia produces different responses in the neural stem cells in the SVZ and DG, which accordingly suggests they might belong to different subtypes of NSCs. Our work has therefore furnished first-time evidence of significant increases in the proliferation of neural stem cells in SVZ and DG in the adult brain following intermittent hypoxia.

BrdU is an analog of thymidine, which is incorporated into DNA of cells during the S phase and has been used to investigate cell proliferation[10]. Our data revealed that the number of BrdU-labeled cells in the SVZ had a significant increase after intermittent hypoxia. However, the number of BrdU-labeled cells returned to normal level in contrast with that in the control group after 4 weeks of hypoxia. Our observation of transient increases in BrdU-labeled cells is consistent with data from global cerebral ischemia, in which the number of BrdU-positive cells peaks at 14 days after ischemia and the number of dividing cells returns to control levels 3–5 weeks after ischemia[11,21]. Transient increases in proliferating progenitor cells in the brain indicate that there is a window for endogenous brain plasticity.

The subgranular zone of hippocampus contains a relatively high density of neural stem cells in adult brain[5]. The cell proliferation in DG was also observed to increase after intermittent hypoxia. We treated male Wistar rats with a mimicked 3000 and 5000 m high altitude in a sealed container for 4 h over 2 weeks. We found that the number of BrdU-labeled cells in the DG increased 35% in the 3000-m group as compared with control groups. Of particular interest is that the number of BrdU-labeled cells induced by intermittent hypoxia had a twofold increase in the DG 4 weeks after hypoxia, and yet the newly generated neurons were not detectable. Consistent with our result, global forebrain ischemia gives rise to enhanced cell proliferation in the rodent subgranule zone[8,11,19]. BrdU-labeled cells

Fig. 3. Photographs of BrdU-labeled cells in the DG after intermittent hypoxia. (A and C) A section in the control; (B and D) a section in the 3000-m group; black granules are BrdU-positive cells, brown are NeuN-positive cells. Panels A and B: scale bar = 100 μm. Panels C and D: scale bar = 200 μm.

Fig. 4. The number of BrdU-positive cells in SVZ and DG after intermittent hypoxia. Left: the number of BrdU-positive cells in SVZ after intermittent hypoxia increased significantly. Right: the number of BrdU-positive cells in DG after 3000 m intermittent hypoxia also increased. Each bar represents the mean ± SEM. *P < 0.05 as compared with the control, n = 4 or 6.
increased approximately sevenfold in the subgranular zone with a peak 10 days after ischemia [6]. The new cells following ischemia migrate into the granule cell layer and become mature neurons [6,18]. This marked discrepancy is possibly ascribable to the difference in the stimuli of ischemia and intermittent hypoxia. Ischemia is a complex stimulus, which contains local tissue injury and tissue hypoxia. The molecular mechanisms regulating ischemia/hypoxia-induced cell proliferation are only partly understood. Perhaps the best example of this process is the hypoxia-induced expression of erythropoietin (EPO), which acts at the EPO receptor to promote proliferation and differentiation of erythroid progenitors and the survival of maturing erythroid cells [20]. Persistent expression of EPO and EPO receptor in the adult CNS, and the upregulation of EPO and EPO receptor in the CNS after hypoxia [3,14] enhance the role of EPO in the brain’s response to ischemia/hypoxia. In vitro studies of cultured CNS neurons have shown that EPO protects against cell death induced by hypoxia or glutamate [14]. Recently, Jin et al. have reported that stem cell factor (SCF), which is synthesized in response to hypoxia, stimulates neurogenesis in the SVZ and SGZ through its receptor c-kit, and the expression of this receptor is increased after ischemia [7]. In addition, VEGF was also believed to be involved in the regulation of neurogenesis after global and focal ischemia [9].

The results from our experiments demonstrate that the neural stem cells in the SVZ and DG respond differently to intermittent hypoxia. Consistent with this, transient global cerebral ischemia significantly increases cell proliferation and neurogenesis in the dentate subgranular zone but not in the SVZ in the adult gerbil [11]. Recent experiments in vivo have provided evidence that the adult rat SVZ contains a population of slowly dividing stem cells that can replenish a more rapidly dividing SVZ population. These data suggest that regional progenitors exhibit distinct profiles of cellular proliferation and differentiation in response to brain injury [13].

In summary, the present study has aimed to verify that intermittent hypoxia induces transient increases in progenitor cell proliferation in the SVZ and a long-term increase in DG. These results may help to kindle interest in future studies focusing on manipulating progenitor cell proliferation to promote recovery of injury brain, and to offer some channels for developing new therapeutic intervention to enhance endogenous neurogenesis following brain injury or disease.

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References
