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Research Paper

Forced running enhances neurogenesis in the hippocampal dentate gyrus of adult rats and improves learning ability

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Abstract: To investigate the effect of forced running in motor-driven wheel on neurogenesis in the hippocampal dentate gyrus (DG) of adult rats, 5-bromo-2-deoxyuridine (BrdU), a thymidine analog was applied to mark cell proliferation. Neuroepthelial stem cell protein (nestin) expression was used to identify neural stem/precursor cells. The BrdU- and nestin-positive cells were examined by immunohistochemical technique. The ability of learning was evaluated by Y-maze test to explore the functional role of the newborn cells in the DG after forced running. It was found that the number of BrdU- and nestin-positive cells in the DG in running groups was significantly increased compared to that in the control group (P<0.05). The effect of forced running on neurogenesis was intensity-dependent. In addition, an improvement of learning ability in Y-maze test was observed after forced running. These findings suggest that forced running in motor-driven wheel could enhance neurogenesis in the hippocampal DG of adult rats and improve learning ability.

Key words: hippocampus; neurogenesis; dentate gyrus; running; 5-bromo-2-deoxyuridine; immunohistochemistry; maze learning; rats

强制运动促进成年大鼠海马齿状回神经发生并提高学习能力

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摘 要：为了探讨强制运动对成年大鼠海马齿状回(dentate gyrus, DG)神经发生的影响，强制大鼠在马达驱动的转轮中跑步，用5-溴-2-脱氧尿苷(5-bromo-2-deoxyuridine, BrdU)标记增殖细胞，巢蛋白(neuroepthelial stem cell protein, nestin)标记神经干细胞/前体细胞，然后用免疫细胞化学技术检测大鼠DG中BrdU及nestin阳性细胞。为了解强制运动后DG增殖细胞的功能意义，采用Y-迷宫检测大鼠的学习能力。结果表明，强制运动组DG中BrdU及nestin阳性细胞数均明显多于对照组(P<0.05)；强制运动对DG神经发生的效应有强度依赖性。Y-迷宫检测结果显示，强制运动能明显改善大鼠的学习能力。结果提示，在转轮中进行强制跑步能促进成年大鼠DG的神经发生，并改善学习能力。

关键词：海马；神经发生；齿状回；跑步；5-溴-2-脱氧尿苷；免疫组织化学；迷宫学习；大鼠
中图分类号：R322

Brain diseases such as Alzheimer's disease, Parkinson's disease and cerebral ischemia or trauma have been considered to cause permanent loss of neurons with no possibility of cell regeneration. This widely held belief has been challenged recently by extensive evidence that certain brain areas retain the capability of generating new neurons in adult mammalian brain[1-4]. Two principal regions within the adult brain have been identified where neural stem/pre-

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cursor cells are able to give rise to new neurons in adulthood, namely the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampal formation and the subventricular zone (SVZ) lining the wall of the lateral ventricles within the forebrain. Experimental studies have demonstrated that a number of physiological and pathological factors can modulate the generation of new neurons (neurogenesis) in adult hippocampus. Enriched environments, physical exercise and learning enhance the adult neurogenesis in the DG, while stress, aging, and the lack of serotonin inhibit it[5-9].

Running, as a kind of physical exercise, is known to be an important positive stimulus for neurogenesis[10-13]. The commonly used type of exercise training in experiment is voluntary wheel running which is ordinarily observed in nocturnal rodents as spontaneously intermittent circadian physical activity. Forced running is another type of training that forces the animal to run (for example, on the treadmill) according to the experiment demands (time, duration and intensity)[12,13]. In the present study, we attempted to examine the effect of a special kind of forced running on the neurogenesis in the hippocampal DG and to determine the different running effects at different intensities.

It is well known that hippocampus is a region of the mammalian brain that is involved in learning and memory. There is evidence that newly generated neurons in the DG of the adult hippocampus lead to an improvement of learning and memory, such as a good performance of behavioral training in Morris water maze and an enhancement of long-term potentiation in the hippocampal slice[14]. However, there was also report that newly generated neurons in the adult hippocampus were not necessarily involved in the performance of learning and memory[15]. Thus, the other aim of this study was to observe the functional significance of cell proliferation on learning ability in the DG using Y-maze test.

1 MATERIALS AND METHODS

1.1 *Animals*

Adult male Sprague-Dawley rats, weighing (200±20) g, were obtained from the Experimental Animal Center of Soochow University. Animals were housed under controlled conditions [07:00~19:00 lighting, (20±2) °C] with free access to food and water. All the rats were habituated in the wheel used in the experiment for running for one week to minimize the influence of non-specific stress.

1.2 *Forced running exercise*

Animals submitted to forced running were placed into a motor-driven rotating wheel which was a cylinder (21-cm diameter, 26-cm long) consisted of metallic grids. When the wheel was driven to rotate by a motor, the rat placed inside the wheel was forced to run against the direction of the wheel revolution (Fig.1). The velocity of wheel revolution was regulated by the motor, from which the speed and intensity of forced running was deduced. In the first part of the present experiment we examined the effects of forced running on neurogenesis at different intensities. Three running intensities (light, moderate and heavy) were set at a running speed of 7, 10 and 13 m/min, respectively. For each rat forced running was conducted twice a day (30 min in the morning from 09:00 and 30 min in the afternoon from 15:00) for 6 consecutive days. The rats in the control group were also put into the wheel for the same schedule as in the running group, but the wheel was not rotated. In the second part of the experiment, the effects of forced running on ability of learning were investigated using Y-maze training.

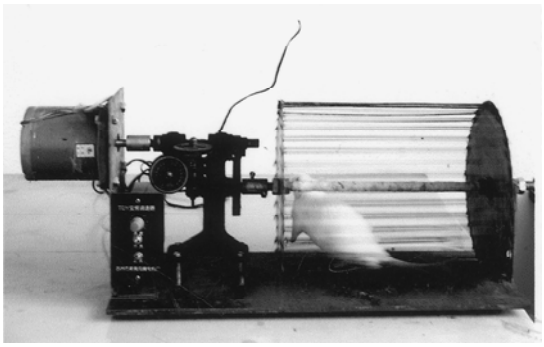

Fig. 1. Picture of motor-driven wheel for forced running.

1.3 *Injection of 5-bromo-2-deoxyuridine (BrdU)*

On the 4th day of forced running, animals in each group received intraperitoneal injection of a cell proliferation marker, BrdU (Roche Diagnostic Co., Germany; 50 mg/kg body weight, dissolved in saline solution) twice a day, 1 h before running (08:00; 14:00) for over 3 consecutive days. In the first part of the experiment, rats were sacrificed 24 h after the last injection of BrdU to evaluate the effect on cell proliferation, while in the second part of the experiment the rats were killed 2 weeks after the last injection of BrdU to examine the effect on cell survival.

1.4 *Y-maze training*

The version of Y-maze used in the present study was to assess the learning ability for active avoidance response in bright-dark discrimination conditioning. The Y-maze was consisted of three identical arms with electrifiable floor made of copper bars. A signal lamp was set at the end of

each arm. The lighting of lamp symbolized the arm being safe. Five seconds after turning on the lamp an electrical current was delivered through the floor of other arms. If the animal ran directly into the safe arm after receiving the electric shock, the response was considered correct; otherwise the response would be wrong. Nine correct responses in 10 consecutive trials were defined as the criterion of learning. For each rat, 1 session including 20 trials was conducted every day for over 5 consecutive days. The number of electric shocks was counted cumulatively and considered as the learning ability. Less shock number, better learning ability. The Y-maze training was conducted a week after the end of forced running.

1.5 *Immunohistochemistry*

Twenty-four hours (in the first part of the experiment) or 2 weeks after (in the second part of the experiment) the last injection of BrdU, rats in all groups were deeply anesthetized using 4% chloral hydrate, and transcardially perfused with 0.01 mol/L PBS followed by 4% paraformaldehyde (PFA). Brains were removed, postfixed in 4% PFA for 8 h and transferred to a 20% sucrose solution overnight until saturated. After saturation, a freezing microtome was used to make serial sections of 40 μm. An one-in-three serial section was selected for immunohistochemistry from the region spanning from bregma -3.30 mm to bregma -5.1 mm. Adjacent sections were taken for the immunohistochemical stainings of BrdU and neuroepthelial stem cell protein (nestin).

Immunohistochemical staining was performed as described previously[16]. Briefly, sections were incubated overnight with an anti-BrdU mouse monoclonal antibody (1: 200; Biosource, Belgium) or anti-nestin mouse monoclonal antibody (1:1 000; Chemicon, Germany) at room temperature. The sections were washed three times with PBS and incubated 2 h with biotinylated mouse secondary antibody (1: 200; Vector Inc., USA). The sections were incubated for another 2 h with an avidin-biotin-peroxidase complex (1: 200; Vector Inc., USA). For staining, the sections were reacted with 0.02% 3,3'-diaminobenzidine (DAB) for 5 min. Finally, the sections were mounted onto gelatinized glass slide and observed under the light microscope. In addition, for BrdU-immunohistochemistry it was necessary to pretreat the section by denaturing in 2 mol/L HCl for 30 min at room temperature and by permeabilizing with 0.1% trypsin at 37 ºC for 10 min.

1.6 *Data analyses*

The number of BrdU- or nestin-positive cells in the granular cell layer (GCL), SGZ and hilus was counted through a light microscope (CX40; Olympus, Japan). All immunopositive cells, regardless of size or shape, were counted through an ×40 objective in bilateral DG. The average number of immunopositive cells was estimated in the DG from 5 sections per animal. The number of immunopositive cells was compared using one-way ANOVA followed by Dunnett *t* test through SPSS 10.0 software. Differences between groups in Y-maze learning were analysed also by one-way ANOVA. Quantitative data were expressed as mean±SD. Differences were considered statistically significant at $P<0.05$.

2 RESULTS

2.1 Effect of forced running on cell proliferation in the DG

2.1.1 Forced running increased BrdU-labeled cells

BrdU was administered intraperitoneally twice daily in the last 3 d of forced running. Animals were killed 24 h after the last injection of BrdU for evaluating the effect on cell proliferation. The results showed that forced running resulted in a significant increase in BrdU-labeled cells in the DG. Typical photomicrographs of BrdU-positive cells in the DG in each group were presented in Fig.2. The mean number of BrdU-positive cells in the DG was 38.08±1.91/section in the control group, 62.80±8.84 in the light-running group, 52.15±6.00 in the moderate-running group and 47.53±6.57 in the heavy-running group ($n=6$). The increase in the number of BrdU-immunopositive cells in all three running groups was significant ($P<0.05$) when compared to that in the control group (Fig.4). The results clearly indicated that forced running could increase the cell proliferation in the adult rat hippocampal DG.

2.1.2 Forced running increased nestin-labeled cells

To identify the nature of the newly generated cells in the DG, adjacent sections to the BrdU staining were taken for the immunohistochemical staining of nestin, a marker of neural stem/precursor cells in the adult brain. Results similar to the BrdU staining were obtained, i.e. the forced running could also increase the number of nestin-positive cells in the DG. Representative photomicrographs showing the nestin-positive cells in the DG were presented in Fig.3. The number of nestin-positive cells in DG was 11.04±0.78/section in the control group, and 43.2±5.63, 35.26±2.74 and 24.85±3.16, in the light-, moderate- and heavy-running groups, respectively ($n=6$). The difference was significant ($P<0.05$) as compared with that in the control group (Fig.4). The result suggested that quite a lot of newly

generated cells in the DG were neural stem/precursor cells, even though the double staining of BrdU/nestin had not been performed in the experiment.

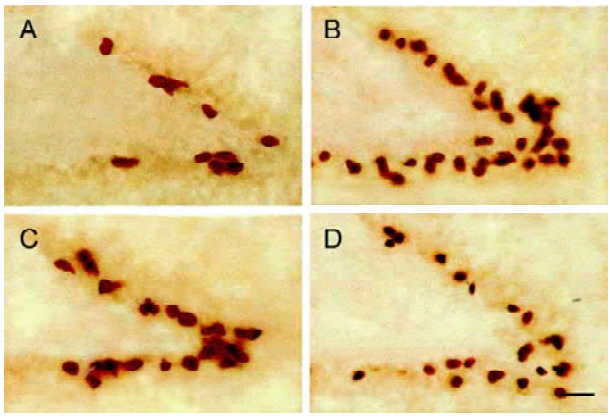

Fig. 2. BrdU-positive cells in the DG of adult hippocampus after forced running (immunohistochemical staining). *A*: Control group. *B*: Light-running group. *C*: Moderate-running group. *D*: Heavy-running group. Scale bar, 50 μm.

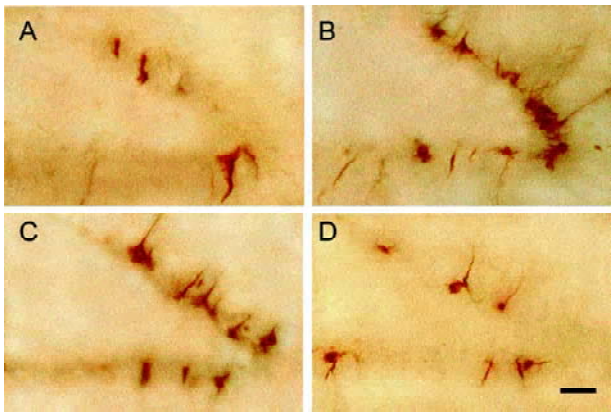

Fig. 3. Nestin-positive cells in the DG of adult hippocampus after forced running (immunohistochemical staining). *A*: Control group. *B*: Light-running group. *C*: Moderate-running group. *D*: Heavy-running group. Scale bar, 50 μm.

2.1.3 The effect of forced running on cell proliferation was intensity-dependent

Although the cell proliferation in the DG increased in all three running groups, the increasing effect was dependent on the intensity of forced running. The greatest increase of BrdU- and nestin-positive cells was observed in light-running group. The number of labeled cells gradually decreased in the moderate- and heavy-running groups. The differences were statistically significant as compared with that in the light-running group (P<0.05) (Fig.4). These results indicated that the effect of forced running on cell proliferation in the DG was intensity-dependent, and running at light intensity was most effective in stimulating cell proliferation.

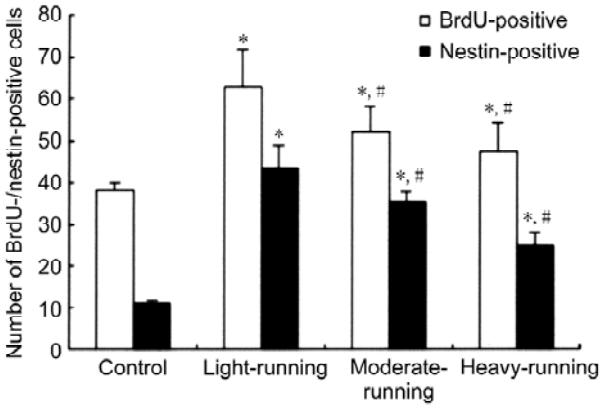

Fig. 4. Intensity-dependent effect of forced running on cell proliferation in the DG. Adjacent slices were selected for BrdU and nestin staining, respectively. It was shown that the number of BrdU- and nestin-positive cells in the DG was increased in all three running groups as compared with that in the control group, but the effect was intensity-dependent and more potent in light-running group. n=6. $^{*}P$< 0.05 *vs* control, $^{\#}P$<0.05 *vs* light-running.

2.2 Effect of forced running on cell survival and learning ability

Since the effect of forced running on cell proliferation in the DG was intensity-dependent, and the light-running was most effective, running at light intensity was used to evaluate the effect in the following part of experiment.

2.2.1 Forced running increased the cell survival in the DG

BrdU was administered intraperitoneally twice a day in the last 3 d of forced running. After a week of rest the learning training was conducted for 5 d. Therefore, the animals were sacrificed 2 weeks after the last injection of BrdU to evaluate the effect on cell survival in the DG. The results showed that the number of BrdU-labeled cells was increased significantly after forced running in Y-maze training [20.74 ±7.15/section in the control group (n=10), 35.62±7.46 in the experimental group (n=10), P<0.05, Table 1]. These findings indicated that forced running could increase the cell survival in the adult rat DG.

2.2.2 Forced running improved the performance of Y-maze learning

After 5 d of Y-maze learning, when the rats in the experimental group (forced running + Y-maze training) attained the criterion of learning, the cumulative number of electric shocks was 26.9±11.4, while in the control group (Y-maze training without running) it was 40.2±15.0. The difference was statistically significant (P<0.05, Table 1). The

result indicated that forced running improved the performance of Y-maze learning.

Table 1. The effects of forced running on cell proliferation in the DG and on Y-maze learning ability

Group	*n*	BrdU-labeled cells/section	Electric shocks
Experimental	10	35.62 ± 7.46*	26.9 ± 11.4*
Control	10	20.74 ± 7.15	40.2 ± 15.0

*$P<0.05$ *vs* control.

3 DISCUSSION

It is well known that BrdU is a thymidine analog, which can be incorporated into the DNA of dividing cells during the S phase in mitotic cycle. It is often used to mark the cell proliferation. In addition, nestin, a class VI intermediate filament protein, is found to be expressed early in the developing central nervous system (CNS) and subsequently decreases as the brain develops[17]. Now nestin-positive cells are viewed as neural stem/precursor cells in the adult CNS. Thus, in the present study, BrdU- and nestin-immunohistochemical staining was adopted to better evaluate the neurogenesis in the DG of hippocampus[18].

Recent studies reported that the cell proliferation and the survival rate of newly formed neurons in the DG of adult rodents could be increased by voluntary physical activity. van Praag *et al*.[10] reported that voluntary wheel running increased cell proliferation and survival rate in mice, and Farmer *et al*.[19] also showed that voluntary wheel running enhanced neurogenesis in the hippocampal DG of adult rats. The cell proliferation in adult DG could also be increased by forced running. Kim *et al*.[12] and Ra *et al*.[13] demonstrated that after forced running on treadmill the cell proliferation in the DG of adult rats was increased. In the present study, forced running was conducted in motor-driven wheel for 6 consecutive days. The immunohistochemical evaluation was performed 24 h or 2 weeks after the last injection of BrdU. The BrdU-positive cells in each running group were more than that in the control group, indicating that this type of forced running, as in the case of voluntary wheel running or forced running on treadmill, could enhance the cell proliferation, as well as the cell survival rate in the hippocampal DG of adult rats.

Experimental studies have demonstrated that forced running on the treadmill may cause stress[20,21] and activate the hypothalamic pituitary adrenocortical axis[22], which can inhibit the neurogenesis in the DG and counterbalance the enhancing effect of forced running on neurogenesis[10]. Therefore, the gradual decrease of the effect on neurogenesis in the case of running at moderate and heavy intensity could be explained by the confounding influence of stress due to the increase in intensity of forced running.

Furthermore, our findings also indicated that the learning ability in running rats was significantly improved with enhanced neurogenesis in the DG. Anderson *et al*.[23] employed the eight-arm radial maze to test the influences of voluntary exercise on spatial learning and also found that wheel running could enhance neurogenesis and improve the performance of maze learning, which emphasized the possibility that increased neurogenesis in the runners might contribute to the improvement of learning. In fact, there exist several factors that elevate production of new neurons in the DG with enhanced learning. For example, living in an enriched environment can double the number of surviving newborn cells and also improve the water maze performance[5]. Moreover, treatment with hormones, such as estrogen, can increase cell proliferation and improve memory function [24]. In contrast, factors that reduce neurogenesis, such as corticosterone treatment, stress, and aging, are associated with impairment of learning ability[24, 25]. Derrick *et al*.[26] reported that the increased granular cell neurogenesis in the adult DG induced by mossy fiber stimulation was sufficient to induce long-term potentiation. In addition, there is extensive evidence that newly generated cells in the adult hippocampus have neuronal morphology and display passive membrane properties, action potentials and functional synaptic links similar to those found in the mature dentate granule cells[27,28]. All these data indicate that the increase of neurogenesis in the DG induced by running might be responsible for the improvement of learning and memory.

Taken together, our findings demonstrate that forced running in motor-driven wheel could enhance the neurogenesis, including cell proliferation and survival rate in the DG of hippocampus in adult rats and improve the learning ability in Y-maze training. Further research will be needed to study the mechanisms underlying the effect of forced running on the neurogenesis and learning ability.

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