



Voluntary Running Enhances Hippocampal Proliferation by Increasing Hippocampal NGF, BDNF, and IGF-1

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Abstract: Neurotrophic factors and growth factors are known to have positive effects on hippocampal proliferation. However, previous findings on the effects of these factors on exercise-induced hippocampal proliferation are inconsistent. In this study, we investigated the effects of voluntary running on hippocampal proliferation and the expression levels of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and insulin-like growth factor 1 (IGF-1) in hippocampus. Twenty-four male C57BL/6 mice were randomly divided into four groups: control groups 1 and 2 (C1, n = 6; C2, n = 6) and running groups 1 and 2 (R1, n = 6; R2, n = 6). Mice from C1 and C2 were placed individually in standard cages for eight weeks; mice from R1 and R2 were housed individually in cages with a running wheel for eight weeks. At the fifth week, C1 and R1 mice were injected with BrdU for 7 consecutive days. On the first day of the ninth week, the C1 and R1 mice were anaesthetized and hippocampal neuronal proliferation was assessed using immunohistochemistry; the mice in C2 and R2 were sacrificed to test the expression levels of NGF, BDNF, and IGF-1 mRNA in hippocampus using RT-PCR. The results indicated that voluntary running significantly increased the number of BrdU-positive cells in the dentate gyrus (DG), and significantly upregulated the levels of NGF, BDNF, and IGF-1 mRNA in the hippocampus. In conclusion, our findings indicated that hippocampal proliferation was enhanced by voluntary running, which was associated with increased NGF, BDNF, and IGF-1 mRNA levels in the hippocampus.

Keywords: Voluntary Exercise, Hippocampal Proliferation, NGF, BDNF, IGF-1

1. Introduction

Many studies have confirmed that neurogenesis continues in discrete regions of the adult brain, especially in the subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricles [1, 2]. During adult hippocampal neurogenesis, newly generated cells can proliferate, differentiate, mature, and integrate into functional neural networks [3]. Aging or dead cells are substituted by new cells. Neuronal proliferation plays a vital role in the maintenance of hippocampal structure and function. Some neurotrophic factors or growth factors have

been demonstrated to be involved in mediating proliferation in the hippocampus. For instance, Birch et al [4] showed that chronic intracerebroventricular infusion of nerve growth factor (NGF) promoted neuronal proliferation in the hippocampal DG. In contrast, conditional knockout brain-derived neurotrophic factor (BDNF) resulted in the reduction of hippocampal proliferation [5]. In addition, Aberg et al [6] found that insulin-like growth factor 1 (IGF-1) had a direct proliferative effect in adult hippocampal progenitor cells.

Some studies also examined the effects of BDNF on exercise-induced hippocampal neurogenesis. For instance,

treadmill exercise increased hippocampal BDNF expression, and promoted hippocampal proliferation [7-9]. However, another study reported that treadmill exercise increased hippocampal proliferation but did not increase BDNF protein [10]. In addition, Marlatt *et al* [11] divided 9-month-old mice into control group, 6-month running group and 1-month running group. Exercise group was housed with a running wheel. All animals were injected with BrdU in the first 10 days. The results showed that 6-month voluntary wheel running increased hippocampal proliferation and BDNF protein, whereas 1-month voluntary wheel running did not enhance hippocampal proliferation and BDNF protein. The reason may be correlated with the duration of voluntary running. Therefore, in the current study we examined the effects of 8-week voluntary wheel running on hippocampal proliferation and hippocampal BDNF mRNA level.

In a previous study, mice were divided into three groups: control mice (C), restraint-stressed mice (RS), and voluntary exercise + restraint-stressed mice (VE + RS). The results showed that the mice in VE+RS group had more BrdU+ cells in the hippocampus and IGF-1 protein in the cerebral cortex than mice of RS group. These indicated that voluntary exercise improved hippocampal proliferation and IGF-1 level in the cerebral cortex of stressed mice [12]. In another study, exercise-induced hippocampal proliferation and IGF-1 protein level in the brain was decreased, if the entrance of circulating IGF-1 into brain was blocked by subcutaneous infusion of a blocking IGF-1 antiserum to rats undergoing exercise training [13].

In addition, wheel running could increase hippocampal NGF level [14], whereas no previous study has examined the effect of exercise-induced hippocampal NGF on hippocampal proliferation. Therefore, the purpose of the present study was to investigate whether or not hippocampal proliferation was enhanced in response to eight weeks of voluntary wheel running in mice. We further investigated the expression levels of NGF, BDNF, and IGF-1 mRNA in the hippocampus.

2. Materials and Methods

2.1. Animals

A total of 24 male C57Bl/6 mice (5 weeks of age; SLAC Laboratory Animal Company, Shanghai, China) were used in this study. The mice were randomly divided into four groups: control groups 1 and 2 (C1, $n = 6$; C2, $n = 6$) and running groups 1 and 2 (R1, $n = 6$; R2, $n = 6$). The mice were housed individually in temperature-controlled conditions at 23°C with a 12-h light/dark cycle. All mice had access to food and water *ad libitum*. All experimental procedures were approved by the Experimental Animal Care and Use Committee at East China Normal University [license number for the use of laboratory animals: SYXK (Shanghai) 2004-0001] and conformed to the guidelines for the use of laboratory animals published by the People's Republic of China Ministry of Health (25 January 1998).

2.2. Exercise Protocol and Injection of BrdU

R1 and R2 mice were exposed to voluntary wheel running (wheel diameter, 10 cm) for 8 weeks. The number of revolutions of the wheel was monitored by a counter and the running distance was determined by multiplying the number of revolutions by wheel circumference [15]. C1 and C2 mice were housed under standard conditions for 8 weeks. At week five, newly generated cells in the DG of the R1 and C1 mice were date-labeled with 50 mg/kg BrdU (intraperitoneal injection after the mice were weighed at 5 PM; Sigma, Hamburg, Germany) for 7 consecutive days. BrdU is a thymine nucleoside analogue that replaces thymidine, and can be incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle).

2.3. Tissue Preparation

On the first day of week 9, BrdU-treated mice (R1 and C1) were anaesthetized with 1% sodium pentobarbital and transcardially perfused with 0.9% NaCl solution followed by 4% paraformaldehyde. The brains were stored in 4% paraformaldehyde overnight at 4°C. After fixation, all brains were transferred to 15% sucrose until the brains sank to the bottom, and then transferred to 30% sucrose for 30 hours. After all brains sank to the bottom, they were cut in the coronal plane (20- μ m sections) with a dry ice-cooled copper block on a sliding microtome (Leica, Bensheim, Germany) and stored in OCT at -80°C until BrdU staining. At the same time, R2 and C2 mice were sacrificed. The hippocampi were collected, frozen in liquid nitrogen immediately, and stored at -80°C until use for gene expression analysis by RT-PCR.

2.4. Immunohistochemistry

For BrdU detection, sections were rinsed in PBS (5 min \times 3), and incubated in 50% formamide for 2 h at 65°C, and hydrolyzed in 2N HCl at 37°C. The sections were then rinsed three times with 0.1 M borate buffer, incubated in 3% H₂O₂ and 0.3% Triton X-100 for 30 min each at room temp, and blocked for 1 h with 3% BSA. The sections were then incubated with sheep anti-BrdU (1:200 dilution, ab1893, Abcam) in 2% BSA overnight at 4°C. On the second day, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-sheep antibodies (1:500 dilution, 6150-02, Southern Biotech) in 2% BSA for 1 hour at RT, kept in the dark, and then incubated in 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) for 2 hours. Finally, fluorescent signals were imaged under a fluorescence microscope (Leica AF6000, Germany).

2.5. RT-PCR

Total RNA was extracted from the hippocampus with Trizol reagent after homogenate. The purity of RNA was measured by an ultraviolet spectrometer. RNA was used for reverse transcription when OD260/OD280 \geq 1.8. cDNA was acquired using the ReverTra Ace qPCR RT kit (Toyobo Co. Ltd., Osaka, Japan). mRNA was measured by the Toyobo

SYBR-green PCR kit (Toyobo Co. Ltd). mRNA abundance was normalized to that of β -actin, which was used as the internal control. The primer sequences are shown in Table 1.

Table 1. Primer Sequence.

Genes	Primer Sequences
NGF	Forward primer: GAC AAC AGA TGA GAA GCA GGC
	Reverse Primer: AGG AGA GTG TGG AGG GGG
BDNF	Forward primer: GTT CCA CCA GGT GAG AAG AGTG
	Reverse Primer: AAA GTG TCA GCC AGT GAT GTCG
IGF-1	Forward Primer: GCTG GACC AGAG ACCC TTTTG
	Reverse Primer : AGGT GCCC TCCG AATG CT
β -actin	Forward Primer: CTC TTT TCC AGC CTT CCT TCT
	Reverse Primer: TGG AAG GTG GAC AGT GAGG

2.6. Statistical Analysis

All statistical analyses were performed using GraphPad Prism (version 5.0; GraphPad Software Inc., San Diego, California) and SPSS version 17.0. The t test was used to compare differences between the control and running groups. All data are presented as means \pm SEM. The level of significance was set at $P < 0.05$ or $P < 0.01$ (two-sided).

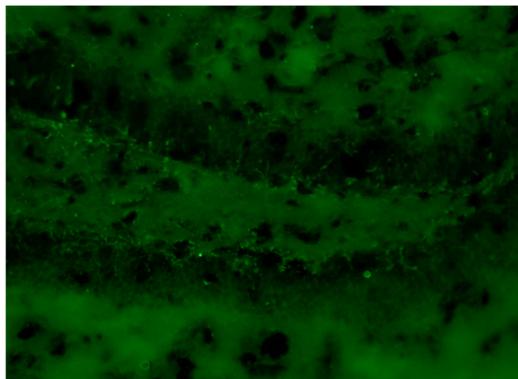
3. Results

3.1. Daily Running Distance

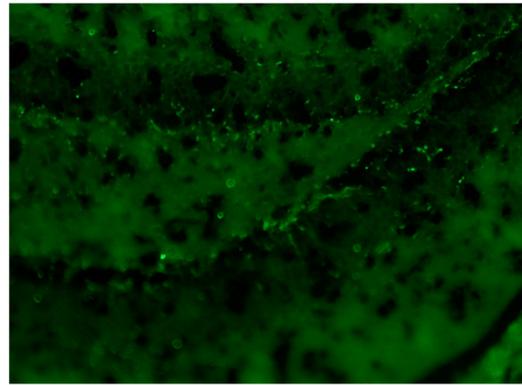
The daily average revolutions of mice from R1 and R2 over the eight-week period were 23625.70 ± 7745.70 . The wheel circumference is 0.314 m. Thus, the daily average running distance was 7418.47 ± 2432.15 m.

3.2. Effect of Voluntary Running on the Number of BrdU+ Cells in the DG

The phenotype of BrdU-positive cells was assessed by single immunofluorescence labeling for BrdU. Fluorescence microscopy was used to count the number of single-labeled BrdU-positive cells in the DG (Fig. 1 a, b). There was a significant difference in the number of BrdU-positive cells in the R1 and C1 groups (C1: 71.83 ± 15.52 , R1: 257.83 ± 56.74 , $P < 0.01$; Fig. 1 c). This result suggested that voluntary running improved cell proliferation in the DG of the hippocampus.



(a)



(b)

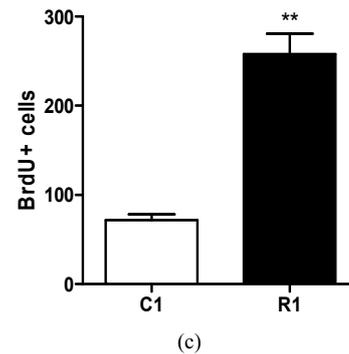


Fig. 1. Effect of voluntary running on the number of BrdU-positive cells in the DG. (a) A representative image showing BrdU+ cells in the dentate gyrus (DG) of the C1 group. (b) A representative image showing BrdU+ cells in the DG of the R1 group. (c) Quantitative data of BrdU+ cells in the two groups. All data are expressed as means \pm SEM ($n = 6$ in each group). ** $P < 0.01$ vs. C1.

3.3. Effect of Voluntary Running on the Expression of NGF, BDNF, and IGF-1 mRNA in the Hippocampus

There was a significant increase in the NGF mRNA (C2: 1.00 ± 0.34 , R2: 3.76 ± 0.47 , $P < 0.01$), BDNF mRNA (C2: 1.00 ± 0.39 , R2: 3.56 ± 1.42 , $P < 0.01$), and IGF-1 mRNA (C2: 1.00 ± 0.73 , R2: 2.51 ± 0.86 , $P < 0.01$) in the hippocampus of the R2 group compared to that in the C2 group (Fig. 2). These results indicated that voluntary running increased the mRNA expression levels of NGF, BDNF, and IGF-1 in the hippocampus.

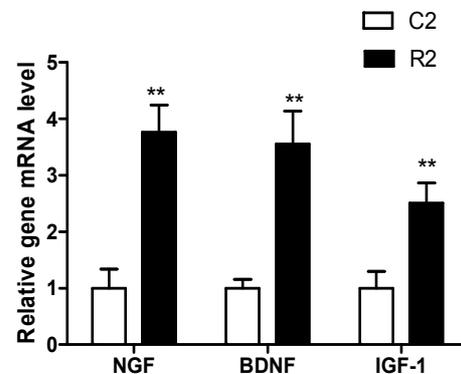


Fig. 2. Effect of voluntary running on the expression of NGF, BDNF, and IGF-1 mRNA in the hippocampus. The expression levels of NGF, BDNF, and IGF-1 between the C2 and R2 groups were determined by RT-PCR. Data are expressed as means \pm SEM ($n = 6$ in each group). ** $P < 0.01$ vs. C2.

4. Discussion

4.1. Voluntary Running Increased Proliferation in the DG

In the present study, we found that the daily running distance was approximately 7.4 km, which was consistent with the results of a previous study of Allen, who found that the average daily running distance of C57BL/6 mice was 6.8 km [16]. We examined the number of proliferative neural cells using BrdU immunofluorescence staining. There were significantly higher BrdU-positive cells in the DG of group R1 than in the DG of group C1 ($P < 0.01$, Fig. 1), suggesting that hippocampal proliferation was increased in mice housed with running wheel. In van Praag's [17] study, adult mice were divided into learner, swimmer, runner, enriched, and control groups, which were given a daily intraperitoneal injection of BrdU. They found that only runner and enriched increased BrdU-positive cells in the DG. In subsequent research, they divided 3-month-old mice into sedentary and running groups. Runners had unlimited access to a running wheel for 45 days. The result revealed that wheel running increased the number of BrdU-positive cells in the DG [18]. These results indicated that voluntary wheel running could increase hippocampal proliferation, which was in accordance with our current results. Another study about treadmill exercise on hippocampal proliferation also showed that treadmill running for 5 weeks promoted neural stem cells (NSC) proliferation in the DG [8]. In another interesting study, rats were randomly divided into four groups: low-intensity, moderate-intensity, and high-intensity exercise groups and control group. The results showed that forced low- or moderate-intensity treadmill exercise for one week enhanced hippocampal neurogenesis in rats [19]. Therefore, low-intensity aerobic exercise, including voluntary exercise and forced exercise, could promote hippocampal proliferation. The positive effect of exercise on hippocampal proliferation may be associated with increased neurotrophic factors or growth factors.

4.2. Voluntary Running Enhanced Proliferation by Increasing NGF, BDNF, and IGF-1

Neurotrophic factors such as NGF and BDNF, are extracellular proteins that play vital roles in neurogenesis. NGF was the first neurotrophic factor to be discovered. It is found in the neurons of the hippocampus, olfactory bulb, and cerebral cortex. In the present study, we found that the NGF mRNA level in the hippocampus was increased following voluntary wheel running for 8 weeks. In previous studies, Neeper *et al* [14] found that voluntary running significantly increased NGF mRNA in the hippocampus, and O'Callaghan *et al* [20] demonstrated that long-term exercise prevented or reversed age-related decline in the expression of NGF mRNA. These results are consistent with our current results. In addition, we also found that there was an increase in NGF accompanied by hippocampal proliferation, indicating that hippocampal NGF has a positive impact on hippocampal proliferation following

voluntary exercise. Regarding subcellular mechanism, Kim *et al* [21] found that NGF activated TrkA receptor, phosphorylated Shc, activated Ras phosphorylation, and induced ERK activity, which in turn phosphorylated CREB to promote proliferation. Therefore, the mechanism may be that exercise increases NGF, which promotes proliferation in the DG through MAPK pathway.

Chronic BDNF injection into the adult DG resulted in increased neurogenesis of granule cells [22], whereas conditional knockdown of BDNF has been shown to reduce the proliferation in the adult DG *in vivo* [5]. Another study found that 3 weeks forced running increased hippocampal proliferation and BDNF protein in one-month old rats [7]. The hippocampal proliferation and BDNF protein were also enhanced in middle age mice by 5 weeks treadmill exercise [8]. In our study, voluntary running increased BDNF mRNA and was accompanied by hippocampal proliferation. However, a previous study reported that short-term treadmill exercise enhanced proliferation in the DG but did not increase BDNF protein in 2-month old Wistar rats [10]. Another study also reported that 1-month running did not enhance hippocampal proliferation and BDNF protein [11]. These discrepancies may be associated with exercise intervention duration. It is likely that the effect of exercise-induced hippocampal proliferation is higher if the duration of voluntary exercise is longer. Hippocampal proliferation is also likely associated with BDNF expression. Two possible subcellular cascades may underlie these changes. First, BDNF may activate the TrkB receptor, which in turn increases CREB phosphorylation via the MAPK pathway, and promotes hippocampal neuronal proliferation. Second, phosphorylation of the TrkB receptor may activate PI3K, which then phosphorylates Akt to promote hippocampal proliferation [23]. Thus, BDNF may be a mediator for exercise-induced neuronal proliferation.

The function of IGF-1 in regulating adult hippocampal proliferation has been demonstrated in previous studies [24, 25]. In the present study, we found that voluntary exercise increased the expression of IGF-1 in the hippocampus, which was in line with other findings [26, 27]. One explanation for this finding could be that exercise enhances IGF-1 production in the liver, and this hepatic IGF-1 might be transported to the brain via the bloodstream, resulting in the increased IGF-1 expression in the hippocampus [13, 28]. Further, we observed that voluntary running not only increased hippocampal IGF-1 mRNA, but also promoted hippocampal proliferation. Therefore, the increase IGF-1 correlated with hippocampal proliferation. IGF-1 activated IGF-1 receptors, which in turn activate the MAPK and PI3K-Akt signal pathways to induce hippocampal proliferation [6, 29]. Thus, the enhancement of IGF-1 following exercise may also play a role in exercise-induced hippocampal proliferation. Consistent with this, the results from a previous study suggest that exercise increases IGF-1 expression in the brain, which enhances BDNF and promotes hippocampal proliferation [30].

5. Conclusion

Voluntary running improved hippocampal proliferation in mice, which was associated with the upregulation of NGF, BDNF, and IGF-1. However, more research is needed to determine which factor is more important in exercise-induced proliferation and to elucidate the interaction between these factors.

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