

ORIGINAL ARTICLE

# Amount of energy consumption during physical activity is a key element in the analysis of neurogenesis in the adult mouse hippocampus

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## Abstract

Hippocampal neurogenesis in the mammalian brain is enhanced by various factors, the most important of which is physical activity. In the study of neurogenic effects of physical activity, a running wheel is generally used to quantify physical activity. Although a running wheel can record the time and distance that mice run, the amount of energy consumed cannot be calculated: fast runners consume more energy than slow ones. To precisely quantify physical activity, amount of energy, an absolute value of physical activity, must be considered. In the present study, we developed a new device consisting of a mouse cage equipped with angled (0°, 45°, and 90°) tunnels of constant length to change the intensity of physical activity, which depended on the angle of the tunnel, with maximum intensity at 90°. To analyze neurogenesis using an immunohistological technique, a water bottle containing 5-bromo-2'-deoxyuridine (BrdU), a thymidine analog that is incorporated into the proliferating cells during DNA synthesis and can be detected by its immunohistochemistry, was set at the end of the tunnel for the 2-week experimental period. The number of passages made by the mice through the tunnels and the amount of BrdU ingested were not significantly different among the different housing conditions. However, a significant difference was detected in the number of BrdU immunopositive (+) cells, which increased in proportion to the angle of the tunnel. All the BrdU(+) cells were doublecortin-expressing neuronal cells. The distance traveled was constant for each round trip in each housing condition, so the energy consumed was dependent on the angle of the tunnel. Our findings indicate that level of hippocampal neurogenesis depends on the amount of energy consumed during physical activity. This study suggests that the upslope exercise in humans is effective regimen to maintain and improve the brain function through factors such as myokines.

**Keywords:** Adult neurogenesis, hippocampus, physical activity, energy consumption, histochemistry

## Introduction

In the adult mammalian brain, the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus is a neurogenic region. Neurogenesis also persists in the human hippocampus throughout life [1]. The hippocampal neural stem cells reside and proliferate in the SGZ, where they differentiate into granular neurons [1]. These newly generated neurons participate in the formation of memories [2].

It is well known that an enriched environment, a type of housing condition provided for animals used in experiments,

enhances neurogenesis and reduces cell death in the DG [3]. In addition, an enriched environment has a positive effect not only on learning ability, but also on recovery potential from certain types of neurological disease [4]. Although various studies have demonstrated the positive effects of an enriched environment on brain function, the defining elements leading to those effects remain obscure. In general, enriched environments provided in experimental conditions include increased opportunities for physical activity and sensory stimulation, including motor activity, sensory stimulation, cognitive stimulation, social interaction, and novelty recognition [5]. However, some of these elements of an enriched environment are difficult to quantitatively analyze and calculate.

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Among these enriched environment elements, physical activity is considered to be a key factor in enhancing neurogenesis in the DG, and running is the simplest experimental paradigm with which to quantify physical activity. At present, there are two devices that are widely used to make experimental animals run: a treadmill and a running wheel. Running on these devices can enhance hippocampal neurogenesis, depending on the time and distance that animals run [6–8]. However, running on a treadmill is a forced exercise: animals are put on the treadmill and forced to run. Forced exercise can cause stress, which has a negative effect on hippocampal neurogenesis [9–12]. To precisely study the neurogenic effects of physical activity, negative factors should be excluded. Running on a running wheel is a voluntary exercise and free of stress: a running wheel is placed in the cage, and animals can access it freely.

Physical activity can be quantitatively expressed using energy consumption depending on body weight, time and intensity of activity. The intensity of activity or the amount of energy consumed have been factors lacking in studies of the neurogenic effects of physical activity. To our knowledge, all studies using the running wheel have discussed the neurogenic effects of the time and/or distance that experimental animals run. For example, Holmes et al. restricted the time that mice could access the running wheel and showed that hippocampal neurogenesis was enhanced following the longest period of access to the running wheel [8]. However, in their experimental paradigm, the precise amount of energy consumed by each mouse was not clear: fast runners likely expend more energy than slow ones over a defined period. The running wheel cannot record acceleration, and only the average running speed (calculated by total time and distance) can be defined. Moreover, several commercial running wheels are available, and the effect of physical activity on neurogenesis cannot be directly compared among studies using different wheels. The amount of energy consumed should be an absolute value in order to compare the effects of different physical activities on neurogenesis. To achieve this aim, the gold standard is the measurement of oxygen consumption, but specialized instruments are needed for this.

To overcome this issue, we developed a new device to change the intensity of voluntary activity. The device is a mouse cage equipped with an angled (0°, 45°, and 90°) tunnel of constant length, where the intensity of activity can be easily adjusted by changing the angle of the tunnel. In human physical activity, metabolic equivalent (MET) is widely used to measure intensity of activity. MET is defined as the ratio of metabolic rate during an activity to the standard

metabolic rate while resting. Sitting quietly, walking on a flat surface at less than 3.2 km/h, walking uphill, and climbing a ladder correspond to 1.0 MET, 2.0 METs, 6.0 METs, and 8.0 METs, respectively [13, 14]. As a rule of thumb, energy consumed (kcal) is represented as  $1.05 \times \text{METs} \times \text{body weight} \times \text{time}$  [13, 14]. Thus, we assumed that the angle of the tunnel in the cage corresponded to MET. Using this device, we investigated the relationship between the amount of energy consumed during voluntary activity and hippocampal neurogenesis in adult mice. Our results clearly show that the proliferation of neural stem/precursor cells was enhanced in proportion to the amount of energy consumed.

## Methods

### Animals

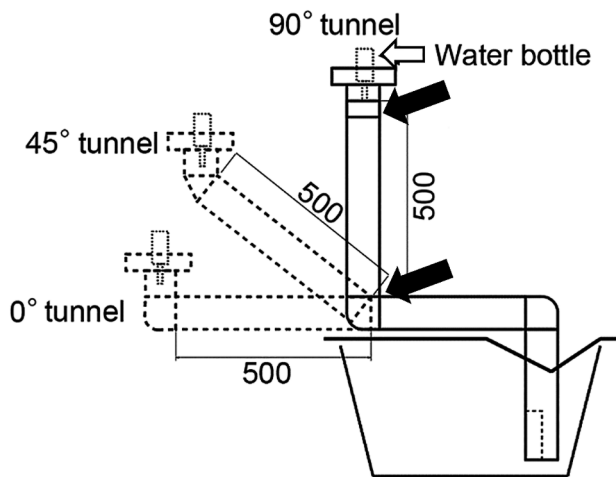
Eight-week-old male C57BL/6J mice (B.W.: 25.0 g, Nippon SLC, Shizuoka, Japan) were maintained in separate cages for 2 weeks. The mice were housed at  $22 \pm 1^\circ\text{C}$  on a 12/12 hr light/dark cycle and provided with freely accessible food and water. All experimental protocols were approved by the animal ethics committee of Kansai Medical University and performed in accordance with the Principles of Laboratory Animal Care (NIH, 1985).

### Devices for adjusting intensity of activity

Three types of cages were prepared, each equipped with an angled tunnel of 0°, 45°, or 90° (**Fig. 1**). The tunnels were made of a vinyl chloride material and were 500 mm in length and 60 mm in diameter. The walls of the tunnels were 2 mm thick, and a net of stainless steel was set inside the tunnel walls. One mouse was housed in each cage. To record the number of times mice passed through the tunnels, they were equipped with two homemade infrared passage counters, one at either end (black arrows, **Fig. 1**). Passage through both counters was considered to be a single passage.

### BrdU labeling

The mice were housed in the angled tunnel-equipped cages for 2 weeks. A water bottle was placed at the end of each tunnel (white arrow, **Fig. 1**). The thymidine analog 5-bromo-2'-deoxyuridine (BrdU; Sigma, St. Louis, MO, USA), which is used to label and trace progenies of proliferating cells, was administered via the drinking water (1 mg/ml) throughout the 2-week experimental period. A standard cage without a tunnel was prepared as a control for water/BrdU intake. The volume of water consumed by the mice each day was measured.



**Fig. 1** Dimensions of cages with angled tunnels. A tunnel was attached to each cage at a different angle (0°, 45°, and 90°). A water bottle was set at the end of the tunnel (white arrow). Black arrows indicate the positions of homemade infrared passage counters.

### Histological analysis

At the end of the experimental period, mice were euthanized using sodium pentobarbital (100 mg/kg, intraperitoneal injection) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.2) followed by 4% formaldehyde in PBS. Brains were removed and immersed in the same fixative for 12 hr. After cryoprotecting the tissues in 20% sucrose in PBS, the brains were embedded in an OCT compound (Sakura Finetek, Tokyo, Japan) and frozen using dry ice. Free-floating coronal sections (25  $\mu$ m thick) were obtained using a cryostat.

BrdU incorporation was detected using a previously described immunohistochemical method [15]. Briefly, sections were treated with 2 M HCl for 30 min at room temperature, followed by two neutralization steps with 0.1 M sodium borate (pH 8.0) for 15 min and three washes with PBS. Sections were successively incubated with rat anti-BrdU antibody (1:200, Abcam, Cambridge, UK, Cat# ab6326, RRID: AB\_305426) for 12 hr at 4°C; biotinylated anti-rat IgG antibody (1:200, Vector Lab, Burlingame, CA, Cat# BA-9400, RRID: AB\_2336202) for 3 hr at room temperature; and an avidin/biotin complex solution (Vectastain Elite ABC kit, Vector Lab, Cat# BA-9200, RRID: AB\_2336827) for 30 min at room temperature. After a chromogenic reaction with 3, 3' diaminobenzidine tetrahydrochloride, the free-floating sections were mounted on glass slides. Double immunofluorescent staining was performed using anti-BrdU and anti-doublecortin (DCX) antibodies to identify BrdU immunopositive (+) cells. Sections were pre-treated with 2 M HCl as described above, followed by incubation with a primary antibody cocktail containing rat anti-BrdU antibody and guinea pig anti-DCX antibody (1:500,

Millipore, Billerica, MA, USA, Cat# AB2253, RRID: AB\_1586992) for 12 hr at 4°C. Primary antibodies were detected using fluorescence-conjugated species-specific secondary antibodies as follows: Cy2-conjugated donkey anti-rat IgG (1:200, Jackson ImmunoResearch, West Grove, PA, USA, Cat# 712-225-153, RRID: AB\_2340674) and Cy3-conjugated donkey anti-guinea pig IgG (1:200, Jackson ImmunoResearch, Cat# 706-165-148, RRID: AB\_2340460) for 3 hr at room temperature. To visualize the nuclei, sections were mounted on glass slides with a medium containing 100 mM dithiothreitol, 5  $\mu$ g/ml Hoechst 33258, and 50% glycerol in PBS. Fluorescence images were acquired using a confocal microscope (LSM 510-META, Carl Zeiss, Oberkochen, Germany).

### Quantification and statistical analysis

A total of 14 coronal sections (one in every five serial sections) was collected from each mouse; these sections included the hippocampus (between 1.2 and 3.0 mm posterior to the bregma). The number of BrdU(+) cells was counted under a microscope (Optiphot-2, Nikon, Tokyo, Japan).

Images were acquired using a digital camera connected to the microscope, and length of the SGZ was measured using Image J software (RRID: SCR\_003070). The number of BrdU(+) cells was evaluated per 100  $\mu$ m of the basal line of the SGZ. Statistical analysis was performed with EZR, a statistical software based on R [16]. For statistical analysis, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was performed. Four to six mice were used for statistical analysis of each housing condition. Significance was defined at the level of  $p < 0.05$ . Error bars represent the standard deviation (SD).

## Results

### Number of passages through the tunnel

The average numbers of passages by the mice over two weeks were not significantly different among the experimental groups (one-way ANOVA,  $p = 0.95$ , **Fig. 2**). The average weight of mice was 25.0 g in each housing condition.

### Water/BrdU intake

The amount of water intake was not significantly different among housing conditions (one-way ANOVA,  $p = 0.32$ , **Fig. 3**). These results indicate that the amount of BrdU ingested was not significantly different among housing conditions.

Number of BrdU(+) cells in the SGZ

BrdU(+) cells were distributed throughout the SGZ (Fig. 4). BrdU(+) cells located outside the SGZ are likely endothelial cells with a flat nucleus shape or glial cells with small nuclei, and these were excluded from the quantitative analysis (Fig. 4, small arrows). The number of BrdU(+) cells per 100 μm of the SGZ was significantly different among housing conditions (one-way ANOVA,  $p = 7.01\text{e-}21$ , Fig. 5). Although the number of BrdU(+) cells not significantly different between mice housed in the control cage and the cage with the 0° tunnel, the number of BrdU(+) cells significantly increased with increasing the tunnel angle (Fig. 5).

Characterization of BrdU(+) cells

Neural stem cells residing in the DG proliferate and generate new granular neurons throughout life. Glial cells and endothelial cells also have a proliferative ability (Fig. 4, small arrows). Thus, we used fluorescent double immunohistochemistry to identify BrdU(+) cells in combination with an anti-DCX antibody, which is a marker of immature neurons. All BrdU(+) cells within the SGZ were DCX(+) in mice from all housing conditions (Fig. 6).

Discussion

In the present study, we defined intensity of physical activity as amount of energy consumed, and we showed that hippocampal neurogenesis in adult mice was enhanced in proportion to amount of energy consumed. To achieve our aim, we introduced the concept of MET into the study of the effects of physical activity on hippocampal neurogenesis, and we developed a new device: a cage with an angled tunnel. Changing the angle of the tunnel leads to changes in intensity of activity of mice under free movement conditions. Thus, we can analyze and discuss the relationship between the amount of energy consumed and hippocampal neurogenesis. The number of passages through the tunnel and amount of water containing BrdU that was consumed were not significantly different among the various housing conditions. However, the number of newly generated BrdU(+) neurons in the SGZ significantly increased in proportion to the angle of the tunnel for the 2-week housing period. Therefore, our results indicate that hippocampal neurogenesis absolutely depends on amount of energy consumed.

Physical activity consumes energy, as do increases in metabolism. Recently, cross talk between hippocampal neurogenesis and whole-body metabolism has attracted consid-

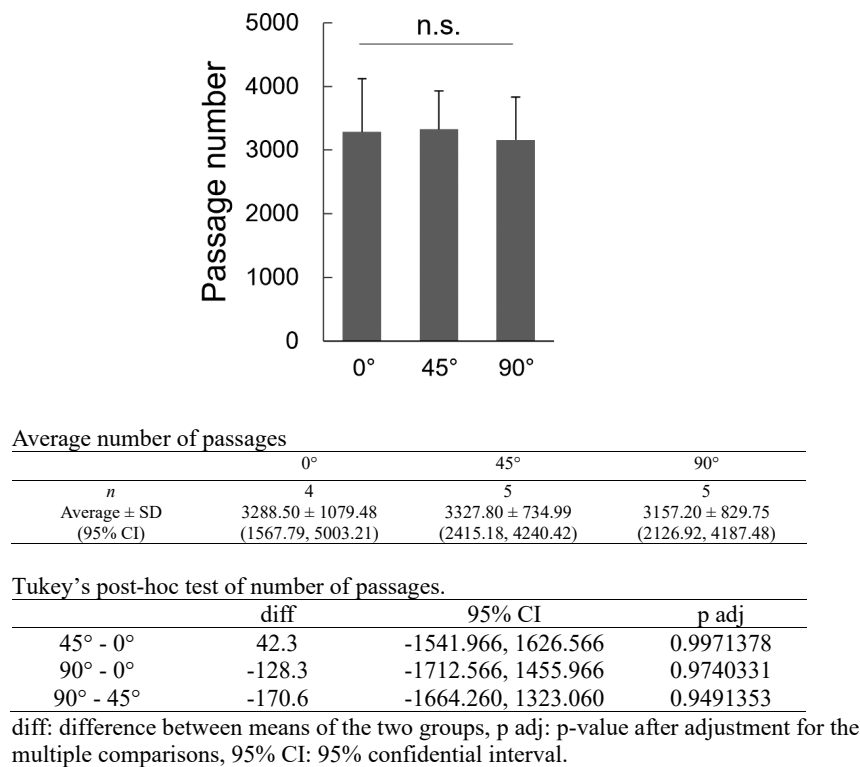
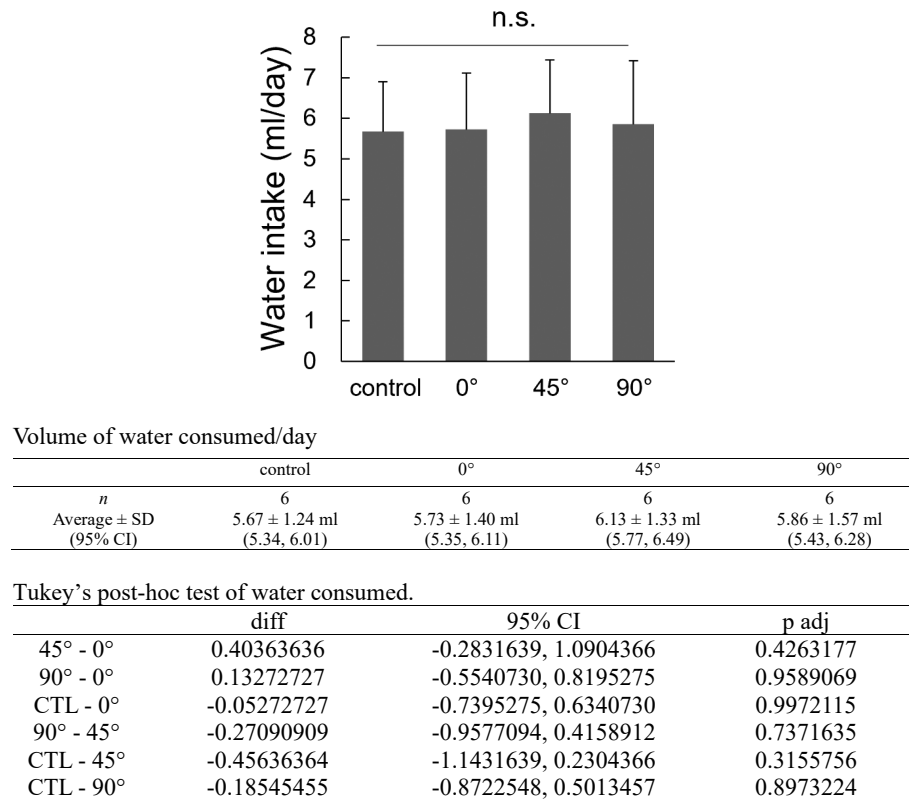
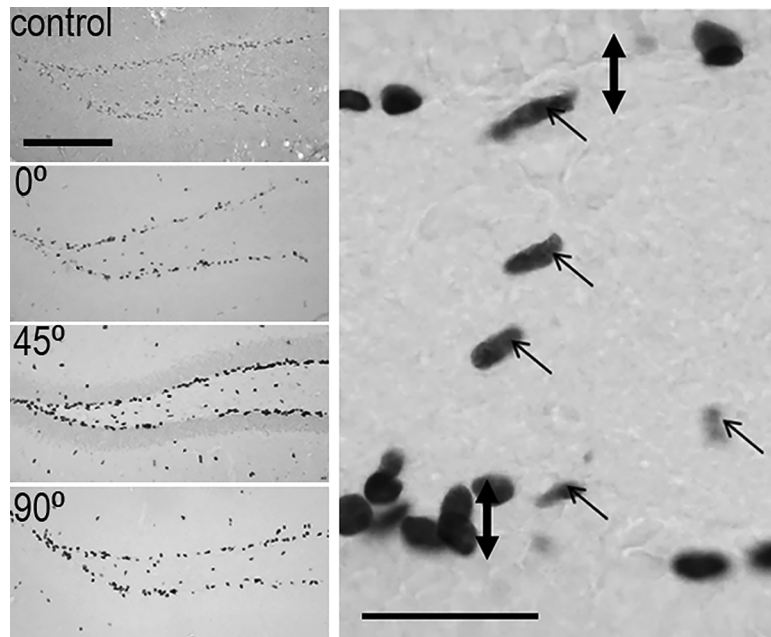


Fig. 2 Average number of passages by the mice throughout the 2-week experimental period. One-way ANOVA ( $p = 0.95$ ). n.s.: not significant, 95% CI: 95% confidential interval.



**Fig. 3** Volume of water consumed by the mice each day. One-way ANOVA ( $p = 0.32$ ).

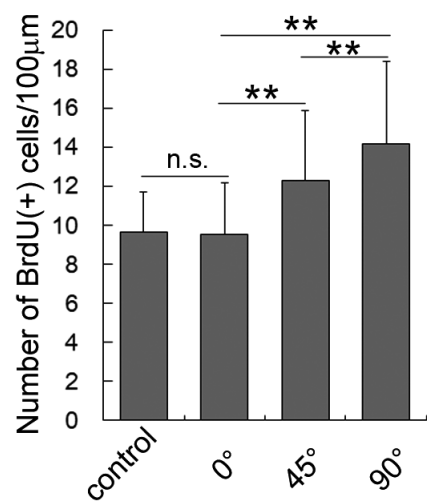


**Fig. 4** BrdU(+) cells in the subgranular zone (SGZ). The left panels show low magnification-microscopy images of BrdU(+) cells from the hippocampus of mice housed in each cage. Most BrdU(+) cells were located in the SGZ. Scale bar: 200  $\mu$ m. A high magnification image of the dentate gyrus (DG) is shown in the right panel. Small arrows indicate endothelial cells (flat-shaped nuclei) or glial cells (small nuclei). Double-head arrows indicate the SGZ. Scale bar: 30  $\mu$ m.

erable attention [17, 18]. For example, ambient temperature has been found to affect hippocampal neurogenesis, which was significantly enhanced in mice maintained at 6°C for 1 or 4 weeks (cold challenge) compared with hippocampal neurogenesis in mice maintained at 20°C [19]. It has also

been shown that running on a wheel at 4.5°C promotes hippocampal neurogenesis in mice, even if actual physical activity (distance and time) is reduced [20]. Under low ambient temperatures, the sympathetic nervous system is activated and metabolism is enhanced to maintain body temperature





Average number of BrdU(+) cells				
	control	0°	45°	90°
<i>n</i>	6	6	6	6
Average ± SD (95% CI)	9.64 ± 2.05 (9.14, 10.15)	9.53 ± 2.62 (8.98, 10.09)	12.32 ± 3.56 (11.61, 13.04)	14.20 ± 4.21 (13.24, 15.15)

Tukey's post-hoc test of number of BrdU(+) cells.			
	diff	95% CI	p adj
45° - 0°	2.7917877	1.554453, 4.029122	0.0000001
90° - 0°	4.6657468	3.354200, 5.977293	0.0000000
CTL - 0°	0.1079545	-1.260642, 1.476551	0.9970045
90° - 45°	1.8739590	0.591121, 3.156797	0.0010980
CTL - 45°	-2.6838332	-4.024943, -1.342723	0.0000025
CTL - 90°	-4.5577922	-5.967662, -3.147922	0.0000000

Fig. 5 Average number of BrdU(+) cells per 100 μm of the SGZ. One-way ANOVA ( $p = 7.01\text{e-}21$ ). \*\*:  $p < 0.01$ .

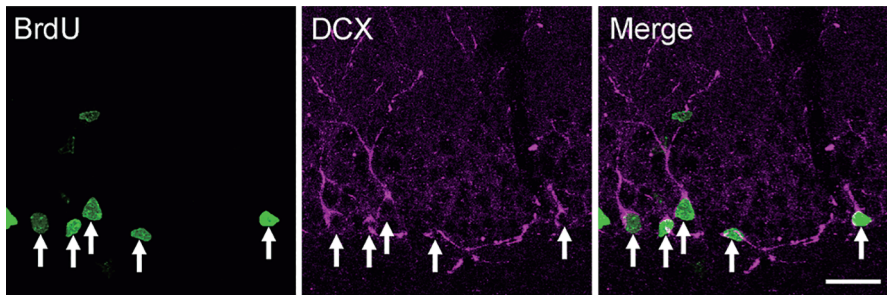


Fig. 6 BrdU and doublecortin (DCX) double-immunopositive cells in the SGZ. All BrdU(+) cells located in the SGZ were DCX(+) young neurons (arrows). Single optical confocal microscopy images are shown. Scale bar: 20 μm.

by consuming energy. On the other hand, decreasing metabolism reduces energy consumption. Obesity is an unbalanced condition between energy intake and consumption. An experiment involving obese experimental mice fed on a high-fat diet indicated a decrease in whole-body metabolism, as well as reduced oxygen consumption, glucose tolerance ability, and body temperature compared with the same parameters in control mice [21–23]. In obese experimental mice, hippocampal neurogenesis decreased [24, 25], but exercise ameliorated this decrease in neurogenesis [26]. Taken together, these studies, including the present study, indicate that energy consumption affects hippocampal neuro-

genesis.

One of the key molecules enhancing hippocampal neurogenesis through increased energy consumption could be the brain-derived neurotrophic factor (BDNF), a well-known neurogenic factor. It is important to note that cold challenge enhances the expression of BDNF in the hippocampus [19], while the expression level of BDNF is low in the hippocampus of obese animals [27, 28]. BDNF is mainly derived from brain tissue, but BDNF, as one of myokines, is released from skeletal muscles into the blood after exercise [17, 29, 30]. And physical activity can increase cerebral blood flow [31, 32]. Thus, physical activity should be an effective way to

enhance hippocampal neurogenesis.

Recently, it was shown that aerobic exercise can increase the volume of the human hippocampus and improve memory not only in younger people [33], but also in older people [34, 35]. Moreover, it is known that the expression level of BDNF, since it is a myokine, is upregulated in human skeletal muscles after exercise [30]. Thus, the present study suggests that an exercise regimen based on the MET-formula should be an effective way to maintain and improve hippocampal function in the adults.

## Conclusion

In conclusion, our study clearly showed that energy consumption is an element that enhances hippocampal neurogenesis. In the past, there was no clear definition of the intensity of voluntary activity in animal experiments; distance and time run on a running wheel do not provide accurate values, and the absolute value should be energy consumed depending on intensity of physical activity. Our new device offers a useful way to study the effects of physical activity on hippocampal neurogenesis and functioning in animal experiments.

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# 運動時の消費エネルギー量は 成体海馬ニューロン新生の鍵因子である

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4. びわこリハビリテーション専門職大学 リハビリテーション学部

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## 要旨

成体海馬におけるニューロン新生を促進する最も重要な要因は身体活動である。消費エネルギー量は身体活動量の基本尺度であるが、消費エネルギー量を用いてニューロン新生を解析した研究は見当たらない。そこで本研究では、成体マウスの飼育ケージに 50 cm 長の角度付きトンネルチューブを設置し、トンネル角度を変化させることで、登坂による消費エネルギー量と海馬ニューロン新生との関係を解析した。トンネル先端にはチミジンアナログの BrdU を含む給水瓶を設置し、2 週間の飼育期間後、新生細胞を組織化学的に検出した。どのトンネル角度の場合でも、走歩距離（トンネル通過回数）・飲水量（BrdU 摂取量）ともに有意差はなかった。2 週間の飼育期間中に新生した細胞数を、BrdU を指標に解析したところ、トンネル角度が大きくなるほど、BrdU 陽性ニューロン数が有意に多かった。また標識された BrdU 摂取細胞はニューロン前駆細胞に分化していることも確認した。トンネル長は一定であるため、トンネル角度増加に比例して登坂による消費エネルギーも増大する。本研究から、海馬ニューロン新生には、消費エネルギーが重要な因子であることが明らかになった。すなわち、消費エネルギーの大きい運動療法が、おそらくは myokine 等の因子を介して脳の機能維持に働く可能性を示唆している。