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ORIGINAL ARTICLE

Acute and long-term treadmill running differentially induce c-Fos expression in region- and time-dependent manners in mouse brain

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Abstract

Acute and long-term exercise differentially affect brain functions. It has been suggested that neuronal activation is one of the mechanisms for exercise-induced enhancement of brain functions. However, the differential effects of acute and long-term exercise on the spatial and temporal profiles of neuronal activation in the brain have been scarcely explored. In this study, we profiled the expression of c-Fos, a marker of neuronal activation, in selected 26 brain regions of 2-month-old male C57/B6 mice that received either a single bout of treadmill running (acute exercise) or a 4-week treadmill training (long-term exercise) at the same duration (1 h/day) and intensity (10 m/min). The c-Fos expression was determined before, immediately after, and 2 h after the run. The results showed that acute exercise increased the densities of c-Fos$^+$ cells in the ventral hippocampal CA1 region, followed by (in a high to low order) the primary somatosensory cortex, other hippocampal subregions, and striatum immediately after the run; significant changes remained evident in the hippocampal subregions after a 2-h rest. Long-term exercise increased the densities of c-Fos$^+$ cells in the striatum, followed by the primary somatosensory, primary and secondary motor cortices, hippocampal subregions, hypothalamic nuclei, and lateral periaqueductal gray; significant changes remained evident in the striatum, hippocampal subregions, hypothalamic nuclei, and lateral periaqueductal gray after a 2-h rest. Interestingly, the densities of c-Fos$^+$ cells in the substantia nigra and ventral tegmental area only increased after a 2-h rest after the run in the long-term exercise group. The densities of c-Fos$^+$ cells were positively correlated with the expression of brain-derived neurotrophic factor in the selected brain regions. In conclusion, both acute and long-term treadmill running at mild intensity induce c-Fos expression in the limbic system and movement-associated cortical and subcortical regions, with long-term exercise involving more brain regions (i.e., hypothalamus and periaqueductal gray) and longer lasting effects.

Keywords Exercise · Frequency · Neuronal activation · c-Fos · Brain pattern

Introduction

Physical activity induces a new dynamic equilibrium of the brain by structurally and functionally changing neural processing at multiple aspects (Holschneider et al. 2007).

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It has been demonstrated that running exercise increases synaptic and cerebrovascular plasticity (Cotman and Berchtold 2002), stimulates adult hippocampal neurogenesis (van Praag 2009), improves cognitive function (Young et al. 2015), attenuates neuroinflammation (Cotman et al. 2007), and decreases the risk of dementia (Ahlskog et al. 2011). The wide variety of physical and chemical factors induced by exercise enhances the overall brain health that helps to preserve neuronal functions and protect brain against neurodegeneration. The exercise-induced benefits could be mediated, at least, via two pathways: directly via enhancing local neuronal activity (Cotman and Engesser-Cesar 2002; Budde et al. 2016) and indirectly via increased secretion of beneficial factors into the circulation by peripheral organs such as muscle and liver (Wrann 2015; Carro et al. 2000). This study was designed to characterize the former pathway.

Repeated neuronal activation induces activity-dependent functional and structural changes in synapses, a process known as neuroplasticity (Flavell and Greenberg 2008). It has been demonstrated that the actions of brain-derived neurotrophic factor (BDNF) signaling pathways are involved in mediating the processes of neuroplasticity (Gomez-Pinilla et al. 2002; Grande et al. 2010), whereas neuronal activity regulates the transcription, transport, and secretion of BDNF. In addition to neuroplasticity, BDNF has also been suggested to play critical roles in exercise-induced beneficial responses in the brain, such as adult hippocampal neurogenesis (Liu and Nusslock 2018) and neuroprotection (Cotman and Engesser-Cesar 2002). These findings suggest that repeated neuronal activation is involved in the exercise-induced modification in the brain. However, the effects of exercise on the brain regional and temporal changes in neuronal activity have never been systemically characterized.

The objective of this study was to characterize the regional and temporal profiles of the exercise-induced neuronal activation in the brains of mice. To accurately control the duration and intensity of exercise, we adapted forced treadmill running as the exercise protocol. The duration and intensity of exercise were set at 1 h/day and 10 m/min, a mild-intensity exercise (Okamoto et al. 2015) known to induce hippocampal neuroplasticity (Tsai et al. 2018a) and neurogenesis (Wu et al. 1985). We used the expression of NeuN, a marker of neuronal cells, to label the neurons and the expression of c-Fos, an immediate-early gene widely accepted as a marker for neuron activation (Clark et al. 2011; Jee et al. 2008), to label the activated neurons. The regional profiles of expression of c-Fos were determined in selected 26 regions throughout the brain. To obtain the temporal profiles of c-Fos expression, the mice were killed before, immediately after, and 2 hours after the treadmill run. Because acute and long-term exercise differentially affect physical and mental functions (Whyte and Laughlin 2010; Hopkins et al. 2012), mice were further divided into acute (a 1-day single bout) and long-term (4 weeks, 5 days/week) exercise group. Finally, because BDNF is known to contribute to the exercise-enhanced brain functions (Cotman and Berchtold 2002) and its expression is regulated by neuronal activity (Liu and Nusslock 2018), the relationship between expressions of c-Fos and BDNF was also determined.

Materials and methods

Animals

All experiments were done in accordance with the National Institute of Health Guideline for Animal Research (Guide for the Care and Use of Laboratory Animals) and approved by the National Cheng Kung University Institutional Animal Care and Use Committee (ethical approval reference number: 104272). Male C57BL/6 N mice at 6 weeks of age were housed five per cage under a 12-h light/12-h dark cycle (lights on at 8 AM) and at constant temperature (25 °C) and humidity in a controlled room at the National Cheng Kung University Animal Center. The mice were given free access to food and water. A total of 30 mice were used and randomly divided into 6 groups (5 mice/group) using the random number generator in Excel (also known as computer-based randomization). The details of grouping are described in the next section.

Treadmill running

The exercise training was performed from 6 pm to 8 pm, the last 2 h of light cycle to harmonize with mouse circadian activity patterns (Yasumoto et al. 2015). To minimize the confounding effect induced by novel environments, each mouse was assigned to run in the same lane of the treadmill during the whole exercise program.

The 6-week-old mice were daily handled for 1 week to reduce handling stress. In the next week (familiarization phase), all of the mice were trained to run on a level, motor-driven treadmill (Model T408E; Diagnostic & Research Instruments Co., Taoyuan, Taiwan) for 10 min/day, 5 day/week to reduce their environmental and training stress. The running speed was set at 8 m/min. The distal end of the runway was shielded from light to attract the mice to run forward, while the proximal end was filled with sponges to stop the mice. No electric shock was given throughout the entire experiment. Two days after the end of familiarization phase, mice were randomly divided to the 1-day single-bout (acute exercise) or the 4-week (long-term exercise) groups. Each group contained 15 mice. After 1 week of handling and 1 week of familiarization, the mice were 8 weeks old.

In the acute exercise group, 15 mice were further randomly divided into 3 groups: 1) without exercise (Basal), 2) exercise for 1 h (E1h), and 3) exercise for 1 h and rest at home cage for 2 h (E1hR2h). The five mice of the Basal group were sacrificed on the experimental day without running. The other ten mice were forced to run for 60 min at a speed of 10 m/min. Five mice were sacrificed immediately after the 1-h run by decapitation (E1h group), while the other five mice were sacrificed after a 2-h rest (E1hR2h group).

In the long-term exercise group, all 15 mice ran at a speed of 10 m/min for 20–60 min/day (an increment of 10 min/day), 5 day/week (weekends off) for the 1st week, followed by 60 min/day at the same speed, 5 day/week (weekends off) for the next 3 weeks. The last 1-h run was given on the following Monday. Similar to the design of acute exercise study, five mice were sacrificed before the last 1-h run (long-term Basal group), five mice were sacrificed immediately after the last 1-h run (long-term E1h), and five mice were sacrificed 2 h after the run (long-term E1hR2h).

Brain specimen preparations

Mice were sacrificed by decapitation and their brains were quickly removed and divided into two hemispheres. The left hemisphere was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 2 days at room temperature. The brain specimens were then dehydrated in graded sucrose solutions (10%, 20%, 30%, and 35%, dissolved in 0.1 M phosphate buffer), embedded with frozen section media (Code no.: 3801480, Leica Biosystems, Wetzlar, Hessen, Germany), and sliced into 25-μm thickness coronal sections using a cryomicrotome. The right hemisphere was used for Western blot. Eight brain regions (M1 cortex, S1 cortex, dorsal hippocampus, ventral hippocampus, amygdala, striatum, thalamus and hypothalamus) were rapidly dissected out, immersed in liquid nitrogen, and homogenized in ice-cold commercial tissue protein extraction reagent (78510; Thermo Fisher Scientific Inc., Waltham, MA, USA) containing protease and phosphatase inhibitors (04693116001 and PHOSS-RO; Roche Diagnostics, Mannheim, Germany). The homogenates were centrifuged at 13,000*g* for 15 min at 4 °C, and the protein concentrations of the supernatants were determined and adjusted to the same concentration for Western blot.

Immunohistochemistry

The 25-μm brain sections containing interested regions were washed with phosphate-buffered saline containing 0.3% Triton X-100 to remove the embedding frozen section media, immersed in 3% H_2O_2 to abolish endogenous peroxidase activity, and blocked with 3% normal goat serum for 1 hour at room temperature. For NeuN and c-Fos double staining, the sections were first probed with primary antibodies against NeuN (1:1000 dilution; MAB377, Merck Millipore, Darmstadt, Germany) for 24 h at 4 °C followed by HRP-conjugated goat anti-mouse IgG (1:1000 dilution; 115-035-166, Jackson ImmunoResearch Laboratories Inc., PA, USA) for 2 h at room temperature. 3,3′-diaminobenzidine tetrahydrochloride hydrate was used as a chromogen for detecting the antigen–antibody complex. Then, the sections were further probed with antibodies against c-Fos (1:500 dilution; sc-52, Santa Cruz Biotechnology, CA, USA) for 48 h at 4 °C, incubated with HRP-conjugated goat anti-rabbit IgG (1:500 dilution; 111-035-144, Jackson ImmunoResearch Laboratories Inc.) for 2 h at room temperature, and reacted with nickel-enhanced 3,3′-diaminobenzidine tetrahydrochloride hydrate. For c-Fos single staining, the sections were blocked, hybridized with primary and secondary antibodies, and reacted with chromogen as aforementioned. The reliability of the selected c-Fos antibody (sc-52, Santa Cruz Biotechnology) has been validated using two other commercially available c-Fos antibodies (#1: ab190289, Abcam, Cambridge, UK; #2: HPA018531, Sigma-Aldrich, St. Louis, MO, USA). The primary antibody was omitted for the detection of nonspecific bindings.

Counting c-Fos immunoreactive cells

The densities of c-Fos immunoreactive (c-Fos$^+$) cells were examined in 26 brain regions, which could be clearly identified in 7 brain sections (Fig. 1; Table 1). According to their stereotaxic coordinates of mouse brain with bregma as the reference point (Paxinos and Franklin 2004), these brain regions, in an anterior–posterior order, were the nucleus accumbens (NAc) at + 0.86 mm; the primary motor cortex (M1), secondary motor cortex (M2), cingulate cortex (Cg), striatum, and septal nucleus (Sep) at + 0.62 mm; the primary somatosensory cortex (S1), dorsal hippocampal CA1 [CA1(D)], dorsal hippocampal CA2/3 [CA2/3 (D)], dorsal hippocampal dentate gyrus [DG(D)], thalamus (TH), amygdala (Amg), piriform cortex (Pir), lateral part of hypothalamus (LH), dorsal medial part of hypothalamus (DMH), and ventral medial part of hypothalamus (VMH) at − 1.82 mm; the substantia nigra (SN) and ventral tegmental area (VTA) at − 3.08 mm; the ventral hippocampal CA1/subiculum [CA1(V)/subiculum], ventral hippocampal CA2/3 [CA2/3 (V)], and ventral hippocampal dentate gyrus [DG(V)] at − 3.40 mm; the superior colliculus (Sc), lateral periaqueductal gray (LPAG), dorsal raphe nucleus (DR), and pontine nucleus (Pn) at − 4.36 mm; and the molecular layer of the anterior lobe of cerebellum (Cb-A) at − 5.68 mm. Therefore, for any given selected region, one section from each mouse was used for c-Fos$^+$ cell counting. The images (900 × 670 μm) of interested brain regions were captured using an AxiocamMRc digital camera (Carl Zeiss, Oberkochen, Germany) driven by the Axiovision 4.8 software (Carl Zeiss) and outlined on each section (Fig. 2a). The outlined area of a selected brain region on the sections of each mouse was stacked (Fig. 2b) and the overlapping area shared by all sections (minimum outline) was used to count the c-Fos$^+$ cells in each section (Fig. 2c). The c-Fos$^+$ cells were counted with the aid of ImageJ software (version 1.49 k, NIH, Bethesda, MD, USA). A consistent signal (background) threshold was set and only the over-threshold signals were counted as the c-Fos$^+$ cells. The selected seven sections from each mouse were at least 200 μm apart to avoid counting the same cell more than once. Within each selected brain region, a plugin of ImageJ software (cell counter) was used to assist initial marking of c-Fos$^+$ cells. The marked cells were examined by a researcher for the final approval.

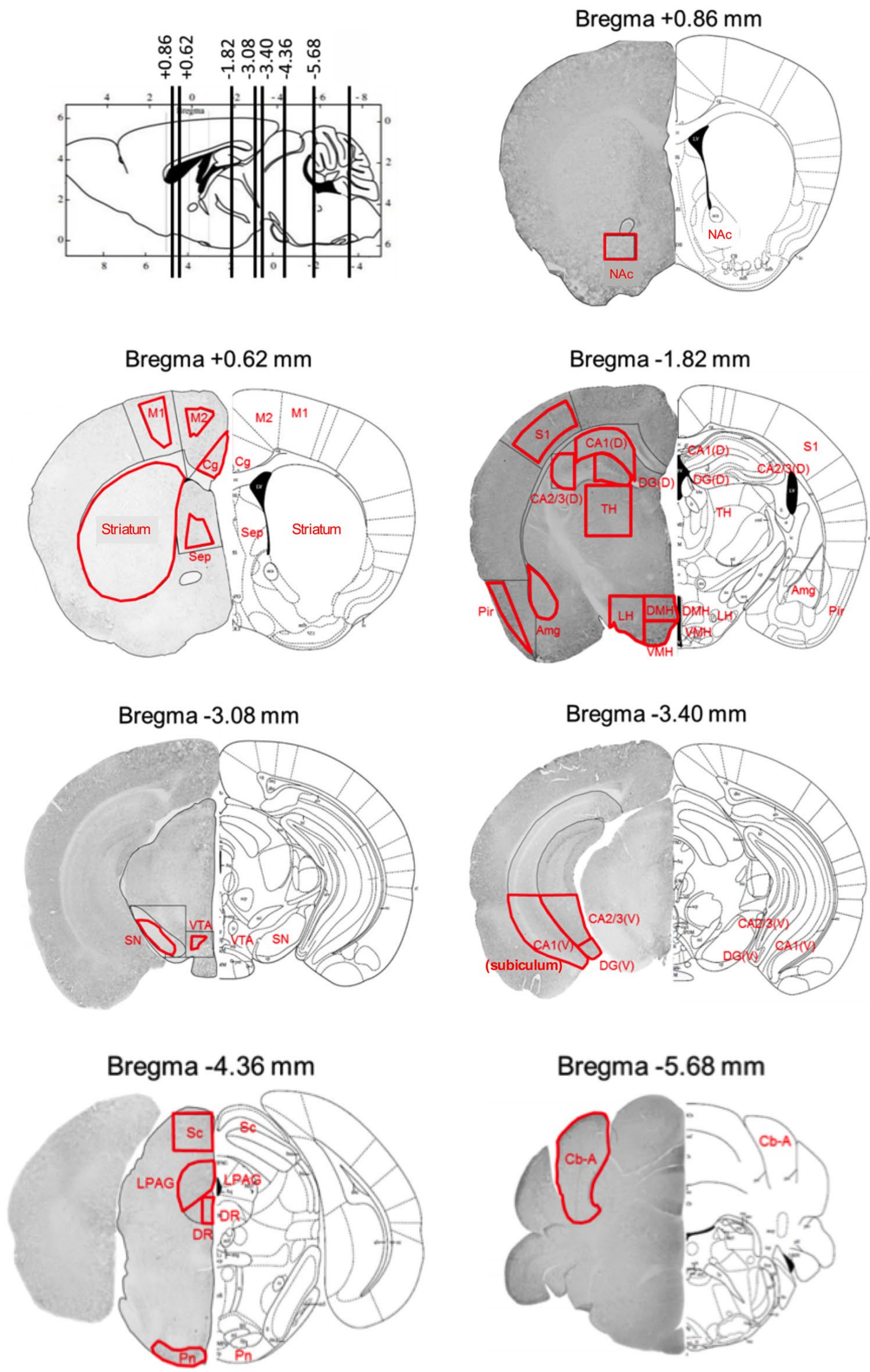

Fig. 1 Stereotaxic unit of mouse brain. Representative diagrams show the selected 26 interested brain regions (framed in red) to quantify the densities of c-Fos^{+} cells

Western blot

The detailed protocol for Western blot has been described elsewhere (Tsai et al. 2018b). In short, relative BDNF levels (versus β-actin) were estimated by immunoblotting using the primary antibody against BDNF (1:500 dilution, sc-546; Santa Cruz Biotechnology) and the HRP-conjugated goat anti-rabbit IgG (1:500 dilution; 111-035-144, Jackson ImmunoResearch Laboratories Inc.). The bound antibodies were detected using an enhanced chemiluminescence detection kit (PerkinElmer, Boston, MA, USA). Band densities were measured using an imaging system (BioChemi; UVP,

Table 1 Stereotaxic coordinates of the selected brain regions

Distances from bregma reference point (mm)	Selected brain regions
+ 0.86	NAc
+ 0.62	M1, M2, Cg, Sep, and striatum
− 1.82	S1, CA1(D), CA2/3(D), DG(D), TH, DMH, VMH, LH, Amg, and Pir
− 3.08	VTA and SN
− 3.40	CA1(V)/subiculum, CA2/3(V), and DG(V)
− 4.36	Sc, LPAG, DR, and Pn
− 5.68	Cb-A

NAc nucleus accumbens, *M1* primary motor cortex, *M2* secondary motor cortex, *Cg* cingulate cortex, *Sep* septal nucleus, *S1* primary somatosensory cortex, *CA1(D)* dorsal hippocampal CA1, *CA2/3(D)* dorsal hippocampal CA2/3, *DG(D)* dorsal hippocampal dentate gyrus, *TH* thalamus, *DMH* dorsomedial part of hypothalamus, *VMH* ventromedial part of hypothalamus, *LH* lateral part of hypothalamus, *Amg* amygdala, *Pir* piriform cortex, *VTA* ventral tegmental area, *SN* substantia nigra, *CA1(V)/subiculum* ventral hippocampal CA1/subiculum, *CA2/3(V)* ventral hippocampal CA2/3, DG(V) ventral hippocampal dentate gyrus, *Sc* superior colliculus, *LPAG* lateral periaqueductal gray, *DR* dorsal raphe nucleus, *Pn* pontine nucleus, *Cb-A* the molecular layer of the anterior lobe of cerebellum

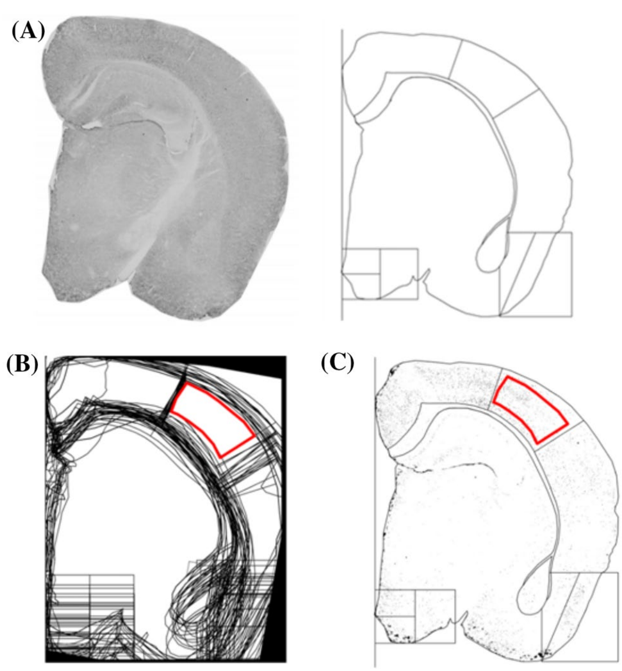

Fig. 2 Procedure of assigning a brain region to calculate the density of c-Fos$^+$ cells. **a** The contour of the selected immunohistochemical micrograph (left panel) and interested brain regions was outlined (right panel) according to the mouse brain atlas. **b** The outlines of every mouse brain sections were stacked to obtain the minimum overlapping area of the interested brain region. **c** The minimum overlapping area was applied on the brain sections containing the interested regions, and then the c-Fos$^+$ cells in the minimum overlapping area were counted for calculating the density of c-Fos$^+$ cells

Upland, CA, USA) and analyzed using ImageJ software (version 1.49 k, NIH). The quantified densities of bands were normalized with a band density gained form the same protein specimen which was extracted from a whole brain lysate and applied to every blot.

Statistical analysis

Results are presented as mean ± SEM. Significance was set at $p < 0.05$. One-way ANOVAs followed by Tukey's multiple comparisons were used to analyzed the densities of c-Fos$^+$ cells in the different brain regions of Basal, E1h and E1hS2h mice in both acute and long-term exercise groups. The correlation between the densities of c-Fos$^+$ cell and the levels of BDNF in the selected regions was analyzed by Pearson correlation.

Results

NeuN/c-Fos immunohistochemistry

Immunohistochemical double staining of NeuN and c-Fos showed that almost all (> 99%) of the c-Fos signals were presented within the NeuN$^+$ cells (Fig. 3), suggesting that c-Fos was primarily expressed in the neurons. In subsequent experiments, single staining of c-Fos was used to perform cell counting to avoid interference from other stained signals.

Effect of a single-bout acute treadmill running on spatial and temporal profiles of c-Fos expression in the brain

The effect of exercise on neuronal activation, determined by the densities of c-Fos$^+$ cells, was evaluated in 26 regions throughout the brain. Based on their anatomic locations, the 26 regions were grouped into 6 main brain areas, including the cortex, limbic systems, basal ganglia, diencephalon, brainstem and cerebellum. One-hour (E1h) acute exercise increased the densities of c-Fos$^+$ cells in multiple brain areas (Table 2, E1h vs. Basal), including the M2 and S1 in the cortex (Figure S1), CA1(D), DG(D), CA1(V)/subiculum, CA2/3(V), DG(V), and Sep in the limbic systems (Figure S2-5), striatum in the basal ganglia (Figure S5), and the Cb-A region (Figure S9). The densities of c-Fos$^+$ cells in the selected areas of the diencephalon and brainstem were unchanged after the 1-h acute exercise (Figure S6-9). After a 2-h rest (E1hR2h), the densities of c-Fos$^+$ cells in three hippocampal subregions [DG(D), CA1(V)/subiculum, and DG(V)] remained significantly higher than those of the Basal group, while other areas resumed to basal levels (Table 2, E1hR2h vs. Basal).

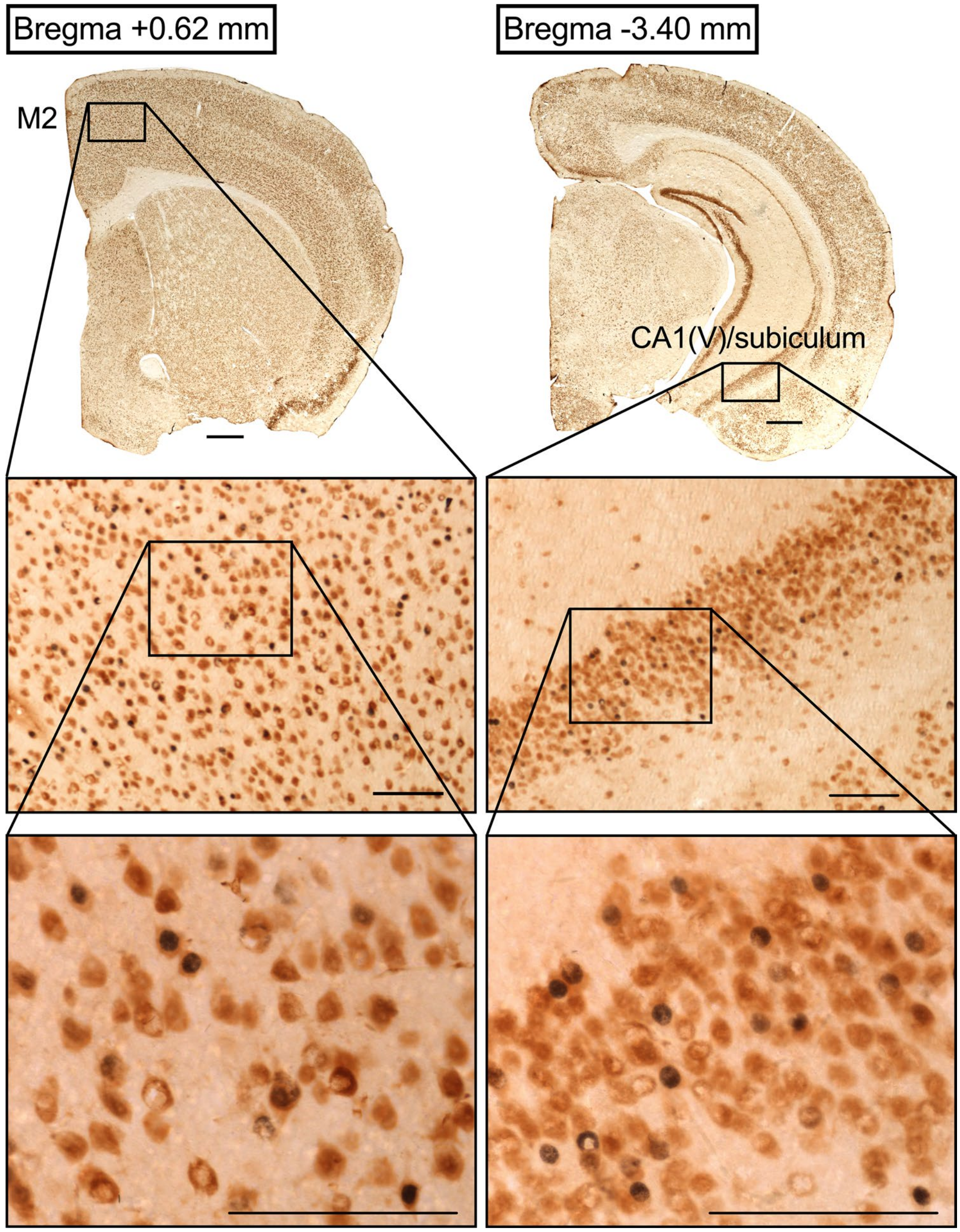

Fig. 3 Representative micrographs of c-Fos/NeuN double immunostaining in the brain. Left panels: M2 region; right panels: CA1(V)/subiculum region. The boxes in the upper panels are enlarged in the respective lower panels. The brown and deep purple signals indicate the $NeuN^+$ and $c\text{-}Fos^+$ cells, respectively. Scale bar: 500 µm in the upper panels; 200 µm in the middle and lower panels

Effect of long-term treadmill running on spatial and temporal profiles of c-Fos expression in the brain

Long-term exercise induced expression of c-Fos in more brain areas than those of the acute exercise group (Table 3, E1h vs. Basal). Compared to the long-term Basal group, the densities of $c\text{-}Fos^+$ cells in the M1, M2, and S1 in the cortex (Figure S1), CA1(D), CA1(V)/subiculum, CA2/3(V), Cg, and Sep in the limbic systems (Figure S2-5), striatum in the basal ganglia (Figure S5), TH, VMH, and LH in the diencephalon, LPAG in the brainstem (Figure S5-8), and Cb-A in the cerebellum (Figure S9) were increased in the E1h group. Two hours after the end of running, the densities of $c\text{-}Fos^+$ cells remained significantly increased in several brain areas (Table 3, E1hR2h vs. Basal), including the CA1(V)/subiculum and CA2/3(V) in the limbic systems, striatum in the basal ganglia, VMH and LH in the diencephalon, and LPAG in the brainstem. Furthermore, the expression of c-Fos in some regions showed a delayed pattern. Compared to the Basal group, changes in the densities of $c\text{-}Fos^+$ cells in the Pir, DMH, SN and VTA did not reach a significant level until 2 h after the run.

Long-term exercise did not dramatically alter the basal expression of c-Fos in the 26 selected brain regions. Comparing the densities of $c\text{-}Fos^+$ cells at basal level in the acute exercise group (Basal in Table 2) and the long-term exercise group (Basal in Table 3), the DG(V) was the only region that

Table 2 Quantitative results (mean ± SD) of the densities of c-Fos$^+$ cell (number/mm^2) in selected brain regions of mice received a single-bout acute treadmill exercise

	Basal	E1h	E1hR2h
Cortex			
M1	26.6 ± 23.7	62.2 ± 60.8	14.7 ± 4.7
M2	94.1 ± 87.7	269.7 ± 119.1*	109.4 ± 41.1
S1	53.9 ± 51.7	703.9 ± 245.4****	126.5 ± 41.3
Limbic systems			
CA1(D)	13.4 ± 16.5	71.1 ± 40.4**	12.5 ± 2.1
CA2/3(D)	27.7 ± 19.1	92.2 ± 63.4	41.5 ± 15.3
DG(D)	59.0 ± 44.2	238.6 ± 99.1**	185.4 ± 21.2**
CA1(V)/subiculum	1.2 ± 1.1	23.6 ± 14.2*	28.7 ± 11.5**
CA2/3(V)	13.4 ± 10.4	72.9 ± 45.9*	43.7 ± 15.2
DG(V)	12.6 ± 3.0	94.1 ± 25.5****	58.2 ± 17.8**
Cg	397.8 ± 257.7	754.4 ± 478.3	518.6 ± 262.2
Sep	119.2 ± 92.1	291.3 ± 95.0*	186.4 ± 76.6
Amg	43.4 ± 13.1	70.1 ± 26.7	47.8 ± 26.1
Pir	179.5 ± 110.6	248.6 ± 157.6	125.5 ± 106.9
Basal ganglia			
Striatum	16.6 ± 19.8	86.9 ± 43.5**	39.8 ± 14.5
NAc	54.4 ± 37.0	38.7 ± 12.7	52.2 ± 18.3
Diencephalon			
TH	6.3 ± 4.7	27.9 ± 25.6	13.0 ± 6.3
DMH	109.8 ± 82.7	193.3 ± 88.1	286.6 ± 125.5
VMH	170.8 ± 209.8	119.9 ± 41.7	118.4 ± 81.4
LH	90.2 ± 67.8	90.4 ± 33.8	119.2 ± 28.8
Brainstem			
SN	13.0 ± 11.0	44.6 ± 50.6	32.9 ± 13.2
VTA	86.4 ± 89.2	132.2 ± 90.6	212.3 ± 148.0
Sc	190.1 ± 112.0	280.3 ± 123.4	283.3 ± 72.6
LPAG	153.7 ± 60.1	154.6 ± 66.6	214.4 ± 45.2
DR	226.7 ± 150.0	301.6 ± 97.8	279.3 ± 86.2
Pn	576.6 ± 510.0	269.4 ± 208.7	381.9 ± 545.0
Cerebellum			
Cb-A	1588.3 ± 224.4	2716.8 ± 356.9*	2046.7 ± 477.8

Basal: without exercise; E1h: immediately after the 1-h run; E1hR2h: rest 2 h after the 1-h run

*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, vs. Basal group, Tukey post hoc test followed by one-way ANOVA

Table 3 Quantitative results (mean ± SD) of the densities of c-Fos$^+$ cell (number/mm^2) in the selected brain regions of mice received long-term treadmill exercise training

	Basal	E1h	E1hR2h
Cortex			
M1	15.8 ± 9.0	196.0 ± 154.4*	48.8 ± 37.6
M2	56.0 ± 40.0	609.4 ± 362.9**	199.2 ± 150.8
S1	74.5 ± 115.2	944.1 ± 563.3**	366.0 ± 173.8
Limbic systems			
CA1(D)	16.0 ± 13.3	145.2 ± 79.9**	53.2 ± 20.8
CA2/3(D)	41.0 ± 24.1	176.2 ± 135.1	149.8 ± 62.1
DG(D)	109.5 ± 32.7	191.7 ± 63.1	144.0 ± 55.7
CA1(V)/subiculum	3.4 ± 3.9	30.2 ± 13.5**	28.7 ± 13.9*
CA2/3(V)	9.3 ± 7.5	55.4 ± 35.4*	81.6 ± 20.4**
DG(V)	43.2 ± 21.1	95.7 ± 66.9	74.5 ± 40.2
Cg	108.7 ± 118.7	894.4 ± 206.2****	289.7 ± 171.7
Sep	125.0 ± 125.1	453.1 ± 172.8*	333.8 ± 174.9
Amg	51.6 ± 34.5	79.3 ± 69.6	46.9 ± 25.5
Pir	169.3 ± 83.4	355.4 ± 224.7	447.4 ± 108.7*
Basal ganglia			
Striatum	3.7 ± 2.2	101.9 ± 16.6****	38.6 ± 17.3**
NAc	59.5 ± 48.4	128.6 ± 51.5	101.8 ± 65.9
Diencephalon			
TH	33.9 ± 27.2	92.5 ± 35.3*	63.5 ± 30.6
DMH	101.6 ± 62.8	308.5 ± 154.2	350.8 ± 93.0*
VMH	45.8 ± 21.8	212.2 ± 54.9**	154.5 ± 69.5*
LH	34.7 ± 25.8	119.8 ± 35.4*	132.7 ± 49.3**
Brainstem			
SN	3.4 ± 3.6	42.9 ± 29.7	68.3 ± 56.2*
VTA	26.5 ± 18.0	116.4 ± 90.4	164.2 ± 90.4*
Sc	125.9 ± 105.6	384.0 ± 234.8	377.8 ± 110.6
LPAG	84.1 ± 66.1	247.2 ± 100.5*	230.3 ± 42.2*
DR	70.4 ± 66.9	499.2 ± 458.9	393.6 ± 200.4
Pn	176.5 ± 232.4	420.2 ± 477.3	505.9 ± 542.2
Cerebellum			
Cb-A	1642.1 ± 252.2	2490.7 ± 320.7***	1738.6 ± 229.3

Basal: without the last 1-h run; E1 h: immediately after the last 1-h run; E1hR2 h: rest 2 h after the last 1-h run

*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, vs. Basal group, Tukey post hoc test followed by one-way ANOVA

showed a significant increase in the density of c-Fos$^+$ cells ($p = 0.012$) among the 26 selected brain regions.

Comparing treadmill running-induced c-Fos expression in different brain regions

In the single-bout acute running study, the brain region with the most pronounced degree of changes in c-Fos$^+$ cell density was the CA1(V)/subiculum, followed by the S1, DG(V), CA2/3(V), CA1(D), striatum, DG(D), M2, Sep and Cb-A [Fig. 4a, Acute (E1h/Basal)]. After a 2-h rest, the degree of changes in c-Fos$^+$ cell density remained prominent in the CA1(V)/subiculum [Fig. 4a, Acute (E1hR2h/Basal)].

In the long-term running study, the brain region with the highest degree of changes in c-Fos$^+$ cell density was the striatum, followed by the S1, M1, M2 and other regions in the limbic systems, diencephalon, brainstem, and cerebellum [Fig. 4b, Long-term (E1h/Basal)]. Two hours later, the highest degree of changes in c-Fos$^+$ cell density occurred in the SN [Fig. 4b, Long-term (E1hR2h/Basal)]. The CA2/3(V),

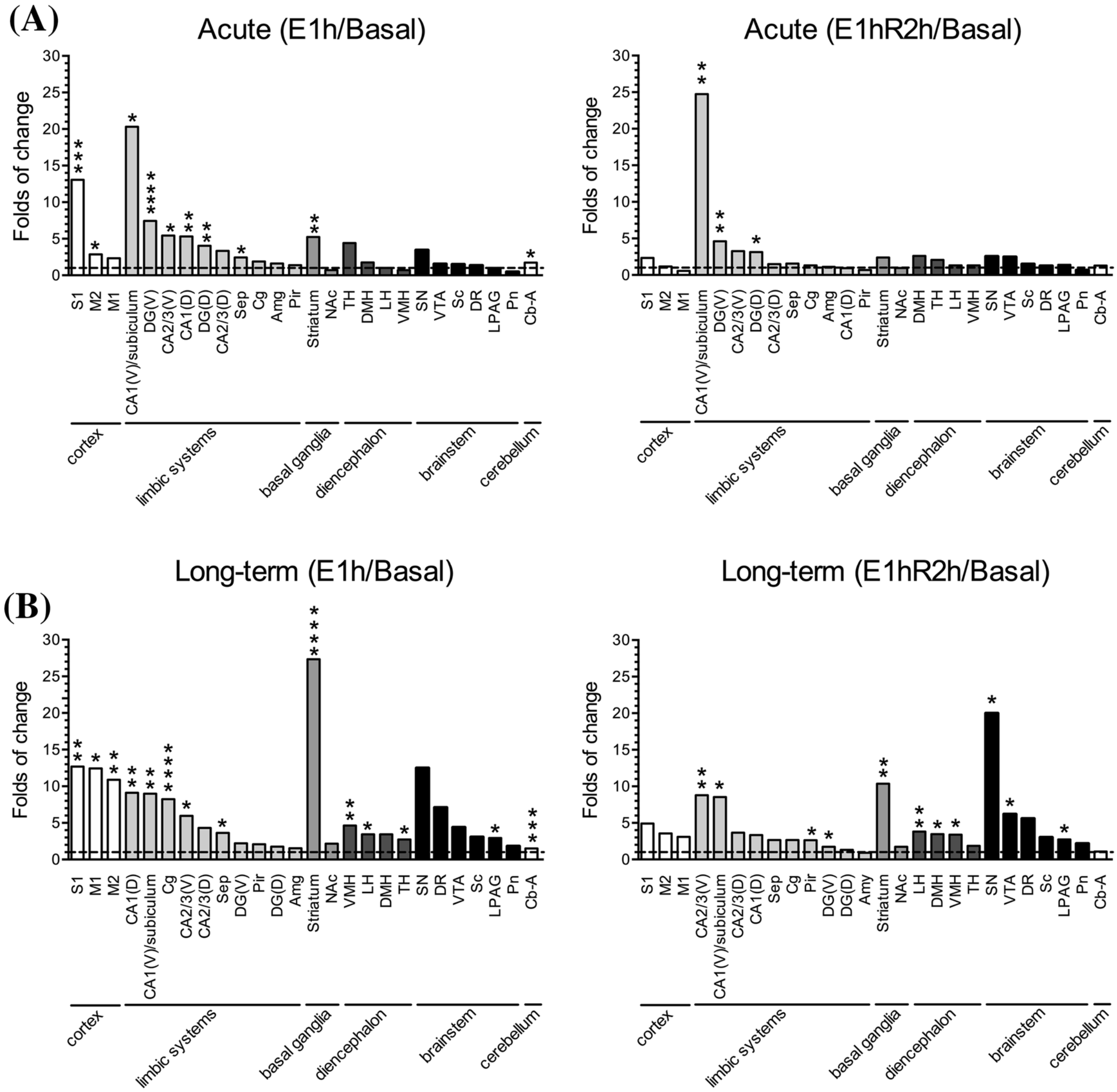

Fig. 4 Folds of change in the densities of c-Fos$^+$ cells in the 26 selected brain regions of mice. **a** Quantified results of the acute exercise group. **b** Quantified results of the long-term exercise group. Folds of change was calculated by dividing the densities of c-Fos$^+$ cells immediately after the 1-h treadmill run (E1h) or the densities of c-Fos$^+$ cells 2 h after the 1-h treadmill run (E1hR2h) by the densities of c-Fos$^+$ cells before the run. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, vs. Basal group, Tukey post hoc test followed by one-way ANOVA

CA1(V)/subiculum, striatum, and VTA also showed high degrees (> fivefold) of changes in the c-Fos$^+$ cell densities. Furthermore, the expression of c-Fos in several nuclei of the hypothalamus and brainstem was shown to be sustained after long-term treadmill running.

Relationships between the expressions of c-Fos and BDNF

The relative expressions of BDNF in the eight regions (M1 cortex, S1 cortex, dorsal hippocampus, ventral hippocampus, amygdala, striatum, thalamus and hypothalamus) of mice before (Basal), immediately after (E1h), and 2 h after (E1hR2h) the acute 1-h running exercise were measured by Western blot. Pearson correlation analysis of these 24 data points (8 brain regions × 3 time points) revealed a positive correlation between the densities of c-Fos$^+$ cells and the BDNF levels in these brain regions (Fig. 5a). A correlation analysis between changed levels of BDNF and changed densities of c-Fos$^+$ cells (E1h—Basal & E1hR2h—Basal) was also performed. The changed levels were defined by the differences between the average levels (5 mice) of the E1 h and Basal groups, as well as E1hR2 h and Basal groups. The results showed a positive correlation between the exercise-related changes in the densities of c-Fos$^+$ cells and the levels of BDNF (Fig. 5b).

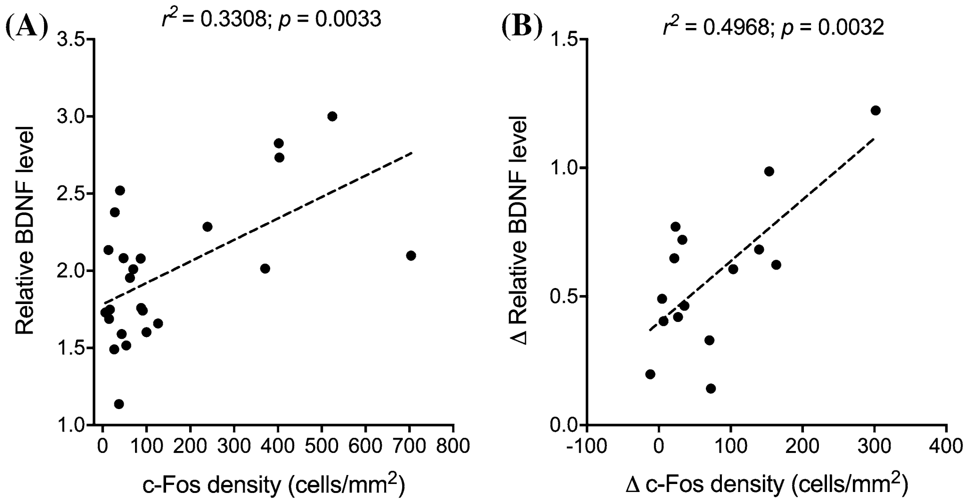

Fig. 5 Correlations between the expressions of c-Fos and BDNF in brains of mice before (Basal), immediately after (E1h), and 2 h after (E1hR2 h) the acute 1-h running exercise. **a** Correlation between the densities of c-Fos$^+$ cells and the BDNF levels in eight brain regions of three time points. Each data point represents an average of five mice. **b** Correlation between the changed densities of c-Fos$^+$ cells and the changed levels of BDNF in eight brain regions. The changed levels are defined by the differences between the E1 h and Basal groups, as well as the E1hR2 h and Basal groups. Pearson r^2 and p values are shown in the figures

Discussion

This study was designed to characterize the regional and temporal changes of c-Fos expression in the brains of mice after a single-bout (acute) and a 4-week (long-term) treadmill run. Our results demonstrate that acute 1-h treadmill running induces c-Fos expression in multiple brain regions within the cortex, limbic systems, basal ganglia, and cerebellum. The exercise-induced c-Fos expression in majority of brain regions resume to basal levels after a 2-h rest, with the exception that the c-Fos expression in the ventral hippocampus remains high even 2 h after the run. Interestingly, acute and long-term treadmill run, at the same intensity (10 m/min) and duration (60 min each run) induce different patterns of expression of c-Fos. In the acute exercise group, the most prominent change in c-Fos expression is the ventral hippocampus, while in the long-term exercise group the striatum has the highest fold of change. Furthermore, expression of c-Fos in the hypothalamus is evident immediately after long-term exercise, while expression of c-Fos in the SN and VTA are only noticed 2 h after the long-term exercise run. These results suggest that the effect of exercise can be accumulated, since long-term exercise has wider (regional) and longer (temporal) impacts on the brain than those of acute exercise.

Physical activity is known to induce structural and functional changes in the central motor control systems, such as the motor cortex (Adkins et al. 1985; Holschneider et al. 2007), basal ganglia (Holschneider et al. 2007; Herrero et al. 2002) and cerebellum (Marques-Aleixo et al. 2015; Cui et al. 2009; Isaacs et al. 1992). The basal ganglia are a group of nuclei of basal forebrain and midbrain involved in the control of motor, cognitive and memory assisting functions (Bolam et al. 2000). The major input to the basal ganglia is projecting from the cerebral cortex, including the supplementary motor area, primary motor and premotor cortices. The cortical information is processed in the striatum and passed via direct and indirect pathways to the output nuclei of the basal ganglia. Subsequently, the output nuclei of the basal ganglia send the signals back to the cortex and then influence the behavioral outputs (Bolam et al. 2000; Smith et al. 1998). These neural circuits are indispensable for the control of voluntary movements (Nambu 2004). The somatosensory cortex is also important to motor functions (Porro et al. 1996). When the motor cortex is impaired, the motor function of the somatosensory cortex becomes predominant and can compensate for the dysfunctions of the motor cortex (Sasaki and Gemba 1984). The cerebellum deeply integrates into the major loop with the cerebral cortex, brainstem, and spinal cord, and takes part in motor control, coordination and learning (D'Angelo 2018). Several human and animal studies have suggested that physical activities generally improve motor functions via modulating cerebellum structures and functions. For example, repeated running induces angiogenesis in the cerebellum (Black et al. 1990), improves the motor performance and prevents the decay of cerebellum cells under various pathological conditions (Huang et al. 1985; Uhlendorf et al. 2011). Therefore, neuronal activation in the cortex (i.e., S1, M1, and M2), basal ganglia (i.e., striatum and SN) and cerebellum after the 1-h treadmill running are expected and well in line with the central motor control systems.

The hippocampus is one of the most sensitive brain regions to the treadmill running stimulations. In agreement with our findings, Rhodes et al. also showed that a 7-day voluntary wheel running induced c-Fos expression in the DG and CA2/3 of the hippocampus and in the sensory cortex (Rhodes et al. 2003). The dorsal hippocampus plays a key role in spatial learning and memory, whereas the ventral hippocampus is preferentially involved in emotional processing, such as stress and depression regulations (Nishijima et al. 2013). It is known that exercise training benefits the hippocampal structure and function, including promoting neuroplasticity (Cotman and Berchtold 2002), improving executive function and spatial memory (Liu et al. 2009; Lin et al. 2012; Byun et al. 2014; Yanagisawa et al. 2010), enhancing adult hippocampal neurogenesis (van Praag et al. 1999; Okamoto et al. 2012), protecting brain from aging-associated adverse effects (Tsai et al. 2018a; Lin et al. 2015), and exerting anti-depression effects (Duman et al. 2008; Strohle 2009). The exercise-induced enhancements of BDNF signals are critical for these exercise benefits in the hippocampus (Gomez-Pinilla et al. 2002; Liu and Nusslock 2018; Russo-Neustadt et al. 1999; Choi et al. 2018). Neuronal activity has been shown to enhance the transcription, transportation, and secretion of BDNF (Liu and Nusslock 2018; Soya et al. 2007). Therefore, a possible mechanism for exercise-induced upregulation of BDNF and beneficial effects in the hippocampus is due to the activation of local neurons. This scenario is supported by a positive correlation between the exercise-related changes in expression levels of c-Fos$^+$ and BDNF in the brain. However, to dissect the role of exercise-induced neuronal activation in the exercise-enhanced brain functions, identifying the regions which first respond to exercise and subsequently activate downstream neural network is critical. Our findings provide an initial insight toward understanding the regional and temporal profiles of brain influenced by exercise.

The hypothalamus is the control center of the autonomic nervous and neuroendocrine systems and governs physiological homeostasis during and after exercise (Porcari et al. 2015). Our results showed that long-term, but not acute, exercise increases expression of c-Fos in the hypothalamus. A previous study characterizing brain regions involved in cardiorespiratory control in rats also found that long-term exercise, but not a single bout of exercise, induced expression of c-Fos in the hypothalamic area (Ichiyama et al. 2002). It has been reported that the caudal hypothalamus modulates the cardiorespiratory responses associated with exercise (Kramer et al. 2000; Horn et al. 2000). Long-term treadmill running resets the resting blood pressure to lower levels by meditating the hypothalamic GABAergic transmission in the normotensive rats (Hsu et al. 2011). If indeed repeated exercise-induced neuronal activation and subsequent neuroadaptation in the hypothalamus are involved in hormonal, autonomic, and metabolic regulations, our results suggest that only long-term exercise could have such beneficial effects. It will be interesting to characterize such an association in the future.

In a few brain regions of the long-term exercise group, the numbers of c-Fos$^+$ cells did not become significant until after rest for 2 h. The delayed expression of c-Fos includes the SN and VTA, both playing important roles in movement and reward controls (Wu et al. 2011; Groenewegen 2003; Morales and Margolis 2017). Parkinson's disease is characterized by a substantial loss of dopamine in the striatum resulting from the gradual degeneration of dopaminergic neurons in the SN (Collier et al. 2011; Gao et al. 2002). Losses of dopaminergic neurons in the SN and nerve terminals in the striatum result in resting tremor, rigidity, bradykinesia and gait disturbance in patients with Parkinson's disease (Jenner 2001). Epidemiologic evidence suggests that moderate to vigorous exercise at earlier ages lower Parkinson's disease occurrence with dose–response relationships (Xu et al. 2010). Animal studies also showed that long-term exercise training attenuates the loss of dopaminergic neuron in the SN induced by inflammation (Wu et al. 2011) and MPP$^+$ toxicity (Tsou et al. 2015) in rodents. On the other hand, the VTA plays an important role in regulating cognitions and motivations (Morales and Margolis 2017). The VTA sends the dopaminergic projections to the NAc and builds up the VTA–NAc reward circuit (Nestler and Carlezon 2006). The dysregulation of this reward circuit has been implicated in the pathogenesis of depression (Nestler and Carlezon 2006). Although the underlying mechanisms are still unclear, accumulated evidence supports an anti-depressant effect of long-term exercise (Ernst et al. 2006; Legrand and Heuze 2007). It is worthwhile to explore whether exercise-induced neuronal activation in the SN and VTA is involved in the protection against Parkinson's disease and depression.

Conclusion

We found that acute (a single bout) treadmill running at mild intensity for 1 h increased the expression of c-Fos in the central motor control system (i.e., somatosensory and motor cortices and striatum) and multiple subregions within the hippocampus, with the ventral CA1/subiculum being the most responsive area in the 26 examined regions. The expression of c-Fos in most of these regions returned to basal levels after resting for 2 h, except the ventral CA1/subiculum whose c-Fos level remained high 2 h after the run. Long-term exercise training did not alter the basal neuronal activation levels in the brain. However, in addition to the central motor control system and the hippocampus, long-term exercise also increased the expression of c-Fos in the hypothalamus, thalamus, and lateral periaqueductal gray. The striatum was the most responsive area after long-term exercise and the c-Fos expression in many of these regions remained high after a 2-h rest. Moreover, delayed upregulations of c-Fos were evident in the midbrain (i.e., substantia nigra and ventral tegmental area). These results indicate that the central motor control system and the hippocampus are involved in physical activity. Long-term exercise at mild intensity induces expression of c-Fos in the basal ganglia, diencephalon and midbrain. Because repeated neuronal activation is known to induce activity-dependent changes in neural networks, our study suggests that long-term exercise may induce neuroadaptation in the aforementioned regions and consequently affect physiological functions related to these brain regions.

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Compliance with ethical standards

Conflict to interest The authors declare that they have no conflict of interest.

Ethical approval All animal experiments were done in accordance with the National Institute of Health Guideline for Animal Research (Guide for the Care and Use of Laboratory Animals) and approved by the National Cheng Kung University Institutional Animal Care and Use Committee (ethical approval reference number: 104272).

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