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RESEARCH ARTICLE | *Physical Activity and Inactivity*

Negative rebound in hippocampal neurogenesis following exercise cessation

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Nishijima T, Kamidozono Y, Ishiizumi A, Amemiya S, Kita I. Negative rebound in hippocampal neurogenesis following exercise cessation. *Am J Physiol Regul Integr Comp Physiol* 312: R347–R357, 2017. First published January 4, 2017; doi:10.1152/ajpregu.00397.2016.—Physical exercise can improve brain function, but the effects of exercise cessation are largely unknown. This study examined the time-course profile of hippocampal neurogenesis following exercise cessation. Male C57BL/6 mice were randomly assigned to either a control (Con) or an exercise cessation (ExC) group. Mice in the ExC group were reared in a cage with a running wheel for 8 wk and subsequently placed in a standard cage to cease the exercise. Exercise resulted in a significant increase in the density of doublecortin (DCX)-positive immature neurons in the dentate gyrus (at *week 0*). Following exercise cessation, the density of DCX-positive neurons gradually decreased and was significantly lower than that in the Con group at 5 and 8 wk after cessation, indicating that exercise cessation leads to a negative rebound in hippocampal neurogenesis. Immunohistochemistry analysis suggests that the negative rebound in neurogenesis is caused by diminished cell survival, not by suppression of cell proliferation and neural maturation. Neither elevated expression of ΔFosB, a transcription factor involved in neurogenesis regulation, nor increased plasma corticosterone, were involved in the negative neurogenesis rebound. Importantly, exercise cessation suppressed ambulatory activity, and a significant correlation between change in activity and DCX-positive neuron density suggested that the decrease in activity is involved in neurogenesis impairment. Forced treadmill running following exercise cessation failed to prevent the negative neurogenesis rebound. This study indicates that cessation of exercise or a decrease in physical activity is associated with an increased risk for impaired hippocampal function, which might increase vulnerability to stress-induced mood disorders.

exercise cessation; hippocampus; neurogenesis; physical activity; ΔFosB

PHYSICAL EXERCISE or a greater than average level of physical activity is known to improve a wide range of brain functions (14, 21, 30). More specifically, the hippocampus is involved in learning, memory, and stress resilience, and it uniquely adapts to the level of physical activity. For example, the volume of the hippocampus in older adults is positively associated with physical activity (54) and aerobic fitness (10, 15). These observations are supported by evidence from randomized control trials demonstrating that long-term exercise interventions (6–12 mo) resulted in hippocampal volume increase (16, 36). In addition, a growing body of animal studies provide unequivocal evidence that exercise increases neurogenesis (41, 53) and expression of trophic factors (26, 43) in the hippocampus, which in turn leads to improvements in hippocampus-dependent functions (18, 28). However, less attention has been paid to the mechanisms underlying the effects of exercise cessation or a decrease in physical activity on the hippocampus. A low level of physical activity increases the risk for neurodegenerative and psychological disorders, including Alzheimer's disease and depressive symptoms (2, 3, 44), and hippocampal dysfunction is a predisposing factor for these disorders (50). Therefore, the hypothesis that a reduction in physical activity impairs hippocampal function warrants further study, which is expected to provide an alternative approach to promoting cognitive and mental health (38).

Because laboratory rodents are generally confined to a standard, small cage, it is practically difficult to decrease their physical activity without any physical restraints. Some previous studies have used hindlimb suspension to model forced physical inactivity and have demonstrated suppressed neurogenesis and brain-derived neurotrophic factor (BDNF) expression in the rat hippocampus (40, 56); however, hind limb suspension is a form of restraint stress, which itself can impair hippocampal function. Recently, studies have examined whether long-term spaceflight affects mouse brain function (25, 34, 48). Although results from those studies are indispensable, it is difficult to obtain reproducible results because of critical limitations such as small sample sizes and a paucity of more detailed information.

Cessation of regular exercise or detraining is another valuable approach to examining how reduced physical activity leads to physiological regression. Several studies have suggested that reduction of physical activity by exercise cessation negatively affects brain function. For example, in healthy individuals who regularly perform aerobic exercise, forced exercise cessation increases depressive symptoms (8) and decreases cerebral blood flow in the hippocampus (1). Moreover, some animal models have suggested that hippocampal adaptation to exercise cessation is not merely a reversal of a prior adaptation to physical exercise (20, 39, 45). Notably, Radak et al. (45) reported that BDNF expression in the hippocampus following exercise cessation was significantly lower than that in sedentary controls; they described this finding as a negative rebound after detraining. Recently, we examined hippocampal neurogenesis following cessation of voluntary wheel running in mice (39). Briefly, after they had been weaned, mice were reared in a cage with a running wheel until early adulthood (from 4 to 11 wk of age) and were subsequently placed in a standard cage for an additional 10 wk. Intriguingly, the rate of neuronal differentiation was suppressed to a level below that

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observed in sedentary control mice, suggesting a negative rebound in neurogenesis following exercise cessation. However, time-course changes in each stage of neurogenesis (i.e., proliferation, differentiation, and survival) have not been examined.

Yutsudo et al. (57) demonstrated that mice lacking the *fosB* gene exhibited impaired neurogenesis, concomitant with a suppression of some neurogenesis-related genes, including those encoding vascular growth factor nerve growth factor inducible (*Vgf*) and galanin prepropeptide (*Gal*). Because VGF and Gal are secretary molecules, it is presumable that neurons expressing ΔFosB [a truncated splice isoform of FosB that mediates long-lasting neural plasticity including neurogenesis (35, 57) and is increased by long-term exercise (37)] may regulate neurogenesis through these peptides in an autocrine/paracrine manner (57). In addition, ΔFosB has a uniquely long half-life (several weeks) (35). BDNF, a well-known regulator of neurogenesis, is demonstrated to revert to sedentary control levels within 2 to 3 wk following exercise cessation (6, 7). In contrast, ΔFosB expression may remain elevated for a longer time following exercise cessation, which might positively regulate neurogenesis; however, it is uncertain how long ΔFosB expression is preserved following exercise cessation. We hypothesize that a decline in ΔFosB expression precedes the suppression of neurogenesis following exercise cessation.

In the current study, we first conducted a thorough examination of neurogenesis over a detailed time course following exercise cessation in mice. In addition, we assessed potential mechanistic roles for the transcription factor ΔFosB. Next, we examined whether the negative rebound in neurogenesis is associated with a stress response (i.e., chronic hypercorticosteronemia) and changes in spontaneous ambulatory activity in the home cage. Finally, we evaluated whether the negative rebound in neurogenesis can be prevented by forced treadmill running.

MATERIALS AND METHODS

Animals. Male C57BL/6N mice (3 wk of age, SLC, Shizuoka, Japan) were housed under controlled temperature (22–24°C) and light (12:12-h light-dark cycle, lights on at 5:00 A.M.) conditions. Food and water were available ad libitum. All experimental procedures were approved by the Animal Experimental Ethics Committee of the Tokyo Metropolitan University.

This study consisted of three experiments (Fig. 1). In the first experiment (Fig. 1*A*), we examined time-course changes in hippocampal neurogenesis and ΔFosB expression following exercise cessation. After a week of initial acclimatization, mice were randomly assigned to either a control (Con, $n = 25$) or an exercise cessation (ExC, $n = 25$) group. Con mice were housed in standard plastic cages (L × W × H = 33.8 × 22.5 × 14.0 cm) throughout the experiment. ExC mice were housed in similarly-sized plastic cages (L × W × H = 27.9 × 21.6 × 15.2 cm) equipped with a running wheel (ENV-3046; Med Associate) for 8 wk (from 4 to 11 wk of age) and were transferred to the standard cages at 12 wk of age, resulting in exercise cessation from then on. All mice were housed in group cages (5 mice per cage), and cage mates were not changed throughout the experiment. Except the cage transfer at 8 wk in the ExC group, the environmental factors (such as wood chip bedding and cage material) were identical between groups. 5-Bromo-2-deoxyuridine (BrdU, 50 mg/kg body wt) dissolved in saline was injected intraperitoneally on the last 2 days of the exercise period to label dividing cells. At 0, 1, 2, 5, and 8 wk after exercise cessation, both Con and ExC mice were euthanized for immunohistochemical examination ($n = 5$ at each time point). The number of wheel rotations was recorded each morning, and body weight was measured weekly. We marked the tails of the mice for identification and regularly observed whether each mouse used the running wheel throughout the experiment.

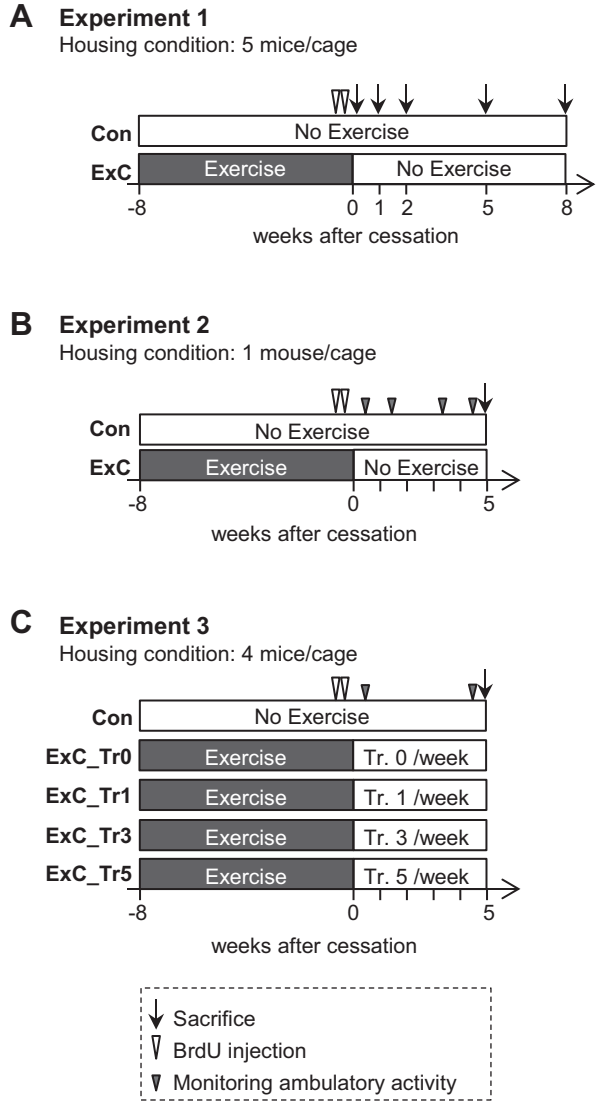

Fig. 1. Experimental setup of the three experiments conducted in the current study. *A*: *experiment 1*. BrdU was intraperitoneally injected on the last 2 days of the exercise period (open arrowheads). Mice in both the control (Con) and exercise cessation (ExC) groups were killed at 0, 1, 2, 5, or 8 wk after exercise cessation (black arrows indicate days of death, $n = 5$ at each time point). *B*: *experiment 2*. BrdU was injected as it was in *experiment 1*. Ambulatory activity in the home cage (from 11:00 A.M. to 9:00 A.M. the next day) was monitored once a week for 5 wk after cessation except for the third week (filled arrowheads). Mice were killed at 5 wk after cessation ($n = 10$ per group). *C*: *experiment 3*. After exercise cessation, mice in ExC_Tr0, ExC_Tr1, ExC_Tr3, ExC_Tr5 groups performed treadmill running (15 m/min for 60 min/day) for 0, 1, 3, or 5 days/week, respectively. Gross activity level per cage was monitored at 1 and 5 wk after cessation. Mice were killed at 5 wk after cessation ($n = 8$ per group).

Experimental design. In *experiment 2* (Fig. 1*B*), we examined whether the negative rebound in hippocampal neurogenesis following exercise cessation observed in the first experiment was caused by chronic stress or reduced ambulatory activity. Mice were randomly assigned to a control (Con) or an exercise cessation (ExC) group ($n = 10$ per group). The experimental design was largely the same as that of *experiment 1*, except that mice in both Con and ExC groups were

individually housed throughout the experiment. Individual housing was necessary to monitor spontaneous ambulatory activity in home cages using a near-infrared beam interruption system (LOCOMO LS-5; Melquest, Toyama, Japan). In addition, to exclude any confounding effects due to a change in housing conditions (i.e., from group to individual housing) during the experiment, all mice were individually housed from the beginning of the experiment. Ambulatory activity was monitored for 22 h (from 11:00 A.M. to 9:00 A.M. the following day) once a week following exercise cessation, except for the third week because of an unexpected mechanical problem. Before injecting anesthetic for death at 5 wk after exercise cessation, blood was collected from the tip of the tail vein and centrifuged [3,000 revolutions per minute for 15 min at 4°C]. Separated plasma was stored at −80°C for corticosterone assay. Concomitant with brain removal, the adrenal glands and thymus were removed and weighed. The relative weight of each organ was calculated as follows: [absolute wet weight (mg)/body weight (g)] × 100.

In *experiment 3* (Fig. 1*C*), we examined whether the negative rebound in hippocampal neurogenesis following exercise cessation could be prevented by forced treadmill running. Mice were randomly assigned to a Con ($n = 8$) or an ExC ($n = 32$) group. All mice were housed in group cages (4 mice per cage). The ExC group was subdivided into four groups according to the frequency of forced treadmill running after cessation (0, 1, 3, or 5 days per week, $n = 8$ per group). Mice were subjected to forced running on a treadmill apparatus (KN-73; Natsume, Tokyo, Japan) at 15 m/min, a moderate speed below the lactate threshold in mice (9), during the dark period (from 6:00 P.M. to 10:00 P.M.) for 5 wk. Running time was set at 60 min/day except for the first session, which was 30 min/day for initial acclimatization. In the first session, the mice were allowed to freely explore the treadmill for 10 min before starting running and experienced a brief electrical shock by the shock grids located at the end of the treadmill. In addition, the front half of the treadmill was covered with a black plastic sheet. This setup allows the mice to spontaneously learn how to avoid the noxious stimuli and effectively motivates them to run at a low to moderate speed. During treadmill running, the mice were sometimes prodded with a stick to encourage them to run; we refrained from using electric shock as much as possible. The gross activity level per cage was tentatively monitored at 1 and 5 wk after cessation using the same system as in *experiment 2*, although the precise activity of each mouse was uncertain because the mice were housed in groups.

Perfusion and tissue processing. At the end of the experimental time course, mice were killed in the morning (9:00–11:00 A.M.) by transcardial perfusion with cold saline while they were deeply anesthetized with pentobarbital sodium (100 mg/kg body weight). Brains were quickly removed and postfixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) overnight. Brains were cryoprotected in 30% sucrose in PBS and frozen until they were sectioned using a freezing microtome to obtain coronal sections (40 μm) to encompass the whole hippocampus. Sections were stored in PBS with 0.01% sodium azide. Brains were coded to enable blind analysis.

Immunohistochemistry. Neurogenesis and ΔFosB immunoreactivity in the hippocampus were examined using methods described in previous reports, with some modifications (37, 39). A one-in-eight series of sections was randomly selected for Nissl staining to estimate the total volume of the granule cell layer (GCL) of the dentate gyrus (DG) based on the Cavalieri principle (29). Adjacent sections (anterior and posterior) were used for either Ki-67 or 5-bromo-2′-deoxyuridine (BrdU)/NeuN immunostaining to assess cell proliferation, cell survival, and neural maturation. Other sections were labeled with doublecortin (DCX), a marker of immature neurons that has been validated for assessing levels of neurogenesis (13, 46), or with ΔFosB, a potential molecular mediator of exercise-induced hippocampal neurogenesis (37, 57).

For Ki-67, DCX, and ΔFosB immunohistochemistry, free-floating sections were first preincubated with 1% H_2O_2 in PBS to quench endogenous peroxidase activity, and then with blocking solution containing 10% normal horse serum in PBS for 2 h. After rinses in PBS, the sections were incubated with rabbit monoclonal anti-Ki-67 antibody (1:1,000, ab16667; Abcam), goat polyclonal anti-DCX antibody (1:500, sc-8066; Santa Cruz Biotechnology), or rabbit polyclonal anti-pan-FosB antibody (1:1,000, sc-48; Santa Cruz Biotechnology) diluted in PBS with 0.5% Triton X-100 and 0.5% bovine serum albumin (PBST-BSA) for 24 h (Ki-67, ΔFosB) or 48 h (DCX) at 4°C. Sections were then incubated with an appropriate biotinylated secondary antibody (anti-rabbit IgG, AP182B; anti-goat IgG, AP180B, both from EMD Millipore) diluted in PBST-BSA (1:1,000) for 2 h at room temperature. Sections were then treated with avidin-biotin-peroxidase complex (Vectastain ABC peroxidase kit; Vector Laboratories) for 2.5 h following the manufacturer's instructions. The antigens were finally visualized with 0.02% 3,3-diaminobenzidine (DAB) in 0.1 M Tris·HCl (pH 7.6) containing 0.001% H_2O_2. For Ki-67 and ΔFosB staining, the reaction was intensified with nickel ammonium sulfate. For Ki-67 and DCX staining, cell nuclei were counterstained with Nissl stain. Sections were mounted onto gelatin-coated slides, dehydrated in a graded ethanol series, cleared in xylene, and coverslips were applied.

For BrdU/NeuN double-immunofluorescence staining, free-floating sections were preincubated with 2 N HCl at 37°C to denature DNA. Following rinses in PBS, the sections were incubated with rat monoclonal anti-BrdU antibody (1:500, ab6326; Abcam) and mouse monoclonal anti-NeuN antibody (1:500, MAB377; EMD Millipore) diluted in PBST-BSA for 24 h at 4°C. Sections were then incubated with secondary antibodies (anti-Rat IgG with Alexa-Fluor 488; anti-mouse IgG with Alexa-Fluor 594, both from Life Technologies) diluted in PBST-BSA (1:1,000) for 2 h at room temperature. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Sections were mounted on gelatin-coated slides and coverslips were applied.

Analysis of neurogenesis. DCX-positive immature neurons were quantified as described previously, with modifications to improve accuracy (37). Because DCX-positive immature neurons in mice that perform long-term exercise are abundant and overlapping, it is difficult to precisely count the DCX-positive soma. Therefore, we previously developed a novel analytical method based on the morphological characteristics of DCX-positive immature neurons that have, on average, a single dendrite when measured within 40 μm of the soma (47). Briefly, fully focused digital images (1,920 × 1,440) of the DG were taken using a BZ-X700 microscope (×20 objective lens; Keyence) throughout the rostral/caudal extent of the hippocampus. With the aid of ImageJ software (National Institutes of Health, Bethesda, MD), a segmented line was drawn along the middle of the GCL and the crossings over dendrites of DCX-positive immature neurons were counted and divided by the length of the line. This was repeated for 8 to 10 images taken from 6 to 8 sections per mouse, and the average was calculated. Because the thickness of the GCL is approximately 60–80 μm, the value thus obtained (crossings/mm) should reflect the density of DCX-positive immature neurons in the DG.

Manual counting to calculate the density of Ki-67-positive or BrdU-positive cells in the DG of the hippocampus was conducted under an optical microscope (×40 objective lens, BX-51; Olympus). The number of Ki-67-positive or BrdU-positive cells counted in each section was divided by the volume of the GCL, as determined from an adjacent Nissl-stained section, and averaged over 6 to 8 sections per mouse.

To assess the differentiation of newly born BrdU-positive cells into NeuN-positive mature neurons, z-stack digital images (960 × 720, 4-μm intervals) were obtained throughout the hippocampus using a BZ-X700 microscope (×20 objective lens, Keyence). All BrdU-positive cells observed in the images were examined for double-staining with NeuN. Data are presented as the ratio of BrdU-positive/NeuN-positive double-labeled cells to BrdU-positive cells.

Quantification of FosB/ΔFosB immunoreactivity. The pan-FosB antibody used in this study cannot discriminate between FosB and ΔFosB isoforms. Therefore, we described the immunostained structures as FosB/ΔFosB-positive nuclei. However, we previously determined by Western blotting that long-term exercise increases the ΔFosB isoform (35–37 kDa) in the hippocampus (37). Levels of FosB/ΔFosB immunoreactivity were obtained using image thresholding methods as described previously (37). Briefly, digital images (4,140 × 3,096) of the DG were taken throughout the hippocampus with an optical microscope (×20 objective lens, BX-51; Olympus) and assessed with imaging software (cellSens, Olympus). The GCL was selected with an irregularly shaped polygon. Moderate-to-strong FosB/ΔFosB-positive nuclei were identified by image thresholding and the FosB/ΔFosB-positive area was automatically calculated as follows: %ROI = (converted area/total ROI area) × 100.

Corticosterone assay. Plasma corticosterone concentration was determined using a commercial corticosterone enzyme immunoassay kit (500655; Cayman) according to the manufacturer's instructions.

Statistical analysis. Results from *experiment 1* were analyzed by two-way repeated-measures ANOVA (changes in body weight during the exercise period, group × time) or two-way factorial ANOVA (neurogenesis and FosB/ΔFosB-positive immunoreactivity, group × time). When a main effect of interaction or group was observed, a two-tailed unpaired *t*-test was used as a post hoc test to determine statistical differences between groups at each time point. Results from *experiment 2* were analyzed by a two-tailed unpaired *t*-test (neurogenesis, chronic stress symptoms, changes in ambulatory activity), two-way repeated-measures ANOVA (time-course profile of ambulatory activity following exercise cessation, group × time), or Pearson's correlation analysis (changes in activity vs. neurogenesis). Results from *experiment 3* were analyzed by one-way factorial ANOVA followed by a Tukey's post hoc test. Data are presented as means ± SE. The threshold for statistical significance was set at $P < 0.05$.

RESULTS

Changes in body weight and running distance during exercise. Changes in body weight during the exercise period in *experiment 1* are shown in Fig. 2*A*. Two-way repeated-measures ANOVA indicated a significant group × time interaction [$F(8, 384) = 11.98$, $P < 0.0001$; $n = 25$ per group] and a main effect of group [$F(1, 48) = 13.52$, $P < 0.001$]. Post hoc analysis revealed a significantly lower body weight in mice in the ExC group, appearing 1 wk after starting exercise. There were no significant differences in body weight between the Con and ExC subgroups (i.e., killed at 0, 1, 2, 5, and 8 wk after exercise cessation). The average daily running distance of all mice per cage (meters/day/cage) was nearly equivalent between the ExC subgroups (Fig. 2*B*). Through regular observation, it was confirmed that all the mice used the running wheel throughout the exercise period, although the precise running distance of each mouse was uncertain. Thus any effects of exercise observed in the hippocampus were not the result of baseline differences between experimental groups.

The average daily individual running distances in *experiment 2* (single housing condition, $n = 10$) are shown in Fig. 2*C*. Although there was an expected individual variation (minimum, 1,131 m/day; maximum, 7,490 m/day in the last week), the average distance increased in the first 3 wk and then reached a plateau at ~4,000 m/day. Changes in body weight in *experiments 2* and *3* and in running distance in *experiment 3* were similar to those in *experiment 1* (data not shown).

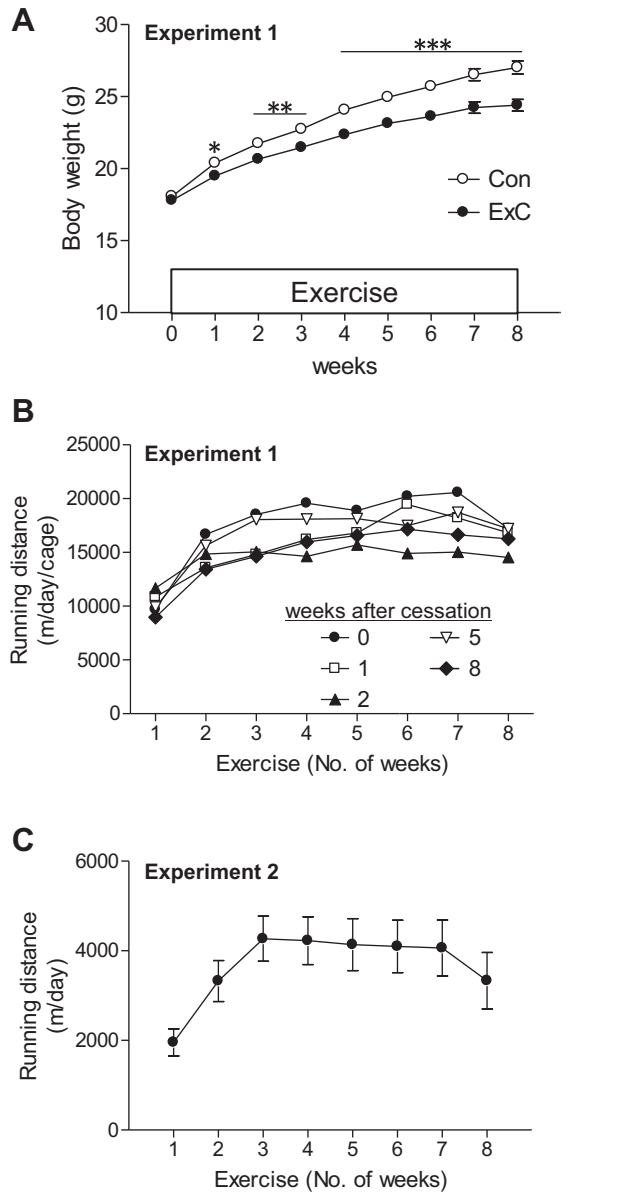

Fig. 2. Body weight changes and running distance during the exercise period. *A*: *experiment 1*. Body weight was significantly lower in mice in the exercise cessation (ExC) group than in the control (Con) group. Values are means ± SE ($n = 25$ per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ between groups at each time point. *B*: *experiment 1*. Average running distance per cage (meters/day/cage) was nearly equivalent between ExC subgroups. Because the mice in each ExC subgroup were housed together ($n = 5$), running distances (meters/day/cage) were plotted as single data. *C*: *experiment 2*. Average running distance per mouse (meters/day) gradually increased in the first 3 wk and then reached a plateau. Values are means ± SE ($n = 10$).

Negative rebound in hippocampal neurogenesis following exercise cessation. Next, we assessed neurogenesis at 0, 1, 2, 5, and 8 wk after exercise cessation. Representative images of immunostaining are shown in Fig. 3. As expected, the number of DCX-positive immature neurons in the ExC group was greater than that in the Con group immediately after the exercise period (*week 0*, Fig. 3, *A* vs. *B*). However, at 8 wk after exercise cessation, the number of DCX-positive immature neurons was apparently decreased in the ExC group (Fig. 3, *B* vs. *D*). We did not observe a clear difference between groups

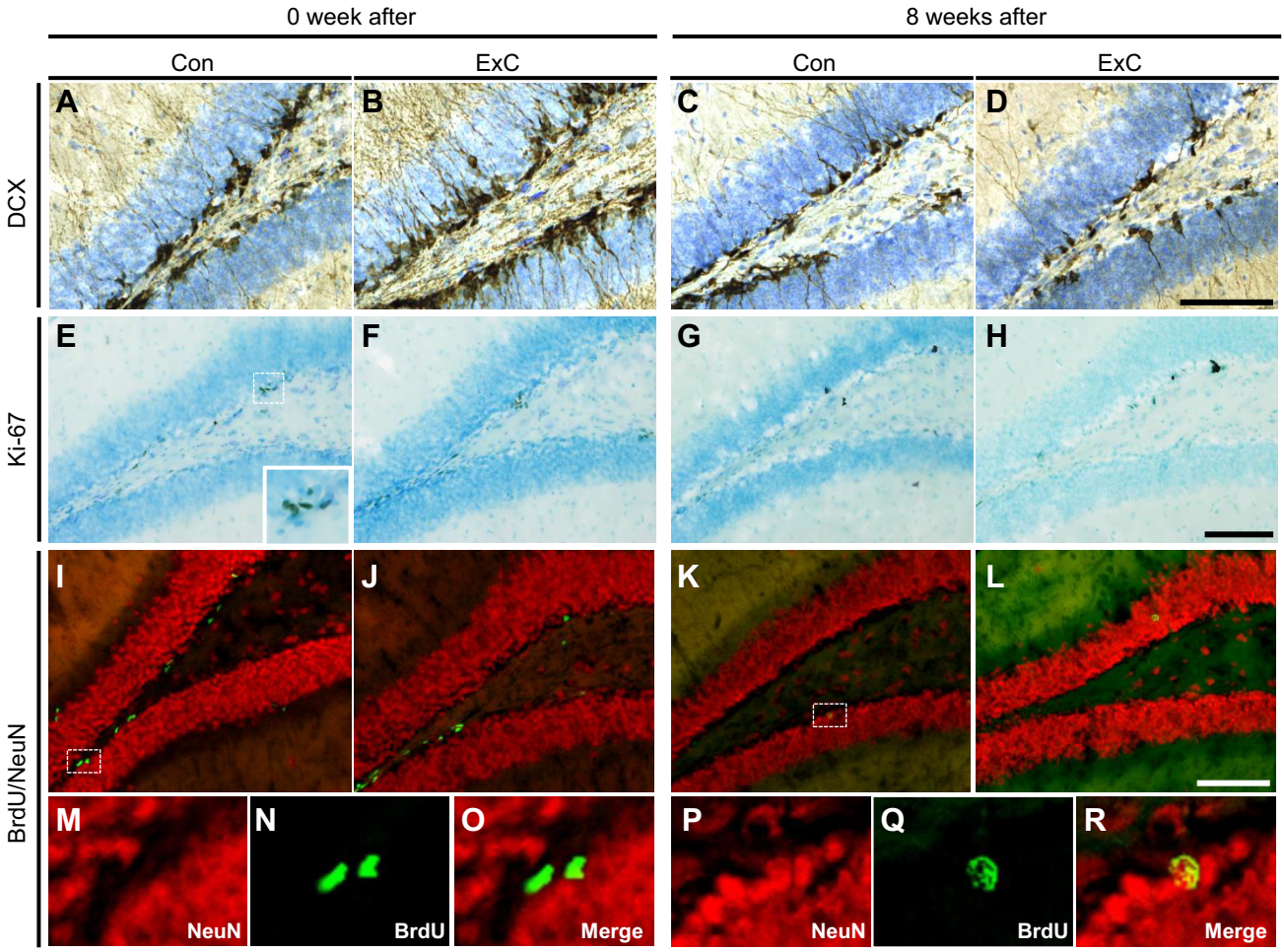

Fig. 3. Representative images of doublecortin (DCX)-positive immature neurons (*A–D*), Ki-67-positive cells (*E–H*), and BrdU-positive/NeuN-positive cells (*I–R*) in the hippocampus at 0 and 8 wk after exercise cessation. A cluster of Ki-67-positive cells is magnified (see *E*, *inset*). Magnifications of BrdU-positive cells (green) in *I* and *K* are shown in *M–O* and *P–R*, respectively. Scale bar = 100 μm. Con, control; ExC, exercise cessation.

in the number of Ki-67-positive nuclei (Fig. 2, *E–H*), most of which were present in clusters in the subgranular zone of the DG (Fig. 3*E*, *inset*). The shapes of BrdU-positive nuclei (green) differed in appearance between *weeks 0* and *8* (Fig. 3, *N* vs. *Q*), and at *week 8*, most BrdU-positive nuclei were double-labeled with the mature neuron marker NeuN (red) in both Con and ExC groups (Fig. 3, *P–R*).

Quantitation of hippocampal neurogenesis is shown in Fig. 4. Two-way repeated-measures ANOVA revealed a significant main effect of group in the estimated total volume of the GCL [Fig. 4*A*, main effect of group, $F(1,40) = 6.29$, $P < 0.05$; group × time interaction, $F(4,40) = 1.93$, $P = 0.124$]. In post hoc analysis, a significant difference between groups was noted only immediately after exercise cessation ($P < 0.05$). Time-course changes in the density of DCX-positive immature neurons, which reflects the overall rate of neurogenesis, were significantly different between groups [Fig. 4*B*, group × time interaction, $F(4,40) = 11.51$, $P < 0.001$]. At *week 0*, the density of DCX-positive immature neurons was significantly higher in mice in the ExC group than in the Con group ($P < 0.01$). However, the density in the ExC group decreased to control levels within 2 wk. Importantly, the density of DCX-positive neurons further decreased and was significantly lower in mice in the ExC group than in the Con group at *weeks 5* ($P < 0.05$) and *8* ($P < 0.001$). These data indicated that cessation of long-term exercise leads to a stark decline in hippocampal neurogenesis, eventually leading to a negative rebound effect (i.e., the rate of neurogenesis is even lower than in exercise-naïve control mice).

The density of Ki-67-positive cells, which is indicative of proliferative activity, was not different between groups at the time of death [Fig. 4*C*, main effect of group, $F(1,40) = 0.97$, $P = 0.331$; group × time interaction, $F(4,40) = 0.11$, $P = 0.978$], suggesting that declines in neurogenesis did not result from suppressed cell proliferation. The initial density of BrdU-incorporated cells did not differ between groups (Fig. 4*D*), confirming the results of Ki-67 staining that proliferative activity was similar between both groups at the end of the exercise period. There were no significant differences in the density of BrdU-positive cells between groups throughout the 8 wk after exercise cessation [main effect of group, $F(1,39) = 0.69$, $P = 0.411$; group × time interaction, $F(4,39) = 0.12$, $P = 0.973$], although the number of BrdU-positive cells did decrease over time in both groups due to normal apoptotic processes [main effect of time, $F(4,39) = 49.98$, $P < 0.001$].

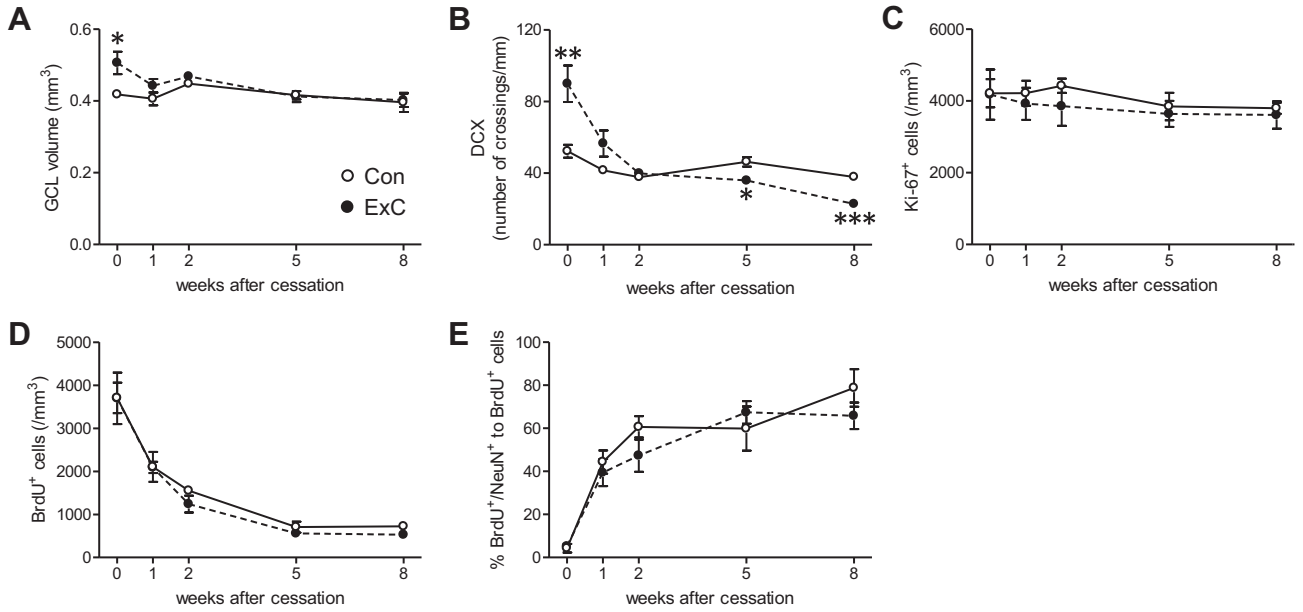

Fig. 4. Time-course changes in hippocampal neurogenesis following exercise cessation (*experiment 1*). Shown are changes in estimated volume of the granule cell layer (GCL) of the hippocampal dentate gyrus (DG) (*A*), density of DCX-positive immature neurons (*B*), Ki-67-positive cells (*C*), BrdU-positive cells (*D*), and the ratio of BrdU-positive/NeuN-positive to BrdU-positive cells (*E*) following exercise cessation. Values are means ± SE ($n = 5$ per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control the (Con) group at each time point.

Next, we assessed the maturation of newly generated neurons during the period following exercise cessation by determining the ratio of BrdU-positive/NeuN-positive to BrdU-positive cells. Over the 8 wk after exercise cessation in the experiment, the proportion of BrdU-positive/NeuN-positive to BrdU-positive cells gradually increased [Fig. 4*E*, main effect of time, $F(4,39) = 34.5$, $P < 0.001$], reaching 78.8% in Con and 65.8% in ExC mice; however, no significant differences were found between the groups [main effect of group, $F(1,39) = 1.28$, $P = 0.265$; group × time interaction, $F(4,39) = 1.01$, $P = 0.415$]. This suggested that neuronal differentiation of cells born before exercise cessation was not suppressed.

Long-lasting elevation in ΔFosB expression following exercise cessation. Next, we sought to determine whether expression of ΔFosB was affected by exercise cessation. Representative images of FosB/ΔFosB immunostaining are shown in Fig. 5*A*. Consistent with our previous study (37), 8 wk of exercise resulted in a clear increase in FosB/ΔFosB immunoreactivity in the GCL of the DG. Image thresholding analysis revealed that the level of FosB/ΔFosB immunoreactivity in ExC mice gradually decreased over time following cessation; however, FosB/ΔFosB immunoreactivity remained significantly elevated even at *week 8* [Fig. 5*B*, main effect of group, $F(1,39) = 95.3$, $P < 0.001$; main effect of time, $F(4,39) = 2.28$, $P = 0.078$; group × time interaction, $F(4,39) = 3.77$, $P < 0.05$]. These results suggested that high levels of ΔFosB are not sufficient to maintain high levels of neurogenesis following exercise cessation.

Chronic stress symptoms and ambulatory activity following exercise cessation. In *experiment 2*, we aimed to determine whether cessation of exercise causes chronic stress, which might be involved in induction of the negative rebound in hippocampal neurogenesis. Thus adrenal gland enlargement, thymus involution, and increase in plasma corticosterone were examined as chronic stress symptoms at 5 wk after exercise cessation. We have previously reported that exploratory activity in the elevated-plus maze, used to assess anxiety-like behavior, was decreased following exercise cessation (39), suggesting that home cage activity might be decreased as well. To test this possibility, mice were housed individually throughout the experiment and ambulatory activity in their home cage was monitored once a week following exercise cessation.

Densities of DCX-positive immature neurons (Fig. 6*A*, $P < 0.001$) were significantly lower in ExC than in Con mice at 5 wk after cessation. The density of Ki-67-positive cells in ExC was slightly lower than in Con mice, but the difference did not reach statistical significance (Fig. 6*B*, $P = 0.106$). These findings confirmed the results of *experiment 1* (Fig. 4, *B* and *C*). On the other hand, the density of BrdU-positive cells was significantly lower in ExC than in Con animals (Fig. 6*C*, $P < 0.01$), which is inconsistent with the result from *experiment 1* (Fig. 4*D*). Here we note that in *experiment 1*, the range of BrdU-positive cell densities (approximately 520–3,800 cells/mm^2) largely exceeded the differences between groups, which may have led to type II errors in the ANOVA. Indeed, *t*-tests for each time point revealed a significant difference between groups at *week 8* ($P < 0.05$, data not shown), suggesting that cessation of exercise partially impairs the survival of cells born before exercise cessation, which might explain the negative rebound in hippocampal neurogenesis.

In Table 1, body and organ weights and chronic stress-related measures are presented. We found no significant differences in the absolute wet weights of the adrenal gland ($P = 0.153$) and thymus ($P = 1.00$) between groups. Because there was no difference in body weight at the end of the experiment ($P = 0.437$), there also were no group differences in the relative weights of both organs (adrenal gland, $P = 0.712$; thymus, $P = 0.550$). No difference was found in plasma corticosterone levels ($P = 0.589$). These results suggest that exercise cessation does not cause chronic stress.

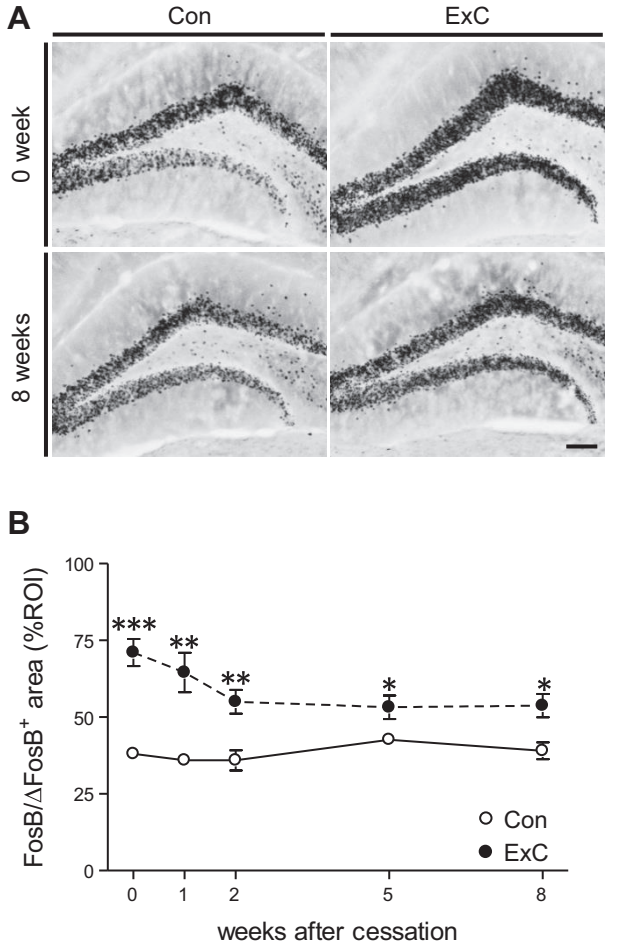

Fig. 5. FosB/ΔFosB expression following exercise cessation. *A*: representative images of FosB/ΔFosB-positive nuclei at 0 and 8 wk after exercise cessation. Scale bar = 100 μm. *B*: changes in FosB/ΔFosB immunoreactive area (%ROI) following exercise cessation. Values are means ± SE (n = 5 per group). *P < 0.05, **P < 0.01, ***P < 0.001 vs. the control (Con) group at each time point.

Time-course profiles of ambulatory activity following exercise cessation significantly differed between groups [Fig. 7*A*, group × time interaction, $F(3,54) = 3.06$, $P < 0.05$]. Thus we calculated changes in activity (between *weeks 1* and *5*); the ambulatory activity was decreased more in ExC than in Con mice, and the difference was nearly statistically significant (Fig. 7*B*, $P = 0.051$). We performed correlation analysis to examine the association between the changes in activity and neurogenesis. There was a significant, positive correlation between changes in activity and DCX-positive immature neuron density (Fig. 7*C*, $r = 0.509$, $P < 0.05$), and a tendency toward positive correlation between activity changes and BrdU-positive surviving cells (Fig. 7*D*, $r = 0.390$, $P = 0.089$). This suggests that hippocampal neurogenesis is suppressed in parallel with the reduction in ambulatory activity.

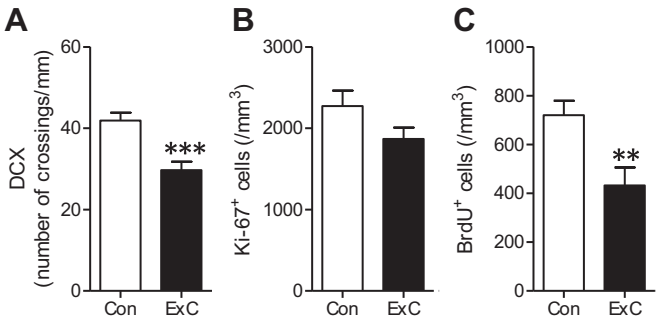

Fig. 6. Confirmation of the negative rebound in hippocampal neurogenesis following exercise cessation (ExC) (*experiment 2*). Shown are densities of DCX-positive immature neurons (*A*), BrdU-positive cells (*B*), and Ki-67-positive cells (*C*). Values are means ± SE (n = 10 per group). **P < 0.01, ***P < 0.001 vs. the control (Con) group.

Table 1. *Body weight and chronic stress-related measures in experiment 2*

	Control	Exercise Cessation
Body weight, g	29.2 ± 0.9	28.3 ± 0.6
Adrenal gland		
Wet weight, mg	3.2 ± 0.07	3.1 ± 0.05
Relative weight, mg/g × 100	11.2 ± 0.46	11.0 ± 0.13
Thymus		
Wet weight, mg	35.5 ± 0.97	35.5 ± 0.75
Relative weight, mg/g × 100	82.9 ± 3.86	80.1 ± 2.44
Corticosterone, ng/ml	95.9 ± 17.3	108.2 ± 14.1

Values are means ± SE (n = 10 per group). Relative weight was calculated as follows: [absolute wet weight (mg)/body weight (g)] × 100.

Forced treadmill exercise following exercise cessation fails to prevent negative rebound in hippocampal neurogenesis. In *experiment 3*, we explored whether forced treadmill exercise can prevent negative rebound in hippocampal neurogenesis following exercise cessation. Mice were forced into moderate-intensity treadmill exercise (15 m/min) for 60 min at different frequencies (0, 1, 3, or 5 days/week). One-way ANOVA revealed that the density of DCX-positive immature neurons differed significantly between groups [Fig. 8*A*, $F(4,39) = 11.88$, $P < 0.001$]. Tukey's post hoc analysis confirmed that exercise cessation resulted in a decreased density of DCX-positive neurons (Con vs. ExC_Tr0, $P < 0.0001$). Treadmill exercise once a week did not prevent this reduction (Con vs. ExC_Tr1, $P < 0.001$). Treadmill exercise at 3 or 5 days/week resulted in a slight increase in DCX-positive neuron density compared with that in ExC_Tr0; however, the density in ExC mice remained significantly lower than in Con mice (Con vs. ExC_Tr3, $P < 0.05$; Con vs. ExC_Tr5, $P < 0.05$). The other parameters of neurogenesis (proliferation, survival, and differentiation) analyzed in this study were not ameliorated by forced treadmill exercise (data not shown).

Although the ambulatory activity of each mouse was uncertain, we tentatively monitored gross activity per cage at 1 and 5 wk after exercise cessation and changes in activity between these two time points were calculated for each cage (Fig. 8*B*). Exercise cessation resulted in decreased gross activity per cage, which was consistent with our findings in *experiment 2* (Fig. 7*B*), and forced treadmill exercise 3 or 5 days/week partially prevented the reduction. Interestingly, changes in activity showed a trend that was similar to that of DCX-positive neuron density, although no correlation could be established because of a lack of individual activity data.

DISCUSSION

Regular exercise is well known to improve hippocampal function; however, the effects of exercise cessation on the

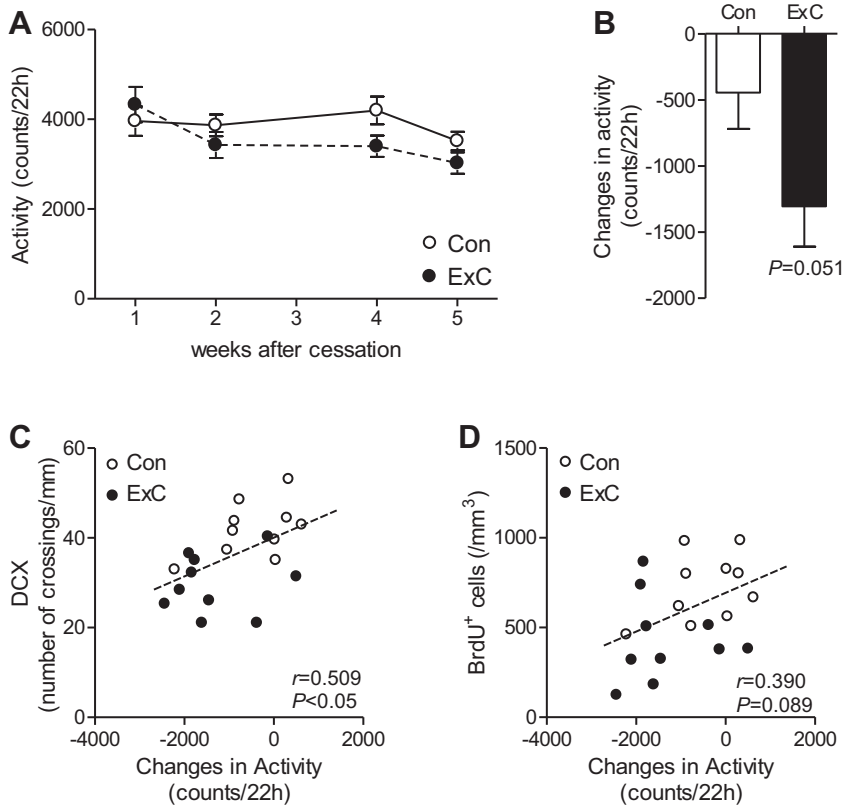

Fig. 7. Ambulatory activity in the home cage decreases following exercise cessation (ExC) (*experiment 2*). *A*: ambulatory activity in mice in the ExC group gradually decreased over a 5-wk period following exercise cessation. *B*: change in ambulatory activity (between the first and fifth weeks) in mice in the ExC group was lower than in the control (Con) group, the difference being nearly significant. *C* and *D*: results of the correlation analysis between changes in activity and density of DCX-positive immature neurons and the density of BrdU-positive cells, respectively. Values are means ± SE (n = 10 per group).

hippocampus have not been established. To extend our previous report, which suggested that forced cessation of voluntary wheel running impairs hippocampal neurogenesis (39), the current study first examined detailed time-course changes in hippocampal neurogenesis following exercise cessation. As expected, voluntary wheel running for 8 wk increased the volume of the GCL of the DG and the density of DCX-positive immature neurons, indicating enhanced adult hippocampal neurogenesis. Cell proliferative activity assessed by both Ki-67 and BrdU immunohistochemistry at the end of the exercise period was unchanged. This finding agrees with the findings of previous studies demonstrating that the stimulating effect of exercise or environment enrichment on cell proliferation is transient and disappears if the duration of the intervention becomes longer (24, 27, 39). Following exercise cessation, the volume of the GCL returned to control levels. Curiously, the density of DCX-positive neurons was not merely reversed, but it decreased to a level that was lower than the control level at 5 and 8 wk after exercise cessation. This result, together with our previous finding, demonstrates that exercise cessation leads to a negative rebound in hippocampal neurogenesis. We further examined the effects on each component of neurogenesis in detail. In *experiment 1*, there was no significant difference in the density of BrdU-positive cells, presumably due to a type II error, whereas in *experiment 2*, exercise cessation resulted in a significant decrease in BrdU-positive cell density. No difference was found in the ratio of BrdU-positive/NeuN-positive to BrdU-positive cells between groups. These results suggested that the negative rebound in neurogenesis following exercise cessation is partially associated with reduced cell survival.

We note here that in *experiment 1*, a continuous exercise group was not included because we previously determined that

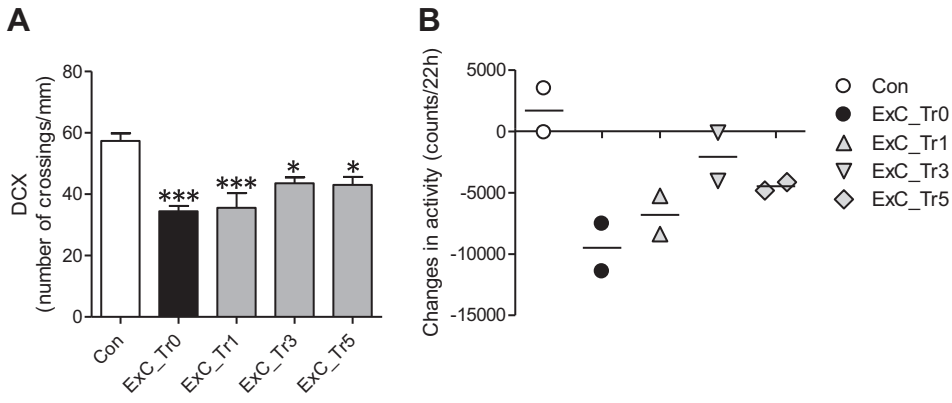

Fig. 8. Treadmill running fails to prevent negative rebound in hippocampal neurogenesis following exercise cessation (ExC). *A*: density of DCX-positive immature neurons significantly decreased in all groups of ExC animals regardless of frequency of treadmill running. Values are means ± SE (n = 8 per group). *P < 0.05, ***P < 0.001 vs. the control (Con) group. *B*: gross ambulatory activity per cage at 1 and 5 wk after ExC were tentatively monitored; the reduction in activity supports the results of *experiment 2*.

long-term voluntary wheel running (from 4 to 21 wk of age, for 18 wk) maintained neurogenic effects (39). The total experimental duration of *experiment 1* (from 4 to 19 wk of age for 16 wk) was almost equivalent to that in the previous report. Eighteen weeks of wheel running resulted in a significant increase in the number of BrdU-positive cells compared with a sedentary control group in which BrdU was injected 2 wk before the animals were killed. Although there was no significant difference in the ratio of DCX-positive/BrdU-positive to BrdU-positive cells between the exercise and sedentary groups, the results (increased cell survival and the same level of neuronal differentiation rate) indicated that long-term running resulted in increased net neurogenesis. Moreover, other studies have demonstrated that voluntary wheel running for more than 6 mo results in a greater density of DCX-positive neurons in mice (24, 32). These results, together with our previous findings, largely exclude the possibility that long-term running abolishes the neurogenic effects.

To understand the mechanisms underlying the negative rebound in hippocampal neurogenesis following exercise cessation, we assessed the potential roles of ΔFosB, which has been shown to be involved in stimulation of hippocampal neurogenesis (57). In accordance with our previous study (37), long-term exercise resulted in increased ΔFosB expression in the DG of the hippocampus. However, ΔFosB remained elevated even at 8 wk after exercise cessation, which was longer than expected and, to the best of our knowledge, would be the longest ΔFosB preservation following stimulus withdrawal ever reported in the literature. This result suggests that the high level of ΔFosB is not sufficient to prevent impairment of neurogenesis following exercise cessation.

We further examined whether the forced interruption of voluntary wheel running is a form of stress, and we found that the mice did not show chronic stress symptoms after exercise cessation. This observation largely excludes the possibility that a sustained increase in circulating corticosterone, a stress hormone that inhibits neurogenesis, is involved in the negative neurogenesis rebound. However, we measured the corticosterone level only at 5 wk after exercise cessation. Because the stress response (i.e., hypothalamus-pituitary-adrenal axis activity) is known to habituate following chronic stress exposure (19), the possibility exists that plasma corticosterone was transiently increased following exercise cessation, which might contribute to the negative rebound in neurogenesis. This possibility must be examined in a future study.

The molecular mechanisms underlying the negative rebound thus remain to be elucidated. Because we found that cell survival was suppressed following exercise cessation, we hypothesize that in our experimental model, neurotrophic factors such as BDNF, which positively regulates cell survival (12), are decreased before the decline in neurogenesis. In addition, the suppression of cell survival implies an increase in apoptosis. Therefore, changes in neurotrophic and proapoptotic factors following exercise cessation should be tested in a future study to understand the molecular mechanisms involved.

We have previously found that exploratory activity in an elevated-plus maze was decreased following exercise cessation (39), which led us to hypothesize that spontaneous ambulatory activity in the home cage would also be decreased by exercise cessation and that the reduction would be associated with the negative neurogenesis rebound. To test this hypothesis, mice were housed individually, and home cage activities were monitored in *experiment 2*. Importantly, we successfully confirmed the results of *experiment 1* that the density of DCX-positive neurons was significantly suppressed following exercise cessation. Intriguingly, the home cage activity of ExC mice gradually decreased over the 5-wk period and the amount of the reduction was greater, albeit not significantly, in ExC mice than in sedentary controls. Furthermore, we found a significant correlation between changes in activity and hippocampal neurogenesis. Although any causality between these variables and mechanisms for suppressing home cage activity remain uncertain, the decrease in activity might explain why the effect of exercise cessation on neurogenesis was long lasting and was suppressed to a level below that of the sedentary controls. A study reported that home cage activities including walking can increase cerebral blood flow in the rat hippocampus (42), indicating that hippocampal neural activation depends on physical activity. Because neuronal activity is a basis for promoting neuronal plasticity (4), home cage activity would be an important mediator in regulating hippocampal function. However, in animal models, the physiological significance of home cage activity has received limited attention. Our finding will open a new avenue of research to understand how home cage activity interacts with brain function.

Forced treadmill running under conditions (intensity, duration, and frequency) similar to those in the current study has been demonstrated to increase hippocampal neurogenesis in mice (11, 31, 51). However, unexpectedly, forced treadmill running, even at 5 days/wk, failed to prevent the negative rebound in neurogenesis following exercise cessation. Importantly, when mice do not cease voluntary wheel running, the density of DCX-positive neurons is expected to be higher than that in control mice. Ironically, this result suggests that the best way to prevent a negative rebound in neurogenesis would be to continue the voluntary wheel running. Another question that remains to be answered is whether impaired hippocampal neurogenesis can be restored if voluntary wheel running is restarted. Berchtold et al. (7) reported that a second experience of voluntary wheel running resulted in a rapidly reinduced BDNF level in the rat hippocampus, warranting further study to test our question.

Reversibility is the principle of exercise training, and it is generally believed that increased physiological function reverts to the pretrained or sedentary control level after detraining. Previous studies have examined how long the effects of exercise on the hippocampus last after exercise cessation and demonstrated that the benefits of exercise on the hippocampus, such as BDNF induction and neurogenesis, are reversible (6, 7, 22, 33, 52). On the contrary, the current and previous results (20, 39, 45) showed that exercise cessation not always merely reverses hippocampal function, but it can worsen compared with sedentary controls. Interestingly, such detrimental effects of exercise cessation have been also found in periepididymal adipose tissue (adipocyte hypertrophy) in rats (49). Because effects of exercise on the hippocampus depend on the form, intensity, and duration of exercise, effects of exercise cessation would presumably depend on these variables as well, which might explain the contradictory findings. In addition, Hopkins et al. (20) reported that the negative effect of exercise cessation on the hippocampal BDNF level was observed only in mice subjected to 4 wk of wheel running during adolescence, but not

during adulthood. In the current study, mice were subjected to 8 wk of wheel running during the growth period (from 4 to 11 wk of age); therefore, we need to replicate the time-course examination with wheel running started from adulthood. Moreover, C57BL/6 mice used in the current study are known to be especially prone to addictive behaviors, including voluntary wheel exercise (23). It would be intriguing to examine whether mouse strains that are not addiction prone, such as BALB/c, also show a negative rebound in neurogenesis following exercise cessation.

Perspectives and Significance

In animal studies, wheel running is considered a form of exercise. Therefore, our results might lead to the conclusion that it is better not to start exercise to avoid the risk of negative effects of exercise cessation on the hippocampus. However, for humans, exercise is intrinsically a health- and fitness-oriented physical activity (5), whereas in laboratory rodents, which are inherently active, voluntary wheel running may reflect their natural level of activity in the wild. Thus our model can be interpreted as an animal model of sudden decrease in daily physical activity or of physical inactivity, rather than of exercise cessation (38). Although physical inactivity is becoming a serious public health concern (5, 55), few animal models have been developed specifically to investigate the effects of physical inactivity on brain function. Because hippocampal function is inversely linked to stress vulnerability (17), our findings led us to hypothesize that physical inactivity impairs hippocampal function and thus might increase vulnerability to stress-induced mood disorders, including depression (38). Given that typical human lifestyles are increasingly sedentary, our results might explain why the prevalence of these disorders is increasing worldwide. Further animal studies are warranted to deepen our understanding of the effects of physical inactivity on the brain.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.N., Y.K., A.I., S.A., and I.K. conceived and designed research; T.N., Y.K., and A.I. performed experiments; T.N., Y.K., and A.I. analyzed data; T.N., Y.K., A.I., S.A., and I.K. interpreted results of experiments; T.N. prepared figures; T.N. drafted manuscript; T.N., S.A., and I.K. edited and revised manuscript; T.N., Y.K., A.I., S.A., and I.K. approved final version of manuscript.

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