Exercise enhances the proliferation of neural stem cells and neurite growth and survival of neuronal progenitor cells in dentate gyrus of middle-aged mice

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Wu CW, Chang YT, Yu L, Chen H, Jen CJ, Wu SY, Lo CP, Kuo YM. Exercise enhances the proliferation of neural stem cells and neurite growth and survival of neuronal progenitor cells in dentate gyrus of middle-aged mice. J Appl Physiol 105: 1585–1594, 2008. First published September 18, 2008; doi:10.1152/japplphysiol.90775.2008.—Aging is an important determinant of adult hippocampal neurogenesis as the proliferation of neural stem/progenitor cells (NSCs) declines dramatically before middle age. Contrary to this, physical exercise is known to promote adult hippocampal neurogenesis. The objective of this study is to investigate the effects of mandatory treadmill running (TR) on neurogenesis, including 1) NSCs proliferation, 2) neurite outgrowth of neuronal progenitor cells, and 3) the survival of newborn neurons in dentate area of middle-aged animals. Compared with 3-mo-old mice, numbers of mitotic cells and neuronal progenitor cells decreased dramatically by middle age and remained at low levels after middle age. Five weeks of TR not only increased NSC proliferation and the number of immature neurons but also promoted the maturation and survival of immature neurons in middle-aged mice. The neurogenic and neurotrophic effects of TR were not due to the reduction of the age-related elevation of serum corticosterone. Significantly, 5 wk of TR restored the age-dependent decline of brain-derived neurotrophic factor and its receptor, TrkB, which are known to promote neuronal differentiation and survival. Taken together, mandatory running exercise alters the brain chemistries of middle-aged animals toward an environment that is favorable to NSC proliferation, survival, and maturation.

middle age; brain-derived neurotrophic factor; survival

ADULT hippocampal neurogenesis, a process of generating new granule neurons from adult neural stem/progenitor cells (NSCs), includes a series of developments: NSC proliferation, neuronal lineage specification, maturation, migration, and synaptic integration (26). These new neurons extend axonal and dendritic projections and establish new synaptic connections to the existing hippocampal circuitry (14, 20, 31). Although it remains debated, neurogenesis in the subgranular zone has been shown to associate with hippocampus-dependent learning and memory (15, 41).

Numerous factors are known to influence adult hippocampal neurogenesis. Among these, aging is an important negative regulator of neurogenesis (19). When compared with young animals, new neuron formation is greatly reduced in the subgranular zone of senescent animals (19, 22, 33). However, the decrease of hippocampal neurogenesis starts long before the animal reaches old age (11, 19, 37). Using rodents as animal models, previous studies have demonstrated that the most prominent diminution of hippocampal neurogenesis is around middle age (17, 33). Therefore, middle age, rather than old age, represents a critical window to investigate changes in age-related decline of neurogenesis.

Nerve growth factors and corticosterone are two factors implicated in the age-related decline of neurogenesis (9, 27). As the levels of nerve growth factor decrease with age, it has been proposed that age-related reduction of neurogenesis is a consequence of reduced neurotrophic stimulation (9, 25, 40). This notion is supported by the observation that administration of neurotrophic factors successfully ameliorates age-related decline of hippocampal neurogenesis (23, 38). The age-related decline of neurogenesis has also been attributed to corticosterone, whose levels are elevated in aged animals (4, 25). Maintenance of corticosterone concentration at a basal level has been shown to reverse the age-related decline of hippocampal neurogenesis in aged rodents (4).

It has been shown that a reduction of neurogenesis in the subgranular zone in aged animals is associated with impairment of hippocampus-dependent learning and memory (43). Therefore, there is an enormous interest in boosting neurogenesis in the hippocampus via antiaging strategies at certain times during aging. Physical exercise represents a nonsurgical and nonpharmacological means that has multiple beneficial effects on the brain, including enhancing adult hippocampal neurogenesis and learning and memory in young and aged animals (42, 43). Thus the objective of this study is to examine the effects of chronic treadmill running (TR) on neurogenesis in middle-aged mice. We chose TR as an exercise paradigm to precisely control the intensity and duration of running exercise. In addition to NSC proliferation, both neurite outgrowth and survival of newborn neurons are critical processes of neurogenesis and are diminished at middle age (32). Hence, we also investigated the effects of chronic TR on the neurite growth and survival of neuronal progenitor cells in the dentate area of middle-aged mice. Initially, we confirmed middle age as a critical stage for the decline of neurogenesis during aging by characterizing the temporal pattern of hippocampal neurogenesis in mice. We then measured the levels of serum corticosterone, hippocampal BDNF, and its receptor, TrkB, during

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aging and attempted to associate their levels with the TR-enhanced neurogenesis in these animals.

METHODS

Animals and Experimental Design

Male C57BL/6J mice obtained from Laboratory Animal Center, National Cheng Kung University were used for all experiments. All experimental protocols were performed according to National Institutes of Health guidelines for animal research (Guide for the Care and Use of Laboratory Animals) and were approved by the National Cheng Kung University Institutional Animal Care and Use Committee. Mice were housed under conditions of controlled temperature (23 ± 1°C) and humidity (55 ± 5%), 12:12-h light-dark cycle (light cycle begins at 0600), and unrestricted access to food and water. In the age-dependent study, cell proliferation, neuronal lineage specification, and neurogenesis were analyzed at five different ages (3, 7, 9, 13, and 24 mo old; n = 9) of mice. To label mitotic cells, five consecutive daily injections of bromodeoxyuridine (BrdU, 50 mg·kg⁻¹·day⁻¹, Sigma, St. Louis, MO) were given intraperitoneally to mice, and neurogenesis was evaluated 2 days after the last BrdU injection. To examine the effect of TR on neurogenesis, mice, at the age of 8 and 12 mo (n = 12), were subjected to a total of 6 wk (1-wk familiarization and 5-wk TR) of training course. Therefore, the ages of animals were labeled as 8 + 1.5 mo old and 12 + 1.5 mo old at the conclusion of TR. Five daily consecutive injections of BrdU (50 mg·kg⁻¹·day⁻¹) were given intraperitoneally to mice at the last 5 days of the 5-wk TR, and the neurogenesis was evaluated 2 days later. Another group of 12-mo-old mice (n = 6) were analyzed for the effects of TR on the survival and maturation of newborn neurons. The animals were subjected to a 9-wk training course (1-wk familiarization and 8-wk TR). Five consecutive daily injections of BrdU (50 mg·kg⁻¹·day⁻¹) were given intraperitoneally to mice at the fifth week of TR training course, and the survival and maturation of newborn neurons were monitored 2 days or 3 wk after the last BrdU injection.

TR exercise. The detailed protocol of TR has been described elsewhere (44). Briefly, at the age of 8 or 12 mo, mice were first subjected to a 1-wk familiarization course to reduce handling- and environment-related stimuli, followed by 5- or 8-wk TR training course. The running speed and time were set at 10 m/min, 20 min for the first day, with an increment of 10 min/day until reaching 60 min/day to fulfill the intensity criteria of ~70% of the animals’ maximal oxygen consumption (39). The same duration and strength of TR were maintained up to the fourth week, when the running speed was elevated to 11 m/min. Mice of the sedentary control group were placed on the same treadmill equipment without training for the same period.

Brain processing. Two days or 3 wk after the last BrdU injection, mice were anesthetized and perfused with ice-cold PBS as previously described (44). The right hemispheres were postfixed in paraformaldehyde, cryoprotected in sucrose solution, sliced into 30-μm sections, and stored in cryoprotectant at −20°C (44). The left hemispheres were quickly submerged in liquid nitrogen for 10 min and then stored at −70°C for biochemical analyses.

Immunohistochemistry. The protocol for immunohistochemical staining of brain section has been described previously (44). The paraformaldehyde-fixed brain section was stained by mouse anti-BrdU (1:1000, Amersharm, Buckinghamshire, UK) for newly proliferated cells, goat anti-doublecortin (DCX, 1:750; Santa Cruz, CA) for immature neurons, and goat anti-rabbit microtubule-associated protein 2 (MAP2, 1:1,000, Chemicon, Temecula, CA) for mature neurons. The immunohistochemical staining was made using the Vectastain ABC system (Vector Laboratories, Burlingame, CA) and nickel-enhanced diaminobenzidine incubation (44). To identify the area of dentate gyrus, slices were counterstained with neutral red. For double immunofluorescence labeling, free-floating tissues were immunostained with respective primary antibodies and incubated either with Alexa Fluor 488 anti-goat (1:1,000, Molecular Probes), Alexa Fluor 594 anti-mouse (1:1,000, Molecular Probes), Alexa Fluor 488 anti-rabbit (1:1,000, Molecular Probes), or Texas Red-conjugated anti-mouse (1:1,000, Vector) secondary antibodies and imaged with an Olympus confocal fluorescent microscope (44).

Cell counting. The entire hippocampal dentate gyrus was cut into an average of 93 coronal sections with a thickness of 30 μm. The numbers of BrdU/DCX+, BrdU−, and DCX-positive cells were counted in every 6th section. The total number of labeled cells per section was determined and divided by the slide selection ratio to obtain the total number of labeled cells per dentate gyrus.

Quantitative analyses of morphology and dendritic length of DCX+ immature neurons. DCX is exclusively expressed in immature neurons up to 4 wk of age and has been widely used as an immature neuronal marker (3, 5). DCX+ immature neurons in dentate gyrus at different stages (A or B, according to the orientation of dendrites in relation to the granular cell layer) were counted in every 6th section throughout the dentate gyrus as aforementioned. Only those stage B cells that had minimal dendritic tree overlap with adjacent cells were included to avoid ambiguity (33). The dendritic lengths were analyzed by National Institutes of Health Image software (http://rsb.info.nih.gov/jj). The experimenter was blind to data collection and quantitative analysis.

Quantification of serum corticosterone. The serum corticosterone levels were analyzed using a corticosterone enzyme immunoassay kit (Assay Designs, Ann Arbor, MI), according to the manufacturer’s protocols. The average sensitivity obtained from eight assays was 35.8 pg/ml. The intra-assay variability (n = 16 wells) was 2.8%, and the interassay variability (n = 8) was 7.8%.

Quantification of hippocampal BDNF. A commercial sandwich ELISA kit (Promega, Madison, WI) was used to quantify the levels of BDNF in the hippocampal homogenate as published (44). In short, hippocampal tissue was homogenized, centrifuged at 15,000 g for 30 min at 4°C, and the protein concentration was adjusted to 2.5 mg/ml before being applied to the microtiter plates for BDNF quantification. The plate was read in an ELISA-spectrophotometer reader with an absorbance wavelength of 405 nm. Standard curves were obtained from values generated from known concentrations of BDNF provided by the kits.

Semiquantification of hippocampal TrkB. Western blotting was used to semiquantify the relative level of hippocampal TrkB. Hippocampal homogenate, containing equivalent total protein concentration, was mixed with sample buffer (Invitrogen, Carlsbad, CA), heated to 70°C for 10 min in the presence of DTT, and separated on a 4–12% Nu-PAGE gel (Invitrogen). The electrophoretically separated proteins were transferred onto PVDF membranes (Bio-Rad, Hercules, CA), blocked with 5% nonfat milk in 20 mM Tris buffer, pH 7.5, containing 0.5 M NaCl and 0.5% Tween-20. The membrane blots were probed with primary antibodies against TrkB (1:3,000, Santa Cruz). Control for protein loading was performed by staining membranes with a monoclonal anti-β-actin antibody (1:10,000, Chemicon). Membranes were incubated with appropriate secondary antibodies (1:10,000), followed by chemiluminescence detection (Perkin Elmer, Boston, MA), and band densities were analyzed with an image analysis system (BioChemi imaging system, UVP, Upland, CA).

Statistical analysis. Data are presented as means ± SE. The Boltzmann sigmoidal nonlinear regression model was used to fit the trend of variables (i.e., BrdU+ cells, corticosterone, etc.) as a function of age. A two-tailed Student’s t-test was applied when variable means were compared between the sedentary and TR groups. Two-way ANOVAs were used to analyze the two main effects (age, TR) and possible interaction. Bonferroni post hoc
tests were performed if significant ($P < 0.05$) main effects or interactions were found.

**RESULTS**

Neurogenesis is dramatically decreased by middle age in mice. To characterize the production of new neurons in dentate gyrus during the aging process, the numbers of mitotic cells (BrdU+), neuronal progenitor cells (DCX+), and newborn immature neurons (BrdU+/DCX+) were analyzed in mice at 3 (young), 7 (adult), 9 (early middle age), 13 (middle age), and 24 (aged) mo of age (Fig. 1A). Compared with 3-mo-old mice, the numbers of BrdU+ cells in dentate gyrus reduced to 44% for 7-mo-old, 27% for 9-mo-old, 16% for 13-mo-old, and 8% for 24-mo-old mice (Fig. 1B). The numbers of DCX+ cells in dentate gyrus also decreased significantly with age. Compared with 3-mo-old mice, the numbers of immature neurons dropped to 20% for 7-mo-old mice, 10% for 9-mo-old mice, 3% for 13-mo-old mice, and 1% for 24-mo-old mice, (Fig. 1C). A similar decline in neurogenesis during aging was also evident. The numbers of BrdU+/DCX+ cells in dentate gyrus were compared with that of 3-mo-old mice, 33% for 7-mo-old mice, 14% for 9-mo-old mice, 5% for 13-mo-old mice, and 1% for 24-mo-old mice (Fig. 1D).

TR enhances neurogenesis in middle-aged mice. We have previously demonstrated that 5 wk of TR enhances neurogenesis in dentate gyrus of young mice (44). As middle age represents a critical turning point for neurogenesis, we then examined the effect of 5-wk TR on neurogenesis in early middle-aged (8-mo-old) and middle-aged (12-mo-old) mice. Our results showed that 5 wk of TR effectively increased the numbers of BrdU+ mitotic cells, DCX+ immature neurons, and BrdU+/DCX+ newborn neurons in dentate gyrus of mice in both age groups (Fig. 2). Two-way ANOVA revealed that there was a significant effect of TR and age ($F = 58.1$, $df = 1/40$, $P < 0.001$) and age ($F = 44.3$, $df = 1/40$, $P < 0.001$) on BrdU+ cells (Fig. 2A). A significant interaction between the effect of TR and age ($F = 15.8$, $df = 1/40$, $P < 0.001$) was revealed, indicating that different ages respond differently to TR. TR also displayed a similar effect on DCX+ immature neurons (Fig. 2B). Both TR ($F = 124.9$, $df = 1/40$, $P < 0.001$) and age ($F = 51.5$, $df = 1/40$, $P < 0.001$) were significant factors in the number of DCX+ cells with a significant interaction between TR and age ($F = 18.9$, $df = 1/40$, $P < 0.001$). Finally, the numbers of BrdU+/DCX+ newborn neurons were also affected by both TR ($F = 19.3$, $df = 1/20$, $P < 0.001$) and age ($F = 38.9$, $df = 1/20$, $P < 0.001$) (Fig. 2C). The significant TR × age interaction ($F = 5.3$, $df = 1/20$, $P = 0.032$) suggests that TR has a more pronounced effect on younger animals than older counterparts.

TR promotes neurite outgrowth of immature neurons in middle-aged mice. We next investigated the effect of TR on neuronal progenitor cell maturation by subcategorizing the DCX+ cells according to their dendritic morphology: stage A, DCX+ cell body located adjacent to subgranular zone with the axis of the cell body parallel to subgranular zone (Fig. 3A, arrowheads); and stage B, DCX+ cell with dendrite projecting to granular cell layer or crossing the granular cell layer to the molecular layer, with the axis of the cell body perpendicular to subgranular zone (Fig. 3A, arrows) (7, 30, 45). Less than one quarter of DCX+ neuronal progenitor cells in dentate gyrus of sedentary middle-aged mice belonged to the relatively mature stage B cells (Fig. 3C). Five weeks of TR drastically increased the fraction of stage B cells to 49% of 8 + 1.5-mo-old mice and 38% of 12 + 1.5-mo-old mice ($F = 28.3$, $df = 1/28$, $P < 0.001$) (Fig. 3C). A significant TR × age interaction ($F = 6.3$, $df = 1/28$, $P = 0.018$) was evident, indicating that the TR-enhanced maturation of neuronal progenitor cells in dentate gyrus was age dependent.

In addition to promoting maturation of neuronal progenitor cells, 5-wk TR also effectively promoted the dendritic outgrowth of immature neurons (Fig. 3B). Quantitative analysis revealed that the dendritic lengths of DCX+ immature neurons were enhanced by 5-wk TR ($F = 73.0$, $df = 1/24$, $P < 0.001$; Fig. 3D). The effect of TR on dendritic outgrowth of DCX+ immature neurons was comparable between 8 + 1.5-mo-old and 12 + 1.5-mo-old mice ($F = 3.1$, $df = 1/24$, $P = 0.090$; Fig. 3D).

TR improves the survival of newborn neurons in middle-aged mice. The majority of adult newborn neurons die within a few weeks after birth, leaving a limited number of newborn neurons that mature successfully (24). To determine whether TR increases the survival of newborn neurons in middle-aged animals, the numbers of BrdU+ cell and BrdU+/MAP2+ cells were counted 2 days and 3 wk after BrdU injections. As expected, the number of BrdU+ cells detected 3 wk after BrdU injections was lower than those 2 days after BrdU injections ($F = 26.5$, $df = 1/16$, $P < 0.001$; Fig. 4A). Compared with the number of BrdU+ cells detected 2 days after BrdU injection, only ~30% of cells survived up to 3 wk (Fig. 4A). TR significantly promoted the 3-wk survival rate of BrdU+ cells to 50% (Fisher’s exact test, $P < 0.05$; Fig. 4A). Furthermore, some of these surviving BrdU+ cells developed into MAP2+ matured neurons 3 wk after BrdU injections. The number of BrdU+/MAP2+ cells was enhanced by TR to more than 2.5 times ($P < 0.01$) that of the sedentary group in middle-aged mice (Fig. 4B).

TR does not change the basal level of serum corticosterone in middle-aged mice. Because corticosterone is known to have strong negative regulatory effects on adult hippocampal neurogenesis (4), we determined the serum corticosterone concentration temporal profile of mice. Based on the Boltzmann sigmoidal regression model, the basal concentrations of serum corticosterone rise at middle age and continue increasing up to 24 mo old (Fig. 5A). One-way ANOVA indicated that levels of serum corticosterone were comparable among mice at 3, 9, and 13 mo of age but increased in 24-mo-old mice. Five weeks of TR did not change the basal level of serum corticosterone in mice at either 8 + 1.5 or 12 + 1.5 mo of age ($F = 0.1$, $df = 1/40$, $P > 0.5$; Fig. 5B), suggesting that TR-induced neurogenic effects are not due to reduction in the basal level of serum corticosterone in middle-aged mice.

TR stimulates BDNF and TrkB expression in hippocampus of middle-aged mice. Numerous studies have shown that BDNF and its downstream signaling pathways are involved in several stages of adult neurogenesis, including promoting differentiation and survival (1, 8, 16). Hence, we investigated the expression profiles of hippocampal BDNF and its receptor, TrkB, during aging and whether their expression patterns were affected by TR in middle-aged animals. Our results showed that levels of hippocampal BDNF revealed a weak trend of decline as age increased (Fig. 5C). Interestingly, a consid-
Fig. 1. Pattern of neurogenesis in the dentate gyrus (DG) of mice at different ages. A: representative micrographs demonstrate immunostaining of bromodeoxyuridine-positive (BrdU⁺) (deep purple) newborn cells, doublecortin-positive (DCX⁺) (deep purple) neuronal progenitor cells, and BrdU⁺ (red)/DCX⁺ (green) newborn immature neurons in dentate gyrus of 3-mo-old (3M), 7-mo-old (7M), 9-mo-old (9M), 13-mo-old (13M), and 24-mo-old (24M) mice 2 days after the last BrdU injection. Scale bar, 75 µm for BrdU⁺ and DCX⁺ panels, 40 µm for BrdU⁺/DCX⁺ graphs. Quantitative analyses of BrdU⁺ cells (B), DCX⁺ cells (C), and BrdU⁺/DCX⁺ cells (D) illustrate that numbers of newborn cells, neuronal progenitor cells, and newborn immature neurons, respectively, in dentate gyrus decrease with increased age. Note that major declines of BrdU⁺, DCX⁺, and BrdU⁺/DCX⁺ cell numbers occur before middle age, which represents a critical turning period of adult hippocampal neurogenesis. Data are expressed as means ± SE; n = 5 for each group.
erable drop of hippocampal full-length TrkB levels was evident before 13 mo of age (one-way ANOVA, \( P < 0.001 \)), whereas the levels were comparable between 13- and 24-mo-old mice (Student’s t-test, \( P = 0.106 \); Fig. 5E). Levels of the truncated form of TrkB, a dominant-negative inhibitor of BDNF signaling, remained unaltered as animals aged (one-way ANOVA, \( P > 0.5 \)).

Five weeks of TR not only enhanced the concentrations of BDNF (\( F = 17.9, df = 1/32, P < 0.001 \); Fig. 5D) but also increased the levels of full-length TrkB (\( F = 31.5, df = 1/26, P < 0.001 \); Fig. 5F) in hippocampi of middle-aged (8 + 1.5- and 12 + 1.5-mo-old) mice. Finally, 5-wk TR did not change the level of truncated TrkB in middle-aged mice (data not shown).

**DISCUSSION**

The present study was designed to characterize the critical time frame of reduction of adult hippocampal neurogenesis in the mouse model. We also attempt to investigate whether chronic TR can ameliorate the age-related reduction of hippocampal neurogenesis at this critical period. Our results provide the following findings. First, adult hippocampal neurogenesis greatly declines before middle age. The number of newborn neuronal progenitor cells in middle-aged mice reduces to \( \sim 5\% \) of that observed in their 3-mo-old counterparts. These results indicate that middle age represents an ultimate stage of life for hippocampal neurogenesis during aging. Second, 5 wk of TR at middle age effectively enhances NSC proliferation and increases the number of neuronal progenitor cells in the hippocampus with a more pronounced effect at younger age. Third, TR improves survival and dendritic outgrowth of newborn immature neurons in middle-aged mice. Finally, chronic TR does not change basal level of serum corticosterone, while expressions of hippocampal BDNF and TrkB are significantly elevated by TR in middle-aged mice. As the function of BDNF signaling has been well established in promotion of neuronal differentiation and survival (1, 8, 16), these results suggest that BDNF signaling pathways play pivotal roles in the TR-elicited neurogenic responses.

Adult hippocampal neurogenesis declines predominately before middle age. In this study, we characterize the age profiles of cell proliferation, neuronal fate specification (DCX\(^{+}\)), and neurogenesis (BrdU\(^{+}\)/DCX\(^{+}\)) in hippocampi of mice and find a steep drop between young adult and middle age in all three parameters. Using BrdU to label dividing cells, our result is consistent with earlier reports that the cell proliferation rate is dramatically reduced in middle-aged mice, yet remains relatively unchanged between middle age and old age (17, 33). A similar trend of decline of the DCX\(^{+}\) immature neurons is also noted in this study. However, using male F344 rat as an animal model, it has been shown that the major decline of DCX\(^{+}\) cell numbers was at 7.5–12 mo of age (33). Such time-pattern discrepancy may be derived from species difference. Previously, results from investigation of four species of wild-living rodents and outbred laboratory mice showed that the onset of the downregulation of cell proliferation and neurogenesis in dentate gyrus was very different within species (2). Furthermore, NSCs derived from rats and mice behaved differently when treated with mitogenic growth factors or differentiation factors in vitro (34). Nevertheless, all the evidence indicates that the major age-related decline of adult hippocampal neurogenesis occurs before middle age. These results also suggest that middle age represents an important turning point of life for investigation of hippocampal neurogenesis in the route to senescence.

**TR stimulates NSC division and increases newborn neuronal progenitor cells in middle-aged mice.** Several reports have shown that physical exercise could robustly induce cell proliferation in the adult hippocampus (18, 42, 43). In this study, we
also demonstrate that 5-wk TR effectively enhances proliferation of NSCs of the subgranular zone in middle-aged mice. Meanwhile, our results also suggest that age strongly determines the potential of exercise-enhanced NSC proliferation. Similar observations have been reported previously even though in a different exercise (running wheel) paradigm (17). Although the exact mechanism of aging-reduced neurogenesis remains unknown, it has been shown that aging does not alter the number or phenotype of NSCs (10). According to these observations, Hattiangady and Shetty (10) argue that the age-related decrease of NSC proliferation is associated with increased quiescence and decreased self-renewal ability of NSCs in the subgranular zone. Along this line, it is possible that only those NSCs still possessing self-renewal ability respond to TR. Thus the TR-enhanced NSC proliferation is also restricted by ages. Interestingly, chronic exercise has been found to maintain the self-renewal capability of NSCs (17). Kronenberg and colleagues (17) showed that 6 mo of voluntary exercise can sustain cell proliferation ability to a level equivalent to that of much younger animals. Therefore, chronic exercise not only stimulates NSC proliferation but also preserves a relatively high self-renewal potential of NSCs.

TR does not alter cell-fate specification in middle-aged mice. TR also increases the total number of immature neurons in middle-aged mice. The increased immature neurons could be due to enhanced NSC proliferation and/or a favored cell-fate specification.

Fig. 3. Chronic TR enhances dendritic outgrowth of DCX<sup>+</sup> neuronal progenitor cells. A: representative micrographs illustrate soma locations and dendritic morphologies during the maturation of neuronal progenitor cells in adult hippocampus. At stage A (green arrowheads), the cell bodies of DCX<sup>+</sup> cells reside in subgranular zone and axes of DCX<sup>+</sup> cell bodies are parallel to the granule layer with horizontal neuritis. At stage B (blue arrows), DCX<sup>+</sup> cells extend vertical dendrites, which shortly extend to granular cell layer or approach the molecular layer and begin branching, thus developing a more complex dendritic morphology. SGZ, subgranular zone; GCL, granular cell layer; MoL, molecular layer. B: representative micrographs of DCX<sup>+</sup> neuronal progenitor cells demonstrate dendritic arborization in the dentate gyrus of 8 + 1.5-mo-old and 12 + 1.5-mo-old mice with or without TR. Examples of tracings (in blue color) are used to measure the dendritic length of these neurons. The bar chart (C) illustrates the percent ratio of stages A and B in BrdU<sup>+</sup> cells in dentate gyrus of 8 + 1.5-mo-old and 12 + 1.5-mo-old mice with or without TR. A total of 250 BrdU<sup>+</sup> cells are counted to categorize stages A and B. D shows the dendritic length of those DCX<sup>+</sup> cells with vertical dendrites (stage B) in 8 + 1.5-mo-old and 12 + 1.5-mo-old mice with or without TR; n = 8 mice per group, 10–12 cells per mouse in 8 + 1.5-mo-old mice and 20–24 cells per mouse in 12 + 1.5-mo-old group. ***P < 0.001: TR vs. respective sedentary (Sed) controls; #P < 0.05: 12 + 1.5-mo-old vs. respective 8 + 1.5-mo-old animals.
neuronal lineage differentiation (rather than glial lineage). In this study, TR does not change the proportions of newborn cells (BrdU\(^+\), Fig. 2A) that develop into neuronal progenitor cells (BrdU\(^+\)/DCX\(^+\), Fig. 2C), suggesting that TR does not change the cell-fate specification in middle-aged mice. Furthermore, the relative cell number increases of TR mice over sedentary groups at 8 + 1.5-mo-old are 2.1-fold for cell proliferation (Fig. 2A), 2.4-fold for immature neurons (Fig. 2B), and 2.3-fold for newborn neurons (Fig. 2C). These results imply that the determinant step for TR-enhanced neurogenesis at middle age is at the initial NSC mitosis stage, rather than the cell-fate specification.

**TR promotes neurite outgrowth of newborn neurons in middle-aged mice.** Besides NSC proliferation and neuronal lineage specification, the maturation, migration, and survival of newborn neuronal progenitor cells are also necessary to warrant successful integration of newborn neuron into existing neuronal networks (26). According to morphological characteristics, the maturation of granule neurons in postnatal dentate gyrus can be divided into four stages: 1) polarization, migration, and initial axonal and dendrite growth; 2) dendritic and axonal growth; 3) dendritic spine growth; and 4) structural modification on dendritic spines (45). Compared with neurons in the brain of a younger animal, the maturation of newborn neurons in aged animals is drastically delayed at stage 2 (32, 45). In this study, approximately three-quarters of immature neurons in middle-aged mice stay in the subgranular zone with the axis of the cell body parallel to the subgranular zone, representing the initial differentiation phase of neuronal progenitor cells. TR effectively promotes the differentiation of immature neurons with their dendrites projecting to granular cell layer or crossing the granular cell layer to the molecular layer and cell bodies migrating into granular cell layer. Furthermore, the age-dependent retardation of dendritic arborization in newborn neurons is greatly improved by TR. The average length of dendrites from immature neurons in middle-aged mice is doubled by 5-wk TR. Taken together, our results show that chronic TR not only increases the quantity but also enhances the quality of newborn neurons.

**TR enhances survival of newborn neurons in middle-aged mice.** It has been shown that most adult newborn neurons die quickly after birth; hence, the survival of newborn neurons critically determines the final outcome of neurogenesis (24). To dissect the net effect of TR-facilitated survival, we analyze BrdU\(^+\) cells at different time points and find that TR enhances the survival of newborn neurons. Using MAP2\(^+\), a marker for mature neurons, to label BrdU\(^+\) newborn cells, we also demonstrated that some of the surviving newborn neurons actually develop into matured neurons in middle-aged animals and the number of the newborn mature neurons is much greater in the TR group. These findings suggest that chronic TR alters the chemistries of middle-aged brain toward an environment resembling younger brains that favors the survival and differentiation of newborn neurons.

**Corticosterone does not associate with age-related decline of neurogenesis.** It has been suggested that corticosterone, a well-known negative regulator of neurogenesis, is involved in age-dependent decline of hippocampal neurogenesis (4, 27). Thus we characterize the changes of serum corticosterone concentration during aging. At first sight, the result of the age-serum corticosterone concentration profile seems to support the assumption that higher basal corticosterone concentrations in aged animals leads to the reduction of neurogenesis. However, detailed analysis indicates that basal levels of serum corticosterone only increase significantly in 24-mo-old mice, which does not associate well with the age-neurogenesis profile that neurogenesis declines predominantly before middle age (Fig. 1). A similar observation has also been reported previously that the basal levels of plasma corticosterone are comparable between young adult (3 mo old) and middle-aged (14 mo old) rats (27). Meanwhile, Heine et al. (11) also failed to find a correlation between the production of hippocampal newborn cells and serum corticosterone levels during aging. In addition, the basal levels of serum corticosterone of middle-aged mice are not changed by 5 wk of TR. Therefore, although corticosterone is a strong negative regulator for adult hippocampal neurogenesis, it contributes little to reducing neurogenesis during aging. However, it is worthy to note that chronic running exercise is known to downregulate the level of hippocampal mineralocorticoid receptor, an innate receptor for glucocorticoids (6, 29). Therefore, the role of the glucocorticoid signaling pathway in neurogenesis and neuronal maturation in middle-aged animals requires further clarification.

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**Fig. 2.** TR mice over sedentary groups at 8 wk. The percentages indicate the number of BrdU\(^+\) cells detected 3 wk after BrdU injection relative to 2 days after BrdU injection. **\(p < 0.01\), ***\(p < 0.001\): 3 wk vs. respective 2 days after the last BrdU injection groups. \#\(p < 0.05\), ##\(p < 0.01\): TR vs. respective sedentary control animals. A: quantitative analyses of the total number of BrdU\(^+\)/H11001 cells detected at dentate gyrus 3 wk after the last BrdU injection in middle-aged mice with or without TR. **\(p < 0.01\).

**Fig. 3.** A, TR does not change the proportions of new- born cells (BrdU\(^+\), Fig. 2A) that develop into neuronal progenitor cells (BrdU\(^+\)/microtubule-associated protein 2-positive (MAP2\(^+\)) cells detected at dentate gyrus 3 wk after the last BrdU injection in middle-aged mice with or without TR. **\(p < 0.01\).**

**Fig. 4.** Chronic TR improves survival of neuronal progenitor cells in hippocampus of middle-aged mice. A: quantitative analyses of the total number of BrdU\(^+\) cells per dentate gyrus 2 days and 3 wk after the last BrdU injection. The percentages indicate the number of BrdU\(^+\) cells detected 3 wk after BrdU injection relative to 2 days after BrdU injection. **\(p < 0.01\), ***\(p < 0.001\): 3 wk vs. respective 2 days after the last BrdU injection groups. \#\(p < 0.05\), ##\(p < 0.01\): TR vs. respective sedentary control animals. B: quantitative analyses of the total number of BrdU\(^+\)/MAP2\(^+\) cells detected at dentate gyrus 3 wk after the last BrdU injection in middle-aged mice with or without TR. **\(p < 0.01\).**
BDNF signaling associates with TR-enhanced neurogenesis and maturation of newborn neurons. Neurotrophic factors have been suggested to correlate with age-dependent decline of neurogenesis (12, 23). Among the assorted neurotrophic factors, BDNF-TrkB signaling pathways have been intimately linked to neuronal differentiation and survival following various insults (1, 8, 16). The levels of hippocampal BDNF reveal a weak trend of decline as age increases, parallel to previous findings (9, 25, 40). However, the relatively small changes in BDNF seem insufficient to explain the dramatic reduction in neurogenesis before middle age. Significantly, levels of hippocampal TrkB demonstrate a great decline in middle age. Therefore, it is reasonable to propose that the age-associated decline of hippocampal BDNF-TrkB signaling takes part in the age-dependent reductions of differentiation, maturation, and survival of newborn neurons.

The role of BDNF-TrkB signaling in aging and neurogenesis deserves further discussion. The roles of BDNF in the control of hippocampal neurogenesis have been well documented. An enhancement of BDNF production has been shown to enhance NSC proliferation in the subgranular zone (13, 28). Furthermore, BDNF is known to promote the survival of newborn granule cells (21, 36). The neurogenic enhancement ability of BDNF has also been demonstrated by intrahippocampal infusion of exogenous BDNF (23, 38). On the other hand, a shortage of BDNF has been linked to a drop in neurogenesis. Mice with heterozygous knockout for BDNF show no response to enriched environment-induced neurogenesis (35). Lower levels of BDNF in the dentate gyrus correlate with reduced granule neuron survival in immune-deficient mice (46). Although the detailed mechanism remains to be elucidated, our results that 5 wk of TR robustly elevates the levels of hippocampal BDNF and TrkB in middle-aged mice suggests that BDNF-TrkB signaling may be involved in the TR-induced neurogenesis. Finally, that the age-associated declines of hippocampal BDNF and TrkB levels are restored by chronic TR also provides a logical explanation for the TR-promoted differentiation, maturation, and survival of newborn neurons in middle-aged animals.

Fig. 5. Effects of age and TR on the changes of concentrations of serum corticosterone and hippocampal brain-derived neurotrophic factor (BDNF) and TrkB. Concentrations of basal serum corticosterone (A) and hippocampal BDNF (C) and TrkB (E) of mice at different ages (3, 9, 13, and 24 mo old) are plotted using Boltzmann sigmoidal nonlinear regression models. Note that basal levels of serum corticosterone increase only after 13 mo of age, hippocampal BDNF expression levels in hippocampus do not alter with age, and hippocampal TrkB levels decrease with age, especially during middle age. Bar charts illustrate the basal levels of serum corticosterone (B), hippocampal BDNF (D), and hippocampal TrkB (F) of 8 + 1.5-mo-old and 12 + 1.5-mo-old mice with or without TR. *P < 0.05, **P < 0.01, ***P < 0.001: TR vs. respective sedentary controls. ##P < 0.01: 12 + 1.5-mo-old vs. respective 8 + 1.5-mo-old animals.
EXERCISE ENHANCES NEUROGENESIS IN MIDDLE-AGED MICE

1593

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