Chronic treadmill exercise in rats delicately alters the Purkinje cell structure to improve motor performance and toxin resistance in the cerebellum

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Submitted 3 November 2011; accepted in final form 19 July 2012

Huang TY, Lin LS, Cho KC, Chen S, Kuo YM, Yu L, Wu FS, Chuang JI, Chen HI, Jen CJ. Chronic treadmill exercise in rats delicately alters the Purkinje cell structure to improve motor performance and toxin resistance in the cerebellum. J Appl Physiol 113: 889–895, 2012. First published July 26, 2012; doi:10.1152/japplphysiol.01363.2011.—Although exercise usually improves motor performance, the underlying cellular changes in the cerebellum remain to be elucidated. This study aimed to investigate whether and how chronic treadmill exercise in young rats induced Purkinje cell changes to improve motor performance and rendered the cerebellum less vulnerable to toxin insults. After 1-wk familiarization of treadmill running, 6-wk-old male Wistar rats were divided into exercise and sedentary groups. The exercise group was then subjected to 8 wk of exercise training at moderate intensity. The rotarod test was carried out to evaluate motor performance. Purkinje cells in cerebellar slices were visualized by lucifer yellow labeling in single neurons and by calbindin immunostaining in groups of neurons. Compared with sedentary control rats, exercised rats not only performed better in the rotarod task, but also showed finer Purkinje cell structure (higher dendritic volume and spine density with the same dendritic field). The exercise-improved cerebellar functions were further evaluated by monitoring the long-lasting effects of intraventricular application of OX7-saporin. In the sedentary group, OX7-saporin treatment retarded the rotarod performance and induced ~60% Purkinje cell loss in 3 wk. As a comparison, the exercise group showed much milder injuries in the cerebellum by the same toxin treatment. In conclusion, exercise training in young rats induced Purkinje cell changes to improve motor performance and protected the cerebellum less vulnerable to toxin insults. After 1-wk familiarization of treadmill running, 6-wk-old male Wistar rats were divided into exercise and sedentary groups. The exercise group was then subjected to 8 wk of exercise training at moderate intensity. The rotarod test was carried out to evaluate motor performance. Purkinje cells in cerebellar slices were visualized by lucifer yellow labeling in single neurons and by calbindin immunostaining in groups of neurons. Compared with sedentary control rats, exercised rats not only performed better in the rotarod task, but also showed finer Purkinje cell structure (higher dendritic volume and spine density with the same dendritic field). The exercise-improved cerebellar functions were further evaluated by monitoring the long-lasting effects of intraventricular application of OX7-saporin. In the sedentary group, OX7-saporin treatment retarded the rotarod performance and induced ~60% Purkinje cell loss in 3 wk. As a comparison, the exercise group showed much milder injuries in the cerebellum by the same toxin treatment. In conclusion, exercise training in young rats increased the dendritic volume and spine density of Purkinje cells, which might play an important role in improving the motor performance. Furthermore, as Purkinje cells in the exercise group were relatively toxin resistant, the exercised rats showed good motor performance, even under toxin-treated conditions.

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THE RELATIONSHIP BETWEEN PHYSICAL activity and brain functions has been widely investigated. In particular, physical activity in older subjects benefits their mental health by protecting the brain against age-related deterioration (6). Many exercise studies primarily focus on brain structural and functional changes related to cognitive improvements (15), with relatively few studies focusing on the motor performance. Consequently, although connections between cognitive deficits and age-associated brain differences have been elucidated, relationships with motor performance are less well understood. Interestingly, aging impacts brain structures and associated behaviors differentially, with the cerebellum showing earlier senescence than the hippocampus (31). As physically active older adults require less error monitoring and show improvements in motor performance (26), it is desirable to explore the beneficial effects of exercise on the cerebellum. Purkinje cells in aged rats show pronounced dendrite degeneration and spine reduction (5), while rats subjected to treadmill exercise for 18 mo show more Purkinje cells and larger soma volume than age-matched sedentary rats (18). Animal studies of this kind, together with the human studies, demonstrate that physical activities generally improve motor functions via modulating Purkinje cell structure in older adults. Whether and how such exercise-induced improvements also happened in young adults remains to be elucidated.

In an early study using 18-day-old mice, increases in physical activity (running, swimming, climbing, and wire walking) induce an increase in Purkinje cell dendritic tree size and spine density during development (24). Later studies using young rats show that a complex paradigm of “acrobat” training or an enriched environment induces morphological changes in Purkinje cells, while repeated running without skill learning induces angiogenesis in the cerebellum (2, 11, 19). However, other animal studies reveal that repeated running with defined intensity and duration improves the motor function and prevents the decay of Purkinje cells under various pathological conditions. For example, chronic treadmill exercise improves motor performance and protects Purkinje cells from degeneration in ataxia animal models (3, 29). Additionally, chronic treadmill running in young rats prevents Purkinje cell loss from traumatic brain injury (27). Therefore, it is necessary to reexamine whether repeated running can modify Purkinje cell morphology and improve motor performance in young rats under healthy and Purkinje-injured conditions.

We hypothesize that chronic treadmill exercise induces adaptive changes in the Purkinje cells of young rats, which not only improve motor functions, but also render the cerebellum relatively resistant to insults from a Purkinje cell toxin. In this study, we compared normal sedentary young rats with age-matched animals subjected to 8 wk of treadmill running at moderate intensity. The motor performance was evaluated by a rotarod test, and the Purkinje cells were examined either in single neurons or in neuron groups. Finally, the exercise-improved cerebellar functions were further evaluated by intraventricular application of OX7-saporin (OX7S, a Thy1 Purkinje cell toxin).
MATERIALS AND METHODS

Animals. All procedures performed were approved by the Institutional Animal Care and Use Committee of National Cheng Kung University (IACUC approved number 98145), whose standards meet the guidelines of the National Institutes of Health (Guide for the Care and Use of Laboratory Animals). Male Wistar rats (5 wk old) from National Cheng Kung University Animal Center were used. All efforts were made to minimize the number of animals used and their suffering. Rats were housed in groups of four at 23°C on a 12:12-h light-dark cycle (lights on at 0600) with ad libitum access to rat chow and water.

Chronic moderate treadmill exercise. Initially, rats ran on a motor-driven leveled treadmill (Model T408E, Diagnostic & Research Instruments, Taoyuan, Taiwan), starting at a very low speed and gradually reaching 9 m/min for 10 min each day for 5 days to become familiar with treadmill running. Additionally, to encourage the rats running forward, the front side of the treadmill was covered to create a dark area. In our hands, the vast majority (>95%) of animals were willing to run at this low speed, if they were handled gently in the beginning. Thus <5% of the rats were excluded because they did not run in the familiarization period. A total of 76 rats were used in this study. They were assigned to sedentary or exercise groups, according to their initial body weight (n = 38 in each group). Animals in the sedentary control group were placed on the treadmill without running for 10 min each day. Animals in the exercise group were subsequently trained at an intensity of ~70% of maximal oxygen consumption for 60 min/day, 5 days/wk for 8 wk (4). The running speed started at 12 m/min, increased 3 m/min every 2 wk, and reached to 21 m/min at the end of the training period. A few rats in exercise groups were unwilling to run on the treadmill when close to the end of training program at 18 or 21 m/min. Those rats were given an additional make-up run, starting from 9 m/min to the designated training speed with 1 m/min increase every 2 min, to reach the same running distance.

Behavioral test. The movement performance was measured with a commercial rotarod instrument (Ugo Basile, model 7750, Linton Instruments). Three days before the test, rats were put on the rotarod four times each day (30 min apart) until they were able to stay on it for at least 120 s at a rotating speed of 8 rpm. The rotating speed increased from 5 to 33 rpm over 5 min during the test, and the time to fall was recorded. Rotarod tests were performed during the early light phase. Animals were transferred to the testing room and habituated for 2 h before the beginning of the experiments. After each individual test session, the apparatus was thoroughly cleaned with 20% alcohol to eliminate the odor and traces of the previously tested animal.

Single-cell labeling of Purkinje cells. Rats were anesthetized with a mixture solution (ketamine 50 mg/ml, xylazine 23.3 mg/ml, atropine 1 mg/ml; 2 ml/kg) intraperitoneally (sedentary group, n = 6; exercise group, n = 6). The anesthetized animals were perfused from the left

Fig. 1. Typical Purkinje cell morphology in sedentary (Sed) and exercise (Ex) rats. Left: compiled images of two Purkinje cells located in lobule 9 (one from each group). The marked regions indicate the dendritic field. Middle: a set of 25 consecutive images, representing the optic sections (250 μm × 250 μm, 1 μm apart) covering the main portion of the dendritic structure. Right: dendritic spines located at the peripheral region of the dendritic structure.
ventricle with 4°C artificial cerebrospinal fluid (ACSF; 117 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 11 mM glucose, 1.2 mM MgCl₂, 25 mM NaHCO₃, 1.2 mM NaH₂PO₄, pH 7.4) containing heparin (1,000 IU) and followed by 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.2). The cerebellum was dissected and postfixed in the same fixative overnight. Single-neuron labeling with the fluorescent dye lucifer yellow was carried out according to methods described in detail previously (23). Briefly, after being embedded in 5% agar, the cerebellar tissue was sectioned into 300-µm sagittal slices. A slice of vermis was transferred to an injection chamber mounted on the stage of a fluorescence microscope. Since cerebellar nodulus/uvula, lobules 9 and 10, are responsible for the computation of various signals related to spatial orientation (32), we focused on examining Purkinje cells located at these two lobules. Specimens were viewed by using differential interference contrast optics under a 100× objective lens (numerical aperture (NA) = 0.8, water immersion). Purkinje cells were identified according to their location and size. We labeled one-half of the cerebellum completely cryosectioned into 40-µm thick sagittal slices. Purkinje cells in tissue slices were labeled with immunostaining and calbindin, a marker of Purkinje cells. Brain slices were incubated with immunoblocking solution (3% normal goat serum, 0.2% Triton X-100 in 0.1 M phosphate-buffered saline) for 1 h, followed by mouse anti-calbindin D-28K (1:3,000, Sigma, St. Louis, MO) overnight at room temperature. After washing, specimens were stained with the fluorescent secondary antibody (Alexa Fluor 488 goat anti-mouse, Invitrogen, Camarillo, CA). The immunostained specimens were preserved in glycerol-based medium (80% glycerol, 20% Na₂CO₃). A conventional fluorescence microscope was used for observing Purkinje cell distribution, while a two-photon microscope was used for more detailed observation of the dendritic structure of Purkinje cells. Total Purkinje cell number in a cerebellar half was obtained by using an upright microscope (Zeiss, Axioskop 2 Puls) equipped with a ×20 lens (plan-NEOFLUAR, NA = 1.3). We sampled one out of every 11 sagittal slices (40-µm thickness) from one-half of a cerebellum, i.e., the cerebellar slices were selected with a random start in the first 11 slices, resulting in a total of 13–17 slices per animal. Each slice was mounted with randomly selected side facing up. The top focal plane (with clear fluorescence images) served as the inbound plane, whereas the bottom focal plane served as the inbound plane. Cell counting was performed from the outbound plane to the inbound plane. A Purkinje cell near the outbound plane was counted only if it was clearly visible when the focal plane moved into deeper layers. In contrast, all fluorescent objects larger than 5 µm in the inbound plane were counted. The total cerebellar slice was surveyed. The total number of Purkinje cells in each rat was estimated as the sum of counted cells × 11 × 2. Twelve rats in each group were counted.

Statistics. Results are expressed as means ± SE in the text and Figs. 2 and 3. The data of rotarod test at different time points were analyzed by repeated ANOVA, followed by Bonferroni post hoc test. Two-tail Student unpaired t-test was used to evaluate the exercise effects on the dendritic fields, dendritic volume, or spine density. Two-way ANOVA with Bonferroni post hoc test was used to evaluate differences of Purkinje cell number between sedentary and exercise groups in OX7S experiments. For correlation studies, Pearson’s correlation analysis (SPSS statistics package, Chicago, IL) was applied.

Intraventricular application of OX7S. OX7S (Advanced Targeting Systems, San Diego, CA) targets Thy1 expressing cells and inactivates their ribosomes by transporting saporin into these cells. Intraventricular injection of OX7S has been shown to kill Purkinje cells in the cerebellum (1, 9, 22). At the end of the 5th wk of exercise training, OX7S (2 µg in 2.3 µl) was locally administered to poison Purkinje cells. Application of an equal volume of ACSF served as the sham control. These reagents were stereotaxically delivered into the lateral ventricle (anterior-posterior: −1.2 mm, medial-lateral: ± 1.6 mm, dorsal-lateral: −4.3 mm). These rats were allowed to rest for 3 days and continued for 3 additional wk of exercise training. Behavioral tests were performed weekly from the end of the 5th wk.

Purkinje cell immunostaining and counting. The paraformaldehyde fixed cerebellum was divided into half at the midsagittal line. Randomly selected one-half of the cerebellum was completely cryosectioned into 40-µm thick sagittal slices. Purkinje cells in tissue slices were labeled with immunostaining and calbindin, a marker of Purkinje cells. Brain slices were incubated with immunoblocking solution (3% normal goat serum, 0.2% Triton X-100 in 0.1 M phosphate-buffered saline) for 1 h, followed by mouse anti-calbindin D-28K (1:3,000, Sigma, St. Louis, MO) overnight at room temperature. After washing, specimens were stained with the fluorescent secondary antibody (Alexa Fluor 488 goat anti-mouse, Invitrogen, Camarillo, CA). The immunostained specimens were preserved in glycerol-based medium (80% glycerol, 20% Na₂CO₃). A conventional fluorescence microscope was used for observing Purkinje cell distribution, while a two-photon microscope was used for more detailed observation of the dendritic structure of Purkinje cells. Total Purkinje cell number in a cerebellar half was obtained by using an upright microscope (Zeiss, Axioskop 2 Puls) equipped with a ×20 lens (plan-NEOFLUAR, NA = 1.3). We sampled one out of every 11 sagittal slices (40-µm thickness) from one-half of a cerebellum, i.e., the cerebellar slices were selected with a random start in the first 11 slices, resulting in a total of 13–17 slices per animal. Each slice was mounted with randomly selected side facing up. The top focal plane (with clear fluorescence images) served as the inbound plane, whereas the bottom focal plane served as the inbound plane. Cell counting was performed from the outbound plane to the inbound plane. A Purkinje cell near the outbound plane was counted only if it was clearly visible when the focal plane moved into deeper layers. In contrast, all fluorescent objects larger than 5 µm in the inbound plane were counted. The total cerebellar slice was surveyed. The total number of Purkinje cells in each rat was estimated as the sum of counted cells × 11 × 2. Twelve rats in each group were counted.

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![Dendritic fields](image_url)

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**Fig. 2. Effects of chronic treadmill exercise on the dendritic field** (A), the dendritic volume (B), and the dendritic spine density (C) of Purkinje cells. Results were summarized from 40 Purkinje cells in lobule 9 and lobule 10 in each group (n = 6). Values are means ± SE. *P < 0.05 calculated according to two-tail Student’s unpaired t-test.
The sample size "n" represented the number of animals in each group. \( P < 0.05 \) was selected for statistical significance.

**RESULTS**

Exercise training improved rotarod performance and delicately altered Purkinje cell morphology. Chronic treadmill exercise at moderate intensity for 8 wk in normal rats improved motor performance, as indicated by staying longer on the rotarod (exercise vs. sedentary, 299.6 ± 10.3 s vs. 168.2 ± 9.2 s, \( n = 8, P < 0.05 \)). Moreover, this exercise training program seemed to delicately alter the morphology of Purkinje cells in the cerebellum (Fig. 1). The summarized results are shown in Fig. 2. Even though the dendritic field (total area covered by dendrites) in individual Purkinje cells remained the same in both groups, cells in the exercise group showed denser dendrites (more dendritic branches and thus higher total dendritic volume), along with higher spine density (more spines per unit dendritic length) than controls.

**Exercise training protected the cerebellum against insults from OX7S.** To evaluate the neuroprotective effects of exercise on the cerebellum, OX7S was applied via intraventricular injection after 5 wk of exercise. Our results in Fig. 3 show that vehicle administration (sham operation) did not affect the rotarod performance in either sedentary group or exercise group (exercise-ACSF vs. sedentary-ACSF, 288 ± 13.7 s vs. 156 ± 8.5 s, \( n = 12, P < 0.05 \)). The exercise-induced improvement of motor performance occurred as early as the 5th wk. Although OX7S progressively retarded the rotarod performance in the sedentary group, it was ineffective in the exercise group (Fig. 3). We further investigated if exercise protected Purkinje cells from OX7S-induced damage. Calbindin immunostaining results show that OX7S treatment in sedentary rats damaged Purkinje cells in almost all lobules (Fig. 4). It is noted that the remaining Purkinje cells in the OX7S-treated specimens were unevenly

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**Fig. 3.** Chronic treadmill exercise prevented the OX7-saporin (OX7S)-retarded rotarod performance. OX7S treatment at the 5th wk progressively hampered the rotarod performance in the Sed group, but not in Ex group. ACSF, artificial cerebrospinal fluid. Values are means ± SE. *\( P < 0.05 \), Ex-ACSF vs. Sed-ACSF, \( n = 12 \). #\( P < 0.05 \), Sed-OX7S vs. Sed-ACSF; \( n = 12 \).

**Fig. 4.** Effects of OX7S treatment on Purkinje cells in Sed or Ex rats. The distribution and morphology of Purkinje cells were revealed by immunostaining of calbindin in the cerebellum slices. Images at top and middle were taken with a conventional fluorescence microscope. Images at bottom were taken with a two-photon microscope. Chronic treadmill exercise apparently made Purkinje cells relatively resistant to the OX7S toxicity.
Effects of OX7S and exercise on Purkinje cell number

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<thead>
<tr>
<th></th>
<th>ACSF</th>
<th>OX7S</th>
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<tr>
<td>Sed</td>
<td>462 ± 12</td>
<td>189 ± 24†</td>
</tr>
<tr>
<td>Ex</td>
<td>473 ± 18</td>
<td>341 ± 30†</td>
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Values are means ± SE; no. of Purkinje cells is in units of 10⁴ cells. Sed, sedentary group; Ex, exercise group; ACSF, injected with artificial cerebrospinal fluid; OX7S, injected with OX7-saporin. †P < 0.05, Ex vs. Sed; n = 12.

Our study is the first to monitor Purkinje cell morphology in detail using single-neuron labeling techniques, combined with two-photon microscopy. Since a Purkinje cell is fan-shaped and almost parallel with neighboring cells of its kind, it was easy to make tissue sections relatively parallel to the plane of Purkinje cells. Overprojection or underprojection should not be a problem for morphometric measurements, because we were able to make optical sections covering the entire cell (~250 μm from the soma to the dendritic tip) within a total thickness ~30 μm, i.e., the estimated maximal tilt would be ~3.5°. The possible tissue shrinkage was estimated to be ~7% in volume, i.e., 2.2% lengthwise. This estimation was based on measuring the volume (not weight) of dissected cerebellum immediately after brain removal and 3 days postfixation (the time of tissue sectioning and single-cell labeling). Besides, the fixation effects are relative mild in the cerebellum (30). However, because the somata of lucifer yellow-labeled neurons were very bright and often contaminated with dye leakage, whether exercise enlarged Purkinje soma or not remained uncertain at present.

Considerable research efforts have focused on whether and how different forms of physical exercise or environmental stimulation remodel neuronal morphology in the brain and modulate corresponding behavioral performances. In cerebellum-related studies, acrobat training (2, 19) or an enriched environment (11), but not repeated running, induces synaptogenesis in Purkinje cells. The apparent discrepancy with regard to running-induced synaptogenesis between the present study and previous reports may be explained by the different running intensities and durations employed. Compared with previous reports (2, 19), our running speed was twice as fast (21 m/min vs. 10 m/min) and the training period was twice as long (8 wk vs. ~4 wk). High running intensity and long training period might thus be required for exercise-induced adaptive changes in Purkinje cell morphology. This viewpoint is further supported by a recent study reporting treadmill running intensity-
dependent changes in the spine number and shape of Purkinje cells (10). Rats after acrobat training showed a mild increase (~20%) in the number of Purkinje synapses in paramedian lobules (2). However, the acrobat-evoked synaptogenesis may predominantly occur in Purkinje cells, since the overall cerebellar level of synaptophysin (a synaptic vesicle fusion protein) in mice was unaltered by similar acrobat training (17). In our work, chronic treadmill running not only increased the synapse density in Purkinje cells (Fig. 3), but also elevated the overall cerebellar level of synaptotagmin I (another synaptic vesicle fusion protein) (L.-S. Lin, unpublished observations).

In the measurement of Purkinje cells, since we counted through the entire thick section, a potential bias was possible due to the lost caps. Although chronic treadmill running did not increase Purkinje cell number in our exercised animals, it delicately altered the Purkinje cell structure and provided neuroprotective effects against OX7S toxicity. Many neurodegenerative conditions in the cerebellum are associated with the degeneration of Purkinje cell structure and function. For example, an early loss of Purkinje neurons in the cerebellum is a common feature in aging and ataxia (18, 29). As aged Purkinje cells show reduced target field for granule cells and spine density (5, 12), morphologically abridged cells are likely to be functionally defective. Life-time exercise (from 5 to 23 mo of age) in rats prevents age-induced Purkinje cell number loss (18). The mean volume of Purkinje cell soma in either exercised or sedentary aged rats is much larger than that in young rats, indicating a life-long growth of the soma of these cells. As the Purkinje cell soma in exercised aged rats is larger than in sedentary aged animals, it is expected that chronic exercise stimulates the growth of Purkinje cell soma, along with the refinement of dendritic tree, and thus improves motor performance in young and aged animals alike.

In response to exercise, the brain produces many neurotrophic factors, especially the brain-derived neurotrophic factor (BDNF) that also serves as a survival factor and a repair factor (33). Modification of BDNF and/or the expression of its receptors (TrkB.FL, TrkB.T1, and TrkB.T2) have been described during normal aging and Alzheimer’s disease (28). Our laboratory’s previous studies have shown that exercise not only transiently elevates BDNF mRNA and protein levels in the hippocampus (13), but also persistently upregulates the hippocampal BDNF receptor TrkB (20, 21). Although acute severe exercise in mice failed to increase the BDNF mRNA level in the cerebellum (25), whether chronic moderate exercise has any effect in this regard remains to be clarified. Interestingly, acute and gradual increases in BDNF concentration elicit distinct signaling in cultured neurons and lead to differential effects on neurite morphology (14). Alternatively, the cerebellar neuroplasticity and toxin resistance may be mediated by other neurotrophic factors instead. For example, insulin-like growth factor I has been reported to mediate the protective effects of exercise against the progression of inherited Purkinje cell degeneration in the cerebellum (3). This growth factor helps the neural protection in culture or in vivo against pro-apoptotic insults (7, 8). Thus insulin-like growth factor I, present in abundance in the plasma under exercised conditions, would exert a neuroprotective effect when it passes through the blood-brain barrier. Taken together, additional studies are needed to clarify the underlying mechanisms of exercise-induced protective effects on the cerebellum, such as to quantify the exercise-induced spatial and temporal changes of specific neurotrophic factors involved.

In conclusion, the exercise training-induced morphological changes in Purkinje neurons play an important role in upgrading both motor performance and neuroprotective capacity. Our findings show the importance of investigating how neuronal structures are fine-tuned by exercise in the cerebellum of young animals.

**GRANTS**

This study was supported by the National Science Council in Taiwan (Grants NSC 95-2320-B-006-045-MY3 and 98-2320-B-006-019-MY3).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


