ORIGINAL PAPER

Effects of Treadmill Exercise on Cell Proliferation and Differentiation in the Subgranular Zone of the Dentate Gyrus in a Rat Model of Type II Diabetes

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Abstract In the present study, we investigated the effects of a treadmill exercise on serum glucose levels and Ki67 and doublecortin (DCX) immunoreactivity, which is a marker of cell proliferation expressed during cell cycles except G0 and early G1 and a marker of progenitors differentiating into neurons, respectively, in the subgranular zone of the dentate gyrus (SZDG) using a type II diabetic model. At 6 weeks of age, Zucker lean control (ZLC) and Zucker diabetic fatty (ZDF) rats were put on a treadmill with or without running for 1 h/day/5 consecutive days at 22 m/min for 5 weeks. Body weight was significantly increased in the control (without running)-ZDF rats compared to that in the other groups. In the control groups blood glucose levels were increased by 392.7 mg/dl in the control-ZDF rats and by 143.3 mg/dl in the control-ZLC rats. However, in the exercise groups, blood glucose levels were similar between the exercise-ZLC and ZDF rats: The blood glucose levels were 110.0 and 118.2 mg/dl, respectively. Ki67 positive nuclei were detected in the SZDG in control and exercise groups. The number of Ki67 positive nuclei was significantly high in exercise groups compared to that in the control groups. In addition, Ki67 positive cells were abundant in ZLC groups compared to those in ZDF groups. DCX-immunoreactive structures in the control-ZDF rats were lower than that in the control-ZLC rats. In the exercise groups, DCX-immunoreactive structures (somata and processes with tertiary dendrites) and DCX protein levels were markedly increased in both the exercise-ZLC and ZDF rats compared to that in the control groups. These results suggest that a treadmill exercise reduces blood glucose levels in ZDF rats and increases cell proliferation and differentiation in the SZDG in ZLC and ZDF rats compared to those in control groups.

Keywords Type II diabetes · Zucker rat · Exercise · Dentate gyrus · Neurogenesis

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Introduction

Diabetes mellitus (DM) is one of the most common endocrine disorders, affecting almost 6% of the world's population. DM is divided according to the action of beta cells: Type I DM is characterized into the degeneration of beta cells, while the main cause of type II DM is a progressive decline in insulin sensitivity with eventual insulin deficiency that results in sustained hyperglycemia [1–3]. The latter is most common in humans and has a proportion of more than 97% in diabetic patients [4]. The Zucker diabetic fatty (ZDF) rat is considered to be an excellent animal model of type II DM that presents a physiological

and metabolic profile similar to those seen in humans, and male ZDF rats develop a phenotype of obesity, insulin resistance, and hyperglycemia [5].

Recently, it has been reported that hippocampal neurons are vulnerable to diabetes [6–9], and the diabetes occurs damages of dendrites, pre- and post-synaptic structures in CA3 neurons, and reduces the expression of insulin growth factors and their receptors in the hippocampus in type I DM model [8, 10–12]. In addition, DM reduces cell proliferation in the dentate gyrus in type I [10, 13, 14] and type II models [15, 16]. Exercise has been focused on metabolic diseases because the exercise reduces predisposing factors induced by diabetes, improving cardiovascular health, lipid-cholesterol balance, energy metabolism, glucose use, insulin sensitivity, inflammation and hippocampal plasticity [17–21].

Neural progenitors in the dentate gyrus proliferate, migrate, and differentiate into granule cells, which extend their axons and contact CA3 pyramidal neurons, becoming integrated into the hippocampal circuitry [22–24]. Although some researchers have reported that treadmill increased cell proliferation in the dentate gyrus of type I DM model [25, 26], there are no studies about correlations between diabetes and neurogenesis after exercise in type II models. Therefore, in the present study, we investigated cell proliferation and neuronal differentiation in the dentate gyrus of Zucker diabetic fatty (fa/fa, ZDF) rats and Zucker lean control (fa/+ or +/+, ZLC) rats using Ki67, a marker of cell proliferation expressed during the late G1, S, M, and G2 phases of the cell cycle [27] and doublecortin (DCX), a marker of progenitors differentiating into neurons.

Experimental Procedures

Experimental Animals

Male and female ZDF (fa/+) were purchased from Genetic Models (Indianapolis, ME) and mated each other. They were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-h light/12-h dark cycle, and free access to food and water. Purina 5008 rodent diets (7.5% fat) were provided as recommended by Genetic Models Co. (Purina, St. Louis, MO). The procedures for handling and caring for the animals adhered to the guidelines that are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985, revised 1996). All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Genotyping of fa Gene and Experimental Design

Genotype of fa gene herein was determined with the strategy described in our previous study [15, 28]. Ten male ZLC and ZDF rats were randomly divided into 4 groups (n = 8 per group): control-ZLC, control-ZDF, exercise-ZLC and exercise-ZDF group. At 6 weeks of age, exercise-ZLC and exercise-ZDF rats were familiarized with the treadmill running on a motorized treadmill (Model 1050 LS Exer3/6; Columbus Instruments, Columbus, OH) for 15 min/day at 15 m/min for 5 consecutive days. After the familiarization, electrical stimulation to encourage the rats to run was disconnected to avoid pain stress. The rats were run for 1 h/day/5 consecutive days at 22 m/min for 5 weeks and the speeds accelerated 2 m/min per 2-weeks. The control ZLC and ZDF rats were put on the treadmill without running for 1 h/day/5 consecutive days at 22 m/min for 5 weeks. All animals were euthanized at 12 weeks of age.

Food Intake, Body Weight, and Blood Glucose Sampling

Total food intake and body weight over the course of the study were determined for each animal by summing the weekly averages. To measure blood glucose concentration, blood was sampled each morning (9:00 am) by "tail nick" using a 27 G needle and analyzed by using a blood glucose monitor (Ascensia Elite XL Blood Glucose Meter, Bayer, Toronto, ON, Canada).

Tissue Processing for Histology

For immunohistological analysis, five animals in each group were anesthetized with sodium pentobarbital and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and postfixed in the same fixative for 6 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter the frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30-μm coronal sections, and they were then collected into six-well plates containing PBS.

Immunohistochemistry for Ki67 and DCX

Immunohistochemical staining was conducted with our previous study [13]. The sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min and 10% normal goat or rabbit serum in 0.05 M PBS for 30 min. They were then incubated with diluted rabbit anti-Ki67 antibody (1:1,000, Abcam, Cambridge, UK) or

diluted goat anti-DCX antibody (1:50, SantaCruz Biotechnology, Santa Cruz, CA) overnight at room temperature and subsequently exposed to biotinylated goat anti-rabbit IgG or rabbit anti-goat IgG and streptavidin peroxidase complex (1:200, Vector, Burlingame, CA). They were then visualized by staining with 3,3′-diaminobenzidine in 0.1 M Tris–HCl buffer (pH 7.2) and mounted on gelatin-coated slides. The sections were mounted in Canada Balsam (Kanto, Tokyo, Japan) following dehydration. A negative control test was carried out using pre-immune serum instead of primary antibody in order to establish the specificity of the immunostaining. The negative control resulted in the absence of immunoreactivity in any structures.

Western Blot Analysis

To confirm changes in DCX levels in the dentate gyrus of control and exercise groups, 3 animals in each group were sacrificed and used for Western blot analysis. After sacrificing them and removing the brain, dentate gyrus were then dissected with a surgical blade. The tissues were homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol bis (2-aminoethyl Ether)-N,N,N′,N′ tetraacetic acid (EGTA) (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylendiamine tetraacetic acid (EDTA) (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). After centrifugation, the protein level was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, USA). Aliquots containing 20 μg of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a 10% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with goat anti-DCX (1:200), peroxidase-conjugated rabbit anti-goat IgG (Sigma, St Louis, MO) and an ECL kit (Pierce Chemical, Rockford, IL).

Quantification of Data

All measurements were performed in order to ensure objectivity in blind conditions, by two observers for each experiment, carrying out the measures of experimental samples under the same conditions.

To elucidate the effects of exercise on cell proliferation in ZLC and ZDF rats, the corresponding areas of the dentate gyrus were measured from 10 sections per animal.

Images of all Ki67 or DCX immunoreactive structures were taken from dentate gyrus through a BX51 light microscope (Olympus, Japan) equipped with a digital camera (DP71, Olympus, Japan) connected to a PC monitor. Dendritic complexity of DCX positive cells was analyzed using the accompanying software (NeuroExplore, MicroBrightField, Inc., VT,), calculating complexity including dendritic length and number of branches. The number of Ki67, DCX positive cells and total granule cells in all groups was counted in dentate gyrus using an image analyzing system equipped with a computer-based CCD camera (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ). Cell counts were obtained by averaging the counts from the sections taken from each animal: A ratio of the count was calibrated as %.

The effects of exercise on cell differentiation in ZLC and ZDF rats were assessed by western blot analysis, and the quantification of the Western blotting was done using Scion Image software (Scion Corp., Frederick, MD), which was used to count the optical density.

Statistical Analysis

The data shown here represent the means of experiments performed for each experimental area. Differences among the means were statistically analyzed by one-way analysis of variance followed by Duncan's new multiple range method in order to elucidate differences between ZLC and ZDF and/or control and exercise group.

Results

Genotyping of the *fa* gene and Changes in Food Intake, Body Weight and Blood Glucose Level

We confirmed the genotype of the *fa* gene in order to identify the homozygote and others such as previous studies [15, 28]. The food intake was not significantly different between ZLC and ZDF group. In addition, the exercise did not give any significant changes in the food intake (data not shown).

Unlike food intake, body weights showed significant difference between ZLC and ZDF group. In the ZDF rats, the body weight was not different at 6 weeks of age. The body weight in the control-ZDF group was much higher than that in the other groups at 10 weeks of age, and this pattern was maintained by 12 weeks of age. In the exercise groups, the body weight was similar between ZLC and ZDF rats (Fig. 1a).

In the control groups, blood glucose level was age-dependently increased and was 392.70 mg/dl and 143.30 mg/dl in the ZDF and ZLC rats, respectively, at

A Body weight

B Blood glucose levels
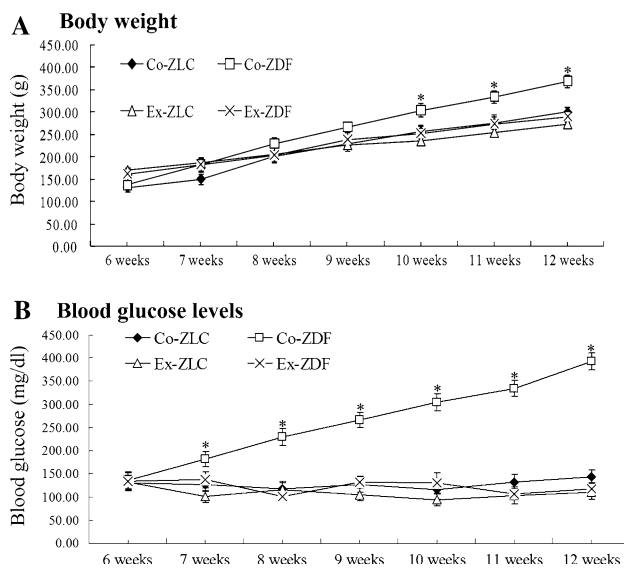
Fig. 1 Changes in body weight (**a**) and blood glucose levels (**b**) in control-ZLC (Co-ZLC), control-ZDF (Co-ZDF), exercise-ZLC (Ex-ZLC) and exercise-ZDF (Ex-ZDF) rats. Blood glucose concentration is significantly increased in the Co-ZLC and Co-ZDF groups with age. However, in the Ex-ZLC and Ex-ZDF group, the blood glucose levels are not significantly changed. The body weight is increased with age in all groups; however in the Co-ZLC, Ex-ZLC and Ex-ZDF groups, the slope of body weight is blunt compared to that in the ZDF group ($n = 5$ per group; *$P < 0.05$, significantly different from the ZLC group, #$P < 0.05$, significantly different from the control group). The bars indicate the means ± SE

12 weeks of age. However, the exercise in ZLC and ZDF rats maintained the blood glucose levels with ages compared to those in the control groups: The blood glucose levels were 110.00 and 118.17 mg/dl in ZLC and ZDF groups, respectively, at 12 weeks of age (Fig. 1b).

Effects of Treadmill Exercise on Cell Proliferation in ZDF and ZLC Rats

In the control groups, Ki67 immunoreactive nuclei were detected in the subgranular zone of dentate gyrus and the number of Ki67 immunoreactive nuclei was abundant in ZLC group compared to that in the ZDF group (Fig. 2a, b and e). The exercise increased the number of Ki67 immunoreactive cells compared to that in the control groups. In addition, the number of Ki67 immunoreactive nuclei in the exercise-ZLC group was more abundant compared to that in the exercise-ZDF group (Fig. 2c, d and e).

Effects of Treadmill Exercise on Neuronal Differentiation in ZDF and ZLC Rats

In all groups, DCX immunoreactive cells were observed in the subgranular zone of the dentate gyrus; however, DCX protein levels were markedly different between groups
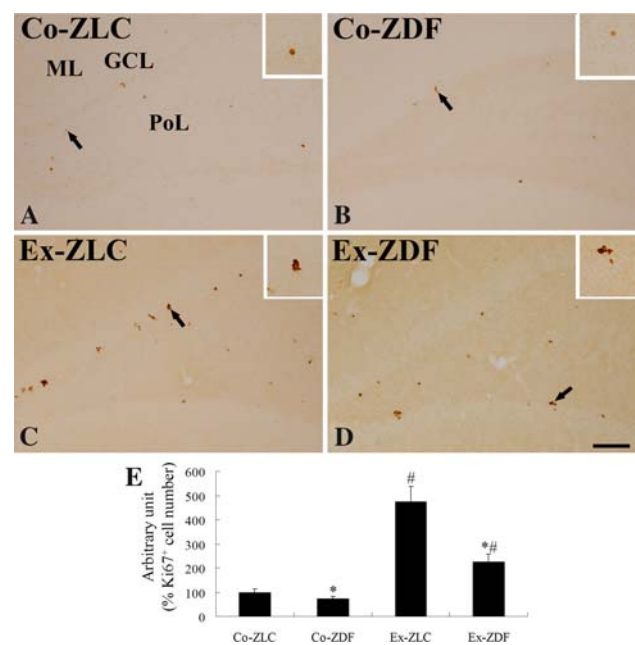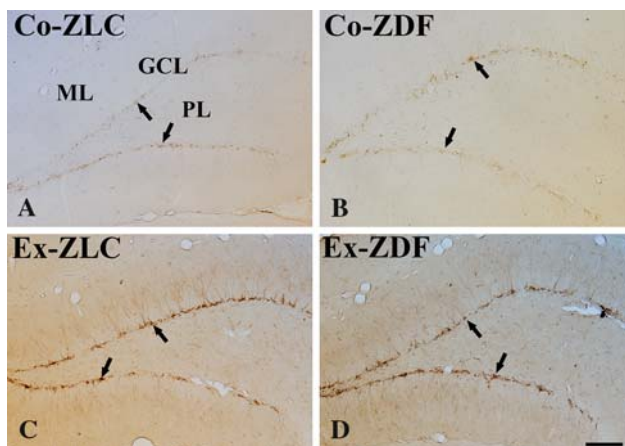

Fig. 2 Microphotographs of Ki67 positive cells in the dentate gyrus in control-ZLC (Co-ZLC), control-ZDF (Co-ZDF), exercise-ZLC (Ex-ZLC) and exercise-ZDF (Ex-ZDF) groups at 12 weeks of age. Ki67 positive cells are detected in the subgranular zone (arrows) of the polymorphic layer (PL). Note that Ki67 immunoreactive cells are abundant in the Ex-ZLC and Ex-ZDF groups compared to those in the Co-ZLC and Co-ZDF groups. GCL, granule cell layer; ML, molecular layer. Bar = 100 μm. (**e**) The relative number of Ki67 positive cells in the dentate gyrus of the Co-ZLC, Co-ZDF, Ex-ZLC and Ex-ZDF groups at 12 weeks of age ($n = 5$ per group; *$P < 0.05$, significantly different from the ZLC group, #$P < 0.05$, significantly different from the control group). The bars indicate the means ± SEM

(Figs. 3, 4, 5). In the control-ZDF group, DCX immunoreactive fibers were decreased compared to those in the control-ZLF group and DCX protein levels in the control-ZLC group were higher than in the control-ZDF group (Figs. 3a, b, 4a, b). In the control-ZDF group, DCX positive cells with tertiary dendrites were fewer than those in the control-ZLC group (Fig. 5). DCX immunoreactive processes in the exercise-ZLC group were well stained with DCX and extended into the outer two-thirds of the molecular layer of the dentate gyrus compared to those in the exercise-ZDF group (Figs. 3c, d, 4c, d). In these exercise groups, the number of DCX positive cells with tertiary dendrites and without tertiary dendrites was significantly increased compared to those in the control groups (Fig. 5). The number of DCX positive with tertiary dendrites was significantly decreased in the exercise-ZDF group compared to that in the exercise-ZLC group (Fig. 5).

In addition, exercise increased the DCX protein levels compared to that in the control groups. The DCX protein levels in the exercise-ZLC group were higher than that in the exercise ZDF-group (Fig. 6).

Fig. 3 Low magnification of DCX immunoreactive cells in the dentate gyrus in control-ZLC (Co-ZLC), control-ZDF (Co-ZDF), exercise-ZLC (Ex-ZLC) and exercise-ZDF (Ex-ZDF) groups at 12 weeks of age. DCX immunoreactive cells are detected in the subgranular zone (arrows) of the polymorphic layer (PL). DCX immunoreactive cells are abundant in the Ex-ZLC and Ex-ZDF groups compared to those in the Co-ZLC and Co-ZDF groups. GCL, granule cell layer; ML, molecular layer. Bar = 100 μm
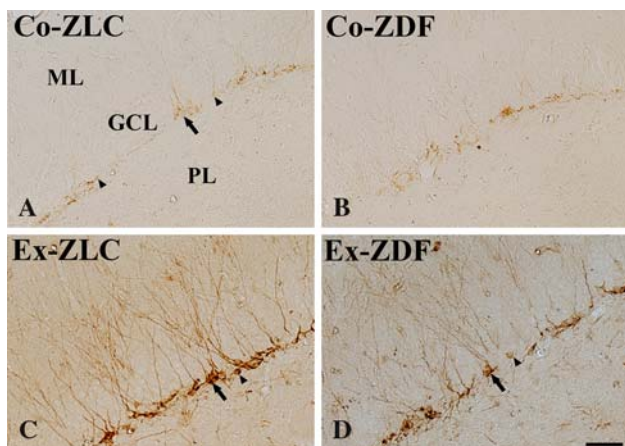
Fig. 4 High magnification of DCX immunoreactive cells in the upper blade of the dentate gyrus in control-ZLC (Co-ZLC), control-ZDF (Co-ZDF), exercise-ZLC (Ex-ZLC) and exercise-ZDF (Ex-ZDF) at 12 weeks of age. DCX immunoreactive cells have many well-stained tertiary dendrites (arrows) and a few poor dendrites (arrowheads) in the Ex-ZLC and Ex-ZDF groups. GCL, granule cell layer; ML, molecular layer. Bar = 25 μm

Discussion

ZDF rats show type II DM pathologies such as glucose tolerance at 12 weeks of age and are maintained for at least 6 months [5, 29, 30]. In a previous [15] and present study, we found that the blood glucose level was much higher in the 12-week-old ZDF rats than that in age-matched ZLC rats. However, the treadmill exercise reduced the blood glucose levels although the food intake was not significantly different between groups. This result was good
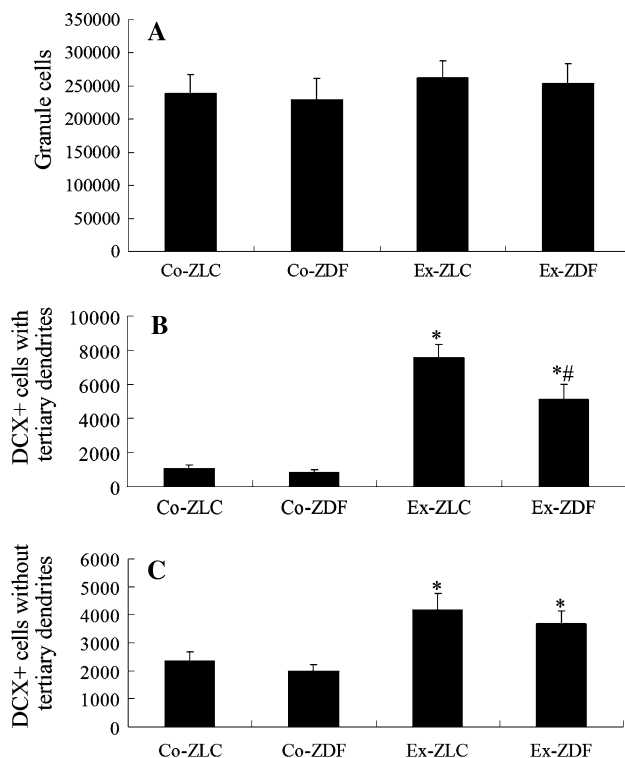
Fig. 5 Analysis of granule cells and DCX positive cells with and without tertiary dendrites in the dentate gyrus of control-ZLC (Co-ZLC), control-ZDF (Co-ZDF), exercise-ZLC (Ex-ZLC) and exercise-ZDF (Ex-ZDF) rats. Note that the number of granule cells is similar in all groups, but DCX positive cells with and without tertiary dendrites are abundant in the Ex-ZLC and Ex-ZDF rats ($n = 5$ per group; *$P < 0.05$, significantly different from the ZLC group, #$P < 0.05$, significantly different from the control group). The bars indicate the means ± SEM
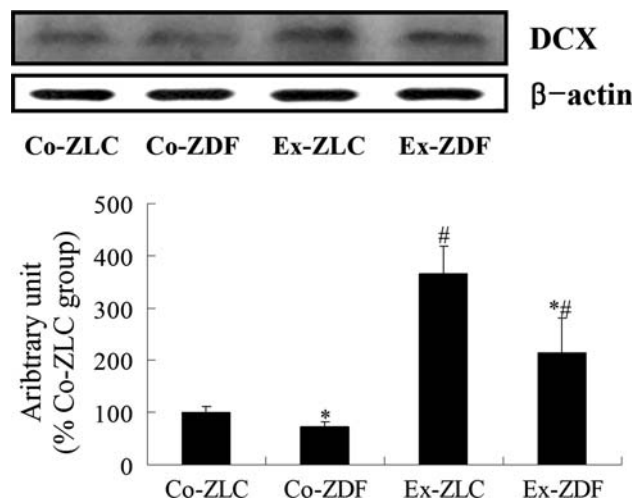
Fig. 6 Western blot analysis of DCX in the dentate gyrus of control-ZLC (Co-ZLC), control-ZDF (Co-ZDF), exercise-ZLC (Ex-ZLC) and exercise-ZDF (Ex-ZDF) rats. Relative optical density (ROD) as % of immunoblot band is also represented ($n = 3$ per group; *$P < 0.05$, significantly different from the ZLC group, #$P < 0.05$, significantly different from the control group). The bars indicate the means ± SEM

agreement with the previous studies that exercise improved insulin sensitivity and attenuated the development of type II diabetes [31–36]. In addition, Király et al. [37] reported that improvement in glucose tolerance with exercise was not a result of reduced food intake, a reduction in body weight, or differences in plasma lipid profiles. The reduction of glucose levels may be associated with a compensatory function in pancreatic beta cells. In a swim exercise model of ZDF rats, regular exercise increased the rates of beta cell proliferation and beta cell mass compared with sedentary control animals [37].

Next, we observed Ki67 immunoreactive nuclei and DCX immunoreactive structures (somata and processes) in the subgranular zone of the dentate gyrus to investigate cell proliferation and differentiation in diabetes and influences after the exercise. For this study, we trained the ZLC and ZDF rats for 5 weeks because it has been reported that most benefits have been associated with longer-term exercise (3–12 weeks) [38–41]. Ki67 immunoreactive nuclei and DCX immunoreactive structures in the dentate gyrus in the control-ZDF rats were more than those in the control-ZLC rats. In addition, the exercise significantly increased the number of DCX positive cells with tertiary dendrites and without tertiary dendrites in the ZLC and ZDF rats. This result coincided with some studies on type I [10, 13, 14] or II [15] DM models. However, in this study, we found that the exercise significantly increased the density of cells in both the ZLC and ZDF rats. These results suggest that an exercise facilitate the neuronal differentiation in the subgranular zone of the dentate gyrus in both control and type II diabetic models. Unlike in the hippocampus, exercise decreased neuronal activity in the posterior hypothalamic area in the spontaneously hypertensive rats [42] and increased glutamic acid decarboxylase gene transcription in the posterior hypothalamic area after physical exercise [43]: This finally reduced sympathetic tone and resting blood pressure.

Exercise increases some neurotrophic factors such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) in the brain [44–46]. An intrahippocampal injection of BDNF receptor antagonist attenuates the beneficial effects of exercise on hippocampus-dependent learning, specifically blocking improvements in both the acquisition and the retention of a spatial learning task [47, 48]. In addition, exercise increases several afferent neurotransmitters systems to the hippocampus, including norepinephrine, serotonin, acetylcholine and GABA [49], which are important factors to modulate the memory function.

A recent report pointed out an association between memory alterations and a decrease in neuronal proliferation in the dentate gyrus in diabetic rodents [10], and some reports also demonstrated that the impairment of cognitive performance in diabetic animals was associated with the hippocampus in terms of both anatomy and phenotype [6, 50]. Therefore, the increase of DCX immunoreactive cells in the dentate gyrus may be related with compensation in the impairment of cognitive performance in diabetic animals because exercise in young and aged animals can facilitate both acquisition and retention in various hippocampus-dependent tasks including the Morris water maze [41, 47], radial arm maze [40], passive avoidance [39] and object recognition [38]. In human study, exercise was found to have a primary effect on cerebral blood volume (CBV) in the dentate gyrus, which is the subregion that supports adult neurogenesis. Moreover, exercise-induced increases in dentate gyrus CBV were found to correlate with postmortem measurements of neurogenesis [51].

In conclusion, neuronal differentiation is markedly decreased in the subgranular zone of the dentate gyrus in type II diabetic rats and treadmill exercise induces cell proliferation and differentiation in the dentate subgranular zone in both control and diabetic rats.

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