

Treadmill exercise ameliorates motor disturbance through inhibition of apoptosis in the cerebellum of valproic acid-induced autistic rat pups

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Abstract. Autism is a neurological disorder that occurs during childhood and is characterized by impairments in social interaction and communication, as well as restricted and repetitive behaviors. Abnormalities of the cerebellum in autism include Purkinje cell loss and motor disturbance. In the present study, we evaluated the effect of treadmill exercise on motor coordination and balance in correlation with reelin expression and the rate of apoptosis in the cerebellum of autistic rat pups. For the induction of the autism-like animal models, 400 mg/kg valproic acid was subcutaneously injected into rat pups on postnatal day 14. Rat pups in the exercise groups were forced to run on a treadmill for 30 min, once a day, five times a week for 4 weeks, starting on postnatal day 28. Motor coordination and balance, as measured using the rotarod test and vertical pole test, were affected by the induction of autism. By contrast, treadmill exercise ameliorated motor dysfunction in the autistic rat pups. The expression levels of reelin, GAD67 and cyclin D1 in the cerebellum of the autistic rat pups were decreased, while the expression levels of these molecules were increased in autistic rat pups who engaged in treadmill exercise. In the cerebellum of the autistic rat pups, Bcl-2 expression was decreased and Bax expression was increased. By contrast, treadmill exercise enhanced Bcl-2 expression and suppressed Bax expression. The therapeutic effect of treadmill exercise on

motor deficits may be due to the reelin-mediated anti-apoptotic effect on cerebellar Purkinje neurons.

Introduction

Autism is a common neurological disorder that occurs during childhood and is characterized by impairments in social interaction and communication, as well as restricted and repetitive behaviors (1,2). In 2008, the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) estimated that the incidence of autism spectrum disorders was 11.4 cases per 1,000 children (8 years of age) compared with 6.4 cases per 1,000 children in 2002 (3). Since autism was first recognized as a disorder in 1943, studies investigating its etiology initially focused on the biological causes of the disorder and gradually shifted to the psychological causes. Recently, the focus of studies has shifted back to the biological etiologies, as data in support of genetic causes have been reported; in particular, a strong correlation between autism and various markers on chromosome 7 (4,5). However, the causes of 80-90% of cases of autism are unknown.

Previous studies have reported neurohistological and anatomical alterations in autistic brains (6-8). Biochemical studies have also reported alterations in the levels of Bcl-2, p53, reelin, brain-derived neurotrophic factor (BDNF) and acetylcholine receptors in the cerebellar and parietal areas of autistic brains (9,10). In the brain of autistic individuals, pre- and post-natal developmental abnormalities involve multiple regions of the brain, including the cerebral cortex, cortical white matter, amygdala, brainstem and cerebellum (8). The cerebellum is considered to be the region of the brain that is vulnerable to autism, as well as an important therapeutic target to improve an autistic-like state. Abnormalities of the cerebellum in autism, Purkinje cell loss in the cerebellar vermis and cerebellar hyperplasia and hypoplasia are strongly associated with the impairment of gait, motor preparation, grip, manual dexterity, ball skills, locomotion and balance (6,7).

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Apoptosis is a form of cell death that constitutes part of a common mechanism in cell replacement, tissue remodeling and the removal of damaged cells (11). Apoptosis consists of two main pathways, the extrinsic and intrinsic pathway. A class of cysteine proteases, including caspase-3, caspase-8 and caspase-9, is commonly involved in these pathways (12). As well as the caspases, the Bcl-2 family of proteins are also important in the regulation of apoptosis. Bcl-2 proteins are classified into anti-apoptotic proteins, which include Bcl-2 and Bcl-2 XL, and pro-apoptotic proteins, including Bax and Bid (13). Excessive neuronal apoptosis contributes to the dysfunction of the central nervous system (14,15).

Reelin is an extracellular glycoprotein that is essential for neuronal migration and brain development (9,16). Reelin glycoprotein is a secretory serine protease that performs dual roles in the mammalian brain. In the embryo, it guides neurons and radial glial cells to their correct positions in the developing brain. In the adult brain, reelin is involved in a signaling pathway that regulates neurotransmission, memory formation and synaptic plasticity (16). The functions of reelin are mediated through the receptors apolipoprotein E receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR), which trigger a complex signaling cascade (17,18). Fatemi *et al* (9) observed deficits in reelin mRNA and protein levels in the brains of patients with schizophrenia, major depression and autism, and suggested that the dysregulation of reelin may be responsible for a number of the structural and behavioral abnormalities observed in the brains of patients with autism (9). Activation of the reelin signaling pathway may inhibit excitotoxic neurotransmission and Tau phosphorylation, and may activate neurogenesis. This may lead to diminished brain injury and an increased rate of brain injury repair (19).

Glutamate decarboxylase p67 (GAD67), a downstream molecule of reelin, is an important enzyme that regulates GABAergic function in the central nervous system. The predominant finding in the postmortem brain of subjects diagnosed with schizophrenia, autism and bipolar illness was a decrease in GAD67 mRNA levels, which affected multiple brain regions (20). Cyclin D1 is a protein encoded by the Bcl-1 gene and is important for regulating the cell cycle. The expression levels of cyclin D1 and reelin are correlated with the morphological development of the cerebellum (21).

Exercise enhances learning ability and memory functions, provides protection from neurodegeneration, delays age-related cognitive decline and alleviates the symptoms of developmental and neuropsychiatric disorders (22-25). Several studies have suggested that exercise may be a critical mediator in treating neuropsychiatric disorders, including autism (24,26-28).

In the present study, we evaluated the effect of treadmill exercise on motor coordination and balance in correlation with reelin expression and the rate of apoptosis in the cerebellum using valproic acid-induced autistic rat pups. Motor coordination and balance were determined using the rotarod test and the vertical pole test. Immunofluorescence, immunohistochemistry and western blot examinations were also conducted.

Materials and methods

Animals and the induction of autism. Male Sprague-Dawley rat pups (weight, 25±5 g; age, 2 weeks old) were used in this

study and all experimental procedures were performed in accordance with the animal care guidelines of the National Institutes of Health (NIH) and the Korean Academy of Medical Sciences (Seoul, Korea). The animals were housed under controlled temperature (23±2°C) and lighting (08:00 to 20:00 h) conditions, with food and water available *ad libitum*. The animals were randomly divided into four groups (n=10 in each group); the control group, the control and treadmill exercise group, the valproic acid-treated group and the valproic acid-treated and treadmill exercise group.

For the induction of the autism-like animal models, 400 mg/kg valproic acid (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline at a concentration of 0.1 ml/kg and was subcutaneously injected into the rat pups on postnatal day 14, according to a previously described method (29). The day of birth was recorded as day 0 and all pups were labeled for individual identification. Rat pups in the control groups received subcutaneous injection of saline at the same volume and on the same schedule as the treadmill exercise groups.

Treadmill exercise. Rat pups in the treadmill exercise groups were forced to run on a treadmill for 30 min, once a day, five times a week for 4 weeks, starting at postnatal day 28. Exercise load for the exercise groups consisted of running at a speed of 2 m/min for the first 5 min, 5 m/min for the next 5 min and then at 8 m/min for the last 20 min, with a 0° inclination.

Rotarod test. We performed the rotarod test (Biological Research Apparatus, Ugo Basile, Varese, Italy) to measure motor coordination and balance, according to a previously described method (30). Each rat pup was placed in a separate compartment on a rotating 7-cm diameter rod. The velocity of the rod was set to a constant 15 rpm. The time of latency until fall-off was automatically recorded by magnetic trip plates. To eliminate stress and fatigue, the rat pups were given a maximum cut-off latency of 180 sec.

Vertical pole test. For the measurement of motor coordination and balance, the vertical pole test was conducted as previously described (31). Each rat pup was placed face-up on a cloth tape-covered pole (3 cm diameter, 150 cm long). The pole was held in a horizontal position and then gradually lifted to a vertical position. The degree of angle until fall off was recorded.

Tissue preparation. The experimental animals were fully anesthetized using Zoletil 50® (10 mg/kg, i.p.; Vibac Laboratories, Carros, France), transcardially perfused with 50 mM phosphate-buffered saline (PBS) and fixed with a freshly prepared solution consisting of 4% paraformaldehyde in 100 mM phosphate buffer (PB; pH 7.4). The brains were dissected, post-fixed in the same fixative overnight and transferred to 30% sucrose for cryoprotection. Sagittal sections (thickness, 40 µm) in each section of the cerebellum were cut with a freezing microtome (Lieca, Nussloch, Germany).

Western blotting for reelin, GAD67, cyclin D1, Bax and Bcl-2 expression levels. Western blotting was performed according to a previously described method (23,32). The cerebellar tissues were collected and immediately frozen at -70°C. When used, the tissues were homogenized on ice and lysed in a lysis

buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl-fluoride, 1 mM EGTA, 1.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 mM sodium orthovanadate and 100 mM sodium fluoride. The protein content was measured using a colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA). Protein samples (30 μg) were separated on sodium dodecyl sulfate-polyacrylamide gels and transferred onto a nitrocellulose membrane.

Mouse β -actin antibody (1:3,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse reelin antibody (1:1,000; Chemicon International, Temecula, CA, USA), rabbit GAD67 antibody (1:1,000; Santa Cruz Biotechnology, Inc.), mouse cyclin D1 antibody (1:1,000; Santa Cruz Biotechnology, Inc.), mouse Bcl-2 antibody (1:1,000; Santa Cruz Biotechnology, Inc.), mouse Bax antibody (1:1,000; Santa Cruz Biotechnology, Inc.) and rabbit cleaved caspase-3 antibody (1:1,000; Cell Signaling Technology, Inc., Beverly, MA, USA) were used as the primary antibodies. Horseradish peroxidase-conjugated anti-rabbit antibody for GAD67 and cleaved caspase-3 (1:4,000; Vector Laboratories, Burlingame, CA, USA) and horseradish peroxidase-conjugated anti-mouse antibody for actin, cyclin D1, Bcl-2 and Bax (each 1:3,000; Amersham Pharmacia Biotechnology GmbH, Freiburg, Germany) were used as the secondary antibodies.

The experiments were performed in normal laboratory conditions and at room temperature, except for the membrane transfer. The membranes were transferred at 4°C using a cold pack and pre-chilled buffer. Band detection was performed using the enhanced chemiluminescence (ECL) detection kit (Santa Cruz Biotechnology, Inc.). In order to compare the relative expression levels, the detected bands were calculated densitometrically using Molecular Analyst™, version 1.4.1 (Bio-Rad).

Immunofluorescence for reelin, calbindin D-28k and caspase-3 expression. Cerebellum samples were embedded and frozen at -20°C, and mounted on positively charged slides. For the visualization of reelin, calbindin D-28k and cleaved caspase-3 expression, immunofluorescence was performed according to a previously described method (33). For double immunofluorescence staining, the sections (40 μm) were fixed with 4% paraformaldehyde and 4% sucrose in PBS at room temperature for 40 min, permeabilized with 0.5% Nonidet P-40 in PBS and blocked with 2.5% horse serum and 2.5% bovine serum albumin for 4 h at room temperature. The sections were incubated with anti-calbindin D-28k rabbit polyclonal antibody (1:800; Santa Cruz Biotechnology, Inc.), anti-reelin mouse monoclonal antibody (1:500; Millipore Corporation, Billerica, MA, USA), anti-cleaved caspase-3 rabbit polyclonal antibody (1:400; Cell Signaling Technology, Inc.) and then incubated with fluorescein-goat anti-mouse (1:600; Molecular Probes, Eugene, OR, USA) or rhodamine-goat anti-rabbit secondary antibodies (1:800; Molecular Probes) in 3% bovine serum albumin for 1 h at room temperature, and coverslipped with gelatin mount medium. The sections were observed using a fluorescence microscope and images were captured using an attached camera.

Immunohistochemistry for caspase-3 expression. Caspase-3 immunohistochemistry was performed according to a previously described method (23). In brief, the sections were incubated overnight with mouse anti-cleaved caspase-3

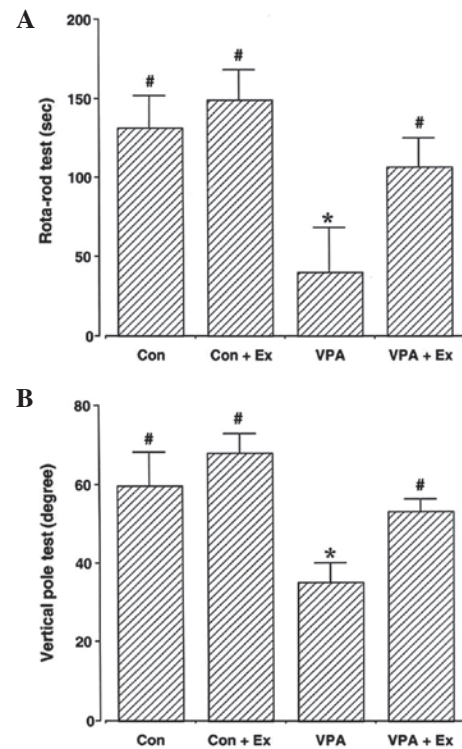


Figure 1. Effect of treadmill exercise on motor coordination and balance in rat pups. (A) Rotarod test; (B) vertical pole test. Values are represented as the mean $\pm$ SEM. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the valproic acid-treated group. Con, control group; Ex, treadmill exercise group; VPA, valproic acid-treated group; VPA + Ex, valproic acid-treated and treadmill exercise group.

antibody (1:500; Santa Cruz Biotechnology, Inc.) and incubated for 1 h with biotinylated mouse secondary antibody. The bound secondary antibody was then amplified using the Vector Elite ABC kit® (1:100; Vector Laboratories). The antibody-biotin-avidin-peroxidase complex was visualized using 0.03% 3,3'-diaminobenzidine. The sections were finally mounted onto gelatin-coated slides. The slides were air-dried overnight at room temperature and the coverslips were mounted using Permount® (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Statistical analysis. The data are expressed as the mean $\pm$ standard error of the mean (SEM). All data were analyzed using SPSS statistical software version 12.0 (SPSS, Inc., Chicago, IL, USA). For comparisons among the groups, one-way ANOVA and the Duncan's post-hoc test were performed. $P < 0.05$ was considered to indicate a statistically significant result.

Results

Effect of treadmill exercise on motor coordination and balance in the rotarod test and vertical pole test. Motor coordination and balance were determined using the rotarod test and vertical pole test (Fig. 1). The results indicated that motor coordination and balance were affected by the induction of autism ($P < 0.05$). By contrast, treadmill exercise ameliorated the disturbance of motor coordination and balance in the autistic rat pups ($P < 0.05$). Under normal conditions, treadmill exercise exerted no significant effect on motor coordination and balance.

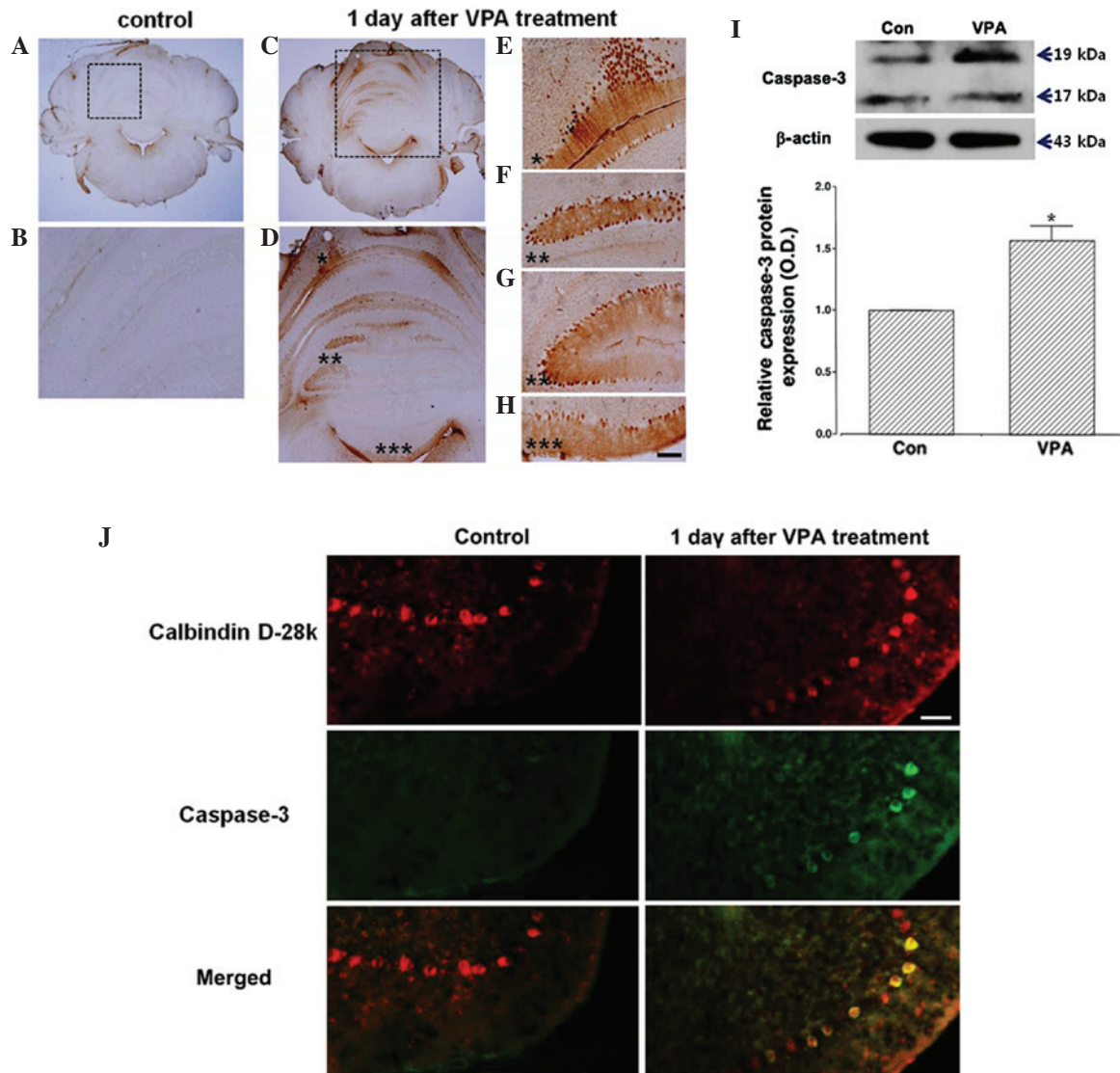


Figure 2. Appearance of caspase-3-positive cells 1 day following valproic acid treatment. (A-H) Caspase-3 immunostaining was performed 1 day following valproic acid treatment. (A) Control group; (B) enlargement of the area denoted by the rectangle in (A); (C) valproic acid-treated group; (D) enlargement of the area denoted by the rectangle in (C); (E-H) enlargement of the areas denoted by the asterisks in (D). The scale bar represents 200 μ m. (I) Level of cleaved caspase-3 expression in the cerebellum, as determined by western blotting and quantitative graph analysis. Values are represented as the mean $\pm$ SEM. * $P < 0.05$ compared with the control group. (J) Purkinje cell death induced by valproic acid treatment. Double immunostaining for calbindin (red) and caspase-3 (green) was performed 1 day following valproic acid treatment. The scale bar represents 200 μ m. Caspase-3 expression was only observed in the Purkinje neurons in the valproic acid-treated group. Con, control group; VPA, valproic acid-treated group.

Caspase-3 expression in the cerebellum 1 day following valproic acid treatment. Representative photomicrographs of cleaved caspase-3-positive cells in the cerebellar vermis are presented in Fig. 2A-H. Numerous caspase-3-labeled cells were observed in the cerebellar Purkinje cell layer of the valproic acid-treated group, while caspase-3-positive cells were not detected in the control group. We quantitatively assessed caspase-3 expression in the cerebellum of the valproic acid-treated group (Fig. 2I). When the band intensity of caspase-3 in the control group was set at 1.00, the level of caspase-3 was 1.57 ± 0.11 in the valproic acid-treated group. To examine the rate of Purkinje cell death by valproic acid treatment, prepared cerebellar tissues were used for double immunostaining with anti-calbindin D-28k and anti-cleaved caspase-3 antibodies. As shown in Fig. 2J, numerous cleaved caspase-3-positive cells in the valproic acid-treated group

overlapped with Purkinje neurons; however, no overlapping was observed in the control group. The results demonstrated the presence of apoptotic cell death in cerebellar Purkinje cells 1 day following valproic acid treatment.

Effect of treadmill exercise on reelin expression in the cerebellum. To examine the effect of treadmill exercise on reelin expression, we performed double immunostaining using anti-calbindin D-28k and anti-reelin antibodies (Fig. 3). Reelin proteins were merged with calbindin-positive neurons in all groups. Valproic acid downregulated the number of Purkinje neurons that overlapped with reelin, while treadmill exercise increased the number of Purkinje neurons, as well as the expression of reelin in the cerebellum of the autistic rat pups. The present results indicated that reelin expression in the cerebellum was decreased by the induction of autism. By

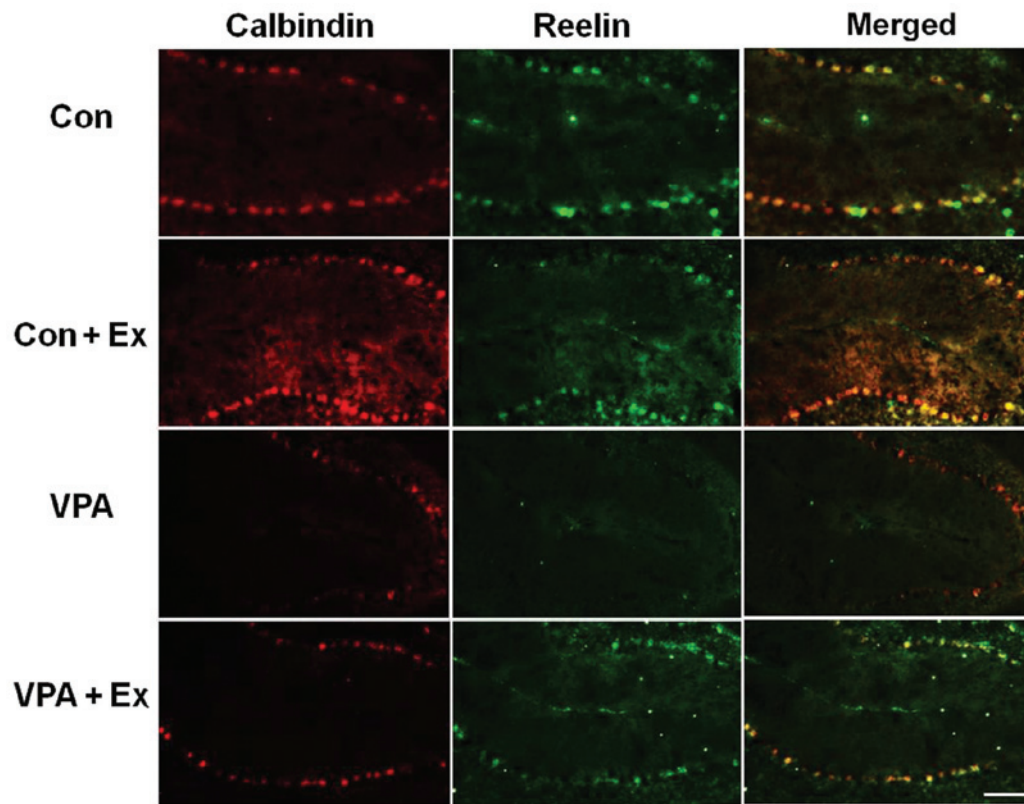


Figure 3. Effect of treadmill exercise on the expression levels of reelin in cerebellar Purkinje cells. Double immunostaining for calbindin (red) and reelin (green) was performed. The scale bar represents 200 μ m. Con, control group; Ex, treadmill exercise group; VPA, valproic acid-treated group; VPA + Ex, valproic acid-treated and treadmill exercise group.

contrast, treadmill exercise upregulated reelin expression in the autistic rat pups.

Effect of treadmill exercise on GAD67 and cyclin D1 expression in the cerebellum. To examine the effect of treadmill exercise on the expression of GAD67 and cyclin D1, both of which are downstream molecules of reelin, we analyzed the relative expression of GAD67 and cyclin D1 proteins by western blotting (Fig. 4). The results indicated that GAD67 and cyclin D1 expression levels in the cerebellum were decreased following the induction of autism ($P < 0.05$). By contrast, treadmill exercise upregulated GAD67 and cyclin D1 expression levels in the autistic rat pups ($P < 0.05$). Under normal conditions, treadmill exercise increased the expression levels of GAD67 and cyclin D1 ($P < 0.05$).

Effect of treadmill exercise on Bcl-2 and Bax expression in the cerebellum. To examine the effect of treadmill exercise on Bcl-2 and Bax expression, we analyzed the relative expression of Bcl-2 and Bax proteins using western blotting (Fig. 5). The results indicated that Bcl-2 expression levels in the cerebellum were decreased and Bax expression levels in the cerebellum were increased by the induction of autism ($P < 0.05$). By contrast, treadmill exercise enhanced Bcl-2 expression and suppressed Bax expression in the autistic rat pups ($P < 0.05$). Under normal conditions, treadmill exercise increased Bcl-2 expression; however, treadmill exercise exerted no significant effect on Bax expression.

Discussion

Cerebellar structural abnormalities in autism include the loss of granular and Purkinje cells (34), and atrophy of Purkinje cells (35). In the present study, the loss of cerebellar Purkinje cells and caspase-3 activation were observed 1 day following 400 mg/kg valproic acid injection. Caspase-3 is the most widely studied member of the caspase family and is a key executor of apoptosis (36). Enhanced caspase-3 activation by valproic acid injection may contribute to Purkinje cell loss in the cerebellum.

Valproic acid has been used as an anticonvulsant and mood stabilizer for seizures and epilepsy (37,38). Exposure to valproic acid during pregnancy leads to the retardation of embryonic motor function and cognition; in a study in which maternal rats received 350 mg/kg of valproic acid on days 11.5 (the day of neural tube closure), 12 or 12.5 of gestation, autism-like brain lesions were detected in the rat pups (39). Postnatal administration of valproic acid induced behavioral and neuroanatomical abnormalities similar to those observed in autism (40). Notably, Wagner *et al* (29) studied the dose-dependent effect of valproic acid on the neurobehavioral alterations representing autism using postnatal day 14 mice. This study demonstrated that mice treated with 400 mg/kg valproic acid exhibited more severe autism-like symptoms. In the present study, a dose of 400 mg/kg valproic acid initiated apoptotic Purkinje cell death and this dosage was sufficient to induce autism in the rat pups.

To examine motor abnormality and imbalance, we used the rotarod test and vertical pole test. The latency in the rotarod

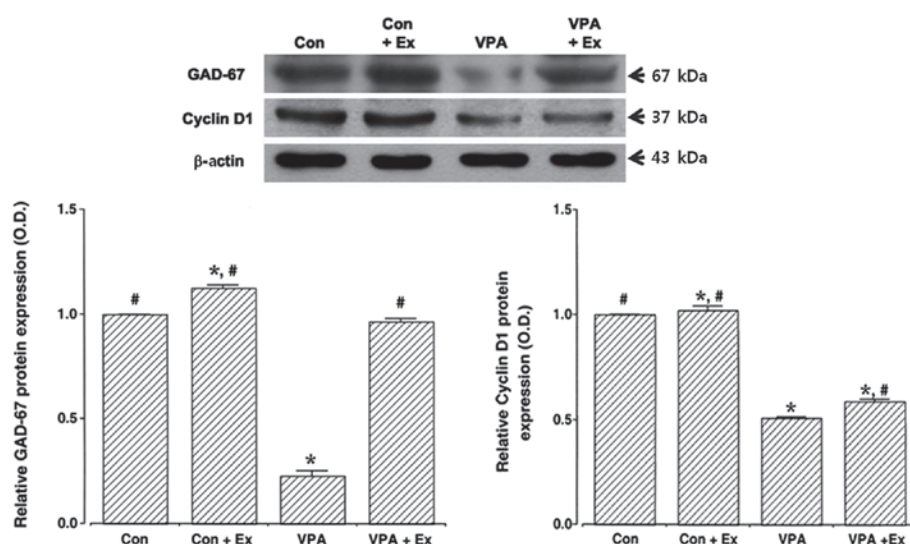


Figure 4. Effect of treadmill exercise on the expression levels of GAD67 and cyclin D1 in the cerebellum. Values are presented as the mean $\pm$ SEM. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the valproic acid-treated group. Con, control group; Ex, treadmill exercise group; VPA, valproic acid-treated group; VPA + Ex, valproic acid-treated and treadmill exercise group; O.D., optical density.

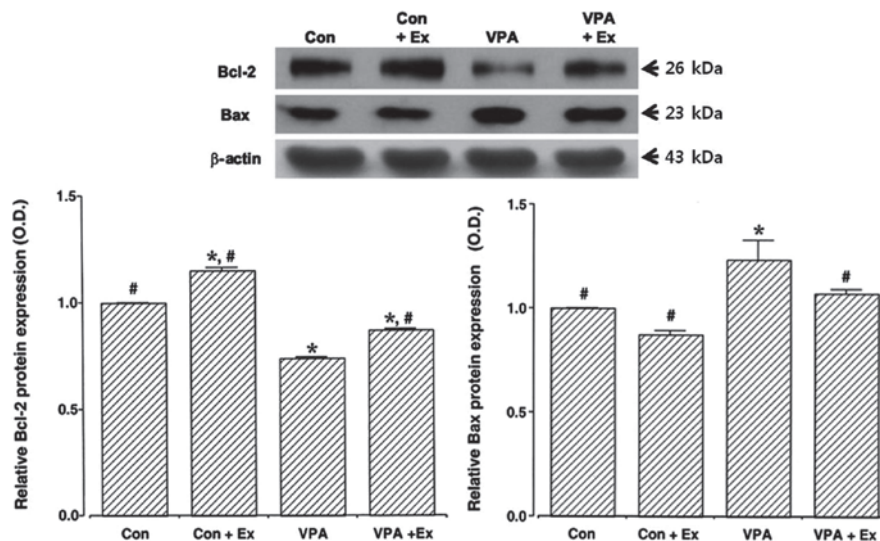


Figure 5. Effect of treadmill exercise on the expression levels of Bcl-2 and Bax in the cerebellum. Values are presented as the mean $\pm$ SEM. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the valproic acid-treated group. Con, control group; Ex, treadmill exercise group; VPA, valproic acid-treated group; VPA + Ex, valproic acid-treated and treadmill exercise group; O.D., optical density.

test and the degree of angle in the vertical pole test prior to fall-off were decreased in the valproic acid-induced autistic rat pups. By contrast, treadmill exercise increased the time of latency in the rotarod test and the degree of angle in the vertical pole test in the autistic rat pups. The cerebellum is important in motor coordination and balance and has been recognized as a prominent contributor to a wide array of cognitive and emotional functions (2,41). Motor function disturbances, including a disturbance of motor anticipation, postural and gait abnormalities, various degrees of dystonia, bradykinesia and hyperkinesia, and abnormality of muscle tone are important aspects of the description of autism (2,42).

The results of the present study also demonstrate that cerebellar Purkinje cell loss was accompanied with a decrease in

reelin expression in the valproic acid-induced autistic rat pups. By contrast, treadmill exercise alleviated Purkinje cell loss with increased reelin expression in the autistic rat pups. Death of cerebellar Purkinje neurons in autism is strongly associated with the reduced activation of the reelin signaling pathway in several psychiatric disorders, including depression, schizophrenia and autism (5,43). Reelin is a serine protease and an important glycoprotein involved in the specific layering of the developing brain (9,44); it is preferentially secreted by cortical GABAergic interneurons. Disruption of the reelin signaling pathway is strongly associated with the onset of neuropsychiatric disorders, including autism and schizophrenia (16).

In the present study, the expression levels of GAD67 and cyclin D1 were suppressed in the cerebellum of valproic

acid-induced autistic rat pups. By contrast, treadmill exercise increased GAD67 and cyclin D1 expression levels in the cerebellum of the autistic rat pups. In a previous study, GAD67 mRNA levels were reduced by 40% in the autistic group, suggesting that reduced Purkinje cell GABA input to the cerebellar nuclei potentially disrupts cerebellar output to higher association cortices, affecting motor and/or cognitive function. The authors of this study suggested that the dysregulation of reelin and GAD67 expression may be responsible for a number of the brain structural abnormalities observed in autism (45). The dysregulation of cyclin D1 expression, resulting in neuro-cardio-facial-cutaneous syndromes, is associated with developmental abnormalities, cognitive deficits and autism (46).

We additionally demonstrated that Bcl-2 expression was decreased and Bax expression was increased in the cerebellum of valproic acid-induced autistic rat pups. By contrast, treadmill exercise increased Bcl-2 expression and decreased Bax expression in the cerebellum of the autistic rat pups. In previous comparative studies of autistic and normal control cerebellar cortices, the expression levels of reelin and Bcl-2 in the autistic cerebellum were reduced compared with the controls (9), and it was suggested that the dysregulation of reelin and Bcl-2 may be responsible for a number of brain structural and behavioral abnormalities observed in autism.

The anti-apoptotic and neuron maturation effects of exercise on various neuropsychiatric diseases are well documented (14,24,32,33,47). Celiberti *et al* (26) reported that regular exercise suppressed autism-like symptoms in a 5-year-old male with autism. Following bouts of physical activity, children with autism experienced a decrease in the levels of negative behaviors, including stereotypy and an incremental change in positive behaviors, including time on task (27,48). Petrus *et al* (28) suggested that exercise provides a reduction of stereotypic behaviors in children with autism spectrum disorder. Children with autism are more physically inactive compared with their non-autistic peers (49).

In conclusion, we demonstrated that treadmill exercise is capable of ameliorating motor dysfunction in autistic rat pups. The therapeutic effect of treadmill exercise on motor deficits may be due to the reelin-mediated anti-apoptotic effect of treadmill exercise on cerebellar Purkinje neurons. These results support the theory that exercise may provide a potential therapeutic strategy for the alleviation of motor symptoms in autistic patients.

Acknowledgements

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