

Swimming exercise alleviates the symptoms of attention-deficit hyperactivity disorder in spontaneous hypertensive rats

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Abstract. Attention-deficit hyperactivity disorder (ADHD) is a neurobehavioral disorder characterized by inattention, hyperactivity and impulsivity. In the present study, we investigated the effects of swimming exercise on the symptoms of ADHD in correlation with the expression levels of dopamine and the dopamine D₂ receptor. Adult male spontaneous hypertensive rats (SHRs) were used as animal models of ADHD and Wistar-Kyoto rats were used as controls. The activity, impulsivity and levels of non-aggressive and aggressive behaviors in rats were measured. The short-term memory in the animal models of ADHD was assessed using an open-field test. The social interaction test, elevated plus maze test and step-through avoidance test were additionally performed. The expression levels of tyrosine hydroxylase (TH), which catalyzes the rate-limiting step of dopamine synthesis, and the dopamine D₂ receptor in the prefrontal cortex, substantia nigra and striatum were evaluated. The expression levels of TH and the dopamine D₂ receptor were detected using immunohistochemistry and western blotting, respectively. In ADHD rats, the activity, impulsivity and levels of non-aggressive and aggressive behaviors were higher than that in control rats. By contrast, short-term memory in ADHD rats deteriorated. Swimming exercise suppressed hyperactivity, impulsivity and non-aggressive and aggressive behaviors, and alleviated the short-term memory impairment observed in ADHD rats. The expression levels of TH and the dopamine D₂ receptor were decreased and increased in ADHD rats, respectively, when compared with control rats. Swimming exercise enhanced the expression of TH and suppressed the expression of the dopamine D₂ receptor in ADHD rats. In the present study, swimming exercise improved the symptoms of ADHD by

upregulating the expression of dopamine and downregulating the expression of the dopamine D₂ receptor.

Introduction

Attention-deficit hyperactivity disorder (ADHD) is a common neurobehavioral disorder that typically arises during childhood. Globally, 5-10% of school-aged children have ADHD and its prevalence is 3 times higher in males than females (1). ADHD is characterized by 3 major behavioral symptoms, inattention, hyperactivity and impulsivity. The exact causes of ADHD are unknown, although genetic, social, physical and environmental factors have been suggested to contribute to the etiology of the disease. ADHD is highly heritable and ~75% of cases are genetically associated (2). Hyperactivity is hypothesized to be caused by a genetic disorder, and the genetic alteration of dopamine transporters has since been suggested as a major cause of ADHD (3).

Dopamine is a neurotransmitter that regulates motor activity, attention and reward-seeking behaviors (4). Clinical and experimental studies have demonstrated a correlation between the transmission of dopamine with inattentive, hyperactive, aggressive and impulsive behaviors (5,6). The axons of dopamine neurons located in the substantia nigra are mainly situated in the dorsolateral striatum, forming the nigrostriatal pathway (7). Dopaminergic activity in the nigrostriatal pathway is affected by social interactions and social status (8). Disrupted dopamine signaling is implicated in the pathogenesis of numerous neurological disorders, including ADHD and Parkinson's disease (9,10). Tyrosine hydroxylase (TH) catalyzes the rate-limiting step in the synthesis of catecholamine neurotransmitters, including dopamine, epinephrine and norepinephrine. TH converts L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). TH activity is progressively decreased in correlation with the loss of dopamine neurons in the substantia nigra and striatum (10,11). TH immunohistochemistry is widely used to detect the injury or death of dopaminergic fibers and cell bodies (12,13). Dopaminergic signaling also affects physical activity (14). Hyperactivity is an important symptom of ADHD and anorexia nervosa, both of which are associated with altered dopaminergic signaling (2,15,16).

Dopamine receptors form the main determinants of the dopamine pathway. Dopamine receptors are divided into

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2 classes, D₁-like (D₁ and D₅) and D₂-like (D₂, D₃ and D₄) dopamine receptors. In particular, the dopamine D₂ receptor is closely associated with neuropsychiatric disorders, including ADHD (3), schizophrenia (17) and depression (18). Dopamine D₂ receptors belong to a family of G protein-coupled receptors and its activation inhibits synaptogenesis (19). Several studies have examined the positive effects of dopamine D₂ receptor activation on physical activity (20-22).

First-line treatment for ADHD consists of the α_2 -adrenoreceptor agonists, clonidine and guanfacin (23). Methylphenidate, the historical gold standard treatment for ADHD, inhibits dopamine and norepinephrine transporters. By contrast, atomoxetine is highly specific for norepinephrine reuptake inhibitors, with a low affinity for other monoamine transporters or neurotransmitter receptors (24). Atomoxetine is a well-tolerated non-stimulant medication that functions differently from other medications approved for the treatment of ADHD (24). However, these medications induce side-effects, including loss of appetite, sleep problems and mood disturbances (25). Thus, there is a need to develop alternative therapeutic drugs that possess fewer side-effects.

Physical exercise improves neurological impairments by increasing neurogenesis and the release of neurotransmitters, facilitating neuronal maturation and enhancing learning ability and memory capability in neuropsychiatric disorders (26-29). In pediatric ADHD patients, physical movement improved working speed and social behavioral problems, and reduced hyperactivity (30). The effect of treadmill exercise in ameliorating the symptoms of ADHD in spontaneous hypertensive rats (SHRs) has been reported (31). Swimming exercise has been demonstrated to be beneficial for the treatment of ADHD, for example, in the case of the Olympic athlete Michael Phelps (32). The possibility that swimming exercise is effective for relieving the symptoms of ADHD has been examined; however, the effects of swimming exercise on ADHD in correlation with the levels of dopamine and dopamine receptor expression have yet to be assessed.

In the present study, we investigated the effects of swimming exercise on several behavioral characteristics, including activity, impulsivity, non-aggressive and aggressive behaviors and short-term memory in ADHD rats. The open-field test, social interaction test, elevated plus maze test and step-through avoidance test were conducted. The effects of swimming exercise on the expression levels of TH and the dopamine D₂ receptor in the prefrontal cortex, substantia nigra and striatum were evaluated. The expression of TH and the dopamine D₂ receptor were detected by immunohistochemistry and western blotting, respectively. The effectiveness of swimming exercise was compared with atomoxetine treatment.

Materials and methods

Experimental animals and treatment. Adult male SHRs and Wistar-Kyoto rats, weighing 180±5 g (6 weeks old), were obtained from a commercial breeder (Orient Bio Co., Seoul, Korea). SHRs were used as animal models of ADHD, since they exhibit the major symptoms of ADHD, including inattention, hyperactivity and impulsiveness (33). In the present study,

SHRs that demonstrated hyperactivity in the open-field test were used as the ADHD rats, while Wistar-Kyoto rats, which did not demonstrate hyperactivity, were used as the control rats, according to previously described methods (31,34).

The 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. The animals were housed under controlled temperature (23±2°C) and lighting (08:00-20:00 h) conditions, with food and water available *ad libitum*. The animals were randomly divided into 4 groups (n=10 in each group): The control group (Wistar-Kyoto rats), the ADHD group (SHRs), the ADHD and swimming exercise group and the ADHD and atomoxetine treatment group. Rats in the atomoxetine treatment group received 1 mg/kg atomoxetine (Strattera®, Eli Lilly Co., Indianapolis, IN, USA) orally once a day for 28 consecutive days.

Open-field test. To confirm hyperactivity in the ADHD rats, the open-field test was conducted 1 day prior to the initiation of the experiment using a previously described method (31). The animals were randomly assigned to an order of testing and placed in a white square open-field arena (100x100 cm) made of wood, enclosed by 40-cm-high walls and exposed to strong illumination (200 lux). The arena was divided into 25 squares (20x20 cm), consisting of 9 central and 16 peripheral squares. The animals were placed in the center of the arena and were allowed to explore the environment for 1 min. Following this time, the number of squares crossed was recorded for 5 min. The activity of each rat was repeatedly assessed using the open-field test at 9, 18 and 26 days following the initiation of the experiment.

Swimming exercise protocol. Rats in the swimming group were forced to swim for 30 min once a day, for a total of 28 days, according to a previously described method (35). The rats were placed individually in cylindrical tanks with a 30-cm diameter and a height of 85 cm. The cylindrical tank was filled with water at a temperature of 33±1°C and to a depth of 75 cm.

Social interaction test. The social interaction test was performed 27 days following the initiation of the experiment, according to a previously described method (36). Two weight-matched rats selected from different cages were examined. While the normal rat was used as the partner rat, the test rat was marked with an oil pen and both rats were placed into the center of a test box (60x60 cm open field with sixteen 15x15 cm squares marked on the floor). The animals were closely observed and the interactions exhibited by the index rat were recorded over a period of 10 min. The social behaviors exhibited by the index rat were measured visually. The number of non-aggressive behaviors (genital investigation, sniffing, following and grooming) and aggressive behaviors (kicking, boxing, biting, crawling under or over the partner and touching the partner's face) were recorded during the test period.

Elevated plus maze test. The elevated plus maze test is a well-established test used to determine the levels of impulsivity in rats. The elevated plus maze test was performed

28 days following the initiation of the experiment, according to a previously described method (37). The plus maze consisted of black plexiglas with two open arms (50x10x36 cm) and two closed arms (50x10x36 cm), arranged so that the two arms were opposite and connected by a central platform (10x10 cm). The whole plus maze was elevated 60 cm above the floor and illuminated by a 100-watt light bulb fixed 2 m above the maze floor. During the test, each rat was placed on the central platform of the maze with their head facing an open arm. The total time spent in the open arms was collected over a period of 7 min.

Step-through avoidance test. Short-term memory was evaluated using step-through avoidance apparatus 29 days following the initiation of the experiment (24 h following the last swimming exercise session), according to a previously described method (26). The apparatus consisted of two compartments, a dark compartment and a light compartment. A guillotine door separated the two compartments. The dark compartment had a stainless steel shock grid floor. During the acquisition trial, each rat was placed in the light compartment. Following a habituation period of 60 sec, the guillotine door was opened. Immediately after the rat had entered the dark compartment, the guillotine door was closed and an electric foot shock (75 V, 0.2 mA, 50 Hz) was delivered to the floor grids for 3 sec. The rat was then removed from the dark compartment 5 sec later and returned to the home cage. The latency of staying in the light compartment was measured 2 h following the acquisition trial; however, a foot shock was not delivered. The latency was recorded to a maximum of 300 sec.

Tissue preparation. The rats were sacrificed immediately following the completion of the step-through avoidance test. The animals were anesthetized using Zoletil 50[®] (10 mg/kg, i.p.; Virbac 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 and post-fixed in the same fixative overnight and transferred into a 30% sucrose solution for cryoprotection. Coronal sections of 40- μ m thickness were cut using a freezing microtome (Leica, Nussloch, Germany). On average, 10 slice sections in the prefrontal cortex, substantia nigra and striatum were collected from each rat.

TH immunohistochemistry. For the immunolabeling of TH in the prefrontal cortex, substantia nigra and striatum of each brain, TH immunohistochemistry was performed as previously described (13). Free-floating tissue sections were incubated overnight with mouse anti-TH antibody (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and the sections were then incubated for 1 h with biotinylated anti-mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). The sections were subsequently incubated with avidin-biotin-peroxidase complex (Vector Laboratories) for 1 h at room temperature. Immunoreactivity was visualized by incubating the sections in a solution consisting of 0.05% 3,3'-diaminobenzidine (DAB) and 0.01% H₂O₂ in 50 mM Tris-buffer (pH 7.6) for ~3 min. The sections were then washed

3 times with PBS and mounted onto gelatin-coated slides. The slides were air-dried overnight at room temperature and coverslips were mounted using Permount[®] (Fisher Scientific, New Jersey, NJ, USA).

Western blotting for the detection of dopamine D₂ receptor expression. Western blotting was performed as previously described (19,27). The prefrontal cortex, substantia nigra and striatum tissues were collected and then immediately frozen at -80°C. The tissues of each brain area were homogenized on ice and lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholic acid, 1% Nonidet P40, 0.1% SDS, 1 mM PMSF and 100 mg/ml leupeptin. The protein content was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA). A total of 30 μ g protein was separated on a SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Mouse anti-actin antibody (1:500; Santa Cruz Biotechnology, Inc.) and mouse anti-dopamine D₂ receptor antibody (1:1,000; Santa Cruz Biotechnology, Inc.) were used as the primary antibodies. Horseradish peroxidase-conjugated anti-mouse antibodies for actin and dopamine D₂ receptor (1:2,000; Vector Laboratories) were used as the secondary antibodies. Experiments were performed in normal lab conditions and at room temperature, with the exception of the membrane transfer. The membrane transfer was performed at 4°C using a cold pack and prechilled buffer. Band detection was performed using the enhanced chemiluminescence (ECL) detection kit (Santa Cruz Biotechnology, Inc.).

Statistical analysis. The number of TH-positive cells in the substantia nigra was counted using a light microscope (Olympus, Tokyo, Japan). The optical densities of the TH-immunoreactive fibers in the prefrontal cortex and striatum were measured in 100x100 μ m square images using an image analyzer (Multiscan, Fullerton, CA, USA). To estimate the TH staining densities, the optical densities were corrected for the non-specific background density, which was measured in completely denervated parts of the prefrontal cortex and striatum. The optical densities of the TH-positive fibers in the prefrontal cortex and striatum were calculated as follows: The optical density in the lesion side/the optical density in the intact side. In order to assess the expression levels of the dopamine D₂ receptor in the prefrontal cortex, substantia nigra and striatum, the detected bands were calculated densitometrically using Image-Pro[®] Plus software (Media Cybernetics Inc., Silver Spring, MD, USA). Statistical analysis was performed using one-way ANOVA followed by the Duncan's post-hoc test. The results are expressed as the mean \pm SEM. P<0.05 was considered to indicate a statistically significant result.

Results

Effects of swimming exercise on activity, impulsivity, short-term memory and non-aggressive and aggressive behaviors. To determine the levels of activity, the open-field test was performed 1 day prior to the initiation of the experiment (Fig. 1A). ADHD rats were hyperactive, demonstrating symptoms of ADHD. The activity score in the open-field test

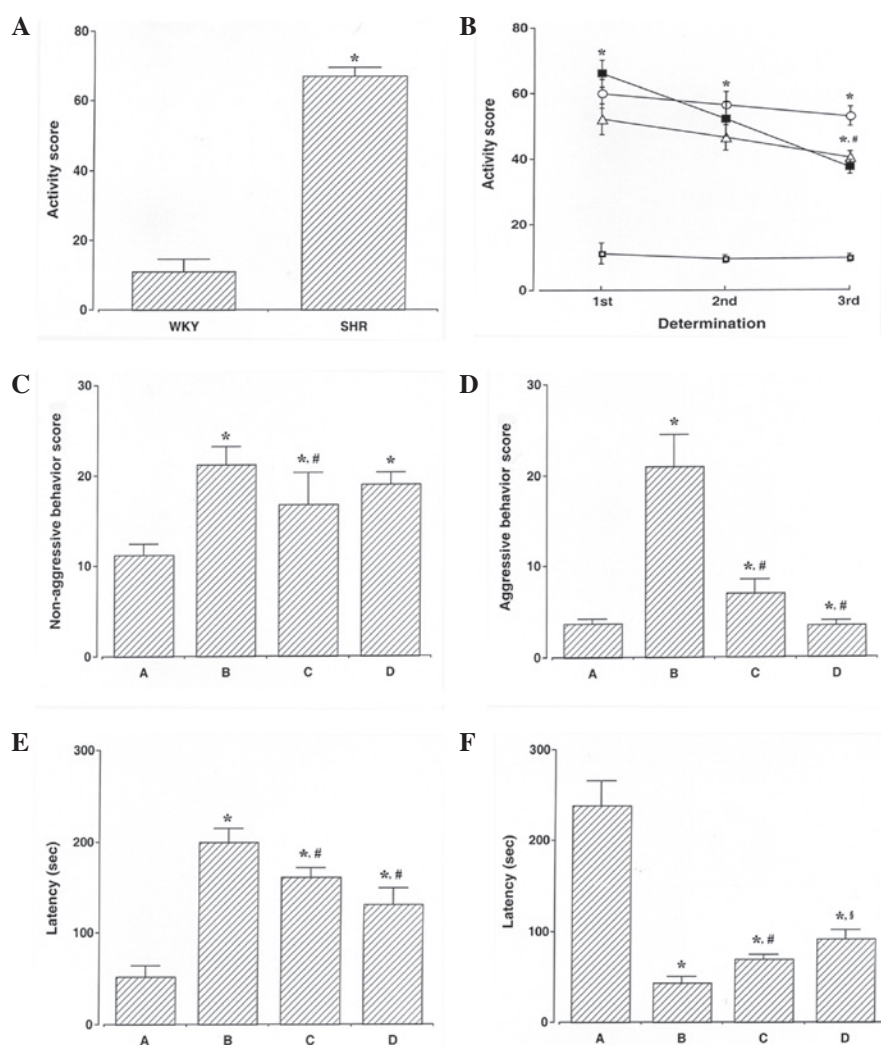


Figure 1. Results of the behavioral tests. (A and B) Open-field test. (A) Open-field pre-test; (B) open-field test during the experimental period. The groups were as follows: control group (open square box), ADHD group (circle), ADHD and swimming exercise group (triangle) and ADHD and atomoxetine treatment group (solid square box). (C and D) Social interaction test. (C) Non-aggressive behavior; (D) aggressive behavior. (E) Elevated plus maze test. (F) Step-through passive avoidance task. The results are presented as the mean \pm SEM. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the ADHD group; § $P < 0.05$ compared with the ADHD and swimming exercise group. A, Control group; B, ADHD group; C, ADHD and swimming exercise group; D, ADHD and atomoxetine treatment group; ADHD, attention-deficit hyperactivity disorder; SHR, spontaneous hypertensive rat; WKY, Wistar-Kyoto rat.

was determined during the experimental period. The activity score in the ADHD rats was higher compared with that of the control rats ($P < 0.05$), whereas swimming exercise and atomoxetine treatment decreased the activity score observed in the ADHD rats ($P < 0.05$; Fig. 1B).

Non-aggressive and aggressive behaviors were assessed using the social interaction test. The scores for non-aggressive behaviors in the social interaction test are presented in Fig. 1C. The score for non-aggressive behaviors in the ADHD rats was higher compared with that of the control rats ($P < 0.05$), whereas swimming exercise decreased the score for non-aggressive behaviors in the ADHD rats ($P < 0.05$). However, atomoxetine treatment exerted no significant effect on the score for non-aggressive behaviors in the ADHD rats. The scores for aggressive behaviors in the social interaction test are presented in Fig. 1D. The score for aggressive behaviors in the ADHD rats was higher compared with the control rats ($P < 0.05$), whereas swimming exercise and atomoxetine treatment decreased the score for aggressive behaviors in the ADHD rats ($P < 0.05$).

Impulsivity was assessed using the elevated plus maze test. The latency of staying in the open arm in the elevated plus maze test is presented in Fig. 1E. The ADHD rats demonstrated an enhanced impulsive activity compared with the control rats ($P < 0.05$), whereas swimming exercise and atomoxetine treatment inhibited impulsivity in ADHD rats ($P < 0.05$).

Short-term memory was assessed using the step-through avoidance test. The latency of staying in the light compartment in the step-through avoidance test is presented in Fig. 1F. ADHD rats demonstrated short-term memory disturbance ($P < 0.05$), whereas swimming exercise and atomoxetine treatment alleviated short-term memory disturbances in the ADHD rats ($P < 0.05$).

Effects of swimming exercise on TH expression levels in the prefrontal cortex, substantia nigra and striatum. Photomicrographs of TH-immunoreactive fibers in the prefrontal cortex, TH-positive cells in the substantia nigra and TH-immunoreactive fibers in the striatum are presented

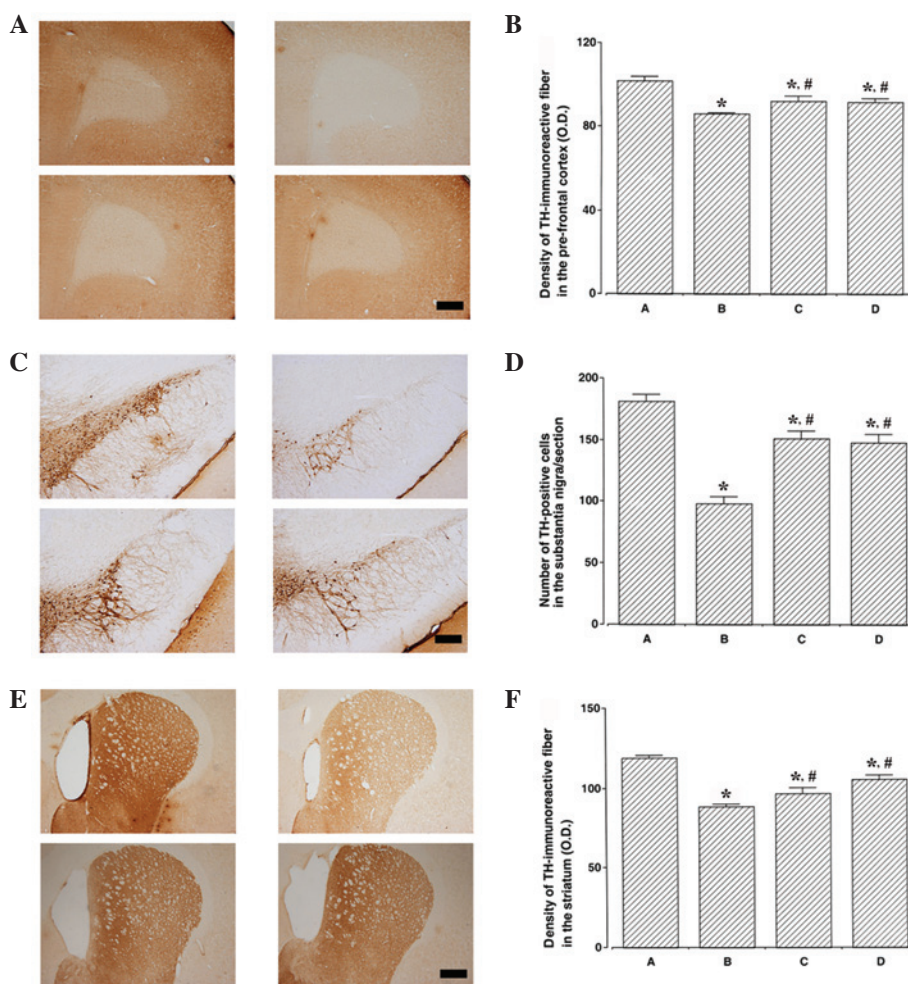


Figure 2. TH expression levels. (A and B) TH expression levels in the prefrontal cortex. (C and D) TH expression levels in the substantia nigra. (E and F) TH expression levels in the striatum. The scale bar represents 200 μ m. The results are presented as the mean \pm SEM. *P<0.05 compared with the control group; #P<0.05 compared with the ADHD group. A, Control group; B, ADHD group; C, ADHD and swimming exercise group; D, ADHD and atomoxetine treatment group; TH, tyrosine hydroxylase; ADHD, attention-deficit hyperactivity disorder; O.D., optical density.

in Fig. 2. TH expression levels were lower in the ADHD rats compared with the control rats (P<0.05). By contrast, swimming exercise and atomoxetine treatment increased TH expression levels in the ADHD rats (P<0.05).

Effects of swimming exercise on dopamine D₂ receptor expression levels in the prefrontal cortex, substantia nigra and striatum. The results of the western blot analysis detecting the expression of the dopamine D₂ receptor in the prefrontal cortex, substantia nigra and striatum are presented in Fig. 3. The dopamine D₂ receptor expression levels were higher in the ADHD rats compared with the control rats (P<0.05). By contrast, swimming exercise and atomoxetine treatment suppressed dopamine D₂ receptor expression levels in the ADHD rats (P<0.05).

Discussion

SHRs were used as animal models of ADHD since they exhibit hyperactivity, impulsivity, impaired ability to withhold responses and poorly sustained attention compared with normotensive Wistar-Kyoto control rats (31,34). Physical exercise improved working speed and social behavioral

disorders, and also diminished hyperactivity in children with ADHD (30). Kim *et al* (31) demonstrated that treadmill exercise ameliorated hyperactivity and enhanced spatial learning memory in ADHD rats. In particular, swimming has been recommended for children suffering from ADHD-induced learning disabilities (32).

In the present study, hyperactivity in ADHD rats was suppressed by swimming exercise. In ADHD rats, non-aggressive and aggressive behaviors, and impulsivity were inhibited by swimming exercise. Swimming exercise also ameliorated ADHD-induced short-term memory impairment. The results of the present study demonstrated that swimming exercise improved the symptoms of ADHD in SHRs (Fig. 1).

Dopamine is an important neurotransmitter in the regulation of attention and cognitive function (16,38). Hypofunction of the dopaminergic system decreased reinforcement of previous behaviors. These conditions led to delayed aversion, the development of hyperactivity in novel conditions, impulsiveness, deficient sustained attention and increased behavioral variability (38). The dysfunction of dopamine signaling in the midbrain is one of the main mechanisms responsible for causing hyperactivity and memory deficits (3,16). Neuronal hypoactivity in the prefrontal cortex induced behavioral

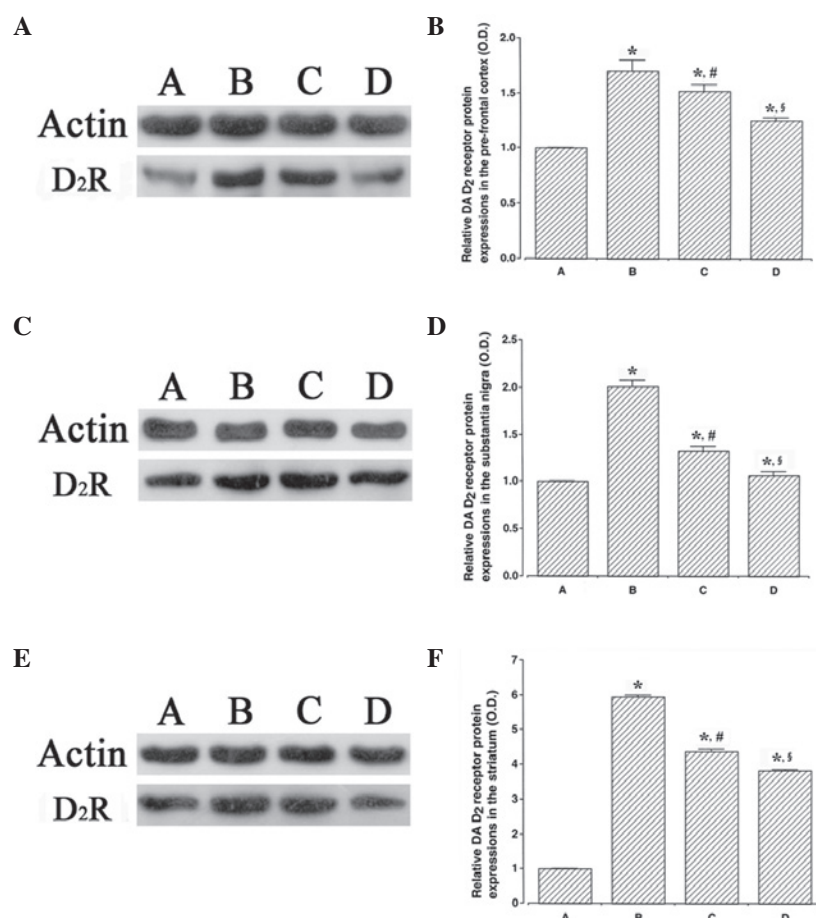


Figure 3. Dopamine D₂ receptor (D₂R) expression levels. (A and B) Dopamine D₂ receptor expression levels in the prefrontal cortex. (C and D) Dopamine D₂ receptor expression levels in the substantia nigra. (E and F) Dopamine D₂ receptor expression levels in the striatum. Actin (43 kDa) was used as the internal control. The results are presented as the mean \pm SEM. **P*<0.05 compared with the control group; #*P*<0.05 compared with the ADHD group; §*P*<0.05 compared with the ADHD and swimming exercise group. A, Control group; B, ADHD group; C, ADHD and swimming exercise group; D, ADHD and atomoxetine treatment group; O.D., optical density.

alterations similar to those observed in ADHD, including attention deficit and hyperactivity (2). Microdialysis studies demonstrated that norepinephrine and dopamine efflux was preferentially decreased within the prefrontal cortex of SHR_s (39,40). Exercise is known to protect dopamine cell loss and increase dopamine levels, resulting in an improvement of symptoms caused by Parkinson's disease and stressful conditions (10,41,42). Exercise also enhanced the recovery from nigrostriatal dopamine injury and altered dopaminergic neurotransmission in the nigrostriatal system (29). Treadmill exercise enhanced TH expression levels in the striatum and substantia nigra of ADHD rats (31).

In the present study, the expression levels of TH, which catalyzes the rate-limiting step of dopamine synthesis, in the prefrontal cortex, substantia nigra and striatum were decreased in ADHD rats. Swimming exercise increased TH expression levels in ADHD rats (Fig. 2). Swimming-induced increments in dopamine expression levels may contribute to the improvement of symptoms observed in ADHD rats.

Swimming exercise induced adaptive changes in the functional responsiveness of dopamine receptors in the nigrostriatal and mesolimbic systems, which were evident from the modification of behavioral responses (43). Aggressive behaviors are closely correlated with expression of dopamine

D₂ receptors in animals and humans, suggesting that meso-corticolimbic dopamine may be closely associated with permission of behaviors (44). Altered dopamine signaling may have a causal association with motor hyperactivity and may be considered as a potential endophenotype of ADHD (45). Chronic activation of dopamine D₂ receptors decreased dopamine release and inhibited synapse formation (19). Dopamine D₁ and D₂ receptor function within the striatum may act to balance behavioral inhibition (46). Alterations in the activity of the dopamine D₂ receptor are implicated in several neurological and psychiatric disorders, including schizophrenia, Parkinson's disease, Huntington's disease, Tourette syndrome, ADHD and drug addiction (47). Dopamine D₂ receptor activation enhanced physical activity (20-22); however, impaired dopamine D₂ receptor function is associated with decreased physical activity (48).

In the present study, the expression levels of the dopamine D₂ receptor in the prefrontal cortex, substantia nigra and striatum were increased in ADHD rats compared with control rats. Swimming exercise decreased the dopamine D₂ receptor expression levels in ADHD rats (Fig. 3). The suppressive effect of swimming exercise on dopamine D₂ receptor expression levels may be associated with the relief of symptoms in ADHD rats.

In this study, we demonstrated that swimming exercise alleviated the symptoms of ADHD in SHR by upregulating the expression of dopamine and downregulating the expression of the dopamine D₂ receptor. The effectiveness of swimming exercise appeared comparable to treatment with atomoxetine. Based on the present results, swimming exercise may be a potential useful therapeutic strategy for the treatment of ADHD.

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