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Moderate-Intensity Exercise Induces Neurogenesis and Improves Cognition in Old Mice by Upregulating Hippocampal Hippocalcin, Otub1, and Spectrin-α

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Abstract

Exercise increases the levels of neurogenic factors and enhances neurogenesis, memory, and learning. However, the molecular link between exercise and neurogenesis is not clear. The purpose of this study was to examine the effects of exercise intensity on cognitive function and protein expression in the hippocampus of old mice. To compare the effects of aerobic exercise intensity on cognition in old mice, we exposed 18-month-old mice to low- and moderate-intensity treadmill exercise for 4 weeks. Moderate-intensity exercise improved cognitive function in the old mice, while low-intensity exercise did not. To investigate the underlying mechanisms, two-dimensional electrophoresis was used to examine protein expression. Using peptide fingerprinting mass spectrometry, we identified 19 proteins that were upregulated in the hippocampus following exercise training, and seven of these proteins were normalized by the control value. Among them, the levels of 14-3-3 zeta and heat shock protein 70, which have been shown to be induced by exercise training and related to neurogenesis, were dramatically increased by moderate exercise. Hippocalcin, α-spectrin, ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1 (otub1), mu-crystallin, serine racemase, and rho GDP dissociation inhibitor 1, which are related to neurogenesis, neuroprotection, and synaptic strength, were upregulated in the hippocampus by moderate exercise. In addition, we confirmed that neurogenic markers, including doublecortin and the neuronal nuclei antigen, and hippocalcin, otub1, and spectrin-α are potential molecular links between hippocampal neurogenesis and exercise in the elderly. Thus, these results showed that steady moderate-intensity exercise delayed the declines in cognitive function in the elderly through the activation of multiple factors.

Keywords Aerobic exercise · Old mouse · Neurogenesis · Cognitive function · Hippocalcin · Otub1 · Spectrin-α · Mu-crystallin

Introduction

Normal aging is accompanied by alterations in brain structure and function and cognition. These changes affect quality of life and are related to an increasing risk for neurodegenerative diseases, such as Alzheimer's disease and other types of dementia. Many efforts are made to delay aging or age in a healthy way. Extensive evidence from animal and human studies [1, 2] has suggested that exercise has a positive impact on cognition. Epidemiological studies have also reported a reduced risk for mild cognitive impairment and dementia in older adults who maintain higher levels of physical activity.

Recent research has found that aerobic physical activity produces the greatest benefits [3–6]. This kind of exercise is performed continuously or at intervals with moderate intensity (60–70% of maximum oxygen consumption). The aerobic energy system is responsible for supplying the oxygen demanded during activities involving large muscle mass that

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last 3 min or more, such as walking, jogging, swimming, and rowing, and that are conducted repeatedly with characteristic frequency. However, few studies have compared the effects of different modes of exercise on cognition.

Exercise increases blood flow to the brain, thereby activating neurotransmitter systems in the brain and inducing neurogenic factor production and synapse formation [7], which promote the binding affinity for cell membrane receptors and positively affect the duration and intensity of downstream signaling cascades. Thus, exercise increases neurogenic factors and affects neurogenesis, memory, and learning [8, 9]. Van Praag et al. [10] have reported that spontaneous wheel exercise in rats not only results in cell proliferation but also increases the survival of existing cells. Aguiar et al. [11] have reported that rats that undergo mild treadmill exercises four times a week for 5 weeks exhibit increased neurogenic factors in the hippocampus and improved spatial learning and memory. Kohl et al. [12] have reported that findings that hippocampal neuronal cell formation is inhibited by the restriction of physical activity of young rats with active nerve cell proliferation suggest that physical activity is an essential factor in the induction of neuronal cell responses. However, the mechanisms underlying the effects of exercise on neurogenesis are not clear.

To compare the effects of aerobic exercise intensity on cognition, we designed a study with two modes, low- and moderate-intensity treadmill exercise, and performed it in 18-month-old mice. Physical training was modeled using a program involving adaptation on the first day and then stepwise increments in exercise intensity to simulate the regular practice of aerobic activity in humans. The present study aimed to investigate (1) the effects of low- and moderate-intensity exercise on cognitive behavior in older adult mice (age 18 months), (2) the intensity dependence of the changes, and (3) the mechanisms that are responsible for the effects on cognitive function of aerobic exercise.

Methods

Experimental Animals

Old male C57BL/6 mice were purchased from the Laboratory Animal Resource Center (Cheongju, Korea) of the Korea Research Institute of Bioscience and Biotechnology. The animals were maintained under constant temperature (21 ± 1 °C) and relative humidity (55 ± 5%) in a 12-h light/12-h dark cycle. To examine nutritional status, weight gain and food intake were recorded every week at the same time for 4 weeks. The animal use protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Dongguk University Ilsan Hospital (No. 201603147) and were in accordance with the National Institutes of Health guidelines.

Exercise Program

In this study, the mice performed aerobic exercise on treadmills. The exercise intensity of the incremental load exercises was manipulated according to a modification of the experimental animal exercise protocol of Kim et al. [13]. Eighteen-month-old male mice were divided into the following three groups: low intensity (low, $n = 6$), moderate intensity (moderate, $n = 6$), and control (control, $n = 6$). The mice were assigned to treadmill exercises that had different speeds that represented low intensity and moderate intensity (Table 1). In brief, the mice in the low-intensity group performed treadmill running at a speed of 4 m/min for 10 min on the first day and then 4 m/min for 20 min on the second to 28th days. The mice in the moderate-intensity group were assigned to run 8 m/min for 10 min on the first day, 8 m/min for 20 min on the second to 14th days, and 20 m/min for 20 min on the 15th to 28th days. The treadmill exercise was performed 5 days per week.

Passive Avoidance Test

The passive avoidance test was performed after the exercise training on days 29 and 30. The step-through passive avoidance test was performed using an apparatus that consisted of two compartments: an illuminated compartment ($100 \times 120 \times 100$ mm^3) with a light (27 W, 3000 lx) on top of the compartment and a dark compartment ($100 \times 170 \times 100$ mm^3; AP model, O'Hara & Co., Ltd., Tokyo, Japan). The compartments were separated by a guillotine door. During the training trial, a mouse was placed in the safe illuminated compartment. Because the compartment was lit, the mouse stepped through the opened guillotine door into the dark compartment. The time spent in the illuminated compartment was defined as the latency time. Three seconds after the mouse entered the dark compartment, a foot shock (0.3 mA, 50 V, 50 Hz AC, 3 s) was delivered to the floor grid in the dark compartment. The mouse could escape from the shock only by stepping back into the safe illuminated compartment. Mice remaining in the light chamber for over 120 s during the learning stage were excluded from the subsequent retention trial. The retention trials were performed 24 h after the training trial to evaluate the retention of avoidance memory. The latency time was measured up to 300 s without delivery of a foot shock, and the mouse was considered to have retained the avoidance memory when it stayed in the safe illuminated compartment for 300 s.

Two-Dimensional Gel Electrophoresis

Thirty days after the behavioral tests were completed, the mice were sacrificed, and their hippocampal tissue was dissected on ice. The hippocampal tissues were directly homogenized by a motor-driven homogenizer (PowerGen 125, Thermo Fisher Scientific Inc., Waltham, MA, USA) in sample lysis solution

Table 1 Exercise program

Intensity	Slope (0°)	Speed (m/min)	Duration (day)	Time spent running (min)	Exercise frequency (day/week)
Low	0	4	1	10	5
	0	4	2–28	20	5
Moderate	0	8	1	10	5
	0	8	2–14	20	5
	0	20	15–28	20	5

that consisted of 7 M urea and 2 M thiourea containing 4% (*w*/*v*) 3-[(3-cholamidopropyl) dimethyammonio]-1-propanesulfonate, 1% (*w*/*v*) dithiothreitol (DTT), 2% (*v*/*v*) Pharmalyte® (MilliporeSigma, Burlington, MA, USA), and 1 mM benzamidine. The proteins were extracted for 1 h at room temperature using vortexing. After centrifugation at 15,000×*g* for 1 h at 15 °C, the insoluble material was discarded, and the soluble fraction was processed by two-dimensional gel electrophoresis (2DE). The protein concentrations were assayed using the Bradford method. Dry immobilized pH gradient strips (4–10 nonlinear immobilized pH gradients, 24 cm, Genomine, Inc., Kyungbuk, Korea) were equilibrated for 12–16 h with 7 M urea and 2 M thiourea containing 2% 3-[(3-cholamidopropyl)dimethyammonio]-1-propanesulfonate, 1% DTT, and 1% Pharmalyte® and then loaded with 200 μg of each sample. Isoelectric focusing was performed at 20 °C using a MultiPhor™ II electrophoresis unit and EPS 3500XL power supply (GE Healthcare Life Sciences, Little Chalfont, UK) according to manufacturer's instructions. For the isoelectric focusing, the voltage was increased linearly from 150 to 3500 V over 3 h for the sample entry, which was followed by a constant 3500 V, with the focusing complete after 96,000 Vh. Prior to electrophoresis, the strips were incubated for 10 min in equilibration buffer (50 mM Tris-Cl, pH 6.8 containing 6 M urea, 2% SDS, and 30% glycerol), first with 1% DTT and second with 2.5% iodoacetamide. The equilibrated strips were inserted into SDS-polyacrylamide gel electrophoresis (PAGE) gels (20 × 24 cm, 10–16%). The SDS-PAGE was performed using a Hoefer DALT 2D system (GE Healthcare Life Sciences) according to the manufacturer's instructions. The 2D gels were run at 20 °C for 1700 Vh. The 2D gels were then stained for Colloidal Coomassie Brilliant Blue.

Image Analysis

The quantitative analysis of the digitized images was conducted using PDQuest software (version 7.0, Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to manufacturer's protocols. The quantity of each spot was normalized using the total valid spot intensity. Protein spots were selected if the variation in their levels of expression deviated over twofold compared with those of the control or normal samples.

Peptide Mass Fingerprinting

For protein identification by peptide mass fingerprinting (PMF), protein spots were excised, digested with trypsin (Promega Corporation, Madison, WI, USA), mixed with α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid, and subjected to matrix-assisted laser desorption/ionization-time of flight analysis (Microflex LRF 20, Bruker Daltonics Inc., Billerica, MA, USA). The spectra were collected from 300 shots per spectrum over the m/z range of 600–3000 and calibrated using two-point internal calibration with trypsin autodigestion peaks (m/z 842.5099, 2211.1046). The peak list was generated using FlexAnalysis (version 3.0, Bruker Daltonics Inc.). The thresholds used for peak-picking were 500 for the minimum resolution of the monoisotopic mass and 5 for signal/noise. The MASCOT search program developed by Matrix Science Inc. (http://www.matrixscience.com/) was used for protein identification by PMF. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic masses, and a mass tolerance of ± 0.1 Da. The PMF acceptance criterion was probability scoring.

Western Blot Analysis

The hippocampi were homogenized in lysis buffer containing 50 mM Tris-base (pH 7.5), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1% glycerol, 10 mM NaF, 10 mM Na-pyrophosphate, 1% NP-40, and protease inhibitors. The proteins were separated by 10% or 15% SDS-PAGE and then transferred to nitrocellulose membranes. The blotted membranes were blocked with 5% skim milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T buffer) for 1 h. After washing the membrane with PBS-T, the blots were incubated with the following primary antibodies: ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1 (otub1), mu-crystallin, hippocalcin (MilliporeSigma, Burlington, MA, USA), p53, serine racemase (SR; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), doublecortin (DCX), neuronal nuclear antigen (NeuN; Cell Signaling Technology, Inc., Danvers, MA, USA), and β-actin (MilliporeSigma). After washing with Tris-buffered saline containing 0.05% Tween 20, the blots were incubated with horseradish peroxidase-conjugated rabbit (catalog PA1-30359, Thermo Fisher Scientific Inc.) and mouse (catalog PA1-30355, Thermo Fisher Scientific Inc.) secondary antibodies. Detection was performed using enhanced chemiluminescence (ECL kit, Thermo Fisher Scientific Inc.), and the images were obtained using Molecular Imager ChemiDoc™ XRS+ (Bio-Rad Laboratories, Inc.). Band intensity was analyzed with Image Lab™ software (ver. 2.0.1, Bio-Rad Laboratories, Inc.).

Statistical Analysis

All statistical analyses were conducted with SPSS (ver. 21.0, IBM Corporation, Armonk, NY, USA). All values are expressed as mean ± standard error of the mean. The data were analyzed using one-way ANOVA analyses of variance, Dunnett's tests, and *t* tests. Differences were considered significant with *p* values less than 0.05.

Results

Moderate-Intensity Exercise Increased Cognitive Activity in the Old Mice

The animals in each group performed treadmill exercise for 4 weeks according to the programs listed in Table 1. The mean body weight of the experimental animals was 51.42 ± 5.57 g in the moderate-intensity exercise group, 46.90 ± 6.60 g in the low-intensity exercise group, and 47.98 ± 4.88 g in the control group ($F = 0.83$, $p = 0.459$), and the difference among the groups was not significant (Fig. 1a).

To investigate the effects of treadmill exercise on cognition in the old mice, a passive avoidance test was performed. The latency time of the moderate-intensity exercise group increased from 81.23 ± 34.73 s before the experiment to 202.60 ± 80.51 s after the experiment, and this difference was statistically significant ($t = 3.19$, $p = 0.033$). The low-intensity exercise group increased from 75.20 ± 33.09 s to 92.60 ± 93.99 s after the experiment, which was not a significant difference ($t = 0.57$, $p = 0.598$) (Fig. 1b).

Hippocampal Protein Profiles of the Control and Moderate-Exercise Mice

The hippocampal protein profiles of the control and exercise groups were compared using 2DE analysis. Ninety-five spots with intensity changes over twofold between the control and exercise groups were selected for image analysis because of the significant differences in expression; 86 spots were increased, and nine spots were decreased compared to the control spot (Fig. 2). Among them, 19 proteins that were induced by moderate exercise training were identified by PMF (Table 2). In addition, an optical density graph was plotted (Fig. 3) to compare seven spot densities. Among them, the levels of 14-3-3 zeta and heat shock protein 70 were dramatically increased by moderate exercise, and these proteins are induced by exercise training and are related to neurogenesis [14, 15]. Hippocalcin, α-spectrin, otub1, mu-crystallin, and rho GDP dissociation inhibitor (GDI) 1 were upregulated by moderate exercise in the hippocampus (Fig. 3).

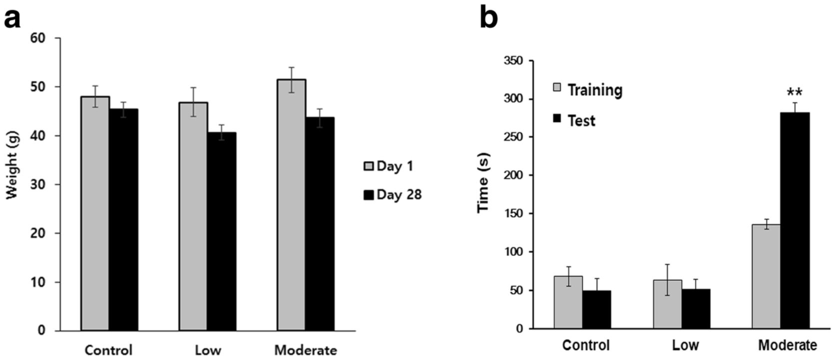
Confirmation of Altered Proteins by Western Blot Analysis

To verify the reliability of the proteomics analysis, hippocalcin, otub1, and mu-crystallin were selected as representative proteins and subjected to western blotting (Fig. 4). The results of the triplicate western blots of the protein extracts from the hippocampus of control, moderate-exercise, and low-exercise mice for otub1 ($F = 11.236$, $p = 0.009$), crystallin ($F = 7.755$, $p = 0.022$), and hippocalcin ($F = 31.348$, $p = 0.001$) (Fig. 4) were consistent with the 2DE results. However, low-intensity exercise did not induce these proteins in the hippocampus. Thus, these results suggest that moderate-intensity exercise may induce hippocalcin, otub1, and crystalline expression in the hippocampus but not low-intensity exercise.

Neurogenic Markers Induced in Old Mice by Moderate-Intensity Exercise

Hippocalcin and spectrin-α are related to nerve regeneration and differentiation [16–18]. Moreover, OUTB1 attenuates neuronal apoptosis after intracerebral hemorrhage [19], and crystallin acts as a survival and differentiation factor in adult hippocampal neurogenesis [20]. Thus, to investigate the relationship between neurogenesis and the increase in proteins by exercise, neurogenic markers, such as NeuN and DCX, were examined by an immunoblotting analysis of the hippocampus of old mice. NeuN ($F = 22.42$, $p = 0.002$) and DCX ($F = 31.68$, $p = 0.001$) were significantly upregulated by moderate exercise training in the hippocampus (Fig. 5). Moreover, we examined the expression of p53 and SR, which is related to antiaging and neuroprotection in the brain [21, 22]. Moderate exercise training increased the levels of p53 ($F = 7.344$, $p = 0.001$) and SR ($F = 2.176$, $p = 0.047$) expression twofold compared to the levels in the hippocampus of old control mice (Fig. 5).

Fig. 1 Moderate-intensity exercise improves the cognition in old mice. **a** Weight change and **b** latency time for control, low intensity, and moderate-intensity exercise groups were measured at in passive avoidance test. Values are reported as means ± SEM. **$p < 0.01$ vs. control group



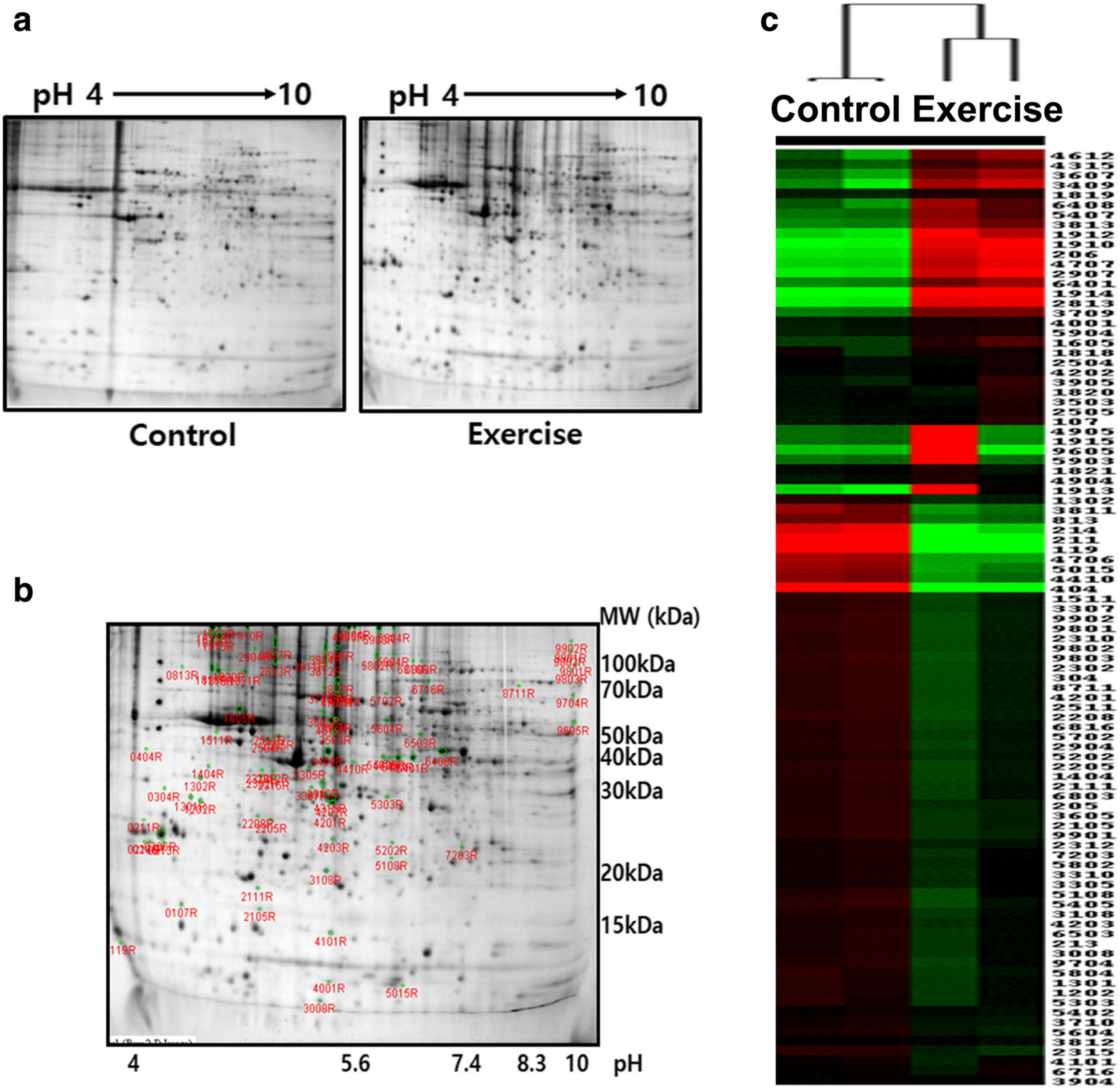


Fig. 2 Protein profiles with differential expression. **a** Hippocampal tissue protein profiles obtained over different pH ranges. **b** Representation of identified differential protein spots in 2D gel templates between moderate intensity and control groups. **c** Heat map from spot data of global gene expression pattern in control and moderate intensity exercise trained hippocampus

Table 2 Upregulated protein spots after moderate intensity of exercise training

	Spot number	Protein name	Protein ID	MW	pI	Sequence coverage (%)	No. of matched peptides
1	107	Hippocalcin	NP_034601. 1	17.7	4.5	67	16
2	206	Zeta polypeptide	EDL08815. 1	25.2	4.3	46	14
3	1301	Otub 1	AAH54410. 1	30.6	4.6	71	17
4	1302	V-type proton ATPase	NP_038505. 2	34.7	4.6	47	21
5	1605	Rab GDP dissociation inhibitor alpha	NP_034403. 1	61.9	4.9	69	32
6	1821	HSP 90-beta	NP_032328. 2	90.4	4.9	38	26
7	1913	Alpha-II spectrin	P 16546.4	148.8	4.7	36	71
8	2504	Protein disulfide-isomerase A6	NP_082235. 1	47.1	5.1	48	19
9	2813	Heat shock protein 4	EDL33603. 1	104.4	5.1	47	36
10	3108	Flavoprotein 2	NP_082664. 1	20.9	5.5	55	13
11	3310	Ketimine reductase mu-crystallin	NP_057878. 1	33.5	5.5	40	10
12	3710	*N*-Ethylmaleimide sensitive fusion protein	EDL34203. 1	75.1	5.5	25	16
13	3812	Heat shock protein 105	BAA11036. 1	104.1	5.5	27	16
14	5407	mCG 126739	EDL18979. 1	39.8	6.4	52	11
15	5802	Matrin-3	NP_034901.2	108.2	6.1	24	22
16	6408	Enolase 1B	NP_001020559. 1	42.1	7.1	61	24
17	6716	Isocitrate dehydrogenase 3 (NAD+) alpha	EDL25822. 1	81.2	6.9	40	14
18	9605	Brain-specific angiogenesis inhibitor 1-associated protein 2	NP_001032843. 1	55.4	9.8	44	18
19	9704	Syn2 protein	AAH66004. 1	69.6	9.8	37	19

Discussion

In this study, we examined the effects of exercise on cognition. We first found that moderate-intensity exercise, and not low-intensity exercise, increased the latency times during the passive avoidance test, which indicated that the mice remembered the previous tests more. Next, we found that moderate-intensity exercise upregulated proteins, such as hippocalcin, otub1, spectrin-α, and mu-crystallin in the hippocampus. These proteins have been reported to be related to neurogenesis and neuroprotection, but they have not been reported to be increased by exercise training. Exercise increases neurogenic factors and affects neurogenesis, memory, and learning [1, 9], but relatively few studies have examined the cellular basis of the improvements in brain function induced by exercise. In the present study, we identified for the first

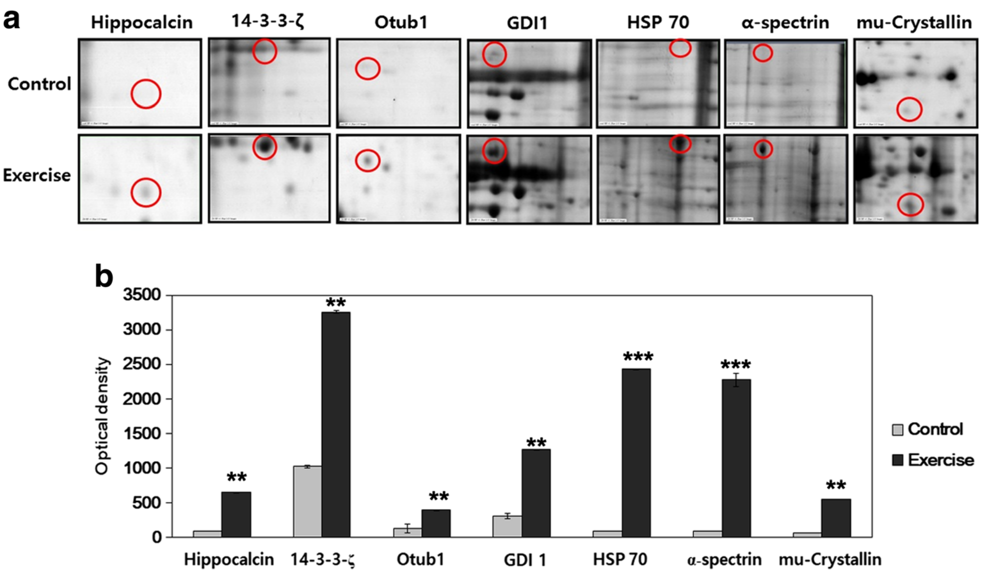


Fig. 3 Differential expression profiles of seven proteins. **a** Red circle on cropped images of 2D gels represent protein spots that showed significantly different changes between the control and exercise groups. **b** Quantitative analyses of upregulated proteins in the groups compared with the exercise group ($n = 3$ for each). The spot intensities were derived from silver-stained 2D gels. Values are reported as means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group

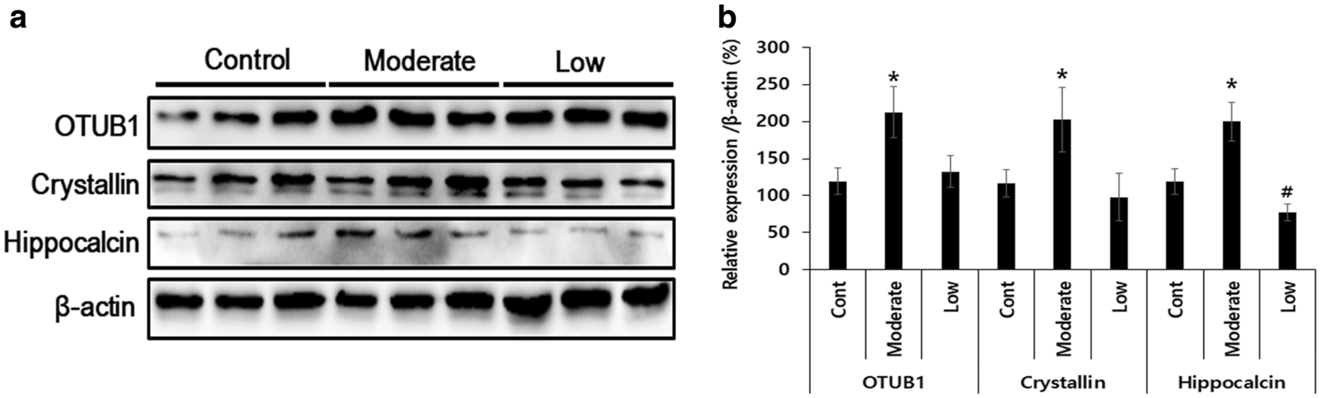


Fig. 4 Validation of proteomic results using western blot analyses of otub1, mu-crystallin, and hippocalcin proteins in the hippocampus of old mice. The hippocampal lysates were electrophoresed in 10% SDS-PAGE and immunoblotted with each antibody. The expression of otub1, mu-crystallin, and hippocalcin proteins band were normalized versus β-actin ($n = 3$ per group). Values are reported as means ± SEM. *$p < 0.05$, **$p < 0.01$ vs. control group

time the proteins induced by exercise and that affect neurogenesis and neuroprotection, which resulted in improved cognition.

Hippocalcin, which is a calcium-binding protein, has been shown to protect neurons against apoptosis [16], promote neuronal differentiation, and inhibit astrocytic differentiation in neural stem cells [17]. Spectrin is a cytoskeletal protein that is considered the main component of intracellular aggregation. Brain spectrins are thought to be involved in neuronal differentiation by way of the cytoskeletal lattice [18]. In this study, we demonstrated that moderate exercise increased the levels of hippocalcin and spectrin-α in the hippocampus of old mice, which suggested that these proteins might be involved in the induction of DCX and NeuN for neurogenesis.

Age-related changes in the central nervous system are well documented and include neuronal loss, demyelination, and deficits in cognitive function, which result from oxidative stress or free radical damage [26–28]. In this study, we examined the upregulation of otub1 and mu-crystallin induced by moderate exercise. Otub1 is a member of the deubiquitinating enzyme family, and it acts as a proteasome-associated deubiquitinase that is involved in ubiquitin-dependent protein degradation. In vitro and in vivo studies have shown that otub1 has anti-apoptotic effects on neural cell apoptosis and protects the brain from secondary damage following intracerebral hemorrhages [19]. Mu-crystallin is a novel mammalian protein that was first identified as a major lens structural protein in many Australian marsupials [23]. Outside the lens, kangaroo mu-crystallin is preferentially expressed in the retina and brain, and it presumably serves in an enzymatic role. In human tissues, mu-crystallin mRNA is present in neural tissues, retina, and the brain, and Vie et al. [24] have shown that it encodes nicodinamide-adenine dinucleotide phosphate-regulated thyroid hormone-binding protein. A recent study has reported that mu-crystallin is a key determinant of the vulnerability of the striatum in patients with Huntington's disease [25]. These observations suggest that exercise-induced cognition improvement may result from neuroprotection by the ubiquitin-dependent degradation of otub1 and the radical scavenging system of mu-crystallin.

GDI1, which encodes alpha Gdi, is one of the proteins that control the activity of the small GTPases in the Rab family in

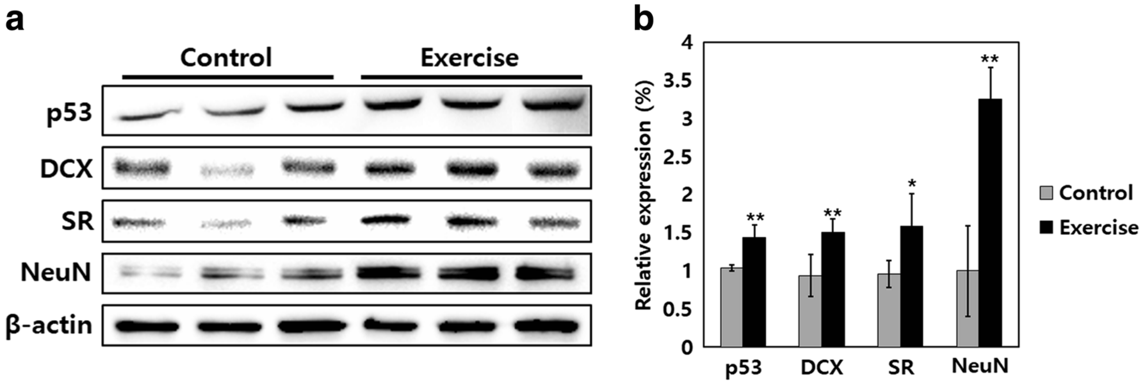


Fig. 5 Moderate intensity exercise increases NeuN, SR, DCX, and p53 expression in the hippocampus of old mice. The brain lysates were electrophoresed in 10% SDS-PAGE and immunoblotted with each antibody. The expression of NeuN, SR, DCX, and p53 proteins band were normalized versus β-actin ($n = 3$ per group). Values are reported as means ± SEM. *$p < 0.05$ vs. control group

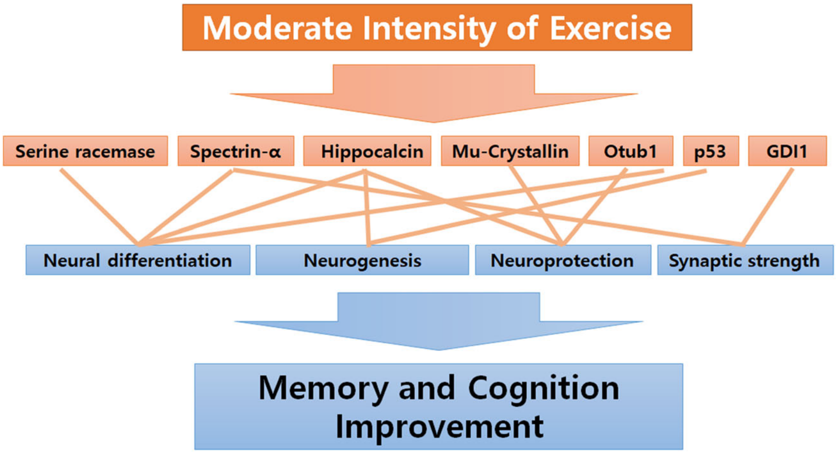


Fig. 6 Schematic diagram of the molecular linkers between moderate exercise and cognitive improvement in the old mice. Moderate intensity of exercise induces hippocalcin, otub1, spectrin-α, p53, GDI1, SR, and mu-crystallin in the hippocampus which is related with neurogenesis, neuroprotection and synaptic strength, resulted in the improvement of memory and cognitive function in the old mice

vesicle fusion and intracellular trafficking. Deletion of the mental retardation gene Gdi1 impairs associative memory and alters social behavior in mice [29]. These results suggest that exercise-induced GDI1 expression may be related to the improvements in memory function.

Impairment in the activity of the brain is a major feature of aging, and this coincides with the decreased function of neural stem cells. Exercise is one of the best-known methods that activate neural stem cells by increasing blood flow to the brain [30] and releasing several neurotrophic factors [31–33] that are related to neurogenesis. Kim et al. [35] have measured neurogenesis by BrdU immunohistochemistry after 6 weeks of treadmill exercise in 5- and 24-month-old mice. The number of BrdU-positive cells in the old group was significantly lower than that of the young group, and the numbers in both the exercise and aged group were significantly higher than that of the non-exercise group. Van praag et al. [36] have also shown by measuring BrdU in 24-month-old mice that 45 days of cyclic exercise induces neurogenesis. These results suggest that exercise increases neurogenesis in young mice as well as in old mice. Among these neuronal markers, DCX is a brain-specific microtubule-associated protein that is considered an immature neuronal marker. In contrast, NeuN can be used as a marker of newly generated neuronal cells in the hippocampus [34]. In this study, we confirmed that moderate-intensity exercise increased the levels of expression of neurogenic markers, such as NeuN and DCX, in the hippocampus, thus suggesting that moderate-intensity exercise activates neural stem cell in the hippocampus of old mice.

Brain blood flow during exercise is also dependent on exercise intensity. Increasing from low- to moderate-intensity exercise (cycling), blood flow through the carotid, vertebral, and middle cerebral arteries increases in healthy humans. At higher exercise intensities, blood flow velocities plateau or decrease (depending on the vessel), whereas carotid artery blood flow continues to rise [40]. This effect is thought to be due to increased blood flow through the external carotid artery, which maintains thermoregulation during higher-intensity exercise [41]. Neurogenesis has been shown to increase more in accordance with the exercise intensity [33, 37], and, in the human studies, neurogenic factor release increases according to the intensity of exercise [38, 39]. Using exercise training as an upregulator for hippocampal neurogenesis, an in vivo imaging study in humans has indicated a positive association between hippocampal-dependent cognitive performance and change of cerebral blood volume (CBV: served as an indirect measure of changes in hippocampal neurogenesis in the human brain) [42]. Furthermore, exercise intervention has been shown to improve performance in a neurogenesis-dependent cognitive test, the visual pattern separation task in human subjects [43]. In spite of the technical limitations associated with the direct measurement of neurogenesis in the human brain, these two studies have suggested that adult-born new neurons in the hippocampus might play a functional role in learning and memory in the human brain. In concert with that, in the present study, moderate-intensity exercise-induced neurogenesis may be through acute increase of blood flow to the brain.

In this study, we found the p53 and SR were induced in the hippocampus of exercise trained mice (Fig. 5). P53 is considered the most relevant tumor inhibitor, but p53 has also been shown to enhance neuromuscular activity, prolong lifespan, and delay age-associated decline of neurogenesis and neuronal differentiation [21], and D-serine is synthesized by the enzyme SR, which converts L- to D-serine and which acts as a neuronal signaling molecule by activating NMDA receptors in the brain. Thus, SR and other components of the serine shuttle are therefore promising targets for neuroprotective drugs and mechanisms that regulate D-serine signaling in health and disease [22], suggesting that exercise controls synaptic plasticity and memory function through the induction of p53 and SR.

In summary, we first found that moderate-intensity exercise induces hippocalcin, otub1, spectrin-α, p53, GDI1, SR, and mu-crystallin in the hippocampus, which is related to neurogenesis, neuroprotection, and synaptic strength (Fig. 6). Although it remains to be elucidated how exercise regulates these proteins in the brain, our novel finding was that the mediating proteins that induce neurogenesis, neuroprotection, and synaptic strength by exercise were identified, and these proteins resulted in the improvements in memory and cognition. Thus, our results showed that steady exercise of moderate intensity can delay the decline of cognitive function in the elderly through the activation of multiple factors.

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Compliance with Ethical Standards

The animal use protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Dongguk University Ilsan Hospital (No. 201603147) and were in accordance with the National Institutes of Health guidelines.

Conflict of Interest The authors declare that they have no conflict of interest.

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