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Exercise ameliorates cognition impairment due to restraint stress-induced oxidative insult and reduced BDNF level

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ABSTRACT

We assessed whether chronic treadmill exercise attenuated restraint stress-induced cognition impairment. Although serum corticosterone was not significantly altered by exercise, the restraint-induced increases in hippocampal malondialdehyde (MDA) and 4-hydroxynonenal (HNE) were reduced by chronic exercise. The exercise paradigm also reversed stress-induced reductions in brain-derived neurotrophic factor (BDNF), which increased cAMP response element-binding protein (CREB) and AKT activation. We verified the relationship between oxidative stress and BDNF signaling by treating primary hippocampal cultures with hydrogen peroxide (H_2O_2), which reduced BDNF and phosphorylated CREB and AKT (p-CREB, p-AKT) in a dose-dependent manner. Notably, pretreatment with *N*-acetylcysteine (NAC) reversed these decreases in a dose-dependent manner. These findings suggest that chronic exercise can ameliorate repeated stress-induced cognitive impairment by detoxifying reactive oxygen species (ROS) in the hippocampus and activating BDNF signaling.

1. Introduction

A growing body of research suggests that chronic stress leads to widespread changes in neural, endocrine, immune, and digestive responses. The hippocampus is particularly susceptible to chronic stress-induced neuronal damage, including atrophy of the CA3 region and decreased neurogenesis and synaptic plasticity, which results in impaired cognitive function [1,2].

Corticosterone that mediates stress response and adaptation is secreted in response to stress via hypothalamic–pituitary–adrenal (HPA) axis activation. Corticosteroid levels are increased by restraint stress in rodent models, and exogenous glucocorticoid result in hippocampal dendritic atrophy and impaired cognition [1,2]. Several studies have demonstrated that restraint stress or corticosterone treatment induce oxidative stress [2,3]. Increased nitric oxide (NO) levels and lipid peroxidation were observed following a 21 days period of restraint stress [2–5]. Others have reported that restraint stress reduces antioxidant defense systems [2,5,6].

Brain-derived neurotrophic factor (BDNF) is highly expressed in the hippocampus and cerebral cortex, where it contributes to neuronal growth, development, plasticity, survival, neuroprotection, and repair [7–9]. Reduced hippocampal BDNF levels are closely linked to chronic stress and psychiatric illnesses, including depression [9–11]. Interestingly, antidepressant treatment increases BDNF serum levels in depressed patients [12]. BDNF deficiency can also result in cognitive impairment [13].

Chronic physical exercise is beneficial for both mood disorders and cognitive impairment [14–16]. Altered long-term potentiation (LTP) expression and cognitive function in aged mice were successfully restored by exercise [16,17]. However, there is little evidence that stress-induced cognitive deficits can be helped by chronic exercise.

Here, we attempted to investigate whether chronic exercise improved restraint stress-induced cognitive impairment, with a specific focus on oxidative stress mechanisms and abnormal BDNF signaling.

2. Materials and methods

2.1. Experimental mice

Male 7 week-old C57BL/6J mice were obtained from Daehan Biolink, Inc. (Eumsung, Chungbuk, Korea) and housed in clear plastic cages in specified pathogen-free conditions under a 12:12 h light-dark cycle (lights on at 0600 and off at 1800). Mice had free access to standard irradiated chow (Purina Mills, Seoul, Korea). All animal procedures were approved by the Institutional Animal

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Care and Use Committee at Sungshin Women's University in accordance with the AAALAC International Animal Care policy.

2.2. Experimental design

The mice were divided into 3 groups (control, restraint stress, exercise combined with restraint stress group; 10–12 mice per group). We employed our previously described exercise protocol [18]. Briefly, treadmill exercise (Myung Jin Instruments Co., Seoul, Korea) was performed at 19 m/min for 60 min/day, 5 days/week from 0 to 12 weeks. Treadmill running was administrated at set times from 1800. To induce restraint stress, 8 week-old mice were individually placed into a well-ventilated 50 mL conical tube, which prevented forward or backward movement. Restraint stress was delivered at set times from 1000 to 1600 for 6 h. Control mice remained undisturbed in their home cages. This was repeated for 21 days unless otherwise indicated. Mice were subjected to restraint stress from 1000 to 1600 for 6 h, followed by exercise intervention 2 h later. Restraint stress was started at 9 weeks and ended at 12 weeks (Fig. 1A).

2.3. Y-maze test

The Y-maze consisted of 3 equal-sized arms that were V-shaped corridors made of white PVC. The arms measured 38.5 cm long, 3 cm wide, and 13 cm high, and were oriented at 60° angles from each other (JEUNG DO Bio & Plant Co. LTD, Seoul, Korea). The Y-maze test was performed under moderate lighting conditions (200 Lux) with moderately loud background white noise (40 db). Mice began a single trial at the end of 1 arm and were allowed to freely explore the Y-maze for 8 min. The number and sequence of arm visits were recorded manually by an observer. Alternation was defined as a consecutive entry in 3 different arms. The alternation percentage was calculated with the following formula: (number of alternations/total number of arm visits) − 2.

2.4. Water maze test

The test was performed using the SMART-CS (Panlab, Barcelona, Spain) program in an air-conditioned room. The water maze experiment was carried out in an air-conditioned room in a 1.5 m diameter plastic circular pool with 22 °C water containing powdered milk to obstruct the platform. Escape latency was monitored by a computer, using the SMART-LD program, which was connected to a ceiling-mounted camera mounted directly above the pool. The training schedule consisted of 2 trials per day over 5 test days, and each trial assessed the ability of the mouse to reach the platform within 60 s. On day 6, the mice were subjected to 3 probe trials, where they would swim for 60 s with no platform. The times

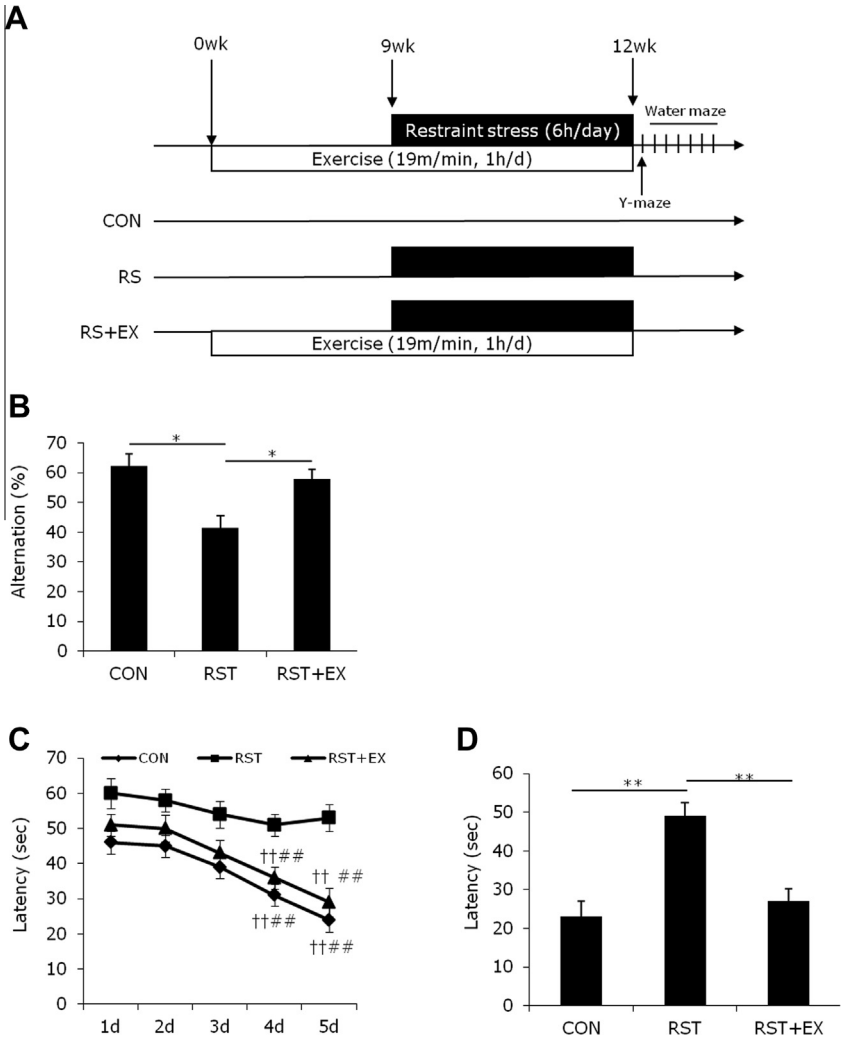

Fig. 1. Chronic exercise ameliorated repeated stress-induced learning and memory impairment. (A) Experimental design. (B) Quantitative analysis of Y-maze test data for short-term memory. (C) Quantitative analysis of water maze test data for learning ability. (D) Quantitative analysis of water maze test data for long-term memory. Data are presented as the means ± SEM ($n = 8$ animals). ∗ and ∗∗ denote differences at $p < 0.05$ and $p < 0.01$, respectively. †† and ## denote differences at $p < 0.01$ compared to day 1 and the restraint group, respectively.

required to reach the previous platform location (escape latency) were recorded. Each trial was stored on videotape for subsequent analysis.

2.5. Western Blot analysis

Protein samples (50 μg) were electrophoretically separated on 10% polyacrylamide gels, transferred to nitrocellulose membranes (Amersham Bioscience, Buckinghamshire, UK), and incubated with primary antibody in a blocking buffer at room temperature overnight. The next day, they were washed in a washing buffer and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The optical density of each band was measured using SCION program (NIH Image Engineering, Bethesda, MD, USA).

Anti-phospho-cAMP response element-binding protein (CREB), anti-CREB, anti-phospho-AKT, anti-AKT, and anti-β-actin antibodies were obtained from Cell Signaling Tech. Inc. (Danvers, MA, USA), and anti-BDNF antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.6. Immunohistochemistry and immunofluorescence

Mice were perfused through the left cardiac ventricle with 100 mM phosphate buffer (pH 7.4) followed by 40 mL of cold 4% paraformaldehyde in 100 mM phosphate buffer. After perfusion, the brains were quickly removed, fixed for 18 h with the same fixative at 4 °C, and subsequently transferred to 10%, 20%, and 30% sucrose solutions. Finally, 40 μm-thick sections were prepared using a vibratome (Leica, Wetzlar, Germany).

Free-floating sections were treating with 0.3% hydrogen peroxide (H_2O_2) in 100 mM phosphate-buffered saline (PBS) containing 0.3% Triton X-100 for 30 min, and nonspecific protein binding was blocked by incubation with 10% normal goat serum in PBS for 1 h. Sections were incubated with anti-NeuN primary antibodies (Millipore, Billerica, MA, USA) at room temperature overnight. Primary antibodies were reacted with biotinylated secondary antibodies diluted 1:1500 in PBS blocking buffer and visualized by the ABC method (ABC Elite kit, Vector Laboratories; Burlingame, CA, USA), and the sections were mounted.

For immunofluorescence, sections were incubated with anti-HNE primary antibodies (Alpha Diagnostic Int'l. Inc., San Antonio, TX, USA) at room temperature overnight. Primary antibodies were reacted with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG secondary antibody diluted 1:200 in PBS blocking buffer. Sections were mounted with VECTASHIELD (Vector Laboratories), and the images were analyzed using an Olympus BX 51 microscope equipped with a DP71 camera and DP-B software (Olympus Co., Tokyo, Japan). Fluorescence intensities were measured using TOMORO ScopeEye program (Techsan Community, Seoul, Korea).

2.7. Corticosterone measurements

Blood was collected from the vena hypogastrica after anesthetization with pentobarbital (40 mg/kg). Blood samples were centrifuged at 1000×*g* for 15 min to obtain serum. Corticosterone levels were measured from serum using enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI, USA).

2.8. Malondialdehyde (MDA) determination

MDA levels were measured in brain samples using the Bioxytech MDA 586 kit (Oxis Research, Portland, OR, USA) in accordance with manufacturer's instructions. MDA data were normalized to sample protein concentrations.

2.9. Primary hippocampal culture and cell death measurement

Primary hippocampal cultures were prepared from E17 ICR mouse hippocampus. Briefly, dissociated single cells were plated in RF media, DMEM with 10% FBS, 1× penicillin/streptomycin, 1.4 mM L-glutamine, and 0.6% glucose, in 12-well plates for 1 day. On day in vitro 1 (DIV1), the cells were incubated in Neurobasal media with 1× B27, 1× penicillin/streptomycin, and 1 × L-glutaMax, and the media was changed every 2 days. DIV7-9 cultures were used for studies. Cell death was assessed by measuring lactate dehydrogenase (LDH) released into the culture medium using a LDH assay kit (Roche Diagnostics Corporation, Indianapolis, IN, USA) according to the manufacturer's instructions. Culture medium collected 18 h after drug treatment. Complete cell death was accomplished by treating cells with 2 mM H_2O_2 for 18 h The results were normalized such that sham-treated cultures and complete cell death were counted as 0% and 100%, respectively.

2.10. Statistical analysis

Significant differences among groups were determined using one-way analysis of variance and repeated measurement (SPSS for Windows, version 18.0, Chicago, IL, USA). Post-hoc comparisons were made using Newman–Keuls tests. All values are reported as mean ± standard error (SE). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Chronic exercise reduced repeated stress-induced learning and memory impairment

First, we assessed whether 12 weeks of treadmill exercise restored stress-induced learning and memory impairment using Y-maze and water maze tests. Y-maze performance was significantly higher in the exercise group compared to restrained mice ($p < 0.05$) but was comparable to the level of control mice (Fig. 1B). The water maze data revealed that control group latency was significantly decreased on days 4 and 5 (both $p < 0.01$) compared with that on day 1 (Fig. 1C). Interestingly, the latency of restrained mice did not change over time, whereas that of the exercise group was significantly reduced on days 4 and 5 (both $p < 0.01$) relative to day 1 (Fig. 1C). The latencies of the control and exercise groups were profoundly reduced on days 4, 5, and 6 (all $p < 0.01$) compared with the restrained group. Expectedly, the latencies of the control and exercise groups were not significantly different on the final day (Fig. 1D).

3.2. Chronic exercise did not affect serum corticosterone

Because the hippocampus is vulnerable to increased corticosterone levels induced by chronic stress, we assessed the serum levels of immediately after the final restraint. There was no significant difference between restrained mice (368.4 ± 21.6 ng/mL) and mice restrained with exercise (412.4 ± 32.6 ng/mL), but both 2 groups were significantly elevated compared with control (75.4 ± 10.5 ng/mL, $p < 0.01$) (Fig. 2A). Despite these differences, the numbers of NeuN-positive neurons in the CA3 region were comparable across all groups (Fig. 2B and C).

3.3. Chronic exercise reduced oxidative stress

Given that repeated stress has been demonstrated to evoke oxidative stress via corticosterone, we measured hippocampal

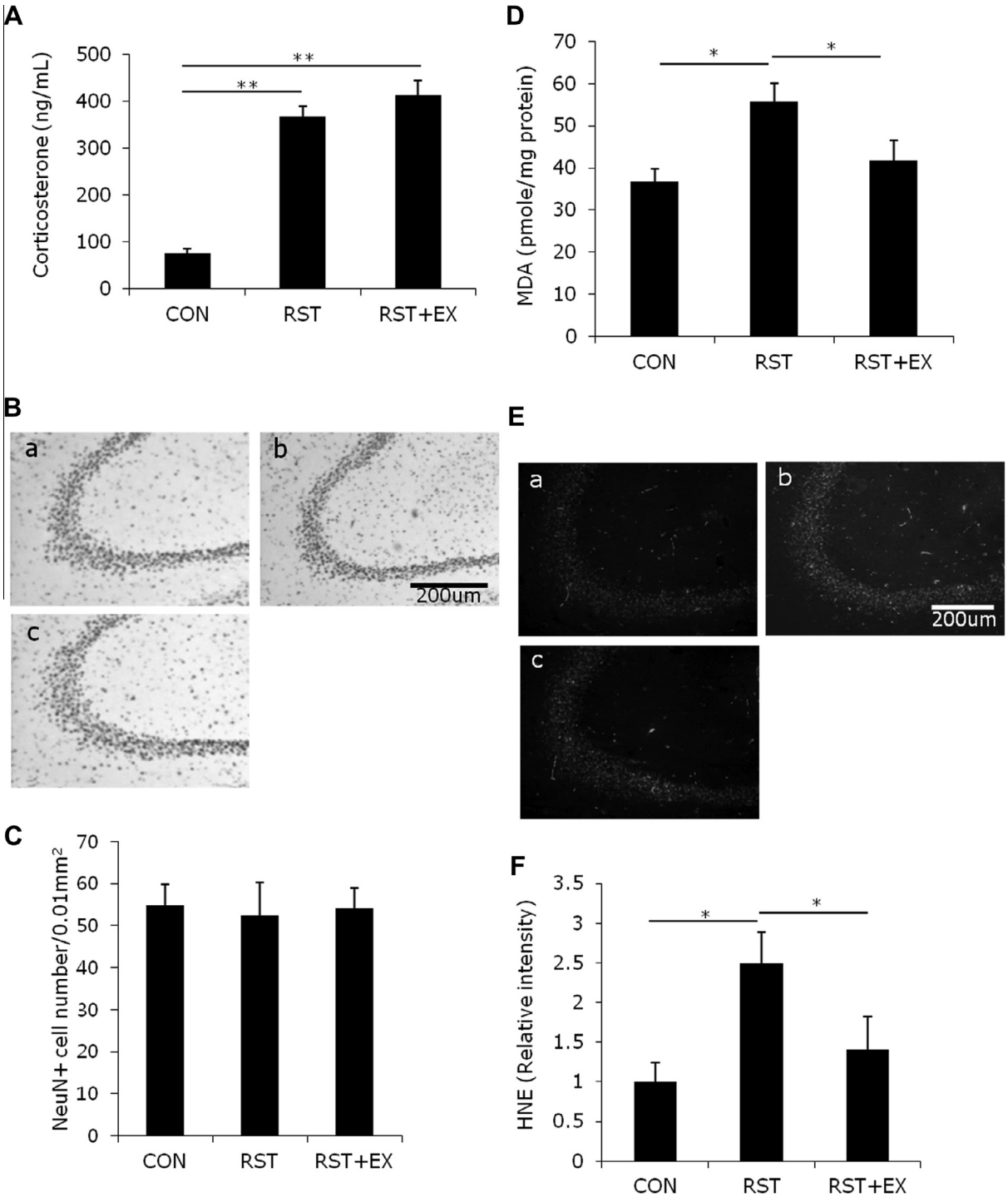

Fig. 2. Chronic exercise attenuated repeated stress-induced hippocampal oxidative stress without neuronal loss. (A) Quantitative analysis of serum corticosterone levels. (B) Photomicrographs showing hippocampal NeuN immunoreactivity (a, Control; b, Restraint; c, Restraint with Exercise). (C) Quantitative analysis of NeuN+ cells. (D) Quantitative analysis for MDA. (E) Photomicrographs showing hippocampal 4-HNE^{+} immunoreactivity (a, Control; b, Restraint; c, Restraint with Exercise). (F) Quantitative analysis for layer density. Data are presented as the means ± SEM ($n = 8$ animals). $*$ and $**$ denote differences at $p < 0.05$ and $p < 0.01$, respectively.

MDA and 4-HNE. Interestingly, the stress-induced MDA increase (55.8 ± 4.2 pmol/mg) was reversed by chronic exercise (41.8 ± 4.8 pmol/mg protein, $p < 0.05$, Fig. 2D). Moreover, 4-HNE immunoreactivity in the CA3 region was significantly lower in the exercise group compared to the restrained only group ($p < 0.05$, Fig. 2E and F).

3.4. Chronic exercise reversed BDNF reductions induced by repeated stress

We used Western Blots to measure the expression of BDNF and downstream signal molecules, including CREB and AKT. We found that BDNF expression was markedly reduced by repeated stress

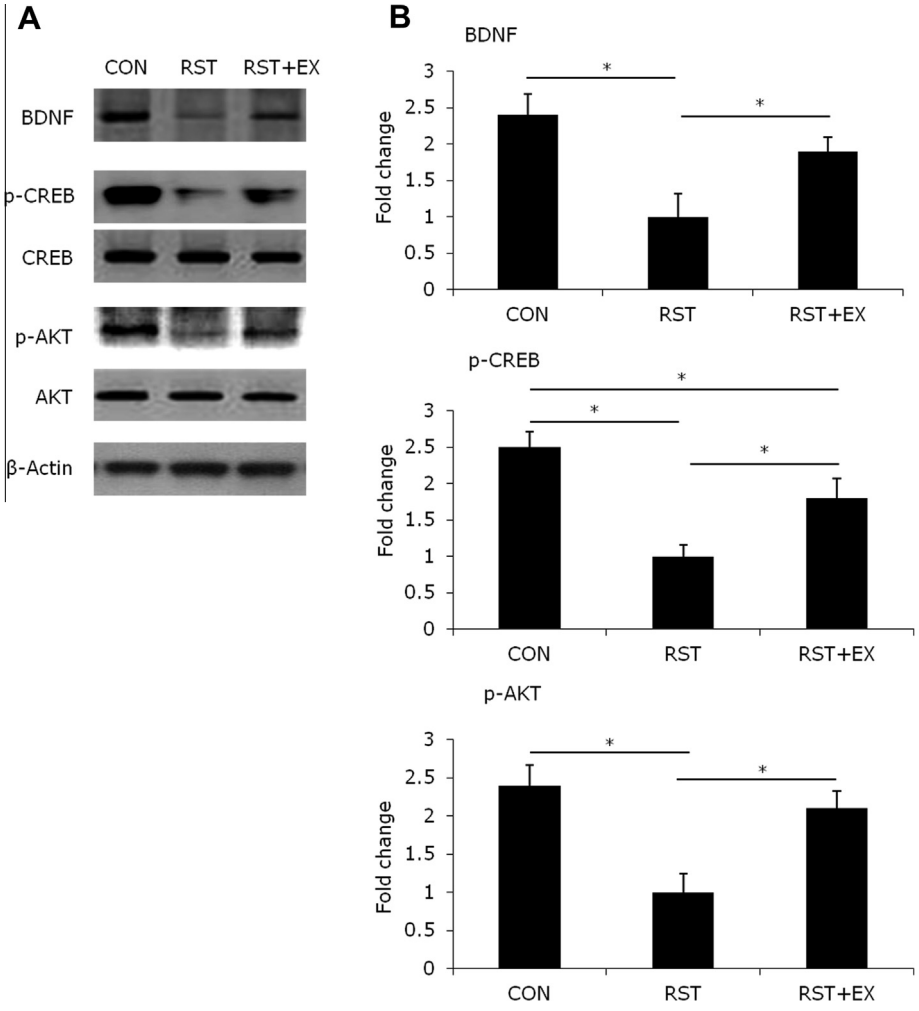

Fig. 3. Chronic exercise reversed BDNF expression decreases induced by repeated stress. (A) Western Blots for BDNF, CREB, AKT, p-CREB, and p-AKT. (B) Quantitative analysis of Western Blots (normalized to β-actin). Data are presented as the means ± SEM ($n = 8$ animals). $*$ and $**$ denote differences at $p < 0.05$ and $p < 0.01$, respectively.

($p < 0.05$), and this was reversed by chronic exercise ($p < 0.05$, Fig. 3A and B). Additionally, the profound reductions in phospho-CREB and phospho-AKT following repeated stress ($p < 0.05$) were attenuated by chronic exercise ($p < 0.05$, Fig. 3A and B).

3.5. Exogenous antioxidant treatment reversed BDNF signaling impairments induced by oxidative stress

The dramatic downregulation of BDNF signaling and increased oxidative stress induced by repeated stress led us to assess the responses of BDNF, p-CREB, and p-AKT to H_2O_2 treatment in primary hippocampal culture. We found that 0 to 2 mM H_2O_2 treatment for 18 h accelerated cell death in a dose-dependent manner; 250 μM H_2O_2 treatment produced about 50% cell death (Fig. 4A). Next, we tested measured BDNF, p-CREB, and p-AKT levels in cells exposed to H_2O_2 (0–250 μM) and found that they were reduced in a dose-dependent manner (Fig. 4B and D). However, primary hippocampal cultures pretreated with 0.5 and 2 mM of the antioxidant *N*-acetylcysteine (NAC) for 2 h enhanced BDNF, p-CREB, and p-AKT expression in a dose-dependent manner (Fig. 4C and E).

4. Discussion

Our results demonstrate that chronic exercise mitigates repeated stress-induced cognitive impairment, likely by reducing oxidative damage and restoring hippocampal BDNF signaling. In this experiment, restraint stress reduced Y-maze and water maze performance, suggesting that the restraint stress paradigm resulted in cognitive impairment.

Body weight of control group was progressively increased throughout 21 day experimental period, and then reached 111.5% of the starting point, whereas that of restraint-alone and of restraint combined with exercise reached 101.9% and 102.6% at the end of restraint stress administration, respectively (Supplementary Fig. 1). Mice administrated with 6 h per day of the 21 consecutive days of restraint stress weighed less than control mice, regardless of exercise treatment. This result suggests that restraint stress induces alteration of body weight and exercise does not effect on body weight change. Animals that were subjected to restraint stress and exercise showed significantly higher learning and memory performance compared to the restraint alone group. Stress can increase corticosterone levels, which may render the hippocampus more susceptible to neuronal damage, including reduced dendritic branching and glutamatergic spine loss, thereby causing cognitive impairment [1,19–20]. However, we found that corticosterone levels in the restraint combined with exercise group were comparable to that of restraint alone, suggesting that corticosterone is not a key mediator in protective effect of exercise.

We did observe differences in ROS levels; MDA and HNE were significantly enhanced in restrained mice compared to the restraint combined with exercise group. Restraint stress or glucocorticoid

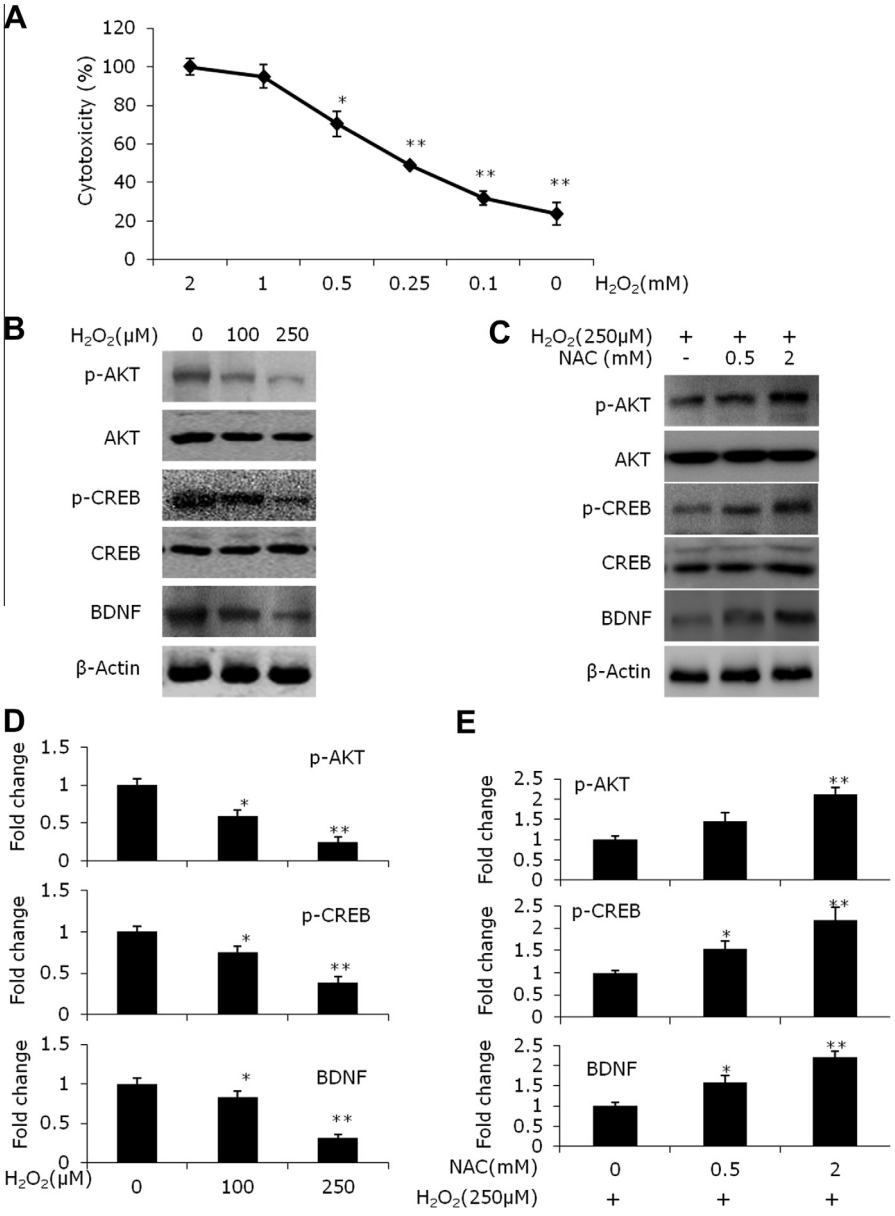

Fig. 4. Oxidative stress downregulated BDNF signaling, which was reversed by exogenous antioxidant treatment. (A) Quantitative analysis of primary hippocampal culture cell death following H_2O_2 treatment. (B) Western Blots for BDNF, CREB, AKT, p-CREB, and p-AKT in primary hippocampal culture treated with H_2O_2. (C) Quantitative analysis of Western Blots (normalized to β-actin). (D) Western Blots for BDNF, CREB, AKT, p-CREB, and p-AKT in primary hippocampal culture with H_2O_2 and NAC. (E) Quantitative analysis of Western Blots (normalized to β-actin). Data are presented as the means ± SEM ($n = 8$ animals). $*$ and $**$ denote differences at $p < 0.05$ and $p < 0.01$, respectively.

administration induces hippocampal oxidative stress [2–4,18,21], and stress-induced redox imbalance may cause structural changes that impair cognition [22–23]. Here, repeated restraint stress-induced ROS accumulation was significantly reversed by exercise. Chronic exercise can increase antioxidant defensive responses. Prolonged exercise prevented increased brain lipid peroxidation in sleep deprivation and chronic cerebral hypoperfusion [24–25]. We previously showed that repeated restraint stress-induced oxidative stress was markedly ameliorated by an 8 week exercise regimen [18]. Emerging evidence suggests that exercise induces the expression of genes that affect antioxidant defense systems. Exercise has been shown to reduce oxidative stress and behavioral anxiety by exercise altering the expression of pro- or antioxidant enzymes, including superoxide dismutases (SOD)-1 and SOD-2, and glutathione peroxidase (GPx) in sleep-deprived and aged mice, suggesting exercise-produced improvement of antioxidant capacity [24–26]. In addition, exercise-mediated induction of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) promotes the expression of antioxidant enzymes, including Mn-SOD, Cu/Zn-SOD, and GPx, thereby decreasing oxidative damage in the brain [26–28]. Collectively, the literature supports our findings that exercise protects against restraint stress-induced oxidative damage in the hippocampus.

A growing body of evidence suggests that restraint stress or corticosterone treatment suppresses hippocampal BDNF levels, which occurs concomitantly with impaired cognition [13,29–31]. Various exercise protocols induce BDNF expression, and AKT/CREB activation plays a crucial role in exercise-induced hippocampal BDNF transcription [18,31–33]. Some studies suggest that the effects of chronic stress on BDNF levels and cognitive impairment are related to oxidative stress; both are improved by enhancing the antioxidant defensive capacity [14,18,28,30]. The results of our

primary hippocampal culture experiments demonstrate a direct relationship between oxidative stress and BDNF signaling, indicating ROS-mediated regulation of hippocampal BDNF signaling.

In conclusion, chronic exercise can rescue repeated stress-induced cognitive impairment through the activation of AKT/CREB/BDNF signaling and ROS detoxification.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.111.

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