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Spontaneous Wheel Running Exercise Induces Brain Recovery via Neurotrophin-3 Expression Following Experimental Traumatic Brain Injury in Rats

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Abstract. [Purpose] The aim of the present study was to investigate the expression of neurotrophin-3 (NT-3) after applying spontaneous wheel running exercises (SWR) after experimental traumatic brain injury (TBI). [Subjects and Methods] Thirty male Sprague-Dawley rats were divided into 3 groups; 20 rats were subjected to controlled cortical impact for TBI, and then, animals were randomly collected from the SWR group and subjected to wheel running exercise for 3 weeks. Ten rats were not subjected to any injury or running exercise to compare with the effect of TBI and SWR. Immunohistochemistry, Western blotting, skilled ladder rung walking test, and 2,3,5-triphenyltetrazolium chloride staining analysis for the evaluation of NT-3 expression were used to assess brain damage and recovery. [Results] The TBI-induced decrease in NT-3 expression was recovered by wheel running exercise. Moreover, decreased ischemic volume and progressive neurobehavioral outcome were observed in the SWR group. [Conclusion] Spontaneous running exercise promotes brain recovery and motor function through an increase in expression of NT-3.
Key words: Neurotrophin-3, Traumatic brain injury, Wheel running exercise

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INTRODUCTION

The majority of deaths and disabilities from trauma occur with traumatic brain injury (TBI)[1)]. TBI is an insult to the brain that results in impairments of cognitive and physical functioning as well as disturbances in behavioral or emotional functioning[2)]. Previous studies have attempted to establish laboratory models of TBI to assess the damage phase and treatment in a variety of primates and conditions[3)]. Among them, controlled cortical impact (CCI), occasionally referred to as the rigid percussion model, is a model of traumatic brain injury in ferrets, rats, and mice that may potentially prove useful in elucidating the mechanisms underlying neurodegeneration using genetically altered animals[4, 5)]. The CCI model allows for ready manipulation and accurate quantification of biomechanical forces[6)]. Injury results in a considerable hematoma under the injury site that is immediately visible, and the impact induces primary necrosis at the center of the contusion. Secondary cellular loss in sensitive regions is observed post injury[1)].

Spontaneous exercise may be therapeutic in the management of CNS injury, by reducing the degree of initiatory damage, limiting the degree of secondary neuronal death, and promoting neural repair and behavioral rehabilitation[7)]. The effects of exercise on genes encoding for neurotrophins and other proteins suggest that exercise could regulate anatomical changes that support brain plasticity. It has been previously demonstrated that exercise increases the number of new neurons in the dentate gyrus[8)]. Epileptic, ischemic, and traumatic insults to the brain induce marked changes in the expression of genes encoding for neurotrophins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) in cortical and hippocampal neurons[9)].

The purpose of the present study was to investigate the expression of NT-3, which provides a neuroprotective effect that promote the maintenance and survival of neurons in rats undertaking spontaneous wheel running exercise after experimental TBI using a CCI model in rats.

SUBJECTS AND METHODS

Thirty male Sprague-Dawley rats weighing between 250 g and 300 g were used and maintained on a 12 h on/12 h off light/dark cycle with *ad libitum* access to food and water. All the experiments were performed in accordance with protocols approved by the University of Daegu Animal Experiment Committee, based on the NIH Guidelines for the Care and Use of Laboratory Animals (NIH publica-

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tion, 1996). Animals were divided into 3 groups randomly: namely, the normal (NRM), TBI, and SWR groups. The TBI and SWR groups were anesthetized with 2 mL/kg 50% Zoletil and 50% Xylazine hydrochloride mixture and secured in a stereotaxic frame. Animals were subjected to a 3-mm diameter craniotomy centered on the left side, 3 mm lateral to the sagittal suture and 1 mm posterior to the bregma. Injury was induced with a weight 3 mm in diameter and weighing 45 g that was dropped through the guide tube from a height of 20 cm. This resulted in an injury with a tissue deformation of 3 mm. The injury device was modified and described in detail by previous studies[10, 11]. The NRM group was not subjected in any injury or treatment.

Animals were collected and assigned randomly to exercise groups. The exercise groups were put on a wheel running exercise regimen for 15 min per day, at the same time every day. The wheel (21 cm diameter and 8 cm width) (JD-A-06 type, JEUNGDO Bio & Plant Co., Ltd., Seoul, South Korea) was fixed on one side, and the rat was permitted to run spontaneously within the wheel. The SWR groups were also made to exercise.

When sacrificed, the animals were anesthetized with a mixture of 2 mL/kg 50% Zoletil and 50% Xylazine hydrochloride and perfused through the heart with 200 mL of 0.9% NaCl solution followed by 4% paraformaldehyde solution. The brains were removed, maintained in post-fixative overnight, and then sectioned to a thickness of 30 μL for immunohistochemistry.

In brief, the sections were washed (3×10 min) in 0.01 M phosphate-buffered saline solution (PBS; pH 7.2) and incubated for 12 h at room temperature with mouse monoclonal anti-NT-3 (Chemicon, Temecula, CA, USA). The antibody was diluted to 1:200 with a solution of Triton X-100 and normal donkey serum. After incubation in primary antibody, the sections were rinsed (3×10 min) in PBS, incubated for 90 min at room temperature with anti-mouse IgG (Vector Laboratories Inc, Burlingame, CA, USA), and diluted to 1:25 with a solution of Triton X-100 and normal donkey serum. After incubation in secondary antibody, the sections were rinsed (3×10 min) in PBS, and then incubated for 1 h at room temperature with a Vectastain Elite ABC-Kit (Vector Laboratories Inc., Burlingame, CA, USA). The sections were rinsed with PBS and incubated for 10 min in 0.04 mg of 3,3′-diaminobenzidine (DAB, in 200 mL distilled water). The sections were then incubated for 1 min in DAB solution with 35% H_2O_2. The DAB sections were rinsed with PBS (3×10 min) to halt the chromogen reaction, wet-mounted onto gelatin/chromium-coated slides, and allowed to air-dry overnight. The sections were then dehydrated through a series of alcohols, soaked in xylene, and cover-slipped with Clarion (Biomedia, USA).

After the animals were anesthetized and sacrificed, their brains were extracted, immersed for 10 min in cold PBS, and sliced into 2.0-mm-thick sections. The sliced brain samples were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO, USA) dissolved in PBS for 20 min at 37 °C and subsequently transferred to 4% paraformaldehyde solution for fixation. Brain damage was revealed by a lack of TTC staining, which indicated that the tissues were dehydrogenase deficient. The volume of brain damage was measured in each slice, and the injured area was determined in terms of a percentage of total brain area pixels.

The brains of each group were collected, washed twice in PBS, and then homogenized and lysed with buffer (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10 μg/ml leupeptin [pH 7.5]) for 30 min on ice. The lysates were centrifuged for 10 min at 15,000 rpm at 4 °C, and the protein concentration was determined as described previously[12]. Equal amounts of protein (40 μg) were resolved via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The blots were washed with TBST (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.05% Tween 20), blocked with 5% skim milk for 1 h, and then, incubated with the appropriate primary antibodies at the dilutions recommended by the suppliers. The membranes were washed, and the primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG or goat-anti mouse IgG. The bands were then visualized via enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

The ladder walk device consisted of a 1 m long wooden-rung walkway with a varying distance of 1.5 cm between rungs. Rats were habituated to the testing ladder before TBI surgery. The animals could traverse the walkway freely and without reinforcement. During the third session, the animals were video-recorded as they traversed the walkway three times. Tapes were later analyzed to obtain pre-operative baseline scores. Each group was tested at week 1 after surgery, and then once per week for the following 2 weeks. In a single testing session, the animals crossed the walkway three times and received a score for the number of fore and hind limb feet faults per each 10 steps. Only the lesion-affected limbs were analyzed, and a foot fault was characterized as a total miss or slip from the rung, or a misplacement of the paw on the rung. The method was modified as described previously[13].

The results were expressed as the means ± standard error (SE) or deviation (SD). All experiments were analyzed via analysis of variance, and some experiments were analyzed via comparisons of the treatment mean with the controls using the Bonferroni-Dunn test. Differences were considered statistically significant when $p < 0.05$. All analyses were performed using SPSS for Windows (v. 12.0 K, SPSS Inc., Chicago, IL, USA).

RESULTS

Skilled ladder rung walking tests were conducted to evaluate the effects of spontaneous wheel running after TBI, which is sensitive to alterations of motor function after sensory motor cortex damage (Table 1). The error ratio was measured in the forelimbs (A) and hind limbs (B). The results for both affected limbs in all three groups showed a significant decrease in the error ratio compared with the before exercise period as time passed significantly ($p<0.05$). Comparison with the NRM group showed that there was the more increase in the error ratio in the TBI group. Moreover,

Table 1. The effect of spontaneous wheel running after TBI on behavior recovery

A

Group	Error ratio (%)			
	Before exercise	1 week	2 weeks	3 weeks
TBI	54±9.67	36±6.70*	34±9.67*	31±8.76*
SWR	56±8.43	41±7.38*	25±8.50*,†	19±8.76*,†,‡,§
NRM	20±8.16	15±5.27	14±5.16	8±7.89*,§

B

Group	Error ratio (%)			
	Before exercise	1 week	2 weeks	3 weeks
TBI	60±8.16	45±8.50*	34±9.66*	32±9.19*,†
SWR	61±8.76	33±6.75*	29±8.76*	19±7.38*,†,§
NRM	18±7.89	17±4.83	15±5.27	11±5.68§

Skilled ladder rung walking test was conducted for the with forelimbs (A) and hind limbs (B). Each example shown is representative of three experiments. The error ratio (%) is the mean ± SD of the values to measure during the test. Statistical analysis was performed by using one-way ANOVA. * $p < 0.05$ versus before exercise between periods; † $p < 0.05$ versus 1 week after exercise between periods; ‡ $p < 0.05$ versus 2 weeks after exercise between periods; § $p < 0.05$ versus CON between the group at 3 weeks after exercise

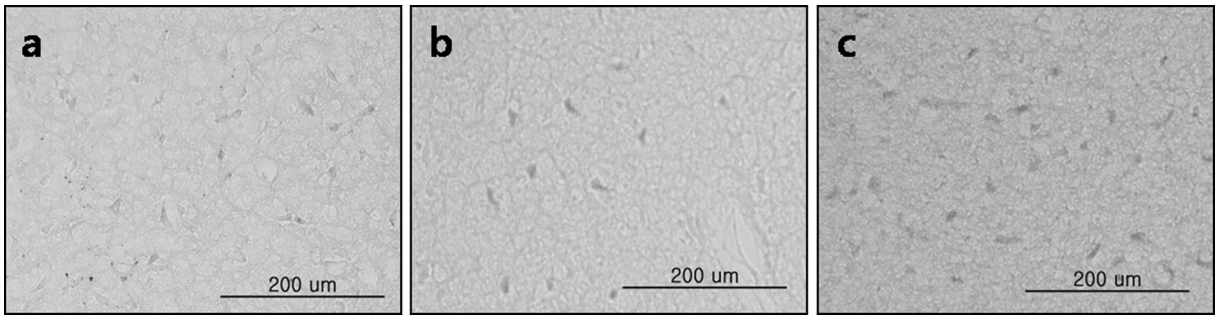

Fig 1. The effect of spontaneous wheel running after TBI on NT-3 expression in immunohistochemistry
To confirm NT-3 expression, immunohistochemistry (A) was conducted in the NRM (a), TBI (b), and SWR (c) groups as described in the Materials and Methods section. Each example shown is representative of three experiments. Scale bar = 200 μm.

Table 2. The effect of spontaneous wheel running after TBI on NT-3 expression in Western blotting analysis

Group	Relative optical density (% of TBI at 3 weeks after exercise)		
	NRM	TBI	SWR
3 weeks	100.00±1.02	79.50±3.48*	120.77±1.87*,§

To confirm NT-3 expression, Western blotting analysis was conducted as described in the Materials and Methods section. Each example shown is representative of three experiments. The optical density values denote the mean ± SE of three experiments for each condition determined from densitometry relative to β-actin, respectively. Statistical analysis was performed by using one-way ANOVA. * $p < 0.05$ versus NRM. § $p < 0.05$ versus TBI.

the increase tended to be less profound in the SWR group than in the TBI group ($p<0.05$).

Immunohistochemistry and immunoblotting for NT-3 expression were performed in each group (Fig. 1 and Table 2). It was observed that the TBI-induced reduction in NT-3 expression increased wheel running for 3 weeks. A more profound significant increase in NT-3 expression was noted in the SWR group than in the NRM group ($p<0.05$).

To confirm the area of the brain injured by TBI, TTC staining was varied out (Table 3). No ischemic areas were detected in the NRM group. In the SWR group, the ischemic area was statistically significant decrease than in the TBI group ($p<0.05$).

Table 3. The effect of spontaneous wheel running after TBI on the brain-injured area

Group	Volume of injury (% of total area at 3 weeks after exercise)		
	NRM	TBI	SWR
3 weeks	0	22.2±3.01	5.3±0.67*

To confirm the ischemic volume of the brain-injured area, TTC staining was conducted in the NRM, TBI, and SWR groups as described in the Materials and Methods section. The results provide the percentage of injured area in total brain area pixels and represent the mean ± SE. Each example shown is representative of three experiments. Statistical analysis was performed by using the independent t-test. * $p < 0.05$ versus TBI.

DISCUSSION

TBI may induce scalp laceration, skull fracture, contusion, intracranial hemorrhage, blood-brain barrier disruption, and diffuse axonal injury according to the features, direction, and degree of force applied to the head[14, 15]. Brain and whole body physiological changes engendered by primary mechanical injuries are associated with a series of morphological and cellular changes as secondary injuries[1, 16]. These brain injuries trigger pathological pathways and auto-protective mechanisms. Pathological pathways that may potentially harm brain cells include excitotoxicity, free radical formation, inflammation, and apoptosis, among others. Auto-protective mechanisms include the formation of heat shock proteins (HSPs), anti-inflammatory cytokines, growth factors (GFs), and endogenous antioxidants[17].

Among endogenous protective mechanisms, growth factors are special endogenous signaling proteins that promote survival, division, growth, and the differentiation and morphological plasticity of neural cells. Moreover, some of these growth factors are required for certain trophic or all of these functions in selected neural populations of the nervous system[18]. Growth factors can generally be divided into four major groups according to their receptor systems and their downstream signal transduction pathways. Many types of growth factors are induced very early after brain lesions, including NGF, BDNF, glial cell line-derived growth factor (GDNF), basic and acidic fibroblastic growth factors (FGF), and members of the transforming growth factor super family (TGF). Other new members that were identified include NT-3 and NT-4/5[17, 18].

NT-3 was detected in the corpus callosum, the hippocampus, cortex (layer V), the primary olfactory cortex, the amygdala, the Purkinje cells of the cerebellum and spinal cord, and so on[19]. It has been suggested that this neurotrophin may serve a neuroprotective function by playing a role in the maintenance and survival of neurons after TBI. Thus, it is important to characterize the spatial and temporal patterns and levels of NT-3 expression following experimental brain injury[20].

In the present study, NT-3 expression was increased in the SWR group and was reduced after TBI. It was reported that physical exercise, such as treadmill and wheel running exercises, induced increases in the levels of neurotrophic factor, and this fact is consistent with our findings[21]. Additionally, these neurotrophic factors prevent programmed cell death and promote cell survival in the progression of brain injury[22]. These imply that increased NT-3 expression as a result of wheel running exercise reduced programmed cell death and tissue ischemic injury and induced brain recovery via the regulation of cellular signaling.

The aim of motor training, such as running exercise, is to diminish functional disability and promote brain recovery. This can be achieved by proper intervention for rehabilitation, which is useful in stimulating therapy-induced recovery in brain-injured patients[23]. Exercise, as a simple and widely practiced behavior, can enhance the activation of molecular and cellular cascades and promote brain vascularization, neurogenesis, and functional changes in neuronal structure, as well as resistance to injury[8].

In conclusion, spontaneous running exercise promotes brain recovery and motor function as the result of NT-3 expression. These facts imply that spontaneous exercise would be needed for rehabilitation of brain-injured patients when properly directed by physical therapist properly. Moreover, it the present study contributes evidence showing that spontaneous rehabilitation training is helpful to biological and functional recovery in brain injured-patients by stimulating the metabolic activation in the brain.

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