Voluntary Exercise Preconditioning Activates Multiple Antiapoptotic Mechanisms and Improves Neurological Recovery after Experimental Traumatic Brain Injury

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Abstract

Physical activity can attenuate neuronal loss, reduce neuroinflammation, and facilitate recovery after brain injury. However, little is known about the mechanisms of exercise-induced neuroprotection after traumatic brain injury (TBI) or its modulation of post-traumatic neuronal cell death. Voluntary exercise, using a running wheel, was conducted for 4 weeks immediately preceding (preconditioning) moderate-level controlled cortical impact (CCI), a well-established experimental TBI model in mice. Compared to nonexercised controls, exercise preconditioning (pre-exercise) improved recovery of sensorimotor performance in the beam walk task, as well as cognitive/affective functions in the Morris water maze, novel object recognition, and tail-suspension tests. Further, pre-exercise reduced lesion size, attenuated neuronal loss in the hippocampus, cortex, and thalamus, and decreased microglial activation in the cortex. In addition, exercise preconditioning activated the brain-derived neurotrophic factor pathway before trauma and amplified the injury-dependent increase in heat shock protein 70 expression, thus attenuating key apoptotic pathways. The latter include reduction in CCI-induced up-regulation of proapoptotic B-cell lymphoma 2 (Bcl-2)-homology 3–only Bcl-2 family molecules (Bid, Puma), decreased mitochondria permeabilization with attenuated release of cytochrome c and apoptosis-inducing factor (AIF), reduced AIF translocation to the nucleus, and attenuated caspase activation. Given these neuroprotective actions, voluntary physical exercise may serve to limit the consequences of TBI.

Key words: controlled cortical impact; exercise; neuroprotection; traumatic brain injury

Introduction

Traumatic brain injury (TBI) represents a significant public health problem, with more than 1.7 million new cases annually in the United States alone and accounting for 60% of all trauma deaths. TBI causes cell death and neurological dysfunction through both direct physical disruptions of tissue or pathways (primary injury), as well as through delayed biochemical changes induced by trauma (secondary injury). Secondary injury mechanisms involve a complex cascade of biochemical changes over time, leading to neuronal cell death and associated neurological deficits.

Human as well as animal studies have suggested that physical activity protects the brain from multiple types of insults by promoting restorative processes, neuroplasticity, and neuronal survival. Physical exercise has been shown to reduce the risks for developing Alzheimer’s disease, improve motor function in animal models of Parkinson’s disease, and offer neuroprotection in animal models of stroke. Exercise also ameliorates neurological dysfunction post-TBI through mechanisms that may include inhibition of apoptosis, attenuation of neuroinflammation and oxidative stress (OS), and improved neurorestoration.

Exercise can induce various growth factors with neuroprotective potential, which may explain, at least in part, the neuroprotection afforded by physical activity. However, not all experimental studies have confirmed the neuroprotective effects of exercise post-TBI. Use of forced exercise and/or acute exercise initiation in the first few days post-TBI may lead to suboptimal induction of neuroplasticity/neuroprotective pathways, such as brain-derived neurotrophic factor (BDNF), and, consequently, poor functional or neuropathological outcomes. Moreover, the exercise-induced increase in metabolic demands when the brain is energetically compromised may negatively impact neurological recovery.

Our previous studies focused on chronic TBI outcomes have used a well-established experimental TBI model, controlled cortical impact (CCI), that leads to chronic cognitive deficits in rodents. We have shown that delayed voluntary exercise initiated as late as 5 weeks after TBI, in contrast to acute intervention (1 week...
post-trauma), results in brain-derived neurotrophic factor (BDNF) and cyclic adenosine monophosphate response element-binding protein (CREB) up-regulation, reduces lesion volume and hippocampal neuronal loss, and improves cognitive function. Although effective, late intervention paradigms do not allow examination of the effects of exercise on neuronal cell death pathways activated early post-TBI and responsible for a considerable proportion of posttraumatic neuronal loss.14

In the present study, we evaluated the effects of voluntary exercise preconditioning (pre-exercise; exercise performed before TBI) on key molecular mechanisms of neuronal cell death induced in the first days post-TBI. We also used a battery of cognitive/affective and sensorimotor outcomes and performed quantitative stereology-based assessment of neuronal loss to characterize exercise-dependent neuroprotection post-TBI.

Methods

Animals

The described experiments were performed in accord with the Animal Welfare Act, Public Health Service Policy on Humane Care and Use of Laboratory Animals, the Guide for the Care and Use of Laboratory Animals, and all other applicable regulations, policies, and procedures and were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee (Baltimore, MD). Ten-week-old male C57BL/6NTac mice (20–25 g) were obtained from Taconic Farms Inc. (Hudson, NY) and were handled as previously described.15

Voluntary motor exercise

Animals were randomly assigned to exercise group \( (n = 23) \) on running wheel (Nalgene, Bend, OR) or sedative control \( (n = 28) \) in home cages for 4 weeks. Habituation was conducted for 3 days on running wheel before starting the exercise. Total revolutions during 4 weeks were recorded by the VitaView Data Acquisition System (Mini Mitter, Bend, OR) and converted to meters run based on the diameter of the running wheel. Mice were weighed before and after pre-exercise; mice without exercise were divided into Sham/No-Ex \( (n = 11) \) and controlled cortical impact (CCI)/No-Ex \( (n = 17) \) groups. Mice subjected to 4-week pre-exercise were randomly divided into Sham/Ex \( (n = 8) \) or CCI/Ex \( (n = 15) \) groups to ensure similar mean revolutions per day for each group. Animal weight was measured before exercise and weekly during the 4-week exercise period.

Controlled cortical impact injury

Twenty-four hours after completion of 4-week exercise, CCI was performed as previously described.15 Surgical anesthesia was induced and maintained with 3% and 2% isoflurane evaporated in a gas mixture containing 70% \( \text{N}_2\text{O} \) and 30% \( \text{O}_2 \) and administered through a nose mask. The injury device consists of a microprocessor-controlled pneumatic impactor, driven by compressed air, with a 3.5-mm diameter tip. A 10-mm midline incision was made over the skull, the skin and fascia were reflected, and a 4-mm craniotomy was made on the central aspect of the left parietal bone between the bregma and lambda. Moderate-level brain injury was induced by an impact velocity of 6 m/sec and a deformation depth of 2 mm, as previously described.15 Mortality was under 10%; no other complications were noted. After injury, the incision was closed using surgical staples, anesthesia was terminated, and the animal was placed onto a heated pad to maintain normal core temperature for 30–60 min postinjury. All animals were monitored carefully for at least 4 h after surgery and then daily. Sham animals underwent the same procedure, including craniotomy, as injured mice except for the impact. Our previous studies in both traumatic spinal cord and brain injuries have shown that sham animals demonstrate molecular changes, compared to naïve animals, but the magnitude and duration of these changes was smaller and shorter than in injured animals.16–18 Thus, sham injury represents an important control for anesthesia and surgical stress.

Motor function evaluation

Chronic motor function was evaluated using a beam walking task, a method that is particularly good at discriminating fine motor coordination differences. The mouse was placed on the beam and the number of foot-faults for the right hindlimb recorded over 50 steps. A basal level of performance was achieved after 3 days training preceding surgery with an acceptance level of less than 10 foot-faults per 50 steps. The test was performed at 0 (immediately before CCI), 1, 3, 7, 14, and 28 days postinjury.

Morris water maze and reversal Morris water maze tests

Spatial learning and memory was assessed using the acquisition phase of the standard Morris water maze (MWM), as previously described.15 A circular tank (100 cm in diameter) was filled with water (23 ± 2°C) that was made opaque with white Crayola nontoxic paint. The maze was surrounded by various extramaze cues on the wall of the room. A transparent platform (10 cm in diameter) was submerged 0.5 cm below the surface of the water. The MWM protocol included standard hidden platform training (acquisition; postinjury day [PID] 14 to PID 17) and standard probe test (PID 18). The swim path, latency to platform, time spent in each zone, and velocity were recorded by a computer-based Any-Maze automated video tracking system (Stoelting Co, Wood Dale, IL). Reference memory was assessed by a probe test on PID 18. The search strategies were analyzed on each of the four trials on day 4 of MWM.15

Tail-suspension test

The tail-suspension (TS) test assesses depression-like behavior in mice and is based on the observation that mice develop an immobile posture when placed in an inescapable hemodynamic stress of being hung by their tail.19,20 The TS was performed on PID 21, as described previously,21 with small modifications. Each mouse was suspended at a height of 50 cm using adhesive tape placed approximately 1 cm from the tip of its tail. Duration of immobility was recorded throughout the 5-min test period. The definition of immobility was passive hanging and complete motionlessness.

Open field test

The open field test was used to measure locomotor activity21 on PID 21. Mice were individually placed in a corner facing the wall of the open-field chamber (22.5 × 22.5 cm) and allowed to freely explore the chamber for 5 min. Distance travelled was recorded by Any-Maze software.

Novel object recognition test

Novel object recognition (NOR), conducted as previously reported,22,23 evaluated nonspatial hippocampal-mediated memory on PID 22 and 23. The apparatus consists of an open field (22.5 × 22.5 cm) with two adjacent located imaginary circular zones. Briefly, mice were habituated to the open field and were allowed to freely explore the area for 5 min (no data were collected). After 24 h, mice were placed into the chamber where two identical objects were placed near the left and right corners of the open field for training (sample phase) and allowed to freely explore until they spent a total of 20 sec exploring the objects (exploration recorded when the front paws or nose contacted the object). Mice...
were then removed and returned to their home cage. After 24 h, object recognition was tested by substituting a novel object for a familiar training object (novel object location counterbalanced across mice). Time spent with each object was recorded; because mice inherently prefer to explore novel objects, a preference for the novel object (more time than chance [10 sec] spent with the novel object) indicates intact memory for the familiar object.

**Histology.** A randomly selected set of animals from the groups that underwent behavior testing were euthanized and transcardially perfused with saline and 10% buffered formalin phosphate solution (containing 4% paraformaldehyde; Fisher Scientific, Pittsburgh, PA) on PID 28 to be used for all histological analysis, including lesion volume and stereology. Frozen brain sections (60-um) were cut and mounted onto glass slides. Microglia were immunostained with anti-Iba-1 (1:1000; Wako Chemicals, Tokyo, Japan) overnight and incubated with biotinylated anti-rabbit immunoglobulin G antibody (Vector Laboratories, Burlingame, CA) and then reacted with 3,3'-diaminobenzidine (Vector Laboratories) for color development.

**Lesion volume assessment**

Lesion volume was determined based on Cavalieri’s method after staining with cresyl violet, as previously described. The lesion area was outlined using the Stereologer 2000 program (MicroBrightField, Williston, VT) to obtain the final volume measurements.

**Assessment of neuronal cell loss in the hippocampal subregions, cortex, and thalamus**

Stereoinvestigator software (MBF Biosciences, Williston, VT) was used to count the total number of surviving neurons in the cortex, thalamus, as well as cornu ammonis (CA) 1, CA2/3, and dentate gyrus (DG) subregions of the hippocampus using the optical fractionator method of unbiased stereology, as previously described.

**Assessment of microglial morphology in the cortex**

Stereoinvestigator software (MBF Biosciences) was used to count the number of cortical microglia in each of the three microglial morphological phenotypes (namely, ramified, hypertrophic, and bushy) using the optical fractionator method of unbiased stereology, as previously described.

**RNA isolation**

Twenty-four hours after injury, animals were euthanized and a 5-mm area surrounding the lesion epicenter on the ipsilateral cortex was rapidly dissected and immediately frozen on dry ice. The cortical tissue extracted 24 h after TBI was divided for homogenization in radioimmunoprecipitation assay (RIPA) buffer or for subcellular fractionation. The RIPA lysate was analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. Immunoblots were probed with anti-α-Spectrin (1:300; Enzo Life Sciences, Ann Arbor, MI), anti-Bid (1:1000; R&D Systems, Minneapolis, MN), anti-PUMA (1:1000; ProSci Incorporated, Poway, CA), and anti-HSP70 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-apoptosis-inducing factor (AIF)-1 (1:1000; Santa Cruz Biotechnology), cytochrome c (1:1000; Santa Cruz Biotechnology), β-actin (1:20,000; Sigma-Aldrich, St. Louis, MO), and Histone H2A.X (1:20,000; Abcam, Cambridge, UK) were used as an endogenous control. Chemiluminescence was captured on a Kodak Image station 4000R station (Carestream Health, Rochester, NY), and protein bands were quantified by densitometric analysis using Carestream Molecular Imaging Software (Carestream Health). The data presented reflect the intensity of the target protein normalized to protein levels of the endogenous control in each sample (expressed in arbitrary units).

**Subcellular fractionation**

Subcellular fractionation was performed as previously described, with some modifications. 5-mm area surrounding the lesion epicenter on the ipsilateral cortex was rapidly dissected and homogenized in ice cold digitonin lysis buffer (20 mM of HEPES [pH 7.4], 80 mM of KCl, 1 mM of ethylenediaminetetraacetic acid, 1 mM of ethyleneglycol tetraacetic acid, 1 mM of dithiothreitol, 250 mM of sucrose, 200 μg/mL of digitonin, and protease inhibitor and phosphatase inhibitor cocktails [P8340; P5726; Sigma-Aldrich]) and

The Verso™ complementary DNA (cDNA) Kit (Thermo Fisher Scientific, Peabody, MA) was used to synthesize cDNA from purified total RNA. RNA (1 μg) was heated to 70°C for 5 min and mixed with 5× cDNA-synthesis buffer, dNTP mix (0.5-nM final concentration), and Verso Enzyme Mix, and, finally, random hexamers (400 ng/μL) were added. Tubes were incubated at 42°C for 30 min, followed by 95°C for 2 min. Quantitative real-time polymerase chain reaction (PCR) amplification was performed by using cDNA TaqMan® Universal Master Mix II (Applied Biosystems, Foster City, CA). In brief, reactions were performed in duplicate containing 2× TaqMan Universal Master Mix II, 1 μL of cDNA (corresponding to 50-ng RNA/reaction), and TaqMan Gene Expression Assay (Applied Biosystems), 20× in a final volume of 20 μL. TaqMan Gene Expression assays for the following mouse genes were performed: glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Mm01205641_m1); PUMA (Mm00457553_m1); Bid (Mm00442037_m1); heat shock protein (HSPA1a; Mm01194864_s1); HSPA1b (Mm03038954_s1); BDNF (Mm01334042_m1); CREB (Mm00501607_m1; Applied Biosystems). Reactions were amplified and quantified using a 7900HT Fast Real-Time PCR System and the corresponding software (Applied Biosystems). The PCR profile consisted of one cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. All reactions were performed twice. Efficiency of reactions for each set gene expression was close to 100%. Efficiency of reactions was measured using the threshold cycle (Ct) slope method. Briefly, serial dilutions of samples were generated and real-time reverse-transcriptase PCR reactions were performed on each dilution. Ct values were then plotted versus the log of the dilution and a linear regression was performed: Efficiency = (10−1/slope)−1×100%.

Samples were confirmed to be free of DNA contamination by performing reactions without reverse transcriptase. Gene expression was normalized to GAPDH, and the relative quantity of messenger RNAs (mRNAs) was calculated based on the comparative Ct method.
incubated for 10 min on ice. The lysate was centrifuged at 1000g for 5 min at 4°C to pellet the nuclei. The supernatant was transferred to a new tube and centrifuged again at 12,000g for 10 min at 4°C to pellet the mitochondria. The resulting supernatant, representing the cytosolic fraction, was recovered. Nuclear and mitochondrial lysates were prepared in RIPA buffer (Teknova, Hollister, CA) with protease and phosphatase inhibitor cocktails.

Statistical analysis

For the beam walk, acquisition trials of the MWM test repeated-measures one-way analyses of variance (ANOVA) was conducted, followed by Student-Newman-Keuls’ post-hoc test to compare the differences between each group. One-way ANOVA analysis, followed by Student-Newman-Keuls’ post-hoc test, was performed for the other behavioral tests and neuronal cell counts, microglia activation, real-time PCR, and Western blot. A two-tailed paired Student’s t-test was used for lesion volume. For the search strategy analysis, chi-square analysis was performed. Statistical analysis was performed using GraphPad Prism software (version 4.00 for Windows; GraphPad Software, Inc., San Diego, CA) or the SigmaPlot Program (Version 12; Systat Software, San Jose, CA). Data are expressed as mean ± standard error of the mean (SEM), and significance was determined at p < 0.05.

Results

Exercise wheel running revolutions and mice weight

Average wheel running revolutions were 5970 ± 1007 for the Sham/Ex group and 6322 ± 482.7 for the CCI/Ex group. Revolutions/day was also converted to meter/day; 4523 ± 763.3 for the Sham/No-Ex group and 6322 ± 365.8 for the CCI/No-Ex group. Average weight of the nonexercise group was 23.88 ± 0.16 at the start and 27.28 ± 0.28 after 4 weeks; average weight of the exercise group was 23.43 ± 0.14 at the start and 24.70 ± 0.31 after 4 weeks of exercise.

Pre-exercise improved cognitive function after traumatic brain injury

To investigate the effect of pre-exercise on spatial learning and memory after moderate-level CCI, we performed the MWM test from PID 14 to PID 18. Mean escape latency on the last day of training was 55.52 ± 4.63 sec for the Sham/No-Ex group, 50.23 ± 5.08 for the Sham/Ex group, 76.37 ± 3.31 for the CCI/No-Ex group, and 66.19 ± 5.33 for the CCI/Ex group (Fig. 1A). Repeated-measures one-way ANOVA showed a significant effect of treatment (F(3,184) = 20.33; p < 0.0001) and day (F(3,184) = 9.76; p < 0.0001). Student-Newman-Keuls’ post-hoc analysis revealed an improvement after pre-exercise with a significant difference between the CCI/No-Ex and CCI/Ex groups on day 4 (p < 0.05) and Sham/No-Ex and CCI/No-Ex groups on days 2 (p < 0.001), 3 (p < 0.05), and 4 (p < 0.001). Swimming speed was measured to rule out the confound of injury-induced impairment on locomotor activity. Repeated-measures one-way ANOVA showed a significant effect of treatment (F(3,184) = 58.50; p < 0.0001), but no significant effect of treatment (F(3,184) = 2.31; p > 0.05) was detected. Student-Newman-Keuls’ post-hoc analysis revealed no significant change of speed among these groups for each day (Fig. 1B). Probe tests were performed on PID 18, and reduced time spent in the target quadrant indicated impaired reference memory. The time spent in the target quadrant showed a significant effect of pre-exercise (Fig. 1C; F(3,49) = 5.08; p < 0.01) by one-way ANOVA, and Student-Newman-Keuls’ post-hoc analysis revealed that the CCI/Ex group spent significantly more time in the target quadrant than the CCI/No-Ex group (p < 0.05). Significant differences were also observed between the Sham/No-Ex and CCI/No-Ex groups (p < 0.01). We then performed search strategy analysis to evaluate the efficiency of locating the platform. Based on previously published criteria,15 three search strategies were evaluated for each of the four trials on PID 17. Occasionally, mice changed search strategies during a trial. When this happened, the strategy that best described the major swimming path was assigned. Search strategy analysis showed statistically significant separation between the CCI/Ex and CCI/No-Ex groups (Fig. 1D; χ² = 419.2; p < 0.001), with the CCI/No-Ex group exhibiting significantly higher reliance on looping search strategies than spatial and systematic search strategies, when compared to the CCI/Ex group.

Hippocampal-mediated nonspatial learning and memory post-TBI was assessed by the novel object recognition test. Sham/No-Ex, Sham/Ex, CCI/No-Ex, and CCI/Ex groups all spent similar times with two identical objects in the sample phase, as shown in Figure 1E. The Sham/No-Ex and Sham/Ex group spent more time than chance (10 sec) with the novel object 24 h after training (choice phase), indicating intact memory. The CCI/No-Ex group spent similar time with the novel and familiar objects, whereas the CCI/Ex group spent more time with the novel than familiar objects (Fig. 1F). Further, one-way ANOVA analysis showed a significant effect of pre-exercise with the novel object (F(3,49) = 4.76; p < 0.05), and Student-Newman-Keuls’ post-hoc analysis revealed that the CCI/Ex group spent significantly more time with the novel object, when compared to the CCI/No-Ex group (p < 0.05). Significant differences were also observed between the Sham/No-Ex and CCI/No-Ex groups (p < 0.05).

Pre-exercise improved motor function, attenuated depressive-like behavior

Mice were tested on the beam walk immediately before sham surgery or TBI and again on PID 1, 3, 7, 14, 21, and 28. Repeated-measures one-way ANOVA showed a significant effect of treatment (Fig. 2A; F(3,321) = 642.6; p < 0.0001) and day (F(6,321) = 74.9; p < 0.0001), and Student-Newman-Keuls’ post-hoc analysis demonstrated significant differences between the Sham/No-Ex and CCI/No-Ex groups on days 1 (p < 0.001), 3 (p < 0.001), 7 (p < 0.001), 14 (p < 0.001), 21 (p < 0.001), and 28 (p < 0.001). Notably, exercises improved motor function after TBI with a significant difference between the CCI/Ex and CCI/No-Ex groups on days 3 (p < 0.05), 7 (p < 0.01), 14 (p < 0.001), 21 (p < 0.001), and 28 (p < 0.01).

To determine the long-term effects on depressive-like behavior after exercise, immobility time was tested in a tail suspension test on PID 21. Increased immobility time in a 5-min test is a measure of the degree of hopelessness and despair. One-way ANOVA analysis showed a significant effect of treatment in the tail suspension test (Fig. 2B; F(3,49) = 15.96; p < 0.001), and Student-Newman-Keuls’ post-hoc analysis demonstrated significantly increased immobility times in the CCI/No-Ex group (p < 0.001), when compared to the Sham/No-Ex group. We observed an improvement following pre-exercise, and there was a significant difference between the CCI/Ex and CCI/No-Ex groups (p < 0.001).

Pre-exercise reduced lesion volume and neuronal loss in the hippocampus, cortex, and thalamus after traumatic brain injury

TBI-induced lesion volume was quantified in cresyl violet-stained coronal brain sections from the CCI/No-Ex and CCI/Ex...
groups at 28 days postinjury by stereological methods. Pre-exercise significantly reduced TBI induced lesion volumes, compared to the sedative group (Fig. 3A; $t(11)=3.39; p<0.01$). Pre-exercise attenuated spatial learning and memory deficit caused by traumatic brain injury, which was demonstrated by a significant decreased latency at day 4 ($p<0.05$). (B) Swimming speed. In order to ensure that latency was not influenced by the locomotor function, swimming speed was measured. No significant difference of swimming speed was found among these four groups in each training day ($p>0.05$). (C) Probe test. Mice in the CCI/Ex group spent significantly more time in the target quadrant than the CCI/No-Ex group ($p<0.05$). Significant differences were also observed between Sham/No-Ex and CCI/No-Ex groups ($**p<0.01$). (D) Search strategy was examined on each of the four trials on acquisition day 4. Search strategy in the MWM showed good separation between Sham/No-Ex, CCI/No-Ex, and CCI/Ex groups ($\chi^2=419.2; p<0.001$). (E and F) Novel Object recognition test. All four groups spent equal time with the two identical objects during the sample phase on postinjury day 22 (dashed line at 10 sec). Twenty-four hours after the sample phase, the time spent with the novel and familiar objects during the choice phase was recorded. Mice subjected to pre-exercise (CCI/Ex) spent significantly more time with the novel object, when compared to the CCI/No-Ex group ($p<0.05$). Significant differences were also observed between Sham/No-Ex and CCI/Ex groups ($**p<0.01$). Analysis by repeated-measures one-way analysis of variance (ANOVA) in (A) and (B), by one-way ANOVA in (C), (E), and (F), followed by Student-Newman-Keuls’ post-hoc test: mean±standard error of the mean. (D) was analyzed by chi-square analysis; $n=8–17$. CCI, controlled cortical impact.
Pre-exercise enhances neuronal synaptic plasticity

To explore the mechanism underlying pre-exercise induced cognitive, affective, and motor functions, expression of genes involved in regulation of synaptic plasticity and apoptosis was analyzed in the ipsilateral cortex 24 h after TBI. We observed significant increase in expression of neurotrophic factors, such as BDNF and CREB (Fig. 5: $F_{(3,24)} = 55.26; p < 0.001$ and $F_{(3,24)} = 73.79; p < 0.001$, respectively) in the Sham/Ex group, when compared to Sham/No-Ex groups. Further, pre-exercise (CCI/Ex) significantly increased BDNF and CREB gene expression, when compared to the CCI/No-Ex group ($p < 0.001$ and $p < 0.001$, respectively).

Pre-exercise down-regulated expression of BID and PUMA and attenuated translocation of apoptosis-inducing factor and cytochrome c from mitochondria to cytoplasm

Quantitative PCR analysis demonstrated that expression of proapoptotic members of the B-cell lymphoma 2 (Bcl-2) family, such as Bid and Puma, were significantly increased in the CCI/No-Ex group, when compared to the Sham/No-Ex group (Fig. 6A; $F_{(3,22)} = 19.68; p < 0.001$ and $F_{(3,22)} = 81.30; p < 0.001$, respectively). Pre-exercise significantly reversed activation of Bid and Puma (Fig. 6B,D; $p < 0.05$ and $p < 0.01$, respectively), when compared to the CCI/No-Ex group.

Levels of Bid and PUMA in the ipsilateral cortex obtained 24 h after TBI with or without exercise were analyzed by Western blotting. Bid and PUMA proteins were significantly increased in the CCI/No-Ex group, when compared to the Sham/No-Ex group (Fig. 6B,D; $F_{(3,19)} = 106.8; p < 0.001$ and $F_{(3,19)} = 156.9; p < 0.001$, respectively). Pre-exercise significantly down-regulated the level of Bid and PUMA (Fig. 6B,D; $F_{(3,19)} = 106.8; p < 0.001$ and

Pre-exercise attenuated microglial activation after traumatic brain injury

TBI results in microglial activation, as demonstrated by the transition of the microglial phenotypes from a ramified (resting form) cellular morphology predominant in noninjured brain, to increasingly more activated phenotypes showing hypertrophic (moderate activation) or bushy (full activation) cellular morphologies. 26 Stereological assessment of microglial cell number and activation phenotype was performed in the ipsilateral cortex at 28 days post-TBI. We observed a significant decrease in ramified microglia in the CCI/No-Ex group (Fig. 4A; $F_{(3,24)} = 5.63; p < 0.05$) and increase in hypertrophic (Fig. 4B; $F_{(3,24)} = 33.47; p < 0.001$) and bushy microglia (Fig. 4C; $F_{(3,24)} = 28.74; p < 0.001$), as compared to the Sham/No-Ex group. Pre-exercise significantly reduced hypertrophic (moderated activated; Fig. 4B) and bushy (highly activated) microglia (Fig. 4C; $p < 0.05$), when compared to the CCI/No-Ex group. Though there was tendency, pre-exercise did not significantly increase the density of ramified microglia (Fig. 4A).

FIG. 2. Pre-exercise improves motor function in beam walk test and reverses depressive-like behavior in the tail-suspension (TS) test. (A) All animals had less than 10 foot faults before CCI. Significant differences were observed between the CCI/No-Ex and CCI/Ex groups on postinjury day (PID) 3 ($p < 0.05$), PID 7 ($p < 0.001$), PID 14 ($p < 0.001$), PID 21 ($p < 0.001$), and PID 28 ($p < 0.01$). Significant differences were also observed between the Sham/No-Ex and CCI/No-Ex groups from PID 1 to PID 28 ($p < 0.001$). (B) The TS test was performed on PID 21. Significantly increased immobility times were observed in the CCI/No-Ex group ($p < 0.001$), when compared to the Sham/No-Ex group. Immobility time was significantly reduced in the CCI/Ex group, as compared to the CCI/No-Ex group ($p < 0.001$). Analysis by repeated-measures one-way analysis of variance (ANOVA) in (A), by one-way ANOVA in (B) followed by Student-Newman-Keuls’ post-hoc test; mean ± standard error of the mean, $n = 8–17$. CCI, controlled cortical impact.

FIG. 3. Pre-exercise reduces lesion size and attenuates neuronal cell loss in the CA1, CA2/3, and DG subregions of hippocampus, cortex, and thalamus. Lesion volume was quantified using Cavalieri’s method. Unbiased stereological assessment of lesion volume at 28 days after traumatic brain injury was performed on cresyl violet–stained brain section. (A) Representative images from each group are shown. (B) Significant reduction of lesion volume was observed in the CCI/Ex group ($p < 0.01$), when compared to the CCI/No-Ex group. Stereological assessment of neuronal cell on postinjury day 28 was performed on cresyl violet–stained sections in the CA1, CA2/3, and DG subregions of the hippocampus, cortex, and thalamus. (C, D, E, F, and G) Significant differences of neuronal density were observed between Sham/No-Ex and CCI/No-Ex groups in the CA1 ($p < 0.01$), CA2/3 ($p < 0.01$), DG ($p < 0.01$) subregions of the hippocampus as well as cortex ($p < 0.005$) and thalamus ($p < 0.001$). Pre-exercise (CCI/Ex) significantly increased neuronal density in the CA1 ($p < 0.05$), CA2/3 ($p < 0.05$), DG ($p < 0.001$), cortex ($p < 0.05$), and thalamus ($p < 0.001$), compared to the CCI/No-Ex group. Analysis by two-tailed paired Student’s $t$-test in (B) and one-way analysis of variance in (C), (D), (E), (F), and (G) followed by Student-Newman-Keuls’ test; mean ± standard error of the mean, $n = 6–8$. CCI, controlled cortical impact; CA, cornu ammonis; DG, dentate gyrus.
Translocation of AIF and cytochrome c from mitochondria to cytoplasm is another well-known marker of apoptotic cell death. Analysis of cytosolic fractions revealed that CCI dramatically increased release of AIF and cytochrome c into the cytosol (Fig. 6C,E; \(F(3,19) = 91.98; p < 0.001\) and \(F(3,19) = 113.4; p < 0.001\), respectively). In contrast, pre-exercise significantly reduced release of AIF and cytochrome c into the cytosol (Fig. 6C,E; \(p < 0.001\) and \(p < 0.001\), respectively).

Pre-exercise up-regulated expression of heat shock protein, attenuated apoptosis-inducing factor translocation to the nucleus and cleavage of \(\alpha\)-Spectrin after traumatic brain injury

We measured expression levels of two genes (Hspa1a and Hspa1b) that encode HSP70, a highly conserved molecular chaperone, which promotes cell survival under various pathological conditions. The coding regions of Hspa1a and Hspa1b are similar, but the promoter and 3' untranslated region sequences differ.30 The CCI/No-Ex group showed significantly increased Hspa1a (Fig. 7A; \(F_{(3,23)} = 32.51; p < 0.01\)) and Hspa1b (\(F_{(3,23)} = 36.83; p < 0.001\)) gene expression, when compared to the Sham/No-Ex group at 24 h after CCI. Notably, pre-exercise further increased HSPA1a (Fig. 7A; \(p < 0.001\)) and HSPA1b (\(p < 0.001\)) gene expression, when compared to the CCI/No-Ex group. HSP70 protein level was also further significantly increased in the CCI/Ex group, when compared to the CCI/No-Ex group (Fig. 7B,E; \(F_{(3,19)} = 35.55; p < 0.001\)).

Western blot analysis of nuclear fractions revealed that CCI dramatically increased translocation of AIF to the nucleus. However, pre-exercise attenuated AIF translocation to the nucleus, compared to the CCI/No-Ex group (Fig. 7C; \(F_{(3,19)} = 32.94; p < 0.001\)).

Cleavage of \(\alpha\)-Spectrin is a well-established marker of cell death; spectrin is a high-molecular-weight (280-kDa) cytoskeletal protein that undergoes degradation catalyzed by activated caspases and/or other proteases during apoptosis, generating C-terminal 120-kDa and N-terminal 145/150-kDa end products.31–33 Quantitative Western blot analysis demonstrated that CCI caused induction of \(\alpha\)-Spectrin cleavage and increased the amount of 150/145- and 120-kDa products of \(\alpha\)-Spectrin cleavage (Fig. 7D,G;...
FIG. 6. Pre-exercise down-regulated expression of Bid and PUMA and attenuated translocation of apoptosis-inducing factor (AIF) and cytochrome c from mitochondria to cytoplasm. (A) Quantitative polymerase chain reaction quantification of expression levels of Bid and PUMA. Ipsilateral cortex was collected 24 h after traumatic brain injury and subjected to Western blotting. (B) Whole-tissue lysates were fractioned on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with antibodies against Bid, PUMA, and β-actin. (C) Cytosolic fractions were fractioned on SDS-PAGE and immunoblotted with antibodies against AIF-1, cytochrome c, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (D) Levels of Bid and PUMA proteins in whole-tissue lysates were quantified as fold change to the Sham/No-Ex group after measurement of band intensity by densitometry and normalization to levels of β-actin. (E) Levels of AIF-1 and cytochrome c in cytosolic fraction c proteins were quantified as fold change to the Sham/No-Ex group after measurement of band intensity by densitometry and normalization to levels of GAPDH. Data are mean ± standard deviation. Analysis by one-way analysis of variance followed by Student-Newman-Keuls’ test; mean ± standard error of the mean, n = 4–6; ***p < 0.001 versus Sham/No-Ex group; #p < 0.05, ##p < 0.001, and ###p < 0.001 versus CCI/No-Ex group. CCI, controlled cortical impact.
Exercise selectively modulates key components of the neuroprotective and cell death pathways in sham and/or injured animals

Table 1 presents an overview of mRNA and/or protein expression levels for select neuroprotective and proapoptotic genes as measured 24 h after CCI. The data quantification is presented as fold change to the Sham/No-Ex group, except p53 protein—owing to undetectable level in shams, fold change was referenced to CCI/No-Ex (mean±SEM; n = 5–8) analysis by one-way ANOVA, followed by Student-Newman-Keuls’ test. Only the neuroprotective molecules, BDNF and CREB (mRNA), were significantly changed (increased) by exercise in noninjured animals. TBI alone had no significant effect, whereas TBI with exercise was similar to exercise alone. Expressions of HSP70 were significantly induced (mRNA levels) by injury alone, but was further elevated (mRNA and protein) in injured animals after exercise. The changes in HSP70 induced by exercise alone did not reach significance. The proapoptotic molecules, Bid and Puma, were induced (mRNAs and proteins) by injury, and these changes were attenuated by exercise. Other proapoptotic molecules, including p53 (mRNA and protein), BCL2-antagonist/killer 1 (Bak1), Noxa, and Bim (mRNAs), were also induced by injury (p < 0.001 for p53, Bak1, Noxa, and Bim), but these changes were not reduced by exercise. The neuroprotective molecule, protein kinase B (Akt), was significantly reduced (protein) by injury (p < 0.001), and these changes were not attenuated by exercise. The changes induced by exercise alone in Bid, Puma, p53, Bak1, Noxa, Bim, or Akt did not reach significance. No significant changes (protein) were detected in the proapoptotic protein, apoptotic protease-activating factor 1 (Apaf-1), in response to any intervention.

Discussion

After mouse CCI, voluntary exercise preconditioning improved post-traumatic sensorimotor performance and enhanced recovery of cognitive/affective functions. The neuroprotective effects of exercise were associated with significant attenuation of lesion volume, reduction of neuronal loss, and decreased neuroinflammation. Four weeks of voluntary exercise significantly increased post-traumatic expression of neurotrophic factors, BDNF and CREB, as well as HSP70, a molecule with robust, pleiotropic neuroprotective potential. Further, exercise reduced trauma-induced expression of the proapoptotic Bcl-2-homology (BH)-3-only proteins, Puma and Bid, and markedly attenuated release of proapoptotic molecules from the mitochondria (cytochrome c, AIF), as well as translocation of AIF to the nucleus and cleavage of caspase substrates.

We examined the effects of exercise preconditioning (pre-exercise) on TBI using a number of different functional outcomes in order to evaluate cognitive, affective, and motor functions. This approach extends previous observations that relied on limited behavioral outcomes that assessed cognitive function. Further, exercise reduced trauma-induced expression of the neurotrophic factors, BDNF and CREB, as well as the motor proteins, heat shock protein; CCI, controlled cortical impact AIF, apoptosis-inducing factor;.
exercise, and its inhibition attenuates the voluntary exercise-dependent cognitive enhancements post-trauma.\textsuperscript{7} Conversely, the lack of activation of BDNF, synapsin I, and/or CREB by acute exercise may explain the relative lack of improvements of neuro-pathological and neurological impairments in this model.\textsuperscript{7,9} Forced exercise, which increases stress and inhibits BDNF signaling, can be detrimental during the acute postinjury period,\textsuperscript{12} although some exercise may explain the relative lack of improvements of neuroplasticity, pre-exercise may also exert neuroprotective effects by impacting other systems, including neurovasculature/perfusion, synaptic density, as well as the brain biomechanical properties, which should be the focus of future investigations. Experimental and clinical data indicate that environmental factors, pre-exercise may also protect neurons from oxidative damage by inducing expression of the DNA repair enzyme, apurinic/apyrimidinic endonuclease 1.\textsuperscript{17}

Previous studies have documented the antiapoptotic effects of exercise.\textsuperscript{8,42,54} Mouse CCI, as well as induction of neuronal death in vitro, sequentially induce p53 activation and up-regulate proapoptotic Bcl-2 family members; the latter include Bax and Bak as well as BH3-only Puma, Noxa, Bim, and Bid, which contribute to mitochondria permeabilization and downstream apoptosis execution cascades.\textsuperscript{55} Our data show that voluntary exercise significantly reduced induction of Bid and Puma after mouse CCI. In contrast to the pattern observed for BDNF/CREB, pre-exercise did not modulate basal expression of Puma/Bid, but attenuated their trauma-induced up-regulation. Our studies are the first to document exercise-dependent attenuation of release of cytochrome c and AIF from mitochondria post-TBI. Importantly, exercise differentially affected various cell death pathways—with no effects on basal levels or trauma-induced up-regulation of p53, Noxa, Bim, or Bak. Moreover, our data suggest that exercise modulates the pathways responsible for injury-dependent Puma and Bid changes, rather than the mechanisms controlling their basal level.

The 70-kDa heat shock proteins (HSP70s) are stress-induced molecules that are expressed in response to various CNS injuries, including stroke, trauma, or chronic neurodegenerative disorders, and appear to have neuroprotective actions.\textsuperscript{56} Increased activity may result in changes in cerebral homeostasis, including modification in temperature, pH, ions, and/or decline in bioenergetics status, which could induce mild cellular stress and lead to exercise-mediated up-regulation of HSP70 in the brain.\textsuperscript{57} In turn, HSP70 elevation may contribute to exercise-mediated neuroprotection.\textsuperscript{58-60}

We have recently demonstrated strong neuroprotective effects of HSP70 up-regulation in vitro as well as after experimental TBI; these actions may reflect HSP70-mediated inhibition of multiple apoptotic mechanisms downstream of the mitochondria—including both the Apaf-1/cytochrome c caspase activation complex (apoptosome) and the caspase-independent pathway that involves AIF translocation to the nucleus.\textsuperscript{55,44} Brain injury induces HSP70 expression,\textsuperscript{44} which may serve as an endogenous neuroprotective mechanism post-TBI.\textsuperscript{74} Here, we show that exercise significantly amplifies HSP70 up-regulation post-TBI, which may contribute to its neuroprotective action, by inhibiting both caspase-dependent and -independent apoptotic pathways. This is supported by our observations that pre-Ex reduces both AIF translocation to the nucleus and cleavage of spectrin, a well-known caspase/calpain substrate.\textsuperscript{31-33}

Exercise preconditioning (forced exercise-treadmill) has been shown to attenuate trauma-induced neuroinflammation and reduce motor impairments in a rat fluid percussion model.\textsuperscript{61} We have previously demonstrated that late voluntary exercise reduces neuroinflammation and cognitive dysfunction after mouse CCI.\textsuperscript{9} It has been hypothesized that sustained microglial activation after CNS trauma may play a role in neuronal cell loss after release of neurotoxic molecules, such as nitrous oxide NO and increased OS.\textsuperscript{14,25,62,63} Our present data demonstrate that exercise preconditioning significantly attenuates trauma-induced activation of neurotoxic microglial phenotypes. The extent to which these findings reflect a direct anti-inflammatory effect or a secondary response owing to reduction in early neuronal cell death remains to be addressed.

In addition to modulation of neuroinflammation, cell death, and neuroplasticity, pre-exercise may also exert neuroprotective effects by impacting other systems, including neurovascular/perfusion, synaptic density, as well as the brain biomechanical properties, which should be the focus of future investigations.

### Table 1. Overview of mRNA and/or Protein Expression Levels for Select Neuroprotective and Proapoptotic Genes as Measured 24 h Post-CCI

<table>
<thead>
<tr>
<th>mRNA, messenger RNA</th>
<th>BDNF</th>
<th>CREB</th>
<th>Bid</th>
<th>Puma</th>
<th>Hspa 1a</th>
<th>Hspa 1b</th>
<th>p53</th>
<th>Bak 1</th>
<th>Noxa</th>
<th>Bim</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham/No-Ex</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Sham/Ex</td>
<td>2.10</td>
<td>2.40</td>
<td>1.12</td>
<td>1.16</td>
<td>1.00</td>
<td>1.00</td>
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<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>CCI/No-Ex</td>
<td>1.17</td>
<td>1.24</td>
<td>1.74</td>
<td>2.88</td>
<td>1.23</td>
<td>1.93</td>
<td>1.93</td>
<td>1.90</td>
<td>2.20</td>
<td>2.12</td>
<td>2.13</td>
</tr>
<tr>
<td>CCI/Ex</td>
<td>2.10</td>
<td>2.60</td>
<td>2.06</td>
<td>2.15</td>
<td>3.66</td>
<td>3.01</td>
<td>3.46</td>
<td>2.06</td>
<td>2.64</td>
<td>2.23</td>
<td>0.57</td>
</tr>
</tbody>
</table>

In addition to modulation of neuroinflammation, cell death, and neuroplasticity, pre-exercise may also induce the expression of a number of proapoptotic genes, such as Bim and Bak, which contribute to mitochondria permeabilization and downstream apoptosis execution cascades.\textsuperscript{55} Our data show that voluntary exercise significantly reduced induction of Bi and Puma after mouse CCI. In contrast to the pattern observed for BDNF/CREB, pre-exercise did not modulate basal expression of Puma/Bid, but attenuated their trauma-induced up-regulation. Our studies are the first to document exercise-dependent attenuation of release of cytochrome c and AIF from mitochondria post-TBI. Importantly, exercise differentially affected various cell death pathways—with no effects on basal levels or trauma-induced up-regulation of p53, Noxa, Bim, or Bak. Moreover, our data suggest that exercise modulates the pathways responsible for injury-dependent Puma and Bid changes, rather than the mechanisms controlling their basal level.
including cognitive/intellectual enrichment, may have neuroprotective effects.38,64

The present study suggests that pre-exercise may provide neuroprotection in TBI, particularly in segments of the general population that do not regularly perform physical activity. Whereas the present data apply to young adult mice, future studies should examine the effects of exercise in aged mice that may better translate to clinical TBI in the elderly, a vulnerable group at high risk from brain trauma.65

In conclusion, the present study demonstrates that exercise preconditioning strongly protects the brain from TBI and results in activation of antiapoptotic and anti-inflammatory pathways. Exercise-induced up-regulation of BDNF and HSP70 serve to inhibit multiple pathways of neuronal apoptosis, both upstream and downstream of the mitochondria. Although the preconditioning exercise model has been used here to identify its potential neuroprotective mechanisms, physical exercise may serve to limit the consequences of TBI in high-risk populations.

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Author Disclosure Statement

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References


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