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Exercise Preconditioning Improves Traumatic Brain Injury Outcomes

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Abstract

Purpose—To determine whether 6 weeks of exercise performed prior to traumatic brain injury (TBI) could improve post-TBI behavioral outcomes in mice, and if exercise increases neuroprotective molecules (vascular endothelial growth factor-A [VEGF-A], erythropoietin [EPO], and heme oxygenase-1 [HO-1]) in brain regions responsible for movement (sensorimotor cortex) and memory (hippocampus).

Methods—120 mice were randomly assigned to one of four groups: 1) no exercise + no TBI (NOEX-NOTBI [n=30]), 2) no exercise + TBI (NOEX-TBI [n=30]), 3) exercise + no TBI (EX-NOTBI [n=30]), and 4) exercise + TBI (EX-TBI [n=30]). The gridwalk task and radial arm water maze were used to evaluate sensorimotor and cognitive function, respectively. Quantitative real time polymerase chain reaction and immunostaining were performed to investigate VEGF-A, EPO, and HO-1 mRNA and protein expression in the right cerebral cortex and ipsilateral hippocampus.

Results—EX-TBI mice displayed reduced post-TBI sensorimotor and cognitive deficits when compared to NOEX-TBI mice. EX-NOTBI and EX-TBI mice showed elevated VEGF-A and EPO mRNA in the cortex and hippocampus, and increased VEGF-A and EPO staining of sensorimotor cortex neurons 1 day post-TBI and/or post-exercise. EX-TBI mice also exhibited increased VEGF-A staining of hippocampal neurons 1 day post-TBI/post-exercise. NOEX-TBI mice demonstrated increased HO-1 mRNA in the cortex (3 days post-TBI) and hippocampus (3 and 7 days post-TBI), but HO-1 was not increased in mice that exercised.

Conclusions—Improved TBI outcomes following exercise preconditioning are associated with increased expression of specific neuroprotective genes and proteins (VEGF-A and EPO, but not HO-1) in the brain.

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Competing Interests:

The authors declare that they have no competing interests.

Keywords

Exercise; Traumatic Brain Injury; Sensorimotor Function; Cognitive Function; Vascular Endothelial Growth Factor; Erythropoietin

1. Introduction

A traumatic brain injury (TBI) results when external mechanical forces (e.g., direct impact to the head, rapid acceleration/deceleration of the head, blast waves, or penetration by a projectile) cause temporary or permanent brain damage and dysfunction. Typically, TBI is divided into primary and secondary injury responses. Primary injury responses are defined by the immediate mechanical damage occurring at the moment of impact and include; brain contusion, laceration, hemorrhage, axon transection, and diffuse axonal injury (Maas et al., 2008). Secondary injury responses including glutamate excitotoxicity, disturbed ionic gradients, metabolic disruption, mitochondrial dysfunction, reactive oxygen species formation, neuroinflammation, and hypoxic-ischemic damage, progress over hours, days, and months following TBI (Maas et al., 2008). These secondary injury responses exacerbate primary injury damage, and are the main cause of death in hospitalized TBI patients (Ghajar, 2000).

TBI induces the transcription of neuroprotective genes (e.g., vascular endothelial growth factor-A [VEGF-A], erythropoietin [EPO], and heme oxygenase-1 [HO-1]) within the hypoxia-inducible factor-1 alpha (HIF-1a) pathway that reduce cellular damage resulting from post-TBI secondary injury responses (Anderson et al., 2009). VEGF-A is a homodimeric glycoprotein that acts as an angiogenic growth factor, and promotes neurogenesis (Anderson et al., 2009; Fabel et al., 2003). VEGF-A is produced and released from neurons and many other cell types during hypoxic cellular conditions (Kovacs et al., 1996), and the production of VEGF-A increases in the brain after stroke (Ma et al., 2013) and TBI (Anderson et al., 2009). Inhibiting the endogenous VEGF-A injury response prevents brain repair by impeding revascularization and astroglial proliferation (Krum and Khaibullina, 2003). In addition, a significantly larger cerebral cortex lesion volume is observed, and increased neuronal and glial cell death result when VEGF-A receptors (VEGFR-2) are blocked prior to, and after TBI (Skold et al., 2006).

EPO is a glycoprotein required for erythropoiesis, but it also has anti-apoptotic, anti-inflammatory, antioxidative, angiogenic, and neurogenic properties (Ponce et al., 2013). The main site of EPO production is the kidneys (Ponce et al., 2013). However, EPO and its receptor are also expressed in neurons, astrocytes, oligodendrocytes, and microglial cells in the brain (Ponce et al., 2013). EPO is neuroprotective in animal models of TBI (Brines et al., 2000), spinal cord injury (Celik et al., 2002), and stroke (Gonzalez et al., 2007). EPO is known to elicit anti-apoptotic actions through inhibition of caspase activity, up-regulation of Bcl-2 family proteins, and by decreasing glutamate excitotoxicity (Anderson et al., 2009). Inhibiting the endogenous EPO response has been shown to worsen neuronal injury resulting from hypoxia and ischemia (Sakanaka et al., 1998).

HO-1 is a neuroprotective enzyme that exhibits anti-oxidative and anti-inflammatory effects (Aztatzi-Santillan et al., 2010; Bae et al., 2010). HO-1 mRNA and protein expression increase in the brain of mice after TBI (Anderson et al., 2009). HO-1 induction occurs in microglia and astrocytes following brain hemorrhage, and increased expression of HO-1 in microglia and astrocytes may improve cellular resistance to oxidative stress and secondary injury after TBI (Fukuda et al., 1995). HO-1 plays a neuroprotective role in other pathophysiological conditions including; 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridinium (MPP+) neurotoxicity (Bae et al., 2010), and stroke (Shah et al., 2010). Disrupting endogenous HO-1 responses has been associated with poor outcomes in many central nervous system disorders (Aztatzi-Santillan et al., 2010).

Exercise alters VEGF-A, EPO, and HO-1 expression in both non-neural and neural tissues. Several studies demonstrate that exercise increases VEGF-A protein production in the skeletal muscles and brain (Ma et al., 2013; Olenich et al., 2013; Viboolvorakul and Patumraj, 2014). Exercise increases EPO gene expression in human skeletal muscle (Ameln et al., 2005), and HO-1 protein expression in human lymphocytes (Thompson et al., 2005), and rat skeletal muscle (Hildebrandt et al., 2003). However, it is not known whether or when EPO and HO-1 increase in the brain after exercise training. Furthermore, no research has investigated whether exercise modulates post-TBI VEGF-A, EPO, and HO-1 responses in the brain.

Exercise is useful in neurorehabilitation after TBI. However, premature initiation of an exercise program after TBI can exacerbate symptoms and worsen outcome (Griesbach, 2011). Thus, we chose to examine the potential prophylactic effects of exercise preconditioning (i.e., voluntary wheel running performed prior to induction of a moderate, focal TBI). The purpose of this study was to determine whether pre-TBI exercise could improve post-TBI sensorimotor and cognitive function in adult mice, and to examine endogenous VEGF-A, EPO, and HO-1 gene and protein responses in the brain after exercise and TBI. We hypothesized that pre-TBI exercise would reduce sensorimotor and spatial learning memory deficits after injury, while simultaneously increasing VEGF-A, EPO, and HO-1 mRNA expression and protein (as indicated by an increase in the average number of positive stained neurons per mm²) in brain regions responsible for movement (i.e., sensorimotor cortex) and memory (i.e., hippocampus).

2. Results

2.1. Exercise

Exercise group mice (i.e., EX-NOTBI and EX-TBI) displayed consistent circadian rhythms during each week of the six week voluntary wheel running protocol (Figure 1A). Mice ran throughout the night, and rarely entered the running wheel during the day. The mice ran on average $4.413 \pm .088$ Km daily during the six week protocol (Figure 1B). Statistical analysis revealed significantly (** $p < .01$) less average daily running distance in week 1 when compared to all other weeks. However, the amount of daily exercise remained steady between weeks 2-6. Physical activity patterns of no exercise group mice (i.e., NOEX-NOTBI and NOEX-TBI) were observed on several occasions throughout the night and day.

The no exercise group mice appeared to be sleeping during the day, and displayed minimal activity (e.g., grooming, eating, drinking) at night.

2.2. Sensorimotor and Cognitive Function

Sensorimotor deficits (i.e., foot faults) were evaluated using the gridwalk task prior to TBI, and at 1, 3, and 7 days post-TBI (Figure 2A). After injury, both NOEX-TBI and EX-TBI mice exhibited significant ($p < .01$, not labeled on graph) increases in the average number of foot faults per minute walking at all post-TBI time points when both groups were compared to the pre-TBI time point. However, 6 weeks of pre-TBI voluntary wheel running exercise decreased post-TBI sensorimotor deficits. EX-TBI mice showed significant ($**p < .01$) reductions in the average number of foot faults per minute walking when compared to NOEX-TBI mice at 1, 3, and 7 days post-TBI.

Spatial learning memory deficits (i.e., average number of non-goal arm entry errors) were assessed during retention testing using the radial arm water maze (RAWM) prior to TBI, and at 7 days post-TBI (Figure 2B). TBI significantly ($*p < .05$) increased the average number of RAWM retention test non-goal arm entry errors in both NOEX-TBI and EX-TBI mice at 7 days post-TBI. However, 6 weeks of pre-TBI exercise training enhanced spatial learning memory prior to, and after TBI. EX-TBI mice demonstrated significant ($*p < .05$) reductions in the average number of RAWM retention test non-goal arm entry errors when compared to NOEX-TBI mice at both the pre-TBI and 7 day post-TBI time points.

2.3. VEGF-A Gene Expression

VEGF-A mRNA expression increased within the right cerebral cortex (Figure 3A) and ipsilateral hippocampus (Figure 3B) in response to both TBI and exercise. Statistically significant ($p < .05$) differences existed between groups for VEGF-A mRNA expression (i.e., fold change relative to control) in both the right cerebral cortex and right hippocampus. Post hoc testing for multiple comparisons revealed several interesting findings between groups. As a result of TBI alone, VEGF-A mRNA expression was significantly ($**p < .01$) increased within the right cerebral cortex of NOEX-TBI mice when compared to NOEX-NOTBI mice at 3 days post-TBI. TBI alone also significantly ($*p < .05$) increased VEGF-A mRNA expression within the right hippocampus of NOEX-TBI mice in comparison to NOEX-NOTBI mice at 1 day post-TBI. After 6 weeks of exercise, EX-NOTBI mice displayed significantly ($**p < .01$) increased right cerebral cortex VEGF-A mRNA expression in comparison to NOEX-NOTBI mice at both 1 and 3 days post-exercise. In addition, exercise alone significantly ($**p < .01$) increased VEGF-A mRNA expression within the right hippocampus of EX-NOTBI mice when compared to NOEX-NOTBI mice at 1 day post-exercise. After 6 weeks of exercise and subsequent TBI, EX-TBI mice demonstrated a significant ($*p < .05$) earlier increase in right cerebral cortex VEGF-A mRNA expression at 1 day post-exercise/post-TBI when compared to NOEX-TBI mice. Furthermore, exercise followed by TBI resulted in significantly ($**p < .01$) greater increases in VEGF-A mRNA expression within the right hippocampus of EX-TBI mice in comparison to NOEX-TBI mice at 1 day post-exercise/post-TBI.

2.4. VEGF-A Protein Localization

Immunohistochemistry revealed VEGF-A protein localization within neurons found in various regions of the right cerebral cortex (Figure 4A) and ipsilateral hippocampus CA1 field (Figure 4B). Minimal staining of right cerebral cortex VEGF-A positive neurons was observed in the sensorimotor cortex (i.e., primary motor cortex, secondary motor cortex, and somatosensory forelimb, hindlimb, and barrel fields) of NOEX-NOTBI mice. NOEX-NOTBI mice also displayed very sparse staining of VEGF-A positive neurons contained in the oriens layer of the right hippocampus CA1 field. However, in contrast to these findings, VEGF-A staining was more apparent within neurons located in the right cerebral cortex and right hippocampus CA1 field of TBI and exercise group mice. NOEX-TBI and EX-TBI mice exhibited an abundance of right cerebral cortex neurons staining positive for VEGF-A in the cingulate cortex (area 1 and 2), and sensorimotor cortex near the injury site. In addition, NOEX-TBI and EX-TBI mice exhibited noticeable VEGF-A staining of neurons located in the oriens layer and pyramidal cell layer of the right hippocampus CA1 field. EX-NOTBI mice displayed many VEGF-A positive stained neurons throughout the right sensorimotor cortex. Furthermore, EX-NOTBI mice demonstrated VEGF-A staining of neurons located in the oriens layer and pyramidal cell layer of the right hippocampus CA1 field.

2.5. VEGF-A Protein Quantification

VEGF-A protein expression (i.e., average number of VEGF-A positive stained neurons per mm²) increased within the right cerebral cortex (Figure 4C) in response to exercise alone, and exercise followed by TBI at 1 day post-TBI/post-exercise. In addition, VEGF-A protein expression increased within the ipsilateral hippocampus CA1 field (Figure 4D) in response to exercise followed by TBI at the 1 day post-TBI/post-exercise time point. Statistically significant ($p < .05$) differences existed between groups for the average number of VEGF-A positive stained neurons per mm² in both the right cerebral cortex and right hippocampus CA1 field. Post hoc testing showed several interesting differences when comparing groups. After 6 weeks of exercise, EX-NOTBI mice demonstrated a significantly ($**p < .01$) increased average number of right cerebral cortex VEGF-A positive stained neurons when compared to NOEX-NOTBI mice. After chronic exercise training and TBI, EX-TBI mice displayed a significant ($*p < .05$) increase in the average number of right cerebral cortex neurons staining positive for VEGF-A protein in comparison to NOEX-TBI mice. A similar response to exercise and subsequent TBI was observed between groups in the hippocampus, as EX-TBI mice exhibited a significantly ($*p < .05$) increased average number of right hippocampus CA1 field VEGF-A positive stained neurons when compared to NOEX-TBI mice.

2.6. EPO Gene Expression

EPO responded in a manner similar to VEGF-A after chronic exercise training. EPO mRNA expression increased within the right cerebral cortex (Figure 5A) in response to exercise. Statistically significant ($p < .05$) differences existed between groups for EPO mRNA expression (i.e., fold change relative to control) in the right cerebral cortex only. As a result of exercise alone, post hoc testing showed that EX-NOTBI mice exhibited significantly

(** $p < .01$) increased right cerebral cortex EPO mRNA expression when compared to NOEX-NOTBI mice at the 1 day post-exercise time point. After chronic exercise training and TBI, post hoc testing indicated significantly (** $p < .01$) increased EPO mRNA expression within the right cerebral cortex of EX-TBI mice in comparison to NOEX-TBI mice at 1 day post-TBI/post-exercise. There were no statistically significant differences between groups in ipsilateral hippocampus (Figure 5B) EPO mRNA expression.

2.7. EPO Protein Localization

EPO protein was localized within neurons throughout various regions of the right cerebral cortex (Figure 6A) and ipsilateral hippocampus CA1 field (Figure 6B). Only slight staining of right cerebral cortex EPO positive neurons was seen in the sensorimotor cortex (i.e., primary motor cortex, secondary motor cortex, and somatosensory forelimb, hindlimb, and barrel fields) of NOEX-NOTBI mice. Furthermore, NOEX-NOTBI mice showed very limited EPO staining of neurons located in the right hippocampus CA1 field. However, in a manner comparable to VEGF-A, staining was more apparent within neurons found in the right cerebral cortex and right hippocampus CA1 field of TBI and exercise group mice. NOEX-TBI and EX-TBI mice demonstrated distinct EPO staining within right cerebral cortex neurons located in the cingulate cortex (area 1 and 2), and sensorimotor cortex near the injury site. Both NOEX-TBI and EX-TBI mice displayed an obvious EPO staining pattern within neurons situated in the pyramidal cell layer of the right hippocampus CA1 field. In addition, NOEX-TBI and EX-TBI mice showed scant EPO staining of right hippocampus CA1 field neurons located in the oriens layer and stratum radiatum. EX-NOTBI mice exhibited abundant EPO staining within neurons found in the right sensorimotor cortex. Light EPO staining of right hippocampus CA1 field neurons located primarily in the oriens layer was also observed in EX-NOTBI mice.

2.8. EPO Protein Quantification

EPO protein expression (i.e., average number of EPO positive stained neurons per mm^2) increased within the right cerebral cortex (Figure 6C) in response to TBI alone, and exercise alone, at 1 day post-TBI/post-exercise. However, EPO protein expression only increased within the ipsilateral hippocampus CA1 field (Figure 6D) in response to TBI at 1 day post-TBI. Statistically significant ($p < .05$) differences existed between groups for the average number of EPO positive stained neurons per mm^2 in both the right cerebral cortex and right hippocampus CA1 field. Post hoc testing was conducted for multiple comparisons between groups, and several interesting findings were revealed. After TBI only, NOEX-TBI mice showed a significantly (** $p < .01$) increased average number of right cerebral cortex neurons staining positive for EPO protein when compared to NOEX-NOTBI mice. This TBI response was also observed in the hippocampus. NOEX-TBI mice displayed a significant (* $p < .05$) increase in the average number of right hippocampus CA1 field EPO positive stained neurons in comparison to NOEX-NOTBI mice. In response to chronic exercise training, EX-NOTBI mice demonstrated a significantly (* $p < .05$) increased average number of right cerebral cortex neurons staining positive for EPO protein when compared to NOEX-NOTBI mice.

2.9. HO-1 Gene Expression

HO-1 mRNA expression increased within the right cerebral cortex (Figure 7A) and ipsilateral hippocampus (Figure 7B) in response to TBI alone. However, in contrast to VEGF-A and EPO, HO-1 mRNA expression did not increase after exercise. Statistically significant ($p < .05$) differences existed between groups for HO-1 mRNA expression (i.e., fold change relative to control) in both the right cerebral cortex and right hippocampus. Post hoc testing was conducted for multiple comparisons between groups. As a result of TBI alone, HO-1 mRNA expression was significantly ($**p < .01$) increased within the right cerebral cortex of NOEX-TBI mice in comparison to NOEX-NOTBI mice at 3 days post-TBI. TBI alone also significantly ($**p < .01$ [3 days post-TBI], and $*p < .05$ [7 days post-TBI]) increased HO-1 mRNA expression within the right hippocampus of NOEX-TBI mice when compared to NOEX-NOTBI mice at both the 3 and 7 day post-TBI time points. Since exercise did not increase HO-1 mRNA expression in either brain region studied, no significant difference in the average number of HO-1 positive stained neurons per mm^2 would be expected between groups. Therefore, immunostaining for HO-1 protein was not performed in this work.

3. Discussion

In the present study, exercise preconditioning improved post-TBI sensorimotor function. EX-TBI mice displayed significant reductions in sensorimotor deficits when compared to NOEX-TBI mice at all three post-TBI time points. This finding supports research by Mota et al., who revealed 4 weeks of pre-TBI treadmill training reduced post-TBI motor deficits in rats, and decreased blood-brain barrier breakdown and neuroinflammation (Mota et al., 2012).

Exercise preconditioning improved post-TBI cognitive performance (i.e. spatial learning memory) in this work. EX-TBI mice exhibited a significant decrease in spatial learning memory deficits when compared to NOEX-TBI mice at 7 days post-TBI. This finding is in agreement with Gu et al., who showed that 3 weeks of pre-TBI voluntary wheel running attenuated spatial learning memory deficits in mice at 15 days post-TBI, and diminished neuron and synaptic density loss resulting from the injury (Gu et al., 2014). Griesbach et al., revealed that a delayed initiation (i.e., 14 days post-TBI) of post-TBI exercise enhanced spatial learning memory after injury (Griesbach et al., 2009). The beneficial effects of exercise on post-TBI cognitive function were attributed to increased levels of the neuroprotective protein brain-derived neurotrophic factor (BDNF) in that study (Griesbach et al., 2009).

Exercise and TBI significantly increased right cerebral cortex and right hippocampus VEGF-A mRNA in this study. The increase in VEGF-A mRNA was present at 1 day post-TBI in the exercise preconditioning group, but an increase in VEGF-A mRNA expression did not occur until 3 days post-TBI in mice that received TBI only. Thus, neuroprotective mechanisms were in place earlier in exercise preconditioned mice, and secondary injury may have been reduced after TBI as a result. It is important to note that VEGF-A mRNA was still significantly increased 3 days after exercise was terminated, and this prolonged transcriptional response to exercise may have protected against neuronal cell death that is

known to peak at 3 days post-TBI (Onyszchuk et al., 2008). Staining for VEGF-A protein was investigated at 1 day post-TBI and/or post-exercise, because this time point coincided with the increased VEGF-A mRNA expression seen in exercise preconditioned mice. Mice that exercised showed prevalent staining patterns for VEGF-A protein localized to neurons in the primary motor cortex, secondary motor cortex, cingulate cortex (area 1 and area 2), and CA1 field of the hippocampus. Exercise preconditioning increased the proportion of neurons staining positive for VEGF-A protein in both the injured cortex and ipsilateral CA1 region of the hippocampus. Neuroprotection may have been enhanced in these brain regions after TBI since exercise induced VEGF-A protein production in neurons. In addition to its effects on neurons, previous studies show that increased VEGF-A protein production after exercise is associated with greater capillary density in the cerebral cortex of rats (Viboolvorakul and Patumraj, 2014), and enhanced post-stroke cerebral blood flow within the ischemic lesion in a rat model of focal cerebral ischemia (Ma et al., 2013). A VEGF-A mediated increase in angiogenesis and blood flow resulting from exercise may augment neuroprotection and accelerate healing by restoring the supply of oxygen rich blood to damaged brain tissues.

Our study showed that exercise preconditioning significantly increased right cerebral cortex EPO mRNA. Mice from both exercise groups displayed significantly greater cortical EPO mRNA expression when compared to mice that did not exercise at 1 day post-TBI and/or post-exercise. EPO mRNA was not significantly elevated in the exercise groups at any other post-exercise time point. This was in contrast to VEGF-A mRNA expression, which remained significantly elevated in the cortex for 3 days after termination of exercise. Taken together, these results suggest that exercise-induced EPO neuroprotection occurs only during the acute post-TBI recovery period, while VEGF-A neuroprotection after exercise extends into the period of significant neuronal cell death that transpires at 3 days post-TBI (Onyszchuk et al., 2008). Furthermore, in contrast to VEGF-A, there were no differences between groups in EPO mRNA expression in the hippocampus. Staining for EPO protein was examined at 1 day post-TBI and/or post-exercise, since EPO mRNA was increased at this time point in exercise preconditioned mice. Mice that exercised displayed dense EPO staining in sensorimotor cortical neurons of the right cerebral cortex, and EPO positive neurons were especially evident around the CCI injury site, and in the pyramidal layer of the right hippocampal CA1 region in both TBI groups. Exercise alone increased EPO protein in cortical brain neurons, but exercise did not increase the post-TBI EPO protein response in the injured cortex at the 1 day time point to a greater extent than that observed in mice who received TBI only. Nonetheless, exercise preconditioned mice would have produced more EPO protein in cortical brain neurons prior to TBI, and it is likely that this prophylactic effect of exercise enhanced neuroprotection and brain repair after injury. The pattern of EPO staining was remarkably similar to VEGF-A in mice that received exercise, TBI, or exercise and TBI. This finding suggests that EPO may work in conjunction with VEGF-A to promote neuroprotection in the brain. Clinical trials are investigating exogenous EPO administration for treatment of TBI (Xiong et al., 2009). Unfortunately, it has been suggested that exogenous EPO administration may increase the risk of thrombosis in TBI patients (Nichol and Cooper, 2009), and evidence is lacking regarding the safety and efficacy of exogenous EPO treatments in TBI patients. Thus, increasing the endogenous production of EPO in the

brain through non-pharmacological interventions such as exercise may be a safer strategy for enhancing neuroprotection and improving TBI outcomes.

TBI significantly increased right cerebral cortex and right hippocampus HO-1 mRNA in this study. However, in contrast to VEGF-A and EPO, exercise preconditioning did not significantly alter HO-1 gene expression in either brain region. HO-1 mRNA expression significantly increased in the injured cortex of TBI group mice at 3 days post-TBI. This finding is in agreement with previously published research (Anderson et al., 2009). The post-TBI increase in cortical HO-1 mRNA expression occurred at the same time point when VEGF-A mRNA was also significantly increased. In the right hippocampus, HO-1 mRNA expression was increased at 3 and 7 days post-TBI. Hypoxia is a common secondary injury response to TBI, and HO-1 is known to be induced in the brain during chronic hypoxia (Neubauer and Sunderram, 2012). Hypoxia may have persisted in the right hippocampus at the 7 day post-TBI time point, and it is possible that the prolonged post-injury HO-1 response observed in this study improved resistance to oxidative stress resulting from TBI (Fukuda et al., 1995). Peripheral HO-1 production is up-regulated after exercise (Thompson et al., 2005), but this response did not occur in the brain. Thus, other treatments that up-regulate HO-1, such as the nutraceutical compounds resveratrol and sulforaphane, may enhance the neuroprotection provided by exercise preconditioning (Lin et al., 2014; Zhao et al., 2007).

Avoidance of exercise and rest are initially recommended to the majority of patients diagnosed with mild TBI (i.e. concussion) because of concerns regarding aggravation of symptoms. Therefore, regular exercise participation prior to the unexpected occurrence of a TBI may be an effective prophylactic measure for optimizing recovery from head injury. We did not monitor exercise intensity in the current study since mice ran voluntarily, but the expression of neuroprotective proteins may be highly dependent on exercise intensity. More specifically, an individual that has performed high intensity exercise (i.e., above the lactate threshold) prior to a TBI may increase the production of neuroprotective proteins in the brain to a greater extent than a TBI patient who participated in low intensity exercise training prior to injury. Support for this notion is garnered from a recent study that investigated VEGF-A responses in humans that performed exercise at various intensities (Wahl et al., 2014). In this study conducted by Wahl et al., high intensity exercise training was shown to increase circulating VEGF-A protein, but low intensity exercise was associated with a decrease, or no change in serum levels (Wahl et al., 2014). Mice that exercised at higher intensities in the present study may have maximized VEGF-A and EPO protein production in brain neurons.

No studies have investigated how various exercise intensities influence the expression of VEGF-A, EPO, and other neuroprotective proteins in the brain. Several mechanisms may be responsible for enhancing neuroprotection and improving recovery if high intensity exercise training is performed prior to TBI. An up-regulated production of neuroprotective hypoxia-inducible proteins such as VEGF-A and EPO may be observed in the brain after high intensity exercise training due to changes in ventilation, blood carbon dioxide levels, blood vessel diameter, blood flow, and pH. High intensity exercise induces hyperventilation, which results in a decreased partial pressure of carbon dioxide ($p\text{CO}_2$) in the blood (Querido

and Sheel, 2007). In response to decreased pCO₂, cerebral vasoconstriction and diminished cerebral blood flow ensue, which reduces the supply of glucose and oxygen to the brain temporarily (Querido and Sheel, 2007). Impaired oxygen supply and delivery to the brain may also occur during high intensity exercise due to reductions in pH and decreased oxyhemoglobin binding (Querido and Sheel, 2007). It is reasonable to speculate that an increased production of neuroprotective hypoxia-inducible proteins (e.g., VEGF-A and EPO) may occur in brain neurons responding to transient decreases in blood flow and oxygen delivery during high intensity exercise. Research is needed to determine if these mechanisms are responsible for inducing neuroprotection, and improving TBI outcomes when high intensity exercise is performed prior to injury.

Findings from our study suggest that pre-TBI fitness level directly impacts post-TBI outcome. Accordingly, humans that are more physically fit prior to the unexpected occurrence of a TBI may display less neurological dysfunction, and faster recovery from injury than sedentary individuals. This idea parallels the cognitive reserve theory that posits the brain may be more resilient to injury, normal aging, and neurodegenerative disease by engaging in challenging mental and physical activities throughout life (Stern, 2002). The clinical manifestation of brain damage may be attenuated by “building” cognitive reserve prior to brain trauma or the onset of neurodegenerative disease. Exercise may build cognitive reserve through the more efficient use of neural networks, or an improved ability to recruit alternate networks within various regions of the brain as needed after injury or disease onset (Stern, 2002). Cognitive reserve may also be built as a result of exercise augmenting neuroprotection, and stimulating neurogenesis in hippocampal brain regions involved in memory (van Praag et al., 1999; van Praag et al., 2005). When compared to a non-exerciser, an individual that has routinely exercised may establish a greater degree of cognitive reserve, which in turn could produce a brain that is more resistant to damage resulting from TBI or neurodegenerative disease.

Exercise positively affects the central nervous system, and displays great potential for enhancing brain fitness (Hillman et al., 2008). Humans participating in regular exercise programs exhibit brain volume increases in the supplementary motor area, right inferior frontal gyrus, left superior temporal gyrus, anterior cingulate, and hippocampus (Colcombe et al., 2006; Erickson et al., 2011). Being more physically active is also associated with reduced Alzheimer's (Lautenschlager et al., 2008) and Parkinson's (Saaksjarvi et al., 2014) disease risk. These are important findings because TBI is a risk factor for the accelerated onset and development of neurodegenerative diseases including Alzheimer's and Parkinson's (Jafari et al., 2013; Sivanandam and Thakur, 2012). Cognitive and motor deficits resulting from the possible accelerated post-TBI onset of these neurodegenerative diseases may be diminished by increasing physical activity since exercise has been shown in many studies to enhance cognition, prevent age-related declines in cognitive function, and improve motor function (Burns et al., 2008; Colcombe et al., 2004; Cotman and Berchtold, 2002; Erickson and Kramer, 2009; Lista and Sorrentino, 2010; van Praag et al., 2005). In addition, the beneficial effects of chronic exercise in mouse models of neurodegenerative disease (i.e., Alzheimer's, Parkinson's, Huntington's) have been reported in numerous studies (Adlard et al., 2005; Nichol et al., 2007; Pang et al., 2006; Parachikova et al., 2008; Petzinger et al., 2007; Tillerson et al., 2003; van Dellen et al., 2008). Results from previous research

discussed in this section, and new findings from the work presented here, indicate that performing regular exercise is a practical measure for increasing neuroprotection, improving TBI outcomes, and enhancing overall brain health across the lifespan.

4. Conclusions

This study showed that improved TBI outcomes were linked to exercise inducing the increased expression of neuroprotective genes and proteins in brain regions responsible for movement (i.e., sensorimotor cortex) and memory (i.e., hippocampus). Long-term pre-TBI exercise attenuated sensorimotor and spatial learning memory deficits after TBI in mice. Improved post-TBI sensorimotor function may have occurred in mice that exercised because pre-TBI exercise increased the production of both VEGF-A and EPO in the sensorimotor cortex. Furthermore, it is quite possible that improved post-TBI spatial learning memory was noticed in mice that exercised since pre-TBI exercise increased the production of VEGF-A in the hippocampus. It is probable that pre-TBI exercise augmented neuroprotection and accelerated the initiation of post-TBI brain repair by up-regulating VEGF-A and EPO production in the brain. Pre-TBI exercise increased VEGF-A and EPO protein production in brain neurons at 1 day post-TBI and/or post-exercise. Consequently, the exercise-induced up-regulation of these proteins at this time point may have protected against neuron cell death that is known to occur in mice at 3 days post-TBI (Onyszchuk et al., 2008). A future study should investigate whether the neuroprotective effect of exercise is diminished by blocking VEGF-A and/or EPO in the brain prior to TBI. We speculate that blocking VEGF-A and/or EPO in the brain of exercising mice would increase post-TBI neuron cell death. Results obtained from this potential future study may confirm that one, or both, of these proteins play a direct role in promoting exercise-induced neuroprotection. Future TBI research should examine how the frequency, intensity, duration, and type of exercise influence the neuroprotective proteins mentioned in this work. In addition, other molecular mechanisms in the brain that may be positively affected by performing pre-TBI or post-TBI exercise should be studied. Determining the appropriate time window for initiating post-TBI exercise is also critically important. This would help to establish exercise guidelines aimed at optimizing recovery from TBI in rehabilitation settings.

In summary, the results of this study demonstrate that exercise preconditioning improves post-TBI sensorimotor and cognitive function in adult mice. Exercise increases VEGF-A and EPO gene expression, and the number of neurons staining positive for VEGF-A and EPO proteins in the brain. Interventions that increase the production of these neuroprotective proteins in the brain prior to TBI, or early after injury during the acute phase of recovery, may reduce neuron death, accelerate brain repair, and improve overall outcome in humans.

5. Experimental Procedures

5.1. Experimental Animals

All mice were obtained from Charles River (Wilmington, MA). A total of 120 adult male C57/BL6 mice (28-32 g, 5 months old) were randomly assigned to one of four groups for this study: 1) no exercise + no TBI (NOEX-NOTBI, n=30), 2) no exercise + TBI (NOEX-TBI, n=30), 3) exercise + no TBI (EX-NOTBI, n=30), and 4) exercise + TBI (EX-TBI,

n=30). The no exercise group mice were individually housed in standard cages without running wheels, and exercise group mice were individually housed in standard cages with free access to running wheels for six weeks prior to sacrifice. Animals were sacrificed at 1, 3, or 7 days post-TBI and/or post-exercise. Mice were exposed to normal 12-hour light-dark cycles inside the University of Kansas Medical Center Laboratory Animal Resources building, and all animals had *ad libitum* access to water and standard rodent chow (8604; Harland Teklad Laboratories, Madison, WI). Animal care and use procedures were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee and conducted according to the Institute of Laboratory Animal Research guidelines.

5.2. Voluntary Exercise/Free Access Wheel Running

A six week voluntary wheel running exercise protocol was used in this study. This six week exercise protocol was chosen because it is similar in length to the 45 day protocol employed by van Praag et al., who demonstrated that exercise improved learning and increased neurogenesis in the hippocampus of non-injured mice (van Praag et al., 2005). The physical activity of mice was monitored via an interface cable that attached each cage containing a running wheel to a computer (Vital View Data Acquisition Software System; Mini Mitter Co. Inc., Bend, OR). The Vital View Data Acquisition Software System automatically recorded the total number of wheel revolutions and distance ran (in kilometers) by each mouse at thirty-minute intervals for the duration of six weeks. Data were expressed as the average daily running distance in kilometers (Km) + SEM.

5.3. Induction of Traumatic Brain Injury via Controlled Cortical Impact (CCI)

A moderate TBI was induced on the last day of each group's respective 6 week no exercise or exercise interventions via a controlled cortical impact (CCI) targeting the sensorimotor cortex of TBI group mice (NOEX-TBI [n=30] and EX-TBI [n=30]). The NOEX-NOTBI [n=30] and EX-NOTBI [n=30] mice received anesthesia (2.5% isoflurane in 30% O₂ and air), a sagittal scalp incision, and sutures only. Exercise responses (e.g., distance ran) and pre-TBI behavioral testing did not influence the selection of which mice received a CCI, or no CCI. Previous studies using magnetic resonance imaging have demonstrated high reliability for use of CCI in mice (Onyszchuk et al., 2007), and CCI reproduces the hallmark features of brain injuries observed in humans including motor deficits, memory loss, and neuron loss. The CCI procedure was performed while TBI group mice were anesthetized with 2.5% isoflurane in 30% O₂ and air as previously described (Onyszchuk et al., 2007; Onyszchuk et al., 2008). Mice were stabilized in a Cunningham stereotaxic frame (Stoelting; Wood Dale, IN), and placed on a heating pad to maintain body temperature at 38°C during the surgical procedure. The skin on top of the head was shaved and scrubbed with iodine, and a longitudinal incision was made with a sterile scalpel blade. Next, the scalp and epicranial aponeurosis were retracted to expose the skull. A 3.5 mm diameter craniotomy was performed with a dental drill on the right side of the mid-sagittal suture, with the following coordinates: Anterior-posterior (AP) coordinates centered at bregma, 2.5 mm lateral to the midline. Saline was routinely applied to the skull surface and drill to prevent heating the underlying brain tissue. Drilling was performed carefully in order to leave the dura intact, and to avoid lacerating blood vessels traversing through the superior sagittal sinus. With the brain exposed, the cortex was impacted with a custom made CCI injury

device. This device was assembled from a linear motor (P01-23x80, LinMot Inc., Zurich, Switzerland) and an electronic servo controller (E100-MT, LinMot Inc., Zurich, Switzerland). The motor consisted of a stator, a shaft, and integrated electronic circuitry. A 3 mm stainless-steel injury tip was attached to the end of the shaft. The impactor tip was slowly lowered to the surface of the dura and center of the injury tip contact zone (2.5 mm lateral to bregma) via a push-button graphical user interface in the impactor control software (Visual Basic 6.0 software), and the CCI was initiated through the following steps: 1) The computer was programmed to control impact velocity (1.5 m/s), impact depth (1.0 mm), and contact time (85 ms), 2) the tip was retracted 20 mm from the dura, and a rapid 21 mm downward strike (20 mm retraction plus the 1.0 operator programmed injury depth) of the impactor tip on the exposed brain followed. After impact, the skin was closed with a sterile suture, and all TBI group mice were returned to standard cages without running wheels. Mice (NOEX-NOTBI [n=30] and EX-NOTBI [n=30]) that did not receive a CCI were also returned to standard cages without running wheels.

5.4. Assessment of Sensorimotor Function

Sensorimotor deficits (i.e., foot faults) were assessed using the gridwalk task. NOEX-TBI and EX-TBI mice were tested on the gridwalk at four time points (pre-TBI [n=24 per group], 1 day post-TBI [n=24 per group], 3 days post-TBI [n=16 per group], and 7 days post-TBI [n=8 per group]). At 1 day prior to TBI, mice performed two gridwalk trials to establish each animal's baseline performance, and to allow familiarization with the gridwalk apparatus. At each subsequent post-TBI time point (i.e., 1, 3, and 7 days post-TBI), mice completed one trial per day. Foot faults were scored by an observer who was blinded to the grouping of mice. Mice were placed on an elevated wire grid (area measuring 32 cm×20 cm×50 cm with 11×11 mm diameter openings) for 5 minute trials. A foot fault was defined as any step passing through a grid opening. The number of foot faults was counted for each foot, and the total time spent walking was calculated during each trial. The total number of foot faults was divided by the total time spent walking during each trial to obtain the number of foot faults per minute of walking. This normalization of the foot fault data was needed to account for differences seen in the duration of walking between trials. Data were expressed as the average number of foot faults per minute walking + SEM.

5.5. Evaluation of Spatial Learning Memory

Spatial learning memory deficits (i.e., number of non-goal arm entry errors) were tested using the radial arm water maze (RAWM). The RAWM has been validated for testing learning and memory in mice (Alamed et al., 2006). 8 mice from the NOEX-TBI and EX-TBI groups were tested in the RAWM at 1 day prior to TBI, and 7 days post-TBI. At each time point, daily sessions consisted of four acquisition trials, followed by a 30-minute delay interval (i.e., mice returned to their respective cages), and a subsequent single retention test trial to evaluate spatial learning memory deficits. Testing began once mice were released into one of the six non-goal arms of the maze; a different semi-randomized start-arm and goal-arm (i.e., platform containing arm) sequence were used each day. Three dimensional objects (i.e., different shapes) erected 24 inches above the water surface on the perimeter of the RAWM served as visual cues for mice navigating the maze. A single trial allowed 120 seconds for mice to swim and find a platform submerged less than 1 centimeter below the

water surface and located in the distal part of the goal-arm. If mice reached the goal-arm platform within the allotted time frame, they were permitted to remain on the platform for 30 seconds, and the trial was ended. If mice failed to find the platform within 120 seconds, animals were gently guided toward the platform and permitted to remain on the platform for 30 seconds. During all trials, non-goal arm entry errors were recorded each time mice swam into an arm of the RAWM that did not contain the goal-arm platform. A non-goal arm entry was scored when the entire body of a mouse (excluding the tail) entered a non-goal arm. At the end of each trial, the total number of non-goal arm entry errors was calculated for each animal, and group averages were figured for the four acquisition trials and the single retention test trial. Water temperature was kept at 24-27°C during all sessions, and the surface of the water was coated with powdered condensed milk to make the water opaque which concealed the location of the goal-arm platform. Retention test data were expressed as the average number of non-goal arm entry errors + SEM.

5.6. mRNA Analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was used for a gene expression time course study as previously described (Anderson et al., 2009). 8 mice from each group (i.e., NOEX-NOTBI, NOEX-TBI, EX-NOTBI, and EX-TBI) were sacrificed via intraperitoneal injection of beuthanasia at 1, 3, and 7 days post-TBI and/or post-exercise. Animals were decapitated and whole brains were removed and stored (for 24 hours at 4°C) in RNA later (Ambion, Austin, TX) to preserve the RNA. Whole brains were dissected in order to collect a 5 mm wide sample (samples from both TBI groups contained the lesion site) of the right cerebral cortex and all of the ipsilateral hippocampus. Total RNA was extracted from these tissues by using a Polytron 2000 homogenizer (Brinkmann Instruments, Westbury, NY), and Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA purity and concentration were determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and samples that did not meet quality standards were discarded. 1 µg of total RNA from each sample, and random hexamers, were used in a Taqman reverse transcription reaction (Applied Biosystems, Foster City, CA, USA) to synthesize complementary DNA (cDNA). 10 ng of cDNA and gene-specific primers (see Table 1 for target genes and sequences of all primer pairs) were combined in a 20 µL reaction volume of SYBR Green PCR master mix (SYBR Green I Dye, AmpliTaqDNA polymerase, dNTPs mixture, dUTP, and optimal buffer components [Applied Biosystems]), 1 µL primer mixture (10 µg forward and 10 µg reverse primers in 150 µL Sigma water) and Sigma water (SigmaAldrich, St. Louis, MO) in a 96 well MicroAmp Optical reaction plate (Applied Biosystems, Foster City, CA). The cDNA/SYBR Green PCR master mix was subjected to PCR amplification (one cycle at 50°C for 2 minutes, one cycle at 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute) using an Applied Biosystems 7300 Real Time PCR System Machine (Applied Biosystems, Foster City, CA). PCR reactions were conducted in duplicate, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. Data were collected and analyzed with Sequence Detection Software 2.0 (Applied Biosystems). VEGF-A, EPO, and HO-1 gene expression were calculated relative to GAPDH by using the delta-delta Ct method (Pfaffl et al., 2002), and reported in accordance with The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)

guidelines (Bustin et al., 2009). Data were expressed as the average fold change + SEM relative to GAPDH in brain samples from the NOEX-NOTBI group.

5.7. Protein Analysis

Immunohistochemistry was performed to investigate the location of neurons staining positive for VEGF-A or EPO proteins within the right cerebral cortex (injured cortex) and CA1 region of the ipsilateral hippocampus as previously described (Onyszchuk et al., 2008). Neurons expressing VEGF-A or EPO proteins were manually counted in the right cerebral cortex and CA1 region of the ipsilateral hippocampus. 6 mice from each group (i.e., NOEX-NOTBI, NOEX-TBI, EX-NOTBI, and EX-TBI) were sacrificed by transcardial perfusion at 1 day post-TBI and/or post-exercise. Brains were fixed by perfusion with 4% buffered formaldehyde, post-fixed for 24 hours, and cryoprotected in 30% sucrose in 0.1 M phosphate buffer (pH 7.2). A microtome was used to cut 35 μ m thick coronal sections of the brain, and frozen sections were stored in ethylene glycol solution at -80°C . Sections were rinsed for 5 minutes in PBS with 0.4 mg/ml glycine, permeabilized with 0.2% Triton X-100, and quenched for peroxidase with 3% H_2O_2 prior to staining. Sections were incubated for 24 hours at 4°C with rabbit anti-mouse primary polyclonal antibodies to VEGF-A (1:5000, ab46154; Abcam Inc., Cambridge, MA) and EPO (1:150, sc-7956; Santa Cruz Biotechnology, Santa Cruz, CA). Next, sections were rinsed (3 washes $\times$ 5 minutes) with PBS, and incubated for 2 hours at room temperature with a biotinylated goat anti-rabbit secondary antibody (1:500, BA-1000; Vector Labs, Burlingame, CA). The immunoperoxidase reaction was produced according to the manufacturer's instructions with an ABC Elite kit (Vector Labs, Burlingame, CA). Color was visualized using diaminobenzidine (DAB) solution (Vector Labs, Burlingame, CA). A Nikon Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY) was used to visualize the location of positively stained neurons in all sections, and digital images were captured at 10X magnification with a Photometrics CoolSNAP ES microscope camera (Photometrics, Tucson, AZ). Image J image analysis software was used to manually count neurons that expressed VEGF-A or EPO proteins. Neurons stained positive for VEGF-A or EPO were labeled in the captured digital images with a blue dot and number after positioning the on-screen cursor over the cell body and clicking the computer mouse. Positively stained neurons were counted (in sections located at Bregma 0.02 mm) within a 3.93721 mm^2 region of the right cerebral cortex that included the primary and secondary motor cortices, and somatosensory fields. In sections containing the TBI lesion cavity, cell counts were normalized to the total area of tissue remaining within the 3.93721 mm^2 target region of the right cerebral cortex. Neurons staining positive for VEGF-A or EPO proteins were also manually counted (in sections located at Bregma -1.94 mm) within an 891.6 μm^2 region of the right hippocampus CA1 field. Data were expressed as the average number of VEGF-A or EPO positive stained neurons per mm^2 + SEM in each brain region.

5.8. Statistical Analyses

SPSS Statistics 20 (IBM, Chicago, IL) software was used for statistical analyses. Exercise and behavioral data were analyzed with one-way and two-way repeated measures ANOVA respectively. Three-way ANOVA (fixed factors; day, TBI, and exercise) was used for analyzing mRNA data, and a two-way ANOVA (fixed factors; TBI and exercise) was

performed for determining significant differences in the number of neurons staining positive for VEGF-A or EPO proteins between groups. Fisher's LSD post hoc testing was conducted for multiple comparisons. Significant differences between groups are represented with asterisks (* $p < .05$ and ** $p < .01$).

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Highlights

- Pre-TBI exercise improved post-TBI sensorimotor and cognitive function
- Exercise increased VEGF-A and EPO gene and protein expression in the brain
- Increasing VEGF-A and EPO early after TBI may improve outcomes

Figure 1.

The average distance ran (in kilometers [Km]) of 5 month old male, adult mice (n=60) during 30 minute sampling intervals throughout the course of one day (A), and the average total daily distance ran during each week of the 6 week exercise training period (B). The mice show profound circadian rhythms in their running patterns (A). Mice ran on average a total of $4.413 \pm .088$ Km daily during the 6 week protocol (B). Mice ran significantly ($**p < .01$) less daily in week 1 when compared to all other weeks (B). However, the average total daily distance ran varied slightly during weeks 2-6 (B). Statistical Analysis: One-way repeated measures ANOVA with Fisher's LSD post hoc testing for multiple comparisons. Mean $\pm$ SEM.

Figure 2.

The gridwalk task was used for investigating sensorimotor deficits (A), and radial arm water maze (RAWM) testing was conducted for evaluation of spatial learning memory deficits (B). 6 weeks of pre-TBI voluntary wheel running exercise reduced post-TBI sensorimotor deficits (i.e., average number of foot faults per minute walking [A]) and spatial learning memory deficits (i.e., average number of non-goal arm entry errors [B]). Exercise + TBI (EX-TBI) group mice demonstrated a significantly (**p<.01) reduced average number of foot faults per minute walking during the gridwalk task at 1, 3, and 7 days post-TBI when compared to no exercise + TBI (NOEX-TBI) group mice (A). TBI significantly (*p<.05) increased the average number of non-goal arm entry errors during the RAWM retention test in both NOEX-TBI and EX-TBI group mice at 7 days post-TBI (B). However, EX-TBI group mice displayed a significantly (*p<.05) decreased average number of non-goal arm entry errors during the RAWM retention test prior to TBI, and at 7 days post-TBI when compared to NOEX-TBI group mice (B). Statistical Analysis: Two-way repeated measures ANOVA (fixed factors; day and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean + SEM for n=24 pre-TBI, n=24 at 1 day post-TBI, n=16 at 3 days post-TBI, and n=8 at 7 days post-TBI per group for the gridwalk task, and n=8 per group at the pre-TBI and 7 days post-TBI time points for RAWM testing.

Figure 3.

qRT-PCR study of right cerebral cortex (A) and right hippocampus (B) VEGF-A mRNA responses after TBI and/or 6 weeks of pre-TBI voluntary wheel running exercise. Y-axis is the average fold change relative to GAPDH (housekeeping gene) in the no exercise + no TBI (NOEX-NOTBI) mouse cortex. Data represents VEGF-A mRNA expression at various post-TBI and/or post-exercise time points (X-axis) for NOEX-NOTBI, no exercise + TBI (NOEX-TBI), exercise + no TBI (EX-NOTBI), and exercise + TBI (EX-TBI) group mice. Right cerebral cortex (i.e., injured cortex in both TBI groups) VEGF-A mRNA increased after TBI, exercise, and the combination of exercise and TBI (A). NOEX-TBI group mice demonstrated significantly ($**p < .01$) increased right cerebral cortex VEGF-A mRNA expression at 3 days post-TBI when compared to NOEX-NOTBI group mice (A). EX-NOTBI group mice displayed significantly ($**p < .01$) increased right cerebral cortex VEGF-A mRNA expression at both 1 and 3 days post-exercise in comparison to NOEX-NOTBI mice (A). EX-TBI group mice showed a significant early ($*p < .05$) increase in right cerebral cortex VEGF-A mRNA at 1 day post-exercise/post-TBI when compared to NOEX-TBI group mice (A). Right hippocampus VEGF-A mRNA increased after TBI, exercise, and the combination of exercise and TBI at the 1 day post-TBI and/or post-exercise time point only (B). NOEX-TBI group mice demonstrated significantly ($*p < .05$) increased right hippocampus VEGF-A mRNA expression at 1 day post-TBI when compared to NOEX-

NOTBI group mice (B). EX-NOTBI group mice displayed significantly (** $p < .01$) increased right hippocampus VEGF-A mRNA expression at 1 day post-exercise in comparison to NOEX-NOTBI mice (B). EX-TBI group mice showed a significant (** $p < .01$) increase in right hippocampus VEGF-A mRNA at 1 day post-exercise/post-TBI when compared to NOEX-TBI group mice (B). Statistical Analysis: Three-way ANOVA (fixed factors; day, TBI, and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean + SEM for $n=8$ per group at each time point.

Figure 4.

VEGF-A protein study (A-D). Coronal immunostained sections (10X magnification, scale bar = 100 μ m for all images) showed localization of VEGF-A protein in right cerebral cortex (A) and right hippocampus CA1 field (B) neurons of no exercise + no TBI (NOEX-NOTBI), no exercise + TBI (NOEX-TBI), exercise + no TBI (EX-NOTBI), and exercise + TBI (EX-TBI) group mice at 1 day post-TBI and/or post-exercise. In the right cerebral cortex (A), NOEX-NOTBI mice exhibited very few neurons staining positive for VEGF-A in the primary motor cortex (M1) and somatosensory (SS) hindlimb region. NOEX-TBI and EX-TBI mice displayed abundant VEGF-A staining within right cerebral cortex neurons located in the cingulate cortex (area 1 [CG1] and area 2 [CG2]), primary motor cortex (not shown), and secondary motor cortex (M2) near the injury site (A). EX-NOTBI mice exhibited very noticeable VEGF-A staining within neurons located in the primary motor cortex (M1), secondary motor cortex (not shown), and somatosensory (SS) hindlimb region of the right cerebral cortex (A). In the right hippocampus CA1 field (B), NOEX-NOTBI mice displayed sparse VEGF-A staining within neurons located in the oriens layer (Or). NOEX-TBI and EX-TBI mice revealed marked VEGF-A staining within neurons located in the oriens layer (Or) and pyramidal cell (Py) layers of the right hippocampus CA1 field (B). EX-NOTBI mice displayed VEGF-A staining within neurons located in the oriens layer (Or) and pyramidal cell (Py) layer of the right hippocampus CA1 field (B). Data in graphs represents the average number of VEGF-A positive stained neurons per mm² in the right cerebral cortex (C) and right hippocampus CA1 field (D) for each group at 1 day post-TBI and/or post-exercise. In the right cerebral cortex (C), EX-NOTBI mice displayed a significantly (** $p < .01$) increased average number of neurons staining positive for VEGF-A protein in comparison to NOEX-NOTBI mice. EX-TBI mice showed a significant (* $p < .05$) increase in the average number of right cerebral cortex neurons staining positive for VEGF-A protein when compared to NOEX-TBI mice (C). In the right hippocampus CA1 field (D), EX-TBI mice showed a significant (* $p < .05$) increase in the average number of neurons staining

positive for VEGF-A protein when compared to NOEX-TBI mice. Statistical Analysis: Two-way ANOVA (fixed factors; TBI and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean + SEM for n=6 per group.

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Figure 5.

qRT-PCR study of right cerebral cortex (A) and right hippocampus (B) EPO mRNA responses after TBI and/or 6 weeks of pre-TBI voluntary wheel running exercise. Y-axis is the average fold change relative to GAPDH (housekeeping gene) in the no exercise + no TBI (NOEX-NOTBI) mouse cortex. Data represents EPO mRNA expression at various post-TBI and/or post-exercise time points (X-axis) for NOEX-NOTBI, no exercise + TBI (NOEX-TBI), exercise + no TBI (EX-NOTBI), and exercise + TBI (EX-TBI) group mice. Right cerebral cortex (i.e., injured cortex in both TBI groups) EPO mRNA increased after exercise, and the combination of exercise and TBI (A). EX-NOTBI group mice displayed significantly (** $p < .01$) increased right cerebral cortex EPO mRNA expression at 1 day post-exercise in comparison to NOEX-NOTBI mice (A). EX-TBI group mice showed a significant (** $p < .01$) increase in right cerebral cortex EPO mRNA at 1 day post-exercise/post-TBI when compared to NOEX-TBI group mice (A). No statistically significant differences in right hippocampus EPO mRNA expression existed between groups at any time point (B). Statistical Analysis: Three-way ANOVA (fixed factors; day, TBI, and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean + SEM for $n=8$ per group at each time point.

Figure 6.

EPO protein study (A-D). Coronal immunostained sections (10X magnification, scale bar = 100 μ m for all images) showed localization of EPO protein in right cerebral cortex (A) and right hippocampus CA1 field (B) neurons of no exercise + no TBI (NOEX-NOTBI), no exercise + TBI (NOEX-TBI), exercise + no TBI (EX-NOTBI), and exercise + TBI (EX-TBI) group mice at 1 day post-TBI and/or post-exercise. In the right cerebral cortex (A), NOEX-NOTBI mice displayed very few neurons staining positive for EPO in the primary motor cortex (M1) and somatosensory (SS) hindlimb region. NOEX-TBI and EX-TBI mice exhibited more obvious EPO staining within right cerebral cortex neurons located in the cingulate cortex (area 1 [CG1] and area 2 [CG2]), primary motor cortex (not shown), and secondary motor cortex (M2) near the injury site (A). EX-NOTBI mice showed EPO staining within neurons located in the primary motor cortex (M1), secondary motor cortex (not shown), and somatosensory (SS) hindlimb region of the right cerebral cortex (A). In the right hippocampus CA1 field (B), NOEX-NOTBI mice demonstrated very limited staining of neurons. NOEX-TBI and EX-TBI mice revealed an obvious EPO staining pattern within neurons located in the pyramidal cell (Py) layer of the right hippocampus CA1 field (B). In addition, NOEX-TBI and EX-TBI mice showed scant EPO staining of right hippocampus CA1 field neurons located in the oriens layer (Or) and stratum radiatum (Rad) (B). EX-NOTBI mice displayed light staining of right hippocampus CA1 field neurons that were primarily located in the oriens layer (Or) (B). Data in graphs represents the average number of EPO positive stained neurons per mm² in the right cerebral cortex (C) and right hippocampus CA1 field (D) for each group at 1 day post-TBI and/or post-exercise. In the right cerebral cortex (C), NOEX-TBI mice demonstrated a significantly (** $p < .01$) increased average number of neurons staining positive for EPO protein in comparison to NOEX-NOTBI mice. EX-NOTBI mice displayed a significantly (* $p < .05$) increased average number of right cerebral cortex neurons staining positive for EPO protein when compared to NOEX-NOTBI group mice (C). In the right hippocampus CA1 field (D), NOEX-TBI mice showed

a significant (* $p < .05$) increase in the average number of neurons staining positive for EPO protein when compared to NOEX-NOTBI group mice. Statistical Analysis: Two-way ANOVA (fixed factors; TBI and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean + SEM for $n=6$ per group.

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Figure 7.

qRT-PCR study of right cerebral cortex (A) and right hippocampus (B) HO-1 mRNA responses after TBI and/or 6 weeks of pre-TBI voluntary wheel running exercise. Y-axis is the average fold change relative to GAPDH (housekeeping gene) in the no exercise + no TBI (NOEX-NOTBI) mouse cortex. Data represents HO-1 mRNA expression at various post-TBI and/or post-exercise time points (X-axis) for NOEX-NOTBI, no exercise + TBI (NOEX-TBI), exercise + no TBI (EX-NOTBI), and exercise + TBI (EX-TBI) group mice. Right cerebral cortex (i.e., injured cortex in both TBI groups) HO-1 mRNA increased after TBI (A). NOEX-TBI group mice displayed significantly (** $p < .01$) increased right cerebral cortex HO-1 mRNA expression at 3 days post-TBI when compared to NOEX-NOTBI mice (A). Right hippocampus HO-1 mRNA increased after TBI in a manner similar to that observed in the right cerebral cortex (B). NOEX-TBI group mice displayed significantly (** $p < .01$, and * $p < .05$) increased right hippocampus HO-1 mRNA expression at 3 and 7 days post-TBI when compared to NOEX-NOTBI mice (B). Statistical Analysis: Three-way ANOVA (fixed factors; day, TBI, and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean + SEM for $n=8$ per group at each time point.

Table 1

Primer sequences used for qRT-PCR

Gene	Primer Sequence	Expected Amplicon Size (bp)
VEGF-A	Forward-5'-TTA-CTG-CTG-TAC-CTC-CAC-C-3' Reverse-5'-ACA-GGA-CGG-CTT-GAA-GAT-G-3'	189
EPO	Forward-5'-CCT-GTC-CCT-GCT-CTC-AGA-AGC-3' Reverse-5'-GTG-GTA-TCT-GGA-GGC-GAC-ATC-3'	177
HO-1	Forward-5'-CGC-CTT-CCT-GCT-CAA-CAT-T-3' Reverse-5'-TGT-GTT-CCT-CTG-TCA-GCA-TCA-C-3'	62
GAPDH (Housekeeping)	Forward-5'-ATG-ACA-TCA-AGA-AGG-TGG-TG-3' Reverse-5'-CAT-ACC-AGG-AAA-TGA-GCT-TG-3'	177