IMPROVING TRAUMATIC BRAIN INJURY OUTCOMES

THROUGH GENE THERAPY AND EXERCISE

BY

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ABSTRACT

Effective interventions that improve traumatic brain injury (TBI) outcomes are lacking, and concerns remain with current surgical and pharmacological treatments. Consequently, there is a significant need for identifying novel non-surgical and non-pharmacological interventions that improve TBI outcomes. Increasing production of neuroprotective molecules such as neuroglobin, vascular endothelial growth factor-A (VEGF-A), erythropoietin (EPO), and heme oxygenase-1 (HO-1) in the brain prior to TBI, or early after injury, may improve outcomes. The purpose of this dissertation was to determine whether gene therapy (i.e., overexpression of the neuroglobin gene) and/or pre-TBI exercise could improve post-TBI sensorimotor and cognitive function in adult mice by increasing brain expression of neuroglobin, VEGF-A, EPO, and HO-1. Additional objectives included determining what cell types and brain regions demonstrated endogenous production of neuroglobin, VEGF-A, EPO, and HO-1 after gene therapy, exercise, and TBI. The central hypothesis of this dissertation is that improved post-TBI sensorimotor and cognitive function are linked to gene therapy and/or pre-TBI exercise increasing the production of these neuroprotective proteins in brain regions responsible for movement (i.e., sensorimotor cortex) and memory (i.e., hippocampus). Study results indicated significantly improved post-TBI sensorimotor function in transgenic mice that overexpressed neuroglobin, and mice that engaged in 6 weeks of voluntary pre-TBI exercise. Improved post-TBI sensorimotor function (i.e., reduction in sensorimotor deficits while walking) was associated with increased neuroglobin production in neurons and glial cells throughout the brain of transgenic neuroglobin overexpressing mice, and increased VEGF-A and EPO in sensorimotor cortex neurons of pre-TBI exercise mice. Pre-TBI exercise mice also showed improved post-TBI cognitive function (i.e., reduction in spatial learning memory errors), and increased VEGF-A production within

hippocampal neurons. These findings suggest that improved post-TBI sensorimotor and cognitive function are linked to gene therapy and/or exercise increasing the expression of neuroglobin, VEGF-A, and EPO in brain regions responsible for movement and memory. An increased production of these neuroprotective proteins was observed within brain neurons before TBI, or early after injury during the acute recovery phase. Interventions that increase neuroglobin, VEGF-A, and EPO production in brain neurons prior to TBI, or early after injury, may improve outcomes by optimizing neuroprotection.

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Introduction

1.1. TRAUMATIC BRAIN INJURY (TBI) BACKGROUND:

1.1.1. Definition, Mechanism of Injury, and Classification

Traumatic brain injury (TBI) is a significant public health concern and major cause of death and disability. A TBI occurs when an external mechanical force (e.g., direct impact to the head, rapid acceleration/deceleration of the head, blast waves, or penetration by a projectile) causes temporary or permanent brain damage and dysfunction. TBI can be classified according to the anatomical involvement (i.e., diffuse or focal), injury mechanism (i.e., closed head trauma or open/penetrating head trauma), and the severity of injury (i.e., mild TBI or concussion, moderate TBI, or severe TBI) [1]. A neurological examination, assignment of a Glasgow Coma Score, and neuroimaging assist with the diagnosis and classification of TBI. The Glasgow Coma Scale (GCS) is frequently used to classify the severity of injury by assessing the level of consciousness in a patient following head trauma. The GCS is a 3-15 point grading scale used to evaluate verbal, motor, and eye-opening reactions to stimuli. Higher GCS scores (i.e., 13-15) are representative of a mild TBI, while lower GCS scores (i.e., 3-8) are associated with severe TBI [1]. Another TBI classification model was developed by the Department of Defense and Department of Veteran Affairs which includes the GCS score, duration of post-traumatic amnesia, duration of loss of consciousness, and findings from neuroimaging to establish severity of injury [2]. Neuroimaging techniques including computerized tomography scanning and magnetic resonance imaging, are useful for visualizing structural brain damage, swelling, and hemorrhage resulting from moderate to severe TBI [3]. However, neuroimaging usually does not reveal any structural brain damage after mild TBI even though brain function is still impaired at the cellular and molecular level [3, 4]. A goal of current and future research is to identify and investigate potential TBI biomarkers in serum, cerebrospinal fluid, and urine [1, 5-7]. The

identification of TBI biomarkers may supplement existing tools (i.e., neurological examination, GCS, and neuroimaging) and further aid in injury classification and determining prognosis following TBI.

1.1.2. Epidemiology

Approximately 1.7 million TBIs occur in the United States annually, of which 1,365,000 people are treated as emergency outpatients, 275,000 people are hospitalized, and an estimated 52,000 people die [8]. Diffuse TBI is more common than focal TBI, and diffuse TBI is highly prevalent in closed head trauma cases [9]. 70%-90% of all treated TBIs are diagnosed as a mild TBI [10, 11], and TBI rates are higher for males when compared to females in all age categories [12]. Children aged 0-4 years, older adolescents aged 15-19 years, and adults aged 75 years or older are more likely to sustain a TBI when compared to persons in other age groups [12]. Adults aged 75 years or older have greater hospitalization and mortality rates in comparison to younger TBI patients [13]. Older aged adults also display worse functional outcome and long-term recovery after TBI in comparison to younger patients [14, 15]. The most common causes of TBI are motor vehicle accidents, domestic violence, assault, and falls [10, 12]. In addition, participation in sporting events is a primary cause of TBI. Approximately 1.6-3.8 million cases of sports-related concussion occur in the United States annually [12]. However, this estimation may be low since many sports-related head injuries are not recognized and medical treatment is not received [16]. Firearm usage and blast injuries from explosions in war zones are the main factors contributing to high TBI rates in active duty military personnel [17]. Child abuse is an unfortunate reason infants sustain a TBI. The violent shaking of a baby can result in “shaken baby syndrome” and may lead to permanent brain damage or death [18, 19]. The incidence of

TBI remains high, and the numerous causes of TBI lead to increased mortality rates and poor recovery in certain populations.

1.2. CLINICAL PICTURE:

1.2.1. Signs and Symptoms of TBI

TBI signs and symptoms are dependent on the type of injury sustained (i.e., diffuse or focal), region(s) of the brain affected, and injury severity. Some signs and symptoms are instantly obvious after TBI, while others may appear during the hours, days, or weeks that follow. Mild TBI is often underdiagnosed, difficult to define, and the symptoms are highly variable [20]. An individual may initially lose consciousness for a few seconds or minutes as a consequence of mild TBI. Loss of consciousness has been suggested to occur due to rotational forces applied at the connection of the upper midbrain and thalamus [21]. These rotational forces cause a temporary disruption of the electrophysiological and intracellular activities of reticular activating system neurons that are important for maintaining alertness [21]. In addition, loss of consciousness may also result from self-limited cortical seizures, or rapid increases in intracranial pressure [21]. In many cases, a loss of consciousness does not occur after mild head trauma [16]. However, many other symptoms of mild TBI including blurred vision, tinnitus, headache, confusion, mood changes, trouble concentrating, amnesia, dizziness, impaired motor coordination, difficulty balancing, fatigue, and alterations in sleep patterns may be evident [10, 16, 22].

A patient diagnosed with moderate or severe TBI may display many of the aforementioned mild TBI symptoms in conjunction with nausea, vomiting, convulsions, aphasia (i.e., word-finding difficulty), slurred speech, dysarthria (i.e., disordered speech due to facial muscle weakness), weakness, numbness, paralysis, behavioral issues, alexithymia (i.e., difficulty

identifying and describing the emotions of others), mydriasis (i.e., dilated pupil), and anisocoria (i.e., unequal pupil size) [4, 23-26]. Hematoma, hemorrhage, and edema combine to exacerbate swelling of the brain and increase intracranial pressure [4, 23, 24, 27]. Increased intracranial pressure may decrease the level of consciousness in a TBI patient, and cause weakness or paralysis on one or both sides of the body [4, 23, 24]. Other classic signs of increased intracranial pressure include unequal pupil size, or a dilated pupil that does not constrict in response to light [4, 23, 28]. These phenomena occur as increased intracranial pressure damages the optic and oculomotor nerves involved in controlling the diameter of the pupil [28]. In addition, Cushing's triad of hypertension, respiratory depression, and bradycardia may manifest in the terminal stages of severe TBI as a result of increased intracranial pressure [4, 29]. Persistent increases in intracranial pressure greater than 20 mm Hg, or decreases in cerebral perfusion pressure to less than 50-60 mm Hg, have been associated with herniation, cerebral infarction, and death after severe TBI [30, 31].

1.2.2. TBI Complications

A host of medical complications may arise after TBI. The risk for and duration of complications is increased with more severe head trauma. A prolonged state of unconsciousness (i.e., coma and vegetative state), and abnormal posturing (i.e., involuntary flexion or extension of the arms and legs) may be evident following severe TBI [4, 26, 32]. Diffuse axonal injury (i.e., widespread damage to axonal tracts projecting to and from the cerebral cortex) is a main cause of coma [33] and persistent vegetative state [34], and lesions in the corpus callosum and brainstem are frequent post-mortem neuropathological findings within the brain of TBI patients that survived for extended periods of time in altered states of consciousness [34]. Prolonged bed rest in conjunction with vascular damage can lead to the development of thrombi and emboli, which

increases the risk of stroke after TBI [35]. Cardiac arrhythmias are frequent [36, 37], and mechanical ventilation may be required to manage primary respiratory failure in severe TBI patients [38]. Head trauma increases the risk of seizures which may ensue during the weeks, months, or years after TBI [4], and patients may experience temporary or permanent alterations in vision, hearing, smell, taste, and touch [4, 22]. Skull fractures and penetrating head wounds tear the meninges that surround and protect the brain. As a result, bacteria may enter the brain causing meningitis and systemic infection [39]. Another consequence of TBI is disrupted pituitary gland function, which can cause transient hypopituitarism [40]. Hypopituitarism occurring secondary to TBI creates systemic hormonal imbalances that are obvious immediately, and have the potential to last for up to one year after injury [40]. TBI survivors may have trouble communicating with, and understanding family, friends, and care providers [41]. Behavioral problems (e.g., risky behavior, verbal and physical outbursts, lack of awareness, and difficulty with self-control) and emotional changes (e.g., altered self-esteem, irritability, anger, anxiety, depression, and suicidal thoughts) are common following TBI [42, 43]. Cognitive and motor dysfunction are devastating long-term complications resulting from TBI. Many human studies have reported reduced long-term (i.e., >3 months) cognitive performance after TBI on tasks that evaluate attention [44-46], memory [47, 48], executive function [46, 49], and information processing [50, 51]. Ataxia (i.e., uncoordinated muscle movements), myoclonus (i.e., involuntary muscle twitching), and reduced range of movement and motor control are movement disorders that may develop due to TBI [52, 53]. TBI is associated with the development of neurological disorders and neurodegenerative diseases later in life. A history of head trauma has been linked to an increased risk of developing psychiatric disorders [54], epilepsy [12], and Parkinson's disease [55]. The incidence of Alzheimer's disease is significantly higher in

individuals previously diagnosed with TBI [56], and sustaining multiple concussions has been correlated to the development of chronic traumatic encephalopathy (CTE) in athletes [8, 57, 58]. The progressive neurological deterioration observed in CTE is due to several brain changes including; atrophy of the cerebral hemispheres, medial temporal lobe, thalamus, mammillary bodies, and brainstem, and enlargement of the ventricles [57, 58]. In addition, post-mortem examination of CTE brain tissue has often revealed an abnormal aggregation of various proteins including; tau, alpha-synuclein, beta-amyloid, transactive response DNA binding protein 43, presinilin-1, and beta-site amyloid precursor protein cleaving enzyme-1 throughout a number of brain regions [57-60]. An abnormal accumulation of these proteins compromises neuronal and synaptic function, impairs axonal transport, and promotes the formation of neurofibrillary plaques and tangles in the brain [57]. These neuropathological findings have been associated with many physical, cognitive, emotional, and behavioral changes in individuals with a history of TBI [57-59].

1.3. TBI PATHOPHYSIOLOGY:

1.3.1. Primary and Secondary Injury Responses

Debilitating post-TBI outcomes can be attributed to the combination of primary and secondary injury responses. Primary injury responses (e.g., contusion, laceration, hemorrhage, axon transection, and diffuse axonal injury) are defined by the immediate mechanical damage occurring at the moment of impact, and secondary injury responses (e.g., 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 the initial trauma [20, 61-63]. These secondary injury responses can exacerbate damage caused by the primary injury, and negatively affect recovery

from TBI [20]. The combination of secondary injury responses is the main cause of death in hospitalized TBI patients [23]. For those patients that survive a TBI, the progression of secondary injury responses is what primarily determines the extent of brain damage, neurological dysfunction, and disability. Therefore, TBI should not be thought of as just an acute event, but rather a prolonged series of pathophysiological responses that ultimately cause neurological dysfunction and poor outcome following injury.

1.3.2. Neurometabolic Cascade of Secondary Injury Responses

A cascade of molecular, biochemical, and cellular events in multiple intracellular pathways promotes neuron death and dysfunction within the brain post-TBI. TBI induces a widespread and massive depolarization of neurons, glial cells, and cerebral vascular endothelial cells. The extensive depolarization of neurons throughout the brain promotes the release of the excitatory neurotransmitter glutamate from pre-synaptic cells. Extracellular concentrations of glutamate increase as stored glutamate is released from pre-synaptic vesicles into the synaptic cleft [64, 65]. Excessive extracellular glutamate contributes to neuron death and dysfunction after TBI [64]. Binding of glutamate to post-synaptic N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors leads to further neuronal depolarization, ionic fluxes (i.e., efflux of potassium [K^+] and influx of calcium [Ca^{2+}]), and adaptations in cellular physiology [64, 65]. As a result of damage to neuronal membranes and axons, voltage-dependent K^+ channels open which substantially increases extracellular K^+ levels [65, 66]. In addition, Ca^{2+} enters the cell as glutamate continues to activate post-synaptic NMDA receptors [67]. Continued neuronal depolarization causes calcium to accumulate in neurons and axons for 2 to 4 days post-TBI [67]. Excessive intracellular calcium may activate apoptotic genetic signals, and initiate neuron death through an over-activation of phospholipases,

plasmalogenase, calpains, protein kinases, nitric oxide synthase, and endonucleases [66]. The widespread and massive depolarization of neurons in the brain is subsequently followed by a wave of neuronal suppression (known as spreading depression) [68]. Various symptoms may be noticeable during a spreading depression state including; loss of consciousness, amnesia, and cognitive dysfunction after sustaining a TBI [66].

Whole brain metabolism is also altered following TBI. Initially, a rapid increase in glucose metabolism is observed as adenosine tri-phosphate dependent sodium-potassium (ATP-Na^+/K^+) pumps are activated in an effort to restore normal intracellular and extracellular ionic balance [65, 66]. This initial acceleration in glucose uptake has been shown to persist for approximately 30 minutes after TBI in the injured cerebral cortex and hippocampus of rats [69]. However, after the initial period of increased glucose uptake, a period of depressed glucose utilization occurs creating a cellular energy crisis [65]. In humans, positron emission tomography scanning has shown that cerebral glucose metabolism decreases for 2-4 weeks after TBI [70]. This decrease in metabolism transpires because TBI diminishes cerebral blood flow, and the supply of glucose cannot meet the cellular demand for glucose within the traumatized brain [66]. When glucose supply is low, cognitive function is impaired [71, 72].

Mitochondrial dysfunction further contributes to the cellular energy crisis after TBI. Mitochondria produce energy (i.e., ATP) in cells via oxidative phosphorylation that occurs in the electron transport chain. After TBI, mitochondrial damage inhibits oxidative phosphorylation, and cells fail to generate ATP aerobically from the catabolism of glucose [73, 74]. Impaired mitochondrial function causes cells to rely more on anaerobic glycolysis for the generation of ATP. When anaerobic glycolysis is accelerated, lactate production increases and lactate accumulates. This phenomenon of increased lactate production and accumulation has been

observed following both ischemic [75] and concussive brain trauma [76]. Accumulation of lactate can disturb neuronal function by promoting acidosis, cell membrane damage, altered blood-brain barrier (BBB) permeability, and cerebral edema [65, 66]. However, lactate may also be beneficial for energy starved cells. It has been suggested that excess lactate produced by glial cells may be transported into neighboring neurons as an alternative fuel source to glucose [77]. A decreased reliance on oxidative metabolism and energy failure also transpire due to excessive intracellular calcium being sequestered in mitochondria [73]. Increased mitochondrial calcium concentrations facilitate the formation of reactive oxygen species (ROS) such as peroxynitrite and superoxide anion [65, 66, 78]. An increased production of ROS disrupts amino acid formation, increases lipid peroxidation, triggers DNA fragmentation, and leads to secondary neuron death [78].

Another component of TBI pathophysiology is neuroinflammation. Post-TBI cerebral inflammatory responses include leukocyte recruitment, up-regulation and release of various cytokines and chemokines, and microglial cell activation [79]. The BBB is damaged following TBI, and circulating neutrophils, monocytes, and lymphocytes infiltrate into the brain parenchyma during the acute post-TBI period [80-83]. These activated blood-borne immune cells secrete a variety of mediators including; prostaglandins, free radicals, complement factors, and pro-inflammatory cytokines and chemokines (e.g., tumor necrosis factor-alpha [TNF-α], interleukin-6 [IL-6], interleukin-one beta, monocyte chemoattractant-one protein, regulated on activation normal T-cell expressed and secreted, and inducible nitric oxide synthase) [84, 85]. These mediators are involved in the communication between resident and peripheral immune cells, which results in the mobilization of more immune cells and glial cells to the injury site(s) [62]. As time passes, increased production of anti-inflammatory cytokines and chemokines

eventually suppresses both humoral and cellular immune activation. Another response that occurs as a result of inflammation after TBI is the activation of resident microglial cells within the brain. Microglial cells are activated to remove cellular debris, inhibit inflammatory processes, and promote tissue remodeling [62]. However, some activated microglial cells release neurotoxic substances including glutamate, and reactive oxygen and nitrogen species [86]. The combination of leukocyte infiltration, increased expression of pro-inflammatory cytokines and chemokines, and exacerbated activation of microglial cells and astrocytes contributes to neuroinflammation, neuron death, and poor outcome following TBI [62, 83, 84].

Endogenous neuroprotective mechanisms are altered after TBI due to hypoxia and ischemia within the brain [87]. Hypotension, hypoxia, and ischemia are common secondary injury responses that have been associated with poor outcome and increased mortality rates post-TBI [87-89]. Cerebral hypotension and hypoxia have been reported to occur with a frequency of 44% in TBI patients [90]. Cellular damage resulting from secondary cerebral ischemia has been observed in more than 90% of patients that died from TBI [89]. Hypoxia and ischemia occur following the initial head trauma (i.e., primary injury response) that disrupts the BBB [91]. Disruption of the BBB allows for penetration of neurotoxic substances into the brain, resulting in vasogenic edema and increased intracranial pressure [89, 92]. Increased intracranial pressure reduces cerebral blood flow leading to secondary hypoxia and ischemia [88, 89]. Active neurons in the brain require an abundant amount of oxygen [93]. When oxygen supply is insufficient, significant stress is placed on cells within the brain since oxygen is required for glucose combustion, ATP production, and cell survival in aerobic organisms [93]. As a result of hypoxic stress, intracellular signal transduction pathways are activated to protect neurons and minimize cellular damage occurring post-TBI. In humans and other mammals, the transcriptional response

to hypoxic stress is regulated via hypoxia inducible factor-1 alpha (HIF-1α) [87, 94, 95]. HIF-1α is a transcription factor that directly activates many genes involved in translating proteins that enhance neuroprotection and brain repair; including, vascular endothelial growth factor – A (VEGF-A), erythropoietin (EPO), and heme oxygenase – 1 (HO-1) [87, 95, 96]. Although the molecular mechanisms are unclear, HIF-1α is also involved in production of the protein neuroglobin, which is thought to supply oxygen and protect neurons during periods of hypoxia [97-100]. An understanding of HIF-1α pathway activity and the functions of potentially neuroprotective proteins following TBI is important since pO_2 monitoring has demonstrated that hypoxia is a common secondary injury response in TBI patients [18].

1.4. NEUROPROTECTIVE PROTEINS AND TBI:

1.4.1. Hypoxia Inducible Factor (HIF)

Hypoxia Inducible Factor (HIF) is a heterodimeric protein consisting of an alpha subunit (HIF-α) and a beta subunit (HIF-β) [94, 95, 101]. Within the HIF family, three HIF-α subunits (HIF-1α, HIF-2α, and HIF-3α) have been identified [102]. HIF-2α and HIF-3α are only expressed within certain cells (e.g., endothelial cells, type II pneumocytes, renal interstitial cells, and liver parenchymal cells) [94]. In contrast, HIF-1α is known to be expressed throughout the body in all cell types, including neurons and glia within the brain [94, 103]. Nuclear factor-kappa beta (NF-κβ) and early growth response protein-1 (Egr-1) are transcription factors that directly regulate HIF-1α expression [104, 105]. HIF-1α is controlled post-translationally by a group of enzymes (prolyl hydroxylase 1 [PHD1], prolyl hydroxylase 2 [PHD2], and prolyl hydroxylase 3 [PHD3]) collectively known as prolyl hydroxylases (PHDs) [94, 101, 104, 106]. When oxygen is sufficiently available to cells (i.e., normoxia), PHDs degrade HIF-1α in the cytoplasm [94, 101, 106]. HIF-1α degradation occurs when PHDs catalyze the hydroxylation of

proline residues within the alpha sub-unit [94, 101, 104]. Following hydroxylation of the proline residues, von-Hippel Lindau (VHL) protein binds to HIF-1α. VHL binding marks the HIF-α sub-unit for proteosome degradation [106]. However, when oxygen supply is low during hypoxic stress, a reduction in the hydroxylation activity of PHDs occurs, and HIF-α accumulates in the cytoplasm of the cell [101, 106]. HIF-1α heterodimers are then formed when HIF-α sub-units escape proteosomal degradation by binding to HIF-β sub-units [101, 104]. HIF-1α may then translocate into the nucleus of cells and bind to hypoxia-response elements in the promoter region of downstream target genes [107]. The binding of HIF-1α to hypoxia-response elements initiates the transcription of many genes (e.g., VEGF-A, EPO, and HO-1) involved in supporting cell survival, neurogenesis, and angiogenesis [87, 95, 96]. In addition, several studies have reported that HIF-1α is required in conjunction with other proteins for inducing the up-regulated transcription of the neuroglobin gene during hypoxic conditions such as TBI [97-100]. HIF-1α has also been shown to increase the expression of cellular glucose transporters (GLUT-1 and GLUT-3), and the glycolytic pathway enzyme phosphofructokinase (PFK) [108-110].

Mouse models of TBI have demonstrated an endogenous increase in HIF-1α expression at 3 days post-TBI in the injured cortex of adult mice [87]. This post-TBI increase in HIF-1α is suggested to be a neuroprotective response [87]. Research has shown that neurons are protected when HIF-1α expression is increased by selectively inhibiting the activity of PHDs [111]. In contrast, inactivating HIF-1α within neurons further aggravates cellular deterioration following hypoxic brain injury [87, 103].

1.4.2. Vascular Endothelial Growth Factor-A (VEGF-A)

Vascular Endothelial Growth Factor-A (VEGF-A) is an important neuroprotective homodimeric glycoprotein in the HIF-1α pathway [94, 112-115]. HIF-1α is the transcription

factor that stimulates the production and release of VEGF-A from many cell types (e.g., endothelial cells, neurons, glial cells, macrophages, and cancer cells) during hypoxic cellular conditions [115-117]. Upon release from hypoxic cells, circulating VEGF-A proteins bind to the tyrosine kinase receptors (i.e., vascular endothelial growth factor receptor-1 [VEGFR-1] and vascular endothelial growth factor receptor-2 [VEGFR-2]), which are located on the surface of cells [118]. VEGFR-1 receptors are located on endothelial cells, neurons, hematopoietic stem cells, monocytes, and macrophages [118-120]. VEGFR-2 receptors are expressed on vascular and lymphatic endothelial cells, megakaryocytes, hematopoietic stem cells, and neurons [118, 119]. Dimerization and phosphorylation occur after VEGF-A binds to its receptor, and a number of intracellular signaling pathways (e.g., phosphatidylinositol-3-kinase/protein kinase B [PI3K/AKT], p38 mitogen-activated protein kinase [p38MAPK], and extracellular signal-regulated kinase [Raf/MEK/Erk]) are activated to promote the proliferation, migration, and survival of endothelial cells [118]. VEGF-A exerts these effects on endothelial cells to facilitate brain repair [87] and angiogenesis when cells are not receiving enough oxygen [114, 121]. In addition to the angiogenic effects of VEGF-A, studies also show that VEGF-A stimulates the growth of new neurons and inhibits neuronal apoptosis [122, 123].

An up-regulation in VEGF-A expression occurs within the brain following stroke [117] and TBI [87] due to cerebral hypoxia and ischemia. At 3 days post-TBI, significant increases in VEGF-A mRNA and protein expression have been observed in the cerebral cortex of mice [87]. Significant increases in VEGF-A protein levels have also been noted in the dentate gyrus of the hippocampus after TBI [124]. The increased production of VEGF-A in response to TBI is critical for promoting neuron survival, neurogenesis, and the development of new blood vessels in and around the lesion site [125, 126]. A VEGF-A mediated increase in angiogenesis restores

the supply of oxygen rich blood to damaged brain tissues, thus enhancing neuronal survival and accelerating healing [117]. Blocking the endogenous VEGF-A response with antibodies has been demonstrated to inhibit brain repair by impeding revascularization and astroglial proliferation [127]. Furthermore, inhibition of VEGFR-2 receptors prior to, and after TBI, leads to significantly larger lesion areas in the cerebral cortex and increased neuronal and glial cell death [128].

1.4.3. Erythropoietin (EPO)

Erythropoietin (EPO) is a glycoprotein comprised of four glycans that are attached to a single 165 amino acid chain [129]. The main site of EPO production is the kidneys [129, 130]. However, various cell types (e.g., neurons, astrocytes, oligodendrocytes, and microglial cells) in the brain also express EPO and its receptor [130-132]. EPO is vital for erythropoiesis, and the protein also has anti-apoptotic, anti-inflammatory, antioxidative, and angiogenic properties [129, 130, 132-134]. In addition, EPO appears to target neural stem cells, and thus has been suggested to play a role in neurogenesis [135]. In hypoxic and ischemic organs and tissues, EPO synthesis is up-regulated via HIF-1α [87, 131-133, 136]. The production of EPO is directly correlated with blood and oxygen supply in brain tissue. When the brain is lacking blood or oxygen, HIF-1α initiates an increased production of EPO to protect neurons [137]. To confer neuroprotection, EPO binds to the extracellular EPO receptor causing its homodimerization. Once EPO is bound to its receptor, Janus Kinase-2 (JAK2) is phosphorylated which leads to the subsequent activation of several downstream neurotrophic and neuroprotective signal transduction pathways including; NF-κβ, Ras-mitogen activated protein kinase (MAPK), phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT), and the transcription factor signal transducers and activators of transcription-5 (STAT-5) [130]. The JAK-STAT pathway plays a critical role in

EPO induced neuroprotection, and *in vivo* studies have shown that administration of a JAK2 inhibitor eliminates the neuroprotective effects of EPO [138, 139].

A large increase in EPO expression occurs in the brain of rodents following TBI [87, 140, 141]. Significant increases in cerebral cortex EPO mRNA and protein expression have been observed 3 days after inducing TBI in mice [87]. EPO has been shown to be neuroprotective in animal models of TBI [142], spinal cord injury [143], stroke [144], and autoimmune encephalomyelitis [145]. 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 [87]. In ischemic tissue, EPO diminishes the production of pro-inflammatory cytokines [146], and inhibiting the endogenous EPO response has been shown to worsen neuronal injury resulting from hypoxia and ischemia [147].

1.4.4. Heme Oxygenase-1 (HO-1)

Heme oxygenase-1 (HO-1) is an anti-oxidative and anti-inflammatory enzyme responsible for converting heme to biliverdin, carbon monoxide, and iron [148, 149]. These products of the HO-1 reaction have several important biochemical roles. Iron is an important substrate involved in the synthesis of new heme, and carbon monoxide and biliverdin function as signaling molecules and antioxidants [150]. HIF-1α, NF-κβ, MAPK, PI3K, and nuclear factor erythroid-2 related factor-2 (Nrf2) are transcription factors capable of inducing expression of the HO-1 gene [150-152]. High concentrations of HO-1 are found in the liver and spleen [153, 154]. During normoxic conditions, HO-1 is not expressed in the brain [150]. However, production of HO-1 occurs within the brain in response to heat shock, oxidative stress (e.g., hypoxia), cerebral ischemia, or glutamate excitotoxicity [150]. Acting as antioxidants, HO-1 and its products protect cells from oxidative stress, hypoxia, and ischemia [87]. It has been suggested that HO-1

protects neurons in the brain from ischemia/reperfusion injury by increasing production of brain derived neurotrophic factor (BDNF), and through up-regulation of the PI3K/AKT anti-apoptosis signal transduction pathway [155]. Additionally, HO-1 prevents cellular apoptosis by suppressing the activation of caspase-3 [155].

The expression of HO-1 increases in the injured cortex of mice after TBI [87]. HO-1 is induced in microglia and astrocytes subsequent to brain hemorrhage, which is a hallmark feature of TBI [156, 157]. Increased expression of HO-1 in microglia and astrocytes may aid in improving resistance to oxidative stress and secondary injury after TBI [157]. HO-1 is neuroprotective in many other pathophysiological conditions including; 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridinium (MPP+) neurotoxicity [149] and stroke [158]. Disruption of endogenous HO-1 responses has been linked to worse outcomes in many central nervous system disorders [148]. However, pharmacological induction of HO-1 has elicited beneficial effects in many pathologic states including; inflammatory processes, atherosclerosis, carcinogenesis, ischemia-reperfusion injury, and degenerative diseases [152]. In addition, the neuroprotective effects of antioxidants including resveratrol and sulforaphane depend on induction of HO-1 [159, 160].

1.4.5. Neuroglobin

Neuroglobin is the most recently characterized member of the globin family (e.g., hemoglobin, myoglobin, cytoglobin, and neuroglobin) of genes that maintain cellular oxygen homeostasis. Neuroglobin is an intracellular monomeric hypoxia-inducible protein that binds and delivers oxygen to brain tissue [161]. In vertebrates, neuroglobin is widely expressed throughout the peripheral and central nervous systems [161-164]. Neuroglobin is present in the cytoplasm of neurons and astrocytes in distinct brain regions including the cerebral cortex,

hippocampus, amygdala, thalamus, hypothalamus, cerebellum, and medulla oblongata [162, 165, 166]. The expression of neuroglobin in neurons and astrocytes is low under normal physiological conditions [161]. However, neuroglobin expression increases in hypoxic neurons [100], and in reactive astrocytes located within regions of the brain near pathologies in mouse models of TBI, cerebral malaria, and experimental autoimmune encephalitis [166]. The mechanisms by which neuroglobin is induced have not been fully elucidated, although correlative evidence suggests that HIF-1α is involved in the induction of neuroglobin in response to hypoxic stress. *In vitro* research has shown that decreasing HIF-1α reduces neuroglobin protein expression, while forced overexpression of HIF-1α increases neuroglobin levels in cultured neurons [98]. An *in vivo* study demonstrated that endogenous HIF-1α suppression significantly attenuates hypoxia-induced increases in neuroglobin protein expression [99]. How HIF-1α modulates neuroglobin gene expression remains unclear because unlike VEGF-A and EPO, there are no conserved hypoxia-response elements for HIF-1α binding in the promoter region of the neuroglobin gene [98]. However, NF-κβ is known to interact with the neuroglobin promoter in mice [99], and it has been proposed that HIF-1α may form a complex with NF-κβ to initiate the transcription of neuroglobin indirectly during hypoxic conditions [97]. Egr1 and specificity protein-1 (Sp1) are other transcription factors capable of regulating neuroglobin expression [99]. Once translated, intracellular neuroglobin protein exerts many neuroprotective effects via different molecular mechanisms which are not completely understood.

An increased endogenous production of neuroglobin is observed after hypoxia and ischemia, and this response augments neuron survival [167]. Thus, neuroglobin is believed to be neuroprotective in experimental models of stroke [100, 168] and TBI [164, 169]. During hypoxic and ischemic conditions, neuroglobin plays a key role in the detoxification of reactive

oxygen and nitrogen species, and apoptosis protection [161, 170-172]. An enhanced formation of reactive oxygen and nitrogen species is known to occur when cytochrome c is released into the cytosol from the mitochondrial electron transport chain [170]. The release of cytochrome c leads to activation of caspase enzymes that interact with apoptotic protease-activating factor 1 (APAF-1) and initiate apoptosis [170]. Neuroglobin protects neurons from reactive oxygen and nitrogen species damage, and inhibits intrinsic apoptosis pathways by preventing the release of cytochrome c from mitochondria [170, 171, 173]. In addition, elevating neuroglobin concentrations in cultured human neuronal cells has been shown to decrease oxidative stress via activation of phosphoinositide-3 kinase (PI3K), and increase ATP levels by activating mitochondrial ATP sensitive potassium channels [174]. Neuroglobin also positively modulates metal homeostasis in hypoxic neurons that exhibit increased intracellular calcium, iron, copper, and zinc levels [175]. Increased concentrations of these metals causes inflammation, mitochondrial dysfunction, uncontrolled reactive oxygen species formation, altered neurotransmitter release, neurotoxicity and cell death [175]. Neuroglobin prevents calcium influx and decreases the cellular uptake of iron, copper, and zinc which ultimately enhances cell viability and inhibits necrosis and apoptosis [175].

1.4.6. Brain-Derived Neurotrophic Factor (BDNF)

Brain-derived neurotrophic factor (BDNF) is an intracellular protein manufactured by the endoplasmic reticulum of cells located within the brain, retina, motor neurons, heart and spleen [176-179]. In the brain, BDNF is highly active in regions that are important for learning and memory including; the cerebral cortex, hippocampus, and basal forebrain [180, 181]. An influx of intracellular Ca^{2+}, and the ensuing membrane depolarization, induces the transcription of BDNF via activation of cyclic adenosine monophosphate response element-binding protein

(CREB) family transcription factors [182]. Furthermore, HO-1 was recently shown to up-regulate BDNF protein production in the hippocampus, although the exact signaling pathway involved in the activation of BDNF via HO-1 remains unknown [155]. Once translated, BDNF proteins can bind to tyrosine kinase B (TrkB) receptors, and several intracellular signaling pathways (i.e., PI3K/AKT and MAPK) are triggered which facilitates neurogenesis, synaptogenesis, neuronal survival, and neuroprotection from trauma and disease [183-185]. In addition, the BDNF protein is involved in nervous system development, and regulating synaptic plasticity in the brain of adults by stimulating the movement of axons in conjunction with adjusting the size and number of dendrite spines projecting from neurons [186].

Rodent models of TBI demonstrate that BDNF mRNA expression is increased in the cerebral cortex and hippocampus hours after brain injury [187, 188]. Increases in BDNF protein have also been observed within the cerebral cortex and hippocampus for several days after TBI [187, 189]. A study by Rostami et al., revealed an even longer period of increased BDNF protein expression in brain regions contralateral to the injury site for up to 2 weeks following penetrating TBI [190]. The up-regulation of BDNF after injury is thought to be a neuroprotective and neuroregenerative response, as BDNF is involved in repairing neurons and restoring neural connectivity in damaged regions of the brain [185, 191].

1.4.7. Insulin-Like Growth Factor-1 (IGF-1)

Insulin-like growth factor-1 (IGF-1) is a growth factor that is structurally related to pro-insulin, and has been shown to be a neuroprotective protein that is involved in brain development and neuron survival [192-194]. The liver, brain, muscles, and skin all produce IGF-1 in response to growth hormone release from the anterior pituitary gland [195, 196]. However, 70% of the total circulating IGF-1 within the body is produced by the liver hepatocytes [195]. Circulating

IGF-1 can enter the brain and bind to both neuronal IGF-1 receptors and insulin receptors [195]. Once IGF-1 is bound to its receptor, multiple intracellular signal transduction cascades involved in cell proliferation, cell survival, and metabolism are activated including the PI3K/AKT pathway, ERK/MAPK pathway, and the c-Src non-receptor tyrosine kinase [197].

There is a direct correlation between a low concentration of circulating IGF-1 and decreased cognitive function in healthy elderly individuals [198]. Gunnell et al., conducted a longitudinal study of children and found that there is a positive correlation between serum IGF-1 concentration and intelligence quotient [199]. The results from this study suggest that IGF-1 is a critical player in brain development and cognitive function. Relationships also exist between IGF-1 levels and cognitive function after TBI. Head trauma can disrupt pituitary gland function, resulting in hypopituitarism, which reduces the secretion of growth hormone into the blood and significantly decreases circulating levels of IGF-1 [40, 200]. The concentration of IGF-1 in both serum and cerebrospinal fluid is decreased in adult patients with severe TBI [201], and hippocampal neuron loss and cognitive dysfunction have been associated with a decreased concentration of circulating IGF-1 after TBI in rats [194]. However, in contrast to the decrease observed in circulating IGF-1 levels post-TBI, the expression of IGF-1 in the brain increases as a neuroprotective response to head injury [194].

1.5. PROGNOSIS:

Prognosis for recovery from TBI is highly dependent upon the severity of injury. Mild TBI symptoms typically resolve within a few weeks or months after injury, and permanent or long-term disability rarely results [202]. Athletes and younger individuals may recover from mild TBI within a few days [22]. Outcomes are worse in patients diagnosed with moderate and severe TBI. After these patients have been discharged from the hospital, a Glasgow Outcome

Scale (GOS) score is generally determined by a clinician at one or multiple time points during the recovery process. The GOS is a five level scale often used to objectively evaluate recovery in patients that sustain more severe head trauma [203, 204]. The GOS also assists with predicting the course of rehabilitation, and return to activities of daily living and work. Possible outcomes on the five level GOS are good recovery (i.e., return to previous level of function), moderate disability (i.e., patient is capable of self-care), severe disability (i.e., patient is incapable of self-care), persistent vegetative state (i.e., no cognitive function), and death. An eight level extended version of the GOS was also developed to further classify global outcome in TBI survivors [205]. The risk of death or severe disability after moderate TBI is estimated to occur in 10%-20% of cases [27]. The mortality rate for patients diagnosed with severe TBI is approximately 25%-33% in the United States [4]. However, greater than 50% of adults diagnosed with severe TBI return to their previous level of function or have moderate disability [4]. Severe TBI patients that are older than 65 years have a 72% increased risk of death in comparison to younger patients [206]. Severe TBI mortality rates are lower in children over the age of 5 years when compared to adults, and children display better immediate and long-term recovery irrespective of injury severity [207]. In TBI survivors, 85% of recovery from moderate and severe TBI occurs during the initial 6 months after injury [63]. Small improvements in neurological function continue over the course of several years [4]. During recovery from TBI, cognitive deficits (e.g., impaired attention, concentration, and memory) are a more common cause of disability in comparison to sensory impairments or motor dysfunction [4]. Finnanger et al., observed significant deficits in cognitive function among moderate and severe TBI patients 1 year after injury [208]. This study also reported that motor function was reduced only in severe TBI patients at 1 year post-injury [208]. Rehabilitation is important for recovery of cognitive

and motor function in TBI patients with persistent deficits. In addition, non-pharmacological and pharmacological treatments aimed at minimizing secondary injury responses can improve overall prognosis.

1.6. POST-TBI TREATMENT:

1.6.1. Treatment Goals and Concerns

Unfortunately, with the exception of routine medical intervention and care, no successful treatment options are currently available for promoting functional recovery from TBI in humans [209]. Preventing the progression of secondary injury responses is the main goal of treatment during the acute stage of TBI since damage occurring from the initial head trauma is not reversible [209]. The primary concerns during this stage are controlling raised intracranial pressure, maintaining adequate cerebral blood flow, and ensuring proper oxygen supply to the brain [4]. Once a patient is medically stable, rehabilitation may be needed if neurological deficits persist. Major persistent neurological deficits have been observed in 50% of TBI patients whose coma exceeds 24 hours, and a prolonged period of rehabilitation is often required for these individuals [4]. Early onset and continuous rehabilitation is critical for optimizing long-term improvements in cognitive and motor function in TBI patients [210]. A multidisciplinary team (e.g., neurologist, neuropsychologist, physical therapist, occupational therapist, and speech and language pathologist) provides the best approach toward rehabilitation. Minimizing the extent of brain injury, preventing complications, and promoting functional recovery through stimulating mental and physical activities are primary rehabilitation goals [210]. Numerous therapeutic approaches including the use of hyperventilation, hypothermia, surgery, pharmacological agents, and physical therapy are currently utilized or being investigated

for TBI management, treatment, and rehabilitation.

1.6.2. Hyperventilation

Hyperventilation may be used initially to decrease rising intracranial pressure [4]. An increase in the rate of breathing results in more carbon dioxide (CO_2) being expired, and this increase in expiration reduces blood CO_2 levels (hypocapnia) and promotes blood vessel constriction. Vasoconstriction decreases blood flow to the brain, which in turn, reduces intracranial pressure. Hyperventilation is only used briefly to treat TBI because hypoxia and ischemia occur if brain blood flow is reduced for an extended period of time [4].

1.6.3. Hypothermia

Between 1980 and 2009, 27 large phase III clinical trials were published for TBI, but the only studies reporting a significant treatment effect involved the use of hypothermia (cooling to 32 °C) and surgery (e.g., decompressive craniectomy) to relieve brain swelling and control increased intracranial pressure [211]. Hypothermia protects the brain through several mechanisms, including; reduction of brain thermopooling, preservation of protein synthesis, blockade of excitotoxic mechanisms, calcium antagonism, reduction in brain metabolic rate and demand for oxygen, altered cerebral blood flow and inflammatory responses, a decrease in edema, neuroprotection of white matter, and inhibition of cell death via apoptosis [209]. Mortality risk is reduced, and neurological outcomes are better when hypothermia is maintained for 48 hours [212]. However, this extended period of hypothermia significantly increases the risk of pneumonia [212]. Therefore, optional and cautious use of hypothermia for adult TBI patients has been recommended by The Brain Trauma Foundation/American Association of Neurological Surgeons guidelines task force [212].

1.6.4. Surgery

In addition to hypothermia, decompressive craniectomy may be performed to reduce dangerously increased intracranial pressure in more severe TBI cases. A bone flap is surgically removed from the skull (bone flap is replaced later) during this procedure which allows for outward brain swelling, and a subsequent reduction in intracranial pressure [4]. Although decompressive craniectomy is effective for managing TBI during the acute stage, one study found that patients who received this surgical intervention had worse extended GOS scores at six months post-TBI when compared to patients who received standard care (i.e., use of barbiturates and mild hypothermia) [213]. In addition, this study reported that patients undergoing decompressive craniectomy had a significantly greater risk of having unfavorable outcomes following TBI versus patients receiving non-surgical treatment [213]. Other surgical procedures may be performed to eliminate large hematomas causing a shift in brain structures, or to remove objects from the brain following a penetrating head injury [27].

1.6.5. Pharmacological Agents

Pharmacological interventions have been used to manage TBI during the acute stage. Although many pharmacological agents have shown beneficial treatment effects in animal models of TBI, no Food and Drug Administration approved drugs currently exist that have been conclusively proven to alleviate secondary injury responses and improve outcomes in TBI patients. Preclinical studies aimed at targeting secondary injury responses in animal models of TBI have investigated the efficacy of various pharmacological agents, including; NMDA antagonists, excitatory amino acid inhibitors, corticosteroids, free radical scavengers, magnesium sulfate, calcium channel blockers, mannitol, and progesterone [214]. The corticosteroids dexamethasone and methylprednisolone were used for three decades in an effort to reduce

intracranial pressure following TBI [209]. However, a randomized clinical trial reported a significantly increased risk of death or severe disability among TBI patients treated with corticosteroids [215]. The results of this study support the conclusion that corticosteroids should no longer be administered to TBI patients [215]. Pharmacological treatment with the osmotic diuretic mannitol is sometimes effective at reducing brain swelling after TBI [209]. However, TBI causes blood-brain barrier (BBB) disruption [91], which can magnify the effects of mannitol, leading to rebound edema and increased intracranial pressure [216, 217]. Research investigating progesterone for the treatment of TBI appears more promising. Animal studies show that progesterone administration decreases brain edema, enhances antioxidant responses, and reduces excitotoxicity after injury, and clinical trials provide evidence that progesterone administration reduces disability and mortality rates post-TBI [218].

Positive effects have been noted in rodents following exogenous VEGF-A treatment for ischemic brain injury and TBI. Intracerebroventricular infusion of recombinant human vascular endothelial growth factor (rhVEGF-A) reduces infarct volume and brain edema in rats subsequent to brief cerebral ischemia [117]. After TBI, research has confirmed significant 90 day improvements in recovery rate and functional outcome in mice treated exogenously (i.e., intracerebroventricular infusion) with rhVEGF-A [122]. Delivering VEGF-A to the central nervous system through other exogenous methods (i.e., intravenously) is very difficult due to the large molecular weight, limited BBB permeability, and extremely short half-life of the protein in plasma [219]. One study did demonstrate that intranasal administration allowed VEGF-A to bypass the BBB and reach many cortical and sub-cortical regions in the rat brain [219].

Exogenous administration of EPO is showing promise as a neuroprotective and neurorestorative treatment approach for brain injuries. Following intraperitoneal injection,

recombinant human erythropoietin (rhEPO) has been shown to cross the BBB via active transport and protect against brain injury [209]. Rats injected with rhEPO after TBI have exhibited a significant decrease in brain edema, and a reduction in the number of infiltrating apoptotic monocyte chemotactic protein-1^{+} and $CD68^{+}$ cells in comparison to saline injected controls [137]. A single dose injection of 5,000 IU/kg of rhEPO administered at 1 and 24 hours post-TBI significantly improved sensorimotor and cognitive recovery, reduced white matter damage, attenuated neuroinflammation, and increased the expression of the EPO receptor in a rat model of diffuse TBI and hypoxia [220]. In addition, neurogenesis within the hippocampus and cortex of mice has been shown to significantly increase following post-TBI rhEPO injections [209]. Exogenous rhEPO treatments have improved histological (i.e., reduced contusion volume and decreased neuron cell death post-TBI) and functional (i.e., improved sensorimotor and spatial learning memory scores) outcomes following TBI in mice [133]. Bouzat et al., determined that intravenous rhEPO administration provided at 30 minutes post-TBI, improved local brain oxygen saturation in rats by reopening collapsed capillaries and redistributing blood flow [221]. In clinical trials, the administration of EPO has been demonstrated to improve neurological outcomes in patients with a variety of neurological conditions including; ischemic stroke [222], multiple sclerosis [223], schizophrenia [224], and out-of-hospital cardiac arrest [225]. Clinical trials are continuing to investigate exogenous EPO administration for treatment of TBI [209, 226]. One phase III clinical trial revealed that a 40,000 IU dose of EPO injected subcutaneously once per week for a duration of three weeks, significantly reduced mortality rates compared to placebo in intensive care unit trauma patients (including many diagnosed with severe TBI) [226]. In contrast to these positive findings, it has been suggested that exogenous EPO administration may increase the risk of thrombosis in TBI patients [227]. Unfortunately,

evidence is lacking regarding the safety and efficacy of exogenous EPO treatments in TBI patients.

Neurological damage may be minimized, and recovery improved, through the use of exogenous pharmacological interventions that up-regulate neuroglobin post-TBI. Unfortunately, direct exogenous administration of neuroglobin is an impractical treatment option because neuroglobin is an intracellular protein that is not capable of crossing cell membranes [228]. However, administration of the iron chelator deferoxamine has been reported to increase endogenous neuroglobin production in previous research [228]. Deferoxamine enhances neuroglobin production by increasing levels of hemin, an oxidation product of heme [228]. Hemin operates as a transcription factor for neuroglobin production through the soluble guanylate cyclase-protein kinase G (sGC-PKG) pathway [229]. Deferoxamine also increases the expression of hypoxia-inducible transcription factors (i.e., HIF-1α and HIF-2α) that play a role in up-regulating neuroglobin production in cortical neurons [228-230]. Although deferoxamine is frequently prescribed to treat iron poisoning in children [231], several studies suggest that the drug may be neuroprotective in animal models of TBI [232-234]. Deferoxamine has been shown to significantly attenuate brain atrophy, improve spatial learning and memory, and down-regulate expression of proteins (i.e., ferritin L, ferritin H, transferrin, and transient receptor potential canonical channel 6) associated with the metabolism of iron after TBI in rats [232]. These pre-clinical studies demonstrate positive results for the use of deferoxamine in TBI treatment. However, none of these studies investigated neuroglobin gene and protein expression after treatment with deferoxamine, and clinical TBI studies need to be conducted.

Cinnamic acid and valproic acid are other chemical compounds that have been previously shown to up-regulate neuroglobin production [228]. Cinnamic acid is obtained from cinnamon

oil, and valproic acid is used as an anti-epileptic drug, and for treating early post-traumatic seizures in TBI patients [235]. *In vitro* studies have demonstrated that cinnamic acid and valproic acid induce neuroglobin protein production in cultured neurons [228]. No *in vivo* TBI studies have been conducted to determine whether exogenous administration of cinnamic acid or valproic acid induces neuroglobin production in the brain, and the mechanisms involved in the induction of neuroglobin after administration of these compounds have not been elucidated. Cinnamic acid has been shown to reduce glutamate toxicity in rat cortical neuron cultures [236], and valproic acid is neuroprotective in animal models of cerebral ischemia [237]. However, neither of these studies determined whether or not induction of neuroglobin was directly responsible for the neuroprotective effects of cinnamic acid and valproic acid. The intracellular production of neuroglobin is increased in response to hypoxia and ischemia, and neuron survival is augmented as a result [167]. Therefore, post-TBI pharmacological interventions that increase intracellular neuroglobin production in the brain may enhance neuroprotection and improve TBI outcomes. More research is needed to identify safe and effective pharmacological treatments that increase endogenous neuroglobin production and improve recovery from TBI.

1.6.6. Physical Therapy

In conjunction with cognitive rehabilitation therapy, speech and language therapy, and treatment of neuropsychiatric symptoms (e.g., emotional distress and clinical depression), physical therapy is an important part of TBI recovery. One hour of physical therapy performed up to three times per week is common during the post-acute phase of TBI recovery [238]. Due to the heterogeneity of TBI symptoms experienced between individuals, a variety of physical therapy approaches are used in rehabilitation. Physical therapy sessions typically include individualized training of gross motor skills, strength, flexibility, and endurance training [238].

The majority of post-TBI physical therapy interventions target specific impairments, including; balance, gait kinematics, spasticity, flexibility, muscle force production, and functional skills [238, 239]. Gait training is an important part of restoring walking ability, and it is imperative that TBI patients train walking ability under everyday conditions. Patients should practice conventional over-ground gait training by walking on a variety of different surfaces; uneven ground, over obstacles, in different lighting conditions, in crowds of people, and up and down slopes [240]. Research has demonstrated that conventional over-ground gait training is more effective than partial body weight supported treadmill training for improving gait symmetry in persons with TBI [241]. However, high intensity partial body weight supported treadmill training is a unique exercise intervention that can improve the often overlooked cardiorespiratory fitness of TBI patients with severe balance and gait deficits [242]. A 20% average increase in aerobic capacity has been reported in TBI patients that performed partial body weight supported treadmill training 2-3 times per week at an intensity approximating 60-75% of age-predicted maximal heart rate [242]. In addition to the use of partial body weight supported treadmill training, virtual reality training is another novel rehabilitative approach that can improve outcomes in TBI patients [243, 244]. Gait and balance training performed in a virtual reality environment has been shown to significantly improve balance and mobility in TBI patients to a greater extent than traditional gait and balance training in a non-virtual reality environment [243]. A study conducted by Grealy et al., revealed that the use of virtual reality and exercise improved cognitive function following TBI in adults ranging from 17-60 years of age [244]. Limited physical therapy options are available for severe TBI patients in a coma or persistent vegetative state. However, sensory stimulation programs may be initiated for these individuals [240]. The goal of sensory stimulation is to accelerate the rate at which a patient

returns to consciousness, although evidence is lacking regarding the efficacy of this method [240]. Currently, there are no standardized physical therapy recommendations for individuals with TBI. More evidence based research needs to be conducted in order to identify viable physical therapy interventions that have the potential to minimize impairments and improve TBI outcomes.

1.7. GENE THERAPY AND TARGETING NEUROGLOBIN TO IMPROVE TBI OUTCOMES:

Neuroglobin is an intracellular protein that is unable to cross cell membranes [228]. Therefore, direct administration of neuroglobin is not a viable treatment approach for TBI and other hypoxic conditions. However, overexpression of the neuroglobin gene is an alternative strategy for stimulating increased intracellular protein production and activity. This form of gene therapy shows potential for enhancing neuroprotection, and improving stroke and TBI outcomes. In a mouse model of ischemic stroke, mice that overexpressed neuroglobin showed a 30% reduction in cerebral infarct volume after middle cerebral artery occlusion when compared to wild type mice [245]. Only a few studies have investigated neuroglobin overexpression and TBI outcomes [164, 169]. Transgenic mice and rats that overexpressed neuroglobin have displayed significantly smaller cortical lesion volumes, and reduced neuron necrosis and apoptosis after TBI in comparison to controls [164, 169]. Interestingly, in TBI patients, genetic polymorphisms in neuroglobin have been shown to positively affect recovery from injury as indicated by better GOS scores at 3, 6, 12, and 24 months post-TBI [246]. In addition to identifying pharmacological interventions that up-regulate intracellular neuroglobin expression, more gene therapy studies are needed to determine whether neuroglobin overexpression improves TBI outcomes. Little is known regarding the regulatory mechanisms of neuroglobin expression

during hypoxic conditions such as TBI. Consequently, transgenic mouse models should be used to further explore neuroglobin, and the molecular mechanisms involved in promoting neuroprotection and improved TBI recovery.

1.8. EXERCISE AND TBI:

1.8.1. Exercise and Brain Fitness

Exercise participation has been associated with the reduction of numerous physical (e.g., cardiovascular disease, obesity, type II diabetes, osteoporosis, colon cancer, and breast cancer), and mental disorders (e.g., depression and anxiety) throughout life [238, 247]. However, members of the scientific community have just begun to understand the benefits of exercise on brain health over the past couple of decades. Exercise positively affects the central nervous system, and displays great potential for enhancing brain fitness throughout life [247]. 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 [248, 249]. Routine exercise improves cognition, prevents cognitive decline, and improves motor function [250-253]. Previous studies have demonstrated that exercise increases hippocampal neurogenesis and vascularization, elevates growth factors in areas of the brain responsible for memory, enhances neuronal survival following brain insults, and promotes functional recovery from central nervous system diseases and injuries including TBI [184, 253-260]. In addition, exercise reduces neuroinflammation [261, 262] and markers of oxidative stress after TBI [263, 264]. Depending on the intensity, exercise can increase cerebral blood flow [265] and tissue oxygenation [93, 266] while simultaneously improving cerebral glucose metabolism [267]. These adaptations and responses to exercise may protect neurons in the brain, enhance brain function, and improve overall outcomes after TBI.

Since all clinical trials have failed to find a safe and effective treatment for TBI, questions have been raised regarding the interventions currently used for TBI treatment. Exercise may be the most practical non-invasive measure for modulating secondary TBI responses, increasing neuroprotection, minimizing post-TBI complications, and improving overall outcome. However, many of the mechanisms underlying the beneficial effects of physical activity on promoting recovery from TBI remain poorly understood due to a lack of research in this area.

1.8.2. Exercise and TBI Complications

It is clear that many long-term health benefits are derived from routine exercise participation throughout life. Therefore, emphasis should be placed on increasing physical activity to prevent the development of complications and chronic disease after TBI. Patients admitted to the hospital with moderate to severe head injuries endure a period of bed rest and immobility which increases stroke risk due to the development of thrombi and emboli [35]. However, there is evidence that exercise reduces the risk of thrombi and emboli formation [268]. Prolonged bed rest has major negative consequences on the cardiovascular, pulmonary, and muscular systems that are involved in oxygen delivery and utilization [269]. Bed rest decreases the efficiency of these systems to deliver and utilize oxygen, and studies have indicated that the peak aerobic capacity of TBI patients is typically between 65-74% of normal [270-272]. In contrast to bed rest, chronic exercise enhances the organ systems involved in oxygen delivery and utilization. Chronic aerobic exercise improves cardiovascular and pulmonary function by decreasing resting heart rate, and increasing stroke volume, cardiac output, pulmonary ventilation, and maximal oxygen consumption (VO2max) [238]. In addition, long-term aerobic exercise training also increases skeletal muscle capillary density and skeletal muscle oxygen

extraction. These peripheral adaptations combine with cardiovascular and pulmonary adaptations to further improve physical and metabolic work capacity [238]. Exercise training adaptations have the potential to reverse or minimize the negative effects observed on oxygen delivery systems as a result of prolonged post-TBI bed rest. Furthermore, an improved fitness level may enable patients to resist fatigue that is common after TBI.

It is important to consider post-TBI endocrine responses and adaptations to exercise since hypopituitarism has been reported in 16-56% of patients diagnosed with moderate to severe TBI [273]. Growth hormone and testosterone levels are known to be deficient in TBI patients [238], but both aerobic endurance exercise and resistance training increase the production of growth hormone and testosterone [274-276]. Long-term exercise training may minimize the effects of hypopituitarism after TBI by positively influencing endocrine system function.

Various psychiatric conditions (e.g., depression, generalized anxiety disorders, and aggressive behavior) are often evident in TBI survivors [42, 43]. However, performing routine aerobic exercise has been associated with reductions in stress, cynical distrust, anger, and depression in a study of over 3200 healthy adults [277]. In TBI patients, it has been suggested that improvements in mood and emotion occur with consistent exercise training [278]. Poor sleep is another common TBI complication [279]. Nonetheless, regular aerobic exercise has been shown to increase self-reported sleep quality scores in older adults [280], but exercise and sleep studies have not been conducted in the TBI population.

TBI is a risk factor for the accelerated onset and development of neurodegenerative diseases including Alzheimer's and Parkinson's [55, 56]. On the other hand, being more physically active is associated with reduced Alzheimer's [281] and Parkinson's [282] disease risk. Cognitive and motor deficits resulting from the 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 [250-255]. Exercise appears to be a viable non-pharmacological approach for minimizing many post-TBI complications and improving overall outcome.

1.8.3. Exercise and Secondary TBI Responses

Glutamate excitotoxicity and the concomitant rise in intracellular calcium contribute to neuron cell death and dysfunction after TBI [64, 66]. However, studies have shown that chronic exercise preconditioning protects the brain from excitotoxic events in various experimental animal models of ischemia and epilepsy [283-285]. In a recent study, chronic exercise (i.e., swimming performed for 30 minutes/day, 5 days/week, for 8 weeks) group rats displayed decreased glutamate release and percentage of cell death in comparison to sedentary group rats after rat hippocampal sections were submitted to *in vitro* oxygen and glucose deprivation [285]. In addition, exercise group rats demonstrated a significant decrease in the hippocampal production and release of pro-apoptotic markers including; caspase-8, caspase-9, caspase-3, and apoptosis-inducing factor (AIF) [285]. This decrease in the production and release of pro-apoptotic markers was associated with the decreased percentage of hippocampal cell death observed in exercise group rats [285]. It is possible that exercise preconditioning enhances the ability of neurons to endure post-TBI disturbances in glutamate production, release, and uptake. No research has been conducted with the purpose of understanding the mechanisms involved in exercise induced neuroprotection and alterations in post-TBI glutamatergic transmission within the brain.

Animal models of TBI demonstrate that cerebral blood flow may be reduced to 50% of normal after injury [66, 286]. As mentioned previously, diminished cerebral blood flow

contributes to hypoxia and ischemia, decreased glucose metabolism, and increased cell death or injury post-TBI [66, 87, 89]. In contrast to TBI, exercise can positively affect cerebral blood flow, oxygenation, and glucose metabolism. Low and moderate intensity (i.e., $\leq$ 60% VO2max) exercise has been shown to increase cerebral blood flow in humans [265]. One major factor contributing to alterations in cerebral blood flow during exercise is carbon dioxide. During low and moderate intensity exercise, an increased partial pressure of carbon dioxide (pCO_2) in the blood causes vasodilation, and cerebral blood flow is concurrently increased [265]. The uptake of oxygen in the brain is important for maintaining cerebral neuronal activity, and exercise has the potential to improve tissue oxygenation within the brain [266]. Significant elevations (i.e., 10-20% above resting level) in regional tissue oxygen pressure (pO_2) within the rat hippocampus have been observed during and after low intensity (i.e., 52% of VO2max) swimming [93]. In addition, exercising rats have shown significantly increased cerebral glucose metabolism in comparison to non-exercising controls [267]. In this study conducted by Kinni et al., exercise rats exhibited significant increases in mRNA and protein expression of GLUT-1 and GLUT-3, glycolytic enzymes (i.e., lactate dehydrogenase and PFK), and phosphorylated adenosine monophosphate kinase (AMPK) activity when compared to sedentary rats [267]. Moreover, cerebral bio-energetic improvements due to exercise training have been noted in animal models of stroke. Rats performing three weeks of treadmill exercise prior to induction of ischemic stroke have displayed significantly increased rates of post-stroke cerebral glucose metabolism accompanied with a more rapid and substantial increase in ATP production when compared to non-exercising rats [287]. Results from this study revealed that cerebral AMPK and HIF-1α mRNA and protein expression were increased in response to both exercise and hypoxia [287]. AMPK is known to play a role in cellular energy homeostasis, and AMPK has been shown to

increase PFK and HIF-1α expression after being induced by exercise or hypoxia [288, 289]. HIF-1α is known to increase the expression GLUT-1, GLUT-3, and PFK [108-110]. GLUT-1 and GLUT-3 are responsible for the uptake of glucose into cells, and PFK plays a key role in regulating glycolysis and ATP production by breaking down glucose into pyruvate [110, 287]. The combined changes occurring in this pathway are an adaptation to exercise that ultimately leads to increased oxidative phosphorylation and ATP production in neurons. Increased cerebral blood flow, tissue oxygenation, and improved glucose metabolism are positive responses to exercise that may lead to greater neuron survival during hypoxic and ischemic conditions such as stroke and TBI.

Mitochondrial function is negatively altered as a result of TBI. Increased reactive oxygen species formation and the impaired antioxidant capacity of damaged mitochondria combine to promote oxidative stress and neurodegeneration after injury [78]. Mitochondrial dysfunction also compromises ATP production in the brain, which ultimately increases neuronal death via apoptotic signaling [73, 74]. However, exercise elicits many beneficial mitochondrial adaptations that oppose TBI responses and lead to neuroprotection and improved brain function. Regular long-term exercise performed at a moderate intensity is known to reduce markers of oxidative stress in the rat brain [290, 291]. Aging rats engaged in long-term (i.e., 15 weeks) aerobic exercise displayed a 28% reduction in the hippocampal level of ROS, and less oxidative damage, when compared to sedentary controls [291]. In this study, the reductions in ROS and oxidative stress following exercise training were attributed to decreased protein carbonyls and increased intracellular levels of the antioxidant enzymes; superoxide dismutase-1 (SOD-1), superoxide dismutase-2 (SOD-2), and glutathione peroxidase [291]. These positive exercise effects have also been noted in TBI studies. Several TBI studies have revealed chronic aerobic

exercise training decreases markers of oxidative stress in the brain post-injury, and protects against TBI induced reductions in antioxidant activity [263, 264]. The AMPK/peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) pathway may be responsible for exercise induced activation of antioxidant enzymes and long-term resistance to oxidative damage [291]. AMPK is known to initiate the transcription of PGC-1α in visual cortical neurons [292], and exercise enhances AMPK activity and increases PGC-1α levels in the brain and skeletal muscles [287, 291, 293, 294]. Bayod et al., demonstrated that long-term exercise training activated AMPK, increased PGC-1α expression, and stimulated mitochondrial biogenesis in the dentate gyrus of rats [294]. These results are consistent with other rodent and human studies showing exercise promotes mitochondrial biogenesis, and energy metabolism is augmented in the brain and skeletal muscles via increased PGC-1α expression [295-298]. Furthermore, rats engaged in 7 days of post-stroke exercise have displayed increased PGC-1α levels and improved mitochondrial biogenesis in the brain [299]. The exercise induced changes observed in this study were associated with smaller cerebral infarct volumes and a decrease in neurological deficits post-stroke [299]. It is likely that exercise mitigates hypoxic and ischemic damage to neurons by increasing the overall number, and improving the function, of mitochondria in the brain [299]. Moreover, exercise may improve recovery from TBI by decreasing ROS formation, increasing antioxidant enzymes, and up-regulating molecular pathways involved in triggering mitochondrial biogenesis and enhanced energy metabolism.

Neuroinflammation is a common post-TBI secondary injury response. Extravasated blood products, increased leukocyte recruitment, release of pro-inflammatory cytokines and chemokines, and microglial cell activation are hallmark features of the post-TBI neuroinflammatory response [79]. Although TBI promotes neuroinflammation, regular exercise

seems to have an anti-inflammatory effect [300]. Exercise is known to decrease inflammation by altering cytokine production and release. IL-6 is released from contracting muscles during exercise, and IL-6 stimulates the appearance of other anti-inflammatory cytokines (e.g., interleukin-1 receptor antagonist [IL-ra] and interleukin-10 [IL-10]) into circulation [300, 301]. Voluntary exercise also increases the expression of IL-6 in the brain, and this response has been demonstrated to protect hippocampal neurons from chemical-induced injury [302]. In addition to increasing anti-inflammatory cytokines, exercise suppresses the production of pro-inflammatory cytokines such as TNF-α [300]. Exercise preconditioning has been shown to protect against brain damage in a model of ischemia/reperfusion injury via TNF-α pathway down-regulation and inhibition of the TNF-α receptor [303]. Furthermore, the exercise induced down-regulation of TNF-α signaling has been linked to improved cognitive function in aged mice [304]. Studies with the purpose of understanding how exercise alters the inflammatory response within the brain following TBI are limited. However, a 2012 study conducted by Mota et al., demonstrated that rats who performed 4 weeks of pre-TBI exercise displayed reduced brain inflammation and better motor function scores at 24 hours post-TBI when compared to sedentary controls [261]. In this study, significantly increased IL-10, and significantly reduced IL-1β and TNF-α protein levels were noted in the injured hemisphere of exercise group rats [261]. Results from this research also revealed significantly reduced plasma fluorescein extravasation, and myeloperoxidase (MPO) activity (i.e., markers of BBB disruption) in exercise group rats when compared to control rats [261]. In addition to pre-TBI exercise, 4 weeks of delayed post-TBI exercise training (i.e., voluntary wheel running initiated at 5 weeks after injury) has been shown to minimize the inflammatory response by attenuating microglial activation, decreasing IL-1β accumulation, and increasing IL-6 and IL-10 cytokine production

[262]. The positive alterations observed in this study were associated with reduced cognitive deficits and brain lesion volumes, and enhanced neuronal survival in the dentate gyrus at three months after TBI in exercise group mice [262]. Post-TBI neuroinflammatory responses coupled with the secretion of various neurotoxic immune mediators exacerbate brain injury. Yet, exercise appears to be an effective non-pharmacological intervention for controlling the acute inflammation that leads to long-term neuron death and disability after TBI.

1.9. NEUROPROTECTIVE PROTEINS AND EXERCISE:

1.9.1. Hypoxia Inducible Factor-1α (HIF-1α)

Exercise alters endogenous HIF-1α activity. Previous studies have demonstrated that exercise increases HIF-1α gene and protein expression in human skeletal muscle [96], and in ischemic skeletal and cardiac muscle tissues of rats [121, 305]. Furthermore, performing three weeks of exercise has been reported to significantly increase HIF-1α gene expression within the cerebral cortex [267]. More studies are needed to determine whether exercise increases HIF-1α protein production in the brain. An increased production of HIF-1α protein in the brain subsequent to exercise may enhance neuroprotection and improve TBI outcomes by initiating the transcription of downstream target genes including; VEGF-A, EPO, HO-1, and neuroglobin.

1.9.2. Vascular Endothelial Growth Factor-A (VEGF-A)

Interventions aimed at increasing the endogenous production of VEGF-A may offer a more practical alternative for improving TBI outcomes than exogenous approaches. Exercise is known to augment endogenous VEGF-A production in both non-neural and neural tissues. The lungs, skeletal muscles, and brain are all exercise responsive organs involved in oxygen transport, and performing just one acute bout of exercise has been shown to increase VEGF-A mRNA expression and protein levels in these organs [306]. Furthermore, Gustafsson et al.

reported that 10 days of endurance exercise training significantly increased VEGF mRNA and protein expression in human skeletal muscle [307]. Exercise has also been shown to increase circulating VEGF-A protein in humans [308]. The production of VEGF-A in response to exercise is a complex process that can ensue independent of the HIF-1α induction that occurs during hypoxia [293]. First, exercise increases nerve activity via β-adrenergic stimulation, and β-adrenegic stimulation induces the production of PGC-1α [293]. Next, PGC-1α coactivates the transcription factor estrogen-related receptor alpha (ERRα) on multiple binding sites found within the promoter region of the VEGF gene [293, 309]. Finally, the VEGF-A protein is translated and angiogenesis is initiated [293, 309]. Several studies have revealed that exercise induces increased VEGF-A protein production, capillary density, and blood flow in the skeletal muscles and brain [310-312]. A 31% increase in skeletal muscle VEGF-A protein expression, in conjunction with a significant increase in muscle capillary density, has been observed in mice that performed voluntary treadmill running for seven days [310]. In addition, male Wistar rats engaged in a swimming protocol (i.e., 60 minutes per day, 5 days per week, over a period of 8 weeks) have demonstrated significantly increased VEGF-A protein levels, and a greater percentage of capillary density in the cerebral cortex when compared to sedentary controls [311]. In a mouse model of focal cerebral ischemia, VEGF-A gene and protein expression were significantly increased within the brain after treadmill training, and cerebral blood flow was enhanced in the ischemic lesion at 16 days post-stroke [312]. Increases in brain capillary density, and improved cerebral blood flow, are favorable circulatory system adaptations to exercise that may aid in promoting functional recovery from TBI by minimizing cellular damage and facilitating brain repair via an enhanced supply of blood to neurons. In addition to these vascular responses, VEGF-A exerts direct positive effects on neural tissue as a result of exercise.

In exercising mice, peripheral VEGF-A is necessary for increased hippocampal neurogenesis, and blocking peripheral VEGF-A eliminates neurogenesis resulting from exercise [113]. Zhang et al., demonstrated that treadmill training increased brain levels of VEGF-A, promoted axon regeneration of newborn corticonigral and striatonigral neurons, and improved motor function after transient middle cerebral artery occlusion in a rat model of ischemic stroke [313]. Although much research has been conducted in animal models of stroke, no research has investigated links between exercise, VEGF-A, and TBI outcomes.

1.9.3. Erythropoietin (EPO)

EPO gene and protein expression are known to be altered in various tissues following acute and chronic bouts of exercise. Interestingly, the response of EPO after exercise is not the same in all tissues. Long-term endurance exercise training has been shown to significantly decrease EPO mRNA and protein levels in the kidneys of mice [314]. In contrast to these findings, previous research in humans has demonstrated a significant increase in intramuscular EPO mRNA expression at 360 minutes after a single exercise bout [96]. Chronic exercise (i.e., 10 weeks of progressive treadmill training) has been shown to significantly increase intramuscular EPO mRNA expression in exercising mice when compared to sedentary controls [314]. However, no significant changes in intramuscular EPO protein production were observed between exercising and sedentary mice in this study [314]. Serum EPO protein levels have been shown to significantly increase when exercise is performed in a hypoxic environment [315]. In order to elevate serum EPO protein levels, increase red blood cell production, and improve oxygen carrying capacity, competitive endurance athletes often choose to live at higher altitudes where the partial pressure of oxygen is lower [316]. In addition, competitive endurance athletes have tried to illegally improve aerobic capacity and endurance by administering rhEPO [317].

This exogenous misuse of rhEPO increases production of red blood cells, and enhances oxygen delivery and uptake in working muscles in a manner similar to the endogenous EPO response that results from living in hypoxic conditions at high altitude [317]. Studies to determine the endogenous response of EPO in the brain following long-term exercise training have not been performed. Since the safety and efficacy of exogenous rhEPO treatment for TBI has not been fully elucidated, exercise may be a more practical non-pharmacological approach for increasing EPO production endogenously. However, no research currently exists exploring whether exercise may improve TBI outcomes by increasing the endogenous production of EPO within the brain.

1.9.4. Heme Oxygenase-1 (HO-1)

HO-1 mRNA and protein expression have been shown to increase in human lymphocytes harvested from the blood after an acute bout of prolonged exercise (i.e., running for 75 minutes at 70% of VO2max) [318]. This acute increase in post-exercise HO-1 is thought to protect cells against oxidative stress and inflammation occurring subsequent to exercise [318]. Depending on the duration and intensity, acute exercise also affects HO-1 transcriptional regulation in skeletal muscles [319]. In a study of rats, greater increases in gastrocnemius HO-1 expression were observed following prolonged low-intensity exercise (i.e., treadmill exercise for 180 minutes at 50% of VO2max) or reduced duration high-intensity exercise (i.e., treadmill exercise for 45 minutes at 75% of VO2max) when compared to rats performing reduced duration low-intensity exercise (i.e., treadmill exercise for 45 minutes at 50% of VO2max) [319]. Steensberg et al., suggested that nitric oxide is responsible for the exercise induced expression of HO-1 in skeletal muscle [320]. It has been demonstrated that both acute (i.e., 2 weeks) and chronic exercise (i.e., 6 weeks) training increase the concentration of HO-1 and endothelial nitric oxide synthase

(eNOS) proteins in the rat aorta [321]. Elevations in HO-1 and eNOS after exercise training were associated with enhanced vasodilation in this study [321]. Moreover, exercise may protect the cardiovascular system by inducing up-regulation of HO-1 in the myocardium [322]. In addition to exercise induced HO-1 responses in lymphocytes, skeletal muscles, and the cardiovascular system, chronic exercise (i.e., 11 weeks of treadmill exercise) has been shown to significantly increase HO-1 protein levels in the liver of rats [323]. Although exercise up-regulates HO-1 in various organs and tissues throughout the body, no data has been published investigating exercise and HO-1 responses in the TBI brain. If exercise increases the endogenous production of HO-1 in the brain, damage occurring as a result of post-TBI secondary injury responses (e.g., hypoxia, ischemia, oxidative stress, and inflammation) may be reduced, and TBI outcomes improved.

1.9.5. Brain-Derived Neurotrophic Factor (BDNF)

Numerous brain and exercise studies have focused on BDNF. In 1995, Neeper et al. suggested that BDNF is involved in brain plasticity, and showed that BDNF expression increased in both the hippocampus and cerebral cortex when rodents were allowed access to a running wheel for 7 days [178]. Neurogenesis resulting from exercise is reduced if BDNF signaling is blocked by using antibodies against the TrkB receptor [324]. Animal studies demonstrate that post-TBI exercise increases BDNF expression within the hippocampus [260, 325], and the increased expression of BDNF is responsible for improvements in object recognition memory [326] and spatial learning memory [260] after injury. These are important findings since BDNF gene and protein expression are altered within the hippocampus in response to TBI [187, 188, 190, 326], and cognitive deficits are often associated with impaired hippocampal function [327]. Exercise induced improvements in post-TBI cognitive function have been suggested to occur as

a result of BDNF facilitating axonal branching and remodeling of neural networks by up-regulating mitogen-activated protein kinase phosphatase-1 (MKP-1) [326, 328]. Studies of MKP-1 knockout mice, and experiments specifically designed to inhibit the BDNF-MKP-1 pathway, lend further support to the notion that BDNF plays a key role in mediating axonal branching and improvements in cognitive function that result from exercise. Neurons from MKP-1 knockout mice lack the ability to sprout new axons in response to BDNF [328], and inhibiting the BDNF-MKP-1 pathway with triptolide abolishes the beneficial effects of exercise on post-TBI object recognition memory [326]. Furthermore, blocking free BDNF from binding to its TrkB receptor with an immunoadhesin chimera (TrkB-IgG) after TBI, and prior to exercise, eliminates improvements in spatial learning memory that are associated with voluntary exercise [260]. Taken together, these studies demonstrate that many of the beneficial effects of exercise on overall brain health and cognitive function are mediated through BDNF.

1.9.6. Insulin-Like Growth Factor-1 (IGF-1)

Exercise is known to increase IGF-1 production in the brain [329], while simultaneously raising the concentration of circulating IGF-1 [330]. Circulating levels of IGF-1 rise because exercise increases the synthesis and release of IGF-1 from the liver and muscles [329]. Long-term aerobic exercise training has been shown to significantly increase and maintain resting serum levels of IGF-1 in competitive collegiate swimmers [331]. A direct correlation exists between elevated serum concentrations of IGF-1 at rest, and increased maximal oxygen uptake (i.e., VO2max), which is an important outcome measure of physical fitness [332]. In addition to aerobic exercise boosting IGF-1 levels, strength training also increases the total concentration of IGF-1 in serum [333]. Circulating IGF-1 can cross the BBB [194], and one study of adult rats revealed that exercise induces neurogenesis and improves cognitive function by increasing the

uptake of circulating IGF-1 into the brain [330]. Carro et al., demonstrated that blocking the entrance of circulating IGF-1 into the brain with an anti-IGF-1 antibody increases neuronal damage, and nullifies the exercise induced recovery of motor coordination in various rat models of brain insults and neurodegenerative disease [330]. The authors of this study concluded that exercise prevents and protects the brain from damage by increasing the uptake of circulating IGF-1 into the brain, and that administering IGF-1 may be a beneficial treatment approach for minimizing neuronal death resulting from brain trauma or disease [330].

1.10. EXERCISE AND RELATIONSHIPS BETWEEN GROWTH FACTOR PROTEINS:

The neurobiological activity of IGF-1 appears to be closely related to the actions of BDNF and VEGF-A in translating exercise into neurological benefits. For example, the levels of IGF-1, BDNF, and VEGF-A all increase in the brain after exercise, and all three of these growth factors elicit neurogenic and neuroprotective effects [113, 330]. IGF-1 signaling is required for the exercise induced up-regulation of BDNF [334], as several studies have shown that blocking IGF-1 signaling with antibodies prevents the exercise induced up-regulation of BDNF [334, 335]. Moreover, blocking BDNF has been demonstrated to prevent the exercise induced up-regulation of IGF-1 within the hippocampus [336]. Both IGF-1 and VEGF-A concentrations increase in the blood after exercise to stimulate repair and growth of the vascular system, which in turn enhances oxygen delivery to muscles and increases aerobic capacity [337, 338]. In addition to these peripheral effects, circulating IGF-1 and VEGF-A can cross the BBB and enter the brain to exert their respective neurogenic and angiogenic actions [194, 324]. Several studies have displayed that neurogenesis resulting from aerobic exercise is significantly inhibited when peripheral IGF-1 or VEGF-A are blocked from entering the brain [113, 339]. These experiments reveal that the beneficial effects of exercise on the nervous and vascular systems are associated

with the coordinated activity of IGF-1, BDNF, and VEGF-A. It is possible that improved post-TBI outcomes may result from IGF-1, BDNF, and VEGF-A interacting and modulating beneficial adaptations at the cellular and molecular level in response to exercise training. More research investigating the response of these growth factors to exercise and TBI is needed.

1.11. RESEARCH QUESTION:

The long-term goal of our research is to improve TBI outcomes. Unfortunately, safe and effective TBI treatments remain limited. There is a critical need for identifying novel preventative and/or therapeutic interventions that improve TBI outcomes. Increasing the production of neuroprotective molecules (e.g., neuroglobin, VEGF-A, EPO, and HO-1) in the brain prior to, or early after TBI, may improve outcomes. Thus, the objective of this dissertation was to determine whether gene therapy and/or pre-TBI exercise could improve post-TBI sensorimotor and cognitive (i.e., spatial learning memory) performance in adult mice by increasing the expression of neuroglobin, VEGF-A, EPO, and HO-1 in regions of the brain that are responsible for movement and memory.

In chapter 2, "Neuroglobin overexpression improves sensorimotor outcomes in a mouse model of traumatic brain injury", results demonstrate that overexpression of neuroglobin reduces sensorimotor deficits after TBI, and increasing neuroglobin expression in the brain during the acute post-TBI recovery phase may improve outcomes.

In chapter 3, "Exercise preconditioning improves traumatic brain injury outcomes", results show that improved post-TBI sensorimotor function and spatial learning memory are linked to pre-TBI exercise increasing the expression of neuroprotective proteins in the sensorimotor cortex (i.e., VEGF-A and EPO) and hippocampus (i.e., VEGF-A only).

In chapter 4, the findings from chapter 2 and chapter 3 are summarized. Discussion focuses on how neuroglobin overexpression and exercise may augment neuroprotection, which in turn, improves TBI outcomes. In addition, the risks and benefits associated with post-TBI and pre-TBI exercise are reviewed. Future research directions are also suggested in this chapter.

Chapter 2

Neuroglobin Overexpression Improves Sensorimotor Outcomes in a Mouse Model of Traumatic Brain Injury

2.1. ABSTRACT:

There is a significant need for novel treatments that will improve traumatic brain injury (TBI) outcomes. One potential neuroprotective mechanism is to increase oxygen binding proteins such as neuroglobin. Neuroglobin has a high affinity for oxygen, is an effective free radical scavenger, and is neuroprotective within the brain following hypoxia and ischemia. The purpose of this study was to determine whether neuroglobin overexpression improves sensorimotor outcomes following TBI in transgenic neuroglobin overexpressing (NGB) mice. Additional study aims were to determine if and when an endogenous neuroglobin response occurred following TBI in wild-type (WT) mice, and in what brain regions and cell types the response occurred. Controlled cortical impact (CCI) was performed in adult (5 month) C57/BL6 WT mice, and NGB mice constitutively overexpressing neuroglobin via the chicken beta actin promoter coupled with the cytomegalovirus distal enhancer. The gridwalk task was used for sensorimotor testing of both WT and NGB mice, prior to injury, and at 2, 3, and 7 days post-TBI. NGB mice displayed significant ($p<.01$) reductions in the average number of foot faults per minute walking at 2, 3, and 7 days post-TBI when compared to WT mice at each time point. Neuroglobin mRNA expression was assessed in the right cortex of WT mice prior to injury, and at 1, 3, 7, and 14 days post-TBI using quantitative real-time polymerase chain reaction (qRT-PCR). Neuroglobin mRNA was significantly ($p<.01$) increased at 7 days post-TBI. Immunostaining showed neuroglobin primarily localized to neurons and glial cells in the injured cortex and ipsilateral hippocampus of WT mice, while neuroglobin was present in all brain regions of NGB mice at 7 days post-TBI. These results showed that overexpression of neuroglobin reduced sensorimotor deficits following TBI, and that an endogenous increase in

neuroglobin expression occurs during the subacute period. Increasing neuroglobin expression through novel therapeutic interventions during the acute period after TBI may improve recovery.

2.2. INTRODUCTION:

Traumatic brain injury (TBI) is a major public health concern with severe consequences that include long-term loss of function, profound disability, and death. With the exception of routine medical intervention and care, no successful treatment options are currently available for promoting functional recovery from TBI in humans. Between 1980 and 2009, 27 large phase III clinical trials were published for TBI, but the only studies reporting a significant treatment effect involved the use of decompressive craniectomy, and hypothermia to relieve brain swelling and control increased intracranial pressure [211]. Accordingly, there is a significant need for novel treatments that will improve TBI outcomes. Improved TBI outcomes may be achieved through treatments that target globin genes (e.g., hemoglobin, myoglobin, cytoglobin, and neuroglobin). The globin gene family translates proteins that are vital for binding oxygen within various body tissues. Following up-regulation, globin proteins also play a neuroprotective role in the brain [170, 340]. The work presented here focuses on neuroglobin, one of the most recently characterized members of the globin gene family. Neuroglobin was first described in 2000 as a protein with a high affinity for binding oxygen that is present in the brain [161]. In addition to its ability to bind oxygen, neuroglobin also functions as an antioxidant and free radical scavenger [172]. Neuroglobin is expressed throughout the central and peripheral nervous systems of vertebrates [161-164]. The primary sites of neuroglobin expression are the limbic system and cerebral cortex [162]. Neuron survival is enhanced via an increased endogenous production of neuroglobin following hypoxia and ischemia [167]. Therefore, neuroglobin has been suggested to be neuroprotective in experimental models of stroke [100, 168] and in TBI [164, 169].

Previous research has demonstrated that transgenic neuroglobin overexpressing (NGB) mice display significantly reduced cortical lesion volumes in comparison to wild-type (WT) mice 21 days after TBI [164]. Furthermore, overexpression of neuroglobin in Wistar rats significantly reduced neuron necrosis and apoptosis after TBI in comparison to controls [169]. In humans, genetic polymorphisms in neuroglobin have been shown to positively influence recovery in TBI patients [246]. The present study was designed to determine whether NGB mice displayed improved sensorimotor recovery after TBI in comparison to WT mice. In addition, related study objectives were to determine if and when an endogenous neuroglobin response occurred following TBI, and in what brain regions and cell types the response occurred. It was hypothesized that overexpression of neuroglobin would reduce sensorimotor deficits following TBI in NGB mice when compared to WT mice.

2.3. METHODS:

Animals

Adult (5 month) male C57/BL6 WT, and transgenic (B6.Cg-Tg(CAG-Ngb,-EGFP)1Dgrn/J; The Jackson Laboratory, ME) NGB mice were used for this study. NGB mice overexpressed neuroglobin via the chicken beta actin promoter coupled with the cytomegalovirus distal enhancer. 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.

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

A controlled cortical impact (CCI) was used to induce a moderate TBI as previously described [341]. The CCI injury model targeted the primary and secondary motor, and primary somatosensory regions of the right cerebral cortex. CCI reproduces many of the features of brain

injuries including motor deficits, memory loss, and neuron loss [342], and the severity of injury can be controlled experimentally by altering the velocity and depth of the impact and the size of the impactor tip [343]. CCI also eliminates a potentially confounding variable of skull thickness, which may change in transgenic mice. Responses following controlled cortical impact include glial activation, increased cytokine expression, blood brain barrier disruption, and neuron loss [62, 91]. Prior to surgery, 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. The CCI procedure was performed while mice were anaesthetized with 2.5% isoflurane in 30% O_2 and air. The skin on top of the head was shaved and scrubbed with iodine. A midline incision was made with a sterile scalpel blade, and the scalp and epicranial aponeurosis were retracted to expose the skull. A Wild operating microscope was used to view the skull at 60X magnification, and 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 periodically applied to the drill and surface of the skull to prevent burning the underlying brain tissue. Drilling was performed in a careful manner to leave the dura intact, and avoid blood vessels traveling through the superior sagittal sinus. Once the brain was exposed, the cortex was impacted with a custom made, electronically controlled, CCI injury device (P01-23x80, LinMot Inc., Zurich, Switzerland). Attached to the shaft of the CCI injury device was a 3 mm stainless-steel injury tip. 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 graphic user interface of the impactor control software (Visual Basic 6.0 software). Using this push-button graphic user interface, the operator initiated the CCI 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, immediately followed by a quick 21 mm downward strike (20 mm retraction plus the 1.0 mm operator programmed injury depth) of the impactor tip. These impact parameters have resulted in a low (5%) mortality rate. Following impact, the skin was closed with a sterile suture and all animals were returned to standard cages. The duration of each CCI procedure was approximately 30 minutes.

Assessment of Sensorimotor Function

Sensorimotor deficits (i.e., foot faults per minute walking) were evaluated using the gridwalk task as previously described [341]. 6 WT and 7 NGB mice were tested on the gridwalk at four time points (1 day pre-TBI, 2 days post-TBI, 3 days post-TBI, and 7 days post-TBI). Two gridwalk trials were performed at 1 day pre-TBI to establish each animal's baseline performance, and to allow the animals to become familiar with the apparatus. Mice were given one trial per day, at the same time of day, and scored by an observer who was unaware of the groups at each subsequent post-TBI time point. Mice were placed on a grid area measuring 32 cm×20 cm×50 cm with 11×11 mm diameter openings. A video camera was placed beside the grid, and foot faults were counted as the mice walked over the elevated wire surface. Mice were allowed to walk on the grid for five minutes, during which their total walking time was measured, and the number of foot faults for each foot counted. Any step passing through a grid hole was considered a foot fault. Foot fault data was normalized (to account for differences in locomotion seen in different trials) by dividing the total counted foot faults by the total time spent walking to obtain a measure of foot faults per minute of walking. Data were expressed as the mean number of foot faults per minute walking $\pm$ SEM. Two-way repeated measures

ANOVA with a Fisher's LSD post hoc for multiple comparisons was used for analyzing significant differences in mean number of foot faults per minute walking at each time-point.

mRNA Analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was utilized for our gene expression time course study as previously described [87]. 6 WT mice were sacrificed via intraperitoneal injection of beuthanasia prior to TBI, and at 1 day, 3 days, 7 days, and 14 days post-TBI. All animals were decapitated at each time point, and whole brains were removed and stored in RNA later (Ambion, Austin, TX) for 24 hours at 4˚C to preserve the RNA. After the 24 hour incubation period, whole brains were dissected in order to collect the injured cortex (right cerebral cortex). Total RNA was extracted from the tissues with a Polytron 2000 homogenizer (Brinkmann Instruments, Westbury, NY), using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To assess RNA quantity, spectrophotometry was performed using a Nanodrop (Nanodrop Technologies, Wilmington, DE). Isolated RNA samples were treated with DNase (Ambion PCR Kit) to remove any contaminating genomic DNA according to manufacturer's instructions. RNA purity and concentration were determined with an Agilent 2100 Bioanalyzer (Agilant Technologies, Santa Clara, CA), and samples not meeting quality standards were discarded. Complementary DNA (cDNA) was synthesized by using 1 μg total RNA from each sample and random hexamers in a Taqman reverse transcription reaction (Applied Biosystems, Foster City, CA, USA). 10 ng of cDNA and gene-specific primers (see Table 1 for target genes and sequences of all primer pairs) were added to 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]), 1μl primer mixture (10 μg forward and 10 μg reverse primers in 150μl Sigma water), and Sigma water

(SigmaAldrich, St. Louis, MO) in a 96 well MicroAmp Optical reaction plate (Applied Biosystems, Foster City, CA). The cDNA 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 GAPDH was used as the housekeeping gene. Sequence Detection Software 2.0 (Applied Biosystems, Foster City, CA) was used to collect and analyze data. Neuroglobin gene expression was calculated relative to GAPDH by the ΔΔCt method, and reported in accordance with The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [344]. A two-way ANOVA was used for analyzing significant differences in mRNA expression between time points. Fisher's LSD was used for post hoc comparisons.

Table 1. Primer sequences used for qRT-PCR

Gene	Primer Sequence
Neuroglobin	Forward-5'-TAC-AAT-GGC-CGC-CAG-TTC-T-3' Reverse-5'-TGG-TCA-CTG-CAG-CAT-CAA-TCA-3'
GAPDH (housekeeping)	Forward-5'-ATG-ACA-TCA-AGA-AGG-TGG-TG-3' Reverse-5'-CAT-ACC-AGG-AAA-TGA-GCT-TG-3'

Protein Analysis

Immunohistochemistry was conducted to assess the location of specific cell types expressing neuroglobin as previously described [91]. 4 WT and 4 NGB mice were sacrificed by transcardial perfusion under anesthesia (isoflurane) at 7 days post-TBI. Brains were fixed by perfusion with 4% buffered formaldehyde, dissected, postfixed overnight, cryoprotected in 30% sucrose in 0.1 M phosphate buffer (pH 7.2), and sectioned through the coronal plane at 50 μm on a microtome. Frozen sections were stored in an ethylene glycol solution at -80°C prior to immunostaining. Sections were rinsed in PBS with 0.4 mg/ml glycine for 5 min, permeabilized

with 0.2% Triton X-100, and quenched for peroxidase with 3% H_2O_2. Sections were incubated overnight at 4°C with the primary antibody (neuroglobin rabbit anti-mouse 1:200, Sigma AB-N7162). After overnight incubation, all sections were rinsed with PBS and incubated with a biotinylated secondary antibody (1:500 goat anti-rabbit; Vector Labs BA-1000) for 2 hours at room temperature. An ABC Elite kit (Vector Labs, Burlingame, CA) was used to produce the immunoperoxidase reaction. Color was visualized using diaminobenzidine (DAB) solution (Vector Labs, Burlingame, CA). A Nikon inverted stage microscope was used to visualize all sections, and digital images were captured with a SPOT microscope camera (Diagnostic Instruments, Sterling Heights, MI).

2.4. RESULTS:

The gridwalk task revealed unilateral sensorimotor forelimb and hindlimb deficits (i.e., foot faults) in both WT and NGB mice at 2 days, 3 days, and 7 days post-TBI (Figure 1). However, NGB mice displayed a significant (*$p<.01$) reduction in the average number of foot faults per minute of walking in comparison to WT mice at all post-TBI time points (2, 3, and 7 days). Furthermore, at 7 days post-TBI, NGB mice demonstrated a reduction in the average number of foot faults per minute of walking that was similar to pre-TBI averages. qRT-PCR analysis (Figure 2) showed a significant (*$p<.01$) increase in neuroglobin mRNA expression in the right cerebral cortex of WT mice at 7 days post-TBI when compared to mRNA levels at all other time points. CCI induced a lesion cavity within the right cerebral cortex that was seen at 7 days post-TBI in both WT and NGB mice (Figure 3 a, d). Immunohistochemistry revealed neuroglobin protein localization within neurons and glial cells in the injured cortex near the injury site, and ipsilateral hippocampus of WT mice at 7 days post-TBI (Figure 3 a, b, c). In

addition, neuroglobin protein was observed in neurons and glial cells throughout the brain of NGB mice at 7 days post-TBI (Figure 3 d, e, f).

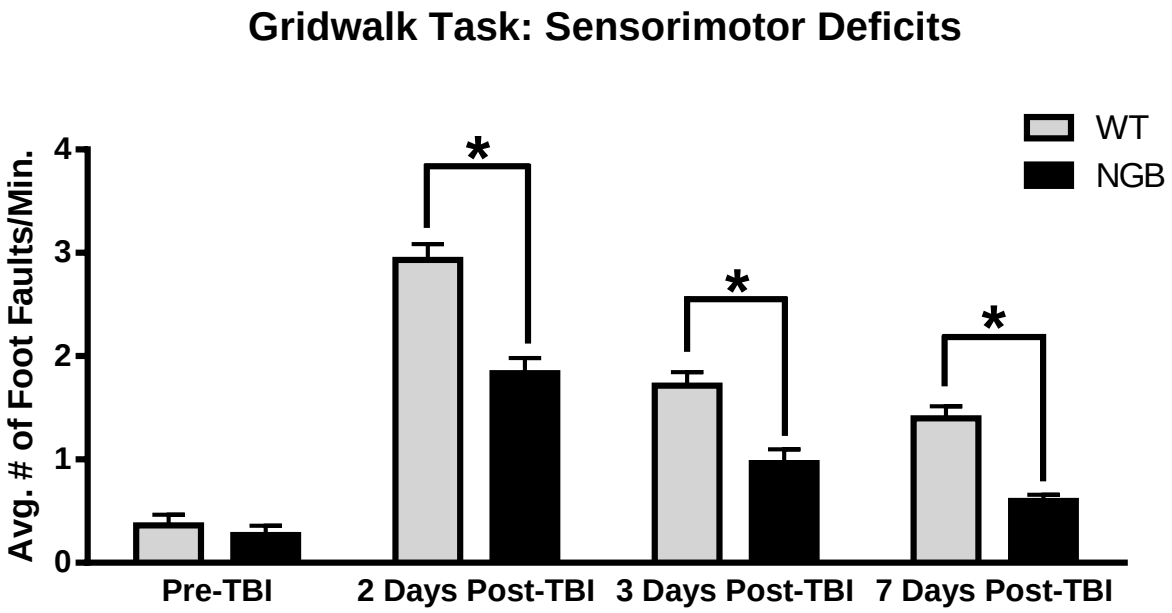

Figure 1. The gridwalk task was used for evaluation of sensorimotor deficits in wild-type (WT) and transgenic neuroglobin (NGB) overexpressing mice. Data represents the average number of foot faults per minute of walking prior to TBI, and at 2, 3, and 7 days post-TBI. NGB mice demonstrated significant reductions in the average number of foot faults per minute of walking at all post-TBI time points when compared to WT controls (*p<.01). Sensorimotor recovery was nearly complete at 7 days post-TBI for NGB mice. Statistical Analysis: Two-way repeated measures ANOVA with Fisher's LSD post hoc testing for multiple comparisons at each time point; n=6 (WT) n=7 (NGB); mean $\pm$ SEM.

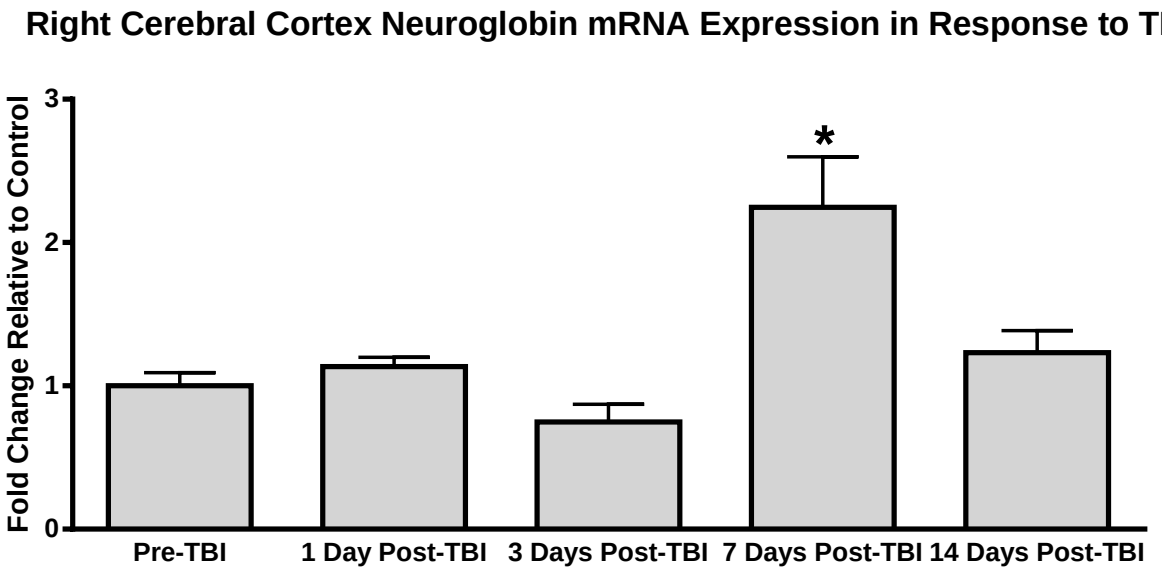

Figure 2. qRT-PCR study of right cerebral cortex (injured cortex) neuroglobin gene expression in WT mice prior to TBI and at various post-TBI time points. Y-axis is the average fold change relative to GAPDH (housekeeping gene) in the right cerebral cortex of pre-TBI (control) group mice. Data represents neuroglobin expression prior to TBI, and at 1, 3, 7, and 14 days post-TBI. A significant (*$p<.01$) late up-regulation in neuroglobin expression occurred at 7 days post-TBI when compared to mRNA levels at all other time points. Statistical Analysis: Two-way ANOVA with Fisher's LSD post hoc testing for multiple comparisons at each time point; n=6 per group at each time point; mean $\pm$ SEM.

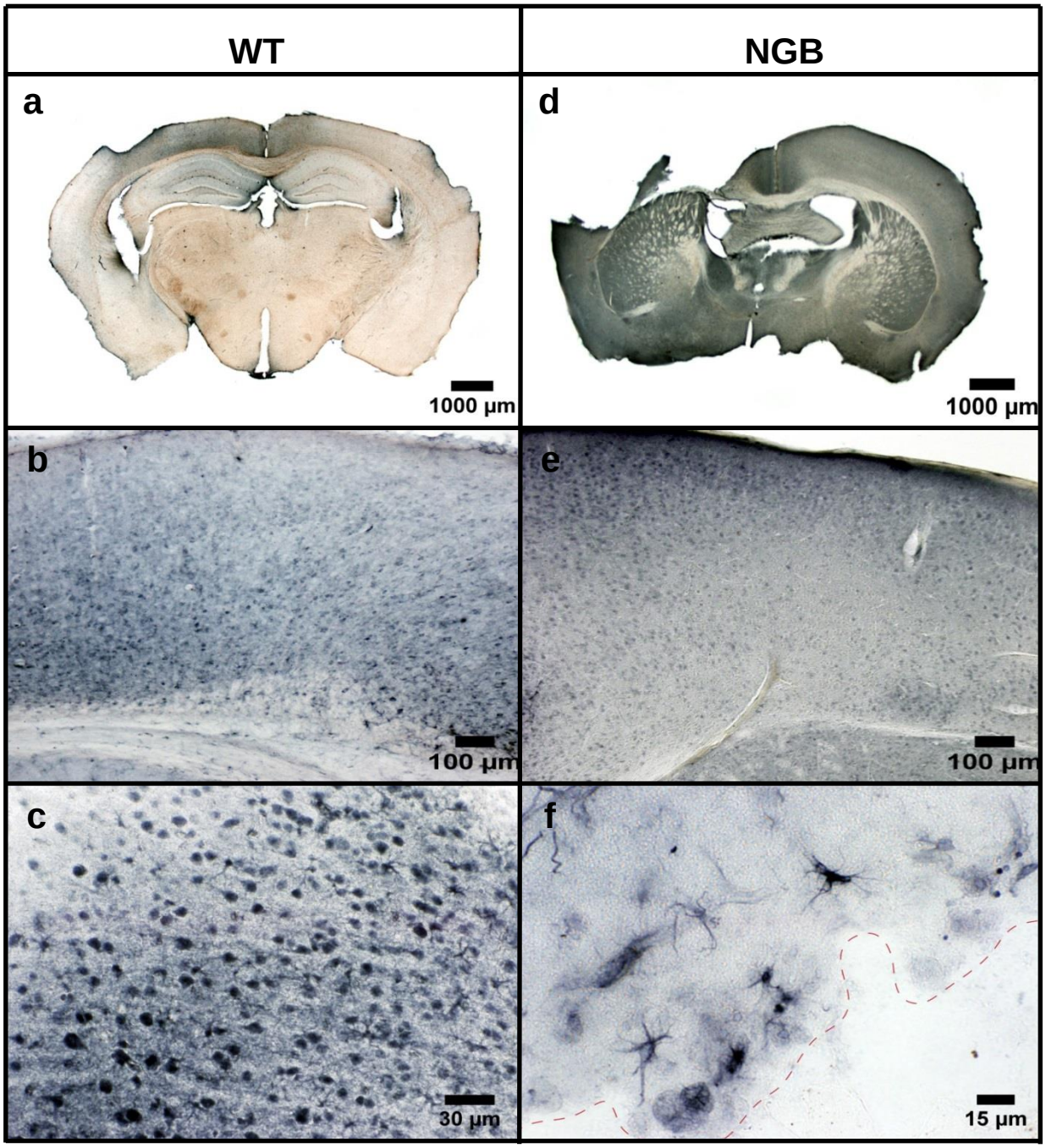

Figure 3. Coronal immunostained sections (50 µm) showing localization of neuroglobin protein in WT (a, b, c) and NGB mice (d, e, f) at 7 days post-TBI. Neuroglobin was present in the injured cortex (right cerebral cortex) and ipsilateral hippocampus of WT mice (a). NGB mice displayed neuroglobin throughout the cortical and subcortical tissues (d). The injured cortex of both WT and NGB mice demonstrated neuroglobin localization in neurons (b, e). Neuroglobin was observed in both neurons and glial cells within the injured cortex of WT mice (c), and the subcortical white matter inferior to the injury site (dashed red line) in NGB mice (f). Magnification at 1X (a, d), 10X (b, e), 40X (c), and 60X (f).

2.5. DISCUSSION:

Neuroglobin Overexpression Improves Post-TBI Sensorimotor Function

The gridwalk task data demonstrated that NGB mice displayed a significant reduction in the number of foot faults (i.e., sensorimotor deficits) per minute walking at all post-TBI time points when compared to WT mice. This result showed a link between neuroglobin overexpression and significantly improved sensorimotor outcomes after TBI. Interestingly, this finding is in contrast to the results of a recent study conducted by Zhao et al., which showed no significant differences in recovery of sensorimotor function between NGB mice and WT mice at multiple post-TBI time points [164]. A disparity in the results may exist because Zhao et al. used a 10-point neurological severity score (NNS) for evaluating post-TBI neurological dysfunction while the present study used the gridwalk task. One limitation of the NNS is that the test cannot evaluate sensorimotor deficits that occur while walking because the NNS only measures the ability of mice to walk on beams of various widths, but it does not evaluate accuracy (i.e., foot faults) of locomotion [345]. The gridwalk task is a well validated test used to assess sensorimotor function following TBI [341, 346], and since the NNS does not measure foot faults, it is less sensitive than the gridwalk task at assessing sensorimotor deficits that occur while walking [345]. In addition, differences in post-TBI sensorimotor function and recovery may have existed because our CCI injury site and impact parameters were different than those of Zhao et al.

Timing of the Endogenous Post-TBI Neuroglobin mRNA Increase and Protein Localization

Neuroglobin is an oxygen-binding protein that supplies oxygen to hypoxic tissue. Hypoxia commonly occurs as a secondary injury response to TBI, and many neuroprotective hypoxia-inducible genes (hypoxia-inducible factor-1 alpha, vascular endothelial growth factor,

heme oxygenase-1, and erythropoietin) are up-regulated after TBI to assist in promoting cell survival [87]. In conjunction with these hypoxia-inducible genes, neuroglobin expression is also known to increase in neurons responding to hypoxia [100]. This increase in neuroglobin expression protects neurons from cell death and reactive oxygen species damage [100, 172]. The qRT-PCR results indicated that there is a late, but significant, increase in neuroglobin mRNA expression at 7 days post-TBI in WT mice. The subacute (i.e., 7 days post-TBI) increase in neuroglobin expression occurred later in comparison to the response of other hypoxia-inducible genes that are known to be up-regulated at 3 days post-TBI [87]. This delayed increase may reduce the neuroprotective potential of the endogenous neuroglobin response because significant neuron cell death occurs in adult mice at 3 days post-TBI [91]. The delayed increase in neuroglobin gene expression observed in our study at the 7 day post-TBI time point is in contrast to the findings of Di Pietro et al., which showed an acute increase in neuroglobin expression [347]. However, it is important to note that in opposition to our study design Di Pietro et al. employed rats instead of mice, and used a diffuse head injury model that induced different injury severities (i.e. mild and severe TBI). Furthermore, neuroglobin expression was quantified at different time points in comparison to our study. Differences in study design and methods make direct comparisons between the results of these studies difficult. The immunostaining performed at 7 days post-TBI confirmed a greater accumulation of neuroglobin protein near the injury site and ipsilateral hippocampus when compared to the uninjured contralateral cortex and hippocampus in WT mice. The presence of neuroglobin protein within the injured cortex of WT mice coincided with the significantly increased right cerebral cortex expression of neuroglobin mRNA observed at 7 days post-TBI. NGB mice demonstrated neuroglobin protein throughout the brain, and neuroglobin was localized in neurons and glial cells of both WT and NGB mice.

Our immunostaining results are in agreement with the findings of DellaValle, et al., who showed similar *in vivo* neuroglobin localization in mouse models of TBI, cerebral malaria, and autoimmune encephalitis [166].

Mechanisms of Neuroprotection

Neuroglobin has multiple neuroprotective effects that operate by different mechanisms, and several studies suggest that neuroglobin may positively affect TBI outcomes. Neuroglobin inhibits the intrinsic apoptosis pathway by maintaining cytochrome c in a non-apoptotic oxidation state [173], protects neurons from nitric oxide toxicity [171], and prevents mitochondrial aggregation in hypoxic neurons [100]. In cell culture models, elevating neuronal neuroglobin reduced oxidative stress and increased intracellular adenosine tri-phosphate (ATP) by activating mitochondrial ATP sensitive potassium channels [174]. Furthermore, neuroglobin is known to positively affect metal homeostasis in neurons during hypoxic conditions. Hypoxic neurons display increased intracellular concentrations of calcium, iron, copper, and zinc [175]. Increased accumulation of these metals promotes inflammation, mitochondrial dysfunction, uncontrolled reactive oxygen species production, altered neurotransmitter release, neurotoxicity, and cell death [175]. Neuroglobin inhibits calcium influx, reduces cellular uptake of iron, copper, and zinc, and inhibits both necrosis and apoptosis [175]. The mechanisms underlying modulation of metal homeostasis by neuroglobin in response to hypoxia have not been clearly defined.

Conclusions

Based upon our observations and prior studies, we postulate that increasing neuroglobin expression prior to 7 days post-TBI would maximize its potential for neuroprotection, thereby improving sensorimotor outcomes. Since neuroglobin is an intracellular protein that is not

capable of crossing cell membranes, direct administration of neuroglobin is not a practical treatment intervention [228]. However, previous research has demonstrated that endogenous neuroglobin production can be up-regulated pharmacologically by deferoxamine, cinnamic acid, and valproic acid [228]. Deferoxamine is an iron chelator known to increase hemin (ferric protoporphyrin IX), an oxidation product of heme [228]. Hemin initiates transcription and translation of neuroglobin in neurons via the soluble guanylate cyclase-protein kinase G (sGC-PKG) signal transduction pathway [229]. In addition, Deferoxamine induces neuroglobin expression by increasing levels of hypoxia-inducible factors (HIF-1α and HIF-2α) in cortical neurons [228-230]. Cinnamic acid and valproic acid induce neuroglobin protein expression in cultured neurons *in vitro* [228], but the mechanisms by which these small molecules enhance neuroglobin production have not been determined. Currently, no *in vivo* TBI studies exist investigating whether administration of cinnamic acid or valproic acid induces neuroglobin in the brain. Increasing neuroglobin via pharmacological treatments during the acute period after TBI may improve sensorimotor outcomes. More research is clearly warranted to determine whether pharmacological treatments are effective, and to define the optimal therapeutic window for neuroprotection after TBI.

Chapter 3

Exercise Preconditioning Improves Traumatic Brain Injury Outcomes

3.1. ABSTRACT:

Devastating long-term effects resulting from traumatic brain injury (TBI) include sensorimotor and cognitive dysfunction. Post-TBI sensorimotor and cognitive function may be improved by increasing the expression of neuroprotective molecules in the brain. Vascular endothelial growth factor-A (VEGF-A), erythropoietin (EPO), and heme oxygenase-1 (HO-1) promote neuron survival, neurogenesis, and angiogenesis in the brain. Exercise has been shown in previous work to increase VEGF-A, EPO, and HO-1 expression in a variety of tissues. However, the response of these neuroprotective molecules in the brain after exercise is not known, and there is a critical need for examining VEGF-A, EPO, and HO-1 responses to exercise and TBI, and whether exercise can improve TBI outcomes. The purpose of this study was to determine whether 6 weeks of voluntary pre-TBI exercise (i.e., exercise preconditioning) could improve post-TBI sensorimotor and cognitive function in adult mice. In addition, we aimed to determine if improvements in post-TBI sensorimotor and cognitive function were associated with pre-TBI exercise increasing the production of VEGF-A, EPO, and HO-1 in regions of the brain responsible for movement (i.e., sensorimotor cortex) and memory (i.e., hippocampus). 120 adult (5 month) male C57/BL6 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]). Behavioral testing was conducted using the gridwalk task for assessment of sensorimotor function, and radial arm water maze (RAWM) for evaluation of spatial learning memory. EX-TBI mice displayed significant ($p<.01$) reductions in the average number of foot faults per minute walking during gridwalk testing when compared to NOEX-TBI mice at 1,3, and 7 days post-TBI. In addition, 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. Quantitative real-time polymerase chain reaction (qRT-PCR) and immunostaining were performed to investigate VEGF-A, EPO, and HO-1 mRNA and protein expression in the right cerebral cortex (injured cortex) and ipsilateral hippocampus. EX-NOTBI and EX-TBI mice displayed significantly ($p<.05$) increased VEGF-A and EPO mRNA expression in the right cerebral cortex at 1 day post-TBI and/or post-exercise. In addition, ipsilateral hippocampus VEGF-A mRNA was significantly ($p<.01$) increased in both EX-NOTBI and EX-TBI mice at 1 day post-TBI and/or post-exercise. EX-NOTBI and EX-TBI mice showed a significantly ($p<.05$) increased number of sensorimotor cortex neurons staining positive for VEGF-A and EPO protein at 1 day post-TBI and/or post-exercise. Furthermore, EX-TBI mice exhibited a significantly ($p<.05$) increased number of right hippocampus CA1 field neurons staining positive for VEGF-A protein at the 1 day post-TBI/post-exercise time point. HO-1 mRNA expression was not different in the right cerebral cortex or ipsilateral hippocampus of mice from either exercise group at any post-exercise time point. However, HO-1 mRNA expression was significantly ($p<.01$) increased in the right cerebral cortex and ipsilateral hippocampus of NOEX-TBI mice at 3 days post-TBI, and ipsilateral hippocampus HO-1 mRNA remained elevated at 7 days post-TBI. Results from this study show that improved TBI outcomes are associated with increased expression of specific neuroprotective genes and proteins (i.e., VEGF-A and EPO, but not HO-1) in the brain following exercise.

3.2. 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 [20, 63]. 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 the initial trauma [20, 61-63]. These secondary injury responses exacerbate primary injury damage, and are the main cause of death in hospitalized TBI patients [23]. Progression of secondary injury responses is a key factor that determines the final extent of brain damage, neurological dysfunction, and disability in those patients that survive a TBI [20]. TBI is not just an acute event, but rather a cascade of pathophysiological responses that ultimately promote neurological dysfunction and poor outcome following injury.

TBI induces the transcription of several genes (e.g., vascular endothelial growth factor-A [VEGF-A], erythropoietin [EPO], and heme oxygenase-1 [HO-1]) that have the potential to protect neurons and reduce cellular damage resulting from post-TBI secondary injury responses [87]. VEGF-A is a homodimeric glycoprotein that acts as an angiogenic growth factor, but it also promotes neurogenesis [87, 113, 117]. VEGF-A is produced and released from many cell types including endothelial cells, neurons, glial cells, macrophages, and cancer cells during hypoxic cellular conditions [115-117]. The production of VEGF-A increases in the brain after stroke [117] and TBI [87]. Increased VEGF-A production is vital for promoting neuron survival, neurogenesis, and angiogenesis in and around the lesion site after TBI [125, 126]. Inhibiting the endogenous VEGF-A injury response prevents brain repair by impeding revascularization and astroglial proliferation [127]. Moreover, a significantly larger lesion volume in the cerebral

cortex, and increased neuronal and glial cell death result when VEGF-A receptors (VEGFR-2) are blocked prior to, and after TBI [128].

EPO is a glycoprotein required for erythropoiesis, but it also has anti-apoptotic, anti-inflammatory, antioxidative, angiogenic, and neurogenic properties [129, 130, 132-135]. The main site of EPO production is the kidneys [129, 130]. However, EPO and its receptor are also expressed in neurons, astrocytes, oligodendrocytes, and microglial cells in the brain [130-132]. EPO is neuroprotective in animal models of TBI [142], spinal cord injury [143], stroke [144], and autoimmune encephalomyelitis [145]. EPO is known to elicit anti-apoptotic actions through inhibition of caspase activity, up-regulation of Bcl-2 family proteins, and by decreasing glutamate excitoxicity [87]. In ischemic tissue, EPO diminishes the production of pro-inflammatory cytokines [146], and inhibiting the endogenous EPO response has been shown to worsen neuronal injury resulting from hypoxia and ischemia [147].

HO-1 is a neuroprotective enzyme that exhibits anti-oxidative and anti-inflammatory effects [148, 149]. Our laboratory has shown in previous work that HO-1 production increases in the brain of mice after TBI [87]. HO-1 induction occurs in microglia and astrocytes following brain hemorrhage [156, 157], and increased expression of HO-1 in microglia and astrocytes may improve cellular resistance to oxidative stress and secondary injury after TBI [157]. HO-1 plays a neuroprotective role in many other pathophysiological conditions including; 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridinium (MPP+) neurotoxicity [149], and stroke [158]. Disrupting endogenous HO-1 responses has been associated with poor outcomes in many central nervous system disorders [148]. In contrast, the pharmacological induction of HO-1 has elicited beneficial effects in many pathologic states including; inflammatory processes, atherosclerosis, carcinogenesis, ischemia-reperfusion injury and degenerative diseases [152]. Furthermore, the

neuroprotective effects of antioxidants including resveratrol and sulforaphane depend on induction of HO-1 [159, 160, 348].

Exercise alters VEGF-A, EPO, and HO-1 expression in both non-neural and neural tissues. Several studies demonstrate that exercise induces increased VEGF-A protein production in the skeletal muscles and brain [310-312]. EPO gene expression is increased in muscle [96, 314] tissue following acute and chronic exercise. Depending on intensity and duration, an acute bout of exercise is known to increase HO-1 protein expression in human lymphocytes [318], and rat skeletal muscle [319]. Chronic exercise has been shown to increase HO-1 expression in the aorta [321] and liver [323] of rats. 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 [349]. Because of this finding, we chose to examine the potential prophylactic benefits offered by performing exercise prior to a TBI. Exploring the novel use of exercise as "pre-habilitation" for improving recovery from TBI is also important since concerns still remain with current surgical and non-surgical treatment options [209]. Exercise training may provide a practical non-invasive intervention for improving TBI outcomes by modulating secondary TBI responses, increasing neuroprotection, and minimizing post-TBI complications. Damage resulting from post-TBI secondary injury responses may be diminished, and TBI outcomes improved, if exercise increases the endogenous production of VEGF-A, EPO, and HO-1 in the brain. Therefore, the purpose of this study was to determine whether pre-TBI exercise (i.e., exercise preconditioning) could improve post-TBI sensorimotor and cognitive function in adult mice, and to examine

endogenous VEGF-A, EPO, and HO-1 responses in the brain after exercise and TBI. We hypothesized that pre-TBI exercise would reduce sensorimotor and spatial learning memory deficits in adult mice, while simultaneously increasing VEGF-A, EPO, and HO-1 gene and protein expression throughout various post-TBI and/or post-exercise time points in brain regions responsible for movement (i.e., sensorimotor cortex) and memory (i.e., hippocampus).

3.3. METHODS:

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 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 being sacrificed. 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.

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 [253]. The voluntary wheel running activities of mice in the exercise groups 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) $\pm$ SEM.

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

A moderate TBI was induced via a controlled cortical impact (CCI) targeting the right sensorimotor cortex of TBI group mice (NOEX-TBI [n=30] and EX-TBI [n=30]). Previous studies using magnetic resonance imaging have demonstrated high reliability for use of CCI in mice [341], and CCI reproduces the hallmark features of brain injuries observed in humans including motor deficits, memory loss, and neuron loss [342]. The CCI procedure was performed on the last day of the six week study, while TBI group mice were anesthetized with 2.5% isoflurane in 30% O_2 and air as previously described [91, 341]. Mice were stabilized in a Cunningham stereotaxic frame (Stoelting; Wood Dale, IN), and placed on a heating pad to maintain body temperature during the surgical procedure. The temperature of all mice was measured throughout the surgery with a rectal thermometer and kept at 38^{o} C. 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. The skull was viewed through a Wild operating microscope at 60X magnification, and 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. Any bleeding from the skull was controlled with bone wax. 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. The injury device was mounted on a platform through a flexible extension piece such that the motor could be oriented with respect to the platform. A 3 mm stainless-steel injury tip was attached to the end of the shaft. Immediately prior to CCI, 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 graphic user interface in the impactor control software (Visual Basic 6.0 software). Using this push-button graphic user interface, 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. Following impact, the skin was closed with a sterile suture, and all TBI group mice were returned to standard cages without running wheels. All control group 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. Each CCI surgery was performed within approximately 30 minutes.

Assessment of Sensorimotor Function

Sensorimotor deficits (i.e., number of foot faults per minute of walking) 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 the pre-TBI time point, 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, mice completed one trial per day, at the same time of day. Sensorimotor deficits (i.e., foot faults) were scored by an observer who was blinded to the grouping of mice at each time point. Mice were placed on an elevated wire grid (area measuring 32 cm×20 cm×50 cm with 11×11 mm diameter openings), and allowed to walk during 5 minute trials to score foot faults. 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 $\pm$ SEM.

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 [350]. RAWM testing combines assessment of spatial working memory via the radial arm maze, with assessment of spatial cognitive deficits provided by the Morris water maze. Furthermore, RAWM testing has been shown to be more sensitive than the Morris water

maze in detecting cognitive deficits in transgenic models of Alzheimer's disease [351, 352]. 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 (separated by 3 minute rest periods for each mouse), followed by a 30-minute delay interval (after completing all acquisition trials the mice were returned to their respective cages during the delay interval), 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. Video was taken during each session (both training and

testing) to record behavior and performance. Retention test data were expressed as the average number of non-goal arm entry errors ± SEM.

mRNA Analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was used for a gene expression time course study as previously described [87]. 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. At each time point, all 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. Spectrophotometry was performed using a Nanodrop (Nanodrop Technologies, Wilmington, DE) to assess RNA quality. In order to remove any contaminating genomic DNA, isolated RNA samples were treated with DNase (Ambion PCR Kit) according to the manufacturer's instructions. RNA purity and concentration were determined with an Agilent 2100 Bioanalyzer (Agilant 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 2 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]), 1µl primer mixture (10 µg forward and 10 µg reverse primers in 150µl 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 [353], and reported in accordance with The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [344]. Data were expressed as the average fold change ± SEM relative to GAPDH in brain samples from the NOEX-NOTBI group.

Table 2. 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

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 [91]. 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 μm thick coronal sections of the brain, and frozen sections were stored in ethylene glycol solution at -80°C. Prior to immunostaining, all 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. 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). Subsequent to overnight incubation, all sections were rinsed (3 washes x 5 minutes each wash) 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 $mm^2 \pm$ SEM in each brain region.

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 **$p<.01$).

3.4. RESULTS:

Exercise

In the present study, exercise group mice (i.e., exercise + no TBI [EX-NOTBI, n=30] and exercise + TBI [EX-TBI, n=30]) displayed consistent circadian rhythms during each week of the six week voluntary wheel running protocol (Figure 4). Mice ran throughout the night, and rarely

entered the running wheel during the day. The mice ran on average 4.413 ± .088 Km daily during the six week protocol (Figure 5). 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 increased and remained steady between weeks 2-6. Physical activity patterns of no exercise group mice (i.e., no exercise + no TBI [NOEX-NOTBI, n=30] and no exercise + TBI [NOEX-TBI, n=30]) were observed on several occasions throughout the day and night. The no exercise group mice appeared to be sleeping during the day, and displayed minimal activity at night that included mainly grooming, eating, drinking, and some walking while exploring the standard cage housing.

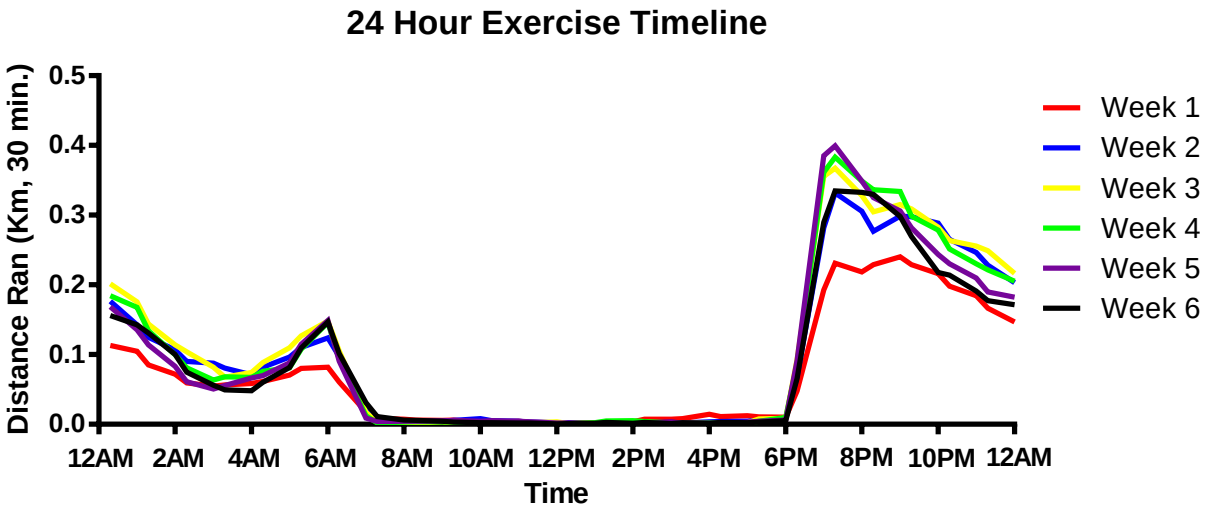

Figure 4. The average distance ran (in kilometers [Km]) of 5 month old male, adult mice (n=60) during 30 minute sampling intervals throughout the course of one day (i.e., 24 hours). The mice show profound circadian rhythms in their running patterns. Data are plotted separately for each week.

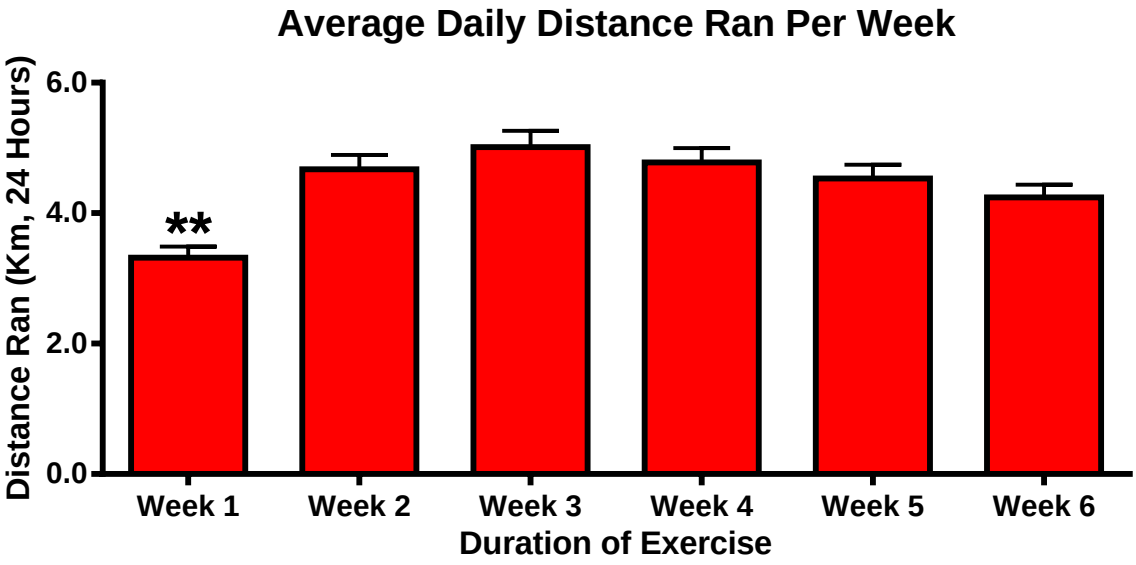

Figure 5. The average total daily distance ran (in kilometers [Km]) during each week of the 6 week exercise training period. Mice ran on average 4.413 $\pm$ .088 Km daily during the 6 week protocol. Mice ran significantly (**p<.01) less in week 1 when compared to all other weeks. However, the average total daily distance ran varied slightly during weeks 2-6. Statistical Analysis: One-way repeated measures ANOVA with Fisher's LSD post hoc testing for multiple comparisons. Mean $\pm$ SEM for n=60.

Behavioral Testing

Sensorimotor deficits (i.e., average number of foot faults per minute walking) were evaluated using the gridwalk task prior to TBI, and at 1, 3, and 7 days post-TBI (Figure 6). Prior to TBI, there were no statistically significant differences between NOEX-TBI and EX-TBI mice in the average number of foot faults per minute walking. After injury, both TBI groups exhibited significant ($p<.01$, not labeled on graph) increases in the average number of foot faults per minute walking during the gridwalk task at all post-TBI time points when both TBI 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 7). 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 decreased spatial learning memory deficits prior to TBI, 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.

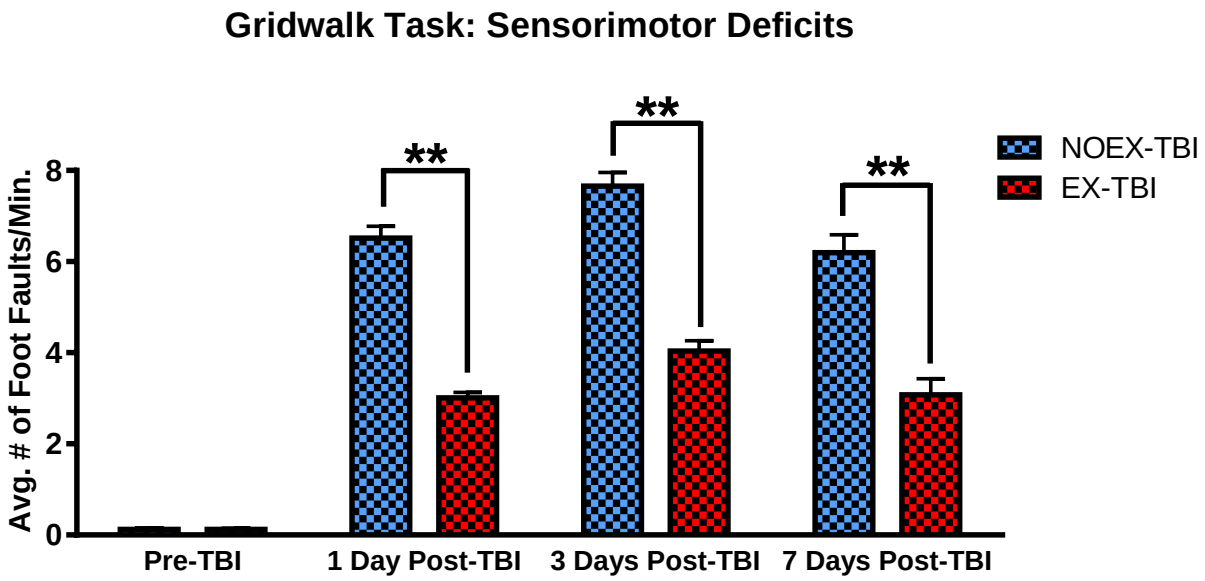

Figure 6. The gridwalk task was used for investigating sensorimotor deficits (i.e., average number of foot faults per minute walking). TBI significantly ($p<.01$, not labeled on graph) increased the average number of foot faults per minute walking during the gridwalk task in both no exercise + TBI (NOEX-TBI) and exercise + TBI (EX-TBI) mice at all post-TBI time points when compared to pre-TBI. However, 6 weeks of pre-TBI voluntary wheel running exercise reduced the average number of foot faults per minute walking at 1, 3, and 7 days post-TBI. EX-TBI mice demonstrated a significantly (**$p<.01$) reduced average number of foot faults per minute walking during the gridwalk task at all post-TBI time points when compared to NOEX-TBI mice. Statistical Analysis: Two-way repeated measures ANOVA (fixed factors; day and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean $\pm$ 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.

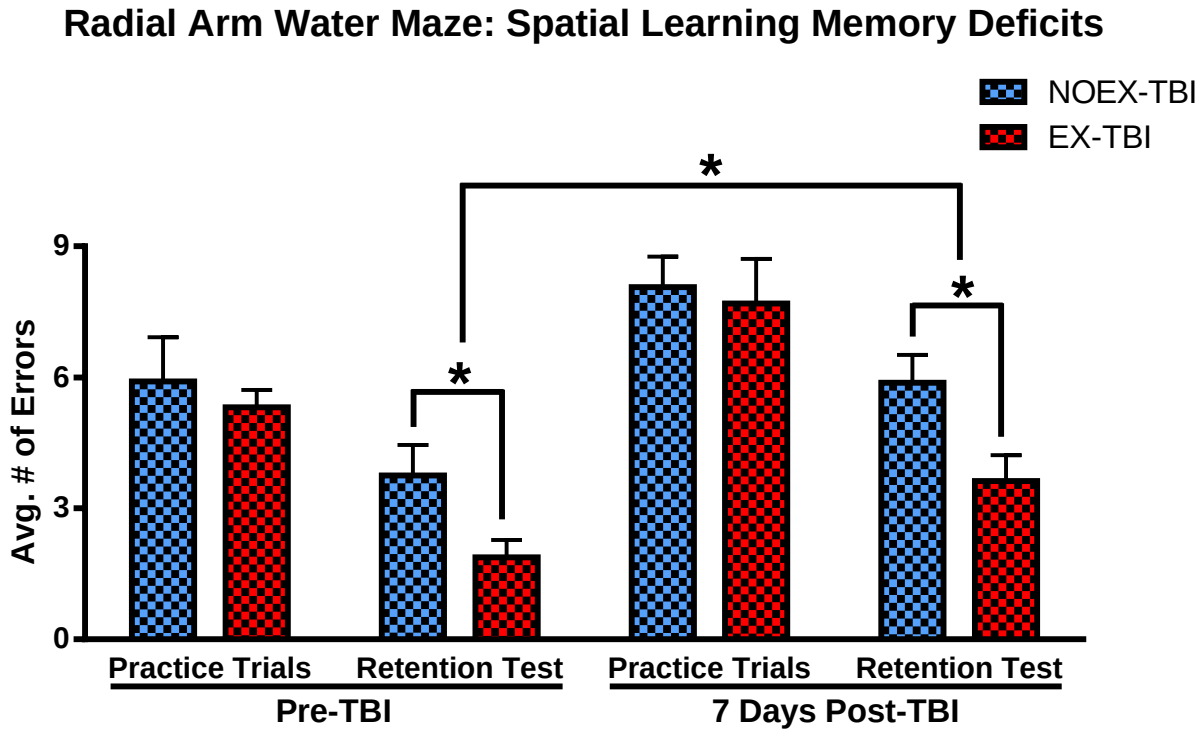

Figure 7. Radial arm water maze (RAWM) testing was conducted for evaluation of spatial learning memory deficits (i.e., average number of non-goal arm entry errors). TBI significantly (*p<.05) increased the average number of non-goal arm entry errors during the RAWM retention test in both no exercise + TBI (NOEX-TBI) and exercise + TBI (EX-TBI) mice at 7 days post-TBI. However, 6 weeks of pre-TBI voluntary wheel running exercise reduced the average number of non-goal arm entry errors prior to TBI, and at 7 days post-TBI. EX-TBI mice displayed a significantly (*p<.05) decreased average number of non-goal arm entry errors during the RAWM retention test at both time points (i.e., pre-TBI and 7 days post-TBI) when compared to NOEX-TBI mice. Statistical Analysis: Two-way repeated measures ANOVA (fixed factors; day and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean $\pm$ SEM for n=8 per group at each time point.

VEGF-A Gene Expression

VEGF-A mRNA expression increased within the right cerebral cortex (Figure 8) and ipsilateral hippocampus (Figure 9) 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 voluntary wheel running exercise but no TBI, 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. In response to the 6 week exercise intervention 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.

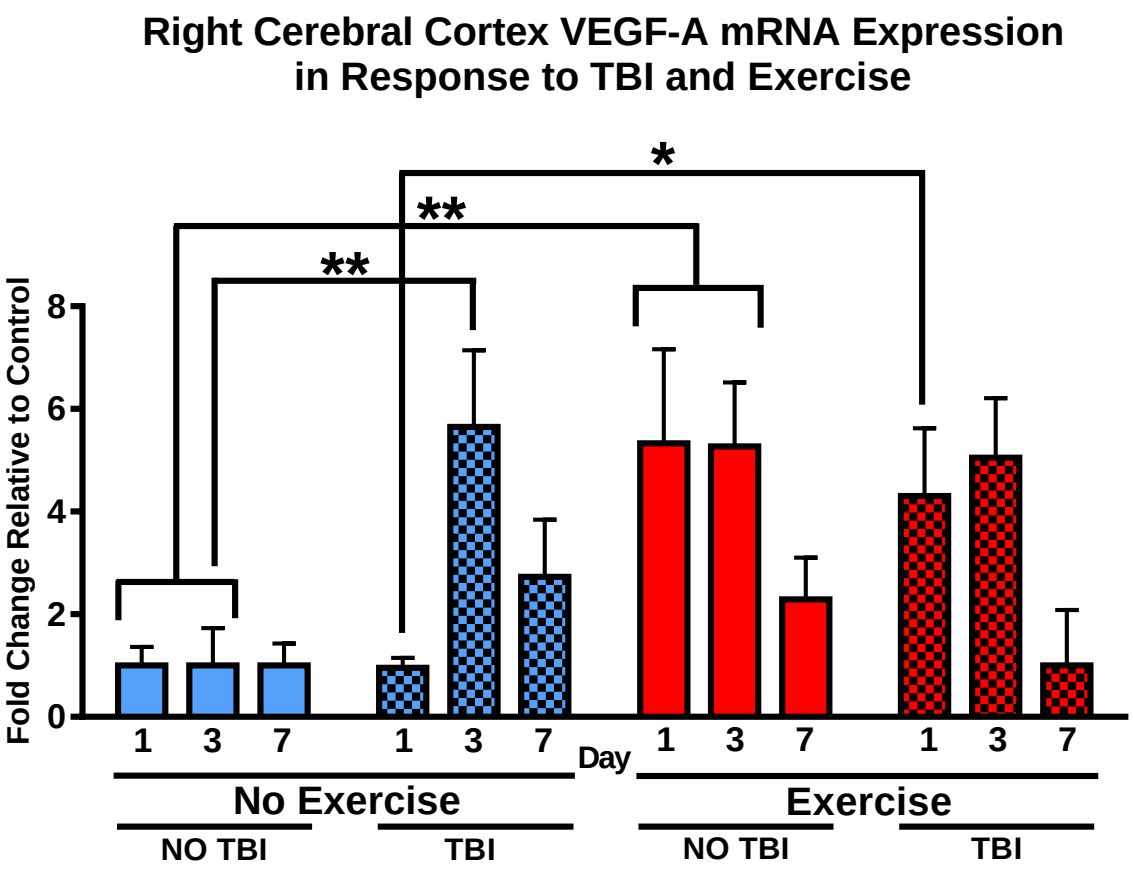

Figure 8. qRT-PCR study of right cerebral cortex VEGF-A mRNA responses after TBI and exercise (i.e., 6 weeks of pre-TBI voluntary wheel running). Y-axis is the average fold change relative to GAPDH (housekeeping gene) in the no exercise + no TBI (NOEX-NOTBI) mouse cortex. Data represents VEGF-A mRNA 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) mice. In response to TBI only, NOEX-TBI mice demonstrated significantly (**p<.01) increased VEGF-A mRNA expression at 3 days post-TBI when compared to NOEX-NOTBI mice. In response to exercise only, EX-NOTBI mice displayed significantly (**p<.01) increased VEGF-A mRNA expression at both 1 and 3 days post-exercise in comparison to NOEX-NOTBI mice. EX-TBI mice showed a significant early (*p<.05) increase in VEGF-A mRNA at 1 day post-exercise/post-TBI when compared to NOEX-TBI mice. Statistical Analysis: Three-way ANOVA (fixed factors; day, TBI, and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean $\pm$ SEM for n=8 per group at each time point.

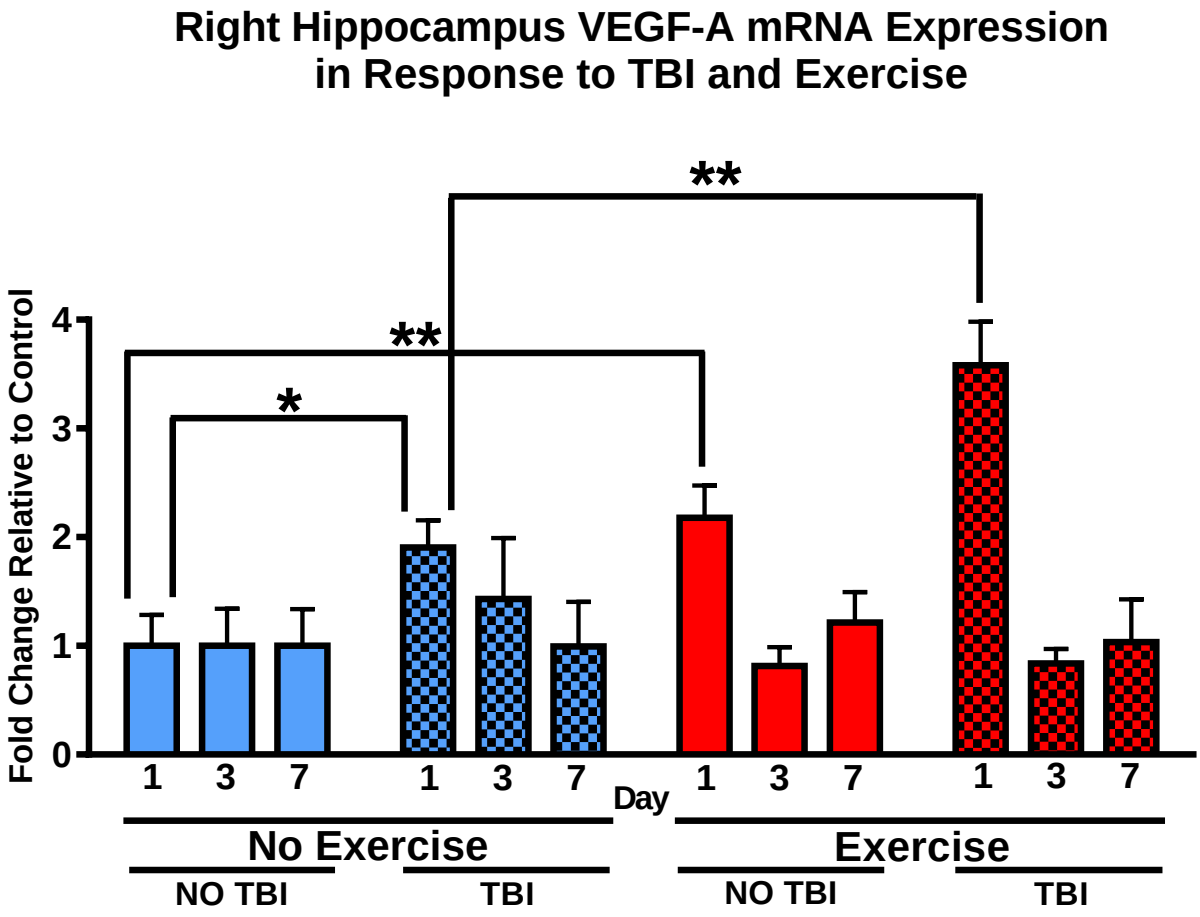

Figure 9. qRT-PCR study of right hippocampus VEGF-A mRNA responses after TBI and exercise (i.e., 6 weeks of pre-TBI voluntary wheel running). Y-axis is the average fold change relative to GAPDH (housekeeping gene) in the no exercise + no TBI (NOEX-NOTBI) mouse cortex. Data represents VEGF-A mRNA expression 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) mice. In response to TBI only, NOEX-TBI mice demonstrated significantly (*p<.05) increased VEGF-A mRNA expression at 1 day post-TBI when compared to NOEX-NOTBI mice. In response to exercise only, EX-NOTBI mice displayed significantly (**p<.01) increased VEGF-A mRNA expression at 1 day post-exercise in comparison to NOEX-NOTBI mice. EX-TBI mice showed a significant (**p<.01) increase in VEGF-A mRNA at 1 day post-exercise/post-TBI when compared to NOEX-TBI mice. Statistical Analysis: Three-way ANOVA (fixed factors; day, TBI, and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean $\pm$ SEM for n=8 per group at each time point.

VEGF-A Protein Localization

Immunohistochemistry revealed VEGF-A protein localization within neurons found in various regions of the right cerebral cortex (Figure 10) and ipsilateral hippocampus CA1 field (Figure 11). 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 layers 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.

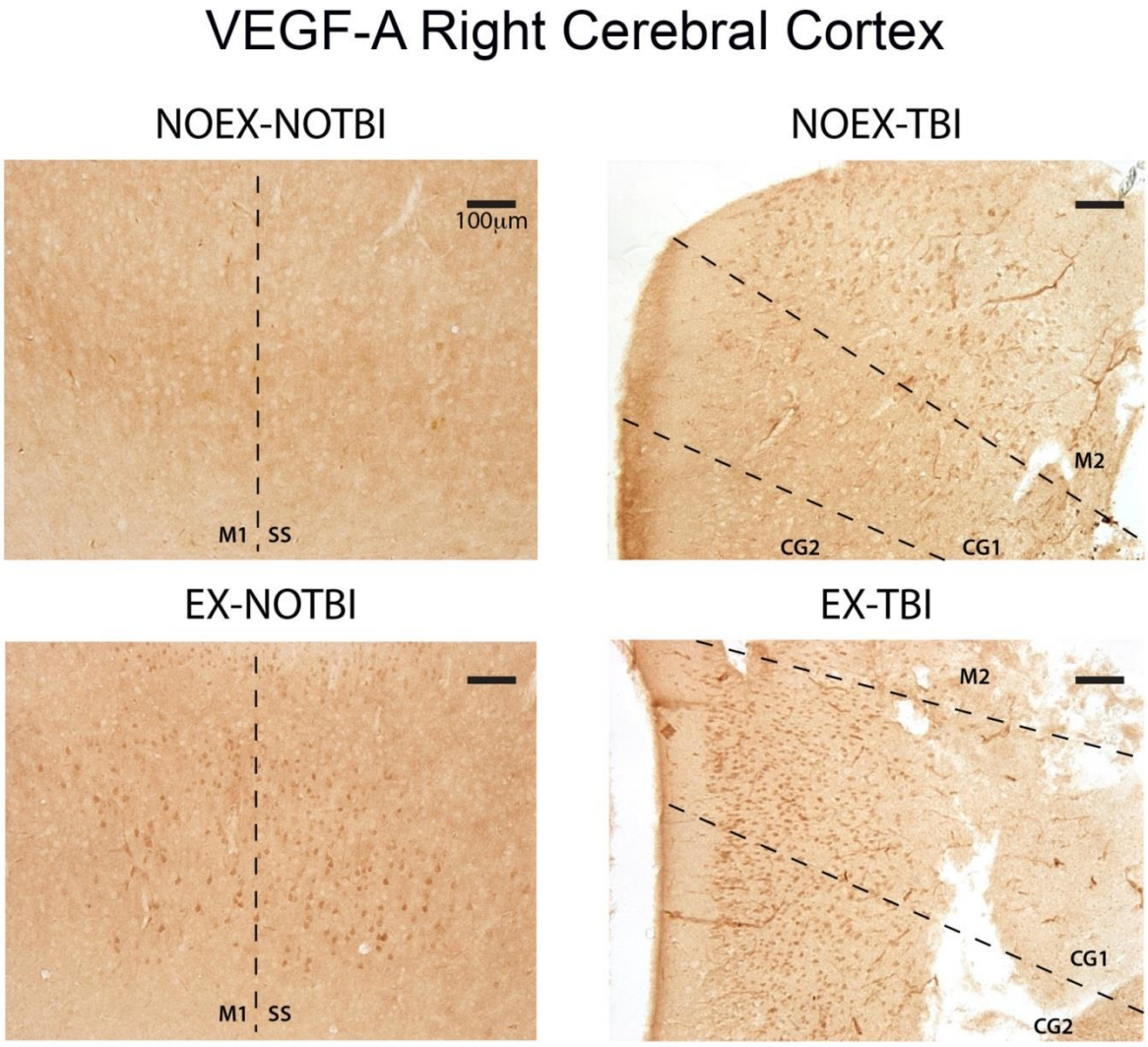

Figure 10. Coronal immunostained sections (35 μm thick sections at Bregma 0.02 mm) showing localization of VEGF-A protein in the right cerebral cortex of no exercise + no TBI (NOEX-NOTBI), no exercise + TBI (NOEX-TBI), exercise + no TBI (EX-NOTBI), and exercise + TBI (EX-TBI) mice at 1 day post-TBI and/or post-exercise. 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 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. EX-NOTBI mice demonstrated very noticeable VEGF-A staining within neurons located in the primary motor cortex (M1), secondary motor cortex (not shown), and somatosensory (SS) hindlimb region. 10X magnification, scale bar = 100 μm for all images displayed.

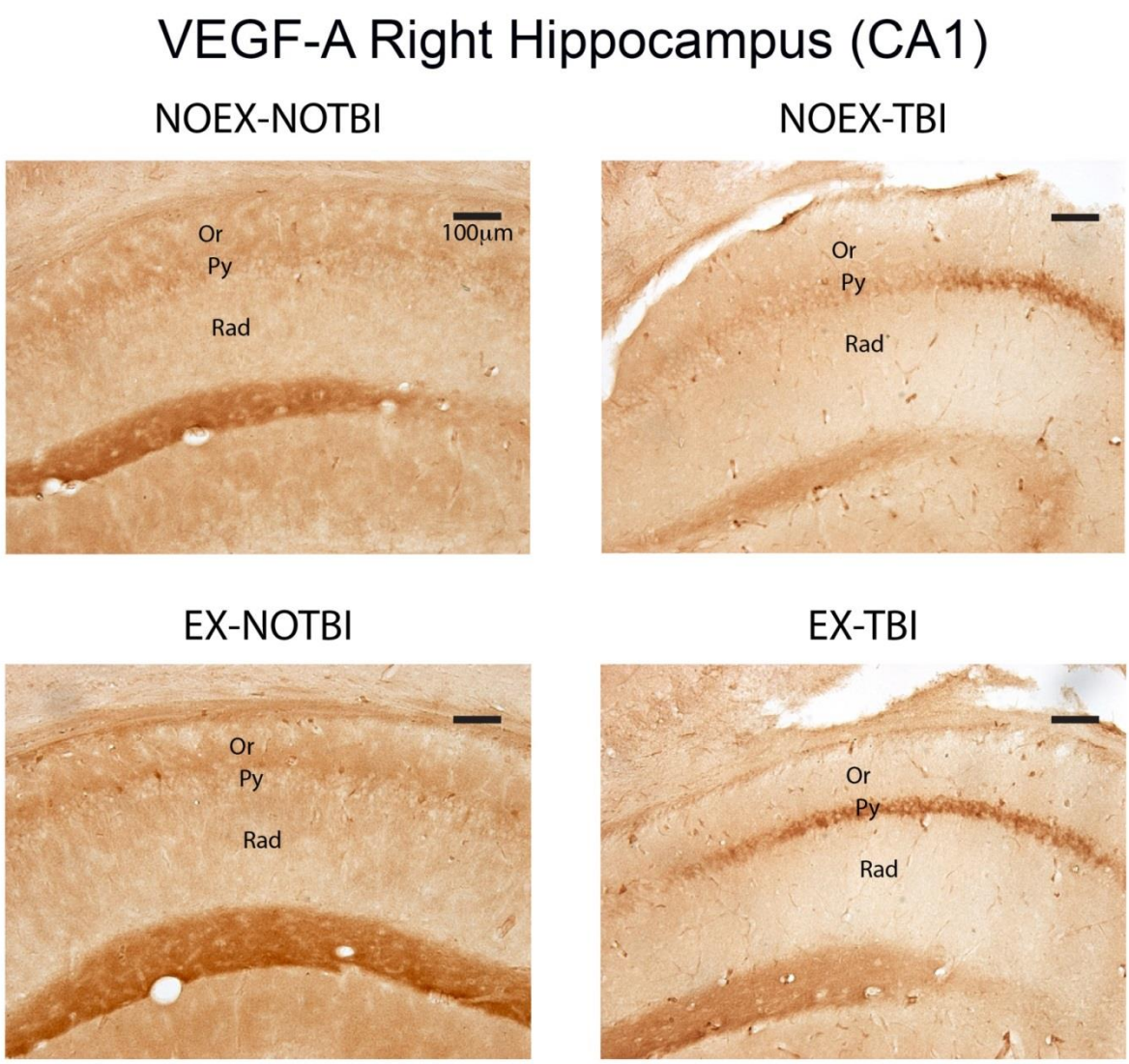

Figure 11. Coronal immunostained sections (35 µm thick sections at Bregma -1.94 mm) showing localization of VEGF-A protein in the right hippocampus (CA1 field) of no exercise + no TBI (NOEX-NOTBI), no exercise + TBI (NOEX-TBI), exercise + no TBI (EX-NOTBI), and exercise + TBI (EX-TBI) mice at 1 day post-TBI and/or post-exercise. 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. EX-NOTBI mice also demonstrated VEGF-A staining within neurons located in the oriens layer (Or) and pyramidal cell (Py) layers. 10X magnification, scale bar = 100 µm for all images displayed.

VEGF-A Protein Quantification

VEGF-A protein expression (i.e., average number of VEGF-A positive stained neurons per mm^2) increased within the right cerebral cortex (Figure 12) 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 13) 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 mm^2 in both the right cerebral cortex and right hippocampus CA1 field. *Post hoc* testing showed several interesting differences when comparing groups. In response to 6 weeks of exercise only, 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.

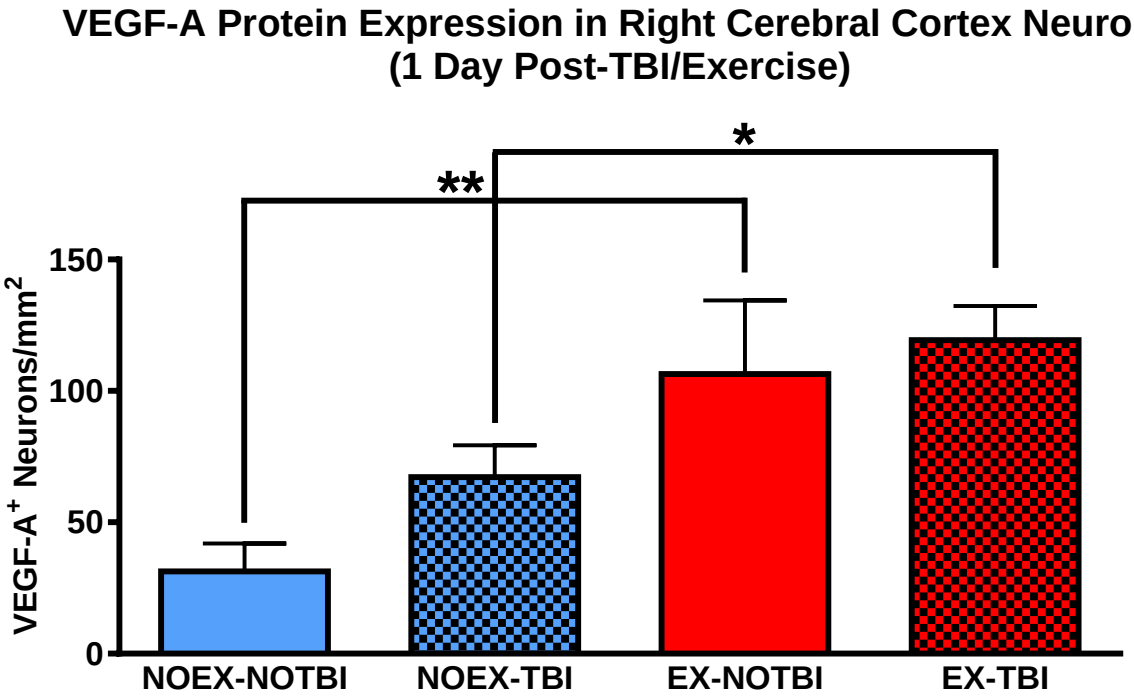

Figure 12. Immunostaining (Anti-VEGF-A antibodies) was performed on coronal cut (35 μm thick) brain sections (Bregma 0.02 mm) to quantify VEGF-A protein expression within neurons of the right cerebral cortex at 1 day post-TBI and/or post-exercise. Data represents the average number of VEGF-A positive stained neurons per mm^2 for no exercise + no TBI (NOEX-NOTBI), no exercise + TBI (NOEX-TBI), exercise + no TBI (EX-NOTBI), and exercise + TBI (EX-TBI) mice. Neurons staining positive for VEGF-A protein were manually counted within a 3.93721 mm^2 region of the right cerebral cortex which 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. In response to exercise only, 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. In addition, 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 group mice. Statistical Analysis: Two-way ANOVA (fixed factors; TBI and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean $\pm$ SEM for n=6 per group.

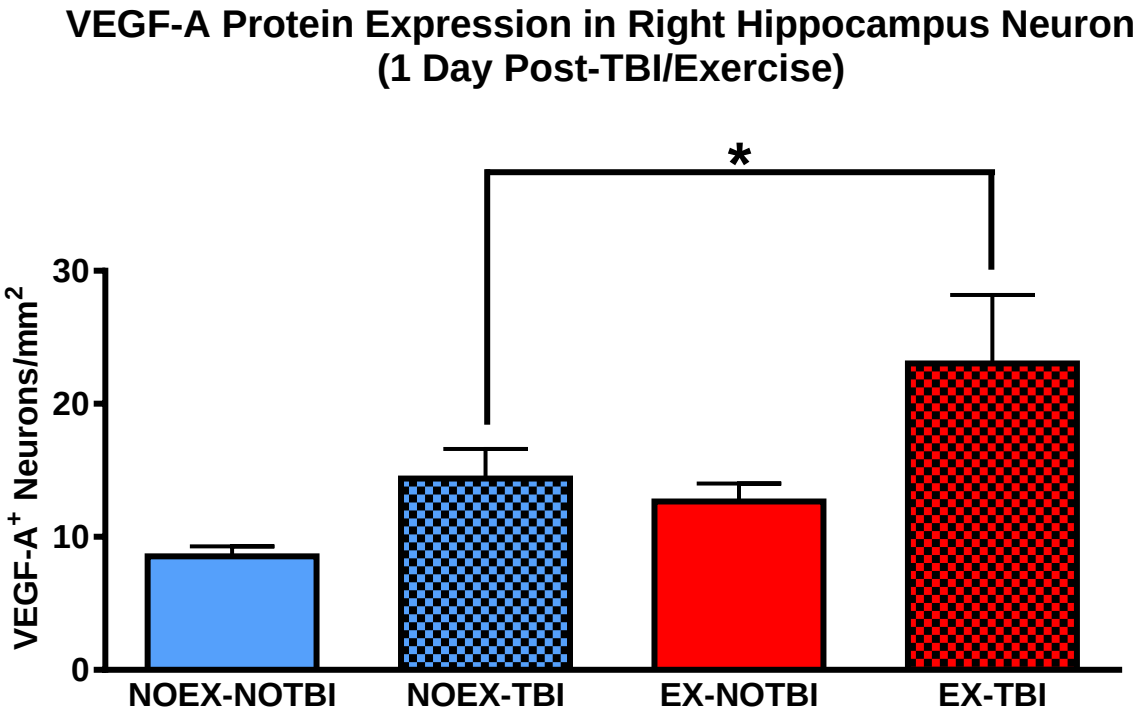

Figure 13. Immunostaining (Anti-VEGF-A antibodies) was performed on coronal cut (35 μm thick) brain sections (Bregma -1.94 mm) to quantify VEGF-A protein expression within neurons of the right hippocampus CA1 field at 1 day post-TBI and/or post-exercise. Data represents the average number of VEGF-A positive stained neurons per mm^2 for no exercise + no TBI (NOEX-NOTBI), no exercise + TBI (NOEX-TBI), exercise + no TBI (EX-NOTBI), and exercise + TBI (EX-TBI) mice. Neurons staining positive for VEGF-A protein were manually counted within an 891.6 μm^2 region of the right hippocampus CA1 field. 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 $\pm$ SEM for n=6 per group.

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 14) 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 15) EPO mRNA expression.

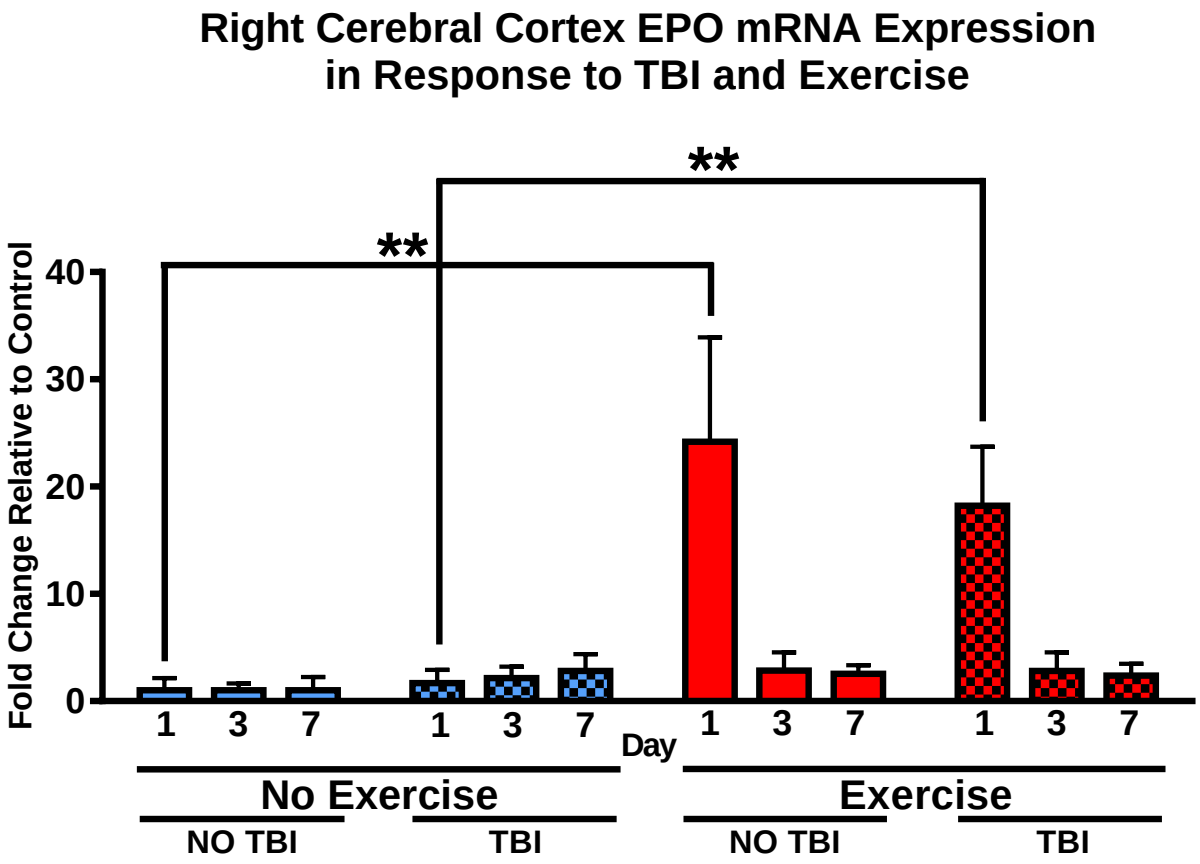

Figure 14. qRT-PCR study of right cerebral cortex EPO mRNA responses after TBI and exercise (i.e., 6 weeks of pre-TBI voluntary wheel running). Y-axis is the average fold change relative to GAPDH (housekeeping gene) in the no exercise + no TBI (NOEX-NOTBI) mouse cortex. Data represents EPO mRNA expression 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) mice. In response to exercise only, EX-NOTBI mice displayed significantly (**p<.01) increased EPO mRNA expression at 1 day post-exercise in comparison to NOEX-NOTBI mice. EX-TBI mice showed a significant (**p<.01) increase in EPO mRNA at 1 day post-exercise/post-TBI when compared to NOEX-TBI group mice. Statistical Analysis: Three-way ANOVA (fixed factors; day, TBI, and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean $\pm$ SEM for n=8 per group at each time point.

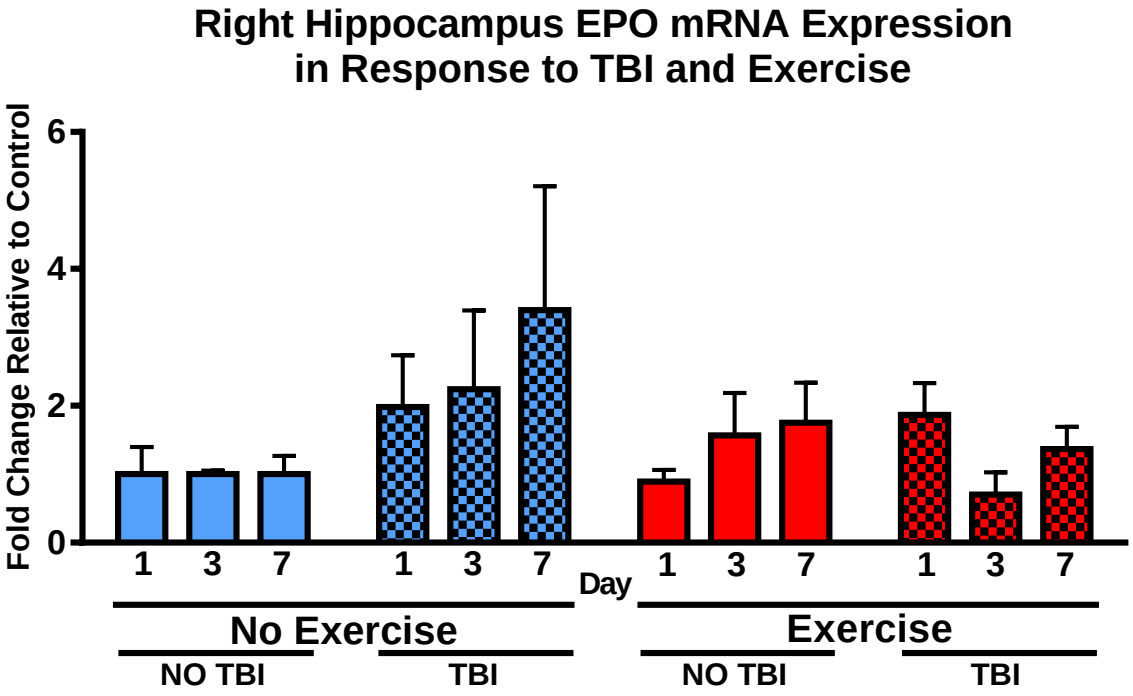

Figure 15. qRT-PCR study of right hippocampus EPO mRNA responses after TBI and exercise (i.e., 6 weeks of pre-TBI voluntary wheel running). Y-axis is the average fold change relative to GAPDH (housekeeping gene) in the no exercise + no TBI (NOEX-NOTBI) mouse cortex. Data represents EPO mRNA expression 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) mice. No statistically significant differences existed between groups. Statistical Analysis: Three-way ANOVA (fixed factors; day, TBI, and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean $\pm$ SEM for n=8 per group at each time point.

EPO Protein Localization

EPO protein was localized within neurons throughout various regions of the right cerebral cortex (Figure 16) and ipsilateral hippocampus CA1 field (Figure 17). 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 staining pattern of EPO positive 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 group 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.

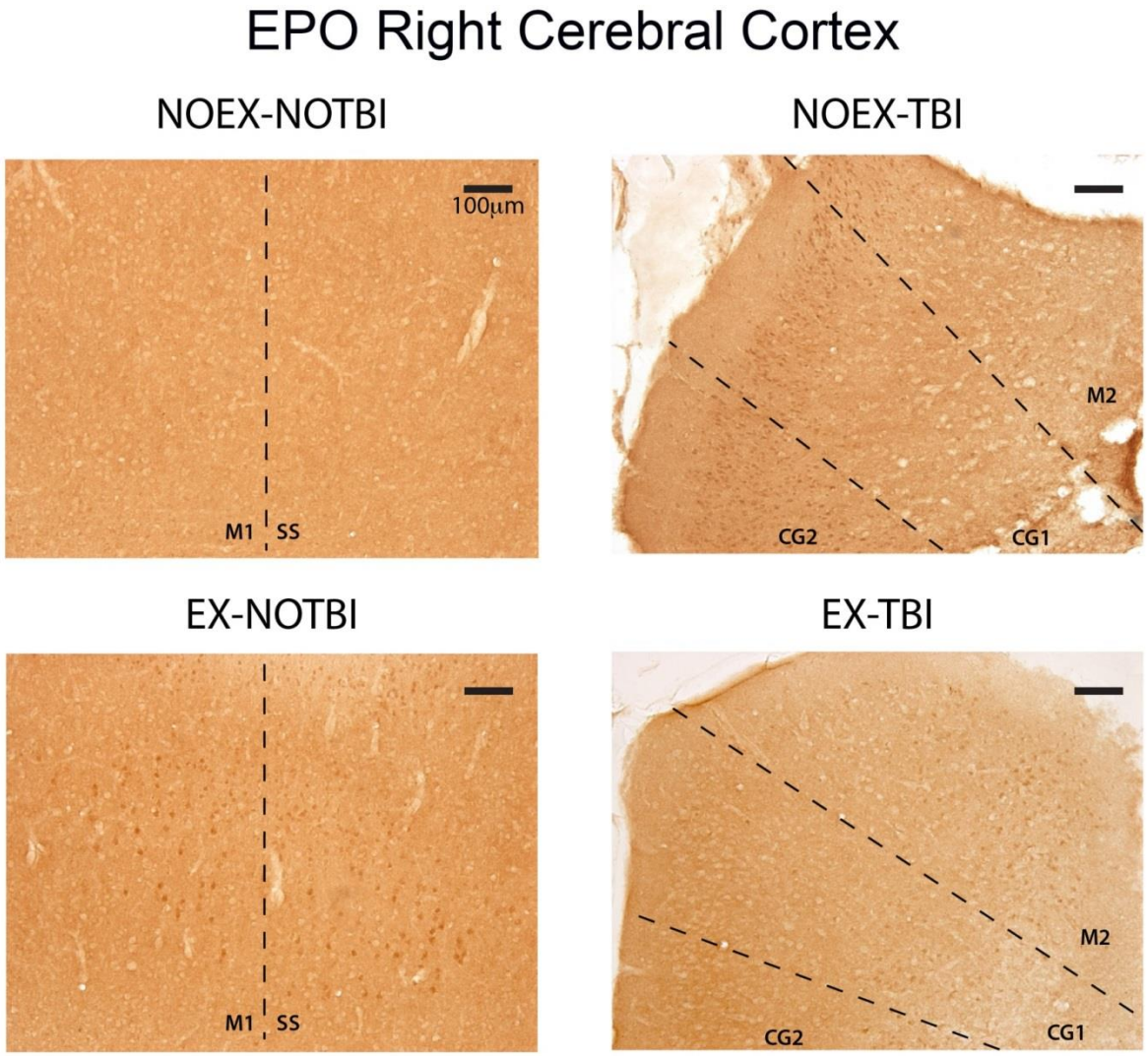

Figure 16. Coronal immunostained sections (35 μm thick sections at Bregma 0.02 mm) showing localization of EPO protein in the right cerebral cortex of no exercise + no TBI (NOEX-NOTBI), no exercise + TBI (NOEX-TBI), exercise + no TBI (EX-NOTBI), and exercise + TBI (EX-TBI) mice at 1 day post-TBI and/or post-exercise. 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 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. 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. 10X magnification, scale bar = 100 μm for all images displayed.

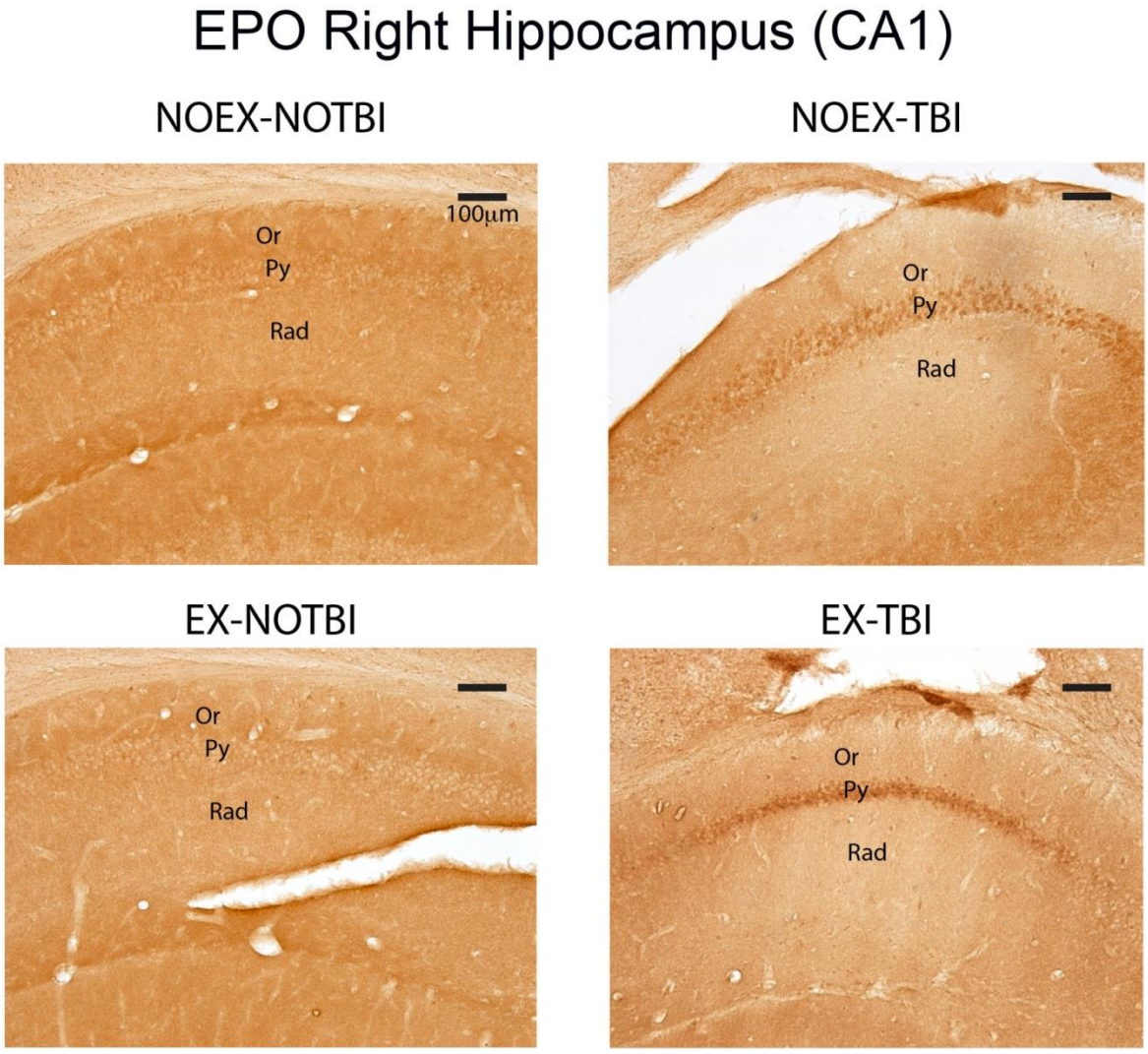

Figure 17. Coronal immunostained sections (35 µm thick sections at Bregma -1.94 mm) showing localization of EPO protein in the right hippocampus (CA1 field) of no exercise + no TBI (NOEX-NOTBI), no exercise + TBI (NOEX-TBI), exercise + no TBI (EX-NOTBI), and exercise + TBI (EX-TBI) mice at 1 day post-TBI and/or post-exercise. NOEX-NOTBI mice demonstrated very few neurons staining positive for EPO. NOEX-TBI and EX-TBI mice revealed an obvious staining pattern of EPO positive neurons located in the pyramidal cell (Py) layer. In addition, NOEX-TBI and EX-TBI mice showed scant EPO staining within neurons located in the oriens layer (Or) and stratum radiatum (Rad). EX-NOTBI mice displayed light staining within neurons that were primarily located in the oriens layer (Or). 10X magnification, scale bar = 100 µm for all images displayed.

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 18) 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 19) 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.

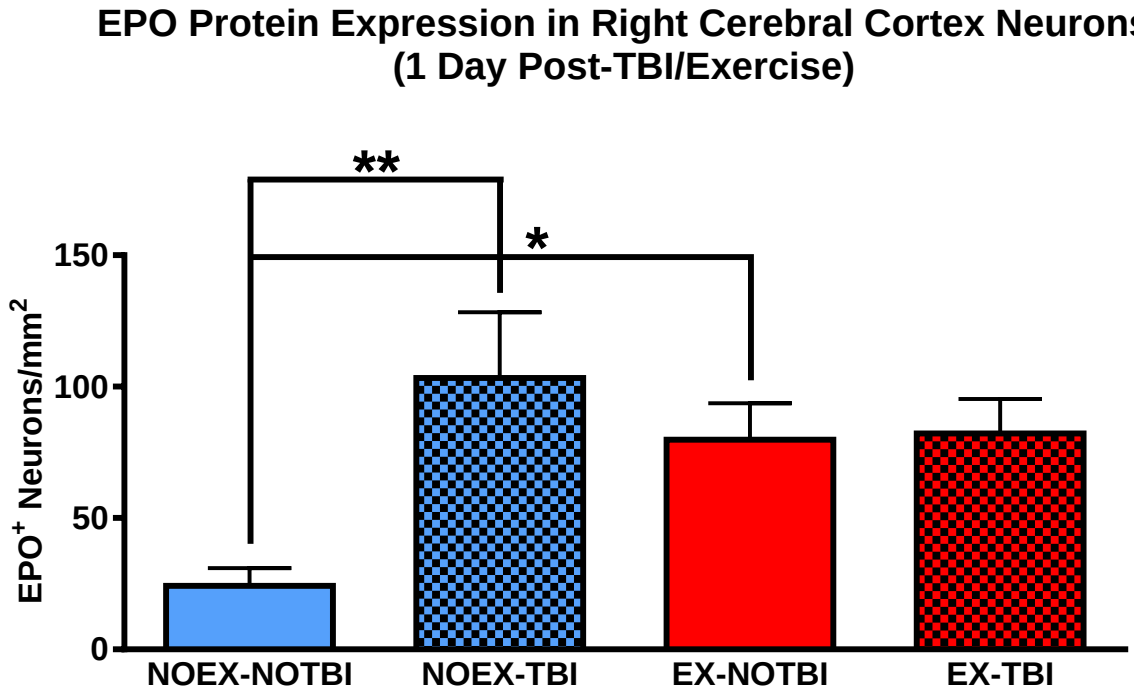

Figure 18. Immunostaining (Anti-EPO antibodies) was performed on coronal cut (35 μm thick) brain sections (Bregma 0.02 mm) to quantify EPO protein expression within neurons of the right cerebral cortex at 1 day post-TBI and/or post-exercise. Data represents the average number of EPO positive stained neurons per mm^2 for no exercise + no TBI (NOEX-NOTBI), no exercise + TBI (NOEX-TBI), exercise + no TBI (EX-NOTBI), and exercise + TBI (EX-TBI) mice. Neurons staining positive for EPO protein were manually counted within a 3.93721 mm^2 region of the right cerebral cortex which 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. In response to TBI only, NOEX-TBI mice demonstrated a significantly (**$p<.01$) increased average number of neurons staining positive for EPO protein in comparison to NOEX-NOTBI mice. In response to exercise only, EX-NOTBI mice displayed a significantly (*$p<.05$) increased average number of neurons staining positive for EPO protein when compared to NOEX-NOTBI mice. Statistical Analysis: Two-way ANOVA (fixed factors; TBI and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean $\pm$ SEM for n=6 per group.

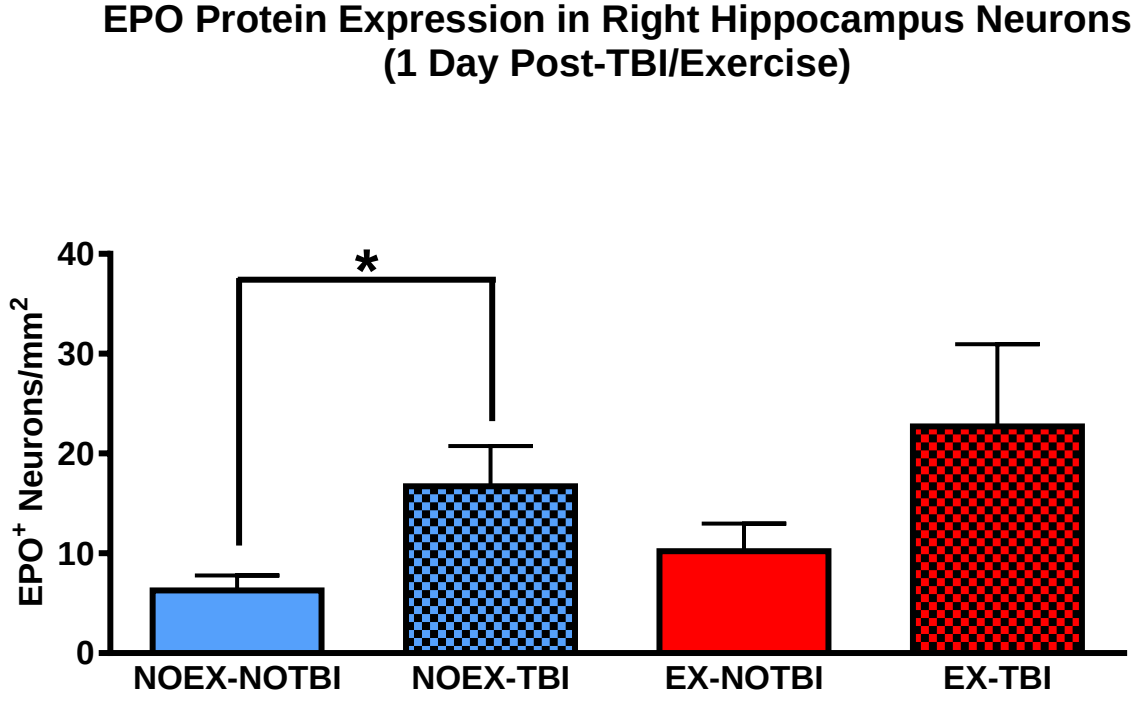

Figure 19. Immunostaining (Anti-EPO antibodies) was performed on coronal cut (35 μm thick) brain sections (Bregma -1.94 mm) to quantify EPO protein expression within neurons of the right hippocampus CA1 field at 1 day post-TBI and/or post-exercise. Data represents the average number of EPO positive stained neurons per mm^2 for no exercise + no TBI (NOEX-NOTBI), no exercise + TBI (NOEX-TBI), exercise + no TBI (EX-NOTBI), and exercise + TBI (EX-TBI) mice. Neurons staining positive for EPO protein were manually counted within an 891.6 μm^2 region of the right hippocampus CA1 field. In response to TBI only, NOEX-TBI group 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 $\pm$ SEM for n=6 per group.

HO-1 Gene Expression

HO-1 mRNA expression increased within the right cerebral cortex (Figure 20) and ipsilateral hippocampus (Figure 21) 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. HO-1 protein was not investigated in this study because exercise did not significantly increase HO-1 mRNA in the right cerebral cortex or ipsilateral hippocampus.

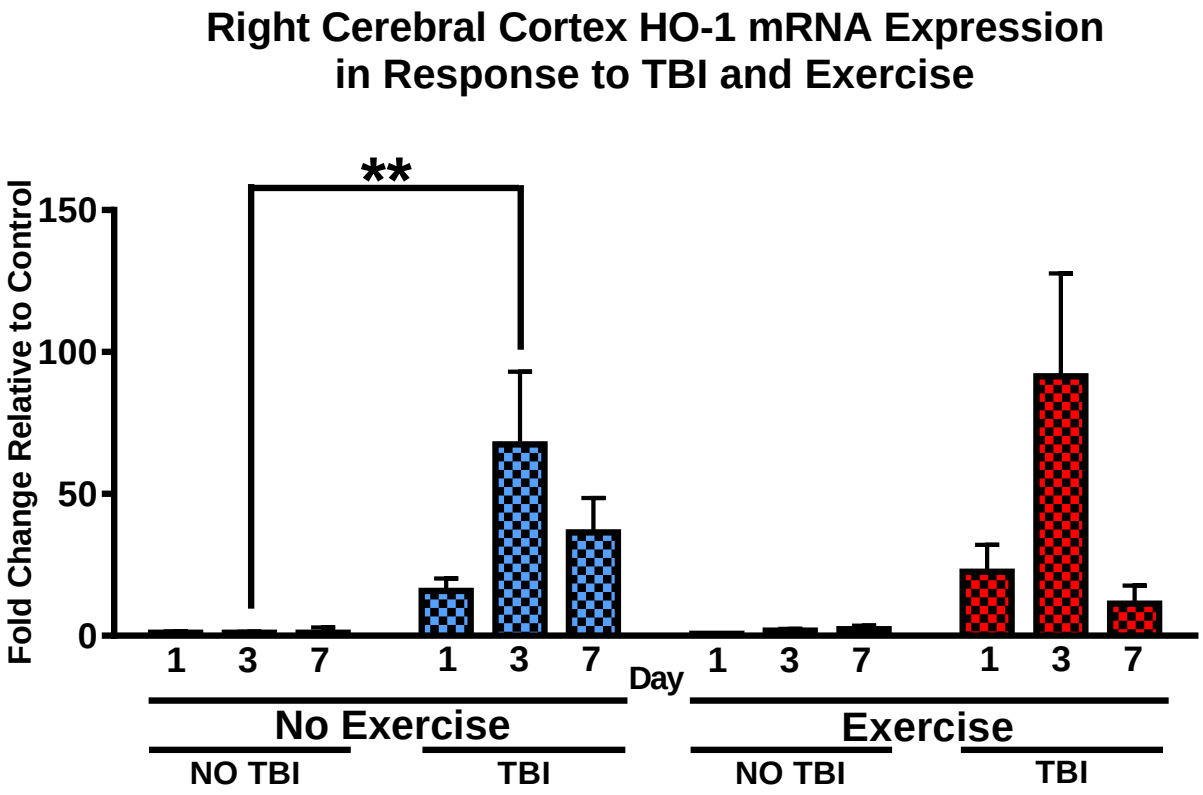

Figure 20. qRT-PCR study of right cerebral cortex HO-1 mRNA responses after TBI and exercise (i.e., 6 weeks of pre-TBI voluntary wheel running). Y-axis is the average fold change relative to GAPDH (housekeeping gene) in the no exercise + no TBI (NOEX-NOTBI) mouse cortex. Data represents HO-1 mRNA expression 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) mice. In response to TBI only, NOEX-TBI mice displayed significantly (**p<.01) increased HO-1 mRNA expression at 3 days post-TBI when compared to NOEX-NOTBI mice. Statistical Analysis: Three-way ANOVA (fixed factors; day, TBI, and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean $\pm$ SEM for n=8 per group at each time point.

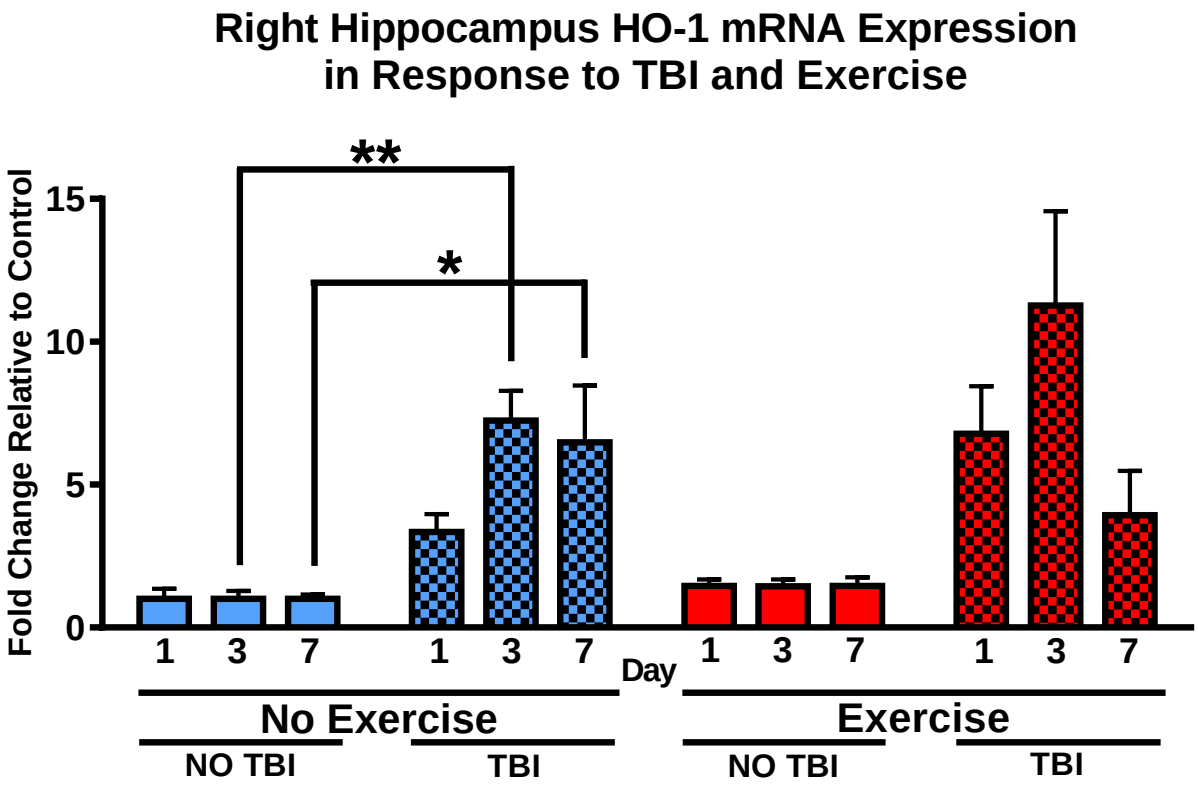

Figure 21. qRT-PCR study of right hippocampus HO-1 mRNA responses after TBI and exercise (i.e., 6 weeks of pre-TBI voluntary wheel running). Y-axis is the average fold change relative to GAPDH (housekeeping gene) in the no exercise + no TBI (NOEX-NOTBI) mouse cortex. Data represents HO-1 mRNA expression 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) mice. In response to TBI only, NOEX-TBI mice displayed significantly (**$p<.01$, and *$p<.05$) increased HO-1 mRNA expression at 3 and 7 days post-TBI when compared to NOEX-NOTBI mice. Statistical Analysis: Three-way ANOVA (fixed factors; day, TBI, and exercise) with Fisher's LSD post hoc testing for multiple comparisons. Mean $\pm$ SEM for n=8 per group at each time point.

3.5. DISCUSSION:

Exercise Improves Post-TBI Sensorimotor Function

In the present study, we demonstrated that chronic pre-TBI exercise training improved post-TBI sensorimotor function. The EX-TBI mice displayed significant reductions in sensorimotor deficits when compared to NOEX-TBI mice at all three post-TBI time points. Other clinical and basic science research studies have also shown exercise can improve post-TBI motor function. Walking speed, mobility, and balance have been demonstrated to improve in humans when intensive mobility training exercise is initiated after TBI [354]. Improved motor function has also been noted in adult patients that performed post-TBI exercise in a virtual reality environment [243]. Mota et al., revealed that chronic pre-TBI aerobic exercise (i.e., 4 weeks of treadmill training) protected against TBI (i.e., fluid percussion injury) induced motor impairment in rats [261]. They also demonstrated that the attenuation of motor deficits seen in exercise group rats was linked to significantly decreased post-TBI neuroinflammation and BBB breakdown [261].

Exercise Improves Post-TBI Cognitive Performance

Results from this study revealed that chronic pre-TBI exercise training improved post-TBI cognitive performance (i.e. spatial learning memory). EX-TBI group mice exhibited a significant decrease in spatial learning memory deficits when compared to NOEX-TBI group mice at 7 days post-TBI. The finding that exercise can decrease spatial learning memory deficits is in agreement with research conducted by Gu et al., which showed 3 weeks of pre-TBI voluntary wheel running exercise attenuated spatial learning memory deficits in mice at 15 days post-TBI, and diminished neuron and synaptic density loss resulting from the injury [355]. The reductions in spatial learning memory deficits, and neuron and synaptic density loss, observed in

exercise group mice were associated with increased cytochrome c oxidase, brain-derived neurotrophic factor (BDNF), snyapsin I, synaptophysin, and growth associated protein 43 levels in the hippocampus [355]. Post-TBI exercise studies have also revealed that exercise enhances object recognition memory [326] and spatial learning memory [260] after injury. The beneficial effects of exercise on post-TBI cognitive function were attributed to increased brain BDNF levels in these studies [260, 326]. It has been suggested that BDNF plays a key role in improving cognitive function by facilitating axonal branching and remodeling of neural networks in brain structures (e.g., hippocampus) that are important for learning and memory [260, 325, 326, 328, 356]. These studies suggest that other neuroprotective molecules including VEGF-A and EPO may be responsible for increasing neuroprotection and improving TBI outcomes.

Exercise Increases VEGF-A Gene Expression in the Brain

Both exercise and TBI significantly increased right cerebral cortex (injured cortex) and right hippocampus VEGF-A gene expression in this study. However, mice from both exercise groups showed a significantly greater increase in VEGF-A mRNA expression in both the right cerebral cortex and right hippocampus when compared to mice from the no exercise and TBI only groups at 1 day post-TBI and/or post-exercise. Interestingly, VEGF-A mRNA expression did not significantly increase in the injured cortex of the no exercise TBI group mice until 3 days post-TBI, and this finding was in agreement with our previously published data [87]. The exercise-induced increase in VEGF-A gene expression observed here at 1 day post-exercise may contribute to improved neuroprotection in the brain, while the delayed increase in VEGF-A gene expression detected 3 days after TBI may reduce its neuroprotective potential. Furthermore, right cerebral cortex VEGF-A mRNA expression was still significantly increased 3 days after exercise was terminated. This increase in VEGF-A mRNA expression at 3 days post-exercise

was nearly equal to the VEGF-A mRNA expression level observed in mice that received TBI only. A prolonged increase in VEGF-A gene expression after chronic voluntary exercise may help to protect neurons in the brain from cell death that has been shown to peak at 3 days post-TBI in adult mice [91].

Exercise Increases VEGF-A Protein Expression in Brain Neurons

VEGF-A protein responses were investigated at 1 day post-TBI and/or post-exercise because this time point coincided with increased VEGF-A mRNA expression in the exercise group mice. Our immunohistochemical results confirmed the presence of VEGF-A protein within neurons located in the right cerebral cortex (i.e., primary motor cortex, secondary motor cortex, and cingulate cortex [area 1 and area 2]) and CA1 field of the right hippocampus. This finding is in agreement with data from other studies demonstrating VEGF-A localization in brain neurons [115-117]. Mice that exercised displayed dense VEGF-A staining in sensorimotor cortex (i.e., primary motor cortex and somatosensory hindlimb region) neurons of the right cerebral cortex. In addition, VEGF-A positive stained neurons were prevalent in the right cerebral cortex around the CCI injury site, and pyramidal layer of the right hippocampus CA1 field in tissue sections of mice from both TBI groups. However, mice that exercised prior to TBI exhibited a heavier staining pattern of neurons in both of these brain regions when compared to mice that did not exercise. We suggest the increased VEGF-A protein in exercising mice at early time points (i.e., prior to TBI, and at 1 day post-TBI) may have provided enhanced neuroprotection near the injury site, and in the CA1 region of the ipsilateral hippocampus.

Results from the current study indicated significantly increased endogenous VEGF-A protein expression in the brain of mice 1 day after termination of chronic voluntary exercise. Specifically, mice from both exercise groups showed a significantly greater increase in VEGF-A

protein expression within neurons of the right cerebral cortex when compared to mice from the no exercise and TBI only groups at 1 day post-TBI and/or post-exercise. Additionally, mice that engaged in exercise prior to induction of TBI exhibited a significant increase in VEGF-A protein expression within right hippocampus CA1 field neurons when compared to mice that did not exercise but received a TBI. We postulate that this increase in VEGF-A protein expression may have allowed more neurons to survive after injury, while also expediting healing of the brain. Several animal studies have demonstrated that VEGF-A is responsible for mediating favorable exercise effects on the brain. In a rat model of ischemic stroke, axon regeneration of newborn corticonigral and striatonigral neurons, and improved motor function after transient middle cerebral artery occlusion, have been linked to an increased production of VEGF-A in the brain after 28 days of treadmill training [313]. Furthermore, Fabel et al. concluded that VEGF-A is necessary for increased hippocampal neurogenesis in exercising mice [113]. In addition to its effects on neurons, increased VEGF-A protein production following exercise has been associated with greater capillary density in the cerebral cortex of exercising rats [311], and enhanced post-stroke cerebral blood flow within the ischemic lesion in a rat model of focal cerebral ischemia [312]. A VEGF-A mediated increase in angiogenesis and blood flow resulting from exercise may augment neuronal survival and accelerate healing by restoring the supply of oxygen rich blood to damaged brain tissues.

We speculate that exercised mice demonstrated improved post-TBI sensorimotor function and spatial learning memory in the current study at least in part as a result of increased VEGF-A protein protecting neurons within the right cerebral cortex and right hippocampus, respectively. Accordingly, it is possible that post-TBI neuron cell death was reduced in both brain regions due to exercise increasing VEGF-A protein production in neurons at 1 day post-TBI and/or post-

exercise. In contrast, the increased VEGF-A mRNA expression observed at 3 days post-TBI in mice that did not exercise may have been too late to augment neuroprotection during the secondary injury response. Further investigation of the roles exercise and VEGF-A could play in preventing neuron death and improving TBI outcomes is clearly warranted. Future studies should also explore whether the neuroprotective effects of exercise may be eliminated by blocking the central production of VEGF-A in the brain prior to, or immediately after injury. In addition, future research should investigate how TBI recovery may be improved by targeting VEGF-A with other non-pharmacological and pharmacological interventions.

Exogenous VEGF-A Administration for Treatment of TBI

The use of exogenous VEGF-A has been studied as a treatment approach for improving ischemic brain injury [117] and TBI outcomes [122]. However, delivering VEGF-A to the central nervous system through exogenous methods (e.g. intravenously) is very difficult due to the large molecular weight, limited BBB permeability, and extremely short half-life of the protein in plasma [219]. Therefore, interventions (e.g., exercise) that increase the endogenous production of VEGF-A in the brain may be a more practical alternative to exogenous VEGF-A TBI treatments.

Exercise Increases EPO Gene Expression in the Brain

Exercise significantly increased right cerebral cortex (injured cortex) EPO gene expression in this study. Mice from both exercise groups showed a significantly greater increase in right cerebral cortex EPO mRNA expression when compared to mice from the no exercise and TBI only groups 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 increased in the right cerebral cortex

3 days after termination of exercise. Taken together, these findings suggest that the post-TBI neuroprotection offered by increased EPO gene transcription may last for a shorter duration in comparison to VEGF-A, once exercise is stopped. Future gene expression time point studies investigating pre-TBI exercise and post-TBI neuron cell death are needed to confirm this. In the present study, EPO mRNA expression was not significantly increased at any post-TBI time point in the injured cortex of mice that received a TBI only. In contrast to VEGF-A, no significant differences in right hippocampus EPO mRNA expression were observed between the exercise or TBI groups in the present study. Results from the current study, and our prior research [87], demonstrate that exercise and TBI appear to increase EPO gene expression in a region specific (i.e., cerebral cortex only) manner within the brain.

Exercise Increases EPO Protein Expression in Brain Neurons

EPO protein responses were examined at 1 day post-TBI and/or post-exercise since we observed increased EPO mRNA at this time point in the exercise group mice. EPO is known to be produced in brain neurons [130], and our immunohistochemical results revealed EPO protein localized to neurons in the sensorimotor cortex of the right cerebral cortex and CA1 field of the right hippocampus. Mice that received exercise only, displayed dense EPO staining in sensorimotor cortex neurons of the right cerebral cortex. In addition, neurons stained positive for EPO were especially evident in the right cerebral cortex around the CCI injury site, and pyramidal layer of the right hippocampus CA1 field in tissue sections of mice from both TBI groups. The pattern of EPO staining observed in the right cerebral cortex and ipsilateral hippocampus was remarkably similar to VEGF-A in mice that received exercise, TBI, or exercise and TBI. In this study, EPO may have worked in conjunction with VEGF-A to promote

neuroprotection and brain repair after TBI in the right cerebral cortex near the injury site, and CA1 region of the ipsilateral hippocampus.

Results from the work presented here revealed that exercise and TBI significantly increased endogenous EPO protein expression in brain neurons of mice at 1 day post-TBI and/or post-exercise. Mice that performed exercise displayed a significantly greater increase in EPO protein expression within neurons of the right cerebral cortex when compared to non-exercising mice at 1 day post-exercise. This up-regulated production of EPO protein subsequent to exercise was comparable to the increase in VEGF-A protein within right cerebral cortex neurons. In mice that received TBI only, EPO protein expression was significantly increased within neurons of the right cerebral cortex and right hippocampus CA1 field at 1 day post-TBI. However, in contrast to VEGF-A, mice that exercised prior to induction of TBI did not exhibit a significant increase in EPO protein expression within neurons of the right cerebral cortex or right hippocampus CA1 field when compared to mice that received TBI only. In other words, our study results indicated that exercise did not increase EPO protein expression in brain neurons to a greater extent than the endogenous injury response at 1 day post-TBI. Nonetheless, exercise alone did increase EPO protein expression in right cerebral cortex neurons at 1 day post-exercise, and neuroprotection and brain repair may still have been enhanced after injury because more EPO protein would have been present in neurons of exercising mice prior to induction of TBI. To our knowledge, this is the first study investigating EPO protein expression within neurons of the brain after exercise and TBI. In comparison to VEGF-A, very few studies have been conducted investigating EPO responses to exercise.

In the present study, EPO protein was increased in right cerebral cortex neurons after long-term exercise in a manner that was analogous to VEGF-A. We suggest that exercised mice

showed improved post-TBI sensorimotor function as a result of increased EPO and VEGF-A protecting neurons within the sensorimotor cortex of the right cerebral cortex. It is conceivable that post-TBI neuron cell death may have been reduced in the right cerebral cortex due to exercise increasing EPO and VEGF-A protein expression in neurons prior to TBI. In contrast to the right cerebral cortex, EPO protein expression was not significantly increased within right hippocampus neurons after exercise. Thus, it is unlikely that EPO played a role in improving the spatial learning memory of exercised mice. Future research should examine the combination of exercise and hypoxia on EPO and TBI outcomes. It is known that performing exercise in a hypoxic environment increases EPO protein in the blood [315]. Elevated serum EPO levels improve oxygen carrying capacity in the body by stimulating increased red blood cell production [316]. An increased supply of circulating red blood cells may augment oxygen transport to the brain. Enhanced brain oxygenation would likely promote increased neuron survival after TBI, but studies are needed to endorse this idea. In addition, future studies should investigate endogenous EPO production in neurons of the brain when exercise is performed in a hypoxic environment. Performing pre-TBI exercise in a hypoxic environment may be a better method for increasing neuronal EPO expression, enhancing neuroprotection in the brain, and improving TBI outcomes. Post-TBI studies could also examine the possible beneficial effects of exercise in hypoxic environments on the brain. However, performing post-TBI exercise in hypoxic conditions too soon after injury would likely exacerbate hypoxic and ischemic responses in the brain, which in turn, could further contribute to neuronal injury and dysfunction. Determining the safety and efficacy of post-TBI exercise under hypoxic conditions would be imperative.

Exogenous EPO Administration for Treatment of TBI

The exogenous administration of EPO has been investigated as a treatment approach for brain injuries, since EPO can cross the BBB via active transport [209]. Injecting rats with recombinant human erythropoietin (rhEPO) after TBI has been shown to significantly decrease brain edema, and reduce the number of infiltrating apoptotic monocyte chemotactic protein-1^{+} and $CD68^{+}$ cells [137]. In addition, a single dose injection of 5,000 IU/kg of rhEPO administered at 1 and 24 hours post-TBI reduced white matter damage, attenuated neuroinflammation, increased the expression of the EPO receptor, and significantly improved sensorimotor and cognitive recovery in a rat model of diffuse TBI and hypoxia [220]. Post-TBI rhEPO injections are known to significantly increase neurogenesis within the cerebral cortex and hippocampus of mice [209]. Exogenous rhEPO treatments have decreased neuron cell death, and improved sensorimotor and spatial learning memory scores after TBI in mice [133]. Clinical trials are also investigating exogenous EPO administration for treatment of TBI [209, 226]. Unfortunately, it has been suggested that exogenous EPO administration may increase the risk of thrombosis in TBI patients [227], and evidence is lacking regarding the safety and efficacy of exogenous EPO treatments in TBI patients. Increasing the endogenous production of EPO in the brain through non-pharmacological interventions such as exercise may be a safer strategy for improving TBI outcomes.

TBI Increases HO-1 Gene Expression in the Brain

TBI significantly increased right cerebral cortex (injured cortex) and right hippocampus HO-1 gene expression in this study. However, in contrast to VEGF-A and EPO, exercise 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, and this

finding was in agreement with our previously published research [87]. This delayed post-TBI increase in HO-1 mRNA expression occurred at the same time point that VEGF-A mRNA significantly increased. In a response similar to the right cerebral cortex, HO-1 mRNA expression significantly increased at 3 days post-TBI in the right hippocampus. Furthermore, right hippocampus HO-1 mRNA expression remained elevated at 7 days post-TBI. Hypoxia is a common secondary injury response to TBI [90], and HO-1 is known to be induced in the brain during chronic hypoxia [150]. It is possible that hypoxia persisted within the right hippocampus at the 7 day post-TBI time point. An increased expression of HO-1 is thought to improve cellular resistance to oxidative stress resulting from TBI [157]. Peripheral HO-1 production may be up-regulated after exercise in an effort to protect cells against oxidative stress and inflammation that occur as a consequence of physical exertion [318]. However, results from this study indicated that exercise alone did not increase HO-1 mRNA in the brain. Therefore, other interventions that up-regulate HO-1 prior to 3 days post-TBI are needed to maximize its neuroprotective properties.

Nutraceutical Compounds Induce HO-1 and Demonstrate Potential for Treating TBI

Several nutraceutical compounds (e.g., sulforaphane, capsaicin, and resveratrol) are known to induce HO-1, enhance neuroprotection, and improve outcomes in animal models of TBI [357-360]. Sulforaphane is a naturally occurring compound found in cruciferous vegetables [348]. Rats that were administered sulforaphane 1 hour after TBI have demonstrated enhanced cognitive function [348]. Capsaicin is an active component of chili peppers, and pre-TBI administration of capsaicin to rats has been shown to significantly reduce motor and cognitive impairments after injury [358]. The antioxidant resveratrol promotes neuroprotection [359, 360]. Resveratrol is a polyphenolic stilbene that exists in grapes and red wine, and a recent study

demonstrated that pre-treating cells with resveratrol prevented rotenone-induced apoptosis of neurons through induction of HO-1 in an *in vitro* Parkinson's disease model [359]. Lin et al., showed that cell survival was improved with resveratrol treatment in a rat model of TBI, and the authors concluded that resveratrol may be beneficial therapeutic agent for treatment of TBI in humans [360]. Sulforaphane, capsaicin, and resveratrol need to be further investigated in TBI animal models and humans to develop a clearer picture of the potential therapeutic benefits these agents offer as a result of HO-1 induction.

Pre-TBI Exercise Intensity and Possible Mechanisms Responsible for Increasing Neuroprotection and Improving TBI Outcomes

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 post-concussion symptoms [361]. Because premature initiation of post-TBI exercise may exacerbate symptoms and lead to worse outcome, regular exercise participation prior to the unexpected occurrence of a TBI may be an effective prophylactic measure for optimizing recovery from head injury. More specifically, an individual that has performed high intensity exercise (i.e., above the lactate threshold) prior to a TBI may maximize the production of neuroprotective proteins in the brain, and this high intensity exercise dependent response could result in a better outcome when compared to a TBI patient that engaged in low intensity exercise 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 [308]. 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 [308]. The expression of other neuroprotective proteins may be highly dependent on exercise intensity.

Exercise intensity was not monitored in the current study since mice ran voluntarily. Nevertheless, mice that consistently exercised at higher intensities may have produced greater amounts of VEGF-A and EPO proteins in brain neurons when compared to mice that exercised at lower intensities. No studies have investigated how various exercise intensities influence the expression of VEGF-A, EPO, and other neuropotective hypoxia-inducible 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 may be observed in brain neurons 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 (pCO_2) in the blood [265]. In response to decreased pCO_2, cerebral vasoconstriction and diminished cerebral blood flow ensue, which reduces the supply of glucose and oxygen to the brain temporarily [265, 266]. Impaired oxygen supply and delivery to the brain may also briefly occur during high intensity exercise due to reductions in pH and decreased oxyhemoglobin binding [265]. It is reasonable to speculate that an increased production of neuroprotective hypoxia-inducible proteins may be detected 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.

Conclusions

This study showed that improved TBI outcomes were linked to exercise inducing the increased expression of neuroprotective genes and proteins in brain regions that are responsible

for movement (i.e., sensorimotor cortex) and memory (i.e., hippocampus). Long-term voluntary wheel running exercise performed prior to TBI 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. Pre-TBI exercise increased VEGF-A and EPO protein production in neurons of the brain at 1 day post-TBI and/or post-exercise. Thus, 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 [91]. In addition, it is probable that pre-TBI exercise accelerated the initiation of post-TBI brain repair by up-regulating VEGF-A and EPO production in the brain. 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. 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.

Chapter 4

Summary of Findings, Discussion and Future Directions

4.1. SUMMARY OF FINDINGS:

Despite being a major cause of death and disability, treating TBI remains difficult since concerns still remain with current treatments [209]. Consequently, novel non-pharmacological and non-surgical interventions that improve TBI outcomes are needed. Results from this dissertation demonstrated that gene therapy (i.e. gene overexpression) and pre-TBI exercise improved outcomes in a mouse model of TBI by increasing the expression of neuroprotective proteins in the brain. Specifically, improved post-TBI motor and cognitive function were associated with an increased endogenous production of neuroglobin, VEGF-A, and EPO in the brain.

Significant improvements in post-TBI sensorimotor function were noted in transgenic mice that overexpressed neuroglobin, and in mice that performed 6 weeks of pre-TBI exercise. Post-TBI reductions in sensorimotor deficits while walking during the gridwalk task, were linked to an increased production of neuroglobin in neurons and glial cells throughout the brain of transgenic mice, and increased VEGF-A and EPO expression in sensorimotor cortex neurons of pre-TBI exercise mice. Results from this dissertation also revealed improved post-TBI spatial learning memory in mice that performed long-term pre-TBI exercise. Pre-TBI exercise mice displayed a decrease in post-TBI spatial learning memory deficits during RAWM retention testing, and increased production of VEGF-A in the hippocampus.

The findings presented here demonstrate that gene therapy and exercise are practical non-pharmacological interventions that have the potential to improve TBI outcomes. Gene therapy and pre-TBI exercise increased the expression of neuroglobin, VEGF-A, and EPO within brain neurons prior to injury, or early after TBI during the acute phase of recovery. Post-TBI improvements in motor and cognitive function were associated with the increased expression of

these neuroprotective proteins in regions of the brain responsible for movement (i.e., sensorimotor cortex) and memory (i.e., hippocampus).

4.2. DISCUSSION AND FUTURE DIRECTIONS:

4.2.1. Improving TBI Outcomes Through Increased Neuroglobin Expression

In chapter 2 of this dissertation, a link between neuroglobin overexpression and improved sensorimotor outcomes after TBI was revealed. Results from this dissertation suggest that transgenic neuroglobin overexpressing mice may have demonstrated improved post-TBI sensorimotor function due to an increased endogenous production of neuroglobin in neurons and glial cells of the sensorimotor cortex. The increased production of neuroglobin in the aforementioned cells may have augmented neuroprotection prior to, and after TBI, thus improving post-TBI sensorimotor function in mice that overexpressed the gene. In contrast to neuroglobin overexpressing mice, wild-type mice displayed a late, but significant, increase in neuroglobin expression at 7 days post-TBI. This increase in neuroglobin expression during the sub-acute phase (i.e. 7 days post-TBI) was observed later in comparison to other neuroprotective hypoxia-inducible proteins that are known to be up-regulated at 3 days post-TBI [87]. Since significant neuron cell death is known to occur in adult mice at 3 days post-TBI [91], it is very possible that neuron cell death could be decreased in the brain if neuroglobin expression is increased prior to this time point. Reduced neuroprotection, greater neuron cell death, and worse post-TBI sensorimotor function may have ensued in wild-type mice due to the delayed increase in neuroglobin expression.

A comparison of differences in post-TBI neuron cell death between neuroglobin overexpressing mice and wild-type mice was not conducted in chapter 2 of this dissertation. However, in a future study, deOlmos aminocupric silver staining (i.e., stains degenerating and

dying neurons) could be used to investigate differences in post-TBI neuron cell death between neuroglobin overexpressing mice and wild-type mice. It is quite possible that post-TBI neuron cell death would be significantly reduced in neuroglobin overexpressing mice when compared to wild-type mice that show a delayed increase in endogenous neuroglobin expression past the time point (i.e., 3 days post-TBI) at which significant neuron cell death peaks. Decreased post-TBI neuron cell death in the brain of neuroglobin overexpressing mice would likely coincide with improved sensorimotor function after TBI. Another potential future study would be to investigate whether sensorimotor outcomes are worse after TBI in neuroglobin deficient (i.e., knock-out) mice when compared to wild-type mice. Results from this study could lend further support to the notion that neuroglobin plays a direct role in post-TBI sensorimotor recovery. In addition, future studies should investigate whether post-TBI cognitive function is improved as a result of increased neuroglobin expression.

Findings from this dissertation and previous studies suggest that increasing neuroglobin expression before TBI, or during the acute phase of recovery, could maximize neuroprotection and subsequently improve sensorimotor outcomes. The neuroglobin protein is produced intracellularly, and it is not capable of crossing cell membranes. Therefore, direct administration (e.g., oral, intramuscular, or intravenous) of neuroglobin is not a feasible treatment approach. However, in addition to gene therapy strategies, pharmacological interventions that increase neuroglobin production in the brain early after TBI may result in improved neuroprotection and better neurological outcome post-injury. Previous research has shown that intracellular neuroglobin production can be increased pharmacologically through administration of deferoxamine, cinnamic acid, and valproic acid [228]. The iron chelator deferoxamine is known to cross the BBB [362], and induce neuroglobin [228]. Interestingly, intranasal administration of

deferoxamine has been shown to protect the brain against stroke in animal models [363], and intraperitoneal injection of deferoxamine improved spatial learning memory performance after TBI in one study of rats [364]. Neuroglobin expression was not investigated in the brain after deferoxamine treatment in either of these studies. An *in vitro* study showed that cinnamic acid and valproic acid induce neuroglobin protein expression in cultured neurons [228]. However, no *in vivo* studies have been conducted to determine whether administration of these small molecules induces neuroglobin within brain neurons.

Future research is needed to determine whether pharmacological treatments aimed at increasing neuroglobin production in the brain after TBI are safe and effective. It is important to remember that although neuroglobin may be induced in neurons via exogenous pharmacological treatments, greater than 98% of small molecule drugs lack the ability to cross the BBB and exert biological effects on cells [365]. There is a critical need for conducting *in vivo* studies with the purpose of determining more pharmacological agents that can safely cross the BBB, and induce neuroglobin within neurons. In addition, alternative methods of delivering neuroglobin-inducing drugs to the brain need to be examined. For example, intranasal administration of neuroglobin-inducing drugs would be an effective non-invasive method for bypassing the BBB via olfactory and trigeminal nerve pathways. This route of delivery could potentially improve TBI outcomes by allowing neuroprotective neuroglobin-inducing drugs greater access to neurons in the brain. Consequently, the intranasal delivery of novel pharmacological agents should be explored in future TBI animal studies. Furthermore, the optimal therapeutic time window for increasing neuroglobin expression in the brain, and thus potentially enhancing neuroprotection after TBI needs to be elucidated. Increasing neuroglobin production in the brain pharmacologically may be an effective alternative approach to gene therapy. Future TBI research is clearly needed to

determine pharmacological treatments that safely and effectively increase neuroglobin production in the brain. Clinical research should be conducted to determine whether monitoring neuroglobin levels in serum or cerebrospinal fluid is useful for predicting outcomes after TBI, since the protein may leak out of damaged neurons and glial cells.

4.2.2. Improving TBI Outcomes Through Exercise

In chapter 3 of this dissertation, improved TBI outcomes (i.e., motor and cognitive function) paralleled an exercise induced increase in the expression of VEGF-A and EPO in regions of the brain responsible for movement and memory. Mice that performed 6 weeks of voluntary wheel running exercise prior to TBI displayed a reduction in sensorimotor and spatial learning memory deficits after TBI. It is likely that improvements in post-TBI sensorimotor function may have resulted from the increased expression of both VEGF-A and EPO that was observed in sensorimotor cortex neurons of mice that exercised. Furthermore, it is probable that the improvements in post-TBI spatial learning memory seen in mice that exercised may have occurred due to an increased expression of VEGF-A in hippocampal neurons. Interestingly, pre-TBI exercise increased VEGF-A and EPO production in brain neurons at 1 day post-TBI. The exercise induced increase in the expression of VEGF-A and EPO was noted prior to the endogenous up-regulation of these proteins seen at 3 days post-TBI in non-exercising mice [87]. The early exercise induced up-regulation of VEGF-A and EPO during the acute phase of TBI recovery may have protected against significant neuron death that has been observed in mice at 3 days post-TBI [91].

In conjunction with gene therapy strategies such as overexpressing neuroglobin, results from this dissertation show exercise is another promising non-pharamacological approach for improving TBI outcomes and increasing the production of proteins that are involved in

protecting neurons and promoting brain repair. Based on findings from chapter 3, it is likely that exercise enhanced neuroprotection in the brain. Better post-TBI sensorimotor function and spatial learning memory performance may have been evident in mice that exercised due to an early increase in VEGF-A and EPO expression after exercise and prior to TBI, or during the acute phase of TBI recovery. Furthermore, increasing the production of these proteins in the brain via non-pharmacological interventions such as exercise may be a safer approach for improving TBI outcomes in comparison to exogenous VEGF-A and EPO administration.

Future efforts should investigate differences in post-TBI neuron cell death between mice that exercise prior to TBI, and mice that do not. In a future study, decreased post-TBI neuron cell death in the sensorimotor cortex and hippocampus of pre-TBI exercise mice would likely correlate with improved post-TBI sensorimotor function and spatial learning memory, respectively. It is conceivable that post-TBI neuron cell death would be significantly reduced in pre-TBI exercise mice since the work presented in chapter 3 showed a significant early up-regulation in the production of neuroprotective proteins (i.e., VEGF-A and EPO). This up-regulation occurred 1 day after exercise, which is prior to the significant neuron cell death that has been previously shown to peak in the mouse brain at 3 days post-TBI [91]. Future research should investigate whether the neuroprotective effects of exercise are abolished by blocking VEGF-A or EPO in the brain prior to, or immediately after injury. In addition, the role VEGF-A and EPO play in exercise induced neurogenesis within the brain needs to be further explored. In addition to its neurogenic properties, VEGF-A is responsible for facilitating angiogenesis. Angiogenesis is necessary for restoring the supply of oxygen rich blood to damaged brain tissues, and this response enhances neuronal survival and accelerates healing [117]. Increased VEGF-A protein expression after exercise has been linked to greater capillary density in the

cerebral cortex of un-injured rats [311]. Thus, a future study could explore differences in post-TBI VEGF-A expression, capillary density, and neuron survival in the brain of mice that engage in pre-TBI exercise, and those that do not. Future TBI animal research should investigate how the frequency, intensity, duration, and type of exercise alter the expression of VEGF-A and EPO in the brain and periphery. There is a need for determining whether monitoring levels of these proteins in the serum and cerebrospinal fluid after exercise and TBI could be useful for predicting outcomes after injury. Furthermore, studies should examine how recovery from TBI may be improved by targeting VEGF-A and EPO with other non-pharmacological and pharmacological interventions.

Results from this dissertation show exercise improves TBI outcomes, and increases the expression of neuroprotective genes and proteins in brain regions that are responsible for movement and memory. However, since multiple biochemical pathways can be activated and manipulated through exercise, the door is open for many future exercise and TBI studies to be conducted. For example, it would be wise to examine and compare the biochemical activity in muscle mitochondria to changes in the brain that occur due to exercise and TBI. Chronic aerobic exercise causes cardiac and skeletal muscle tissue to adapt and become more oxidative, which protects these tissues from oxidative stress [366]. This is an important finding, because future work could be aimed at determining whether pre-TBI exercise attenuates oxidative stress and secondary neuron death that occurs within the brain following TBI. Alterations in the activity of cytochrome c oxidase may contribute to mitochondrial dysfunction, increased oxidative stress, secondary neuron death, and long-term damage and disability following TBI [367]. Cytochrome c oxidase would be an interesting target to investigate within brain and muscle cell mitochondria, since this enzyme plays a critical role in oxidative phosphorylation [367]. Interestingly, the

activity of this enzyme is known to decrease at seven days post-TBI within the injured cortex [368], but significantly increase activity by 52% (in non-TBI animals) in muscle tissue that has received aerobic exercise training [366]. Exercise has many positive effects on brain health, and many biochemical pathways are involved in the response to physical activity. It is important to understand the effect of exercise on complex biochemical pathways, because future research may lead to the development of potent pharmacological interventions that mimic the positive prophylactic effects of pre-TBI exercise within the brain.

Findings from chapter 3 have important implications for humans. Faster recovery from TBI, and an overall improvement in neurological outcome, may be apparent in humans that are more physically fit prior to the unexpected occurrence of a TBI. Future basic science and clinical TBI studies should investigate how the frequency, intensity, duration, and type of exercise influence TBI recovery and overall outcome. Although pre-TBI exercise improves outcomes, many questions remain regarding the use of post-TBI exercise. The appropriate time window for initiating post-TBI exercise in rehabilitation settings has not been defined. Therefore, future studies should be conducted to determine the effects of post-TBI exercise on the brain, and to establish exercise guidelines that optimize recovery from TBI in humans.

4.2.3. Possible Benefits and Risks Associated with Using Post-TBI Exercise to Improve TBI Outcomes

Post-TBI exercise displays potential for enhancing recovery or exacerbating neurological deficits depending on the timing of exercise initiation, exercise intensity, and injury severity. Early post-TBI treadmill exercise performed for seven days has been shown to increase neural stem cell proliferation near the injury site in the brain of rats [369]. The authors concluded that exercise performed in the acute phase after TBI is important for recovery from cerebral

dysfunction, but no behavioral studies were conducted, and histological data (i.e., neuron cell counts, contusion volume measurements) were not obtained [369]. Two weeks of low intensity exercise (initiated 1 day after induction of a severe TBI) has been reported to significantly improve spatial learning memory and reduce neurologic deficits when compared to control group rats [370]. This finding is in agreement with clinical studies that have demonstrated early onset rehabilitation can improve cognitive and motor function in TBI patients [210, 371]. Moderate intensity exercise (i.e., approximately 60% of maximal oxygen consumption) initiated 2 days post-TBI, and performed for a duration of one or two weeks, has been shown to restore object recognition memory and prevent progressive neuronal loss and activation of microglia in mice that received a moderate TBI [326]. In contrast to low and moderate intensity exercise, early post-TBI initiation of high intensity exercise does not reduce cognitive deficits in rats, and secondary injury responses may be exacerbated [370].

Magnetic resonance spectroscopy studies have shown that vigorous exercise significantly increases glutamate levels within the brain of humans [372]. If high intensity exercise is initiated too soon after TBI, it is conceivable that excessive glutamate production and release may worsen neurologic outcome and hinder recovery. Positron emission tomography (PET) studies have demonstrated that performing high intensity (i.e., 75% of VO2max) exercise decreases global brain glucose uptake [373], and reductions in cerebral glucose uptake are known to occur for 2-4 weeks after TBI [70]. Therefore, high intensity exercise performed during the first month post-TBI may further impair whole brain metabolism and exacerbate the cellular energy crisis that transpires after injury. It is accepted that low and moderate intensity (i.e., $\leq$ 60% of VO2max) exercise increases cerebral blood flow and oxygen supply [265]. However, decreases in cerebral blood flow have been noted with exercise intensities above 60% of VO2max [265]. In addition,

performing 20 minutes of exercise at 70% of the age-predicted maximal heart rate was shown to significantly reduce grey matter cerebral blood flow by 11% at 10 minutes post-exercise in a recent study of healthy young adults [374]. Oxygen uptake is also altered in the brain during strenuous exercise. Rats that swam for 2 hours at 75% of VO2max have exhibited significant reductions in hippocampal oxygen pressure after the completion of exercise [93]. However, a maximal intensity exercise study demonstrated increased brain oxygen, glucose, and lactate uptake in humans despite a reduction in cerebral blood flow [375]. The increase in brain uptake of energy substrates (i.e., glucose and lactate) and oxygen observed in this study may have occurred to compensate for diminished cerebral blood flow, and reduced glucose and oxygen supply [375]. Mismatches in the cellular supply and demand for glucose and oxygen during vigorous exercise may exacerbate secondary injury responses after TBI. Post-TBI exercise performed at high intensities greater than the aforementioned may intensify hypoxic and ischemic responses, and further contribute to neuronal injury and dysfunction if exercise is performed too soon after injury.

4.2.4. Pre-TBI Exercise Benefits and Potential Mechanisms Involved in Increasing the Production of Neuroprotective Proteins within the Brain

In order to limit post-TBI symptoms, avoidance of exercise and rest are initially recommended to most patients diagnosed with a mild TBI (i.e., concussion) [361]. Premature initiation of post-TBI exercise may exacerbate symptoms and lead to worse outcome. However, engaging in routine exercise prior to the unexpected occurrence of a TBI may be an effective prophylactic measure for optimizing TBI recovery. More specifically, individuals that have 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 those that engaged

in low or moderate intensity exercise prior to injury. This idea is supported by a recent study that investigated VEGF-A responses in healthy humans performing exercise at various intensities [308]. Wahl et al., showed that high intensity exercise training increased circulating VEGF-A protein, but low intensity exercise was associated with a decrease or no change in serum levels [308]. These findings suggest that the expression of VEGF-A and other neuroprotective proteins may be highly dependent on exercise intensity. If high intensity exercise maximizes the production of neuroprotective proteins in the brain, better TBI outcomes may be evident in a patient that was performing a vigorous exercise program before sustaining a TBI.

The exercise intensity of mice was not monitored in the chapter 3 study of this dissertation work. Unfortunately, no other studies have investigated how various exercise intensities influence the expression of VEGF-A, EPO, and other neuropotective hypoxia-inducible proteins in the brain. Nevertheless, 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 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 (pCO_2) in the blood [265]. In response to decreased pCO_2, cerebral vasoconstriction and diminished cerebral blood flow ensue, which reduces the supply of glucose and oxygen to the brain temporarily [265, 266]. Impaired oxygen supply and delivery to brain neurons may also momentarily occur during high intensity exercise due to reductions in pH and decreased oxyhemoglobin binding [265]. It is reasonable to speculate that an increased production of neuroprotective hypoxia-inducible proteins may be noticed in brain neurons that

are responding to transient decreases in blood flow and oxygen delivery during high intensity exercise. Research is needed to determine if these mechanisms induce neuroprotection, and improve TBI outcomes when high intensity exercise is performed prior to injury. In addition, the exercise intensity that maximizes the production of VEGF-A, EPO, and other neuroprotective proteins in the brain needs to be determined.

4.3. OVERALL SUMMARY:

Non-pharmacological and non-surgical interventions for improving TBI outcomes are desirable since many pharmacological and surgical treatments pose risks, and may exacerbate secondary injury responses. This dissertation demonstrated that TBI outcomes were improved in a mouse model of TBI via the use of gene therapy and exercise. Improvements in post-TBI sensorimotor function and spatial learning memory were linked to gene therapy and exercise increasing the production of neuroprotective proteins (i.e., neuroglobin, VEGF-A, and/or EPO) in regions of the brain that control movement (i.e., sensorimotor cortex) and memory (i.e., hippocampus). 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.

REFERENCES

1. Saatman, K.E., A.C. Duhaime, R. Bullock, A.I. Maas, A. Valadka, G.T. Manley, T. Workshop Scientific, and M. Advisory Panel, *Classification of traumatic brain injury for targeted therapies.* J Neurotrauma, 2008. **25**(7): p. 719-38.

2. Management of Concussion/m, T.B.I.W.G., *VA/DoD Clinical Practice Guideline for Management of Concussion/Mild Traumatic Brain Injury.* J Rehabil Res Dev, 2009. **46**(6): p. CP1-68.

3. Harmon, K.G., J.A. Drezner, M. Gammons, K.M. Guskiewicz, M. Halstead, S.A. Herring, J.S. Kutcher, A. Pana, M. Putukian, and W.O. Roberts, *American Medical Society for Sports Medicine position statement: concussion in sport.* Br J Sports Med, 2013. **47**(1): p. 15-26.

4. Parikh, S., M. Koch, and R.K. Narayan, *Traumatic brain injury.* Int Anesthesiol Clin, 2007. **45**(3): p. 119-35.

5. Strathmann, F.G., S. Schulte, K. Goerl, and D.J. Petron, *Blood-based biomarkers for traumatic brain injury: evaluation of research approaches, available methods and potential utility from the clinician and clinical laboratory perspectives.* Clin Biochem, 2014. **47**(10-11): p. 876-88.

6. Kou, Z., R. Gattu, F. Kobeissy, R.D. Welch, B.J. O'Neil, J.L. Woodard, S.I. Ayaz, A. Kulek, R. Kas-Shamoun, V. Mika, C. Zuk, F. Tomasello, and S. Mondello, *Combining biochemical and imaging markers to improve diagnosis and characterization of mild traumatic brain injury in the acute setting: results from a pilot study.* PLoS One, 2013. **8**(11): p. e80296.

7. Yokobori, S., K. Hosein, S. Burks, I. Sharma, S. Gajavelli, and R. Bullock, *Biomarkers for the clinical differential diagnosis in traumatic brain injury--a systematic review.* CNS Neurosci Ther, 2013. **19**(8): p. 556-65.

8. Chauhan, N.B., *Chronic neurodegenerative consequences of traumatic brain injury.* Restor Neurol Neurosci, 2014. **32**(2): p. 337-65.

9. Alwis, D.S., V. Johnstone, E. Yan, and R. Rajan, *Diffuse traumatic brain injury and the sensory brain.* Clin Exp Pharmacol Physiol, 2013. **40**(7): p. 473-83.

10. Cassidy, J.D., E. Boyle, and L.J. Carroll, *Population-based, inception cohort study of the incidence, course, and prognosis of mild traumatic brain injury after motor vehicle collisions.* Arch Phys Med Rehabil, 2014. **95**(3 Suppl): p. S278-85.

11. Ahman, S., B.I. Saveman, J. Styrke, U. Bjornstig, and B.M. Stalnacke, *Long-term follow-up of patients with mild traumatic brain injury: a mixed-method study.* J Rehabil Med, 2013. **45**(8): p. 758-64.

12. Langlois, J.A., W. Rutland-Brown, and M.M. Wald, *The epidemiology and impact of traumatic brain injury: a brief overview.* J Head Trauma Rehabil, 2006. **21**(5): p. 375-8.

13. Rutland-Brown, W., J.A. Langlois, K.E. Thomas, and Y.L. Xi, *Incidence of traumatic brain injury in the United States, 2003.* J Head Trauma Rehabil, 2006. **21**(6): p. 544-8.

14. Mosenthal, A.C., D.H. Livingston, R.F. Lavery, M.M. Knudson, S. Lee, D. Morabito, G.T. Manley, A. Nathens, G. Jurkovich, D.B. Hoyt, and R. Coimbra, *The effect of age on functional outcome in mild traumatic brain injury: 6-month report of a prospective multicenter trial.* J Trauma, 2004. **56**(5): p. 1042-8.

15. Thompson, H.J., W.C. McCormick, and S.H. Kagan, *Traumatic brain injury in older adults: epidemiology, outcomes, and future implications.* J Am Geriatr Soc, 2006. **54**(10): p. 1590-5.

16. Theadom, A., N.J. Starkey, T. Dowell, P.A. Hume, M. Kahan, K. McPherson, V. Feigin, and B.R. Group, *Sports-related brain injury in the general population: an epidemiological study.* J Sci Med Sport, 2014. **17**(6): p. 591-6.

17. Belanger, H.G., S.G. Scott, J. Scholten, G. Curtiss, and R.D. Vanderploeg, *Utility of mechanism-of-injury-based assessment and treatment: Blast Injury Program case illustration.* J Rehabil Res Dev, 2005. **42**(4): p. 403-12.

18. Geddes, J.F. and H.L. Whitwell, *Inflicted head injury in infants.* Forensic Sci Int, 2004. **146**(2-3): p. 83-8.

19. Duhaime, A.C., T.A. Gennarelli, L.E. Thibault, D.A. Bruce, S.S. Margulies, and R. Wiser, *The shaken baby syndrome. A clinical, pathological, and biomechanical study.* J Neurosurg, 1987. **66**(3): p. 409-15.

20. Park, E., J.D. Bell, and A.J. Baker, *Traumatic brain injury: can the consequences be stopped?* CMAJ, 2008. **178**(9): p. 1163-70.

21. Ropper, A.H. and K.C. Gorson, *Clinical practice. Concussion.* N Engl J Med, 2007. **356**(2): p. 166-72.

22. Kushner, D., *Mild traumatic brain injury: toward understanding manifestations and treatment.* Arch Intern Med, 1998. **158**(15): p. 1617-24.

23. Ghajar, J., *Traumatic brain injury.* Lancet, 2000. **356**(9233): p. 923-9.

24. Heegaard, W. and M. Biros, *Traumatic brain injury.* Emerg Med Clin North Am, 2007. **25**(3): p. 655-78, viii.

25. Williams, C. and R.L. Wood, *Alexithymia and emotional empathy following traumatic brain injury.* J Clin Exp Neuropsychol, 2010. **32**(3): p. 259-67.

26. Stevens, R.D. and R. Sutter, *Prognosis in severe brain injury.* Crit Care Med, 2013. **41**(4): p. 1104-23.

27. Kolias, A.G., M.R. Guilfoyle, A. Helmy, J. Allanson, and P.J. Hutchinson, *Traumatic brain injury in adults.* Pract Neurol, 2013. **13**(4): p. 228-35.

28. Chen, J.W., Z.J. Gombart, S. Rogers, S.K. Gardiner, S. Cecil, and R.M. Bullock, *Pupillary reactivity as an early indicator of increased intracranial pressure: The introduction of the Neurological Pupil index.* Surg Neurol Int, 2011. **2**: p. 82.

29. Fodstad, H., P.J. Kelly, and M. Buchfelder, *History of the cushing reflex.* Neurosurgery, 2006. **59**(5): p. 1132-7; discussion 1137.

30. Marino, R., R. Gasparotti, L. Pinelli, D. Manzoni, P. Gritti, D. Mardighian, and N. Latronico, *Posttraumatic cerebral infarction in patients with moderate or severe head trauma.* Neurology, 2006. **67**(7): p. 1165-71.

31. Vik, A., T. Nag, O.A. Fredriksli, T. Skandsen, K.G. Moen, K. Schirmer-Mikalsen, and G.T. Manley, *Relationship of "dose" of intracranial hypertension to outcome in severe traumatic brain injury.* J Neurosurg, 2008. **109**(4): p. 678-84.

32. Davis, R.A. and P.S. Cunningham, *Prognostic factors in severe head injury.* Surg Gynecol Obstet, 1984. **159**(6): p. 597-604.

33. Gennarelli, T.A., L.E. Thibault, J.H. Adams, D.I. Graham, C.J. Thompson, and R.P. Marcincin, *Diffuse axonal injury and traumatic coma in the primate.* Ann Neurol, 1982. **12**(6): p. 564-74.

34. Adams, J.H., D.I. Graham, and B. Jennett, *The neuropathology of the vegetative state after an acute brain insult.* Brain, 2000. **123 (Pt 7)**: p. 1327-38.

35. Burke, J.F., J.L. Stulc, L.E. Skolarus, E.D. Sears, D.B. Zahuranec, and L.B. Morgenstern, *Traumatic brain injury may be an independent risk factor for stroke.* Neurology, 2013. **81**(1): p. 33-9.

36. Sriganesh, K., R. Rajgopal, and G.S. Rao, *Hemodynamically compromising cardiac arrhythmia during surgery for acute traumatic brain injury: management and outcome.* J Neurosurg Anesthesiol, 2012. **24**(4): p. 427-8.

37. Bourdages, M., J.L. Bigras, C.A. Farrell, J.S. Hutchison, J. Lacroix, and G. Canadian Critical Care Trials, *Cardiac arrhythmias associated with severe traumatic brain injury and hypothermia therapy.* Pediatr Crit Care Med, 2010. **11**(3): p. 408-14.

38. Maung, A.A. and L.J. Kaplan, *Mechanical ventilation after injury.* J Intensive Care Med, 2014. **29**(3): p. 128-37.

39. Weisbrod, A.B., C. Rodriguez, R. Bell, C. Neal, R. Armonda, W. Dorlac, M. Schreiber, and J.R. Dunne, *Long-term outcomes of combat casualties sustaining penetrating traumatic brain injury.* J Trauma Acute Care Surg, 2012. **73**(6): p. 1525-30.

40. Richmond, E. and A.D. Rogol, *Traumatic brain injury: endocrine consequences in children and adults.* Endocrine, 2014. **45**(1): p. 3-8.

41. Temkin, N.R., J.D. Corrigan, S.S. Dikmen, and J. Machamer, *Social functioning after traumatic brain injury.* J Head Trauma Rehabil, 2009. **24**(6): p. 460-7.

42. Rutherford, G.W. and J.D. Corrigan, *Long-term consequences of traumatic brain injury.* J Head Trauma Rehabil, 2009. **24**(6): p. 421-3.

43. Velikonja, D., E. Warriner, and C. Brum, *Profiles of emotional and behavioral sequelae following acquired brain injury: cluster analysis of the Personality Assessment Inventory.* J Clin Exp Neuropsychol, 2010. **32**(6): p. 610-21.

44. Mangels, J.A., F.I. Craik, B. Levine, M.L. Schwartz, and D.T. Stuss, *Effects of divided attention on episodic memory in chronic traumatic brain injury: a function of severity and strategy.* Neuropsychologia, 2002. **40**(13): p. 2369-85.

45. Chan, R.C., *Sustained attention in patients with mild traumatic brain injury.* Clin Rehabil, 2005. **19**(2): p. 188-93.

46. Catale, C., P. Marique, A. Closset, and T. Meulemans, *Attentional and executive functioning following mild traumatic brain injury in children using the Test for Attentional Performance (TAP) battery.* J Clin Exp Neuropsychol, 2009. **31**(3): p. 331-8.

47. Vanderploeg, R.D., G. Curtiss, and H.G. Belanger, *Long-term neuropsychological outcomes following mild traumatic brain injury.* J Int Neuropsychol Soc, 2005. **11**(3): p. 228-36.

48. Chen, J.K., K.M. Johnston, A. Collie, P. McCrory, and A. Ptito, *A validation of the post concussion symptom scale in the assessment of complex concussion using cognitive testing and functional MRI.* J Neurol Neurosurg Psychiatry, 2007. **78**(11): p. 1231-8.

49. Erez, A.B., E. Rothschild, N. Katz, M. Tuchner, and A. Hartman-Maeir, *Executive functioning, awareness, and participation in daily life after mild traumatic brain injury: a preliminary study.* Am J Occup Ther, 2009. **63**(5): p. 634-40.

50. Kinnunen, K.M., R. Greenwood, J.H. Powell, R. Leech, P.C. Hawkins, V. Bonnelle, M.C. Patel, S.J. Counsell, and D.J. Sharp, *White matter damage and cognitive impairment after traumatic brain injury.* Brain, 2011. **134**(Pt 2): p. 449-63.

51. Lachapelle, J., J. Bolduc-Teasdale, A. Ptito, and M. McKerral, *Deficits in complex visual information processing after mild TBI: electrophysiological markers and vocational outcome prognosis.* Brain Inj, 2008. **22**(3): p. 265-74.

52. Krauss, J.K., R. Trankle, and K.H. Kopp, *Posttraumatic movement disorders after moderate or mild head injury.* Mov Disord, 1997. **12**(3): p. 428-31.

53. Sartor-Glittenberg, C. and L. Brickner, *A multidimensional physical therapy program for individuals with cerebellar ataxia secondary to traumatic brain injury: a case series.* Physiother Theory Pract, 2014. **30**(2): p. 138-48.

54. Hesdorffer, D.C., S.L. Rauch, and C.A. Tamminga, *Long-term psychiatric outcomes following traumatic brain injury: a review of the literature.* J Head Trauma Rehabil, 2009. **24**(6): p. 452-9.

55. Jafari, S., M. Etminan, F. Aminzadeh, and A. Samii, *Head injury and risk of Parkinson disease: a systematic review and meta-analysis.* Mov Disord, 2013. **28**(9): p. 1222-9.

56. Sivanandam, T.M. and M.K. Thakur, *Traumatic brain injury: a risk factor for Alzheimer's disease.* Neurosci Biobehav Rev, 2012. **36**(5): p. 1376-81.

57. Blennow, K., J. Hardy, and H. Zetterberg, *The neuropathology and neurobiology of traumatic brain injury.* Neuron, 2012. **76**(5): p. 886-99.

58. Gavett, B.E., R.A. Stern, and A.C. McKee, *Chronic traumatic encephalopathy: a potential late effect of sport-related concussive and subconcussive head trauma.* Clin Sports Med, 2011. **30**(1): p. 179-88, xi.

59. Smith, D.H., V.E. Johnson, and W. Stewart, *Chronic neuropathologies of single and repetitive TBI: substrates of dementia?* Nat Rev Neurol, 2013. **9**(4): p. 211-21.

60. McKee, A.C., B.E. Gavett, R.A. Stern, C.J. Nowinski, R.C. Cantu, N.W. Kowall, D.P. Perl, E.T. Hedley-Whyte, B. Price, C. Sullivan, P. Morin, H.S. Lee, C.A. Kubilus, D.H. Daneshvar, M. Wulff, and A.E. Budson, *TDP-43 proteinopathy and motor neuron disease in chronic traumatic encephalopathy.* J Neuropathol Exp Neurol, 2010. **69**(9): p. 918-29.

61. Sandhir, R. and N.E. Berman, *Age-dependent response of CCAAT/enhancer binding proteins following traumatic brain injury in mice.* Neurochem Int, 2010. **56**(1): p. 188-93.

62. Sandhir, R., G. Onyszchuk, and N.E. Berman, *Exacerbated glial response in the aged mouse hippocampus following controlled cortical impact injury.* Exp Neurol, 2008. **213**(2): p. 372-80.

63. Maas, A.I., N. Stocchetti, and R. Bullock, *Moderate and severe traumatic brain injury in adults.* Lancet Neurol, 2008. **7**(8): p. 728-41.

64. Belousov, A.B., Y. Wang, J.H. Song, J.V. Denisova, N.E. Berman, and J.D. Fontes, *Neuronal gap junctions play a role in the secondary neuronal death following controlled cortical impact.* Neurosci Lett, 2012. **524**(1): p. 16-9.

65. Barkhoudarian, G., D.A. Hovda, and C.C. Giza, *The molecular pathophysiology of concussive brain injury.* Clin Sports Med, 2011. **30**(1): p. 33-48, vii-iii.

66. Giza, C.C. and D.A. Hovda, *The Neurometabolic Cascade of Concussion.* J Athl Train, 2001. **36**(3): p. 228-235.

67. Fineman, I., D.A. Hovda, M. Smith, A. Yoshino, and D.P. Becker, *Concussive brain injury is associated with a prolonged accumulation of calcium: a 45Ca autoradiographic study.* Brain Res, 1993. **624**(1-2): p. 94-102.

68. Sugaya, E., M. Takato, and Y. Noda, *Neuronal and glial activity during spreading depression in cerebral cortex of cat.* J Neurophysiol, 1975. **38**(4): p. 822-41.

69. Yoshino, A., D.A. Hovda, T. Kawamata, Y. Katayama, and D.P. Becker, *Dynamic changes in local cerebral glucose utilization following cerebral conclusion in rats: evidence of a hyper- and subsequent hypometabolic state.* Brain Res, 1991. **561**(1): p. 106-19.

70. Bergsneider, M., D.A. Hovda, S.M. Lee, D.F. Kelly, D.L. McArthur, P.M. Vespa, J.H. Lee, S.C. Huang, N.A. Martin, M.E. Phelps, and D.P. Becker, *Dissociation of cerebral glucose metabolism and level of consciousness during the period of metabolic depression following human traumatic brain injury.* J Neurotrauma, 2000. **17**(5): p. 389-401.

71. Gailliot, M.T. and R.F. Baumeister, *The physiology of willpower: linking blood glucose to self-control.* Pers Soc Psychol Rev, 2007. **11**(4): p. 303-27.

72. Bushman, B.J., C.N. Dewall, R.S. Pond, Jr., and M.D. Hanus, *Low glucose relates to greater aggression in married couples.* Proc Natl Acad Sci U S A, 2014. **111**(17): p. 6254-7.

73. Verweij, B.H., J.P. Muizelaar, F.C. Vinas, P.L. Peterson, Y. Xiong, and C.P. Lee, *Mitochondrial dysfunction after experimental and human brain injury and its possible reversal with a selective N-type calcium channel antagonist (SNX-111).* Neurol Res, 1997. **19**(3): p. 334-9.

74. Xiong, Y., P.L. Peterson, B.H. Verweij, F.C. Vinas, J.P. Muizelaar, and C.P. Lee, *Mitochondrial dysfunction after experimental traumatic brain injury: combined efficacy of SNX-111 and U-101033E.* J Neurotrauma, 1998. **15**(7): p. 531-44.

75. Richards, T.L., M.A. Keniry, P.R. Weinstein, B.M. Pereira, B.T. Andrews, E.J. Murphy, and T.L. James, *Measurement of lactate accumulation by in vivo proton NMR spectroscopy during global cerebral ischemia in rats.* Magn Reson Med, 1987. **5**(4): p. 353-7.

76. Meyer, J.S., A. Kondo, F. Nomura, K. Sakamoto, and T. Teraura, *Cerebral hemodynamics and metabolism following experimental head injury.* J Neurosurg, 1970. **32**(3): p. 304-19.

77. Tsacopoulos, M. and P.J. Magistretti, *Metabolic coupling between glia and neurons.* J Neurosci, 1996. **16**(3): p. 877-85.

78. Cornelius, C., R. Crupi, V. Calabrese, A. Graziano, P. Milone, G. Pennisi, Z. Radak, E.J. Calabrese, and S. Cuzzocrea, *Traumatic brain injury: oxidative stress and neuroprotection.* Antioxid Redox Signal, 2013. **19**(8): p. 836-53.

79. Morganti-Kossmann, M.C., M. Rancan, V.I. Otto, P.F. Stahel, and T. Kossmann, *Role of cerebral inflammation after traumatic brain injury: a revisited concept.* Shock, 2001. **16**(3): p. 165-77.

80. McIntosh, T.K., K.E. Saatman, R. Raghupathi, D.I. Graham, D.H. Smith, V.M. Lee, and J.Q. Trojanowski, *The Dorothy Russell Memorial Lecture. The molecular and cellular sequelae of experimental traumatic brain injury: pathogenetic mechanisms.* Neuropathol Appl Neurobiol, 1998. **24**(4): p. 251-67.

81. Clark, R.S., J.K. Schiding, S.L. Kaczorowski, D.W. Marion, and P.M. Kochanek, *Neutrophil accumulation after traumatic brain injury in rats: comparison of weight drop and controlled cortical impact models.* J Neurotrauma, 1994. **11**(5): p. 499-506.

82. Kato, H. and W. Walz, *The initiation of the microglial response.* Brain Pathol, 2000. **10**(1): p. 137-43.

83. Kubes, P. and P.A. Ward, *Leukocyte recruitment and the acute inflammatory response.* Brain Pathol, 2000. **10**(1): p. 127-35.

84. Sandhir, R., V. Puri, R.M. Klein, and N.E. Berman, *Differential expression of cytokines and chemokines during secondary neuron death following brain injury in old and young mice.* Neurosci Lett, 2004. **369**(1): p. 28-32.

85. Werner, C. and K. Engelhard, *Pathophysiology of traumatic brain injury.* Br J Anaesth, 2007. **99**(1): p. 4-9.

86. Kreutzberg, G.W., *Microglia: a sensor for pathological events in the CNS.* Trends Neurosci, 1996. **19**(8): p. 312-8.

87. Anderson, J., R. Sandhir, E.S. Hamilton, and N.E. Berman, *Impaired expression of neuroprotective molecules in the HIF-1alpha pathway following traumatic brain injury in aged mice.* J Neurotrauma, 2009. **26**(9): p. 1557-66.

88. Bohman, L.E., G.G. Heuer, L. Macyszyn, E. Maloney-Wilensky, S. Frangos, P.D. Le Roux, A. Kofke, J.M. Levine, and M.F. Stiefel, *Medical management of compromised brain oxygen in patients with severe traumatic brain injury.* Neurocrit Care, 2011. **14**(3): p. 361-9.

89. Maloney-Wilensky, E., V. Gracias, A. Itkin, K. Hoffman, S. Bloom, W. Yang, S. Christian, and P.D. LeRoux, *Brain tissue oxygen and outcome after severe traumatic brain injury: a systematic review.* Crit Care Med, 2009. **37**(6): p. 2057-63.

90. Yan, E.B., L. Satgunaseelan, E. Paul, N. Bye, P. Nguyen, D. Agyapomaa, T. Kossmann, J.V. Rosenfeld, and M.C. Morganti-Kossmann, *Post-traumatic hypoxia is associated with prolonged cerebral cytokine production, higher serum biomarker levels, and poor outcome in patients with severe traumatic brain injury.* J Neurotrauma, 2014. **31**(7): p. 618-29.

91. Onyszchuk, G., Y.Y. He, N.E. Berman, and W.M. Brooks, *Detrimental effects of aging on outcome from traumatic brain injury: a behavioral, magnetic resonance imaging, and histological study in mice.* J Neurotrauma, 2008. **25**(2): p. 153-71.

92. Jia, F., Y.H. Pan, Q. Mao, Y.M. Liang, and J.Y. Jiang, *Matrix metalloproteinase-9 expression and protein levels after fluid percussion injury in rats: the effect of injury severity and brain temperature.* J Neurotrauma, 2010. **27**(6): p. 1059-68.

93. Gegentonglaga, H. Yoshizato, Y. Higuchi, Y. Toyota, Y. Hanai, Y. Ando, and A. Yoshimura, *Variable alteration of regional tissue oxygen pressure in rat hippocampus by acute swimming exercise.* Life Sci, 2013. **93**(21): p. 773-7.

94. Majmundar, A.J., W.J. Wong, and M.C. Simon, *Hypoxia-inducible factors and the response to hypoxic stress.* Mol Cell, 2010. **40**(2): p. 294-309.

95. Umschweif, G., A.G. Alexandrovich, V. Trembovler, M. Horowitz, and E. Shohami, *Hypoxia-inducible factor 1 is essential for spontaneous recovery from traumatic brain injury and is a key mediator of heat acclimation induced neuroprotection.* J Cereb Blood Flow Metab, 2013. **33**(4): p. 524-31.

96. Ameln, H., T. Gustafsson, C.J. Sundberg, K. Okamoto, E. Jansson, L. Poellinger, and Y. Makino, *Physiological activation of hypoxia inducible factor-1 in human skeletal muscle.* FASEB J, 2005. **19**(8): p. 1009-11.

97. Cutrupi, S., G. Ferrero, S. Reineri, F. Cordero, and M. De Bortoli, *Genomic lens on neuroglobin transcription.* IUBMB Life, 2014. **66**(1): p. 46-51.

98. Haines, B., M. Demaria, X. Mao, L. Xie, J. Campisi, K. Jin, and D.A. Greenberg, *Hypoxia-inducible factor-1 and neuroglobin expression.* Neurosci Lett, 2012. **514**(2): p. 137-40.

99. Liu, N., Z. Yu, S. Xiang, S. Zhao, A. Tjarnlund-Wolf, C. Xing, J. Zhang, and X. Wang, *Transcriptional regulation mechanisms of hypoxia-induced neuroglobin gene expression.* Biochem J, 2012. **443**(1): p. 153-64.

100. Khan, A.A., X.O. Mao, S. Banwait, C.M. DerMardirossian, G.M. Bokoch, K. Jin, and D.A. Greenberg, *Regulation of hypoxic neuronal death signaling by neuroglobin.* FASEB J, 2008. **22**(6): p. 1737-47.

101. Mimura, I., T. Tanaka, Y. Wada, T. Kodama, and M. Nangaku, *Pathophysiological response to hypoxia - from the molecular mechanisms of malady to drug discovery: epigenetic regulation of the hypoxic response via hypoxia-inducible factor and histone modifying enzymes.* J Pharmacol Sci, 2011. **115**(4): p. 453-8.

102. Gordan, J.D. and M.C. Simon, *Hypoxia-inducible factors: central regulators of the tumor phenotype.* Curr Opin Genet Dev, 2007. **17**(1): p. 71-7.

103. Baranova, O., L.F. Miranda, P. Pichiule, I. Dragatsis, R.S. Johnson, and J.C. Chavez, *Neuron-specific inactivation of the hypoxia inducible factor 1 alpha increases brain injury in a mouse model of transient focal cerebral ischemia.* J Neurosci, 2007. **27**(23): p. 6320-32.

104. van Uden, P., N.S. Kenneth, and S. Rocha, *Regulation of hypoxia-inducible factor-1alpha by NF-kappaB.* Biochem J, 2008. **412**(3): p. 477-84.

105. Sperandio, S., J. Fortin, R. Sasik, L. Robitaille, J. Corbeil, and I. de Belle, *The transcription factor Egr1 regulates the HIF-1alpha gene during hypoxia.* Mol Carcinog, 2009. **48**(1): p. 38-44.

106. Coulon, C., M. Georgiadou, C. Roncal, K. De Bock, T. Langenberg, and P. Carmeliet, *From vessel sprouting to normalization: role of the prolyl hydroxylase domain protein/hypoxia-inducible factor oxygen-sensing machinery.* Arterioscler Thromb Vasc Biol, 2010. **30**(12): p. 2331-6.

107. Manalo, D.J., A. Rowan, T. Lavoie, L. Natarajan, B.D. Kelly, S.Q. Ye, J.G. Garcia, and G.L. Semenza, *Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1.* Blood, 2005. **105**(2): p. 659-69.

108. Bergeron, M., A.Y. Yu, K.E. Solway, G.L. Semenza, and F.R. Sharp, *Induction of hypoxia-inducible factor-1 (HIF-1) and its target genes following focal ischaemia in rat brain.* Eur J Neurosci, 1999. **11**(12): p. 4159-70.

109. Jones, N.M. and M. Bergeron, *Hypoxic preconditioning induces changes in HIF-1 target genes in neonatal rat brain.* J Cereb Blood Flow Metab, 2001. **21**(9): p. 1105-14.

110. Kim, B.J. and N.S. Forbes, *Flux analysis shows that hypoxia-inducible-factor-1-alpha minimally affects intracellular metabolism in tumor spheroids.* Biotechnol Bioeng, 2007. **96**(6): p. 1167-82.

111. Siddiq, A., I.A. Ayoub, J.C. Chavez, L. Aminova, S. Shah, J.C. LaManna, S.M. Patton, J.R. Connor, R.A. Cherny, I. Volitakis, A.I. Bush, I. Langsetmo, T. Seeley, V. Gunzler, and R.R. Ratan, *Hypoxia-inducible factor prolyl 4-hydroxylase inhibition. A target for neuroprotection in the central nervous system.* J Biol Chem, 2005. **280**(50): p. 41732-43.

112. Jin, K., Y. Zhu, Y. Sun, X.O. Mao, L. Xie, and D.A. Greenberg, *Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo.* Proc Natl Acad Sci U S A, 2002. **99**(18): p. 11946-50.

113. Fabel, K., B. Tam, D. Kaufer, A. Baiker, N. Simmons, C.J. Kuo, and T.D. Palmer, *VEGF is necessary for exercise-induced adult hippocampal neurogenesis.* Eur J Neurosci, 2003. **18**(10): p. 2803-12.

114. Nor, J.E., J. Christensen, D.J. Mooney, and P.J. Polverini, *Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression.* Am J Pathol, 1999. **154**(2): p. 375-84.

115. Liu, Y., S.R. Cox, T. Morita, and S. Kourembanas, *Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer.* Circ Res, 1995. **77**(3): p. 638-43.

116. Kovacs, Z., K. Ikezaki, K. Samoto, T. Inamura, and M. Fukui, *VEGF and flt. Expression time kinetics in rat brain infarct.* Stroke, 1996. **27**(10): p. 1865-72; discussion 1872-3.

117. Ma, Y., Y. Qu, and Z. Fei, *Vascular endothelial growth factor in cerebral ischemia.* J Neurosci Res, 2011. **89**(7): p. 969-78.

118. Holmes, K., O.L. Roberts, A.M. Thomas, and M.J. Cross, *Vascular endothelial growth factor receptor-2: structure, function, intracellular signalling and therapeutic inhibition.* Cell Signal, 2007. **19**(10): p. 2003-12.

119. Takahashi, H. and M. Shibuya, *The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions.* Clin Sci (Lond), 2005. **109**(3): p. 227-41.

120. Rosenstein, J.M., J.M. Krum, and C. Ruhrberg, *VEGF in the nervous system.* Organogenesis, 2010. **6**(2): p. 107-14.

121. Leosco, D., G. Rengo, G. Iaccarino, E. Sanzari, L. Golino, G. De Lisa, C. Zincarelli, F. Fortunato, M. Ciccarelli, V. Cimini, G.G. Altobelli, F. Piscione, G. Galasso, B. Trimarco, W.J. Koch, and F. Rengo, *Prior exercise improves age-dependent vascular endothelial growth factor downregulation and angiogenesis responses to hind-limb ischemia in old rats.* J Gerontol A Biol Sci Med Sci, 2007. **62**(5): p. 471-80.

122. Thau-Zuchman, O., E. Shohami, A.G. Alexandrovich, and R.R. Leker, *Vascular endothelial growth factor increases neurogenesis after traumatic brain injury.* J Cereb Blood Flow Metab, 2010. **30**(5): p. 1008-16.

123. Carmeliet, P., *Blood vessels and nerves: common signals, pathways and diseases.* Nat Rev Genet, 2003. **4**(9): p. 710-20.

124. Lee, C. and D.V. Agoston, *Inhibition of VEGF receptor 2 increased cell death of dentate hilar neurons after traumatic brain injury.* Exp Neurol, 2009. **220**(2): p. 400-3.

125. Skold, M.K., C. von Gertten, A.C. Sandberg-Nordqvist, T. Mathiesen, and S. Holmin, *VEGF and VEGF receptor expression after experimental brain contusion in rat.* J Neurotrauma, 2005. **22**(3): p. 353-67.

126. Lee, C. and D.V. Agoston, *Vascular endothelial growth factor is involved in mediating increased de novo hippocampal neurogenesis in response to traumatic brain injury.* J Neurotrauma, 2010. **27**(3): p. 541-53.

127. Krum, J.M. and A. Khaibullina, *Inhibition of endogenous VEGF impedes revascularization and astroglial proliferation: roles for VEGF in brain repair.* Exp Neurol, 2003. **181**(2): p. 241-57.

128. Skold, M.K., M. Risling, and S. Holmin, *Inhibition of vascular endothelial growth factor receptor 2 activity in experimental brain contusions aggravates injury outcome and leads to early increased neuronal and glial degeneration.* Eur J Neurosci, 2006. **23**(1): p. 21-34.

129. Jelkmann, W., *Physiology and pharmacology of erythropoietin.* Transfus Med Hemother, 2013. **40**(5): p. 302-9.

130. Ponce, L.L., J.C. Navarro, O. Ahmed, and C.S. Robertson, *Erythropoietin neuroprotection with traumatic brain injury.* Pathophysiology, 2013. **20**(1): p. 31-8.

131. Bernaudin, M., A. Bellail, H.H. Marti, A. Yvon, D. Vivien, I. Duchatelle, E.T. Mackenzie, and E. Petit, *Neurons and astrocytes express EPO mRNA: oxygen-sensing mechanisms that involve the redox-state of the brain.* Glia, 2000. **30**(3): p. 271-8.

132. Maslov, L.N., *[Role of erythropoietin in the ischemic preconditioning. Postconditioning and regeneration of brain after ischemia]*. Ross Fiziol Zh Im I M Sechenova, 2010. **96**(1): p. 26-42.

133. Xiong, Y., A. Mahmood, C. Qu, H. Kazmi, Z.G. Zhang, C.T. Noguchi, T. Schallert, and M. Chopp, *Erythropoietin improves histological and functional outcomes after traumatic brain injury in mice in the absence of the neural erythropoietin receptor.* J Neurotrauma, 2010. **27**(1): p. 205-15.

134. Zhang, Y., M. Chopp, A. Mahmood, Y. Meng, C. Qu, and Y. Xiong, *Impact of inhibition of erythropoietin treatment-mediated neurogenesis in the dentate gyrus of the hippocampus on restoration of spatial learning after traumatic brain injury.* Exp Neurol, 2012. **235**(1): p. 336-44.

135. Shingo, T., S.T. Sorokan, T. Shimazaki, and S. Weiss, *Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells.* J Neurosci, 2001. **21**(24): p. 9733-43.

136. Zhang, F., J. Xing, A.K. Liou, S. Wang, Y. Gan, Y. Luo, X. Ji, R.A. Stetler, J. Chen, and G. Cao, *Enhanced Delivery of Erythropoietin Across the Blood-Brain Barrier for Neuroprotection against Ischemic Neuronal Injury.* Transl Stroke Res, 2010. **1**(2): p. 113-121.

137. Xu, F., Z.Y. Yu, L. Ding, and S.Y. Zheng, *Experimental studies of erythropoietin protection following traumatic brain injury in rats.* Exp Ther Med, 2012. **4**(6): p. 977-982.

138. Zhang, F., A.P. Signore, Z. Zhou, S. Wang, G. Cao, and J. Chen, *Erythropoietin protects CA1 neurons against global cerebral ischemia in rat: potential signaling mechanisms.* J Neurosci Res, 2006. **83**(7): p. 1241-51.

139. Zhang, F., S. Wang, G. Cao, Y. Gao, and J. Chen, *Signal transducers and activators of transcription 5 contributes to erythropoietin-mediated neuroprotection against hippocampal neuronal death after transient global cerebral ischemia.* Neurobiol Dis, 2007. **25**(1): p. 45-53.

140. Ning, R., Y. Xiong, A. Mahmood, Y. Zhang, Y. Meng, C. Qu, and M. Chopp, *Erythropoietin promotes neurovascular remodeling and long-term functional recovery in rats following traumatic brain injury.* Brain Res, 2011. **1384**: p. 140-50.

141. Grasso, G., A. Sfacteria, F. Meli, V. Fodale, M. Buemi, and D.G. Iacopino, *Neuroprotection by erythropoietin administration after experimental traumatic brain injury.* Brain Res, 2007. **1182**: p. 99-105.

142. Brines, M.L., P. Ghezzi, S. Keenan, D. Agnello, N.C. de Lanerolle, C. Cerami, L.M. Itri, and A. Cerami, *Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury.* Proc Natl Acad Sci U S A, 2000. **97**(19): p. 10526-31.

143. Celik, M., N. Gokmen, S. Erbayraktar, M. Akhisaroglu, S. Konakc, C. Ulukus, S. Genc, K. Genc, E. Sagiroglu, A. Cerami, and M. Brines, *Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury.* Proc Natl Acad Sci U S A, 2002. **99**(4): p. 2258-63.

144. Gonzalez, F.F., P. McQuillen, D. Mu, Y. Chang, M. Wendland, Z. Vexler, and D.M. Ferriero, *Erythropoietin enhances long-term neuroprotection and neurogenesis in neonatal stroke.* Dev Neurosci, 2007. **29**(4-5): p. 321-30.

145. Cerami, A., *Beyond erythropoiesis: novel applications for recombinant human erythropoietin.* Semin Hematol, 2001. **38**(3 Suppl 7): p. 33-9.

146. Villa, P., P. Bigini, T. Mennini, D. Agnello, T. Laragione, A. Cagnotto, B. Viviani, M. Marinovich, A. Cerami, T.R. Coleman, M. Brines, and P. Ghezzi, *Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis.* J Exp Med, 2003. **198**(6): p. 971-5.

147. Sakanaka, M., T.C. Wen, S. Matsuda, S. Masuda, E. Morishita, M. Nagao, and R. Sasaki, *In vivo evidence that erythropoietin protects neurons from ischemic damage.* Proc Natl Acad Sci U S A, 1998. **95**(8): p. 4635-40.

148. Aztatzi-Santillan, E., F.E. Nares-Lopez, B. Marquez-Valadez, P. Aguilera, and M.E. Chanez-Cardenas, *The protective role of heme oxygenase-1 in cerebral ischemia.* Cent Nerv Syst Agents Med Chem, 2010. **10**(4): p. 310-6.

149. Bae, J.W., M.J. Kim, C.G. Jang, and S.Y. Lee, *Protective effects of heme oxygenase-1 against MPP(+)-induced cytotoxicity in PC-12 cells.* Neurol Sci, 2010. **31**(3): p. 307-13.

150. Neubauer, J.A. and J. Sunderram, *Heme oxygenase-1 and chronic hypoxia.* Respir Physiol Neurobiol, 2012. **184**(2): p. 178-85.

151. Surh, Y.J., J.K. Kundu, M.H. Li, H.K. Na, and Y.N. Cha, *Role of Nrf2-mediated heme oxygenase-1 upregulation in adaptive survival response to nitrosative stress.* Arch Pharm Res, 2009. **32**(8): p. 1163-76.

152. Ferrandiz, M.L. and I. Devesa, *Inducers of heme oxygenase-1.* Curr Pharm Des, 2008. **14**(5): p. 473-86.

153. Sass, G., R. Barikbin, and G. Tiegs, *The multiple functions of heme oxygenase-1 in the liver.* Z Gastroenterol, 2012. **50**(1): p. 34-40.

154. Watanabe, T., G. Hasegawa, T. Yamamoto, K. Hatakeyama, M. Suematsu, and M. Naito, *Expression of heme oxygenase-1 in rat ontogeny.* Arch Histol Cytol, 2003. **66**(2): p. 155-62.

155. Qi, D., C. Ouyang, Y. Wang, S. Zhang, X. Ma, Y. Song, H. Yu, J. Tang, W. Fu, L. Sheng, L. Yang, M. Wang, W. Zhang, L. Miao, T. Li, X. Huang, and H. Dong, *HO-1 attenuates hippocampal neurons injury via the activation of BDNF-TrkB-PI3K/Akt signaling pathway in stroke.* Brain Res, 2014. **1577**: p. 69-76.

156. Turner, C.P., M. Bergeron, P. Matz, A. Zegna, L.J. Noble, S.S. Panter, and F.R. Sharp, *Heme oxygenase-1 is induced in glia throughout brain by subarachnoid hemoglobin.* J Cereb Blood Flow Metab, 1998. **18**(3): p. 257-73.

157. Fukuda, K., S.S. Panter, F.R. Sharp, and L.J. Noble, *Induction of heme oxygenase-1 (HO-1) after traumatic brain injury in the rat.* Neurosci Lett, 1995. **199**(2): p. 127-30.

158. Shah, Z.A., R.C. Li, A.S. Ahmad, T.W. Kensler, M. Yamamoto, S. Biswal, and S. Dore, *The flavanol (-)-epicatechin prevents stroke damage through the Nrf2/HO1 pathway.* J Cereb Blood Flow Metab, 2010. **30**(12): p. 1951-61.

159. Sakata, Y., H. Zhuang, H. Kwansa, R.C. Koehler, and S. Dore, *Resveratrol protects against experimental stroke: putative neuroprotective role of heme oxygenase 1.* Exp Neurol, 2010. **224**(1): p. 325-9.

160. Ping, Z., W. Liu, Z. Kang, J. Cai, Q. Wang, N. Cheng, S. Wang, J.H. Zhang, and X. Sun, *Sulforaphane protects brains against hypoxic-ischemic injury through induction of Nrf2-dependent phase 2 enzyme.* Brain Res, 2010. **1343**: p. 178-85.

161. Burmester, T., B. Weich, S. Reinhardt, and T. Hankeln, *A vertebrate globin expressed in the brain.* Nature, 2000. **407**(6803): p. 520-3.

162. Hundahl, C.A., G.C. Allen, J. Hannibal, K. Kjaer, J.F. Rehfeld, S. Dewilde, J.R. Nyengaard, J. Kelsen, and A. Hay-Schmidt, *Anatomical characterization of cytoglobin and neuroglobin mRNA and protein expression in the mouse brain.* Brain Res, 2010. **1331**: p. 58-73.

163. Shang, A., K. Liu, H. Wang, J. Wang, X. Hang, Y. Yang, Z. Wang, C. Zhang, and D. Zhou, *Neuroprotective effects of neuroglobin after mechanical injury.* Neurol Sci, 2012. **33**(3): p. 551-8.

164. Zhao, S., Z. Yu, G. Zhao, C. Xing, K. Hayakawa, M.J. Whalen, J.M. Lok, E.H. Lo, and X. Wang, *Neuroglobin-overexpression reduces traumatic brain lesion size in mice.* BMC Neurosci, 2012. **13**: p. 67.

165. Hundahl, C.A., G.C. Allen, J.R. Nyengaard, S. Dewilde, B.D. Carter, J. Kelsen, and A. Hay-Schmidt, *Neuroglobin in the rat brain: localization.* Neuroendocrinology, 2008. **88**(3): p. 173-82.

166. DellaValle, B., C. Hempel, J.A. Kurtzhals, and M. Penkowa, *In vivo expression of neuroglobin in reactive astrocytes during neuropathology in murine models of traumatic brain injury, cerebral malaria, and autoimmune encephalitis.* Glia, 2010. **58**(10): p. 1220-7.

167. Greenberg, D.A., K. Jin, and A.A. Khan, *Neuroglobin: an endogenous neuroprotectant.* Curr Opin Pharmacol, 2008. **8**(1): p. 20-4.

168. Yu, Z., N. Liu, J. Liu, K. Yang, and X. Wang, *Neuroglobin, a Novel Target for Endogenous Neuroprotection against Stroke and Neurodegenerative Disorders.* Int J Mol Sci, 2012. **13**(6): p. 6995-7014.

169. Shang, A., X. Feng, H. Wang, J. Wang, X. Hang, Y. Yang, Z. Wang, and D. Zhou, *Neuroglobin upregulation offers neuroprotection in traumatic brain injury.* Neurol Res, 2012. **34**(6): p. 588-94.

170. Fiocchetti, M., E. De Marinis, P. Ascenzi, and M. Marino, *Neuroglobin and neuronal cell survival.* Biochim Biophys Acta, 2013. **1834**(9): p. 1744-9.

171. Jin, K., X.O. Mao, L. Xie, A.A. Khan, and D.A. Greenberg, *Neuroglobin protects against nitric oxide toxicity.* Neurosci Lett, 2008. **430**(2): p. 135-7.

172. Li, W., Y. Wu, C. Ren, Y. Lu, Y. Gao, X. Zheng, and C. Zhang, *The activity of recombinant human neuroglobin as an antioxidant and free radical scavenger.* Proteins, 2011. **79**(1): p. 115-25.

173. Fago, A., A.J. Mathews, and T. Brittain, *A role for neuroglobin: resetting the trigger level for apoptosis in neuronal and retinal cells.* IUBMB Life, 2008. **60**(6): p. 398-401.

174. Antao, S.T., T.T. Duong, R. Aran, and P.K. Witting, *Neuroglobin overexpression in cultured human neuronal cells protects against hydrogen peroxide insult via activating phosphoinositide-3 kinase and opening the mitochondrial K(ATP) channel.* Antioxid Redox Signal, 2010. **13**(6): p. 769-81.

175. Duong, T.T., P.K. Witting, S.T. Antao, S.N. Parry, M. Kennerson, B. Lai, S. Vogt, P.A. Lay, and H.H. Harris, *Multiple protective activities of neuroglobin in cultured neuronal cells exposed to hypoxia re-oxygenation injury.* J Neurochem, 2009. **108**(5): p. 1143-54.

176. Gatta, C., L. Castaldo, A. Cellerino, P. de Girolamo, C. Lucini, and L. D'Angelo, *Brain derived neurotrophic factor in the retina of the teleost N. furzeri.* Ann Anat, 2014. **196**(4): p. 192-6.

177. Gajewska-Wozniak, O., M. Skup, S. Kasicki, E. Ziemlinska, and J. Czarkowska-Bauch, *Enhancing proprioceptive input to motoneurons differentially affects expression of neurotrophin 3 and brain-derived neurotrophic factor in rat hoffmann-reflex circuitry.* PLoS One, 2013. **8**(6): p. e65937.

178. Neeper, S.A., F. Gomez-Pinilla, J. Choi, and C. Cotman, *Exercise and brain neurotrophins.* Nature, 1995. **373**(6510): p. 109.

179. Yamamoto, M., G. Sobue, K. Yamamoto, S. Terao, and T. Mitsuma, *Expression of mRNAs for neurotrophic factors (NGF, BDNF, NT-3, and GDNF) and their receptors (p75NGFR, trkA, trkB, and trkC) in the adult human peripheral nervous system and nonneural tissues.* Neurochem Res, 1996. **21**(8): p. 929-38.

180. Yamada, K. and T. Nabeshima, *Brain-derived neurotrophic factor/TrkB signaling in memory processes.* J Pharmacol Sci, 2003. **91**(4): p. 267-70.

181. Bekinschtein, P., M. Cammarota, I. Izquierdo, and J.H. Medina, *BDNF and memory formation and storage.* Neuroscientist, 2008. **14**(2): p. 147-56.

182. Tao, X., S. Finkbeiner, D.B. Arnold, A.J. Shaywitz, and M.E. Greenberg, *Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism.* Neuron, 1998. **20**(4): p. 709-26.

183. Rothman, S.M. and M.P. Mattson, *Activity-dependent, stress-responsive BDNF signaling and the quest for optimal brain health and resilience throughout the lifespan.* Neuroscience, 2013. **239**: p. 228-40.

184. Vaynman, S. and F. Gomez-Pinilla, *License to run: exercise impacts functional plasticity in the intact and injured central nervous system by using neurotrophins.* Neurorehabil Neural Repair, 2005. **19**(4): p. 283-95.

185. Kaplan, G.B., J.J. Vasterling, and P.C. Vedak, *Brain-derived neurotrophic factor in traumatic brain injury, post-traumatic stress disorder, and their comorbid conditions: role in pathogenesis and treatment.* Behav Pharmacol, 2010. **21**(5-6): p. 427-37.

186. Martinez-Levy, G.A. and C.S. Cruz-Fuentes, *Genetic and Epigenetic Regulation of the Brain-Derived Neurotrophic Factor in the Central Nervous System.* Yale J Biol Med, 2014. **87**(2): p. 173-186.

187. Griesbach, G.S., D.A. Hovda, R. Molteni, and F. Gomez-Pinilla, *Alterations in BDNF and synapsin I within the occipital cortex and hippocampus after mild traumatic brain injury in the developing rat: reflections of injury-induced neuroplasticity.* J Neurotrauma, 2002. **19**(7): p. 803-14.

188. Hicks, R.R., S. Numan, H.S. Dhillon, M.R. Prasad, and K.B. Seroogy, *Alterations in BDNF and NT-3 mRNAs in rat hippocampus after experimental brain trauma.* Brain Res Mol Brain Res, 1997. **48**(2): p. 401-6.

189. Oyesiku, N.M., C.O. Evans, S. Houston, R.S. Darrell, J.S. Smith, Z.L. Fulop, C.E. Dixon, and D.G. Stein, *Regional changes in the expression of neurotrophic factors and their receptors following acute traumatic brain injury in the adult rat brain.* Brain Res, 1999. **833**(2): p. 161-72.

190. Rostami, E., F. Krueger, S. Plantman, J. Davidsson, D. Agoston, J. Grafman, and M. Risling, *Alteration in BDNF and its receptors, full-length and truncated TrkB and p75(NTR) following penetrating traumatic brain injury.* Brain Res, 2014. **1542**: p. 195-205.

191. Gao, X., G.M. Smith, and J. Chen, *Impaired dendritic development and synaptic formation of postnatal-born dentate gyrus granular neurons in the absence of brain-derived neurotrophic factor signaling.* Exp Neurol, 2009. **215**(1): p. 178-90.

192. Aksu, I., M. Ates, B. Baykara, M. Kiray, A.R. Sisman, E. Buyuk, B. Baykara, C. Cetinkaya, H. Gumus, and N. Uysal, *Anxiety correlates to decreased blood and prefrontal cortex IGF-1 levels in streptozotocin induced diabetes.* Neurosci Lett, 2012. **531**(2): p. 176-81.

193. Aleman, A. and I. Torres-Aleman, *Circulating insulin-like growth factor I and cognitive function: neuromodulation throughout the lifespan.* Prog Neurobiol, 2009. **89**(3): p. 256-65.

194. Ozdemir, D., B. Baykara, I. Aksu, M. Kiray, A.R. Sisman, F. Cetin, A. Dayi, T. Gurpinar, N. Uysal, and M.N. Arda, *Relationship between circulating IGF-1 levels and traumatic brain injury-induced hippocampal damage and cognitive dysfunction in immature rats.* Neurosci Lett, 2012. **507**(1): p. 84-9.

195. Torres-Aleman, I., *Toward a comprehensive neurobiology of IGF-I.* Dev Neurobiol, 2010. **70**(5): p. 384-96.

196. Junnila, R.K., E.O. List, D.E. Berryman, J.W. Murrey, and J.J. Kopchick, *The GH/IGF-1 axis in ageing and longevity.* Nat Rev Endocrinol, 2013. **9**(6): p. 366-76.

197. Martin, B., R. Brenneman, E. Golden, T. Walent, K.G. Becker, V.V. Prabhu, W. Wood, 3rd, B. Ladenheim, J.L. Cadet, and S. Maudsley, *Growth factor signals in neural cells: coherent patterns of interaction control multiple levels of molecular and phenotypic responses.* J Biol Chem, 2009. **284**(4): p. 2493-511.

198. Arwert, L.I., J.B. Deijen, and M.L. Drent, *The relation between insulin-like growth factor I levels and cognition in healthy elderly: a meta-analysis.* Growth Horm IGF Res, 2005. **15**(6): p. 416-22.

199. Gunnell, D., L.L. Miller, I. Rogers, J.M. Holly, and A.S. Team, *Association of insulin-like growth factor I and insulin-like growth factor-binding protein-3 with intelligence quotient among 8- to 9-year-old children in the Avon Longitudinal Study of Parents and Children.* Pediatrics, 2005. **116**(5): p. e681-6.

200. Greco, T., D. Hovda, and M. Prins, *The effects of repeat traumatic brain injury on the pituitary in adolescent rats.* J Neurotrauma, 2013. **30**(23): p. 1983-90.

201. Sanus, G.Z., T. Tanriverdi, A. Coskun, H. Hanimoglu, M. Is, and M. Uzan, *Cerebrospinal fluid and serum levels of insulin-like growth factor-1 and insulin-like growth factor binding protein-3 in patients with severe head injury.* Ulus Travma Acil Cerrahi Derg, 2007. **13**(4): p. 281-7.

202. Wojcik, S.M., *Predicting mild traumatic brain injury patients at risk of persistent symptoms in the Emergency Department.* Brain Inj, 2014. **28**(4): p. 422-30.

203. Jennett, B., J. Snoek, M.R. Bond, and N. Brooks, *Disability after severe head injury: observations on the use of the Glasgow Outcome Scale.* J Neurol Neurosurg Psychiatry, 1981. **44**(4): p. 285-93.

204. Jennett, B. and M. Bond, *Assessment of outcome after severe brain damage.* Lancet, 1975. **1**(7905): p. 480-4.

205. Wilson, J.T., L.E. Pettigrew, and G.M. Teasdale, *Structured interviews for the Glasgow Outcome Scale and the extended Glasgow Outcome Scale: guidelines for their use.* J Neurotrauma, 1998. **15**(8): p. 573-85.

206. Hukkelhoven, C.W., E.W. Steyerberg, A.J. Rampen, E. Farace, J.D. Habbema, L.F. Marshall, G.D. Murray, and A.I. Maas, *Patient age and outcome following severe traumatic brain injury: an analysis of 5600 patients.* J Neurosurg, 2003. **99**(4): p. 666-73.

207. Alberico, A.M., J.D. Ward, S.C. Choi, A. Marmarou, and H.F. Young, *Outcome after severe head injury. Relationship to mass lesions, diffuse injury, and ICP course in pediatric and adult patients.* J Neurosurg, 1987. **67**(5): p. 648-56.

208. Finnanger, T.G., T. Skandsen, S. Andersson, S. Lydersen, A. Vik, and M. Indredavik, *Differentiated patterns of cognitive impairment 12 months after severe and moderate traumatic brain injury.* Brain Inj, 2013. **27**(13-14): p. 1606-16.

209. Xiong, Y., A. Mahmood, and M. Chopp, *Emerging treatments for traumatic brain injury.* Expert Opin Emerg Drugs, 2009. **14**(1): p. 67-84.

210. Andelic, N., E. Bautz-Holter, P. Ronning, K. Olafsen, S. Sigurdardottir, A.K. Schanke, U. Sveen, S. Tornas, M. Sandhaug, and C. Roe, *Does an early onset and continuous chain of rehabilitation improve the long-term functional outcome of patients with severe traumatic brain injury?* J Neurotrauma, 2012. **29**(1): p. 66-74.

211. Maas, A.I., B. Roozenbeek, and G.T. Manley, *Clinical trials in traumatic brain injury: past experience and current developments.* Neurotherapeutics, 2010. **7**(1): p. 115-26.

212. Peterson, K., S. Carson, and N. Carney, *Hypothermia treatment for traumatic brain injury: a systematic review and meta-analysis.* J Neurotrauma, 2008. **25**(1): p. 62-71.

213. Cooper, D.J., J.V. Rosenfeld, L. Murray, Y.M. Arabi, A.R. Davies, P. D'Urso, T. Kossmann, J. Ponsford, I. Seppelt, P. Reilly, and R. Wolfe, *Decompressive craniectomy in diffuse traumatic brain injury.* N Engl J Med, 2011. **364**(16): p. 1493-502.

214. Narayan, R.K., M.E. Michel, B. Ansell, A. Baethmann, A. Biegon, M.B. Bracken, M.R. Bullock, S.C. Choi, G.L. Clifton, C.F. Contant, W.M. Coplin, W.D. Dietrich, J. Ghajar, S.M. Grady, R.G. Grossman, E.D. Hall, W. Heetderks, D.A. Hovda, J. Jallo, R.L. Katz, N. Knoller, P.M. Kochanek, A.I. Maas, J. Majde, D.W. Marion, A. Marmarou, L.F. Marshall, T.K. McIntosh, E. Miller, N. Mohberg, J.P. Muizelaar, L.H. Pitts, P. Quinn, G. Riesenfeld, C.S. Robertson, K.I. Strauss, G. Teasdale, N. Temkin, R. Tuma, C. Wade, M.D. Walker, M. Weinrich, J. Whyte, J. Wilberger, A.B. Young, and L. Yurkewicz, *Clinical trials in head injury.* J Neurotrauma, 2002. **19**(5): p. 503-57.

215. Alderson, P. and I. Roberts, *Corticosteroids for acute traumatic brain injury.* Cochrane Database Syst Rev, 2005(1): p. CD000196.

216. Gross, A.K., J. Norman, and A.M. Cook, *Contemporary pharmacologic issues in the management of traumatic brain injury.* J Pharm Pract, 2010. **23**(5): p. 425-40.

217. Meyer, M.J., J. Megyesi, J. Meythaler, M. Murie-Fernandez, J.A. Aubut, N. Foley, K. Salter, M. Bayley, S. Marshall, and R. Teasell, *Acute management of acquired brain injury part II: an evidence-based review of pharmacological interventions.* Brain Inj, 2010. **24**(5): p. 706-21.

218. Junpeng, M., S. Huang, and S. Qin, *Progesterone for acute traumatic brain injury.* Cochrane Database Syst Rev, 2011(1): p. CD008409.

219. Yang, J.P., H.J. Liu, S.M. Cheng, Z.L. Wang, X. Cheng, H.X. Yu, and X.F. Liu, *Direct transport of VEGF from the nasal cavity to brain.* Neurosci Lett, 2009. **449**(2): p. 108-11.

220. Hellewell, S.C., E.B. Yan, D.S. Alwis, N. Bye, and M.C. Morganti-Kossmann, *Erythropoietin improves motor and cognitive deficit, axonal pathology, and neuroinflammation in a combined model of diffuse traumatic brain injury and hypoxia, in association with upregulation of the erythropoietin receptor.* J Neuroinflammation, 2013. **10**: p. 156.

221. Bouzat, P., A. Millet, Y. Boue, K. Pernet-Gallay, T. Trouve-Buisson, L. Gaide-Chevronnay, E.L. Barbier, and J.F. Payen, *Changes in brain tissue oxygenation after treatment of diffuse traumatic brain injury by erythropoietin.* Crit Care Med, 2013. **41**(5): p. 1316-24.

222. Ehrenreich, H., M. Hasselblatt, C. Dembowski, L. Cepek, P. Lewczuk, M. Stiefel, H.H. Rustenbeck, N. Breiter, S. Jacob, F. Knerlich, M. Bohn, W. Poser, E. Ruther, M. Kochen, O. Gefeller, C. Gleiter, T.C. Wessel, M. De Ryck, L. Itri, H. Prange, A. Cerami, M. Brines, and A.L. Siren, *Erythropoietin therapy for acute stroke is both safe and beneficial.* Mol Med, 2002. **8**(8): p. 495-505.

223. Ehrenreich, H., B. Fischer, C. Norra, F. Schellenberger, N. Stender, M. Stiefel, A.L. Siren, W. Paulus, K.A. Nave, R. Gold, and C. Bartels, *Exploring recombinant human erythropoietin in chronic progressive multiple sclerosis.* Brain, 2007. **130**(Pt 10): p. 2577-88.

224. Ehrenreich, H., D. Hinze-Selch, S. Stawicki, C. Aust, S. Knolle-Veentjer, S. Wilms, G. Heinz, S. Erdag, H. Jahn, D. Degner, M. Ritzen, A. Mohr, M. Wagner, U. Schneider, M. Bohn, M. Huber, A. Czernik, T. Pollmacher, W. Maier, A.L. Siren, J. Klosterkotter, P. Falkai, E. Ruther, J.B. Aldenhoff, and H. Krampe, *Improvement of cognitive functions in chronic schizophrenic patients by recombinant human erythropoietin.* Mol Psychiatry, 2007. **12**(2): p. 206-20.

225. Cariou, A., Y.E. Claessens, F. Pene, J.S. Marx, C. Spaulding, C. Hababou, N. Casadevall, J.P. Mira, P. Carli, and O. Hermine, *Early high-dose erythropoietin therapy and hypothermia after out-of-hospital cardiac arrest: a matched control study.* Resuscitation, 2008. **76**(3): p. 397-404.

226. Corwin, H.L., A. Gettinger, T.C. Fabian, A. May, R.G. Pearl, S. Heard, R. An, P.J. Bowers, P. Burton, M.A. Klausner, M.J. Corwin, and E.P.O.C.C.T. Group, *Efficacy and safety of epoetin alfa in critically ill patients.* N Engl J Med, 2007. **357**(10): p. 965-76.

227. Nichol, A.D. and D.J. Cooper, *Can we improve neurological outcomes in severe traumatic brain injury?: Something old (early prophylactic hypothermia) and something new (erythropoietin).* Injury, 2009. **40**(5): p. 471-478.

228. Jin, K., X.O. Mao, L. Xie, V. John, and D.A. Greenberg, *Pharmacological induction of neuroglobin expression.* Pharmacology, 2011. **87**(1-2): p. 81-4.

229. Zhu, Y., Y. Sun, K. Jin, and D.A. Greenberg, *Hemin induces neuroglobin expression in neural cells.* Blood, 2002. **100**(7): p. 2494-8.

230. Sun, Y., K. Jin, X.O. Mao, Y. Zhu, and D.A. Greenberg, *Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury.* Proc Natl Acad Sci U S A, 2001. **98**(26): p. 15306-11.

231. Mann, K.V., M.A. Picciotti, T.A. Spevack, and D.R. Durbin, *Management of acute iron overdose.* Clin Pharm, 1989. **8**(6): p. 428-40.

232. Zhang, L., R. Hu, M. Li, F. Li, H. Meng, G. Zhu, J. Lin, and H. Feng, *Deferoxamine attenuates iron-induced long-term neurotoxicity in rats with traumatic brain injury.* Neurol Sci, 2013. **34**(5): p. 639-45.

233. Zhang, R., E. Shohami, E. Beit-Yannai, R. Bass, V. Trembovler, and A. Samuni, *Mechanism of brain protection by nitroxide radicals in experimental model of closed-head injury.* Free Radic Biol Med, 1998. **24**(2): p. 332-40.

234. Erkan Ustun, M., A.D. Md, C. Oztin Ogun, F. Sumer, and M. Gurbilek, *Effects of deferoxamine on tissue superoxide dismutase and glutathione peroxidase levels in experimental head trauma.* J Trauma, 2001. **51**(1): p. 22-5.

235. Temkin, N.R., S.S. Dikmen, G.D. Anderson, A.J. Wilensky, M.D. Holmes, W. Cohen, D.W. Newell, P. Nelson, A. Awan, and H.R. Winn, *Valproate therapy for prevention of posttraumatic seizures: a randomized trial.* J Neurosurg, 1999. **91**(4): p. 593-600.

236. Kim, S.R. and Y.C. Kim, *Neuroprotective phenylpropanoid esters of rhamnose isolated from roots of Scrophularia buergeriana.* Phytochemistry, 2000. **54**(5): p. 503-9.

237. Ren, M., Y. Leng, M. Jeong, P.R. Leeds, and D.M. Chuang, *Valproic acid reduces brain damage induced by transient focal cerebral ischemia in rats: potential roles of histone deacetylase inhibition and heat shock protein induction.* J Neurochem, 2004. **89**(6): p. 1358-67.

238. Mossberg, K.A., W.E. Amonette, and B.E. Masel, *Endurance training and cardiorespiratory conditioning after traumatic brain injury.* J Head Trauma Rehabil, 2010. **25**(3): p. 173-83.

239. Bland, D.C., C. Zampieri, and D.L. Damiano, *Effectiveness of physical therapy for improving gait and balance in individuals with traumatic brain injury: a systematic review.* Brain Inj, 2011. **25**(7-8): p. 664-79.

240. Hellweg, S. and S. Johannes, *Physiotherapy after traumatic brain injury: a systematic review of the literature.* Brain Inj, 2008. **22**(5): p. 365-73.

241. Brown, T.H., J. Mount, B.L. Rouland, K.A. Kautz, R.M. Barnes, and J. Kim, *Body weight-supported treadmill training versus conventional gait training for people with chronic traumatic brain injury.* J Head Trauma Rehabil, 2005. **20**(5): p. 402-15.

242. Mossberg, K.A., E.E. Orlander, and J.L. Norcross, *Cardiorespiratory capacity after weight-supported treadmill training in patients with traumatic brain injury.* Phys Ther, 2008. **88**(1): p. 77-87.

243. Sveistrup, H., J. McComas, M. Thornton, S. Marshall, H. Finestone, A. McCormick, K. Babulic, and A. Mayhew, *Experimental studies of virtual reality-delivered compared to conventional exercise programs for rehabilitation.* Cyberpsychol Behav, 2003. **6**(3): p. 245-9.

244. Grealy, M.A., D.A. Johnson, and S.K. Rushton, *Improving cognitive function after brain injury: the use of exercise and virtual reality.* Arch Phys Med Rehabil, 1999. **80**(6): p. 661-7.

245. Khan, A.A., Y. Wang, Y. Sun, X.O. Mao, L. Xie, E. Miles, J. Graboski, S. Chen, L.M. Ellerby, K. Jin, and D.A. Greenberg, *Neuroglobin-overexpressing transgenic mice are resistant to cerebral and myocardial ischemia.* Proc Natl Acad Sci U S A, 2006. **103**(47): p. 17944-8.

246. Chuang, P.Y., Y.P. Conley, S.M. Poloyac, D.O. Okonkwo, D. Ren, P.R. Sherwood, M. Hravnak, and S.A. Alexander, *Neuroglobin genetic polymorphisms and their relationship to functional outcomes after traumatic brain injury.* J Neurotrauma, 2010. **27**(6): p. 999-1006.

247. Hillman, C.H., K.I. Erickson, and A.F. Kramer, *Be smart, exercise your heart: exercise effects on brain and cognition.* Nat Rev Neurosci, 2008. **9**(1): p. 58-65.

248. Erickson, K.I., M.W. Voss, R.S. Prakash, C. Basak, A. Szabo, L. Chaddock, J.S. Kim, S. Heo, H. Alves, S.M. White, T.R. Wojcicki, E. Mailey, V.J. Vieira, S.A. Martin, B.D. Pence, J.A. Woods, E. McAuley, and A.F. Kramer, *Exercise training increases size of hippocampus and improves memory.* Proc Natl Acad Sci U S A, 2011. **108**(7): p. 3017-22.

249. Colcombe, S.J., K.I. Erickson, P.E. Scalf, J.S. Kim, R. Prakash, E. McAuley, S. Elavsky, D.X. Marquez, L. Hu, and A.F. Kramer, *Aerobic exercise training increases brain volume in aging humans.* J Gerontol A Biol Sci Med Sci, 2006. **61**(11): p. 1166-70.

250. Cotman, C.W. and N.C. Berchtold, *Exercise: a behavioral intervention to enhance brain health and plasticity.* Trends Neurosci, 2002. **25**(6): p. 295-301.

251. Erickson, K.I. and A.F. Kramer, *Aerobic exercise effects on cognitive and neural plasticity in older adults.* Br J Sports Med, 2009. **43**(1): p. 22-4.

252. Colcombe, S.J., A.F. Kramer, K.I. Erickson, P. Scalf, E. McAuley, N.J. Cohen, A. Webb, G.J. Jerome, D.X. Marquez, and S. Elavsky, *Cardiovascular fitness, cortical plasticity, and aging.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 3316-21.

253. van Praag, H., T. Shubert, C. Zhao, and F.H. Gage, *Exercise enhances learning and hippocampal neurogenesis in aged mice.* J Neurosci, 2005. **25**(38): p. 8680-5.

254. Burns, J.M., M.S. Mayo, H.S. Anderson, H.J. Smith, and J.E. Donnelly, *Cardiorespiratory fitness in early-stage Alzheimer disease.* Alzheimer Dis Assoc Disord, 2008. **22**(1): p. 39-46.

255. Lista, I. and G. Sorrentino, *Biological mechanisms of physical activity in preventing cognitive decline.* Cell Mol Neurobiol, 2010. **30**(4): p. 493-503.

256. Pereira, A.C., D.E. Huddleston, A.M. Brickman, A.A. Sosunov, R. Hen, G.M. McKhann, R. Sloan, F.H. Gage, T.R. Brown, and S.A. Small, *An in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus.* Proc Natl Acad Sci U S A, 2007. **104**(13): p. 5638-43.

257. Stranahan, A.M., Y. Zhou, B. Martin, and S. Maudsley, *Pharmacomimetics of exercise: novel approaches for hippocampally-targeted neuroprotective agents.* Curr Med Chem, 2009. **16**(35): p. 4668-78.

258. Fuss, J., N.M. Ben Abdallah, M.A. Vogt, C. Touma, P.G. Pacifici, R. Palme, V. Witzemann, R. Hellweg, and P. Gass, *Voluntary exercise induces anxiety-like behavior in adult C57BL/6J mice correlating with hippocampal neurogenesis.* Hippocampus, 2010. **20**(3): p. 364-76.

259. Holmes, M.M., L.A. Galea, R.E. Mistlberger, and G. Kempermann, *Adult hippocampal neurogenesis and voluntary running activity: circadian and dose-dependent effects.* J Neurosci Res, 2004. **76**(2): p. 216-22.

260. Griesbach, G.S., D.A. Hovda, and F. Gomez-Pinilla, *Exercise-induced improvement in cognitive performance after traumatic brain injury in rats is dependent on BDNF activation.* Brain Res, 2009. **1288**: p. 105-15.

261. Mota, B.C., L. Pereira, M.A. Souza, L.F. Silva, D.V. Magni, A.P. Ferreira, M.S. Oliveira, A.F. Furian, L. Mazzardo-Martins, M.D. Silva, A.R. Santos, J. Ferreira, M.R. Fighera, and L.F. Royes, *Exercise pre-conditioning reduces brain inflammation and protects against toxicity induced by traumatic brain injury: behavioral and neurochemical approach.* Neurotox Res, 2012. **21**(2): p. 175-84.

262. Piao, C.S., B.A. Stoica, J. Wu, B. Sabirzhanov, Z. Zhao, R. Cabatbat, D.J. Loane, and A.I. Faden, *Late exercise reduces neuroinflammation and cognitive dysfunction after traumatic brain injury.* Neurobiol Dis, 2013. **54**: p. 252-63.

263. Silva, L.F., M.S. Hoffmann, R. Gerbatin Rda, S. Fiorin Fda, F. Dobrachinski, B.C. Mota, A.T. Wouters, S.P. Pavarini, F.A. Soares, M.R. Fighera, and L.F. Royes, *Treadmill exercise protects against pentylenetetrazol-induced seizures and oxidative stress after traumatic brain injury.* J Neurotrauma, 2013. **30**(14): p. 1278-87.

264. Lima, F.D., M.S. Oliveira, A.F. Furian, M.A. Souza, L.M. Rambo, L.R. Ribeiro, L.F. Silva, L.T. Retamoso, M.S. Hoffmann, D.V. Magni, L. Pereira, M.R. Fighera, C.F. Mello, and L.F. Royes, *Adaptation to oxidative challenge induced by chronic physical exercise prevents Na+,K+-ATPase activity inhibition after traumatic brain injury.* Brain Res, 2009. **1279**: p. 147-55.

265. Querido, J.S. and A.W. Sheel, *Regulation of cerebral blood flow during exercise.* Sports Med, 2007. **37**(9): p. 765-82.

266. Ogoh, S. and P.N. Ainslie, *Cerebral blood flow during exercise: mechanisms of regulation.* J Appl Physiol (1985), 2009. **107**(5): p. 1370-80.

267. Kinni, H., M. Guo, J.Y. Ding, S. Konakondla, D. Dornbos, 3rd, R. Tran, M. Guthikonda, and Y. Ding, *Cerebral metabolism after forced or voluntary physical exercise.* Brain Res, 2011. **1388**: p. 48-55.

268. Adams, M., A. Williams, and J. Fell, *Exercise in the fight against thrombosis: friend or foe?* Semin Thromb Hemost, 2009. **35**(3): p. 261-8.

269. Greenleaf, J.E. and S. Kozlowski, *Physiological consequences of reduced physical activity during bed rest.* Exerc Sport Sci Rev, 1982. **10**: p. 84-119.

270. Bhambhani, Y., G. Rowland, and M. Farag, *Reliability of peak cardiorespiratory responses in patients with moderate to severe traumatic brain injury.* Arch Phys Med Rehabil, 2003. **84**(11): p. 1629-36.

271. Jankowski, L.W. and S.J. Sullivan, *Aerobic and neuromuscular training: effect on the capacity, efficiency, and fatigability of patients with traumatic brain injuries.* Arch Phys Med Rehabil, 1990. **71**(7): p. 500-4.

272. Hunter, M., J. Tomberlin, C. Kirkikis, and S.T. Kuna, *Progressive exercise testing in closed head-injured subjects: comparison of exercise apparatus in assessment of a physical conditioning program.* Phys Ther, 1990. **70**(6): p. 363-71.

273. Lieberman, S.A., A.L. Oberoi, C.R. Gilkison, B.E. Masel, and R.J. Urban, *Prevalence of neuroendocrine dysfunction in patients recovering from traumatic brain injury.* J Clin Endocrinol Metab, 2001. **86**(6): p. 2752-6.

274. Rezaee, S., S. Kahrizi, and M. Hedayati, *Hormonal responses of combining endurance-resistance exercise in healthy young men.* J Sports Med Phys Fitness, 2014. **54**(2): p. 244-51.

275. Rahimi, R., M. Qaderi, H. Faraji, and S.S. Boroujerdi, *Effects of very short rest periods on hormonal responses to resistance exercise in men.* J Strength Cond Res, 2010. **24**(7): p. 1851-9.

276. Copeland, J.L., L.A. Consitt, and M.S. Tremblay, *Hormonal responses to endurance and resistance exercise in females aged 19-69 years.* J Gerontol A Biol Sci Med Sci, 2002. **57**(4): p. B158-65.

277. Hassmen, P., N. Koivula, and A. Uutela, *Physical exercise and psychological well-being: a population study in Finland.* Prev Med, 2000. **30**(1): p. 17-25.

278. Gordon, W.A., M. Sliwinski, J. Echo, M. McLoughlin, M.S. Sheerer, and T.E. Meili, *The benefits of exercise in individuals with traumatic brain injury: a retrospective study.* J Head Trauma Rehabil, 1998. **13**(4): p. 58-67.

279. Masel, B.E., R.S. Scheibel, T. Kimbark, and S.T. Kuna, *Excessive daytime sleepiness in adults with brain injuries.* Arch Phys Med Rehabil, 2001. **82**(11): p. 1526-32.

280. King, A.C., R.F. Oman, G.S. Brassington, D.L. Bliwise, and W.L. Haskell, *Moderate-intensity exercise and self-rated quality of sleep in older adults. A randomized controlled trial.* JAMA, 1997. **277**(1): p. 32-7.

281. Lautenschlager, N.T., K.L. Cox, L. Flicker, J.K. Foster, F.M. van Bockxmeer, J. Xiao, K.R. Greenop, and O.P. Almeida, *Effect of physical activity on cognitive function in older adults at risk for Alzheimer disease: a randomized trial.* JAMA, 2008. **300**(9): p. 1027-37.

282. Saaksjarvi, K., P. Knekt, S. Mannisto, J. Lyytinen, T. Jaaskelainen, N. Kanerva, and M. Heliovaara, *Reduced risk of Parkinson's disease associated with lower body mass index and heavy leisure-time physical activity.* Eur J Epidemiol, 2014. **29**(4): p. 285-92.

283. de Souza Wyse, A.T., E.L. Streck, P. Worm, A. Wajner, F. Ritter, and C.A. Netto, *Preconditioning prevents the inhibition of Na+,K+-ATPase activity after brain ischemia.* Neurochem Res, 2000. **25**(7): p. 971-5.

284. Souza, M.A., M.S. Oliveira, A.F. Furian, L.M. Rambo, L.R. Ribeiro, F.D. Lima, L.C. Dalla Corte, L.F. Silva, L.T. Retamoso, C.L. Dalla Corte, G.O. Puntel, D.S. de Avila, F.A. Soares, M.R. Fighera, C.F. de Mello, and L.F. Royes, *Swimming training prevents pentylenetetrazol-induced inhibition of Na+, K+-ATPase activity, seizures, and oxidative stress.* Epilepsia, 2009. **50**(4): p. 811-23.

285. Mourao, F.A., H.R. Leite, L.E. de Carvalho, E.V.T.H. Ferreira, M.C. Pinto, D. de Castro Medeiros, I.L. Andrade, D.F. Goncalves, G.S. Pereira, M.F. Dutra Moraes, and A.R. Massensini, *Neuroprotective effect of exercise in rat hippocampal slices submitted to in vitro ischemia is promoted by decrease of glutamate release and pro-apoptotic markers.* J Neurochem, 2014. **131**(1): p. 65-73.

286. Yuan, X.Q., D.S. Prough, T.L. Smith, and D.S. Dewitt, *The effects of traumatic brain injury on regional cerebral blood flow in rats.* J Neurotrauma, 1988. **5**(4): p. 289-301.

287. Dornbos, D., 3rd, N. Zwagerman, M. Guo, J.Y. Ding, C. Peng, F. Esmail, C. Sikharam, X. Geng, M. Guthikonda, and Y. Ding, *Preischemic exercise reduces brain damage by ameliorating metabolic disorder in ischemia/reperfusion injury.* J Neurosci Res, 2013. **91**(6): p. 818-27.

288. Lee, M., J.T. Hwang, H.J. Lee, S.N. Jung, I. Kang, S.G. Chi, S.S. Kim, and J. Ha, *AMP-activated protein kinase activity is critical for hypoxia-inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells.* J Biol Chem, 2003. **278**(41): p. 39653-61.

289. Marsin, A.S., L. Bertrand, M.H. Rider, J. Deprez, C. Beauloye, M.F. Vincent, G. Van den Berghe, D. Carling, and L. Hue, *Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia.* Curr Biol, 2000. **10**(20): p. 1247-55.

290. Ogonovszky, H., I. Berkes, S. Kumagai, T. Kaneko, S. Tahara, S. Goto, and Z. Radak, *The effects of moderate-, strenuous- and over-training on oxidative stress markers, DNA repair, and memory, in rat brain.* Neurochem Int, 2005. **46**(8): p. 635-40.

291. Marosi, K., Z. Bori, N. Hart, L. Sarga, E. Koltai, Z. Radak, and C. Nyakas, *Long-term exercise treatment reduces oxidative stress in the hippocampus of aging rats.* Neuroscience, 2012. **226**: p. 21-8.

292. Yu, L. and S.J. Yang, *AMP-activated protein kinase mediates activity-dependent regulation of peroxisome proliferator-activated receptor gamma coactivator-1alpha and nuclear respiratory factor 1 expression in rat visual cortical neurons.* Neuroscience, 2010. **169**(1): p. 23-38.

293. Chinsomboon, J., J. Ruas, R.K. Gupta, R. Thom, J. Shoag, G.C. Rowe, N. Sawada, S. Raghuram, and Z. Arany, *The transcriptional coactivator PGC-1alpha mediates exercise-induced angiogenesis in skeletal muscle.* Proc Natl Acad Sci U S A, 2009. **106**(50): p. 21401-6.

294. Bayod, S., J. Del Valle, A.M. Canudas, J.F. Lalanza, S. Sanchez-Roige, A. Camins, R.M. Escorihuela, and M. Pallas, *Long-term treadmill exercise induces neuroprotective molecular changes in rat brain.* J Appl Physiol (1985), 2011. **111**(5): p. 1380-90.

295. Hood, D.A., I. Irrcher, V. Ljubicic, and A.M. Joseph, *Coordination of metabolic plasticity in skeletal muscle.* J Exp Biol, 2006. **209**(Pt 12): p. 2265-75.

296. Little, J.P., A. Safdar, D. Bishop, M.A. Tarnopolsky, and M.J. Gibala, *An acute bout of high-intensity interval training increases the nuclear abundance of PGC-1alpha and activates mitochondrial biogenesis in human skeletal muscle.* Am J Physiol Regul Integr Comp Physiol, 2011. **300**(6): p. R1303-10.

297. Steiner, J.L., E.A. Murphy, J.L. McClellan, M.D. Carmichael, and J.M. Davis, *Exercise training increases mitochondrial biogenesis in the brain.* J Appl Physiol (1985), 2011. **111**(4): p. 1066-71.

298. Liang, H. and W.F. Ward, *PGC-1alpha: a key regulator of energy metabolism.* Adv Physiol Educ, 2006. **30**(4): p. 145-51.

299. Zhang, Q., Y. Wu, P. Zhang, H. Sha, J. Jia, Y. Hu, and J. Zhu, *Exercise induces mitochondrial biogenesis after brain ischemia in rats.* Neuroscience, 2012. **205**: p. 10-7.

300. Petersen, A.M. and B.K. Pedersen, *The anti-inflammatory effect of exercise.* J Appl Physiol (1985), 2005. **98**(4): p. 1154-62.

301. Steensberg, A., C.P. Fischer, C. Keller, K. Moller, and B.K. Pedersen, *IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans.* Am J Physiol Endocrinol Metab, 2003. **285**(2): p. E433-7.

302. Funk, J.A., J. Gohlke, A.D. Kraft, C.A. McPherson, J.B. Collins, and G. Jean Harry, *Voluntary exercise protects hippocampal neurons from trimethyltin injury: possible role of interleukin-6 to modulate tumor necrosis factor receptor-mediated neurotoxicity.* Brain Behav Immun, 2011. **25**(6): p. 1063-77.

303. Ding, Y.H., M. Mrizek, Q. Lai, Y. Wu, R. Reyes, Jr., J. Li, W.W. Davis, and Y. Ding, *Exercise preconditioning reduces brain damage and inhibits TNF-alpha receptor expression after hypoxia/reoxygenation: an in vivo and in vitro study.* Curr Neurovasc Res, 2006. **3**(4): p. 263-71.

304. Parachikova, A., K.E. Nichol, and C.W. Cotman, *Short-term exercise in aged Tg2576 mice alters neuroinflammation and improves cognition.* Neurobiol Dis, 2008. **30**(1): p. 121-9.

305. Giusti, B., M. Marini, L. Rossi, I. Lapini, A. Magi, A. Capalbo, R. Lapalombella, S. di Tullio, M. Samaja, F. Esposito, V. Margonato, M. Boddi, R. Abbate, and A. Veicsteinas, *Gene expression profile of rat left ventricles reveals persisting changes following chronic mild exercise protocol: implications for cardioprotection.* BMC Genomics, 2009. **10**: p. 342.

306. Tang, K., F.C. Xia, P.D. Wagner, and E.C. Breen, *Exercise-induced VEGF transcriptional activation in brain, lung and skeletal muscle.* Respir Physiol Neurobiol, 2010. **170**(1): p. 16-22.

307. Gustafsson, T., A. Knutsson, A. Puntschart, L. Kaijser, A.C. Nordqvist, C.J. Sundberg, and E. Jansson, *Increased expression of vascular endothelial growth factor in human skeletal muscle in response to short-term one-legged exercise training.* Pflugers Arch, 2002. **444**(6): p. 752-9.

308. Wahl, P., F. Jansen, S. Achtzehn, T. Schmitz, W. Bloch, J. Mester, and N. Werner, *Effects of high intensity training and high volume training on endothelial microparticles and angiogenic growth factors.* PLoS One, 2014. **9**(4): p. e96024.

309. Shoag, J. and Z. Arany, *Regulation of hypoxia-inducible genes by PGC-1 alpha.* Arterioscler Thromb Vasc Biol, 2010. **30**(4): p. 662-6.

310. Olenich, S.A., N. Gutierrez-Reed, G.N. Audet, and I.M. Olfert, *Temporal response of positive and negative regulators in response to acute and chronic exercise training in mice.* J Physiol, 2013. **591**(Pt 20): p. 5157-69.

311. Viboolvorakul, S. and S. Patumraj, *Exercise Training Could Improve Age-Related Changes in Cerebral Blood Flow and Capillary Vascularity through the Upregulation of VEGF and eNOS.* Biomed Res Int, 2014. **2014**: p. 230791.

312. Ma, Y., L. Qiang, and M. He, *Exercise therapy augments the ischemia-induced proangiogenic state and results in sustained improvement after stroke.* Int J Mol Sci, 2013. **14**(4): p. 8570-84.

313. Zhang, Q.W., X.X. Deng, X. Sun, J.X. Xu, and F.Y. Sun, *Exercise promotes axon regeneration of newborn striatonigral and corticonigral projection neurons in rats after ischemic stroke.* PLoS One, 2013. **8**(11): p. e80139.

314. Baker, J.M., M. De Lisio, and G. Parise, *Endurance exercise training promotes medullary hematopoiesis.* FASEB J, 2011. **25**(12): p. 4348-57.

315. Wahl, P., A. Schmidt, M. Demarees, S. Achtzehn, W. Bloch, and J. Mester, *Responses of angiogenic growth factors to exercise, to hypoxia and to exercise under hypoxic conditions.* Int J Sports Med, 2013. **34**(2): p. 95-100.

316. Wehrlin, J.P., P. Zuest, J. Hallen, and B. Marti, *Live high-train low for 24 days increases hemoglobin mass and red cell volume in elite endurance athletes.* J Appl Physiol (1985), 2006. **100**(6): p. 1938-45.

317. Diamanti-Kandarakis, E., P.A. Konstantinopoulos, J. Papailiou, S.A. Kandarakis, A. Andreopoulos, and G.P. Sykiotis, *Erythropoietin abuse and erythropoietin gene doping: detection strategies in the genomic era.* Sports Med, 2005. **35**(10): p. 831-40.

318. Thompson, D., S. Basu-Modak, M. Gordon, S. Poore, D. Markovitch, and R.M. Tyrrell, *Exercise-induced expression of heme oxygenase-1 in human lymphocytes.* Free Radic Res, 2005. **39**(1): p. 63-9.

319. Hildebrandt, A.L., H. Pilegaard, and P.D. Neufer, *Differential transcriptional activation of select metabolic genes in response to variations in exercise intensity and duration.* Am J Physiol Endocrinol Metab, 2003. **285**(5): p. E1021-7.

320. Steensberg, A., C. Keller, T. Hillig, C. Frosig, J.F. Wojtaszewski, B.K. Pedersen, H. Pilegaard, and M. Sander, *Nitric oxide production is a proximal signaling event controlling exercise-induced mRNA expression in human skeletal muscle.* FASEB J, 2007. **21**(11): p. 2683-94.

321. Sun, M.W., M.F. Zhong, J. Gu, F.L. Qian, J.Z. Gu, and H. Chen, *Effects of different levels of exercise volume on endothelium-dependent vasodilation: roles of nitric oxide synthase and heme oxygenase.* Hypertens Res, 2008. **31**(4): p. 805-16.

322. Marini, M., R. Lapalombella, V. Margonato, R. Ronchi, M. Samaja, C. Scapin, L. Gorza, T. Maraldi, P. Carinci, C. Ventura, and A. Veicsteinas, *Mild exercise training, cardioprotection and stress genes profile.* Eur J Appl Physiol, 2007. **99**(5): p. 503-10.

323. Zhao, J.X., Y. Tian, J.M. Cao, L. Jin, and M.H. Xie, *[Effect of treadmill exercise and nutrition supplement on activity and gene expression of rate-limiting enzyme of heme metabolism and globin]*. Zhongguo Ying Yong Sheng Li Xue Za Zhi, 2009. **25**(4): p. 440-4.

324. Cotman, C.W., N.C. Berchtold, and L.A. Christie, *Exercise builds brain health: key roles of growth factor cascades and inflammation.* Trends Neurosci, 2007. **30**(9): p. 464-72.

325. Griesbach, G.S., F. Gomez-Pinilla, and D.A. Hovda, *Time window for voluntary exercise-induced increases in hippocampal neuroplasticity molecules after traumatic brain injury is severity dependent.* J Neurotrauma, 2007. **24**(7): p. 1161-71.

326. Chen, M.F., T.Y. Huang, Y.M. Kuo, L. Yu, H.I. Chen, and C.J. Jen, *Early postinjury exercise reverses memory deficits and retards the progression of closed-head injury in mice.* J Physiol, 2013. **591**(Pt 4): p. 985-1000.

327. Wilde, M.C., R.J. Castriotta, J.M. Lai, S. Atanasov, B.E. Masel, and S.T. Kuna, *Cognitive impairment in patients with traumatic brain injury and obstructive sleep apnea.* Arch Phys Med Rehabil, 2007. **88**(10): p. 1284-8.

328. Jeanneteau, F., K. Deinhardt, G. Miyoshi, A.M. Bennett, and M.V. Chao, *The MAP kinase phosphatase MKP-1 regulates BDNF-induced axon branching.* Nat Neurosci, 2010. **13**(11): p. 1373-9.

329. Cetinkaya, C., A.R. Sisman, M. Kiray, U.M. Camsari, C. Gencoglu, B. Baykara, I. Aksu, and N. Uysal, *Positive effects of aerobic exercise on learning and memory functioning, which correlate with hippocampal IGF-1 increase in adolescent rats.* Neurosci Lett, 2013. **549**: p. 177-81.

330. Carro, E., A. Nunez, S. Busiguina, and I. Torres-Aleman, *Circulating insulin-like growth factor I mediates effects of exercise on the brain.* J Neurosci, 2000. **20**(8): p. 2926-33.

331. Koziris, L.P., R.C. Hickson, R.T. Chatterton, Jr., R.T. Groseth, J.M. Christie, D.G. Goldflies, and T.G. Unterman, *Serum levels of total and free IGF-I and IGFBP-3 are increased and maintained in long-term training.* J Appl Physiol (1985), 1999. **86**(4): p. 1436-42.

332. Poehlman, E.T. and K.C. Copeland, *Influence of physical activity on insulin-like growth factor-I in healthy younger and older men.* J Clin Endocrinol Metab, 1990. **71**(6): p. 1468-73.

333. Kraemer, W.J., L. Marchitelli, S.E. Gordon, E. Harman, J.E. Dziados, R. Mello, P. Frykman, D. McCurry, and S.J. Fleck, *Hormonal and growth factor responses to heavy resistance exercise protocols.* J Appl Physiol (1985), 1990. **69**(4): p. 1442-50.

334. Ding, Q., S. Vaynman, M. Akhavan, Z. Ying, and F. Gomez-Pinilla, *Insulin-like growth factor I interfaces with brain-derived neurotrophic factor-mediated synaptic plasticity to modulate aspects of exercise-induced cognitive function.* Neuroscience, 2006. **140**(3): p. 823-33.

335. Chen, M.J. and A.A. Russo-Neustadt, *Running exercise- and antidepressant-induced increases in growth and survival-associated signaling molecules are IGF-dependent.* Growth Factors, 2007. **25**(2): p. 118-31.

336. Gomez-Pinilla, F., S. Vaynman, and Z. Ying, *Brain-derived neurotrophic factor functions as a metabotrophin to mediate the effects of exercise on cognition.* Eur J Neurosci, 2008. **28**(11): p. 2278-87.

337. Cappon, J., J.A. Brasel, S. Mohan, and D.M. Cooper, *Effect of brief exercise on circulating insulin-like growth factor I.* J Appl Physiol (1985), 1994. **76**(6): p. 2490-6.

338. Kraus, R.M., H.W. Stallings, 3rd, R.C. Yeager, and T.P. Gavin, *Circulating plasma VEGF response to exercise in sedentary and endurance-trained men.* J Appl Physiol (1985), 2004. **96**(4): p. 1445-50.

339. Trejo, J.L., E. Carro, and I. Torres-Aleman, *Circulating insulin-like growth factor I mediates exercise-induced increases in the number of new neurons in the adult hippocampus.* J Neurosci, 2001. **21**(5): p. 1628-34.

340. Tian, S.F., H.H. Yang, D.P. Xiao, Y.J. Huang, G.Y. He, H.R. Ma, F. Xia, and X.C. Shi, *Mechanisms of neuroprotection from hypoxia-ischemia (HI) brain injury by up-regulation of cytoglobin (CYGB) in a neonatal rat model.* J Biol Chem, 2013. **288**(22): p. 15988-6003.

341. Onyszchuk, G., B. Al-Hafez, Y.Y. He, M. Bilgen, N.E. Berman, and W.M. Brooks, *A mouse model of sensorimotor controlled cortical impact: characterization using longitudinal magnetic resonance imaging, behavioral assessments and histology.* J Neurosci Methods, 2007. **160**(2): p. 187-96.

342. Colicos, M.A., C.E. Dixon, and P.K. Dash, *Delayed, selective neuronal death following experimental cortical impact injury in rats: possible role in memory deficits.* Brain Res, 1996. **739**(1-2): p. 111-9.

343. Dixon, C.E., G.L. Clifton, J.W. Lighthall, A.A. Yaghmai, and R.L. Hayes, *A controlled cortical impact model of traumatic brain injury in the rat.* J Neurosci Methods, 1991. **39**(3): p. 253-62.

344. Bustin, S.A., V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, and C.T. Wittwer, *The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments.* Clin Chem, 2009. **55**(4): p. 611-22.

345. Shelton, S.B., D.B. Pettigrew, A.D. Hermann, W. Zhou, P.M. Sullivan, K.A. Crutcher, and K.I. Strauss, *A simple, efficient tool for assessment of mice after unilateral cortex injury.* J Neurosci Methods, 2008. **168**(2): p. 431-42.

346. Baskin, Y.K., W.D. Dietrich, and E.J. Green, *Two effective behavioral tasks for evaluating sensorimotor dysfunction following traumatic brain injury in mice.* J Neurosci Methods, 2003. **129**(1): p. 87-93.

347. Di Pietro, V., G. Lazzarino, A.M. Amorini, B. Tavazzi, S. D'Urso, S. Longo, R. Vagnozzi, S. Signoretti, E. Clementi, B. Giardina, G. Lazzarino, and A. Belli, *Neuroglobin expression and oxidant/antioxidant balance after graded traumatic brain injury in the rat.* Free Radic Biol Med, 2014. **69C**: p. 258-264.

348. Dash, P.K., J. Zhao, S.A. Orsi, M. Zhang, and A.N. Moore, *Sulforaphane improves cognitive function administered following traumatic brain injury.* Neurosci Lett, 2009. **460**(2): p. 103-7.

349. Griesbach, G.S., *Exercise after traumatic brain injury: is it a double-edged sword?* PM R, 2011. **3**(6 Suppl 1): p. S64-72.

350. Alamed, J., D.M. Wilcock, D.M. Diamond, M.N. Gordon, and D. Morgan, *Two-day radial-arm water maze learning and memory task; robust resolution of amyloid-related memory deficits in transgenic mice.* Nat Protoc, 2006. **1**(4): p. 1671-9.

351. Arendash, G.W., M.N. Gordon, D.M. Diamond, L.A. Austin, J.M. Hatcher, P. Jantzen, G. DiCarlo, D. Wilcock, and D. Morgan, *Behavioral assessment of Alzheimer's transgenic mice following long-term Abeta vaccination: task specificity and correlations between Abeta deposition and spatial memory.* DNA Cell Biol, 2001. **20**(11): p. 737-44.

352. Chen, G.H., Y.J. Wang, X.M. Wang, and J.N. Zhou, *Accelerated senescence prone mouse-8 shows early onset of deficits in spatial learning and memory in the radial six-arm water maze.* Physiol Behav, 2004. **82**(5): p. 883-90.

353. Pfaffl, M.W., G.W. Horgan, and L. Dempfle, *Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR.* Nucleic Acids Res, 2002. **30**(9): p. e36.

354. Peters, D.M., S. Jain, D.M. Liuzzo, A. Middleton, J. Greene, E. Blanck, S. Sun, R. Raman, and S.L. Fritz, *Individuals with chronic traumatic brain injury improve walking speed and mobility with intensive mobility training.* Arch Phys Med Rehabil, 2014. **95**(8): p. 1454-60.

355. Gu, Y.L., L.W. Zhang, N. Ma, L.L. Ye, X. Wang de, and X. Gao, *Cognitive improvement of mice induced by exercise prior to traumatic brain injury is associated with cytochrome c oxidase.* Neurosci Lett, 2014. **570**: p. 86-91.

356. Griesbach, G.S., D.A. Hovda, R. Molteni, A. Wu, and F. Gomez-Pinilla, *Voluntary exercise following traumatic brain injury: brain-derived neurotrophic factor upregulation and recovery of function.* Neuroscience, 2004. **125**(1): p. 129-39.

357. Zhao, J., A.N. Moore, J.B. Redell, and P.K. Dash, *Enhancing expression of Nrf2-driven genes protects the blood brain barrier after brain injury.* J Neurosci, 2007. **27**(38): p. 10240-8.

358. Vink, R., A. Young, C.J. Bennett, X. Hu, C.O. Connor, I. Cernak, and A.J. Nimmo, *Neuropeptide release influences brain edema formation after diffuse traumatic brain injury.* Acta Neurochir Suppl, 2003. **86**: p. 257-60.

359. Lin, T.K., S.D. Chen, Y.C. Chuang, H.Y. Lin, C.R. Huang, J.H. Chuang, P.W. Wang, S.T. Huang, M.M. Tiao, J.B. Chen, and C.W. Liou, *Resveratrol partially prevents rotenone-induced neurotoxicity in dopaminergic SH-SY5Y cells through induction of heme oxygenase-1 dependent autophagy.* Int J Mol Sci, 2014. **15**(1): p. 1625-46.

360. Lin, C.J., T.H. Chen, L.Y. Yang, and C.M. Shih, *Resveratrol protects astrocytes against traumatic brain injury through inhibiting apoptotic and autophagic cell death.* Cell Death Dis, 2014. **5**: p. e1147.

361. Leddy, J.J., K. Kozlowski, M. Fung, D.R. Pendergast, and B. Willer, *Regulatory and autoregulatory physiological dysfunction as a primary characteristic of post concussion syndrome: implications for treatment.* NeuroRehabilitation, 2007. **22**(3): p. 199-205.

362. Palmer, C., R.L. Roberts, and C. Bero, *Deferoxamine posttreatment reduces ischemic brain injury in neonatal rats.* Stroke, 1994. **25**(5): p. 1039-45.

363. Hanson, L.R., A. Roeytenberg, P.M. Martinez, V.G. Coppes, D.C. Sweet, R.J. Rao, D.L. Marti, J.D. Hoekman, R.B. Matthews, W.H. Frey, 2nd, and S.S. Panter, *Intranasal deferoxamine provides increased brain exposure and significant protection in rat ischemic stroke.* J Pharmacol Exp Ther, 2009. **330**(3): p. 679-86.

364. Long, D.A., K. Ghosh, A.N. Moore, C.E. Dixon, and P.K. Dash, *Deferoxamine improves spatial memory performance following experimental brain injury in rats.* Brain Res, 1996. **717**(1-2): p. 109-17.

365. Pardridge, W.M., *The blood-brain barrier: bottleneck in brain drug development.* NeuroRx, 2005. **2**(1): p. 3-14.

366. Vainshtein, A., L. Kazak, and D.A. Hood, *Effects of endurance training on apoptotic susceptibility in striated muscle.* J Appl Physiol, 2011. **110**(6): p. 1638-45.

367. Dai, W., H.L. Cheng, R.Q. Huang, Z. Zhuang, and J.X. Shi, *Quantitative detection of the expression of mitochondrial cytochrome c oxidase subunits mRNA in the cerebral cortex after experimental traumatic brain injury.* Brain Res, 2009. **1251**: p. 287-95.

368. Harris, L.K., R.T. Black, K.M. Golden, T.M. Reeves, J.T. Povlishock, and L.L. Phillips, *Traumatic brain injury-induced changes in gene expression and functional activity of mitochondrial cytochrome C oxidase.* J Neurotrauma, 2001. **18**(10): p. 993-1009.

369. Itoh, T., M. Imano, S. Nishida, M. Tsubaki, S. Hashimoto, A. Ito, and T. Satou, *Exercise increases neural stem cell proliferation surrounding the area of damage following rat traumatic brain injury.* J Neural Transm, 2011. **118**(2): p. 193-202.

370. Shen, X., A. Li, Y. Zhang, X. Dong, T. Shan, Y. Wu, J. Jia, and Y. Hu, *The effect of different intensities of treadmill exercise on cognitive function deficit following a severe controlled cortical impact in rats.* Int J Mol Sci, 2013. **14**(11): p. 21598-612.

371. Franckeviciute, E. and A. Krisciunas, *[Evaluation of factors influencing effectiveness of kinesitherapy in patients after traumatic brain injury]*. Medicina (Kaunas), 2006. **42**(9): p. 732-7.

372. Maddock, R.J., G.A. Casazza, M.H. Buonocore, and C. Tanase, *Vigorous exercise increases brain lactate and Glx (glutamate+glutamine): a dynamic 1H-MRS study.* Neuroimage, 2011. **57**(4): p. 1324-30.

373. Kemppainen, J., S. Aalto, T. Fujimoto, K.K. Kalliokoski, J. Langsjo, V. Oikonen, J. Rinne, P. Nuutila, and J. Knuuti, *High intensity exercise decreases global brain glucose uptake in humans.* J Physiol, 2005. **568**(Pt 1): p. 323-32.

374. MacIntosh, B.J., D.E. Crane, M.D. Sage, A.S. Rajab, M.J. Donahue, W.E. McIlroy, and L.E. Middleton, *Impact of a single bout of aerobic exercise on regional brain perfusion and activation responses in healthy young adults.* PLoS One, 2014. **9**(1): p. e85163.

375. Ide, K., I.K. Schmalbruch, B. Quistorff, A. Horn, and N.H. Secher, *Lactate, glucose and O2 uptake in human brain during recovery from maximal exercise.* J Physiol, 2000. **522 Pt 1**: p. 159-64.