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Environmental Enrichment-Mediated Neuroprotection Against Traumatic Brain Injury: Role of Brain-Derived Neurotrophic Factor

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Environmental Enrichment-Mediated Neuroprotection Against Traumatic Brain Injury: Role of Brain-Derived Neurotrophic Factor

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

KYLE LEANN TRAVER
B.S., The University of Toledo, 2009

2011
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Kyle Leann Traver ENTITLED Environmental Enrichment-Mediated Neuroprotection Against Traumatic Brain Injury: Role of Brain-Derived Neurotrophic Factor BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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Traver, Kyle Leann. M.S., Department of Neuroscience, Cell Biology and Physiology, Wright State University, 2011. Environmental Enrichment-Mediated Neuroprotection Against Traumatic Brain Injury: Role of Brain-Derived Neurotrophic Factor

Each year there are over 1.7 million traumatic brain injuries (TBI) in the United States causing long-term health deficits and significant medical costs. This growing epidemic has led researchers to find methods for treating and preventing these injuries. Environmental Enrichment (EE) is an innovative technique shown to improve cognitive and functional outcomes following a TBI; however, the beneficial effects before injury have not been established. Brain-Derived Neurotrophic Factor (BDNF) has been determined as a neuroprotective biomolecule in TBI. This research examined these two protective components and their roles in TBI. It was hypothesized that BDNF mRNA and protein levels would be elevated in animals environmentally enriched prior to TBI compared to non-enriched animals. Results indicated that EE applied before TBI improved cognitive performance, but BDNF mRNA and protein levels were not elevated significantly. These findings require further examination to determine a more definitive relationship between these mechanisms.

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Dedication

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Thank you for all of your love and for always standing beside me when I need you the most.

I love you.
Background

Traumatic Brain Injury

The Center for Disease Control and Prevention has reported that each year approximately 1.7 million people sustain a Traumatic Brain Injury (TBI), leading to long-term health deficits as well as significant direct and indirect medical costs\textsuperscript{1}. Of these 1.7 million injuries, nearly 50,000 patients die and 90,000 individuals experience permanent disability\textsuperscript{2}. According to the World Health Organization, TBI will surpass many diseases as the major cause of death and disability by the year 2020, with an estimated 10 million people affected annually\textsuperscript{2}. In 2000, the cost of direct and indirect care for patients suffering from a TBI accounted for nearly 60 billion dollars in the United States alone\textsuperscript{1}. Traumatic Brain Injury is a costly, devastating condition which can generate long-lasting social and occupational disabilities in behavior, memory, and executive function, and the need for preventative measures against this epidemic are crucial\textsuperscript{3}.

TBI is commonly categorized into three levels of severity. These levels include mild, moderate, and severe TBI. Studies using rodents have determined that the severity of a TBI directly correlates with the amount of histological manipulation, locomotor and cognitive deficits seen post-injury\textsuperscript{4,5}. Consequently, as TBI severity is increased, damage to the central nervous system (CNS) tends to extend beyond the initial site of injury and spread to other regions of the brain, particularly the hippocampus\textsuperscript{4,5}. This structure is considered part of the limbic system and is located in the basal medial part of the temporal lobe\textsuperscript{6}. The hippocampus is configured in a curved formation and is composed of various layers of neurons\textsuperscript{6}. Functions of the hippocampus include learning, memory and integration of information. Following a TBI, these functions have been shown to be disrupted\textsuperscript{7}. Although the hippocampus may not be impacted directly during the injury,
the processes which occur after a TBI may result in deleterious effects on this vital brain component.

*Mechanisms of TBI*

There are three main stages (acute, sub-acute & chronic) which occur following a TBI. The first stage (acute) occurs at or near the site of injury and results in direct tissue damage, impaired regulation of cellular metabolism, penetration of the blood brain barrier and cellular death\(^8,9\). This stage can only be treated by preventative measures prior to the injury, not therapeutic treatments after the TBI\(^8\). The second stage (sub-acute) is characterized by membrane depolarization which leads to the excessive release of excitotoxic neurotransmitters, such as glutamate, and cytokines\(^8\). These biomolecules act on the brain to induce cell necrosis and heighten the initial damage. The third stage (chronic) following a TBI is delayed cell death which is caused by apoptotic influences such as inflammation\(^9\). Each stage plays a vital role in the outcome of patients suffering the deleterious effects of a TBI (Figure 1).

During the acute stage, direct tissue damage may occur depending on the severity of the TBI\(^8\). This trauma can cause cellular necrosis at the site of injury and activate the hypothalamic-pituitary-adrenal axis (HPA axis). The TBI can also disrupt the integrity of the blood brain barrier and impair cellular metabolism\(^8,10\). Necrosis causes the release of excitatory neurotransmitters and induces cellular metabolic failure\(^8\). This metabolic failure causes a loss of adenosine triphosphate (ATP) production, initiating energy-dependent ion pump malfunction and leading to detrimental effects on the cells of the brain\(^8\).
Figure 1: Mechanistic effects of a traumatic brain injury distinguished by stages. During the acute stage, numerous physical and biochemical disruptions occur which can lead to neuronal degeneration. These effects have the ability to carry over into the sub-acute stage which increases degeneration by increasing apoptosis. The chronic stage includes inflammation and advanced apoptosis, causing detrimental effects to the CNS.

Once the HPA axis is activated, corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) are released from the paraventricular nucleus of the hypothalamus\textsuperscript{5,10}. The expression of these hormones causes the release of adrenocorticotropic hormone (ACTH) from the pituitary, and in turn, ACTH stimulates the biosynthesis and release of glucocorticoids (GCs), such as cortisol, from the adrenal cortex\textsuperscript{10} (Figure 2).
Figure 2: Normal HPA Axis Response to Stress. Once a stress transpires, the hypothalamus and PVN activate and release CRH and AVP. These hormones activate the anterior pituitary which leads to the release of ACTH. Discharging that hormone causes the adrenal cortex to expel cortisol. The adrenal cortex has a negative feedback loop to both the hypothalamus and anterior pituitary which aids in the regulation of the system, and assists in regaining homeostasis.

Under normal conditions, these GCs act under a feedback loop with the hypothalamus to reduce the amount of CRH produced and prevent over-activation of the system\textsuperscript{11}. Following a TBI, there is dysregulation of the HPA axis, providing persistent exposure to GCs and numerous detrimental effects throughout the CNS\textsuperscript{10-12}.

The sub-acute stage occurs hours to days following injury. During this stage, neurons near the site of injury swell, shrink and eventually die, resulting in neuronal degeneration\textsuperscript{8,9}. This stage is characterized by the excessive release of excitotoxic neurotransmitters, increased lesion size and number of neuronal apoptotic cells and the activation of voltage-dependent ion channels\textsuperscript{8,11}. Fluxes in ionic concentrations cause calcium (Ca\textsuperscript{2+}) and sodium (Na\textsuperscript{+}) imbalances which can result in detrimental effects
throughout the brain. These effects may include the release and upregulation of cytokines, further blood brain barrier breakdown and the need for more energy to compensate for the dysfunction of the ionic gradients\textsuperscript{8,11,13}.

The chronic stage of a TBI includes delayed cell death due to processes such as inflammation and can occur instantaneously through weeks following insult\textsuperscript{14}. This inflammatory response involves the release of cytokines like tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interleukin-4 and interleukin-1beta (IL-1\(\beta\)). These cytokines produce positive effects including the elimination of injured and adjacent tissue as well as negative effects like increases in apoptotic neurons in the cortex and hippocampus, activation and accumulation of immune cells such as lymphocytes and leukocytes and impaired neuronal function\textsuperscript{8,10,13}. Studies have shown that neuronal reduction and dystrophy due to the accumulation of immune cells may account for the long-term deficits which occur after a TBI\textsuperscript{14-16}. These effects highlight that the inflammatory process is a balance between the cytokines which play important regulatory functions during a TBI. If this balance becomes disrupted, detrimental effects may occur which could lead to vast cognitive and physical deficits\textsuperscript{14}. During this stage, the subfields of the hippocampus have also demonstrated upregulation of dystrophic neurons due to inflammation, which can lead to cell death, providing evidence of the vulnerability of the hippocampus to the inflammatory process\textsuperscript{17}. The dentate gyrus (DG), CA1 and CA3 regions of the hippocampus are particularly sensitive to cognitive function and learning, and damage to these components could lead to detrimental effects (Figure 3).
Biomolecules Involved in TBI

Other biomolecules shown to be involved in the brain’s response to TBI are neurotrophic factors. Neurotrophic factors are a unique family of growth factors which are locally produced in the nervous system and influence the survival, proliferation, differentiation and death of cells in the CNS\(^\text{18}\). They are also able to mediate higher-order activities such as learning, memory and behavior\(^\text{18}\). There are numerous neurotrophins which have been distinguished and examples of these include: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Neurotrophic factors have the ability to influence the CNS in diverse ways based on their availability, their functionality once phosphorylated, their affinity to the appropriate receptors and the signaling cascades they stimulate\(^\text{18}\). These factors have been shown to provide neuroprotective effects by influencing synaptic plasticity and neuronal function using cytokine pairing\(^\text{16}\). Due to the fragility of this balance and the possibility of enhanced neuroprotective effects, many studies have been performed to determine ways which the levels of these growth factors may be regulated following injury. One potential intervention that has been examined is environmental
enrichment (EE), which confers numerous positive effects following an insult, including changes in behavior and biochemical outcomes.

**Environmental Enrichment**

Environmental Enrichment was first established by Hebb et al. (1947) in the late 1940’s. It is a complex sensory-motor stimulation that provides animals with an increased opportunity for physical exercise, various learning experiences, and social interactions which may result in a variety of neuroplastic changes in the brain. In his experiments, Hebb found that early experiences and environments provide permanent effects on the problem-solving abilities of adult rats, and increasing task complexity led to better cognitive performance. Hebb attributed this phenomenon to neuronal plasticity, a term which refers to the brain transforming its elements or connectivity because of learning and cognitive function. This discovery led to a vast increase in experiments which have aided in the understanding of the connection between enrichment and plasticity. Rosenzweig et al. (1996) determined that EE led to measurable changes in brain chemistry and weight. In his work, enriched rodents had increased cortical volume, most commonly due to the development of dendritic branching. Enriched housing also caused increased rates of messenger RNA (mRNA) and protein synthesis, which are required for long-term memories in various areas the brain. Following a brain insult, Rosenzweig also observed that EE improved motor skills and increased memory performance in rodent subjects. In a study by Kempermann et al. (1997), EE was found to produce a significant amount of new neurons and a larger granule cell layer in the hippocampus compared to standard housed animals. All of these positive effects collectively provided support for the idea that EE
enhances functional recovery after brain injury by increasing neurogenesis in the hippocampus\textsuperscript{19-23}.

When applied following TBI, EE facilitates learning and memory and improves motor skills performance\textsuperscript{24,25}. Models of injury include fluid percussion injury (FPI) and controlled cortical impact (CCI). FPI induces an injury by rapidly injecting fluid volumes into the cranial cavity while CCI uses a pneumatic impactor to impact exposed brain\textsuperscript{26,27}. EE attenuates some of the detrimental effects that both FPI and CCI induce\textsuperscript{26,27}. Common trends of neuroprotection provided by enrichment after these injuries include; attenuation of hippocampal CA3 cell loss, reduction of overall lesion volume, induction of cell proliferation, reduction of inflammatory processes, production of growth factors and improved cognitive performance\textsuperscript{28-30}. Following an injury, rodents exposed to EE performed better on spatial memory tasks, including the Morris Water Maze (MWM), compared to their standard counterparts\textsuperscript{24,30}. Experiment models using rodents have also demonstrated attenuation of deficits following injury on the beam walking task (locomotor recovery assessment) and the sawdust maze (a test of learning) when EE was applied\textsuperscript{22}. EE is consequently able to influence not only the cellular processes when applied following injury, but also provide behavioral and physical task enhancements.

The influence of environment on numerous biochemical and physical aspects following an injury may be modulated by transcription factor activation as well as growth factor expression\textsuperscript{31}. Transcription factors control and modulate the transcriptional activity of genes and respond to changes in brain dynamics, such as a TBI. Growth factors generally act as signaling molecules between cells and once bound to their
specific receptors can initiate or inhibit cellular processes. During development, growth factors play a role in cell differentiation and neural outgrowth while in adulthood their function switches to tissue homeostasis and neurogenesis following injury. These hormones are part of the neurotrophin family and are needed for the regulation and differentiation of neurons.

**Neurotrophic Factors**

Neurotrophins aid in the regulation and control of neuron development, function, survival and plasticity. Some of the responsibilities these regulators maintain include; control of cellular Ca\(^{2+}\) homeostasis, inhibition of programmed cell death, upregulation of enzymes for metabolism and amelioration of negative morphological and functional effects caused by brain injury. Neurotrophins are synthesized and packaged into vesicles in the soma, and are then transported to presynaptic axon terminals and released to postsynaptic dendrites by regulated or constitutive secretion. Most neurotrophins are released using the regulatory pathway and require a stimulus to induce their secretion. Once these biomolecules are discharged, they autophosphorylate to induce functionality and activate signaling pathways throughout the CNS using either tropomyosin-related kinase (trk) receptors or a tumor necrosis family receptor, p75\(^{NTR}\).

The trk receptors, part of the tyrosine kinase family, are found throughout the CNS. Once stimulated, these receptors aid in the regulation of neuronal proliferation and survival, axonal and dendritic growth, synapse formation and function and repair of damaged neurons. Tyrosine Receptor Kinase (trk) A is specific for NGF while trkB binds to both BDNF and NT-4. Brain-derived neurotrophic factor can also bind with low affinity to the p75\(^{NTR}\) receptor, which may cause negative effects such as apoptosis and...
long-term depression, as well as positive functions like signal transduction and cell survival\textsuperscript{33}.

**Brain-Derived Neurotrophic Factor (BDNF)**

Each neurotrophin influences the brain following injury, including promotion of neuronal survival and plasticity of neuronal circuits, or neurotoxicity of the neurons from overexposure\textsuperscript{34}. One neurotrophin in particular, BDNF, is especially effective in promoting the survival of neurons following a TBI and is upregulated with the application of enrichment\textsuperscript{35,36}. These tendencies have led researchers to believe that BDNF may be an important neuroprotective component in the CNS.

BDNF is a 27kDa activity-dependent modulator of neurogenesis, neuronal structure and function, synaptic plasticity and hippocampal function\textsuperscript{37}. BDNF is synthesized, stored and released from the glutamatergic neurons and distributed throughout the nerve terminals and dense-core vesicles\textsuperscript{30,36,37}. BDNF binds specifically to both the trkB and p75\textsuperscript{NTR} receptors\textsuperscript{37,38}.

BDNF is released following a stimulus, such as a TBI\textsuperscript{36}. Once released, BDNF can transport both anterogradely and retrogradely throughout the neurons\textsuperscript{39}. This bi-directional movement of the neurotrophin allows for optimal exposure and binding with the trkB receptor\textsuperscript{39}. Binding of BDNF to its receptor activates the tyrosine kinase domain which can then stimulate various intracellular substrates\textsuperscript{39}. One of these substrates includes the lipid kinase phosphoinositide 3-kinase (PI3K), which upon activation localizes to the cell membrane\textsuperscript{39,40}. The PI3K pathway will cause phosphorylation of the survival serine/threonine protein kinase Akt by recruiting it to the membrane as well\textsuperscript{40,41}. Once activated, Akt serves to phosphorylate multiple proteins and is a regulator of cell proliferation, glucose metabolism and cell survival\textsuperscript{41}. This mechanism (Figure 4) is a key
component and pathway for the beneficial effects that BDNF has on neuronal survival. BDNF, as well as the other neurotrophic factors, also have the capability to stimulate the activation of the mitogen-activated protein kinase (MAPK) pathway in hippocampal neurons. While this pathway is able to phosphorylate many of its downstream targets and produce an upregulation of BDNF mRNA, Zheng et al. (2004) were able to demonstrate that it was not as involved in the protective effects of BDNF compared to the effects seen with the PI3K/Akt pathway.

**Figure 4: BDNF Mechanism using PI3K Pathway.** BDNF causes the phosphorylation of its trkB receptor which causes the phosphorylation of PI3K. The PI3K moves to the cell membrane and causes the phosphorylation of Akt. Activation of Akt leads to cell survival and increased neurogenesis.

Through the PI3K/Akt pathway, BDNF is able to induce multiple beneficial changes in the brain not only in the neurons, but also the synapses by being a mediator of synaptic transmission and plasticity. BDNF is involved both pre- and post-synaptically.
and is consequently able to enhance synaptic transmission in the hippocampus by strengthening excitatory synapses, causing long term potentiation (LTP)\textsuperscript{37,39}. LTP is believed to be the mechanism behind learning and memory\textsuperscript{37}. The induction and maintenance of LTP is highly sustained by BDNF as shown by Bramham et al. (2005) when LTP was unable to be induced in BDNF knock-out mice\textsuperscript{37}. This study provides evidence of the crucial involvement of BDNF in processes involving synaptic plasticity\textsuperscript{37}.

BDNF is needed for neurogenesis, synaptic plasticity and hippocampal function\textsuperscript{38}. Following a TBI, changes to the CNS may prohibit BDNF from completing these crucial tasks\textsuperscript{26,43}. Pro-inflammatory cytokines can inhibit the secretion of BDNF and certain physiological and psychological stressors have also reported decreasing the amount of BDNF protein found in the hippocampus\textsuperscript{43}. Reduction in BDNF under these conditions can result in a loss of synaptic activity and neuronal proliferation.

Following a TBI, the levels of BDNF mRNA and protein in the neurons vary based on the effects of the injury on the hippocampus\textsuperscript{26}. Studies have shown that following a TBI, BDNF mRNA levels are increased contralateral to the injury for a short period (12-36 hours after injury) of time before returning to control levels whereas these levels are decreased significantly ipsilateral to the injury during this same time frame\textsuperscript{26,27,43,44}. These changes are seen most commonly in the CA3 and DG of the hippocampus\textsuperscript{27}. The DG granule cells show a more pronounced increase in BDNF levels and become selectively more resilient to cell death following injury\textsuperscript{44}. Increased BDNF in the CA3 region of the hippocampal pyramidal cells also correlated with an enhanced cell survival rate\textsuperscript{44}. These findings provide further evidence of the neuroprotective role.
that BDNF plays following TBI in compensating for the loss of neurons and synapses in the hippocampus.

Along with aiding in the recovery of hippocampal components, BDNF also plays a role in other functional areas. When elevated, BDNF has been shown to enhance performance on the MWM task, illustrating the ability of this neurotrophic factor to positively influence cognitive performance\textsuperscript{51}. This research group also determined that changes induced by BDNF in synaptic plasticity, including enhanced connectivity throughout the brain, were associated with functional recovery following brain injury\textsuperscript{51}.

BDNF plays an important role in the recovery of function following a TBI, and when EE is applied the benefits of neuroprotection are increased\textsuperscript{45,46}. Enrichment alone enhances BDNF mRNA and protein levels in the rat hippocampus, cerebral cortex and hindbrain hours to days following application\textsuperscript{45,47}. By increasing these levels of BDNF, the amount of hippocampal neurons can elevate due to their improved survival tendencies and increased ability to differentiate\textsuperscript{45,46}. Both EE and BDNF play crucial roles in neuronal and synaptic survival and differentiation, and plasticity throughout the CNS. By combining these components in an appropriate paradigm, the brain may be able to enhance its synaptic and neuronal connections, providing added neuroprotection against a TBI.

Knowing the positive effects of both enrichment and BDNF following an injury has fueled research of how these components work together to provide cognitive enhancements if applied before injury. The current research focuses on BDNF as a neuroprotective mechanism when paired with enrichment. It is hypothesized that BDNF mRNA and protein levels will be elevated in animals which are enriched prior to TBI.
compared to non-enriched animals. Identifying the EE-induced mechanism behind neuroprotection will provide cellular targets of intervention to reduce the negative effects of TBI.
Materials & Methods

Subjects
Animals used in this study were male Sprague Dawley rats approximately 90 days of age from Charles River Laboratories. Forty-two animals were used for behavioral testing while 21 were utilized for biochemical analysis. Upon arrival, animals were separated into various housing conditions in a room with stable temperature (25°C) and a 12 hour light/dark cycle. The animals were provided ad libitum access to food and water and handled for 5 min/day or more depending on their housing condition. Animals were weighed on a daily basis and were given 7 days to acclimate prior to testing. These animals were part of a larger study which was focused on the effects of enrichment prior to TBI, and in that experimental design, behavioral testing was a focal point (Figure 5).

![Figure 5: Study Layout Timeline. Animals were housed appropriately 15 days prior to injury or surgery and were then exposed to various behavioral tests before begin sacrificed at 33-days post-injury.](image-url)
**Housing Conditions**

*Enriched*

Environmental enrichment rats were housed 8 per cage in a large wire mesh cage (~1m³) with a variety of items such as a running wheel, plastic tubes, ladders, ropes and mirrors (Figure 6) for 15 days before TBI. Each day the toys were rearranged at feeding and twice weekly the items were replaced by new toys in order to provide the animals with novel stimuli. Enriched animals also received acrobatic training (Figure 7) and were exposed to olfactory stimulation twice a day for 10 days (two 5-day blocks) following the acclimation period.

![Image](image.jpg)

**Figure 6:** Environmental Enrichment Cage. Animals are socially housed and given novels toys to interact with. Other stimulators include ropes, ladders, running wheel, olfactory stimulation and motor skills training

The EE cages were kept out of sight from the standard and control animal cages using wall dividers. Enriched animals were subjected to a medial pre-frontal TBI following the 15-days of EE housing.
Figure 7: Environmental Enrichment Acrobatic Training. Animals are exposed to an obstacle course consisting of beams, ropes, and ladders which are elevated above the ground

Standard and Control

Standard (ST-TBI) and control (Sham) animals were housed 2 per standard polycarbonate shoebox cage for 15 days before TBI. These animals did not receive contact with other animals or additional training and were handled with minimal contact (5min/day) during routine cage changing. Standard animals were subjected to a medial pre-frontal TBI following the 15-days of standard housing. The control group consisted of sham animals which underwent anesthesia and a craniotomy but did not receive an injury.

Controlled Cortical Impact

Enriched and ST-TBI animals were subjected to TBI via a controlled cortical impact (CCI) device following the 15 days of appropriate housing (Figure 5). Animals were anesthetized with isoflurane (5% induction, 2% maintenance) and secured with their heads fixed in a horizontal position. Blood saturation of peripheral oxygen (SpO₂) and body temperature were closely monitored and maintained throughout the surgery. A 6mm craniotomy was performed 5mm anterior to bregma and the bone segment removed was discarded. An electrical contusion impactor (Custom Design and Fabrication, Virginia Commonwealth University Medical Center, Richmond, Virginia) with a velocity of 2.25m/sec and a depth of 3mm was used to inflict the TBI at the prefrontal cortex.
(total of 50msec of contact with the brain). Following the procedure, the scalp was sutured with 7mm surgical staples. Sham controls were anesthetized and received a craniotomy but not a CCI. After surgery and recovery, animals were returned to their original cages and cage mates. The EE group no longer received additional enrichment and the extra stimuli that had been dispersed in their cages were removed. By discontinuing EE at this stage, data would reflect the effects of enrichment prior to injury only.

**Behavioral Testing**

All animals were subjected to behavioral testing starting 11 days post-injury (Figure 5). The Morris Water Maze (MWM) was implemented to test spatial memory and learning. Although other behavioral tests were performed, this paper will focus on the MWM because of its specificity in learning and memory which are correlated with hippocampal function as well as BDNF levels.

*Morris Water Maze*

The MWM was used 11 days after surgery to test spatial learning and memory. Animals were placed in a 70 inch diameter dark circular tank filled with water. The tank was divided evenly into four quadrants (A, B, C, D) with appropriate marking of the quadrants on the top edge of the tank (Figure 8). The platform consisted of 4 inch diameter clear plexiglass submerged 0.8 inches beneath the water level and placed approximately 11 inches from the wall of the pool in quadrant C. Throughout the experiment, the position of the platform was not changed.
Latency to find the hidden platform was recorded using Ethovision software (Noldus; Leesburg, VA). The test was performed in two 5-day blocks over 10 days. Each animal performed 2 trials per day, except for the 10th day during which the platform was removed from the tank and animals were only tested once for the amount of time spent in quadrant C. For each trial, the animal was placed in the pool in random quadrants, facing the wall. If the animal was unable to locate the platform in 90 seconds, they were physically guided to it and upon reaching the platform were allowed to remain there for 10 seconds. They were then removed for a 30 second interval before the start of the second trial.

**Sacrifice**

Animals were sacrificed 33 days post-injury (Figure 5). They were briefly anesthetized with 5% isoflurane until a toe pinching test received no reaction and decapitated via guillotine. The brains were removed and split in half sagittally. Alternating left and right sides were kept intact and flash frozen in isopentane. For in-situ hybridization analysis, coronal sections of the brain hemispheres (14 µm) through the prefrontal cortex and hippocampus were cut on a cryostat and stored at -20°C.
contralateral side was dissected to extract the hippocampus for Enzyme-Linked Immunosorbent Assay (ELISA) techniques and stored at -80°C.

**Biochemical Analysis**

Protein quantification techniques were used to determine the amounts of BDNF and trkB mRNA and protein throughout various areas of the brain. In-situ hybridization techniques were carried out at The University of Cincinnati under the supervision of Dr. Kim Seroogy while a Promega® BDNF Immunoassay Kit was used to determine BDNF protein concentrations in the hippocampus.

**In-Situ Hybridization**

Localization of BDNF and trkB mRNA was performed using *in-situ* hybridization as described by Hicks et al (1998)\(^{38}\). Briefly, the unperfused slide-mounted sections were pretreated by removing the slides from the -20°C freezer and quickly drying them under a stream of cold air. The slides were immersed in 4% paraformaldehyde for 10 minutes, washed twice for 5 min in 0.1M phosphate buffer saline (PBS), washed twice for 5 min in 0.2% PBS/Glycine, washed again in 0.1M PBS, and then placed in 0.25% acetic anhydride/0.1M triethanolamine for 10 minutes. The slides were then dehydrated using various concentrations of ethanol, delipidated in chloroform and air dried. The BDNF and trkB cRNA probes (Kindly provided by Christine Gall, University of California-Irvine and Kathryn Albers, University of Pittsburgh) were prepared using in vitro transcription from a linearized cDNA construct with T3 polymerase in the presence of \(^{35}\)S-UTP (NEG039H, Perkin Elmer). Hybridization was performed at 60°C for 18-24 hours using hybridization buffer [1M Tris-HCL + 0.5M EDTA + 50x Denhardt’s Solution + 5M NaCl + 10mg/mL denatured salmon sperm DNA + 15mg/mL yeast tRNA + 5M DTT + 1.0x10^6 cpm/50uL/slide \(^{35}\)S-labeled cRNA probe]. Following hybridization
treatment, slides were washed with 4x saline-sodium citrate (SSC) + 10mM sodium thiosulfate at 37°C twice for 30 minutes each, then incubated in ribonuclease A for 30 minutes at 45°C. Slides were washed in 2X SSC and 0.5X SSC two times each at 37°C for 20 minutes, and 0.1X SSC at 37°C for 30 minutes. Sections were dipped in dH₂O followed by 95% EtOH, air-dried and loaded into cassettes with β-Max Hyperfilm (Amersham). The films were exposed at room temperature for 10 days for BDNF and 7 days for trkB in order to generate film autoradiograms. Following film development, films were dipped in GPX developer and fixer (Kodak), air dried and then exposed in light-tight boxes at 4°C for 2-4 weeks. Upon completion of the autoradiographic development, slides were analyzed using a Nikon Optiphot-2 microscope and Scion software (NIH). Analysis compared the density of hybridization for BDNF mRNA in the Piriform Cortex, Prefrontal Cingulate, DG and CA3 subfields. Background hybridization from the proximal tissue was subtracted from the hybridization in the hippocampal subfields to obtain corrected optical density (OD) measurements. Gray scale values were calculated by taking area of the region of interest into account as well.

Hippocampal Preparations
For immunoblot analysis of proteins, hippocampi (frozen at -80°C) were thawed on wet ice. During the thawing, 400µL of cold 0.3M sucrose buffer [102g sucrose + 4.3g Sodium Chloride + 1.21 g Tris Base + 40mL 500mM EDTA + 40mL 500mM EGTA] was placed in a 1.5mL microcentrifuge tube for each sample. 100µL of 0.2% Triton x-100 (EW-88230-41; Cole-Parmer) and 4µL Protease Inhibitor (PI) Cocktail (P178415; VWR) were added to the sucrose buffer and mixed thoroughly. The tissue sample was added to the appropriate microcentrifuge tube and minced using scissors for approximately 100 strokes. The tube was placed back on wet ice for 10 minutes to allow
the PI to penetrate the sample fully. Following the incubation period, samples were homogenized using the Misonix S-4000® sonicator (Fisher Scientific, Pittsburgh PA). Timed intervals of 30 seconds were used per tube with replacement of the sample back onto ice during settling. Samples were sonicated until homogenous. Following sonication, the tubes were placed in the Legend 17R Sorvall® (Thermo Scientific, Asheville, NC) tabletop centrifuge and spun for 8 minutes at 4°C and 6000xg. The supernatant of each sample was transferred to a fresh microcentrifuge tube (200uL per tube) and the pellet was discarded.

**BCA Protein Assay**

A protein assay was performed using the instructions from the Thermo Scientific Pierce® BCA Protein Assay Kit (23227; Fisher Scientific, Pittsburgh PA). Briefly, wash reagent was created [5.9mL Reagent A + .118mL Reagent B]. The albumin standard (2mg/mL) was diluted according to the test tube procedure and placed in duplicate into a 96-well plate. Supernatants were diluted 1:10 [6μL sample + 54μL milli-q H₂O] and added to the plate in duplicates. 200μL of wash reagent was introduced into each well and incubated at 37°C for 20 minutes. The plate was cooled to room temperature and read on a spectrophotometer (Spectramax190, Molecular Devices; Silicon Valley, CA) at a wavelength of 562nm. The results were recorded in SoftMax Pro 5.2 software for data analysis.

**BDNF E_{max}® ImmunoAssay**

The BDNF ELISA was completed using the BDNF E_{max}® ImmunoAssay System (G7611; Promega, Madison WI). Samples were normalized using Block & Sample 1X Buffer [42.4mL of milli-q water + 10.6mL of Block & Sample 5X Buffer] to a concentration of 6.75 mg/mL total protein and stored on ice. Plate coating was carried
out the afternoon before running the ELISA. 10uL of anti-BDNF mAb was added to 9.99mL of carbonate coating buffer [0.025M sodium bicarbonate (AC21712-0010; Acros Organics, New Jersey) + 0.025M sodium carbonate (AC20680-0010; Acros Organics); adjust pH to 9.7] and mixed thoroughly by inversion. 100µL of this mixture was dispensed into each well of a polystyrene ELISA plate (6005600; Perkin Elmer, Waltham, Massachusetts) using a multichannel pipettor. The plate was incubated overnight at 4°C and the following day the wells were vigorously washed three times with tris-buffered saline and tween-20 (TBST) [20mM Tris-HCl (IC816124; VWR) + 150mM NaCl (VWR) + 0.05% Tween 20 (H5151; Promega)] using an automated plate washer (ELx405 Biotek, Winooski, Vermont). 200µL of Block & Sample 1X Buffer was added to each well and the plate was incubated for one hour. To prepare the standard, the supplied BDNF (1 µg/mL) was diluted 1:40 [10µL of undiluted standard + 390µL of Block & Sample 1X Buffer], then diluted to 1:2000 [10µL of the first dilution + 490µL of Block & Sample 1X Buffer] in a fresh tube and stored at 4°C. The normalized supernatants were acid treated by adding approximately 4µL of 1N HCl to lower the pH below 3.0, in order to dephosphorylate and to measure total BDNF protein. The samples were incubated for 15 minutes at room temperature, and using 1N NaOH were neutralized to a pH of approximately 7.6 and placed back on ice. Following plate blocking, the wells were washed one time vigorously with TBST. A serial dilution of the standard was performed from a concentration of 125pg/mL to 1.95pg/mL, including a blank, and samples were added to the plate in duplicate using 100µL of sample per well. The plate was sealed using a coverslip and incubated for 2 hours with shaking at room temperature. After incubation, the plate was vigorously washed with TBST. BDNF
primary antibody (pAb; 1:50) was prepared and mixed [9.98mL of Block & Sample 1X Buffer + 20µL Anti-Human BDNF pAb] and 100µL of the solution was added to each well. The plate was sealed as before and incubated for an additional 2 hours with shaking. After incubation, the plate was vigorously washed with TBST. 100µL of Horseradish Peroxidase (HRP) Conjugate [9.95mL of Block & Sample 1X Buffer + 50µL of Anti-IgY HRP conjugate] was added to each well, followed by another 1-hour long incubation with shaking and a repeated washing. 100µL of room temperature 3,3,5,5-tetramethylbenzidine (TMB) One solution (Promega) was added to each well and the plate was covered and shaken for 10 minutes. The wells were a blue tint following this incubation. 100µL of 1N HCL was placed into each well, which turned the solutions to a yellow tint, and the plate was immediately read on the spectrophotometer at a wavelength of 450nM. Data was collected for analysis using the SoftMax Pro 5.2 software.

**Statistical Analysis**

All measurements were analyzed with Statistical Analysis Software (SAS) and expressed as mean ± SEM. If group (Sham, ST-TBI, and EE-TBI) was the only factor, a one-way analysis of variance (ANOVA) was performed. If day was included as a factor, as done for the MWM data, a mixed design ANOVA was performed. Post-hoc paired comparisons among the groups used the Tukey procedure with a 0.05 experimentwise error level. For completeness, two-tailed two-sample t-tests were also performed. If the variance between the groups being tested was significantly different, approximate t-tests were performed.
Results

One animal from the sham control group was removed from all statistical analysis. It was determined during sacrifice that the animal suffered a large hematoma which resulted in various behavioral, cognitive and biochemical abnormalities. The sham group contained n=13 for MWM, n=6 for the ELISA and n=7 for in-situ statistical analysis after removing that animal.

MWM

Analysis of the Morris Water Maze data revealed significant differences during the second week of testing among groups and days (Figure 9). Latency to the platform (seconds) was used as the dependent variable in a mixed-design ANOVA with both group and day serving as the factors.

![Figure 9: Morris Water Maze Trends (Mean latency to find platform ± SEM). During the last 4 days of testing (Days 18-21 Post-Injury), the EE-TBI group was able to perform as well as the sham controls while the standard housed animals did not, suggesting that EE is able to build resistance prior to injury.](image)

F-tests showed a significant difference among the ST-TBI, EE-TBI and Sham groups ($F_{2,41} = 4.56, p = 0.0162$) as well as among the days ($F_{3,121} = 4.20, p = 0.0073$) but there was not a significant group/day interaction ($F_{6,121} = 0.41, p = 0.8697$). Post hoc analysis
by way of the Tukey method showed that ST-TBI was significantly different than both Sham and EE-TBI. Table 1 exhibits pair-wise differences between groups at each day.

<table>
<thead>
<tr>
<th>Day</th>
<th>Latency to Platform (sec)</th>
<th>Two-Tailed Two-Sampled t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level 1</td>
<td>Level 2</td>
</tr>
<tr>
<td></td>
<td>Group</td>
<td>Mean</td>
</tr>
<tr>
<td>8</td>
<td>Sham</td>
<td>20.2</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>20.2</td>
</tr>
<tr>
<td></td>
<td>ST-TBI</td>
<td>40.9</td>
</tr>
<tr>
<td>9</td>
<td>Sham</td>
<td>15.9</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>15.9</td>
</tr>
<tr>
<td></td>
<td>ST-TBI</td>
<td>35.7</td>
</tr>
<tr>
<td>10</td>
<td>Sham</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>ST-TBI</td>
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</tr>
<tr>
<td>11</td>
<td>Sham</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>ST-TBI</td>
<td>28.7</td>
</tr>
</tbody>
</table>

Table 1: Results of Two-Tailed Two-Sampled t-test for the MWM

Figure 10 displays the trends of the second week of testing, excluding the last day which was used as a probe. There were no significant differences between the standard housed sham and the enriched conditions, but the ST-TBI group took significantly longer to reach the platform.

Figure 10: Morris Water Maze Results (Mean latency to hidden platform ± SEM)
In-Situ Hybridization

BDNF and trkB mRNA levels were used as the dependent variable in a one-way ANOVA. The factor was group and levels used were Sham, ST-TBI and EE-TBI. Results of the f-tests are shown in Table 3.

<table>
<thead>
<tr>
<th>Location</th>
<th>F-test of Group</th>
<th>DF</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prefrontal Cingulate</td>
<td>BDNF</td>
<td>2,18</td>
<td>0.01</td>
<td>0.9966</td>
</tr>
<tr>
<td>Piriform Cortex</td>
<td>BDNF</td>
<td>2,18</td>
<td>0.18</td>
<td>0.8377</td>
</tr>
<tr>
<td>DG of Hippocampus</td>
<td>BDNF</td>
<td>2,18</td>
<td>0.99</td>
<td>0.3921</td>
</tr>
<tr>
<td>CA3 of Hippocampus</td>
<td>BDNF</td>
<td>2,17</td>
<td>0.68</td>
<td>0.5215</td>
</tr>
<tr>
<td>Prefrontal Cingulate</td>
<td>trkB</td>
<td>2,13</td>
<td>0.13</td>
<td>0.8810</td>
</tr>
<tr>
<td>Piriform Cortex</td>
<td>trkB</td>
<td>2,13</td>
<td>0.06</td>
<td>0.9460</td>
</tr>
<tr>
<td>DG of Hippocampus</td>
<td>trkB</td>
<td>2,13</td>
<td>7.54</td>
<td>0.0067</td>
</tr>
<tr>
<td>CA3 of Hippocampus</td>
<td>trkB</td>
<td>2,13</td>
<td>1.42</td>
<td>0.2769</td>
</tr>
</tbody>
</table>

Table 2: Results of in-situ hybridization f-test for Sham, ST-TBI and EE-TBI Groups. No significant differences were found in the various brain regions except for trkB in the DG (p=0.0067)

Analysis of the film autoradiograms revealed that there were no statistically significant differences in BDNF mRNA in any of the measured brain regions among the groups; however, in the Dentate Gyrus (DG) there was a significant difference found (F_{2,13}=7.54, p=0.0067) for trkB mRNA. The Tukey procedure revealed Sham was significantly different than ST-TBI. Using the corrected grey scale values from the densitometric measurements, it was determined that neither housing conditions nor injury alter the levels of BDNF (Figures 11a/11b and 12a/12b/12c) mRNA when observed 33 days post-injury.
Figure 11: BDNF mRNA levels: (a) BDNF mRNA in Prefrontal Cingulate, (b) BDNF mRNA in Piriform Cortex

Figure 12: BDNF mRNA Analysis: (a) BDNF mRNA Levels in Hippocampus Dentate Gyrus, (b) BDNF mRNA in CA3, and (c) autoradiogram image of in situ showing the hippocampus

Throughout the various modules of the brain which were observed, the aforementioned tests revealed that trkB mRNA levels (Figures 13a/13b and 14a/14b/14c) did not exhibit a significant difference between groups except in the DG of the hippocampus.
Here, it was observed that there was a noteworthy distinction between the Sham and ST-TBI group; however, the Tukey procedure did not show significant differences between Sham and EE-TBI for this same region. Using t-tests as shown in Table 4 (Appendix),
ST-TBI versus EE-TBI for trkB in the DG reveals a low p-value (p=0.0357). Thus, more analysis is required to verify this distinction and significance.

**ELISA**

The differences in the quantity of BDNF in the hippocampus among the varying conditions were minimal. BDNF protein concentration (pg/mL) was used as the dependent variable in a one-way ANOVA with the group being the factor and did not show a significant difference among the EE-TBI, ST-TBI, and Sham groups (F_{2,17}=0.36, p=0.6995). Table 2 contains paired testing among the groups.

<table>
<thead>
<tr>
<th>Corrected BDNF Protein Concentration (pg/mL)</th>
<th>Two-Tailed Two-Sample t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1 Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Sham</td>
<td>10.9</td>
</tr>
<tr>
<td>Sham</td>
<td>10.9</td>
</tr>
<tr>
<td>ST-TBI</td>
<td>12.9</td>
</tr>
</tbody>
</table>

**Table 3: Results of Two-Tailed Two-Sample t-test for BDNF ELISA**

Figure 15 illustrates the trends between the conditions and verifies that changes in BDNF protein concentrations among the groups were negligible.

**Figure 15: BDNF Protein Concentration Results (Mean protein concentrations ± SEM)**
Discussion

Environmental enrichment and BDNF can provide cognitive enhancements post-injury, but little is known about the mechanism of these neuroprotective effects when applied prior to injury. The current research sought to establish a foundational understanding of pre-injury EE effects on post-TBI cognitive performance. In this paper, it was hypothesized that EE, when applied prior to injury, would provide protection against TBI and that this protection would be accompanied by elevated BDNF mRNA and protein levels. Results determined that EE attenuated cognitive deficits when applied prior to injury and that the beneficial effect of EE on cognitive performance was present even without detectable changes in BDNF mRNA or protein. The following discussion compares results from the current study to the existing body of knowledge on neuroprotective mechanisms, particularly those involving EE and protective biomolecules like BDNF. Identifying techniques which can confer resilience to the brain before injury and support neuronal survival could provide the framework for a new area of study in neuroprotection.

Environmental Enrichment Improves Cognitive Performance when Applied Before TBI

EE applied prior to injury improved spatial memory in rodents compared to standard housed animals. From our research, results for MWM testing of spatial memory revealed that during the second week of testing (days 18-21 post-surgery), the effect of TBI on decreasing cognitive performance was completely prevented in animals which had been exposed to EE (Figure 9). These results are consistent with studies observing the effects of TBI on performance as well as research exploring the effects of enrichment when applied after injury. In particular, Sozda et al. (2010) determined that advances in performance on the MWM following TBI and EE introduction may have
been caused by attenuation of CA3 hippocampal cell loss as well as a decrease in overall lesion volume\textsuperscript{28}. Hamm et al. (1996) and de Witt et al. (2010) found that EE applied after injury significantly improved spatial memory performance on the MWM compared to injured animals in standard housing which did not receive EE. Passineau et al. (2001) also observed morphological changes near the site of injury and determined that enrichment played a role in cell proliferation, growth factor expression and cellular metabolism; however, the exact mechanism behind these neuroprotective effects was not established\textsuperscript{29}. These studies reiterate the original findings by Rosenzweig et al. (1996) which determined that EE has the ability to induce neurochemical, neuroanatomical and behavioral alterations\textsuperscript{20,30}. Combined, all the findings show that EE before and after injury may improve cognitive performance as indicated by the results in the MWM. Our study, however, was the first to establish data which provided evidence of these neuroprotective effects prior to TBI.

Because decreases in cognitive performance were prevented in the current research, additional insight may be gained on which parts of the brain are affected by EE, such as the hippocampus. The hippocampus is important in research involving TBI as this structure is especially vulnerable to TBI and is associated to cognitive and behavioral dysfunctions, which may include impairments in learning and memory\textsuperscript{49,50}. Yu et al. (2009) discovered that rodents displayed significant memory recall impairments in the MWM following a CCI injury. The deficits were more prominent when the injury was severe enough to cause damage to the hippocampus, re-establishing the importance of this structure in learning and memory\textsuperscript{4,27,49,50}. In our research, EE-TBI animals had no
memory deficits compared to ST-TBI, suggesting EE builds resilience when applied prior to injury to areas beyond the site of injury, such as the hippocampus.

**TBI Stages Induce Various BDNF Responses**

In addition to the effect EE has on cognitive performance, enrichment has elevated BDNF levels\(^4\). Combined, EE and elevated BDNF levels could provide compounded neuroprotective effects towards the mechanisms following injury. Understanding the timing of the stages following a traumatic insult is vital in considering techniques to improve function and recovery. There are three distinct stages of TBI response and recovery that induce a variety of biochemical changes in the CNS (Figure 1)\(^8,9,52,53\). In the current study, observation of BDNF levels were limited to the chronic stage, in order to obtain behavioral data, but were not found to be significantly different between groups. A more in-depth look at previous findings of BDNF levels during these stages may help to explain the current results.

**Acute Stage**

During the acute stage of injury, BDNF levels are shown to be prominently elevated following a short delay. Yang et al. (1996) observed BDNF mRNA and protein level elevations occurred one hour following TBI in the hippocampus, but no changes were observed in BDNF levels prior to this time point\(^5\). The largest increases in these levels occurred in the granule cells of the DG followed by the CA3 region of the hippocampus. In the DG, increased mRNA levels for the BDNF receptor, trkB, were also observed\(^2\). This suggests that a delay in BDNF elevation may be caused by the biochemical processes following a TBI. These processes include elevations in Ca\(^{2+}\) levels as well as neuronal excitation which could activate signaling pathways, such as cAMP response element-binding (CREB)\(^5\). Additional research completed by Hicks et al.


and Oyesiku and colleagues (1999) reported animals exposed to a TBI experienced large increases in BDNF mRNA and protein levels which peaked at 3-12 hours post-injury. These elevations in BDNF levels may be eliciting neuroprotective effects throughout the brain in an attempt to counteract the effects of the mechanism of TBI.

The effects seen during the acute phase of injury cannot be counteracted by EE unless the enrichment is applied prior to injury. Currently, no published studies have shown the effects of EE during the acute stage of TBI when it is applied post-injury. This is because there is a latency for the neuroprotective effects of EE, so when applied post-injury the ‘window of opportunity’ to disrupt the cascade of detrimental effects caused by a TBI during the acute phase is missed. The current study shows that when EE is applied prior to TBI, changes in cognition are relevant weeks after the injury using the MWM. Although these changes were observed during the chronic stage of TBI, disruption of the cascade of events during the acute phase by the neuroprotection enforced by EE, and possible increases in BDNF, may have played a role in these enhancements. Increases in BDNF during this stage, may have improved performance on certain tasks as displayed by BDNF knock-out (KO) mice. Bramham et al. (2005) found these rodents to have severe deficits in MWM tasks attributed to the lack of BDNF, reiterating the role BDNF plays in learning and memory. Although these KO mice studies did not investigate behavioral changes related to TBI, one can surmise that elevated levels of BDNF protects against the detrimental effects of TBI by restoring LTP capabilities and improving executive function. To confirm these ideas, future studies will focus on the effects of this EE/BDNF model during the acute phase of injury.
Sub-Acute Stage

During the sub-acute stage of TBI, hours to days following injury, BDNF mRNA and protein levels begin to decline. Research performed by Oyesiku and colleagues (1999) determined BDNF was prominently elevated during the acute phase of TBI and significant BDNF levels were still observed at 24 hours post-injury, but by 36 hours had returned to near control levels\(^{43}\). Hicks et al (1997) found similar results with decreasing BDNF and trkB levels starting at 24 hours post-injury which returned to control levels at 72 hours\(^{48}\). A study completed by Griesbach et al. (2002) observed the trends following a TBI and established that BDNF mRNA was still elevated at 24 hours post-injury compared to control subjects. These levels were not as significant as those observed during the acute phase\(^{54}\). The findings above provide evidence that BDNF levels begin to decline during the sub-acute stage which may have accounted for the results of the current research, occurring in the chronic stage.

Environmental enrichment has been shown to enhance BDNF protein levels and neurogenesis in the adult rat brain, especially during the sub-acute stage of TBI\(^{25,45,46}\). It is determined that when EE is applied directly following injury, neurogenesis is increased which may occur by disrupting some of the effects in these stages such as influencing the inflammation process by decreasing the amount of pro-inflammatory cytokines\(^{45,46}\). By using enrichment to enhance the amount of BDNF during the sub-acute phase, exposure of this neuroprotective component to the hippocampus and area of injury may produce cellular enhancements such as dendritic branching, cell proliferation, reduced inflammation and enhanced resilience to injury\(^{27,54,57-60}\). Recall in this stage that cytokine production normally increases which can lead to inflammation. Both BDNF and EE have
been shown to decrease inflammation which may interrupt the mechanism of TBI and account for results in this study by inducing cellular processes which could affect the executive function of the subject.

**Chronic Stage**

The current research, performed during the chronic stage, revealed no changes in BDNF mRNA and protein levels and only one significant difference was found in the trkB levels. These results are supported by previous research observing the trends of BDNF during this stage. Gobbo et al. (2005) found no significant differences in BDNF levels between enriched and standard housed injured animals at 3 weeks post-injury; however, BDNF levels were elevated in comparison to the standard housed animals\(^6\). In a similar study by Bindu et al. (2007) the effects on BDNF levels 3 weeks post-injury showed that animals enriched after TBI experienced enhanced dendritic morphology and spine density, but the levels of BDNF remained insignificant between housing conditions\(^6\). Both of these studies demonstrate the positive effect EE may have on neuroprotection by elevating BDNF (although not significantly) and enhancing neuronal morphology especially when applied after injury.

The chronic stage was the only stage observed in the current study, and the existing results of our research parallel some of the findings above. In the current study, EE exposure prior to a TBI did not significantly elevate BDNF mRNA and protein levels in the cortex or hippocampus when observed 33-days following the injury using the ELISA and *in-situ* hybridization quantification techniques. One finding was that trkB levels were reduced significantly in the DG for the ST-TBI group compared to the Shams. Recall during the acute stage of injury, Hicks et al (1999) observed elevated levels of trkB in the DG for injured animals. These levels began to decrease during the
sub-acute stage due to cell dystrophy and inflammatory processes, which may have resulted in the decreases seen in the current research\textsuperscript{48}. These decreases in trkB receptor may have accounted for the deficits seen in the ST-TBI group on the MWM task, a measure of learning and memory. A reduction in receptors will limit BDNF binding sites and thus, result in a lack of neuroprotective benefits. It should be noted that the EE-TBI levels were not as diminished as the ST-TBI group, suggesting EE provided some neuroprotection against cell death. There was not a significant difference found between the EE-TBI and ST-TBI groups however, and the BDNF levels did not follow the same trend as the trkB receptor (Figures 11a and 13a). This contradiction may be further validated by a larger group of samples. Future investigations using a different stage of TBI are required to observe the trends of BDNF, as well as its receptor, in neuroprotection.

\textit{TBI Stages Induce Various BDNF Responses}

All of these findings provide supporting evidence that BDNF plays a role following a TBI, at least during a particular ‘window of opportunity’. From the referenced studies described above, it can be verified that BDNF has a delayed reaction to the impact of injury and that following a certain time-point (~24-72 hours) the levels will return to control amounts. These trends may explain why BDNF levels in our study, observed only in the chronic stage, were not significantly different between groups. Based on the above data, it is possible our research was past the time point of measuring a change in the mRNA and protein intensities. Future research should include shorter time points (possibly 1, 4, 8, 12, 24, 36, or 72 hours) of sacrifice following TBI to obtain the mechanism of BDNF in this series of events.
The aforementioned studies, as well as the results of our research, illustrate that EE helps mitigate the detrimental effects of TBI and that BDNF plays a role in the recovery of cognitive function. The exact mechanism of BDNF in repair and recovery is unknown and requires further investigation. It is accepted that following a TBI, BDNF increases until a particular time point to improve cognitive learning and neurogenesis\textsuperscript{26,27,43,44}. Enrichment alone can improve cognitive function when applied both pre- and post-injury as well as stimulate BDNF production to provide numerous neuroprotective advantages\textsuperscript{24,25,57,59}. Without fully understanding the role of BDNF and other neuroprotective biomolecules when paired with EE, it is difficult to understand the exact mechanism of recovery.

**Hypothesized BDNF Mechanism of Recovery**

Although changes in the levels of BDNF mRNA and protein were not distinguishable in this study, BDNF may still have been a factor in neuroprotection. Future studies using this enrichment/injury model to observe the BDNF ‘window of opportunity’ will provide a more definitive conclusion regarding the recovery mechanism. Until then, a possible mechanism behind this model is hypothesized based on the literature and the current results.

From the current findings and previous research, it is hypothesized that the proposed mechanism (Figure 16) is an appropriate model for the trends of BDNF if the proper stages are observed. It should be noted that prior to the induction of injury, BDNF levels may have been elevated due to animal exposure to EE. Recall that EE has been shown to enhance BDNF protein levels and neurogenesis in the adult rat brain\textsuperscript{25,45,46}. From these findings it is also hypothesized that increased BDNF levels, induced by EE
prior to the injury, may have caused plasticity in the brain which could result in enhanced resilience to injury. The current study did not allow observation of the levels of BDNF prior to injury; however, a study completed by Klein et al. (2011) has shown that there is a strong and significant correlation between BDNF levels found in blood plasma and BDNF in brain tissue for rats. Therefore, future studies could observe BDNF trends during various key experimental points, including prior to injury, to determine if BDNF plays a role in neuroprotection both prior to and following a TBI.

Figure 16: Possible CREB Influence in TBI and BDNF Mechanism. A TBI results in HPA axis and ATP production dysregulation which activated excess excitatory neurotransmitters as well as NMDA receptors. The excess of neurotransmitters leads to the start of BDNF production, activating the MAPK or PI3K pathways. The PI3K pathway causes the phosphorylation of Akt which causes neurogenesis in the brain. Excitation of the MAPK pathway causes the increased production of CREB, which can also be stimulated by the excess calcium produced by the activation of the NMDA receptors. Once CREB is produced, it can fabricate elevated amount of BDNF as well as stimulate the BCL2 family to enhance plasticity and neurogenesis in the CNS.

Once the TBI is induced, a cascade of physical and biochemical changes occur in the CNS including activation and dysregulation of the HPA axis and ATP production...
dysfunction\textsuperscript{5,8}. These catastrophic effects may result in a loss of homeostasis throughout the brain which then produces a compensatory response\textsuperscript{8}. There is an excess release of excitatory neurotransmitters which then activates the glutamatergic neurons to produce BDNF. BDNF can phosphorylate and bind to its trkB receptor in order to activate various signaling pathways, including the PI3K pathways discussed earlier. Another pathway that BDNF can stimulate is the MAPK pathway, which then has the ability to phosphorylate transcription factor CREB. This response is also triggered by an increase in \( \text{Ca}^{2+} \). The influx in \( \text{Ca}^{2+} \) can also cause the phosphorylation of CREB\textsuperscript{8,9}. Upon activation, CREB regulates neuronal excitation and neurotrophic factor gene transcription thus playing a role in neuroprotection\textsuperscript{31,64}.

CREB has anti-apoptotic effects and can reduce cell death in rats introduced to EE following an injury\textsuperscript{31,64}. In the hippocampus of these rodents, Walton et al. (2000) observed that the neurons resistant to the effects of injury had higher levels of phosphorylated CREB\textsuperscript{56}. Once phosphorylated, CREB can induce and increase BDNF production, become co-localized with BDNF in the neurons of the hippocampus and has the ability to regulate the transcription of BDNF directly through the MAPK and PI3K/Akt pathways (Figure 4 & 16)\textsuperscript{64,56}. Walton et al. (2000) demonstrated that Akt can be directly phosphorylated by CREB, providing further neuroprotective effects by continuing the cascade in the B-cell lymphoma 2 (BCL2) family which promotes cell survival and blocks apoptosis (Figure 16)\textsuperscript{52,56}. These changes then increase BDNF mRNA and protein levels.

This intricate series of events aids in understanding the results of the current study. From this cascade, it can be determined that the induction of BDNF production is
not immediate, but requires a specific series of events to occur prior to being influenced. Yang et al. (1996) reported similar trends and found that BDNF levels were not elevated following a TBI until one hour post-injury. BDNF levels will elevate and fall in a time-dependent manner with the highest expression levels occurring once mRNA and protein production has been initiated; however, following the acute stage of TBI, levels will begin to decrease due to the loss of stimulation both in the PI3K pathway and CREB. This decrease in BDNF may take between hours and days due to the extent of damage, stimulation and inflammatory influence.

Neurotrophins may provide neuroprotective benefits during the sub-acute and chronic stages of TBI, specifically in inflammation (not included in Figure 15). Piehl et al. (2001) determined that neurotrophic factor production by the invading immune cells may diminish some of the negative effects of inflammation. Kerschensteiner et al. (1999) found that immune cells, such as T cells and B cells, possess the ability to produce BDNF. It is therefore also hypothesized that increasing BDNF production during the immune response may aid in minimizing neuronal damage through down-regulation of other degenerative immune responses. Inflammatory processes can last from days to weeks, but the current study showed no increase in BDNF protein or mRNA levels throughout the hippocampus. This may have been due to a reduced inflammatory response in the hippocampus caused by the positive influence EE may have played in making the brain more resilient to these neurodegenerative effects. To determine if this theory is accurate, observing alterations in cytokines using multiplexing techniques would help to determine the role EE and BDNF have during these stages and processes.
By observing trends of numerous biomolecules (BDNF, CREB, BCL2, Akt) in this hypothesized model, verification of this model will be elucidated. These molecules should be observed in both the cortex and hippocampus using techniques such as microarray, RT-PCR, ELISA or immunohistochemistry. In addition to observing gene expression changes, the phosphorylated proteins can be analyzed via western blot techniques to verify activation of the proposed signaling pathways. By determining activation of these biomolecules and expression levels, a more pronounced picture of the mechanism of recover following TBI will be elucidated.

Verifying that this model is correct and determining the influence the BDNF has prior to injury as well as throughout the stages of TBI will help to develop new technologies and conditions for improved neuroprotection and neurogenesis.
Conclusion
The hypothesis of this research was that BDNF mRNA and protein levels would be elevated in animals which were environmentally enriched prior to TBI compared to non-enriched animals. It was revealed that EE attenuated the injury-induced deficits in memory when applied prior to injury. This study also revealed that exposure to EE prior to a TBI did not elevate BDNF mRNA and protein levels in the cortex or hippocampus when observed 33-days following the injury. There was however a significant difference found among trkB levels in the DG of the hippocampus. The findings from this study were critical in establishing the benefit of neuroprotection that EE provides when applied prior to TBI.

Future research is necessary to focus on the biochemical changes of this repair mechanism. To do so, specific times of sacrifices would be developed in order to observe trends in BDNF and trkB during the opportune time, and determine if enriched animals show more resilience following an injury as measured by increasing levels of BDNF. Other studies may determine the most effective models of enrichment which will result in enhanced resistance and optimal levels of BDNF.

Determining the underlying neuroprotective mechanisms will have a significant impact on developing effective preventative measures to mitigate the debilitating effects of TBI. These techniques will provide valuable understanding of the stages of TBI which will allow for the disruption of neurodegenerative processes and improved cognitive function. This research will continue to benefit the 1.7 million people affected yearly by this debilitating action and has exceptional potential for aiding the resilience of soldiers prior to engaging in war.
### Appendix

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**Table 4: In-Situ Paired Comparisons of Groups**
References


