The Effects of 7,8-Dihydroxyflavone (7,8-DHF) on Neuroprotection and Neuroplasticity Following a Traumatic Brain Injury

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The Effects of 7,8-Dihydroxyflavone (7,8-DHF) on Neuroprotection and Neuroplasticity Following a Traumatic Brain Injury

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

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April 29th, 2015
Acknowledgements

During my time here at VCU, I have come to appreciate many things. I have learned the importance of having a valuable PI and being able to collaborate with others when the solution to a situation isn’t obvious. I want to thank my research advisor, Dr. Dong Sun, for giving the opportunity to work in her lab under her expertise and my committee members, Dr. Linda Phillips, Dr. Liya Qiao, and Dr. Andrew Ottens, for providing valuable feedback and insight.

I would also like to thank Andy Rolfe, our lab technician, for having the patience to guide me through protocols used in this study and providing some comic relief on several occasions. In addition, I would like to thank Dr. Ottens’ lab for the camaraderie and “therapy” sessions.

Lastly, I would like to thank my husband, mother, and son for always being supportive and patient as I made my way through this journey.
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# Abbreviations

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<tr>
<td>7,8-DHF</td>
<td>7,8-Dihydroxyflavone</td>
</tr>
<tr>
<td>Akt</td>
<td>AKT8 Virus Oncogene Cellular Homolog</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell Lymphoma-2</td>
</tr>
<tr>
<td>BDA</td>
<td>Biotinylated Dextran Amine</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain Derived Neurotrophin Factor</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxy Uridine</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu Ammonis 1</td>
</tr>
<tr>
<td>CA3</td>
<td>Cornu Ammonis 3</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CCI</td>
<td>Controlled Cortical Impact</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP Response Element-Binding Protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral Spinal Fluid</td>
</tr>
<tr>
<td>dpi</td>
<td>Days Post Injury</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular Signal-Regulated Kinase</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular Domain</td>
</tr>
<tr>
<td>FPI</td>
<td>Fluid Percussion Injury</td>
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<tr>
<td>HCL</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>ICD</td>
<td>Intracellular Domain</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-Terminal Kinase</td>
</tr>
<tr>
<td>LSD</td>
<td>Least Significant Difference</td>
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<tr>
<td>LTP</td>
<td>Long-Term Potentiation</td>
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MAPK ........................................................... Mitogen-Activated Protein Kinase
MEK .......................................................... MAPK/Erk Kinase
MRI .......................................................... Magnetic Resonance Imaging
mRNA ......................................................... Messenger Ribonucleic Acid
MWM ......................................................... Morris Water Maze
NGF .......................................................... Nerve Growth Factor
NT-3 .......................................................... Neurotrophin-3
NT-4 .......................................................... Neurotrophin-4
p75(NTR) ..................................................... p75 Neurotrophin Receptor
PBS .......................................................... Phosphate Buffer Saline
PI3K .......................................................... Phosphatidylinositol 3-kinase
PLC .......................................................... Phospholipase C
PNS .......................................................... Peripheral Nervous System
SGZ .......................................................... Subgranular Zone
TBI .......................................................... Traumatic Brain Injury
Trk .......................................................... Tropomyosin-Related Kinase
Abstract

The Effects of 7,8-Dihydroxyflavone (7,8-DHF) on Neuroprotection and Neuroplasticity Following a Traumatic Brain Injury

By

Jennifer Marie Romeika

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2015

Advisor: Dong Sun, MD, PhD, Department of Neurosurgery

Aside from preventing traumatic brain injuries (TBIs) altogether, treatment options for TBI typically focus on the secondary biochemical processes that occur in response to the primary mechanical insult. These secondary injuries can lead to apoptosis and necrosis in the days and weeks that follow a TBI. Therefore, finding a treatment that can prevent, reduce, and repair secondary damage is instrumental in the recovery of TBI patients. The flavonoid 7,8-dihydroxyflavone (7,8-DHF) has been identified as a TrkB agonist that mimics the effects of brain derived neurotrophin factor (BDNF). Upon binding to the TrkB receptor, signaling cascades are initiated that can promote neuronal survival and neural differentiation. The use of 7,8-DHF in the treatment of TBI is favorable due to its long half-life and ability to pass the blood-brain barrier (BBB). In this study, we evaluated the dosage time frame of 7,8-DHF that would allow for the greatest impact in recovery after a focal TBI. Adult Sprague-Dawley rats were subjected to a moderate cortical impact injury and administered a 5mg/kg dose of 7,8-
DHF i.p. for five days starting on day 0, 2, 3, or 5 post injury. Sensorimotor function was evaluated with beam walk and rotarod test. Morris Water Maze (MWM) and fear conditioning test were used to analyze cognitive function. Biotinylated dextran amine (BDA) was injected into the contralateral cerebral cortex 14 days after injury and animals were sacrificed 28 dpi. Brain sections were processed for Giemsa histological staining to assess cortical lesion volume and the total number of surviving neurons. Parallel sections were processed for BDA staining to assess changes of axon sprouting in the injured cortex. VGlut-1 staining of the hippocampus was used to identify presynaptic plasticity. We found that the administration of 7,8-DHF starting at one hour after TBI could provide protection against motor and cognitive dysfunction. Histological examination showed a significant reduction of cortical lesion volume and higher number of survival neurons in the injured hippocampus when 7,8-DHF administration began one hour and two days after injury. BDA staining of intracortical axon sprouting and VGlut-1 staining of the hippocampus highlighted a trend that 7,8-DHF administration starting day five post brain injury may enhance neuronal plasticity. Collectively, the results indicate that 7,8-DHF can provide the better neuronal protection when administration begins one hour after TBI.
1.1 Definition/Pathophysiology of Traumatic Brain Injury (TBI)

Traumatic brain injury (TBI) can be described as a loss of brain function due to an external mechanical force. The cells and tissue that are directly injured upon impact, including blood vessels, axons, neurons, and glia, are considered primary damage (Tsao, 2012). Secondary damage is delayed and includes neuronal degeneration in the distal and surrounding regions of the impact site. This neuronal degeneration is predominately programmed cell death, or apoptosis (Stoica & Faden, 2010) and can be observed in the cortex, hippocampus, and thalamus after TBI (Keane et. al., 2001; Stoica & Faden, 2010; Raghupathi et. al., 2000). Many biochemical processes, such as excess excitotoxin release, increased intracellular calcium concentration, lipid peroxidation, inflammatory mediators, and the production of free oxygen radicals, contribute to the secondary damage (Veenith et. al., 2009; Chiaretti et. al., 2008; S. Liu et. al., 2014). A great deal of research and funding has been funneled into TBI research, yet treatment options are still limited with much left to be understood about the pathobiology of TBI (Yu et. al., 2009).

1.1.1 Neurodegeneration after TBI

Secondary damage can occur over days to weeks after TBI (Wagner & Zitelli, 2013) and result in diffuse neurodegeneration that affects motor and cognitive function (Hall et. al., 2005). Anterograde degeneration can be observed in axons that originate in the ipsilateral injury
hemisphere and project via the corpus callosum into the contralateral hemisphere. Retrograde degeneration of axons can be seen in neuronal cell bodies that reside in the contralateral cortex and hippocampus (Hall et. al., 2005). Caspases, mediators of apoptosis, have been found in the thalamus and subcortical region of the injured hemisphere as early as one hour after brain injury (Keane et. al., 2001) and at increased levels in the cortex at 24 hours (Clark et. al., 2000). Cortical projection neurons in thalamus (Natale et. al., 2002) and predominately immature neurons in the granule cell layer of the hippocampus can be observed undergoing apoptosis within 24 hours (Zhou et. al., 2012). Widespread hippocampus degeneration occurs at 48 hours, affecting the axons that arise from the entorhinal cortex to the dentate gyrus and cornu ammonis 3 (CA3) region, the mossy fibers that extend from the dentate gyrus to CA3 pyramidal cells, and the axons in the Schaffer collateral pathway that originate in the CA3 region and project to the cornu ammonis 1 (CA1) region. Diffuse neurodegeneration in the ipsilateral cortex, hippocampus and thalamus has been found to peak between 48-72 hours after brain injury (Hall et. al., 2005). Seven days after injury, dying cells in the hippocampus are predominately mature neurons (Zhou et. al., 2012). This culmination of neurodegeneration substantially contributes to the tissue damage (Faden, 2002), as well as to spatial memory deficits and motor dysfunction that occur after TBI (Hall et. al., 2005).

1.1.2 Neurogenesis after TBI

There are two main locations of neurogenesis in the adult brain: the subventricular zone of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) (Gomazkow, 2012; Balu & Lucki, 2009). The first stage of neurogenesis in the hippocampus is proliferation in the
SGZ followed by differentiation where the cells become immature neurons and commit to neuronal lineage. Then immature neurons will migrate to the granule cell layer (GCL) where the neuron will start to make connections by extending an axon towards the pyramidal layer of the CA3 and dendrites toward the molecular layer of the dentate gyrus (Bohlen und Halbach, 2007). These connections can occur within 4-10 days of birth (Hastings & Gould, 1999). The last step is synaptic integration into the hippocampal network (Bohlen und Halbach, 2007) that happens around 14 days after birth (Hastings & Gould, 1999). Seven weeks after division, action potentials can be generated by the new granule cells (Balu & Lucki, 2009). Cell death of the immature neurons has also been observed within 1-2 weeks after birth. If the neuron reaches maturity, it can last several months (McDonald & Wojtowicz, 2005).

Several studies have demonstrated that there is an increase in cell proliferation in the hippocampus after TBI (Chirumamilla et. al., 2002; Dash et. al., 2001; Sun et. al., 2005; Rice et. al., 2003). This TBI-induced proliferation reached a peak at 2 days post brain injury and returned to basal levels by day 14 in fluid percussion injury (FPI) model (Figure 1.1, Sun et. al., 2005). While a peak was observed at 3 days post brain injury in a cortical contusion injury (CCI) model (Rice et. al., 2003).
Figure 1.1. Cell Proliferation in the Ipsilateral Hippocampus in Juvenile and Adult Rats Following TBI. Graphs showing increased cell proliferation in the ipsilateral hippocampus at 2 and 7 days post injury with peak of proliferation occurring at 2 days (Sun et. al., 2005).
1.2 Epidemiology of TBI

TBI is not only a growing concern in the United States, but affects established and emerging countries worldwide (Wagner & Zitelli, 2013). It is a leading cause of disability and death in the developed world (Mustafa et. al., 2010). TBI accounts for 50% of the deaths among 15 to 35 year olds (Kim, 2011). The National Institutes of Health has reported that about 5 million new head injuries occur in the United States every year. Two million of these cases result in permanent difficulties in employment, education, and family. Around 300,000 individuals will require hospitalization and 80,000 will have long-term impairments. Overall, TBIs create an economic burden of more than $56 billion yearly (Yu et. al., 2009). Physical impairments due to brain injury can limit an individual’s daily functions. Cognition can also be greatly disrupted, effecting language, concentration, memory, and attention (Kline et. al., 2002). The causes of TBI vary within different age groups and demographics: children under the age of four years old have a high rate of TBI due to ‘shaken baby syndrome’, automobile accidents are the prominent cause of TBI for 15 – 19 year olds; and the elderly are more likely to have fall-related TBIs (Wagner & Zitelli, 2013).

1.3 Traumatic Brain Injury Model

Simulation models have been created to gain a better understanding of what occurs during and after a TBI. The fluid percussion injury (FPI) and the controlled cortical impact (CCI) injury are two of the most common models used because they both induce impairments that resemble that of motor and cognitive abnormalities observed in individuals that have experienced a TBI (Yu et. al., 2009). Both models are able to produce cell loss in the CA3 and the hilus region of
the hippocampus (Fujimoto et.al., 2004), which are common areas to evaluate after a TBI due
to their sensitivity to injury, role in cognitive functions, and area of neurogenesis throughout a
lifetime (Gao et. al., 2009a). The learning and memory deficits that occur as a result of brain
injury induced hippocampal damage are often considered the hallmarks of TBI (Richardson et.
al., 2007).

1.3.1 Fluid Percussion Injury

The FPI model consists of a fluid pulse that is applied to the dura through a craniotomy window,
allowing for injury without fracturing the skull (Chauhan et. al., 2010). FPI is able to create
alterations in intracranial pressure, cerebral blood flow, cerebral metabolism, and disruption of
blood-brain barrier. The severity of TBI can also be manipulated by adjusting the magnitude of
impact (Hicks et. al., 1996). However, this method can cause higher rates of morbidity (Chauhan
et. al., 2010) and has indirect indices from the mechanical loading on the brain produced by the
fluid pulse, creating more variables and less accuracy with results (Fujimoto et. al., 2004).

1.3.2 Controlled Cortical Impact

In the CCI model, a piece of the skull is removed followed by an impact from a electromagnetic
driven metallic rod. The severity of the TBI is determined and controlled by the depth in which
the impact rod is driven and the velocity at which it moves (Goodman et. al., 1994). This model
allows for a focal area of injury that can be easily distinguished (Fujimoto et. al., 2004). Other
clinical presentations of TBI such as increased intracranial pressure, compression of the brain,
and brain edema can also be reproduced with the CCI model (Puritt et. al., 2014). The CCI
model is able to replicate a more controlled TBI with cortical deformation as would be seen in the clinic, making it a good model for preclinical research (Chauhan et. al., 2010).

1.3.2.1 Determining the Severity of Controlled Cortical Impact

Impairments in behavioral and cognitive tasks increase with the severity of the TBI. A CCI can replicate many clinical symptoms of mild, moderate, and severe TBI just by adjusting the depth and velocity of the impact (Yu et. al., 2009). Depth of impact is often the guide to adjusting injury severity. Generally speaking, a 2.0 mm impact is considered mild, a 2.5 mm impact is moderate, and a 3.0 mm impact is considered severe. However, a reduction of velocity, such as 3m/sec to 1 m/sec in a 2.5 mm impact could create a deformation not much greater than a 2mm impact at 5m/sec, demonstrating that these parameters are more guidelines than exact predictions (Goodman et. al., 1994).

1.3.2.2 Cognitive and Motor outcomes with Controlled Cortical Impact

Animals were able to manifest a range of cognitive and motor tasks that matched the degree of brain damage in CCI-injured rat and mouse models. Comparison tests of TBI severity and performance in rotarod and Morris water maze (MWM) were able to demonstrate distinct levels of cognitive behavioral deficits that corresponded with mild, moderate, and severe TBI. Both rats and mice had longer escape latency and impaired recall ability in the probe test of MWM and shorter lengths of time on the rotarod as the TBI injury went from mild to severe (Yu et. al., 2009).
The ability of the CCI model to replicate a range of clinical symptoms in an animal model makes it valuable in testing experimental TBI therapeutic drugs. The CCI model also provides a way to target a certain patient population, decreasing variables, and ensure that laboratory findings can be translated to a clinical population. In addition, the CCI model has provided a way to standardize outcomes and parameters of TBI animal models providing validation of experimental data and comparison among various research studies (Yu et al., 2009).

1.4 Neurotrophins

A group of polypeptides, called neurotrophins, have been shown to have great influence over the survival, development, and functioning of structures in the central nervous system (CNS) and peripheral nervous system (PNS). They participate in every stage of creating a neuronal network, including the release of neurotransmitters, trafficking of receptors, growth of axons and dendrites, maintaining synapses, and aiding in long-term potentiation (LTP). Neurotrophins have the ability to moderate single cells and networks through their influences on synaptic plasticity and protection. The differentiation, survival, and synaptogenesis of nerve cells is influenced by neurotrophins (Gomazkow, 2012). Synthesis of neurotrophins in the CNS occurs predominantly by neurons during certain physiological conditions. Both neuronal and non-neuronal cells synthesize neurotrophins in a precursor, or proneurotrophin, form. These immature proteins are then either secreted or cleaved by plasmin or extracellular proteases into the mature form (Marco-Salazar et al., 2014). Cells are able to survive apoptosis, or cell death, due to the retrograde flow of neurotrophin from the nerve terminal, up the axon, and
into the cell body. The neuron would not be able to maintain a functional differentiated state
without this continuous retrograde flow of neurotrophin (Skaper, 2012).

1.4.1 Neurotrophins and Their Receptors

The diverse effects that occur from the presence of neurotrophins are a result of the ligand-
receptor binding and the specific signal cascades that follow. This includes two transmembrane-
receptor signaling systems that consist of the p75 neurotrophin receptor (p75\textsuperscript{NTR}) and the
tyrosine kinase receptors: TrkA, TrkB, and TrkC. The p75\textsuperscript{NTR} belongs to the tumor necrosis factor
receptor super family while the tyrosine kinase receptors belong to the tropomyosin-related
kinase (Trk) family (Marco-Salazar et. al., 2014). The neurotrophin family of signaling molecules
include nerve growth factor (NGF), brain-derived neurotrophin factor (BDNF), neurotrophin-3
(NT-3) and neurotrophin-4 (NT-4). These neurotrophins have binding specificity for particular
receptors among the Trk family receptors. NGF has a higher affinity for TrkA, BDNF and NT-4
prefer TrkB, and TrkC binds with NT-3. All of the neurotrophins are able to bind to p75\textsuperscript{NTR}.
(Marco-Salazar et. al., 2014).

1.4.2 The Endogenous Response of Neurotrophins after Traumatic Brain Injury

A significant up-regulation of neurotrophic factors has been observed after an injury to the
brain (Chiaretti et. al., 2002). Children that demonstrated better outcomes after experiencing a
TBI had elevated levels of neurotrophins in their cerebral spinal fluid (CSF) (Malik et. al., 2011).
More specifically, increased levels of the neurotrophin BDNF were found in the CSF of TBI
patients after injury (Chiaretti et. al., 2002). TBI animal models were also able to replicate this
increase of BDNF after a brain injury (Chiaretti et. al., 2008). Studies that have used the direct
treatment of neurotrophins to injured brains have been able to demonstrate an upregulation of protective genes, such as Ttr, Trh, and CCL-2 (Malik et. al., 2010). However, neurotrophins also have their drawbacks due to their limited ability to diffuse in the CNS parenchyma, short half-lives, and difficulty with being able to permeate the blood-brain barrier (BBB) (Blaya et. al., 2015).

1.5 Brain Derived Neurotrophin Factor (BDNF)

The neurotrophin BDNF has been identified as one of the signaling molecules for neurogenesis, promoting differentiation and proliferation of neurons, as well as neuronal survival (Lu et. al., 2008). Presynaptic BDNF signaling can promote the release of neurotransmitters and postsynaptic BDNF signaling can enhance ion channel function (Autry & Monteggia, 2012). BDNF has the ability to encourage the branching and elongation of neurons and neurite differentiation into axons (Gill et. al., 2013). Overall, BDNF has become one of the most important regulators of the survival and differentiation of neurons in the CNS. Many psychiatric disorders and neurodegenerative diseases have been associated with changes in BDNF and its TrkB receptor, including Alzheimer’s disease, Rett syndrome, Parkinson’s disease, schizophrenia, Huntington’s disease, major depressive disorder, and TBI (Bernard-Gauthier et. al., 2013).

1.5.1 BDNF Prevalence and Production in CNS

BDNF mRNA has the highest expression in the hippocampus, but can be found in many places in the brain (Gomazkow, 2012). More precisely, the dentate gyrus of the hippocampus is one of the main areas of BDNF production (Lee et. al., 2002). The precursor form of BDNF is
synthesized in the endoplasmic reticulum and is hydrolyzed by the matrix metalloproteinases plasmin and furin in the Golgi network. At this point, BDNF is packed into secretory vesicles and can be found in the presynaptic axon terminals and postsynaptic dendrites (Gomazkow, 2012). In an activity-dependent manner, BDNF can be secreted from the post- and pre-synaptic spines and terminals (Waterhouse & Xu, 2009).

1.5.2 BDNF and Its Receptors

Two different classes of receptors are responsible for mediating BDNF signaling: p75NTR and TrkB (Lu et al., 2008). BDNF has a $K_d = 9.9 \times 10^{-10}$ M for the TrkB receptor and a $K_d \sim 10^{-9}$ M for the p75NTR demonstrating its binding selectivity and affinity for each of the receptor types (Bernard-Gauthier et al., 2013). It is through its high affinity for TrkB that BDNF is able to provide neuronal survival, neuronal plasticity, and neurogenesis (Lu et al., 2008). The p75NTR receptor is more associated with apoptosis. ProBDNF binds more to the p75NTR receptor while the mature form of BDNF has a high affinity to TrkB (Bollen et al., 2013). However, the mature form of BDNF can bind to p75NTR receptor when there are high concentrations of BDNF (Boyd & Gordon, 2003). Both of the BDNF receptors can be found in the same cell, coordinating and modulating neuronal responses. Furthermore, the signals generated by each receptor can augment each other or go against each other, fluctuating between a enhancing and suppressing relationship (Kaplan & Miller, 2000).

The binding of BDNF to p75NTR induces cell death, or apoptosis. This induced cell death has been demonstrated with hippocampal neurons. Neurons lacking Trk receptors are even more prone to p75NTR cell death, demonstrating the relationship of TrkB receptor protection and p75NTR apoptosis. The p75NTR leads to Jun amino-terminal kinase (JNK) activation and the
following activation of Bad and Bim, which eventually leads to the release of caspase-3, -6, and -9 (Ichim et. al., 2012).

BDNF and TrkB can be found throughout the CNS (Baseri et. al., 2012). The positive effects that BDNF has on the synapse are regulated through the TrkB receptor. More specifically, BDNF is able to bind to the CC-2 domain, Ig2 domain, and a leucine-rich motif of amino acid residues 103-181 of the TrkB receptor (Jang et. al., 2010). The TrkB receptor becomes a homodimer upon BDNF binding. Then tyrosine and serine residues on the intracellular domain are phosphorylated, activating signaling pathways (Lai et. al., 2012). The axons, nerve terminals, and dendritic spines of the granule and pyramidal neurons of hippocampus all express the TrkB receptor, elucidating the idea that BDNF works both on the pre-and post-synaptic sides (Leal et. al., 2014; Waterhouse & Xu, 2009), but in particular, pyramidal cells have high levels of TrkB receptor expression (Dinocourt et. al., 2006).

1.5.3 BDNF/TrkB Signaling Pathways

BDNF and TrkB binding induces the signaling pathways that include mitogen-activated protein kinase (MAPK)/Ras pathway, phosphatidylinositol 3-kinase (PI3k)/ serine threonine kinase (Akt) pathway, and/or phospholipase C-gamma (PLC-gamma) pathway. These signal cascades play important roles in plasticity and survival of neurons as well as neurogenesis (Zhang et. al., 2014).

The MAPK pathway stimulates anti-apoptotic proteins, such as B-cell lymphoma-2 (Bcl-2) and cAMP-response-element binding protein (CREB). In particular, CREB is required by neurotrophins for mediating neuronal survival (Kaplan & Miller, 2000; Wang et. al., 2010).
MAPK can also activate extracellular signal related kinase (ERK) (Autry & Monteggia, 2012). MAPK pathway leads to further phosphorylation of synapsin I, a protein involved in synaptic vesicle clustering and release (Griesbach et al., 2002). Interfering with the MAPK pathway has shown to decrease BDNF-induced synapsin I phosphorylation; thus inhibiting the release of neurotransmitters (Waterhouse & Xu, 2009). Ras, a small Guanosine 5’-Triphosphate-binding protein, is often responsible for neurotrophin-dependent survival, but it does not have a direct effect on survival. Ras turns neurotrophin initiated signals into other multiple signaling pathways, including PI3k/Akt and MEK/MAPK (Kaplan & Miller, 2000; Patapoutian & Reichardt, 2001). Overall, MAPK activation leads to neuronal survival and differentiation (Baseri et al., 2012).

PI3K activates Akt and ERK (Autry & Monteggia, 2012). Akt is able to promote neuronal cells by regulating proteins that influence cell survival: including Bad, which inhibits the anti-apoptotic protein Bcl-2, pro-caspase-9, and the transcription factor Forkhead that influence apoptosis. Overall, Akt’s influence is based on its ability to inhibit apoptotic proteins (Kaplan & Miller, 2000). Similar to MAPK, PI3K activation also leads to neuronal survival and differentiation (Baseri et al., 2012).

PLC-gamma leads to an increase in intracellular calcium levels and can activate the calcium/calmodulin kinase pathway, which can then activate CREB (Baseri et al., 2012). When activated, PLC-gamma can regulate the development of neurons and the plasticity of synapses (Zhang et al., 2013).
1.5.3.1 Current Research on TrkB

The activation of the TrkB pathways has been shown to improve cognition and has also been correlated with an increase in synaptic density (Castello et. al., 2013). Administration of BDNF to murine cell lines that were transfected with TrkB showed an activation of the PI3K/Akt pathway and reduced apoptosis (Jang et. al., 2010). In the hippocampus, TrkB signaling can affect the maturation of dendritic trees and synaptic terminals. TrkB signaling in the hippocampus modulates synaptogenesis of the mossy fibers extending from the dentate granule cell layer to the hilus and the CA3 region (Otal et. al., 2005). Studies with mice have shown that the lack of the TrkB receptor or mutation of the TrkB receptor greatly influences synapses in the hippocampus negatively impact learning and memory (Lai et. al., 2012).

1.5.4 BDNF Influences Synaptic Plasticity

BDNF and TrkB are upregulated in areas where there is neuronal plasticity occurring. Due to this relationship, BDNF is considered a molecular mediator in the function and structure of synaptic plasticity and plays a pivotal role in memory formation as well as memory consolidation (Zeng et. al., 2012). Even a disruption in the pathway that transports and produces BDNF can result in the clinical symptoms of deteriorating memory and cognitive dysfunction (Gomazkow, 2012). Many studies have also shown a relationship between lower levels of BDNF and cognitive decline observed in aging, schizophrenia, and Rett’s syndrome (Bollen et. al., 2013).
1.5.4.1 BDNF Influences the Synapse

The cellular basis for learning and memory is considered to be the synapses in the hippocampus. It is the formation of these synapses through dendritic spines that have been shown to correlate with the ability to learn and recall (Zeng et al., 2012). Strengthening or weakening of the synapses in neuronal networks, which occurs at excitatory synapses, allows for information to be represented, processed, and stored (Waterhouse & Xu, 2009). BDNF influences the morphology of mature granule cells and increases the excitatory transmission in CA1, CA3, and dentate gyrus (Lee et al., 2002). BDNF has the ability to regulate this synaptic activity and plasticity through structural functional changes in the neurons (Waterhouse & Xu, 2009), such as axon and dendrite growth and increased exocytosis of synaptic vesicles (Park & Poo, 2013).

1.5.4.2 BDNF and Long-term Potentiation (LTP)

Long-term potentiation (LTP) is a specific form of plasticity that occurs in the hippocampus and is greatly influenced by BDNF (Bollen et al., 2013; Leal et al., 2014). The synaptic plasticity that occurs at the CA3 and CA1 synapses is involved in the establishment of memories and learning. An important characteristic recognized is the increase in secretion and local synthesis of BDNF with the increase of synaptic activity. The induction of LTP is also associated with the enlargement and formation of spine heads (Lai et al., 2012). BDNF has the ability to convert early LTP to late LTP as well as potentiating subthreshold activation to create LTP (Autry & Monteggia, 2012). Much research has been able to demonstrate the importance of BDNF in inducting and maintaining LTP. Studies have shown an increase in BDNF mRNA in the
hippocampus when learning spatial tasks like that exhibited during the Morris water maze and radial maze tests (Lu et. al., 2008). Studies with mice have shown that the absence of BDNF or its receptor, TrkB, can have a great impact on the formation of LTP in hippocampal synapses, leading to poorer results in certain learning tasks (Leal et. al., 2014; Lai et. al., 2012). In fact, hippocampal synaptic plasticity relies heavily on the activation of TrkB receptors (Yang et. al., 2014).

1.5.5 BDNF and Its Influence on Neurogenesis

Neurogenesis and neuronal protection signal cascades area initiated when BDNF binds to its TrkB receptor. Research has demonstrated that with the treatment of BDNF, neurogenesis can be stimulated in the dentate gyrus (Gomazkov, 2012). Rats that were infused with BDNF were observed to have neurogenesis of the granule cells, which occurred contralaterally to infusion site, indicating a wide spread effect on the hippocampus (Scharfman et. al., 2005). Neurodegenerative disorders, TBI, and brain ischemia studies have been able to show the initiation of neurogenesis. In these studies, new neuronal cells could be found in the areas of damage, attempting to replace tissue structures that were no longer viable (Gomazkov, 2011). BDNF also has the ability to activate the synthesis of proteins that will protect the differentiation of progenitor stem cells into mature neurons, making BDNF a valuable asset in the survival of cells created during neurogenesis (Gomazkov, 2011). Neuronal survival has also been shown to decrease with the reduction of BDNF and TrkB activity (Salranen et. al., 2005). This relationship of BDNF and neurogenesis provides another reason to study the promising therapeutic effects of BDNF and its receptor TrkB (Gao et. al., 2009b).
1.5.6 BDNF and Its Relationship with TBI

TBI research continues to demonstrate the neuronal protective and neuronal plasticity effects of BDNF. The presence of increased levels of BDNF after TBI is indicative of the brain’s attempt to provide neuroprotection against biochemical and molecular changes induced by a TBI (Chiaretti et. al., 2002). BDNF binding to the TrkB receptor has shown to have neuroprotective effects after several types of brain injury (Hicks et. al., 1998). Mice knockouts models indicate that BDNF can provide neuroprotective and repair function, trying to regain the connections. Studies have shown an increase in cortex and hippocampal BDNF mRNA after an experimental TBI of moderate severity, lasting from several hours to days after the injury (Kaplan et. al., 2010). After a unilateral FP injury, expression of BDNF mRNA has also been observed to increase bilaterally in the dentate gyrus granule cell layer and CA3 pyramidal cell layer (Hicks et. al, 1997). BDNF mRNA has been observed to increase in the hippocampus as soon as one hour after a FPI or CCI and can remain elevated for as long as 72 hours after the TBI (Hicks et. al., 1998). BDNF and TrkB expression have also been found to be upregulated after Schaffer collateral injury. Axonal sprouting of the pyramidal neurons in the CA3 region of the hippocampus can occur near the site of injury. Blocking BDNF attenuates these morphological and functional changes (Gill et. al., 2013).

1.6 Vulnerability of the Hippocampus after TBI

The hippocampus is often a common area to evaluate after a TBI due to its vulnerability, role in cognitive function, and being one of the few areas in the brain that undergo neurogenesis throughout a lifetime (Gao et. al., 2009a). Bilateral loss of hippocampal neurons has been
observed in 85% of fatal human TBI cases, indicating that hippocampal atrophy is a major consequence of TBI. This neuronal was repeatedly demonstrated in animal studies where TBI’s were induced, including nonhuman primates, rodents, and pigs (Royo et. al., 2007). Magnetic resonance imaging (MRI) studies demonstrated this hippocampal reduction in both children and adults after a TBI. Secondary damage can be attributed to this pathological response (Ariza et. al., 2006).

In particular, a major area of concern when it comes to cell loss in the hippocampus is the CA3 region. Hippocampal CA3 pyramidal cells are the most sensitive to damage. Cell death can lead to deficits in learning, memory, and cognitive impairment (Malik et. al., 2011). It is the hippocampus that is responsible for long-term potentiation (LTP), involved with memory and learning. As mentioned before, BDNF plays a role in this hippocampal signaling and contributes to the mediation of LTP (Leal et. al., 2014).

Neural stem/progenitor cells in the SGZ of the dentate gyrus are continuously generating new neurons. These neural stem/progenitor cells have the potential to repair damage done to the hippocampus after a TBI (Gao et. al., 2009a). However, these hippocampal progenitor cells are also more vulnerable to apoptosis (Gomazkov, 2011). Permanent deficits in learning, memory, and cognitive abilities can result from the cell death that occurs in the hippocampus after a TBI. Protection and regeneration of these cells has become one of the focuses of TBI research due to the debilitating effect of their loss. However, at this time there is no proven therapeutic technique or medicine in treating a TBI and preventing the cognitive impairments that may result (Malik et. al., 2010).
1.7 The Molecule 7,8-Dihydroxyflavone (DHF)

Insufficient BDNF has also been thought to play a role in neurodegenerative diseases including Parkinson’s, Huntington’s, Depression, and Alzheimer’s (Baseri et. al., 2012). Due to its valuable role in neuronal survival, neurogenesis, and neuronal plasticity, BDNF has become an attractive target in the use of therapeutic treatment for cognitive disorders and TBI. However, it also has a poor pharmacokinetic profile that restricts its therapeutic potential, causing the research community to find an alternative molecule that can stimulate the positive effects initiated by BDNF (Bollen et. al., 2013). Compounds known as flavonoids have become a focus of interest due to their potential to provide neuronal protection, enhance existing neuronal function, and stimulate neurogenesis (X. Liu et. al., 2010). The screening of chemical libraries has resulted in the discovery of a flavone derivative, 7,8-dihydroxyflavone (7,8-DHF), which mimics BDNF (Zeng et. al., 2012).

The molecule 7,8-DHF can bind to TrkB receptors with high affinity and has the added advantage of being able to enter the brain with more efficacy than BDNF, making it a viable alternative to BDNF in therapeutic treatment (Zeng et. al., 2012; Wu et. al., 2014). The flavonoid, 7,8-DHF is part of a polyphenolic compound family found in fruits and vegetables. Other flavonoids, such as 3,6-dihydroxyflavone, 3,7-dihydroxyflavone, have also shown to protect against neuronal cell death, stimulate the process of neuronal regeneration, and also enhance the function of neurons. However, 7,8-DHF mimics BDNF due to its ability to bind to TrkB, resulting in neuronal survival, differentiation, and neurogenesis (Chen et. al., 2011). Not only does 7,8-DHF have the potential to be a therapeutic tool in the treatment of neurological
disease (Jang et. al., 2010) it could also become valuable in the treatment of TBI (Wu et. al., 2014).

1.7.1 Pharmacokinetics of 7,8-DHF

BDNF is not a viable therapeutic agent due to its short half-life, poor delivery and inability to easily cross the BBB (Wang et. al., 2014). BDNF is quickly cleared from circulation with a half-life of less than ten minutes. The BBB has the ability to be permeable to molecules with the size of about 180 daltons or less, unfortunately, BDNF with its molecular weight of 27 kilodaltons is far too big to easily cross the BBB (Baseri et. al., 2012). Compared to BDNF, 7,8-DHF is not only smaller but also very potent. It is able to pass through the BBB with ease (Bollen et. al., 2013) when administered orally or intraperitoneal (Wang et. al., 2014). The molecular mass of 7,8-DHF is less than 1% of the size of active BDNF, measuring at 254 Da. The binding of 7,8-DHF to the TrkB extracellular domain activates signal cascades that can last for hours without causing degradation of the internalized receptor (X. Liu et. al., 2014). In one study, the bioavailability of 7,8-DHF was about 5% with a half-life of 134 minutes given orally at 50mg/kg (Zhang et. al., 2014). It has a low cellular EC$_{50}$ of 35 nM with a 5 mg/kg dosage. The ligand to receptor ratio resulted in 1:1 ratio (Jang et. al., 2010). In another study that used surface plasmon resonance (SPR), the $K_d$ for BDNF was 1.7 nM while 7,8-DHF was 15.4 nM. Fluorescence spectroscopy also revealed a $K_d$ of about 12 nM. Both experimental studies demonstrated 7,8-DHF’s specificity for the TrkB receptor (X. Liu et. al., 2014).
1.7.2 TrkB and 7,8-DHF

7,8-DHF is a selective TrkB agonist. It is able to activate TrkB receptors without binding to p75 receptors, allowing for the mediation of signaling pathways that influence neuroprotection and neurogenesis neglecting the activation of the apoptotic processes (Bollen et al., 2013). 7,8-DHF does not bind to the extracellular domain (ECD) or intracellular domain (ICD) of the p75 receptor. It is able to autophosphorylate TrkB selectively without the autophosphorylation of TrkA or TrkC. Overall it has a high affinity for the ECD of TrkB indicating a strong binding specificity (Jang et al., 2010).

When administered intraperitoneal (IP), 7,8-DHF was able to activate brain TrkB, establishing that it can cross the brain-blood barrier. 7,8-DHF was able to induce the autophosphorylation of TrkB, which lead to the activation of MAPK, PI3/Akt, and Erk1/2 signal pathways in a time frame that was comparable to BDNF and in a dose-dependent manner (Jang et al., 2010; Z. Liu et al., 2010). Furthermore, the activation of TrkB receptors through 7,8-DHF did not result in the ubiquitination or degradation of receptor which occurs when TrkB is activated by BDNF. Binding results in the internalization of the TrkB receptor and ligand complex and eventual formation of signaling endosomes (X. Liu et al., 2014). Daily administration of 7,8-DHF to mice in an Alzheimer’s model exhibited a partial rescue of down-regulated TrkB receptors in the hippocampus. However, the wild-type litter mates in this study did experience a down-regulation of TrkB receptors, indicating that there may be a compensatory reaction to continuous exposure to agonist (Devi & Ohno, 2012).
In murine cell lines transfected with TrkB, the binding of 7,8-DHF to the TrkB receptor prevented apoptosis, with similar results to BDNF binding in the same cells. Mice hippocampal cells presented a decrease in apoptosis to kainic acid when also administered with 7,8-DHF. TrkB receptor was activated by 7,8-DHF in a knock-out mouse model that had a BDNF-depleted cortex. Reduced infarct volumes were also observed in a stroke model mice that received 7,8-DHF. This reduction of apoptosis and decrease infarct volume demonstrate the protective ability of TrkB signal pathways via 7,8-DHF (Jang et. al., 2010).

1.7.3 Current Experimental Findings of 7,8-DHF

In particular, 7,8-DHF has neurotrophic activities that are associated with Parkinson’s disease (Chen et. al., 2011), Alzheimer disease (Castello et. al., 2013), Huntington disease, stroke, (X. Liu et. al., 2014), Depression, and Rett syndrome(Liu et. al., 2012). Reduce tissue damage, apoptosis, brain edema, and an increase in neuronal protection was observed in a TBI mouse model that administered 7,8-DHF at various time points after brain injury (Wu et. al., 2014). Overall several studies have been able to show the diversity of neuroprotection and plasticity that 7,8-DHF can play in various neurological diseases and disorders (Liu et. al., 2012).

1.7.3.1 Neuronal Protection and 7,8-DHF

In several experimental models, 7,8-DHF has demonstrated that it was able to enhance neuronal survival through anti-apoptotic means. These models include the survival of retinal ganglion cells, motorneurons, dopaminergic neurons in Parkinson disease (X. Liu et. al., 2014), as well as cerebral neurons in focal ischemia model (Wang et. al., 2014). 7,8-DHF has been shown to exhibit potent protective effects in a oxygen-glucose deprivation environment in a
dose-dependent manner. It was able to protect rodent and human neurons from hydrogen peroxide-induced apoptosis. 7,8-DHF blocked glutamate-provoked caspase-3 activation in mice, again showing that is able to protect through the repression of neuronal apoptosis via TrkB (Jang et. al., 2010). In addition, 7,8-DHF has also been demonstrated to attenuate ischemic cerebral injury by inhibiting cell apoptosis, via down-regulation of caspase-3 and Bax and up-regulation of Bcl-2. Caspase-3, the executioner of programmed cell death, when activated will lead to DNA fragmentation. Bax is a pro-apoptotic interacellular protein while Bcl-2 is a anti-apoptotic protein (Wang et. al., 2014).

1.7.3.2 The Effects of 7,8-DHF on Cognition and Motor Function

Deficits in memory due to age, stress or genetic background, have been shown to improve with the treatment of 7,8-DHF, with dosages of i.p. 5mg/kg. However, memory also improved in healthy, young rats that received low doses of 7,8-DHF (p.o. 0.1-3 mg/kg) (Bollen et. al., 2013). Chronic application of 7,8-DHF was able to restore behavioral performances and spatial learning and memory in aged rats that had impaired cognitive and motor capabilities (Zeng et. al., 2012). In a Alzheimer mouse model, 7,8-DHF mice showed improved hippocampus-dependent behavior via the MWM (Castello et. al., 2013). Animal models demonstrated that 7,8-DHF plays an important and positive role in learning and memory. An injection of 7,8-DHF was able to strengthen their emotional learning ability in normal rats. Chronic administration of 7,8-DHF was able to restore deficits in memory in aged rats and in Alzheimer rat models. Even pretreating with 7,8-DHF in rats that were immobilized to provoke stress had improved long-term spatial memory compared to their untreated counterparts. The working learning deficits
that are seen in schizophrenia rat models were also reversed with chronic treatment with 7,8-DHF (Yang et. al., 2014).

1.7.3.3 The Effects of 7,8-DHF on Neuronal Plasticity

Changes in neuronal synapses are often used to measure the plasticity of the brain. The synapse is also considered to be the cellular component of learning and memory. The number and density of dendritic spines along the pyramidal neurons in the hippocampal CA1 region had increased in aged rats treated with 7,8-DHF, suggesting that 7,8-DHF assists in the formation of synapse. 7,8-DHF increased the induction of LTP, indicating that 7,8-DHF could be used to target plasticity in the hippocampus and improve memory. 7,8-DHF administration also rescued spatial memory and synaptic plasticity in cognitively impaired aged rats (Zeng et. al., 2012).

High-frequency stimulus-induced synaptic potentials in the hippocampus were enhanced with 7,8-DHF. TrkB receptor phosphorylation in hippocampal neurons, which are associated with plasticity, learning, and memory were increased with 7,8-DHF (Yang et. al., 2014). Activated TrkB receptors observed in the dentate gyrus after chronic treatment of 7,8-DHF indicate that the TrkB agonists ability to promote neurogenesis by promoting the proliferation of progenitor cells (X. Liu et. al., 2010).

1.8 Summary and Hypothesis

TBI is a leading cause of disability and death worldwide (Wagner& Zitelli, 2013) with 5 million new head injuries occurring in the US every year (Yu et. al., 2009). After the initial mechanical impact, secondary damage continues to evoke neurodegeneration in the form of apoptosis that
further affects motor and cognitive function (Zhou et. al., 2012). A rise in BDNF and its TrkB receptor has been considered to be an endogenous response to the damages that occur with brain injury (Chiaretti et. al., 2002). This increase of BDNF and TrkB leads to the initiation of signal cascades that mediate neuronal protection, neuronal plasticity, and neurogenesis (Lu et. al., 2008).

The flavone derivative 7,8-DHF was discovered in hopes of finding a molecule that could mimic BDNF and bind to the TrkB receptor. Since its discovery, 7,8-DHF has been documented in providing neuroprotection and neuroplasticity in various neurological diseases and disorders (Liu et. al., 2012). Unlike BDNF, 7,8-DHF has a much longer half-life and can easily pass the BBB, making it a possible alternative in treating TBI (Zeng et. al., 2012; Wu et. al., 2014).

This study addressed the effects of 7,8-DHF on neuroprotection and neuroplasticity after TBI. It is hypothesized that 7,8-DHF will have the ability to provide neuroprotection and neuroplasticity after a TBI and this will vary according to time given after TBI. 7,8-DHF will provide neuronal protection when administration begins one hour and two days after brain injury, based on the time frame of previous documented neurodegeneration in the brain after TBI. The protective aspect of DHF treatment will be quantified using contusion volume, motor and cognitive recovery, and hippocampal cell numbers. 7,8-DHF will provide neuronal plasticity when administration begins three days and four days after brain injury, based on the time frame of synaptic regeneration. The plasticity aspect of DHF will be quantified using neuronal tracers/axon sprouting, motor and cognitive recovery, and synaptic markers.
Chapter Two - Methods and Materials

2.1 Experimental Animals

All the animal care and experimental procedures were in accordance with the National Institute of Health Guide for Care and Use of Animals and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. Male Sprague Dawley rats (300-350g; Harlan Laboratories) were housed in pairs, maintained in a 20-22 degree Celsius environment with a 12 hour light/dark cycle and fed *ad libitum* with standard rodent chow. Animals were acclimated to environment for at least one week prior to behavioral tests and surgeries. A total of 52 Sprague Dawley rats were used in this experiment. Animals were randomly assigned to one of six groups (Table 2.1): Sham group (n= 8), Vehicle group which received a CCI injury and vehicle treatment (n=13), Group 1 which received CCI + DHF treatment starting day one post injury (n=10), Group 2 which received CCI + DHF treatment starting day two post injury (n=8), Group 3 which received CCI + DHF treatment starting day three post injury (n=8), or Group 4 which received CCI + DHF treatment starting day five post injury (n=5).
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Table 2.1 Animal Treatment Groups in 7,8-Dihydroxyflavone Study. Table showing the number of animals in each treatment group.
2.2 Controlled Cortical Impact (CCI) Injury

A total of 44 male Sprague-Dawley rats at the age of 3 months old received a controlled cortical impact injury (CCI) using a Benchmark Stereotaxic Impactor (Figure 2.1). Animals were anesthetized with isoflurane (2.5% in 30% O2 and 70% Nitrogen) and remained sedated throughout surgical procedure. To ensure sedation, a tail-pinch or paw-pinch reflex was administered every ten to fifteen minutes. Once sedated, the scalp was shaved where injury would be made. Rats were placed in a stereotactic frame attached to the impactor. The head was maintained at a horizontal position via ear bars and jaw fixed to biting plate. To protect corneal membranes during surgery, an ophthalmic lubricant ointment was applied to eyes. Betadine was applied prior to making a midline incision to the skin above the skull with a sterile scalpel-blade. The skin, fascia, and temporal muscles were reflected bilaterally and held back with hemostats, keeping skull exposed during procedure. A 3.5-mm circular craniotomy was performed on the left side at 2mm lateral to the mid-sagittal suture and 2-mm below the bregma with a stainless steel trephine and Burr-Drill. A 3-mm rigid impactor (Leica Benchmark Stereotaxis Impactor) was driven by an electromagnetic piston and applied perpendicularly to the exposed dura mater, moving at 3.5 m/sec. The zero point was obtained by lowering the impactor tip until it reached the dura mater. The injury was induced by a 2.5-mm depression that remained in cortical tissue for 500ms. After the injury, the incision was closed with an absorbable suture, single stitch and Lidocaine Hydrochlorid Jelly USP 2% and Bacitracin was applied to the sutured area. Animals that were part of the sham group, received an identical anesthesia and surgery without brain injury. Animals maintained a body temperature of 37 degrees using a thermostatically controlled heating pad. Once paw and tail reflexes were
regained and normal breathing was observed, animals was transferred to cage to be monitored for discomfort or distress before returning to housing area.
Figure 2.1 Controlled Cortical Impact Injury Device. Photograph of the device used to deliver the controlled cortical impact injury (CCI) to Sprague-Dawley rats.
2.3 TrkB agonist, 7,8-Dihydroxyflavone (7,8-DHF), Administration

The small molecule flavonoid, 7,8-Dihydroxyflavone (7,8-DHF,) has the therapeutic potential for TBI and other neurological diseases such as amyotrophic lateral sclerosis, Parkinson disease, and Alzheimer’s disease (Jang et. al., 2010; Wu et. al., 2014). Due to its ability to mimic BDNF by binding to TrkB receptors and initiating signal cascades, it can influence neuronal survival, neuronal differentiation, and neurogenesis (Chen et. al., 2011).

Animals receiving DHF treatment were administered 5mg/kg of 7, 8 DHF intraperitoneal (IP) of single doses for 5days/doses starting at the following 4 regimes: Group 1 received DHF given 0-4 dpi, starting at 1 hour post injury (n=10), Group 2 received DHF given 2-6 days post injury (n=8), Group 3 received DHF given 3-7 days post injury (n=8), Group 4 received DHF given 5-9 days post injury(n=5). A total of 31 injured animals received 7,8-DHF.

To prepare 7,8-DHF for injection, 7,8-DHF was weighed out and dissolved in DMSO, then added to sterile PBS. The final vehicle composition was 17% DMSO in sterile PBS (v/v). 7,8-DHF was mixed with the vehicle at the concentration of 10mg/ml. Animals received intraperitoneal administrated of 5mg/kg of 7,8-DHF using a 26 gauge needle. The Sham Group received a vehicle(n=8). The Sham group and the CCI + Vehicle group received vehicle (17% DMSO) injection only. Lastly the CCI with vehicle group received 15% DMSO given 0-4 days post injury (n=13).
2.4 Motor Function Tests: Beam Walk and Rotarod Tests

Behavior tests add another level of measurement when determining the severity of a TBI and the effects of therapeutic treatment. Any motor deficit consequences were measured by evaluating simple reflexes with the beam walk and rotarod. These tests are able to evaluate the balance, coordination, and walking of animals, and mimic everyday activities. Since these are not reflexive behaviors, they require pre-surgery training to ensure that the animal has learned the task and has become acclimated to environment of the test. The pre-injury results become the baseline for the animal. The beam walk task is able to assess the animal’s coordination of muscular movements in order to maintain equilibrium. It is able to test simple, gross vestibulomotor function and balance/cerebellar function (Fujimoto et. al., 2004). The rotarod task also has the ability to detect motor impairments. It has also been able to demonstrate a higher sensitivity in motor analysis and dysfunction after a TBI compared to the beam walk task due to the increasing challenge of the animal to maintain equilibrium at increasing speeds (Hamm et. al., 1994). All animals were administered behavioral tests.

2.4.1 Beam Walk

During the beam-walk task, the rats were trained to move away from a bright light paired with a loud white noise. The rats would move horizontally across an elevated 100-cm long and 1.5cm wide narrow beam with the light and noise on one end and a darkened box at the other end. At equal distances and alternately placed on either side of beam, four pegs of 3mm diameter protrude upwards from beam, forcing the rat to move around these pegs to get to darkened box. Rats were first trained on beam by placing them facing and closer to the darkened box.
until the rat could travel the entire length of the beam. During training, once the box was reached, the rat was allowed to stay in box for 60 seconds. The rat was considered trained in the beam walk task when he was able to cross the beam under 10 seconds for three consecutive times. For the testing part of the beam-walk, the rats were placed on the end of the beam near the light and noise source and timed until it reached the darkened box. During testing, the animal was allowed to remain in box for 30 seconds. The behavior was assessed by the latency of the animal being able to reach the darkened box. Again, this was tested three consecutive times. The timer was stopped when rat’s body entered the box. The maximum amount of time that a rat could attempt beam-walk was 60 seconds. If the animal fell off or jumped off beam, his score was documented at 60 seconds. Training was administered one day prior to CCI. Testing of beam-walk occurred at 1, 3, 5, and 7 days post injury.

2.4.2 Rotarod

The rotarod instrument consists of a wheel made of 1mm stainless steel rods that spin around via motor. There are 18 rods that create a cylindrical shape. When placed on the wheel, the animal is able to grip the rods with his paws, changing his footing on the rods while it is rotating. A Plexiglas disc is on either side of the wheel, framing the area where the animal is to walk. Adjacent to the wheel is a control box that can be used to control speed, direction, rotational direction, and brake. The goal of the task is for the animal to walk on the rods/rungs while the rods/rungs are rotating without falling off at an increasing speed until 30rpm is reached. Initially, the animal is placed on the wheel for ten seconds at 0 rpm. After the wheel begins to rotate, it increases in speed at about every 15-20 seconds, adding to the sensitivity of
the task. The animal is timed, starting when the wheel begins to move, until he falls completely off the rotating wheel or is able to continue walking on wheel for 120 seconds. If the animal turns around on the wheel twice or grips rungs and rotates with wheel for two consecutive rotations, it is considered a fall and the timer was stopped, indicating the animal’s score. Training of the rotarod occurred day prior to CCI. Testing of rotarod was administered at 1, 3, 5, and 7 days post TBI.

2.5 Cognitive Function Tests: Morris Water Maze (MWM) and Fear conditioning

Cognitive dysfunction is another layer of trauma that occurs after a TBI. Two common methods to evaluate cognitive dysfunction are the Morris water maze (MWM) and the fear conditioning test. A total of 52 Sprague-Dawleys were administered cognitive tests. Of those 52 rats, 8 were part of the Sham group, 13 were part of the CCI + Vehicle group, 10 were part of Group 1 (CCI + DHF given 1-4 dpi), 8 were part of Group 2 (CCI + DHF given 2-6 dpi), 8 were part of Group 3 (CCI + DHF given 3-7 dpi), and 5 were part of Group 4 (CCI + DHF given 5-9).

2.5.1 Morris Water Maze (MWM)

For the TBI model that uses a CCI in rats, mazes are sensitive enough to detect the severity of the injury and the efficacy of therapy. Behavioral tasks, such as mazes and boxes, allow for non-verbal animals to be assessed for cognitive deficiencies. In order to complete a maze, there must be a visuospatial memory created and then recalled during completion. In the case of the MWM, it is a versatile maze that can be used to test retrograde amnesia or memory, anterograde amnesia or learning, spatial reference memory, spatial working memory, and motor deficit. The ability to use spatial navigation and create episodic memories is
hippocampally mediated. The prefrontal cortex mediates the functions involved with learning the ways to solve a problem, for example swimming to platform. Therefore, the MWM can be used to evaluate damage that has occurred in several areas of the brain due to TBI (Fujimoto et. al., 2004).

The MWM consists of a round water tank that is six feet in diameter and three feet deep. Permanent extra-maze cues were placed in room on the walls i.e. book shelf and pictures on walls. The tank was filled with 12cm of water and made opaque with 500ml of white latex paint. The water temperature was maintained at approximately 22.2 degrees Celsius. A platform that is 11cm tall is placed in one of the four equal quadrants. The appearance of the platform is concealed due to the opaque water. To allow for recovery of residual motor deficits, animals were tested on days 12 through 16 after TBI. The animal was tested by placing him in tank, facing the wall of the tank in one of the four quadrants, consisting of north, south, east, and west, which was randomly picked. The platform was placed in the south-west quadrant 25cm from the rim of the tank. For each trial, a new starting point, or quadrant was picked until the animal had completed four trials for each of the five days. The animal was allotted 120 seconds to find the platform and once it was reached the animal would rest on the platform for 30 seconds. If the animal was unable to find the platform in 30 seconds, then he would be taken out of the water and manually placed on the platform for 30 seconds. A computerized video tracking system monitored and recorded the paths of the animal. That latency time to find the platform and the path length were analyzed to determine cognitive deficits. The longer the animal took to reach platform indicated a deficient in learning function. A probe trial is a measurement for memory function which was conducted 24 hours after the last of latency test
which involved removing the platform and letting the animal swim in tank for 60 seconds. Measurements of the time that the animal spent in the quadrant where the platform was prior were recorded.

2.5.2 Fear Conditioning

Fear conditioning uses associative learning to determine the degree of cognitive dysfunction. Associative learning is pairing of a conditioned stimulus (CS) with a unconditioned stimulus (US) that leads to conditioned response (CR), which can be elicited without the presence of the US. In the case of fear conditioning, a tone (the CS) is paired with a footshock (the US) and freezing behavior, or a refraining of movement, is evoked (CR). The freezing behavior, with the exception of movement due to respiration, has been documented as a behavioral response to fear in rodents. Initially, the animal has no fear-like response to the tone and continues to move around the cage in an un-effected manner. After pairing, the animal will freeze when the tone is heard even when the footshock does not occur. It is an indication that the animal has learned that the sound of a tone will result in an aversive stimulus, a shock. Measuring the freezing time when the animal is in the box and when he hears the tone, allows for the evaluation of fear memory (Lai et. al., 2012).

Animals were placed in a Plexiglas chamber with a stainless steel grid bottom. The chamber is equipped with a light, video camera, and speaker. The stainless steel bars are able to administer a shock to the animals’ feet. The video camera allows for the monitoring and recording of the animal’s movement. On day one of fear conditioning, animals were allowed to acclimatize themselves with the chamber for about 120 seconds. After 120 seconds, a tone was presented for 30 seconds. Delay conditioning is conducted by presenting a mild electric shock
with duration of 0.5 seconds administered to the animal’s feet through the grid floor at the end of the tone on the first day of training. On day two of fear conditioning, the animal was placed in the chamber again for a total of 300 seconds with no tone or administration of shock. Four hours after the test with no tone or shock, the animal is placed in chamber again for a total of 180 seconds. A tone is presented towards the end of the time in the chamber during this time, starting at 120 seconds and lasting for 60 seconds. Experimenters remained silent throughout all experiments to avoid associative learning with additional sounds other than the tone. The amount of time that the animal refrained from moving, or freezing behavior, was measured on day two of experiment, including the 300 seconds without tone or shock and the 180 seconds with tone. Respiration movement was exempt from calculations. Testing of Fear Conditioning was administered on days eight through nine post CCI.

### 2.6 Tissue Processing

After completing behavior tests, animals were sacrificed at 16 or 28 days post injury. Animals were deeply anesthetized with isofluorane inhalation and transcardially perfused 400ml of 1x phosphate buffer solution (PBS) and then perfused with 400 ml of 4% paraformaldehyde in PBS. Solutions were made on the day of sacrifice. After opening the animal’s skull, brains were removed and post-fixed with 4% paraformaldehyde in PBS at 4°C. Post-fixation occurred for a minimum of 48 hours, after which, brains were sliced using a Leica vibratome. Starting at the rostral end of the brain, coronal sections were cut into 60 or 50 µm thick sections for a total of 120 sections collected from each brain. Once sliced and collected, brain tissue sections were placed in five 24-well plates with each well containing PBS + 0.02% sodium azide. All tissue sections were stored at 4°C in a refrigerator until needed for staining procedures.
2.7 Immunohistochemistry: Giemsa, Biotinylated Dextran Amine (BDA), and VGlut1

2.7.1 Giemsa Staining

Giemsa is a dye that is able to stain the phosphate groups of DNA. It more predominately attaches to regions that have adenine-thymine bonding and is often used to identify chromosomes (Prainsack et. al., 2014). Giemsa histological staining was used to determine the total lesion volume induced by CCI and the number of neurons that were present in different areas of the hippocampus.

Brain tissue sections spanning the rostro-caudal extent of the hippocampal dentate gyrus area were selected for staining. These sections were relative to the coordinates of -2.5 to -5.2 from the Bregma according to the Paxinos and Watson stereotaxic rat atlas (Paxinos & Watson, 1986).

The tissue sections were mounted on microscope slides and dried overnight, then processed with Giemsa histochemical staining. The tissue sections were immunostained with Giemsa in the following manner: 10% Giemsa solution made from Giemsa stock solution (EMS, #26156-01) 30ml, distilled water 270ml, and 400ul of 0.5% acetic acid. Giemsa solution was filtered, heated to 60°C, and poured into a staining pot. Tissue sections were first soaked in running tap water for three minutes before putting them in warmed Giemsa solution and incubated for 45 minutes at 60°C. Then sections were rinsed in running tap water for two minutes. After rinse, tissue sections were dipped in a differentiation bath of 0.5% acetic acid for one minute, then a quick rinse of deionized water. Sections were dipped in 95% alcohol, 100% alcohol, and 100%
alcohol for thirty seconds in each bath consecutively. Lastly, sections were placed in two Xylene baths for five minutes each then coverslipped.

2.7.1.1 Cortical Injury Volume

A total of 33 Sprague-Dawleys were evaluated for lesion volume (Table 2.2). Of those 33 rats, 9 were part of the CCI + Vehicle group, 10 were part of Group 1 (CCI + DHF given 1-4 dpi), 8 were part of Group 2 (CCI + DHF given 2-6 dpi), 4 were part of Group 3 (CCI + DHF given 3-7 dpi), and 4 were part of Group 4 (CCI + DHF given 5-9).

Using a 4x objective, the injury site of the moderate controlled cortical impact was outlined. The area of the injury site from five sections was measured and added together. The total injury site area was multiplied by four and the sliced width of the section (i.e. 60µm) to calculate a mean in µm$^3$ and then converted to mm$^3$ to determine the volume of injury in mm$^3$. 
<table>
<thead>
<tr>
<th>Giemsa</th>
<th>Sham</th>
<th>Vehicle + CCI</th>
<th>Group 1, DHF 0-4 dpi</th>
<th>Group 2, DHF 2-6 dpi</th>
<th>Group 3, DHF 3-7 dpi</th>
<th>Group 4, DHF 5-9 dpi</th>
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<tr>
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<td>8</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>33</td>
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<tr>
<td><strong>Hippocampal Cell Counts</strong></td>
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<td>5</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>25</td>
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</tbody>
</table>
Table 2.2 Number of Animals Used from Each Treatment Group to Determine Lesion Volume and Hippocampal Cell Counts. Table showing the total number of animals used for Giemsa staining according to the animal treatment group and area of interest.
2.7.1.2 Hippocampal Neuronal Cell Counts

Sections from a total of 25 rat brains (Table 2.2) were evaluated for hippocampal cell counts. Neuronal cell counts in the hippocampus were determined using the same brain tissue sections selected for measuring volume of injury site (i.e. -2.5 to -5.2 from Bregma). A stereological optical fractionator method was used to assess the number of neurons in the ipsilateral CA3, hilus, and granular region of the hippocampus. A total of 5 sequential sections 240um apart were assessed for each brain. These areas are vulnerable to TBI injury and can be easily quantified. Using a 4x objective, the area to be counted was outlined i.e. CA3, hilus, or granular region. A 60x oil immersion objective was used to count the cells in the outlined area. The Giemsa-stained neuronal nuclei cells with predetermined $x$, $y$ intervals were counted using an optical dissector counting frame. The stage-stepping intervals that moved over the section were relative to the known area ($a$) of the counting frame. The sampling fraction ($asf$) is equal to the $a$ (frame)/$a$ ($x,y$ step). The section thickness ($t$) was relative to the dissector height ($h$). Given the parameters, total cell counts could be estimated with $n = \Sigma Q\bar{\cdot} \frac{t}{h} \cdot \frac{1}{asf} \cdot \frac{1}{ssf}$. The $\Sigma Q$ is the number of cells counted and the $ssf$ is the section-sampling fraction, which is 0.25. Neuronal cell counts in the CA3 and granular region were determined using the $n = \Sigma Q\bar{\cdot} \frac{t}{h} \cdot \frac{1}{asf} \cdot \frac{1}{ssf}$ method, while the entire outlined hilus region (area between granule cell layer and CA3/4 with scattered neurons) was scanned under 60x objective and each hilus neuron cell body was counted individually. The neurons that were counted all demonstrated clear nucleoli defined in a nuclear membrane.
2.7.2 Biotinylated Dextran Amine (BDA) Staining

BDA is a commonly used neuronal tracer when injected into the nervous system. After injection, BDA can be taken up by cell bodies and dendrites of neurons then transported anterogradely or taken up by the axon terminals and transported retrogradely back to cell body (Xue et. al., 2004). High molecular weight BDA (10k) provides labeling of axons and terminals, while low molecular weight BDA (3k) provides labeling of neuronal cell bodies. It can later be visualized through the use of an avidin-biotinylated HRP, or ABC, procedure and enhanced with diaminobenzindine (DAB) (Reiner et. al., 2000). In this study BDA was used for anterograde labeling of inter- and intracortical axon sprouting in the cerebral cortex around the injury site. A total of 9 rats were studied for BDA labeling to asses axon sprouting (Table 2.3).
### Immunohistochemistry

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Vehicle + CCI</th>
<th>Group 1, DHF 0-4 dpi</th>
<th>Group 2, DHF 2-6 dpi</th>
<th>Group 3, DHF 3-7 dpi</th>
<th>Group 4, DHF 5-9 dpi</th>
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<td>0</td>
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<td>2</td>
<td>16</td>
</tr>
</tbody>
</table>
Table 2.3 Number of Animals used for Biotinylated Dextran Amine (BDA) and VGlut1 Labeling.

Table showing the total number of animals used for biotinylated dextran amine (BDA) and VGlut1 labeling according to the animal treatment group.
The protocol for the use of biotinylated Dextran Amine (BDA) in inter- and intracortical neuronal labeling after stroke was successfully used by X. Liu et. al. (2010) and similarly used in this experiment. BDA was injected into the injury contralateral cortex 14 days post injury. Rats were placed in a stereotactic frame and a cranionectomy was performed on the injury contralateral side. A BDA solution consisting of 100nl (a 10% solution in 0.1 mol/L PBS, 10,000 MW) was injected through a finely drawn glass capillary into a forelimb motor cortex with the stereotactic coordinates of 0.5, 1.5mm rostral to the bregma, 2.5 lateral to the midline, at a depth of 1.5 to 1.7 mm from the cortical surface and being administered for a duration of three minutes. Animals were sacrificed two weeks after BDA injection at 28 days post-TBI.

After perfusion and fixation, a vibratome was used to cut 60 or 50 µm brain tissue sections from each brain. Four sections from each brain injected with BDA were used to detect labeling with relative coordinates of 1.2 to -2.0 from the bregma according to the Paxinos and Watson stereotaxic rat atlas (Paxinos & Watson, 1986). A mean pixel intensity, determined from the four sections of each BDA labeled brain, was used to analyze inter- and intracortical staining. The tissue sections were immunostained in the following manner: sections were rinsed with phosphate buffer saline (PBS) twice, five minutes each rinse and placed on shaker during rinses. Then sections were quenched with 3% hydrogen peroxide for one hour and placed on shaker. After one hour in hydrogen peroxide, sections were rinsed with PBS for ten minutes and placed on shaker. Due to the Biotin-conjugated antibodies, sections were then incubated with avidin-biotin complex (ABC) for two hours. ABC was made 30 minutes in advance and made at a 1:200 dilution in PBS. After ABC step, sections were washed again with PBS for ten minutes, three times, on shaker. A DAB solution was made with 10mg of DAB in 40ml of 0.1M phosphate
buffer saline and 17.5 µl of 30% hydrogen peroxide. The sections were incubated with DAB solution for five to fifteen minutes at room temperature while being monitored and controlled under a microscope. Reaction with DAB was stopped with PBS and additional washes with PBS.

Tissue sections were then mounted onto slides and counterstained with Nissel.

Nissel staining was achieved in the following manner: place mounted tissue sections in 75% alcohol for one minute two times. Then place sections in 0.1% Nissel for one minute. Next wash sections with running tap water. Differentiate by dipping sections in acid alcohol a few times. Acid alcohol solution consisted of 75% alcohol and a few drops of 2N hydrochloric acid. Evaluate color change. Place sections in 95% alcohol for one minute, then in 100% alcohol for one minute (two times), and then in Xylene for five minutes (two times). Coverslip sections with permount and glass cover.

After Nissel staining, sections were examined by a light microscopy. Images were photographed at 10x and than evaluated with ImageJ program. A mean pixel intensity, determined from the four sections of each BDA labeled brain, was used to analyze inter- and intracortical staining.

2.7.3 VGlut-1 fluorescent staining

In the brain, specific vesicular glutamate transporters (VGLUTs) load glutamate into excitatory neurotransmitters into synaptic vesicles. More specifically, VGLUT1 is predominately found in the hippocampus. The regulation of VGLUT expression can affect glutamate release and may play a role in presynaptic plasticity (Hel et. al., 2009).
A total of 16 Sprague-Dawleys were evaluated for Vglut 1 staining (Table 2.3). After animal sacrifice, a vibratome was used to cut 60 or 50 µm brain tissue sections from each brain. Brain tissue sections spanning the rostro-caudal extent of the hippocampal dente gyrus area were selected for staining. These sections were relative to the coordinates of -2.5 to -5.2 from the bregma according to the Paxinos and Watson stereotaxic rat atlas (Paxinos & Watson, 1986). These selected tissue sections were immunostained with VGlut1 in the following manner: sections were rinsed with phosphate buffer saline (PBS) twice, five minutes each rinse and placed on shaker during rinses. Then sections were quenched with 3% hydrogen peroxide for one hour and placed on shaker. After one hour in hydrogen peroxide, sections were rinsed with PBS for ten minutes and placed on shaker. Tissue sections were blocked overnight at 4˚C on shaker in a blocking buffer that consisted of 5% normal horse serum and PBS + 0.3% triton. The following day (day 2 of procedure), the primary antibody, consisting of guinea pig anti-VGlut1 (1:1000; Millipore, Temecula, CA) was placed on the tissue sections at 300µl per well/tissue. Sections were incubated at 4˚C on shaker for 48 hours. On day four of the procedure, sections were brought back to room temperature and rinsed with PBS + 0.3% triton for ten minutes three times. The sections were blocked again using the same blocking buffer as before (5% normal horse serum and PBS + 0.3% triton) for three hours on shaker. After three hours, the secondary antibody, consisting of Alex Fluor 568 goat anti-guinea pig IgG (1:1000, Life Technologies), was placed on sections at 4˚C on shaker overnight covered with tin foil. On day five of staining procedure, tissue sections were brought back to room temperature and washed with PBS for ten minutes, three times. Then the tissue sections were incubated with DAPI at a dilution of 1:1000 for 15 minutes on shaker. Again, tissue sections were washed with PBS for
ten minutes three times followed by a quick rinse with water twice. Tissue sections were mounted on glass slides and coverslipped with vectorshield sealed with nail polish and stored in a -20°C freezer.

After fluorescent staining, the hippocampus on the ipsilateral injury side was examined by a fluorescent microscope. Images were photographed at 4x. The intensity of staining was evaluated with ImageJ program. Mean pixel intensity was used to analyze excitatory synapse stained by VGlut1.

2.8 Statistical Analysis

The data gathered from behavioral, cognitive, and immunohistochemistry procedures was analyzed using SPSS software. Data is presented as the mean and standard error of the mean. A one-way analysis of variance (ANOVA) followed by a post-hoc Fisher least significant and difference (LSD) test or the Duncan Multiple Range test was performed to identify significance between groups. Significance was determined by p-values less than 0.05.
Chapter Three – Results

3.1 The Effect of 7,8-DHF Treatment on Motor Function

The tests that evaluated behavioral and motor skills included the beam walk task and the rotarod. Training of beam walk task and rotarod was administered one day prior to CCI. Testing of behavioral/motor tasks occurred on days 1, 3, 5, and 7 post-CCI. An ANOVA was used to determine significance among all treatment groups, followed by a post hoc Fisher least significant difference (LSD) to determine the pairwise significant differences among group means.

3.1.1 Beam Walk

Using ANOVA, it was determined that there was an overall significant difference between all treatment group mean scores of the beam walk task on days 1 (p = .001), 3 (p = .001), 5 (p = .000), and 7 (p = .013) post-CCI. Post hoc comparisons, using Fisher LSD, were used to evaluate pairwise differences among group means.

Post hoc comparisons of day 1 post-CCI of beam walk task showed that the Sham group mean (5.68 ± 1.53 seconds) was significantly shorter than the mean difference scores of the Group 1 (9.32 ± 2.13 seconds, p = .036), Group 2 (11.53 ± 4.13 seconds, p = .002), Group 3 (11.14 ± 2.05 seconds, p = .004), Group 4 (10.20 ± 2.42 seconds, p = .030), and the CCI + Vehicle group mean (13.23 ± 5.40 seconds, p = .000). In addition, there was also a significant difference between Group 1 and the CCI + Vehicle group, p = .012, on day one post brain injury of beam walk task.
At the time of testing of day 1 post CCI of beam walk test, the only group to have received 7,8-DHF treatment was Group 1.

Post hoc comparisons of day 3 post CCI of beam walk task also showed similar results. The Sham group (3.97 ± .94 seconds) spent significantly less time to transverse the beam compared to Group 2 (7.47 ± 2.27 seconds, p = .009), Group 3 (7.95 ± 2.13 seconds, p = .003), Group 4 (7.76 ± 2.01 seconds, p = .012), and the CCI + Vehicle group mean (9.66 ± 4.12 seconds, p = .000). In addition, the CCI + Vehicle group continued to have a significant difference compared to the mean difference score of Group 1 (6.35 ± .79 seconds, p = .004).

All animals continued to exhibit improvement on beam walk task on day 5 of testing. However, the Sham group (3.34 ± .90 seconds, p = .000), Group 1 (4.20 ± .42 seconds, p = .000), Group 2 (5.04 ± .90 seconds, p = .008), and Group 3 (5.37 ± .50 seconds, p = .024) were all significantly different from the CCI + Vehicle group (7.15 ± 2.58 seconds, # p < .05). This trend of only Group 4 (3.84 ± .39 seconds) not having a significant difference compared to CCI + Vehicle group (4.85 ± 1.20 seconds, #p < .05) continued into day 7 of beam walk testing (Sham, 3.06 ± .78, p = .004; Group 1, 3.01 ± .46, p = .002; Group 2, 3.52 ± .544, p = .040; Group 3, 3.12 ± .35, p = .005).

For all time points post CCI of the beam walk task, there was an improved score among all experimental groups (Figure 3.1). However, 7,8-DHF treatment Group 1 performed significantly better at every time point of the beam walk task compared to CCI + Vehicle (*p < .05). Treatment Group 1 also had a mean score on day 7 of beam walk testing comparable to the Sham group (3.01 ± .46, 3.06 ± .78 respectively). 7,8-DHF treatment Group 4 did perform better than the CCI + Vehicle treatment group but not enough to be significant at any time point (p >
These results indicate that 7,8-DHF can provide neural protection after a TBI when starting administration one hour after injury, leading to better motor recovery and even possible extinction of motor deficits.

3.1.2 Rotarod

Using ANOVA, it was determined that there was an overall significant difference between treatment group mean scores of the Rotarod test on days 1 (p < .001), 3 (p =.001), and 5 (p =.074) post-CCI. Post hoc comparisons, using Fisher LSD, were used to evaluate pairwise differences among group means. Compared to all other treatment groups, the Sham group performed significantly better on day 1, 3, and 5 of Rotarod test (*p < .05). All animals that received a CCI, including those that received 7,8-DHF and Vehicle doses, improved in their ability to stay on the Rotarod at every time point; however, the only significance was in relation to those animals that did not receive a CCI (Figure 3.1). No difference was found among injured animals.
A  
**Beam Walk**

- Sham
- CCI + Veh
- Group 1
- Group 2
- Group 3
- Group 4

B  
**Rotarod**

- Sham
- CCI + Veh
- Group 1
- Group 2
- Group 3
- Group 4
Figure 3.1 Administration of 7,8-DHF Starting One Hour after TBI Significantly Improved Beam Walk Results but 7,8-DHF Did Not Improve Rotarod Results. (A) In the beam walk test, injured animals with vehicle treatment took a longer time to transverse the beam than sham group, demonstrating motor deficits ($p<.05$). Among injured animals, 7,8-DHF treatment group 1 spent less time to transverse the beam than the vehicle group, indicating improved motor function ($p<.05$). 7,8-DHF treatment group 4 did not show a difference compared to vehicle. (B) In the rotarod test, all injured animals spent less time on rotating wheel than the sham group, indicating motor deficits ($p<.05$).
3.2 The Effect of 7,8-DHF Treatment on Cognitive Functions

The cognitive tests used included the Morris Water Maze (MWM) and fear conditioning test. The MWM was administered on days 12-15 post CCI. Latency was tested days 12-15 and the probe trial was administered on day 16 post CCI with a 24 hour delay after the last latency test. The fear conditioning test was administered days 8 and 9 post CCI. An ANOVA was used to determine significance among all treatment groups, followed by a post hoc Fisher LSD to determine the pairwise significant differences among group means in the probe test of the MWM and for fear conditioning results. The Duncan Multiple Range test was used to determine specific group differences in the latency testing of MWM.

3.2.1 Morris Water Maze (MWM): Latency

A group x day split-plot ANOVA test was used to analyze MWM tests. The Duncan Multiple Range test was used to determine specific group differences. Comparisons of days 13, 14, and 15 post-CCI of MWM showed that the Sham group was significantly different compared to all other groups (*p<.05). On day 14 of the MWM latency testing, 7,8-DHF treatment Group 2 mean (35.82 ± 7.89 seconds) and Group 3 mean (35.10 ± 3.46) were also significant compared to Group 4 (47.24 ± 10.40 seconds, *p < .05). On day 15 of MWM latency testing, both Group 1 and Group 2 performed better and were able to reach the submerged platform sooner than Group 3, Group 4, and CCI + Vehicle group; however, the differences between means was not significant (p > .05) (Figure 3.2). Overall, administration of 7,8-DHF starting two and three days after TBI appears to provide some mild protection against hippocampal spatial memory and prefrontal cortex learning deficits.
Comparisons of proximity to platform in MWM during latency testing indicated that there was a significant difference on day 15 of testing (p < .05). This was determined to be the Sham Group (22.25 +/- 2.50, *p<.05), being statistically closer in proximity to hidden platform than all other groups [Group 1 (28.85 +/- 5.25), Group 2 (28.75 +/- 2.36), Group 3 (32.69 +/- 4.27), Group 4 (31.40 +/- 7.99), and CCI + Vehicle group (32.35 +/- 7.18)]. 7,8-DHF treatment Group 2 had a closer proximity to the platform days 13-15 of MWM compared to all other animals that received a CCI; however, this was not significant (p > .05). This trend in closer proximity to the platform by Group 2 suggests that administration of 7,8-DHF starting two days after brain injury may provide some protection against deficits in hippocampal learning and memory (Figure 3.2).
Figure 3.2 Administration of 7,8-DHF Starting Two and Three Days after TBI Improves Morris Water Maze Latency

(A) In MWM latency testing, the injured animals with vehicle treatment took longer to find the hidden platform compared to sham on days 13-15 dpi, demonstrating cognitive deficits (*p<.05). On day 14 dpi, 7,8-DHF treatment Group 2 and Group 3 had shorter latency times compared to Group 4 (*p < .05), indicating some improved memory function. 7,8-DHF treatment groups 1, 2, 3 and 4 did not have difference compared to vehicle group.

(B) In the MWM proximity to platform during latency testing, sham animals had a smaller proximity to hidden platform than the injured vehicle treatment group on day 15 post injury (*p<.05), indicating cognitive deficits after brain injury. All 7,8-DHF treatment groups did not show a difference compared to vehicle.
3.2.2 Morris Water Maze (MWM): Probe Test

ANOVA results indicated that there was a significant difference between treatment group means for the MWM probe test, time spent in the goal quadrant, or 4th quadrant, where the platform was previously placed for latency testing on days 12-15 post CCI (p < .05). Further evaluation using the post hoc comparisons, LSD, determined that the average time that Group 4 (24.46 ± 6.91 seconds, *p < .05) spent in the goal quadrant was significantly less than the Sham Group (37.38 ± 5.84, p = .000), Group 1 (36.19 ± 5.53, p = .001), and Group 3 (32.51 ± 2.28, p = .020). The CCI + Vehicle group mean (25.04 ± 7.68) was significantly less than (*p < .05) all other groups except Group 4 (Figure 3.3).

During the probe test, the proximity to the missing platform was also measured. ANOVA results indicated that there was a significant difference between treatment group means for the MWM probe test, proximity to where the platform was previously placed for latency testing on day’s 12-15 post CCI (p = .002). Further evaluation using the post hoc comparisons, LSD, determined that the average proximity that Group 1 (43.79 ± 6.99, *p = .019) and Group 3 (44.54 ± 4.51, *p = .048) had to the once placed platform was significantly less than the CCI + Vehicle group (51.04 ± 9.55). Group 2 (47.20 ± 4.64) and Group 4 (47.44 ± 10.16) also had mean proximities less than the CCI + Vehicle group; however, these were not significant (p > .05) (Figure 3.3). In addition, the Sham group’s mean proximity (36.41 ± 3.47) during the probe test was also significantly less compared to all groups (p < .05). The results from the probe portion of the MWM testing indicate that 7,8-DHF administration starting at one hour or three days after brain injury can provide protection against hippocampal memory loss associated with TBI.
A

MWM Probe Trial

Time in the Goal Quadrant (seconds)

Sham  CCI + Veh  Group 1  Group 2  Group 3  Group 4

0  5  10  15  20  25  30  35  40  45

B

MWM Probe Trial

Proximity to Missing Platform (cm)

Sham  CCI + Veh  Group 1  Group 2  Group 3  Group 4

0  10  20  30  40  50  60

*
Figure 3.3 Administration of 7,8-DHF Starting One Hour and Three days after TBI Spent More Time in Goal Quadrant and Closer Proximity to Missing Platform. (A) In probe trial tests, injured animals with vehicle treatment spent much shorter time in the goal quadrant compared to sham, demonstrating significant memory deficits (*p<.05). Among injured animals, 7,8-DHF treated Group 1, 2, and 3 spent longer time in the goal quadrant that the injured vehicle group demonstrating improved memory function (*p<.05). 7,8-DHF treatment Group 4 did not show difference compared to Group 2 and vehicle group. (B) Uninjured animals were closer to the location of the previously placed platform than all injured animals, indicating significant memory deficits (*p<.05). Among injured animals, 7,8-DHF treatment groups 1 and 3 had closer proximities than injured vehicle group, indicating improved memory deficits (*p<.05). 7,8-DHF treatment groups 2 and 4 did not show difference compared to vehicle.
3.2.3 Fear Conditioning: Contextual and Cued

Fear Conditioning was measured through contextual and cued approaches. ANOVA scores for the contextual aspect of fear conditioning indicated that there was a difference between the sham group mean (37.20 ± 10.68) and CCl + vehicle group mean (16.29 ± 4.08, *p<.05), indicating that there is a deficit in hippocampal dependent memory function. Looking at the trend in the freezing percentages a slight pattern can be observed. Animals that received 7,8-DHF had a slightly higher freezing percentage during the contextual testing of fear conditioning compared to the CCl + Vehicle group, indicating there may be an improvement in hippocampal dependent memory function (Figure 3.4).

The ANOVA results for the cued portion of the fear conditioning test indicated that there was significant difference among groups (p < .05). Further post hoc, LSD, analysis showed that injured animals with vehicle treatment froze less than sham, demonstrating significant amygdala dependent memory deficits (*p<.05). Among injured groups, 7,8-DHF treatment groups 1 and 2 had a slightly higher but not significant freezing percentage compared to the CCl + Vehicle group, indicating there may be some improvement in amygdala dependent memory function. 7,8-DHF treatment group 3 did not show a difference compared to vehicle and froze significantly less than sham (*p<.05) (Figure 3.4).
**A**

Fear Conditioning - Contextual

- Sham
- CCI + Veh
- Group 1
- Group 2
- Group 3
- Group 4

**B**

Fear Conditioning - Cued

- Sham
- CCI + Veh
- Group 1
- Group 2
- Group 3
- Group 4
Figure 3.4: Administration of 7,8-DHF After TBI May Protect Against Hippocampal Dependent Memory Loss. (A) In the contextual part of fear conditioning test, injured animals with vehicle treatment froze less than sham, demonstrating significant hippocampal dependent memory deficits (*p<.05). Among injured groups, all animals that received 7,8-DHF had a slightly higher but not significant freezing percentage compared to the CCI + Vehicle group, indicating there may be some improvement in hippocampal dependent memory function. (B) In the cued portion of the fear conditioning test, injured animals with vehicle treatment froze less than sham, demonstrating significant amygdala dependent memory deficits (*p<.05). Among injured groups, 7,8-DHF treatment groups 1 and 2 had a slightly higher but not significant freezing percentage compared to the CCI + Vehicle group, indicating there may be some improvement in amygdala dependent memory function. 7,8-DHF treatment group 3 did not show a difference compared to vehicle and froze significantly less than sham (*p<.05).
3.3 The Effect of 7,8-DHF Treatment on Neuroprotection

Due to the fact that Giemsa dye is able to stain the phosphate groups of DNA (Prainsack et. al., 2014), it was used to determine the cerebral cortex lesion volume induced by CCI and the number of neurons that were present in different areas of the hippocampus.

3.3.1 Cortical Lesion Volume

The ANOVA results of the lesion volumes indicated that there was a significance among groups (p < .05). Further post hoc, LSD, analysis showed 7,8-DHF treatment Group 2 (1.13 ± 1.03) had a reduced lesion volume compared to the vehicle group (2.80 ± 1.64, *p = .019), indicating neuronal protection. 7,8-DHF treatment Group 1 (1.52 ± 1.23, *p = .040) and Group 2 (1.13 ± 1.03, *p = .014) had smaller lesion volumes compared to Group 4 (3.33 ± 1.39) (Figure 3.5). These results indicate administration of 7,8-DHF starting one hour and two days after a TBI can provide neuronal protection against cortex tissue loss after a TBI. 7,8-DHF treatment Group 3 did not have a difference compared to vehicle.
A

Lesion Volume

**Lesion Volume (mm³)**

- **Group 1 Ipsilateral Cortical Lesion**
- **Group 2**
- **Group 3**
- **Group 4**
- **CCI + Vehicle Ipsilateral Cortical Lesion**

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**Group 1 Ipsilateral Cortical Lesion**

**CCI + Vehicle Ipsilateral Cortical Lesion**
Figure 3.5 Cerebral Cortex Lesion Volume Significantly Reduced in Treatment Groups That Received 7,8-DHF Administration One Hour and Two Days After Injury Incident. (A) 7,8-DHF treatment Group 2 had a reduced lesion volume compared to the vehicle group, indicating neuronal protection (*p < .05). 7,8-DHF treatment Group 1 and Group 2 had smaller lesion volumes compared to Group 4 (*p < .05). These results indicate administration of 7,8-DHF starting one hour and two days after a TBI can provide neuronal protection against cortex tissue loss after a TBI. 7,8-DHF treatment Group 3 did not have a difference compared to vehicle. (B) Cortex tissue damage on the ipsilateral side of CCI animal that received 7,8-DHF within one hour of brain injury. (C) Cortex tissue damage on the ipsilateral side of CCI animal that received vehicle.
3.3.2 Hippocampal Neuronal Cell Counts

Giemsa staining allowed for the neurons to be counted in the CA3, hilus, and granular region (Figure 3.6). Areas were outlined with 4x objective and cells were counted with a 60x oil immersion objective. Neuronal cell counts in the CA3 and granular region were determined using the $n = \Sigma Q_t \cdot t/h \cdot 1/asf \cdot 1/ssf$ method, while the entire outlined hilus region (area between granule cell layer and CA3/4) was scanned under 60x objective and each hilus neuron cell body was counted individually. Neuronal cell counts in the CA3 of the hippocampus indicated that the injured animals with vehicle treatment (26,660.33 ± 623.75) had less amount of neurons compared to the sham group (33,074.23 ± 2839.32), indicating neuronal cell loss in CA3 (*p=.022). There was no difference among 7,8-DHF treatment animals and vehicle treated animals (p > .05) (Figure 3.6). In the hilus region, post hoc, LSD, revealed that injured animals with vehicle treatment (2,651.97 ± 73.07) had a smaller amount of cells compared to the sham group (4,580.10 ± 595.02, *p=.010), indicating neuronal cell loss. Group 2 (4,203.97 ± 1,269.09, *p = .032) was significantly higher than the CCI + Vehicle group, indicating neuronal protection. 7,8-DHF treatment Group 1 (3,736.89 ± 459.35), Group 3 (3,227.49 ± 693.81), and Group 4 (2,612.6) were not different from vehicle group (Figure 3.7). In the granular region, LSD analysis revealed that 7,8-DHF treatment Group 1 (160,995.32 ± 5,901.52, *p = .03), Group 2 (160,280.05 ± 20,562.50, *p = .034), and Group 3 (164,269.26 ± 36,402.41, *p = .025) had a larger amount of cells compared to the CCI + Vehicle group (129,721.43 ± 10,997.14), indicating neuronal protection. Group 4 (120,965.64) did not have a difference compared to vehicle (Figure 3.7). These results indicate that 7,8-DHF administration starting at one hour, two days, or three days post brain injury could provide neuronal cell protection.
**Figure 3.6 Giemsa Staining of Neurons in Hippocampus.**

(A) Giemsa stained neurons in the CA3 region of hippocampus. (B) Giemsa stained neurons in the hilus region of the hippocampus. (C) Giemsa stained neurons in the granular region of the hippocampus. (D) Neuronal cell count CA3 indicated that the injured animals with vehicle treatment had less amount of neurons compared to the sham group, indicating neuronal cell loss in CA3 (*p<.05). There was no difference among 7,8-DHF treatment animals and vehicle treated animals (p > .05) (Figure 3.6).
Figure 3.7 Administration of 7,8-DHF Starting Day Two After TBI had an Effect on Neuronal Cell Counts Hilus and Granular Region. (A) In the hilus region, post hoc, LSD, revealed that injured animals with vehicle treatment had a smaller amount of cells compared to the sham group (*p<.05), indicating neuronal cell loss. Group 2 was significantly higher in cell numbers than the CCI + Vehicle group, indicating neuronal protection (*p<.05). 7,8-DHF Groups 1, 3, and 4 did not show a difference compared to vehicle. (B) In the granular region, 7,8-DHF treatment Group 1, 2, and 3 had a larger amount of cells compared to the CCI + Vehicle group (*p<.05), indicating neuronal protection. 7,8-DHF Group 4 did not show a difference compared to vehicle. These results indicate that 7,8-DHF administration starting at one hour, two days, or three days post brain injury could provide neuronal cell protection.
3.4 The Effect of 7,8-DHF Treatment on Neural Plasticity

3.4.1 Biotinylated Dextran Amine (BDA)

When injected into the nervous system, biotinylated dextran amine (BDA) can provide detailed neuronal labeling. After injection, BDA can be taken up by cell bodies and dendrites of neurons then transported anterogradely (Xue et. al., 2004). In this study BDA was used for anterograde labeling of inter- and intracortical axon sprouting in the cerebral cortex around the injury site.

BDA was injected through a finely drawn glass capillary into the forelimb motor contralateral cortex for duration of three minutes 14 days post injury. Animals were sacrificed two weeks after BDA injection at 28 days post-TBI. Four sections from each brain injected with BDA were used to detect labeling with relative coordinates of 1.2 to -2.0 from the bregma according to the Paxinos and Watson stereotaxic rat atlas (Paxinos & Watson, 1986). A mean pixel intensity, determined from the sections of each BDA labeled brain, was used to analyze inter- and intracortical staining.

Through SPSS software, an ANOVA was used to analyze the mean relative density percentages of treatment groups. This revealed that there was a trend of slightly higher, but not significant, density readings, which may indicate increased intracortical axon sprouting in the ipsilateral injured cortex of animals that received 7,8-DHF treatment starting three days after brain injury (Figure 3.9).
Figure 3.8 BDA Labeled Intracortical Axon Sprouting of Ipsilateral Injury Cortex. (A) BDA labeling highlighted a slight trend that the axons of animals treated with 7,8-DHF three days after injury had slightly higher, but not significant, density readings, which may indicate increased intracortical axon sprouting. (B) Intracortical axon sprouting in the ipsilateral injured cortex of a Group 3 brain section. (C) Intracortical axon sprouting in the ipsilateral injured cortex of a CCI + vehicle brain section.
3.4.2 Vglut1

In the brain, specific vesicular glutamate transporters (VGLUTs) load glutamate into excitatory neurotransmitters into synaptic vesicles. The regulation of VGLUT expression can affect glutamate release and may play a role in presynaptic plasticity (Hel et. al., 2009). In this study Vglut1 was used to label synapse in the ipsilateral hippocampus. After fluorescent staining, images were photographed at 4x.

Only the CA3 region of the hippocampus showed Vglut1 staining. This region was further evaluated with ImageJ program. Mean pixel intensity was used to analyze excitatory synapse stained by VGlut1. ANOVA results indicated that there was no statistical difference between groups, p > .05. However, Group 4 (39.88 ± 6.46) had a higher mean score than Group 1 (29.01 ± 16.97), Group 2 (31.73 ± 9.87), Group 3 (33.48 ± 5.53) and the CCI + Vehicle group (33.07 ± 5.73), indicating that some neuronal plasticity is taken place in CA3 of the hippocampus (Figure 3.9).
A  VGlut1 in CA3 of Hippocampus

B  VGlut-1 Stain of Group 3 Hippocampus
**Figure 3.9 VGlut-1 Labeling of the Hippocampus (A)** A slight, but not significant, increase of VGlut-1 staining in treatment group receiving 7,8-DHF starting day five post brain injury compared to other brain injury groups, indicating that there may be some neuronal plasticity.

**(B)** Image of VGlut1 fluorescent labeling of CA3
Since the discovery of 7,8-DHF, it has been documented to provide neuroprotection and neuroplasticity in various neurological diseases and disorders (Liu et. al., 2012). 7,8-DHF has a much longer half-life and can easily pass the BBB, making it also a possible alternative in treating TBI (Zeng et. al., 2012; Wu et. al., 2014). This study addressed the effects of 7,8-DHF on neuroprotection and neuroplasticity after TBI when administered for 5 doses/daily starting at 0, 2, 3, and 5 days after a CCI. Sensorimotor function was evaluated with beam walk test and rotarod. Morris Water Maze (MWM) and fear conditioning test were used to analyze cognitive function. Brain sections were processed for Giemsa histological staining to calculate cortical lesion volume and the total number of surviving neurons. Parallel sections were also processed for BDA staining to identify axon sprouting in the injured cortex and VGlut-1 staining to assess presynaptic plasticity in the hippocampal region.

Sensorimotor tests revealed that animals receiving 7,8-DHF, starting one hour after brain injury, performed significantly better and were able to transverse the beam in a shorter length of time than vehicle treated animals at all time points. By day seven of the beam walk test, animals that had received 7,8-DHF treatment beginning one hour to three days after CCI performed better than those that started receiving 7,8-DHF at 5 days post injury. Our findings are similar to Wu et. al. (2014) who demonstrated that four doses of 20mg/kg of 7,8-DHF given to mice ten minutes after a CCI could significantly reduce neurobehavioral deficits. Results from this study and previously mentioned study coincide with a therapeutic window that could protect against
neurodegeneration. It is within the first hour of brain injury that caspases, the mediators of apoptosis, can be found in the thalamus and subcortical regions (Keane et. al., 2001). Cortical projection neurons in thalamus can be observed undergoing apoptosis in the following 24 hours (Natale et. al., 2002) and diffuse neurodegeneration in the ipsilateral cortex and thalamus has been found to peak between 48-72 hours. Damage to these cortical and thalamic neurons can result in motor dysfunction (Hall et. al., 2005). The results from this study indicate that the administration of 7,8-DHF starting at one hour after injury and before the peak of cortical and thalamic neurodegeneration at 48-72 hours can provide optimal neuroprotection against secondary damage and significantly decrease motor dysfunction due to TBI.

According to Hamm et. al. (1994) the rotarod is sensitive enough to evaluating motor function in even mild TBI cases. Animals that received a CCI in our study exhibited similar lengths of time on the rotarod at all time points. This is contrary to what was observed by the Wu et. al. (2014) study that found 7,8-DHF administration after brain injury resulted in better performance on Rotarod compared to vehicle treated animals. It is important to note that all CCI animals in our study did improve in their ability to stay on the rotarod over time, indicating that the rotarod was able to measure motor function and a natural motor recovery process can occur after brain injury. Conflicts in findings could be due to the 4x greater dose of 7,8-DHF administered in the Wu et. al. study and the type of animal used in the CCI model.

Previous animal models have concluded that 7,8-DHF can reduce cognitive deficits that result from synaptic loss in the hippocampus and cortex. Improved spatial learning and memory in the MWM was observed in an Alzheimer’s mouse model (Castello et. al., 2013) and in an aged rat
model that both administered 5mg/kg of 7,8-DHF doses for several days (Zeng et. al., 2012). Our results from the MWM latency testing did not corroborate previously mentioned studies that report 7,8-DHF improves spatial learning. Treatment groups that received 7,8-DHF starting one hour and two days after brain injury only showed a trend of better latency times than other CCI injury groups. However, animals that started receiving 7,8-DHF treatment at one hour or three days after brain injury performed significantly better on the MWM probe test. These results indicate that the administration of 7,8-DHF starting at one hour or three days after TBI can provide protection against hippocampal memory loss. This treatment regimen coincides with neurodegeneration in the hippocampus where apoptosis can start to occur within the first 24 hours (Zhou et. al., 2012) and peak between 48-72 hours after brain injury (Hall et. al., 2005).

According to the time frame of neurodegeneration, 7,8-DHF administration starting two days after brain injury should have also provided protection against hippocampal memory loss; however, we did not see this in our results. The hippocampus is one of the main areas of BDNF production (Lee et. al., 2002) and it has been demonstrated that BDNF levels increase after brain injury (Chiaretti et. al., 2003). In addition, the mature form of BDNF can bind to p75NTR receptor when there are high concentrations of BDNF (Boyd & Gordon, 2003). Perhaps the poor protection in memory loss observed in animals that received 7,8-DHF starting at two days post injury reflects the activation of the apoptotic pathway via the BDNF/p75NTR due to 7,8-DHF and BDNF competing for the TrkB receptors. In addition, the differences observed in spatial learning compared to Castello et. al. and Zeng et. al. could also be a result of the duration of 7,8-DHF.
treatment. This study provided 7,8-DHF for five days where it was given for two weeks in the Alzheimer’s model and for 34 days in aged rats.

In further evaluation of cognitive function, contextual results from the fear conditioning test also highlighted a trend that 7,8-DHF may provide some relief to hippocampal memory deficits observed with TBI, especially when 7,8-DHF administration begins one hour after injury. The cued portion of the fear conditioning test indicated that amygdala induced memory deficits were not significantly affected by 7,8-DHF treatment. Our findings contradict the Andero et. al. (2011) study that found that mice receiving 7,8-DHF had improved non-hippocampal memory, suggesting 7,8-DHF targets TrkB receptors in the amygdala and are critical for fear learning. Both studies used the same dose of 7,8-DHF; however, Andero et. al. (2011) used a mouse model that inflicted foot shock with tone for five trials, where we only conducted one trial.

Contusion measurements indicated that the administration of 7,8-DHF starting one hour or two days after brain injury resulted in significantly smaller lesion volumes compared to vehicle treated animals. This reflects reduced infarct volumes observed in a mouse stroke model that received 7,8-DHF two hours after injury (Jang et. al., 2010). Wu et. al. (2014) also found a significant reduction in lesion volume of ipsilateral hemisphere when 7,8-DHF was administered ten minutes after a CCI in mice. As mentioned previously, our results coincide with a possible therapeutic window that could protection against neurodegeneration seen within the first hour of brain injury (Keane et. al., 2001) and peaking between 48-72 hours (Hall et. al., 2005).

The effect that 7,8-DHF has on neuroprotection after TBI was also evaluated through examination of neuronal cell numbers in different regions of the hippocampus, an area most
vulnerable following brain injury. In the Baldwin et. al. (1997) study that looked at total number of neurons in the CA3 region of the rat hippocampus after a CCI, they predict a therapeutic window of less than 24 hours after brain injury to provide neuroprotective treatment. It is within this time period that they saw the most neuron cell death. According to their prediction, 7,8-DHF starting at one hour after brain injury should have resulted in more CA3 neuronal cells compare to other treatment groups in our study. Our findings of the CA3 region did not follow this prediction. There was no significant difference in CA3 cell numbers among 7,8-DHF treatment or vehicle groups. Comparatively, our CCI model inflicted a smaller contusion than the Baldwin et. al. (1997) study, which could explain our CA3 neuronal cell numbers.

Despite the fact that there was no significant neuronal protective effect on neuronal survival in the CA3 region, neuronal cell counts in the hippocampal hilus and the granular regions did show significant differences among 7,8-DHF treatment groups and the CCI + Vehicle group. In the hilus region, animals that received 7,8-DHF administration starting two days after injury had significantly more neuronal cells than the vehicle group. Neuronal cell numbers were also significantly higher in the granular region of 7,8-DHF treatment groups that started receiving doses one hour, two days, and three days post injury compared to the vehicle group. The results from the neuronal cell counts reflect 7,8-DHF’s effect on two different mechanisms that occur after TBI: neurodegeneration and neurogenesis. Again, the treatment regiment starting at one hour, two or three days after brain injury overlaps the time course of neurodegeneration in the hippocampus. However, it also coincides with TBI induced neurogenesis where it has been documented that there is increased cell proliferation after injury, (Chirumamilla et. al., 2002; Dash et. al., 2001; Sun et. al., 2005) peaking at two days after brain injury (Sun et. al.,
2005). The higher neuronal cell counts in the granular region suggest neuroprotective and neurogenic effects of 7,8-DHF after TBI.

In this study, biotinylated dextran amine (BDA) and VGlut1 were used to determine if varied times of 7,8-DHF administration after TBI had any effect on neuronal plasticity. BDA labeling highlighted a slight trend that the axons of animals treated with 7,8-DHF three days after injury had slightly more, but not significant, intracortical axon sprouting in the ipsilateral injured cortex compared to vehicle animals. This could reflect a better treatment window for neuroplasticity. However, due to variances in staining at site of injection, further analysis would need to be conducted to normalize labeling, ensuring that density readings were a result of labeled axon sprouting and not excess dye. VGlut1 results also highlighted a trend of neuronal plasticity taking place in the CA3 region of the hippocampus with the administration of 7,8-DHF. The area of VGlut1 staining is the same region where immature neurons extend their axons to create connections with the pyramidal neurons in CA3 (Bohlen und Halbach, 2007). These connections can occur within 4-10 days of birth (Hastings and Gould, 1999) which could explain why there was a slight increase in VGlut-1 staining in 7,8-DHF treatment groups that received treatment starting on day five post brain injury compared to treatment groups that received 7,8-DHF starting at one hour, two, and three days post injury.

Results from this study indicate that the administration of 7,8-DHF starting one hour after brain injury can provide neuroprotection evidenced by attenuated motor and cognitive impairments, reduce lesion volume and increased neuronal cells in the granular cell layer of the hippocampus. Administration of 7,8-DHF starting two days and three days post TBI also
exhibited some protection against neurodegeneration. Neurogenesis was not directly assessed in this study; however, we found indications that 7,8-DHF may promote neurogenesis via the increased number of neurons in the granule cell layer. Our BDA and VGlut-1 labeling highlighted a possible trend that neuronal plasticity could be enhanced with 7,8-DHF administration starting day five post injury. Further animals in the treatment groups that received 7,8-DHF starting day three and five post brain injury could provide the statistical significance needed to clearly indicate 7,8-DHF’s effect on neuronal plasticity. Additional staining with immature and mature neuronal markers could indicate the effect that 7,8-DHF has on neurogenesis after TBI.
List of References


