The Effects of 7,8-Dihydroxyflavone on Hippocampal
Neurogenesis Following Traumatic Brain Injury

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The Effects of 7,8-Dihydroxyflavone on Hippocampal Neurogenesis Following 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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Abbreviations

7,8-Dihydroxyflavone ................................................................. 7,8- DHF; DHF

Traumatic Brain Injury ................................................................. TBI

Subgranular Zone ........................................................................... SGZ

Granular Cell Layer ...................................................................... GCL

Controlled Cortical Impact ......................................................... CCI

Brain-derived neurotrophic factor ............................................. BDNF

Central Nervous System ............................................................. CNS

Blood brain barrier ...................................................................... BBB
Abstract

The Effects of 7,8-Dihydroxyflavone (7,8-DHF) on Hippocampal Neurogenesis Following Traumatic Brain Injury

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

Virginia Commonwealth University 2016

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

Following traumatic brain injury (TBI), the hippocampus is particularly vulnerable to damage, and BDNF, an endogenous neurotrophin that activates the TrkB receptor, has been shown to play a key role in the brain’s neuroprotective response. Activation of the TrkB signaling pathway by BDNF in the CNS promotes cell survival and aids in cell growth. However, due to its inability to cross the blood brain barrier (BBB), the therapeutic advantages of BDNF treatment following TBI are limited. 7,8-Dihydroxyflavone (7,8-DHF) is a flavonoid that mimics the effects of BDNF, is a potent TrkB receptor agonist, and can successfully cross the BBB. Our lab has previously demonstrated that administration of 7,8-DHF post-TBI results in improved cognitive functional recovery, increased neuronal survival, and reduced lesion volume.

The current study examined the effects of 7,8-DHF on neurogenesis and neuronal migration in the dentate gyrus following TBI. In this study, adult male Sprague-Dawley rats were subjected to moderate controlled cortical impact injury (CCI) or sham surgery. Injured animals received 5 daily single doses of 7,8-DHF treatment (i.p) or vehicle starting either 60 mins after injury or 2 days after injury. BrdU was administered in 3 doses at 2 days post-injury for animals sacrificed at day 15, and single daily doses at days 1-7 post-injury for animals sacrificed at day 28 to label cell proliferation. Animals were sacrificed at 15 days or 28 days post-injury to examine cell proliferation, generation of new neurons, and differentiation of newly generated
cells using proliferation marker Ki67, immature neuronal marker DCX, and BrdU double-labeling with markers for mature neurons (NeuN), astrocytes (GFAP) and microglia (Iba1).

We found that administration of 5 doses (5mg/kg) of 7,8-DHF beginning two days post-injury had the strongest effect on neurogenesis and migration, but did not have a significant prolonged effect on cell proliferation at 15 days post-injury. We also found that 7,8-DHF treatment given early or 2 days post-TBI did not affect the neuronal differentiation in the granule cell layer. However, a higher percentage of BrdU/GFAP+ and BrdU/Iba1+ cells were found in the hilus regions in 7,8-DHF treated animals, suggesting newly generated cells in this region are mostly glial cell types. Our results suggest that 7,8-DHF has neurotrophic-like therapeutic effects following injury, and due to increased neurogenesis (compared to injured animals treated with vehicle), may effectively contribute to greater cell survival long-term. Additionally, potential long-term survival coupled with increased outward migration from the subgranular zone may result in increased integration of newly formed neurons into existing hippocampal circuitry, further contributing to cognitive recovery.
Chapter 1: Introduction

1.1 Definition and Epidemiology of Traumatic Brain Injury

Traumatic Brain Injury (TBI) is the loss or alteration of brain function generated by an external force (Menon et al., 2010). TBI can be diagnosed with symptoms and signs that are temporally close to the external insult, including damage to blood vessels, axons, neurons, and glia, which are considered primary damages. However, it is critical to recognize delayed manifestations of injury, which can arise as complications of primary injury, and include hypoxia, ischemia, edema, and central nervous system (CNS) infection, among others (Menon et al., 2010; Tsao et al., 2010). Neuronal cell death following TBI can also be categorized into primary and secondary injury, where primary refers to the immediate death of cells on impact from the external disruption and secondary refers to delayed cell death as a result of physiological and biochemical changes in the surrounding area as a result of the injury (Stoica and Faden, 2010).

Traumatic Brain Injury is a global public health issue with few treatment options available (Chauhan, 2014). With approximately 10 million people affected by TBI annually, it is a major cause of death and disability worldwide, and the World Health Organization projects that it will surpass the mortality and morbidity of many diseases by the year 2020. Low and middle-income families, as well as developing countries bear the greatest burdens of TBI, as they may not possess the financial means or access to adequate health systems to properly handle its outcomes. It is difficult to quantify the full magnitude of TBI, as multiple factors influence it being underreported, including mild head trauma, which is the most common brain injury, not being reported, its “silent” effects, which may not be physically observed, but present in the form
of memory or cognitive deficits, and the fact that it is commonly mislabeled as a different cause of death or injury in statistics (Hyder et al., 2007).

According to the CDC, children aged 0-4 and 15-19 experience the highest rates of TBI, with adults over the age of 75 trailing close behind (Laskowitz and Grant, 2015). For young people, athletes, and members of the military, the risk of recurrent head trauma, especially mild TBI, presents an additional concern, as this may increase the risk of cognitive deficits or neurodegenerative disorders later in life (Guskiewicz et al., 2005). In addition to the long-term physical impairments caused by TBI, which 80,000-90,000 Americans experience annually, the economic cost of TBI, which can present in the form of missed work, hospital bills, and lost productivity, exceed $60 billion annually (Thurman et al., 1999; CDC Injury Fact Book, 2006).

1.2 Traumatic Brain Injury Model: Controlled Cortical Impact (CCI) Injury

To gain a better understanding of the clinical outcomes and potential treatment options following TBI, several injury models have been created. The two most commonly used TBI models are controlled cortical impact (CCI) injury and fluid percussion injury (FPI), the former of which will be used in this study. These models have been shown to produce motor and cognitive impairments which resemble those seen in TBI patients (Yu et al., 2009). It has been demonstrated that mild TBI from the CCI model causes profound neuronal apoptosis in the anterior thalamus and cortex, as well as significant axonal injury in subcortical white matter tracts (Dikranian et al., 2008). Furthermore, activation of Microglia, which comprise the innate immune system of the CNS, has been shown to be significantly upregulated in the hippocampus immediately following CCI injury, contributing to a pronounced inflammatory response and exacerbating neural damage. The highest levels of microglial activation have been observed at 3 days post injury (Sandhir et al., 2008).
1.3 Pathophysiology Following TBI

Primary cell injury is the damage that occurs immediately following injury. In the moments following impact, there is a significant release of a variety of neurotransmitters that is associated with the mechanical disturbance of neurons. Within the large release of neurotransmitters are glutamate and other excitatory neurotransmitters, which causes an influx of extracellular calcium into the cell. This, in turn, leads to the release of intracellular calcium stores, and results in high intracellular calcium levels that are sufficient to cause cytotoxic injury and cell death. The mechanical disruption of the axolemma and neuronal cell membrane also causes an influx of extracellular calcium and has the same aforementioned effects. The temporal length and degree of distortion, however, determine the fate of the membranes, as cells that are not severely damaged may be able to repair and reseal, while severely damaged cells may fall into an increasingly disrupted state or cell death (McAllister, 2011).

High levels of intracellular calcium activate caspases and calpains, and also produce free radicals, which disrupt cytoskeletal elements (McAllister, 2011; Farkas and Povlishock, 2007). Caspases require energy to activate apoptotic pathways slowly over hours and weeks. Calpains rapidly activate the necrosis pathway in an energy-independent manner and trigger an inflammatory response. The activation of this pathway is thought to be related to energy failure, and the collapse of cellular homeostasis that ensues. Calpains are proteases whose primary substrates include neurofilaments and spectrins, which, when acted upon by calpains, disrupt cell transport and destroy cytoarchitecture, ultimately leading to cell death (Farkas and Povlishock, 2007; Friedlander, 2003; Raghupathi, 2004).

Beyond the intracellular events caused by the disruption of calcium homeostasis and the release of free radicals, the rupturing of lysosomal membranes caused by the mechanical force of
the injury results in intracellular release of hydrolytic enzymes (Farkas and Povlishock, 2007). In addition to the significant release of glutamate described above, the excessive release of acetylcholine may exacerbate the negative effects of excitatory amino acids, which are also present in excess following injury. This can have especially harmful effects in the hippocampus and frontal cortices, where excitatory amino acids and acetylcholine are colocalized in high concentrations (Phillips and Reeves, 2001).

1.3.1 Neuronal death following TBI

While neuronal cell death is a normal part of CNS development, as a process to remove dysfunctional cells, it can have detrimental cognitive effects and result in neurodegenerative disorders if it occurs in excess, as it does in TBI. Caspases, or cysteine-dependent aspartate-specific proteases, are a family of proteases responsible for mediating various components of apoptosis, or programmed cell death. Necrosis (passive cell death) and apoptosis are associated with caspase-1 and caspase-3, respectively (Eldadah and Faden, 2000). In lateral Fluid Percussion Injury (FPI) and Controlled Cortical Impact (CCI) injury models in rats, and also demonstrated in humans, there is an increase of caspase-1 and caspase-3 activity in the cortex and hippocampus following TBI (Yakolev et al., 1997; Clark et al., 2000; Clark et al., 1999), which is consistent with an increase in cell death in those regions (Basil and Faden, 2000). Neurodegeneration after TBI can have adverse effects in many regions of the brain, leading to deficits in cognition, motor function, and psychological function, but the hippocampus is considered one of the most vulnerable regions to injury, as damage to this region can result in memory and learning deficits (Chauhan, 2014; Smith et al., 1991).
1.3.2 Neurogenesis Following Traumatic Brain Injury

Adult neurogenesis is the process of generating new cells in a mature brain, and it is known to take place in the subgranular zone (SGZ) of the dentate gyrus (DG) and subventricular zone (SVZ) of the lateral ventricles in the mammalian CNS. Although many cells, particularly progenitor stem cells, are more susceptible to apoptosis during neurogenesis, BDNF synthesis during this period promotes the viability of new neurons (Gomazkov, 2012). Progenitor cells from the SGZ migrate to the granular cell layer (GCL) and differentiate into mature granular neurons. This migration is normally guided by radial glial fibers during development, and is likely guided by these same fibers in the mature brain, as their presence persists in adulthood (Kornack and Rakic, 1999).

It has been demonstrated in multiple studies that TBI (either fluid percussion injury or controlled cortical impact) leads to an endogenous increase in stem cell proliferation in the hippocampus (Sun et al., 2005; Chirumamilla et al., 2004; Dash et al., 2001). Neural stem cell-supported adult neurogenesis may provide a compensation method for neuronal loss following TBI (Wang et al., 2015). In a FPI model, both juvenile and adult rats demonstrated robust neuronal proliferation of the ipsilateral dentate gyrus, specifically in the SGZ and hilus. The proliferative response was strongest at 48 hours post-injury, and continued for 7 days in the SGZ, but ultimately declined by day 14 to the same levels as sham animals, as shown in Figure 1.1 (Sun et al., 2005).
A  BrdU cell counts in the SGZ

Number of BrdU+ cells

![Graph A: BrdU cell counts in the SGZ](image)

B  BrdU cell counts in the hilus

Number of BrdU+ cells

![Graph B: BrdU cell counts in the hilus](image)
Figure 1.1: Cell Proliferation in the Ipsilateral Dentate Gyrus following Fluid Percussion Injury. Graphs demonstrate significantly increased cell proliferation at 2 and 7 days post-injury in the SGZ, and at 2 days post-injury in the hilus (Sun et al., 2005).
It has been established that adult neurogenesis in the hippocampus, specifically adult-born granule cells of the dentate gyrus, plays an important role in hippocampal dependent learning and memory function (Deng et al., 2009; Clelland et al., 2009). Our lab and others have also demonstrated that TBI enhances hippocampal neurogenesis, and newly generated neurons are able to successfully integrate into existing hippocampal circuitry (Emery et al., 2005; Sun et al., 2007). The successful integration of new neurons into surrounding circuitry coincides with the time of innate cognitive recovery, suggesting a link between post-TBI hippocampal neurogenesis and cognitive recovery (Sun et al., 2007). Furthermore, studies that augment post-TBI hippocampal neurogenesis with growth factors, such as epidermal growth factor and basic fibroblast growth factor, have shown improved cognitive functional recovery (Sun et al., 2009; Sun et al., 2010). These studies suggest that manipulation of the brain’s endogenous cell response may provide a unique opportunity for brain regeneration following TBI.

However, severity of injury has been shown to affect adult neurogenesis of the hippocampus. Moderate TBI has been shown to promote neural stem cell (NSC) proliferation, but not affect neurogenesis, while severe TBI increased the three stages of neurogenesis: proliferation, migration, and differentiation, in the hippocampal dentate gyrus (Wang et al., 2015).

1.4 Neurotrophins

Neurotrophins are endogenous peptides secreted from neuronal and glial cells, and are associated with regulating the function, survival, and development of individual cells and neuronal networks across the entire brain. More specifically, neurotrophins regulate synaptic plasticity, protect neurons from oxidative stress and apoptosis, and can aid in the replacement of damaged neurons by stimulating progenitor stem cells, which leads to neurogenesis. The
neurotrophin family of growth factors includes Nerve Growth Factor (NGF), Brain-derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin-4/5 (NT-4/5), which are classified together based on their structural similarity to NGF, the first neurotrophin discovered. Although neurotrophins are present in multiple structures across the brain, they have the highest level of expression in the hippocampus, particularly NGF, NT-3, and BDNF, with BDNF showing the greatest level of expression following an initial development period with high NT-3 expression. Within the hippocampus, activated glutamate receptors stimulate the production of neurotrophins during changing environmental conditions in the brain (Gomazkov, 2012).

Due to their role in regulating neuronal survival, function, and development, neurotrophins provide valuable protective and repair functions that attenuate neuronal damage. Neuroprotective functions of neurotrophins range from cytoskeleton stabilization, suppressed amyloid deposition, and balancing neurotransmitter levels, to preventing neurodegeneration and promoting neurogenesis (Gomazkov, 2012). Neurotrophins have also been implicated in multiple neurodegenerative disorders and pathophysiological states, as defects in their expression can contribute to diseases, such as Alzheimer’s Disease and Parkinson’s Disease (Price et al., 2007; Gomazkov, 2012). Early, preclinical stages of dementia are often accompanied with lower than normal levels of NGF in the frontal cortex, but as the disease progresses, there is a compensatory increase in NGF. This pattern of disrupted NGF regulation is characteristic of a variety of diseases, ranging from Alzheimer’s Disease to diabetic neuropathy (Gomazkov, 2012; Shaub et al., 2014; Siegel and Chauhan, 2000).

Neurotrophins are able to exert their neuroprotective effects through the transmembrane receptors they bind to and the signaling cascades they initiate. There are two main classes of transmembrane neurotrophin receptors, which include tropomyosin-related kinase (Trk) family
of tyrosine kinase receptors, TrkA, TrkB, and TrkC, and the p75 Neurotropin receptor (p75NTR), a member of the tumor necrosis-factor family (Marco-Salazar et al., 2014). NGF preferentially binds to TrkA, BDNF and NT-4/5 to TrkB, and NT-3 to TrkC, all with high affinity, while each of these neurotrophins binds with low affinity to p75NTR receptors (Gomazkov, 2012; Marco-Salazar et al., 2014). Additionally, p75NTR contributes to proper Trk receptor function, and promotes ligand binding of neurotrophins with their correct Trk receptor (Skaper, 2012). Once bound to their Trk receptors, neurotrophins activate a cascade of events through Ras, phosphatidylinositol 3-kinase (PI3K), phospholipase-Cγ (PLCγ), and mitogen-activated protein kinase (MAPK) signaling pathways (Skaper, 2012).

1.4.1 Neurotrophins in Response to Traumatic Brain Injury

By virtue of their role in neuronal growth, differentiation and replenishment, it is no surprise that neurotrophins play a physiologically important role in protecting the brain following TBI (Gomazkov, 2012). Neurotrophic therapy first became a concept following the discovery of NGF’s ability to induce neuronal survival and repair after injury (Cohen et al., 1954; Levi-Montalcini, 1987). This led to studies investigating the presence of additional growth factors following brain injury or insult and the potential therapeutic uses of neurotrophins for TBI and in neurodegenerative diseases, such as Parkinson’s Disease (Gomazkov, 2012).

Immediately following TBI, mRNA expression of NGF and BDNF is transiently and significantly increased. As early as 12 hours post-injury, levels of BDNF and NGF mRNA are significantly upregulated at the site of injury and in remote regions of the brain (non-injury sites), but levels of BDNF begin to decline at 24 hours post-injury, and are no longer significantly elevated at 36 hours post-injury in both regions. NGF mRNA levels, conversely, are significantly elevated at 12 hours post-injury, increase at 24 hours post-injury, and begin to decline at 36
hours post-injury, at which point their levels are three-times that of controls, but not statistically significant in both regions. These results demonstrate that although BDNF reacts faster following TBI, its response ends sooner than the response of NGF (Oyesiku, 1999). In addition to an upregulation of neurotrophins at the site of injury and in remote areas, TrkB receptors, which bind BDNF, are also transiently upregulated in the hippocampus and dentate gyrus following neural insults (Merlio et al., 1993).

1.4.2 Brain-derived Neurotrophic Factor (BDNF) and its receptors

Brain-derived Neurotrophic Factor (BDNF) is a neurotrophin that is produced in the endoplasmic reticulum (ER) as a precursor protein, known as prepro-BDNF, transferred to the Golgi apparatus for further processing where it becomes pro-BDNF, and finally is placed as mature BDNF in secretory vesicles, from which it can be released at the presynaptic terminal via an active calcium-dependent manner (Gomazkov, 2012). Pro-BDNF is secreted in an activity-dependent manner, and is cleaved by enzyme tissue plasminogen activator to produce mature BDNF (Waterhouse and Baoji, 2009). BDNF is known to activate intracellular signaling pathways through both TrkB and p75NTR receptors (Gomazkov, 2012). Additional studies have shown that pro-BDNF primarily acts through binding the lower affinity p75NTR receptor, while mature BDNF acts through the higher-affinity TrkB receptor (Roux and Barker, 2002; Lessmann et al., 2003).

Although it is found only at very low levels within the CNS, BDNF has been shown to have profound effects in regulating cell survival and other biological processes that ensure proper mental function (Dieni et al., 2012). BDNF signals for neurite and axonal growth (Yoshii and Constantine-Paton, 2010), is required for the survival and development of dopaminergic, GABAergic, serotonergic, and cholinergic neurons (Pillai, 2008), and is essential to learning and
memory, and the synaptic plasticity required by both functions (Lu et al., 2008). While BDNF offers great therapeutic potential because of its endogenous functions, it is limited by its poor pharmacokinetic profile, namely that it has a short half-life (BDNF half-life < 10 minutes), degrades rapidly in blood, and also diffuses poorly across the blood-brain barrier (BBB) (Price et al., 2007).

1.4.3 BDNF-TrkB Signaling Pathway

Upon binding to the TrkB receptor, BDNF induces dimerization and autophosphorylation of the receptor, which causes internalization of the TrkB receptor and initiates intracellular signaling cascades (Figure 1.2; Levine et al., 1998). These signaling cascades include the phosphatidylinositol 3 kinase (PI3K) pathway, the phospholipase C γ (PLCγ) pathway, and the mitogen-activated protein kinase (MAPK) pathway. The PI3K pathway activates protein kinase B (Akt), which ultimately promotes cell survival by inhibiting Bad and consequently allowing the expression of anti-apoptotic proteins, such as Bcl2 (Yoshii and Constantine-Paton, 2010). Phosphorylation of Akt at the proper site also results in the suppression of pro-apoptotic proteins, pro-caspase-9 and Forkhead (Kaplan and Miller, 2000). Upregulated Bcl2 levels are correlated with positive outcomes, such as attenuated cell death and a better prognosis (Nathoo et al, 2004). The PLCγ pathway leads to the release of intracellular calcium stores via activation of the inositol triphosphate (IP3) receptor, and helps to increase calmodulin kinase (CamK) activity, and thus synaptic plasticity via the transcription factor CREB (cyclicAMP response element binding protein). The MAPK pathway, also referred to as extracellular related signal kinase (ERK) pathway, aids in cell growth and differentiation. A PLCγ mediated response is likely
responsible for quick, short-term actions, while MAPK and PI3K pathways involve long-term transcriptional effects (Yoshii and Constantine-Paton, 2010).
Figure 1.2: The activation pathways of the TrkB receptor.

The MAPK pathway activation stimulates anti-apoptotic proteins, including Bcl2 and cAMP Response-Element Binding protein (CREB), the latter of which is required by neurotrophins for mediating neuronal survival. Activation of the MAPK pathway also stimulates extracellular signal related kinase (ERK), which is responsible for transcriptional regulation, and causes the phosphorylation of Synapsin I, mediating the clustering and release of synaptic vesicles. TrkB receptor activation also leads to the activation of the PI3K pathway, which activates Akt. Akt inhibits apoptosis by inhibiting the protein Bad, which, when active, inhibits anti-apoptotic protein, Bcl2. Pro-caspase 9 and Forkhead are pro-apoptotic proteins that are suppressed when Akt is phosphorylated at the apoptosis protein on the Akt consensus phosphorylation site. (PI3K = phosphatidylinositol 3 kinase; Akt = Protein Kinase B, PKB; Bad = Bcl2 associated death promoter) PI3K does not interact directly with Trk receptors. However, adaptor proteins such as GRB-associated binder-1 (GAB1), insulin-receptor substrate 1 (IRS1) and IRS2, which are recruited to activated TrkB through GRB2 binding, mediate the association and activation of PI3K with TrkB. Finally, the Phospholipase C-gamma (PLC γ) pathway is also activated by the TrkB receptor. PLC-gamma can lead to an increase in intracellular calcium levels, which activates the calcium/calmodulin pathway, which can then activate CREB.
1.4.4 BDNF-TrkB Receptor Signaling and Neurogenesis

Data suggests that neurotrophins play a crucial role in neurogenesis in the CNS (Gomazkov, 2012). An increase in BDNF alone will not result in neurogenesis, as presence of its TrkB receptor is necessary for the proper organization and growth of dendrites and spines, as well as neurogenesis-dependent long-term potentiation (LTP), which is essential for memory processes (Bergami et al., 2008; Cooke and Bliss, 2006). Neurogenesis is critical to normal brain and psychological functions, and the lack of TrkB signaling, and thus neurogenesis, has implications in mood-related behavior, specifically increased anxiety. This underscores the importance of neurogenesis and neurotrophins restoring proper function (Bergami et al., 2008).

The role of BDNF in neurogenesis regulation plays out at multiple steps in the maturation process. While depleting central BDNF in mice increases hippocampal cell proliferation, and does not affect cell survival or fate, the newly proliferated cells are not able to fully mature and lack a neurogenic response to GABA(A) receptor stimulation (Chan et al., 2008). These studies indicate that without BDNF, immature neurons are not able to properly differentiate in the adult hippocampus. Additional studies have demonstrated that BDNF is crucial to neural stem cell proliferation, and when given in conjunction with a restricted diet, promotes the survival of newly generated neurons (Lee et al., 2002).

Furthermore, when BDNF is administered directly to the dentate gyrus (and does not need to cross the BBB), there is an increase in neurogenesis of granule cells on both the ipsilateral and contralateral hemispheres of the hippocampus (Scharfman et al., 2005). Conversely, conditionally knocking-out BDNF in the hippocampal dentate gyrus causes a significant increase in adult-born immature neuron death after moderate TBI (Gao and Chen,
This suggests that BDNF plays a critical role in regulating mechanisms of survival of immature neurons in the hippocampus, particularly following a brain insult.

1.4.5 BDNF in Response to Traumatic Brain Injury

Due to their valuable role in neuronal survival and proliferation, it is logical that neurotrophins act as an endogenous neuroprotective response following TBI, and work to attenuate secondary cell damage related to the pathophysiology of brain injury (Mattson and Scheff, 1994). Multiple studies have demonstrated that TBI modulates BDNF and TrkB mRNA levels bilaterally in the hippocampus. Both BDNF and TrkB mRNA levels increase bilaterally in the hippocampus for several hours following injury, while NT-3 mRNA expression decreases and TrkC, the high-affinity binding partner of NT-3, remains unchanged within the hippocampus (Hicks et al. 1999; Hicks et al., 1998). These alterations in neurotrophin levels are representative of molecular and chemical changes following TBI, and serve as early biomarkers for injury (Chiaretti et al., 2003).

Rising BDNF levels in the hours and days after injury are thought to play a crucial role in ameliorating the effects of secondary brain injury. Increased levels of expression have been found at the site of injury, in distant areas damaged by secondary mechanical stress, and areas connected via fiber pathways to the injured area. BDNF provides neuroprotection and works to restore proper connectivity following TBI, but if the injury induces chronic stress or prolonged glucocorticoid exposure, as in post-traumatic stress disorder (PTSD), BDNF levels can fall and lead to hippocampal dysfunction by causing dendritic disconnection, retraction, and rewiring (Kaplan et al., 2010).
1.5 Hippocampus following Traumatic Brain Injury

Along with the frontal cortex and sub-frontal white matter, the hippocampus, among other deep midline structures, is particularly vulnerable to traumatic brain injury. Mesial temporal structures, which include the hippocampus and dentate gyrus, are subject not only to injuries from physical impact, but also cytotoxic effects from secondary injury (McAllister, 2011). The hippocampus is of particular concern as it contains the subgranular zone (part of the hippocampal dentate gyrus), which is one of the two known locations in the brain to undergo adult neurogenesis (Gomazkov, 2012).

Learning and memory dysfunction are common consequences of damage to the hippocampus. Parasagittal fluid percussion injury has been demonstrated to cause severe spatial memory deficits in the Morris Water Maze, a behavioral navigation task used to test spatial learning and memory (Smith et al., 1991). The primary and secondary insult to the hippocampus elicit multiple inflammatory events, which ultimately result in neuropsychiatric, cognitive, and motor issues. One study was able to show that although hippocampal-dependent learning and memory were measured as normal 7 days post-injury, at one month post-injury anterograde learning impairments developed. These impairments coincided with an acute immune challenge at 30dpi to demonstrate that microglia are still sensitized to the primary insult and overreact to smaller secondary insult to contribute to cognitive decline. This exhibits the fragile state of the hippocampus following the initial injury, and how additional cognitive deficits may develop if the brain has not yet fully recovered (Muccigrosso et al., 2016).
1.6 The Molecule 7,8-Dihydroxyflavone

Although BDNF possesses a great deal of therapeutic potential due to its numerous neurological targets and key roles in neurogenesis and neuroprotection, it has a short half-life (<10 minutes), is cleared quickly from the circulatory system by the liver, and lacks the ability to cross the BBB due to its large size (27 kDa), and consequently lacks an adequate means of administration in a clinical setting (Baseri et al., 2012). Because of its poor pharmacokinetic profile, researchers completed a cell-based drug screening to find a small molecule that can act as a TrkB agonist. 7,8-Dihydroxyflavone (7,8-DHF) is a flavonoid discovered in this search that not only specifically binds to the TrkB receptor with high affinity and induces its dimerization, but it also induces the receptor’s downstream signaling, is orally bioactive, and can cross the BBB (Liu et al., 2016).

Flavonoids are found in fruits and vegetables, and some are known to have antioxidant and anti-inflammatory properties (Harborne and Williams, 2000). Other studies have shown positive effects of flavonoids in protecting and enhancing neural function, as well as promoting neuronal regeneration (Spencer, 2008). 7,8-DHF in particular mimics the effects of BDNF, and has been demonstrated to have positive effects in axon regeneration and a protective role in the pathogenesis of Alzheimer's Disease (Liu et al., 2016). Furthermore, 7,8-DHF has been shown to protect neurons from apoptosis; neurons that lack TrkB, the receptor for 7,8-DHF and BDNF, cannot be rescued from apoptosis by 7,8-DHF (Jang et al., 2010).
1.6.1 Pharmacokinetics of 7,8-Dihydroxyflavone

In contrast to the short half-life of BDNF (<10 minutes), 7,8-DHF has a half-life of 134 minutes in plasma (50 mg/kg oral administration), oral bioavailability of 5%, and can still be detected in the brain 4 hours after administration, despite first-pass metabolism by the liver (Zhang et al., 2014; Liu et al., 2016). Glucuronidation and sulfation are the two main modifications that result in the clearance of 7,8-DHF (Liu et al., 2016). 7,8-DHF is also considerably smaller than BDNF, with a molecular weight of 254 Da compared to BDNF’s 27 kDa, which allows for greater permeability across the BBB when given orally or intraperitoneally (Liu et al., 2014).

1.6.2 7,8-Dihydroxyflavone interactions with TrkB Receptor

As stated above, 7,8-DHF binds the TrkB receptor with high affinity. Although BDNF has a lower dissociation constant (high binding affinity) (K_d = 1.7nm) than 7,8-DHF (K_d = 15.4nm) with the TrkB receptor, 7,8-DHF TrkB receptor phosphorylation lasts for many hours longer than BDNF receptor phosphorylation. Additionally, TrkB receptors activated by 7,8-DHF are not degraded, but instead are recycled to the cell surface after internalization, as opposed to BDNF activated TrkB receptors, which are tagged for ubiquitination and degraded after internalization. Internalization is a vital part of initiating signal transduction for the neurotrophin-Trk complex. 7,8-DHF can successfully mimic BDNF-TrkB internalization in neurons, producing endosomes with TrkB as early as 10 minutes, as BDNF does, and producing a more robust endocytic response than BDNF at 60 minutes (Liu et al., 2014).

Not only does 7,8-DHF selectively promote the homodimerization of TrkB receptor and autophosphorylation of tyrosine residues as strongly as BDNF, it also influences its downstream effectors. In the downstream pathways of TrkB, binding of 7,8-DHF to the TrkB receptor
resulted in significant activation of Akt and ERK/MAPK pathways. In this manner, 7,8-DHF is able to promote the same neuroprotective effects as BDNF (Jang et al., 2010).

1.6.3 Current Experimental Findings of 7,8-DHF in the Treatment of TBI: Behavioral Level Response

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 blood-brain barrier, making it a possible alternative in treating TBI (Zeng et. al., 2012; Wu et. al., 2014). In mouse models employing controlled cortical impact (CCI) injury, there was significant improvement in neurological function at days 14, 21, and 28 post-injury in mice given 7,8-DHF (20mg/kg) at four days after CCI compared to the control group (Wu et al., 2014).

This was demonstrated in rotarod and beam-walking tests, which test motor and coordination function, and resulted in 7,8-DHF-treated mice performing significantly better on the rotarod test (not falling off or remaining on the rotarod for a longer period of time) and requiring significantly less time to cross in the beam-walk test as early as 7 days post-injury for both (Wu et al., 2014). Additionally, rats subject to fluid percussion injury (FPI) had a significant increase in latency time on the Barnes maze, used to assess spatial memory and cognitive function, as opposed to rats treated with 7,8-DHF (5mg/kg). Latency in the Barnes maze test is indicative of memory impairment, suggesting that the significant reduction in latency time for animals treated with 7,8-DHF beginning the day of injury is due a protective effect 7,8-DHF offers against TBI-induced memory deficits (Agrawal et al., 2015).
1.6.4 Current Experimental Findings of 7,8-DHF in the Treatment of TBI: Gross Anatomic Level Response

Tissue loss and brain edema are also common occurrences following brain damage that can be minimized with 7,8-DHF. Initial contusion volume after injury significantly decreased by day 28 compared to controls, and also restricted further tissue loss through atrophy, suggesting, again, a neuroprotective effect of 7,8-DHF. Even 4 days post-injury, when neurodegeneration that is normally observed at the acute stage, 7,8-DHF ameliorates neuronal damage and significantly decreases contusion volume (Yu et al., 2010; Wu et al., 2014). The preservation of brain tissue also likely contributes to long-term functional recovery (Wu et al., 2014). Brain edema is characterized by excess fluid and swelling (Fishman, 1975). A decrease in tissue volume may give way to greater brain water content. CCI induces a significant increase in water content of the affected/ipsilateral hemisphere compared to the contralateral hemisphere, an effect that is significantly lessened by the treatment of 7,8-DHF given 4 days post-injury (Wu et al., 2014).

Tissue preservation by 7,8-DHF after brain injury also reflects a decrease in cell apoptosis. Cleaved caspase-3 is a main effector of the apoptotic cascade (Gown and Willingham, 2002), and by 4 days post-injury its levels in 7,8-DHF-treated brains had significantly decreased compared to controls (Wu et al., 2014). Additionally, the ratio between anti-apoptotic and pro-apoptotic Bcl-2 proteins increases with 7,8-DHF, implying that its protective effects include preventing apoptosis after injury (Wu et al., 2014).
1.6.5 Current Experimental Findings of 7,8-DHF in the Treatment of TBI: Metabolic Response

Mitochondrial biogenesis is the growth and division of preexisting mitochondria, and requires the co-transcription factor PGC-1α (peroxisome-proliferator-activated receptor γ co-activator-1α) to activate several other transcription factors which will activate mitochondrial transcription factor A (TFAM) and induce mitochondrial biogenesis. PGC-1α is important in linking the internal metabolic response to external stimuli (Jornayvaz and Shulman, 2010). TBI negatively affects PGC-1α levels in the brain, and corresponds with increased latency times in the Barnes maze test, suggesting its absence may contribute to poor memory function. PGC-1α levels and a lower latency time are restored when injured animals are given 7,8-DHF (Agrawal et al., 2015).

In addition to the restoration of PGC-1α levels, 7,8-DHF also lessens the impact of TBI on TFAM protein, cytochrome oxidase, and SIRT2 protein levels through activation of the TrkB receptor (Agrawal et al., 2015). TFAM is the final component driving transcription and replication of mitochondrial DNA, so it would follow that appropriate levels of TFAM would allow for proper mitochondrial biogenesis following environmental stress, including cell division and renewal and oxidative stress that result from TBI (Jornayvaz and Shulman; 2010). Mitochondrial cytochrome oxidase II (COII), which is important in mitochondrial oxidative phosphorylation, and serves as an indicator of the mitochondrial function to mass ratio, also has reduced levels following brain injury. The enzyme’s levels are maintained after injury, however, if 7,8-DHF is given the day of injury. As shown with reduced levels of PGC-1α, lower levels of COII are also reflected in an increased latency time in the Barnes maze test, suggesting that memory function may rely on the function and levels of mitochondrial proteins (Agrawal et al.}.
Sirtuin 1 (SIRT2) protein is induced during fasting, and activates PGC-1α via deacetylation. Deficits in SIRT2 would result in an inability to respond to urgent metabolic needs that result from sensing energy deficits during fasting (Jornayvaz and Shulman; 2010). Like TFAM and COII levels, SIRT2 protein levels were restored with the treatment of 7,8-DHF (Agrawal et al., 2015).

Cellular energy levels are closely monitored by AMP activated protein kinase (AMPK), which is an important regulator of mitochondrial biogenesis. Mitochondrial biogenesis, in turn, activates the metabolic response to cellular energy crises (Jornayvaz and Shulman; 2010). Following TBI, AMPK phosphorylation decreases, reducing or inhibiting the transcription of mitochondrial DNA. 7,8-DHF treatment significantly raises the levels of AMPK phosphorylation after TBI, and also increases AMPK levels in sham animals. Presumably this response will aid in meeting the energy demands of neuronal growth following injury (Agrawal et al. 2015).

Cognitive and emotional disturbances are coupled with brain damage, as well, and often present in the form of depression and anxiety (Cohen et al., 2007; Signoretti et al., 2010; Pandey et al., 2009). These disorders have been linked to metabolic disturbances, which may underscore the importance of 7,8-DHF restoring PGC-1α, COII, and SIRT2 to homeostatic levels (Vagnozzi et al., 2010; Agrawal et al., 2015). BDNF has been shown to participate in glucose utilization and energy management (Yamanaka et al., 2007), so it follows that using the TrkB agonist 7,8-DHF to stimulate the pathway should restore metabolic functions to homeostatic levels and relieve the cognitive and behavioral disturbances associated with its imbalance (Agrawal et al., 2013; Zhang et al, 2015). After 8-12 hours of 7,8-DHF treatment (200nM), neuro-2a (N2a) cells show increased cellular respiration, as measured by a higher oxygen consumption rate (OCR),
suggesting that the activation of this pathway increases the mitochondrial reserve capacity of
cells, improving mitochondrial function (Agrawal et al., 2013).

1.6.6 Current Experimental Findings of 7,8-DHF in the Treatment of TBI: Molecular Level
Response

The presence of 7,8-DHF also has quantitative effects on a molecular level. Under
normal conditions, the binding of neurotrophins to Trk receptors induces the phosphorylation of
tyrosine residues on the intracellular domain, specifically TrkB Tyr\textsubscript{515}, Tyr\textsubscript{706}, and Tyr\textsubscript{816}. 7,8-
DHF also induced phosphorylation of the same residues, but elicited weaker signals than BDNF
(Xia et al; 2014). Sham animals not subject to TBI, however, had a significant increase in TrkB
phosphorylation when treated with 7,8-DHF. Animals subject to FPI had a significant reduction
in the phosphorylation of TrkB, which was reversed with the treatment of 7,8-DHF, suggesting a
potential therapeutic use of the flavonoid (Agrawal et al; 2015). Downstream signaling pathways
of TrkB, such as PI3K/Akt pathways, were also restored when TBI was followed with 7,8-DHF,
increasing Akt Ser\textsubscript{473} phosphorylation by as much as 198% compared to vehicle treatments. The
phosphorylation of TrkB receptors following TBI has been analyzed further within the
hippocampus, and has shown a qualitative decrease in phosphorylation, particularly within the
CA3 region. This molecular-level phenotype corresponded with the observation that TrkB
phosphorylation is inversely related to latency time in the Barnes maze, suggesting a link

Blocking TrkB phosphorylation with K252a, a TrkB antagonist, prevents the beneficial
cognitive, motor, and cellular effects observed with 7,8-DHF administration following brain
injury. Injecting K252a into the hippocampus counteracts memory improvement demonstrated in
the Barnes maze test, and reduces TrkB phosphorylation, CREB phosphorylation, and PGC-1α
levels in animals treated with 7,8-DHF (Agrawal et al., 2015). Additionally, K252a completely reverses the reduction in contusion volume induced by 7,8-DHF. This has been replicated using the PI3K inhibitor LY294002, which also abolishes the protective effect of 7,8-DHF, leading to an increased injury volume compared to the animals treated with only 7,8-DHF (Wu et al., 2014).

In addition to an increase in phosphorylation of TrkB and its downstream targets, which results in activation of several proteins leading to the mediation of neuronal survival and vesicle release (Kaplan & Miller, 2000; Wang et. al., 2010; Waterhouse & Xu, 2009), 7,8-DHF also induces an increase in CREB phosphorylation level at day 4 post-injury. Phosphorylated CREB is a key transcription factor for the production of BDNF, and consequently, as levels of CREB phosphorylation increase, so do levels of BDNF. This indicates that the endogenous increase of BDNF likely contributes to 7,8-DHF’s protective effects via a positive feedback loop (Wu et al; 2014). In the neocortex, BDNF has been shown to play a critical role in fear memory and learning, and when treated with 7,8-DHF, memory and learning deficits induced by low levels of BDNF are rescued (Choi et al; 2009).

In addition phosphorylating key components of the TrkB pathway, 7,8-DHF also restores GAP-43 and syntaxin-3 levels. GAP-43 is a protein associated with axonal regeneration, serves as a growth and plasticity indicator, and is highly expressed during development (Benowitz and Routtenberg; 1997). Syntaxin-3 is a protein charged with transporting vesicles for exocytosis and assisting with the growth of neuritis (Martin-Martin et al., 1999). Restoration of both of these proteins’ levels has important implications for the reestablishment of brain plasticity and recovery following injury. Differences in these proteins’ activity following treatment with
vehicle versus 7,8-DHF are also qualitatively different in subregions of the hippocampus (Agrawal et al. 2015).

1.7 Summary and Hypothesis

Traumatic brain injury is a serious medical issue worldwide. Primary and secondary damages caused by TBI have long-lasting effects due to the activation of calpains and caspases, as well as inflammatory responses, which can lead to overactivation of microglia, ultimately leading to neurodegeneration (Wood, 2002).

This study seeks to elucidate the therapeutic potential for 7,8-Dihydroxyflavone in the context of neurogenesis following traumatic brain injury. Based on previous unpublished data, we have found that 7,8-DHF has neuroprotective and neural plasticity effects in the injured brain, and that post-TBI treatment can improve cognitive functional recovery, reduce lesion volume, and increase neuronal survival. In this study, we will explore the neurogenic effect of 7,8-DHF following TBI by examining levels of cell proliferation, generation of new neurons, and cell type differentiation in two treatment groups.
Chapter 2: Methods

2.1 Experimental Animals

Male Sprague-Dawley rats (300-350g) aged 3 months old were used in this experiment. Rats were housed in pairs in the Division of Animal Resource (DAR) facilities at a temperature between 20-22°C on a 12-hour light/dark cycle, and fed ad libitum. Animals acclimated to this environment for one week prior to beginning behavioral testing and surgery. A total of 32 animals were included in this study. Animals were randomly divided into two sets: Day 15 (D15) animals (n=20), which were sacrificed 15 days post-injury, and Day 28 (D28) animals (n=12), which were sacrificed 28 days post-injury. Within each experimental group, D15 and D28, animals were randomly assigned to a treatment group (Table 2.1). The D15 group was comprised of 4 treatment groups, including sham animals (n = 3), animals which received CCI and vehicle treatment (n = 7), animals which received CCI and 7,8-DHF beginning the same day as injury (DHF Group 1; n = 5), and animals which received CCI and 7,8-DHF beginning two days post-injury (DHF Group 2; n = 5). The D28 animals were divided into 4 treatment groups, which were the same as the D15 experimental group. Animals were sacrificed at D15 to examine survival and migration, and at D28 to examine differentiation and survival of new neurons in the hippocampus.
<table>
<thead>
<tr>
<th>Animal Treatment Groups</th>
<th>Sacrificed</th>
<th>Sham</th>
<th>CCI + Vehicle</th>
<th>CCI + DHF Group 1</th>
<th>CCI + DHF Group 2</th>
<th>Total</th>
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<tr>
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<td>3</td>
<td>7</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>20</td>
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<td>12</td>
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Table 2.1 Animal Treatment Groups for Day 15 (D15) and Day 28 (D28) Animals in 7,8-Dihydroxyflavone Study; Comparing post-injury endogenous neurogenesis response to post-injury neurogenesis with 7,8-DHF administration.
2.2 Controlled Cortical Impact (CCI) Injury

A total of 28 (+4 sham) animals received controlled cortical impact (CCI) injury for this experiment using a Benchmark Stereotaxic Impactor. The animals were sedated with 2.5% isofluorane (in 30% O\textsubscript{2} and 70% N\textsubscript{2}), and remained under sedation for the duration of the procedure. A paw-pinchn reflex was tested every 10-15 minutes throughout the procedure to ensure continued sedation. Once sedated, the top of the animal’s head was shaved where the incision to open the scalp would be made. The rats were then placed in a stereotactic frame attached to the impactor, where their head was held in place by ear bars and a biting plate, which their jaw was attached to. To prevent their eyes from drying out, an ophthalmic lubricant ointment was applied to both eyes once the animal was set in the stereotactic frame. To reduce the possibility of infection, betadine, an iodine antiseptic, was applied along the scalp where the incision would be made. Rats were placed on thermostatically controlled heating pad to maintain a stable body temperature.

The incision was made along the midline of the scalp with a sterile scalpel blade. Skin and fascia were held apart with hemostats on either side of the head to keep the skull exposed throughout the procedure. To gain access to the brain tissue, a 3.5mm craniotomy was performed on the left hemisphere, 2mm lateral to the mid-sagittal suture and 2mm below the coronal suture. A 3mm rigid impactor (Leica Benchmark Stereotaxic Impactor) was placed perpendicularly to the exposed dura mater, and driven by an electromagnetic piston at 4 m/s. The impactor was driven into the cortical tissue to make a 2.5mm depression, and held there for 500ms. Following the injury, the scalp was closed with an absorbable suture, and lidocaine hydrochloride jelly and a triple antibiotic were applied to the area to provide a local anesthetic and prevent infection, respectively. Animals’ reflexes and breathing were monitored as they recovered and regained
consciousness prior to being placed back in their appropriate cage and back to the housing area.

Sham animals received the same anesthesia and surgery (incision) without injury.

2.3 Drug Administration

Animals in DHF Group 1 and 2 received 5 single daily 5 mg/kg doses of 7,8-DHF intraperitoneally. Group 1 DHF animals received their first injection 1 hour post-CCI injury, and continued to receive treatment until day 4 post-injury (day 0-4 post-TBI), while Group 2 DHF animals received their first injection starting on day 2 post-injury, and continuing through day 6 (day 2-6 post-TBI). Vehicle animals had the same treatment schedule as Group 1 DHF animals, but instead were administered 15% DMSO (dimethyl sulfoxide) intraperitoneally day 0-4.

7,8-DHF was prepared immediately before injection to ensure that 7,8-DHF did not come out of solution. The proper amount of 7,8-DHF that was needed for a particular day was weighed out and dissolved in 100% DMSO first, then sterile PBS was added to make DMSO at 15%. The final 7,8-DHF concentration in vehicle for injection was 5 mg/ml, and was administered to the rats i.p. with a 26-gauge needle. The vehicle treatment was prepared by diluting 100% DMSO to 15% DMSO with sterile PBS, and was administered in the same manner (26-gauge needle, i.p.), dosage, and concentration as 7,8-DHF treatments.

All animals were also injected with 5-Bromo-2’-deoxyuridine (BrdU) to label dividing cells. D15 animals (sacrificed 15 days post-injury) received three doses of BrdU injection two hours apart (50mg/kg, i.p) at two days post-TBI. D28 animals received a single daily BrdU injection (50mg/kg, i.p.) for 7 days starting at 1 day post-TBI. BrdU was made in 0.9% saline with 0.007N NaOH at the concentration of 10mg/ml.
2.4 Tissue Processing

Animals were sacrificed at day 15 or day 28 post-injury. Animals were anesthetized with 5% isofluorane inhalation and transcardially perfused with 400 ml of 1x phosphate buffer solution (PBS), followed by 400 ml of 4% paraformaldehyde in PBS. The paraformaldehyde was prepared fresh on the day of perfusion. Once the perfusion was complete, the animal’s skull was opened and the brain was removed, and post-fixed in 4% paraformaldehyde in PBS at 4°C for 48 hours. The brains were then sliced into 60µm sections using a Leica Vibratome. Sections were sliced coronally in a rostro-caudal direction, and collected in five 24-well plates containing PBS with 0.02% sodium azide. Tissue sections were kept at 4°C until used for staining procedures.

2.5 Immunohistochemistry Staining: Doublecortin (DCX) and Ki67

Doublecortin (DCX) is a microtubule-binding protein associated with the migration of neuroblasts. DCX is an effective marker for adult neurogenesis as it has been demonstrated to be transiently expressed in proliferating progenitor cells and neuroblasts. As these newly generated cells develop and differentiate, their DCX expression declines and is replaced by mature neuronal markers, such as NeuN, a nuclear antigen (Brown et al., 2003). Ki-67 is a nuclear protein that is expressed in proliferating cells, and has been linked to ribosomal RNA (rRNA) synthesis (Bullwinkel et al., 2006). As such, it was employed in this experiment to assess cell proliferation in the hippocampus post-injury.

To assess the number of DCX+ and Ki67+ cells, every eighth section extending from the rostro-caudal hippocampal dentate gyrus region from D15 animals was processed for DCX or Ki67 immunohistochemistry. The selected sections, contained individually in 24-well plates, were first washed with 1X PBS twice, and then quenched with 3% hydrogen peroxide for 1 hour at room temperature. Sections were then washed with a PBS-Triton mix (1X PBS + 0.3% Triton X-100).
Triton100) once before blocking overnight with blocking buffer (5% normal horse serum in PBS- 0.3% Triton mix, as described previously) at 4°C on a shaker. The following day, the blocking buffer was removed and 250µl of primary antibody solution containing goat anti-DCX (1:1000, Santa Cruz) or rabbit anti-Ki67 (1:500, Abcam) in blocking buffer were added to each section, and allowed to incubate at 4°C on the shaker for 48 hours.

After incubation with the primary antibody, sections were returned to room temperature, primary antibodies were removed, and sections were rinsed with the PBS- 0.3% Triton solution 3 times. The sections were then blocked with the same blocking buffer for 3 hours at room temperature. Following blocking, 250µl/well of secondary antibody solutions (biotin-conjugated anti-goat 1:200 dilution for DCX; biotin-conjugated anti-rabbit 1:200 dilution for Ki67) in the same blocking buffer were added to the sections and allowed to incubate overnight at 4°C on a shaker. The next day, sections were brought back to room temperature and washed with 1X PBS 3 times. An avidin-biotin complex (ABC; 1:200A, 1:200B) solution was prepared 30 minutes before use, and then added to the tissue sections for a 2 hour incubation at room temperature. The sections were then washed 3 times with 1X PBS.

To prepare the DAB (Vector lab) solution, 10 mg of DAB powder were weighed out and added to 40 ml of 1X PBS and 17.5µl of 30% H₂O₂. Sections were incubated with the DAB solution for 2-5 minutes (or until staining could be seen in dentate gyrus under the light microscope), and then washed 3 times with 1X PBS. After staining was complete, the sections were mounted on glass slides, with 6 sections per slide placed in rostrocaudal order, and allowed to dry completely. Sections were counterstained with Nissl and coverslipped with Permount.
2.5.1 Stereological Cell Quantification

A total of 20 brains (Table 2.1) were evaluated for DCX+ and Ki67+ hippocampal cell counts. To quantify the number of DCX+ cells in the ipsilateral and contralateral dentate gyrus and hilus regions, sections were manually counted with an Olympus Image System CAST program (Olympus, Denmark) using the optical fractionator sampling scheme. This method allows counting particles within a uniform and systematic sample that is part of a known fraction of the region to be analyzed to estimate the total number of neurons in a particular section of the hippocampus.

Five sequential sections per brain, 240µm apart, were examined. The region of interest (hippocampal dentate gyrus) was outlined while at the 4x objective, and the cells were counted at the 40x objective. For each section, an optical dissector was used to count the number of neurons within a predetermined grid of x,y positions. The area of the unbiased counting frame ($a / f$) was known, and the rest of the positions in the grid were determined by stage-stepping intervals. Together these measurements are used to determine the area sampling fraction, ($asf$) = $a/f / (Δx × Δy)$.

The dissector height was also known ($h = 15µm$) relative to the section thickness ($t$), and with all of these parameters, the total number of neurons in the object could be estimated using the following equation: $n = ΣQ^- · t/h · 1/asf · 1/ssf$, where $ΣQ^-$ is the number of cells counted, and $ssf$ is the section-sampling fraction (0.125 in this study).

DCX+ neurons were accounted for by their location in the hilus and location relative to the SGZ. The SGZ and inner third of the granular cell layer (GCL), the middle third of the GCL, and the outer third of the GCL were the three regions cells were counted to examine the extent of neuron migration (Figure 2.1). Ki67+ cells were located at 4x magnification and counted at 40x magnification, focusing through the entire section to account for cells that may have been darker.
or lighter within different planes. Ki67+ cell counts were divided only by their presence within either the hilus or the SGZ + GCL region.

Type 1 early precursor cells, also referred to as radial glial cells, initiate the process of adult neurogenesis in the SGZ, and divide asymmetrically to produce type 2 (non-radial) cells. Type 2 cells proliferate rapidly, and have the potential to differentiate into neurons or glial cells. Type 2 cells generate a more fate-committed type 3 cell (neural progenitor cell), which transitions from a proliferating neuroblast to an immature neuron (Ehninger and Kempermann, 2008; Aimone et al., 2014). Immature neurons in the SGZ then migrate into the GCL; adult-born neurons, specifically, have been shown to preferentially migrate to the inner and middle layers of the GCL as they differentiate into mature granular cells, develop dendritic branches reaching towards the molecular layer, and develop axonal processes extending to the CA3 region (Aimone et al., 2014). This integration and migration pattern can be disrupted in pathological conditions, such as schizophrenia, epilepsy, and neurodegenerative diseases (Ehninger and Kempermann, 2008).

Injury has also been shown to affect the outward migration of adult-born neurons in the SGZ. The migration of immature neurons into the GCL is not only accelerated in injury, but outward migration into the GCL is also increased, with some new neurons migrating beyond the GCL into the molecular layer following CCI. In addition to influencing outward migration, CCI injury has also been shown to increase complexity of dendrites in adult-born neurons, with an increase in the number of branch points closer to the soma (Villasana et al., 2015).
Figure 2.1 Regions of GCL and Hilus in Hippocampal Dentate Gyrus to Determine Neuron Migration. DCX+ cell counting in the GCL was divided into three parts: inner, middle, and outer, with the inner $\frac{1}{3}$ including the SGZ, the site of adult neurogenesis. Image A shows a 4x magnification of the regions of interest within the DG. Image B shows a 40x magnification of the three layers within the DG, demonstrating individual cells within each layer. Red: DCX staining; Blue: DAPI staining. Image C shows the TBI-induced migration of immature adult-born neurons from the SGZ into the GCL demonstrated by Villasana et al. (2015).
2.6 Differentiation Study

2.6.1 Immunofluorescent Staining

Incorporation of 5-Bromo-2’-deoxyuridine (BrdU) into dividing cells is generally used to detect adult neurogenesis and survival (Brown et al., 2003). The maturational fate of newly generated cells was determined by processing the sections for immunofluorescent double labeling with antibodies against BrdU and mature cell markers for neurons (NeuN), astrocytes (GFAP), or microglia (Iba1). To assess for BrdU+ cells, the sections were first washed with PBS, and then the DNA was denatured with 50% formamide for 60 minutes at 65°C. The sections were then rinsed with 2X SCC and incubated with 2N HCL for 30 minutes at 37°C.

Following the denaturation process, sections were rinsed with 1X PBS, and subsequently quenched with 3% H2O2 for 1 hour at room temperature. Prior to blocking the sections overnight at 4°C (5% normal horse serum in 0.3% Triton), sections were rinsed with PBS. Following blocking, the sections were incubated with either mouse-anti BrdU primary antibody (1:200 Dako) or rat-anti BrdU (1:200, Serotec) and mouse-anti NeuN (1:500, Chemicon), rabbit-anti GFAP (1:2000, Dako), or rabbit-anti Iba1 (1:1000, Wako) in PBS-Triton solution with 5% normal horse serum for 48 hours at 4°C. After 48 hours, sections were brought to room temperature, washed with PBS-Triton solution, and blocked overnight with the same blocking buffer.

Secondary antibodies in blocking buffer were added the following day as follows: Alexa Fluor 488 anti-rat (1:200) and Alexa Fluor 568 anti-mouse (1:200) for BrdU/NeuN labeling, and Alexa Fluor 488 anti-mouse (1:200) and Alexa Fluor 568 anti-rabbit (1:200) for BrdU/GFAP and BrdU/Iba1 labeling. Sections incubated for 3 hours at room temperature, were washed with PBS, mounted on glass slides, and coverslipped with Vector shield.
2.6.2 Quantification of fluorescent double-labeled cells

To quantify the percentage of BrdU+ cells in the dentate gyrus that co-labeled with cell type specific markers, sections were examined with a Zeiss 710 confocal microscope. BrdU cells were examined for the presence of co-labeling with cell specific markers, NeuN, GFAP, or Iba1. Only BrdU+ cells, for which the nucleus was unambiguously associated with a certain cell-type specific marker were considered co-labeled. The percentage of double-labeled cells was determined as the number of BrdU+/NeuN, BrdU+/GFAP, or BrdU+/Iba1+ cells versus the total number of BrdU+ cells. For each brain, a minimum of 100 BrdU+ cells were examined for their co-labeling with cell type specific markers in each subregion.

2.7 Statistical Analysis

The data gathered from immunohistochemistry procedures was analyzed using SPSS software. Data presented shows the mean and standard error of the mean. A two-way analysis of variance (ANOVA), followed by a Fisher least significant difference (LSD) test was performed to identify significance between treatment groups. P-values less than 0.05 determined significance.
Chapter 3: Results

3.1 The effect of 7,8-DHF treatment on cell proliferation at subacute stage following TBI

In this study, we use the proliferation marker Ki67 to assess whether DHF treatment can have a lasting effect on cell proliferation in the DG at 15 days post-injury. The common staining pattern for Ki67+ cells within the DG is shown in Figure 3.1. Ki67 stained cells are located in the SGZ and inner 1/3 of the GCL, mostly in clusters as shown in Fig. 3.1B.

Our previous studies have found that injured juvenile and adult rats have a significant increase in cell proliferation in the DG at 2 and 7 days post-injury compared to sham (Figure 1.1), but no longer have a significant increase in proliferation by 14 days post-injury (Sun et al., 2005). Using proliferation marker Ki67, we tested whether at the subacute stage following TBI (15 days), 7,8-DHF treatment has an effect on cell proliferation in the DG. We found that the number of Ki67 cells in the injured groups, including vehicle and both DHF treatment groups, are slightly higher than the sham group in the ipsilateral side of SGZ+GCL, however, the difference did not reach statistical significance. In the contralateral side of SGZ+GCL, slightly, but not significantly, higher number of Ki67+ cells are observed in the injured animals with DHF treatment (Fig 3.2). In the hilus region, similar trend of Ki67+ cells are found as in the SGZ+GCL in both hemispheres (Fig. 3.3). The Ki67 data suggest that DHF did not affect cell proliferation at this time point.
Figure 3.1: 4x magnification (A) and 40x magnification (B) of Ki67+ staining within the hippocampal dentate gyrus. There was no significant difference in Ki67 staining, and hence, no significant difference in cellular proliferation, between treatment groups. Figure 3.7B illustrates the clumping pattern of proliferating cells that is characteristic for Ki67+ staining.
Ki67+ Cells in SGZ+GCL 15dpi

Cell Counts

- Sham
- CCI-Veh
- DHF G1
- DHF G2

SGZ+GCL (Ipsilateral) vs SGZ+GCL (Contralateral)
Figure 3.2: Comparing Ki67+ cells in the ipsilateral and contralateral SGZ+GCL 15 days post-injury. There was no significant difference between treatment groups, however, all injured groups, particularly the DHG G2 group had a slightly higher number of Ki67+ cells.
Ki67+ Cells in Hilus 15dpi

Cell Counts

Hilus(Ipstitial)

Hilus(Contralateral)

Sham
CCI-Veh
DHF G1
DHF G2
Figure 3.3: Comparing Ki67+ cells in the ipsilateral and contralateral hilus regions 15 days post-injury. There was no significant difference between treatment groups.
3.2 The effect of 7,8-DHF on generation of new neurons at subacute stage following TBI

DCX, an immature neuronal marker that labels neuroblast and migration new neurons, is widely used to assess generation of new neurons. In this study, to assess whether DHF treatment has an effect on neurogenesis in the DG, we processed brain sections taken from 15 days post-injury with DCX staining and quantified the total number of DCX+ cells in the SGZ+GCL and hilus regions using unbiased stereological method. The DCX+ staining patterns that were commonly observed for each treatment group are shown in figure 3.4. In both DHF treatment groups, the cell body of DCX+ cells were densely distributed throughout the SGZ of the DG, with their dendrites extending outward to the molecular layer. There was also an increase in DCX+ cell density in vehicle DG, however, it was visibly less dense than either DHF treatment group.

Within the SGZ+GCL region, a two-way ANOVA revealed a significant increase ipsilaterally in DCX+ cells in DHF Group 2 compared to sham (p = 0.0003), vehicle (p = 0.0009), and DHF Group 1 (p = 0.0009) animals. Post-hoc comparisons confirmed these significant values (Figure 3.5). Contralaterally, there was a significant increase in DCX+ cells in DHF Group 2 compared to vehicle (p = 0.01) and DHF Group 1 (p = 0.001), which was confirmed by a post-hoc comparison of pairwise differences among group means (Figure 3.5). In the hilus, a two-way ANOVA showed a significant increase in DCX+ cells ipsilaterally in DHF Group 2 compared to sham (p = 0.001) and DHF Group 1 (p = 0.02) animals, and in vehicle compared to sham (p = 0.01) animals (Figure 3.6). Figure 3.1 illustrates the differences in DCX+ staining within the four treatment groups.
Figure 3.4: Difference in DCX+ staining within the ipsilateral hemisphere of the hippocampal dentate gyrus of sham, vehicle, DHF Group 1, and DHF Group 2 following CCI injury. Both DHF treatment groups show increased density of DCX+ cells in the SGZ and into the GCL compared to sham and vehicle groups. Sham animals display the lowest density of DCX+ staining, and most DCX+ cells are restricted to the SGZ. DHF Treatment Group 2 had the most densely stained DCX+ cells within the ipsilateral SGZ+GCL and hilus regions.
DCX+ Cells SGZ+GCL 15dpi

Cell Counts

- Sham
- CCI-Veh
- DHF G1
- DHF G2

SGZ+GCL (Ispilateral)
SGZ+GCL (Contralateral)
Figure 3.5: Total DCX+ Cells in Subgranular Zone and Granular Cell Layer in the ipsilateral and contralateral hemispheres 15 days post-injury. There is a significant increase in DCX+ cells in the ipsilateral SGZ+GCL region of DHF Group 2 compared to all other treatment groups (*p<0.05). Contralaterally, there were significantly more DCX+ cells in sham animals compared to DHF Group 1, and significantly more DCX+ cells in DHF Group 2 compared to DHF Group 1 (*p<0.05).
**DCX+ Cells Hilus 15dpi**

- **Hilus (Ipsilateral)**
- **Hilus (Contralateral)**

- **Cell Counts**

- **Sham**
- **CCI-Veh**
- **DHF G1**
- **DHF G2**

*Significant difference*
Figure 3.6: Comparing DCX+ cells of the hilus region in the ipsilateral and contralateral hemispheres 15 days post-injury. Ipsilaterally, there are significantly more DCX+ cells in DHF Group 2 and vehicle animals hilus region compared to sham animals, and more DCX+ cells in DHF Group 2 compared to DHF Group 1 (*p<0.05). Contralaterally, there were no significant differences.
3.2.1 7,8-DHF Treatment Group 2 increases migration of new neurons

Adult-born neurons generated in the SGZ migrate outward to the granular cell layer where they become dentate granule cells and integrate into the existing circuitry (Zhao et al., 2008). Migration of new neurons to the hilus or beyond the GCL to the molecular layer have been associated with pathological conditions, such as schizophrenia and epilepsy (Duan et al., 2007; Gong et al., 2007). TBI has also been shown to increase the outward migration of new neurons following injury, however, unlike neuronal migration in schizophrenia or epilepsy, these new neurons are able to functionally integrate into the hippocampal cell network, and mature to have normal electrophysiological properties. These studies have suggested that newly generated neurons are able to participate in information processing once they have migrated from the SGZ into the surrounding circuitry (Villasana et al., 2015).

In this study, we wanted to see if DHF influenced the migration of newly generated neurons from the SGZ to the GCL. There is a possibility that if more neurons migrate away from the SGZ and are able to functionally integrate into hippocampal circuitry, it may contribute to greater cognitive recovery. To examine this, the SGZ and GCL were divided into 3 regions to examine neuronal migration, with the inner third region containing the SGZ, which is a site of adult neurogenesis (Figure 2.1). Within the inner third of the SGZ + GCL, there was a significant increase in DCX+ cells ipsilaterally in DHF Group 2 compared to sham (p = 0.001), vehicle (p = 0.002), and DHF Group 1 (p = 0.003) animals (Figure 3.4), which was confirmed in a post-hoc comparison. Contralaterally, there were significantly more DCX+ cells in DHF Group 2 compared to vehicle (p = 0.04) and DHF Group 1 (p = 0.003) animals (Figure 3.7).

Within the middle third of the GCL, DHF Group 2 once again had significantly more DCX+ cells than sham (p = 0.001), vehicle (p = 0.006), and DHF Group 1 (p = 0.004) animals,
ipsilaterally, and significantly more cells compared to vehicle (p = 0.004) and DHF Group 1 (p = 0.01), contralaterally. Sham animals also had significantly more DCX+ cells in the middle third of the GCL compared to vehicle (p = 0.003) and DHF Group 1 (p = 0.009) animals contralaterally, indicating that the injury may have disrupted normal neuron migration in these groups (Figure 3.8). In the outer third layer, a two-way ANOVA and post-hoc comparison showed no significance between treatment groups (Figure 3.9).
DCX+ Cells Inner 1/3 Layer of SGZ+GCL 15dpi
Figure 3.7: Comparing the number of DCX+ cells of the ipsilateral and contralateral inner third of the SGZ+GCL 15 days post-injury. There was a significant (*p < 0.05) increase in DCX+ cells on the ipsilateral side in DHF Group 2 compared to all other treatment groups. On the contralateral side, there were significantly (*p < 0.05) more DCX+ cells in DHF Group 2 compared to vehicle and DHF Group 1.
DCX+ Cells Middle 1/3 layer of SGZ+GCL 15dpi

Cell Counts

Ipsilateral(middle)  Contralateral(middle)

Sham  CCI-Veh  DHF G1  DHF G2

*
Figure 3.8: Comparing the number of DCX+ cells in ipsilateral and contralateral middle third of GCL 15 days post-injury. There was a significant (*p < 0.05) increase in DCX+ cells in DHF Group 2 compared to all other treatment groups, ipsilaterally. Contralaterally, there was a significant (*p < 0.05) increase in DCX+ cells for both sham and DHF Group 2 animals compared to vehicle and DHF Group 1.
DCX+ Cells Outer 1/3 Layer of SGZ+GCL 15dpi

- Ipsilateral (outer)
- Contralateral (outer)

Cell Counts

- Sham
- CCI-Veh
- DHF G1
- DHF G2

Graph showing cell counts for different groups and locations.
Figure 3.9: Comparing the number of DCX+ cells in the ipsilateral and contralateral outer third of GCL 15 days post-injury. There was no significant difference in the number of DCX+ cells among all groups.
3.3 The effect of 7,8-DHF treatment on cell type differentiation of newly generated cells following TBI

Immunofluorescent double-labeling is an immunohistochemistry technique that localizes two specific antigens within a cell using fluorescently-labeled antibodies. For the purposes of this experiment, double-labeling allows us to determine the maturational fate of progenitor cells by observing the proteins the cell begins expressing as it matures, while it is still expressing its immature protein profile (Chen et al., 2010). We have demonstrated that the DHF has a significant effect on the generation of new neurons in the SGZ following TBI, and in this portion of the study we sought to determine the maturational fate of newly generated cells. Previous studies in our lab have shown that injured juvenile rats have a significant increase in newly generated cells differentiating into mature neurons, as demonstrated by higher percentage of co-localization of BrdU+/NeuN+ cells in the SGZ (Sun et al., 2005).

NeuN is a widely used marker for mature neurons which labels neuronal nuclei. Co-labeling of BrdU and NeuN indicates neuronal maturation fate of newly generated cells. Under confocal microscopy, most of cells co-labeled with BrdU (green) and NeuN (red) are located in the SGZ and inner 1/3 of the GCL (Fig. 3.10). Scattered BrdU+/NeuN+ cells are also observed in the middle and outer 1/3 of the GCL. Co-labeling of two markers resulted in a yellow tone (Fig. 3.10C). Using a two-way ANOVA and post-hoc analysis, it was determined that within the SGZ+GCL and hilus regions of the hippocampus there was no significant difference in the percentage of BrdU/NeuN co-localization between treatment groups. No BrdU/NeuN co-localization was present for sham animals within the hilus region. As 3-month old adult rats were used in this study, the findings for vehicle-treated animals are consistent with previously
described studies (Sun et al., 2005), and also demonstrated that 7,8-DHF does not significantly increase differentiation of injury-induced proliferating cells into mature neurons.
Figure 3.10: Individual staining patterns of NeuN+ (3.10A) and BrdU+ (3.10B) cells and their merged colocalization (3.10C). There were no significant differences in the percentage of BrdU/NeuN colocalization between treatment groups (3.10D) within the SGZ+GCL or hilus regions. Regardless of injury or treatment, roughly the same percentage of proliferating cells differentiated into mature neurons.
Glial fibrillary acidic protein (GFAP) is an intermediate filament protein present in astrocytes within the CNS. Astrocytes are a type of star-shaped glial cell in the CNS that facilitate communication between neurons by absorbing and secreting neurotransmitters within the tripartite synapse, and maintaining the BBB (Araque et al., 1999). Studies have shown the fate of proliferating cells proximal to the injury site in mice is predominantly astrocytic at 60 days post-CCI injury, forming a astrogliotic scar at the site of injury, and demonstrating that newly generated cells contribute to cellular remodeling following TBI (Kernie et al., 2001). A similar increase in BrdU+/GFAP+ cells is observed in the SGZ following injury in both adult rats and mice (Kernie et al., 2001; Sun et al., 2005). In this portion of the study, we sought to determine if 7,8-DHF treatment affected the differentiation of newly generated cells into astrocytes following injury.

Astrocytes are star-shaped, with multiple processes extending from the cell body, and wrapping around surrounding neurons (Figure 3.11 C). The staining pattern of GFAP+ cells is demonstrated in figure 3.11 A. Progenitor cells that co-expression GFAP and BrdU following CCI injury will likely differentiate into astrocytes. Type 1 progenitor cells in the SGZ also express GFAP, but can be differentiated from astrocytes in the SGZ as Type 1 progenitors also express Nestin (Kriegstein and Alvarez-Buylla, 2009). The total number of independent BrdU+ and colocalized BrdU+/GFAP+ cells was counted within the SGZ+GCL and hilus regions to determine the percent co-localization of BrdU+/GFAP+. A two-way ANOVA revealed no significant difference between treatment groups within the SGZ+GCL region, but a significant increase in DHF Treatment Group 2 compared sham (*p = 0.031) and vehicle (*p = 0.023) animals within the hilus region. A Fisher least significant difference (LSD) post-hoc comparison confirmed these results. Once again, sham animals had no co-localization within the hilus region.
BrdU/GFAP Percent Colocalization in SGZ+GCL and Hilus at 28dpi
Figure 3.11: Individual staining patterns of GFAP+ (3.11A) and BrdU+ (3.11B) cells and their merged colocalization (3.11C). There was no significant difference in the percentage of BrdU+/GFAP+ co-labeling within the SGZ+GCL region (3.11D), however there were significantly (*p<0.05) higher percentage of BrdU+/GFAP+ cells within the hilus region of DHF Treatment Group 2 animals compared to sham and vehicle.
Finally, we assessed the percentage of co-localization of BrdU+/Iba1+ cells in the DG to determine if 7,8-DHF influenced the differentiation of injury-induced proliferating cells into microglia. Iba1 is a widely used marker for microglia. It labels ionized calcium-binding adaptor molecule 1 expressed by microglial cells. Microglia are a type of glial cell commonly activated by inflammatory pathways following injury, and are crucial to the central nervous system’s immune response. Over-activation of microglia, however, can have negative effects, including chronic pain and destruction of neurons, leading to neurodegenerative disorders (Wood, 2002).

Progenitor cells that co-express BrdU and Iba1 suggest differentiation into microglia following injury. Microglia exist in ramified (or resting) and activated forms, which are reflected in their morphology. Ramified microglia have a small cell body and long, intricate cellular processes or arborization, while microglia activated by disruptions in homeostasis have shortened projections with reduced complexity and a larger cell body (Kettenmann et al., 2011). Activated microglia were observed in this study, as shown in red in figure 3.12 A, as a result of the CCI injury.

The percent BrdU/Iba1 co-localization was measured by counting independent BrdU+ cells and cells co-labeled with BrdU and Iba1+ cells. In the SGZ+GCL region, a two-way ANOVA and Fisher LSD post-hoc analysis has shown that injured animals with vehicle treatment had a significantly higher percentage co-localization of BrdU+/Iba1+ neurons compared to sham animals within the SGZ-GCL region (*p=0.037). Within the hilus, vehicle (*p=0.01) and both treatment groups (DHF G1 *p= 0.012; DHF G2 *p=0.007) had a significantly higher percentage of BrdU+/Iba1+ co-localization compared to sham animals. No BrdU/Iba1 co-labeled cells were found within the SGZ-GCL region in sham.
BrdU/Iba1 Percent Colocalization in SGZ+GCL and Hilus at 28dpi
Figure 3.12: Individual staining patterns of Iba1+ (3.12A) and BrdU+ (3.12B) cells and their merged colocalization (3.12C). There were significantly (*p<0.05) more BrdU+/Iba1+ cells in vehicle animals compared to sham animals within the SGZ-GCL region. There was no colocalization within the hilus region of sham animals. Every other treatment group (including vehicle) had significantly (*p<0.05) higher percentage of BrdU+/Iba1+ cells than the sham animal.
Chapter 4: Discussion

4.1 Summary

The goal of this study was to examine the effects of 7,8-DHF on proliferation, neurogenesis, neural migration, and neural differentiation within the hippocampal DG following CCI injury. Injury-induced cellular proliferation, neurogenesis, and migration were examined at 15 days post-injury, and differentiation of newly generated cells was examined 28 days post-injury. In this study we found that 7,8-DHF did not have a significant effect on proliferation in the SGZ+GCL or hilus regions in the subacute stage. However, there was an increasing trend in the SGZ+GCL region among treatment groups, demonstrated in figure 3.8, in which all injured animals experience increased proliferation, and both DHF treatment groups had increased proliferation compared to vehicle animals. We also found that 5 doses of 7,8-DHF treatment starting 48hrs post-CCI injury caused a significant increase in neurogenesis in the SGZ+GCL and hilus regions of the dentate gyrus, and an increase in migration in the SGZ+GCL compared to sham and vehicle animals, as well as animals treated with 5 doses of 7,8-DHF beginning 1hr post-injury.

In the differentiation study, neither vehicle nor DHF-treated animals demonstrated an increase in differentiation of newly generated cells into mature neurons within both SGZ-GCL and hilus regions. Conversely, all injured animals, regardless of vehicle or DHF treatment, had an increase in BrdU/GFAP and BrdU/Iba1 labeled cells in the hilus, suggesting that the injury-induced increase of BrdU-labeled cells in the hilus region are mainly glial cell types, regardless of treatment.
4.2 Discussion

Our study found that 7,8-DHF did not have a significant effect on proliferation at day 15 post-injury. Previous studies in our lab have shown that there is a significant increase in proliferation of the SGZ at 2 and 7 days post-injury in adult adults, but levels are no longer significantly elevated by day 14 post-injury (Figure 1.1; Sun et al., 2005). Our results for sham and vehicle at 15 days post-injury are consistent with the 14 day post-injury data of this study, and the lack of significantly elevated Ki67+ cells in both DHF treatment groups suggest that by 15 days post-injury, 7,8-DHF does not significantly influence proliferation at this time point post-TBI. This is consistent with another group that studied DHF effects on proliferation following TBI, which found that DHF did not cause any significant increase in cell proliferation at 2 weeks post-injury (Zhao et al., 2015).

Multiple studies have shown that growth factors play an important role in mediating neurogenesis, and have elevated expression during brain development (Caday et al., 1990). Experiments that supplied exogenous basic fibroblast growth factor (bFGF) following lateral fluid percussion injury have shown a significant increase in proliferation of the SGZ and subventricular zone (SVZ) at 7 days post-injury compared to both sham and vehicle animals (Sun et al., 2009). Although 7,8-DHF is not a growth factor, it binds to the same receptor, TrkB, as growth factor BDNF, and mimics its downstream effects. Our study did not include assessment at 7 days post-injury, but given previous studies and 7,8-DHF’s growth-factor like effects, one would expect that it may affect cellular proliferation at early time points post-injury. Future studies in our lab will include an assessment of proliferation at 7 days post-injury to confirm or reject this hypothesis.
It is worth noting that although injured brains have increased proliferation up to 2 weeks post-injury, long-term cell survival is diminished in injured brains compared to sham. This is supported by the fact that only 46% of newly generated neurons in the dentate gyrus survive up to 10 week post-injury, while sham animals exhibit 65% survival of new neurons in the same time frame, suggesting that the injured brain environment is not well-suited for long-term cell survival (Sun et al., 2007). One explanation for the poor environment an injured brain provides for cell survival may be an insufficient level of neurotrophins. Such environments are typical of brains in the initial stages of neurodegenerative diseases such as Alzheimer’s, but limited neurotrophic support is also observed in cognitive impairments and brain trauma (Sun et al., 2007).

According to our results, intraperitoneal administration of 5mg/kg of 7,8-DHF, starting at 2 days post-injury for 5 daily doses significantly increased neurogenesis and migration in the hippocampal dentate gyrus compared to sham, vehicle, and DHF Group 1 animals. Previous studies in our lab have shown that injured juvenile (vehicle) brains experience a more robust neurogenic response than adult or aged rats (Sun et al., 2005). Several groups have had conflicting results on whether TBI produces a significant increase in neurogenesis, which is due, in large part, to the variation in injury severity in different TBI models. In a mouse CCI model, a recent study has reported that mild TBI did not affect cell proliferation in the DG, while moderate TBI caused an increase in cell proliferation, but no neurogenesis. Only severe TBI resulted in an increase of both proliferation and neurogenesis (generation of new mature neurons) (Wang et al., 2015).

In the current study, we did not observe a significant increase in neurogenesis in vehicle animals, which is consistent with the moderate CCI injury we performed and Wang et al’s 2015
study. Although the lack of significant increase in proliferation is consistent with our lab’s previous studies (Sun et al., 2005), because we did not test proliferation at day 7 post-injury, we cannot be certain that the moderate TBI, by Wang’s description above, was performed, as we did not measure proliferation during a time period (day 2-7) that TBI-induced proliferation is known to be significantly increased.

A recent study examined the effects of 7,8-DHF on adult mice also found that DHF significantly increased the number of DCX+ cells in the SGZ and inner ⅓ layer of the GCL at 2 weeks post-injury (Zhao et al., 2015). This group employed a different DHF treatment strategy and a mouse CCI model that was different from our study. In this study, injured animals received daily DHF injections for the weeks following injury until they were sacrificed. It was reported that DHF-treated injured animals have significantly more DCX+ cells compared to sham animals, and sham animals treated with DHF did not have a significant change in DCX+ cell density, suggesting that DHF’s neurogenic effects are injury-(or pathology) related (Zhao et al., 2015).

By increasing the number of surviving cells at 15 days post injury, it is possible that injured brains treated in this manner will have more surviving cells long-term that can successfully integrate into the hippocampal circuitry. Adult neurogenesis is involved in hippocampal-dependent learning, thus the combination of injury and 7,8-DHF-induced neurogenesis may result in better cognitive outcomes (Gould et al., 1999). This is supported by the fact that enhanced neurogenesis in this region from injections of basic fibroblast growth factor (bFGF), a neurotrophic factor, post-injury has been shown to contribute to cognitive improvement in the Morris Water Maze (Sun et al., 2009).
DHF treatment group 2 consistently having significantly more DCX+ cells in the DG than DHF group 1, which received DHF injections beginning 1 hour post-injury, may be due to a variety of reasons. As described above and shown in Figure 1.1, the proliferative response in the DG following injury is highest at 48 hours post-injury and continues for up to 7 days (Sun et al., 2005). It is possible that complementing the endogenous proliferative response with 7,8-DHF over the same time period (day 2-6) of increased proliferation contributes to the significant increase in DCX+ cells at 15 dpi.

Additionally, endogenous BDNF levels are significantly upregulated at 12 hours post-injury, but start declining at 24 hours, and are not at significantly elevated levels by 36 hours post-injury. The necrosis pathway, associated with calpain proteases, is also quickly activated in the hours immediately following injury, and employs neurofilaments and spectrins as primary substrates, leading to the disruption of cell transport and structure, and ultimately cell death (McAllister, 2011). Beginning treatment once initial BDNF and calpain pathways have subsided may aid in harnessing 7,8-DHF’s neurotrophic-like effects, as surviving cells may be able to properly utilize it once the inflammatory responses are reduced. The initial response of BDNF following injury is to initiate a neuroprotective response, rather than a regenerative effect (Zhao et al., 2015). Beginning DHF treatment during the initial spike in BDNF levels may help protect existing dendrites from injury, but may not contribute as much to the regenerative response that is established later on. Finally, BDNF binds to the TrkB receptor with a higher affinity (Kd = 1.7nm) than 7,8-DHF (Kd = 15.4nm), so it is possible that BDNF outcompetes 7,8-DHF for TrkB receptors within the first 24-36 hours following injury.

TBI has been shown to accelerate the outward migration of newly generated neurons post-injury, migrating from the subgranular zone (SGZ) to the granular cell layer (GCL),
specifically in the ipsilateral hemisphere. Injury, however, does not increase migration into the hilus region (Villasana et al., 2015). Our results confirmed this outward migration in DHF Group 2 animals, as this treatment group exhibited significant increase in DCX+ cells ipsilaterally in the middle third layer of the GCL, and though the increase was not significant in the outer third layer, there was a noticeable increase in DCX+ cells for DHF Group 2 animals in this layer compared to sham and DHF Group 1 animals. This outward migration from the dentate gyrus towards the outer GCL region may aid in the integration of dendrites into the existing CA1 hippocampal circuitry, and contribute to cognitive recovery (Gould et al., 1999).

Vehicle animals did not have a statistically significant increase in DCX+ cells in the middle and outer layers of the GCL, but did have increased cell counts compared to sham and DHF Group 1 animals. One study has shown that untreated injured animals have significantly increased migration into the ipsilateral GCL region compared to sham animals. (Villasana et al., 2015). Our results are not consistent with that study. Although vehicle animals had increased levels of DCX+ cells in all three regions of the SGZ+GCL compared to sham, none of the differences were statistically significant. This discrepancy may be attributed to differences in measurement strategies (distance from SGZ).

The maturational fates of the neural progenitor cells following injury and DHF treatment provide insight into the role new neurons will play in the weeks following injury. Previous studies in our lab have demonstrated that newly generated cells post-injury have a higher percentage differentiating into glial cells in adult rats and a higher percentage differentiation into mature neurons in juvenile rats (Sun et al., 2005). Our results were consistent with these findings, as there was no statistically significant difference in percentage co-localization of BrdU+/NeuN+ between treatment groups within the SGZ+GCL or hilus regions, but there was a
significant increase in BrdU+/GFAP+ cells in the hilus region of DHF Group 2 animals and a significant increase in BrdU+/Iba1+ cells in the hilus region of all injured animals. If these mature neurons survive, they will functionally incorporate into the surrounding circuitry (Villasana et al., 2015).

While there was no statistically significant difference in the percentage of BrdU+/GFAP+ co-localization within the SGZ+GCL region, DHF Treatment Group 2 had significantly a significantly higher percentage of colocalization than both sham and vehicle in the hilus region. GFAP is an astrocytic marker, and cells expressing this protein will differentiate into astrocytes. Astrocytes are a type of glial cell found in both the brain and spinal cord, and function as part of the tripartite synapse, facilitating the passage of neurotransmitters to and from synapses, and providing support to endothelial cells that line the BBB. Although astrocytes were once thought of as space fillers in the brain, they have been found to play a crucial role in neuronal network’s communication (Araque et al., 1999). In addition to its role in signaling, astrocytes have also been shown to promote oligodendrogenesis following damage to white matter through the BDNF signaling pathway. Oligodendrogenesis promotes the growth of oligodendrocytes, which contribute to the creation of myelin that wraps around axons to ensure rapid signaling (Miyamoto et al., 2015).

The significant increase in BrdU+/GFAP+ cells in DHF Treatment Group 2 in the hilus region may aid in the reestablishment of communication between new and/or existing neuron and oligodendrogenesis. The entire dentate gyrus surrounds the CA3 region of the hippocampus, but the hilus in particular outlines it closely. It is possible that an increase in astrocytes in this region promotes the integration of axons from neurons in the SGZ with neurons of the CA3 region.
Finally, there was a significant difference in BrdU+/Iba1+ colocalization in vehicle animals compared to sham in the SGZ+GCL, and also for all treatment groups (including vehicle) compared to sham within the hilus region. Cells that co-express BrdU/Iba1 indicate that they are proliferative microglia cells. Microglia are related to cells from the macrophage lineage, and perform similar functions to their relatives within the CNS, phagocytosing debris and intruders. Microglia are also the first cells to respond to injury within the CNS; they subsequently release cytotoxic mediators and generate an inflammatory response (Gehrmann et al., 1995). Following an injury, or in some autoimmune diseases, microglia can rapidly proliferate, over activate, and ultimately lead to neurodegeneration (Wood, 2002).

In both the SGZ-GCL and hilus regions, it is not surprising that the sham animal had very few Iba1+ cells and no co-localization at all. The animal had not experienced injury, so presumably, there was no experimental reason for microglia to be in the activated form. Vehicle animals, understandably, had a statistically significant increase in BrdU+/Iba1+ percent co-localization compared to sham. While it was not statistically significant, vehicle animals did have a greater percentage of co-localization within the SGZ-GCL region compared to both DHF treatment groups. This suggests that, within this region, 7,8-DHF may keep the inflammatory response low.

4.3 Conclusions

The findings in the current study support the therapeutic potential for the use of 7,8-dihydroxyflavone in post-TBI treatment. Due to its small size (254Da) and substantial half-life (134 minutes), 7,8-DHF is able to cross the BBB and effectively act as a TrkB agonist, supporting the generation and migration of new neurons, astrocytes, and microglia. Finding
feasible treatment options, like 7,8-DHF, that can combat neuronal loss following injury is crucial to the recovery and cognitive outcomes of TBI patients.


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