The induction of traumatic brain injury by blood brain barrier disruption

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entitled
The Induction of Traumatic Brain Injury by Blood Brain Barrier Disruption
by
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Submitted to the Graduate Faculty as partial fulfillment of the
requirements for the Doctor of Philosophy Degree in Engineering

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Animal models of traumatic brain injury (TBI) are utilized for the study of underlying mechanisms and for the development of potential therapeutics. Traditional TBI models apply concussive forces to the cranium or provide a direct injury to neuronal tissue to model penetrating head wounds. The observation of mild to moderate TBI symptoms in personnel exposed to blast waves has led to the hypothesis that the disruption of the blood-brain barrier (BBB) can cause the symptoms commonly associated with TBI. Using intracarotid injection of hyperosmotic fluid, we assessed the histological and behavioral consequences of a transient disruption of the BBB and compared the results to the classical controlled-cortical impact (CCI) model of penetrating head injuries in rats. Our results demonstrate that the disruption of the BBB causes similar deficits in spatial memory as measured by the Barnes maze compared to other classical TBI models. Furthermore, a computational model was developed to investigate how different types of neural injuries affect a biological network capable of neurogenesis. These findings suggest that a disruption of the BBB can contribute to the neuronal dysfunction associated with brain injury and novel treatments that prevent plasma extravasation during BBB disruption could have therapeutic benefits.
For my wife, Jenny. Three decades, two Memorial Days, and one water project finally brought us together.
Acknowledgements

This dissertation would not have been possible without the support of Drs. Scott Molitor and Ragheb Assaly. I would like to thank the Department of Laboratory Animal Research and especially Tamara Phares at the Department of Bioengineering for their assistance in the intracarotid surgeries. I would like to also thank Steven Wolfe for his work on the Barnes Maze and assisting during surgeries, and Aaron Matyas and Anthony Webb for their help on the controlled-cortical impact device. Many thanks are also given to Dr. David Dignam for the albumin fluorophores.

I am grateful for the constant love and encouragement of my parents, Richard and Katherine, and the rest of my family throughout my life. My success can be attributed to them as much to my hard work and dedication. I also thank Cynthia Mayo, Bernard Scanlon, and the Silcott family for their support throughout the years. Special thanks are also given to my ex-roommates Julie and Ray Bogusz.

I am grateful to Dr. Andrew Heydinger, Clifford Gordon, and others from Engineers Without Borders for enriching my graduate education. My work with them in the south of Honduras provided me with a life changing experience.
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List of Abbreviations

Aβ ..................... amyloid beta
ADEM .................. acute disseminated encephalomyelitis

BBB .................. blood-brain barrier
CCI ..................... controlled-cortical impact
CNS .................... central nervous system
CSF ..................... cerebrospinal fluid

EAE ..................... experimental autoimmune encephalomyelitis
EC ..................... endothelial cells
EPSP .................. excitatory postsynaptic potential

GCS .................... Glasgow Coma Scale
HSA ..................... human albumin serum

IED ..................... improvised explosive device
IFN-γ .................... interferon-γ
IL-1β .................... interleukin-1β

LTD ..................... long-term depression
LTP ..................... long-term potentiation

MS ..................... multiple sclerosis

NO ..................... nitric oxide

PEG ..................... polyethylene glycol
PPF ..................... pair pulse facilitation
PTSD .................... post-traumatic stress disorder

ROS ..................... reactive oxidative species

SGZ .................... subgranular zone

TBI ..................... traumatic brain injury
TGF-β.................transforming growth factor-β
TJ......................tight junctions
TNF-α..................tumor necrosis factor-α
Chapter 1

Introduction

Traumatic brain injury (TBI) is a complex disorder that has lifelong effects on an estimated 5.3 million people in the United States (Marr and Coronado, 2004). The direct and indirect medical costs by factors such as loss of productivity are estimated to be $60 billion a year (Finkelstein et al., 2006). However, TBI has also become an unexpected epidemic among veterans returning from Iraq and Afghanistan affecting an estimated 25% of wounded personnel (Fisher, 2008). TBI is defined as damage to the brain with varying severity from an external force such as rapid acceleration, penetration, and impact caused by falls, motor accidents, bomb blasts, head strikes, and violence. Brain function can be impaired temporarily or permanently and might not necessarily be detectable with current imaging technology and biomarkers. Most TBI studies have examined how a concussive blow to the head can produce diffuse axonal and focal injuries and lead to necrosis and apoptosis. However, few experiments have investigated how dysfunction of the neurovascular unit might contribute to TBI symptoms. In this dissertation, we demonstrate that a disruption of the blood-brain barrier (BBB) can produce behavioral and pathological symptoms associated with TBI and present
computational models to provide additional evidence of the behavioral mechanisms such as learning and memory deficiencies.

The BBB separates the blood from the central nervous system (CNS) by restricting the passage of molecules through a high density of endothelial cells (ECs) bound together by tight junctions (TJs). However, when a disruption occurs in this barrier, solutes from either the blood or CNS can freely exchange causing a shift from normal homeostatic equilibrium. The consequences of this disruption are demonstrated in other pathologies such as epilepsy, Alzheimer’s disease, and multiple sclerosis (Bennett et al., 2010; de Vries et al., 1997; Deane and Zlokovic, 2007; Engelhardt, 2008; Iadecola, 2004; Ifergan et al., 2008; Janigro, 2010; Moor et al., 1994; Oby and Janigro, 2006; Ransohoff, 2009). Due to recent increases in bomb blast induced TBI, researchers have proposed the disruption of the BBB to be central to symptoms experienced following a wider range of TBI. In this study, experiments demonstrated that the disruption in the BBB induce memory deficits comparable to classical animal models of TBI. In addition, a therapeutic treatment hypothesized to restore the integrity of BBB showed the attenuation of symptoms. Additional computational modeling provided evidence of possible mechanisms causing these symptoms. A novel animal model of TBI induced by the disruption of the BBB offers researchers a new experimental method to investigate brain pathologies.

1.1 Traumatic Brain Injury

Symptoms of TBI are dependent on the severity and magnitude of the injury, and often the only signs of injury are manifested by cognitive dysfunction. Clinicians employ
the Glasgow Coma Scale (GCS) to measure the extent of injury to the CNS. The GCS is a universal clarification system that measures the level of consciousness based on verbal, motor and eye-opening response to stimuli based on the scale 3 to 15 (Teasdale and Jennett, 1974). Mild TBI (GCS 13-15) and moderate TBI (GCS 9-12) will be investigated in this dissertation. Mild TBI is defined by an injury that causes loss of consciousness and post-traumatic amnesia lasting less than 24 hours. Some forms of mild TBI such as concussions exhibit no loss of consciousness. Moderate TBI produces loss of consciousness lasting between 1 and 24 hours or post-amnesia for 1 to 7 days. Severe TBI causes loss of consciousness for more than 24 hours or post-amnesia for more than one week. Individuals who experience mild TBI often do not exhibit abnormalities on brain imaging. Patients who die of TBI, 90% die within the first 48 hours from intracranial pressure to the brainstem (Sahuquillo and Arikan, 2006).

Most TBI studies have examined how a concussive blow to the head can produce diffuse axonal injury and lead to TBI symptoms. Although neurodegeneration produced by diffuse axonal injury has a rapid time course, many victims who commonly never seek medical attention exhibit TBI symptoms days after the initial incident. The delayed manifestation of symptoms suggests diffuse axonal injury is not the only mechanism responsible for mild- to moderate-TBI.

Primary injuries are caused by mechanical forces to the CNS where differing tissues have a wide range of susceptible to injury. The results of immediate injury include but are not limited to hemorrhages in multiple regions, axonal stretching, and cerebral lacerations and contusions (Greve and Zink, 2009). Rupture to capillaries and
axons produce cell death in a nonspecific manner. The time course for primary injury is short and therefore little intervention is possible.

Secondary injury of cellular processes is the indirect result of the primary insult and can occur days to weeks later (Faden, 2002; Faden and Stoica, 2007). Metabolic cascades result in necrosis and apoptosis in cells not injured in the initial TBI. These pathways can cause edema, ischemia, alterations in blood pressure, hypoxia, and increases in intracranial pressure. Physiological conditions can often worsen after the primary injury and may result in mortality. Other common cellular changes are depolarization, release of free radicals, further BBB disruption, excitotoxicity, and imbalances of ion homeostasis. Inflammatory pathways are initiated and cytokines are released signaling phagocytotic responses by microglia. In addition, exogenous T-cells extravasate the BBB and can chronic inflammation.

Cognitive deficits after mild-TBI include lack of abstract though processes, loss of memory, impaired attentions, and poor judgment (Hall et al., 2005). Victims can also struggle with emotional and behavioral problems such as depression, anxiety, apathy, and anger. In addition, many symptoms persist as dizziness, headaches, and difficulties concentrating (Hoge et al., 2008; McIntosh et al., 1996; Zitnay et al., 2008). Multiple TBIs further exacerbate symptoms shown by higher risks by career boxers for dementia, Parkinson’s disease, and memory difficulties (Deane and Zlokovic, 2007; Jordan, 2000). Psychiatric consequences of TBI predispose individuals to alcohol and drug abuse, bipolar disorder, schizophrenia, phobia, and obsessive compulsion disorder (Adibhatla and Hatcher, 2008). Often the ability for the victim of TBI to relate emotionally to family and friends has been drastically altered demonstrated sociologically by a spike in
divorces during the first few years after the injury. The increase is attributed to the eventual realization by the spouse that victim is permanently altered as an individual (Arango-Lasprilla et al., 2008; Wood and Yurdakul, 1997).

1.2 Blood-Brain Barrier

The disruption of BBB is a common hallmark of CNS injury and recently therapeutics have targeted the BBB (Shlosberg et al., 2010). The BBB regulates the transport of solutes from the blood into the central nervous system (CNS) by restricting the passive diffusion of large molecules and smaller hydrophilic molecules. The distinctive feature of the BBB is tight junctions (TJs) that connect the polarized endothelial cells (ECs) to create a barrier that is impermeable to plasma and associated solutes. ECs line the luminal surface of the BBB and inhibit the free diffusion of molecules from the vascular space into the parenchyma (Mitic et al., 2000). The CNS primarily maintains homeostasis by highly regulating the exchange of molecules through endothelial transcellular transport. The BBB allows small hydrophobic molecules such as hormones, CO₂, and O₂ to diffuse across ECs, whereas other molecules are subject to active transport, facilitated diffusion, and ion exchange. The BBB in the CNS also is comprised of astrocytic end feet called glia limitans which affect blood flow in the brain, regulate ionic homeostasis, synaptic transmission, and mobilize to assist in cellular repair following injury. The BBB is also surrounded by a large number of pericytes, also called perivascular macrophages, and the basement membrane, which makes up the basic vascular entity (Figure 1-1).
TJs and adherens junctions connect ECs to each other creating the BBB. TJs are the transmembrane complexes that form an interconnected lattice between ECs. The major proteins present in the TJ complex are claudin and occludin which is bound to the cytoplasmic membrane surface proteins, ZO-1 and ZO-2. Occludin has been demonstrated to be the key regulator of BBB permeability (Anderson and Van Itallie, 1995) and dysfunction has been implicated in numerous diseases (Forster, 2008; Wolburg and Lippoldt, 2002). Adherens junctions are more basal and permeable than TJs and make up the primary contacts of ECs. Cadherins constitute the major connections of adherens junctions and are coupled with the cytoskeleton.

Astrocyte end feet surround the ECs and can maintain certain aspects of BBB along with pericytes which have smooth-muscle characteristics. A multifunctional glial cell type, astrocytes have long unbranching cellular processes that envelope synapses. Residing mostly in grey matter, astrocytes have three phenotypes such as radial, fibrous, and protoplasmic within the CNS. Recently, the concept of a “tripartite” synapse has gained traction in the neuroscience community. The presynaptic and postsynaptic terminals are joined by an astrocytic process that is capable of sensing and releasing neurotransmitters (Halassa and Haydon, 2010). The astrocytic signaling mechanism relies on G-protein coupled and other metabotropic receptors, and this interaction has particular significance when astrocytes are injured at the perivascular unit. The release of neurotransmitters such as glutamate by astrocytes uses Ca^{2+} signaling pathways and can contribute to persistent plasticity in the hippocampus (Perea and Araque, 2007). Astrocytes also have a high density of K^{+} channels and can clear extracellular concentration of K^{+} quickly. If this function is hindered, levels of extracellular K^{+}
cause a shift in neuronal membrane potentials and depolarization is more readily obtained. In addition, astrocytes are also coupled to cerebral capillaries and response to neuronal signals to regulate blood flow by changing arteriole diameters (Straub and Nelson, 2007).

**Figure 1-1 The Blood-Brain Barrier (BBB).** The BBB consists of endothelial cells that surround the brain capillary and are joined together by tight junctions. Pericytes support the endothelial cells by providing nutrients, paracrine signals and also serve as contractile cells to regulate blood flow. Astrocytic feet, also called glia limitans, project to the basement membrane which completely surrounds the endothelial process. It is unclear if neurons project to the basement membrane.
Astrocytes have a broad milieu in the CNS as regulators of neuronal plasticity and signaling, neurosecretion, and metabolism. Once thought to be only a support cell to neurons, glia have distinctive signaling roles in the CNS. However, it is still unclear if astrocytes exhibit negative or positive effects following TBI and neuroinflammation (Floyd and Lyeth, 2007; Mahesh et al., 2006). Astrocytes have been shown to assist in the determination of neural stem cells differentiation fate in the hippocampus which could provide a beneficial response (Song et al., 2002). Interestingly, the resident phagocytotic cells, microglia, are activated prior to astrocytic activation following animal models of TBI (Lee et al., 1999; Sandhir et al., 2008). This might indicate that astrocytes are not the primary determinant of the immune response, but rather follow signals released by other CNS cells.

1.3 Pathologies of the Blood-Brain Barrier

The BBB is implicated in several neuronal pathologies such as epilepsy, Alzheimer’s disease, and multiple sclerosis (MS). These neurological disorders can provide researchers with evidence of the BBB pathologies along with a better understanding of normal BBB physiological functions. Most treatments and therapeutics target neuron survival in order to stop deterioration within CNS. However, recent studies have shown the etiology of many neurology diseases might be found in the dysfunctions of some once-called “supporting” cells: glia and ECs.

Epilepsy is a neurological disease that is characterized by chronic seizures brought on by little or no warning. Approximately 50 million people in the world at any given moment have some type of epilepsy, most common being post-traumatic epilepsy
(Kale, 2002). Not a single disorder, the common symptom of epilepsy is abnormal electrical activity in the brain in which action potential lack proper synchronization (Fisher et al., 2005; Prince et al., 2009). In animal experimental models, the administration of pilocarpine can also induce acute seizures by opening the BBB and is the common method of generating status epilepticus in rodent models (Fabene et al., 2008; Marchi et al., 2007). Evidence demonstrates that the chronic disruption of BBB can lead to seizures by creating astrocytic dysfunction by allowing the extravasation of plasma proteins (Seiffert et al., 2004). The hyperexcitability induced by the reduction of potassium and glutamate uptake by glia have been demonstrated to be caused by altered TGF-β signaling (Cacheaux et al., 2009; David et al., 2009; Janigro et al., 1997). The breakdown of the BBB and an increase in angiogenesis in the hippocampus has also been shown to be the result of chronic seizures (Rigau et al., 2007). Moreover, epileptic seizures can be caused by the extravasation of leukocytes during BBB disruption (Kim et al., 2009; Oby and Janigro, 2006).

Alzheimer’s disease is a degenerative neurological disease that is the most common form of dementia. The accumulation of amyloid-beta plaques and neurofibrillary tangles, the histological hallmarks of Alzheimer’s disease, may initiate a pathogenic cascade that leads to neuronal dysfunction in the CNS. A defective BBB is a common characteristic of patients diagnosed with Alzheimer’s disease. The progression of the pathology has been demonstrated by the ability of blood-borne amyloid beta proteins (i.e. Aβ-42 and Aβ-40) to extravasate through a disrupted BBB and bind to specific neuronal subtypes (Clifford et al., 2007). Along with the endogenous production of amyloid beta proteins, these molecules form senile plaque and overwhelm the
clearance mechanisms of neurons and glia. Furthermore, the BBB has an important role of facilitating the clearance of plaques from the CNS interstitial fluid to the blood plasma thus decreasing neurotoxicity (Deane et al., 2004; Deane and Zlokovic, 2007). The activation of microglia has been shown to facilitate in extravasation of amyloid beta proteins (Ryu and McLarnon, 2009). In addition, *in vivo* and *in vitro* studies show that reactive microglia collect around senile plaques (Carpenter et al., 1993).

The migration of T-lymphocytes across the BBB is considered the hallmark of chronic autoimmune diseases such as MS and acute disseminated encephalomyelitis (ADEM). However, studies have demonstrated the dysfunction of the tight junction proteins and endothelial cells may contribute to the decrease in BBB integrity (Bennett et al., 2010; Noubade et al., 2008; Wolburg et al., 2003). BBB disruption results in the infiltration of leukocytes and the activation of microglia (Morganti-Kossmann et al., 2007). Once immune cells extravasate across the BBB, axons are demyelinated causing permanent deficits in CNS signaling (Deb et al., 2010; Huseby et al., 2001). Therefore, BBB disruption can produce a range of neurological disorders, and maintenance of an intact BBB is essential for normal neuronal function.

Widely used, the experimental autoimmune encephalomyelitis (EAE) model is most commonly induced by inoculating the animal with myelin proteolipid protein. EAE can also be induced by whole CNS tissue, purified myelin basic protein, and myelin oligodendrocyte glycoprotein. The demyelination effects of EAE are similar to both MS and ADEM, and EAE is consequently commonly used by researchers to model these diseases despite multiple differences.
The chronic prevalence of the neuroinflammatory pathways has been demonstrated in several studies of TBI and spinal cord injury for up to a year after trauma (Bramlett and Dietrich, 2007). It is known that disruption of the BBB can activate neuroinflammatory pathways; it is hypothesized that neuroinflammation can lead to the neuronal degeneration and dysfunction that underlies TBI symptoms (Lotocki et al., 2009). The CNS has long been regarded as an immunologically privileged organ because of the lack of lymphatic vessels and classical antigen presenting cells such as dendritic cells. The CNS was previously thought to be absent of the immune system in order to maintain a proper homeostasis, but it is now accepted that immune cells gain access to the CNS during both physiological and pathological conditions. Under normal physiological conditions, a limited number of leukocytes cross the BBB and enter into the CNS in a process termed immunosurveillance (Engelhardt, 2006). Immune surveillance is the theory that leukocytes (specifically T-cells) patrol the CNS to check for cancer cells and other abnormalities. By understanding this phenomenon, the mechanisms of BBB extravasation by lymphocytes can be further investigated (Hailer, 2008).

The immune system, specifically leukocytes, utilizes unique initiation and pathogenic entry points in order to extravasate through the BBB into the CNS parenchyma (Figure 1-2). Leukocytes must first be activated by chemokines or signaling molecules released by ECs, and then arrest movement on the EC via integrin binding. Only after these steps can the leukocytes transmigrate through the BBB by disassembling the tight junctions which are complexes that cause the BBB to be virtually impermeable. After extravasation through the BBB, activated leukocytes result in further activation of integrins. In the CNS parenchyma, the activity of leukocytes is mainly dictated by
molecular signals released by microglia. Once activated by neurodegeneration and the release of cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ), microglia can quickly change from a ramified to a macrophage-like morphology. Microglia constitute 20% of the total glial population in the brain. Once activated from neurons and glia during neurodegeneration caused by BBB disruption, microglia change their phenotype depending on the severity and means of the injury. Even small changes in neurophysiology cause microglia to become antigen-presenting such as an increase in extracellular potassium (Dissing-Olesen et al., 2007). Extravasation of albumin across the BBB has also been shown to further lead to microglia activity (Ivens et al., 2007).

![Figure 1-2](image)

**Figure 1-2 Transmigration of Leukocytes Across the BBB.** Leukocytes extravasate the BBB by receptor-mediated signaling. Selectin and chemokine signaling on the surface of the EC is induced by cytokine expression. Leukocytes arrest on the EC via integrin binding and cell adhesion molecules such as PECAM-1 pull the leukocytes across the BBB (Ley et al., 2007).

Leukocytes are activated by recognizing the reactive microglia by the antigens presented on the cell surface and fulfills a variety of phagocytic roles (Dheen et al.,
2007; Glezer et al., 2007; Rock et al., 2004). After transendothelial migration, leukocytes respond to signals presented by activated microglia. Once activated by microglia, leukocytes act as macrophages by phagocytosing neurons and glia. In addition, cells in the CNS can also undergo programmed cell death. It is hypothesized that following BBB disruption, the pro-inflammatory molecules secreted by astrocytes within the CNS parenchyma further propagate the immune response (Aloisi, 2001).

Extravasation of the blood plasma in the CNS has been implicated in numerous neuronal diseases. Blood serum albumin, the key regulator of blood volume and plasma oncotic pressure, is also extravasated with glutamate resulting in a shift from normal vascular homeostasis (Neuwelt, 2004; Salahuddin et al., 1988a). Previous studies have shown that extravasated albumin results in the release of cytokines such as transforming growth factor β (TGF-β) and in the down-regulation of glial K+ inward rectifier channels (Ivens et al., 2007). The increase in extracellular K+ further exacerbates the hyperexcitability of localized neurons. Excessive albumin in the CNS parenchyma can trigger proliferate and activate astrocytes (Nadal et al., 1995; Tigyi et al., 1995; Tomkins et al., 2008). Chronic seizures have been shown to result in angiogenesis causing the formation of porous vessels that have high BBB permeability. In addition, chronic pain can lead to changes in BBB permeability and TJ alterations (Brooks et al., 2005). The disruption of BBB appears to perpetuate the progression of hyperexcitability in CNS.

The acute or chronic progression of MS, epilepsy, and Alzheimer’s disease is important when trying to understand the relationship of BBB disruptions and the immune system. These diseases demonstrate the fundamental functions of BBB physiology that may have a role in BBB disruptions and furthermore, TBI. Secondary injury mechanisms
commence immediately after TBI such as the release of reactive oxidative species (ROS) which may have a significant role demonstrated by evidence of matrix metalloproteinase activation and TJ disassembly (Pun et al., 2009; Schreibelt et al., 2007). The role of nitric oxide (NO) as a vasodilator and modulator of BBB permeability has made it a subject of investigation in TBI (Guzik et al., 2003). In addition, NO has a role in the establishment of long-term potentiation in memory formation. NO is a lipid- and water-soluble gas produced from arginine by nitric oxidase synthase, acts as a paracrine signal transducer, and activates guanylyl cyclase. Leukocytes generate NO and inducible nitric oxide (iNOS) as part of the immune response by IFN-γ or TNF. TGF-β, IL-4, and IL-10 are inhibitors of iNOS (Ahn et al., 2004; Alagarsamy et al., 1998; Cherian et al., 2004). Interestingly, nitric oxide (NO) is used as a treatment for neuroinflammation (Handa et al., 2008; Papadimos et al., 2009).

During secondary injury, inflammatory mediators have an important role in regulating the immunological response. In particular, phospholipase A2 (PLA₂) is activated by cytokines resulting in the release of arachidonic acid (AA) leading to the synthesis of eicosanoids such as prostaglandins and leukotrienes (Farias et al., 2009). Receptor for cys-LTs (CysLT1 and CysLT2) are overexpressed (Hu et al., 2005; Zhang et al., 2004) and the formation of cysteinyl leukotrienes in the CNS has been documented following TBI (Schuhmann et al., 2003). Consequently, inhibitors of PLA₂ is a potential therapeutic target for several neurological disorders (Farooqui and Horrocks, 2006).

Excitotoxicity originating from the original BBB disruption is hypothesized to cause a secondary BBB disruption via astrocytic signaling along neuron fiber tracts (Hartwick et al., 2008; Maier and Watkins, 1998). Glutamate, an endogenous excitatory
molecule, does not cross the BBB without active transport. Following BBB disruption, glutamate is free to diffuse down its osmotic gradient into the CNS parenchyma extracellular space (Bullock et al., 1991; Smith, 2000; Zhang et al., 2000). Glutamate concentrations in plasma are 50-100 μM/L; in whole brain, they are 10,000-12,000 μM/L but only 0.5-2 μM/L in extracellular fluids (Hawkins, 2009). The increased glutamate concentration leads to increased NMDA receptor expression resulting to the increase in the influx of Ca^{2+} ions into neurons and glia resulting in neuronal excitability (Gouix et al., 2009). Due to the effects of glutamate receptors mobilization on neuronal plasticity and synaptic formation, it is possible minor and local disruptions of the BBB could have profound effects on memory and learning (Hawkins, 2009; Le Vasseur et al., 2008). Furthermore, GLT-1 and GLAST are astrocytic glutamate transporters that regulate extracellular glutamate by clearing excess glutamate. After TBI, both GLT-1 and GLAST are downregulated and result in neuronal excitotoxicity (Laird et al., 2008). However, it is not known if increases in either transient or persistent BBB permeability result in the neuropathological manifestations of TBI including memory loss, cognitive dysfunction, and behavioral problems.

1.4 Memory and Learning

Clinical and experimental data has shown that TBI-induced cognitive deficits present as hippocampus-dependent changes in memory and learning, such as spatial information processing (Dixon et al., 1999; Fox and Faden, 1998). The hippocampus is part of the limbic system which is responsible for behavior, emotion, long-term memory, and olfaction. The major regions of the hippocampus are the dentate gyrus and Ammon’s
horn which is denoted as CA1 through CA3 (Figure 1-3). The circuitry in the hippocampus is often simplified to follow in a unilateral pathway, even though, feedback and bypass connections are present. The perforant pathway is the major output of the layer 2 of entorhinal cortex which has connects with different cortical regions and thalamus. The dentate gyrus receives the perforant pathway and passes along the signal through the axons of the granule cells to CA3 region via mossy fibers. The Schaffer collaterals of CA3 axons connect to the dendrites of the CA1, and the axons of the CA1 extend to the subiculum and the deep layer of the entorhinal cortex. This loop is called the trisynaptic loop (Anderson et al., 1971).

Figure 1-3 Hippocampus. The hippocampus is a part of the limbic system and has roles in learning and memory along with spatial navigation. Synaptic plasticity in the form of long-term potentiation (LTP) is often studied in the hippocampus because of three distinct areas: dentate gyrus (DG), CA3 and CA1 (Harry and Lefebvre d'Hellencourt, 2003).
Long-term potentiation (LTP) is thought to be the basis of learning and memory and is defined as the increase in synaptic transmission between two neurons caused by synchronous stimulation. The synaptic strength between presynaptic and postsynaptic neurons is strengthened by the binding of glutamate to N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Activation of the NMDA receptor is constitutively blocked by Mg\(^{2+}\) until activity in both the presynaptic and postsynaptic neuron coincide, resulting in the release of Mg\(^{2+}\) when the membrane potential is sufficiently depolarized and glutamate is bound to the NMDA receptor. The result is the influx in extracellular Ca\(^{2+}\) through the NMDA receptor. Calcium-dependent kinases (Ca\(^{2+}/\)calmodulin and protein kinase C) is signaled by Ca\(^{2+}\) and phosphorylates AMPA receptors. The increase in sensitivity to glutamate by the postsynaptic cell assists in the induction of LTP. Furthermore, retrograde release of NO from the postsynaptic cell causes an enhancement of neurotransmitter release from the presynaptic cell (Figure 1-4).

The mechanisms for the alterations in memory and learning are suggested to be caused by neural degeneration, elevated glutamate levels, ischemia, and disruptions in plasticity (McIntosh et al., 1998; Park et al., 2008; Raghupathi et al., 2000). In particular, the dentate subgranular zone (SGZ) of the hippocampus has been identified as a region complicit in deficits resulting from injuries to the CNS (Herrera et al., 2003; Monje et al., 2003). Furthermore, insults to the CNS create alterations in hippocampal neurogenesis of precursor cells in the SGZ (Rola et al., 2006).
The induction of long-term potentiation (LTP) is caused by the synchronous activity of neurons. Activity from the presynaptic cell signals the release of glutamate resulting in the activation of the postsynaptic NMDA and AMPA receptors. Calcium signaling cascade in the postsynaptic dendrite causes the further activation of postsynaptic receptors by the release of Mg^2+. An increase in synaptic strength is facilitated between the neurons by LTP mechanisms. (Kandel et al., 2000).

The hippocampus has been identified as a key facilitator of learning and memory within the CNS. Mild- to moderate-TBI has been shown to damage the dentate gyrus more than any other region of the hippocampus (Anderson et al., 2005). The hippocampus is particularly vulnerable to neurodegeneration following TBI (McCarthy, 2003; Sato et al., 2001). LTP is greatly diminished shown by smaller NMDA potentials and glutamate-induced glutamate excitatory potentials in the CA1 region (Schwarzbach et al., 2006). Furthermore, inhibitory inputs such as GABAergic neurons decrease following TBI (Reeves et al., 1997; Sanders et al., 2000). Mossy cells in the dentate hilus are vulnerable to various types of injuries such as epilepsy, ischemia, and TBI (Buckmaster and Schwartzkroin, 1994). The glutamatergic mossy cells act as a feedback
system for granule cells in the dentate gyrus and also innervate GABAergic interneurons. The loss of mossy cells has been associated with hyperexcitability within the hippocampus. However, the survival of mossy hilar cell may also have a significant role in memory deficits (Santhakumar et al., 2000). It is also hypothesized that alteration is dopamine signaling following TBI cause deficits in memory, attention, and motor learning (Bales et al., 2009; Wagner et al., 2009; Wagner et al., 2005).

1.5 Mechanisms of Blood-Brain Barrier Disruption

Exposure to the bomb blasts and other explosions have been demonstrated to cause TBI (Elsayed, 1997; Stuhmiller, 1997). Pressure waves produced during a blast are greatly attenuated upon interaction with solid or liquid interfaces such as the skull and brain (Moore et al., 2009; Stuhmiller, 2008). Therefore it is not likely that TBI from a blast is produced by direct mechanical damage to neural tissue unless a concussive force is delivered by a projectile or some other secondary mechanical insult. One possibility is that TBI from a blast is produced by BBB disruption. One mechanism of BBB disruption is theorized to be caused by a vascular surge through the carotid arteries (Chavko, 2007; Knudsen and Oen, 2003). Another mechanism is pulmonary inflammation leading to blood-borne inflammatory agents (Cernak et al., 2001a; Elsayed and Gorbunov, 2003, 2007). Recently, studies of blast TBI have studied BBB pathologies more in depth (Readnower et al., 2010).

High explosives generate an instantaneous increase in atmospheric pressure resulting in blast overpressure waveform (Figure 1-5). The impact of these pressure waveforms is greatest within air filled cavities of the body, such as the lungs, digestive
tract and ears (Bala et al., 2008; Ling et al., 2009; Mayorga, 1997). The pressure causes a surge in fluid within thoracic blood vessels to propagate from the thorax up toward the head (Cernak, 2010; Courtney and Courtney, 2009). It is theorized that vascular route of the carotid arteries provides a conduit for a transfer of energy into cerebral blood vessels. The result is an increase in arterial pressure and the potential damage to endothelial cells of the cerebral capillaries.

![Idealized Blast Overpressure Waveform](image)

**Figure 1-5 Idealized Blast Overpressure Waveform.** Also called a Friedlander waveform, the initial overpressure and subsequent negative pressure is hypothesized to cause a “whiplash” of bodily organs. The overpressure can cause shearing forces of tissues on the interface of air-filled organs such as the lungs. In addition, drastic changes in pressure have also been theorized to cause a vascular surge via the carotid arteries originating in the thorax.

Alternatively, damage to the lungs from a blast wave can cause a diffuse inflammation leading to the apoptosis of endothelial cells. This phenomena has been observed in pulmonary leak syndrome (Assaly et al., 2001). The blast wave causes the most damage to air-filled organs such as the ears, gastrointestinal tract, and lungs. Shear forces at high density tissue interfaces are most susceptible to the negative pressure that
follows the initial overpressure (DePalma et al., 2005; Moore et al., 2009). Inflammation of the CNS after a blast TBI may initiate in the lungs and might be caused by transmigration of innate immune system and the activation of cell death cascade within the neurovascular unit (Agoston et al., 2009; Elsayed et al., 1996; Gorbunov et al., 2004; Mayorga, 1997; Svetlov et al., 2009; Zunic et al., 2000). The resulting inflammation causes cell death in the CNS and disturbances in normal neuronal functions such as learning and memory.

Regardless of the origin, the disruption of the BBB induces the symptoms of TBI by the extravasation of blood plasma. The imbalance of oncotic pressure allows plasma molecules such as albumin and glutamate to passively cross into the CNS. The primary injury of the overpressure results in the release of ROS and cytokines that causes the activation of phagocytic cells such as leukocytes and microglia. The secondary injury leads to the cell death and deficits in behavioral tests similar to the rodent model of blast-induced TBI (Long et al., 2009). Physiological changes in astrocytes and neuronal signaling following mild- to moderate TBI are associated with changes in glutamate signaling (Gouix et al., 2009; Yi and Hazell, 2006) and can diminish LTP (D'Ambrosio et al., 1998).

1.6 Validation of the Blood-Brain Barrier Hypothesis

The disruption of the BBB is hypothesized to cause the symptoms of TBI. The extravasation of blood plasma and immunological cells may lead to chronic pathologies that cause secondary inflammatory injuries through an unknown mechanism. The novel BBB disruption model of TBI was compared to the classical controlled-cortical impact
(CCI) model. A hyperosmotic mannitol solution was used to create a transient opening of the BBB by inducing shrinkage of ECs lining the cerebral capillaries. The injection rate of the mannitol was also explored to determine the specific effects of tonicity on the cerebrum.

In order to investigate the progression of symptoms following BBB disruption, behavioral assays were conducted to measure deficits in spatial navigation of a rodent model. Histological studies of fluorescently-labeled plasma molecules were also studied to establish the magnitude and location of BBB disruption. Furthermore, electrophysiological field potentials were measured \textit{in vitro} from brain slices to determine changes in the ability to induce LTP within the hippocampus. In exploration of synaptic changes associated with learning and memory, a biological neural network was also developed to study neural connectivity and cell survival after TBI. Finally, a therapeutic treatment of polyethylene glycol (PEG)-modified albumin was investigated to determine if the restoration of oncotic pressure across the BBB could attenuate symptoms.

The goal of the above mentioned experimentation is to provide significant evidence that BBB disruption causes the manifestations commonly related to TBI. Traditional TBI models examine how diffuse axonal and focal injuries lead to inflammation and cell death. However, few studies have demonstrated how dysfunction of the BBB might contribute to TBI symptoms. In addition, the current development of treatments focus on cellular cascade mechanisms related to necrosis and apoptosis. In this dissertation, we constructed an experimental design to investigate the disruption of
the BBB in terms of behavioral and pathological changes commonly associated with learning and memory.
Chapter 2

Methods and Materials

In this study, we utilize intracarotid injection of hyperosmotic saline in rats to test the hypothesis that transient blood-brain barrier (BBB) disruption can lead to mild- to moderate traumatic brain injury (TBI). The injection of concentrated hypertonic solutions to induce significant disruption was first proposed by Rapoport et al. (Rapoport, 1970; Rapoport et al., 1972) by transiently shrinking endothelial cells (ECs). In order to validate this hypothesis, we compared the performance of animals injected with hyperosmotic saline during a spatial localization test to animals subjected to an established TBI model known as controlled-cortical impact (CCI). We used in vivo injections of fluorescently-labeled albumin and Lucifer yellow to determine the extent of BBB permeability. These molecules provided a variable range of molecule size to qualitatively assess the extravasation of plasma constituents with fluorescent microscopy. We found the deficiency in behavioral performance of rodents was comparable in the BBB disruption and CCI models. In addition, we determined the extravasation of plasma into the central nervous system (CNS) might be the basis of brain injury pathologies demonstrated by histological results. Together, this data suggests BBB disruption can
produce TBI symptoms in animal models, and therapeutics that promote BBB integrity might attenuate symptoms commonly associated with TBI.

In order to address the possibility of restoring the BBB, we also administered polyethylene-glycol (PEG)-modified albumin (PEG-Alb) as a potential therapeutic following hyperosmotic saline and CCI models. Behavioral outcomes were compared to injury-only animals to determine the efficacy of both treatments. Furthermore, \textit{in vitro} hippocampal electrophysiological field potential recordings were investigated to show changes the induction of long-term potentiation (LTP). Lastly, a biological neural network was employed to model the synaptic remodeling of CNS neurons following TBI.

### 2.1 Animal Injury Models

#### 2.1.1 Surgery Preparation

Male Sprague–Dawley rats weighing 270-430 g were used for both behavioral and histological studies. Animals were subject to intracarotid injection or controlled-cortical impact (CCI) in addition to behavioral testing. Animals are initially anesthetized with 5\% isoflurane and maintained at 1.5-2\% isoflurane in an 80\%/20\% air/O\(_2\) mixture at a flow rate of 3 L/minute. A subcutaneous injection of 0.05 mg/kg buprenorphine (1:5 dilution of standard 0.3 mg/ml with sterile saline; Bedford Laboratories, Bedford, OH) was made prior to incision in order to minimize discomfort during and after surgery. Surgical sterile technique and animal care were performed according to the regulations of the University of Toledo Institutional Animal Care and Use Committee.
2.1.2 Intracarotid Injection of Mannitol and Therapeutics

In this current study, the disruption of the BBB was induced by a hyperosmotic injection of mannitol (Figure 2-1) to compromise the structurally integrity of the neurovascular unit and to induce the effects of TBI (Hiesiger et al., 1986; Lu et al., 2004; Rapoport, 1978, 2000). In the clinical setting, high molarity mannitol is commonly used to relieve intracranial pressure (Berger et al., 1995; Zornow et al., 1990) and to deliver therapeutic drugs that otherwise would not cross the BBB (Kroll and Neuwelt, 1998; Neuwelt et al., 2008; Neuwelt et al., 1986). The rate and duration of hypertonic infusion is an important factor on the effectiveness of BBB disruption (Bellavance et al., 2008; Cosolo et al., 1989; Remsen et al., 1999). Fast and slow mannitol injection rates used in the current study were 0.17 and 0.11 ml·s⁻¹ based on the results from these studies.

**Figure 2-1 Blood-Brain Barrier (BBB) Disruption.** BBB disruption is commonly used as a therapeutic tool to allow the extravasation of drugs into the CNS that could not cross otherwise. After the administration of a hyperosmotic solution, interendothelial tight junctions are estimated to widen to a radius of 200 Å caused by EC shrinkage (Kroll and Neuwelt, 1998). (a) normal cerebrovascular unit; (b) the effect of hyperosmotic shrinkage on endothelial cells. (Bellavance et al., 2008)
A hyperosmotic insult to the CNS was induced by an intracarotid injection of 25% mannitol (Abbott Laboratories, Abbott Park, IL). Following isoflurane induction, the hair over the thorax was clipped and the surgical field was scrubbed with povidone-iodine followed by 70% EtOH. A 2-3 cm vertical incision was made from the suprasternal notch to below the chin. After exposure of the sternothyroid muscle, the common carotid artery (CCA) was exposed and is separated from the posteriorly placed vagal nerve. After exposure of the bifurcation of internal carotid artery (ICA) and external carotid artery (ECA), 4-0 sutures were looped around the each of the carotid branches. Hemostats were clamped to the end of the sutures and were extended to stop the flow of arterial blood from all branches. The ECA was partially ligated with a fourth suture near the bifurcation, and a 45º incision was made to the ECA 3 mm proximal from the bifurcation. A modified 27 gauge blunt tip needle was inserted into the incision of the ECA toward the bifurcation and the suture was tightened around the needle tip. The suture around the ICA was released and hyperosmotic mannitol (1.5 M in sterile 0.9 % NaCl, 6 ml/kg) or saline sham (sterile 0.9 % NaCl only, 6 ml/kg) was injected with a syringe pump at either 0.17 or 0.11 ml·s⁻¹. After the fluid injection, the flow from the ICA was stopped, the needle was removed, the suture around the proximal ECA was tightened and the suture around CCA was released. Following removal of the CCA suture before suturing the incision, the ECA was checked for bleeding and additional ligation was provided as required. The incision was sutured and topical antibiotic was applied.

Special considerations must be taken to avoid precipitation of mannitol. Crystallization occurs at temperatures near 37 °C, therefore, the mannitol solution needs
to be heated to 60-80 °C, allowed to cool, and filtered (at least 0.45 mm pore diameter) prior to infusion to avoid microinfarcts (Hsieh et al., 2005; Salahuddin et al., 1988a).

In order to test the efficacy of PEG-Alb, the therapeutic along with the control, human serum albumin (HSA), was administered immediately (approximately 1 minute) after the induction of injury and before the removal of the intracarotid cannula. A three valve manifold was used to minimize the occurrence of air bubbles in the infusion and to provide a 0.1 ml saline (0.9 % NaCl) buffer in between the mannitol and PEG-Alb. The dose of PEG-Alb and HSA were 0.6 g/kg animal weight at a concentration of 40 mg/ml. The administration rate of the saline buffer and therapeutics (PEG-Alb and HSA) was 4.12 ml/min (0.07 ml/sec).

2.1.3 Controlled-Cortical Impact

In addition, a controlled-cortical impact (CCI) device was utilized to model TBI that results from a penetrating head injury (Figure 2-2). The CCI device was constructed in-house and consisted of a four tension-spring driven piston triggered by a manual release mechanism. A compression spring and collar were used to gauge the depth of the impact. The device was attached to a stereotaxic frame via a right angled steel arm. The impact depth and velocity was calibrated through the use of high speed image acquisition.
Figure 2-2 Controlled-Cortical Impact (CCI) Device. The position of the CCI device will be repeatable by using a stereotaxic frame (left panel). The CCI device has a spring-loaded piston (head diameter of ~2 mm) that is designed to penetrate 2 mm at a velocity of 4 mm/ms into the brain (right panel).

The animal was placed in a stereotaxic frame, hair over cranium was clipped and the surgical field was scrubbed with povidone-iodine followed by 70% EtOH. A 1-2 cm incision was made in the scalp and the fascia was excised. A 3-4 mm hole was drilled in the left hemisphere of the cranium equidistant from the bregma and lambda and 3 mm lateral to the sagittal suture. The dura mater was left intact and a 2 mm small spring-loaded piston was inserted into the hole until contact was made. An impact with a depth of 2 mm and a velocity of 4 mm/ms was delivered. The hole was then filled with a Gelfoam® sponge (Pfizer Pharmaceutical, New York, NY), the incision closed, and topical antibiotic was applied. The sham surgeries were performed without the delivery of an impact with all other steps remaining the same.
2.1.4 Post-Surgery Monitoring

Animals were allowed to recover in a warm environment and were regularly monitored during recovery. Incision sites will be monitored for infection and a topical antibiotic were applied as needed. If post-surgical discomfort or distress were observed, buprenorphine was administered via subcutaneous injection once every 12 hours as needed.

2.1.5 Administration of Fluorescent-Labeled Molecules

Fluorescent-labeled molecules were used to determine the extravasation of plasma after BBB disruption caused by mannitol and CCI. Human serum albumin (HSA) and polyethylene-glycol albumin (PEG-alb) were labeled with fluorescein and Texas Red, respectively. The development of the fluorophores was previously described (Assaly et al., 2004). Lucifer yellow (Molecular Probes®, Invitrogen, Eugene, Oregon) was also used due the small molecular size and ability to extravasate into the CNS parenchyma. Fluorophores were added to mannitol and saline shams solutions to maximize extravasation. For CCI surgeries, fluorophores were administered intravenously in the tail vein immediately after injury. Doses of PEG-alb and HSA were 0.6 mg/kg and lucifer yellow was 60 μg/kg.

2.1.6 Transcardial Perfusion

Animals were immediately transcardial perfused after intracarotid and CCI surgery with 500 ml of 0.9% NaCl followed by 25 ml of 1% lanthanum (III) chloride
(Sigma-Aldrich, St. Louis, Missouri) and 500 ml of 4% paraformaldehyde. During surgery preparation, two 1L bottles were labeled SALINE and FIX and filled with 500 mL of the appropriate solution. A 100 ml media bottle label LaCl₃ was filled with 25 mL with LaCl₃. The perfusion pump (Vera Varistaltic Pump Plus, Monostat, New York, NY) was set up by filling the infusion line with saline solution and placing a 2 inch 18 gauge blunt stainless steel needle at the end of the line. The line was then checked to make sure no air bubbles were present. The perfusion instruments were set out: 1 pair of large tissue forceps, 5 hemostats, 2 scissors (one large and one small), one #10 scalpel, a pair of rongeurs, and a narrow spatula. An overturned wire rat cage cover was placed on the necropsy table which proved proper drainage and downward ventilation.

Approximately 5 minutes later intracarotid injection or CCI, animals were given 10 ml/kg of ketamine (87 mg/ml) / xylazine (13 mg/ml). The animal was positioned over the incline of the overturned cage cover with the abdomen faced upwards. Four hemostats were then fastened at the loose skin at the proximal ends of each limb and let to fall in between the grates of the cage cover to firmly secure the animal. Pinch tests were preformed on the base of the tail to determine if the animal was properly sedated. Once sedated, an incision was made with the small scissor through the skin inferior to the xyphoid process and along the bottom of each side of the ribcage. The larger scissors were then used to cut along the same line but through the cartilage and bone of the ribcage. The inferior aspect of the diaphragm was exposed and carefully removed with the small scissors. The large tissue forceps were attached to the xyphoid process and placed over the head of the animal completely exposing the thoracic cavity. Fascia attaching the heart was then cut.
A perfusion line with blunt needle was inserted into the left ventricle at the apex of the heart and pushed up through the aortic valve. Saline is initially used to exsanguinate the animal. The needle was only inserted once to prevent fluid leakage from the heart. The perfusion pump filled with saline was turned on to the setting of 30-50 ml/min (4-5 on pump) and the tubing was taped down. The right atrium was cut with #10 scalpel tip to allow the liquid to escape. The needle was pointed toward the left at about a 10º angle from the midline. Approximately 1.5 inches of the needle was in the heart when the needle was correctly placed within the atrium.

After all blood was rinsed out (clear liquid), the pump was turned off. Complete exsanguination was confirmed by visual inspection of the liver which turns from dark brown to light pink when perfused with saline. The perfusion line was then inserted into the media bottle containing LaCl₃. The rationale for the use of LaCl₃ before the fixative was due to the ability of the low molecular weight molecule to cross the BBB, enter the cytoplasm of ECs, and distinguish damaged TJs (Nag et al., 1982; Sheikov et al., 2008). For this reason, LaCl₃ is commonly used in studies concerning TJs and the BBB. The pump was re-started to intermediate solution flowing and the line was again checked for air bubbles. After the solution was depleted, the same procedure was repeated but fixative was used. The fixative ran through the pump until all of the 500 mL was used. During the fixation the body would became stiff.

After the fixation was complete the needle was removed from the heart and the head was severed using scissors. The skull was removed to expose the brain using rongeurs. The skull nearest to the brainstem was removed first and the process continued anteriorly. The head was then flipped to visualize the ascending nerves. The optic,
auditory and olfactory nerves were severed using the scalpel. The brain fell easily from
the remaining skull when accurate pressure from a spatula was added. The brain was
placed in a 50mL tube filled with fixative and let incubate at 4°C overnight.

2.1.7 Detection of Fluorescent-Labeled Molecules

Whole brains were immediately excised and were kept in 4% paraformaldehyde
at 4 °C for at least 7 days prior to sectioning. Coronal 50 µm slices were sectioned with a
vibrating microtome (Vibratome, TPI Systems, St. Louis, MO), mounted on slides and
imaged with a CoolSnap ES camera (Roper Scientific, Tuscon AZ) mounted on an
inverted fluorescent microscope (IX-71, Olympus, Tokyo, Japan). Images at 4x
resolution were taken of ipsilateral hemispheres and whole brain coronal slices. Filter
cubes (Chroma) were used to image Lucifer yellow (LY; excitation 405 – 445 nm, 460
nm dichroic, emission 515 – 565 nm), fluorescein-labeled PEG-albumin (FL-PEG-Alb;
excitation 465 - 495 nm, 505 nm dichroic, emission 515 – 555 nm) and Texas Red-
labeled albumin (TR-Alb; excitation 540 - 580 nm, 590 nm dichroic, emission 600 - 660
nm). Images were acquired using a custom-made MATLAB software interface
(MathWorks, Natick, Massachusetts). Separate images were constructed into a complete
view of the slice with imaging software (Photoshop® 6.0, Adobe Systems Incorporated,
San Jose, California).

2.2 Behavioral Testing

The Barnes maze was used to test spatial memory and learning (Barnes, 1979).
Animals survived 7 days post-injury and were not used for histology. The spatial
memory test gives reinforcement for animals to escape an open platform to a recessed box called a target box. The platform is 4 feet in diameter and contains 20 four inch diameter escape holes near the perimeter with only one target box in which the animal can hide. To provide reference landmarks for the animal, the maze was located in the middle of a room containing numerous landmarks outside the platform, such as doors, wall hangings, or office furniture. Special considerations were taken so the environment and personnel present are the same for each animal.

The animal was kept in its housing cage for 2 minutes prior to each trial. The animal was then placed in a cylindrical container with a 10 inch diameter in the middle of the maze for 1 min. A buzzer (85 dB) was pressed and high intensity lights directed at the maze were turned on, and after 10 seconds, the container was raised by a string attached to an arm over the maze. The animal was allowed to find the target box. However, if the animal was unable to find the target box, the animal was guided by the personnel to the target box and allowed to remain in the box for 1 min. The buzzer is stopped upon the entry of the animal into the box. Pre-training consisted of 5 trials per day until animals could complete all trials under 12 seconds during a single training session. At that point, the animals were considered trained and were ready for either intracarotid injection or CCI surgeries.

After each trial, the entire maze was cleaned with 10% EtOH solution to minimize intramaze cues. At the end of the day and between each testing of animals, the maze was cleaned with Spor-Klenz® (Steris, Mentor, Ohio) to disinfect and prevent against cross contamination. Periodically the maze was also rotated and target box moved to remain relative to spatial cues in the testing room. Each trial was recorded to by measuring
errors, distance from target box, and search strategies along with time that the animal took to escape into the target box (Patil et al., 2009).

2.3 Statistical Analysis

Immediately before surgery, animals were tested for their latency, search pattern, and search rate to find the target box. Latency was determined as the amount of time the animal took to enter into the target. The search pattern was the measurement of the first box each animal checked during the trial. Once proficient, animals would move towards spatial cues in the room to outer portion of the maze and subsequently check boxes until the target box was located. This behavior was constant among properly trained animals. Search rate was also measured by determining the difference in the initial hole checked to the target box and dividing by the latency. Following recovery, animals were subsequently tested 1, 3 and 7 days after surgery to determine whether intracarotid injections or CCI surgery altered an animal’s behavior to locate the target box.

A two-way analysis of variance (ANOVA) was completed for all samples to elucidate the differences between the day 0 baseline latency and all subsequent days tested post-surgically and any differences between the experimental groups and their respective sham groups. Significance levels were set at (p < 0.05) and the ANOVA analysis was performed using Minitab 16 (Minitab, State College, Pennsylvania). Individual treatment groups were classified by confidence intervals and Tukey’s comparison; significant differences from the ANOVA tests were confirmed using an unpaired Student’s t-test.
2.4 Electrophysiology

2.4.1 Preparation of Slices

Male Sprague–Dawley rats weighing 270-430 g were given 10 ml/kg of ketamine (87 mg/ml) / xylazine (13 mg/ml). These animals were the same rats sacrificed on day 7 after Barnes maze testing. Animals were quickly decapitated, the cerebrum removed, and placed in Modified Standard Ringer’s (MSR) solution that contained (in mM) 117.4 NaCl, 4.4 KCl, 1.1 KH$_2$PO$_4$, 26.2 NaHCO$_3$, 10.0 glucose, 3.2 CaCl$_2$, and 1.3 MgSO$_4$. The left hemisphere ipsilateral to intracarotid injection or CCI injury were cut into 450 μm thick slices in the coronal plane using a vibratory microtome (Vibratome, TPI Systems, St. Louis, MO) and were placed into an incubation chamber containing the MSR maintained at 34 °C. Coronal cortical sections produce hippocampal slices in the same orientation as the hippocampal section shown in Figure 1-2. Slices remained in incubation at least 1 hour before being transferred to the recording chamber, and were superfused with the MSR (2 – 4 ml/min) maintained at 32 °C throughout all recordings.

2.4.2 Electrophysiological Recordings

Extracellular field potentials were obtained from the CA1 region of hippocampal slices placed within a heated recording chamber on the stage of an upright microscope with water immersion optics (Olympus BX51WI). Recording pipettes are pulled with a horizontal puller from borosilicate glass capillaries, filled with an extracellular electrode solution that consisted of 250 mM NaCl and have a final resistance of 5 - 8 MΩ. To evoke postsynaptic responses, a bipolar stimulating electrode with 25 μm tip diameter is
placed on the surface of the slice and stimulus pairs (0.1 ms bipolar pulses, 40 ms
interpulse intervals) are delivered at 0.1 Hz. The stimulating electrode was placed in the
stratum moleculare on the Schaffer collaterals, and the recording electrode was placed in
stratum radiatum on the dendrites of the CA1 pyramidal cells. The field was visualized
with infrared video microscopy (MacVicar 1984) on an Olympus BX51W1 fixed-stage
upright microscope with a 10X objective.

Recordings were obtained using an Axon Instruments Multiclamp 700A amplifier
(Axon Instruments, Union City CA) in fast current clamp mode. Recording traces were
filtered at 10 kHz and digitized at 50 kHz with a 12-bit A/D converter (National
Instruments, Austin TX) using customized software developed under MATLAB
(Mathworks, Natick MA). Recording pipettes were pulled from borosilicate glass
capillary tubing (KG33, Garner Glass, Claremont, CA) and have a final resistance of 5 - 8
MΩ. Field potential responses to presynaptic stimuli were obtained once every 10 sec for
up to 30 minutes. A tetanic stimulus of 100 Hz for 1 sec was then presented following
this 30 minute baseline. Field potential responses following this tetanus were then
additional obtained once every 10 sec for an additional 60 minutes.

2.4.3 Data Analysis

The synaptic response obtained by field potentials are analyzed for population
spike amplitude, excitatory postsynaptic potential (EPSP), spike volley, and EPSP slope.
The population spike is the axonal response of the presynaptic neurons to the stimulus
and is first to be measured by the recording electrode. The EPSP is the collective
response of the postsynaptic dendrites to neurotransmitters release by the presynaptic
fibers responding to the electrical stimulus. If LTP is induced, the amplitude of the EPSP (b in Figure 2-3) will be greater than before the tetanic stimulus. However, the amplitude of the population spike (a in Figure 2-3) should remain relatively constant during the entire protocol to insure changes in the EPSP are not a result of changes to presynaptic input. A spike volley of the postsynaptic neurons (not shown) is generated if the EPSP is large enough to elicit a coordinated postsynaptic action potential and would be superimposed on the EPSP. The EPSP slope is calculated in mV/ms and is representative of the sensitivity of postsynaptic neurons to response to neurotransmitters released from presynaptic neurons.

These measures were also compared as a ratio of responses to paired pulses spaced 50 ms apart (Figure 2-3). The comparison of the paired pulses also provides evidence of synaptic facilitation in the form of calcium priming in the presynaptic terminal. This is demonstrated by a larger EPSP response in the second stimulus. The analysis of these field potential characteristics enables the quantification of the LTP and LTD of neuronal subsets. In particular, differential changes in EPSP amplitude or slope in response to the first or second pulse of the pulse pair can indicate changes in presynaptic neurotransmitter release.
Figure 2-3 Example of Excitatory Postsynaptic Potential (EPSP). The synaptic response obtained by \textit{in vitro} field potentials in the rat hippocampus. A paired pulse at 40 ms apart was delivered to the stratum moleculare and recorded in stratum radiatum. (a) The amplitude of the population spike is the axonal response to the stimulus. (b) The amplitude of the EPSP is the postsynaptic response to the presynaptic excitation. Note that the stimulus artifact at 10 and 50 ms was removed.

2.5 Computational Model

A biological neural network was utilized to investigate neuronal activity following TBI in order to provide evidence for possible mechanisms leading to secondary injury and deficits in learning and memory. All neurons within the network receive inputs identically and output to other neurons in either the form of excitation or inhibition. The presynaptic activity leads to postsynaptic activity which dictates changes
in synaptic strength. Excessive or low activity results in the neuron being removed from the network. New neurons are added to the network and can form synaptic contacts in a prescribed fashion.

2.5.1 McCulloch-Pitts Model

The model uses a bottom-up approach to create a non-Hebbian activity-dependent formation of synaptogenesis based on a McCulloch-Pitts neuron which uses a weighted-sum to generate an all-or-none response. Morphogenetic changes occur in the network periodically in attempt to maintain stability over time. The network provides a platform for which neurons, axons, and synapses can be instantaneously removed as experimentally shown in TBI. In addition, firing thresholds of neurons can also be manipulated to stimulate the effects of a compromised BBB and the extravasation of glutamate into the CNS parenchyma.

One of the first artificial neurons was proposed by Warren McCulloch and Walter Pitts in 1943 (McCulloch and Pitts, 1943). The McCulloch-Pitts model is a simple logic neuron were each individual input and output is either zero or one. If the weighted sum of the inputs is greater than the threshold, then the neuron will be one (Figure 2-4). If the weighted sum is less than the threshold, then the neuron will not fire and will be zero. The neuron constitute the elements in network used which is developed as an input constant network with random connectivity among the neurons (Griffith, 1971).
The McCulloch and Pitts defined the network by the following variables: $N$, $NE$, $C$, $\theta$, $\Phi$, $\alpha$, and $\beta$. The network consists of $N$ number of neurons of which $NE$ is excitatory neurons. The neurons will follow a notation of index numbers $(1, 2, \ldots, N)$ with $(1, 2, \ldots, NE)$ as the subset of excitatory neurons and $(NE+1, \ldots, N)$ as the subset of inhibitory neurons. Each neuron is connected to each other and to itself in a $N \times N$ connectivity matrix, $C$. The connectivity strength between $j$ cell and $i$ cell is denoted as the coefficient $c_{ij}$ in the matrix (Equation 2.1). For example, an excitatory neuron $i$ with $1 \leq i \leq NE$ receives synaptic input from all neurons in row $i$ and projects axon terminals to all neurons in column $i$ of the $C$-matrix. $\theta$ is the common threshold of all neurons; $\Phi$ is the relative weight of inputs from inhibitory neurons; $\alpha$ is the afference or external activity of the network; and $\beta$ is the noise level in the threshold function (see Equation 2.6 below).

\[
C = \begin{pmatrix}
c_{1,1} & \cdots & c_{1,NE} & c_{1,NE+1} & \cdots & c_{1,N} \\
\vdots & & \vdots & & \vdots & \\
c_{N,1} & \cdots & c_{N,NE} & c_{N,NE+1} & \cdots & c_{N,N}
\end{pmatrix}
\]

(Figure 2-4 McCulloch-Pitts Logical Neuron.)
The state of the network at time $t$ is a vector, $z^t$, where $z^t_i \in \{0,1\}$ and $1 \leq i \leq N$ (Equation 2.2). The activity of $z^t_i$ is the output of the weight sum of all postsynaptic inputs as determined by the ability of neuron $i$ to reach the membrane potential, $MP^t_i$ (Equation 2.3).

$$ z^t = (z^t_1, z^t_2, ..., z^t_N) \quad (2.2) $$

$$ MP^t_i = \sum_{j=1}^{NE} c_{i,j} z^t_j - \phi \sum_{j=NE+1}^{N} c_{i,j} z^t_j \quad (2.3) $$

The deterministic function (Equation 2.4) provides $z^{t+1}_i$ of neuron $i$:

$$ z^{t+1}_i = \begin{cases} 1 : MP^t_i \geq \theta \\ 0 : otherwise \end{cases} \quad (2.4) $$

The mean activity of the network at time $t$:

$$ a^t = \frac{1}{N} \sum_{k=1}^{N} z^t_k \quad (2.5) $$

Subsequent network activity, $a^{t+1}$, cannot be predicted from $a^t$. All inputs into a cell remain constant and outputs will vary with time.

The probability of neuron $i$ to be active in the next time step is given by the threshold function:

$$ \text{prob}(z_i^{t+1}) = 1 / \left(1 + e^{(MP_i^t - \alpha - \theta)/(-\beta)}\right) \quad (2.6) $$

where $\beta$ is the threshold noise and $\alpha$ is the percentage afference to the network. The threshold noise, $\beta$, determines the sigmoid slope of the threshold function. For example, $\beta < 1$ requires only a small change in the membrane potential, $MP$, to induce a neuron to fire. Whereas, $\beta < 1$ needs a large shift in $MP$ to induce an active neuron. The afference,
\(a\), is the external input into the network and allows for the representation of changes in external activity.

The optimal initial connectivity matrix, \(C\), is determined by previous extensive analysis (Dammasch and Wagner, 1984). A weak input-constant matrix where constant number of inputs is distributed equally among a neuron (Equation 2.7) provides the strongest random connectivity. During the generation of the \(C\)-matrix, a constant synaptic weight, \(n\), is randomly dispersed along each row of matrix where each synaptic connectivity is equal to one. The postsynaptic input of any given neuron will be equal to the constant, \(n\).

\[
n = \sum_{j=1}^{N} c_{ij}
\]  

(2.7)

For this model, the common threshold of all neurons, \(\theta = 1\), and the relative weight of inputs from inhibitory neurons, \(\Phi = 8\), which corresponds to a ratio of 9:1 (excitatory : inhibitory). However, the ratio will remain greater than 9:1 under normal physiological conditions since the desired activity of each neuron corresponds to a postsynaptic potential slightly above the threshold value, \(\theta\). This behavior is necessary because the excitatory inputs must override the weighted inhibitory inputs in Equation 2.3.

With the parameters given above, the reproducibility of the network has been shown to be independent of the number of neurons, \(N\), in the network (Dammasch and Wagner, 1984). The number of neurons does not affect the qualitative outcome of the network. The determinate factor is rather the ratio of excitatory and inhibitory neurons. The initial synaptic weight was determined to 2/3 of the total number of network neurons (Dammasch et al., 1986, 1988). Therefore, network size of \(N = 30\) neurons and
excitatory neurons of $NE = 27$ was chosen. The initial synaptic weight, $n$, is set at 20. Lastly, the initial $z^0$ vector is randomly chosen with 50% of neurons firing in order to provide a network that is neither excessive nor low on activity.

2.5.2 Morphogenetic State and Compensation Algorithm

The modified McCulloch-Pitts neural network by Dammasch *et al.* (1986, 1988) utilizes a logic neuron and compensation algorithm to model synaptogenesis (Dammasch *et al.*, 1986, 1988). The morphogenetic state descriptions were utilized from a previous modified model (Butz *et al.*, 2006). However, the compensation algorithm was modified to incorporate other synaptic developmental theories that involve TBI and astrocytic modulation (Cohen and Fields, 2008; D'Ambrosio *et al.*, 1998; Eroglu and Barres, 2010; Mattson, 2008).

**Figure 2-5 Schematic of Compensation Algorithm.** The compensation algorithm can be schematically described by this figure. Activity in a presynaptic neuron will move a postsynaptic neuron to a similar activity by increasing or decreasing presynaptic terminals (increases for high-activity; decrease for low-activity). The postsynaptic neuron will compensate for the induced low activity by strengthening connectivity with all excitatory neurons and weaken connectivity with all inhibitory neurons in the network. Likewise, a high activity neuron will decrease excitatory connectivity by decreasing connectivity with all excitatory neurons and increase connectivity with all inhibitory neurons in the network (Butz *et al.*, 2006).
Individual neuronal activity as defined in Equation 2.8 determines connectivity among the network. For example, an excitatory neuron that has low activity will decrease postsynaptic connectivity with inhibitory neurons and increase postsynaptic connectivity with excitatory neurons. Vice versa, if an excitatory neuron has high activity, the neuron will increase postsynaptic connectivity with inhibitory neurons and decrease postsynaptic connectivity with excitatory neurons. Presynaptic terminals will also decrease for low activity neurons and increase for high activity neurons. Inhibitory neurons follow the same logic. The behavior of presynaptic terminals can be described as Hebbian plasticity. Furthermore, if neurons experience activity at either extremes of high or low activity, the neuron will be eliminated from the network (Figure 2-5).

In this model, non-Hebbian plasticity was utilized to describe the postsynaptic changes in the dentate gyrus. Modifications to existing synapses are dictated by competitive neuronal behaviors. Hebbian types of synaptic connectivity results from interdependent competition among connected neurons. Synaptic connections are strengthened with the presynaptic and postsynaptic neurons that have high synchronous activity. However, non-Hebbian plasticity occurs due to independent competition and results from the individual activity of a given neuron. There is theorized to be interactions among these two types of synaptic plasticity such as non-Hebbian changes lower the threshold for inducing Hebbian plasticity. This type of synaptic remodeling was first identified in the synapses of the dentate gyrus-CA3 (Urban and Barrionuevo, 1996).

The effects of changing morphogenetic states (approximately 300 steps) is illustrated by the activity transition function which calculates the expected activity at the
next time step \( (Ea^{t+1}) \) as a function of the current network activity state \( (a^t, \text{Figure 2-6}) \). As stated before \( Ea^{t+1} \) cannot be directly calculated from \( a^t \), so multiple non-morphogenetic simulations at were randomly initiated over the range, \( a^t = [0, 1] \). The activity of the next time step activity were averaged together to determine \( Ea^{t+1} \).

The deviation for a neuron from the normal physiological state (0.5) is called the morphogenetic state (Equation 2.8).

\[
\Delta s_i = s_i - 0.5, \text{ with } s_i = \frac{\sum_{t_0}^{t_0+\Delta t} \text{prob}(z'_i)}{\Delta t}
\]

(2.8)

where \( t_0, \Delta t \in \mathbb{N}, i = 1, \ldots, N \). When \( s_i \) becomes greater than 0.85 or less than 0.25, the cell is eliminated from the network due to excitotoxicity or inactivity, respectively. In addition, if the physiological state is \( 0.45 \leq s_i \leq 0.55 \), no changes are made to the connectivity of the neuron.
Figure 2-6 Example of Activity Transition Function Pre- and Post-Morphogenesis. A network makes morphogenetic changes towards an average activity near $a' = 0.5$. When the network is initially generated (black line), high activity results in lower activity. After morphogenesis (red line), high activity will result in higher activity; low activity will result in low activity. Most importantly, approximate values within $0.2 < a' < 0.8$ will converge towards a value near 0.5. This behavior will create a network that will remain stable even in the case of disturbances. However, large variations can cause divergence towards in $a' = 0$ or 1.

The synaptic offers of any neuron $i$ is described as either bound or free.

Presynaptic and postsynaptic bound offers for a given neuron $i$ is defined as the sum of the corresponding column $i$ and row $i$, respectively, in the corresponding $C$-matrix. Free offers are created as neurons are eliminated from the network due to excessive or low activity or most commonly when morphogenetic steps decrease synaptic elements in order to compensate for shifts from normal activity. As the network undergoes morphogenetic steps, postsynaptic elements are removed for either high or low activity
The presynaptic counterparts are then considered a free offer and can bind to any postsynaptic neuron in need of a presynaptic offer. Similarly, low-state neurons will decrease the presynaptic element which allows of postsynaptic elements to find new presynaptic counterparts.

**Table 2.1 Morphogenetic Changes.**

<table>
<thead>
<tr>
<th>Low-Activity Neuron</th>
<th>High-Activity Neuron</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta \sigma^{bpr}<em>i = -k^{L}</em>{bpr} \cdot \sigma^{bpr}_i \cdot</td>
<td>\Delta s_i</td>
</tr>
<tr>
<td>( \Delta \sigma^{bipo}_i = 0 )</td>
<td>( \Delta \sigma^{bipo}<em>i = -k^{H}</em>{bipo} \cdot \sigma^{bipo}_i \cdot</td>
</tr>
<tr>
<td>( \Delta \sigma^{fpr}<em>i = -k^{L}</em>{fpr} \cdot \sigma^{fpr}_i \cdot</td>
<td>\Delta s_i</td>
</tr>
<tr>
<td>( \Delta \sigma^{fipo}<em>i = k^{L}</em>{fipo} \cdot</td>
<td>\Delta s_i</td>
</tr>
<tr>
<td>( \Delta \sigma^{fepo}<em>i = -k^{L}</em>{fepo} \cdot \sigma^{fepo}_i \cdot</td>
<td>\Delta s_i</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Low-Activity Rate Constants</th>
<th>High-Activity Rate Constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k^{L}_{bpr} = 1 )</td>
<td>( k^{H}_{bepo} = 1 )</td>
</tr>
<tr>
<td>( k^{L}_{bipo} = 1 )</td>
<td>( k^{H}_{fpr} = 30 )</td>
</tr>
<tr>
<td>( k^{L}_{fpr} = 6 )</td>
<td>( k^{H}_{fepo} = 6 )</td>
</tr>
<tr>
<td>( k^{L}_{fepo} = 27 )</td>
<td>( k^{H}_{fipo} = 3 )</td>
</tr>
<tr>
<td>( k^{L}_{fipo} = 6 )</td>
<td></td>
</tr>
</tbody>
</table>

Bound excitatory postsynaptic (bepo) elements are the sum of all excitatory inputs into a neuron \( \sigma^{bepo}_i = \sum_{j=1}^{NE} c_{ij} \). Bound inhibitory postsynaptic (bipo) elements are the sum of all inhibitory inputs into a neuron \( \sigma^{bipo}_i = \sum_{j=NE+1}^{N} c_{ij} \). Bound presynaptic (bpr) elements are the sum of all outputs of a neuron \( \sigma^{bpr}_i = \sum_{i=1}^{N} c_{ij} \). The free elements \( \sigma^{fepo}_i, \sigma^{fipo}_i \), and \( \sigma^{fpr}_i \) are initially set to zero.

Shifts in synaptic offer elements, \( \Delta \sigma_i \), were made as described in Table 2.1.

Note that high- and low-activity states have differing rules. Changes to the \( C \)-matrix for presynaptic elements were made by adding the current \( c_{ij} \) value to the product of the ratio
\( (\Delta \sigma_j / \sigma_j) \) and \( c_{ij} \). The loss of postsynaptic elements was determined similarly to the presynaptic elements by adding the current \( c_{ij} \) value to the product of the ratio \( (\Delta \sigma_j / \sigma_j) \) and \( c_{ij} \). However, note in Table 2.1 that excitatory and inhibitory postsynaptic elements must be calculated separately and change the \( C \)-matrix accordingly. Losses to postsynaptic elements (and their presynaptic counterparts) were transferred to both the pre- and postsynaptic neurons by Equation 2.9-11.

\[
\sigma_j^{f_{pr}} = \sigma_j^{f_{pr}} + \Delta \sigma_j^{f_{pr}} + \sum_{i=1}^{N} |\delta_{ij}^{post}|
\]  

(2.9)

\[
\sigma_i^{f_{epo}} = \sigma_i^{f_{epo}} + \Delta \sigma_i^{f_{epo}}
\]  

(2.10)

\[
\sigma_i^{f_{ipo}} = \sigma_i^{f_{ipo}} + \Delta \sigma_i^{f_{ipo}}
\]  

(2.11)

where \( \delta_{ij}^{post} = c_{ij} \cdot \Delta \sigma_i^{bepo} / \sigma_i^{bepo} \) for excitatory postsynaptic elements \( (j = 1, \ldots, NE) \) and \( \delta_{ij}^{post} = c_{ij} \cdot \Delta \sigma_i^{bipo} / \sigma_i^{bipo} \) for inhibitory postsynaptic elements \( (j = NE + 1, \ldots, N) \).

For each neuron \( i \), the product of free pre- and postsynaptic elements was divided by greatest summation of either free excitatory pre- or postsynaptic elements in the entire network. The calculation \( (\gamma_{ij}^{free}) \) was then added to the current \( c_{ij} \) value. The free synaptic elements were updated to account for the recombination.
Figure 2-7 Example of Structure State Profile Pre- and Post-Morphogenesis. The ratios of bound excitatory postsynapses (bepo) and bound inhibitory postsynapses (bipo) are shown here for pre- and post- morphogenetic changes for a network. Ratios converge towards the line, $n_e = \Phi n_i + \theta$ but remain just left due to stability at slightly higher ratios. The post line fit (dashed line) is also shown to illustrate this tendency.

The steady state of the network is the average activity per cell over a given amount of time. The network is expected to have steady state near 0.5 during normal physiological behavior. Some synaptic loss is also the consequence of the compensation algorithm and is also expected as the network stabilizes. Lastly, if the network is stable, the number of postsynaptic excitatory and inhibitory synapses will converge to the line:

$$n_e = \phi n_i + \theta$$  \hspace{1cm} (2.12)

where $n_e$ is equal to the sum of all excitatory synapses and $n_i$ is equal to the sum of all inhibitory synapses. From the free offers, synaptic elements were able to recombine and form new synapses. Figure 2-7 demonstrates how randomly distributed synaptic offers
converge towards the line defined in Equation 2.7 as the network experiences approximately 300 morphogenetic steps and the sequential compensation algorithm at each step.

2.5.3 Model of Traumatic Brain Injury

The neural network model by Butz et al. (2006) allows for mechanistically accurate simulations of TBI regarding synaptogenesis and cell survival (Butz et al., 2006). Changes were made to the compensation algorithm and an injury scenario was developed to mimic the effects of the plasma extravasation. In addition, complete axons were removed (all postsynaptic output; eliminate column in $C$-matrix), individual cells undergo necrosis (eliminate both row and column), and extravasation of glutamate (decrease in threshold value, $\theta$) are simulated in this model. These parameters were systematically altered to represent a variety of injury severities. All code was written with MATLAB software (MathWorks, Natick, Massachusetts).
Chapter 3

Experimental Results

The results of the following studies demonstrate the ability of BBB disruption to produce symptoms of TBI. Behavioral data displayed deficits in learning and memory after BBB disruption. Memory assays exhibited changes in spatial search pattern and latency without significant alterations in search rates for BBB disruption. Animals experimentally induced with BBB alterations demonstrated focal extravasation of blood plasma in the hippocampus, thalamus, basal ganglia, and parietal cortex which all have roles in processing spatial information. Furthermore, treatments to restore BBB integrity showed improved behavioral outcomes. However, electrophysiological measures of synaptic efficacy in a hippocampal circuit associated with spatial memory did not show any deficits following BBB disruption.

3.1 Extravasation of Blood Plasma

The histological data demonstrated that intracarotid injection of mannitol disrupted the BBB, and the rate of injection determined the magnitude of BBB disruption. Further analysis indicated that the disruption was most prevalent in areas
proximal to the internal carotid arteries and to the Circle of Willis. Contralateral fluorescence of labeled albumins and Lucifer yellow was minimal and was only present in cortical regions. Consistent with spatial locale of middle cerebral and anterior choroidal arteries, the thalamus, basal ganglia, hippocampus, and parietal regions of the cortex exhibited the largest increase in fluorescence (Figure 3-1). Only small traces of fluorescence were observed in the distal anterior areas of the cerebrum such as frontal lobe and striatum. Slightly more was observed in the posterior regions of the cerebrum and cerebellum, but not as substantial as the medial regions. The relative magnitude of the plasma extravasation was assessed by the amount of fluorophores that crossed the BBB.

The BBB was disrupted with internal carotid artery injections of a hyperosmotic mannitol solution. *In vivo* fluorescent double-labeling of Texas Red-conjugated human serum albumin (HSA) and fluorescein-conjugated polyethylene glycol modified albumin (PEG-Alb) was added to the injection solution. Lucifer yellow (LY), a much smaller hydrophilic fluorophore than the labeled albumin, was also used to assess BBB disruption. Because LY is a much smaller molecule than HSA or PEG-Alb, it would be expected to penetrate smaller openings of the BBB and diffuse more extensively throughout regions where BBB disruption occurred. Yellow labeling in the left column indicates colocalization of both albumin fluorophores. Furthermore, different injection rates of slow and fast (0.11 and 0.17 ml·s⁻¹, respectively) were investigated in order to create symptoms comparable to mild- to moderate-TBI. In Figure 3-1, sham mannitol injections were with the fast injection rate. In addition, fluorophores were administered to controlled-cortical impact (CCI) animals via intravenous injections.
The left hemisphere (ipsilateral) is shown in Figure 3-1 after different injury types. The medial region of the cerebrum where the transverse hippocampus has the greatest profile was selected from each animal. Sham saline injection with PEG-Alb and HSA demonstrated little fluorescence (Figure 3-1A). Sham saline injection with LY demonstrated more fluorescence but relative less than injury animals (Figure 3-1B). Inset in Figure 3-1B corresponds to Figure 3-4D. Fast mannitol injection with PEG-Alb and HSA is shown in Figure 3-1C. Insets (left to right) correspond to Figures 3-3B, D, and C which are the parietal cortex, hippocampus, and thalamus, respectively. Fast mannitol injection with LY in Figure 3-1D has similar fluorescence than the albumin-labeled fluorescence. Left and right insets (parietal cortex and hippocampus) correspond to Figures 3-4C and B, respectively. In Figure 3-1E, slow mannitol injection with PEG-Alb and HSA showed limited extravasation and was demonstrated by the fluorescence of only the HSA fluorophore which has a smaller molecular size than PEG-Alb. Slow mannitol injection with LY is shown in Figure 3-1F. Slightly more diffuse LY labeling is observed compared to sham injections in Figure 3-1B; however far less LY label is observed with slow mannitol injection rates compared to the fast mannitol injection results show in Figure 3-1D. CCI with PEG-Alb and HSA had strong fluorescence at the impact site and at the hippocampal fissures posterior to the cortex in Figure 3-1G. Inset corresponds to Figure 3-3A. In Figure 3-1H, CCI with LY had similar fluorescence as the albumin fluorophores but also had some fluorescence at the thalamus. Inset corresponds to Figure 3-4A.

Slow mannitol injections induced limited EC shrinkage and only allowed HSA but not the PEG-Alb to cross the BBB, as demonstrated by the red ipsilateral label in
Figure 3-2A. In particular, Texas Red HSA is observed most extensively in deeper brain regions proximal to the internal carotid artery and Circle of Willis. In contrast to the ipsilateral extravasation of the HSA, whole brain sections demonstrated the bilateral extravasation of smaller molecular weight compound, Lucifer yellow, particular in cortical and thalamic regions (Figure 3-2B). This is attributed to the ability of LY to diffuse more widely throughout neuronal tissues once the BBB is penetrated. The small fluorescence increases observed following slow mannitol injections does indicate some BBB disruption; however, behavioral studies indicated that a wide-scale BBB disruption only observed with the fast mannitol injection rate are necessary to induce behavioral changes. Results from spatial memory assays are discussed in Section 3.2.
The hippocampal region of the CA3 (Figure 3-3D) showed considerably more fluorescence of all fluorophores in a fast mannitol injection. These results are consistent with previous studies that found higher CA3 capillary density than the CA1 region (Cavaglia et al., 2001). The double-labeling of HSA and PEG-Alb was seen in the fast mannitol injections, except some pial arterioles in the cortex where only HSA is labeled (Figure 3-2). PEG-Alb is approximately 16x larger than HSA, and therefore, does not cross the BBB as readily (Assaly et al., 2004; Assaly et al., 2008). Lucifer yellow is substantially smaller than HSA and can easily extravasate the BBB even with slow mannitol injections. Moreover, HSA was able to enter the CNS parenchyma whereas PEG-Alb only minimally extravasated in circumventricular organs (Figure 3-3E and 3-3F).
Figure 3-2 Whole Brain Sections of Slow Mannitol Injections. Panels A and B show the same animal and section that was injected with PEG-Alb, HSA, and Lucifer yellow. (A) Labeled PEG-Alb and HSA is shown. HSA is located in the thalamus and basal ganglia along with small amounts of fluorescence in the parietal cortex. PEG-Alb did not show significant amounts of fluorescence. In addition, contralateral fluorescence of both PEG-Alb and HSA was minimal. The left and right insets correspond to Figure 3-3E and F, respectively. (B) Only Lucifer yellow is shown. Due to the relatively smaller molecular size of Lucifer yellow, the fluorophore is able to extravasate bilaterally with only a limited BBB disruption. Scale bar is 2 mm.

Extravasation of fluorophores following CCI was typical of other classical TBI animal models of the same type (Brody et al., 2007; Cernak, 2005; Fox and Faden, 1998). BBB breakdown has been shown to increase over 24 hours after CCI injury. Additionally, a second peak of BBB permeability was observed at 3 hours post-injury suggesting BBB breakdown is a biphasic process (Baskaya et al., 1997). Increases in TBI induced axonal biomarkers have also been demonstrated to have peaks at 24 and 48 hours post-injury (Anderson et al., 2008).

All panels in Figure 3-3 show double-labeling of HSA and PEG-Alb. Figures 3-3A through 3-3D are after fast mannitol injections. Figures 3-3E and 3-3F are after slow mannitol injections. All figures are at a higher magnification than Figures 3-1 and 3-2.

Extensive fluorescence at the impact site and the fissures on the dorsal and ventral region of the hippocampus suggested massive hematomas (Figure 3-3A). The hippocampus itself remained primary undamaged and the BBB of capillaries appeared to
have maintained their integrity. However, at the lesion sites, a graded extravasation of albumin fluorophores indicated varying BBB disruption. HSA was able to cross the BBB at proximal locations, whereas, the PEG-Alb only was able to extravasate distal to the impact site. The arrows indicate the areas of hematomas. The figure corresponds to the inset from Figure 3-1G.

The animal was subjected to fast mannitol injection in Figures 3-3B and 3-3C. In Figure 3-3B, the auditory and somatosensory regions of the parietal cortex showed fluorescence; the arrow indicates albumin co-labeled vasculature. The figure corresponds with the left inset from Figure 3-1C. In Figure 3-3C, left arrow indicates the basal ganglia. Center arrow indicates the thalamus. Right arrow indicates an apparent hematoma at the hippocampal-thalamic fissure. Figure corresponds with right inset in Figure 3-1C.

In Figure 3-3D, extensive HSA and PEG-Alb labeling of pericapillary regions is observed in the CA3 region of the hippocampus. Arrows point at limited BBB disruption in the vasculature of the CA3 exhibited by only Texas Red HSA fluorescence. All other fluorescence is co-labeled with both types of albumins. Figure corresponds to center inset of Figure 3-1C. In Figure 3-3E, arrow points to the choroid plexus in lateral ventricle after slow mannitol injection. HSA extravasation was present along with limited PEG-Alb fluorescence. Figure from left inset of Figure 3-2A. In Figure 3-3F, the arrow indicates the habenular nucleus which along with the choroids plexus has a weak BBB. Figure from right inset of Figure 3-2A.

Lucifer yellow had minimal extravasation during sham and slow injections demonstrated by fluorescence located within the vasculature (Figure 3-4). In fast
mannitol injections, extravasation was diffuse in regions proximal to the middle cerebral artery (Figure 3-4C) and lacked the specificity that other injection types had (Figure 3-4B and D). A lamination at layer IV (arrow in Figure 3-4C) suggests an increase in BBB disruption at a region with dendrites that have axonal projections to the thalamus. The CCI model had similar results for both the PEG-Alb/HSA fluorophores and Lucifer yellow shown by labeling at the impact site and fissures of the hippocampus (Figure 3-4A). For sham injections, the lucifer yellow molecule could not enter into high cell body dense regions such as the granular layer of the dentate gyrus (Figure 3-4B).
Figure 3-3 Regions of Blood-Brain Barrier (BBB) Disruption with Albumin Fluorophores. All panels show double-labeling of human serum albumin (Texas Red) and PEG-albumin (fluorescein). Panels A through D are after fast mannitol injections. Panels E and F are after slow mannitol injections. (A) CCI injury occurred on the fissures on the dorsal and ventral regions of the hippocampus along with the surface of the cortex at the impact site. The size difference of the labeled albumin allowed HSA to extravasate further into the injury site. The arrows indicate the areas of hematomas. The figure corresponds to the inset from Figure 3-1G. The animal was subjected to fast mannitol injection in B and C. (B) The ectorhinal cortex above the rhinal sulcus exhibited BBB disruption. The auditory and somatosensory cortices (not shown) also had fluorescence. The arrow indicates albumin co-labeled vasculature. The figure corresponds with the left inset from Figure 3-1C. (C) Left arrow indicates the basal ganglia. Center arrow indicates the thalamus. Right arrow indicates an apparent hematoma at the hippocampal-thalamic fissure. Figure corresponds with right inset in Figure 3-1C. (D) Arrows point at limited BBB disruption in the vasculature of the CA3 exhibited by only HSA fluorescence. All other fluorescence is co-labeled with both types of albumins. Figure corresponds to center inset of Figure 3-1C. (E) Arrow points to the
choroid plexus in lateral ventricle after slow mannitol injection. HSA extravasation was present along with limited PEG-Alb fluorescence. Figure from left inset of Figure 3-2A. (F) The arrow indicates the habenular nucleus which along with the choroids plexus has a weak BBB. Figure from right inset of Figure 3-2A. Scale bar for panels A-D is 1 mm. Scale bar for panels E and F is 200 \( \mu m \).

**Figure 3-4 Extravasation of Lucifer Yellow.** All panels show extravasation of Lucifer yellow in either mannitol or CCI injury models. Lucifer yellow has a small molecular size and can easily cross the BBB even with the slightest loss of permeability. (A) CCI injury produced substantial fluorescent at the site of impact along with focal regions on the dorsal hippocampal fissure of ruptured vasculature. Arrows indicate apparent hematomas. Figure corresponds to inset from Figure 3-1H. (B) Sham injections (slow rate) displayed focalized fluorescence without labeling dense cell body layers (arrows) suggesting Lucifer yellow did not extravasate in the hippocampus. (C) Fast mannitol injection showed a diffusion of fluorescence in the auditory and motor cortices suggesting a global disruption of the BBB. A lamination at layer IV (arrow) suggests an increase in BBB disruption to an area that projects to the thalamus. Figure corresponds to Figure 3-1D. (D) The same of location as panel C shows minimal disruption in the sham injection and a failure to label layer IV. Scale bar for panels A-D is 1 mm.

A summary of the histological results are described in Table 3.1. Each category has only one animal and injury types, dyes, and regions of fluorescence are quantified. Individual dye fluorescence was independent of other dyes present. Overall trends
indicated that Lucifer yellow would extravasate more readily and demonstrated more
diffuse labeling than HSA and PEG-Alb demonstrated by more diffuse labeling.

Fluorescence of only the habenular nucleus and circumventricular organs such as the
choroid plexus by HSA and not the PEG-Alb exhibited the difference in molecular size of
the two compounds. In addition, these regions of the CNS have a high BBB
permeability. Generally, the thalamus and cortex were fluorescent more than any other
regions suggesting that these regions are more susceptible than other regions.

<table>
<thead>
<tr>
<th>Injury Type</th>
<th>Dyes</th>
<th>Hippocampus</th>
<th>Cortex</th>
<th>Thalamus</th>
<th>Choroid Plexus</th>
<th>Habenular Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast Mannitol</td>
<td>HSA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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3.2 Spatial Memory and Barnes Maze

The disruption of the BBB with intracarotid injection of hyperosmotic mannitol
was manifested as behavioral changes in spatial memory. Barnes maze testing revealed
that memory retention was significantly altered in fast mannitol injections (5 animals),
but there was not a significant change in fast and slow sham injections (3 and 4 animals,
respectively) and slow mannitol injections (3 animals). CCI injury and CCI sham (2
animals each) were used to compare results from a traditional penetrating head injury model of TBI to results using BBB disruption to induce TBI. Some animals were administered HSA (3 animals) and PEG-Alb (3 animals) following fast mannitol injections. These results suggested that both HSA and PEG-Alb were effective treatments for the attenuation of memory deficits.

Three different metrics (latency, search pattern, and search rate) were analyzed for Barnes maze testing in Panels A-C, respectively, in Figures 3-5 to 3-8. Latency was defined as the time in seconds the animal took to escape into the target box. Search pattern was measured as the relative hole location the animal would look into first before finding the target box. The search rate was defined as the ratio of search pattern to latency in holes per second to provide a measure of the rate the animal would explore before find the target box. Two-factor ANOVA were used to find statistical differences in the various measures across different injury groups; across post-surgery days 1, 3 and 7; and across individual combinations of injury and post-surgical day. Tukey’s post-test comparison was used to determine which groups differed if statistical significance was found.

Statistical analysis revealed that latencies and search patterns were the most altered by fast mannitol injections (Panels A-B of Figures 3-5 to 3-8). In general, search rates were not significantly altered in any type of injury which provided evidence that differences in latencies were not due to lethargy or decreased aversion to noise and light (Panel C of Figures 3-5 to 3-8). The rate of mannitol injections (0.11 ml/s and 0.17 ml/s) was determined to be significant among latency and search pattern measures. Further
analysis also revealed fast mannitol injuries were comparable to CCI injuries, and shams of all injury types did not induce any memory deficits.

All intracarotid injection groups (fast mannitol*, slow mannitol, fast sham, and slow sham) were analyzed (Figure 3-5). Asterisk denotes the group which comparisons are made and will be used for the remainder of this section. Fast mannitol latencies (Figure 3-5A) were significantly higher than other intracarotid injections ($p = 0.004$). No significant differences were observed in search latencies across post-surgery days ($p = 0.607$), which indicates that the performance of animals did not significantly change following recovery from surgery. Individual combinations of injury and days were also not significant ($p = 0.777$). Tukey comparisons showed that fast mannitol had the largest deficits and were followed by slow mannitol, fast sham, and slow sham in no particular order.

Animals that were subjected to fast mannitol injections also exhibited the biggest differences in search strategy (Figure 3-5B) pre- and post-surgery compared to other intracarotid injection groups ($p = 0.011$). There was not significance across all days and all combinations of injury and day ($p = 0.717$ and 0.887, respectively). The Tukey comparison found mannitol having the largest change in search pattern and was significantly greater than fast sham and slow mannitol ($p < 0.05$) and was nearly significant compared to slow sham ($p = 0.065$). No statistical differences could be observed from search rates (Figure 3-5C) across all intracarotid injection types ($p = 0.084$), across all days ($p = 0.695$) and across all combinations of injury and day ($p = 0.951$).
The classical CCI*, CCI sham and novel mannitol injury models (fast and slow injection rates) were compared in order to determine if behavioral deficits were comparable between traditional TBI models and BBB disruption (Figure 3-6). There was significant changes in latency (Figure 3-6A) when comparing across all injury groups (p = 0.041), although the most significant difference as identified by the Tukey comparison was between the fast and slow mannitol groups (p = 0.0575). There were no significant changes in latency across all days (p = 0.799) and for individual combinations of injury and day (p = 0.885). We interpreted these results to indicate the latencies were fast mannitol > CCI > CCI sham and slow mannitol, although the small numbers of CCI and CCI sham animals (N = 2) precluded statistical significance. No significant differences across injuries (p = 0.099), days (p = 0.601) or combination of injuries and day (p = 0.916) were observed in the search pattern (Figure 3-6B), although the Tukey comparison did suggest that fast mannitol search direction changes were almost significantly higher than slow mannitol (p = 0.0575). ANOVA across all injuries (p = 0.060), all days (p = 0.960), all combinations of injury and day (p = 0.978) revealed no significance differences in search rates (Figure 3-6C), although the Tukey comparison showed a significantly slower fast mannitol search rate compared to CCI sham (p = 0.047).
**Figure 3-5 Mannitol Barnes Maze Performance.** Deficiencies in memory recall were shown when comparing different rates and solutions of intracarotid injections. (A) Fast mannitol injections caused substantial increases in latency time. (B) Fast mannitol animals consistently seek new search patterns whereas slow, fast sham, and slow sham mannitol animals maintain approximate patterns. (C) No significance was found in search rates.
Figure 3-6 CCI Barnes Maze Performance. The classical CCI and novel mannitol injury models were compared to determine if slow mannitol injury provided any deficits in memory. (A) Slow mannitol and CCI sham was significantly different than CCI when latencies from all days were averaged. (B) Changes in search pattern between the CCI and slow mannitol groups were also significantly different. (C) No significance was found in search rates.
The sham surgical groups CCI sham*, fast sham, and slow sham were compared to determine if the surgical procedures themselves produced any spatial memory deficits (Figure 3-7). Given the invasive nature of intracarotid injections, it is possible that this procedure would result in behavioral deficits without BBB disruption produced by hyperosmotic mannitol. However, latency changes (Figure 3-7A) across all injuries \( (p = 0.720) \), all days \( (p = 0.222) \), and all combinations of injury and day \( (p = 0.351) \) showed no significant differences. A notable difference was observed in search pattern changes (Figure 3-7B), with a significantly higher change in search direction for CCI sham animals compared to fast sham \( (p < 0.01) \) and slow mannitol \( (p < 0.05) \) as indicated by Tukey’s comparison. No significant differences were observed across all days \( (p = 0.095) \) and across all combinations of injury and day \( (p = 0.612) \). These results could be interpreted as having exploratory animals in the CCI sham group that lacked a response to the aversive stimuli. However, search rate ANOVA across all injuries \( (p = 0.391) \), across all days \( (p = 0.218) \), and across all combinations of injury and day \( (p = 0.962) \) found no significance changes to suggest CCI sham animals conducted their search for the correct hole at a slower rate.

The results from the comparison of fast mannitol*, fast sham, PEG-Alb, and HSA gave support to the hypothesis that either HSA or PEG-Alb may attenuate TBI symptoms that occur following BBB disruption (Figure 3-8). The latency for fast mannitol was significantly higher than fast sham, PEG-Alb and HSA groups \( (\text{ANOVA } p = 0.005, \text{ Tukey comparisons all } p < 0.05) \). Furthermore, there was no significant increase in latency of the PEG-Alb and HSA animals when compared to the fast sham animals \( (\text{Tukey comparisons all } p >> 0.05) \). As with previous comparisons, there were no
significant changes in latency across days (p = 0.724) or combinations of injury and day
(p = 0.783). However, a comparison of search pattern and search rate revealed that there
was no significance changes across injury types (p = 0.083 and p = 0.077, respectively),
post-surgery day (p = 0.408 and p = 0.789) or combinations of injury and day (p = 0.781
and p = 0.854). Therefore these results provide additional evidence that both HSA and
PEG-Alb may restore the BBB after disruption and attenuate symptoms of TBI.
Figure 3-7 Sham Barnes Maze Performance. No significance was found among experimental shams when comparing the behavioral measures of latency and search rate. However, panel B results suggest that search pattern was altered in the CCI sham group. These results are interpreted to be caused by exploratory animals in the CCI sham group who lacked the normal aversive response to light and noise stimuli.
Figure 3-8 Treatment Barnes Maze Performance. (A) Both human serum albumin (HSA) and PEG-Albumin (PEG-Alb) were significantly different when all days where averaged. (B) In the search pattern (direction change) only the fast sham and PEG-Alb had significantly lower changes. This provides evidence that PEG-Alb supplies sufficient recovery following fast mannitol injections. (C) The search rate times for the fast sham and PEG-Alb are significantly faster than the fast mannitol suggesting recovery as well.
3.3 Physiological Changes

It is possible that poor performance in spatial localization following intracarotid injection of hyperosmotic mannitol could be the result of physiological changes produced by hyperosmotic fluids and not a result of BBB disruption. Analysis among all injury groups determined significance in weight loss (p = 0.028) when compared to CCI impact (Figure 3-8). These results demonstrate that the weight loss might be due the diuretic effects of mannitol. However, behavioral tests suggest that weight loss has little effect on memory deficiencies as shown by comparison between fast and slow mannitol injection which delivered equal amounts. In addition, no significant changes were determined in the time from the animal was removed from isoflurane administration until the animal was ambulatory (Figure 3-9). Therefore it is reasonable to conclude that neurological deficits in our BBB disruption model of TBI were responsible for the degraded performance of fast mannitol animals in the spatial localization task.
Figure 3-9 Weight Changes in Injury and Treatment Models. Animals were weighed prior to surgery and at day 7 post-surgery before euthanasia. (A) All animals injected with mannitol and saline shams experienced significant weight loss when compared to CCI injury animals. (B) Treatment animals of PEG-Albumin and human serum albumin differed significantly from fast mannitol injection animals.
Figure 3-10 Recovery Time in Injury and Treatment Models. The time immediately after isoflurane administration to when the animal was ambulatory was measured. No significance was found, but trends indicate that recovery times on average for animals receiving fast mannitol injections were the longest. Most interestingly, the animals that received PEG-Albumin treatment after fast mannitol on average had a shorter recovery time. This data provides additional data that PEG-Alb attenuates TBI symptoms.

3.4 Electrophysiology

Field potentials from the hippocampus were evoked by electrical stimulation of the Schaffer collaterals and recorded in the basal dendrites of CA1 pyramidal cells. The amplitude of excitatory postsynaptic potentials (EPSPs) was used to measure synaptic efficacy and as an assay for long-term potentiation (LTP) after tetanic stimulation of Schaffer collaterals. The amplitude of presynaptic fiber volleys were also measured to differentiate between changes in Schaffer collateral excitability and synaptic efficacy (refer to EPSP Figure 2-3).

Furthermore, field potentials were elicited in response to pairs of Schaffer collateral stimuli separated by 40 ms to investigate whether changes in synaptic strength
were a result of changes in neurotransmitter release or postsynaptic responses to neurotransmitter. When the action potentials elicited by electrical stimulation of the Schaffer collaterals propagate toward the presynaptic axon terminals, Ca\(^{2+}\) influx leads to the exocytosis of glutamatergic vesicles. If another action potential invades the presynaptic terminal within a short time window (usually < 50 ms), presynaptic Ca\(^{2+}\) levels do not have time to recover to resting levels and a larger increase in Ca\(^{2+}\) concentration is observed within the presynaptic terminal, leading to the exocytosis of more vesicles, a larger release of neurotransmitter and a larger postsynaptic response. Therefore the ratio of EPSP amplitudes evoked by the stimulus pair (S2/S1) provides a measure of paired-pulse facilitation (PPF). Changes to PPF following tetanic stimulation in LTP protocols are traditionally associated with increases in neurotransmitter release, whereas LTP induction without changes in PPF are generally interpreted as a change in the postsynaptic response to an unaltered release of neurotransmitter.
Figure 3-11 Fast Mannitol and Fast Sham EPSP and Presynaptic Fiber Volley. (A) Fast mannitol EPSP had a late LTP. (B) The fiber volley remained constant. $X = S1$ and $O = S2$ in top, $X = S2/S1$ in bottom. (C) Fast sham EPSP and (D) fiber volley increased at a similar rate. These results suggest that no LTP took place. These results suggest that no LTP took place.
Figure 3-12 Slow Mannitol and Slow Sham EPSP and Presynaptic Fiber Volley. (A) Slow mannitol. (B) Slow Sham. X = S1 and O = S2 in top, X = S2/S1 in bottom.
Figure 3-13 CCI and CCI Sham EPSP and Presynaptic Fiber Volley. (A) Slow mannitol. (B) Slow Sham. (C) CCI. (D) CCI Sham. X = S1 and O = S2 in top, X = S2/S1 in bottom.
Figure 3-14 HSA and PEG-Alb EPSP and Presynaptic Fiber Volley. (A) EPSP of HSA treated animals. (B) The fiber volley increases with the EPSP suggesting no LTP. (C) The EPSP of PEG-Alb animals also increased in a late LTP. (D) There was not an increase in fiber volley. X = S1 and O = S2 in top, X = S2/S1 in bottom.
Fast mannitol (Figure 3-11A) shows late LTP in the EPSP amplitude evoked by
the second stimulus (S2) after initial short-term potentiation (STP) of both responses (S1
and S2). Given the late increase in S2 relative to S1, the PPF S2/S1 also shows slight
increase. These changes can be attributed to a post-tetanic change in synaptic efficacy, as
they are not accompanied by any changes in the presynaptic fiber volley (Figure 3-11B).
Fast sham (Figure 3-11C) results were inconclusive, with LTP in both S1 and S2
responses after tetanic stimulation after a small STP, this time without PPF changes.
However, fiber volley (Figure 3-11D) shows a corresponding increase as well. The
oscillation in S2 amplitudes is probably a result of saturating responses that were
removed from the analysis.

Both slow mannitol (Figure 3-12A) and slow sham (Figure 3-12C) showed
sustained LTP that develops after tetanus; neither was associated with significant PPF
changes. Interestingly, there was a decrease in fiber volley amplitude (Figure 3-12B and
3-12D) after tetanus. Since it is unlikely that fiber volleys could rundown while EPSPs
get larger, it is possible that this decrease in fiber volley magnitudes is an artifact of
increasing EPSP magnitudes, because both appear as negative deflections adjacent to one
another in the field potential waveform.

EPSPs from CCI animals (Figure 3-13A) respond similarly to slow mannitol and
slow sham with LTP and fiber volley (Figure 3-13B) decrease following tetanus. In
contrast, EPSPs from CCI sham animals (Figure 3-13C) showed no significant STP or
LTP. These results are confounding from a standpoint that they were obtained from a
sham surgery group with no behavioral evidence of spatial memory impairment, therefore
the ability to respond to tetanic stimuli with LTP was expected. It is possible that these
results are due to poor quality slices (CCI shams were the earliest experiments), although fiber volley amplitudes (Figure 3-13D) would have also been expected to decrease if slices were of poor quality.

The EPSP magnitudes of fast mannitol treated with either HSA (Figure 3-14A) or PEG-Alb (Figure 3-14C) changed in a similar fashion to previous results from other intracarotid injection groups (Figures 3-11 and 3-12), with a small post-tetanic STP followed by late LTP with little or no PPF changes. An increase in fiber volley amplitudes (Figures 3-14B and 3-14D) was only observed in the last 10 minutes of the PEG-Alb group, and clearly cannot account for the LTP that develops within 20 minutes of the tetanus.

Average post-tetanic EPSP amplitudes evoked by S1 and S2 stimuli are shown in Figure 3-15 A and 3-15B normalized by pre-tetanus levels. Note asterisks in Figures 3-15 and 3-16 are comparisons relative to fast mannitol EPSP and volley amplitudes, which were averaged at different time windows (5-10, 25-30, and 55-60 min) after tetanus in order to determine the temporal development of LTP. S1 and S2 EPSP show delayed LTP (> 25 min post tetanus) for most cases except CCI sham.

A two-factor ANOVA was used to infer statistical differences between EPSP and fiber volley amplitudes across different injuries, across different post-tetanus time windows (5-10, 25-30 and 55-60 min), and across different combinations of injury and post-tetanus time window. No significant changes in S1 EPSP amplitudes (Figure 3-15A) were found across injury groups (p = 0.113) or different combinations of injury and time window (p = 0.907). There was a significant increase in EPSP amplitudes from 5-10 to 55-60 min post-tetanus (p = 0.002), which indicates the slow onset of LTP
following tetanus. strong significance across different windows for S1 (p = 0.002), and no significance across combinations of surgery type and windows for S1 (p = 0.907). In contrast, post-tetanic S2 EPSP amplitudes (Figure 3-15B) were significantly larger for the fast mannitol animals compared to CCI sham (p = 0.022). A similar development of LTP was observed from the 5-10 to 55-60 min post-tetanus time windows (p = 0.008), and no combination of injury and time window yielded significantly different S2 EPSPs (p = 0.800).

Presynaptic fiber volley amplitudes were generally unchanged following tetanic stimulation with the exception of CCI sham and CCI animals (Figure 3-16A and 3-16B). For the post-tetanic S1 volleys, CCI and CCI sham were significantly smaller than fast mannitol and fast sham (p = 0.001). It should be noted that this was not due to a significant increase in fast mannitol and fast sham post-tetanus fiber volley amplitudes above control levels (note the fast mannitol and fast sham averages had large standard error bars). Instead, it appears that there was a significant rundown in CCI and CCI sham fiber volleys after tetanus. No time-dependent changes in fiber volley amplitudes were observed during the 5-10, 25-30 and 55-60 min post-tetanus time windows (p = 0.730), nor were any significant differences observed with a particular combination of injury and time window (p = 0.950). Similar results were seen in the S2 fiber volley amplitudes, with a significant decrease in CCI and CCI sham relative to fast mannitol and fast sham (p = 0.006), without any changes across post-tetanus time window (p = 0.643) or combination or injury and time window (p = 0.937).
Figure 3-15 EPSP After All Injury Types. Most injury types showed delayed LTP except for the CCI sham. (A) S1 and (B) S2. Asterisks are comparisons relative to fast mannitol.
Figure 3-16 Presynaptic Fiber Volley After All Injury Types. Most fiber volleys were unchanged except for CCI sham and CCI. Fast mannitol had large standard error bars. (A) S1 and (B) S2. Note asterisks are comparisons relative to fast mannitol.
No clear conclusions can be drawn from these LTP results. Unlike the behavioral results which showed clear deficits in animals receiving intracarotid injections of hyperosmotic mannitol at a rate of 0.17 ml/sec, the electrophysiological results did not show a similar deficit in the plasticity of Schaffer collateral – CA1 pyramidal cell synapses. Since long-term changes in strength at these synapses can be viewed as the neurophysiological correlate of spatial memory assays, this appears to be an unexpected if not contradictory result.

It should be noted that we observed a large variability in field potentials evoked by the electrical stimulation of Schaffer collaterals. Hippocampal field potentials can show substantial variability, and the typical approach is to record from a large number of slices and provide strict exclusion criteria to limit averages to slices that show more stable responses. Given the limited number of animals used for these studies, it was not possible to exclude unstable responses. Although we limited our recordings to the CA1 region, there may be substantial variability in the responses of slices taken from different CA1 locations along the rostral-caudal axis of the hippocampus. Again, the limited number of animals used in these studies precluded the use of slices obtained from a narrow region along the rostral-caudal axis of the hippocampus.

A more consistent assay for hippocampal LTP studies can be obtained using intracellular recordings from CA1 pyramidal neurons. In these studies, LTP can be more reliably induced by pairing presynaptic and postsynaptic action potentials as a substitute for the tetanic stimulation of presynaptic fibers. However, intracellular recordings can be more reliably obtained from juvenile animals. The surgical manipulations used in the
present study required the use of fully grown adults which would make intracellular recordings difficult to obtain.

Finally, field potential recordings from Schaffer collateral – pyramidal cell synapses in the CA1 region of the hippocampus traditionally used in LTP studies may not provide the best assay of neurophysiological deficits related to spatial memory in the BBB disruption model. The granule cell – perforant pathway synapses in the dentate gyrus and/or the mossy fiber- pyramidal cell synapses in the CA3 region may be more susceptible to damage following TBI. However, field potential assays are not suitable at these synapses given the more diffuse nature of the afferent innervation, and assays at these synapses would have required the use of single-cell recording techniques. As previously stated, the use of adult animals required for the surgical manipulations in this study are not conducive to these types of electrophysiological recordings.
Chapter 4

Modeling Results

Computational models of biological systems provide dynamic descriptions of normal physiological function and of pathophysiological changes induced by injuries. Simulations with a biological neural network exhibited an inability of synaptic connectivity to maintain maximum strength and stability after changes in firing thresholds and axon degeneration. Relevance of simulated injuries provides insight into the behavior of the dentate gyrus in vivo, which exhibits an increase in neurogenesis after TBI. The interactions between BBB permeability, immune response, cell proliferation, and afference were investigated by modeling the qualitative relationships of these properties from the literature. The results of these modeling studies suggest that excessive activity by networks that result in cell death and poor synaptic connectivity could be attenuated by the selective removal of hyperactive axons, control of cell proliferation rates, and maintenance of proper BBB homeostasis.
4.1 Computational Simulations

A theoretical neural network by Butz et al. (2006) was modified to allow for manipulations that represent axon degeneration and excitotoxicity in both neurogenetic and non-neurogenetic CNS tissues (Butz et al., 2006). Furthermore, an afferent input was added to the network in order to model external disturbances. Computational models allowed for a systematic and deliberate control of parameter values following a simulated TBI. The outputs of cell survival, synaptic changes, and overall activity of network provided a descriptive representation of pathological downstream events.

The purpose of this model was to explore how cell proliferation rates of the dentate gyrus and different types of injuries to the overall hippocampus might affect cell survival and synaptic integrity. TBI induces BBB disruption and diffuse axonal injury resulting in pathological cascades which are poorly understood. Neuronal death and changes in synaptic plasticity causes substantial deficits in the ability of a hippocampal network to learn and recall. Neurons experience apoptosis in an activity-dependent manner and can also remove themselves from a network if activity is excessive or low. Moreover, neurons entering a network will alter the synaptic connectivity of all the neurons. The model provides the ability to simulate and observe pathological events such as axonal degeneration and excitotoxicity induced by glutamate extravasation.

Each simulation of the neural network consisted of 300 morphogenetic steps by means of the compensation algorithm. The initial run was analogous to early neural development in an animal system. No cell deletion took place until 30 morphogenetic steps after either the start of the simulations. No cell deletion of added cells occurred until 30 morphogenetic steps. The functional state—no changes in $C$-matrix—followed
each morphogenetic change and consisted of 50 time steps. This allowed for the network to reach an equilibrium point. In addition, the average activity of each cell during the functional state dictated changes in the morphogenetic state occurred with the compensation algorithm.

Neurogenesis was also incorporated into the model to study the behavior of the dentate gyrus after TBI. Adult neurogenesis was first discovered in the mammalian CNS in the 1960s by Joseph Altman, but his findings were largely dismissed by the scientific community (Altman, 1962, 1963, 1969). However, after several studies in the 1980s and 1990s, neurogenesis was shown to be present in the rat and canary, and finally won favor in mainstream science (Gage et al., 1995; Goldman and Nottebohm, 1983).

The dentate gyrus was used as the basis for the neural network because of its known adult neurogenesis and role in memory and learning (Ehninger and Kempermann, 2008; Jin et al., 2001). Considered the processing point of the hippocampus, the dentate gyrus receives major input from the entorhinal cortex by the perforant pathway. The subgranular layer—the location of adult neurogenesis—is situated between the granule cell body layer and the hilus. This neurogenic niche is highly regulated by the vasculature, astrocytes, and neural precursor cells (Riquelme et al., 2008; Tavazoie et al., 2008)

4.2 Optimization of the Synaptogenetic Rate Constant

Optimization simulations were employed using networks without cell proliferation to find the best synaptogenetic rate constant (v). As mentioned before, each morphogenetic step consists of synaptic reorganization in order to compensate for high
and low activity within the network. The synaptogenetic rate constant \( (v) \) alters the magnitude of this compensation. Previously described in Table 2.1, the morphogenetic rate constants \( (k) \) needed to be globally adjusted while maintaining the same ratios to determine the optimal rate at which synapses change connectivity. The relationship of each \( k \) was maintained by finding the product of the constant \( v \) and each activity-dependent \( k \). Three outputs—cell survival, steady state, and synaptic decay—were averaged over 100 randomly generated simulations per rate constant value \( (v) \) to determine the optimal rate. The optimization simulations revealed that \( v = 0.1 \) gave the most stability and greatest conservation during the network developmental stage and represent experimental observations of neural development.

Cells were deleted from the network when activity was less than 0.25 or greater than 0.85. On a logarithmic scale, the percentage of cell survival follows a sigmoid curve (Figure 4-1A) which reaches a 100% cell survival average at \( v > 0.1 \). Further analysis revealed that 96% of simulations for \( v = 0.1 \) returned cell survival of 100%.

Average steady state did not significantly vary with changes in the rate constant (Figure 4-1B). This result was expected because the compensation algorithm was designed to maintain the overall activity to be within the range of 0.45 to 0.55 (100% of simulations at \( v = 0.1 \) were within the range). However, when there was significant cell deletion caused by a slow synaptogenetic rate \( (v < 0.01) \), 57% of steady states were less than 0.45.

Synaptic decay was defined at the sum of the original connectivity matrix, \( C_{pre} \), divided by the final connectivity matrix, \( C_{post} \), after morphogenetic changes. This provided an overall description of synaptic changes during the initial morphogenetic
steps. A certain level of synaptic decay is expected and represents the physiological
theory of synaptic maturation called, developmental selection, where synapses are pruned
away (i.e. retinal development). The average synaptic change demonstrated a parabolic
nature (Figure 4-1C). The least amount of synaptic decayed was determined to be near $v$
$= 0.1$. Synapses remained above 70% of their original connectivity for 95% of all
simulations at $v = 0.1$. 
Figure 4-1 Optimization of Synaptogenetic Rate Constant. Output measure (y-axis) were calculated by averaging 100 network simulations per rate constant value (v). The optimal rate constant was determined to be 0.1 (red dashed line). A logarithmic x-axis provided the best description of changes of the outputs. (A) The percentage of cell survival
followed a sigmoid curve and reached maximal value (100%) approximately at \( v = 0.1 \).

(B) The steady state does not change significantly with variations in the synaptogenetic rate constant. This behavior is expected because the network was designed to maintain a steady state within the range 0.45 to 0.55. (C) The least amount of synaptic decay occurred at \( v = 0.1 \) with approximately 85% synaptic strength remaining for the initial connectivity matrix. Synaptic decay is analogous to the synaptic physiological phenomenon called, developmental selection.

4.3 BBB Disruption and Axonal Injury Model

The neural network was manipulated in order to represent the effects of BBB disruption and TBI. When the blood plasma extravasates the BBB, neuronal cells often undergo a focal excitotoxicity by high concentrations of the neurotransmitter, glutamate. Mechanical damage to the axons within the CNS also disrupts signaling pathways. Excitotoxicity was modeled by shifting the firing threshold, \( \theta \), for all neurons—both excitatory and inhibitory. Additionally, complete axons are removed from the network at random so that \( \sum_{i=1}^{N} c_{ij} = 0 \) for a neuron \( j \).

Permutations of random axonal deletions and threshold shifts were constructed to measure changes in cell survival, synaptic connectivity, and steady state activity. After 300 morphogenetic steps of a network of zero cell proliferation, the firing threshold parameter (Equation 2.6) was changed as follows: \( \theta = \{-10,-9.5,-9,\ldots,5\} \). The default is \( \theta = 1 \) and is identified with a dashed line in Figures 4-2 to 4-5. At the same step as the change in firing threshold, presynaptic elements for an entire cell were eliminated:

\[ \text{number of axons} = \{0,1,2,3,\ldots,N\}, \text{ with } N = \text{ total number of cells in the network.} \]

For this model, \( N \) is equal to 30 neurons. Postsynaptic elements (\( \sigma^{fepo} \) and \( \sigma^{fepo} \)) of deleted synapses were able to connect to free presynaptic elements (\( \sigma^{fpr} \)). Five simulations at
each firing threshold and axonal deletion combination were used to determine average cell survival, steady state, synaptic weight, and synaptic ratios.

The neural network was allowed to experience an additional 300 morphogenetic steps following the simulated injury at different cell proliferation rates ($rCP = 0, 1, 2, 5, 10, 50, 100$) in terms of morphogenetic cycles per cell. Figures 4-2 to 4-5 demonstrate changes in cell survival, steady state, average synaptic weight, and average synaptic ratio of the network after injury. Cell survival and average synaptic weight were most affected by changes in cell proliferation. Steady state and average synaptic ratio were the least affected except for alterations resulting from zero to the smallest cell proliferation rates.

Cell survival (Figure 4-2) was dependent on the amount of axon deletion and the shift in firing threshold. When axonal deletion and firing threshold was low, very few neurons survived in the network. However, more neurons survived by decreasing hyperexcitability as more axons were deleted. This behavior was not as effective as cell proliferation rates were increased. Axonal deletion had limited effect as firing thresholds increased above the normal value of 1, and eventually created the complete elimination of the network due to inactivity. Interestingly, a network without proliferation has clusters of high cell survival near complete and zero axonal deletion (Figure 4-2A). Hyperexcitability induced cell deletion is minimized by removing axons that would perpetuate this behavior. This suggests that a complete elimination of axonal outputs could be a method to maximize cell survival after the firing threshold is decreased.

Steady state (Figure 4-3) was minimally affected by changes in cell proliferation rates. As mentioned previous, a steady state of 0.5 is considered optimal because activity is neither excessive nor low. However, a network without cell proliferation (Figure 4-
maintained stability throughout the range of shifts in firing threshold and axonal deletion. The inability of network to replace eliminated neurons with new neurons facilitated the attenuation of hyperactivity. Networks with neurogenesis had high activity with a low firing threshold and axonal deletion. Cell survival was minimal in this region—only one or two excitatory neurons—and a high steady state can be contributed to recurrent activity.

Average synaptic weight (Figure 4-4) was affected by the cell proliferation rate. The synaptic weight is defined as the sum of both excitatory and inhibitory postsynaptic elements of every neuron in the network. The network is initiated with each neuron receiving an average synaptic weight of 20. As the network progresses via morphogenetic steps, the average synaptic weight reduces to 75-80% of the original. As the firing threshold is shifted and axons are deleted, the loss of postsynaptic input is greatly increased. This phenomenon was further exacerbated by increases in cell proliferation rate. Competitiveness among new neurons limited the retention of existing synapses by established neurons in the system. However, cell proliferations rates of 100 and 50 cycles per cell (Figures 4-4B and 4-4C) appeared to increase average synaptic weights when compared to no proliferation at slightly lower firing thresholds and minimal axon deletion. This behavior may be descriptive of how homeostasis is maintained within the dentate gyrus after mild disturbances. Recent research has shown exercise increases new neurons in the adult brain and may be beneficial to cellular recovery following TBI (Erickson et al., 2011; Itoh et al., 2011).

Minimal changes occurred in averaged postsynaptic ratios of excitatory and inhibitory inputs after shifts in firing thresholds and axon deletion (Figure 4-5). The
compensation algorithm (Section 2.5.2) was designed to make changes in synaptic connectivity in order to sustain moderate activity. Based on Equation 2.3, each neuron offered and accepted synaptic elements to generate a membrane potential slightly above the firing threshold. The ratio of 9 : 1 (excitatory to inhibitory) is optimal and is defined by Equation 2.12 based on the parameters set. A network without cell proliferation was at this optimal ratio near zero axonal deletion and small changes in firing threshold (Figure 4-5A). As the network experiences cell proliferation of various rates, the optimal ratio was preserved along ranges of the largest cell survival and synaptic weights approximately 9 or greater. As the network approached a cell proliferation rate of 1 cycle per cell (Figure 4-5B), synaptic ratios become greater than 9 near zero axonal deletion and normal firing threshold. This behavior is due to the addition of only excitatory neurons to the network.
Figure 4-2 Percentage of Cell Survival Post-Injury. (A) No Cell Proliferation. (B) 100 cycles per cell. (C) 50 cycles per cell. (D) 20 cycles per cell. (E) 10 cycles per cell. (F) 5 cycles per cell. (G) 2 cycles per cell. (H) 1 cycle per cell.
Figure 4-3 Steady State Post-Injury. (A) No Cell Proliferation. (B) 100 cycles per cell. (C) 50 cycles per cell. (D) 20 cycles per cell. (E) 10 cycles per cell. (F) 5 cycles per cell. (G) 2 cycles per cell. (H) 1 cycle per cell.
Figure 4-4 *Average Synaptic Weight Post-Injury.* (A) No Cell Proliferation. (B) 100 cycles per cell. (C) 50 cycles per cell. (D) 20 cycles per cell. (E) 10 cycles per cell. (F) 5 cycles per cell. (G) 2 cycles per cell. (H) 1 cycle per cell.
Figure 4-5 Average Excitatory-Inhibitory Ratio Post-Injury. (A) No Cell Proliferation. (B) 100 cycles per cell. (C) 50 cycles per cell. (D) 20 cycles per cell. (E) 10 cycles per cell. (F) 5 cycles per cell. (G) 2 cycles per cell. (H) 1 cycle per cell.
4.4 BBB Permeability and Immune Response Model

The model of the dentate gyrus was extended to incorporate the immune response within the neural network after mild-TBI. Qualitative relations between BBB breakdown, afference, cell proliferation rates, and immune response were made based on published observations and data. Following mild-TBI, there is not an immediate axonal disconnection and dysfunction. Only after a secondary injury do symptoms of TBI become apparent. Based on the histological results of this study, instantaneous BBB disruption may be the initiator of the cellular signaling cascades that result in changes in immunological levels, neurogenesis, recurrent pathways, and further BBB breakdown. In addition, these physiological changes could be the basis for the manifestation of behavioral deficits demonstrated in the memory assay of the current study.

In the neural network, cell proliferation rates were varied in the set: \{1, 2, 3, …, 10, 12, 14, …, 30, 35, 40, …, 50\} morphogenetic steps per cell. Intrinsic activity \{-0.2 to 0.5\} was also modeled and affected the system by shifting the firing threshold as shown in Equation 2.6. In contrast to intrinsic responses to synaptic input, an afference value of 0 is interpreted as normal physiological external input into the network outside of normal synaptic transmission. Excitatory afference or external activity that induces firing, such as excess glutamate transport across the BBB, is denoted as negative values. External activity that inhibits the network and decreases the chances of neuronal firing is denoted as positive values. The behavior of randomly generated networks with neurogenesis and afferent activity proved to be too variable to average metrics such as cell survival and synaptic measures. Therefore, a common connectivity \( C \)-matrix was chosen and parameter modulations were conducted (Figure 4-6). Observations of randomly
generated matrices, however, exhibited similar overall behavior, such as a plateau centered near or at zero afference and little effect of changing cell proliferation rates.

Abnormal activity was present at afference greater than 0.1 and less than -0.1. As previously discussed, afference affects the probability that a given neuron will fire (Equation 2.6). Note that the firing threshold ($\theta$) used in Section 4.3 has the same effect on firing probability. However, the range of afference used was considerably smaller in order to model how normal external input into network might affect overall behavior. It should be mentioned again that the results of Section 4.3 are average behavior of different randomly generated connectivity $C$-matrices. Therefore, those results do not exhibit the qualitative behavior shown in Figure 4-6 because the drastic afference-dependent change was blurred by averaging different neural networks.

A small range of afference values between -0.1 and 0.1 were required for normal ranges of cell survival (Figure 4-6A), steady-state activity (Figure 4-6B), synaptic weight (Figure 4-6C) and synaptic ratio (Figure 4-6D). In particular, these results show that the cell proliferation rate of 1 to 100 time steps per cell had little effect on these network measures as long as the afference values remained near zero. Once outside this small range of afference values, the measures of network behavior shown in Figures 4-6A to 4-6D generally took abnormal values regardless of cell proliferation. The only exception to this is the observation of near-normal survival, activity and synaptic connectivity at low afference levels (-0.2 to -0.1) with moderate cell proliferation rates (5 to 20 steps per cell).
Figure 4-6 Cell Survival, Steady State, Synaptic Weight, and Synaptic Ratio of Neurogenic Network. (A) Cell survival. (B) Steady state. (C) Synaptic weight. (D) Synaptic ratio.

The interactions of cell proliferation $\mu$, afference $\alpha$, immune response $\varepsilon$, and BBB permeability $\gamma$ were explored with qualitative representations. Theoretical equations were developed to depict the relations based on empirical data obtained in the literature. Using the generic rate equation for variable, $x$:

$$ \frac{dx}{dt} = \frac{(x_\infty - x)}{\tau_x} \tag{4.1} $$

The numerical expression of the equation without time dependency:

$$ x_{n+1} = x_n + \frac{(x_\infty - x_n)}{\tau_x} \tag{4.2} $$

where $x_\infty$ is the steady state value of the parameter and $\tau_x$ is a time constant. Therefore:
Immune Response ($\varepsilon$): 
\[ \varepsilon_{n+1} = \varepsilon_n + \frac{(\varepsilon_\infty - \varepsilon_n)}{\tau_\varepsilon} \]  
(4.3)

BBB Permeability ($\gamma$): 
\[ \gamma_{n+1} = \gamma_n + \frac{(\gamma_\infty - \gamma_n)}{\tau_\gamma} \]  
(4.4)

Afference ($\alpha$): 
\[ \alpha_{n+1} = \alpha_n + \frac{(\alpha_\infty - \alpha_n)}{\tau_\alpha} \]  
(4.5)

Cell Proliferation ($\mu$): 
\[ \mu_{n+1} = \mu_n + \frac{(\mu_\infty - \mu_n)}{\tau_\mu} \]  
(4.6)

where $\tau_\varepsilon = 10$, $\tau_\gamma = 50$, $\tau_\alpha = 45$, and $\tau_\mu = 50$. The time constants were chosen to reflect that the immune response develops more rapidly than changes in BBB permeability, afference or cell proliferation. Simulations were conducted to optimize these time constants to obtain stability at the initial conditions. Furthermore, the time constants were chosen so BBB disruption of 0.6 or less would only produce transient responses (see Figure 4-10).

Non-dimensional steady state equations ($x_\infty$) were developed according to experimental relationships described below. The immune response $\varepsilon_\infty$ is a function of the average afference $\alpha$ into the network. The BBB permeability parameter $\gamma_\infty$ is a function of immune response $\varepsilon$. Lastly, the functions $\alpha_\infty$ and $\mu_\infty$ are functions of both the BBB permeability $\gamma$ and the immune response $\varepsilon$.

The immune response is dependent on the magnitude of the afference activity into the network. Excitotoxicity has been shown to initiate the immune response (Dugan and Choi, 1994; Obrenovitch et al., 2000). Also, inactivity can cause apoptosis through caspase-mediated mechanisms following TBI (Cernak et al., 2002). In this model, afference is defined as the activity of the neural network by both external disturbances.
and intrinsic shifts in network behavior caused by changes in ion channel expression or membrane potentials. Equation 4.7 was developed to represent the findings that both high and low activity (i.e., afference) creates inflammatory responses within the network.

\[
\varepsilon_\infty = \left| 1 - e^{-\frac{1}{\alpha_e} \left( e^{-\frac{x}{c_e}} \right)^{\alpha_e}} \right| \quad (4.7)
\]

where \( \alpha_{half} = 0.1 \) and \( c_{\alpha_e} = 12 \). This relationship is shown in Figure 4-7A.

In this model, BBB permeability is dependent on the immune response into the system. Extravasation of innate immunological cells such as leukocytes from the vasculature via transcellular mechanisms will dismantle tight junctions and enter into the CNS, resulting in the compromise of BBB integrity (Lossinsky and Shivers, 2004). The sigmoidal relationship was represented in Equation 4.8 and Figure 4-7B.

\[
\gamma_\infty = 1 - e^{-\frac{1}{\epsilon_{half} \left( e^{-\frac{x}{c_{\epsilon}}} \right)^{\gamma_e}}} \quad (4.8)
\]

where \( \epsilon_{half} = 0.6 \) and \( c_{\epsilon_e} = 3 \).

**Figure 4-7** Relationship of BBB Permeability, Immune Response, and Afference. (A) Immune response as a function of afference. (B) BBB permeability as a function of the immune response.

Afference can be dependent on the immune response by the inhibition of cytokines on glutamate transporters which have a critical role in the removal of excess extracellular glutamate (Miksa et al., 2005; Morganti-Kossmann et al., 2007). As
mentioned before, BBB permeability also controls the afference by allowing the extravasation of blood plasma into the CNS parenchyma resulting in hyperactivity. However, excessive concentrations of glutamate by the extravasation of blood plasma can inhibit the propagation of an action potential by maintaining a constant refractory period (Figure 4-8B).

\[ \alpha_a = 0.5\gamma^{d_r} \sin(2\pi\gamma) - 0.5\varepsilon^{d_e} \sin(2\pi\varepsilon) \]  

(4.9)

where \( d_{r_a} = 0.05 \) and \( d_{e_a} = 0.05 \).

At the extremes of the immune response and BBB permeability, afference is zero. The network receives no afference when the BBB is completely intact and the immune system is inactive. BBB permeability within the network causes afference to increase, which causes inhibition and a decrease in firing probability. Retrograde signaling induced by glutamate excitotoxicity in the network causes afferent neurons to experience axonal degeneration. The decrease in external axonal input results in the decrease in the overall afference into the network (Ross and Ebner, 1990). However, if the BBB breakdown is too great, the extravasation of plasma will spread beyond the network and affect neurons that provide external input. The afference then causes the increase in firing probability within the network and induces activity similar to a seizure where activity is uncontrollable. The afference returns back to zero as input neurons experience a persistent refractory period caused by hyperactivity.

As the immune system initially responds to injuries, afference becomes more negative and causes the network to become hyperactive. The cytokine, IL-1β has been demonstrated to induce excitotoxicity (Fogal and Hewett, 2008). When the immune response increases and phagocytosis occurs, afference increases causing the firing
probability to be decreased. The afference becomes zero again as the immune system eliminates the remaining synaptic input.
Figure 4-8 Afference as a Function of the Immune Response and BBB Permeability. (A) Function of $\varepsilon$ and $\gamma$. (B) When $\varepsilon = 0$. (C) When $\gamma = 0$.

Cell proliferation in the form of adult neurogenesis is dependent on both BBB permeability (i.e. disruption) and the immune response. The immune response has been shown to increases neurogenesis after acute mild inflammation but severe inflammation cause negative effects (Whitney et al., 2009). Neurogenesis has also been shown to be caused by glutamate which is extravasated following BBB disruption (Schlett, 2006). Therefore, Equation 4.10 was developed to represent these cellular responses (Figure 4-9). When $\varepsilon = 0$, cell proliferation is only partially activated (Figure 4-9B). It is also
notable that cell proliferation is kept at $\mu = 0.1$ during when $(\gamma, \varepsilon) = (0, 0)$ to model the constitutive neurogenesis within the dentate gyrus.

$$\mu_\infty = d_\varepsilon \varepsilon (p(\varepsilon)) + d_\gamma \gamma \left(1 - e^{-\left[p(\gamma)^{-1}\right]}\right) + \mu_0 (1 - \varepsilon)$$

(4.10)

and where $p$ is a 4th-order polynomial:

$$p(\varepsilon) = 17.22\varepsilon^4 - 35.15\varepsilon^3 + 18.83\varepsilon^2 - 0.98\varepsilon + 0.1$$

(4.11)

and also where $d_\varepsilon = 0.75$, $d_\gamma = 0.525$, $c_\gamma = 0.525$, and $\mu_0 = 0.1$.

**Figure 4-9** Cell Proliferation as a Function of the Immune Response and BBB Permeability. (A) function of $\varepsilon$ and $\gamma$. (B) when $\varepsilon = 0$. (C) when $\gamma = 0$. 
Table 4.1 Summary of the Relationships of Model Parameters

<table>
<thead>
<tr>
<th>Parameter Function</th>
<th>BBB Permeability</th>
<th>Immune Response</th>
<th>Afference</th>
<th>Cell Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBB Permeability</td>
<td>0 + 0</td>
<td>0 + 0</td>
<td>0 +/-/+</td>
<td>0</td>
</tr>
<tr>
<td>Immune Response</td>
<td>0 0 +/-/+</td>
<td>0 +/-/+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Afference</td>
<td>+/- -/+</td>
<td>0 +/-/+</td>
<td>0 +/-/+</td>
<td>0</td>
</tr>
<tr>
<td>Cell Proliferation</td>
<td>+ +/-</td>
<td>+ +/-</td>
<td>0 +/-/+</td>
<td>0</td>
</tr>
</tbody>
</table>

The immune response ($\epsilon$), BBB permeability ($\gamma$), and the average afference ($\alpha$) are initialized at 0. The cell proliferation rate ($\mu$) during normal physiological conditions is 1 cell per 10 time steps (0.1). Therefore $\epsilon(0) = 0$, $\gamma(0) = 0$, $\alpha(0) = 0$, and $\mu(0) = 0.1$. The stimulus is an instantaneous increase in BBB permeability ($\gamma$) that is given at $t = 100$ for $\Delta t = 1$, or $\gamma_{stim} = \{0, 0.2, 0.4, 0.6, 0.8, 1\}$. The transient opening of the BBB could be caused by a large number of physiological conditions such as hypoxia, cessation, hemorrhage, TBI, encephalitis, Alzheimer’s disease, multiple sclerosis, and epilepsy. These pathologies are commonly associated with immune cell infiltration indicated by the presence of cytokines within the CNS.

The parameters demonstrate a bifurcation in the network parameters that depends on BBB permeability (Figure 4-10). When there is no BBB permeability, all parameters remain at zero activity except for cell proliferation which remains at $\mu = 0.1$.

Constitutive rates of adult neurogenesis have been shown to be present in the dentate gyrus under normal physiological conditions (Mongiat and Schinder, 2011). Small changes in BBB permeability, $\gamma \leq 0.6$, cause only transient changes in the parameter. However, when $\gamma > 0.6$, the model reaches a perpetually active state where the immune response, BBB permeability, and cell proliferation do not return to the initial state. The afference oscillates at negative values which represents hypoactivity into the network.
In the model, a substantial disruption of the BBB can cause chronic state of immune response, excitatory afference, high rates of adult neurogenesis, and further irreversible BBB disruption. The immune system (Figure 4-10A) reacted to moderate to severe BBB disruptions ($\gamma \geq 0.4$) by initially reaching a near full response. This reaction was attributed to the endogeneous immune system such as microglia and activated astrocytes. When $\gamma \geq 0.8$, a constant immunological state occurred as an oscillation suggesting a limit cycle was present. This behavior could be analogous to certain chronic inflammatory diseases such as MS and Alzheimer’s where T-cells extravasate the BBB from the blood plasma.

The reaction of the BBB (Figure 4-10B) following a disruption of $0.4 \leq \gamma \leq 0.6$ was consistent with studies that observed a biphasic opening of the BBB after TBI (Baskaya et al., 1997). Mild disruptions ($\gamma \leq 0.2$) cause a transient breakdown of BBB which might only cause reversible alterations in cytoskeleton molecules and TJs. A large instantaneous breakdown of the BBB caused a chronic BBB disruption similar to the immune response except it had smaller oscillations.

The behavior of the afference exhibits transient positive deviation from $\alpha = 0$ if $\gamma \leq 0.2$ creating a decrease in firing probability of the network (Figure 4-6C). If the BBB disruption was large enough $0.4 \leq \gamma \leq 0.6$, a short inhibition by the external input is followed by an increase in firing probability and then a return to an inhibitory input. BBB disruptions $\gamma \leq 0.6$ returned back to $\alpha = 0$ after approximately 600 time steps. When $\gamma \geq 0.8$, the network immediately experiences excitatory external inputs and maintains this behavior.
Increases in cell proliferation rates (Figure 4-10D) occurred at $\gamma \geq 0.4$. For $0.4 \leq \gamma \leq 0.6$, a small increase precedes a larger transient in cell proliferation. This activity was biphasic similar to the response of BBB breakdown following TBI. Adult neurogenesis has been shown to be induced by glutamate (Schlett, 2006) which is extravasated following BBB disruption. At $\gamma \geq 0.8$, cell proliferation increases and remains at a rate of 0.6 (~2 cycles per cell).

![Graphs showing temporal responses](image1)

**Figure 4-10** Temporal Responses Induced by Instantaneous BBB Disruptions. (A) Immune response. (B) BBB permeability. (C) Afference. (D) Cell proliferation.

The output graphs in Figure 4-11 were used to represent the steady state, cell survival, average postsynaptic weight (excitatory : inhibitory), and postsynaptic ratio in a time scale. Figure 4-11 was generated by correlating the afference and cell proliferation
vs. time from the temporal responses (Figure 4-10) to the original network outputs of steady state, cell survival and postsynaptic connectivity which are shown in Figure 4-6. Baseline values where determined by setting cell proliferation rate $\mu$ to 0.1 and afference $\alpha = 0$. After various instantaneous changes in BBB permeability, network properties were graphed in order to insight to how network changes through time.

The steady state (Figure 4-11A) leaves the baseline value of 0.504 and increases in overall activity after disruptions of the BBB. A transient breakdown of $\gamma \leq 0.2$ caused a short deviation from the baseline. When $0.4 \leq \gamma \leq 0.6$, steady state becomes more hyperactive but returns back to the baseline. If $\gamma > 0.6$, the steady state remained at a constant higher value after a very short decrease in activity.

Percentages of cell survival (Figure 4-11B) were shown to decrease after BBB disruptions. Slight increases following large decreases were present after disruptions of $0.4 \leq \gamma \leq 0.6$, but returned back to baseline of 75%. If the disruptions were $\gamma > 0.6$, cell survival entered an oscillatory state suggesting alternating periods of apoptosis.

Average postsynaptic weight (Figure 4-11C) had a baseline of approximately 12. After a slight disruption $\gamma < 0.2$, synaptic weight experienced a small increase and quickly returned to baseline. For $0.4 \leq \gamma \leq 0.6$, a large decrease occurred followed by an increase in synaptic weight. This phenomenon may be analogous to how synapses reorganize after an insult to the CNS. However, postsynaptic weight decreased significantly after a disruption of $\gamma \geq 0.8$ and oscillated around 9.

The average ratio of postsynaptic excitatory and inhibitory elements (Figure 4-11D) was shown to increase after substantial BBB disruption. The baseline ratio was determined to be approximately 10 which facilitated a network to be excitable when
needed. As mentioned previously, a ratio slightly higher than 9 is required in order to
overcome inhibition in the network. However, if the ratio is too great, the network will
be excessively hyperactive and result in decreased cell survival. After a disruption of $\gamma < 0.2$, the average ratio became less than 10 but soon returned back to baseline. The
average ratio increased following moderate BBB breakdowns ($0.4 \leq \gamma \leq 0.6$). When the
disruption was $\gamma \geq 0.8$, the network remained at a persistent oscillation centered around
an excitatory ratio.

Figure 4-11 Temporal Changes in Network Metrics. (A) Steady state. (B) Cell Survival.
(C) Average postsynaptic weight. (D) Average Postsynaptic Ratio.
Chapter 5

Discussion

5.1. Overview of Study

In this current study, the disruption of the BBB by hyperosmotic mannitol was
demonstrated as a model of mild- to moderate TBI in the rodent. The behavioral
performance on the Barnes maze and histological observations of the BBB disruption
model were comparable or exceeded those from the traditional CCI model. Different
sized fluorescence-labeled albumin and Lucifer yellow determined the extent of BBB
permeability. In addition, the extravasation of plasma into the CNS might be the basis of
brain injury pathologies demonstrated by histological results. The results of this study
demonstrate that the disruption of BBB is an appropriate model of TBI, and suggests
therapeutics that reduces extravasation of plasma through the disrupted BBB might
diminish hyperexcitability and inflammation associated with TBI.

The computational results produced by a biological neural network provided
supplementary evidence that a disruption in the BBB causes the pathological
consequences associated with TBI. Based on empirical and theoretical experiments, the
neural network enabled the modulation of system parameters and a quantification of
cellular properties that cannot be measured in vivo. Changes in synaptic connectivity and
cell survival were quantified over time following a simulated TBI caused by the deletion of axonal output and shifts in firing threshold. The model gave insight how changes in connectivity may compensate for the increase in membrane potentials induced by the extravasation of excitatory neurotransmitters, or by the neuronal apoptosis induced by inflammatory responses. Furthermore, neurogenesis within the network explored potential behavior of the dentate gyrus following TBI.

5.2 Relationship of BBB Disruption and TBI

5.2.1 Disruption of the Blood-Brain Barrier

The brain is not completely protected by the BBB during normal physiological conditions. The choroid plexi, pineal gland, and circumventricular organs have a weak BBB and are able to transport specific molecules blood without facilitated transport (Pritchard and Alloway, 1999). The already compromised BBB makes these areas more susceptible to hyperosmotic effects demonstrated by a significant increase in fluorescence (Figures 3-2E and 3-2F).

Following injury, the opening of the BBB has been shown to be biphasic in pathology. Maximum BBB disruption has been shown to occur 4-6 hours and 3-days post-TBI and is demonstrated by increases in Evans Blue staining (Baskaya et al., 1997). Evans blues has a high affinity for serum albumin and is commonly used as a marker for the permeability of the BBB for macromolecules. Extravasation of the blood plasma in the CNS has been implicated in numerous neuronal diseases. Blood serum albumin, the key regulator of blood volume and plasma oncotic pressure, is also extravasated with glutamate resulting in a shift from normal vascular homeostasis. Previous studies have
shown that extravasated albumin results in the release of cytokines such as transforming growth factor β (TGF-β) and in the down-regulation of glial K⁺ inward rectifier channels. The increase in extracellular K⁺ further exacerbates the hyperexcitability of localized neurons (Friedman et al., 2009). Chronic seizures have been shown to result in angiogenesis causing the formation of porous vessels that have high BBB permeability. The disruption of BBB appears to perpetuate the progression of hyperexcitability in CNS.

After the administration of hyperosmotic solution, interendothelial TJs are estimated to widen to a radius of 200 Å caused by EC shrinkage (Kroll and Neuwelt, 1998). However, the disruption of the BBB can cause physical stress on the cytoskeleton of ECs and may initiate a signaling cascade involving calcium influx and nitric oxide (Berk et al., 1995; Dascalu et al., 1995; Lai et al., 2005). The cellular injury has been observed in vitro studies of rapid stress and strain forces on EC (Owatverot et al., 2005). Neurons and cortical astrocytes also have similar responses (Bain and Meaney, 2000; LaPlaca et al., 2005; Pfister et al., 2003; Smith et al., 1999). Conversely, there is evidence that osmotic opening of the BBB does not affect ECs (Farrell and Shivers, 1984).

There are structural changes and damages to the CNS parenchyma following a BBB disruption caused by a mannitol injection. Specifically, multifocal astrocytic swelling and the presence of macrophages indicate that mannitol-induced BBB disruption is not without immunological effect (Salahuddin et al., 1988a). Evidence of perivascular lymphocytic infiltration is present after 4 days following hypertonic infusion (Spigelman et al., 1986). After infusion of mannitol, acidophilic neurons with pyknotic nuclei present themselves in the infused hemisphere (Salahuddin et al., 1988b). Early evidence
of transient BBB disruption showed alteration in uptake and metabolism of glucose and in changes of local cerebral blood flow (Pappius et al., 1979; Rapoport et al., 1981). Alternatively, previous studies have demonstrated that little or no significant changes occurred after a hypertonic infusion exhibited by lack of necrotic cell labeling (Rapoport et al., 1972; Spigelman et al., 1986; Tomiwa et al., 1982).

5.2.2 Blast-Induced Traumatic Brain Injury

The conflicts in Iraq and Afghanistan have produced an increasing number of patients afflicted with TBI. IEDs have accounted for over 60% of combat casualties (Kelly et al., 2008; Okie, 2005; Shanker, 2007). It is also estimated at that 20% to 25% of the deployed force or 320,000 service members suffer from TBI induced by an IED (Elder and Cristian, 2009; Lew et al., 2008; Warden, 2006). Approximately 50% of combat-related TBIs can be clinically categorized as mild (Tanielian and Jaycox, 2008). The high rate is partly attributed to lower mortality rates and advancements in body armor and medical techniques (Okie, 2005). The symptoms, either permanent or temporary, include deficiencies and disorders in motor sensory function, memory, learning, vision, sleep, cognition, and behavior (Newman et al., 2003; Schneiderman et al., 2008).

Despite the increase in the rate of survival attributed to improvements in medical treatment and protective equipment, technological advancements may have unintentionally caused an increased amplification of overpressure created by IEDs in military personal (Mathis and Clutter, 2007; Thom and Cronin, 2009). Considerable resources by the federal government have been allocated to research and medical
treatment of TBI. Pressure sensors are now integrated into the combat helmets of some service personnel in order to better characterize the dynamics of bomb blasts (Wang et al., 2009). In addition, the Department of Defense has recently opened a center directed at the rehabilitation of veterans inflicted with TBI (Hoffman et al., 2010).

TBI is often not the only wounded sustained during deployment. Along with permanent disabilities such as loss of limbs and blindness, psychological wounds persist long after returning home. The symptoms of TBI and post traumatic stress disorder (PTSD) have been shown to overlap (Cernak et al., 1999; Elder and Cristian, 2009; Guy et al., 2000; Schneiderman et al., 2008; Trudeau et al., 1998). A decline in general health, difficulties sleeping, forgetfulness, and distracted attention are often associated with both pathologies. “Shell shock” is a term coined almost a century ago by Sir F.W. Motts in 1917 when first reported death caused by bomb blasts without any external injury and at the time was commonly referred as a psychiatric illness (Mott, 1916, 1917). Combat veterans with PTSD have been shown to have altered neuroendocrine signaling and memory dysfunction with finding of reduced hippocampal volume (Yehuda et al., 2007). However, some recent studies suggest there is not an association of PTSD with mild-TBI (Hoge et al., 2008).

5.2.3 Vascular Surge Model of Traumatic Brain Injury

Even though no organs experience a blunt force, the overpressure created by the blast is hypothesized to cause substantial tissue damage to vital organs which have significant changes in tissue mass density (i.e. brain and lungs) in response to the blast wave (Bala et al., 2008; Cernak et al., 2001b; Chavko, 2007). The symptoms of the
primary injury to the CNS are often unobserved in the field and commonly subside before medical treatment is administered. Once undiagnosed and untreated, non-ballistic bomb blast victims are becoming the subject of further research and more importantly is increasingly recognized by the public as a physical injury.

The uncoordinated contraction of the heart resulting from the blast overpressure propagating through the thorax can cause an instantaneous increase in intracarotid pressure (Chavko, 2007; Knudsen and Oen, 2003). The vascular surge is hypothesized to provide the most pressure around the Circle of Willis via the internal carotid and can cause disruptions of the BBB (Armonda et al., 2006; Harders and Gilsbach, 1987). Evidence from Cernak et al. suggests that this cause is the primary injury indicated by the similarities between rats with and without protective head shielding (Cernak et al., 2001b). Studies by Kato et al suggests that a higher threshold is required to induce an equal criteria of brain injury when the overpressure is directed at only the head (Kato et al., 2007). Vagotomy has also shown a reduction in apnea and bradycardia but does not eliminate damage to the CNS due to blast overpressure to only the thorax. This suggests that injuries to the brain is induced by a thoracic mechanism (Courtney and Courtney, 2009). In addition, soldiers also experience these same cardiac and pulmonary pathologies following an exposure to blast overpressure (Okie, 2005).

A previous study had demonstrated the protective effects of Kevlar® vest in the prevention of axonopathy and cell loss following the bomb blast model (Long et al., 2009). In this study, similar regions of the brain exhibiting BBB disruptions (i.e. subthalamic area and temporal cortex) were present in our study (Figure 3-2). These results are consistent with other previous studies that theorize the vasculature as a conduit
of high energy to the brain in bomb blasts and blunt behind-armor TBIs (Cernak et al., 2001b; Gryth et al., 2008). This current study provides evidence that the disruption of the BBB may be central to TBI symptoms similar to those manifested after bomb blasts. Other recent studies support this hypothesis that vascular surges generated by overpressure to the thorax can cause BBB disruptions. Potential therapeutics could target mechanisms that restore BBB in a variety of TBI patients.

5.2.4 Pulmonary Inflammation Model of TBI

In addition to the vascular surge model of blast-TBI, overpressure damage to the alveoli of lungs is hypothesized to cause the activation of leukocytes resulting in a systemic inflammation throughout the vasculature. As mentioned previously, hollow organ systems such as the auditory, lung, and gastrointestinal systems are particularly susceptible to blast waves. The energy emitted as the overpressure transitions from high density tissue to an air-filled space causes shearing at the interface. The result is hypothesized to be activation of the blood-borne immune system.

Inflammatory pathways signal activated immune cells which extravasate the BBB and phagocytosis CNS cells through cytokine signaling mechanisms. Moderate or severe TBI patients of bomb-blasts have punctuated hemorrhages in the corpus callosum (Finkel, 2006; Okie, 2005). Microglia have been shown to be activated after bomb-blast related injuries specifically in the gray matter as well as the choroid plexus which both have high densities of capillaries (Kaur et al., 1995, 1996). The infiltration of the CNS by blood-borne species compromise the integrity of BBB initiating a cascade of events associated with secondary injury. Along with leukocytes, hemoglobin may be more
readily interactive with ROS after blast exposure (Elsayed et al., 2000; Elsayed and Gorbunov, 2003; Gorbunov et al., 1997). NO production has been shown to be altered following overpressure in animal models (DeWitt and Prough, 2009; Elsayed et al., 1996; Zunic et al., 2000).

It is possible that both vascular surge and pulmonary inflammation contribute to BBB disruption after a blast, and therefore contribute to the symptoms of TBI. This current study provides evidence that the disruption of the BBB may be central to TBI symptoms similar to those manifested after bomb blasts. Other recent studies support this hypothesis that vascular surges generated by overpressure to the thorax can cause BBB disruptions (Cernak and Noble-Haeusslein, 2010). Additional evidence has demonstrated that injury to the lungs by blast overpressure cause inflammation throughout the vasculature (Elsayed and Gorbunov, 2007). Sepsis has also shown to cause damage to the CNS with similar mechanisms (Semmler et al., 2008). As shown in Section 3.2, potential therapeutics could target mechanisms that limit the extravasation of BBB by plasma molecules and immunological cells could benefit a spectrum of TBI patients.

5.3 TBI Biomarkers and Treatments

5.3.1 Biomarkers

One of the major difficulties in the treatment of TBI is often the inability to detect if a TBI even occurred. Especially for mild- to moderate TBIs, imaging techniques only have a limited resolution. Currently, the most common criterion for the diagnosis of TBI is the GCS which only uses cognitive data. The disruption of protein synthesis following TBI suggests that protein and lipid levels should be observed. Two aspects of TBI are
commonly investigated by measuring the levels of biomarkers: those concerning structural changes following the primary TBI, and those resulting for the repair phase or the secondary TBI.

A common approach to measuring the disruption of the BBB is the quantification of CNS protein levels in the peripheral blood. The glial proteins such as glial fibrillary acidic protein (GFAP) and S100B, and neuronal proteins such as neuron specific enolase (NSE) are brain specific biomarkers that have been shown to be predictors of outcome after severe TBI (Herrmann et al., 2001; Kochanek et al., 2008; Vos et al., 2004).

S100B is a calcium binding protein localized predominately in astroglia and Schwann cells (Donato et al., 2009; Jauch et al., 2006; Pelinka et al., 2004; Rothermundt et al., 2003). The family of proteins was given the name, “S100”, because they are soluble in 100% saturated ammonium sulfate solution (Moore, 1965). When released from glia, S100B has an autocrine and paracrine effect similar to the immunological effects of IL-α, IL-β, and human endothelial growth factor shown by an influx of free Ca\(^{2+}\) into glia and neurons (Barger and Van Eldik, 1992). Micromolar extracellular concentrations of S100B have detrimental and degeneration consequences on the CNS, however, nanomolar concentrations both extracellular and intracellular contribute to the proliferation and differentiation of neurons and glia. In vitro evidence has shown that S100B may have a neural protective following TBI (Ellis et al., 2007).

The majority of the known functions of S100B have been determined by in vitro and cell culture studies. However, in vivo studies has shown that S100B effectively determines the magnitude and outcome of TBI (Korfias et al., 2007). S100B inhibits the protein phosphorylation of many proteins such as GFAP, p53, and tau protein. The
elevation of intracellular free Ca\(^{2+}\) by S100B may increase the phosphorylation state of these target proteins (Donato, 2001). However, increased levels of Ca\(^{2+}\) also decrease the firing threshold of neurons which could induce excitotoxicity.

GFAP is an intermediate filament for specifically glia and is better characterized than S100B (Sandhir et al., 2008). This protein is particularly important because it is a part of the cytoskeleton of astrocytes which are key regulator of the BBB. GFAP is also the only biomarker known to be specific to astrocytes. During CNS pathologies that induce chronic inflammation, increased concentrations of GFAP are present in the plasma along with glial scarring surrounding injury sites. GFAP also has an important role coordinating white matter architecture (Liedtke et al., 1996) and neurogenesis (Garcia et al., 2004).

Neuron specific enolase (NSE) is a glycolytic enzyme localized in predominantly in neuronal cytoplasm and is sensitive and specific in TBI, stroke, and cardiac arrest (Beers et al., 2007; Wunderlich et al., 2006). NSE has also been found in red blood cells and platelets leaving the possibility of cross-contamination (Johnsson et al., 2000). However, NSE is an alterative to S100 in the detection of TBI in patients.

Neuropeptides have been identified as potential determinants of functional deficits following TBI (Nimmo et al., 2004). Substance P is a neuropeptide released from both peripheral and central primary afferent neurons and is known to function as a neurotransmitter in pain perception. Substance P belongs to the tachykinin neuropeptide family and was first identified in the early 1930s to have potential contractile properties (Von Euler and Gaddum, 1931).
The pathologies of TBI and BBB disruption are part of an interdependent relationship which results in the manifestation of secondary injury caused by molecular signals. In this study, BBB disruption has been shown to induce symptoms of TBI by the extravasation of plasma. Likewise, all severities of TBI have been shown in numerous studies to induce BBB disruption. This relationship results in the presence of plasma biomarkers that can be used to diagnosis TBI before any symptoms appear. Imaging with traditional methods makes the detection of mild-TBI and BBB disruption difficult. Biomarkers may provide a means to diagnosis increases in BBB permeability before symptoms of TBI and other neurological diseases emerge.

5.3.2 Treatments

Currently, there are no effective treatments of TBI in humans. In vitro studies have shown multiple treatments of cell death following TBI models. However, many of these advancements fail during in vivo studies to impede secondary injury in animal models. Moreover, the treatments that exhibit attenuation of TBI symptoms in animals have not been able to pass Phase III in FDA trials. Drugs that target individual molecular pathways often unmask other pathways leading to further cell death (Gottrone et al., 1997). Approaches are being made to target multiple secondary injury factors including those occurring later in time (Loane and Faden, 2010). The regulation of endogenous neuroprotective factors such as thyrotropin releasing hormone (TRH) were the first of these factors to be identified. TRH-related peptides improve blood flow after trauma, regulate ionic changes, and inhibit multiple secondary injury factors. TRH has been shown to improve outcome after TBI or spinal cord injury (Faden et al., 2005).
The pretreatment of magnesium sulfate (MgSO₄) attenuates the diffuse disruptive effects of mannitol (Kaya et al., 2004). In addition, the treatment of MgSO₄ at 30 minutes post-diffuse acceleration-impact model attenuates the symptoms of TBI (Fromm et al., 2004). The neuroprotective effects of magnesium have been well studied in ischemic models. Interestingly, the use of mannitol to access the CNS along with the co-administration of magnesium in combination with other therapeutics by the has been suggested as a treatment for TBI (Sen and Gulati, 2010). As mentioned in Section 1.4, Mg²⁺ constitutively blocks NMDA receptors from allowing an influx of Ca²⁺ into the postsynaptic neuron. Excess Mg²⁺ in the CNS parenchyma may limit the induction of Ca²⁺ influx through NMDA receptors and could reduce excitotoxicity.

In this study, PEG-Alb was shown to attenuate TBI symptoms in the rodent model by regaining the BBB homeostasis. Moreover, polyethylene glycol (PEG) alone has been shown to assist in the repair in cellular membranes following TBI (Koob et al., 2008; Liu-Snyder et al., 2007). Albumin is an important plasma protein that regulates oncotic pressure in the blood. The approximate length of human serum albumin is 160 Å (Carter et al., 1989). These results suggest that PEG-Alb might have cellular interactions with ECs and the CNS along oncotic forces across the BBB (Abbas et al., 2010). In addition, unmodified albumin was shown to be equally effective to reduce the severity of behavioral deficits following BBB disruption and TBI. It is hypothesized that the large amount of unmodified albumin which was approximately a quarter of normal levels may have saturated the blood (Johnson-Delaney, 1996; Lee and Blaufox, 1985). In future studies, the concentration of both the modified and unmodified albumin should be decreased in order to differentiate between the efficacies of the two compounds.
By understanding the pathways involved in the transendothelial migration of leukocytes into the parenchyma of the CNS during EAE, researchers hope to develop a treatment for CNS-related autoimmune diseases such as MS. Therapeutics that treats MS may be beneficial towards disease with similar symptoms such as TBI. One therapy that has been the subject of much controversy is the humanized anti-α4 integrin antibody, natalizumab. While undergoing phase III clinic trials, three patients had incidences of progressive multifocal leukoencephalopathy which resulted in a voluntarily withdrawal of natalizumab (Davenport and Munday, 2007). However, after slightly over a year later and further safety reviews, natalizumab was approved by the FDA in June 2006 for patients for relapsing MS and have no beneficial response to alternative treatments (O'Connor, 2007).

Another method to minimize neuroinflammation is the restoration of normal BBB permeability after a disruption event. In this current study, it is hypothesized that the PEG-modified of albumin (PEG-Alb), which theoretically can not cross the BBB because of physical size, decreases serum protein extravasation by restoring oncotic pressure and may also result in the facilitation of TJ reassembly due to the hypotonic effects of PEG-Alb (Assaly et al., 2004; Assaly et al., 2008). It is not known if the reassembly of the endothelial TJ decreases the severity of the immunological response following BBB disruption. This current study also did not investigate the extent of inflammation or if the immune response was attenuated following a mannitol-induced BBB disruption.

The intracarotid injection of mannitol may not have provided a significant disruption of the BBB demonstrated by lack of significance of behavioral results between PEG-Alb and unmodified albumin (see Section 3.2). The disruption of the BBB induced
by a vascular surge is presumably more severe than the hyperosmotic insult induced in this study. Pulmonary inflammation might also produce a BBB disruption that might be irreversible caused by the phagocytosis of ECs. The efficacy of unmodified albumin would be lessened if the BBB was greater.

The development of edema is a major clinical concern following TBI that is severe enough to affect intracranial pressure. Therefore, most actions following TBI address alleviating intracranial pressure through the administration of hyperosmotic agents (i.e. mannitol) and barbiturates, or induction of hyperventilation and hypothermia, as well as the drainage of CSF and decompressive craniotomy (Latorre and Greer, 2009). In this study, mannitol was delivered at a much higher concentration and rate when used clinically to alleviate intracranial pressure. In the present study, these therapies are not intended to improve patient functional outcome; instead they are applied in a manner consistent with the hypothesis that BBB disruption results in TBI.

5.4 Learning and Memory

5.4.1 Hippocampus and Traumatic Brain Injury

The hippocampus has been identified as a key facilitator of learning and memory within the CNS. Furthermore, mild- to moderate-TBI has been shown to damage the dentate gyrus more than any other region of the hippocampus (Anderson et al., 2005) which may contribute to memory deficits commonly associated with TBI. The hippocampus is particularly vulnerable to neurodegeneration following TBI (McCarthy, 2003; Sato et al., 2001). Long-term potentiation (LTP) is greatly diminished shown by smaller NMDA potentials and glutamate-induced glutamate excitatory potentials in the
CA1 region (Schwarzbach et al., 2006). Inhibitory inputs such as GABAergic neurons decrease following TBI (Reeves et al., 1997; Sanders et al., 2000).

Behavioral results in the current study indicate dysfunction of learning and memory in the rodent model. Signs of BBB disruption in the dorsal hippocampus in the CA3 and dentate region provide additional histological support to a possible mechanism for deficits (Figure 3D). The dentate gyrus is particularly susceptible to TBI probably due to its role in the entorhinal-hippocampal regulation. Mossy cells in the dentate hilus are vulnerable to various types of injuries such as epilepsy, ischemia, and TBI (Buckmaster and Schwartzkroin, 1994). The glutamatergic mossy cells act as a feedback system for granule cells in the dentate gyrus and also innervate GABAergic interneurons. The loss of mossy cells has been associated with hyperexcitability within the hippocampus. However, the survival of mossy hilar cell may also have a significant role in memory deficits (Santhakumar et al., 2000). It is hypothesized that alteration is dopamine signaling following TBI cause deficits in memory, attention, and motor learning (Bales et al., 2009; Wagner et al., 2009; Wagner et al., 2005).

5.4.2 Hippocampus and Blood-Brain Barrier Disruption

Histological results provided evidence that the initial BBB disruption was the major cause for memory discrepancies. Equal volume mannitol injections differed in respect to injection rate suggesting immunological reactivity of the vasculature was less likely the origin of the primary injury. Possible mechanism affecting learning and memory are hypothesized to be related to the extravasation of blood plasma, in particular, the excitatory neurotransmitter, glutamate. Consequences from the initial BBB
disruptions include excitotoxicity which can cause a secondary BBB disruption via astrocytic signaling along neuron fiber tracts (Hartwick et al., 2008; Maier and Watkins, 1998). Glutamate does not cross the BBB without active transport and is free to diffuse down its osmotic gradient into the CNS parenchyma extracellular space (Bullock et al., 1991; Smith, 2000; Zhang et al., 2000). Concentrations of glutamate in plasma are 50-100 micromol/L; in whole brain, they are 10,000-12,000 micromol/L but only 0.5-2 micromol/L in extracellular fluids (Hawkins, 2009).

The increased glutamate concentration leads to increased NMDA receptor expression resulting to the increase in the influx of Ca^{2+} ions into neurons and glia resulting in neuronal hyperexcitability (Gouix et al., 2009). Due to the effects of glutamate receptors mobilization on neuronal plasticity and synaptic formation, it is possible minor and local disruptions of the BBB could have profound effects on memory and learning (Hawkins, 2009; Le Vasseur et al., 2008). Furthermore, GLT-1 and GLAST are astrocytic glutamate transporters from the EAAT family that regulate extracellular glutamate by clearing excess glutamate. After TBI, both GLT-1 and GLAST are downregulated and result in neuronal excitotoxicity (Laird et al., 2008).

Search rates were not significantly altered following the induction of injury by either CCI or mannitol injections. This result suggests that animals did not experience lethargy and were responsive to aversive stimuli during Barnes maze testing. Rather deficits in memory retention were demonstrated by significant differences in latency and search pattern for animals administrated a fast mannitol solution. Behavioral changes provide evidence that BBB disruption and the extravasation of plasma proteins effect synaptic plasticity mechanisms resulting in memory and learning deficiencies.
5.4.3 Electrophysiological Studies

The results produced by electrophysiology appeared to contradict the behavioral deficits found in Barnes maze data (see Section 3.2). The LTP protocol administered to the synapses between the Schaffer collateral and CA1 neurons. The plasticity of these synapses is dependent upon NMDA receptor activation; whereas perforant pathway - dentate granule cell and mossy fibers - CA3 pyramidal cell synapses produce LTP through NDMA-independent pathways (Harris and Cotman, 1986). This suggests that TBI and BBB breakdown may not produce any changes in this region. Further studies should be performed in the dentate gyrus where neurogenesis and synaptogenesis may alter information processing. However, electrophysiological recordings in the dentate gyrus are difficult because intracellular recordings are required. In addition, the significant increase in glial cells and extracellular matrix molecules in the adult animal make intracellular recordings difficult.

The induction of LTP may not be altered following TBI and BBB disruption. In this study, it is apparent that the maintenance of LTP or the memory of a certain task was altered by brain injury. Future studies should investigate the ability of normal and injured animals to learn a new task. Variability among the rates in which animals learn may prove this approach insignificant unless large amount of animals are used. *In vivo* recordings of the hippocampus may also reveal changes in the activity of place cells which exhibit specific spatial awareness in the environment of the animal. This approach
was yet to be explored in animals induced with TBI and are theorized to generate changes in place cells similar to status epilepticus (Liu et al., 2003).

5.5 Applications of Computational Model

In Chapter 4, the dentate gyrus was modeled with a neural network that allowed the morphogenetic change of synaptic connectivity that was dependent on the activity of individual neurons. TBI and BBB disruption were modeled with the manipulation of connectivity and activity by the deletion of axons and the shift in firing threshold (Section 4.3). The network outputs of cell survival, overall activity, average synaptic weight, and average synaptic ratio were evaluated at various cell proliferation rates. A second adaptation of neural network (Section 4.4) incorporated adult neurogenesis, afference, immune response, and BBB permeability into the model. Qualitative relationships between these properties were determined from previous studies and represented in non-dimensional manner. These two types of network utilizations allowed the analysis of synaptic changes and cell survival which both have profound consequences in the CNS.

The modeling results suggest a few novel implications of BBB disruption and axonal degeneration (Section 4.3). The firing threshold of neurons in the network was systematically altered to represent the effects of glutamate that extravasate the BBB after TBI. Complete axons were also removed to model the effects of diffuse axonal injury and TBI. In non-neurogenic tissues, the greatest cell survival was present near zero axonal removal along with complete axonal removal (Figure 4-2A). This result suggests the Wallerian degeneration may be beneficial to cell survival in networks that experience injury by eliminating outputs that would otherwise cause excitotoxic cell death. In
general, high rates of neurogenesis were shown to cause impaired synaptic remodeling and cell survival. This result is hypothesized to be caused by the inability of proper synaptic connections to be made while new neurons were added to the network. Competition caused by new neurons weakened the connectivity among existing neurons which often resulted in elimination from the network due to inactivity. Cell proliferation rates of 50-100 cycles per new cell were shown to be optimal to repair the most damage caused by a lowered firing threshold and axonal deletion.

Changes in BBB permeability resulted in either temporary or permanent changes to model outputs (Section 4.4). The interaction of inflammation, hyperexcitability, and BBB disruption was demonstrated to be the determining factor if the network experienced permanent dysfunction in cell survival and synaptic connectivity. Unlike the model used in Section 4.3, cell proliferation did not appear to have a large effect on the outcome of the network, and the magnitude of the afference had the most effect on state of the network (Figure 4-6). The implications of these relationships suggest that the external input into an injured network might be the determinant of cell survival and proper connectivity among neurons.

The temporal responses induced by various magnitudes of BBB disruption (Figure 4-10) produced some interesting results. Small changes in BBB permeability resulted in only a temporary change in the physiological state before returning to the baseline values. Figure 4-10B even exhibited the biphasic nature of BBB permeability often observed as edema in TBI studies in adult animals (Barzo et al., 1997; Baskaya et al., 1997; Cernak et al., 2004). Low afference which represents a decrease in firing threshold for the network was shown in Figure 4-10C for large BBB disruption. This
behavior might be analogous to the events leading to epileptic seizure. In addition, the
decrease in afference was preceded by an increase in the immune response which has
been implicated in the manifestation of epilepsy (Ransohoff, 2009).

Along with the results from Figure 4-11, the BBB permeability-immune response
model demonstrated how excitotoxicity, inflammation, and BBB disruptions can affect
the cell survival and synaptic connectivity of a neural network. If one element is reduced
or eliminated, then the result might be the preservation of synaptic connectivity and
maximized cell survival. Novel treatments should be developed to target these elements.
In fact, the rationale of using PEG-Alb in the experimental methods was to minimize the
extravasation of blood plasma into the CNS parenchyma. The hypothesis was the
reduction of BBB permeability by PEG-Alb following a disruption would limit the
amount of glutamate and albumin crossing the BBB. In turn, neurons in injured regions
of the brain would not experience excitotoxicity and inflammation commonly associated
with TBI and BBB disruption.

5.6 Conclusions

In this study, hyperosmotic mannitol was demonstrated to be a model of mild- to
moderate TBI shown by the disruption the BBB and behavioral symptoms associated
with TBI. Histological data exhibited the extravasation of fluorescence-labeled albumin
and Lucifer yellow across the BBB following a high concentration and rate injection of
mannitol. The memory performance of behavioral assay demonstrated the inability of
animals induced with BBB disruption to recall a trained task. Furthermore, the modeling
results produced by a biological neural network provided additional evidence that a
disruption in the BBB causes the pathological consequences associated with TBI. Decreases in cell survival following a simulated TBI provided evidence how changes in connectivity may compensate for the increase in membrane potentials, inflammation, and axonal degeneration. In addition, adult neurogenesis within the network explored the potential behavior of the dentate gyrus following TBI. The results of this study demonstrate that the disruption of BBB is an appropriate model of TBI, and suggests therapeutics that reduces extravasation of plasma through the disrupted BBB might diminish hyperexcitability and inflammation associated with TBI.
References


