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The Effects of Blast-Induced Neurotrauma on Blood-Brain Barrier Permeability, Cell-Cell Junction Protein Levels and the Role of Peroxynitrite

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The Effects of Blast-Induced Neurotrauma on Blood-Brain Barrier Permeability, Cell-Cell Junction Protein Levels and the Role of Peroxynitrite

by

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Dedication

This dissertation is dedicated to my family, Edward and Carolyn Ruppert, and Charles, Allison, Emma and Colin Brownlee. Without your patience, prayers and support, none of this would have been possible.

The Effects of Blast-Induced Neurotrauma on Blood-Brain Barrier Permeability, Cell-Cell Junction Protein Levels and the Role of Peroxynitrite

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Traumatic brain injury is one of the most common injuries presented in emergency departments in the U.S.. While mild traumatic brain injury (TBI) is often not reported or treated it may still result in significant pathophysiological disruptions for the patient. As a result of the ongoing military Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF), service men and women are returning home after deployment with mild TBI (mTBI). This has resulted in mild TBI becoming the signature injury of these operations and has presented a substantial challenge for researchers and clinicians alike. Blast-induced Neurotrauma (BINT), often caused by improvised explosive devices (IEDs), is an injury that affects military personnel, however, the increasing threat of worldwide terrorist attacks makes this a concern for all individuals. Many of those who

are subjected to blast overpressures and underpressures experience deficits in cognition, memory, sleep, behavior and mood. Because the effects of blast exposure are not well defined on a cellular level, there is still a need for effective interventions and therapeutics. Here we will discuss a novel rodent model for the study of blast-induced neurotrauma, which we used to investigate the effects of BINT on the blood-brain barrier, cognitive and sensorimotor function and reactive nitrogen species formation. We hypothesize that BINT increases the permeability of the BBB, decreases the presence of the tight junction protein, occludin, in cerebral blood vessels, causes deficits in sensorimotor function and spatial learning and working-memory, and increases the formation of potent oxidant, peroxynitrite. A better understanding of the direct and indirect effects of blast exposure on the brain will contribute to our understanding of the mechanisms responsible for the unique pathology associated with BINT.

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List of Abbreviations

3-NT	3-Nitrotyrosine
4-HNE	4-Hydroxynonenal
ABC	ATP-Binding Cassette
AJ	Adherens Junction
AOC	Alteration of Consciousness
BBB	Blood-Brain Barrier
BINT	Blast Induced Neurotrauma
CBF	Cerebral Blood Flow
CCI	Controlled Cortical Impact
CDH5	Cadherin 5
CNS	Central Nervous System
CPP	Cerebral Perfusion Pressure
CT	Computerized Tomography
CVR	Cerebral Vascular Resistance
DOD	Department of Defense
EAE	Experimental Autoimmune Encephalomyelitis
EB	Evans Blue dye
FPI	Fluid Percussion Injury
GCS	Glasgow Coma Scale
GSBS	Graduate School of Biomedical Science
HIFU	High Intensity Focused Ultrasound
HIV	Human Immunodeficiency Virus

HRP	Horseradish Peroxidase
IA	Impact Acceleration
IC	Intravascular Coagulation
ICP	Intracranial Pressure
IED	Improvised Explosive Device
iNOS	Inducible Nitric Oxide Synthase
IVIS	*In vivo* Imaging System
LDF	Laser Doppler Flowmetry
LOC	Loss of Consciousness
MAP	Mean Arterial Pressure
MCA	Middle Cerebral Artery
MMP	Matrix Metalloproteinases
MS	Multiple Sclerosis
mTBI	Mild Traumatic Brain Injury
MWM	Morris Water Maze
NINDS	National Institute of Neurologic Disorders and Stroke
NO	Nitric Oxide
OEF	Operation Enduring Freedom
OIF	Operation Iraqi Freedom
$ONOO^-$	Peroxynitrite
PCS	Post Concussive Syndrome
PECAM	Platelet-Endothelial Cell Adhesion Molecules
PenMe	Penicillamine Methyl Ester

PPE	Personal Protective Equipment
PSS	Physiologic Saline Solution
PTA	Post Traumatic Amnesia
PTSD	Post Traumatic Stress Disorder
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SLC	Solute Carriers
SOD	Superoxide Dismutase
SSS	Superior Sagittal Sinus
TBI	Traumatic Brain Injury
TDC	Thesis and Dissertation Coordinator
TEER	Transendothelial Electrical Resistance
TJ	Tight Junction
UTMB	University of Texas Medical Branch
VA	Veterans Affairs
VDB	Vandenberg

THE EFFECTS OF BLAST-INDUCED NEUROTRAUMA ON BLOOD-BRAIN BARRIER PERMEABILITY, CELL-CELL JUNCTION PROTEIN LEVELS AND THE ROLE OF PEROXYNITRITE

Chapter 1: Introduction

Traumatic brain injury (TBI), as defined by the National Institute of Neurological Disease and Stroke (NINDS), is an acquired brain injury resulting from a sudden trauma to the brain (Vital, 2002). TBI is among the most prevalent causes for disability and death each year. In the United States, 1.7 million TBI cases occur each year with over 52,000 of those cases resulting in death (Faul, Xu et al., 2010). The main risk factors for TBI are age, gender and socioeconomic status with the rate of TBI being highest amongst young males and the elderly (Bruns and Hauser, 2003). Accounting for nearly 85% of the 1.7 million TBI cases annually, mild traumatic brain injury (mTBI) has a significant impact on public health (Levin and Robertson, 2013). Of all reported TBI cases, the most common causes were falls (28%), motor-vehicle accidents (19%) and assaults (11%) (DeCuypere and Klimo, 2012). Of those hospitalized and discharged for acute TBI, 43% will develop long-term disability (Rutland-Brown et al., 2006, Ma et al., 2014b).

Currently, mTBI is a focus of intensive research, due to the rise in sports-related concussions and a relatively new group of mTBI patients, the returning military personnel who have been subjected to blast exposure during deployment for Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF) (Stuhmiller,

2010). TBI is a chronic condition, beginning with a trauma event and evolving over time as a cascade of pathophysiological responses revealing temporally dependent symptoms and exacerbating other symptoms (Masel and DeWitt, 2010). TBI requires research in basic mechanisms in acute, subacute and chronic phases of injury, rehabilitation and palliative care, so the pathological sequelae are better understood, leading to improved interventions and enhanced functional recovery.

CLASSIFICATIONS OF TRAUMATIC BRAIN INJURY

TBI can be classified into groups based upon the mechanism of the injury, such as a closed-head or penetrating TBI. A closed-head injury occurs when a blunt-force impacts the head but does not penetrate the skull (Centers for Disease Control and Prevention, 2003). This impact may cause an acceleration and deceleration of the brain within the skull, resulting in brain-skull contact at two opposing points, often referred to as a coup-countercoup injury (Centers for Disease Control and Prevention, 2003). A penetrating TBI occurs when an object perforates the skull, entering brain tissue (Vital, 2002). In addition to mechanism of injury, TBI can also be classified based upon the presentation and severity of symptoms. The qualifiers mild, moderate or severe TBI indicate the presence of a particular set of symptoms at each distinct injury level. As mentioned previously, mTBI is the most common grade of injury and is often referred to as concussion (Centers for Disease Control and Prevention, 2003). Symptoms of mTBI may include a brief loss of consciousness, headache, nausea and vomiting, difficulty with motor coordination and balance, changes in sleep patterns and difficulty with memory, concentration or attention (Vital, 2002). Moderate and severe TBI encompass a vast range of symptoms, including many of those listed for mTBI, but also longer periods of unconsciousness, aphasia, dysarthria, and longer-lasting changes in cognitive function (Vital, 2002, Wang et al., 2005). While mTBI is

less severe than moderate or severe TBI, this level of injury can manifest with lasting effects such as posttraumatic stress disorder (PTSD) and/or depression, rendering many of these individuals disabled (Macgregor et al., 2010).

Behavior	Response	Score
Eye opening response	Spontaneously	4
	To speech	3
	To pain	2
	No response	1
Best verbal response	Oriented to time, place, person	5
	Confused	4
	Inappropriate words	3
	Incomprehensible sounds	2
	No response	1
Best motor response	Obeys commands	6
	Moves to localized pain	5
	Flexion withdrawal from pain	4
	Abnormal flexion	3
	Abnormal extension	2
	No response	1
Total score		**3-15**

Table 1: Glasgow Coma Scale. The Glasgow Coma Scale (GCS) is one of the assessment tools utilized in presentation of TBI. The GCS is a gross assessment of neurological function and is very useful in classifying a patient at any stage of intake or recovery (Teasdale and Jennett, 1976).

While the scenarios may seem similar, each TBI is unique due to myriad variables involved in obtaining the injury and the individual himself. This is a well-known, confounding factor that researchers and clinicians face in the study and treatment of TBI that can be further complicated by difficulty in communication between physicians, therapists and patients. For these reasons, protocols for the assessment and diagnosis of TBI have been established. The Glasgow Coma Scale (GCS) is one such method for the assessment and ranking of TBI patients (Table 1), providing reliable, standardized information to physicians regarding the severity of each individual injury. The GCS

focuses on consciousness immediately and subsequently following a TBI, examining and scoring the patient's abilities in eye opening, verbal response and motor response tasks. Eye opening is scored with a value of 1-4; a score of 1 is given to patients exhibiting no ability to open eyes, while a score of 4 indicates the ability to spontaneously open eyes. Verbal responses are scored on a scale of 1-5; 1 indicates no response and 5 indicates normal conversation. Motor responses are measured as both movement and postural abnormalities on a scale of 1-6; a score of 1 is given for lack of movement and 6 is given for normal movement. The scores from each area of assessment are summated and ranked according to injury severity (Table 1). Severe TBI patients have a GCS of 3-8, moderate TBI is a score of 9-12 and mild TBI is scored 13-15. Mild TBI may result in temporary or permanent neurological symptoms but may not show physical damage in CT scans or MRIs. Moderate and severe TBI often result in permanent cognitive and behavioral impairments (Teasdale and Jennett, 1974).

Aside from GCS, other methods for examination of head injuries may include loss of consciousness (LOC), alteration of consciousness (AOC) and posttraumatic amnesia (PTA). These three categories of criteria, along with GCS, comprise the standard protocol for acute TBI assessment by Veterans Affairs (VA) and the Department of Defense (DoD) which is shown in Table 2. Mild traumatic brain injury (mTBI) accounts for nearly 1.44 million reported TBI cases annually, making it the most common type of head injury (Levin and Robertson, 2013). While the term 'mild' indicates a less serious injury, as opposed to 'moderate' or 'severe', it may not accurately describe the long-term consequences of the injury. According to Veterans Affairs and the Department of Defense, a patient is classified as having mTBI if they have experienced a loss of consciousness (LOC) < 30 minutes, an alteration of consciousness (AOC) for ≤ 24 hours, posttraumatic amnesia for ≤ 24 hours and a Glasgow Coma Score (GCS) of 13-15. There are numerous classification systems (for review see DeWitt, et al., J Neurotrauma, 2013) and questionnaires for evaluation of

acute head injury however the methods utilized by the VA and DoD are especially relevant to the focus of the projects discussed in the present study.

	Glasgow Coma Score (GCS)	**Loss of Consciousness (LOC)**	**Alteration of Consciousness (AOC)**	**Posttraumatic Amnesia (PTA)**
Mild TBI	13-15	0-30 min	≤ 24 hours	≤ 24 hours
Moderate TBI	9-12	30 min – 24 hrs	> 24 hrs *	24 hrs > 1 week
Severe TBI	≤ 8	> 24 hrs	> 24 hrs *	> 1 week

Table 2: VA/DoD Classification of TBI Severity. Widely used methods of assessment for patients suspected of acute TBI examine potential loss of consciousness (LOC), alteration of consciousness (AOC) and posttraumatic amnesia (PTA), based upon presence and duration of symptoms. Table modified from U.S. Department of Veterans Affairs website (Defense, 2009).

MECHANISMS OF EXPLOSIVE BLASTS

An explosion occurs when incendiary materials are ignited, instantaneously converting the explosive materials into high-pressure and high-temperature gases (Atiyeh et al., 2007). As high-pressure gases rapidly expand they act as a spherical piston, driving the compression wave through the air. The propagating shockwave front increases in pressure, moving away from the fireball, in the direction of the wave movement. The pressure increase at the shockwave front occurs almost instantaneously with a microsecond rise time and is then followed by the expansion phase at 5 atm/msec (Elert, 1998-2015). This brief pulse of increased air pressure produces a positive pressure blast wave front, which is immediately followed by the suction of a negative pressure front (Owen-Smith, 1981). At velocities greater than the speed of sound, a propagating blast wave creates a front of high pressure that compresses

surrounding air – producing positive overpressure- and rapidly falling to produce negative underpressure. The type of explosives used and the distance an object is from the blast origin determines the blast wave duration effect on the object (Clemedson, 1956). Specifically, blast wave duration is defined as the amount of time an object or person is subjected to the effects of over- and underpressures associated with blast (Cernak and Noble-Haeusslein, 2010). Because the blast wave envelops the whole body and interacts with thoracoabdominal organ systems and brain simultaneously, it is capable of producing multiple injuries and eliciting local, systemic and cerebrovascular responses (Cernak, 2010). A single blast exposure may result in instant death, injuries with immediate manifestation of symptoms, or latent injuries that may manifest over an extended period after blast exposure (Ling et al., 2009). Blast exposure occurs in mere milliseconds but may produce injuries that will impact a person for a lifetime.

Much remains unclear about the mechanisms by which blast-induced neurotrauma (BINT) occurs, and both the acute and long-term effects of this type of TBI. Experimental BINT models have been designed to study various components of blast injury, however, comparison across models remains very difficult due to variation among models. Blast is more subtle than other forms of TBI, both in mechanism and clinical outcome, and occurs much more rapidly than most of the previously studied forms of TBI (Stuhmiller, 2010). The unique pathology of BINT is presumed to be, at least in part, due to blast wave exposure (Baalman et al., 2013, Cernak and Noble-Haeusslein, 2010, Cernak et al., 1999, Ling et al., 2009, Masel et al., 2012, Risling, 2010). Because explosions may cause a variety of injuries, blast injury effects have been categorized as: (1) primary blast injury, caused by the blast wave itself; (2) secondary injury, caused by the fragments propelled by the explosion; (3) tertiary injury, due to the acceleration and sudden deceleration, when the body or part of the body hits the ground or a solid object; and (4) quaternary injury, caused by burns or toxic gas exposure from the burning propellant (Owen-Smith, 1981, Mellor, 1988, Cernak,

2010). There is also much controversy regarding the concept of direct versus indirect transfer of energy from the blast wave (Cernak et al., 1999, Chavko et al., 2007, Livingston et al., 1945).

Table 3 indicates primary BINT is a non-impact brain injury, resulting from exposure to rapid but transient blast overpressure followed by underpressure of lower intensity but longer duration (Cernak et al., 2001c). Primary blast produces barotrauma of air- or fluid-filled organs and tissues (Luethcke et al., 2011b). Exposure to a blast wave passing through an individual's head may cause BINT, presumably by processes such as spallation (disruption that occurs when the compression wave is reflected at the boundary of a less dense medium), gas bubble implosion (the blast wave passes through liquid, compressing gas bubbles which then re-expand explosively)(Phillips, 1986) and inertial effects (lighter tissue is accelerated more than a denser tissue, resulting in stress at the boundary of the two densities)(Cooper et al., 1991);(Gorbunov, 2004). Therefore, the eyes, tympanic membrane of the ears, lungs, brain and spinal cord are vulnerable to these effects and are often damaged in blast exposure (Finkel, 2006a).

Debris or shrapnel that is propelled through the air by the explosion causes secondary blast injuries. Because IEDs are crafted to cause the most harm possible, they often contain small metal objects such as nails or ball bearings that act as projectiles, penetrating skin and tissue. Shrapnel and debris are propelled by the blast wind, which immediately follows the blast wave and result in secondary blast injuries.

Tertiary blast injury, caused by the force of the blast wind, may be of greater magnitude than naturally occurring winds. For example, a hurricane may have a peak overpressure of 1.75 kPa (0.25 psi) and velocity of 200 km/h, capable of picking-up and moving objects in its path (Stuhmiller et al., 1991). A lethal blast, on the other hand, may contain a peak overpressure of 690 kPa (100 psi) and velocity of about 2,414 km/h (Cernak and Noble-Haeusslein, 2010, Owen-Smith, 1981). Soldiers experiencing explosions may be thrown into other objects or structures, resulting in the acceleration

and deceleration of the head similar to what is seen in many civilian TBIs (automobile accidents, falls, etc.) and is considered to be the tertiary mechanism of blast injury (Cernak et al., 1999).

Quaternary blast injuries consist of flash burns or damage caused by the inhalation of gases. The subtlety of primary and tertiary blast injuries makes them much more difficult to examine and diagnose. With no visible signs of injury, a person suffering from primary and/or tertiary blast injury may not be aware of the origin of their symptoms or may not believe they are injured without physical evidence. Understanding blast injuries and how to prevent and treat them is increasingly complicated due to the infrequency of individuals reporting their injuries.

However, given the increased incidence in chronic loss of executive function from survivors of the Middle East conflict, greater attention is being given to long-term consequences of blast injury. Thus, model development to determine mechanisms in the acute, subacute and chronic phase after blast injury are critical for developing rational therapeutic interventions.

Category	Definition	Mechanism	Associated
Primary	The effect of the blast wave enveloping the body; damage caused solely by blast wave	Shear stress, spallation, cavitation, air emboli	Blast lung; tympanic membrane rupture; laceration to kidneys, liver, spleen; cardiac contusions; brain injury without physical indication.
Secondary	Injury caused by debris or shrapnel from explosion impacting the body	Dependent upon projectile mass and velocity	Penetrating head injury; embedded shrapnel, debris or ballistics; any injury associated with contact of a high-speed object.
Tertiary	Injury by force of blast wind; body is thrown into object(s) or structures.	Blunt impact-like injury	Non-penetrating head injury; concussion; musculoskeletal injury; bruising; swelling.
Quaternary	Burns; injury sustained by inhalation of toxic gases released during explosion.	Burning of body surface or respiratory tract	Asphyxia; various degrees of burned skin; damage to mucosal linings of respiratory tract.

Table 3: Blast Injury Taxonomy. Blast-induced neurotrauma occurs via multiple injury mechanisms. These four types of blast mechanism are commonly used to more accurately describe the type of injuries sustained from blast exposure (Cernak et al., 1999).

PATHOLOGY OF BLAST-INDUCED NEUROTRAUMA

Explosive blasts have been a phenomenon observed and studied throughout time; however, thorough investigations into understanding the nature and mechanisms of blasts did not arise until World War II and the unveiling of nuclear bombs (Masel et al., 2012).

In 1945, Livingston and colleagues proposed the idea that the kinetic energy transfer of explosion-generated pressure waves could result in damage to the central nervous system (Livingston et al., 1945). The concept of damage from exposure to the pressure waves alone was novel and provided a basis for future investigation of blast effects, specifically on the CNS. Prior to modern warfare, injuries sustained by military personnel had much higher rates of mortality, reportedly approaching 90% in cases of TBI (Masel et al., 2012). Advancements in personal protective equipment (PPE) and body armor have decreased the risk of penetrating injuries and primary blast injuries, and dramatically increased the rate of survival after blast exposure (Okie, 2005, Hicks et al., 2010, Faul et al., Faul et al., 2010b). However, the increase in survival rates also corresponds to an increase in reports of severe injuries and long-term complications (Cernak and Noble-Haeusslein, 2010). It is estimated that approximately 20% of wounded military personnel have sustained a head injury (Carey, McCrea et al., 2008).

In addition to increased survival due to PPE, the increased number of TBI cases can be attributed to the use of improvised explosive devices (IEDs). The use of IEDs as a common tactic in modern military and civilian terrorism began in the early 1980's (Stuhmiller et al., 1991). Used as a weapon, IEDs are designed to generate maximum physiological injuries as well as incite the psychological stresses of terror and panic among the targeted group (Finkel, 2006b). The Department of Defense reports that greater than 73% of military casualties during recent Operation Enduring Freedom/Operation Iraqi Freedom (OEF/OIF) were caused by explosions (Defense Manpower Data Center, 2008) with the majority of combat-related TBIs due specifically to IED explosions (Galarneau et al., 2008). The pervasive use of IEDs during OEF/OIF has resulted in an estimated 15% to 23% of military personnel experiencing TBI (Bogdanova and Verfaellie, 2012).

The exact number of cases is difficult to define due to the high incidence of unreported or untreated injuries (McCrea et al., 2008), however estimates indicate that

the majority of sustained TBIs were classified as mild (Brenner et al., 2009, Warden and al, Bogdanova and Verfaellie, Warden, 2006). TBI is known as the signature injury of modern warfare with blast-induced TBI becoming recognized as a disease distinct from penetrating TBI and other forms of closed-head TBI (Elder and Cristian, 2009, Ling et al., 2009). Many soldiers who have sustained a mild injury from blast exposure may exhibit problems with daily functions, including but not limited to irritability, memory deficits, sleep disturbances, increased anger and aggression and exhibit increased potential for developing additional conditions later in life (DePalma et al., 2005).

Blast-induced damage to the CNS has been studied in various models over the past few years, providing critical information about the underlying mechanisms of blast injury and its physiological and psychological repercussions. Commonly reported effects of blast exposure include inflammation, infiltration of immune cells, microglia activation, oxidative and nitrosative damage, ultrastructural changes, diffuse axonal injury and cognitive and neurological deficits (Ling and Ecklund, 2011, Nakagawa et al., 2011, Perez-Polo et al., 2015). With focus on the many aspects of BINT pathophysiology, the characterization of blast injury is gradually developing and is now recognized as a distinct form of mTBI.

HISTOPATHOLOGY AND IMMUNOLOGY

In impact/concussive TBI, the initial impact (primary injury) is followed by inflammation, excitatory neurotransmitter release, Ca^{++} influx, reactive oxygen/nitrogen species formation (secondary injury) in which multiple cell damage pathways are activated, affecting both the injury site and the surrounding tissue (a.k.a the penumbra) (Hernandez-Ontiveros et al., 2013, Bramlett and Dietrich, 2004, Adams and Graham, 1994, Maas et al., 2008). Early immune-inflammatory responses include local and systemic complement activation, edema, inflammatory cell infiltration and pro-

inflammatory cytokine production (Hernandez-Ontiveros et al., 2013). The initial immune response following TBI is also associated with a loss of BBB integrity and neuronal injury (Smith et al., 1997, Nagamoto-Combs et al., 2007, Namas et al., 2009, Hernandez-Ontiveros et al., 2013). Accumulation and activation of microglia is another hallmark of pathological conditions of the CNS, which occurs prior to damage of neuronal and glial cells in CNS trauma. This response results in the activation of microglia, prompting them to act as antigen-presenting cells (APCs), expressing pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), chemokines, neurotrophic factors (BDNF, TGF- β, NGF), nitric oxide (NO) and superoxide ($O_2^{\bullet}$) (Giunta et al., 2012, Hernandez-Ontiveros et al., 2013, Rieske et al., 1989, McGeer et al., 1993, Giulian et al., 1995). This process creates a dynamic environment with the potential for increased secondary cell death of surrounding cells.

Rat models of experimental BINT show evidence of an active immune response in cerebral and cerebellar cortices in the first few weeks following injury. Mild/moderate levels of blast exposure (120 kPa) generate significant systemic complement release 3 to 48 hrs post-BINT, with increases in C3 (complement activation protein) and C5b-9 (complement membrane attack complex protein), which are associated with cortical vasculature (Lucca et al., 2012), and activation of microglia and increased MHC I and II expression within 14 days post-BINT (Kaur et al., 1995). Hippocampal accumulations of the same markers have been associated with neurons, suggesting multiple sources of complement proteins and results in varied distribution patterns (Lucca et al., 2012). In other areas of the CNS, microglia are readily activated via upregulation of their surface CR3 receptors and MHC antigens following chemical lesions and injuries, resulting in neurodegeneration (Graeber et al., 1988, Akiyama and McGeer, 1989, Kaur and Ling, 1992). Reactive glia and myelin debris are found in the corpus callosum and hippocampus at 2 hrs (McCabe et al., 2014), 24 hrs (McCabe et al., 2014, Cernak et al., 2001c) and 5 days post-injury (Cernak et al., 2001c). Microglia activation is also found

in the substantia nigra following BINT (Readnower et al., 2010a), that may be correlated to the high density of NMDA receptors in these regions, as they have been shown to be more vulnerable to blast injury and associated excitotoxic insults (Butler et al., 2009, Readnower et al., 2010a).

The early BBB breakdown in the outer layer of the cortex (Readnower et al., 2010b) may contribute to the complement proteins' ability to deposit in the cortex, indicating systemic complement activation. However, neural cells produce complement proteins (Wang et al., 2010, Arumugam et al., 2009, Ingersoll et al., 2010), resulting in the accumulation of these proteins after injury. Increases in leukocyte infiltration, complement activation and accumulation in BINT tissues is consistent with non-blast TBI models (Keeling et al., 2000). Additionally, increases in plasma TNF-α can be directly correlated to injury severity (Lucca et al., 2012), which has also been reported in human BINT patients (Surbatovic et al., 2007). While activation of the complement system was examined in non-blast, closed-head TBI models (Stahel et al., 2001, Bellander et al., 1996, Bellander et al., 2001b, Bellander et al., 2004, Wood et al., 1993, Anderson et al., 2004), Dalle Lucca et al., were the first to characterize these responses following mild/moderate BINT in rodents (Lucca et al., 2012).

Many ultrastructural changes are also observed following BINT. Ultrastructural changes are detected in perivascular microglia (Kaur et al., 1995) and macroglia (Kaur et al., 1997) at 24 hrs and 7 days post-injury, and in the hippocampus at 24 hrs and 5 days post-injury (Cernak et al., 2001c). Astrocytes appear hypertrophied and display swollen end-feet as early as 24 hrs post-BINT (Cernak et al., 2001c), at 7 days (Kaur et al., 1997), and as late as 14 days post-injury (Kaur et al., 1995). Apical cytoplasm leakage peaks 7 days post-injury, allowing the migration of monocytes and lymphocytes across the epithelium (Kaur et al., 1996). The migration of immune cells across the BBB supports the hypothesis that BINT compromises the BBB, allowing serum-derived substances entry to the parenchyma, potentially causing astrocytic hypertrophy. Blast-induced

activation and recruitment of macrophages/microglia may also be triggered by a local release of IFN-γ at the site of injury (Kaur et al., 1997). At 24 hrs, BBB disruption is indicated by the presence of activated microglia and reactive astrocytes in parietal and temporal regions of the cerebral cortex, corpus callosum and hippocampus (McCabe et al., 2014). Treatment with aminoguanidine (AG) attenuates neurodegeneration when administered either before or after BINT (Moochhala et al., 2004). Aminoguanidine (AG) is a nitric oxide synthase (NOS) inhibitor that reduces advanced glycation end products (AGEs) via interaction with 3DG. 3DG is known to induce ROS and oxidative DNA damage and can be responsible for producing intracellular oxidative stress. Both AGEs and 3DG are associated with the modification and cross-linking of proteins such as collagen, contributing to vascular complications associated with hypertension and inflammation. Aminoguanidine has been shown to reduce AGE associated neural pathologies in animal studies (Cash D, Iadecola C, 1995, Lu et al., 2003, Stoffel M, 2000, Wada K, 1998, Zhang FY, 1996) and its effects after blast injury provide insight that NOS may play a detrimental role in BINT pathology (Moochhala et al., 2004).

EFFECTS OF PRESSURE WAVES

The standards set for performance decrement as a result of blast injury previously depended upon criteria for damage to pulmonary and gastrointestinal tracts, as well as the threshold for tympanic membrane rupture (Mayorga, 1997, Elsayed and Gorbunov, 2007). Studies using porcine models of blast injury have shown that exposure to 45 kPa overpressure is sufficient to produce brain hemorrhage and edema (Saljo et al., 2008). Because the threshold for BINT in humans is unclear, many have investigated the effects of various peak overpressures to compare and contrast the effects on the brain, attempting to determine a standard maximal peak overpressure at which human brain injury occurs. To determine the effects of low to moderate levels of blast, animals were exposed to

maximum peak overpressures of 10, 30 and 60 kPa in a closed-end shock tube and their intracranial pressure and cognitive function tested post-injury (Saljo et al., 2009). A dose-dependent rise in ICP and an increasing time delay in elevation with decreasing levels of exposure were reported, which is consistent with previous reports (Engelborghs et al., 1998). At 60 kPa the initial rise in ICP occurred within 30 minutes of blast exposure while it did not appear until 2 hrs after the 30 kPa blast and 6 hrs after the 10 kPa blast, which correlates to the time course of glial activation post-BINT (Saljo et al., 2001). All animals experienced a return to ICP comparable to that of control animals after 7 days. Cognitive impairment was detected 2 days post-injury for animals exposed to 10 and 30 kPa blasts with a 100% increase in latency compared to pre-injury latencies. This study supported previous evidence (Moochhala et al., 2004) that even low-level blast exposures can produce increased ICP and cognitive impairments, suggesting that the brain is highly sensitive to injury as a result of blast exposure. However, the pressure level within the skull must be assessed.

Implanting miniature fiber optic pressure transducers within the skull prior to blast exposure allows for the direct measurement of blast waves traversing the brain (Chavko et al., 2007). A micro-optical mechanical system (MOMS) is a full order of magnitude smaller than the piezoelectric sensors commonly used to measure pressure waves and is verified to withstand harsh conditions associated with explosions (Pinet et al., 2005). Analysis of 40 kPa blast-injured brain tissues reveals no gross pathology is present. Recordings indicate a slight dampening of the pressure waves, presenting lower peak overpressures, but exhibit longer durations of the positive pressure phase. Even with a low-level blast injury, there are significant performance deficits and cortical neurodegeneration after injury, as is also reported by Moochhala (Moochhala et al., 2004), suggesting that perhaps the threshold for neuronal damage by blast injury is much lower than that of pulmonary damage. Thus, indirect, peripheral blast trauma is capable

of initiating functional, biochemical and morphological changes in the brain (Cernak et al., 1996, Kaur et al., 1997).

The vulnerability of CNS tissue has been shown by reports of long-term EEG changes and persistent neurological deficits in humans following blast exposure (Cernak et al., 1999). Direct pressure measurement within the brains of blast-exposed animals indicates significant increases in ICP and that the size and age of the rats directly affects the change in ICP, with older and heavier rats experiencing the highest ICP, possibly due to variations in skull flexure (Leonardi et al., 2011).

Understanding the underlying mechanisms associated with BINT requires validated and reproducible experimental models to simulate the key events responsible for human BINT. A controversial question in model development is whether whole body versus head-only exposure to a blast wave is the better approach. Blast overpressures ranging from 120 to 250 kPa result in a significant decrease in body weight, cell immunoreactive to IgG that appear to be neurons in the cerebral cortex and hippocampus, evidence of "blast lung" as moderate pulmonary hemorrhage with edema and vascular damage (Skotak, Wang et al. 2013). Even at low peak blast overpressures, animals receiving either a whole-body exposure or head-only exposure display cortical neurodegeneration (Moochhala et al., 2004) and evidence of vacuole formation in the nerve terminals of the hippocampus and myelin at 24 hrs and 5 days post-injury (Cernak et al., 2001a). The medial mesodiencephalic reticular formation and the dorsal hippocampus also show neuronal swelling, reactive glia, myelin debris and increases in pinocytotic activity on day 5 post-BINT, with a significant increase in nitric oxide production at 3 hrs and 24 hrs post-injury, which is consistent with the previously observed changes in expression of iNOS mRNA (Cernak et al., 2001a). Additional studies to examine blast effects on BBB and vessel dysfunction at multiple levels of overpressure reveal lesions throughout both hemispheres, despite being a direct lateral injury (Yeoh et al., 2013). This detail makes blast-related TBI distinct from most

conventional TBI models and is a critical component in accurately simulating the global nature of BINT.

Another component of blast model development criteria is the type of injury generated by the device itself. For example, the literature on blast injury focuses on the use of shock or blast tubes (Cernak et al., 2011, Cernak et al., 2001a, Cernak et al., 2001b, Desmoulin and Dionne, 2009, Readnower et al., 2010a, Risling et al., 2011, Goldstein et al., 2012, Moochhala et al., 2004, Chavko et al., 2007, Lucca et al., 2012). The blast tubes are known to simulate a primary blast injury, replicating a pressure waveform similar to that of the Friedlander wave, with the signature instantaneous peak in positive pressure (overpressure) followed by a phase of negative pressure (underpressure)(Friedlander, 1946). While primary blast injury exposure defines BINT, it is not the only factor to consider and model from blast injuries sustained by humans.

Exposure to open-field explosions, like those generated by IEDs in warfare, encompasses primary, secondary, tertiary and quaternary blast injury mechanisms (Cernak et al., 1999). Composite or compound blast injury models generate two or more of these mechanisms. For example, Svetlov et al., use a blast device that produces primary blast injury via the blast wave, as well as tertiary blast injury through a gas venting jet that streams from the device and produces a blunt impact-like injury (Svetlov et al., 2010). Silver staining in deep brain regions can be seen following composite blast injury (Svetlov et al., 2010). In contrast to the studies performed by Cernak et al., head-only blast exposure results in different pathophysiological responses than do whole body exposure (Cernak et al., 2001b, Svetlov et al., 2012). It has also been shown minimal head/cervical acceleration occurs in the "off-axis" position when compared to the "on-axis" position in which the animal is directly in line of the venting gas and peak overpressure generated by the shock tube (Svetlov et al., 2012). In a comparison of primary blast versus composite blast injury, composite blast produces significant changes in neuro-glial markers after injury while primary blast induces systemic vascular

reactions (Svetlov et al., 2012). To date, Svetlov and associates are the only other researchers to investigate composite blast injury in this manner, making the data generated in the present study a valuable contribution to the field. Because modern warfare includes personal protective equipment, it is critical to incorporate these measures into model development and the characterization of blast injury models, keeping in mind that protection from lethal blasts is highly dependent upon the types of materials used to make the protective equipment and may actually increase the severity of brain injury sustained (Cernak and Noble-Haeusslein, 2010).

A unique component of shock wave-induced brain injury is the response of the brain and skull to shock waves. High- and low-pressure micro-explosions within a brain cavity reveal that the direct effects of blast exposure are dark and shrunken cortical, hippocampal and cerebellar neurons with distorted dendrites, with elevated NeuN (neuronal biomarker) reactivity (Nakagawa et al., 2008). This set of experiments provides insight into the effects of blast waves on tissues of varied densities, interaction with the skull and the occurrence of cavitation. Cavitation is the transient formation of gas bubbles in fluid at low-pressure, which then collapse when subjected to high-pressure, causing a shock wave and high-speed jet that can damage surrounding surfaces (Benzinger, 1950, Chiffelle, 1966, Phillips, 1986, Cernak, 2009, (Goeller et al., 2012). It is proposed - and computational models have suggested - that the cerebrospinal fluid surrounding the brain may exhibit cavitation and subsequent tissue damage in response to blast exposure (Chafi et al., 2010, Moore et al., 2009, Moss et al., 2009, Nyein et al., 2010, Nakagawa et al., 2011). The collapse of cavitation bubbles generates considerable force, thereby presenting potential for significant damage following BINT (Wu and Nyborg, 2008).

Shear stress is another established mechanism of cellular injury resulting from blast exposure. Shear stress is caused by the interface of high frequency, low-amplitude stress waves and low frequency, high-amplitude shear waves creating motion between

two tissues that extends beyond normal elasticity(Cooper et al., 1991, Gorbunov, 2004). TBI-induced shear stress results in diffuse axonal injury (DAI), presenting as lesions of white matter and at white and grey matter interfaces. DAI is observed within 2 weeks of BINT, with substantive global fiber tract degeneration detected via silver staining and immunohistochemistry (Saljo et al., 2000, Long et al., 2009, Bauman et al., 2009). Contrary to previous studies reporting that rotation/acceleration injury induces axonal changes detectable by immunolabeling with anti-APP antibody (Davidsson et al., 2009), Risling and associates reported no evidence of such injury after blast exposure, even in the presence of motor deficits (Davidsson et al., 2009). The use of diffusion tensor imaging (DTI) allows examination of axonal tracts, outlining distinct differences in the brains of those subjected to blast exposure versus civilian TBI (MacDonald et al., 2014, Risling, 2010). DTI is a more sensitive technique for detecting diffuse axonal injury (DAI) following blast exposure compared to conventional MRI where little to no injury is visible (MacDonald et al., 2014, Ropper, 2011).

In our model, we chose to focus on a head-only, single blast exposure injury. Head-only exposure allowed us to observe the effects of blast exposure on the brain without input from pulmonary and cardiovascular responses to torso loading. We designed the experiments in the present study to focus on key mechanisms of BINT pathophysiology, 1) blood-brain barrier, 2) inter-endothelial cell junctions, 3) production and effect of peroxynitrite on the BBB, and 4) motor and cognitive function, that we hypothesize to be involved in worsened outcomes.

CEREBRAL VASCULATURE

Blood-Brain Barrier

The studies that lead to the discovery of a physiological barrier between circulating blood and the central nervous system (CNS) were performed in the late

1800's by Paul Ehrlich, where he observed that acidic dyes injected into blood vessels stained all of the organs except the brain (Ehrlich, 1904). His student, Edwin Goldmann, performed similar studies by injecting acid-azo dyes into the blood vessels, confirming Ehrlich's results, then showed that the injection of basic dyes readily stained CNS tissue but did not stain other organs (Goldmann, 1913). Goldmann went on to describe the hematoencephalic barrier as a component of the CNS that prohibits the passage of acidic compounds from entering tissue of the CNS. Additional studies revealed that some brain regions did not possess this ability, such as the choroid plexus, pineal body and areas where lesions had previously occurred (Wislocki and Leduc, 1952, Wislocki and King, 1936, King, 1938).

The anatomical structure described by Reese, Karnovsky and Brightman is frequently used to illustrate the basic organization of the blood-brain barrier (BBB) and its components (Hawkins and Davis, 2005, Reese and Karnovsky, 1967). Their research proposed that the BBB functions as a selective diffusion barrier composed of cerebral vascular endothelial cells bound together by tight junctions with astrocytic endfeet providing structural support to endothelial cells (Reese and Karnovsky, 1967). The development of electron microscopy provided the first visible evidence of tight junctions restricting paracellular movement. Horseradish peroxidase (HRP, a 44 kDa glycoprotein) and ionic lanthanum (La^{3+}, 4.6 Å hydrated radius) were intravenously injected into naïve mice, revealing a dark-colored reaction product located exclusively within the paracellular cleft of the endothelial cells (Brightman and Reese, 1969). As a result of these observations, it was concluded that tight junctions (TJs) between endothelial cells were responsible for preventing open passage between ventricular and vascular spaces (Brightman and Reese, 1969, Karnovsky, 1968, Bouldin and Krigman, 1975). Brain endothelial cells have a high mitochondrial concentration, low levels of pinocytic activity and tight junctions but lack fenestrations (Fenstermacher et al., 1988). In addition to the inter-endothelial cell junctions, the structural components involved in the BBB are also

important in maintaining its function. The astrocytes, pericytes and glia that surround the endothelial cells act as scaffolding to support the vascular cells. The functional relationship between brain endothelial cells and these supporting cells has contributed to the concept of a vascular unit (Chodobski et al., 2011, Abbott et al., 2006).

Inter-endothelial Cell Junctions

The physical barrier property of the BBB relies heavily on the junctions to limit paracellular transport. TJs located at the intercellular cleft of the endothelium are composed of multiple intramembranous proteins that span the cleft near the apical membrane (Abbott et al., 2010). Adherens junctions (AJs) are similar to TJs in that they are formed by intramembranous proteins and span the intercellular cleft, however, they are located closer to the basal lamina and are integral in adhesion of endothelial cells (Hawkins and Davis, 2005). Both TJ and AJ are composed of protein complexes in the intercellular cleft and within the adjacent cells. The measurement of transendothelial electrical resistance (TEER) in cerebral blood vessels is a useful method for measuring cellular and paracellular ion transport (Olesen, 1989, Olesen, 1986, Crone and Olesen, 1982, Butt, 1988, Butt et al., 1990). The tight junctions of a normal BBB allow passage of molecules <4.1 Å and have a TEER >1000 Ωcm^2, while a typical peripheral capillary has a TEER of 2-20 Ωcm^2 (Abbott et al., 2006, Butt et al., 1990). In pathologies such as pain, brain tumors, HIV, multiple sclerosis and trauma-related inflammation, increased permeability and breakdown of the BBB is attributed to dysfunction of junction proteins (Abbott et al., 2010, Huber et al., 2001, Willis and Davis, 2008, Minagar and Alexander, 2003, Dallasta et al., 1999) therefore, it is important to examine these proteins. Two major proteins involved in cell-cell adhesion are cadherin and catenins.

The isoform of cadherin that localizes to the BBB AJs, Vascular endothelial cadherin (VE-cadherin), is a classical type II cadherin of the cadherin superfamily and is

sometimes referred to as cadherin 5 (CDH5) (Cardoso et al., 2010). VE-cadherin is a 130kDa Ca^{2+} -dependent adhesion protein with five extracellular Ca^{2+} binding domains, a transmembrane domain and a cytoplasmic tail domain. In early studies, VE-cadherin was identified as an endothelial cadherin that localized at intercellular junctions. *In vitro and in vivo* studies determined that this protein possessed specific adhesive qualities, operated in a Ca^{2+} -dependent manner and decreased intercellular permeability to large molecules (Breviario et al., 1995, Vestweber, 2008). VE-cadherin binds to partner proteins, p120, β-catenin and plakoglobin, via its cytoplasmic tail (Stamatovic et al., 2008, Dejana et al., 2008). β-catenin and plakoglobin bind to α-catenin, which may then bind to the actin cytoskeleton via ZO-1 or vinculin (Weis and Nelson, 2006, Dejana et al., 2008). Binding to the cytoskeleton provides a connection, through which forces generated within the cell can be transmitted to the periphery and induce movement (Hawkins and Davis, 2005). AJs contain both VE-cadherin and platelet-endothelial cell adhesion molecules (PECAM). In addition to AJs, TJs provide a significant barrier to paracellular transport (Butt et al., 1990, Crone and Olesen, 1982, Abbott et al., 2010, Hawkins and Davis, 2005). TJs are very similar to AJs in that they are also composed of several proteins working together to span the intercellular cleft and maintain the physical barrier properties of the BBB (Abbott et al., 2010, Hawkins et al., 2013).

Occludin and claudin make up the protein strands of TJs, each forming two extracellular loops that interact with homologous structures on adjacent cells (Forster, 2008). Occludin is a 60kDa protein with four transmembrane domains and both the N and C-termini located within the cytoplasm (Forster, 2008). Claudin isoforms found in endothelial TJs are claudins 1, 3, 5 and 12. Claudins are smaller proteins, 20-27kDa, but form similar structures to those of occludin, two extracellular loops with four transmembrane domains and both termini in the cytoplasm (Forster, 2008). Claudins are also considered to be the main contributor to the high TEER of TJs, while occludin is more often associated with supporting and maintaining the integrity of the TJ (Wolburg

and Lippoldt, 2002, Hawkins and Davis, 2005). Occludin-knockout and knockdown experiments have indicated that occludin is not essential to TJ formation, however, decreased expression or truncated C-terminal occludin does result in increased paracellular permeability (Balda et al., 1996, Bolton et al., 1998, Huber et al., 2002, Brown and Davis, 2005). The extracellular loops of claudin contain charged primary amino acid sequences, enabling claudin to form ion selective pores in the TJ, although ion selectivity is dependent upon which claudin isoform is present (Hawkins and Davis, 2005). This isoform-dependent ion selectivity is the mechanism by which claudin is able to modulate the TEER of TJs (Colegio et al., 2003, Forster, 2008). Associated with occludin and claudin are accessory proteins responsible for connecting the TJ protein strands to the actin cytoskeleton (Hawkins and Davis, 2005). These accessory proteins include the zonula occludens family, ranging from 130-225 kDa. Isoforms of zonula occludens, ZO-1, ZO-2 and ZO-3, are localized in endothelial cells of the BBB and perform various functions necessary to maintain TJ integrity, such as providing stability, transmitting intracellular signaling and facilitating the binding of occludin and claudin to the actin cytoskeleton (Abbott et al., Liu et al., 2008). ZO-1 is predominantly located at the TJ while ZO-2 and ZO-3 are more diffusely distributed within endothelial cells (Islas et al., 2002, Traweger et al., 2002). Subtle changes intracellularly or extracellularly can initiate signaling cascades, which may then contribute to migration of cytoskeleton-associated proteins and decreased TEER.

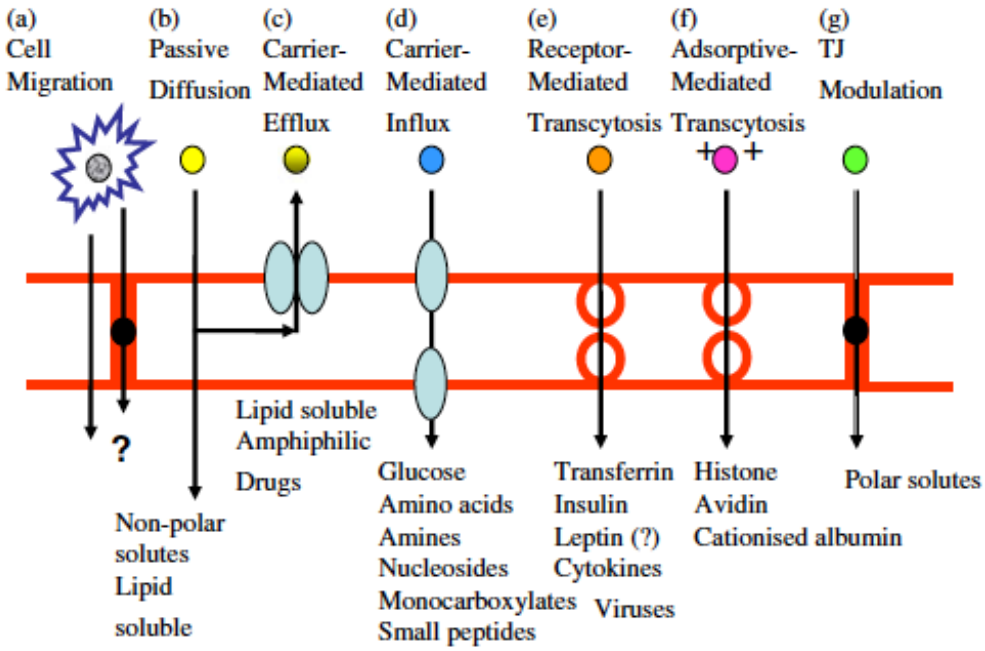


Figure 1. Blood-brain barrier transport mechanisms. The blood-brain barrier possesses multiple dynamic methods of transport and diffusion to maintain brain homeostasis. Diagram modified from (Abbott, 2013).

Transport Mechanisms

Although the BBB serves to separate CSF and circulating blood, it is also a selective transport system (Stamatovic et al., 2008, Abbott et al., 2010). A variety of ions, molecules and proteins necessary to regulate this environment require specialized transport pathways (Abbott et al., 2010, Forster, 2008). Biologically relevant molecules require tight regulation, such as the opening or closing of ion channels, to maintain optimal synaptic function (Kandel et al., 2000). There are several types of transport that take place at the BBB, each type providing a transport mechanism that is specific to the properties of the molecules being transported (Abbott et al., 2010). Passive diffusion is the process for transporting lipid soluble, non-polar molecules that generally have a low affinity for binding to plasma proteins and require little energy to move across the membrane, such as water, blood gases and ions (Pardridge, 1998). The transport of

glucose, heparin, select amino acids and other polar molecules occurs via solute carriers (SLCs) (Tsuji and Tamai, 1999). Similarly, ATP-binding cassette (ABC) transporters function to transport non-polar, lipid-soluble compounds across the endothelium (Loscher and Potschka, 2005). Transcytosis provides transport of macromolecules across the BBB via two mechanisms, receptor mediated or adsorptive mediated transcytosis (Pardridge, 1988). Lastly, the movement of whole cells across the BBB, diapedesis, allows the passage of cells (often macrophages) through the capillary wall, rather than paracellular transport, eliminating the need for tight junction rearrangement. This transport pathway is generally associated with infection and inflammation (Abbott et al., 2010). Evidence suggests that the increased permeability of the BBB leads to further damage to brain tissue by the infiltration of leukocytes, microglia activation and inflammation (Morganti-Kossmann et al., 2007).

Effects of Blast Exposure on Cerebral Vasculature

Impairment of brain microvasculature and increases in BBB permeability are frequently associated with both clinical and experimental TBI. A compromised BBB following TBI has been associated with neuronal cell loss, alterations in consciousness, memory and motor function (Chodobski et al., 2011). Trauma may also result in the disruption of tight junction proteins (Schreibelt et al., 2007), oxidative stress (Hall et al., 2010, Haorah et al.), infiltration of immune cells (DiStasi and Ley, 2009, Szmydynger-Chodobska et al., 2010, Semple et al., 2010), and the influx of proinflammatory mediators and inflammatory cells. It has been reported in many studies that an increase in BBB permeability is a frequent consequence of TBI (Habgood et al., 2007, Povlishock et al., 1978, Baskaya et al., 1997, Shapira et al., 1993, Alves, 2014, Chodobski et al., 2011, Skotak et al., 2013, Chen and Huang, 2011). With a leaky BBB, there is an increased possibility of developing edema, potentially causing an increase in intracranial

pressure (ICP). Edema brought on by dysfunction of the BBB is likely vasogenic in nature, caused by an extracellular accumulation of fluid rather than intracellular accumulation associated with cytotoxic/cellular edema. Edema may result in increased ICP, possibly impairing cerebral perfusion and aiding in the development of secondary ischemic injuries (Unterberg et al., 2004). Cerebral perfusion may also be reduced due to the formation of intravascular coagulation (IC), even in mild TBI (Schwarzmaier et al., 2010, Schroder et al., 1998, von Oettingen et al., 2002, Stein et al., 2002). A thorough review of the effects of BINT on the BBB has not been described yet, likely due to the diversity of blast injury models. Here we will discuss the literature to date that investigates the effects of BINT on the BBB and neurovascular unit in rodent models.

Readnower et al studied the effects of exposure to blast overpressure using an air-driven shock tube, focusing on the progression of neuropathological processes after injury. Animals subjected to blast exposure at 120 kPa, a level of blast shown to produce moderate pulmonary damage and acute inflammatory response for one week post-injury (Chavko et al., 2007), demonstrate significant reflex suppression (Readnower et al., 2010a). Neurological and neuropathological responses to blast exposure are measured using acute reflex suppression, as it has been used to validate injury severity in other diffuse TBI models (Denny-Brown and Russel, 1941, Dixon et al., 1987, Fijalkowski et al., 2007). Animals subjected to either sham or blast injury and sacrificed at 30 mins, 3 hrs, 24 hrs and 72 hrs revealed a significant increase in IgG immunoreactivity at 3 and 24 hrs post-BINT ($p < 0.05$), indicating BBB disruption, as well as increases in staining for 4-HNE and 3-NT, and activated microglia in both the hippocampus and substantia nigra (Readnower et al., 2010a). It is suggested that oxidative stress and microglial activation may play a role in the neuropathology of BINT (Readnower et al., 2010a). Previous reports from non-blast TBI models indicate BBB dysfunction can occur as soon as minutes after injury and is usually resolved after a few days (Tanno et al., 1992, Enters et al., 1992, Kelley et al., 2007). Readnower et al., observed blast-induced transient BBB

breakdown, resulting in increased permeability to IgG that is significantly elevated at 3 and 24 hrs post-injury but resolved by 72 hrs, a pattern similar to that seen by Whalen et al., (Whalen et al., 1999). Increased binding of PK11195 (microglial activation) occurs in the dentate gyrus, substantia nigra and ventral hippocampus at 5 and 10 days after blast injury in both the ipsilateral and contralateral hemispheres (Readnower et al., 2010a). This pattern of distribution is consistent with that observed after traumatic cerebral vasospasm (TCV) which may have been caused by blast wave propagation through brain tissue (Armonda et al., 2006). TCV is a condition in which a blood vessel spasms and is followed by vasoconstriction, frequently leading to cerebral ischemia and tissue death. Vasospasm is more commonly seen in moderate and severe TBI patients, occurring in the first 48 hrs or may be delayed until 10 – 14 days post-injury. This phenomenon has been observed in mild intensity blast exposure (172.67 kPa) of pigs in a shock tube (Bauman et al., 2009).

Abdul-Muneer et al., was the first in the field to describe the effects of single and repeated blast exposure on the initiation of cerebral vascular injury by oxidative stress and associated neuroinflammation (Abdul-Muneer et al., 2013). Animals exposed either to a single shock-tube generated blast wave or two exposures to the blast wave, both at 123 kPa, display significantly increased oxidative stress marker 4-HNE levels from 1 hr to 24 hrs while levels of nitrosative 3-NT are significantly increased as early as 1 hr and maintain elevation through day 8 (Abdul-Muneer et al., 2013). While the full profile varies, both 4-HNE and 3-NT peaked at 6 – 24 hrs post-BINT. In an effort to correlate the oxidative damage observed to the components of the BBB, immunofluorescent staining and Western blotting can be used to examine changes in tight junction (TJ) proteins, such as occludin, claudin-5 and ZO-1. In a similar time frame to the oxidative/nitrosative stress profile, there is a decrease in expression of TJ proteins in cerebral microvessels. Also concurrent with these changes is decreased expression of PDGFR-β (a pericyte-specific marker), indicating a breakdown of perivascular units of

the BBB basement membrane, potentially contributing to the loss of BBB integrity and neuroinflammatory response observed after BINT (Abdul-Muneer et al., 2013). It is suggested that the biochemical activation of NADPH oxidase 1 and inducible nitric oxide synthase (iNOS) leads to tight junction protein reduction in occludin, claudin-5 and zona occludens-1 and that oxidative stress induced activation of matrix metalloproteinases (MMPs) and aquaporin-4 leads to promoting vascular fluid cavitation and edema, BBB leakage and neuroinflammation (Abdul-Muneer et al., 2013). Matrix metalloproteinases (MMPs) are involved in the degradation of TJ and basement membrane proteins as a result of oxidative stress (Desmoulin and Dionne, 2009). Both single and repeated blast exposures result in increases in the expression of MMP-2, MMP-3 and MMP-9 in the cerebral microvasculature, with MMP-3/MMP-9 gradually increasing up to 24 hrs while MMP-2 exhibits a transient profile, decreasing after 6 hrs post-BINT (Desmoulin and Dionne, 2009). Examination of aquaporin-4 (AQP-4), a water channel protein, can indicate if MMP-induced permeability increases are due to a disruption of water-channel proteins. BINT results in an increase in the expression of AQP-4 in the perivascular region and cortical tissue of BINT animals (Desmoulin and Dionne, 2009). The co-localization of GFAP and AQP-4 indicate that AQP-4 is indeed associated with astrocytes near the end-feet, in the perivascular region and suggests that AQP-4 activation may be involved in cerebrovascular edema formation, possibly contributing to fluid cavitation and inflammation after BINT. Examination of the tightness of the BBB after blast injury reveals significant increases in leakage to both low and high molecular weight tracers (Na-Fl and Evans Blue, respectively) at 24 hrs post-injury. This is consistent with other reports of blast-induced BBB permeability increases (Readnower et al., 2010a). The loosening of the TJs is described as being mediated by oxidative-stress induced MMPs and AQP-4 activation in the perivascular region (Abdul-Muneer et al., 2013). Degradation of the BBB TJs and MMPs leads to BBB leakage, vascular fluid cavitation, edema and neuroinflammation. A strong association can be made between the

damage to the BBB and leakage of neuronal-specific proteins into the bloodstream, with the permeable BBB allowing leakage of degenerated neuroglia components surrounding the perivascular unit (Abdul-Muneer et al., 2013). This concept is also supported by the reports of Goldstein et al., regarding the appearance of chronic traumatic encephalopathy (CTE) in military personnel who have experienced BINT (Goldstein et al., 2012). They proposed that all of these changes occurred within a window of 6-24hrs post-blast with only the repeated exposure group exhibiting persistent vascular damage. If accurate, the window of opportunity for a therapeutic intervention for cerebral vascular damage and neuroinflammation is very brief. They also suggested that the cognitive and behavioral changes associated with BINT must be generated by repeated exposure to blast waves, as there is an absence of chronic injury induced by their single exposure model.

In regards to the production of free radicals and oxidative stress, Cernak and colleagues have shown that high levels of blast overpressure results in an increase in lipid peroxidation and nitric oxide production in the brain starting at 3hrs post-injury and lasting through 5 days post-injury (Cernak, 2010). The effects of mild and moderate levels of blast injury on the formation of free radicals and oxidative damage was unclear until Readnower and associates reported a significant increase in both 3-NT and 4-HNE levels at both 3 hrs and 24 hrs following a 120 kPa blast injury (Readnower et al., 2010a). This data suggests that even moderate BINT can produce a rapid induction of oxidative stress. Additionally, cortical neurodegeneration associated with oxidative stress has been shown to be attenuated with the treatment of aminoguanidine (AG) either before or after (Moochhala et al., 2004). Aminoguanidine (AG) is a NOS inhibitor that reduces advanced glycation end products (AGEs) via interaction with 3DG. 3DG is known to induce ROS and oxidative DNA damage and can be responsible for producing intracellular oxidative stress. Both AGEs and 3DG are associated with the modification and cross-linking of proteins such as collagen, contributing to vascular complications associated with hypertension and inflammation. Aminoguanidine has been shown to

reduce AGE associated neural pathologies in animal studies (Cash D, Iadecola C, 1995, Lu et al., 2003, Stoffel M, 2000, Wada K, 1998, Zhang FY, 1996). It has been suggested that the fact that both oxidative stress and BBB breakdown occur at these time points indicates that the free radicals responsible for the oxidative stress may be generated by the same vascular alterations that result in BBB dysfunction (Pun et al., 2009, Kontos and Wei, 1986).

The diversity of the studies mentioned here represents the variation among researchers in the field of BINT at the moment. With multiple models, various mechanisms of injury associated with blast exposure are examined and contribute to the overall knowledge of blast-induced TBI. The literature indicates a strong association of the cerebral vasculature in the acute pathophysiological response to BINT, with disruption of the BBB, immune response, neuroinflammation, edema, glial injury and oxidative stress consistent amongst the experiments performed to date. The addition of behavior analysis will also contribute to our understanding of the symptomology associated with human BINT. Continued investigation of the cellular mechanisms at play in this injury response will enable researchers and clinicians to design effective therapeutics and interventions that may control and/or reverse the damage to the cerebral vasculature and mitigate the injurious effects mentioned in the present study.

Thus, we have chosen to focus on a head-only model, examining BBB as measured by Evans blue extravasation, vascular occludin as a measure of endothelial leakage and the effect of BINT on behavioral outcomes such as vestibulomotor tasks and the spatial working memory paradigm for Morris water maze.

SPECIFIC AIMS

Traumatic brain injury (TBI) results in central nervous system (CNS) and cerebral vascular disruptions associated with deficits in cognition, memory and behavior. Blast induced neurotrauma (BINT), often referred to as the signature injury of Operations Iraqi and Enduring Freedom, is associated with cognitive deficits, sleeplessness and behavior changes that may manifest weeks to months after injury and persist for months to years (Cernak et al., 2001c). We designed and constructed a custom made device that fires blank cartridges from powder-driven nail guns to generate reproducible, graded levels of blast over- and underpressures similar to those observed after open-field blasts. We have observed immediate, significant increases in cerebral vascular resistance and reductions in cerebral perfusion after BINT in rats. These results indicate that BINT is associated with acute cerebral vascular injury and, perhaps, damage to the blood-brain barrier (BBB). Although there have been several excellent studies of the effects of blast on neuronal and glia injury, as well as neurological and behavioral outcomes in rats and other animals, the effects of blast injury on the BBB has not been studied extensively. Our central hypothesis is that BINT results in cerebral microvascular disruption and impaired BBB function and is associated with behavioral dysfunction. We will test specific mechanisms that contribute to vascular, neurological and cognitive deficits associated with BINT.

Specific Aim 1 is to determine the anatomical locations and temporal sequence of blast-induced BBB permeability increases.

Aim 1*:* We will determine the anatomical locations and temporal sequence of BBB permeability changes by injecting Evans Blue dye (EB) 30 minutes, 2 hours and 1, 3 and 7 days post-blast. Extravasated EB will be quantified by the *in vivo* imaging system (IVIS).

Specific Aim 2 is to test the hypothesis that mild BINT causes decreased expression of junction protein occludin in cerebral vascular endothelial cells.

Aim 2*:* We will analyze the effects of mild BINT on the quantities of occludin protein expression by stereological counting of occludin-positive, immunolabeled cerebral vessels.

Specific Aim 3 will test the hypothesis that blast injury produces changes in peroxynitrite production and changes in behavioral outcome.

Aim 3.1*:* We will analyze the effects of BINT on the production of peroxynitrite by 3-nitrotyrosine (3-NT) immunoreactivity.

Aim 3.2*:* To determine an association between ROS/RNS and BBB permeability following BINT, we will use peroxynitrite scavenger, penicillamine, at 30 minutes and 24 hours post-injury.

Aim 3.3: We will examine the effects of BINT on spatial working memory using location-match tests in the Morris water maze. We will also examine neurological deficits using a short neuroscore test, beam balance and beam walking tests.

Chapter 2: Materials and Methods

ANIMALS

All animal protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas Medical Branch. Adult, male Sprague-Dawley rats weighing 350-500g were anesthetized with 4.0% isoflurane in an anesthetic chamber, intubated and mechanically ventilated with 1.5-2.0% isoflurane in O_2 and room air (70:30) using a volume ventilator (EDCO Scientific, Chapel Hill, NC). A polyethylene cannula was inserted in the caudal vein for monitoring of arterial pressure. Rectal temperature was monitored using a telethermometer (Yellow Springs Instruments, Yellow Springs, OH) and maintained using a thermostatically controlled water blanket (Gaymar, Orchard Park, NY). Rats were prepared for blast injury and measurements of laser Doppler perfusion (LDF), opto-acoustic measurements of oxygen saturation of the sagittal sinus and mean arterial pressure (MAP) as described below. The rat's head was shaved, placed in a stereotaxic frame and the scalp incised in the midline and reflected. Rats were then prepared for the placement of a fiber optic laser Doppler flow probe (see below). Isoflurane was lowered to 1.5% and the animal was positioned to receive a sham-injury or blast-induced neurotrauma (BINT).

Animals received either low-mild, high-mild or moderate-severe blast injury, determined by the length of righting reflex suppression (Table 4). Righting reflex suppression is used as an indirect measure of injury severity. For model characterization, multiple levels of BINT were studied, however, we eventually focused on high-mild BINT. Specifically, BBB permeability experiments (chapter 3) compared sham animals to high-mild BINT animals. The effects of BINT on cerebral vascular occludin immunoreactivity were examined in sham and high-mild BINT animals (chapter 4). Performance in beam balance, beam walking and neurological response tests were

measured and compared in sham, low-mild, high-mild and moderate-severe BINT animals (chapter 5). Morris water maze performance was assessed in sham, low-mild and high-mild animals, with low-mild and high-mild animals grouped together as mild BINT (chapter 5). Immunoreactivity to 3-nitrotyrosine antibody and the effect of penicillamine treatment on Evans blue extravasation was assessed in sham and high-mild BINT animals (chapter 6). Model characterization experiments were performed in sham, low-mild, high-mild and moderate-severe BINT animals (appendix A).

Injury Level	**Suppressed Reflex**
Sham	$\leq$ 4 mins
Low-Mild	6 mins $\geq$ 10 mins
High-Mild	10 mins $\geq$ 16 min
Moderate-Severe	$\geq$ 16 mins

Table 4: Righting reflex suppression. Righting reflex is measured by lying the animal in a supine position and measuring the length of time required for the animal to independently return to a prostrate position (righting).

BLAST INJURY

All blast injuries were produced by the Vandenberg custom-made blast device, which fires 0.27 caliber blank cartridges used in power-driven nail guns (Vandenberg Custom, Webster, TX). The Vandenberg device produces a combined blast injury: 1) a primary blast injury is produced by the blast wave as it passes over the animal, 2) the gas venting jet following the blast wave causes an impact-like acceleration/deceleration brain injury.

For each blast injury, the animal was anesthetized, intubated and ventilated on 2% isoflurane. The head was shaved and covered with acoustic coupling gel and a 1/16”

silicone spacer to protect the animal from flash burn injury. The ears were plugged, the animal was placed on a piece of dense foam and positioned underneath the barrel of the Vandenberg device. A safety switch was toggled and held in the active position and the trigger button depressed to fire the cartridge. The animal was positioned 15 mm below and 5mm to the right of the exit hole in the bottom of the barrel. The injury site of 5mm lateral to midline was chosen to resemble the injury site used in our previous fluid percussion studies (Hawkins & Davis, 2005).

JUGULAR VEIN CANNULATION

The animal was anesthetized, intubated and ventilated on 2% isoflurane. With the animal in a supine position, an incision was made above the clavicle, toward the larynx to reveal the underlying jugular vein. A polyethylene cannula with heparinized saline was inserted into a small incision made on the vessel surface and secured in place.

EVANS BLUE EXTRAVASATION

Rats were anesthetized with 4.0% isoflurane in an anesthetic chamber, intubated and mechanically ventilated on 2% isoflurane as described above. A polyethylene cannulae filled with heparinized saline was placed in a jugular vein for injection of Evans Blue, an azo-dye to which is impermeable in the intact BBB. After injury, Evans blue (2% in saline, 2 mg/kg) was injected into the jugular vein cannula, allowed to circulate for one hour, isoflurane levels were increased to 4.0% and the rat was decapitated.

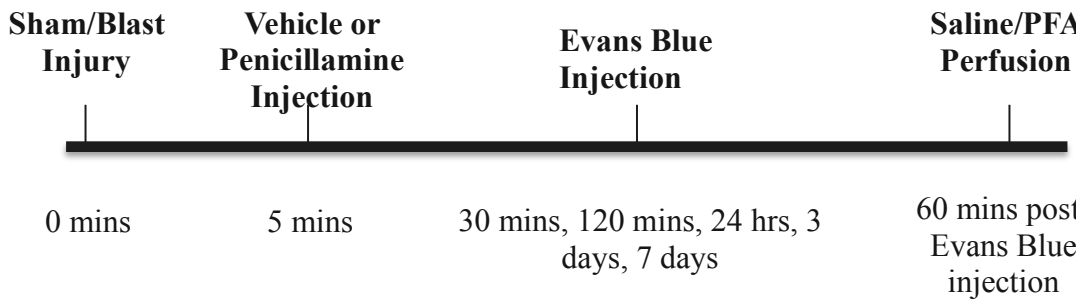


Figure 2. Injection Schedule. At the indicated time points following sham- or blast-injury, animals received various injections of EB, saline or Penicillamine, dependent upon the goal of the individual study.

PENICILLAMINE ADMINISTRATION

For studies involving the scavenging of reactive nitrogen species, peroxynitrite (ONOO-), the intravascular ONOO- scavenger, penicillamine, was administered. Rats were anesthetized with 4% isoflurane in an anesthetic chamber and penicillamine (10 mg/kg in saline) was injected via the jugular vein cannula 5 minutes post-injury, as indicated in Figure 2. Both the dose and time of administration were selected based upon efficacy observed in a previous study performed in weight-drop TBI mice (Hall et al., 1999).

EVANS BLUE QUANTIFICATION

Evans blue extravasation studies were quantified by using the fluorescence system of an *in vivo* imaging system, also referred to as IVIS. The IVIS Spectrum Pre-clinical *in vivo* Imaging System (Perkin Elmer, Waltham, MA) performs high-sensitivity, high throughput, high resolution imaging of fluorescent and bioluminescent specimens *in vivo*. For our purposes, we used the IVIS and coordinating Living Image software for acquisition and analysis of the fluorescence emitted by Evans blue at an excitation of 640

nm and emission of 680nm. The IVIS quantifies fluorescence in units of radiant efficiency, essentially, the number of activated photons reflected by the specimen.

$$\text{Total Radiant Efficiency} = \frac{\text{Emission light (photons/sec/cm}^2\text{/str)}}{\text{Excitation light } (\mu\text{W/cm}^2)}$$

IMMUNOHISTOCHEMISTRY

Rats were anesthetized with 4% isoflurane and pentobarbital (1 mg/kg intraperitoneally) either 30 minutes or 24 hours post sham- or blast-injury, and decapitated. The brains were removed and immediately placed on dry ice for freezing. Once frozen, brains were stored at -80°C until further processing. Brains were removed, embedded in OCT and coronally sectioned at 35 μm in a cryostat (Leica Biosystems, IL) onto Superfrost Plus glass slides (Thermo Fisher Scientific, MA). Sections were then incubated ice-cold methanol for 20 minutes and allowed to air dry. Sections were rinsed in TBS 1X (3 times, 10 mins) and blocked in 10% normal donkey serum in TBS 1X + 0.3% Triton X-100 for 1 hour at room temperature to block non-specific binding. Following blocking, sections were incubated in primary antibody (anti-occludin goat monoclonal antibody, 1:200, Santa Cruz; anti-nitrotyrosine mouse monoclonal antibody, 1:200, Millipore; anti-RECA-1 mouse monoclonal antibody, 1:50, Bethyl) solution overnight at 4°C. After primary antibody incubation, sections were rinsed in TBS 1X (3 times, 10 mins) and incubated in secondary antibody solution for 3 hours at room temperature. Sections were then rinsed with dH2O and mounted with Vectashield HardSet Mounting Media with DAPI (Vector Labs, CA) and coverslipped for viewing. Occludin immunoreactivity was measured quantitatively through stereological counting of positively stained blood vessels (vessels were co-labeled with ant-RECA-1 for vessel localization) within the penumbra. Vessels immunofluorescent for anti-occludin antibody were counted using an epifluorescent microscope (Olympus BX51) and Stereo

Investigator stereological counting software (MicroBrightField Bioscience), which generated 10 counting frames of uniform size (15 cm^2) that were randomly overlaid onto 20x images of the cortex surrounding the injury site. The area fraction was calculated by the area of positively stained vessels divided by total area of the counting frame. Nitrotyrosine immunoreactivity was assessed quantitatively using Fiji for ImageJ2 software. Two uniform regions of interest were created to measure fluorescence intensity in the penumbra of both sham and high-mild BINT animal tissues harvested at 24 hours post-injury.

BEHAVIOR ANALYSES

Beam balance

The balance beam apparatus consists of a beam 22.5" L x 1.5" H x .75" W and a barrier 10.5" H x 13" W. The beam was secured to a table and the barrier attached to the beam so that 10.5" of the beam protrudes from the barrier away from the table over a padded safety box. Animals underwent two training sessions and one pre-assessment prior to injury (day 0) beginning on day –2. On day –2 the animal was placed on the balance beam for 60 second trials. The animal was removed from the beam for a 15 second resting period between each trial in order to disorient him from the beam. If the animal could not balance, it was allowed to fall from the beam into a padded box. Animals were trained until able to remain on the beam for three consecutive 60-second trials. Trials were scored numerically, 1-6. Each trial was scored as follows: (1) Balances with steady posture (grooms, climbs barrier) (2) Grasps sides of beam and/or has shaky movements (3) Hugs the beam or slips or spins on beam (4) Attempts to balance, but falls off after ten seconds (5) Drapes over beam or hangs from beam and falls off in less than ten seconds (6) Falls off, makes no attempt to balance or hang from

beam. On the day of injury (day 0), the animal underwent a pre-assessment consisting of three trials. The animals were assessed on various days post injury, 1, 2 and 3 days – three trials each day (Dixon et al., 1987, Hamm, 2001, Long et al., 1996, Sell et al., 2008). Two-way ANOVA of beam balance scores indicated an extremely significant variation due to injury level on post-injury days 1-3 ($p < 0.0001$) and a significant variation among days post-injury ($p = 0.0274$). Bonferroni posttest for multiple comparisons revealed very significant ($p < 0.01$) and significant ($p < 0.05$) variation among Sham vs. High-Mild BINT animals on post-injury days 2 and 3, respectively. Sham vs. Moderate-Severe BINT animals exhibited very significant ($p < 0.01$) variation on post-injury day 1 and extremely significant variation ($p < 0.001$) post-injury days 2 and 3. All values are represented as mean ± SEM. Sham n=4, Low-Mild BINT n=7, High-Mild BINT n=5 and Moderate-Severe BINT n=7.

Beam-walking

The beam walk apparatus consists of a beam 40" L x 1" W. One end of the beam is stabilized by a stand (starting end) and the opposite end is attached to the goal box that is on a table. The goal box is a black box with a hinged lid, for accessing the animal. The box is 11"L x 7.25" H x 7.25" W. The beam leads to a doorway (4.25" square) in the goal box. Four pegs (.75" H) are inserted at 9.5", 18.5", 18.5", and 38.25" from the starting end. Peg placement alternates along the outer edges of the beam beginning on the right edge. A light source and white noise source are positioned on a cart at the starting end of the beam walk. Training on the beam walk begins on day –2. The animal is placed in the goal box for two minutes at the start of the training session and the pegs are removed from the beam. At the end of two minutes, the handler turns on the white noise and light and removes the animal from the goal box by means of the hinged lid. The animal is placed on the beam at the location of the peg closest to the goal box and

allowed to walk to the goal box. As soon as the animal's front feet cross the threshold of the goal box the light and noise source are turned off. The animal is allowed to rest in the goal box for 30 seconds between each run. The animal is not allowed to fall from the beam; the handler assists him if he starts to fall off. This procedure is repeated twice at each peg location and from the starting position. After two training runs from each position, the pegs are inserted and one complete beam walk is done for practice. Three timed beam walk trials are then recorded to conclude training. The timer is started once the animal is securely positioned at the starting point and the first step is taken in the direction of the goal box. On the day of injury (day 0), the animal undergoes a pre-assessment consisting of three timed trials. The animals are assessed on various days post-injury days 1, 2 and 3 with three trials each day (Dixon, 1987; Lyeth, 2001; Sell, 2008). Two-way ANOVA indicated a very significant variation in beam-walking latency on post-injury days 1-3 ($p = 0.0016$). Using the Bonferroni posttest for multiple comparisons revealed a significant variation of Sham vs. Moderate-Severe BINT animals on day 3 post-injury ($p < 0.05$). All values are represented as mean ± SEM. Sham n=4, Low-Mild BINT n=7, High-Mild BINT n=5 and Moderate-Severe BINT n=7.

Brief neurological assessment

Each day, starting 2 days prior to injury, neurological outcomes were assessed using a neuroscore system (McIntosh et al., 1989). The short functional neuroscore consists of five tests: (1) forelimb flexion test, (2) hind limb flexion test, (3) visually triggered placing test, (4) contact triggered placing test, and (5) hind paw grasping reflex test. Forelimb flexion was tested by lifting the rat by the tail and holding approximately 12 inches above the table surface, observing for flexion or extension of forelimbs. Flexion is abnormal and receives a score of (1). No flexion, (0). The hind limb flexion test is done the same as the flexion test, scoring (1) for hind limb flexion or (0) for no

flexion. Visual triggered placing tests were performed by lifting the rat by the tail and slowly lowering toward the table edge, up to approximately 10 cm from nose to table edge. Moving the rat towards the edge, observation of the presence or absence of extending forepaws was scored. Extension is a normal behavior with a score of (0), no extension (1). Contact triggered placing tests were performed by holding the rat with body in hand, parallel to table edge, with forelegs free. Slowly the rat was lowered to the table until the whiskers on one side touch the edge of the table. Forelimb extension on the same side as the touching whiskers is normal, scoring (0), while the absence of extension in response to tactile stimulation is abnormal (1). Repeat for opposite side. Hindpaw grasping reflex was tested by holding the rat in hand, thumb and index finger around the chest, under the forelimbs and lowering the rat toward the table edge until whiskers touch the table. Gently touching the palm of each hind paw with right forefinger and observing whether the rat grasps the finger. The presence of grasp is normal and receives a score of (0), no grasp (1). Each test was performed on both left and right sides. All scores were tallied for a possible total of 21. A score of 0 indicated normal reflex function. Two-way ANOVA and the Bonferroni posttest for multiple comparisons were used for statistical analysis. All values are represented as mean ± SEM.

Morris Water Maze

The procedures for using the Morris water maze (MWM) to assess working memory are described in detail by Hamm, et al., 1996 (Hamm, et al., 1996). Briefly, the MWM is a white tank (180 cm diameter, 28 cm depth) filled with water and containing a clear plastic platform beneath the surface of the water. The hidden platform version of the Morris water maze assesses the animal's ability to learn spatial relations between distal cues and the escape platform. Acquisition blocks consisted of four daily trials over three

consecutive days. On each acquisition trial, rats were placed by hand in the pool facing the wall. Animals were given a maximum of 120 sec to find the hidden platform. If the rat failed to find the platform after 120 sec, it was placed on the platform by the experimenter. All rats were allowed to remain on the platform for 30 sec before being placed in a heated incubator between trials. There was a 4-min inter-trial interval (Hamm, et al., 1996, 2001; Morris, et al., 1982; Sell, et al., 2008). Unpaired t test with Welch's correction were used for statistical analysis of the data. All values are presented as mean ± SEM.

STATISTICAL ANALYSIS

All statistical analyses were evaluated at the alpha level of significance of 0.05 ($\alpha = 0.05$). Experimental data were tested for statistical significance using two-way ANOVA or unpaired t tests, followed by tests of factors including pair-wise comparisons where appropriate with either the with Bonferroni and Welch's correction posttests, Evans blue extravasation (Fig. 4), beam balance (Fig. 7), beam walking (Fig. 8) and neurological response (Fig. 9) data was analyzed for statistical significance using a two-way ANOVA and Bonferroni multiple comparisons posttest. Morris water maze and immunoreactivity to 3-nitrotyrosine (Fig. 5) data was analyzed for statistical significance using an unpaired t test and Welch's correction.

Chapter 3: BBB Permeability

INTRODUCTION

The BBB performs many important functions to maintain brain homeostasis: ion regulation, separation of central and peripheral nervous system neurotransmitter pools, mediated transcytosis of large molecules, regulation of innate immune response, regulation of leukocyte recruitment and the regulation of small solutes (Abbott, et al., 2010). To examine the ability of the BBB to act as a physical barrier to large molecules following experimental BINT, we used a standard method of dye extravasation.

Evans blue (EB, 960.8 Da) is a blue azo-dye that binds irreversibly to serum albumin (65 kDa). EB and HRP are commonly used large markers that bind to albumin proteins (Habgood et al., 2007) and are considered as standard methods for examination of BBB permeability. Both can be injected intravenously to circulate through the vasculature and be taken up by neurons and their processes, should the dye be able to cross the BBB, as is the case in many injuries and illnesses. HRP requires a prolonged time of circulation (Nag, 2003) and has been shown to induce hypotension in Sprague-Dawley rats (Deimann et al., 1976); therefore, we used EB in our permeability studies. To quantify the extravasation of EB, we used an in vivo imaging system (IVIS) to scan 2mm sections of brain tissue from both sham and BINT animals, using fluorescence to detect EB at an excitation of 640nm and emission of 680nm. Using analysis software created for use with IVIS, the amount of EB detected was measured in total radiant efficiency (TRE), which is essentially the number of activated photons within the set excitation and emission spectrum. Brain tissue was examined at 30 mins, 120 mins, 24 hrs, 3 days and 7 days post sham or blast injury.

MATERIALS AND METHODS

Methods applied in the experiments of this chapter are reported in chapter 2.

RESULTS

BINT Results in Increased BBB Permeability

Compared to animals that received a sham injury, BINT animals exhibited significant increases in the amount of EB detectable in extravascular brain tissue. Gross examination of the brains revealed noticeable regions of increased BBB permeability at 30 mins, 120 mins, 24 hrs and 3 days post-BINT (Fig. 3). Brain tissues were sliced into 2 mm sections and laid flat for scanning with the IVIS (Fig. 4). Analysis of the scanned images resulted in significantly increased EB extravasation in tissues from BINT animals at 30 mins, 120 mins, 24 hrs and 3 days after injury when compared to sham-injured animals ($p < 0.0001$) (Fig. 5).

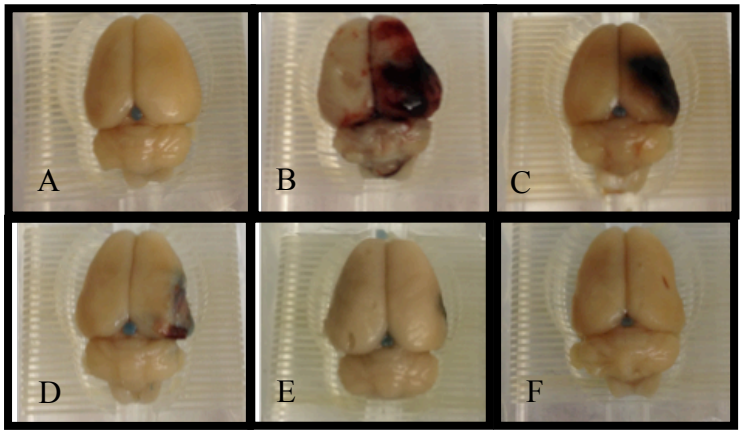


Figure 3. Sham and High-Mild BINT Brains with Evans Blue. Following sham (A) or blast injury (B-E), EB was injected at 30 mins (B), 120 mins (C), 24 hrs (D), 3 days (E) and 7 days (F).

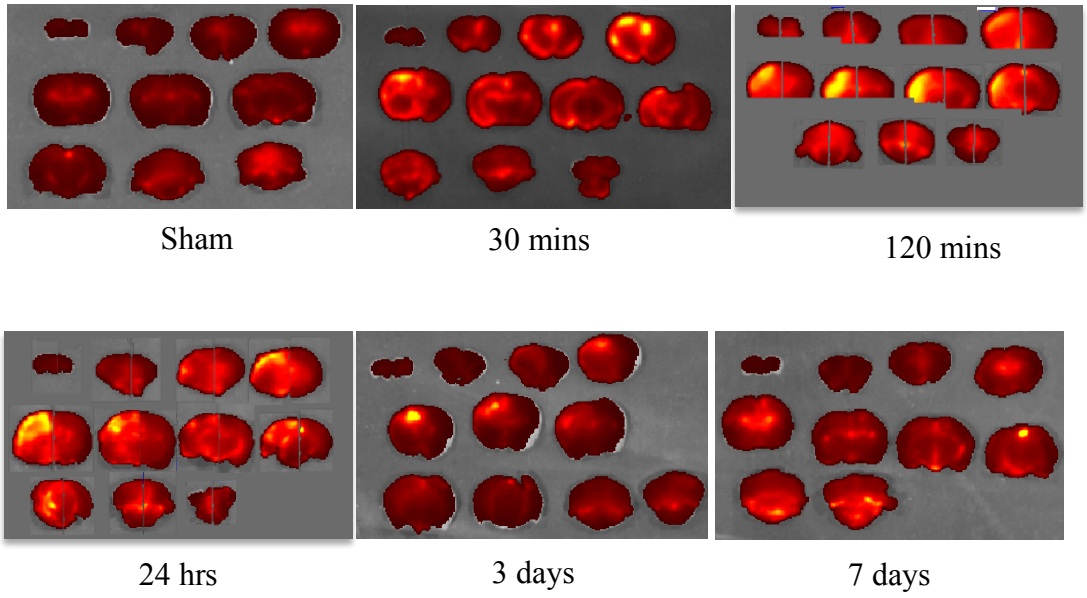


Figure 4. IVIS Scans of Evans Blue Following BINT. Prior to scanning with the in vivo imaging system (IVIS), brain tissues were cut into serial, coronal sections of 2 mm thickness. Sections were arranged side-by-side on a piece of Parafilm for simultaneous imaging.

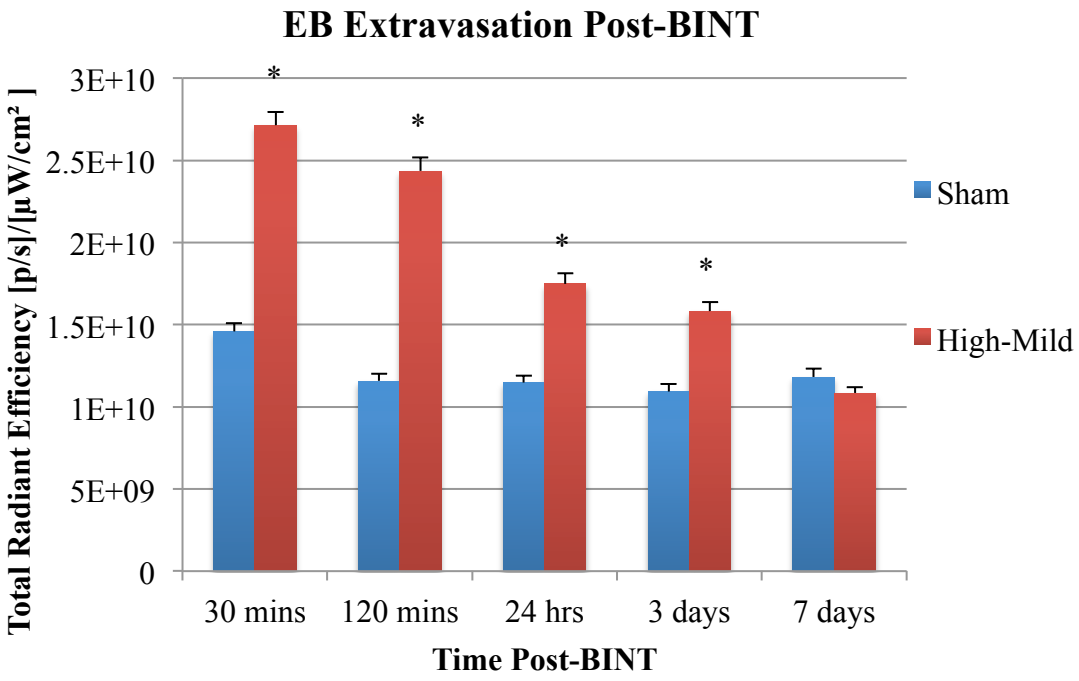


Figure 5. Evans Blue Extravasation. Evans Blue was quantified using an IVIS to detect the presence of Evans Blue following sham BINT or BINT. Total radiant efficiency (TRE) is the number of activated photons scanned within a predetermined excitation and emission range. Here the excitation was 640 nm and the emission was 680 nm, based upon the ability of Evans Blue to fluoresce between 620-680 nm. A two-way ANOVA indicated significant increases in TRE at 30 mins, 120 mins, 24 hrs and three days (*, p-value < 0.0001).

DISCUSSION

The BBB is a critical component in the maintenance of a functional and healthy CNS, acting as a physical, transport, metabolic and immunologic barrier. Increased BBB permeability is a frequent result of both clinical (Bellander et al., 2001a, Vajtr et al., 2009, Hawkins and Davis, 2005) and experimental TBI (Abdul-Muneer et al., 2013, Svetlov et al., 2009, Readnower et al., 2010a). TBI-induced brain edema can be initiated by a damaged BBB, allowing an influx of protein-rich exudate to by-pass diminished or degenerated tight junctions and enter brain parenchyma (Hue et al., 2013).

The present study provides evidence of an acute, transient increase in BBB permeability after high-mild BINT in rats. We report significant increases in BBB permeability that occur as early as 30 mins post-BINT and persist through 72 hrs post-BINT ($p < 0.0001$) (Fig. 5). Increases in BBB permeability peak 30 mins post-BINT and steadily decline over one week, returning to levels that were not significantly different when compared to sham values, indicating partial recovery of BBB function.

It was previously thought that the BBB remains open up to 48 hrs after TBI, however, we show that following BINT, the BBB is significantly more permeable for 72 hrs. Other reports of BBB permeability differ in the temporal window of injury-induced permeability. In experiments by Readnower et al., (Readnower et al., 2010a) tissue from shock tube-blasted animals was analyzed for BBB permeability at 30 mins, 3 hrs, 24 hrs and 72 hrs following injury. Those experiments indicated a significant increase in BBB permeability at 3 hrs and 24 hrs, however at 30 mins and 72 hrs post-injury there was no significant increase in permeability, which contrasts with our observations. Yeoh et al., observed generally small, focal regions of perivascular IgG immunoreactivity that were scattered throughout sections, showing no correlation to site or direction of blast (Yeoh et al., 2013). Increased BBB permeability is also reported at 24 hours in contralateral cortical areas following single exposure to blast (Garman et al., 2011). BINT causes an acute opening of the BBB, which is resolved within one to two weeks after injury (Readnower et al., 2010a, Garman et al., 2011, Abdul-Muneer et al., 2013). Our observations of increased BBB permeability as evidenced by increased Evans blue extravasation 24 hrs post-BINT are consistent with the current literature (Yeoh et al., 2013, Readnower et al., 2010a, Garman et al., 2011, Abdul-Muneer et al., 2013). Direct comparison of experimental results remains difficult due to the variation in blast models. The consistency of the reported findings and similar data in the literature suggest that acute BBB impairment is a common result of both TBI and BINT.

Quantification of EB extravasation via IVIS is innovative, however, it does present some challenges. Potential miscalculations may arise because this method relies upon imaging, whereas other quantification methods rely upon spectrophotometric analysis of extravasated samples. IVIS images are two-dimensional representations of fluorescence detected in hand-sectioned brain tissues. Reflections from surface moisture may also vary depending upon the duration of air exposure. Procedures are in place to maintain tissue quality and consistency, although some human error is eminent. Images included in the studies described here were taken at specific excitation and emission wavelengths (640 and 680, respectively) to provide standardized conditions for each set of images. Adjusting these parameters will provide recalculated measurements of detected fluorescence.

Chapter 4: Endothelial Cell-Cell Junctions

INTRODUCTION

As a major component of the BBB, endothelial cells are responsible for protecting the microenvironment of the brain. Brain endothelial cells are connected at the junctional complex via tight junctions (TJ) and adherens junctions (AJ) (Hawkins and Davis, 2005, Zlokovic, 2008). These junctions span the intercellular cleft, regulating paracellular diffusion of molecules. Composed of occludin, claudins and zonula occludens accessory proteins, tight junctions maintain the integrity of the BBB by preventing the entrance of blood-borne molecules into the brain parenchyma, however these proteins may be affected by injury or disease.

As mentioned previously, TBI is often accompanied by a compromised BBB, resulting in vulnerability of brain homeostasis to blood-borne molecules and immune cells. One possibility for the increase in BBB permeability is the degeneration or loss of tight junctions. In a study performed by Bamforth and associates, a deletion construct lacking the N-terminus and extracellular domains of occludin had a powerful effect on TJ integrity (Bamforth et al., 1999). Using freeze-fraction electron microscopy, they observed gaps in the P-face that were associated with TJ strands, an increase in paracellular flux of small molecular tracers and low TEER (Bamforth et al., 1999). These findings indicate that the N-terminus of occludin does play an important role in maintaining the TJ complex, and thereby the integrity of the BBB. A study in an experimental multiple sclerosis (MS) model, experimental autoimmune encephalomyelitis (EAE), showed that dephosphorylation of occludin occurs prior to an increase in BBB permeability (Morgan et al., 2007). These results indicate that occludin may contribute to the cascade of events affecting the integrity of the BBB in an inflammatory environment such as that seen in MS. In cases of human

immunodeficiency virus-1 (HIV-1) it has been shown that the loss of occludin and TJ accessory protein, ZO-1, leads to the breakdown of the BBB which then increases the brains vulnerability to HIV-1 in the blood (Pu et al., 2007). The effect of blast injury on occludin in the tight junction assembly still requires more investigation. It is indicated that occludin levels decrease following BINT (Abdul-Muneer et al., 2013). To determine the effect of primary and tertiary blast on the presence of occludin in brain microvasculature, we performed immunohistochemical staining with a primary anti-occludin antibody to stain brain sections taken from both sham- and blast-injured animals at 30 mins, 120 mins, 24 hrs, 3 days and 7 days post-injury. We counted the immunoreactive blood vessels in the area of the cortex associated with the injury site using stereological analysis software (Stereoinvestigator).

MATERIALS AND METHODS

Methods applied in the experiments of this chapter are reported in chapter 2.

RESULTS

Mild BINT Does Not Decrease Occludin-Positive Cerebral Vessels in Cortex

Blood vessels immunoreactive for anti-occludin antibody were visualized using epifluorescent microscopy and counted using stereological methods. A counting frame of 15 cm2 was applied randomly in ten locations over an image of the injury site taken at 20x magnification. Within each counting frame, vessels exhibiting immunoreactivity to the anti-occludin antibody were selected and counted. This method was applied to tissue sections from both sham and BINT animals sacrificed at 30 mins, 120 mins, 24 hrs, three days and seven days after injury (Fig. 6). All tissue sections were incubated with

monoclonal goat anti-occludin primary antibody (1:200, Santa Cruz). Representative images of sham and BINT tissue sections at 24 hrs and three days are included in fig.7. In these experiments we did not see a statistically significant difference in the number of immunoreactive vessels in BINT sections compared to sham sections.

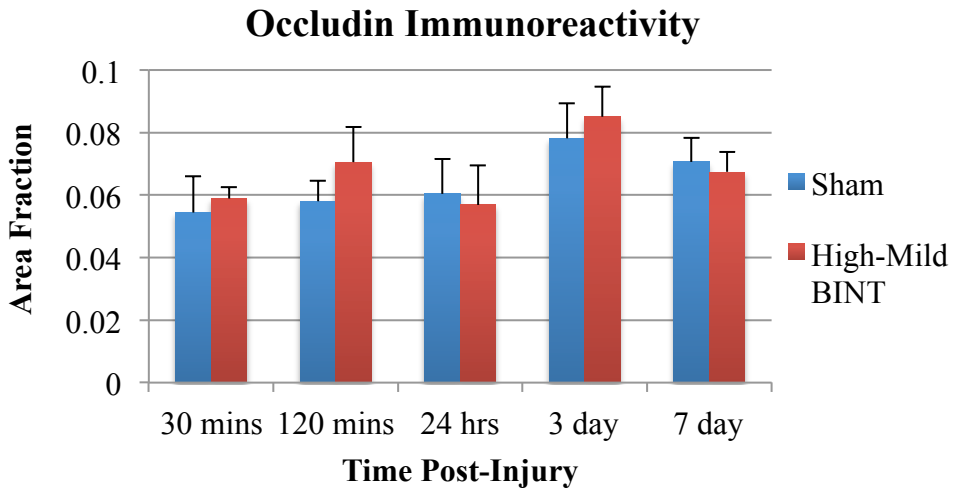


Figure 6. Mild BINT Does Not Alter Occludin Expression in Cerebral Vessels. Cerebral vessels of the cortex were immunolabeled for occludin, were visualized with epifluorescence and stereologically counted. Two-way ANOVA revealed there was no significant change in the amount of occludin-positive vessels following BINT.

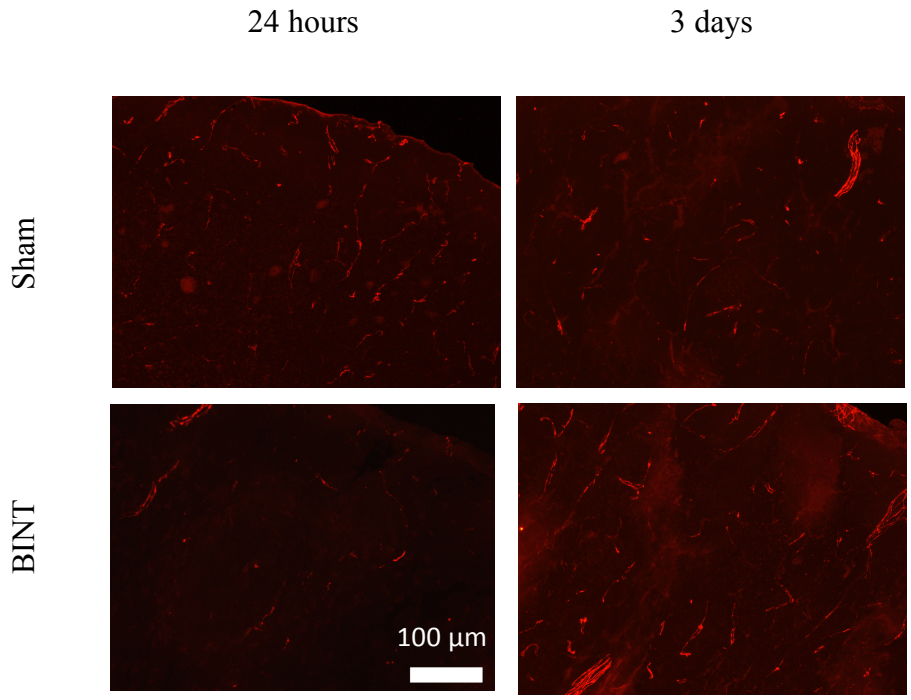


Figure 7. Occludin Immunoreactivity in Cortex. Occludin-positive vessels at 24 hrs and 3 days in the cortex of sham- and blast-injured animals. All images at 20x.

DISCUSSION

In 1993, Furuse et al. discovered the integral membrane protein, occludin (Furuse et al., 1993). The structure of occludin contains many functional and regulatory domains, particularly the extended C-terminus. The C-terminus has been shown to be critical in the interactions between occludin and ZO-1, signaling and dimerization functions (Chen et al., 1997, Furuse et al., 1994, Li et al., 2005, Walter et al., 2009a, Walter et al., 2009b). The first extracellular loop is also of interest, as it is composed of nearly 60% tyrosine and glycine residues (Feldman et al., 2005). These residues may play an important role in the regulation of endothelial TJs through the different states of occludin phosphorylation, a process that has been shown to correlate to vessel barrier dysfunction (Pang et al., 2005, DeMaio et al., 2001, Antonetti et al., 1999, Morgan et al., 2007,

DeMaio et al., 2006). As a prominent protein in the maintenance of TJ adhesiveness, occludin spans the intercellular cleft to form homodimers, providing the physical barrier that is characteristic of TJs. While the literature contains many studies of the effects of other types of TBI on occludin protein expression (Huber et al., 2001, Bolton et al., 1998, Morgan et al., 2007, Liu et al., 2012), the effects of combined primary and tertiary blast mechanisms remain unclear.

The results of this study did not indicate a significant change in occludin protein expression at the predetermined time points of 30 mins, 120 mins, 24 hrs, 3 days and 7 days. If this is indeed the case, the increased permeability that we see after BINT is not likely associated with the amount of occludin protein present in the vessels. Occludin may still play a role in the increased permeability of the BBB following BINT however it is purely speculation until further tests are performed.

There is some evidence in the literature that supports the hypothesis that barrier-disruptions can stimulate the phosphorylation of occludin tyrosine residues, attenuating the occludin-ZO-1 interaction, causing occludin to delocalize from the membrane and resulting in barrier disruption (Elias et al., 2009, Kale et al., 2003). Other possible contributors to the increase in BBB permeability are 1) physical disruption of the junction assembly due to shearing effects, 2) oxidative stress in or around the neurovascular unit, 3) change in receptor mediated transport mechanisms.

If the BBB permeability increases evident after BINT are associated with a physical disruption to the junction assembly, one might expect to see large molecules moving through the intercellular cleft with little or no restriction by tight junctions. Fluorescently labeled dextrans of varying molecular weight could also be introduced via the blood circulation. Observing these dextrans in brain tissue sections would indicate whether they were able to pass through the barrier, also indicating the size of molecule to which the barrier has become permeable. Using more sensitive, high-resolution

microscopy methods may also be useful in further examination of the tight junctions and associated proteins after BINT.

Electron microscopy would allow visualization of the junctions themselves, providing visual evidence of the integrity and localization of the TJ assembly and components, which, if disrupted could potentially contribute to the increases in BBB permeability. If the increase in BBB permeability is associated with oxidative stress, immunofluorescence staining of brain tissue sections with antibodies for known indicators of ROS/RNS damage may provide insight.

The stereological counting technique described here provides evidence of whether occludin protein is present in the vasculature at the time of interest. Unfortunately, this method does not provide information regarding the level of protein present. A Western blot analysis of tissue samples probed with anti-occludin primary antibody and a conjugated secondary antibody would indicate the level of occludin present at the time of interest.

It is puzzling that we report an increased permeability to Evan blue after BINT compared to sham control values in the absence of significant changes in occludin as determined by immunohistochemical reaction products. As suggested above, the occludin protein conformation may be altered following BINT but not effect the antibody-binding site. Alternate explanations are increased transport of Evans blue by caveolli, receptor mediated transport or passive diffusion (Abbott et al., 2006).

Chapter 5: Behavior

INTRODUCTION

As mentioned in the introduction, BINT has become a major concern for health professionals and researchers, particularly due to the recent military operations in Iraq and Afghanistan. An estimated 15%-23% of service members experience TBI during deployment in Iraq, the majority of which are mild TBI (Hoge et al., 2008a). Complicating the issue is that fact that mTBI is often comorbid with post-traumatic stress disorder or depression, with many symptoms that seemingly overlap (Elder and Cristian, Hoge et al., Warden) (Elder and Cristian, 2009, Hoge et al., 2008b, Warden, 2006). Symptoms often referred to as post-concussive syndrome (PCS) include amnesia, compromised executive function, headache, confusion, anxiety, mood changes and disturbance of sleep patterns (Ling and Ecklund, 2011). These symptoms may develop immediately following injury or may surface months to years afterwards. While much is known about the behavioral and neurological effects of other TBI models, the neurobehavioral deficits associated with BINT have yet to be clearly defined.

In the present study we report increased BBB permeability localized to cortical and hippocampal regions. To test for neurological and behavior deficits associated with regions of increased BBB permeability, we utilized beam walking and balancing tasks, neurological assessment and the Morris water maze (MWM).

The beam-walking task that we employed was similar to that used in previous studies in our lab (Hulsebosch et al., Sell et al., 2008) and elsewhere (Dixon et al., 1991, Bramlett et al., 1995, Szmydynger-Chodobska et al., 2010, Fox et al., 1998). Animals were trained on pre-injury days one and two. The baseline was recorded the morning of the injury day, prior to sham or blast injury. Animals were tested again on post-injury

days one, two and three. In addition to the beam-walking task, we tested vestibulomotor function with the beam balance task. Also a common tool for measuring deficits in balance and sensorimotor function, the beam balance task is also commonly used to detect and measure deficits in balances and sensorimotor function (Dixon et al., 1987, Sell et al., 2008, Sherbel et al., 1999, Hamm, 2001). Training occurred for two days pre-injury and a baseline score was recorded the morning of the injury day, prior to sham or blast injury. Animals were then scored on post-injury days one, two and three.

Animals were also examined for neurological deficits following sham or blast injury. A short neuroscore was performed once each day, for the entire six-day behavior study (two days prior to injury, injury day and three days post-injury). The assessment consisted of five tests: hindpaw grasp, forelimb flexion, hindlimb flexion, visually triggered placement and contact triggered placement. Each test was performed in triplicate, testing both the right and left sides, on a daily basis. These tests were scored with 0 = normal response and 1 = abnormal response. The scores were tallied and averages were reported.

To examine the effects of primary and tertiary blast injury on spatial learning and working-memory we used the Morris water maze (MWM). Animals were tested on post-injury days one through three, allowing them to rest for 24 hours immediately following sham or blast injury. Additional descriptions of behavior tasks are included in chapter two.

MATERIALS AND METHODS

Methods applied in the experiments of this chapter are reported in chapter 2.

RESULTS

Beam Balance Performance Declines Following BINT

Animals were trained for two days then a baseline score was taken in the morning, just prior to sham or blast injury. The beam balance test required placing the animal on an elevated beam, timing their stay on the beam and scoring their postures while balancing. Higher scores indicate a greater difficulty in balancing on the beam. On days two and three post-injury, animals that were subjected to high-mild BINT displayed significantly higher scores ($p<0.01$, 0.05) when compared to sham-injured animals. Animals that were subjected to moderate-severe BINT injury displayed significantly higher scores when compared to shams on post-injury days one, two and three ($p<0.01$, $p<0.001$, $p<0.001$, respectively).

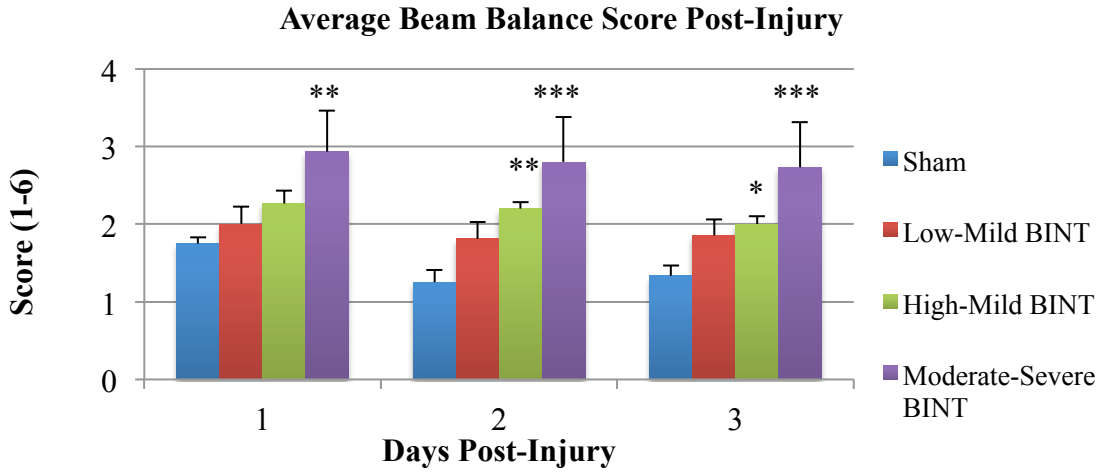


Figure 8. Beam Balance Performance. Two-way ANOVA of beam balance scores indicated significant variation due to injury level on post-injury days 1-3 (P value <0.0001) and a significant variation among days post-injury (P value = 0.0274). Bonferroni posttest for multiple comparisons revealed significant increase in score among Sham vs. High-Mild BINT animals on post-injury days 2 (**) and 3 (*). Sham vs. Moderate-Severe BINT animals exhibited significant increase in score on post-injury day 1 (**) and post-injury days 2 and 3 (***), (*, P value <0.05; **, P value <0.01; ***, P value < 0.001). All values are represented as mean ± SEM. Sham n=4, Low-Mild BINT n=7, High-Mild BINT n=5 and Moderate-Severe BINT n=7.

Effects of BINT on Beam-walking Task

Animals were again trained for two days then a baseline was recorded in the morning, just prior to sham or blast injury. The beam-walking task required animals to be placed on an elevated beam with white noise and bright lights as adverse stimuli and the animals were expected to traverse the beam to reach the goal box, where the adverse stimuli would cease. The time it took for the animal to traverse the beam and enter the goal box was recorded as beam-walking latency. Animals subjected to moderate-severe BINT displayed significantly higher latencies in the beam-walking test when compared to shams on day three post-injury ($p < 0.05$).

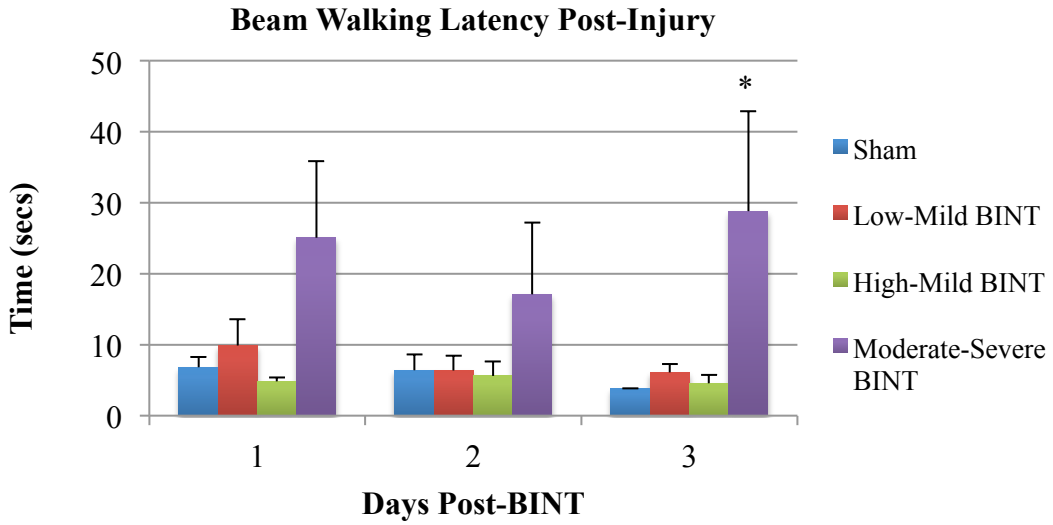


Figure 9. Beam-walking Latency Following BINT. Two-way ANOVA indicated a significant increase in beam-walking latency on post-injury days 1-3 (P value = 0.0016). Using the Bonferroni posttest for multiple comparisons revealed a significant increase in balance time of Sham vs. Moderate-Severe BINT animals on day 3 post-injury (*, P value <0.05). All values are represented as mean ± SEM. Sham n=4, Low-Mild BINT n=7, High-Mild BINT n=5 and Moderate-Severe BINT n=7.

Minor Neurological Deficits as a Result of BINT

A short neuroscore was used to detect potential neurological deficits as a result of BINT. Five tests were performed everyday, beginning two days prior to sham or blast injury and lasting until post-injury day three. The tests were designed to reveal deficits in forelimb and hindlimb flexion, visual and contact triggered placement and hindpaw grasping ability. The contact triggered placement test revealed abnormal responses in all of the BINT injured animals. Low-mild and high-mild BINT animals displayed deficits on days two and three post-injury while the moderate-severe BINT animals displayed significant deficits on days one, two and three post-injury when compared to shams ($p < 0.05$, $p < 0.01$, $p < 0.01$).

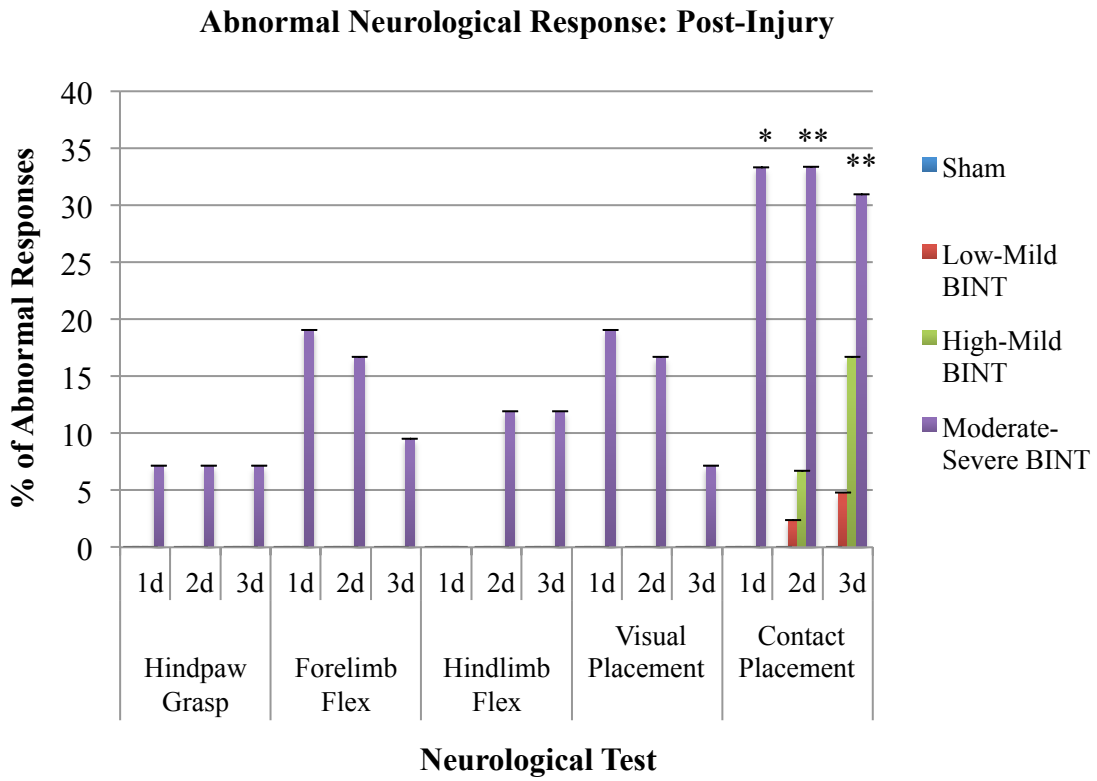


Figure 10. Neurological Assessment Following BINT. Two-way ANOVA of the results of a short neurological assessment on day 1 post-injury revealed significant increase in abnormal response due to injury level (P value = 0.0042), a significant increase in abnormal response on day 2 (P value = 0.0001) and day 3 (P value = 0.0026). On post-injury day 3, there was also a significant variation among test type (P value = 0.0209). Using the Bonferroni posttest for multiple comparisons, the contact triggered placement test revealed significant variation between Sham vs. Moderate-Severe BINT animals on post-injury day 1 (*, P < 0.05), day 2 (**, P value < 0.01) and day 3 (**, P value < 0.01). All values are represented as mean ± SEM. Sham n=4, Low-Mild BINT n=7, High-Mild BINT n=5 and Moderate-Severe BINT n=7.

Effects of BINT on Acute Morris Water Maze Performance

Animals were tested for deficits in spatial learning and working-memory using the Morris water maze (MWM). On days one, two and three post-injury, both sham and high-mild, blast-injured animals were tested using a spatial learning and working-memory paradigm for the MWM, which is described in detail in the methods section.

This paradigm utilizes a location-match system with four trials per day, each with a new starting point and new platform location. Animals were placed in a starting quadrant and allowed to swim until they found the submerged platform, or until 120 sec had passed. After a brief intertrial interval, the animal was placed in the same starting quadrant and allowed to find the platform, also in the same location as the previous trial. The time it took the animal to locate the platform on the second trial was recorded as latency. On post-injury days one and two, high-mild BINT animals exhibited significantly longer latencies than sham-injured animals ($p = 0.0353$, $p = 0.0045$).

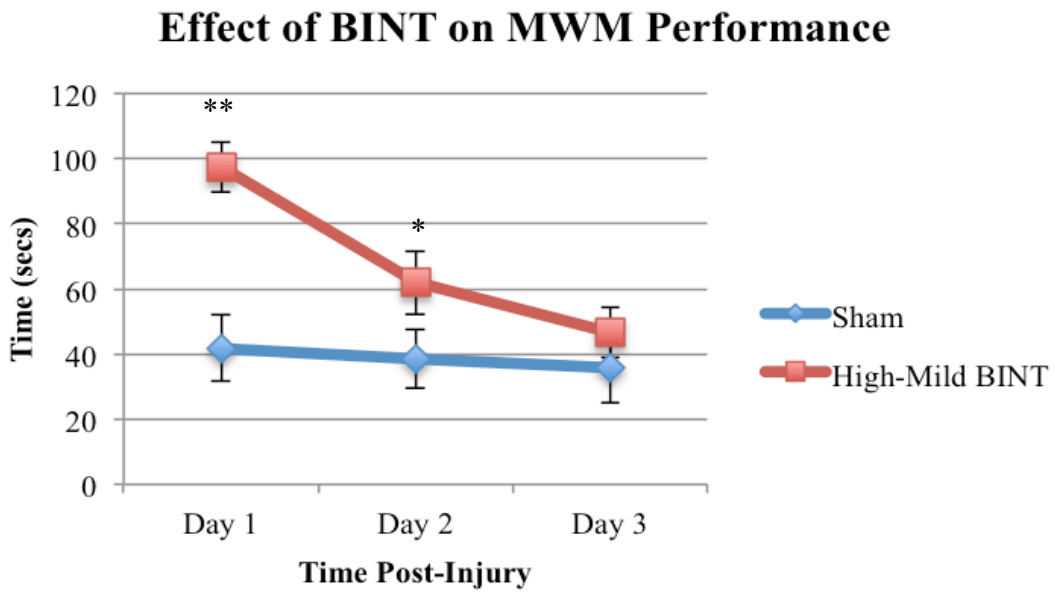


Figure 11. MWM Performance Following BINT. Sham and BINT animals were tested for deficits in spatial working memory and learning on post-injury days 1-3. An unpaired t test with Welch's correction indicated a significant (**, P value = 0.0045) increase in latency on day 1 and a significant increase (*, P value = 0.0353) in latency on day 2. All values are presented as mean ± SEM. Sham n=4, BINT n=16.

DISCUSSION

Our beam balance results reveal a significant increase in score ($p < 0.001$), with higher scores indicating increased difficulty balancing) for animals that receive either high-mild or moderate-severe levels of BINT at 24 hrs and 72 hrs post-injury compared to shams. Animals receiving a low-mild BINT do not display a significant increase in beam balance score when compared to sham-injured animals.

Beam-walking tests reveal a significantly longer latency ($p < 0.05$) between sham-injured and moderate-severe BINT animals on day three post-injury. Beam-walking latencies were five times greater in moderate-severe animals compared to shams. These results are similar to those found in a recent similar study examining vestibulomotor function in shock tube-blasted rats within 72 hrs of injury (Baalman et al., 2013). Both beam balance and beam-walking scores indicate that BINT results in significant, measurable vestibulomotor deficits.

A brief neurological assessment was employed to identify neurological impairments following blast injury. The forelimb flexion test involves suspending the animal by its tail and observing the posture of the forelimbs. A normal response is to extend the forelimbs toward the table above which they are suspended. An abnormal response would be the extension of one limb, flexing the contralateral limb and spinning towards the side contralateral to the injury (Modo et al., 2000, Markgraf et al., 1992). The contact triggered placement task tests the highly sensitive vibrissae of the rat and the response triggered by their stimulation. While holding the animal with forelimbs free from obstruction, the vibrissae are brushed along the edge of a table. A normal response is the extension of the coordinating forelimb towards the source of stimulation. An abnormal response is the extension of the contralateral forelimb or no response at all. The forelimb flexion test is used in other models of experimental brain injury and is sometimes referred to as the forelimb-placing test (Hua et al., 2002, Freret et al., 2006,

Leasure and Shallert, 2004, Schaar et al., 2010). Results of the neurological assessment indicate that moderate-severe BINT produces significant impairment in the contact triggered placement test 24 hrs ($p < 0.05$) and 48-72 hrs ($p < 0.01$) post-injury. Both low-mild and high-mild BINT animals demonstrate neurological deficit in the contact triggered placement test 48 and 72 hrs post-injury but these results are not significant when compared to sham-injured animals. The number of abnormal responses to neurological stimuli increases as injury level increases, indicating a direct association between BINT and neurologic impairment.

Morris water maze tests revealed that BINT animals required more time to locate the platform in match trials compared to sham-injured animals, indicating impairment in their ability to use spatial cues to match platform location from the previous location trial. In a recent study by Budde et al., animals subjected to primary blast exposure were tested with a visuo-spatial learning MWM paradigm on days one through four post-injury (Budde et al., 2013). Their results indicate a significant increase in latencies of blast-injured animals compared to sham-injured animals, with a main effect of blast severity of $p < 0.019$ (Budde et al., 2013). These results are similar to our findings and are consistent with other experimental investigations of shockwave-induced TBI (Kovesdi et al., 2012, Kwon et al., 2011).

Our data indicate that a combined primary and tertiary blast injury results in neurological, sensorimotor, vestibulomotor and spatial learning and working-memory deficits for at least 72 hours following injury. Gross examination of the brains from the EB extravasation study indicate that the most frequent regions of increases in EB extravasation are the cingulum, primary and secondary motor cortex, primary and secondary somatosensory cortex, hippocampus, corpus callosum and striatum. A correlation may exist between damage to sensorimotor cortex and hippocampus, and deficits in motor function and cognition (Liu et al., 2014). Moderate CCI studies by Hamm et al., show an injury effect up to three days post-injury in both beam-walking and

beam balance tasks, which is similar to our results (Hamm et al., 1992). Complex motor pathways and sensorimotor integration are commonly altered or damaged in TBI, resulting in behavior deficits (Fujimoto et al., 2004). Data from our permeability study and behavior analyses validate our hypothesis that increases in BBB permeability and behavior deficits are both associated with BINT, with increases in permeability potentially impacting behavior.

Chapter 6: Reactive Nitrogen Species

INTRODUCTION

In addition to edema, BBB leakage can lead to further complications for the TBI patient. Oxidative stress, inflammation and cell signaling pathways are disrupted as a result of a compromised BBB. The main function of the BBB is to protect the brain microenvironment from the potentially harmful increase of reactive oxygen- or nitrogen species (ROS/RNS), inflammatory mediators or invading immune cells. ROS/RNS production increases after injury, as part of the normal function of recruiting specific cell types, such as leukocytes or platelets, to the injury site for repair. In excess, ROS/RNS enhances oxidative stress, which is linked to cognitive decline associated with neurodegenerative diseases like dementia. After injury, formation of the powerful oxidant, peroxynitrite ($OONO^-$), is of particular concern. In the event of increased oxidative stress and inflammation, an abundance of nitric oxide radicals and superoxide radicals ($\bullet O_2^-$) combine to produce peroxynitrite. Increased production of peroxynitrite adjacent to the neurovascular unit may contribute to further dysfunction of the BBB.

Using scavengers to reduce radicals limits the formation of reactive species, thereby attenuating oxidative damage by ROS/RNS. It is evident that penicillamine is a capable peroxynitrite scavenger, as it reacts stoichiometrically with peroxynitrite (Fig. 12) (Althaus et al., 2000). In a mouse study of severe diffuse TBI, penicillamine treatment after injury was reduced protein nitration, evidenced by 3-NT staining, and improved grip scores (Hall et al., 1999). Not only does penicillamine reduce nitration of proteins, such as nitrotyrosine, but it is implicated as a protectant of brain mitochondria after neural injury (Singh et al., 2007). Singh et al., investigated the effects of peroxynitrite scavengers, including penicillamine, to protect against the effects of peroxynitrite (Singh et al., 2007). Applied to isolated brain mitochondria, SIN-1 (3-

morpholinosydnonimine), a peroxynitrite donor, results in increased lipid peroxidation as well as increased protein nitration. Penicillamine treatment provided partial but significant ($p < 0.05$) protection against these effects of the peroxynitrite donor. As a known, effective peroxynitrite scavenger, penicillamine is a good candidate for the present blast studies.

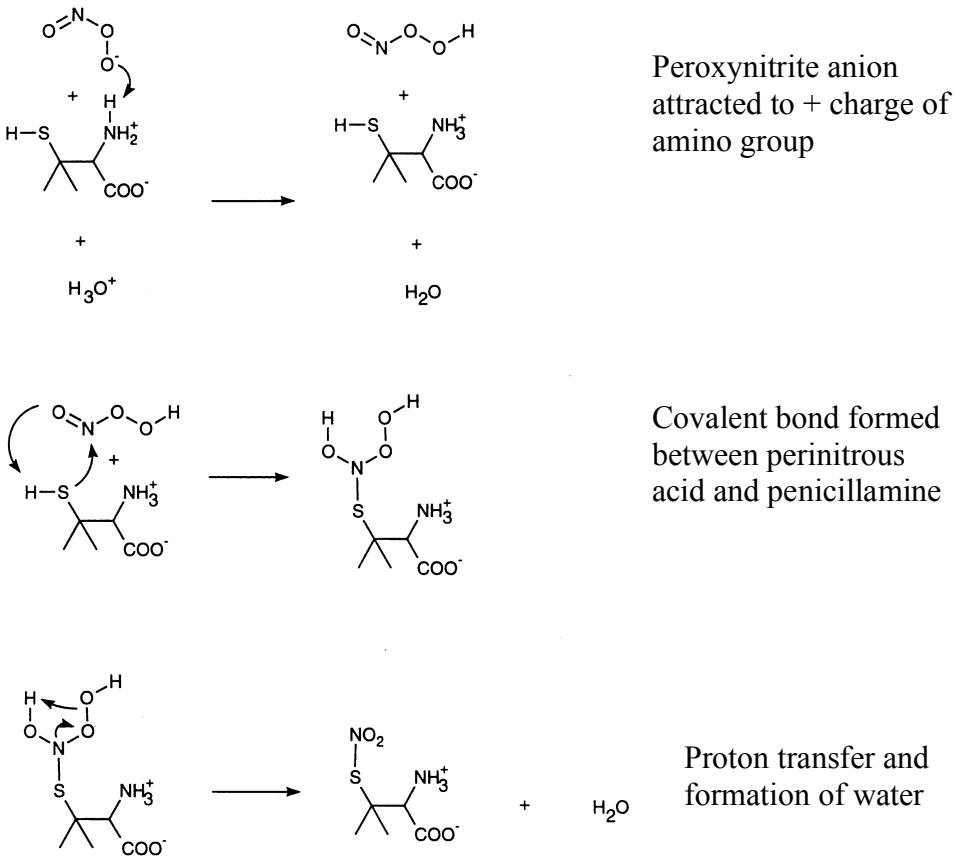


Figure 12. Penicillamine Scavenging Peroxynitrite. The reaction mechanism by which penicillamine scavenges peroxynitrite. This is a stoichiometric reaction with peroxynitrite (1:1). (Hall et al., 1999)

To determine the effects of BINT on peroxynitrite production and the role it plays in the increase in BBB permeability we immunolabeled blast-injured tissue for the presence of tyrosine nitration, a selective marker of peroxynitrite (Beckman, 1993,

Beckman, 1991), and imaged tissue sections of penicillamine treated animals on the IVIS (*in vivo* imaging system) for EB extravasation. EB extravasation regions were quantitatively measured and reported as total radiant efficiency means ± SEM. Statistical analysis of the data was performed using a two-way ANOVA.

MATERIALS AND METHODS

Methods applied in the experiments of this chapter are reported in chapter 2.

RESULTS

Peroxynitrite Formation Increases Following BINT

Tissue sections from animals subjected to either sham or blast injury were taken 24 hrs after injury and stained for immunohistochemical analysis with monoclonal mouse anti-nitrotyrosine antibody (1:200, Millipore). Quantitative analysis was performed on the injury site cortex in sham and blast-injured tissue sections (representative images are in Fig. 13). Two, uniform regions of interest were measured in each section, at the injury site for mean fluorescence intensity using analytical software (ImageJ2) and indicated a significantly increased intensity (P value = 0.0041) in BINT tissue samples when compared to sham tissue samples. Results were statistically analyzed using an unpaired t test with Welch's correction.

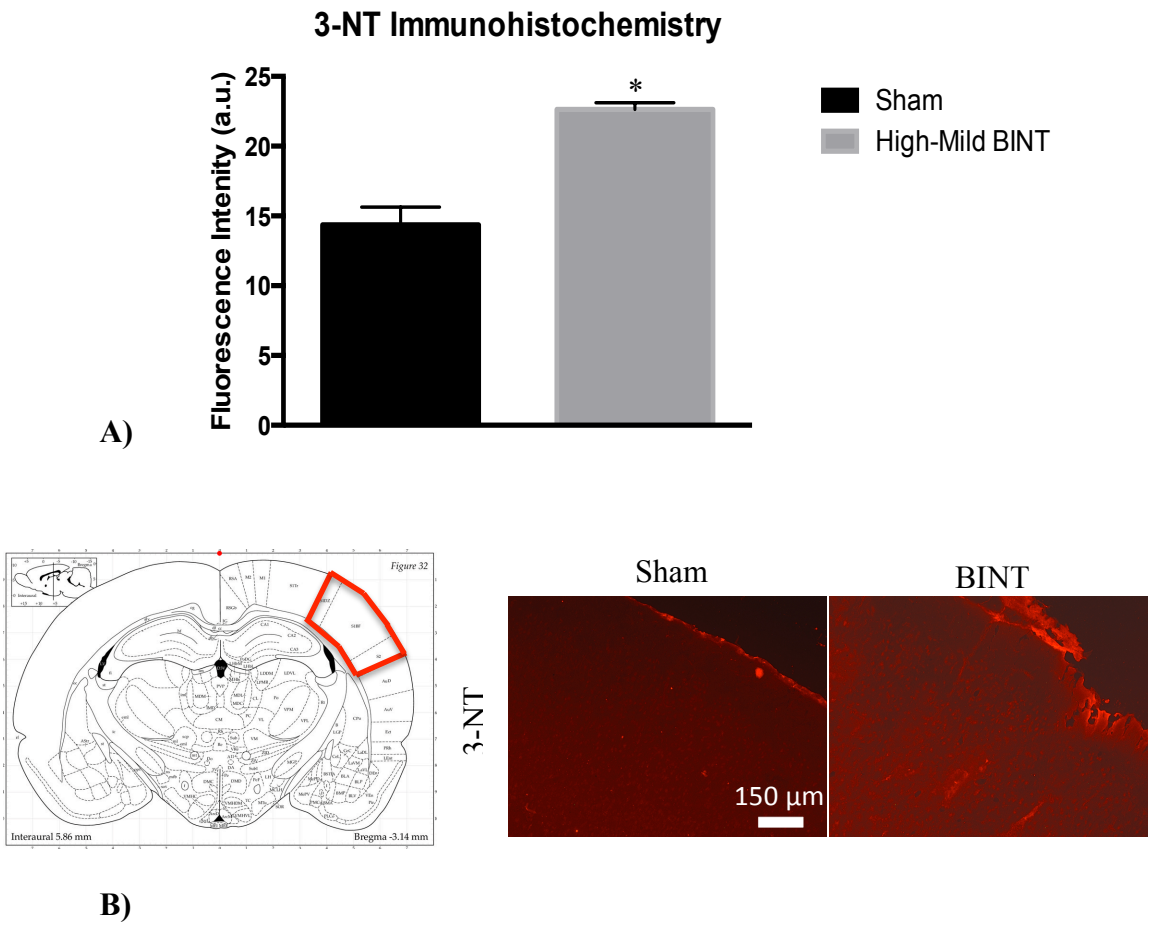
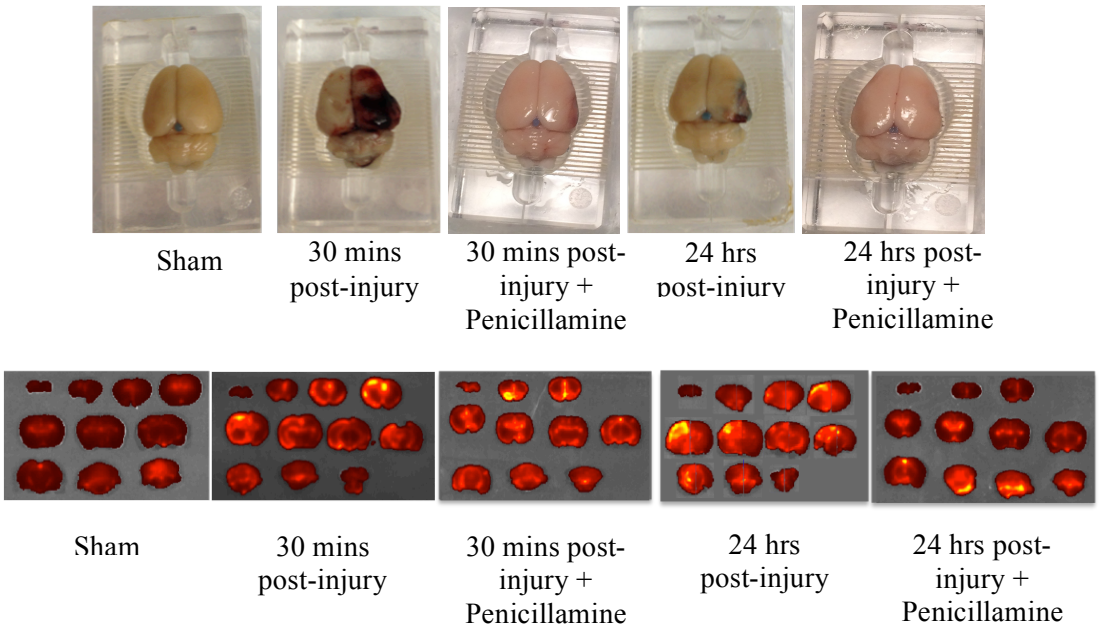


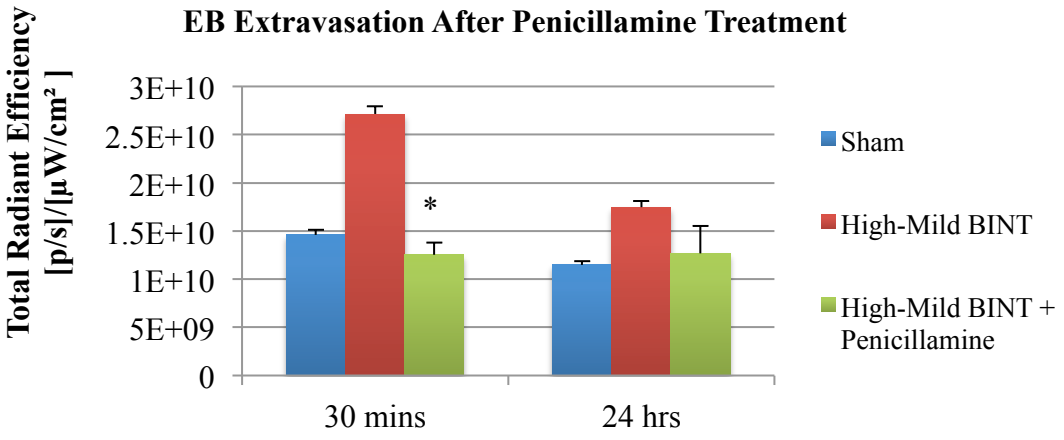
Figure 13. Increased Nitrotyrosine Immunoreactivity at Injury Site. Fluorescence intensity was measured in tissue sections from both sham and high-mild BINT animals at 24 hrs using ImageJ2 software. Two uniform regions of interest were randomly selected and intensity measured. Results are shown as mean ± SEM. (Sham, n=2; High-Mild BINT, n=6). Unpaired t test with Welch's correction revealed fluorescence intensity was significantly increased (*) after high-mild BINT when compared to sham tissues (*, P value = 0.0041)(A). At 24 hrs after sham or blast injury, immunoreactivity to 3-NT antibody was increased in the cortex adjacent to the injury site. Images are representative of 10X magnification of tissue sections used for quantification (B). Brain regions examined for immunoreactivity are highlighted in red in the coronal section diagram (B). All sections stained for IHC with 3-NT primary antibody (1:200, Millipore).

Penicillamine Attenuates BINT-related BBB Permeability Increases

Peroxynitrite scavenger, penicillamine, was intravenously administered to both sham and BINT animals at 30 mins or 24 hrs post-injury (10 mg/kg). Treatment with penicillamine was effective in diminishing the blast-induced increase in BBB permeability, as was obvious in both gross visual assessment and high-resolution IVIS (*in vivo* imaging system) scans of tissue sections from sham and blast-injured animals, receiving either vehicle or penicillamine injections five minutes after injury (Fig. 14).



A)



B)

Figure 14. Penicillamine Treatment Attenuates Post-BINT Evans Blue Extravasation. Five minutes after sham- or blast-injury, animals were injected with either vehicle or penicillamine treatments. At 30 mins or 24 hrs post-injury, the animal was injected with 2% EB (4 mg/kg) which circulated for 1 hour. Brains were scanned with the IVIS at 640 nm excitation, 680 nm emission to determine the quantity of EB extravasation (A). Using a two-way ANOVA to analyze the data, the treated BINT group exhibited an extremely significant variance of EB extravasation at 30 minutes post-injury when compared to untreated, BINT animals (B) (*, $p < 0.0001$).

DISCUSSION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) production is part of the acute response to CNS injury, occurring within minutes of mechanical impact (Bains and Hall, 2011, Deng et al., 2007). Immediately following the primary injury, ion homeostasis, mitochondrial function and microvascular function become disrupted and glutamate excitotoxicity develops as part of the secondary injury cascades (Bains and Hall, 2011). Oxidative damage, another characteristic process of CNS injury, is initiated by the rapid formation of free radicals in response to and perpetuated by secondary injury cascades. Peroxynitrite generated by the microvascular system is a key component of the early phase of TBI-induced oxidative pathophysiology and may contribute to the damage generated by secondary CNS injury (Hall et al., 1993, Bains and Hall, 2011, Saran et al., 1990).

Oxidative stress is a result of an accumulation of ROS exceeding the available antioxidants, causing function-altering modification of nucleic acids, proteins and lipids, resulting in irreversible cellular damage. A transient increase of superoxide radical occurs within minutes of injury in experimental rodent TBI models (Hall et al., 1993, Hall et al., 1994). Endothelial, neuronal and inducible NOS are all up regulated during the first 24 hrs following non-blast TBI (Gahm et al., 2000, Cobbs et al., 1997, Rao et al., 1999), however, it is not yet known if this is true for BINT. Up-regulation of NOS is a result of increased production of both superoxide and nitric oxide, thereby creating an ideal environment for peroxynitrite production. Increased peroxynitrite is detected as early as one hour and lasting as long as several days following non-blast experimental rodent TBI, as indicated by the quantification of tyrosine nitration, a commonly accepted method for detecting peroxynitrite activity (Hall et al., 2004, Hall et al., 1999, Hall et al., 2010, Bayir et al., 2005, Mesenge et al., 1998). TBI-induced increases in brain levels of nitrotyrosine are decreased when nitric oxide synthase inhibitors are administered,

producing a therapeutic effect that is associated with attenuation of peroxynitrite generation (Mesenge et al., 1998). To decrease damage associated with peroxynitrite, a species-specific scavenger may be used. In the present study, penicillamine was chosen because we had preliminary evidence of BBB damage and the presence of ROS/RNS. While penicillamine is incapable of penetrating an intact BBB (Hall et al., 2004), BINT-related BBB dysfunction provides an opportunity to administer penicillamine effectively. The exact mechanism of scavenging peroxynitrite with penicillamine is shown in Fig. 12.

In the present study, quantitative assessment of 3-NT immunolabeled tissue sections indicates that BINT enhances peroxynitrite production. Increased 3-NT immunoreactivity is visible in the cortex and hippocampus adjacent to the injury site at 30 mins and 24 hrs after injury. Treatment with penicillamine, a peroxynitrite scavenger, attenuates increased 3-NT immunoreactivity, as evidenced in BINT + penicillamine treated animals at 30 mins and 24 hrs post-injury. All reported increases in 3-NT immunoreactivity are based upon comparison to naïve and sham + penicillamine animals, 30 mins and 24 hrs post-injury. While the results are subjective in nature, they are sufficient to warrant testing the effect of penicillamine on blast-induced increases in BBB permeability. Using the IVIS, we scanned sections of brain tissues from animals in each of the four groups (sham, sham + penicillamine, BINT, BINT + penicillamine) and animals each received EB injections at either 30 mins or 24 hrs post-injury. The IVIS scans revealed a significant decrease in EB extravasation in BINT + penicillamine animals when compared to sham animals that were perfused with EB at 30 mins post-injury ($p < 0.0001$). Decreased EB extravasation was also apparent in the 24 hr BINT + penicillamine group, however it was not statistically significant when compared to sham animals. Our data suggests a correlation between blast-induced peroxynitrite formation and BBB permeability. Oxidative stress-induced microvascular damage potentially weakens cerebral vasculature, allowing increased permeability by paracellular passage or reduced restriction of molecules via transcytosis.

To further elucidate the role of peroxynitrite following BINT, additional studies are required. While immunohistochemical analysis offers information regarding localization of enhanced nitrotyrosine staining, more sensitive methods of protein quantification should be performed. Western blot analysis of tissue samples probed with anti-nitrotyrosine primary antibody at the selected time points will reveal fluctuations in nitrotyrosine levels, thereby revealing fluctuations in peroxynitrite production. Western blot results may also indicate more appropriate time points to test for penicillamine efficacy after BINT.

Chapter 7: Conclusions and Future Studies

The recent military operations in Iraq and Afghanistan demand significant focus on mTBI as nearly half of all injured soldiers are exposed to blast(s) and subsequently experience mTBI; hence mTBI has been termed the "signature wound" of these operations (Martin et al., 2008, Hoge et al.). Injured personnel often do not exhibit physical signs of injury and are redeployed into the field, potentially experiencing additional blast exposure and aggravating an existing condition (Hoge et al., 2008a, Hoge, 2014, Vanderploeg et al., 2012). Consquently, soldiers with undiagnosed injuries and self-medicate with drugs and/or alcohol, masking and further delaying proper diagnosis and treatment for BINT and mental health issues (Santiago et al., 2010, Wilk et al., 2010, Otis et al., 2011). The high comorbidity of BINT and PTSD complicates attempts to study BINT and PTSD independently. The combination of psychological and physiological stressors contribute strongly to the development of cognitive and behavior changes in chronic BINT patients, observed as short-term learning and memory deficits in animal blast models (Kamnaksh et al., 2012, Trudeau et al., 1998, VandeVord et al., 2012).

Few individuals experience isolated primary blast injury, thus, the generation of a combined injury animal model may be more relevant in producing a complex polytrauma that is clinically relevant (Hicks et al., 2010). Unfortunately, the field of blast injury research has yet to agree upon a standard model of experimental BINT, producing great variation within the literature and complicating comparisons. Shock and blast tubes are the most commonly used experimental BINT models. These devices are used to study primary blast injury, although one could argue that subjects are exposed to multiple wave exposure rather than a single blast wave that is eliminated at the end of the tube, a key

factor in model design. Generally, shock and blast tubes are cylindrical in shape with a compressed-gas driver on one end and an opening on the opposite end. Because open-field blasts expand in a hemispherical shape, the cross section of the hemisphere is conical. The conical geometry correlates to the natural expansion of a blast wave, which has a 1° angle of expansion. Without a proper expansion angle, the wave cannot expand freely and will produce reflection waves at various points along the length of tube. Open-end tubes also produce inverted waves that then travel back up the tube, passing the animal a second time. As time passes, waves moving within the tube will interact with one another, producing additional waves. It may be more accurate to say that tubes of this nature produce exposure to primary blast wave and various reflected waves, which is more similar to blast exposure within a structure rather than an open-field blast. These dynamics are more thoroughly described by Svetlov et al., elsewhere (Svetlov et al., 2010), however, this brief description serves as evidence of the importance of properly designing and characterizing experimental BINT devices.

The difference between blast or shock tubes and the VDB device used in the present study is that the VDB-blasted animal experiences a single blast wave exposure. Because the VDB contains the combustion and generation of the shock wave within the chamber of the device, the animal is only exposed to the wave that emits from the end of the device barrel thus eliminating the opportunity for the animal to be subjected to multiple reflection waves within the barrel. A single, head-only blast exposure is unique to the VDB device. In addition to the primary blast injury generated by the blast wave, a tertiary blast injury is produced by the venting gas jet. The gases that are released from the end of the VDB barrel are high pressure and are focused by the small opening of the barrel (0.27 in). The focused jet of gas produces an impact similar to that of a blunt object impact, producing a tertiary blast injury (Svetlov et al., 2010). In preliminary experiments performed for VDB characterization, hallmarks of BINT pathophysiology were produced. Cerebral vascular injury such as CBF suppression, transient decrease in

MAP, impaired pressure autoregulation and neurobehavioral deficits are evidence that the VDB rodent model reproducibly generates graded levels of primary and tertiary blast injury.

Composed of specialized brain endothelial cells, the cerebral vasculature is the main line of defense in protecting the sensitive homeostasis of the brain from blood-borne molecules. The endothelial cells, however, do not work alone. Supporting neural cells (astrocytes, pericytes and glia) and neurons comprise the neurovascular unit, interacting with the endothelium to achieve and maintain a tightly regulated barrier (Abbott et al., 2006, Abbott et al., 2010). In healthy individuals, the BBB is actively engaged in maintaining the separation of brain and circulation through active and passively mediated transport. In injury and disease, the BBB becomes, allowing passage of a variety of immune cells and blood-borne pathogens into the parenchyma. TBI is known to produce cerebral vascular dysfunction, thus causing the brain to be more vulnerable to secondary injury and increased mortality and morbidity (DeWitt and Prough, 2003).

Increased permeability of the BBB is reported as early as minutes after blast injury and lasting as long as several days (Readnower et al., 2010a, Skotak et al., 2013, McCabe et al., 2014, Yeoh et al., 2013). Non-blast, TBI-induced BBB impairment results in edema, which leads to increased ICP and suppression of cerebral perfusion, eventually resulting in secondary injury (Unterberg et al., 2004). The present permeability studies were performed to elucidate temporal BBB permeability changes following mild BINT. Some have reported BBB permeability increases as early as 30 mins (Whalen et al., 1999) while others witness a slightly delayed increase in permeability around 3 hrs (Readnower et al., 2010a). A biphasic opening of the BBB has also been reported following CCI experiments, indicating an initial opening at 4-6 hrs and a second opening at 3 days post-injury(Baskaya et al., 1997), however, our experiments indicate a significant increase in BBB permeability at 30 mins, 120 mins, 24 hrs and 3

days post-BINT. While there is a significant increase in permeability during the first 72 hrs, there is a trending decrease at each time point after 30 mins (Fig. 13). Reasons for an increase in BBB permeability are varied. Readnower et al., observed an increase in BBB permeability following exposure to blast overpressure at 3 and 24 hrs, which they proposed is due in part to heightened inflammatory response (Readnower et al., 2010a). A Baskaya CCI study revealed a biphasic opening of the BBB with the first peak at 4-6 hrs and the second on post-injury day three. They concluded that the openings of the BBB may be the result of either acute ischemia or physical tearing of microvessels and disruption of endothelial membranes (Baskaya et al., 1997). In another CCI study, Whalen et al., observed an increase in BBB permeability starting at 30 mins post-injury and recovery by 4 hrs with a peak of inflammation and white blood cell accumulation at 24 hrs (Whalen et al., 1999). Contrary to other studies, Whalen concluded that the observed increase in BBB permeability did not correlate to an increase in inflammation at 24 hrs. Knowledge of when the BBB begins to breakdown would allow for interventions to prevent additional loss of BBB integrity, potentially limiting the damage of secondary injuries such as edema and ischemia/hypoxia. We know that injured microvessels activate a coagulation cascade, leading to the aggregation of platelets and leukocytes which may slow blood flow in pericontusional areas (von Oettingen et al., 2002, Schwarzmaier et al., 2010). Our CBF monitoring data and BBB permeability results support the idea that blast-injury results in almost immediate disruption of the BBB and suppression of CBF, potentially setting the stage for secondary injury.

The junctional assemblies of brain endothelium have been shown to be critical in the maintenance of BBB integrity. As one of the major intercellular cleft-spanning proteins, occludin plays a significant role in providing the tightness that is characteristic of BBB tight junctions. Animal studies have demonstrated that increased BBB permeability may be brought on by inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β), degrading tight junction proteins, including

occludin (Liu et al., 2012). A previous preliminary immunohistochemical analysis of our BINT rat brain tissue indicated the localization of IL-1β in both microglia and astrocytes of the hippocampus at 12 hrs and 72 hrs post-injury, which do not express IL-1β endogenously. An inflammatory response as early as 12 hrs post-injury is consistent with reports from other TBI studies. The stereological analysis of brain tissues immunolabeled for occludin revealed little variation between sham and blast-injured animals. The blood vessels in the cortex and hippocampus adjacent to the injury site showed no significant difference in immunoreactivity to occludin at any of the predetermined time points. As mentioned previously, it is possible to have an increase in BBB permeability without affecting the amount of tight junction proteins present. The junction assembly may exhibit changes, such as junction disassembly and cytoskeletal reorganization, due to the phosphorylation states of proteins like occludin and the associated proteins of ZO-1, ZO-2 or ZO-3. Such changes may result in increased paracellular passage of larger, blood-borne molecules.

The neurologic and behavior deficits we observed in BINT animals have not yet been reported in a similar model. Beam balance and beam-walking tasks revealed deficiencies in BINT animals with increased difficulty in traversing the beam as injury level increased. The same effect was seen in beam balance tests. Animals with BINT were less capable of balancing on the beam and, in more severely injured animals, incapable of staying on the beam at all. These findings suggest that the increased BBB permeability in the motor and somatosensory cortex may indeed have an impact on the functional abilities associated with these regions. Based upon the neuroscore we used, BINT animals displayed deficits in the contact triggered placement task, which appeared to be dependent upon injury severity. These results were not surprising considering that the injury site correlates to the somatosensory cortex for the barrel field. The brushing of the whiskers on the table may not be processed properly with damage to this cortical area, causing the stimulation of the barrel receptors not to elicit the normal response. An early

study of blast injury in rhesus monkeys showed memory and performance deficits (Bogo et al., 1971). Blast-related impairments in MWM performance have been shown in animal studies with moderate blast overpressure exposure (Long et al., 2009). Motor and sensory function deficits were seen in rats exposed to mild blast overpressures (Moochhala et al., 2004, Saljo et al., 2010).

Oxidative stress has been shown to be a key factor in secondary injury after TBI. In other models of TBI, a rapid increase in 4-hydroxynonenal (4-HNE), a lipid peroxidation marker, and 3-nitrotyrosine (3-NT), a protein nitration marker, has been reported as early as 30 mins after injury and returning to control levels by 24 hrs (Hall et al., 2004, Deng et al., 2007). Readnower et al., reported significant increases in 4-HNE and 3-NT at 3 hrs post-BINT which returned to near control values by 24 hrs (Readnower et al., 2010a). Similar to these findings, we saw an increase in 3-NT immunoreactivity at 30 mins and 24 hrs post-BINT in both the hippocampus and cortex adjacent to the injury site (Fig.20). The presence of peroxynitrite at these time points suggests that mild BINT is sufficient to cause oxidative stress similar to that found in other TBI models. The effects of excessive peroxynitrite, and likely other ROS/RNS, are underway in the acute phase of injury and are likely contributing to the impairment of vascular permeability, pressure autoregulation and behavior that we observed at later time points. The results from the penicillamine study show a significant decrease in BBB permeability at 30 mins post-injury when penicillamine is administered five minutes after injury. This effect was not seen in control animals or BINT animals that were given a vehicle injection. These results suggest that peroxynitrite does indeed play a role in the breakdown of the BBB. By scavenging some of the peroxynitrite generated as a response to blast injury, we were able to demonstrate that the BBB damage seen immediately after blast injury was directly associated to the presence of peroxynitrite. This conclusion points to vascular or perivascular peroxynitrite production as a key contributor to the observed increase in

BBB permeability. This mechanism has been observed in both mouse (Hall et al., 2004, Mesenge et al., 1998) and rat models of TBI (Coeroli et al., 1998).

Future studies of the effects of BINT on the BBB should focus on defining the size of molecules to which the barrier is permeable and a time-course that coordinates to changes in the sizes of passable molecules. Additional studies should also be performed to investigate the link between increased permeability of the BBB and neurobehavioral deficits. Further investigation of the role of peroxynitrite after BINT should also be considered, specifically, mechanisms by which ROS/RNS scavengers attenuate BBB permeability. Blast injury will continue to be a relevant area of research for both military personnel and civilians, as there is continuous military unrest all over the world (MacDonald et al., 2014).

Appendix A Development of a Rat Model of Blast-Induced Neurotrauma

EPIDEMIOLOGY OF TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI), as defined by the National Institute of Neurological Disease and Stroke (NINDS), is an acquired brain injury resulting from a sudden trauma to the brain (Vital, 2002). TBI is among the most prevalent causes for disability and death each year. In the United States, 1.7 million TBI cases occur each year with over 52,000 of those cases resulting in death (Faul et al., 2010a). The main risk factors for TBI are age, gender and socioeconomic status with the rate of TBI being highest amongst young males and the elderly (Bruns and Hauser, 2003). Accounting for nearly 85% of the 1.7 million TBI cases annually, mild traumatic brain injury (mTBI) has a significant impact on public health (Levin and Robertson, 2013). Of all reported TBI cases, the most common causes were falls (28%), motor-vehicle accidents (19%) and assaults (11%) (DeCuypere and Klimo, 2012). Of those hospitalized and discharged for acute TBI, 43% will develop long-term disability (Ma et al., 2014a, Rutland-Brown et al., 2006).

In the past decade, mTBI has become a focus of intensive research, consequently due to the rise in sports-related concussions and a relatively new group of mTBI patients, returning military personnel who have been subjected to blast exposure during deployment for Operations Enduring Freedom and Iraqi Freedom (Stuhmiller, 2010). The estimated total cost of TBI in 2013 was $78.1 billion, proving to be a substantial financial burden and ranking third in a review of the eight most common conditions requiring rehabilitation, after back pain at $119-238 billion, and osteoarthritis at $161.8 billion (Ma et al., 2014a). TBI is a new kind of medical crisis and a more troubling

condition than most comprehend. TBI is a chronic condition, beginning with a trauma event and evolving over time as the cascade of pathophysiological responses unfold, inevitably revealing new symptoms and exacerbating others (Masel et al., 2012). TBI needs to be researched and studied at every level, from basic science through rehabilitation and palliative care so that patients and physicians alike can better understand the challenges of living with brain injury.

CLASSIFICATIONS OF TRAUMATIC BRAIN INJURY

TBI can be classified into groups based upon the mechanism of the injury, such as a closed-head or penetrating TBI. A closed-head injury occurs when a blunt-force impacts the head but does not penetrate the skull. This impact may cause an acceleration and deceleration of the brain within the skull, resulting in brain-skull contact at two opposing points, often referred to as a coup-countercoup injury. A penetrating TBI occurs when an object perforates the skull, entering brain tissue (Vital, 2002). In addition to mechanism of injury, TBI can also be classified based upon the presentation and severity of symptoms. The qualifiers mild, moderate or severe TBI indicate the presence of a particular set of symptoms at each distinct injury level. As mentioned previously, mTBI is the most common grade of injury and is often referred to as concussion (Centers for Disease Control and Prevention, 2003). Symptoms of mTBI may include a brief loss of consciousness, headache, nausea and vomiting, difficulty with motor coordination and balance, changes in sleep patterns and difficulty with memory, concentration or attention (Vital, 2002). Moderate and severe TBI encompass a vast range of symptoms, including many of those listed for mTBI, but also longer periods of unconsciousness, aphasia, dysarthria, and longer-lasting changes in cognitive function (Vital, 2002, Wang et al., 2005). While mTBI is less severe than moderate or severe TBI, this level of injury can

manifest with lasting effects such as posttraumatic stress disorder (PTSD) and/or depression, rendering many of these individuals disabled (Macgregor et al., 2010).

Behavior	Response	Score
Eye opening response	Spontaneously	4
	To speech	3
	To pain	2
	No response	1
Best verbal response	Oriented to time, place, person	5
	Confused	4
	Inappropriate words	3
	Incomprehensible sounds	2
	No response	1
Best motor response	Obeys commands	6
	Moves to localized pain	5
	Flexion withdrawal from pain	4
	Abnormal flexion	3
	Abnormal extension	2
	No response	1
Total score		3-15

Table 5: Glasgow Coma Scale The Glasgow Coma Scale (GCS) is one of the assessment tools utilized in presentation of TBI. The GCS is a gross assessment of neurological function and is very useful in classifying a patient at any stage of intake or recovery (Teasdale and Jennett, 1976).

While the scenarios may seem similar, each TBI is unique due to myriad variables involved in obtaining the injury and the individual himself. This is a well-known,

confounding factor that researchers and clinicians face in the study and treatment of TBI that can be further complicated by difficulty in communication between physicians, therapists and patients. For these reasons, protocols for the assessment and diagnosis of TBI have been established. The Glasgow Coma Scale (GCS) is one such method for the assessment and ranking of TBI patients, providing reliable, standardized information to physicians regarding the severity of each individual injury. The GCS focuses on consciousness immediately and subsequently following a TBI, examining and scoring the patient's abilities in eye opening, verbal response and motor response tasks. Eye opening is scored with a value of 1-4; a score of 1 is given to patients exhibiting no ability to open eyes, while a score of 4 indicates the ability to spontaneously open eyes. Verbal responses are scored on a scale of 1-5; 1 indicates no response and 5 indicates normal conversation. Motor responses are measured as both movement and postural abnormalities on a scale of 1-6; a score of 1 is given for lack of movement and 6 is give for normal movement. The scores from each area of assessment are summated and ranked according to injury severity, as is seen in Table 1. Severe TBI patients have a GCS of 3-8, moderate TBI is a score of 9-12 and mild TBI is scored 13-15. Mild TBI may result in temporary or permanent neurological symptoms but may not show physical damage in CT scans or MRIs. Moderate and severe TBI often results in permanent cognitive and behavioral impairments (Teasdale and Jennett, 1974). Aside from GCS, other methods for examination of head injuries may include loss of consciousness (LOC), alteration of consciousness (AOC) and posttraumatic amnesia (PTA). These three categories of criteria, along with GCS, comprise the standard protocol for acute TBI assessment by Veterans Affairs (VA) and the Department of Defense (DoD) which is shown in Table 2. Mild traumatic brain injury (mTBI) accounts for nearly 1.44 million reported TBI cases annually, making it the most common type of head injury (Levin and Robertson, 2013). While the term 'mild' indicates a less serious injury, as opposed to 'moderate' or 'severe', it may not accurately describe the long-tem consequences of the

injury. According to Veterans Affairs and the Department of Defense, a patient is classified as having mTBI if they have experienced a loss of consciousness (LOC) < 30 minutes, an alteration of consciousness (AOC) for ≤ 24 hours, posttraumatic amnesia for ≤ 24 hours and a Glasgow Coma Score (GCS) of 13-15. There are numerous classification systems and questionnaires for evaluation of acute head injury however the methods utilized by the VA and DoD are especially relevant to the focus of the projects discussed here.

	Glasgow Coma Score (GCS)	**Loss of Consciousness (LOC)**	**Alteration of Consciousness (AOC)**	**Posttraumatic Amnesia (PTA)**
Mild TBI	13-15	0-30 min	≤ 24 hours	≤ 24 hours
Moderate TBI	9-12	30 min – 24 hrs	> 24 hrs *	24 hrs > 1 week
Severe TBI	≤ 8	> 24 hrs	> 24 hrs *	> 1 week

Table 6: VA/DoD Classification of TBI Severity. Widely used methods of assessment for patients suspected of acute TBI examine potential loss of consciousness (LOC), alteration of consciousness (AOC) and posttraumatic amnesia (PTA), based upon presence and duration of symptoms. Table modified from U.S. Department of Veterans Affairs website (Defense, 2009).

BLAST-INDUCED NEUROTRAUMA

Explosive blasts have occurred for the past 2000 years with reports of use in military combat as early as the 1864 Battle of the Crater during the American Civil War

(Stuhmiller, 2010). With many coal miners fighting for the Union in the siege at Petersburg, Virginia, knowledge and experience in using gunpowder explosives to clear areas underground was abundant. Underground explosives were used in an attempt to distract and disassemble the opposing Confederate troops. The battle proved unsuccessful for the Union, however it was later named the Battle of the Crater in reference to the massive pit that was formed by the explosion (Corrigan, 2006). During the Vietnam War fuel-air explosives were utilized to clear large forest areas, structures and personnel. In the early 1980's, it was gas-enhanced explosives that were employed in the conflict between Lebanon and the Israelis. The tactic of using roadside bombing has played a role in many historical conflicts, including Belorussia on the Nazis, Northern Ireland against the British, Afghanistan against Russia, Lebanon and the Israelis and now, amidst the continual strife in the Middle East (Stuhmiller, 2010). Explosive blasts have been a phenomenon observed and studied throughout time, however, serious investigations into understanding the nature and mechanisms of blasts did not arise until World War II and the unveiling of nuclear bombs (Masel et al., 2012).

Prior to modern warfare, injuries sustained by military personnel had much higher rates of mortality, reportedly approaching 90% in cases of TBI (Masel et al., 2012). During World War I, common medical intervention and treatment for TBI called for aggressive debridement and removal of penetrating bone fragments, provided the soldier survived long enough to be transported to a care facility (Cushing, 1918). This approach remained standard throughout the war in Vietnam and was actually the suggested intervention until the technological developments of the 1980's (Hammon, 1971). The use of computerized tomography (CT) for the initial evaluation of combat injuries was first reported in a study of a large group of patients evacuated from the ongoing Lebanese conflict occurring from 1982-1985 (Brandvold, 1990). Brandvold and associates (Brandvold, 1990) observed that preservation of cerebral tissue by limited debridement and leaving penetrating bone fragments in place proved to be unassociated with infection

and seizures upon reevaluation nearly 6 years later. This method soon became the standard operating procedure for acute evaluation and treatment of combat head trauma until further advancements in prevention and treatment arose.

The advancements in personal protective equipment (PPE) and body armor have decreased the risk of penetrating injuries and primary blast injuries, and dramatically increased the rate of survival after blast exposure (Okie, 2005, Hicks et al., 2010, Faul et al., 2010a). However, this increase in survival rates also corresponds to an increase in reports of severe injuries and long-term complications (Cernak and Noble-Haeusslein, 2010). Of the Korean, Vietnam and Persian wars, it was reported that 1 in every 4 injuries was fatal, compared to the recent Operation Enduring Freedom and Operation Iraqi Freedom (OEF/OIF) where 1 in every 10 injuries was fatal (Luethcke et al., 2011b, Leland et al.). It has also been estimated that approximately 20% of wounded military personnel had sustained a head injury (Carey, McCrea et al., 2008). In addition to increased survival due to PPE, much of the increase in TBI cases can be attributed to the use of improvised explosive devices (IEDs). The use of IEDs as a common tactic in modern military and civilian terrorism began in the early 1980's (Stuhmiller et al., 1991). Used as a weapon, IEDs are designed to generate maximum physiological injuries as well as incite the psychological stresses of terror and panic amongst the targeted group (Finkel, 2006b). The Department of Defense has reported that greater than 73% of military casualties during recent OEF/OIF were caused by explosions (Defense Manpower Data Center, 2008) with the majority of combat-related TBIs due specifically to IED explosions (Galarneau et al., 2008). This pervasive use of IEDs during OEF/OIF has resulted in an estimated 15% to 23% of military personnel experiencing TBI (Bogdanova and Verfaellie, 2012). The exact number of cases is difficult to define due to the high incidence of unreported or untreated injuries (McCrea et al., 2008), however estimates indicate that the majority of sustained TBIs were classified as mild (Brenner et al., 2009, Warden and al, Bogdanova and Verfaellie, Warden, 2006).

For these reasons, TBI has become known as the signature injury of modern warfare with blast-induced TBI becoming recognized as a disease distinct from penetrating TBI and other forms of closed-head TBI (Elder and Cristian, 2009, Ling et al., 2009). Many soldiers who have sustained what appears to be a mild injury from blast exposure may exhibit problems with daily functions, including but not limited to irritability, memory deficits, sleep disturbances, increased anger and aggression and exhibit increased potential for developing additional conditions later in life (DePalma et al., 2005).

MECHANISMS OF EXPLOSIVE BLASTS

A single blast may result in instant death, injuries with immediate manifestation of symptoms, or latent injuries, which may manifest over an extended period after the initial blast exposure (Ling et al., 2009). With so much variation from one blast to another, we should closely examine the physical properties and mechanisms of explosive blasts to better understand the physiological repercussions of blast exposure. The dynamic process of an explosive blast occurs when explosive materials are ignited and instantaneously convert the explosive materials into high-pressure and high-temperature gases. The high-pressure gases rapidly expand, acting as a spherical piston, driving the compression wave through the air. The propagating shockwave front increases in pressure, moving away from the fireball, in the direction of the wave movement. The pressure increase at the shockwave front occurs almost instantaneously with a microsecond rise time and is then followed by the expansion phase at 5 atm/msec. This brief pulse of increased air pressure produces a positive pressure blast wave front, which is immediately followed by the suction of a negative pressure front (Owen-Smith, 1981). At velocities greater than the speed of sound, a propagating blast wave creates a front of high pressure that compresses surrounding air – producing positive overpressure- and

rapidly falling to produce negative underpressure. Another important feature of the mechanisms of blast is the duration of the blast wave. The type of explosives used and the distance an object is from the blast origin determines the blast wave duration (Clemedson, 1956). Blast wave duration is defined as the amount of time an object or person is subjected to the effects of over- and underpressures associated with blast (Cernak and Noble-Haeusslein, 2010). Because the blast wave envelops the whole body and interacts with thoracoabdominal organ systems and brain simultaneously, it is capable of producing multiple injuries and eliciting local, systemic and cerebrovascular responses (Cernak et al., 2001a). The injuries sustained by exposure to the blast wave occur in mere milliseconds.

While we know that blast-induced neurotrauma (BINT) is a major concern facing our military and civilian populations, much remains unclear about the mechanisms by which BINT occurs and the acute and long-term effects of this type of TBI. Experimental BINT models have been designed to study various components of blast injury but are so varied that comparison across models and forming conclusions about the experiments performed becomes very difficult. To further complicate our understanding of blast injury is the nature of blast injury itself. Blast is more subtle than of other forms of TBI, both in mechanism and clinical outcome, and occurs much more rapidly than most of the previously studied forms of TBI (Stuhmiller, 2010). Attempting to elucidate a primary injury mechanism that is not mechanical in nature is an entirely new challenge for TBI research, therefore model creation must be even more carefully designed, focusing on specific characteristics of both the injury mechanisms and the resultant clinical pathology. In an effort to better understand blast injury we can begin by classifying the injury into four classes, highlighting the distinct mechanisms and effects of blast. The unique pathology of BINT is presumed to be, at least in part, due to blast wave exposure. Because explosions may cause a variety of injuries, blast injury effects have been categorized as: (1) primary blast injury, caused by the blast wave itself; (2)

secondary injury, caused by the fragments propelled by the explosion; (3) tertiary injury, due to the acceleration and sudden deceleration when the body or part of the body hits the ground or a solid object; and (4) quaternary injury, caused by burns or toxic gas exposure from the burning propellant (Owen-Smith, 1981, Cernak, 2010, Mellor, 1988).

As table 3 indicates, primary BINT is a non-impact brain injury, resulting from exposure to rapid but transient blast overpressure followed by underpressure of lower intensity but longer duration (Cernak et al., 2001c). Primary blast produces barotrauma of air- or fluid-filled organs and tissues (Luethcke et al., 2011a). Exposure to a blast wave passing through an individual's head may cause BINT, presumably by processes such as spallation (disruption that occurs when the compression wave is reflected at the boundary of a less dense medium), gas bubble implosion (the blast wave passes through liquid, compressing gas bubbles which then re-expand explosively)(Phillips, 1986) and inertial effects (lighter tissue is accelerated more than a denser tissue, resulting in stress at the boundary of the two densities) (Cooper et al., 1991, Gorbunov, 2004)). Therefore, the eyes, tympanic membrane of the ears, lungs, brain and spinal cord are vulnerable to these effects and are often damaged in blast exposure (Finkel, 2006b). Debris or shrapnel that is propelled through the air by the explosion causes secondary blast injuries. Because IEDs are crafted to cause the most harm possible, they often contain small metal objects such as nails or ball bearings that become projectiles and act as projectiles, penetrating skin and tissue with ease. Shrapnel and debris are propelled by the blast wind, which immediately follows the blast wave. The force of a blast wind may be of greater magnitude than naturally occurring winds. For example, a hurricane may have a peak overpressure of 1.75 kPa (0.25 psi) and velocity of 200 km/h, capable of picking-up and moving objects in its path (Stuhmiller et al., 1991). A lethal blast, on the other hand, may contain a peak overpressure of 690 kPa (100 psi) and velocity of about 2,414 km/h (Cernak and Noble-Haeusslein, 2010, Owen-Smith, 1981). Soldiers experiencing explosions may be thrown into other objects or structures, resulting in the acceleration

and deceleration of the head similar to what is seen in many civilian TBIs (automobile accidents, falls, etc.) and is considered to be the tertiary mechanism of blast injury (Cernak et al., 1999). Quaternary blast injuries consist of flash burns or damage caused by the inhalation of gases. The subtlety of primary and tertiary blast injuries makes them much more difficult to examine and diagnose. With no visible signs of injury, a person suffering from primary and/or tertiary blast injury may not be aware of the origin of their symptoms or may not believe they are truly injured without physical evidence. This again complicates understanding blast injuries and how to prevent and treat them because they are so frequently unreported.

Category	Definition	Mechanism of Injury	Associated Injuries
Primary	The effect of the blast wave enveloping the body; damage caused solely by blast wave	Shear stress, spallation, cavitation, air emboli	Blast lung; tympanic membrane rupture; laceration to kidneys, liver, spleen; cardiac contusions; brain injury without physical indication.
Secondary	Injury caused by debris or shrapnel from explosion impacting the body	Dependent upon projectile mass and velocity	Penetrating head injury; embedded shrapnel, debris or ballistics; any injury associated with contact of a high-speed object.
Tertiary	Injury by force of blast wind; body is thrown into object(s) or structures.	Blunt impact-like injury	Non-penetrating head injury; concussion; musculoskeletal injury; bruising; swelling.
Quaternary	Burns; injury sustained by inhalation of toxic gases released during explosion.	Burning of body surface or respiratory tract	Asphyxia; various degrees of burned skin; damage to mucosal linings of respiratory tract.

Table 7: Blast Injury Taxonomy. Blast-induced neurotrauma occurs via multiple injury mechanisms. These four types of blast mechanism are commonly used to more accurately describe the type of injuries sustained from blast exposure (Cernak et al., 1999).

Materials and Methods

ANIMALS

The Institutional Animal Care and Use Committee of The University of Texas Medical Branch approved all animal protocols prior to beginning these studies. Adult, male Sprague-Dawley rats weighing 350-500g were used for all of the following experiments. Rats were prepared for blast injury and measurements of laser Doppler perfusion, optoacoustic measurements of oxygen saturation of the superior sagittal sinus (SSS) and mean arterial blood pressure (MAP) as described below. The rat's head was shaved, placed in a stereotaxic frame and the scalp incised in the midline and reflected. Rats were then prepared for the placement of a fiber optic laser Doppler flow probe (see below).

BLAST INJURY

All blast injuries were produced by a Vandenberg (VDB) custom-made blast device, which fires 0.27 caliber blank cartridges used in powder-driven nail guns (Vandenberg Custom, TX). Although this has not been confirmed, we believe that this device produces a combined blast injury; a primary blast injury is produced by the blast wave as it passes over the animal, while the gas venting jet following the blast wave causes an impact-like acceleration/deceleration brain injury.

For each blast injury, the animal was anesthetized, intubated and ventilated on 2% isoflurane. The head was shaved and covered with acoustic coupling gel and a 1/16" silicone spacer to protect the animal from flash burn injury. The ears were plugged, the animal was placed on a piece of dense memory foam and positioned underneath the barrel of the Vandenberg device. A safety switch was toggled and held in the active position and the trigger button depressed to fire the cartridge. The animal was positioned

15 mm below and 5mm to the right of the exit hole in the bottom of the barrel. The injury site of 5mm lateral to midline was chosen to resemble the injury site used in our previous fluid percussion studies (DeWitt et al., 1997)

Injury severity was determined by righting reflex suppression following BINT. By selecting the cartridge size and power (0.27 caliber, #5 charge), and the distance of the animal head from the end of the device barrel (15 mm), we repeatedly produced high mild BINT with suppression of righting reflex lasting between 10 and 16 minutes. We have previously determined that a low mild injury, with righting reflex suppression lasting 6-10 minutes, is produced by using 0.27 caliber blank cartridges with #5 charge and positioning the animal 17mm from the end of the barrel. Positioning the animal 13mm below the end of the barrel and using 0.27 caliber blank cartridges with #5 charge produced a moderate-severe injury, with righting reflex suppression lasting longer than 16 minutes.

LASER DOPPLER FLOWMETRY

Laser Doppler flowmetry (LDF) was used to measure relative cerebral perfusion. Anesthetized rats were surgically prepared for measurement of relative cerebral perfusion as described elsewhere (Haberl et al., 1989). Briefly, rats were anesthetized with 4.0% isoflurane, intubated and mechanically ventilated on 2% isoflurane. The animals were then placed in a stereotaxic frame (Stoelting Co., Wood Dale, IL), the scalp incised in the midline, reflected and the skull lateral to the midline over the left, frontal-parietal cortex was thinned using an air-cooled dental drill (Dremel, Racine, WI). All stereotaxic coordinates were recorded to ensure accurate repositioning after the injury. A fiber-optic needle probe (Perimed, Stockholm, Sweden) was placed over the thinned parietal calvaria. Baseline cerebral perfusion was measured using LDF. After recording baseline

LDF measurements, the incised scalp edges were approximated and fixed together with surgical tape just prior to subjecting the animal to sham injury or BINT.

Immediately following injury, sham-injured and BINT animals were repositioned in the stereotaxic frame at pre-recorded coordinates and the surgical tape removed from the scalp. The LDF probe was repositioned over the previously thinned parietal calvaria. The LDF probe emits monochromatic red light (632.8 nm) that is reflected by erythrocytes moving below the probe. Detectors located in the probe head monitor the power and speed of the reflected signal, both of which are proportional to blood volume and blood velocity, respectively. Red blood cells moving in the area perfused by the probe laser and reflected back to the probe's receiver create a Doppler shift, allowing calculation of blood velocity. By multiplying blood volume by velocity, we can calculate the perfusion in a 1 mm^3 tissue volume beneath the probe (Haberl et al., 1989). Measurements were recorded by a PeriFlux PF3 Laser Doppler Perfusion Monitor (Perimed, Stockholm, Sweden) and values were reported as cerebral blood flow (CBF). In an effort to measure cerebral perfusion as soon after blast injury as possible, righting reflex testing was not performed.

MEAN ARTERIAL BLOOD PRESSURE MONITORING

Animals were anesthetized with 4.0% isoflurane in an anesthetic chamber, intubated and mechanically ventilated with 1.5-2.0% isoflurane in O_2 and room air (70:30) using a volume ventilator (EDCO Scientific, Chapel Hill, NC). A polyethylene cannulae filled with heparinized saline was inserted into the tail artery for continuous monitoring of arterial blood pressure. Rectal temperature was monitored using a telethermometer (Yellow Springs Instruments, Yellow Springs, OH) and maintained using a thermostatically controlled water blanket (Gaymar, Orchard Park, NY). A computer-based physiological monitoring system (BioPac MP150, BioPac Systems, CA)

and analysis software (Acqknowledge, BioPac Systems, CA) were used to record LDF and arterial blood pressure measurements.

MEASUREMENT OF MIDDLE CEREBRAL ARTERY DIAMETER

Immediately following sham or BINT, anesthetized rats were decapitated, brains were removed and the middle cerebral arteries (MCAs) were harvested as described previously (Mathew et al., 1999, DeWitt et al., 2001). Briefly, MCAs were cleaned of connective tissue, mounted on glass micropipettes in an arteriograph (Living Systems Instrumentation, St. Albans, VT) and perfused with physiologic salt solution (PSS). Vessels were pressurized to 50 mm Hg and bathed in PSS for 60 minutes to allow equilibration. Pressure transducers between micropipettes were used to monitor intravascular pressure. Outer arterial diameters were measured with using a video scaler on an inverted microscope. After the equilibration period, intravascular pressure was increased to 100 mmHg. Arterial diameter measurements were made as the intravascular pressures were reduced from 100 to 20 mmHg in 20 mmHg increments.

OPTOACOUSTIC MONITORING OF OXYGEN SATURATION

Baseline and 5-minute incremental measurements were recorded post-injury, rats were anesthetized and cerebral venous oxygen saturation was monitored following sham or Vandenberg BINT. For the acute time-point measurements, animals were prepared according to the procedure described in the Blast Injury section, up to the point of discontinuing isofluorane and testing for a toe-pinch withdrawal reflex. Acute venous oxygen saturation measurements were made at 15, 30, 45, 60 and 120 minutes post injury. These animals remained under anesthesia from the point in which they were prepared for injury until measurements were complete and the animal was sacrificed by

decapitation. Because optoacoustic measurements required placement of the rat in a stereotaxic frame, we did not measure righting reflex suppression. The optoacoustic probe, also mounted to the stereotaxic frame, was positioned over the sagittal sinus of the animal, an area covered with acoustic coupling gel. The animal's skin and skull remained intact, as optoacoustic measurements are completely noninvasive. Positioning coordinates were recorded for accurate repositioning following injury, a baseline recording was taken and the probe was removed for the animal to receive sham injury or BINT. After injury, the optoacoustic probe was repositioned at the previously recorded coordinates over the sagittal sinus and oxygen saturation recordings were taken at the time-points described above. Measurements were taken for up to 2 hours after blast. After measurements were complete the animal was sacrificed by decapitation.

THE VANDENBERG DEVICE (VDB)

Device Properties and Mechanism

The VDB device was designed by Dr. Douglas DeWitt and Mr. Edward Vandenberg and constructed by Vandenberg Custom (Webster, TX) to simulate an explosive blast in an experimental setting. Vandenberg device is based on powder-driven nail guns that fire .22 and .27 caliber nail gun cartridges.

The VDB (Fig. 15) uses an electric solenoid -driven firing pin to strike the rim of the cartridge in the firing chamber, producing a small explosion that generates a blast wave and following hot gas jet flow. The VDB contains a safety switch that must be toggled simultaneously while depressing the trigger switch. Pressure measurements and high-speed video recordings of blasts from this device verify production of a waveform similar to that of the waveform of an open field blast, which will be explained in more detail in a later section. The blast wave is followed by a concentrated jet flow that makes

contact with the animal's head and accelerates it downward into a memory foam bed that decelerates the head and prevents it from rebounding into the device barrel.

Establishing Blast Parameters

Blast wave amplitudes and durations were measured using a pressure transducer positioned perpendicular to the direction of the blast wave. The VDB reliably produced a Friedlander-like waveform (Friedlander, 1946)(Fig. 16), the waveform generated in open-field explosions. Once we verified that the VDB generated a highly reproducible waveform, we tested various materials for their ability to accurately transmit the blast wave while protecting the animal's head from thermal injury during the blast. The first material tested was a self-mixed silicone gel (Silastic, Dow Corning) poured into glass petri dishes to produce discs 1.0 cm thick and approximately 3.5 inches in diameter. After testing several silicone discs, we concluded that the material was not dense enough to prevent penetration of unburned powder and debris. The next material tested was sheet silicone rubber of ¼" thickness that were cut into 3" by 3" squares. While this material prevented particle penetration, the blast wave profile was altered. Finally, we observed that a 1/16" thick sheet of silicone rubber remained impenetrable to debris and still allowed the unmodified passage of the blast waveform.

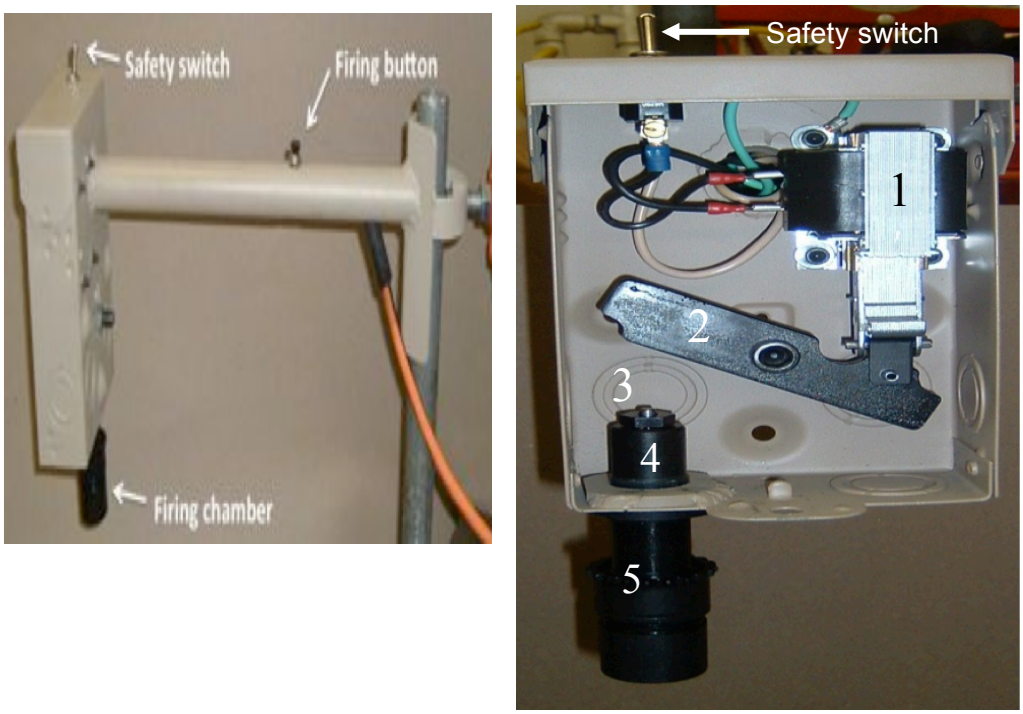


Figure 15. VDB Device and Components. The image on the left indicates the operational firing button and safety toggle switch. The image on the right shows the internal components that work together to produce the blast. Electric solenoid (1), hammer (2), firing pin (3), standing breech (4) and firing chamber (5).

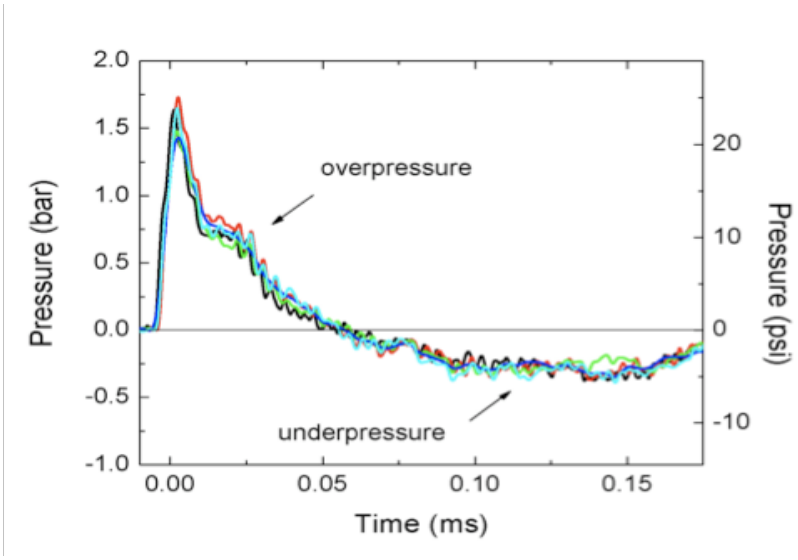


Figure 16. VDB Pressure Waves. Pressure waves generated by the sequential firing of three blank cartridges of the same power levels are nearly identical in shape, amplitude and duration of overpressure and underpressure waves.

Next, we tested the effects of the VDB on dead, adult, male, Sprague-Dawley rats, previously sacrificed in other experiments. This step was necessary to identify the appropriate placement of the animal in relation to the device as well as the logistics of performing blasts with specimens that would be intubated and ventilated during the procedure. During this time we also tested various foam materials that would be placed underneath the rat to counteract acceleration of the head. Commercially available memory foam sufficiently absorbed the impact of the blast and slowed the movement of the animal enough to avoid any rebound injury.

To determine the appropriate method for generating graded levels of injury in live, anesthetized, adult, male Sprague-Dawley rats, we used righting reflex suppression times as a surrogate endpoint. When conscious, rats, like most animals, will immediately assume a prone position on all four legs if placed on their backs. In practice, the rat is placed in a prone position and the time required for the rat to right itself is recorded. The righting reflex is a brainstem reflex that returns prior to thalamocortical function during recovery from unconsciousness due to anesthesia or brain injury (Bignall, 1974). The time required for the return of the righting reflex is a measure of the duration of unconsciousness that is widely used in studies of the effects of anesthetics (Nguyen, et al., 2009) or brain injury (Henninger, et al., 2005; Raghupathi, et al., 2007). There is some inherent variability in all models of experimental brain injury. All models use some type of "input" variable to select the injury level. For example, rats that are randomized to a mild injury group are positioned 15 mm from the end of the firing chamber and injured using a level 5, .27 caliber blank cartridge. However, due to variability in the model, two rats injured using those "input" variables may not sustain equal levels of brain injury. Unfortunately, the differences in the actual brain injury sustained may not be apparent until histopathological or behavioral outcomes are assessed. If the input variables (distance from the firing chamber, cartridge caliber and power level) were the only measures of injury, both rats would be included in the

moderate injury group. However, in this example of two rats receiving "mild" blast injury, one rat may exhibit righting reflex suppression of 15 min, consistent with mild TBI, while the other may recover the righting reflex in 4 minutes, consistent with very mild TBI or no TBI at all since 4 minutes is the average time required for an uninjured rat to recover sufficiently from the effects of the isoflurane anesthesia to right itself. The inclusion of both rats in the mild injury group would significantly increase variability and the numbers of rat needed to achieve statistical significance. Therefore, the goal of measurements of righting reflex suppression is to determine, immediately after injury, whether the rat actually received the desired level of injury. By using the righting reflex as a surrogate endpoint, we can assign the rat with the 15-minute righting reflex suppression to the mild blast injury group and the other to the sham group.

Injury Level	**Distance Below Barrel (cm)**	**Righting Reflex Suppression Range (mins)**
Sham	--	0-6
Low-Mild BINT	1.7	6-10
High-Mild BINT	1.5	10-16
Moderate-Severe BINT	1.3	>16

Table 8: VDB Injury Level Parameters. To create graded blast injuries, righting reflex suppression was used as a surrogate end point. Sham-injured animals typically exhibit a righting reflex suppression that lasts up to 6 minutes. To produce a mild BINT, the range of righting reflex suppression should be 1- to 16 minutes. This suppression range can be achieved when placing the animal head 1.5 cm below the bottom surface of the VDB barrel. This distance produces a mild injury when using the .27 caliber, #5 power cartridge.

Cerebral Vascular Effects of VDB Injury

SUMMARY

TBI often causes cerebral vascular injuries and disruptions such as impaired autoregulation, edema, hypoxia/ischemia, impaired O2 regulation, mitochondrial dysfunction, impaired glucose utilization, elevated intracranial pressure and hypoperfusion (Golding et al., 1999, Bouzat et al., 2013). Understanding the effects of TBI on the cerebral vasculature is important for determining underlying mechanisms involved in both acute and chronic pathology.

The cerebral circulation possesses the ability to regulate vasodilatory and vasoconstrictory mechanisms in response to changes in local and systemic conditions. This discovery was made in 1890 when Roy and Sherrington described that the brain has an intrinsic mechanism to vary its local vascular supply in response to local functional activity (Roy and Sherrington, 1890). The concept of the brain protecting its cerebral microenvironment from circulatory changes elsewhere in the body was novel and critical to the advancement of our understanding of cerebral vascular dynamics, especially pertaining to injury and disease. Following the work of Roy and Sherrington, many others contributed to the development of this concept of cerebral autoregulation. With the use of a cranial window, studies of in vivo pial circulation and its responses to various stimuli became possible. Fog observed that the pial circulation was capable of reducing cerebral vascular resistance in response to reductions in systemic arterial blood pressure (Fog, 1937). He continued to study this relationship between arterial blood pressure and vasomotor responses of the pial circulation, later describing that these pial vessels could not only dilate but also constrict (Fog, 1939). This compensation is, in part, due to adjustments in cerebral vascular resistance, dilating or constricting the vessels to accommodate increases or decreases in pressure and perfusion. Several clinical and

experimental studies have shown these mechanisms working to respond to changes in the brain microenvironment, such as variations in PaO2 , PaCO2, pH, and CBF. CBF is an important indicator of cerebral vascular function and is routinely monitored in TBI patients. Hypoperfusion, or decreased blood flow, to the brain after TBI can easily result in ischemic damage if left untreated. Bouma et al. observed that many severely injured TBI patients exhibited significant reductions in CBF within hours of injury (Bouma et al., 1991, Bouma and Muizelaar, 1992). CBF reductions have also been reported in experimental TBI models of fluid-percussion injury (FPI) (Lewelt et al., 1980, Yuan et al., 1988, Prough et al., 2006), controlled cortical impact (CCI) (Bryan et al., 1995, Cherian et al., 1996) and impact acceleration (IA) (Sawauchi et al., 2003).

The reactivity of cerebral blood vessels, especially the large vessels like the middle cerebral artery, can be tested to reveal the condition of and vasoconstrictory responses to changes in transmural pressure. In accordance with our understanding of cerebral autoregulation, an increase in transmural pressure results in vasodilation while a decrease in intravascular pressure results in vasoconstriction of the vessel(s). The mechanisms involved in autoregulation are metabolic, neurogenic and/or myogenic in nature (Rangel-Castilla et al., 2008). The intrinsic ability of the vessel smooth muscle to respond to changes in transmural pressure by vasodilation or vasoconstriction is described as the myogenic mechanism of autoregulation (Golding et al., 1999). The response of cerebral vessels has been measured in fluid-percussion injured rats in a previous study in our lab. Mathew et al. (Mathew et al., 1999) harvested MCA segments from both sham and FPI rats, mounted them on an arteriography and subjected them to variations in intraluminal pressure while measuring vessel diameter. Step-wise reductions in transmural pressure revealed normal vasodilatory responses, however, FPI appears to reduce this response for at least 30 minutes after injury (Mathew et al., 1999).

Hematoma formation, brain edema and BBB disruption are common and serious consequences of TBI (Okubo et al., 2013). While extracranial hematoma formation can

occur, it is less likely to be associated with a poor outcome for TBI patients and therefore, we chose to focus on intracranial hematomas in this study. Intracranial hematomas can be subdural or epidural in nature, with subdural describing the area between the arachnoid and dura maters and epidural as the area between the dura mater and skull (Petrov et al., 2014). These intracranial hematomas can present significant risk to TBI patients as they occupy space within an already confined cranium, possibly creating further impairment of CBF and damage to tissue in contact with the hematoma, thus worsening patient outcome (Petrov et al., 2014). Areas of compromised CBF in contact with the hematoma are especially vulnerable to hypoxia and/or ischemia. Addressing this concern, the detection of these hematomas would provide critical information to physicians and better direct therapeutic intervention. Through collaboration with the Laboratory for Optical Sensing and Monitoring, High-resolution Ultrasound Imaging Core of UTMB and the Center for Biomedical Engineering, we examined BINT rats using a prototype optoacoustic system for the presence of hematoma and monitored cerebral venous blood oxygenation (Petrov et al., 2014). This prototype has been validated previously in a large animal TBI study where the non-invasive measurements were verified with invasive measurements (Petrov et al., 2009, Petrov et al., 2012b, Petrov et al., 2012a). Together, the examination of CBF, MAP, vasodilatory and vasoconstrictory capabilities and the detection of hematomas would provide a more thorough understanding of the acute effects of BINT on the cerebral vasculature as produced by the VDB device. In our studies, the VDB produced BINT with a significant, sustained suppression of CBF to levels approximately 50% of baseline along with a transient suppression of arterial blood pressure. MAP returned to baseline levels within a few minutes of BINT.

RESULTS

Cerebral perfusion

Relative cerebral perfusion was monitored using Laser Doppler flowmetry before and after sham or BINT. Baseline measurements of cerebral perfusion were made the rats were removed from the stereotaxic frame, subjected to BINT or sham BINT and, immediately animals returned to the stereotaxic frame. The LDF probe was realigned and recordings continued for 60 minutes following injury. At 15 and 30 minutes, significant reductions in CBF were recorded. At 45 and 60 minutes, the reduction of CBF was even greater and therefore, very statistically significant.

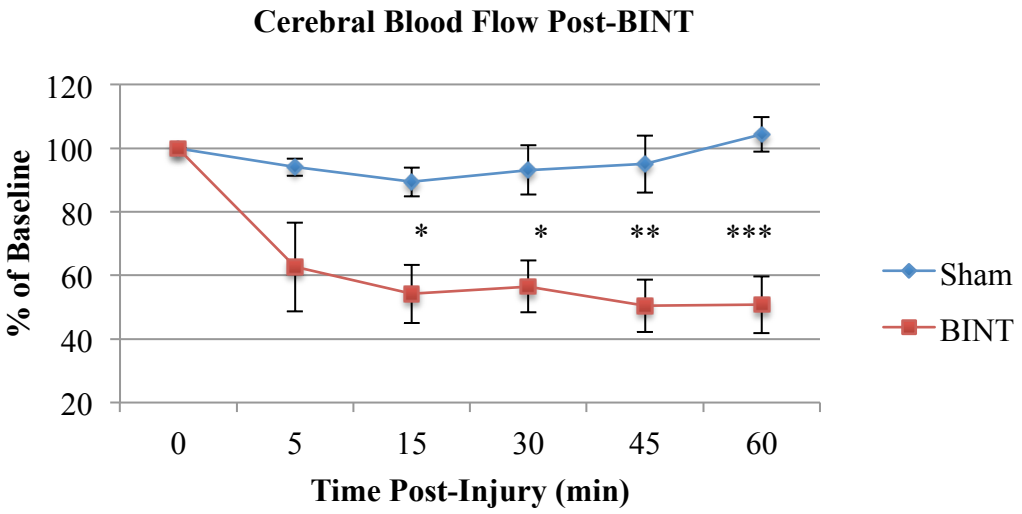


Figure 17. Suppression of CBF Following BINT. Relative cerebral perfusion was monitored using laser Doppler flowmetry for 60 minutes following mild BINT. Cerebral perfusion was reduced to 50% of baseline in animals with mild BINT. The suppression was sustained over the entire recording period. Mild BINT values are statistically significant when compared to sham values in a two-way ANOVA: (*, p-value <0.05), (**, p-value <0.01), (***, p-value <0.001). Sham, n=6. Mild BINT, n=12.

Mean Arterial Blood Pressure

We examined the effects of BINT on MAP in sham and BINT animals for a period of 60 minutes post-injury. MAP was monitored using a tail artery cannula that was connected to a pressure transducer and physiological monitoring system (BioPac) with acquisition software (Acqknowledge), continuously recording MAP. There was a significant ($p < 0.0001$) but transient decrease in MAP to 39% of the baseline immediately following blast-injury, however, MAP values returned to near-baseline levels within minutes. There was no statistically significant, sustained decrease in MAP as a result of BINT (Fig. 18).

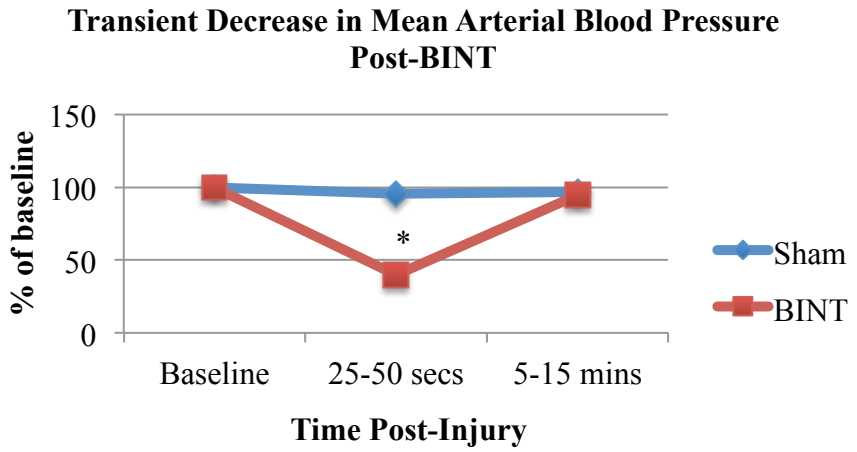
Vascular Reactivity of Isolated Middle Cerebral Arterial Segments

We examined myogenic responses to intraluminal pressure changes in isolated, pressurized middle cerebral arterial segments (MCAs) harvested from rats exposed to mild, moderate or severe BINT in vivo. Step-wise reductions in transmural pressure were made in 10-minute intervals, decreasing 20 mmHg at a time, starting at 100 mmHg and decreasing to 20 mmHg. Statistically significant reductions of dilator responses to reduced intravascular pressure were observed in after mild, moderate and severe BINT (Fig. 19).

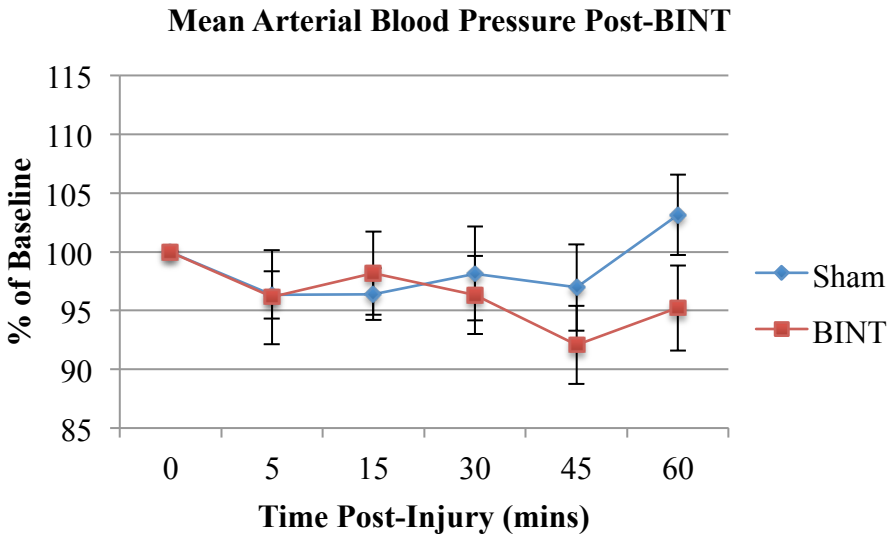
Hematoma Detection

To examine the presence of intra- and extracranial hematoma formation in response to BINT, we employed a novel optoacoustic monitoring system and focused on

the superior sagittal sinus (SSS). Animals were monitored before and after receiving either sham- or blast-injury from the VDB device. Baseline values were recorded for comparison after injury (Fig. 20-22).



A)



B)

Figure 18. Mean Arterial Blood Pressure Following BINT. Recording of arterial blood pressure from baseline to 15 mins post-blast. Transient decrease in MAP between 25-50 secs post-BINT. Difference between baseline and recorded values at 25-50 sec was significant ($p < 0.0001$) although transient (A). Plotted percentage of baseline mean arterial blood pressure values recorded at 0, 5, 15, 30, 45 and 60 minutes post-injury (B). A two-way ANOVA did not reveal any statistical significance when comparing sham and BINT-injured values for MAP.

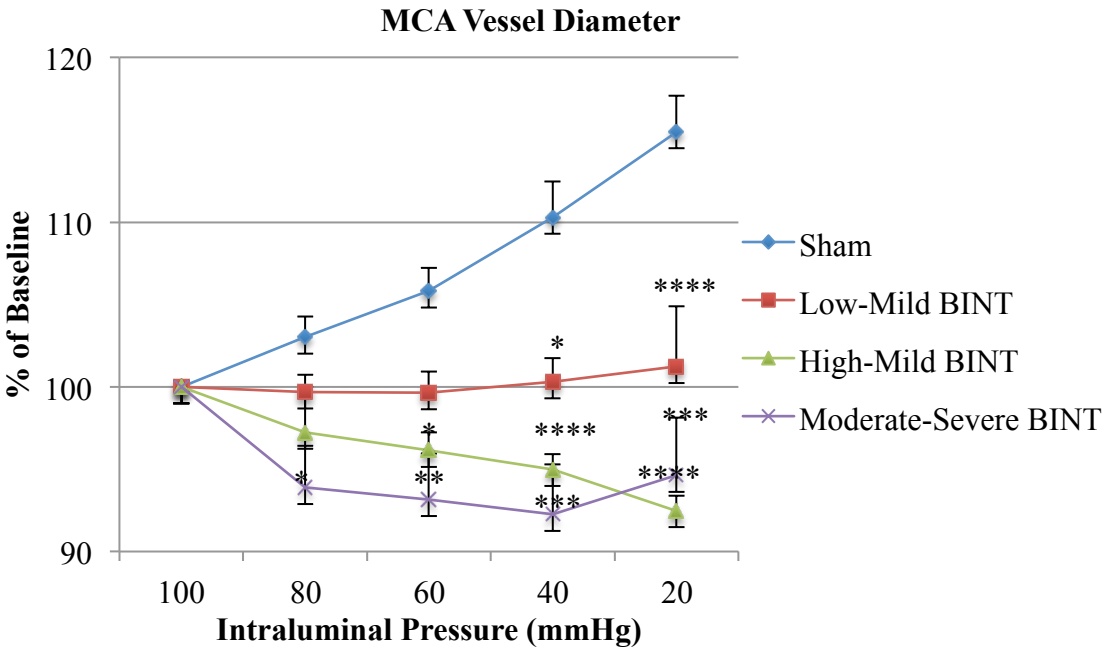


Figure 19. Myogenic Response of MCA Following BINT. Using a two-way ANOVA to compare to Sham at 80, 60, 40 and 20 mmHg, mild, moderate and severe BINT exhibited significant differences and are indicated as follows: (*, p-value <0.05), (**, p-value<0.01),(***, p-value <0.01) and (****, p-value <0.0001). n = 4 per group.

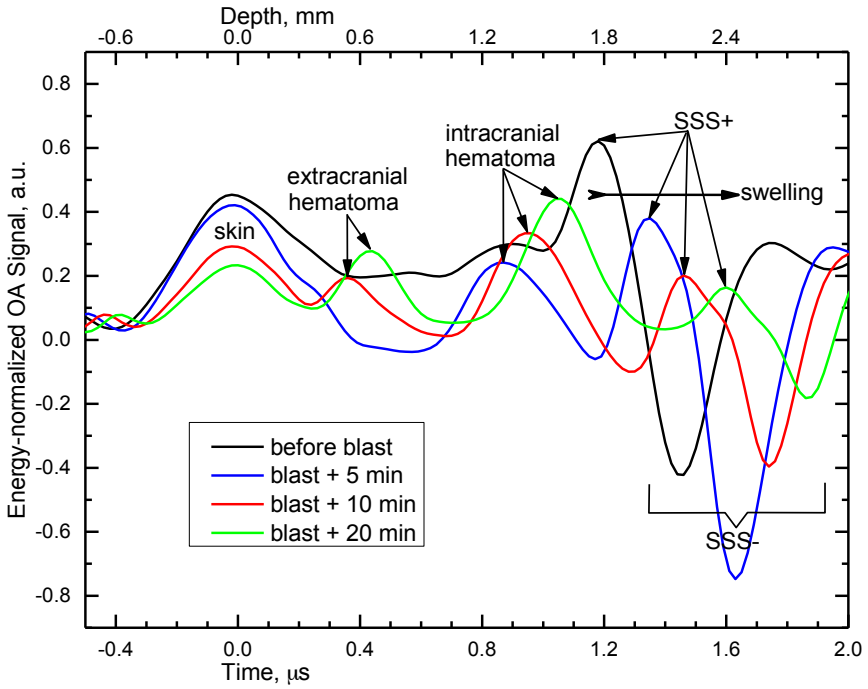


Figure 20. Optoacoustic signals from a rat's head before and after blast injury. Measurements of skin and SSS registered prior to blast however, immediately following the injury, intracranial and extracranial hematomas appeared (indicated above with arrows) and continued to grow. From (Petrov et al., 2014)

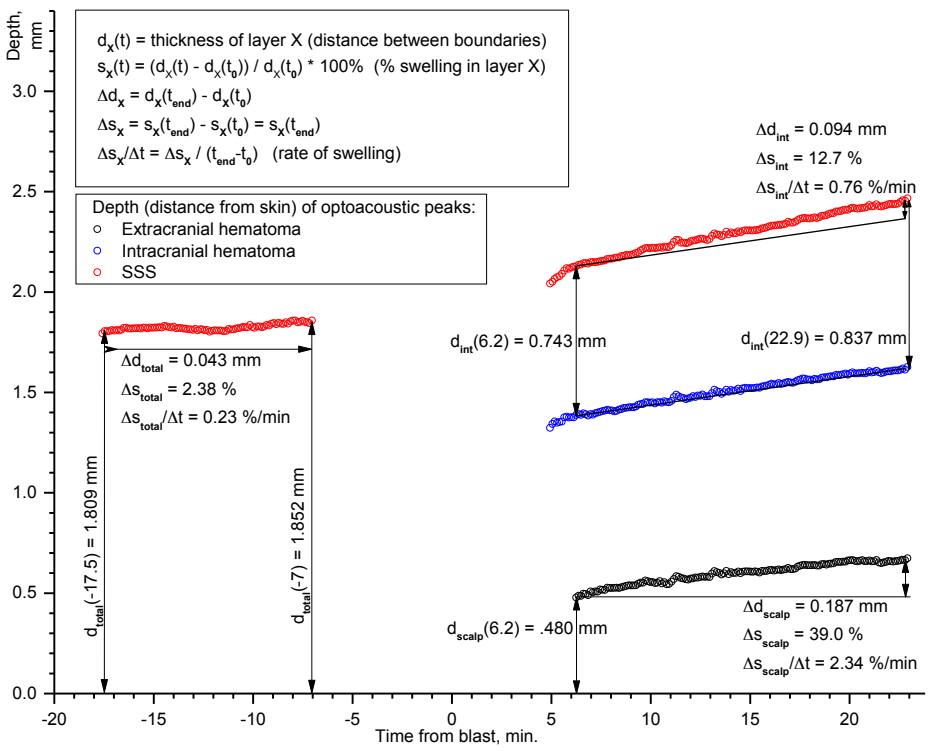


Figure 21. Depths of the optoacoustic peaks, before and after blast, and definitions of swelling parameters. The tissue was divided into 3 hypothetical layers for measurement purposes, scalp, skull and intracranial. Compared to pre-blast measurements, the rate of swelling post-BINT is significantly increased. The swelling of the intracranial layer was likely limited to the physical confinement of the cranium. From (Petrov et al., 2014)

Layer	**d(start), mm**	**d(end), mm**	**Δd, mm**	**Δs, %**	**Δs/Δt, %/min**
Pre-blast total	1.809	1.852	0.043	02.4	0.23
Post-blast total	2.128	2.459	0.331	15.6	0.93
Scalp	0.480	0.667	0.187	39.0	2.34
Skull	0.905	0.955	0.050	05.5	0.33
Intracranial	0.743	0.837	0.094	12.7	0.76

Table 9. Blast-induced swelling rates by layer. d(start) and d(end) refer to the thickness at the start and end of the monitoring period (either pre- or post-blast). Δd, Δs and Δs/Δt are defined in Fig.7. From (Petrov et al., 2014)

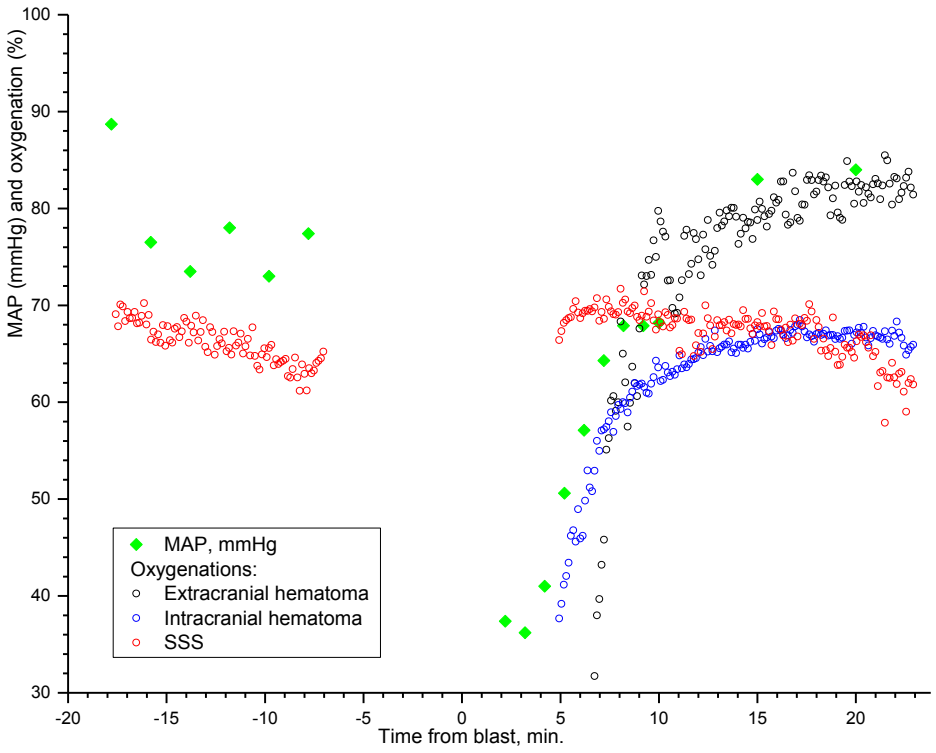


Figure 22. MAP and oxygenation of the SSS and hematomas, before and after blast. The blood oxygenation of the SSS and both hematomas following BINT. SSS oxygenation decreased from 70% to 60%, MAP gradually increased and ICP rapidly increased, resulting in lower CPP and declining SSS oxygenation. These findings correlate with the fact that CPP=MAP-ICP and that SSS oxygenation increases with CPP. From (Petrov et al., 2014)

DISCUSSION

We observed that Vandenberg blast injury produced significant reductions in CBF without significant reductions in arterial blood pressure. Accordingly, cerebral vascular resistance was significantly elevated by BINT. BINT also was associated with significantly reduced dilator responses in step-wise reductions in intravascular pressure in isolated middle cerebral arterial segments.

There has been little investigation of the cerebral vascular effects of blast injury. However, a previous report of reduced cerebral perfusion after blast injury in rats (Bir et al., 2012) is consistent with ours. The MRI studies of Bir, et al.,revealed

significant reductions in relative cerebral perfusion in the range of 0-21% at blast overpressures in the 17 - 28 psi range. Although we didn't measure blast pressure in these studies, the cartridges used for these studies (0.27 caliber, #5) typically produce overpressures of 15 - 20 psi. This level of Vandenberg BINT was associated with 40 - 50% reductions in relative cerebral perfusion that persisted for at least two hours. In contrast, Bir et al., observed reduction in relative perfusion of about 20% but this level of hypoperfusion persisted for at least 72 hrs post-blast. Our results and those of Bir, et al., indicate that blast exposure is followed by a 50% reduction in cerebral perfusion acutely, followed by a period of cerebral hypoperfusion that is longer in duration but lower in magnitude.

As stated above, the cerebral vascular effects of blast have received little attention but the vascular effects of impact TBI are well known. CBF reductions have been extensively reported in experimental TBI models of FPI (Lewelt et al., 1980, Yuan et al., 1988, Prough et al., 2006), controlled cortical impact (CCI) (Bryan et al., 1995, Cherian et al., 1996) and impact acceleration (IA) (Sawauchi et al., 2003). Posttraumatic hypoperfusion may be caused by impairment or destruction of a cerebral vasodilatory mechanism such as nitric oxide (DeWitt et al., 2001). This hypothesis is supported by evidence that the inhibition of NO synthesis results in significant reductions in CBF (Beckman, 1991, DeWitt et al., 2001, Pelligrino et al., 1993). Another mechanism of blast-induced cerebral vascular injury is oxidative/nitrative stress. Impact TBI is associated with significant increases in the production of the superoxide anion radical (Kontos & Wei, 1986; Fabian, et al., 1995). Another free radical produced by impact TBI is NO, which increases immediately post-injury but then decreases markedly (Cherian et al., 2000). Posttraumatic reductions in NO may be due to the combination of NO with superoxide to form peroxynitrite, a potent oxidant that impairs dilator responses to reduced intravascular pressure in middle cerebral arteries (DeWitt, et al., 2001). We have observed that Vandenberg blast injury is associated with increases in cortical 3-

nitrotyrosine staining (Masel, et al., 2012 and see below), a marker of peroxynitrite production (Beckman, 1991). The hypothesis that blast-induced, like impact-induced, cerebral vascular dysfunction is due, in part, to the effects of reactive oxygen/nitrogen species is supported by evidence that blast injury in humans results in increases is oxidative stress (Cernak, et al., 1999).

A second potential mechanism of blast-induced cerebral vascular dysfunction is primary blast injury to vascular smooth muscle cells. Alford, et al., reported phenotypic switching mechanism that results in a prolonged hypercontractile state within the vascular smooth muscle cells subjected to blast injury *in vitro* (Alford et al., 2011). Cerebral arterial hypercontractility after blast exposure would be consistent with the results of Armonda et al., who reported that vasospasm was observed in nearly 50% of military personnel admitted with head trauma and that it was associated with all types of intracranial bleeding (Armonda et al., 2006). Approximately 10% of those presenting with vasospasm had experienced BINT. The study also suggested that the incidence of vasospasm was correlated to delayed ischemic deficits and outcomes. Armonda projected that the duration and severity of vasospasm observed might be due, at least in part, to the blast overpressure that occurs with explosions (Bell et al., 2010).

In addition to intracranial mechanism for blast-induced cerebral hypoperfusion hypoperfusion, extracranial mechanisms may play a role. Cernak et al., subjected rabbits to pulmonary blast exposure to determine the role of vagal afferentation in cardiorespiratory and metabolic control after blast injury. One group of rabbits received a bilateral transection of the vagus, glossopharyngeal and hypoglossal nerves. Animals that did not receive deafferentation had increased lactate/pyruvate ratios in brain tissue while those that did undergo vagal deafferentation did not. These data suggest that blast induced vagal stimulation may contribute to cerebral hypoperfusion..

This study is among the first to describe hypoperfusion following experimental BINT with blunt impact-like injury, providing valuable information for future studies and

potential interventions. This information, combined with our data of peroxynitrite formation after BINT, supports the idea that TBI-induced oxidative stress, vasospasm or abnormal vagal stimulation may be contributors to the observed reductions in CBF immediately following injury.

Hematoma formation is another common result of TBI, especially in the first several hours after injury. While extracranial hematoma formation can occur, it is less likely to be associated with a poor outcome for TBI patients and therefore, we chose to focus on intracranial hematomas in this study. Intracranial hematomas can be subdural or epidural in nature, with subdural describing the area between the arachnoid and dura maters and epidural as the area between the dura mater and skull. These intracranial hematomas can present significant risk to TBI patients as they may create further impairment of CBF and damage to tissue in contact with the hematoma, thus worsening patient outcome (Petrov et al., 2014). It stands to reason that areas of compromised CBF, in contact with the hematoma, are especially vulnerable to hypoxia and/or ischemia. Addressing this concern, the detection of these hematomas would provide critical information to physicians and better direct therapeutic intervention. Through collaboration with the Laboratory for Optical Sensing and Monitoring, High-resolution Ultrasound Imaging Core of UTMB and the Center for Biomedical Engineering, we were able to perform thorough examinations on BINT rats using a prototype optoacoustic system for hematoma diagnosis and cerebral venous blood oxygenation monitoring (Petrov et al., 2014). This prototype has been validated previously in a large animal TBI study where the non-invasive measurements were verified with invasive measurements (Petrov et al., 2009, Petrov et al., 2012b, Petrov et al., 2012a). Together, the examination of CBF, MAP, vasodilatory and vasoconstrictory capabilities and the detection of hematomas provide a more thorough understanding of the acute effects of BINT especially on the cerebral vasculature as produced by the VDB device.

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Vita

Katherine Amelia Ruppert was born in Metairie, Louisiana on November 16, 1983 to Edward and Carolyn Ruppert. Katherine graduated from Saint Agnes Academy located in Houston, Texas in 2001. After high school, she pursued a bachelor's of science degree in College Station, Texas at Texas A&M University, which was awarded in August 2005. Katherine enrolled in the Ph. D. program at the University of Texas Medical Branch and moved to Galveston, Texas in the summer of 2006.

During her time at UTMB, Katherine was involved in student leadership roles of the Cell Biology Graduate Program society as a member (2006-2009), vice-president (2009-2011) and president (2011-2012). She has also maintained membership in several professional societies including Society for Neuroscience, National Neurotrauma Society, Sigma Xi and American Association for the Advancement of Science. Katherine has also been an active volunteer for the Houston Livestock Show and Rodeo (2013-present) in an effort to support the show's mission to award millions of dollars annually in scholarships to area youth.

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