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## TRAUMATIC BRAIN INJURY: CYCLOPHILIN D AS A THERAPEUTIC TARGET AND THE NEUROPATHOLOGY CAUSED BY BLAST

Ryan Douglas Readnower  
*University of Kentucky*, [rdread2@uky.edu](mailto:rdread2@uky.edu)

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ABSTRACT OF DISSERTATION

Ryan Douglas Readnower

The Graduate School  
University of Kentucky

2011

TRAUMATIC BRAIN INJURY: CYCLOPHILIN D AS A THERAPEUTIC TARGET  
AND THE NEUROPATHOLOGY CAUSED BY BLAST

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the  
College of Medicine  
at the University of Kentucky

By  
Ryan Douglas Readnower

Lexington, Kentucky

Director: Dr. Patrick Sullivan, Professor of Anatomy and Neurobiology

Lexington, Kentucky

2011

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## ABSTRACT OF DISSERTATION

### TRAUMATIC BRAIN INJURY: CYCLOPHILIN D AS A THERAPEUTIC TARGET AND THE NEUROPATHOLOGY CAUSED BY BLAST

With an estimated incidence of 1.5 million each year, traumatic brain injury (TBI) is a major cause of mortality and morbidity in the United States. Opening of the mitochondrial permeability transition pore (mPTP) is a key event contributing to TBI pathology. Cyclophilin D (CypD), a matrix peptidyl-prolyl cis-trans isomerase, is believed to be the regulating component of the mPTP. Cyclosporin A, an immunosuppressant drug, inhibits CypD and blocks mPTP formation and has been shown to be neuroprotective following TBI. However, it is unclear if CsA's neuroprotective mechanism is due to inhibition of CypD and/or immuno-suppression. Therefore to directly assess the contribution of CypD to TBI pathology, CypD knockout mice were subjected to a controlled cortical impact model of TBI. CypD ablation resulted in increased tissue sparing, hippocampal protection, and improved mitochondrial complex I driven respiration. Next a dose-response study of the Cyclophilin D inhibitor, NIM811, was performed. NIM811 administration following TBI resulted in improved cognition, increased tissue sparing, and improved mitochondrial function. These results suggest a major role for CypD in TBI pathology and validate CypD as a potential therapeutic target for TBI.

TBI has been proposed to be the signature injury of the current Middle Eastern conflicts with an estimated prevalence of 15-60 % among combat soldiers. Although the brain does appear to be vulnerable to blast, the exact mechanisms underlying the injury remain unclear. Adult male Sprague-Dawley rats were exposed to a moderate level of blast overpressure. Following blast, blood brain barrier disruption was evident at 3 and 24 h post-exposure, oxidative damage increased at 3 h post-exposure, and microglia were activated in the hippocampus at 5 and 10 days post-exposure. This may widen future neuroprotective avenues for blast since blast brain injury appears to share similar mechanisms of injury with other TBI models.

KEYWORDS: Traumatic Brain Injury, Cyclophilin D, NIM811, Blast Overpressure,  
Blast Traumatic Brain Injury

Ryan D Readnower

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Student's Signature

28 June 2011

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Date

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By

Ryan Douglas Readnower

Patrick Sullivan, Ph.D.

---

Director of Dissertation

Wayne Cass, Ph.D.

---

Director of Graduate Studies

28 June 2011

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DISSERTATION

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TRAUMATIC BRAIN INJURY: CYCLOPHILIN D AS A THERAPEUTIC TARGET  
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2011

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For my mom and dad, whose unquestioning love inspires.

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## 1. CHAPTER 1

### **Introduction and Background**

#### 1.1 An overview of traumatic brain injury

##### *1.1.1 Traumatic brain injury in the US*

In the United States, approximately 1.5 million individuals each year sustain a traumatic brain injury (TBI) severe enough to warrant medical attention (Corrigan et al. 2010). This estimate is conservative since many individuals with mild TBIs do not seek medical attention. Following hospitalization with TBI, as many as 3.2 million Americans are currently living with an injury-related disability (Corrigan et al. 2010). The high incidence and devastating consequences of TBI produce an enormous economic burden in the US with total costs in excess of \$60 billion a year. Unfortunately there are currently no approved pharmacological treatments for TBI and care remains aimed at acute clinical management and chronic rehabilitation therapy (Hatton 2001).

Major risk factors for civilian TBI (cTBI) are age and gender (Corrigan et al. 2010). The incidence of cTBI is increased in the elderly and the young, with 659 and 900 per 100,000, respectively. Increased risk-taking behavior in males contributes to them being twice as likely as females to sustain a cTBI. Falls, motor vehicle accidents, incidents involving the head being struck by or against an object, and assaults are the major causes of cTBI (Fig. 1.1) (Langlois et al. 2006). Another major cause of cTBI are the approximately 300,000 sports-related TBIs reported yearly (Thurman et al. 1998).



## Causes of Civilian Traumatic Brain Injury

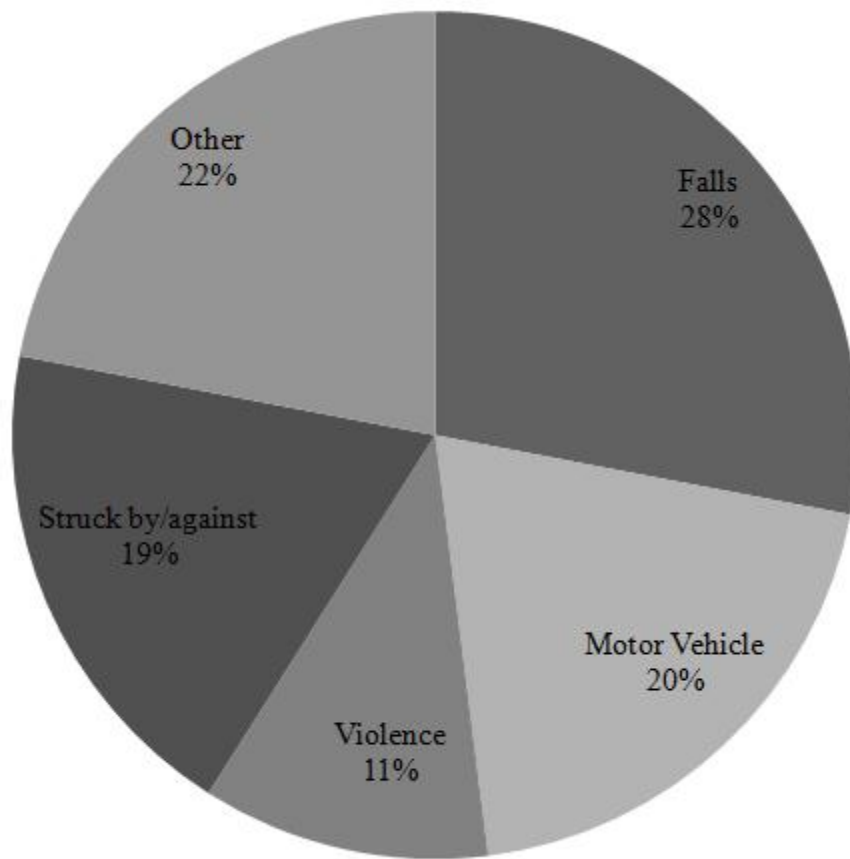


Figure 1.1. Causes of civilian TBI (cTBI) in the United States. Falls, at 28 %, are the most common cause of cTBI. The second most common cause of cTBI is motor vehicle accidents (20 %). Followed by other (i.e. suicide attempt, unknown, bicycle crashes) at 22 %, struck by or against at 19 %, and violence at 11 %. (Modified from Langlois et al. 2006)

TBI is a clinically heterogeneous neurological disorder which, depending on injury severity, may produce symptoms ranging from a brief disruption in mental status to long-lasting physical and mental disability. The Glasgow Coma Scale (GCS) is the most widely used diagnostic tool for initial assessment of the severity of TBI (Andriessen et al. 2010). The GCS is scored based on an individual's eye response, verbal response, and motor response. A GCS score of 13-15 represents a mild TBI, 9-12 moderate TBI, and a  $\leq 8$  as a severe TBI.

### *1.1.2 Military TBI*

Recent military operations in the Middle East have resulted in an increased public awareness of TBI. However, military TBI isn't a new phenomena and reports of "shell shock" date back to World Wars I and II (WWI and WWII) (Jones et al. 2007). During WWI soldiers without head wounds presented with amnesia, headache, and poor concentration (Jones et al. 2007). Some of these soldiers had been in close proximity to a blast event, while others with similar symptoms had not been anywhere near an explosion. This observation lead a British psychologist to postulate that since soldiers are exposed to large amounts of stress while in combat, that some cases of shell shock are psychological in origin rather than the result of a brain lesion (Jones et al. 2007). Whether or not the neuropathological symptoms following blast exposure are the result of head trauma or psychological in origin continues to be debated.

Better battlefield technology (i.e. personal body armor) and medical care allow present day soldiers to survive blasts that would have been deadly in other wars. As such TBI has been proposed to be the signature injury of Operation Iraqi Freedom (OIF) and

Operation Enduring Freedom (OEF) (Elder and Cristian 2009). One recent study reported that approximately 15% of 2525 Army infantry soldiers surveyed had experienced either injuries with a loss of consciousness or injuries with altered mental status while serving in Iraq, injuries which are indicative of mild traumatic brain injury (Hoge et al. 2008). However some symptoms associated with postconcussive syndrome, such as headaches, difficulty with concentration, and memory loss, overlap with post traumatic stress disorder further complicating diagnosis of mild TBI in troops (Bryant 2001) (Table 1.1). Many of these troops presenting with postconcussive symptoms experienced no overt head trauma but share a common link, blast exposure.

Table 1.1. Symptoms of posttraumatic stress disorder and traumatic brain injury.  
 N/A=not applicable (modified from Bryant 2001)

<b>Symptom</b>	<b>PTSD</b>	<b>TBI</b>
Depersonalization	Present	Present
Amnesia	Present	Present
Social Detachment	Present	Present
Nightmares	Present	N/A
Insomnia	Present	Present
Irritability	Present	Present
Concentration Deficits	Present	Present
Hypervigilance	Present	N/A

### *1.1.3 TBI pathology and the secondary injury cascade*

TBIs result from a mechanical input that directly damages tissue (primary response) which initiates a complex secondary injury cascade (secondary response) (LaPlaca et al. 2007). The primary response involves blood vessel damage, axonal shearing, and cellular damage (Gaetz 2004; Smith et al. 2003). Vascular changes are a key event and may result in decreased cerebral blood flow leading to ischemic tissue and subsequent neuronal death (Nolan 2005). Brain edema is hypothesized to be the primary contributor to the high mortality and morbidity associated with TBI (Donkin and Vink 2010). Edema can be classified as cytotoxic or vasogenic depending on the source of the water. Cytotoxic edema represents an increase in intracellular water content with a corresponding decrease in extracellular water. Vasogenic edema is the result of movement of water from the vasculature into the extracellular space which may occur as a result of blood brain barrier (BBB) breakdown following trauma. Vasogenic, but not Cytotoxic, edema results in an increase in intracranial pressure.

Following TBI, there is progressive neurodegeneration that peaks between 48-72 h post-injury (Hall et al. 2005). Associated with this secondary injury is a several fold increase in extracellular glutamate (Faden et al. 1989; Matsushita et al. 2000; Yi and Hazell 2006) (Fig. 1.2). Increased glutamate results in the activation of  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which cause depolarization (Rho et al. 2010). This increase in glutamate can also lead to the activation of voltage dependent  $\text{Na}^+$  channels, leading to further depolarization resulting from increased  $\text{Na}^+$  entry into the cell and osmotic swelling of the neuron as  $\text{Cl}^-$  passively

enters the cell, causing an influx of water. Aberrant depolarization can also result in the removal of the  $Mg^{2+}$  block from *N*-methyl-daspartate (NMDA) receptors, allowing them to be activated by glutamate. Activation of NMDA receptors and group I metabotropic glutamate receptors allows  $Ca^{2+}$  influx into the neuron, subsequently activating downstream mediators of programmed cell death (Sullivan et al. 2005; Walker 2007). This disruption of  $Ca^{2+}$  homeostasis is believed to underlie glutamate-mediated toxicity (excitotoxicity). The increase in intracellular calcium may cause sustained depolarization, ionic imbalance, increased intracranial pressure (ICP), cerebral edema, calpain activation, decreased ATP production, and increased reactive oxygen species (ROS) (Sullivan, 2005; Yi and Hazell, 2006). In order to maintain neuronal  $Ca^{2+}$  homeostasis during periods of excitotoxicity mitochondria serve as high-capacity  $Ca^{2+}$  sinks. The role mitochondria play in TBI neuropathology will be discussed in detail in a later section.

In addition to excitotoxicity, oxidative damage and inflammation are important components of the secondary injury cascade. The brain is especially vulnerable to oxidative damage as it accounts for approximately 20 % of basal  $O_2$  consumption, is rich in peroxidizable membranous polyunsaturated fatty acids, and contains large amounts of iron (Halliwell and Gutteridge 2007; Patel 2002; Rho 2004). Oxidative damage has been shown to occur early (30 min-6 h) and spatially overlaps but temporally precedes peak neurodegeneration (48 h); indicating a role for oxidative damage in secondary injury (Deng et al. 2007). Post-TBI there are numerous sources of superoxide which include: microglia, macrophages, arachidonic acid cascade (i.e. 5-lipoxygenase activity), mitochondria, and dopamine oxidation (Lim and Lajtha 2006). Through a series of reactions superoxide can form either the hydroxyl radical, nitrogen dioxide, or carbonate

radical which can initiate lipid peroxidation or protein carbonylation. Additionally, the formation of 3-nitrotyrosine, by nitrogen dioxide, can alter protein structure and function and therefore contributes to post-trauma pathology (Reed et al. 2009). Taken together, it is evident that protein nitration and lipid peroxidation are early hallmarks of CNS injury (Hall and Braugher 1993). Other evidence that implicates oxidative damage in TBI neuropathology is the observation that antioxidant therapy is neuroprotective and improves behavioral outcome (Hall et al. 2010; Mustafa et al. 2010; Singleton et al. 2010).

Immune activation is common among neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, stroke, multiple sclerosis, and TBI (Amor et al. 2010). The notion that the CNS is "immune privileged" has been challenged recently and evidence exists that demonstrates that the CNS is not isolated from the immune system following TBI (Morganti-Kossmann et al. 2007). Circulating leukocytes have been shown to transmigrate into the CNS following vascular disruption and activate resident immune cells (microglia and astrocytes) (Hickey et al. 1991). Activation of microglia occurs rapidly (6-48 h) following diffuse TBI and is followed by persistent activation (7-28 days) with phagocytic activity (Kelley et al. 2007). Activated microglia secrete pro-inflammatory cytokines and cytotoxic factors which exacerbate the immune response and promote cell death (Morganti-Kossmann et al. 2002). Tumor necrosis factor (TNF) is rapidly released after injury and acts initially as a pro-inflammatory cytokine (Morganti-Kossmann et al. 2002). Interestingly the production of TNF stimulates production of interleukin-6, an anti-inflammatory cytokine, which in-turn inhibits TNF synthesis. Immunosuppression using various immunosuppressants including cyclosporin A, FK506,

rapamycin, and methylprednisolone following neurotrauma has been shown to be neuroprotective (Hailer 2008). Therefore, inflammation represents one component of the secondary injury cascade that may be amenable to therapeutic intervention.



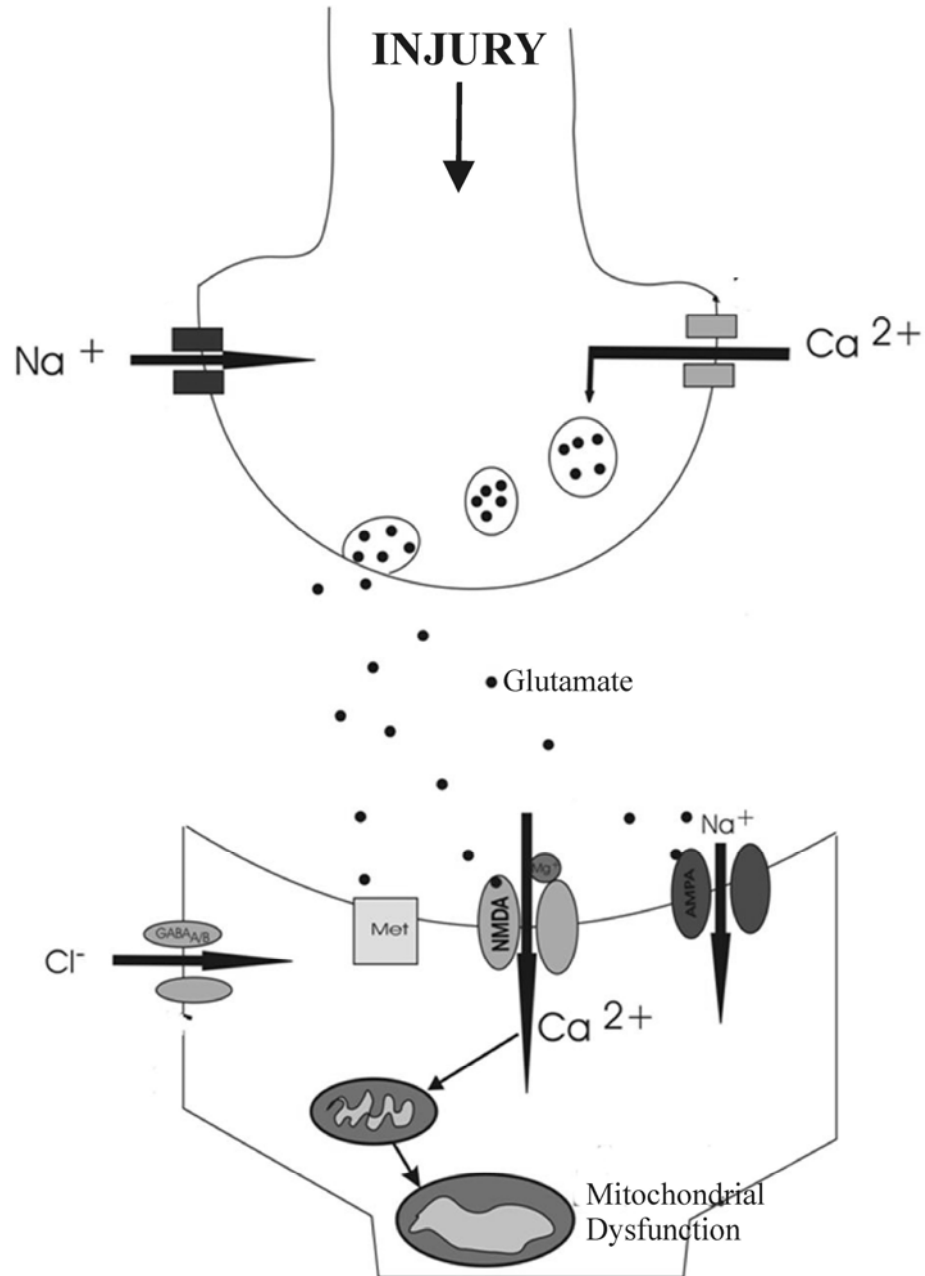


Figure 1.2. The excitotoxic cascade following injury. Injury results in increased levels of extracellular glutamate causing influxes of Ca<sup>2+</sup> into the dendrite via NMDA receptor activation. If unchecked, this Ca<sup>2+</sup> influx into neurons can result in a subsequent loss of neuronal Ca<sup>2+</sup> homeostasis. This disruption of Ca<sup>2+</sup> homeostasis is believed to be a defining factor of glutamate-mediated toxicity. Excessive mitochondrial Ca<sup>2+</sup> results in mitochondrial dysfunction.

## 1.2 Mitochondria and TBI

Approximately 800 million years ago a symbiotic relationship between two cells led to the uptake of one cell by the other and made the development of multicellular organisms possible (Scheffler 2008). Chondros (Greek), Korn (German) or grain (English) were early (mid-1800s) descriptions of the morphology of distinct intracellular structures that later (1898) became known as mitochondria. In the early 1900s Kingsbury, Lehninger, and Warburg hypothesized that mitochondria were associated with cellular respiration. Later, Mitochondria would become known as the reliable “powerhouse of the cell” (Nicholls and Ferguson 2002). Now it appears that mitochondria not only produce cellular energy but are also involved in the life and death of the cell. Recently mitochondrial dysfunction has been implicated in a host of neurodegenerative disorders, such as stroke, spinal cord injury, TBI, and Alzheimer’s disease (Sullivan et al. 2005).

### 1.2.1 *Mitochondrial function*

Mitochondria have an inner and outer membrane, each with its own specific functions. The outer mitochondrial membrane contains porins which are non-specific pores for molecules less than 10 kDa. The inner membrane is energy transducing and contains components of the electron transport chain (ETC) along with specific carrier proteins. Additionally the inner membrane exhibits folds, known as cristae, which act as independent compartments (Zick et al. 2009). The space between the membranes is referred to as the inter-membrane space and the internal compartment is known as the matrix. The matrix contains enzymes related to the citric acid cycle. The citric acid cycle reduces  $\text{NAD}^+$  to NADH. Electrons are then passed from NADH to the ETC.

Mitochondria sustain the activity of neurons by producing ATP via the ETC (Fig. 1.3). The ETC is a series of redox reactions involving the transfer of electrons from one carrier to the next (Alberts 2002). Electrons are transferred from carriers with low redox potentials to carriers with high redox potentials. ETC chain carriers include cytochromes, iron-sulfur proteins, and coenzyme Q. Complex I (NADH dehydrogenase) catalyzes the transfer of two electrons from NADH to coenzyme Q (Nicholls 2002). Complex III (ubiquinol-cytochrome *c* oxio-reductase) catalyzes the transfer of electrons from coenzyme Q to cytochrome *c*. Complex IV (cytochrome *c* oxidase) reduces one molecule of oxygen to two molecules of water. As electrons are transported through Complexes I, III, and IV protons are pumped into the inner membrane space, generating an electrochemical gradient. This store of energy is used to generate ATP via the ATP synthase.

Superoxide production is a byproduct of electron transport and occurs when electrons escape or slip from the complexes and reduce O<sub>2</sub> due to a backup of electrons in the ETS. ROS production is linked to  $\Delta\psi$  such that a high  $\Delta\psi$  promotes increased ROS production (Sullivan and Brown 2005). High  $\Delta\psi$  results in altered redox potential of ETC carriers and an increase in the half-life time of ubisemiquinone leading to increased ROS production. Also, any damage to components of the ETC could lead to a stalling of reduced intermediates of the ETC which would increase the probability of an electron slipping and reducing O<sub>2</sub> to form ROS.

In addition to producing the majority of ATP for the cell mitochondria also regulate intracellular calcium levels. Mitochondrial Ca<sup>2+</sup> accumulation is dependent upon a

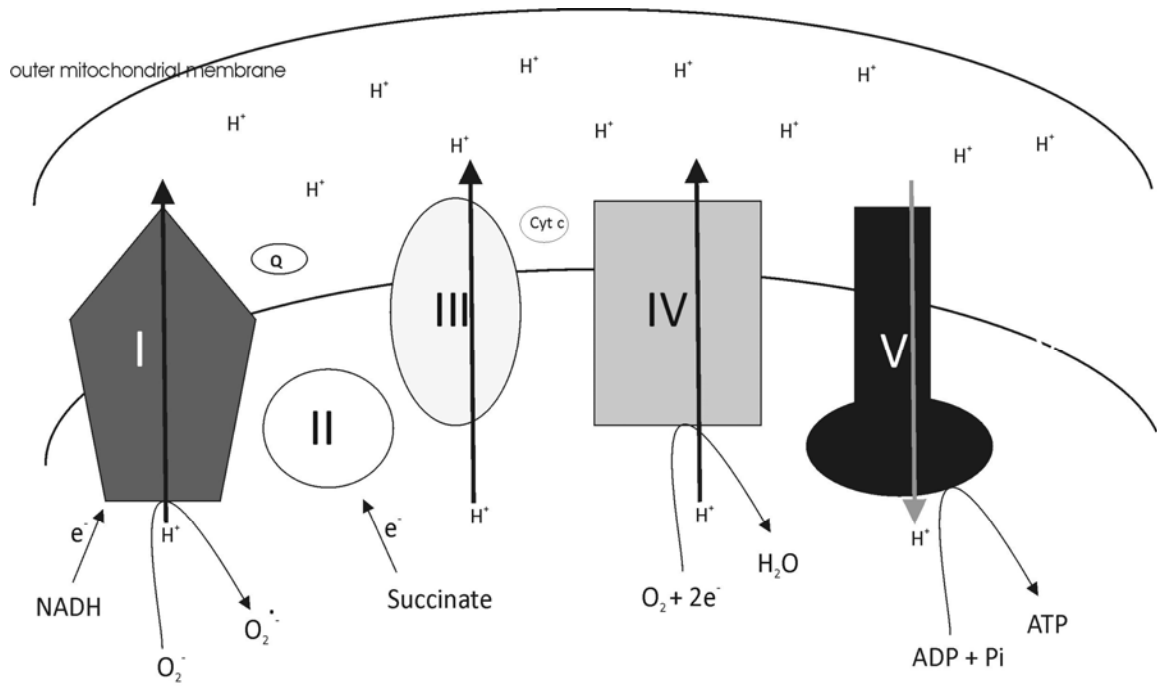


Figure 1.3. The mitochondrial electron transport chain (ETS). As electrons are transported down the ETS, complexes I, III, and IV translocate protons from the matrix, generating an electrochemical gradient. This store of energy can then be coupled to ATP production as protons move down the gradient and back into the matrix via F<sub>0</sub>F<sub>1</sub> ATP synthase. Superoxide production is a byproduct of electron transport.

$\text{Ca}^{2+}$  uniporter,  $\text{Na}^+/\text{Ca}^{2+}$  antiporter, and  $\text{H}^+/\text{Na}^+$  antiporter (Gunter and Gunter 1994; Scheffler 2008). The  $\text{Ca}^{2+}$  uniporter transports  $\text{Ca}^{2+}$  into the matrix in response to  $\Delta\psi$ .  $\text{Ca}^{2+}$  efflux is driven by  $\text{Na}^+/\text{Ca}^{2+}$  exchange which is driven by  $\text{H}^+/\text{Na}^+$  exchange. Matrix  $\text{Ca}^{2+}$  is buffered by forming a complex with phosphate (calcium-phosphate gel) (Nicholls and Ferguson 2002). Physiologically, mitochondrial  $\text{Ca}^{2+}$  acts as an activator of several key enzymes involved in oxidative phosphorylation (i.e. pyruvate dehydrogenase) (Gunter and Gunter 2001; Scheffler 2008). In response to rises in intracellular  $\text{Ca}^{2+}$  levels mitochondria can act as a temporary sink for  $\text{Ca}^{2+}$  and maintain cytoplasmic  $\text{Ca}^{2+}$  homeostasis. However, excessive mitochondrial  $\text{Ca}^{2+}$  loading (i.e. during periods of excitotoxicity) results in cell death initiation and will be discussed in detail in subsequent sections.

### 1.2.2 Mitochondrial permeability transition

Excessive mitochondrial  $\text{Ca}^{2+}$  cycling/loading can result in mitochondrial permeability transition (mPT) (Crompton and Costi 1990; Haworth and Hunter 1979). mPT is the sudden increase in the inner membrane permeability to solutes of molecular mass less than 1500 Da and was originally described by Hunter and Haworth in the 1970s (Haworth and Hunter 1979; Hunter and Haworth 1979a; Hunter and Haworth 1979b; Zoratti and Szabo 1995). mPT results in a loss of  $\Delta\psi$  which uncouples electron transport from the production of ATP (Halestrap 2009). Also mPT results in further mitochondrial swelling, rupture of the outer mitochondrial membrane, and increased ROS production (Mazzeo et al. 2009; Sullivan et al. 2005). mPT is thought to be mediated by opening of a mega channel known as the mitochondrial permeability transition pore (mPTP) (Sullivan

et al. 2005). Although the exact composition of the mPTP remains to be elucidated, the three most characterized components include the adenine nucleotide translocase (ANT), cyclophilin D (CypD), and the voltage dependent anion channel (VDAC) (Fig. 1.4).

CypD is an 18 kDa matrix peptidyl-prolyl cis-trans isomerase (PPIase) encoded by the nuclear gene *PPIF* that binds to the matrix face of ANT (Halestrap 2009; Halestrap and Brenner 2003). PPIase activity is associated with an ability to catalyze the isomerization of protein proline residues, which has dramatic effects on protein structure and function. This binding of CypD, in the presence of high levels of  $\text{Ca}^{2+}$ , results in a conformational change in ANT that converts ANT into a non-specific pore located in the inner membrane (Griffiths and Halestrap 1991; Halestrap and Brenner 2003). The interaction of the CypD/ANT complex with VDAC results in the formation of the mPTP. In addition to  $\text{Ca}^{2+}$  induced mPTP, oxidative stress has been shown to promote mPTP by enhancing the binding of CypD to ANT (Halestrap et al. 1997). This interaction between ANT and CypD is blocked by cyclosporin A (CsA) (Crompton et al. 1988). Recent studies in CypD knockout mice have demonstrated that mitochondria lacking CypD are resistant to  $\text{Ca}^{2+}$  and ROS induced mPTP; further validating CypD involvement in mPTP. Additionally, CypD knockout does protect against cardiac ischemia-reperfusion injury (Nakagawa et al. 2005). However, CypD knockout did not protect against cell death induced by pro-apoptotic Bcl-2 family members (Baines et al. 2005). This indicates that cell death mediated by mPTP may occur through two mechanisms; either CypD-dependent mechanism or CypD-independent mechanism (Fig. 1.5).

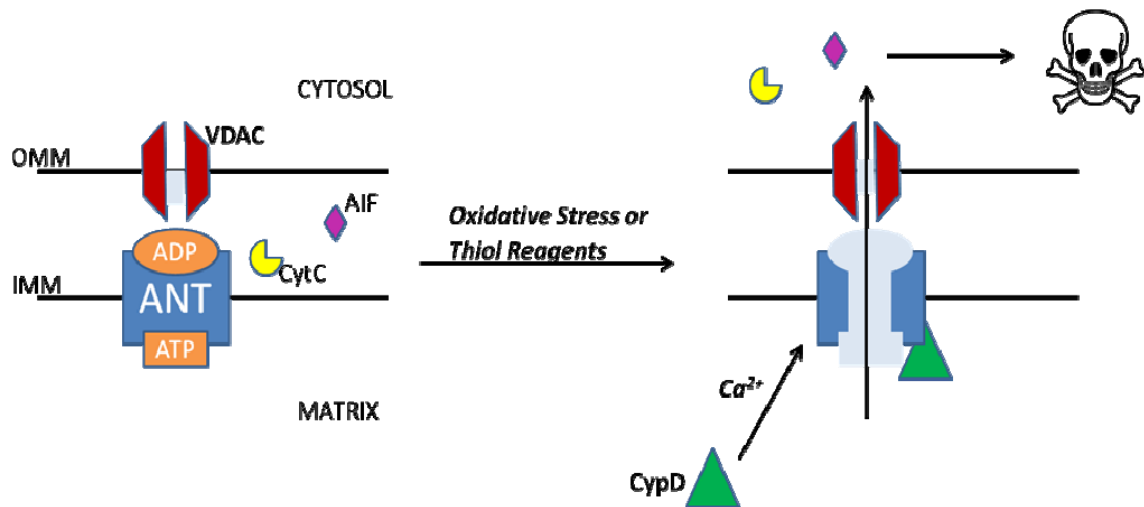


Figure 1.4. Mitochondrial permeability transition pore. The mPTP is a conductance pore that spans the inner and outer mitochondrial membranes and is putatively composed of the adenine nucleotide translocase (ANT) of the inner membrane, matrix cyclophilin D (CypD), and the voltage-dependent anion channel (VDAC) of the outer membrane. (Right) Under normal conditions bound adenine nucleotides inhibit the opening of the mPTP. (Left) Under pathophysiological conditions oxidative stress results in a conformational change in ANT that prevents the binding of adenine nucleotides. In the presence of high Ca<sup>2+</sup> CypD binds to ANT and promotes formation of the mPTP. Opening of the mPTP results in the release of apoptotic proteins (i.e. cytochrome C (CytC) and apoptosis inducing factor (AIF)).

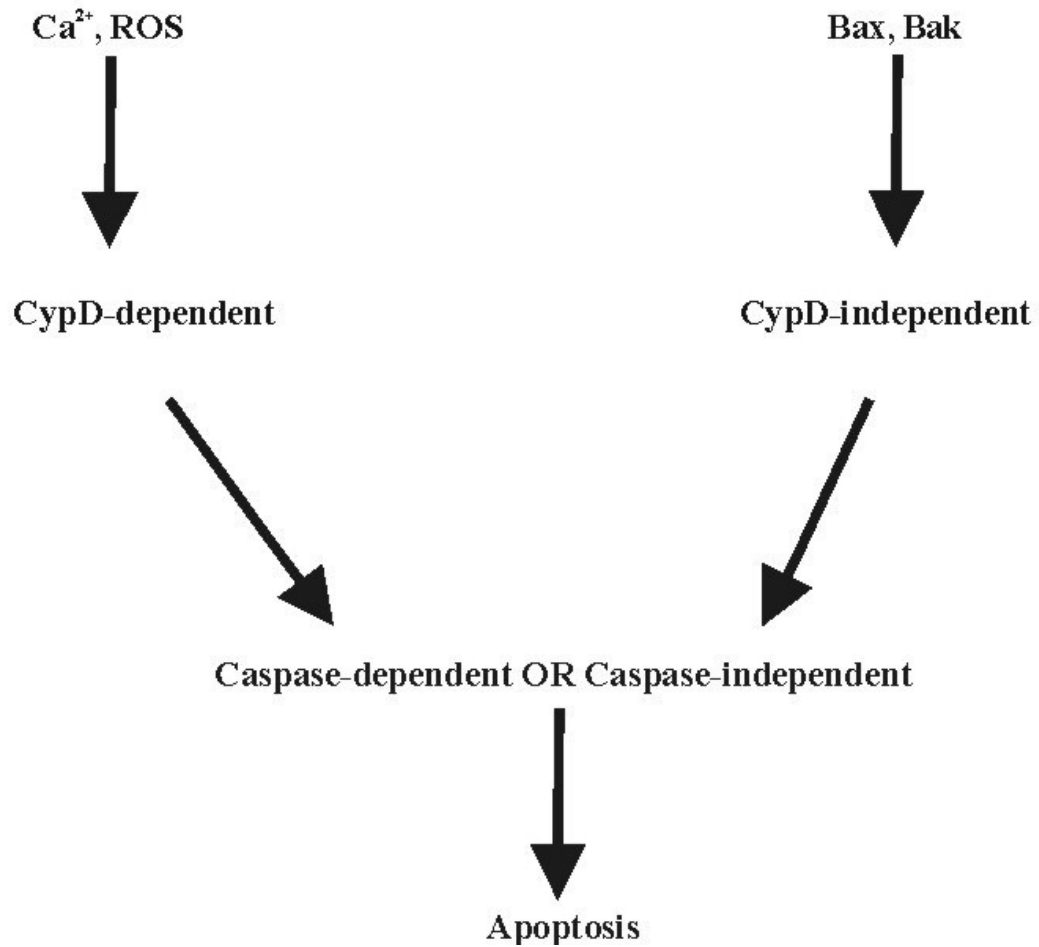


Figure 1.5. The role of CypD in cell death.  $\text{Ca}^{2+}$  and ROS have been shown to promote mPT and cell death through a CypD-dependent mechanism. Pro-apoptotic Bcl-2 family members (i.e. Bax and Bak) induce mPT and cell death in a CypD-independent mechanism. Likely, both pathways coexist.



Following mPTP, pro-apoptotic proteins such as Smac/DIABLO, apoptosis inducing factor (AIF), and cytochrome *c* can be released from the mitochondria, leading to apoptosis (Fig. 1.6). Once released, cytochrome *c* forms a ATP-dependent complex with apoptosis-inducing factor (Apaf-1) that in turn will activate caspase-9 and caspase-3 and results in caspase-dependent cell death (Stoica and Faden 2010). In contrast, following release AIF translocates to the nucleus where it causes DNA fragmentation in a caspase-independent mechanism. The latter cell death pathway does not require ATP and can proceed under conditions of bioenergetic compromise, such as in TBI.

### *1.2.3 Mitochondrial dysfunction following TBI*

It is well established that mitochondrial dysfunction occurs early after TBI (Robertson et al. 2007; Singh et al. 2006; Xiong et al. 1997). Associated with this early mitochondrial dysfunction is an increase in ROS production, protein nitration, and lipid peroxidation (Azbill et al. 1997; Braugher and Hall 1992; Hall et al. 1992). Specifically oxidative damage to cortical and hippocampal mitochondria has been shown to occur as early as 30 min following TBI (Singh et al. 2006). Administration of antioxidants following TBI has been demonstrated to improve mitochondrial function (Mustafa et al. 2010; Xiong et al. 1999; Xiong et al. 2005). As a consequence of increased mitochondrial  $Ca^{2+}$  and oxidative damage mitochondrial bioenergetics are compromised following TBI (Sullivan et al. 2005). Additionally, mitochondrial calcium sensitivity has been shown to decrease following TBI (Lifshitz et al. 2003). Also cytochrome *c* release is well documented in TBI (Robertson et al. 2007; Sullivan et al. 2002).

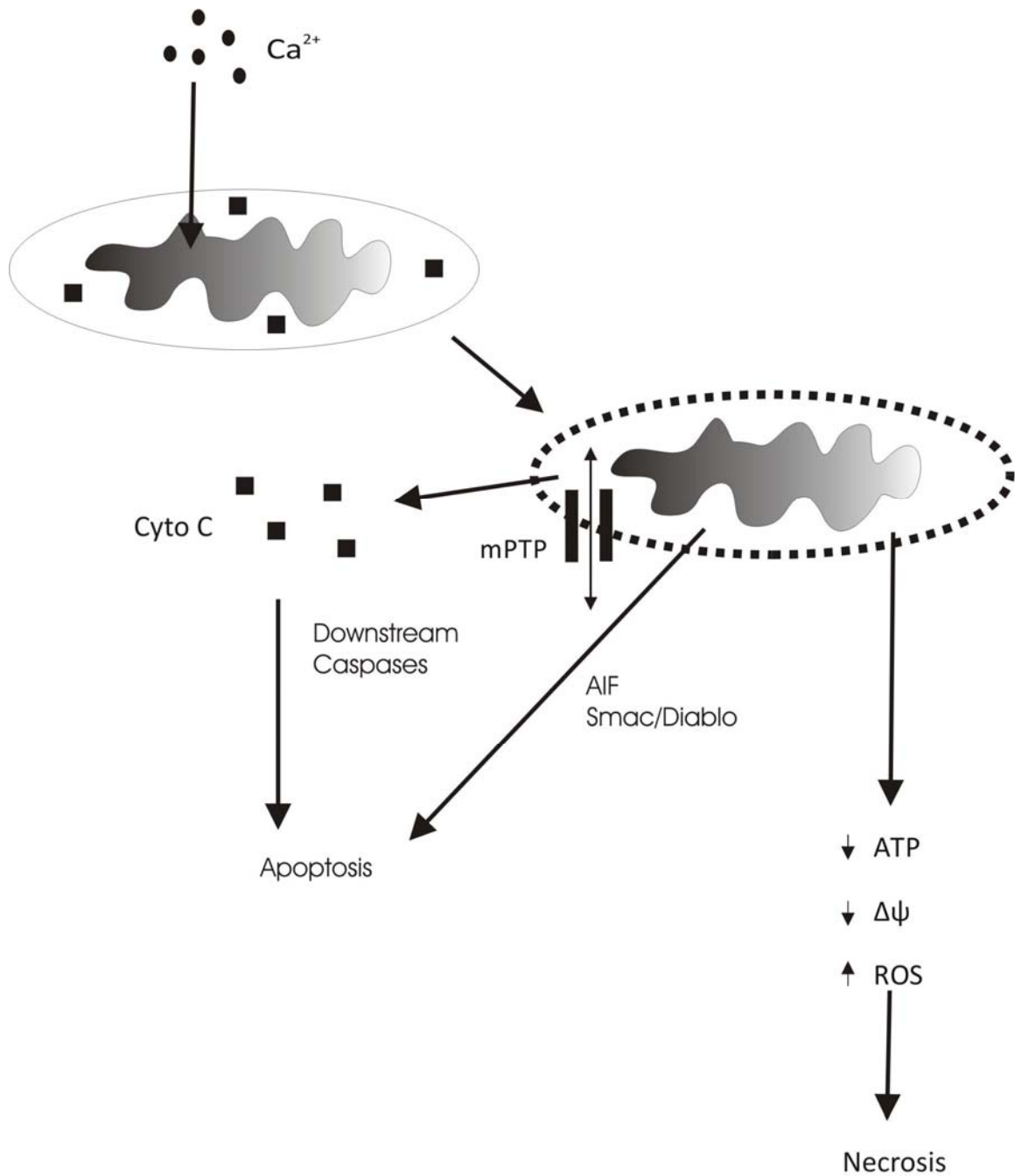


Figure 1.6. Highly simplified schematic diagram illustrating the pivotal role of mitochondria in apoptotic and necrotic neuronal cell death following excitotoxic insult. The excitatory amino acid glutamate increases  $\text{Ca}^{2+}$  influx into neurons. Mitochondria sequester the  $\text{Ca}^{2+}$  in an effort to maintain cytosolic  $\text{Ca}^{2+}$  homeostasis. Excessive mitochondrial  $\text{Ca}^{2+}$  cycling/loading can result in mitochondrial permeability that can be mediated by the mitochondrial permeability transition pore (mPTP) opening or via

increased permeability of the outer membrane without mPTP opening. In either case, pro-apoptotic proteins such as Smac/DIABLO, apoptosis-inducing factor (AIF), and cytochrome *c* (Cyto C) can be released from the mitochondria, leading to activation of downstream caspases and apoptosis. Ca<sup>2+</sup>-induced mPT results in increased conductance of the inner membrane, mitochondrial swelling, loss of ATP production due to decreased membrane potential ( $\Delta\psi$ ), and increased reactive oxygen species (ROS) production. These events promote mPT and can lead to necrosis depending on the extent and severity of the insult or injury.

There is a wealth of evidence that demonstrates that mPTP inhibition following TBI is neuroprotective. Administration of CsA following TBI has proven to be efficacious in multiple models of TBI (Albensi et al. 2000; Alessandri et al. 2002; Buki et al. 1999; Kilbaugh et al. 2011; Okonkwo et al. 1999; Okonkwo and Povlishock 1999; Scheff and Sullivan 1999; Sullivan et al. 2000b; Sullivan et al. 2000c; Sullivan et al. 1999) as well as in other CNS injury models (Friberg et al. 1998; Yoshimoto and Siesjo 1999). Recently the non-immunosuppressive CsA analog, NIM811, was shown to improve motor function, improve mitochondrial function, decrease oxidative damage, and decrease neurodegeneration following TBI (Mbye et al. 2009; Mbye et al. 2008).

### 1.3 Blast physics and injury pathology

The *Friedlander* wave form mathematically describes the blast overpressure (BOP) event generated by an explosion which includes a shock wave that is generated by an instantaneous rise in atmospheric pressure (high-pressure shock wave) that decays exponentially from the origin of the blast (Elsayed 1997) (Fig. 1.7). Following the initial shock wave the pressure may drop below ambient pressure before returning to baseline.

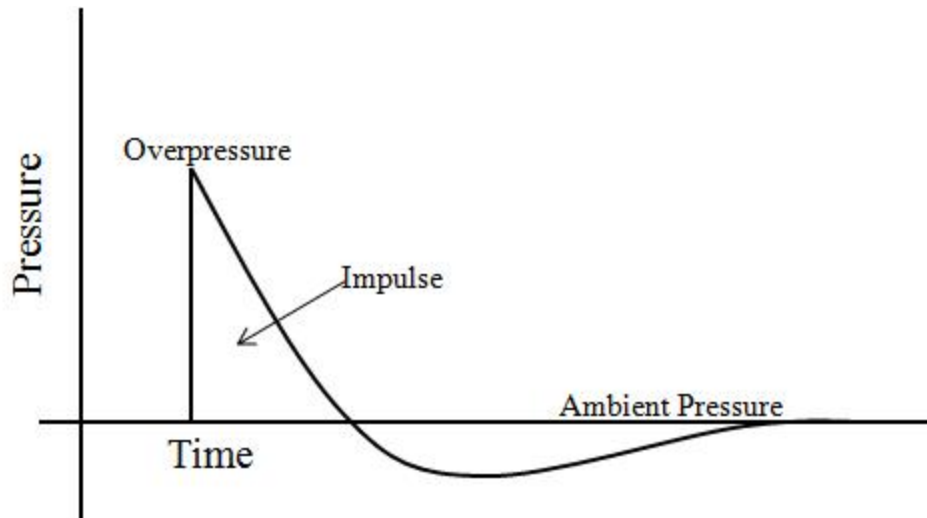


Figure 1.7. Friedlander wave. Open-field waveform characterized by an instantaneous rise in ambient pressure (positive phase), followed by exponential decay that may drop below ambient pressure (negative phase).

It is important to note that this wave form only applies to blasts that occur in open air environments while blasts that occur in enclosed environments display more complex wave forms (Mayorga 1997). The duration of overpressure, the time between the initial increases in pressure until its return to baseline, has been shown to have a direct impact on injury severity (Elsayed 1997). Primary blast injuries are caused by the direct effects of either overpressurization or underpressurization (barotrauma) (DePalma et al. 2005). Air-filled organs and air-fluid interfaces are the most common sites of primary blast injury, this is due to dynamic pressure differences at tissue density (air-fluid) interfaces caused by the interaction of a high frequency stress wave and lower frequency shear wave (Finkel 2006). Pulmonary barotrauma is one of the most common and life threatening primary blast injuries and often results in pulmonary edema (Finkel 2006). As a consequence of lung injury hypotension and hypoxemic secondary events are likely contributors to overall outcome following blast (Dewitt and Prough 2008). Secondary blast injuries are caused by objects set in motion by the shock wave that come in contact with people. Tertiary blast injuries result from individuals being propelled away from the site of the blast; these injuries are caused by individuals coming in contact with objects. Quaternary blast injuries are considered injuries not associated with primary, secondary, or tertiary injuries; such as burns, radiation exposure, and toxic inhalation (DePalma et al. 2005). Secondary and tertiary injuries are not specific to air filled organs and are capable of producing brain injury, however little is known about the possibility of primary brain blast injuries.

BOP is commonly modeled using blast tubes or shock tubes and less often in an open-field environment (Cernak and Noble-Haeusslein 2010). In both tubes the animal is

positioned in a holder that prevents secondary and tertiary blast injuries. Blast tube shockwaves are generated by an explosion (i.e. combustion of a flammable gas or detonation of an explosive charge). Whereas shockwaves generated in a shock tube are produced by compressed air or gas. A shock tube is composed of a compression chamber separated from an expansion chamber by a rupturable membrane (i.e. mylar membrane). The compression chamber is loaded with air or gas until the failure point of the membrane is reached, at which time the membrane ruptures and releases the gas/air into the expansion chamber. The thickness of the membrane determines the peak overpressure such that the thicker the membrane the higher the peak overpressure (Elsayed 1997). The rapidly expanding gas/air results in a shockwave.

High levels of BOP, even without skull fracture, are capable of producing cerebral contusion and subdural hemorrhage (Mayorga 1997). Recently pressure waves with intensities capable of damaging nervous tissue were detected in the brains of rats exposed to low level (40 kPa) BOP (Chavko et al. 2006). Although animal studies evaluating the effect of BOP on the CNS are limited, some have shown the susceptibility of the CNS to primary blast forces. One group studied the effect of BOP on the CNS with rats exposed to a severe level of BOP; they reported cognitive deficits, neuronal swellings, glial reaction, and oxidative stress (Cernak et al. 2001a; Cernak et al. 2001b). Another group showed that electroencephalogram activity was depressed in pigs exposed to a mild BOP event (Axelsson et al. 2000). More recent experimental reports in rats and pigs have further characterized the neuropathology that ensues following BOP exposure. In order to delineate the systemic effects versus the direct effects of BOP on brain injury, Long and colleagues recently equipped rats with Kevlar vests in order to protect the abdomen and

thorax from BOP. This study found that rats equipped with vests had less fiber degeneration than those without vests. Also this study reported that rats exposed to 126 kPa BOP showed less neuropathology than those exposed to 147 kPa, indicating a dose response between level of blast and neuropathology (Long et al., 2009). A recent swine model of BOP indicated that pigs exposed to BOP exhibited cortical fiber degeneration and astrocytic activation (Bauman et al., 2009). Behavioral changes and neuropathological signs of brain injury as a result of exposure to low levels of BOP have also been reported (Saljo et al. 2008; Saljo et al. 2009c).

#### 1.4 Specific Aims and Hypotheses

Inhibition of CypD with CsA and NIM811 following TBI has been shown to be neuroprotective. However, both NIM811 and CsA have inverted U dose responses following TBI. It remains to be determined whether the observed dose responses are due to the interaction with CypD and/or other off-target interactions. Therefore CypD null mice will be used to determine the contribution of CypD to secondary injury and to provide further validation of CypD's potential as a therapeutic target following TBI (Fig. 1.8). We hypothesize that genetic knockout of CypD will attenuate formation of the mPTP, which will translate into increased cortical tissue sparing, increase hippocampal neuron survival, and improved mitochondrial bioenergetics.

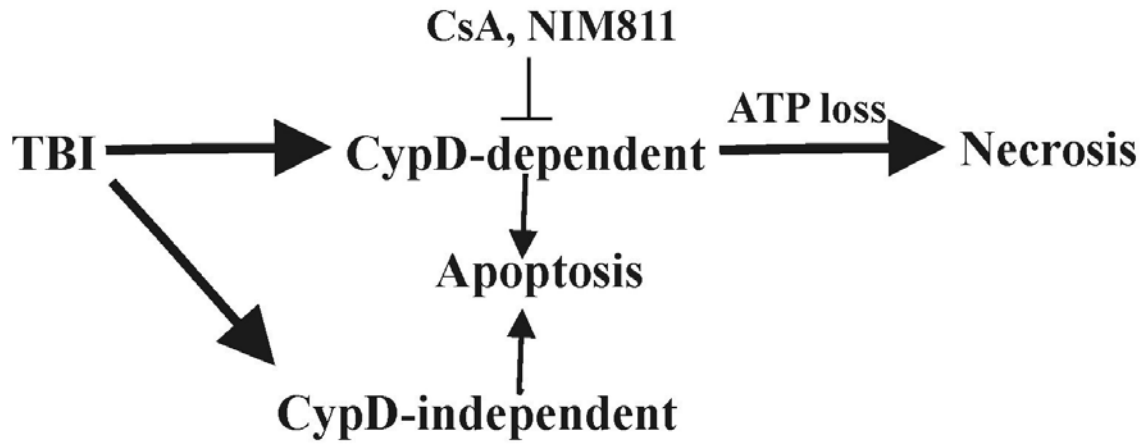


Fig. 1.8. Model of the hypothesis that CypD is an essential mediator of cell death following TBI. CypD knockout studies have demonstrated that, depending on the cell death signal, mPT mediated cell death may proceed through either a CypD-dependent mechanism or a CypD-independent mechanism. TBI is proposed to engage both mechanisms. Therefore, pharmacological inhibition and genetic knockout of CypD is hypothesized to provide neuroprotection following TBI.



Because there may be a prerequisite for CypD for normal physiological functioning, genetic ablation of CypD could prove detrimental. As such partial pharmacological inhibition of CypD, rather than total inhibition, could prove to be a better therapeutic strategy. The utility of CsA is limited due to its interaction with calcineurin and resultant toxicity. Since NIM811 is less toxic than CsA and can be administered *in vivo* at higher levels it may have greater therapeutic potential. NIM811 has been shown to attenuate mitochondrial dysfunction, decrease neurodegeneration, and improve motor function following TBI, thus we hypothesize that administration of NIM811 will improve cognition, increase tissue sparing, and improve mitochondrial function.

Clinical and experimental data have shown that blast exposure does result in neuropathology. In order to begin to understand the mechanism (s) of injury following blast traumatic brain injury (bTBI) and to develop treatment strategies for bTBI it is important to develop clinically relevant bTBI models. Therefore, we aim to characterize the time-course of “classical” TBI markers of pathology following bTBI with the eventual goal of selecting therapeutic targets for bTBI.

In order to test these hypotheses we have formulated the following specific aims:

*Specific aim 1 will evaluate the hypothesis that genetic ablation of CypD will improve behavioral function and provide neuroprotection following TBI.* The effects of CypD knockout on neuroprotection following TBI will be assessed using cortical tissue sparing analysis and stereological assessment of hippocampal cell counts. Additionally, the effects of CypD knockout on mitochondrial function following TBI will be determined.

*Specific aim 2* will examine the hypothesis that pharmacological inhibition of CypD with NIM811 will reduce tissue damage, attenuate mitochondrial dysfunction, and improve functional outcome following TBI. MWM will be used to determine the effect of NIM811 on cognitive function following TBI. Following TBI, isolated mitochondria from NIM811 and vehicle administered rats will be assayed to determine the effects of treatment on mitochondrial function. Neuroprotective effects of NIM811 will be determined using cortical tissue sparing analysis.

*Specific aim 3* will test the hypothesis that BOP exposure will result in oxidative damage, inflammation, and blood brain barrier (BBB) breakdown. Immunohistochemistry using antibodies against 3-nitrotyrosine, 4-hydroxynonenal, IgG, and Cd11b/c will be used to determine the profile of oxidative damage, inflammation, and blood brain barrier (BBB) breakdown following TBI. Additionally, [3H]-PK11195 autoradiography will be used to determine the extent of microglial activation following TBI.

## 2. CHAPTER 2

### **Genetic Approach to Elucidate the Role of Cyclophilin D in TBI Pathology**

#### 2.1 Introduction

Cell death following TBI occurs in two waves; an immediate wave of cell death as a consequence of physical injury and a secondary wave of cell death resulting from biochemical and/or physiological signals (Stoica and Faden 2010). Excitotoxicity following injury results in disrupted intracellular  $\text{Ca}^{2+}$  homeostasis. In an effort to maintain cytosolic  $\text{Ca}^{2+}$  levels mitochondria buffer rises in intracellular  $\text{Ca}^{2+}$ . However, mitochondrial  $\text{Ca}^{2+}$  buffering is finite and excessive mitochondrial  $\text{Ca}^{2+}$  loading results in mitochondrial dysfunction. As such mitochondrial dysfunction has been implicated as a key pathological event following TBI. Mitochondria regulate both necrotic and apoptotic cell death pathways likely via modulation of the mPTP (Green and Reed 1998; Schneider 2005). Although the exact molecular identity of the mPTP is unknown the adenine nucleotide translocase (ANT) of the inner membrane, the voltage-dependent anion channel (VDAC) of the outer membrane, and the matrix-specific peptidyl-prolyl *cis-trans* isomerase cyclophilin D (CypD) appear to be mPTP components. Cyp-D translocates from the matrix to the mPTP where it interacts with ANT and promotes pore formation (Lemasters, et al., 2009).

Recently,  $\text{Ca}^{2+}$  and ROS were shown to promote pore formation by a CypD dependent mechanism (Baines et al. 2005; Basso et al. 2005). Also, CypD knockout resulted in remarkable protection from cardiac ischemia-reperfusion injury (Nakagawa et

al. 2005). However, not all cell death pathways initiated by the mitochondria are CypD dependent (i.e. Bcl-2 family members induce apoptosis by a CypD independent mechanism) (Green and Kroemer 2004). CypD knockout did not protect against cell death mediated by Bcl-2 family members (Baines et al. 2005).

The connection between CypD and cell death following TBI has been supported by studies demonstrating that inhibition of mPTP formation with NIM811 and CsA is neuroprotective. Presumably, the neuroprotective actions of CsA and NIM811 are attributable to their inhibition of CypD, which prevents the binding of CypD to ANT and formation of the mPTP (Nicolli, et al., 1996). However, it is difficult to discern the protective mechanisms of NIM811 and CsA given that both have targets other than CypD. Since cell death may occur independent of CypD the overall role CypD plays in cell death following TBI remains largely unknown.

To further elucidate the role CypD plays in neuropathology following TBI mice lacking the CypD encoding gene *Ppif* were subjected to a controlled cortical impact model of TBI. We hypothesized that genetic knockout of CypD would confer neuroprotection following TBI. At 18 days post-injury cortical tissue sparing assessment was performed. To determine the effects of CypD knockout on hippocampal cell survival, neurons were counted for regions dentate gyrus (DG), CA3, and CA1 using the optical dissector method. Next we determined the effects of CypD knockout on mitochondrial function following TBI. The data demonstrate that knockout of CypD, likely via maintenance of mitochondrial homeostasis, spares cortical tissue and protects

CA3 neurons following TBI and support the hypothesis that CypD is an important mediator of cell death following TBI.

## 2.2 Methods

### 2.2.1 *Animals, injury, and experimental design*

All experimental animal procedures were approved by the Animal Care and Use Committee at the University of Kentucky. Adult wild-type (C57BL/6) and CypD knockout mice were subjected to a severe (1.0 mm) unilateral controlled cortical contusion TBI or sham-operation (n=18). Briefly, the skull was exposed through a midline incision and an approximately 4 mm craniotomy was made lateral to the midline and centered between bregma and lambda. A cortical contusion was produced using a pneumatically driven injury device with a 3 mm tip as previously described (Mbye et al. 2008). Following injury a prosthetic skull cap was glued over the craniotomy site. Animals remained on a 37° heating pad until their righting reflex had returned.

### 2.2.2 *Tissue processing and measurements of tissue sparing*

At 18 days post-injury, wild-type and CypD knockout mice were anesthetized by an overdose of pentobarbital and transcardially perfused with physiological saline followed by 4% paraformaldehyde (n=4-5/group). After removal, the brains were placed in 4% paraformaldehyde-sucrose (15%) for an additional 24 h. Coronal sections (30 µm) were then cut using a freezing microtome, throughout the rostrocaudal extent of the brain, extending through the septal area to the most posterior extent of the hippocampus.

### 2.2.3 *Optical Fractionator Method*

All sampling was conducted using an Olympus BX51 microscope with a 60 X oil objective, with an ASI automated stage (Eugene, OR). Bioquant Image analysis software (R and M Biometrics, Memphis, TN) was used to estimate the total cell number in ipsilateral hippocampal regions CA1, CA3, and DG using the optical fractionator method as previously described (Baldwin et al. 1997). Briefly, this method involves sampling a known fraction of the section thickness, under a known fraction of the sectional area, in a known fraction of the sections that contain the structure. The total number of neurons (N) is estimated by:  $N = \Sigma Q * t/h * 1/asf * 1/ssf$ , where  $\Sigma Q$  is the number of neurons counted in the optical dissectors,  $t$  is the tissue thickness (30  $\mu\text{m}$ ),  $h$  is the height of the dissector (25  $\mu\text{m}$ ),  $1/asf$  is the counting grid area (100 X 100)/the dissector area (CA3, CA1-15  $\mu\text{m}$  X 15  $\mu\text{m}$ ; DG-10  $\mu\text{m}$  X 10  $\mu\text{m}$ ), and  $1/ssf$  is the sampling section fraction (12).

#### *2.2.4 Mitochondrial isolation and respiration analysis*

At 6 h post-injury wild-type and CypD knockout mice were asphyxiated with CO<sub>2</sub> until unconscious, decapitated, and the brains were rapidly removed and placed in isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, and 1 mM EGTA; pH 7.2) (n=3/group). The cortices were dissected with a 3 mm-diameter punch centered on the site of impact. The cortical tissue punch contained tissue from the site of the impact and the surrounding penumbra. The tissue punches were homogenized and isolated by differential centrifugation as previously described (Singh, et al., 2006). Briefly, the homogenate was centrifuged twice at 1300 xg for 3 min. The pellet was discarded, and the supernatant was further centrifuged at 13,000 xg for 10 min. In order to release synaptic mitochondria, the crude mitochondrial pellet was subsequently

subjected to nitrogen decompression using a nitrogen cell disruption bomb (1200 psi, 10 min). After nitrogen disruption, the mitochondria were suspended in 3.5 mL of 15% percoll and placed on a percoll gradient consisting of 3.5 mL of 24% percoll and 3.5 mL of 40% percoll in 13-mL ultraclear tubes. The gradient was centrifuged in a fixed-angle rotor at 30,400  $\times g$  for 10 min at 4°C. The fraction accumulated at the interphase of the 40% and 24% percoll layers was carefully removed and diluted with isolation buffer without EGTA, then centrifuged at 12,300  $\times g$  for 10 min at 4°C. The supernatants were carefully removed, and the pellet was resuspended in isolation buffer without EGTA and centrifuged at 13,000  $\times g$  for 10 min at 4°C. The mitochondrial pellets were recentrifuged at 10,000  $\times g$  for 5 min at 4°C to yield tighter pellets. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a concentration of ~10 mg/mL. The protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Mitochondrial respiration was measured using a Seahorse Biosciences XF24 Flux Analyzer (North Billerica, MA) as previously described (Sauerbeck et al. 2011). Briefly, pyruvate plus malate plus ADP, oligomycin, FCCP, and rotenone plus succinate were injected sequentially through ports A–D, respectively, in the Seahorse Flux Pak cartridges to yield final concentrations of 5 mM (pyruvate), 2.5 mM (malate), 1 mM (ADP), 1  $\mu g/ml$  (oligomycin), 1  $\mu M$  (FCCP) and 100 nM (rotenone), 10 mM (succinate), respectively.

### *2.2.5 Statistics*



For all statistical comparisons, significance was set at  $p < 0.05$ . Tissue sparing assessment data were analyzed using unpaired  $t$ -test. Cell count data were analyzed using a two-factor ANOVA followed by Bonferroni post-hoc analysis. Mitochondrial data were analyzed using one-way ANOVA followed by SNK post-hoc analysis.

## 2.3 Results

### 2.3.1 *Cyclophilin D knockout increases tissue sparing following TBI*

In order to determine the effects of CypD knockout on tissue sparing following TBI, wild-type and CypD knockout mice were subjected to a severe (1.0 mm) CCI TBI. In these experiments cortical tissue sparing was determined at 18 days post-injury utilizing the Calvalieri method. CypD knockout mice demonstrated a significant increase in tissue sparing ( $84.1 \pm 2.1$ ) compared to wild-type mice ( $76.2 \pm 2.248$ ) ( $p < 0.03$ ) (Fig. 2.1).

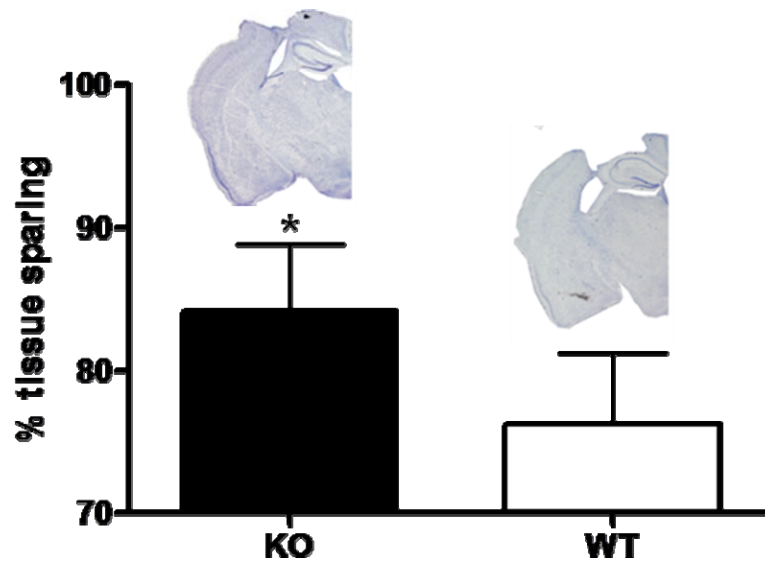
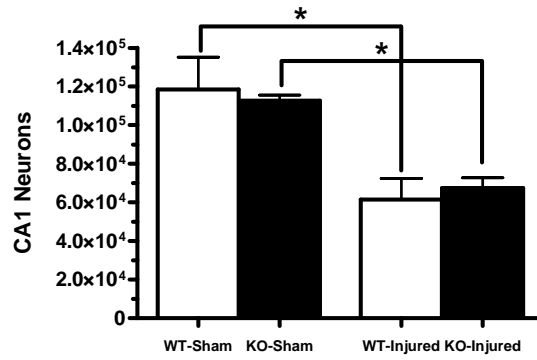


Figure 2.1. CypD knockout increases tissue sparing following TBI. Quantitative assessment of tissue sparing revealed that CypD knockout animals demonstrated a significant increase in tissue sparing when compared to wild-type animals. Data points represent group means  $\pm$  SD. \* $p = 0.033$ .

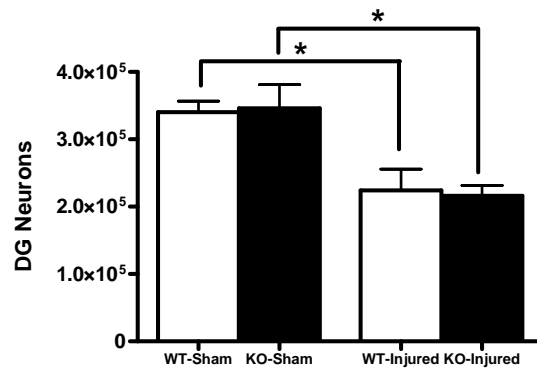
### 2.3.2 Cyclophilin D knockout protects CA3 hippocampal neurons

The number of surviving CA1, CA3 and DG neurons were estimated using the optical fractionator method at 18 days post-injury (Fig. 2.2). In wild-type mice, injury significantly decreased the number of neurons in ipsilateral CA1 [ $F(1,16) = 110.7$ ;  $p < 0.001$ ], CA3 [ $F(1,16) = 21.6$ ;  $p < 0.001$ ], and DG [ $F(1,16) = 120.1$ ;  $p < 0.01$ ]. In CypD knockout mice, injury significantly decreased the number of neurons in ipsilateral CA1 [ $F(1,16) = 120.1$ ;  $p < 0.001$ ] and DG [ $F(1,16) = 120.1$ ;  $p < 0.001$ ]. In ipsilateral CA3 of CypD knockout TBI mice, numbers of surviving neurons were not significantly different than CypD knockout sham mice.

A.)



B.)



C.)

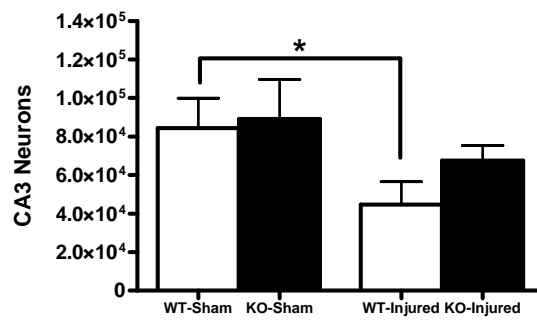


Figure 2.2. CypD knockout protects CA3 hippocampal neurons following TBI. Injury significantly decreased the number of neurons in CA1 (A), DG (B), and CA3 (C) in wild-type mice. CypD knockout protected CA3 neurons from injury-induced cell loss. Data points represent group means  $\pm$  SD. \* $p < 0.05$ .

### *2.3.3 Cyclophilin D knockout improves mitochondrial complex I driven respiration*

In order to determine the effects of CypD knockout on mitochondrial respiration following TBI, mitochondria were isolated at 6 h post-injury from the ipsilateral cortex of wild-type and CypD knockout mice. In the presence of pyruvate plus malate plus ADP, injury-induced impairments in complex I driven respiration (State III respiration) were attenuated by CypD knockout ( $p < 0.05$ ) (Fig. 2.3). Complex II driven respiration was significantly reduced in both CypD knockout and wild-type mice ( $p < 0.05$ ).

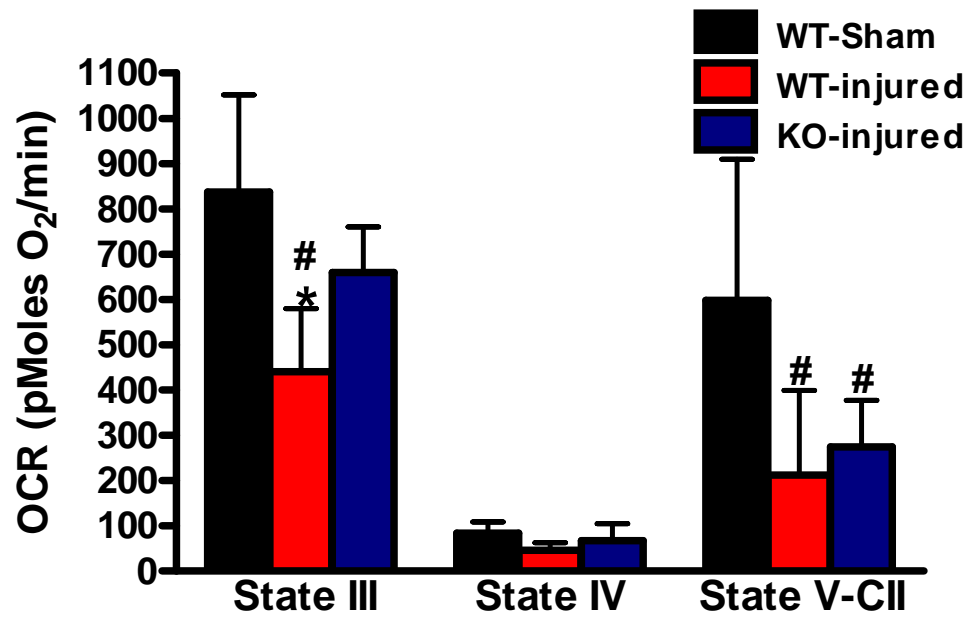


Figure 2.3. CypD knockout attenuates mitochondrial dysfunction following TBI. At 6 h post-injury wild-type mice (white bars) complex I driven respiration was significantly reduced compared to sham (black bars); CypD knockout attenuated this dysfunction. Data points represent group means  $\pm$  SD. # $p < 0.05$  v WT-sham, \* $p < 0.05$  v KO-injured.

## 2.4 Discussion

We investigated the role of Cyp-D in TBI, in which Cyp-D dependent cell death mechanisms have been implicated including  $\text{Ca}^{2+}$  dishomeostasis and increased ROS production. The present data for the first time demonstrates that genetic ablation of Cyp-D is neuroprotective following TBI. Importantly, Cyp-D knockout attenuated injury-induced inhibition of mitochondrial complex I respiration. Therefore, the present results confirm the key role Cyp-D plays in cell death following TBI.

Pharmacological studies have suggested an important role for Cyp-D in neurodegeneration and TBI. Administration of CsA has been shown to be neuroprotective following multiple experimental models of TBI (Buki et al. 1999; Kilbaugh et al. 2011; Okonkwo et al. 1999; Scheff and Sullivan 1999; Sullivan et al. 2000c; Sullivan et al. 1999). Since CsA interacts with Cyp-D and inhibits calcineurin, the neuroprotective mechanism(s) of CsA is greatly debated. NIM811, which inhibits Cyp-D but doesn't affect calcineurin, has also been shown to have neuroprotective properties following TBI and spinal cord injury. However, NIM811 has a U-dose response curve following TBI which may indicate that NIM811 is acting through other potential off-target mechanisms. Genetic knockout of Cyp-D provides a novel approach for directly examining the role that Cyp-D plays without the confounding off-target effects of pharmacological studies.

The observed improvement in mitochondrial complex I activity following TBI in Cyp-D knockout mice likely reflects a secondary effect of inhibition of mPTP formation by loss of Cyp-D (Mbye et al. 2008). The attenuation of decreases in mitochondrial complex I respiration rates suggest that loss of Cyp-D increases mitochondrial resistance



to TBI. These findings are in line with studies involving mitochondria isolated from CypD deficient mice which showed that mitochondria lacking CypD are resistant to  $\text{Ca}^{2+}$  induced mPTP formation (Baines et al. 2005).

Although it is generally accepted that the mPTP plays a role in cell death the mechanisms underlying induction of mPTP following TBI remain unclear. There appears to be two alternative routes to cell death, one that is Cyp-D dependent and an alternative route that is Cyp-D independent; both pathways have been implicated in TBI. An important question is if inhibition of one pathway is sufficient to overcome cell death or if inhibition of one pathway could enhance/promote the other pathway, with the same result either way-cell death. There is growing evidence that suggests that Cyp-D knockout is protective in neurodegenerative disorder models (Du et al. 2008; Du et al. 2011; Wang et al. 2009). Interestingly, one study found that the prevalent cell death pathway after hypoxic-ischemic brain injury is developmentally regulated such that Cyp-D dependent pathway is more critical in the development of brain injury in the adult (Wang et al. 2009). This study demonstrated that Cyp-D knockout significantly reduced hypoxic-ischemic brain injury in adult mice while knockout exacerbated the injury in neonatal mice. Related to this, our results indicate that maintenance of mitochondrial homeostasis, by the absence of Cyp-D, following TBI is protective.

We have previously demonstrated that continuous infusion of CsA for up to 7 days produces greater protection than a single dose administration of CsA following TBI (Sullivan et al. 2000c). Also, we have recently shown that the earlier the treatment with CsA is initiated following TBI the greater the neuroprotection that is afforded (Sullivan et

al. 2011). Taken together these results suggest that the mechanisms underlying cell death following TBI are active for a prolonged period of time and that early intervention provides the greatest benefit. Consistent with these findings, genetic knockout of Cyp-D indicated that following TBI complete absence of Cyp-D from the onset was indeed beneficial.

Cyp-D knockout was recently shown to protect neurons against glutamate triggered cell death *in vitro* (Li et al. 2009). Importantly, the protection was dependent on the severity of the glutamate insult such that protection was not observed following the more severe insult. This may indicate that following more severe insults that Cyp-D independent cell death mechanisms are more prevalent. In unpublished data from our lab we have shown that following a mild (0.5 mm) CCI TBI there is modest neuroprotection. Our results suggest that Cyp-D dependent cell death mechanisms are more prevalent in TBI, as Cyp-D ablation resulted in protection from mild and severe TBI.

This is the first study to report the effects of Cyp-D knockout in a model of traumatic brain injury and we showed that the lack of Cyp-D significantly decreased the damage associated with TBI. Thus, Cyp-D dependent cell death is critically involved in TBI, supporting the hypothesis that Cyp-D is a valid target for neuroprotection following TBI.

### 3. CHAPTER 3

#### **Post-injury administration of the mitochondrial permeability transition pore inhibitor, NIM811, is neuroprotective and improves cognition following traumatic brain injury in rats**

*(Accepted for publication in Journal of Neurotrauma)*

##### 3.1 Introduction

Traumatic brain injury (TBI) is characterized by significant neurological dysfunction which results from a primary rapid necrosis of tissue at the site of injury followed by a delayed secondary injury. Mechanisms believed to play a role in cell death following TBI are numerous and include excitotoxicity, inflammation, mitochondrial dysfunction, oxidative stress, calpain activation, and caspase activation (Deng-Bryant et al. 2008; Deng et al. 2007; Hall et al. 2004; Kelley et al. 2007; Lifshitz et al. 2003; Lifshitz et al. 2004; McGinn et al. 2009; Morganti-Kossmann et al. 2007; Saatman et al. 2010; Singh et al. 2006). Following TBI, excessive activation of *N*-methyl-D-aspartate (NMDA) receptors by excitatory amino acids results in an elevation in cytoplasmic  $Ca^{2+}$  (Hinzman et al. 2010). Under normal conditions mitochondria primarily act to regulate energy metabolism and as high-capacity  $Ca^{2+}$  sinks (Giacomello et al. 2007). However, excessive mitochondrial  $Ca^{2+}$  loading following TBI may result in formation of the mitochondrial permeability transition pore (mPTP) (Sullivan et al. 2005). A consequence of mPTP formation is a loss of membrane potential, which supports the uncoupling of electron transport from ATP production. The release of pro-apoptotic molecules (i.e., cytochrome C, Smac/Diablo, and apoptosis inducing factor) from the mitochondria is, in part, orchestrated by mPTP and leads to the activation of cell death pathways (Jordan et al. 2003). An additional consequence of mPTP formation is the production of reactive

oxygen species (ROS) which contribute to cellular damage by oxidizing cellular proteins and lipids (Mazzeo et al. 2009).

Well-known components of the mPTP include the adenine nucleotide translocase (ANT) of the inner membrane, the voltage-dependent anion channel (VDAC or Porin) of the outer membrane, and the matrix-specific peptidyl-prolyl *cis-trans* isomerase cyclophilin D (Cyp-D). Cyp-D translocates from the matrix to the mPTP where it interacts with ANT and promotes pore formation (Lemasters et al. 2009). Cyclosporin A (CsA) and NIM811, inhibit the opening of mPTP by binding Cyp-D, which prevents the binding of Cyp-D to ANT (Nicolli et al. 1996).

Abundant evidence demonstrates the neuroprotective efficacy of mitochondrial permeability transition pore inhibition following TBI (Cook et al. 2009; Vink et al. 2001). Administration of CsA following TBI significantly improves mitochondrial function and subsequently decreases neuronal damage (Buki et al. 1999; Okonkwo and Povlishock 1999; Scheff and Sullivan 1999; Sullivan et al. 2000b; Sullivan et al. 2000c; Sullivan et al. 1999). However, studies investigating the effects of CsA administration on cognition following TBI are limited and contradictory. In one study CsA's neuroprotection directly translated into improved cognition (Alessandri et al. 2002). In contrast another study reported that CsA administration did not result in improved cognition following experimental TBI (Riess et al. 2001). Therefore the absence of cognitive data supporting the use of CsA following TBI warrants further investigation of mPTP inhibitors.

The non-immunosuppressive cyclosporin A analog, N-methyl-4-isoleucine-cyclosporin (NIM811), lacks the ability to inhibit calcineurin and therefore can be administered *in vivo* at much higher doses than CsA (Rosenwirth et al. 1994; Waldmeier

et al. 2002). NIM811 has been shown to have a favorable pharmacokinetic profile with oral bioavailability similar to that of CsA (Rosenwirth et al. 1994). The toxicity of NIM811 is significantly less than CsA as indicated by inducing fewer nephrotoxic effects. Recently we demonstrated that a 10-mg/kg dose of NIM811 was as effective as a 20-mg/kg dose of CsA in attenuating mitochondrial dysfunction and neurodegeneration and improving motor function following experimental controlled cortical impact (CCI) TBI in mice (Mbye et al. 2009; Mbye et al. 2008).

In the present study, we used the CCI TBI model to assess whether or not the effects of NIM811 administration to rats would duplicate the neuroprotective effects of NIM811 previously observed with mice and to determine if these effects would translate into improved cognition. Firstly, we determined the dose-response of NIM811 for increased tissue sparing. Secondly, we determined the effect of the optimal dose of NIM811 for improved cognitive performance using the Morris Water Maze (MWM). Thirdly, we assessed the effect of the optimal dose of NIM811 for improving mitochondrial function and decreasing mitochondrial oxidative damage. The results demonstrate that NIM811 administration is capable of both conferring neuroprotection and improving cognition following TBI.

## 3.2 Methods

### 3.2.1 *Animals, injury, and experimental design*

All experimental animal procedures were approved by the Animal Care and Use Committee at the University of Kentucky. Adult male Sprague-Dawley rats weighing approximately 300-350 g were subjected to a severe (2.0 mm) unilateral controlled cortical contusion TBI or sham-operation (n=66). Surgical procedures were performed as previously described under 2% isoflurane (Davis et al. 2008). For tissue sparing assessment studies, animals were administered vehicle (100 % DMSO) or NIM811 (*N*-methyl-4 isoleucinecyclosporin; Novartis Pharma Ltd., Basal, Switzerland) (5, 10, 20, or 40 mg/kg) at 15 min and 24 h post-injury depending on group designation (n=5/group). Animals for behavioral studies were administered vehicle or NIM811 (10 mg/kg) at 15 min and 24 h post-injury. Animals designated for mitochondrial studies were administered either vehicle or NIM811 (10 mg/kg) at 15 min post-injury (n=5/group).

### 3.2.2 *Tissue processing and measurements of lesion volume*

At 15 days post-injury, animals were anesthetized by an overdose of pentobarbital and transcardially perfused with physiological saline followed by 4% paraformaldehyde. After removal, the brains were placed in 4% paraformaldehyde-sucrose (15%) for an additional 24 h. Coronal sections (50  $\mu$ m) were then cut using a freezing microtome, throughout the rostrocaudal extent of the brain, extending through the septal area to the most posterior extent of the hippocampus.

The extent of tissue sparing following TBI was assessed blindly with respect to treatment group. The assessment employed an unbiased stereological protocol, the

Cavalieri method (Michel and Cruz-Orive 1988). A systematic random subset of sections from the region of interest (minimum of 12), separated by a known distance, is then selected for analysis. Cortical tissue sparing was measured using Image J (NIH) software. On each section, the total cortical area, defined as the dorsal aspect of lamina I to the dorsal aspect of the corpus callosum, was determined for the entire ipsilateral hemisphere. The cortical area of the contralateral hemisphere was also determined in an identical fashion using the Cavalieri method. The volume of ipsilateral tissue spared was compared with the volume of tissue spared contralateral to the injury, and the results were expressed as the percentage tissue spared [(ipsilateral/contralateral) X 100].

### *3.3.3 Cognitive assessment*

Two separate cohorts of animals were used for MWM testing. Cohort I involved NIM811 treated and vehicle treated rats (n=5/group) and only involved training. Cohort II included sham (n=5), NIM811 treated (n=8), and vehicle treated (n=8) rats and involved training and a probe trial. For training data, cohorts I and II were averaged. For probe data, only cohort II was used. Beginning 10 days after the injury, spatial memory was assessed using an adapted MWM paradigm (Morris 1984) as previously described (Guseva et al. 2008). The water maze was divided into four quadrants, with the platform submerged in one of the quadrants. Visual cues located throughout the testing room aided in spatial orientation. Swimming during the “transfer” trials was videotaped and processed using a video motion analyzer (Columbus Instruments, Columbus, OH). Briefly, for each trial quadrant, entry was randomized for different starting positions, and animals were allowed to swim until they found the platform, where they remained for 15

sec after the trial. If the platform was not located within the allotted 60-sec trial, the animal was placed on the platform for 15 sec. Each day consisted of four acquisition trials separated by 5-min intervals. Over 5 days a total of 20 acquisition trials were administered for each animal. The average time to reach the platform each day was calculated for individual groups. For cohort II, training was followed by one 15 sec probe trial. The platform was removed from the pool for the probe trial. The number of times each animal crossed the platform was recorded.

#### *3.3.4 Mitochondrial isolation, mitochondrial calcium buffering capacity, and measurement of mitochondrial function*

All steps of the mitochondrial isolation protocol were performed on ice. At 6 h post-injury rats were asphyxiated with CO<sub>2</sub> until unconscious, decapitated, and the brains were rapidly removed and placed in isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, and 1 mM EGTA; pH 7.2). The cortices were dissected with a 5 mm-diameter punch centered on the site of impact. The cortical tissue punch contained tissue from the site of the impact and the surrounding penumbra. The tissue punches were homogenized and isolated by differential centrifugation as previously described (Singh et al. 2006). Briefly, the homogenate was centrifuged twice at 1300 xg for 3 min. The pellet was discarded, and the supernatant was further centrifuged at 13,000 xg for 10 min. In order to release synaptic mitochondria, the crude mitochondrial pellet was subsequently subjected to nitrogen decompression using a nitrogen cell disruption bomb (1200 psi, 10 min). After nitrogen disruption, the mitochondria were suspended in 3.5 mL of 15% percoll and placed on a percoll gradient consisting of 3.5 mL of 24%



percoll and 3.5 mL of 40% percoll in 13-mL ultraclear tubes. The gradient was centrifuged in a fixed-angle rotor at 30,400 xg for 10 min at 4°C. The fraction accumulated at the interphase of the 40% and 24% percoll layers was carefully removed and diluted with isolation buffer without EGTA, then centrifuged at 12,300 xg for 10 min at 4°C. The supernatants were carefully removed, and the pellet was resuspended in isolation buffer without EGTA and centrifuged at 13,000 xg for 10 min at 4°C. The mitochondrial pellets were recentrifuged at 10,000 xg for 5 min at 4°C to yield tighter pellets. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a concentration of ~10 mg/mL. The protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Mitochondria isolated from naïve animals were used to assess the effect of *in vitro* NIM811 treatment (1 µM) on mitochondrial Ca<sup>2+</sup> buffering capacity and mPTP. Mitochondrial Ca<sup>2+</sup> buffering capacity was assessed using a fluorescent spectrofluorometer assay as previously described (Brown et al. 2006). 100 nM CaG5N (excitation, 506 nm, emission, 532 nm) was used to measure extramitochondrial Ca<sup>2+</sup> in a Shimadzu RF-5301PC spectrofluorometer (Kyoto, Japan). Ca<sup>2+</sup> was infused using a KD Scientific model 310 series infusion syringe pump (Holliston, MA) (160 nmol of Ca<sup>2+</sup>/mg of protein/min).

Mitochondrial respiration was assessed using a miniature Clark-type oxygen electrode (Hansatech Instruments, Norfolk, UK) in a sealed, thermostatically controlled chamber at 37°C as described previously (Patel et al. 2010; Sullivan et al. 2004). Mitochondrial respiration was assessed using standard polarographic methods with pyruvate and malate (5 and 2.5 mM, respectively) as oxidative substrates (Sullivan et al.

2003). Following the addition of pyruvate and malate (state II respiration), 120 nmol ADP was added to the chamber to induce state III respiration; this was followed by the addition of 1  $\mu$ m oligomycin to block ATP synthesis (state IV respiration). The respiratory control ratio (RCR) was used as an overall metric of mitochondrial functionality. The RCR was calculated by dividing the slope of the response of isolated mitochondria to state III respiration by the slope of the response to state IV respiration.

### *3.3.5 Mitochondrial Oxidative Damage Assessment*

Mitochondrial lipid peroxidation was assessed as previously described by performing slot blots, using an antibody directed against 4-hydroxynonenal (4-HNE) (Sullivan et al. 2002; Sullivan et al. 2004). Mitochondrial protein oxidation was assessed by measuring protein carbonyls using the OxyBlot Protein Oxidation Detection Kit (OxyBlot; Intergen Company, Purchase, NY) as previously described (Pandya et al. 2007). The films were scanned and the densitometric measurements were quantified by outlining the reactive bands to obtain average pixel intensities, using NIH Image software.

### *3.3.6 Statistical Analysis*

For all statistical comparisons, significance was set at  $p \leq 0.05$ . Tissue sparing assessment data were analyzed using a one-factor analysis of variance (ANOVA) followed by post-hoc analysis (Fisher PLSD test). MWM training data were analyzed using a two-factor repeated-measure ANOVA, with the day of training as the repeated measure, followed by Bonferroni post-hoc analysis. MWM probe data was analyzed by

one-factor ANOVA followed by SNK post-hoc analysis. Mitochondrial RCR and oxidative damage data were analyzed using unpaired *t*-tests.

### 3.3. Results

#### 3.3.1 *Dose-response analysis of NIM811 on cortical tissue sparing following TBI*

To establish a dose-response curve for NIM811, adult rats received a severe unilateral 2-mm cortical contusion and were administered various doses (5, 10, 20, or 40 mg/kg) of NIM811 or vehicle at 15 min post-injury with a subsequent injection 24 h later. In these experiments, cortical tissue sparing was determined at 15 days post-injury utilizing the Cavalieri method. An ANOVA revealed a significant group effect in the percentage of the cortex damaged following TBI [ $F(4, 20) = 9.359$ ;  $p < .0002$ ] (Fig. 3.1). Post-hoc comparisons indicated that all groups administered NIM811 demonstrated significantly greater tissue sparing ( $p < 0.05$ ) compared to vehicle-treated animals. However, animals receiving a 10-mg/kg dose of NIM811 demonstrated a significant ( $p < 0.0001$ ) increase in tissue sparing compared to all other doses of NIM811.

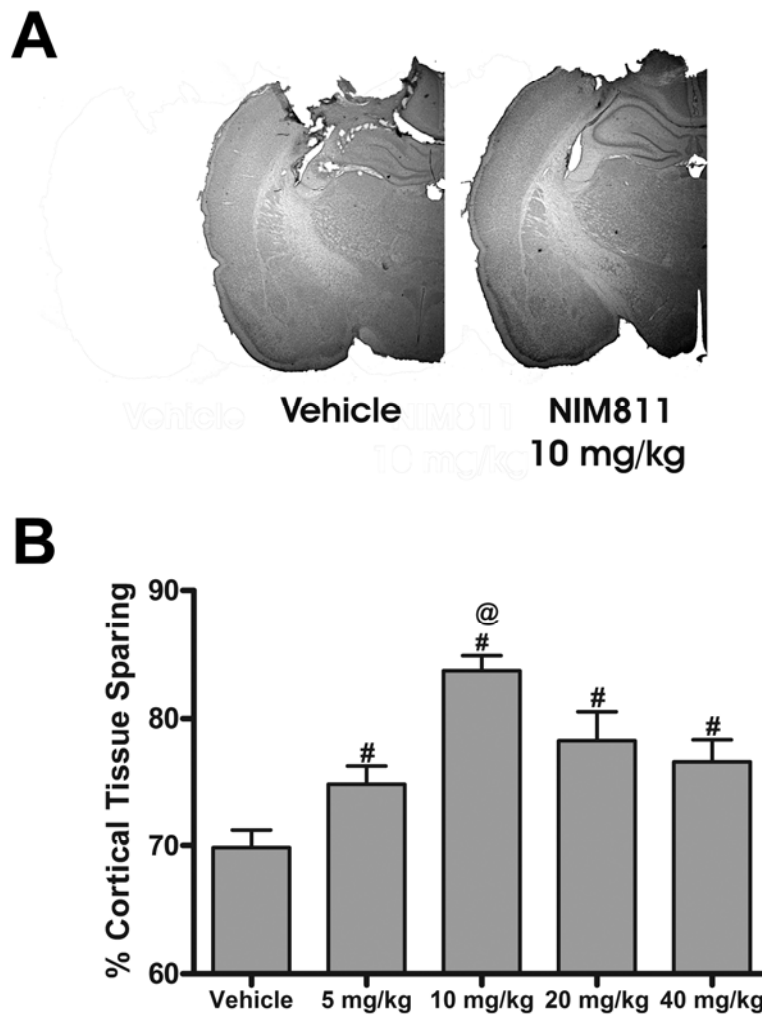


Figure 3.1. NIM811 administration increases tissue sparing following TBI. Tissue sparing was differentially affected depending on the dose of NIM811 administered following a severe (2 mm) CCI TBI. Adult rats were administered various dosages of NIM811 (5, 10, 20, or 40 mg/kg) or vehicle at 15 min and 24 h post-injury, with subsequent tissue sparing assessment at 15 days post-injury. (A) Representative sections from a vehicle-treated animal and a 10-mg/kg NIM811-treated animal (B) Quantitative assessment of tissue sparing revealed that all animals receiving NIM811 demonstrated a significant increase in tissue sparing when compared to vehicle-treated animals. Data points represent group means  $\pm$  SEM. # $p < 0.05$  vs. vehicle; @ $p < 0.0001$  vs. all other doses of NIM811.

### 3.3.2 NIM811 improves behavioral outcome following TBI

Experimental CCI TBI produces significant deficits in both acquisition and retention of the Morris water maze (MWM) spatial memory task (Scheff, et al., 1997). Spatial memory was assessed beginning 10 days post-injury using the MWM. In this experiment, the effect of the 10-mg/kg dose of NIM811 on MWM performance was compared to vehicle. NIM811 administration was associated with significant improvement in the acquisition phase of the MWM. ANOVA results revealed significant effects of the day of testing [ $F(4, 28) = 23.95; p < 0.0001$ ], treatment [ $F(2, 28) = 4.766; p = 0.0165$ ] and a day X treatment interaction [ $F(8, 28) = 2.51; p=0.0152$ ] (Fig. 3.2A). On day 13, post-hoc analysis of daily latencies indicated that NIM811-treated animals had significantly improved MWM performance compared to vehicle-treated animals ( $p < 0.01$ ). Also, on day 14 vehicle treated rats demonstrated significantly increased escape latency compared to sham animals ( $p < 0.01$ ). During the probe trial, NIM811 treated rats crossed the platform significantly more times than vehicle treated animals [ $F(2, 20) = 3.555; p = 0.050$ ] (Fig. 3.2B). No significant group differences in swim speed were present during the acquisition phase of testing (data not shown).

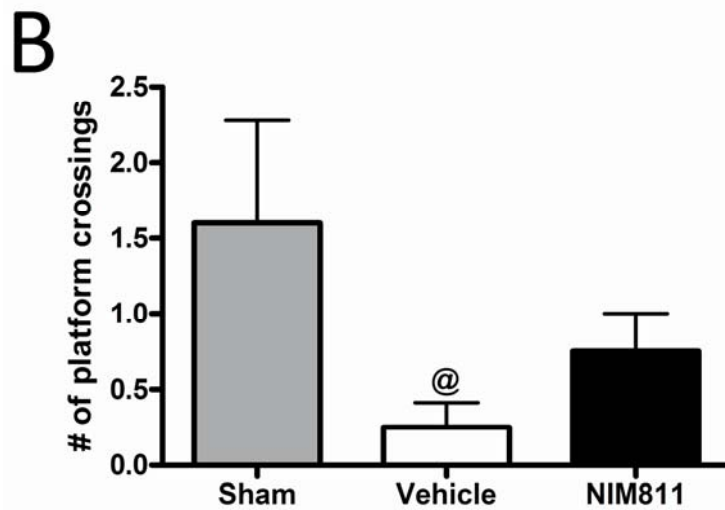
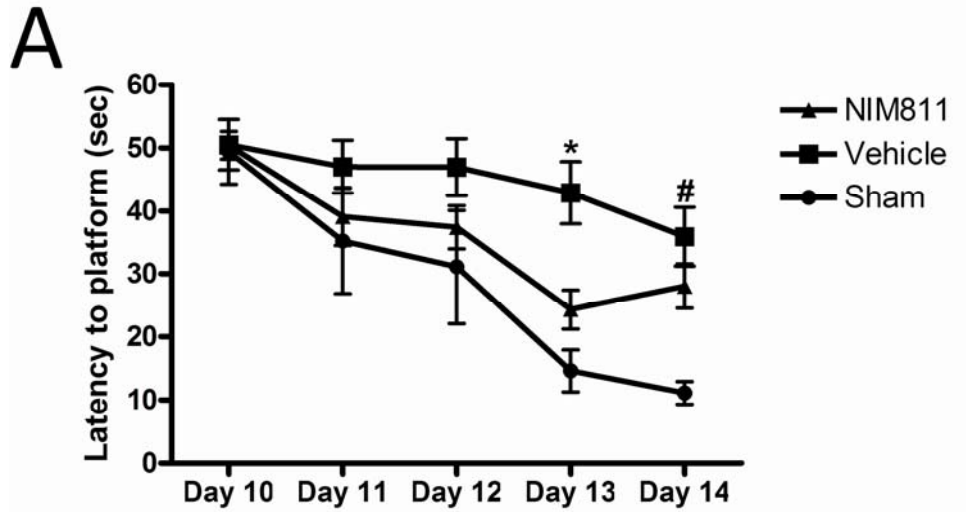


Figure 3.2. NIM811 treatment improves MWM performance following TBI. (A) Animals that received 10 mg/kg of NIM811 (15 min and 24 h post-injury) (triangles) found the submerged platform significantly faster on day 13 than vehicle-treated animals (squares). (B) Number of platform crossings over the 15 sec memory retention test. Vehicle treated animals significantly found the platform fewer times than sham animals; NIM811 treatment attenuated this deficit in memory retention. Data points represent group means  $\pm$  SEM. \* $p$ <0.01 vs. sham and NIM811; # $p$ <0.01 vs. sham, @ $p$ <0.05 vs. sham.

### 3.3.3 NIM811 attenuates post-traumatic mitochondrial dysfunction and oxidative damage

The ability of NIM811 to inhibit mPTP in isolated mitochondria from naïve animals was assessed using a calcium infusion assay. *In vitro* treatment of naïve mitochondria with NIM811 indicated that NIM811 increases the Ca<sup>2+</sup> buffering capacity of isolated mitochondria and delays the onset of mPTP compared to vehicle treatment (Fig. 3.3A). In order to elucidate the effects of NIM811 administration on mitochondrial bioenergetic function, mitochondria were isolated at 6 h post-injury from the ipsilateral hemisphere of animals administered NIM811 (10 mg/kg) or vehicle at 15 min post-injury. Mitochondrial respiration was measured using a Clark-type oxygen electrode. Respiratory control ratios (RCR) were used as a metric for overall mitochondrial functionality. RCR values (state III/state IV) represent the extent of coupling between proton transport and ATP production. As expected, vehicle-treated rats showed significantly lower RCR values ( $3.06 \pm 0.18$ ) than NIM811-treated rats ( $5.65 \pm 0.27$ ) ( $p < 0.0001$ ) following TBI (Fig. 3.3B).



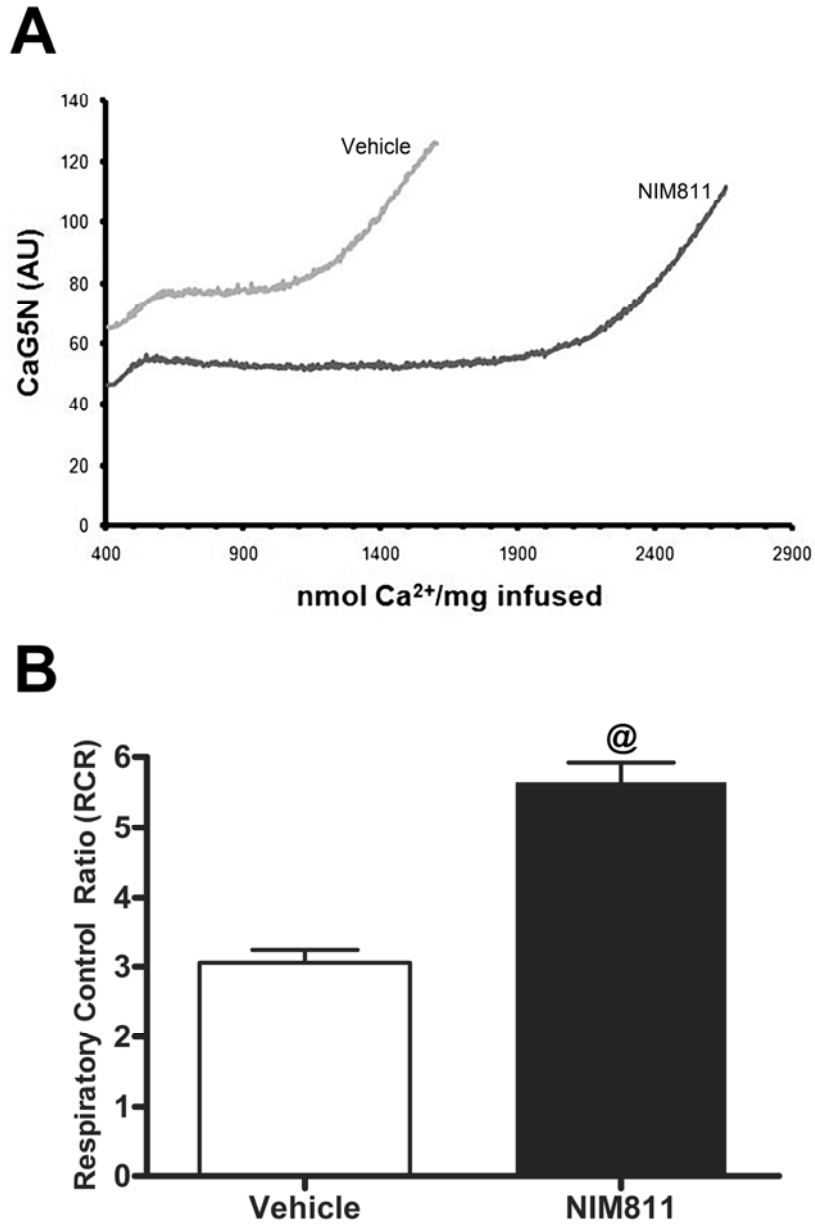


Fig. 3.3. NIM811 is a potent inhibitor of mPTP and NIM811 treatment attenuates injury-induced impairments in mitochondrial bioenergetics following TBI. (A) Representative Ca<sup>2+</sup> infusion traces from isolated naïve mitochondria in the presence and absence of NIM811 (1  $\mu$ M). In the presence of NIM811, mitochondria buffered more of the infused Ca<sup>2+</sup> than in the absence of NIM811. Animals were subjected to a severe CCI TBI and administered the optimal dose of NIM811 (10 mg/kg) or vehicle at 15 min post-injury. (B) At 6 h post-injury, NIM811-treated rats demonstrated significantly higher RCRs than vehicle-treated rats, indicating that coupling of the electron transport system to oxidative phosphorylation was maintained. Bars are group means  $\pm$  SEM. @ $p$ <0.0001 vs. vehicle.

Mitochondrial oxidative stress and deficits in mitochondrial functioning have been shown to occur simultaneously following experimental TBI (Opii, et al., 2007, Singh, et al., 2006). In order to determine the effects of NIM811 treatment on mitochondrial oxidative damage, a portion of the mitochondria isolated from the ipsilateral cortex were used for quantitative measurement of 4-HNE, as an index of lipid peroxidation, and protein carbonyls, as an index of protein oxidation, using slot blots and OxyBlots, respectively. Analysis of oxidative damage markers at 6 h post-injury demonstrated that mitochondria isolated from NIM811-treated rats had significantly lower levels of 4-HNE ( $p < 0.01$ ) and protein carbonyls ( $p < 0.02$ ) (Fig. 3.4). These data indicate that treatment with NIM811 attenuates TBI-induced mitochondrial oxidative damage.

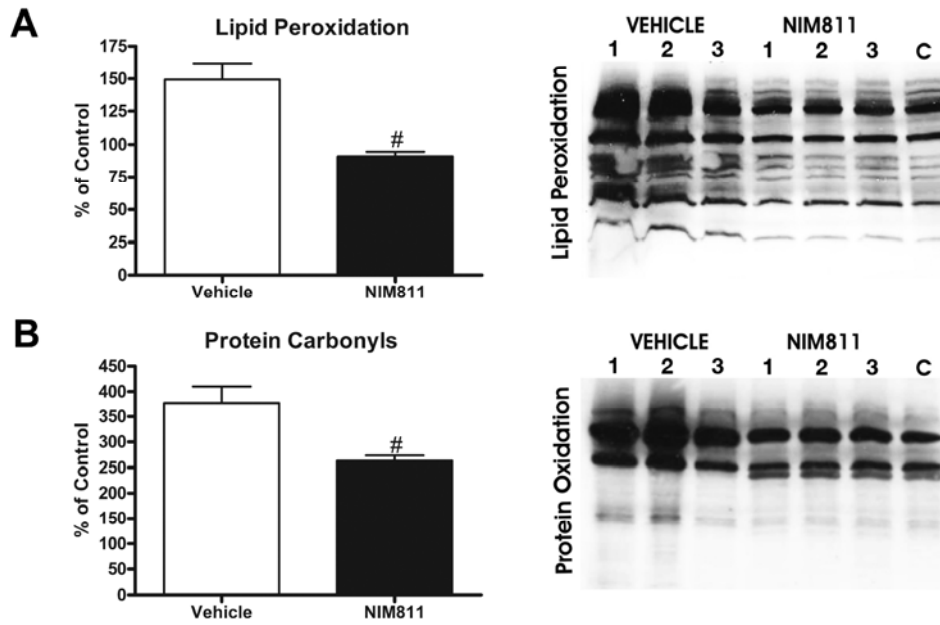


Figure 3.4. Administration of NIM811 (10 mg/kg) reduces mitochondrial oxidative damage following TBI. NIM811 administration significantly decreased levels of mitochondrial (A) lipid peroxidation and (B) protein carbonyls at 6 h post-injury. Bars are group means  $\pm$  SEM. <sup>#</sup> $p < 0.05$  vs. vehicle.

### 3.4 Discussion

Following TBI, mitochondrial dysfunction precedes secondary cell death (Robertson 2004). The overall hypothesis for the current study was that attenuation of this dysfunction with NIM811 would result in neuroprotection. In the present study, NIM811, which inhibits mPTP without affecting calcineurin, significantly increased tissue sparing when compared to vehicle-treated animals. The dose-response of NIM811 is an inverted U-shaped curve, with 10 mg/kg as the most effective dose. The 10-mg/kg dose of NIM811 was selected as the optimal dose based on these results and is in line with a previous report of Mbye and colleagues (Mbye et al. 2008).

Human TBI results in significant memory dysfunction and potential TBI therapeutics should be aimed at attenuating this common clinical observation. The neuroprotective efficacy of NIM811 has been recently demonstrated following severe TBI. However, prior to the current study the neuroprotective effects of NIM811 had yet to be translated into improved cognitive endpoints. In the present study we determined if the neuroprotective effects of NIM811 would translate into significant improvements in MWM performance. Indeed the neuroprotection provided by the 10-mg/kg dose of NIM811 translated into a significant reduction in brain injury-induced spatial learning deficits in the acquisition phase and memory phase of the MWM. While there was a general trend of NIM811 improving memory on every day of training, only on day 13 did NIM811 treatment significantly decrease escape latency compared to vehicle treatment. Also, on day 14 of training vehicle treated rats had significantly increased escape latencies compared to sham animals (on day 14 NIM811 treated animals were not significantly different than sham). During the probe trial on average approximately 3 out

4 NIM811 treated animal crossed the platform at least once, whereas only 1 in 4 vehicle treated animals crossed the platform.

Since mitochondrial dysfunction occurs as early as 15-30 min post-injury, it has been suggested that therapeutic interventions targeting mitochondria must be initiated early after TBI (Gilmer et al. 2009; Singh et al. 2006; Sullivan et al. 1998). In previous studies, mice showed a significant improvement in mitochondrial function following a single IP injection of NIM811 administered 15 min post-injury. Based on these findings, it was determined that a single injection at 15 min post-injury may be sufficient to attenuate the rapid mitochondrial dysfunction occurring following TBI. Based on our dose-response studies, we decided to investigate the effects of treatment with the 10-mg/kg dose of NIM811 on mitochondrial respiration compared to vehicle. We chose to evaluate the effect of NIM811 or vehicle at 6 h post-injury based on previous studies showing that mitochondrial dysfunction peaks between 6 and 12 h post-injury (Singh et al. 2006; Xiong et al. 2007). Results from our mitochondrial studies indicated that 10 mg/kg of NIM811 was effective in attenuating trauma-induced mitochondrial dysfunction compared to vehicle treatment. This improvement in mitochondrial function most likely represents maintained mitochondrial electron transport as a result of NIM811-mediated inhibition of mPTP formation (Sullivan et al. 1999).

Although the mechanisms of secondary injury are not completely understood, increased free radical generation post-TBI and subsequent lipid peroxidation and protein nitration appear to play a role in the progressive neuropathology associated with TBI (Deng-Bryant et al. 2008; Deng et al. 2007; McCall et al. 1987; Readnower et al. 2010; Singh et al. 2006). mPTP formation and mitochondrial oxidative damage occur

simultaneously following TBI (Sullivan et al. 2005). Here, we demonstrate that a 10-mg/kg dose of NIM811 significantly reduced levels of protein oxidation and lipid peroxidation in rats following TBI. These findings are in line with the results observed in mice by Mbye and colleagues (Mbye et al. 2008). Because NIM811 and CsA lack the chemical structure to act as direct antioxidants or free radical scavengers, the reduction in oxidative damage is most likely an indirect effect of maintained mitochondrial homeostasis, which would result in fewer free radicals and less oxidative damage (Mbye, et al., 2008).

It is well established that  $\text{Ca}^{2+}$  increases mitochondrial ROS production; however, the mechanism through which  $\text{Ca}^{2+}$  induces ROS formation is largely unknown (Komary et al. 2010; Votyakova and Reynolds 2005). Since mPTP decreases mitochondrial  $\text{Ca}^{2+}$  levels, it may be hypothesized that inhibition of mPTP would increase mitochondrial ROS production. But, here, we show that mPTP inhibition actually decreases mitochondrial oxidative stress. This finding may be explained by two possible mechanisms: (i) mPTP formation results in inner membrane structural alterations leading to increased ROS production or (ii) mPTP results in decreased clearance of ROS due to impairment of antioxidant systems (Crompton 1999; Kowaltowski et al. 1995). In isolated mitochondria, mPTP formation increases mitochondrial ROS production, and inhibition of mPTP with CsA decreases ROS production (Maciel et al. 2001). These findings suggest that the mechanism by which mitochondrial  $\text{Ca}^{2+}$  uptake promotes ROS production is actually mediated through mPTP formation. This notion is supported by the findings that  $\text{Ca}^{2+}$  does not increase ROS production without mPTP formation (Hansson et al. 2008).

The clinical utility of Cyp-D inhibitors, such as NIM811 and CsA, may be limited by the overall role mPTP plays in both apoptosis and necrosis. In studies involving Cyp-D knockout mice,  $\text{Ca}^{2+}$  and oxidative stress insults required mPTP for induction of cell death while Bcl-2 mediated cell death did not require mPTP (Baines et al. 2005). Taken together this may indicate that mPTP inhibition itself may not overcome all cell death pathways initiated by the mitochondria.

To date, the efficacy of NIM811 has been demonstrated in multiple models of neurodegeneration, including transient focal cerebral ischemia, spinal cord injury, and TBI (Korde et al. 2007; Mbye et al. 2009; Mbye et al. 2008; McEwen et al. 2007; Ravikumar et al. 2007). Despite the proven efficacy of CsA, NIM811 may have better potential as a TBI therapeutic due to its reduced toxicity and specificity as a mPTP inhibitor relative to CsA (Mazzeo et al. 2009). The substitution of isoleucine for leucine at the fourth amino acid of CsA allows NIM811 to bind CyP-D and cyclophilin A (CyP-A) but prevents NIM811 from inhibiting calcineurin. Like other cyclophilins, CyP-A exhibits peptidylprolyl isomerase (PPIase) activity which is associated with chaperone-like actions which aid in *de novo* protein folding and protein repair/refolding following stress (Andreeva et al. 1999). This PPIase activity may be especially important following brain injury and may aid in recovery. One recent study demonstrated that CyP-A protein expression was increased following CCI TBI and that exogenous administration of CyP-A following a stab wound decreased blood-brain barrier permeability and reduced tissue damage (Redell et al. 2007). Our observed U-shaped dose-response could reflect this; although inhibition of mPTP with 10 mg/kg of NIM811 was protective, perhaps the higher doses became cytotoxic due to further inhibition of other cyclophilin family

members, which indicates isoform-specific cyclophilin inhibitors may provide even greater neuroprotection than either CsA or NIM811. Nevertheless, the current data supports the involvement of the mPTP in TBI neuropathology and validates mPTP inhibition as a potential neuroprotective target for TBI.

In summary, 10 mg/kg of NIM811 is the most effective dose for achieving neuroprotection following TBI. NIM811 treatment is capable of improving mitochondrial function and reducing mitochondrial oxidative stress when compared to vehicle treatment following TBI. This is one of the first studies demonstrating that mPTP inhibition following TBI can be directly translated into improved cognitive behavior. These data highlight the importance of maintaining mitochondrial homeostasis following TBI. Since NIM811 is less toxic than CsA, it may be administered at higher doses which may confer a greater degree of neuroprotection following trauma.



## 4. CHAPTER 4

### **Increase in Blood–Brain Barrier Permeability, Oxidative Stress, and Activated Microglia in a Rat Model of Blast-Induced Traumatic Brain Injury**

*(Published in Journal of Neuroscience Research, 88, 3530-3539)*

#### 4.1 Introduction

Accumulating clinical evidence as well as experience in contemporary military operations suggests that substantial short-term and long-term neurologic deficits can be caused by blast exposure without a direct blow to the head (Cernak et al. 1999; DePalma et al. 2005; Elder and Cristian 2009; Ling et al. 2009; Trudeau et al. 1998). With an estimated 15% of troops serving in Iraq sustaining some level of neurological impairment due to blast exposure, TBI has become the signature injury of this war (Hoge et al. 2008). Hoge and colleagues reported a high correlation between service members who reported symptoms consistent with mild TBI and those who received exposure to blast. Although sharing common clinical features with both, brain injury caused by blast (bTBI) is clinically distinct from closed head and/or penetrating TBI (Ling et al., 2009). While the exact biophysics underlying bTBI are not completely understood, interaction with a fast moving, transient pressure wave is generally accepted as the primary cause of brain injury that results from blast exposure (DePalma et al. 2005; Elsayed 1997; Taber et al. 2006). Experimental studies in rodents have demonstrated a correlation between memory dysfunction and distribution of neuropathological damage in the brain after exposure to blast overpressure (BOP) (Cernak et al. 2001a; Cernak et al. 2001b).

Previous studies have also demonstrated that rats exposed to low and moderate intensity BOP (83 kPa or 112 kPa) had lowered food intake and exercise performance (Bauman et al. 1997) with similar findings being reported in sheep (Mundie et al. 2000).

Rats exposed to low intensity BOP (20 kPa) displayed significant performance deficits on rotametric and grip-strength tests (Moochhala et al. 2004; Saljo et al. 2009b) whereas animal studies using moderate levels of BOP (126 kPa) have shown Morris water maze performance impairment, gliosis, and fiber degeneration (Long et al. 2009). Reactive gliosis, neuronal swelling, and cytoplasmic vacuolation have also been observed in the hippocampus of rats subjected to thoracic blast injury (Cernak et al. 2001b). Rhesus monkeys exposed to high BOP (207 kPa, 276 kPa, or 345 kPa) demonstrated significant, albeit transient, memory and performance deficits (Bogo et al., 1971).

TBI can result in brain microvasculature and blood-brain barrier (BBB) breakdown leading to increased BBB permeability. Disruption of the BBB following TBI results in brain edema, a primary event that affects both morbidity and mortality following TBI (Unterberg et al. 2004). Edema increases intracerebral pressure (ICP), and leads to secondary ischemic injuries by impairing cerebral perfusion and oxygenation (Unterberg et al., 2004). Following TBI, various mediators are released which enhance vasogenic and/or cytotoxic brain edema. These include glutamate, lactate,  $H^+$ ,  $K^+$ ,  $Ca^{2+}$ , nitric oxide, arachidonic acid and its metabolites, free oxygen radicals, histamine, and kinins (Unterberg et al., 2004). BBB permeability to endogenous proteins, such as immunoglobulins (IgG), is increased following experimental TBI (Sullivan et al. 2000b; Tanno et al. 1992). An additional consequence of BBB disruption is the infiltration of leukocytes into brain tissue, activation of microglia and inflammation (Morganti-Kossmann et al. 2007). There is some indication of a widespread activation of microglia 1-14 days after exposure to blast, however, it remains unclear if this activation was a direct result of blast wave or it was caused indirectly by an increase in permeability in

blood-brain barrier and transfer of inflammatory mediators from circulation (Kaur et al. 1995). Oxidative stress has been shown to play a critical role in the secondary injury process following TBI. In recent work, our colleagues demonstrated that following both controlled cortical impact and weight drop models of TBI there is a rapid increase in the lipid peroxidation marker, 4-hydroxynonenal (4-HNE), and protein nitration marker, 3-nitrotyrosine (3-NT) (<30 min post injury), which returned to control levels 24 h post-injury (Deng et al. 2007; Hall et al. 2004).

PK11195, an isoquinoline derivative, is an antagonist of the translocator protein (TPSO) (18 kDa) (Banati et al., 1997). TPSOs, previously referred to as the peripheral benzodiazepine receptor (PBR), play an important role in steroidogenesis by acting as cholesterol pores in the outer mitochondrial membrane (Scarf et al. 2009). TPSOs are absent from neurons but are expressed in high density in glial and ependymal cells (Chen and Guilarte 2008). Increased PK11195 binding has been localized to activated microglia in several animal models of CNS injury including TBI, ischemia, and excitotoxic lesion models (Dubois et al. 1988; Gotti et al. 1990; Grossman et al. 2003). The use of TPSO autoradiographic analysis as a marker of inflammation is advantageous over other methods due to the high sensitivity and anatomical resolution of the technique (Benavides et al. 2001).

To begin to elucidate the mechanism(s) involved in bTBI neuropathology the current study was designed to assess acute changes in neurological function and to characterize acute changes in BBB permeability and oxidative damage, and the subacute inflammatory response of the brain following BOP exposure. The results demonstrate the novel findings that following exposure to a moderate BOP (120 kPa) there is significant

reflex suppression, disruption of the BBB, oxidative stress, and widespread microglia activation. Taken together, these data suggest that bTBI shares mechanisms of pathobiology that are hallmarks of injury in other models of TBI, which may widen future avenues of neuroprotective interventions for blast injuries.

## 4.2 Methods

### 4.2.1 *Animals and exposure to blast*

Adult virus-free male Sprague-Dawley rats (250 - 300 g; n = 49) were randomly divided into blast-exposed (n = 44) and control group (n = 14). Animals were anesthetized with isoflurane and positioned in a holder that prevented secondary and tertiary blast injuries as previously described (Chavko et al. 2006). Animals were then placed into the end of the expansion chamber of an air-driven shock tube (2.5 ft compression chamber connected to a 15 ft expansion chamber) with their right side ipsilateral to the direction of the BOP. Animals assigned to exposures were subjected to a peak overpressure of 120 kPa. Control animals received anesthesia and were treated in the same way except for the exposure to BOP.

### 4.2.2 *Acute Neurological Assessment*

Acute neurological function was assessed using a battery of tests that are analogous to motor components of the Glasgow Coma Scale (GCS) as previously described (Dixon et al. 1987). These tests measure the duration of suppression of a response due to injury. Immediately following BOP exposure or control treatment, the duration of suppression of three reflexes were acutely evaluated. The reflexes of control animals were evaluated in order to determine the effects of anesthesia on reflex suppression. The corneal reflex was assessed by lightly touching the eye to elicit a blinking response. The paw flexion reflex was assessed by briefly applying a pinch (approximately 0.2 kg/mm<sup>2</sup>) to the hind paw to elicit a withdrawal response. The righting

response was tested by placing the animal on its back and timing the return to a spontaneous upright position. Evaluation continued until all reflexes had returned.

#### *4.2.3 Immunohistochemistry*

For IgG immunohistochemical assessment a total of 24 animals were exposed to blast and allowed to recover for either 0.5, 3, 24, or 72 hours following BOP exposure (n=6/group). A total of 6 control animals were handled in a similar manner and did not receive any BOP exposure. At the designated survival intervals animals were anesthetized with pentobarbital (95 mg/kg body weight) and transcardially perfused with phosphate buffered saline (PBS) followed by 4 % paraformaldehyde, pH 7.4. After removal, the brains were placed in 4 % paraformaldehyde-sucrose (15%) for an additional 24 hrs. Coronal sections (50  $\mu$ m) were then cut using a freezing microtome throughout the rostral caudal extent of the brain, extending through the septal area to the most posterior extent of the hippocampus. BBB permeability was assayed on every twelfth section from each animal with biotinylated anti-rat IgG antibody as previously described (Hoane et al. 2006). Sections were rinsed with PBS and incubated in 0.3% H<sub>2</sub>O<sub>2</sub> in order to block endogenous peroxidase activity. Next sections were blocked in 10% normal goat serum with 0.2% Triton X-100 in PBS for 1 h at room temperature followed by incubation in primary antibody overnight at 4°C (1:1000; biotinylated goat anti-rat IgG, Vector Labs, Burlingame, CA). Sections were rinsed and incubated in avidin-horseradish peroxidase complex (Vectastain ABC, Burlingame, CA) for 1 hour. IgG immunoreactivity was visualized by incubation of sections in 0.05% diaminobenzidine, 0.01% H<sub>2</sub>O<sub>2</sub>, and 0.3% imidazole for 10 minutes. Lastly, sections were

rinsed with PBS, mounted on gelatinized slides, and coverslipped with permount. IgG immunoreactivity was quantified using a gray-level-index (GLI) as previously described (Bisler et al. 2002). Quantification was performed using Image-Pro Plus software (Mediacybernetics, Bethesda, MD) such that GLI values corresponded to the area of immunoreactivity in relation to an area of interest. The GLI value for each section (~12 sections per animal) were calculated and averaged in order to determine the percent total brain area immunoreactive for IgG. These values were then averaged for each group.

For immunohistochemical detection of brain oxidative stress levels a total of 6 animals were subjected to BOP and allowed to recover for either 3 h or 24 h following BOP exposure. A total of 3 control animals were handled in a similar manner and did not receive any BOP exposure. At the designated survival intervals animals were anesthetized with pentobarbital and perfused with PBS and 4 % paraformaldehyde. Brains were placed in 4 % paraformaldehyde-sucrose (15%) for an additional 24 hrs. Coronal sections (50  $\mu$ m) were then cut using a freezing microtome throughout the rostral caudal extent of the brain, extending through the septal area to the most posterior extent of the hippocampus. Free floating sections were double-labeled for 3-NT (1:1000; mouse anti-3NT monoclonal antibody, Upstate, Lake Placid, NY) and 4-HNE (1:5000; rabbit anti-HNE polyclonal antibody, Calbiochem, San Diego, CA) and visualized by infrared immunohistochemistry as previously described with few modifications (Hawes et. al., 2005). Briefly, sections were washed three times for 5 min each in PBS, followed by adduct reduction in 0.1 M NaBH<sub>4</sub> in 0.1 MOPS (Sigma) for 10 min. Following reduction sections were rinsed three times for 5 min each in PBS, followed by blocking in 10% normal goat serum with 0.2% Triton X-100 in PBS for 1 hour at room temperature.

Sections were incubated in primary antibodies overnight at 4°C. Before incubation in secondary antibody, sections were washed three times for 5 min each in PBS. Sections were then incubated with secondary antibodies for 2 h at room temperature (1:20,000; IRDye700DX conjugated anti-mouse IgG; Gilbertsville, PA) (1:10,000; IRDye800 conjugated anti-rabbit IgG; Gilbertsville, PA). All sections were rinsed three times for 5 min each in PBS and mounted onto Fisher Superfrost Plus Slides. Slides were scanned simultaneously on a LICOR Odyssey Infrared Imager (LI-COR Biosciences). The fluorescence intensity for each section (~12 sections per animal) was quantified using an area of similar size throughout each section. In order to obtain a value for each animal the levels of 3-NT and 4-HNE for each section were averaged. These values were then averaged for each group.

For assessment of microglia morphology antibodies against CD11b/c that recognize microglia-specific complement type 3 receptor were utilized. Briefly, endogenous peroxidase activity was blocked using 0.3% H<sub>2</sub>O<sub>2</sub>. Next sections were blocked in 10% normal goat serum with 0.2% Triton X-100 in PBS for 1 h at room temperature followed by incubation in primary antibody overnight at 4°C (1:1000; mouse anti-rat CD11b/c; BD Pharmigen, San Diego, CA). Sections were then incubated in secondary antibody for 2 h at room temperature (1:500; biotinylated goat anti-mouse; Vector Laboratories, Burlingame, CA). Sections were rinsed and incubated in avidin-horseradish peroxidase complex (Vectastain ABC, Burlingame, CA) for 1 hour. IgG immunoreactivity was visualized by incubation of sections in 0.05% diaminobenzidine, 0.01% H<sub>2</sub>O<sub>2</sub>, and 0.3% imidazole for 10 minutes. Lastly, sections were rinsed with PBS, mounted on gelatinized slides, and coverslipped with permount.



#### 4.2.4 [<sup>3</sup>H]PK11195 Autoradiography

For autoradiography studies a total of 14 animals were subjected to BOP and allowed to recover for either 5 or 10 days following exposure (n=7/group). A total of 5 control animals were given the same treatment except for BOP exposure. Animals were euthanized and their brains were immediately removed and frozen in isopentane on dry ice. Brains were sectioned (16 µm) on a cryostat and mounted on Fisher Superfrost Plus Slides. TPSO autoradiography was performed using 1 nM [<sup>3</sup>H]PK11195 ligand (PerkinElmer, Boston, MA, specific activity=85.5Ci/mmol) as previously described (Kelso et al., 2009). Brain sections were preincubated in 50 mM Tris- HCl, pH 7.4 at 4°C for 15 minutes, followed by incubation with radioactive ligand at 4°C for 2 hours. The binding was terminated by washing sections in 50 mM Tris- HCl, pH 7.4 (3 x 3 min), followed by dipping in ice cold water. The sections were exposed to Kodak BioMax Autoradiography Film (Kodak, Rochester, NY) for 55 days. All films were developed using a Kodak D-19 developer and analyzed using NIH image v1.59 on a Power Macintosh connected to a Sony XC-77 CCD camera via a Scion LG-3 frame-grabber (Scion, Inc., Frederick, MD). Autoradiograph data was quantified by densitometry.

#### 4.2.5 Statistics

Individual reflexes (corneal, paw flexion, and righting) were analyzed by one-tailed t-test. Data from immunohistochemical assays were analyzed by one-way ANOVA followed by Student Newman-Keuls (SNK) post-hoc analysis. [<sup>3</sup>H]PK11195 autoradiography results were analyzed by two-way repeated measures (hemisphere)

ANOVA followed by Bonferroni post-hoc analysis. Significance was accepted as  $p < 0.05$ .

All values are presented as means  $\pm$  SEM.

### 4.3 Results

#### 4.3.1 *Effect of blast overpressure on acute neurological function and brain morphology*

Recently Hamm (2001) demonstrated that reflex suppression is a sensitive predictor of injury effect, therefore acute neurological deficits as a result of BOP exposure were assessed using a battery of reflexes which test somatomotor function (Hamm 2001). The survival rate of animals following BOP exposure was 98%. The return of the corneal reflex in BOP exposed animals ( $23.6 \pm 2.2$  sec) was significantly delayed compared to control animals ( $14.33 \pm 6.6$  sec) ( $p < 0.05$ ). Additionally, BOP exposure resulted in a significant suppression of the paw flexion reflex ( $47.7 \pm 4.0$  sec) compared to control animals ( $21.8 \pm 4.5$  sec) ( $p < 0.01$ ) (Fig. 4.1). However, no significant differences were measured between the righting reflexes of either group. There was no evidence of tissue disruption or tissue loss in BOP exposed animals (Fig. 4.2).

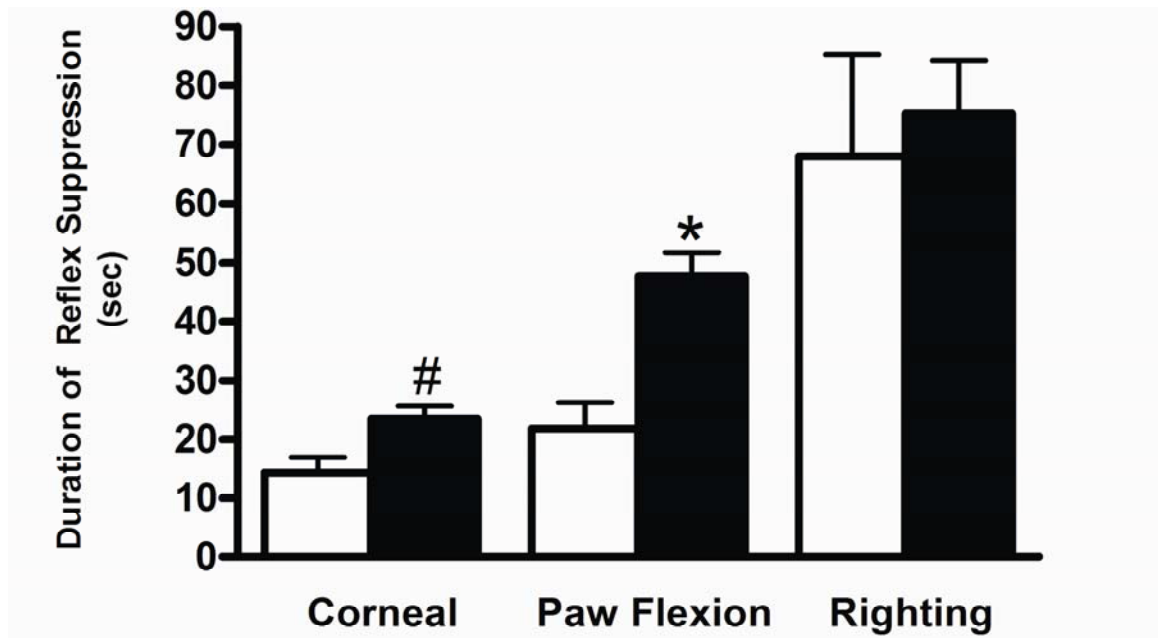
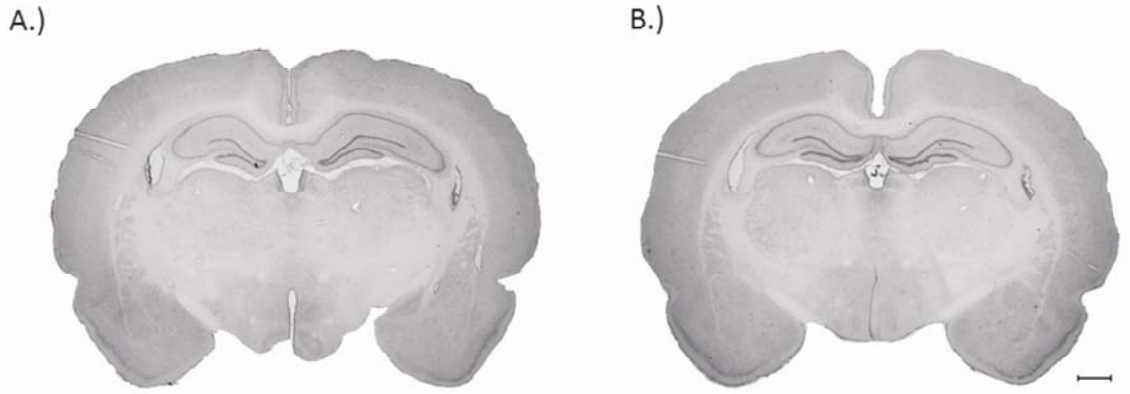


Figure 4.1. Effect of BOP on acute neurological functioning immediately following exposure. Exposure to blast (full columns) significantly delayed the return of corneal and paw flexion reflexes compared to control (open columns). The data represents the mean duration of suppression  $\pm$  SEM. Significance (t-test) is denoted as follows: # $p < 0.05$ , \* $p < 0.01$  vs. control.



**Figure 4.2.** Representative images of H & E stained brain sections of control (A) and at 3 days post blast 120 kPa (B). There were no overt signs of tissue disruption or cell loss. Scale bar denotes 1 mm.

#### *4.3.2 Effect of blast overpressure on IgG immunoreactivity*

The spatial extravasation of endogenous IgG immunoreactivity was used as an index for BBB breakdown following BOP exposure. BBB disruption is a well-established hallmark following brain injury and has been assessed in several models of TBI. Qualitative assessment of IgG extravasation revealed that at 3 and 24 h following BOP exposure there was a conspicuous increase in IgG immunoreactivity in the outer most layer of the cortex. An ANOVA revealed a significant group difference ( $F[4,22]= 12.77, p<0.0001$ ) in IgG immunoreactivity following BOP exposure. Following BOP exposure, post-hoc comparisons revealed a significant increase in IgG immunoreactivity at 3 ( $p<0.001$ ) and 24 h ( $p<0.01$ ) post BOP exposure (Fig. 4.3 & 4.4). Although not significant there was a trend towards increased IgG immunoreactivity at 0.5 h post injury surrounding the lateral ventricles.

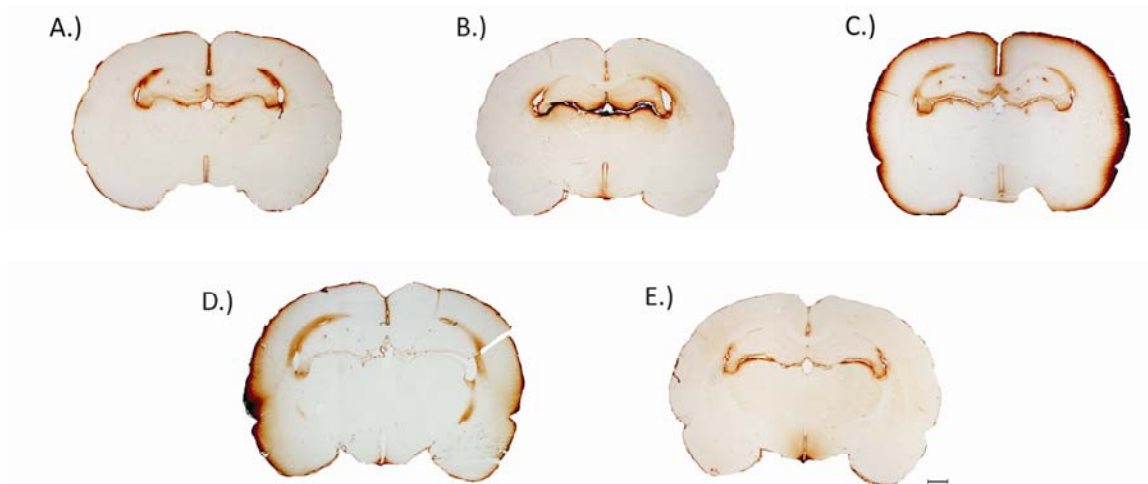


Figure 4.3. Effect of 120 kPa BOP on brain IgG immunoreactivity. The images are representative for A - Control animals; B - 0.5 h post blast; C - 3 h post blast; D - 24 h post blast; E - 3 days post blast. Increased IgG immunoreactivity in layer I of the cortex was observed at 3 and 24 h following exposure. Although not significant there was increased staining in the area surrounding the lateral ventricles (4 out of 6 brains) at 0.5 h post blast. There was no difference in IgG staining in brains 3 days post exposure compared with controls. Scale bar denotes 1 mm.

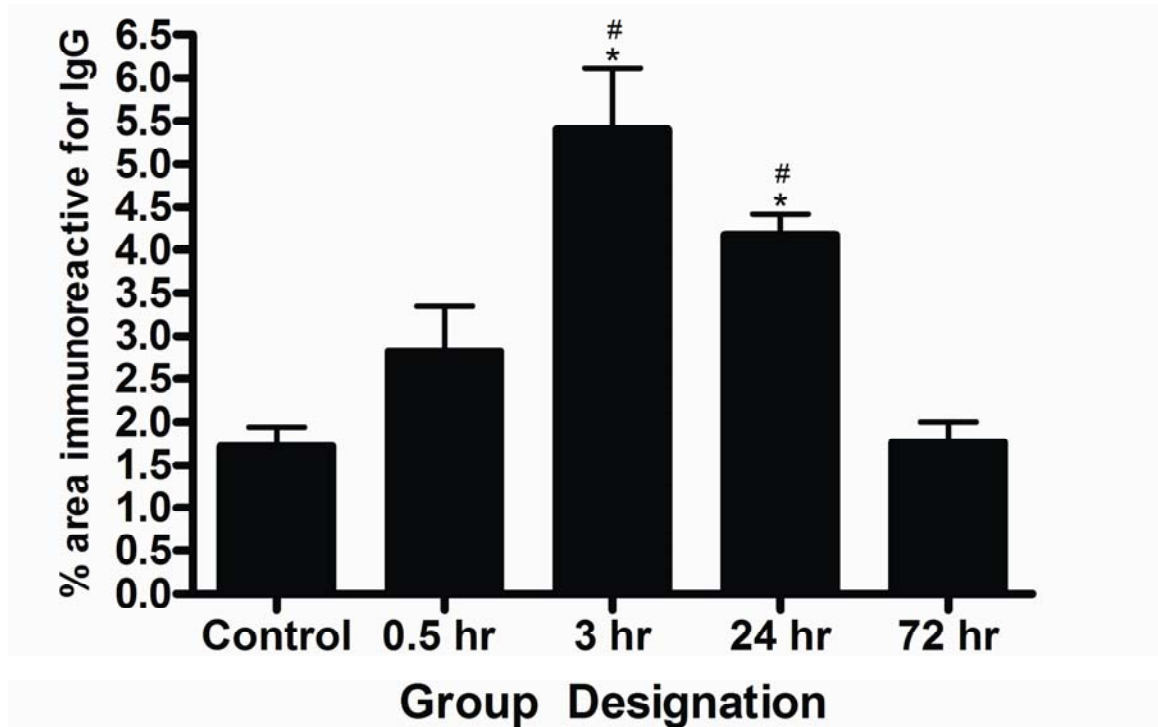


Figure 4.4. Quantification of IgG immunoreactivity in brain sections after exposure to blast. Significant increases in IgG staining were observed at 3 and 24 h after exposure compared to control. The data represents the percentage of brain area stained for IgG  $\pm$  SEM. Significance with ANOVA followed by post hoc analysis (SNK) as follows: \* $p$ <0.01 vs. control; # $p$ <0.05 vs. 72 hr.



#### *4.3.3 Effect of blast overpressure on oxidative stress*

Oxidative stress is known to play a role in the secondary injury cascade following TBI; in the present study we determined 3-NT and 4-HNE levels following BOP exposure using quantitative immunohistochemistry. An ANOVA revealed a significant group difference ( $F[2,6]= 11.24, p<0.01$ ) in 4-HNE levels following BOP exposure at 3 h post-exposure ( $p<0.01$ ). 4-HNE levels returned to control values at 24 h post-exposure. Similarly, there was a significant group difference ( $F[2,6]= 11.55, p<0.01$ ) in 3-NT levels at 3 h following exposure ( $p<0.05$ ) which returned to control values at 24 h post-exposure (Fig. 4.5).

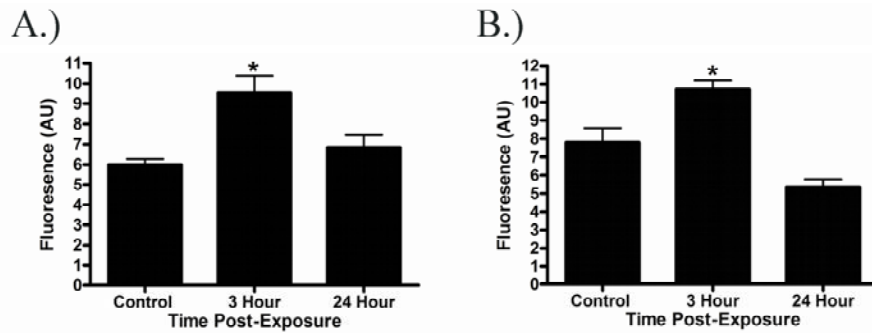


Figure 4.5. Quantification of 4HNE (A) and 3NT (B) levels in brain sections after exposure to blast. Significant increases in 3NT and 4HNE levels were observed at 3 h after exposure compared to control. At 24 h after exposure, 3 NT and 4HNE levels returned to control values. The data represents the mean fluorescence  $\pm$  SEM. Significance with ANOVA followed by post hoc analysis (SNK) as follows: \* $p < 0.05$ .

#### 4.3.4 Effect of blast overpressure on TPSO binding and microglia morphology

Inflammation has been demonstrated in multiple models of TBI; in the present study microglial activation was determined using TPSO autoradiographic localization. The effects of blast exposure on TPSO density were evaluated using [<sup>3</sup>H]PK11195 autoradiography in several brain regions at 5 and 10 days post blast (Fig. 4.6). A two-way ANOVA was significant for effect of BOP exposure ( $F[2, 16] = 2.287, p < 0.001$ ), but not for hemisphere ( $F[1, 16] = 11.20, p > 0.05$ ) (Table 4.1). No significant interaction between BOP exposure and hemisphere existed ( $F[2, 16] = 1.494, p > 0.05$ ). At 5 days post blast, post-hoc testing revealed a significant increase in the density of [<sup>3</sup>H]PK11195 binding in the ipsilateral dentate gyrus ( $p < 0.001$ ) and contralateral dentate gyrus ( $p < 0.05$ ). At 10 days post blast, post-hoc comparisons revealed a significant increase in [<sup>3</sup>H]PK11195 binding in the contralateral dentate gyrus ( $p < 0.001$ ), ipsilateral dentate gyrus ( $p < 0.001$ ), contralateral substantia nigra ( $p < 0.01$ ), and contralateral ventral hippocampus ( $p < 0.05$ ).

In order to further demonstrate the response of microglia to BOP exposure antibodies against CD11b/c were used to visualize microglia morphology. In the cortex, hippocampus, and substantia nigra of control animals microglia demonstrated a resting morphology characterized by highly ramified processes (Fig. 4.7). In contrast microglia in the hippocampus from animals exposed to blast displayed an activated morphology characterized by clustering. In addition to this microglia in the substantia nigra of BOP exposed animals revealed activated morphology characterized by rounded cell bodies and fewer ramifications.

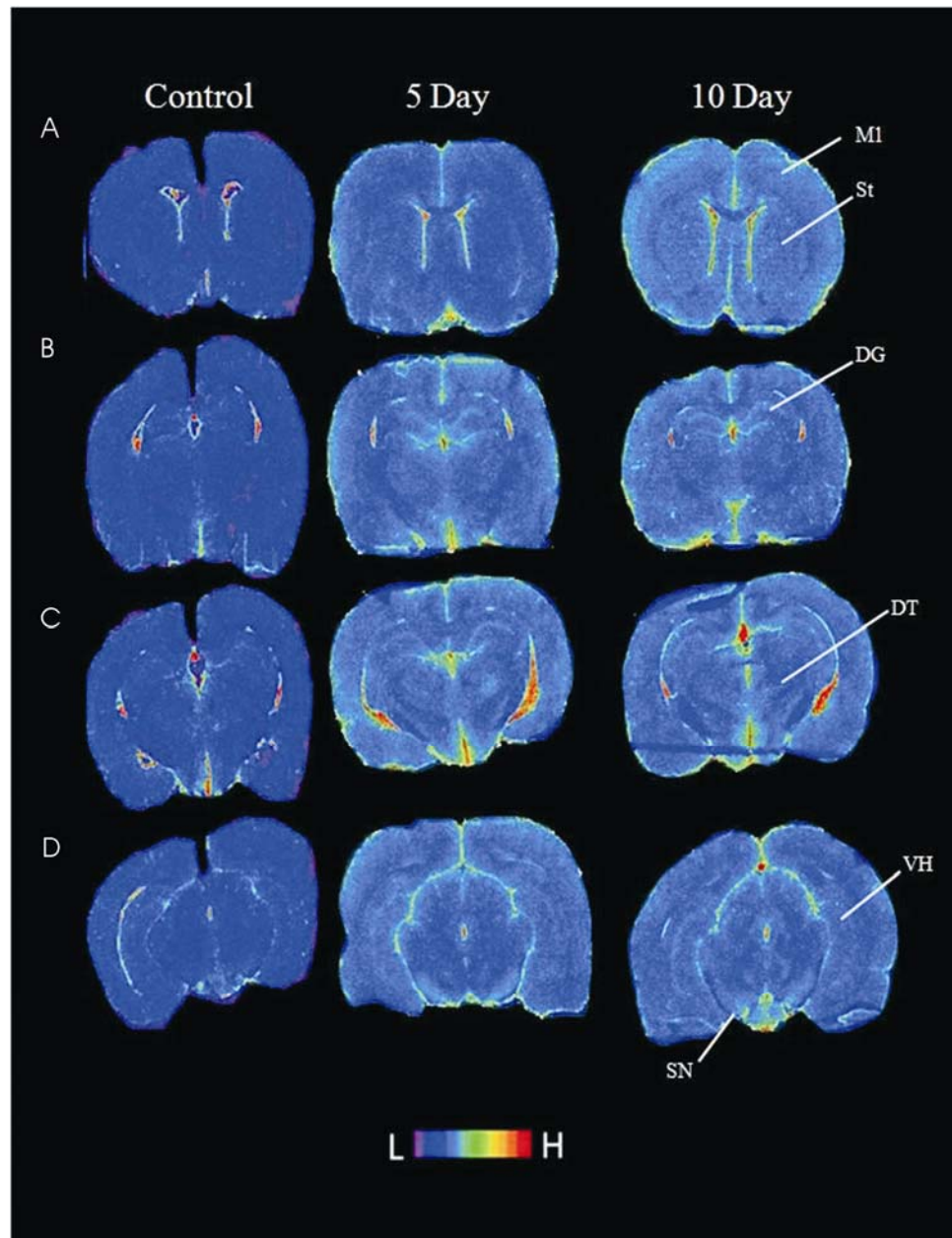


Figure 4.6. Effect of exposure to 120 kPa BOP on TPSO expression. Pseudocolored autoradiogram of brain sections labeled with [ $^3\text{H}$ ]PK11195 in control and in 5 or 10 days post exposure animals. Four different levels are shown: A - anterior striatum; B - dorsal hippocampus; C - dorsolateral thalamus; D - ventral hippocampus. Brain regions abbreviated as follows: primary motor cortex (M1), striatum (St), dentate gyrus (DG), dorsolateral thalamus (DT), ventral hippocampus (VH), and substantia nigra (SN). Prominent areas of high TPSO density in control animals are restricted to ventricular/choroid plexus regions. High density areas of TPSO in exposed animals are

the dentate gyrus and substantia nigra. The scale shown at bottom shows the range from low to high expression.

Region	Control	5 Day		10 Day	
		Ipsilateral	Contralateral	Ipsilateral	Contralateral
Primary motor cortex	41.25 (6.8)	41.53 (5.5)	43.79 (3.6)	44.89 (1.9)	48.32 (3.0)
Striatum	38.22 (4.9)	37.29 (7.3)	38.47 (5.8)	42.68 (6.1)	43.99 (3.7)
Dentate gyrus	37.07 (5.4)	43.95 (4.4)*	45.34 (3.3)**	48.04 (3.4)*	47.68 (2.5)*
Dorsolateral thalamus	37.69 (11.0)	39.15 (6.5)	39.88 (7.2)	41.84 (3.1)	42.09 (3.0)
Substantia nigra	39.28 (8.0)	42.80 (6.0)	45.64 (3.5)	45.70 (3.5)	49.74 (3.1)**
Ventral hippocampus	40.41 (11.3)	42.06 (3.4)	43.45 (4.9)	42.97 (4.7)	48.28 (3.5)**

Table 4.1. Quantification of TPPO expression in selected brain regions. There was a significant increase in PK11195 binding in the dentate gyrus at both survival intervals. In addition the TPPO expression was increased in both the contralateral substantia nigra and ventral hippocampus 10 days after blast. The data represents the mean density of [<sup>3</sup>H]PK11195 binding (arbitrary optical units)  $\pm$  SEM. Significance from control is denoted as follows: \* $p$ <0.001; \*\* $p$ <0.05.

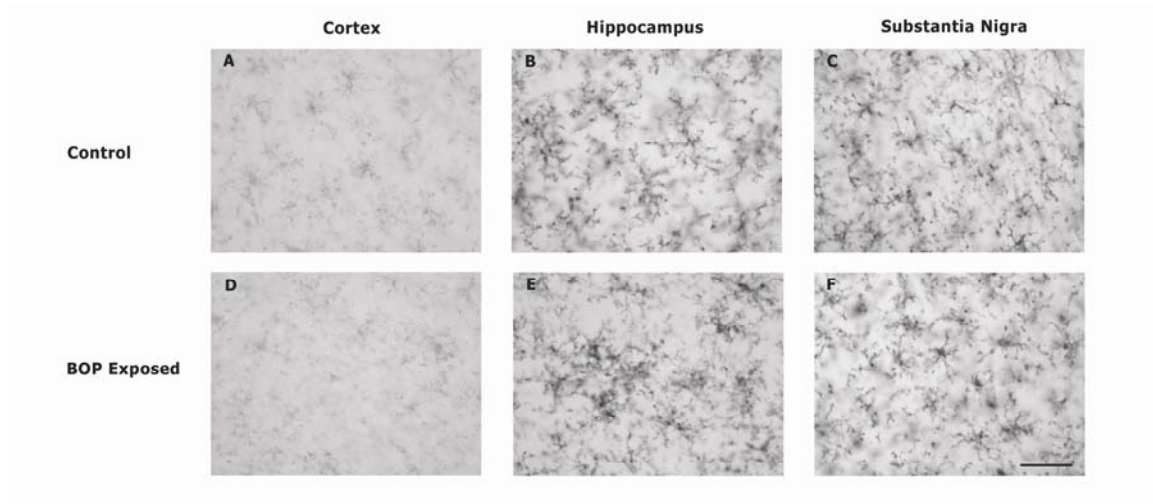


Figure 4.7. Effect of 120 kPa BOP on microglia morphology in selected brain loci. In order to confirm PK11195 autoradiographic data demonstrating microglial activation in the dentate gyrus and substantia nigra, antibodies against CD11b/c were employed to visualize microglial morphology. In sham animals all regions that were evaluated contained microglia with resting morphology characterized by highly ramified processes (A-C). In the cortex of animals exposed to blast microglia also displayed resting morphology (D). Exposure to blast resulted in activated microglial morphology characterized by microglial clustering in the hippocampus (dentate gyrus, E). In addition activated microglia morphology in the substantia nigra is revealed by microglia with rounded cell bodies and reduced ramifications (F). Scale bar = 50  $\mu$ m.

#### 4.4 Discussion

Brain injury in rats as a result of exposure to BOP using an air-driven shock tube was used to study the progression of neuropathological processes that could be relevant to blast exposed populations. The systemic effects of the level of blast (~120 kPa) used in the present study were previously characterized and it was shown to induce moderate pulmonary damage with an acute inflammatory responses that were resolved by 8 days (Chavko et al., 2006). The exposure to this level of BOP does not produce lethal damage and the survival rate of rats at 120 kPa under isoflurane anesthesia is more than 90%. In the present study we looked for some basic neurological and neuropathological changes in the brain and demonstrated that 120 kPa BOP exposure produced acute reflex suppression in rats which was similar to suppression observed in other models of diffuse brain injury (Denny-Brown and Russell 1940; Dixon et al. 1987; Fijalkowski et al. 2007). This extent of reflex suppression is consistent with concussion and mild TBI which closely resembles the clinical manifestation of bTBI (Bruns and Jagoda 2009; Jones et al. 2007). Previous studies have demonstrated a dose response relationship between the extent of acute neurological impairment and overall cognitive outcome following experimental diffuse brain injury (Beaumont et al. 1999). Long and colleagues (2009) reported that following 126 kPa BOP exposure there is transient cognitive dysfunction; the observed reflex suppression in the present study predicts these findings by Long. The results also demonstrate increased IgG immunoreactivity in the brain 3 and 24 h after exposure indicating increased early permeability of BBB followed by microglia activation at 5 and 10 days post-exposures.



BBB breakdown has been shown to occur early after TBI; it is mostly transient and the time course of the BBB opening varies in different models. In a fluid percussion model of injury, BBB damage in the rat brain was most pronounced within the first hour after TBI and was reestablished by 6 h after injury (Tanno et al. 1992). Others also reported that the BBB sealed within a few hours after fluid-percussion injury (Enters et al. 1992). We observed significant BBB breakdown following blast which was limited to layer I of the cortex and was considerably smaller in comparison to diffuse fluid percussion brain injury, where overt BBB breakdown was observed throughout the neocortex, hippocampus, and thalamus (Kelley et al. 2007). Similarly to other TBI models BBB disruption was transient and appeared recovered by 3 days.

It is generally thought that the BBB opening is a major factor contributing to the presence of brain edema observed in TBI. However, in some TBI models, edema formation clearly does not correspond with BBB opening (Beaumont et al. 2000). Instead, cytotoxic mechanism is thought as the major mechanism of edema formation in TBI. Despite this a permeable BBB can provide a route for passive fluid movement and thus worsening the cytotoxic cell edema. Recently, Saljo et al (2009) reported a blast dose-dependent rise in ICP in rats exposed repetitively 3 times to blast, and an increasing time delay in elevation with decreasing intensity of exposure. While the initial elevation took place within 30 min after exposure to 60 kPa, it did not appear until after 2 h and 6 h after exposure at 30 and 10 kPa, respectively. The delayed BBB opening and similar time course in ICP increase may indicate a contribution of vasogenic component to brain damage after exposure to blast.

BBB breakdown in TBI can be a result from the initial insult or the product of secondary injuries, such as accumulation of inflammatory cytokines. Whalen et al. (1997) found a pattern of transient BBB opening that was independent of inflammation; the BBB was leaky within the first 30 minutes to 4 hours after controlled cortical impact in rats whereas white blood cell accumulation peaked at 24 hours (Whalen et al. 2000). It was concluded that BBB damage was not related to the white blood cell accumulation and inflammation. It was previously shown that exposure to BOP produced early (1-3 h post exposure) recruitment of polymorphonuclear leukocytes (PMNs) in peripheral blood at the level of blast in the present study (Gorbunov et al. 2004). Therefore, contribution of inflammation to the BBB opening cannot be ruled out and needs further investigation.

In virtually all pathological conditions of the CNS, accumulation and activation of microglia precedes or is concomitant to neuronal and glial cell damage. Microglia as the principal immune cells in the CNS are known as a source of highly cytotoxic substances including proinflammatory cytokines, reactive oxygen intermediates, proteinases, and complement proteins (Giulian et al. 1995; McGeer et al. 1993; Rieske et al. 1989). Microglia derived oxygen free radicals as well as subsequent reaction products, hydrogen peroxide and peroxynitrite, have the potential to harm cells and have been implicated in contributing to oxidative damage and neurodegeneration in neurological diseases. Our findings show the inflammatory response in the brain long after initial impact and with the absence of detectable cell loss. It has been suggested that microglial activation in TBI cannot be exclusively attributable to the infiltration of blood-borne macrophages or molecules from the circulating blood. This implies that the microglial activation occurring is caused largely by intrinsic mechanisms within brain tissue, e.g. as a response

to diffuse axonal injury. Several recent reviews have suggested that inflammation may serve as a biomarker for bTBI (Agoston et al. 2009; Svetlov et al. 2009). Following a single severe blast, Kaur and colleagues previously demonstrated that there is widespread microglial activation up to 14 days post blast (Kaur et al. 1995). In addition, ultrastructural findings by Cernak and colleagues demonstrated glial reaction in the hippocampus of rats exposed to high levels of BOP (Cernak et al. 2001b). Recently Long and colleagues reported that following moderate levels of BOP (126 kPa) there was widespread gliosis throughout all levels of the brain (Long et al. 2009). However it was unclear whether microglia and/or astrocytes were activated and the regional pattern of inflammation remained uncertain.

It was recently shown that increased PK11195 binding in some brain areas corresponds with microglial activation following TBI (Benavides et al. 2001; Raghavendra Rao et al. 2000). In order to provide higher anatomical resolution of regional microglial activation following a moderate level of BOP we selected PK11195 binding as a surrogate marker of microglial activation. We demonstrated increased PK11195 binding in the dentate gyrus, substantia nigra, and ventral hippocampus 5 days and 10 days after exposure to blast. At 10 days the inflammatory response appeared to be evolving further as increased binding was observed compared to 5 days. While at 5 days after exposure the distribution of lesions was localized bilaterally in both hemispheres, latter at 10 days increased binding density increase was distributed more contralaterally to the site of impact. This distribution of microglia has some analogy with previously published data on the vasospasm distribution after exposure to blast. Unlike conventional TBI, traumatic cerebral vasospasm (TCV) often occurred in vessels far distal to the area

of fragment penetration in the hemisphere contralateral to the injury. It was suggested that, in addition to the fragment injury, propagation of the blast wave through the tissue could have caused TCV (Armonda et al. 2006). Another possibility is migration of toxic components of microglia activation products from the site of injury into distant regions (Gong et al. 2000).

The results presented clearly illustrate the selective vulnerability of the hippocampus and substantia nigra to BOP exposure. The high density of NMDA receptors present in the hippocampus may be partially responsible for the vulnerability of the hippocampus to excitotoxic insults (Butler et al. 2009). It was shown by positron electron tomography (PET) that PK11195 can visualize excitotoxic lesions in the living human brain (Groom et al. 1995). High vulnerability to oxidative stress of dopaminergic neurons in the substantia nigra and resultant mitochondrial dysfunction may contribute to the observed increase in microglia activation in substantia nigra (Hastings 2009).

Cernak and colleagues demonstrated that high levels of BOP result in an increase in brain nitric oxide production and lipid peroxidation as early as 3 h post-exposure that resolves by 5 days post-exposure. However, prior to the current study, oxidative stress following a moderate level of blast remained undetermined. Here we report that as early as 3 h post-exposure there is a significant increase in 3-NT and 4-HNE levels. At 24 h, 4-HNE and 3-NT levels had returned to control values. This indicates that there is a rapid increase in oxidative stress following moderate BOP exposure and that oxidative stress is at least partially occurring concomitantly with BBB breakdown. It has been postulated that vascular abnormalities following TBI are dependent on the production of oxygen radicals (Kontos and Povlishock 1986; Pun et al. 2009). Therefore antioxidants may

represent one potential neuroprotective target for therapeutic intervention following bTBI, which may in turn reduce other downstream pathological processes (i.e. BBB breakdown).

In summary, our data demonstrate that bTBI likely share some common mechanisms of injury with other TBI models. In this study we demonstrated that inflammation likely plays a role in the neuropathology associated with bTBI. We report significant BBB breakdown that resolved by 3 days post exposure. Oxidative damage markers increased rapidly and resolved by 24 h post exposure. Taken together our results clearly show that the brain is susceptible to BOP exposure and that specific brain regions are more susceptible to blast than others.

## 5. CHAPTER 5

### Discussion and Conclusions

#### 5.1 Review

The wars in Iraq and Afghanistan have resulted in a high prevalence of TBI in military populations. Estimates of incidence of blast TBI range from 15-60 % of all deployed US military combatants (Ling et al. 2009). Since traumatic brain injury is often an “invisible wound”, its diagnosis can be extremely difficult, especially in the context of explosive blast injury. Currently, there is a great deal of investigation involving the mechanism(s) by which blast causes brain injury. In addition to the military contribution, mild TBI (concussion) in the US is gaining popular attention in the context of athletic injuries. In fact, over 18 % of all TBIs in the US are attributable to athletic injuries (Meehan and Bachur 2009). While concussion results in symptoms that usually resolve over the first few weeks following injury, some symptoms may persist for months to years.

The pathology associated with TBI occurs biphasically, with initial (primary) mechanical damage followed by a delayed secondary injury cascade. The secondary injury process is complex and involves multiple pathological events such as excitotoxicity, oxidative damage, mitochondrial dysfunction, and inflammation. Although the pathological processes following TBI are well defined an effective therapeutic for TBI remains elusive.

The results from the experiments in this dissertation have demonstrated that: (I) Cyclophilin D plays a key role in TBI pathology and is a valid target for neuroprotective intervention; (II) Administration of NIM811, a CypD inhibitor, is neuroprotective and improves cognition following TBI; and (III) Blast exposure results in neuropathology similar to other models of TBI.

## 5.2 Cyclophilin D's involvement in TBI

Until now, CypD's involvement in TBI pathology was entirely based on indirect evidence. This evidence was based on the assumption that the neuroprotective mechanism of CsA was through inhibition of CypD. Here we have demonstrated that genetic knockout of CypD increases tissue sparing and protects CA3 hippocampal neurons following TBI. These data suggest that CypD is a valid therapeutic target for TBI.

The selective protection of CA3 neurons observed in CypD knockout mice may be explained by the relative vulnerability of individual hippocampal subfields following insult. Selective vulnerability of CA1 neurons is well-documented following hypoxia-ischemia insult (Kirino 1982; Kirino and Sano 1984). In fact, it has been shown that inhibition of complex II with malonate results in a selective loss of CA1 neurons in rats (Davolio and Greenamyre 1995). This may be especially relevant to these studies given that we observed only partial mitochondrial protection and that complex II driven respiration was significantly decreased in both wild-type and CypD knockout mice following TBI. Therefore, CA3 neurons maybe intrinsically less susceptible to insult and in turn maybe more amenable to therapy.

Protection of CA3 neurons may have important implications for behavioral function following TBI. The CA3 region has been shown to play a critical role in spatial memory acquisition and in the formation of long-term spatial memory (Florian and Rouillet 2004). Since CypD knockout resulted in CA3 protection this suggests that modulation of CypD may improve memory following TBI.

Next we choose to evaluate the efficacy of the CypD inhibitor, NIM811, following TBI. NIM811 is a more specific mPTP inhibitor than CsA and therefore has greater therapeutic potential. NIM811 resulted in significant neuroprotection which translated into improved behavioral function. Although we did not directly assess the effects of NIM811 treatment on hippocampal integrity, we speculate that the improvement in memory acquisition in NIM811 treated animals is related to a selective protection of CA3 neurons like we observed in our CypD knockout studies. Future studies may address the effects of NIM811 treatment on hippocampal integrity following TBI.

We also report that the protection produced by both genetic ablation of CypD and pharmacological inhibition of CypD with NIM811 is likely mediated through maintenance of mitochondrial function. An important phenomena associated with glutamate excitotoxicity is delayed calcium dysregulation (DCD) which refers to the delayed loss of  $\text{Ca}^{2+}$  homeostasis that leads to cell death (Starkov et al. 2004). Following glutamate exposure, initially intracellular  $\text{Ca}^{2+}$  levels of cultured neurons rapidly increase and fall (as a result of mitochondrial buffering of  $\text{Ca}^{2+}$ ), this is followed by a delayed irreversible increase in intracellular  $\text{Ca}^{2+}$  (Manev et al. 1989). This secondary rise in  $\text{Ca}^{2+}$



is thought to be mediated by an increase in mitochondrial ROS production (Castilho et al. 1999). Induction of mPTP, through an unknown mechanism, has been shown to result in increased ROS production (Kowaltowski et al. 1998). This increase in ROS production is thought to promote an increase in plasma membrane  $\text{Ca}^{2+}$  permeability (Wehage et al. 2002). Inhibition of mPTP, through CypD knockout or pharmacological inhibition, is hypothesized to be the underlying mechanism of protection in the studies highlighted in this dissertation. In fact, the NIM811 studies demonstrated that inhibition of CypD results in decreased mitochondrial oxidative damage. Maintenance of mitochondrial homeostasis through modulation of CypD likely decreased ROS production and prevented DCD and subsequent cell death in these studies.

It is interesting that the observed dose-response curve for NIM811 and CsA are both U-shaped and that the optimal dose of NIM811 (10 mg/kg) is less than the optimal dose of CsA (20 mg/kg) (Sullivan et al. 2000b). Since the optimal dose of NIM811 is less than CsA this may indicate that it is a more potent inhibitor of mPTP than CsA *in vivo*.

Although these studies provide evidence that CypD plays a role in TBI the question related to the neuroprotective mechanism of CsA remains unanswered. In fact, these studies in which wild-type mice and CypD knockout mice are being administered CsA are ongoing in our laboratory. If knockout of CypD and administration of CsA results in an additive affect it would indicate that CsA is providing protection at least partially in a mechanism independent of CypD.

To date, CypD knockout has proven beneficial in multiple neurodegenerative models including hypoxic-ischemic brain injury and Alzheimer's disease (Du et al. 2008;

Wang et al. 2009). Also, NIM811 has been shown to be neuroprotective in multiple CNS injury models including TBI, spinal cord injury, and transient focal forebrain ischemia (Korde et al. 2007; Mbye et al. 2008; McEwen et al. 2007). These studies, along with those contained in this dissertation, demonstrate that CypD is critically involved in TBI and that modulation of CypD following TBI has great therapeutic potential.

### 5.3 Blast TBI

Because of improvements in body armor soldiers are surviving greater explosions and as such TBI has become a military epidemic. Also, the use of improvised explosive devices (IEDs) in Iraq and Afghanistan has contributed to the increased prevalence of TBI in the military. A renaissance in blast TBI research has occurred since the start of the current conflicts and it is increasingly evident that the brain is susceptible to primary blast forces. The development of relevant models of blast injury has shed new light on the pathology of blast TBI.

The studies presented here have attempted to characterize the time-course for well-established markers of TBI pathology in a clinically relevant blast TBI model. We showed that blast overpressure results in an early transient impairment of neurological function. The modest impairment likely indicates that our blast model is modeling a concussion-like injury. In fact, soldiers exposed to blast often report symptoms (i.e. confusion, amnesia, headache) consistent with concussion injury (Lippa et al. 2010). The similarity between the neurological impairment in our model and the clinical manifestation of blast are striking. In fact, studies by Long and colleagues have demonstrated that our blast model results in cognitive dysfunction as determined by

MWM performance (Long et al. 2009). These observations help validate the clinical relevancy of our model.

Our results demonstrate that brain oxidative damage occurs rapidly (<3 h) following TBI and resolves by 24 h post-injury. This increase in oxidative stress partially occurred concomitantly with increases in BBB permeability. Oxidative stress is known to promote BBB breakdown, which suggests a causal role for ROS in BBB dysfunction (Pun et al. 2009). Thus, antioxidant therapy may be one potential therapeutic strategy for blast TBI that could attenuate rises in ROS and in turn decrease alterations in BBB permeability.

Increased PK11195 binding has been shown to correlate with activated microglia following TBI (Woodcock 2009). As such we used PK11195 as a marker for activated microglia and showed that there is increased neuroinflammation in both the hippocampus and substantia nigra following blast. Since the hippocampus is intimately involved in memory, damage to the hippocampus could explain cognitive dysfunction following blast.

### 5.3.1 Blast commentary

In studies which only subjected the head to blast (i.e. abdomen and chest shielded with a Kevlar vest) neuropathology was reduced compared to whole body blast (Koliatsos et al. 2011; Long et al. 2009). These data indicate a systemic involvement in brain injury following blast. One hypothesis is that forces from blast waves impacting the thorax are transmitted via the large blood vessels to the brain, resulting in damage

(Cernak and Noble-Haeusslein 2010). Also systemic injury could result in a production of proinflammatory cytokines which could enter the brain following BBB breakdown and promote inflammation. This may be true in our blast model since it has been shown to result in moderate pulmonary damage (Chavko et al. 2006). Therefore in our studies we cannot rule out a systemic (polytrauma) contribution to the observed brain injury in our blast model.

Clinical and experimental data suggests that repetitive mild TBI may cause cumulative damage to the brain (Weber 2007). Animal models have shown learning and cognitive dysfunction following repeated mild TBI that was absent following a single injury (DeFord et al. 2002) (Creeley et al. 2004). However the time lapse between the first injury and second injury appears to be an important determinant of overall outcome, if given enough time the brain may recover and the cumulative effect of the injuries may diminish (Weber 2007). Occupationally military personnel have an increased risk of repetitive BOP exposure and therefore may have a higher incidence of repetitive injury (Elsayed and Gorbunov 2007). The hypothesis that damage caused by one mild BOP exposure would have cumulative effects on the brain if BOP exposure is repeated needs attention. One group found that following three repeated low level blasts there was a dose-dependent increase in intracranial pressure (Saljo et al. 2009a). However, this study did not include any histopathological evidence of a cumulative effect of blast. These repeated blast studies would have important implications for return to work/combat guidelines for soldiers.

The rationale behind pretreatment seems especially pertinent to military populations. Since the mechanisms of injury following TBI evolve rapidly, having a compound “on-board” at the time of injury may produce the greatest protection. Creatine is a commonly used dietary supplement that has neuroprotective efficacy and could be taken prophylactically by warfighters (Dedeoglu et al. 2003; Rabchevsky et al. 2003; Scheff and Dhillon 2004; Sullivan et al. 2000a). Creatine likely provides protection by maintaining mitochondrial homeostasis through increasing levels of phosphocreatine and maintaining mitochondrial structural integrity. Antioxidants, such as resveratrol, could be administered prophylactically to those at greater risk of TBI. Resveratrol has been shown to be neuroprotective and improve cognition following TBI (Singleton et al. 2010).

In summary, blast TBI represents a major public health problem with social and economic implications. Research initiatives elucidating the mechanisms of injury following blast will lead to the development of therapeutic strategies and better diagnostic tools.

#### 5.4 Concluding Remarks

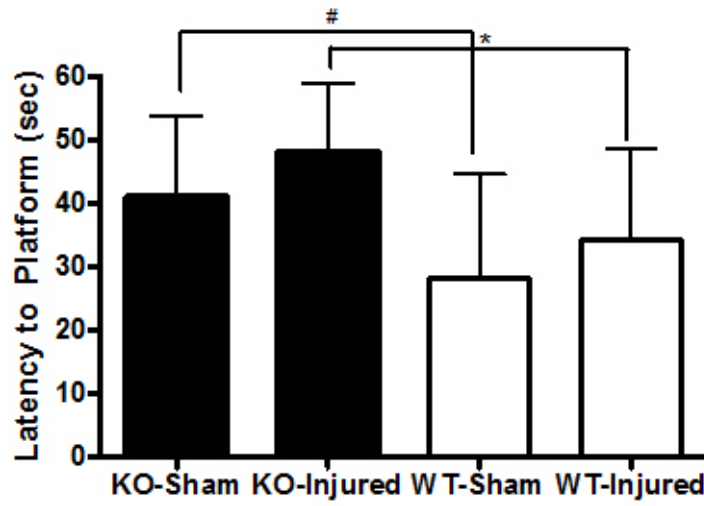
Based on the results highlighted in this dissertation, along with reports from others, CypD plays a critical role in cell death following TBI and indicates that pharmacological inhibition of CypD remains a plausible therapeutic strategy for TBI. We chose to use NIM811 in our studies based on its specificity for CypD in relation to CsA. Indeed NIM811 administration conferred neuroprotection following TBI. These results warrant further studies evaluating the therapeutic window for NIM811. Also, NIM811

efficacy needs to be determined in other models of TBI. Since our results indicate that blast causes neuropathology in the rat brain, further investigation is needed to evaluate neuroprotective compounds in blast TBI.

APPENDIX A: list of acronyms and abbreviations

3-NT	-	3-nitrotyrosine
4-HNE	-	4-hydroxynonenal
AIF	-	Apoptosis inducing factor
AMPA	-	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ANT	-	Adenine nucleotide translocase
BBB	-	Blood brain barrier
BOP	-	Blast overpressure
bTBI	-	Blast traumatic brain injury
CCI	-	Controlled cortical impact
CsA	-	Cyclosporin A
cTBI	-	Civilian traumatic brain injury
CypD	-	Cyclophilin D
DCD	-	Delayed calcium dysregulation
ETC	-	Electron transport chain
GCS	-	Glasgow Coma Scale
mPTP	-	Mitochondrial Permeability transition pore
MWM	-	Morris Water Maze
NIM811	-	N-methyl-4-isoleucine-cyclosporin
NMDA	-	<i>N</i> -methyl-daspartate
PBR	-	Peripheral benzodiazepine receptor
ROS	-	Reactive oxygen species
TBI	-	Traumatic brain injury
TPSO	-	Translocator protein
VDAC	-	Voltage-dependent anion channel

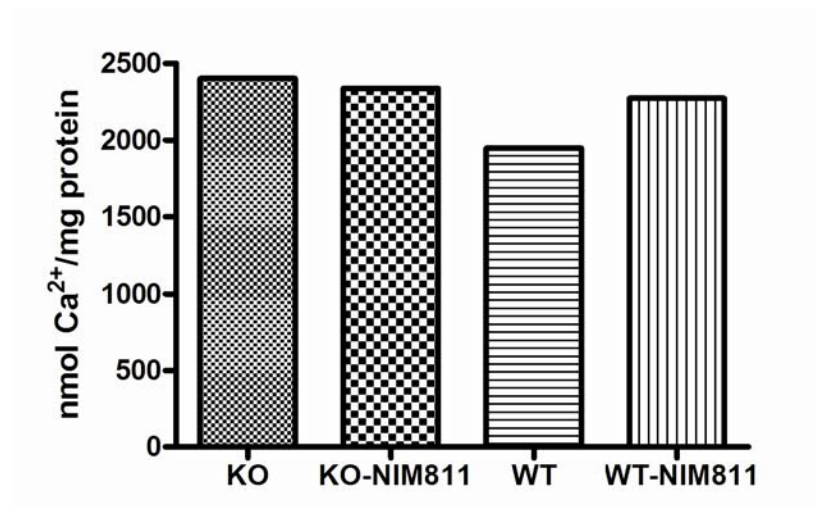
APPENDIX B: Effects of CypD on spatial memory



Morris Water Maze training data collapsed across all training days. CypD knockout resulted in a genotype-specific decrease in MWM performance compared to wild-type. Similar results were reported by Mouri and colleagues (Mouri et al. 2010). In these studies, inhibition of CypD with CsA produced similar MWM deficits as CypD knockout.



APPENDIX C: Ca<sup>2+</sup> buffering capacity of CypD knockout mitochondria and wild-type mitochondria



Mitochondria isolated from naïve wild-type and CypD knockout animals were used to assess the effect of *in vitro* NIM811 treatment (1  $\mu$ M) on mitochondrial Ca<sup>2+</sup> buffering capacity and mPTP. CypD knockout mitochondria were capable of buffering approximately 20 % more Ca<sup>2+</sup> before undergoing mPTP than wild-type mitochondria. CypD knockout and wild-type + NIM811 mitochondria demonstrated similar Ca<sup>2+</sup> buffering capacity.

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## VITA

### **Ryan Douglas Readnower**

#### Personal Information

*Date of birth:* 11-19-1983

*Place of birth:* Lexington, KY

#### Education

Doctorate of Philosophy, Anatomy and Neurobiology

University of Kentucky, Lexington, KY, USA (expected-August 2011)

B.S., Agricultural Biotechnology with a double major in biology

University of Kentucky, Lexington, KY, USA (May 2006)

#### Professional Positions

Research Assistant/Doctoral Candidate; June 2006 to July 2011

University of Kentucky, Lexington, KY

Teacher Assistant-Anatomy & Physiology for Nursing; August 2008-May 2009

University of Kentucky, Lexington, KY

#### Publications

**RD Readnower**, JD Pandya, ML McEwen, JR Pauly, JE Springer, PG Sullivan. (2011) Post-injury administration of the mitochondrial permeability transition pore inhibitor, NIM811, is neuroprotective and improves cognition following traumatic brain injury in rats. *Journal of Neurotrauma*, accepted.

**RD Readnower**, AD Sauerbeck, PG Sullivan. (2011) Mitochondria, Amyloid- $\beta$ , and Alzheimer's Disease. Invited review-*International Journal of Alzheimer's Disease* (PMID 21547208).

A Sauerbeck, J Gao, **R Readnower**, M Liu, JR Pauly, G Bing, PG Sullivan. (2011) Pioglitazone attenuates mitochondrial dysfunction, cognitive impairment, cortical tissue



loss, and inflammation following traumatic brain injury. *Experimental Neurology*, 277, 128-135 (PMID 20965168).

A Sauerbeck, J Pandya, I Singh, K Bittman, **R Readnowor**, G Bing, PG Sullivan. (2011) Analysis of regional brain mitochondrial bioenergetics and susceptibility to mitochondrial inhibition utilizing a microplate based system. *Journal of Neuroscience Methods*, 227, 128-135 (PMID 21402103).

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**R Readnowor**, J Pandya, P Sullivan. A novel, nano-scaled device to increase metabolism via mitochondrial uncoupling. May 2011, Poster presentation at the Barnstable Brown Obesity and Research Day.

**RD Readnowor**, JW Geddes, JR Pauly, PG Sullivan. Cyclophilin D as a therapeutic target following traumatic brain injury. May 2011, Poster presentation at the 17<sup>th</sup> Annual Kentucky and Spinal Cord and Head Injury Research Trust Symposium.

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A Sauerbeck, P Sullivan, **R Readnowor**, G Bing, J Gao. Therapeutic efficacy of pioglitazone following traumatic brain injury. *Journal of Neurotrauma Abstracts*; May 2010, A-66.

**RD Readnower**, R McCarron, M Chavko, S Adeeb, P Sullivan. Blast induced neurotrauma in the rat brain. *Journal of Neurotrauma Abstracts*; August 2009, A-87.

**RD Readnower**, M Chavko, R McCarron, P Sullivan. Neurotrauma in the rat brain caused by blast overpressure. June 2009, poster presentation at the 15<sup>th</sup> annual Kentucky and Spinal Cord and Head Injury Research Trust Symposium.

**RD Readnower**, R McCarron, M Chavko, P Sullivan. Blast induced traumatic brain injury: characterizing the injury pathology. *Journal of Neurotrauma Abstracts*; July 2008, Vol. 25, No. 7: 904.

**RD Readnower**, AH Sebastian, LM Davis, GA Goldstein, PG Sullivan. Following traumatic brain injury antioxidant therapy, TOVA (a glutathione precursor), increases tissue sparing and reduces oxidative stress. June 2008, poster Presentation at the 14<sup>th</sup> Annual Kentucky Spinal Cord and Head Injury Research Trust Symposium.

LM David, JR Pauly, AH Sebastian, **RD Readnower**, JM Rho, PG Sullivan. Acute post injury fasting is neuroprotective following traumatic brain injury: potential mechanisms and implications for treatment. November 2007, Poster presentation at the Society for Neuroscience annual meeting.

LM Davis, AH Sebastian, **RD Readnower**, JM Rho, PG Sullivan. Acute post injury fasting is neuroprotective following traumatic brain injury; potential mechanisms and implications for treatment. *Journal of Neurotrauma Abstracts*; July 2007, Vol. 24, No. 7: 1270.

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