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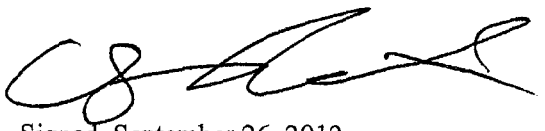

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THE EFFECT OF DIAZOXIDE AND DIMETHYL SULFOXIDE ON BEHAVIORAL OUTCOMES AND MARKERS OF PATHOLOGY FOLLOWING CONTROLLED CORTICAL IMPACT IN THE MOUSE.

Major Craig S. Budinich

Thesis directed by Joseph T. McCabe, Ph.D., Professor & Vice Chair, Department of Anatomy, Physiology & Genetics, F.E. Hébert School of Medicine, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD

Abstract

Traumatic brain injury (TBI) is a significant worldwide problem with an estimated incidence of at least 200 cases per 100,000 people per year. Due to recent military conflicts in the Middle East, a tremendous spike in the incidence of combat related brain injury has occurred over the past decade. Over the last 30 years, preclinical research focused on evaluating potential pharmacologic therapeutic agents has produced multiple promising candidates; however, to date no single drug intervention has consistently shown benefit when administered in the clinical setting. Fortunately, new potential therapeutic targets are being identified at a staggering rate as technology advances and our understanding of the pathology behind brain injury improves. In an effort to advance the therapeutic and molecular target realms of basic research, our team tested two hypotheses. First, that diazoxide (DZ), a putative mitochondrial K_{ATP} opener, administered after brain injury will result in short and long-term improvement in

memory, motor, and behavioral function; and second, that moderate and severe traumatic brain injury will result in early and sustained widespread alterations of polysialylated neural cell adhesion molecule (PSA-NCAM) expression.

To evaluate the first hypothesis, male C57BL/6 mice were subjected to moderate controlled cortical impact brain injury and were administered DZ 2.5 mg/kg 30 minutes after injury and once a day for three days thereafter. Behavioral and motor performance were evaluated on post injury days 1, 7, and 14, and memory function was assessed on post procedure days 17 – 21. Western blotting and immunohistochemistry evaluation were also utilized to monitor changes in the expression levels of select biomarkers of healing and pathology. *In toto*, neither dimethyl sulfoxide, the vehicle used to dissolve diazoxide, alone or in combination with diazoxide produced a consistent and significant improvement in behavioral and memory performance on any of the functional parameters measured. DZ treatments produced a modest improvement in motor performance. Protein expression evaluation revealed ambiguous results indicating enhancement of both pro survival and pro apototic marker expression and variable results regarding cytoskeletal and inflammatory markers.

In an attempt to identify a novel marker of altered brain function following TBI, evaluation of short and long term PSA-NCAM level changes following graded-CCI was conducted using immunoblot and immunohistochemical analysis. Both short (2, 24, and 48 hour) and long term (1 and 3 week) alterations in PSA-NCAM expression were noted throughout the brain following moderate and severe-CCI. These changes in protein expression levels may represent pathological alterations or restorative efforts by the brain. This work may serve as a foundation for future efforts aimed at understanding the

relationship between PSA-NCAM and neuropathology as well as identifying potential therapeutic windows for interventional therapy.

THE EFFECT OF DIAZOXIDE AND DIMETHYL SULFOXIDE ON BEHAVIORAL OUTCOMES AND MARKERS OF PATHOLOGY FOLLOWING CONTROLLED CORTICAL IMPACT IN THE MOUSE.

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Doctoral Dissertation submitted to the faculty of the Graduate Program in Neuroscience of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 2012

DEDICATION

I dedicate this work to the following people:

Tracy Budinich, my beautiful and infinitely supportive wife. I love you.

Nina and **Avery**, my lovely children.

Carl Arsane Budinich, my Grandfather and true hero. I love you "Paw Paw".

Rest in peace.

Senior Airman Jason D.Cunningham

You have inspired me to pursue my dream. Your sacrifice will never be forgotten!

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AA	Arachidonic acid
Aβ	Amyloid beta
AD	Alzheimer's disease
AIF	AIF
Akt	Protein kinase B
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANT	Adenine nucleotide translocase
sAPP	Soluble amyloid precursor protein
APO-1	Fas/Apolipoprotein-1
ASK1	Apoptosis signal regulating kinase 1
ATP	Adenosine triphosphate
β-APP	β-amyloid precursor protein
BACE 1	β-secretase
Bak	Bcl-2 antagonist/killer
Bax	BCL-2-associated protein X
BBB	Blood-brain barrier
BCL-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BH3	BCL-2 homology domain 3
Bid	BH3 interacting-domain
CaMKIIα	Calmodulin-dependent protein kinase IIα

CBF	Cerebral blood flow
CCI	Controlled cortical impact
CSF	Cerebrospinal fluid
CSPG	Chondroitin sulfate proteoglycans
CT	Computed tomography
Cyp D	Cyclophilin D
Daxx	Fas/ Death-associated protein 6
$\Delta\psi m$	Mitochondrial transmembrane potential
DMSO	Dimethyl sulfoxide
DZ	Diazoxide
EGF	Epidermal growth factor
ERKs	Extracellular signal-regulated kinases
FGFR	Fibroblast growth factor receptor
GAP-43	Growth-associated protein-43
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GSK3β	Glycogen synthase kinase 3β
5-HD	5-hydroxydecanoate
H_2O_2	Hydrogen peroxideHSP
HSP	Heat-shock protein
HSPG	Heparin sulfate proteoglycans
Iba-1	Ionized calcium binding adaptor molecule 1
ICAM-1	Intercellular adhesion molecule 1

ICP	Intracranial pressure
IFN-γ	Interferon gamma
IL-6	Interleukin-6
IL-18	Interleukin-18
IL-1β	Interleukin- 1β
IMS	Intermembrane space
i.p.	Intraperitoneal
JNKs	Jun N-terminal kinases
K_{ATP}	Adenosine 5'-triphosphate–sensitive potassium channel
K_{ir}	Inwardly-rectifying potassium channel
LANP	Leucine-rich acidic nuclear protein
LTP/D	Long-term potentiation and depression
M&M	Morbidity and mortality
MAP-2	Microtubule associated protein-2
MCP-1	Monocyte chemoattractant protein-1
MEKs	Mitogen-activated protein kinases
MgADP	Magnesium-adenosine diphosphate
mK_{ATP}	Mitochondrial K_{ATP}
MMP	Mitochondrial membrane permeabilization
MOMP	Mitochondrial outer-membrane permeabilization
MRI	Magnetic resonance imaging
mTBI	Mild Traumatic brain injury
MWM	Morris water maze

MyoNAP	Myogenesis-related and NCAM-associated protein
NADH	Superoxide dismutases
NBD	nucleotide binding domains
NMDA	N-Methyl-D-aspartate
NO	Nitric oxide
$\cdot O_2$-	Superoxide
OGD	Oxygen glucose deprivation
Omi/HtrA2	Omi stress-regulated endoprotease/high temperature requirement protein A2
$ONOO^-$	Peroxynitrite
OXPHOS	Oxidative phosphorylation complexes
p38MAPK	p38 Mitogen-activated protein kinase
p53	Tumor protein 53
PACAP	Pituitary adenylate cyclase-activating polypeptide
PC	Preconditioning
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PI3Ks	Phosphatidylinositol 3-kinases
PKC/G	Protein kinase C or G
PLCγ	Phospholipase C-gamma
Post-C	Postconditioning
PP2A	Protein phosphatase 2A
PSA-NCAM	Polysialylated neuronal cell adhesion molecule

PSD-95	Post-synaptic density-95
PST	Polysialyltransferases
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PTPC	Permeability transition pore complex
ROS	Reactive oxygen species
RPTPα	Receptor protein tyrosine phosphatase α
SBDPs	Spectrin breakdown products
SDF-1α	Stromal cell-derived factor-1 alpha
SDH	Succinate dehydrogenase
Smac/DIABLO	Second direct activator of caspases/direct IAP-binding protein with a low pI
SOD	Superoxide dismutase
SUR	Sulfonylurea receptor
SVZ	Subventricular zone
TAI	Traumatic Axonal Injury
TBI	Traumatic brain injury
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TOAD-64	Turned on after division-64
VDAC	Voltage-dependent anion channel

Chapter 1

Introduction

The incidence of traumatic brain injury (TBI) in civilian and military populations has reached epidemic proportions throughout the world. In spite of improvements in the technologies directed toward preventing TBI (e.g., seatbelts, helmets, and airbags), over 1.7 million individuals in the United States will experience trauma related brain injury [1]. Unfortunately, a rapid rise in motor vehicle related TBI is also occurring in developing countries as the use of motorized transportation outpaces the ability to develop an adequate safety infrastructure [2, 3]. Analysis conducted by the Centers for Disease Control and Prevention reveals that the majority of TBI occurs in a bimodal distribution including predominantly young adult males (15 to 24 years-old) and the elderly (≥75 years-old) [4]. In addition to an approximately two-percent rate of mortality associated with civilian TBI, long-term implications translate into nearly 2% of the American population experiencing chronic neurological symptoms that place not only an enormous emotional burden on the victims and their loved ones, but also result in a greater than $60-billion annual liability placed on American society due to lost productivity and disability [5].

Combat related TBI has become a prominent military health care issue over the last 15 years. Advances in protective armor and lifesaving medical interventions during combat operations have allowed soldiers to survive exposure to blast and mechanical forces that historically would have produced lethal brain injury [6]; however, this has translated into a 'silent epidemic' with an estimated 20% or at least 300,000 or more Iraq

and Afghanistan veterans suffering from TBI [7, 8]. Because penetrating brain injuries produce mortality as high as 91% [9], the majority of survivable combat related TBI is classified as blast-induced mild TBI (mTBI). Of note, 16% of soldiers who have experienced TBI report loss or alteration of consciousness subsequent to the injury [10, 11]. Even though blast related TBI has been, and will likely continue to be, responsible for the majority of TBI experienced by military service members, a greater than 14,000 annual worldwide terrorist attack incidence will place civilians at increased risk of blast related TBI [12].

The range of outcomes following TBI, particularly mTBI, is remarkably variable not only depending on the severity of injury but also on the diagnostic criteria and assessment tools used to manage victims [13, 14]. The impact of severe TBI on an individual's ability to function following injury is well appreciated, but approximately 15% of mTBI victims are considered the "miserable minority" and also suffer long-term disability [15]. Interestingly, conventional diagnostic mechanisms may fail to accurately identify patients who could suffer long term deficits and "post concussive syndrome" resulting from relatively mild head trauma as evidenced by the fact that up to 80% of these patients demonstrate normal magnetic resonance imaging (MRI) and computed tomography (CT) studies at the time of injury [16, 17]. The heterogeneity of outcomes resultant to TBI reflects the complexity of processes that interact to produce immediate and long-term pathology following brain injury. Considering the lack of an existing therapy that reliably improves post-TBI outcomes, and the prevalence and immense impact that TBI has on the individual and society as a whole, continued research that investigates novel approaches to treat post-TBI neuropathology is desperately needed.

Over 130 monotherapy *in vitro* and *in vivo* TBI animal studies have demonstrated efficacy; however, the translation of neuroprotective results from the laboratory to the clinical arena has not been wholly successful [18]. Nearly all Phase II/III clinical trials have failed to show any consistent improvement in outcome for TBI patients [19]. The reasons are many. First, none of the animal models of TBI adequately replicate the heterogeneity of TBI in humans [20, 21]. The precision used in inflicting damage, the lack of secondary systemic injury or hemorrhage, and the use of a pretreatment or early treatment time paradigm have all contributed to the poor translation to clinical practice. Animal trials have, however, improved our understanding of the pathophysiology underlying TBI, and we can use this knowledge to modify our approach to improving TBI treatment efforts. As more is understood, different agents are being evaluated. For example, knowledge about the permeability transition pore complex (PTPC) and its role in ischemic injury has served as the inspiration promoting research involving the mitochondrial ATP-sensitive potassium channel opener, diazoxide (DZ), and its ability to provide neuroprotection [22]. The promising results related to that research has served as the inspiration for the work presented here.

The heterogeneous presentation of TBI must be addressed with an equally diverse treatment paradigm. Pharmacologic treatments aimed at addressing key bedrock pathological processes that promulgate subsequent pathological pathways or agents that simultaneously address more than one pathologic process appear to be the next step in preclinical and clinical evaluation. For example, drugs such as magnesium demonstrate the ability to address multiple pathways in secondary TBI such as edema formation, excitotoxicity, vasospasm, apoptosis, and mitochondrial ATP utilization [23]. Although a

recent TBI clinical trial actually demonstrated a worsened outcome with low dose magnesium administration after TBI [24], perhaps adjusted dosing and timing schedules should be investigated. In animal studies, thyrotropin-releasing hormone has also been shown to address multiple secondary injury pathways [25]. As will be revealed shortly, our selected therapy (DZ) is believed to focus on preventing the initiation and progression of a core process that leads to multiple subsequent pathological events.

The dissertation contained within is composed of the following five sections: (1) an introductory chapter containing an exploration of the mechanisms involved in TBI related neuropathology. This chapter will also introduce a novel drug intervention, diazoxide administration, and discuss the putative mechanisms of action that make it a viable candidate for evaluation. In addition to a survey of the established markers of injury and/or healing that were used to evaluate the efficacy of the drug intervention, polysialylated neuronal cell adhesion molecule (PSA-NCAM), a novel marker of injury and/or healing following TBI was evaluated. Its relevance will be examined; (2) a chapter presenting an examination of the effects of post-injury administration of DZ and DMSO, the vehicle used to deliver DZ, on short and long-term neurobehavioral outcomes following TBI; (3) a chapter presenting a detailed examination of the short and long-term changes in expression levels of PSA-NCAM throughout the brain following graded-controlled cortical impact (CCI); (4) a concluding chapter summarizing the implications of the presented work and recommended future directions of study; and (5) an appendix presenting data related to the effects of DZ and DZ+DMSO pre and post-injury treatment on select biomarkers of injury. It is the author's wish that the work presented here may

serve to contribute to the knowledge pool regarding TBI neuropathology and potential treatment.

Pathophysiology of Traumatic Brain Injury

Significant strides in understanding the pathological changes that occur with the various degrees of TBI, from mild/concussive to severe penetrating injury, have been made over the past decade. Improvements in molecular techniques and imaging technology have allowed for the identification of likely key players in the immediate and late secondary injury cascade and repair events that follow the primary physical event. Classification of TBI is generally made using factors such as the physical mechanism of injury, prognostic modeling, injury severity, pathoanatomic changes, and pathophysiologic mechanisms initiated by the primary physical events [26]. Using pathophysiologic and pathoanatomic approaches, the major processes or events that are initiated include contusion, hemorrhage/hematoma, a 'Neurometabolic Cascade' [27], vascular disruption, edema, inflammation, oxidative stress, traumatic axonal injury (TAI), and apoptotic and/or necrotic cell death [28-30]. Because mitochondrial function is a critical element at the center of restorative or pathological processes that occur following TBI, and because the pharmacologic therapy that was evaluated during this work is believed to exert its putative protective effect via mitochondrial mechanisms, special examination of this topic will be addressed. The clear majority of TBI victims present with mTBI [31], therefore the following discussion will center on the processes associated with it; however, it should be appreciated that more exaggerated and widespread abnormality can be associated with severe TBI.

Hemorrhage and Contusion

Various neuroimaging techniques are used to identify and classify intracranial abnormalities, such as hemorrhage and contusion, following injury. The "gold standard" imaging technique used to evaluate TBI victims when they first present to the hospital is CT [32]. Surprisingly, up to 80% of patients presenting to the emergency room within 24-hours following blunt mTBI do not have positive findings on CT scans [32]. Although there is not a direct correlation between outcome and the presence of an intracranial abnormality following TBI, identification of hemorrhages, contusions, or edema via CT scanning generally has a less favorable outcome that patients without such findings [33]. The rapid development and deployment of more advanced imaging techniques, such as susceptibility weighted MR imaging, may improve prognostic capabilities due to the ability to detect microhemmorhages and contusions with never before seen accuracy [34]. In fact, in the absence of obvious pathology following mTBI, MR can be used to identify the presence of minute amounts of hemosiderin, a breakdown product of blood, with extreme accuracy [35]. This information is useful because it serves as evidence that considerable mechanical injury to microvessels (< 20 μm diameter) and the surrounding parenchyma has taken place [36].

Neurometabolic Cascade

The metabolic alterations that take place in the 'Neurometabolic Cascade' are initiated by a disruption of the neural membrane that results in an unregulated flux of ions (see [30] for review). **Figure 1** depicts a graphic representation of the complex interaction of these alterations. The initial flux results in unregulated release of neurotransmitters (i.e., excitatory amino acids such as glutamate), an increased intensity of ionic fluxes (i.e., K^+, Ca^{2+}), and widespread depolarization and depression of neurons

[30]. Glucose utilization increases rapidly as the Na^+/K^+ ATP-dependent pump functions at maximum capacity in an attempt to correct the ionic imbalance. However, because cerebral blood flow (CBF) autoregulation and CO_2-reactivity are perturbed [37], an uncoupling of glucose utilization and CBF may lead to ischemic injury [27, 30]. This scenario becomes more likely considering the fact that the threshold for permanent injury is three-times higher in TBI vs. ischemic stroke ($15 \text{ ml} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$) [38]. Reductions in CBF following mild to moderate TBI is between 70-80% and even more pronounced with severe injury [39]. If these TBI induced changes return to baseline within a short, as of yet undefined, interval then transient hypofunction usually results and permanent injury is avoided [40]. In some cases, these alterations take place over several days or weeks, and make the neurons and other supporting cells more vulnerable to the development of long-term pathology [41].

Vascular Disruption

Altered vascular function of different durations has been shown to take place in all levels of brain injury [42, 43]. For example, significant alterations in microvascular density and blood flow following graded fluid percussion injury in rats was seen at significant distances from the impact epicenter for at least two-weeks following injury [43]. In the grand scheme, TBI produces varying degrees of blood-brain barrier (BBB) breakdown and microvascular dysfunction [44]. Increased permeability of the BBB will produce edema and may acutely increase intracranial pressure (ICP). Elevated ICP accentuates injury by causing cerebral hypoperfusion, secondary ischemia, and brain herniation [45]. In fact, refractory elevations of ICP > 20 mmHg have been linked to a significantly higher morbidity and mortality (M&M) than matched patients with normal

ICP measurements (< 20 mmHg) [46]. A sobering statistic reveals that 90% of patients who die within two days of injury do so due to brain stem herniation resulting from inability to control an acute increase in ICP [2]. The violation of BBB integrity following TBI has been shown to be a biphasic event in experimental animal models [47]. After reaching a peak of permeability within hours of injury, a second phase of increased permeability begins 3 – 7 days following injury and may persist for months or years although most cases return to normal within weeks [48-50]. It is believed that the secondary BBB breakdown period is treatable and can have substantial influence on patient outcome [51].

Although a thorough discussion of the recognized contributions of BBB breakdown to early and long-term neuropathology is beyond the scope of this work (see [51] for a detailed review), key points will be presented. Authorities believe that immediate and delayed alterations in BBB integrity are due to compromised integrity of endothelial tight-junctions and/or disruption of the neuron, astrocyte, and microvessel complex known as the gliovascular unit [51-53]. For example, serum levels of S-100B, a small calcium binding protein found in astroglia that indicates damage, are elevated following TBI, and this elevation is correlated with other structural changes indicative of compromised BBB function, such as enlargement of astrocyte foot processes, appearance of microvilli on the luminal face of endothelial cells, and an increase in the number of endothelial pinocytic vesicles [36, 54]. Additionally, it is now recognized that dysfunction of the BBB is a major contributing factor to the development of neuropathology associated with TBI, Alzheimer's disease (AD), and Parkinson's disease (PD) [55]. Initial disruption of the BBB produces a robust activation of the coagulation

cascade and results in a significant decrease in pericontusional blood flow due to thrombi formation in the microcirculation [56]. The unregulated influx of fibrinogen, albumin, and thrombin have profound effects on microglia, astrocytes, and neurons (see below) [57]. Invasion of systemic inflammatory cells occurs and exert multiple influences such as neutrophil-driven injury to damaged neurons and endothelial cells via release of large amounts of ROS and multiple proteolytic enzymes [58]. The goal of restoring BBB function post-TBI serves as a valuable target because it may provide the ability to "balance" the invasion of systemic inflammatory cells, activation of resident inflammatory cells, and propagation of inflammatory and pro-growth signaling cascades to promote optimal recovery.

Edema

As previously mentioned, post-TBI edema is a significant contributor to secondary injury and long-term morbidity and mortality. The two primary forms of edema seen following injury are vasogenic and cytotoxic. Vasogenic edema results from initial mechanical or delayed biologic (i.e., reactive oxygen species (ROS) generation) initiated increases in endothelial cell permeability or tight junction disruption that results in the accumulation of water and osmotically active molecules from the plasma [57, 59]. Cytotoxic edema results when primarily astrocytes and endothelial cells [60] accumulate excessive H_2O and ions due to energy depletion and failure of membrane bound ion pumps [38]. Vasogenic edema is thought to contribute to early onset edema following TBI; however diffusion-weighted magnetic resonance imaging has been used to demonstrate that vasogenic edema may not be as significant a contributor to post-TBI edema as previously thought [61]. Nevertheless, disruption of the BBB may persist for

years following TBI and apparently plays a critical pathogenic role in disorders such as post-traumatic epilepsy [48]. Cytotoxic edema in both gray and white matter may be acutely caused by mechanical injury to the cell membrane, while delayed edema may result from intracellular water accumulation due to excitotoxic stimulation producing Na^+, Cl^-, and Ca^{2+} influx [59].

Inflammation

The neuroinflammatory response of the brain following TBI may be partitioned into acute and chronic phases that involve the release of cytokines, chemokines and the recruitment, migration, and activation of immune cells. Initial violation of the BBB allows for the movement of serum fibrinogen, albumin and thrombin into the brain parenchyma. Fibrinogen participates in the immediate and delayed post-injury neuroinflammatory response by contributing to the activation of microglia, most likely via activation of toll-like receptors (TLRs), that increase phagocytic activity and release chemokines that attract systemic neutrophils, monocytes, and leukocytes to the injury site [62]. The presence of fibrinogen in the parenchyma may also affect neuronal repair by promoting the formation of glial scars and inhibiting neurite outgrowth; most likely via transforming growth factor beta (TGF-β) induced phosphorylation of Smad-2 [63, 64]. Extravasated albumin is also believed to contribute to the inflammatory response by increasing microglial proliferation [65] and enhancing release of interleukin- 1β (IL-1β) and reactive oxygen species from activated microglia [57]. The presence of the serine protease thrombin in the brain parenchyma may be protective or injurious depending on concentration as evidenced by the fact that high concentrations promote apoptosis of cultured neurons and astrocytes [66], promote astrocyte and microglial activation,

proliferation, and generation of nitric oxide (NO) [57], and even contribute to early vasogenic edema [67].

In addition to the movement of serum components, infiltration of leukocytes occurs via interaction with endothelial intercellular adhesion molecule 1 (ICAM-1) and various chemokines (see [68] for review) and activate resident microglia. Activated resident microglia, astrocytes, macrophages and lymphocytes release both pro- and anti-inflammatory cytokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-18 (IL-18) and interleukin-6 (IL-6)], chemokines [i.e., stromal cell-derived factor-1 alpha (SDF-1α) and monocyte chemoattractant protein-1 (MCP-1)], neurotransmitters (i.e., glutamate) and reactive oxygen species (i.e., nitric oxide) [69]. Microglial activation is one of the central forces of post-traumatic inflammation, and this activation may continue for over a year in animal models and beyond a decade in humans, thus perpetuating a chronic inflammatory response that may be linked to neurodegenerative disorders such as AD and PD and behavioral disorders such as depression [70, 71]. One must note that the implications of the TBI-associated inflammatory response on clinical outcome have not been fully appreciated or understood, but it is generally accepted that the acute phase is required for repair and reorganization while the chronic phase may lead to detrimental clinical outcomes [51].

Oxidative Stress

Because the brain has the highest oxygen metabolic rate in the body, consuming 20% of the oxygen in the body, it is exceptionally vulnerable to oxidative stress [72]. Oxidative stress associated with TBI occurs as a culmination of other concurrent

pathological processes, including excitotoxicity due to excessive EAA release [73], maximum energy consumption by Na^+/K^+-ATPase activity attempting to balance ionic gradients [27], and CBF uncoupling [37]. It has been placed "on the top of the list" of pathophysiological mechanisms that potentiate secondary injury in TBI [57]. The generation of ROS such as nitric oxide (NO), peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2), and superoxide ($\cdot O_2$-) occurs due to exhaustion of the existing antioxidant system composed of superoxide dismutase (SOD), catalase, and glutathione peroxidase [38, 72]. Widespread detrimental effects occur in neurons, astrocytes, and endothelial cells via peroxidation of membrane polyunsaturated fatty acids [57], DNA cleavage [72], oxidation of proteins [72], and inhibition of the mitochondrial electron transport chain [38].

Axonal Injury

Human and animal studies, whether they be blast, penetrating, or concussive mechanisms of injury, have demonstrated that in the majority of cases not only is there extensive damage localized around the epicenter of force, but that disparate regions of axonal injury are often seen due to inertial effects that are transmitted throughout the brain [59, 74]. This traumatic axonal injury (TAI) is a pathologic alteration that is considered to be unique to TBI [75]. Early studies suggested that shearing forces acting upon white matter tracts would result in the acute formation of large numbers of axonal retraction balls that could be used to diagnose the severity of traumatic axonal injury [76]. More recent studies contradict these early findings [77]. **Figure 2** presents a schematic presentation of the time course of key events seen in axonal injury following varying degrees of strain injury. It is currently believed that, particularly in mild and

moderate TBI, rapid and widespread proteolysis of axonal cytoskeletal components does not occur and that most axonal degradation is the result of a delayed (by several hours or days) "secondary axotomy" that is produced via a disruption of ionic homeostatic mechanisms (i.e., excessive Ca^{2+} influx) due to loss of axolemma integrity [41]. Anterograde and retrograde transport is affected as neurofilaments are compacted, microtubule networks are destroyed, and mitochondrial bioenergetic capability is reduced [78]. If the level of physical insult exceeds a "point of no return," then axonal viability is threatened as Ca^{2+} induced calpain activation, mitochondrial cyt *c* release, and caspase activation occurs, and ultimately Wallerian degeneration and disconnection will result [79]. Of note, the axons may maintain a reactive, vulnerable state for several months following injury [41]. This places the neuron at increased risk of secondary injury if a subsequent traumatic event were to occur, but it also presents as an extended window for therapeutic interventions. Of note, even following mTBI, in mice, a progression of axonal damage was noted to spread throughout various cortical and subcortical structures over a 4 – 6 week period, and these changes correlated with poor spatial learning and memory performance [30, 80].

Necrosis and Apoptosis

The two generally accepted mechanisms by which neuronal death occurs following TBI includes necrosis and apoptosis. The irreversible membrane and metabolic disturbances that immediately follow the primary injury event result in necrosis, which has traditionally been considered to be a passive process [36, 81]. Evidence of significant necrosis following focal and diffuse TBI models is abundant [79, 82, 83]. However, recent evidence has shown that the extent of necrosis that occurs with TBI may not be as

significant as previously thought because more recent studies show that neuronal necrosis does not follow diffuse TBI in the rat, but rather reparative responses occur without mitochondrial or cytoskeletal pathological alterations [36, 79]. Apoptosis is a long-term, energy dependent form of programmed cell death that, unlike necrosis, displays nuclear condensation and fragmentation and preservation of organelle structure during the process [81]. Apoptotic pathways lead to activation of cysteine aspartate-specific proteases, called caspases, or the release of caspase-independent proteins that modulate cell death [83]. An imbalance of pro- and anti-apoptotic proteins leads to initiator caspases (i.e., caspase-8 and -9) activating downstream effector (i.e., caspase-3 and –7), which leads to amplification of the caspase cascade [83]. Although the precise molecular mechanisms of caspase-independent cell death are not as well understood as caspase-dependent death mechanisms, caspase-independent cell death can occur in the face of ATP depletion and may therefore play a more prominent role in more severe forms of TBI [84]. Multiple mitochondrial proteins, such as cytochrome *c*, apoptosis inducing factor (AIF), and Smac/DIABLO have been shown to be intimately involved in caspase dependent, caspase independent, or both forms of apoptosis, respectively [81]. In scenarios where ATP availability is tenuous, the term aponecrosis has been adopted to describe cells where necrotic and apoptotic morphological changes overlap [85].

Mitochondrial Dysfunction

The heterogeneity of the above described TBI initiated pathomechanisms illustrates the difficulty encountered by investigators attempting to evaluate possible pharmacologic therapeutic interventions aimed at moderating secondary injury cascade pathology and bolstering reparative mechanisms. However, mitochondrial dysfunction is

understood to be a key critical initial event leading to apoptotic and necrotic cell death [86-88]. There are two recognized biochemical pathways responsible for apoptosis: the extrinsic and intrinsic (mitochondrial) pathways [89]. In the extrinsic pathway, cell membrane receptors initiate apoptosis. This discussion will focus on the mitochondrial pathway because it is considered the primary pathway of apoptosis following neuronal injury [90]. In particular, activation of the mitochondrial PTPC, is believed to be a "critical switch" that determines if a cell survives or perishes following TBI [75]. It is important to note that the extrinsic and mitochondrial pathways are not mutually exclusive entities, but are linked (primarily by the BCL-2 homology domain 3 (BH3)-only protein called BID) via mitochondrial membrane permeabilization (MMP) [89].

If, in the face of injury, activation of pro-death pathways exceeds that of pro-survival pathways, then MMP will result (see **Figure 3**). As discussed above (see "Neurometabolic Cascade" and Fig. 1), traumatic injury or ischemic conditions can lead to the excessive generation of ROS and excessive influx of Ca^{2+} that will initiate pro-death and pro-survival pathways (see [90] and [91] for review). These pathways composed of various kinases, redox-active molecules, and pro- and anti-apoptotic members of the BCL-2 family will enhance or block each other's activity and ultimately converge upon the mitochondria. If pro-death mechanisms predominate, then MMP will occur and, if enough mitochondria are affected, will commit the affected cell to death [90].

Because MMP is the common final pathway that initiates the mechanisms of programmed cell death, a further investigation of the two accepted molecular mechanisms that are responsible for this event is warranted. The first mechanism is

referred to as mitochondrial outer-membrane permeabilization (MOMP). In this scenario, large lipidic pores formed by the proapoptotic proteins BCL-2-associated protein X (Bax) or Bcl-2 antagonist/killer (Bak) lead to the release of cytochrome *c* and other intermembrane space proteins which may result in uncoupling of oxidative phosphorylation, generation of excessive ROS, and dissipation of the mitochondrial transmembrane potential ($\Delta\psi m$) [90-92]. Normally, Bax and Bak are inactive and located in the cytosol and loosely associated with the outer mitochondrial membrane, respectively, but in an apoptotic environment activator type BH3-only proteins (such as Bid) will form a heterodimer with Bax and Bak and promote conformational changes in these proteins to induce formation of this pore [90]. Recent evidence suggests that products of sphingomyelin metabolism may also induce the conformational changes required for MOMP [93]. Sensitizer type BH3-only proteins such as Bad, may also dislocate inhibitory Bcl-2 proteins that are bound to Bax and Bak and lead to pore formation [90]. Release of cyt *c* and other caspase activators marks the point of no return for programmed cell death [94].

The second method of MMP is induced by the opening of a supramolecular structure that spans the inner and outer membrane called the permeability transition pore complex (PTPC) [95]. Controversy remains regarding the composition of the PTPC, but most authorities agree that a voltage-dependent anion channel (VDAC) is associated with the outer membrane, an adenine nucleotide translocase (ANT) in the inner membrane, and cyclophilin D (Cyp D), which may modulate pore opening, in the matrix [91, 94]. **Figure 4** depicts the proposed mechanism of action of the PTPC. Normally, the conductance state of the PTPC is tightly regulated to ensure controlled exchange of ions

between the mitochondrial matrix and cytosol [90]. Factors such as oxidative stress and excessive Ca^{2+} sequestration in the mitochondrial matrix will significantly increase conductance through the complex and allow solutes and water to enter the mitochondrial matrix, thus decreasing the $\Delta\psi m$ and producing osmotic swelling of the matrix until eventual membrane rupture occurs [95-97]. Implications of the release of select inner mitochondrial space proteins on cell death are presented in **Table 1**.

Therapeutic Intervention

It is apparent that following TBI, mitochondrial dysfunction is the principal factor in determining cell survival, or death and ultimately the severity of signs and symptoms and functional disability experienced by the victim [98]. The majority of work focusing on therapies attempting to prevent or correct mitochondrial dysfunction related to CNS injury has been *in vitro* and *in vivo* ischemic/oxygen and glucose deprivation situations [99-104]. As would be expected, studies directed at preemptive therapy aimed at preventing the initiation of MMP and apoptosis have demonstrated the most efficacy [82, 104-107]. Although not as abundant, research investigating the effect of post injury administration of therapies targeting mitochondrial dysfunction or the effectors of apoptosis is promising [108-110]. As will be discussed, DZ, the therapeutic intervention evaluated in this thesis, has been shown to provide robust protective effects *in vitro* and *in vivo* when administered before OGD stress in both heart and CNS scenarios [102, 111-113]. Post-insult administration has not been as thoroughly investigated [114-116]. Surprisingly, DZ has not been evaluated in TBI. In the following section diazoxide, its putative mechanism of action and the existing evidence surrounding its use in OGD

models will be presented as evidence used for the decision to evaluate the drug in the TBI setting for this project.

Diazoxide (DZ)

Diazoxide is a benzothiadiazine (3-methyl-7-chloro-1,2,4-benzo-thiadiazine,-1,1-dioxide) that has been in clinical use since around 1961 for its antihypertensive and antihypoglycemic effects [117-119]. Physically it is a white crystalline material that is odorless, has a molecular weight of 230.7 g/mol, a melting point of 330.5°C, and is soluble in alkali solutions and various organic solvents [118]. Regarding the pharmacokinetic and pharmacodynamics properties of the drug, it has a rapid onset of antihypertensive effects at 2 minutes following an intravenous bolus injection, a volume of distribution approximately at steady state of 0.2 liter/kg, a half-life of 32 – 63 hours, and a plasma clearance rate of 202–5.3 ml/kg/hr^{-1} [120]. DZ may also be administered via continuous intravenous infusion at 7.5 mg/kg/hr^{-1} to maintain a 16 – 30 % decrease in mean arterial pressure and orally at 10 mg/kg/day^{-1} for chronic hypoglycemia treatment [120, 121]. The hypotensive and diabetogenic clinical effects result from DZ's ability to open K_{ATP} channels in the plasma membrane while the recognized neuroprotective effects of DZ are believed to be due to the ability of the drug to open mitochondrial K_{ATP} (mK_{ATP}) channels. Of note, DZ has an approximately 2000 fold higher affinity for mK_{ATP} over K_{ATP} channels in the sarcolemma [122, 123].

mK_{ATP} Channels

Adenosine 5'-triphosphate–sensitive potassium channels (K_{ATP}) were initially identified by Akinori Noma in 1983 when cyanide treated mammalian heart cells revealed a highly selective K^+ channel that was inhibited by ATP application [124].

Much work, particularly focusing on cardioprotection, regarding these sarcolemmal K_{ATP} channels ensued [125, 126]. However, after evidence demonstrating that this particular channel was not responsible for the cardioprotective effects observed, new investigations targeting channels within the cell were launched. In 1991, the existence of mK_{ATP} channels was confirmed by patch clamping of mitoplasts from rat liver [127]. Although the exact composition of the channel is not known, **Figure 5** presents the accepted structure of the mK_{ATP}. The channel is an octameric structure composed of four inwardly-rectifying potassium channels (K_{ir}) that make-up the pore and four sulfonylurea receptor (SUR) units each containing two nucleotide binding domains (NBD) (see [128, 129] for review). The precise subtype of K_{ir} and SUR subunits composing the mK_{ATP} are not known, but because Kir 6.1 subunits seem to be more concentrated in mitochondria versus whole brain tissue, it may be a likely candidate [130, 131]. Although some authors claim that the structure of mK_{ATP} is not similar to the plasmalemmal K_{ATP} channel [132], most published investigations support the above stated structural construct.

The mK_{ATP} is a critical component of the mitochondrial K^+ cycle. As reviewed by Garlid and Paucek [133], protons (H^+) are ejected by the electron transport chain and results in the generation of an electrical membrane potential that drives K^+ from the intermembrane space (IMS) into the matrix via a K^+ leak and the mK_{ATP}. The exchange of H^+ for K^+ leads to alkalinization of the matrix, and inorganic phosphate (Pi^-) will enter with H^+ through the Pi^-/H^+ symporter. The gain of K^+ to the matrix osmotically draws water with it to result in matrix swelling. The K^+/H^+ antiporter will eject excess K^+ from the matrix in order to maintain proper matrix volume and high $\Delta\psi m$ [133]. During normal energy states, these actions help to ensure that oxidative phosphorylation occurs

without impairment. The mK_{ATP} is greatly influenced by the energy state of the mitochondria. In low or high energy states ADP or ATP presumably bind to the NBDs and result in increased or decreased K^+ influx into the matrix, respectively [122], and this results in the moderation of matrix volume and the pH gradient required to drive ATP synthesis. **Figure 6** presents a schematic of the beneficial effects of K^+ uptake into the matrix.

Pre and postconditioning

Evidence supports the assertion that mK_{ATP} activity protects the mitochondria during OGD conditions. This protective capability is a component of phenomena referred to as ischemic preconditioning (PC) and ischemic postconditioning (Post-C). Briefly, ischemic preconditioning occurs when one or more brief episodes of ischemia or introduction of a therapeutic agent known to possess preconditioning characteristics (i.e., DZ) close to the threshold of damage protects the brain from a subsequent lethal ischemic episode [134]. This phenomenon may be further subdivided into early or delayed preconditioning where the sub-lethal ischemic signal or drug administration precedes the lethal episode by minutes or days, respectively [135]. Naturally, the clinical applicability of this form of therapy is limited due to the unpredictable nature of ischemic episodes. Postconditioning occurs when an ischemic episode is introduced following injury but before the onset of perfusion in order to protect tissue from ischemic-reperfusion injury [135]. Immediate postconditioning is defined as the induction of a brief, sublethal ischemic episode or pharmacologic intervention immediately before reperfusion. Delayed postconditioning occurs when the brief ischemic episode or drug administration occurs hours or even days after the initial injury. Pre- and postconditioning were initially shown

to exist in the heart [136-138], but recent evidence has demonstrated this phenomenon in the brain as well [139-142]. It has been suggested that the mK_{ATP} is the key channel involved in both pre- and postconditioning in the brain [134, 140]. The fact that the brain contains 6 – 7 times more mK_{ATP} channels than the heart makes investigation of this possibility a logical undertaking [143]. The reader is advised to remember that although the pathological mechanisms involved with TBI are rather complicated and may involve additional factors such as shear, strain, compression, and other mechanical forces, extensive evidence demonstrates that ischemic brain conditions related to TBI have a significant effect on patient prognosis [144-146]; therefore the novel application of a drug intervention such as DZ which has been proven to be of benefit in pre-clinical animal models of ischemic brain injury may improve survival and neurological outcome following TBI.

The pathological mechanisms set in motion during ischemia and the reperfusion period ultimately may result in the opening of the PTPC and initiation of cell death mechanisms associated with it (see **Pathophysiology of TBI** section for details). During ischemic conditions, anoxic depolarization results in a significant influx of Ca^{2+} into cells that is then sequestered in mitochondria. This accumulation of Ca^{2+} increases the generation of ROS, which interferes with oxidative phosphorylation and results in a dramatic decrease in ATP production and cell death. There is now evidence that a primary mechanism of PTPC activation occurs in response to increased ROS and Ca^{2+} mediated Cyp D attachment to the ANT component of the PTPC [147]. Although the reoxygenation experienced with reperfusion will result in rescue of a large percentage of ischemic cells, the rapid correction of acidosis by the reinvigorated Na^{+}/H^{+} exchanger

will cause secondary activation of the Na^+/Ca^{2+} exchanger in reverse mode, which leads to an exacerbation of the existing cytosolic Ca^{2+} accumulation [148]. Additionally, in the presence of O_2 the previously ischemia-inhibited respiratory chain will establish a $\Delta\psi m$ and synthesize ATP, but also lead to an excessive level of Ca^2+ in the matrix with a resultant increase in ROS [148].

The precise mechanisms involved in ischemic and DZ mediated PC and Post-C are not fully illuminated, but multiple mechanisms of action have been revealed. Initial investigation into the PC and Post-C abilities of DZ were conducted using ischemic scenarios in the myocardium, however the findings have transferred to CNS tissue as well. In an ischemic state, the ratio of AMP and ADP to ATP increases and induces the mK_{ATP} to open and allow K^+ to enter the mitochondrial matrix. This sudden increase in K^+ stimulates the K^+/H^+ exchanger which uncouples the mitochondrion because it allows H^+ to bypass the ATP synthase (F1 apparatus) [149]. As a result, mitochondrial energy consumption is temporarily reduced, and MMP is delayed or does not occur. Additionally, opening of the mK_{ATP} maintains mitochondrial matrix volume and decreases the $\Delta\psi m$ and K^+ influx, which allows for the normal state of low membrane permeability to ADP and ATP, thus limiting ATP hydrolysis during ischemic states [150]. Likewise, the decreased $\Delta\psi m$ may also increase the rate of the electron transport chain and increase ATP production [151]. The maintenance of $\Delta\psi m$ and inhibition of ATP hydrolysis may also quell mitochondrial Ca^{2+} uptake [100]. The concomitant matrix alkalization that occurs with mK_{ATP} opening induces superoxide generation by complex I (NADH: ubiquinone oxidoreductase) [152]. Dismutation of superoxide by superoxide dismutases (SODs) generates ROS. These reactive oxygen species then proceed to

activate protective kinases such as protein kinase G (PKG) and C (PKC), which prevent the opening of the PTPC and even enhance further activation of the mK_{ATP} [153]. In fact, strong evidence exists that mK_{ATP} induced ROS generation activates PI3K, Akt, and PKC species that act as antagonists of pro-death mediators such as p53, Bad, the apoptotic initiator Caspase-9, and Gsk3β [154]. It should be noted that the level of ROS is a finely tuned and dangerous balancing act because with prolonged ischemia, ROS generation is massively increased which, along with excessive H^+ influx into the matrix and loss of $\Delta\psi m$, results in mitochondrial degradation, membrane rupture and release of cyt *c* and other harmful intermembrane proteins [155]. Although one of the ostensible protective mechanisms of action of mK_{ATP} channel opening is the generation of ROS, conflicting evidence demonstrates that activation of mK_{ATP} reduces ROS generation *in vitro* [155]. Importantly, in addition to an ability to mediate ROS generation induced by mK_{ATP} channel opening, it was seen that high doses of DZ (750 μM solution) administered to *in vitro* rat primary hippocampal neurons exposed to OGD stress, led to an increase in the generation of protective levels of ROS via inhibition of succinate dehydrogenase (SDH: complex II of electron transport chain) [104]. As molecular techniques improve, perhaps additional information regarding protective mechanisms of action in addition to mK_{ATP} activity will be revealed.

Studies demonstrating the protection that DZ confers in preconditioning paradigms are abundant. Pretreatment of primary neuron cell cultures with DZ before glutamate excitotoxicity [107, 156], OGD conditions [104, 157], and hypoxia-ischemia-reperfusion conditions [158] demonstrate strong antiapoptotic effects. Pretreatment of rodents with DZ before focal or global ischemia reduced lesion size [113], decreased

apoptosis [159, 160], attenuated expression of markers of inflammation [102, 115], and improved performance on memory tasks compared to vehicle treated ischemic animals [115]. Although there are limited human neuroprotective investigations evaluating DZ preconditioning effects, one study with particular relevance to the endothelial dysfunction that occurs following TBI was discovered [105]. Healthy volunteers were exposed to forearm ischemia-reperfusion injury via extended blood pressure cuff occlusion of circulation. Pretreatment with DZ (800 μg/min for 20 minutes prior to the 20 minute ischemic period) preserved endothelial function during the reperfusion period as evidenced by a robust vasodilation response to acetylcholine infusion. This response was absent in untreated ischemic limbs and was blocked in DZ subjects by an infusion of the mK_{ATP} antagonist glibenclamide. Preservation of hypercapnia-induced arteriolar vasodilation was also observed in piglets that were administered DZ prior to global cerebral ischemia while pigs administered vehicle had an approximately 70% loss of response to CO_2 [161]. Considering the CBF abnormalities and endothelial and BBB dysfunction observed following TBI, the preceding two studies provide incentive to explore the effects of DZ administration on endothelial function and BBB integrity in the setting of TBI.

Although not as numerous as preconditioning studies, the postconditioning effects of DZ has received some attention. For example, a five-day course of DZ dissolved in dimethyl sulphoxide (DMSO) (5 mg/kg), administered following permanent bilateral common carotid occlusion in rats, decreased long term microglial activation and restored hippocampal-related learning tasks evaluated three months following injury [106]. Adamczyk , et al. [140] revealed that rats exposed to one hour of cerebral ischemia

followed by 24 hours or 7 days of reperfusion experienced improved motor function and decreased cerebral infarct size when a single DZ dose (10 mg/kg) was administered 30-minutes before the onset of reperfusion. Domoki , et al. [99] exposed piglets to 10 minutes of global ischemia, administered intravenous DZ (3 mg/kg) at the conclusion of the ischemic period, and then subjected the pigs to 15 minutes of reperfusion. At the conclusion of the reperfusion, the animals were sacrificed. Using oxalate-pyroantimonate electron cytochemistry combined with quantitative morphometry to examine changes in the mitochondrial structure and total intracellular Ca^{2+} levels in CA1 hippocampal neurons. These neurons displayed a lack of mitochondrial swelling and Ca^{2+} accumulation comparable to non-infarct animals. This effect was blocked by 5-hydroxy-decanoate (5HD), a selective antagonist of the mK_{ATP}. Neurons in the hippocampus are extremely susceptible to ischemic injury and also have been shown to display a high concentration of SUR1 labeling [128]. This serves as evidence that these neurons likely contain high concentrations of mK_{ATP} channels and that robust protection is afforded when they are open. Finally, Robin , et al. [135] administered a single intraperitoneal (i.p.) dose of DZ (10 mg/kg) 20 minutes before the onset of reperfusion following one hour of cerebral ischemia in rats to evaluate effects on lesion size, behavioral, and mitochondrial function 24-hours following injury. Infarct size was reduced, neuro-score was improved, $\Delta\psi m$ was maintained, and mitochondrial Ca^{2+} retention was decreased relative to vehicle treated ischemic animals.

Although the initiating event of TBI is significantly dissimilar to an initiating ischemic episode, there is much similarity in the pathophysiological events that follow. For example, metabolic and ionic perturbations ($\downarrow$ ATP, $\uparrow$ lactate, Ca^{2+}, Na^{+}, and K^{+}

fluctuations), cytokine/chemokine release, vascular flow and BBB abnormalities, vasogenic and cytotoxic edema, inflammation, apoptosis, cytoskeletal/axonal damage, and behavioral abnormalities are observed in both scenarios [162]. In the ischemic setting, DZ has been shown to possess remarkable effectiveness in preventing or decreasing the severity of many of the perturbations listed. The next logical step is to evaluate its effectiveness in treating TBI pathology via evaluating the effect of pre- and post-TBI DZ administration on behavior and novel and traditional markers of injury and recovery following TBI.

Behavioral Outcomes and Biological Markers of Interest

Behavioral Outcomes

Chapter 2 presents our findings regarding the effect of post-injury administration of DZ and its vehicle DMSO on short and long-term behavioral and motor disorders following moderate-TBI in the mouse.

Cognitive, motor, and behavioral impairments following TBI often lead to long-term functional impairment and a high incidence of psychiatric disorders [163]. In fact, disorders such as depression, anxiety, and changes in personality (aggression, disinhibition, and emotional lability) are ranked among the most distressing sequelae of TBI (see [164] for review of psychiatric disorders associated with TBI). Although mild and moderate concussive TBI (patients presenting with a Glasgow Coma Scale of 9 or greater), which accounts for 78% of all reported TBI [49], generally is marked by successful recovery, an average of 34 % may experience some of the above mentioned behavioral symptoms [164].

Constructing a link between injury severity and behavioral and cognitive outcomes has proven to be difficult. Washington, et al. [165] exposed mice to sham, mild, moderate, and severe CCI (injury depth 0, 1.5, 2.0, or 2.5mm, respectively) and then evaluated if emotional and cognitive impairments increased as a function of injury severity. Cognitive impairments, as assessed by the MWM, worsened with increased injury severity, but emotional disturbances as measured by the forced swim test and prepulse inhibition of acoustic startle did not worsen with increased injury severity. The fact that mild and severe injury animals displayed comparable behavioral deficits led the authors to postulate that the threshold for emotional difficulties following TBI is low. Clouding the picture further are recent results published by authors investigating the behavioral and emotional effects of a closed head minor TBI model that produced no skull fracture, BBB damage, or other noted structural damage to the brain of mice [166]. Following injury all motor, pain threshold, and anxiety tests were normal. However, an interesting pattern was revealed on learning integration tasks (MWM, passive avoidance, and swim T-maze). The learning deficits noted on these tasks did not begin until 30 days post injury and extended to the 90 day end point of the study. As suggested by the authors, this result illustrates that even in very subtle brain injury, a delayed and long term apoptotic and secondary injury process is occurring in the brain regions that are responsible for memory acquisition and processing, namely the hippocampus and cortex. Other studies support this finding [167-169]. Specifically, levels of AIF and cyt *c* in the ipsilateral hippocampus were increased at 24 and 72 hours, but amyloid precursor protein (APP), a protein that is considered a link between TBI and Alzheimer's Disease-like pathology [170] , levels were not affected by injury. In all, these studies indicate that

behavioral and memory outcomes following TBI are extremely sensitive indicators of subtle pathology that, if apoptosis is a primary factor, may be amenable to mK_{ATP} treatment. Finally, please refer to [11] for an outstanding review of the literature regarding brain region dysfunction and the relationships between regional abnormalities and cognitive or behavioral deficits.

Novel Marker of Injury and Recovery

Chapter 3 presents our findings regarding the short and long-term changes in expression of neuronal cell adhesion molecules carrying the linear homopolymer of α2,8-linked sialic acid (PSA-NCAM) expression following graded-CCI in the mouse.

A large number of accepted blood and cerebrospinal fluid (CSF) biomarkers of brain injury have been identified in animal models and human subjects using conventional and novel methods (see [45, 171] for review), however classification of alterations in PSA-NCAM levels following TBI has yet to be investigated. To understand the possible effects of altered PSA-NCAM expression and function following TBI, an appreciation of the identified functions of the molecule is necessary. The importance of PSA-NCAM during ontogeny is undisputed; nevertheless, the more subtle mechanisms of function and interactions with other molecules have only recently been appreciated. The actions of PSA-NCAM and NCAM in general can broadly be partitioned into neuronal development, synaptic formation and maintenance, and regeneration via cell adhesion, intracellular signaling, and participation in cytoskeletal dynamics [172]. Although not an exhaustive survey, this section illustrates the importance of PSA-NCAM function.

Of the 20-30 forms of NCAM that can be generated by alternative splicing and posttranslational modification of the NCAM gene, the 180, 140, and 120 kDa isoforms

are expressed throughout the CNS [173]. These NCAMs have an identical extracellular N-terminal structure comprised of five immunoglobulin (Ig 1-5) and two fibronectin type III (FN 1-2) domains [174]. NCAM 140 and 180 differ in the composition of the C-terminal intracellular portion, and NCAM 120 lacks a cytoplasmic component, and PSA is attached to the fifth immunoglobulin domain [172]. Regarding neuronal and synaptic development and maintenance, it is understood that the molecule is associated with developing synapses in an activity dependent manner, but it is neither required nor sufficient for synapse formation as evidenced by the fact that knockout of all three isoforms of NCAM is nonlethal and produces viable mice [175]. However, upon closer investigation these animals display decreased olfactory bulb size [176], altered hippocampal cytoarchitecture [177], increased inter-male aggression [178], and synaptic abnormalities including atypical mossy fiber sprouting and impaired long-term potentiation and depression (LTP/D) in the CA1 region of the hippocampus [179], synaptic vesicle defects with generalized impaired synaptic plasticity [180], and poor performance on memory tasks when compared to wild type peers [180, 181]. The dynamic and acute effects that NCAMs have on synaptic maintenance is demonstrated by the substantial reduction of LTP/D in rat hippocampus slices following NCAM antibody application [182].

The mechanisms responsible for the acute and long-term effects of alterations in NCAM and PSA-NCAM expression and function may be understood by examining the signaling cascades initiated by NCAM hetero or homophilic activation on neurons and the neighboring neuroglia, particularly astrocytes whose distal processes encapsulate the synapse. Heterophilic partners of NCAM include: *cis* binding to the Ig superfamily single

pass transmembrane adhesion molecule L1 or the fibroblast growth factor receptor (FGFR) [183], glial cell line-derived neurotrophic factor (GDNF) and GFRα receptors [184], TAG-1/axonin-1 [185], and a variety of extracellular matrix- associated molecules such as heparin and chondroitin sulfate proteoglycans (HSPG/CSPG) or agrin and neurocan [186]. The known intracellular protein ligands understood to directly bind to NCAM include β1 spectrin, receptor protein tyrosine phosphatase α (RPTPα), leucine-rich acidic nuclear protein (LANP), phospholipase C-gamma (PLCγ), and myogenesis-related and NCAM-associated protein (MyoNAP) [187]. The majority of these interactions probably take place in lipid rafts that serve as organizers for signaling complexes [172]. All three isoforms of NCAM interact with varying affinity to the ubiquitous cytoskeletal organizer, spectrin [172]. One predominate NCAM 140 and NCAM 180 association appears to be dependent on NCAM-mediated FGFR activation. FGFR activation creates spectrin-linked NCAM-RPTPα and NCAM-protein kinase C β_2 ($PKC\beta_2$) complexes that result in neurite outgrowth and pre- or postsynaptic complex reorganization that most likely occurs via Fyn/FAK-initiated activation of MAPK [188, 189]. Additionally, NCAM-spectrin-NMDA receptor complexes appear to be required in the postsynaptic density for initiation of NMDA receptor-dependent LTP at excitatory synapses [187]. Other spectrin mediated interactions include: NCAM-180-spectrin-growth associated protein 43 (GAP 43) complex signaling [190] and NCAM–spectrin–α-actin or β-actin complexes [191]. Calcium-dependent association of NCAM with calmodulin-dependent protein kinase IIα (CaMKIIα) and RPTPα also produces neurite outgrowth via CaMKIIα-mediated phosphorylation of RPTPα [192]. Other methods by which NCAM-180 and 140 contribute to the cytoskeletal mobility and flexibility required

for neurite outgrowth, synapse formation, and/or synaptic plasticity include the linkage to multiple isoforms of scaffolding microtubule-associated protein (MAP) via LANP [193], arachidonic acid (AA) mediated Ca^{2+} influx following NCAM - FGFR mediated activation of PLCγ [194], and association with α-/β-tubulin and brain spectrin [191, 195]. Molecules involved in disparate mechanisms such as growth cone formation in early neuronal development (MyoNap and turned on after division-64 (TOAD-64)) or clathrin-mediated endocytosis of synaptic vesicles (Syndapin) have also been shown to associate with NCAM either directly or via linker molecules [187, 196]. Allowing for the expansion in molecular techniques used to dissect signaling pathways, it is not unreasonable to expect identification of an even greater number of molecules as binding partners and members of NCAM affiliated signaling cascades that are essential from embryonic age to senescence. Please refer to **Figure 7** in Chapter 3 for an overview of the accepted signaling pathways of NCAM.

Having a general understanding of the signaling mechanisms and interaction partners of NCAM in general allows for an appreciation of PSA-NCAM. Heavily expressed during development, a time in which it promotes the separation and migration of cells and axons, PSA-NCAM levels rapidly fall throughout the CNS and are predominantly restricted to regions of morphofunctional plasticity and neurogenesis during adulthood [197]. These regions include the olfactory bulb, hippocampus, suprachiasmic nucleus, and subventricular zone [198]. Importantly, PSA-NCAM is heavily expressed in neural precursors and is heavily re-expressed during axon regeneration [199].

Unlike with NCAM deficiency, a lack or absence of PSA-NCAM has catastrophic consequences. A lack of either one of the sialyltransferases (ST8Sia IV/PST or ST8Sia II/STX) will limit the degree of sialylation of NCAM and produce significant decreases in long- term potentiation/depression, axon guidance, and synapse formation in the hippocampus [197, 200, 201]. The absence of both sialytransferases in mice is a lethal defect that results in death within a four week postnatal period [202]. It is believed that the lack of PSA on NCAM allows for unregulated and excessive NCAM hemophilic and heterophilic binding [172], prevents efficient pro-survival NCAM-mediated FGFR activation [203], PDGF [204], and BDNF [205] signaling in neurons.

How may acute or long term alterations in PSA-NCAM levels affect neuropathology following TBI? In addition to the well-known effects of PSA-NCAM on synaptic health, ample evidence exists that demonstrates PSA-NCAM's pro survival capabilities. For example, PSA-NCAM has been shown to reduce glutamate-induced excitotoxic death *in vitro* via inhibition of NR2B subunit containing NMDA receptors [206]. On the other hand, the lack of PSA-NCAM has also been shown to increase the rate of apoptosis in dissociated neuroblasts from the subventricular zone and rostral migratory stream by almost threefold [207]. Precursor cells in neurogenic zones express high levels of PSA-NCAM [208]. The presence of PSA-NCAM may allow these cells to mount a survival response to stress and then migrate and integrate into existing neuronal circuits, such as the trisynaptic circuit in the hippocampus, so that memory or behavioral function may be preserved. Additionally, the natural increase of PSA-NCAM expression on the axons of injured motor neurons has been shown to improve the interaction of the growth cone with the extracellular environment in order to increase axon targeting,

collateral sprouting, and decrease the occurrence of misprojections [209]. Recent research has demonstrated that the engineered expression of PSA-NCAM during injury promotes a permissive growth and repair environment without interfering with "normal" tissue function. For example, the engineered expression of PSA-NCAM by astrocytes [199] and grafted Schwann cells [210] has been shown to improve the prohibitive environment of the scar tissue and improve axonal growth. Naturally, much work is required in this field, but an understanding of the expression level changes of PSA-NCAM following TBI may help researches to better categorize contributing factors to functional deficiencies and perhaps target windows of therapeutic opportunity to improve recovery.

Various Markers

Chapter 5 presents our findings regarding short and long-term changes in select traditional and novel markers of injury and healing following TBI with pre and post-injury DZ treatment.

Evaluation of the effects of DZ pre- and post-injury treatment on expression levels of selected markers in the left and right hippocampus included immunoblot and limited immunohistochemical analysis of selected proteins categorized into the following groups: cytoskeletal/axonal, synaptic markers, inflammation, and pro-survival or pro-death. These markers were selected either because previous ischemic injury studies demonstrated significant effects of DZ treatment on expression levels of these proteins, because noteworthy alterations in expression levels have been associated with TBI injury, or because they are novel markers whose expression levels following TBI have not previously been examined.

Cytoskeletal/Axonal

One significant component of the pathology that follows TBI is axonal injury and the cytoskeletal dysfunction accompanying it. TAI, a pathomechanism unique to TBI [75], may be focal or diffuse, and its distribution and degree of dysfunction coincides with injury severity. In moderate or severe TBI, it may progress from early (12–24 hours) features of axolemmal swelling, cytoskeletal changes, and mitochondrial dysfunction to eventual Wallerian degeneration over months and possibly years following injury [79]. Four markers reflecting cytoskeletal and axonal pathology that were measured in this work were αII-spectrin, β-amyloid precursor protein (β-APP), microtubule associated protein-2 (MAP-2), and Tau.

αII-spectrin (280 kDa) is a cytoskeletal linker protein essential to maintaining axonal shape and is considered a reliable marker of the extent of neuronal injury and lesion size [45, 211]. Extracellular levels of Ca^{2+} are approximately 10,000 times greater than that found within the cell, so when axolemmal integrity is violated by mechanoporation. A sudden increase in intra-axonal Ca^{2+} will initiate αII-spectrin proteolysis mediated by the cysteine protease calpain [41]. Proteolysis by caspase-3 may also occur, but is related to programmed cell death [212]. Western blotting reveals an increase in the signature spectrin breakdown products (SBDPs) at 150 kDa and 120 kDa with calpain and caspase-3 mediated proteolysis, respectively. This distinction allows for investigators to not only characterize the time course of changes, but to determine if the dominant process of degradation is related to intracellular Ca^{2+} levels or apoptotic pathways, and to develop therapeutic strategies that target these mediators [2].

Prior investigations using CCI and lateral fluid percussion injury in rodents demonstrated acute (< 24-hours) increases in the hippocampal expression of 120 kDa SBDPs and a long-term (2-week) increase in 150 kDa SBDPs, and this time course was characterized by multiple peaks [41, 213]. In mice, the 14-day peak in degradation products paralleled peak neurodegeneration in the hippocampus [214]. In addition to being a marker of the general progression of post-TBI recovery, recent evidence presented by Bukalo , et al. [180] indicated that a postsynaptic spectin-NCAM-180-NMDA receptor interaction may be critical to establish normal LTP in the CA1 subfield of the hippocampus. Interfering with this interaction appeared to have detrimental effects on establishing LTP and on MWM performance mutant mice displaying this defect. Direct inhibitors of calpain (such as cyclosporinA and pituitary adenylate cyclase-activating polypeptide (PACAP)) inhibit proteolytic breakdown of spectrin [5]. The effect of DZ on spectrin breakdown following TBI has not previously been evaluated. The ability of DZ, through its action at the mK_{ATP}, to prevent or reduce MMP may not only decrease the likelihood of initiation of apoptotic pathways but may also allow the mitochondria to sequester intracellular Ca^{2+} without detrimental results. Decreased SBDPs may result.

Microtubule associated protein-2 (MAP-2) is predominately located in the perikarya and dendrites of neurons and plays a critical role in stabilizing microtubules so that they may efficiently provide a pathway for directional transport of various intracellular constituents [215]. Previous investigators have discovered interesting patterns in MAP-2 staining following CCI in rodents. Of note, a decrease in MAP-2 levels in the hippocampus following mild and moderate injury (0.5–1.0 mm impact

depth) are observed as early as 15 minutes and persist for up to 2-weeks following injury [216, 217], the loss in staining occurs only ipsilateral to the injury site and occurs even following mild injury [215, 216], decreased staining was not necessarily associated with neuronal loss [216], the rebound increase in MAP-2 staining was not believed to necessarily be an adaptive or plastic response because many of the cells with increased post-injury staining were of abnormal pathology [218]. Due to the extreme variability of MAP-2 staining within the hippocampus following TBI, and the lack of a strong correlation with behavioral changes, previous investigators were only comfortable stating that this may reflect transient neuronal dysfunction and may not indicate a commitment to cell death [216, 218]. Importantly, when therapeutic agents were given *after* post-injury, MAP-2 changes were noted still helped levels to return to near baseline levels compared to vehicle treated injured animals [217, 219]. These observations suggest an extended window of post-injury intervention may exist.

Axonal damage will result in varying degrees of impaired axonal transport, and this will result in the accumulation of proteins that are carried along the axon. One of the widely recognized markers of traumatic axonal damage is amyloid precursor protein (APP) accumulation [79]. Because it is transported bidirectionally via fast axonal transport, when disruption of the axon occurs axonal swelling/bulbs are seen within 30-minutes of injury [59]. Regarding APP in general, it is a 770 amino acid Type I transmembrane protein of unknown function; however, it is believed to participate in adhesion, neuroproliferative and neurotrophic activity, intercellular communication, and various intracellular signaling pathways [220, 221]. Different proteases cleave the molecule, and the end products may be associated with pathology or survival. Cleavage

by β-secretase (BACE 1) may either be followed by lysosomal degradation or γ-secretase cleavage of the remaining carboxyl-terminal fragment (C99) [220]. In this scenario, amyloid beta (Aβ) is generated, and this molecule may aggregate and form amyloid fibrils or exert neurotoxic effects independent of fibril formation [222]. There is some evidence linking TBI and Aβ pathology to Alzheimer's disease and other neurodegenerative disorders [170, 223]. The more common form of cleavage within the β-amyloid sequence is by α-secretase, and this results in the production of, among other things, a non-amyloidogenic soluble form of APP (sAPP) which has been linked to neurogenic and synaptic plasticity abilities [224, 225].

Although significant deposition of amyloid-β (Aβ) peptide following TBI only occurs in transgenic mice overexpressing human APP [30, 226], the appearance of APP bulbs or nodules in white fiber tracts indicates axonal disruption. Even following mTBI in the rodent, white matter tracts display varying degrees of β-APP nodules and bulbs. Specific regions are more susceptible to axonal strain, including the corpus callosum, cingulum, internal and external capsule, and dorsal thalamus [2, 80, 227, 228]. Interestingly, the appearance of acute and delayed axonal injury correlates well with noted cognitive changes but not necessarily with increased cell death, thus indicating that connectivity, not neuronal death, may be a significant contributing factor to the post-TBI phenotype.

In addition to the implications of β-APP axonal staining, changes in the overall levels of β-APP may have implications for post-injury recovery. Generally, APP mRNA increases acutely following TBI [226]. Although the overexpression of APP following TBI may increase the likelihood of Aβ plaque formation [229], it may also indicate a

restorative effort by affected neurons. Both *in vitro* and *in vivo* work has demonstrated that APP, particularly sAPP, protects neurons against oxidative and excitotoxic insults [230-232]. Additionally, APP expression may be a significant factor in the ability of progenitors in neurogenic zones to survive traumatic insult. For instance, Caille , et al. [224] demonstrated that the subventricular zone (SVZ) of mice contains a large number of sAPP binding sites on PSA-NCAM positive neuroblasts. They also established that *in vitro*, sAPP appears to participate in epidermal growth factor (EGF) mediated proliferation of these neuroblasts as evidenced by the fact that the addition or removal of sAPP from culture medium significantly decreased or increased the proliferation rate of the EGF-responsive progenitors from the SVZ. Further work characterizing the APP response to injury and its effects on recovery is needed.

Synaptic Markers

Growth-associated protein-43 (GAP-43) and post-synaptic density-95 (PSD-95) are important participants in the development, maturity, and refinement of CNS synapses. GAP-43 is a presynaptic phosphoprotein that plays a key role in guiding the growth of axons and modulating the formation of new connections via interaction with spectrin and reorganization of the cytoskeleton [233, 234]. Expression of GAP-43 has been noted to acutely decrease in the ipsilateral hippocampus of rats following TBI and may therefore be a target for pharmacologic treatment. For example, Darrah , et al. [235] noted that following CCI a single dose of phenytoin (75 mg/kg ip) given 24-hrs after injury resulted in an increased expression of GAP-43 and preserved hippocampal neurons and improved novel arm exploration in the y-maze one week following injury. Of particular interest to our work is the revelation that GAP-43 and NCAM interact extensively during neurite

outgrowth. NCAM-180 induced phosphorylation of GAP-43 results in a significant increase in neurite outgrowth *in vitro* [172, 190].

PSD-95 is an excitatory post synaptic density modular scaffolding protein of the guanylate kinase family that is involved in receptor anchoring and clustering, particularly of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-Methyl-D-aspartic acid (NMDA) receptors [236]. Significant decreases in the expression of this protein in the hippocampus for at least 24-hours following lateral fluid percussion in the rat have been noted [237]. Considering the importance of this protein in regulating the structure and plasticity of excitatory synapses, most likely via its role in the regulation of AMPA receptor trafficking [238], perturbation of the expression level of this protein following TBI may have a profound effect on synaptic integrity and behavioral function.

Inflammation

Glial fibrillary acidic protein (GFAP) is only found in the CNS as it is an intermediate filament protein found in the cytoskeleton of astrocytes. GFAP is a marker of activated astrocytes. It has generated much interest as a serum and CSF marker of TBI and a predictor of morbidity and mortality [239]. In fact, high levels of serum GFAP released from injured cells following severe TBI in humans has been shown to predict mortality with sensitivity approximately 75% when measured within 4-days of the injury [240]. Following trauma, the soma and main processes of astrocytes become hypertrophic and increase expression of GFAP and other components of the intermediate filament family such as vimentin and nestin [241]. There is no consensus regarding what level of reactive gliosis following trauma is beneficial; however it is understood that a degree of reactive gliosis may be necessary to promote adequate healing following TBI [242].

Reactive astrocytes possess several neuroprotective properties, including uptake of glutamate, protection from oxidative stress via glutathione production, degradation of Aβ peptides, reducing vasogenic edema, and stabilization of extracellular fluid and ion balance [243]. On the other hand, $GFAP^{-/-}$ astrocytes display poor migration abilities and are not able to induce blood-brain barrier properties in aortic endothelial cell cultures [244]. Severe and persistent astrogliosis results in extensive overlap of astrocyte domains and results in formation of a glial scar, which may impede neurite outgrowth, but may also serve to isolate viable tissue from the spread of inflammatory cells and toxins (see [242] for a thorough review of astrocyte biology).

Following an ischemic event, DZ administration has been shown to improve astrocyte health and GFAP expression. Rajapakse , et al. [102] discovered a delayed preconditioning effect when cultured astrocytes were treated with DZ for three days before a 12 hour exposure to OGD and H_2O_2. Untreated astrocyte cultures experienced a 76% death rate with OGD and a 40% death rate with H_2O_2 exposure while the DZ groups experienced no death. This effect was blocked with 5-hydroxydecanoate (5-HD), and the mechanisms of protection were believed to be due to mKATP activation that resulted in maintenance of $\Delta\psi m$, moderate free radical production and, PKC activation. *In vivo* evidence reveals that the response of astrocytes to OGD is a regionally dependent phenomenon. For example Frakas , et al. [110] administered DZ (0.5 mg/kg) for five consecutive days following permanent bilateral occlusion of the common carotid arteries and noted neither an increase or decrease in GFAP staining in the hippocampi in the ischemic groups or any of the DZ treated groups at 13 weeks following injury. On the other hand, Farkas, et al. [116] noted that using the same injury model but increasing the

dose of DZ to 5 mg/kg for 5 days post-injury, GFAP staining in the corpus callosum was reduced 33% at 13 weeks in both the ischemic and sham animal groups. The authors did not give specific explanations for DZ's effect on astrocyte proliferation.

Ionized calcium binding adaptor molecule 1 (Iba-1) is an EF hand calcium-binding protein that is specifically located in activated microglia/macrophages in the brain. Ischemic injury produces widespread microglial activation. Microglial activation following TBI occurs rapidly (< 24 hours), is pronounced near the core of injury, and may extend for weeks after injury [245]. As discussed in the **Pathophysiology of Traumatic Brain Injury** section of this document, activated microglia release multiple pro-inflammatory mediators including various cytokines, ROS, and matrix metalloproteinases (MMPs) and are considered to be the main initiators and propagators of the prolonged inflammatory response to CNS injury.

Regarding DZ's effect on microglial activation following injury, there is some evidence that administration of the drug reduces microglial activation in the ischemic setting. Using the 5 mg/kg dosing paradigm discussed in the GFAP section, Farkas , et al. [116] noted a significant reduction in microglial activation to control levels in the corpus callosum 13 weeks following injury. An earlier study conducted by the same authors using the same injury and dosing scheme demonstrated the ability of DZ to reduce long-term microglial activation in the hippocampus by 200–300 % compared to vehicle treated ischemic mice [106]. The authors surmised that DZ interacts with cell surface potassium channels, but they admit that the intracellular pathways involved in DZ's effect need to be investigated [116]. Existing evidence of DZ's effect on microglial activation in the ischemic arena lays the groundwork for investigation in the TBI setting.

Bcl-2 Family

Bcl-2 is an antiapoptotic protein involved in the intrinsic pathway of apoptosis, and it exerts its effect on the outer mitochondrial membrane by stabilizing membrane integrity, preventing the translocation of Bax (a proapoptotic member of the Bcl-2 family) to the outer membrane, and ultimately preventing the release of cyt *c* [169]. The beneficial and detrimental effects may be induced by interaction of Bcl-2 or Bax with the voltage-dependent anion channel of the PTPC [87]. Following TBI, the ratio of Bax to Bcl-2 is increased both immediately (< 3hrs) and long term (at least 1 week) [246], and this will eventually lead to an inability of Bcl-2 to suppress the effects of Bax on the outer membrane. Similar events occur following ischemic injury, and DZ's effect on the Bcl-2 family of proteins has been evaluated in this setting. Liu , et al.[160] administered DZ (5 mg/kg) administration 30 minutes before middle cerebral artery occlusion in mice decreased infarct size at 24 hours by 65%. They also administered DZ to primary hippocampal neuron cultures exposed to OGD and noted an increase in Bcl-2 expression, decreased Bax association with the outer membrane, decreased cyt *c* release and an almost 50% decrease in apoptotic neurons at 24 hours.

HSP-25 and 70

Heat-shock proteins (HSPs) are molecular chaperones that are upregulated following various forms of CNS injury and have the capability of protecting neurons and glia by preventing or correcting abnormal protein folding [90]. Additional protective roles related to mitochondrial function have been discovered. For example, HSP-70 has potent caspase dependent and independent anti-apoptotic effects by interfering with the ability of procaspase-9 to bind with Apaf-1, and thereby preventing assembly of a

functional apoptosome [247]. HSP-70 also prevents the release of procaspase-9 and decreases its nuclear accumulation [90, 248]. HSP-27 is believed to have antiapoptotic effects via interference with Fas/Apolipoprotein-1 (APO-1) and Fas/ Death-associated protein 6 (Daxx) signaling in the extrinsic pathway of apotosis [249, 250]. Our laboratory has previously demonstrated that DZ administered in a delayed preconditioning and postconditioning paradigm in hemorrhagic shock significantly increases the expression of HSP-27 and -70 at the 24 hour post injury collection point [114]. Evaluation of the effect of DZ pre or post injury administration on HSP expression following TBI has not been previously conducted.

Vasogenic and Cytotoxic Edema

Both vasogenic and cytotoxic edema are significant pathologies that contribute to post-TBI morbidity and mortality. As discussed above, DZ's effect on vascular function in ischemic injury has been evaluated; however its effect on vascular integrity following TBI has not been explored. Assessment of BBB integrity following CCI in the mouse has been evaluated by tracking the extravasation of various markers into the parenchyma. We have chosen to monitor the effects of pre- and post-injury DZ administration on edema formation by performing blinded reviewer evaluation of the degree of extravasation of IgG (150 kDa) in the cortex and hippocampus at 24 hours following injury because it is at this time point that acute edema is believed to peak [251]. Additionally, a modified version of the wet/dry technique of determining brain water content as decribed by Tait , et al. [252] was utilized. To better categorize the regional distribution of edema, we dissected the brain into five regions including the left and right cortices, hippocampi, and the diencephalon.

Research Goals

Traumatic brain injury is a worldwide burden. In spite of decades of research aimed at preventing or decreasing the pathology and ultimate morbidity and mortality, efforts have failed to produce a therapy that has demonstrated consistent effectiveness. Of particular importance to the progression of pathology is the immediate and late bioenergetic status of mitochondria, because it is widely acknowledged that mitochondrial dysfunction is at the heart of post-TBI neuropathological deterioration. The ability of a drug to preserve mitochondrial health, and as a result prevent or decrease the severity of pathology and ultimately promote restoration of function following TBI, would prove to be an important component of an ever-expanding pharmacologic collection of drugs demonstrating promising preclinical results. Diazoxide may be the drug that has such an effect. Additionally, identification of novel markers of injury and healing following TBI adds to the knowledge base of potential therapeutic targets and markers that can be evaluated to understand and explain the post-TBI clinical course. PSA-NCAM may be such a marker. The specific aims of this investigation were the following: (1) evaluate the effect of post moderate-CCI administration of DZ on short and long-term motor and behavioral outcomes in the mouse (Chapter 2); (2) Categorize and quantify the short- and long-term change in PSA-NCAM expression throughout the mouse brain following graded-CCI (Chapter 3); and (3) evaluate the effects of CCI and pre- or post-CCI administration of DZ on early and late expression levels of select markers of injury and recovery in the mouse hippocampus (Chapter 5).

Experimental Approach

Considering the existing evidence regarding DZ's protective effect in ischemia when given both pre and post injury, investigation into the effects of DZ, a putative mK_{ATP} channel opener, on neuropathology and recovery following TBI is warranted. **Chapter 2** presents the results of such an effort. Because behavioral, memory, and motor outcomes characterize the post-TBI phenotype and reflect the severity of underlying injury or neurological recovery, the first goal of this project was to evaluate the effect of DZ administration on both the short and long-term manifestations of these parameters. Since post injury administration of a pharmacologic intervention is a clinically applicable dosing scheme, the following plan that was adopted. An initial dose 30 minutes following injury falls within the average medical care response time both in the civilian and military settings [253], and a subsequent 3-day dosing scheme allows for maximal brain penetration because this is well within the window of BBB violation. **Figure 7** illustrates the dosing scheme and the motor, behavioral, and memory evaluation schedules that were instituted. Moderate CCI was utilized because mild and moderate TBI account for the majority of injuries experienced in humans and because this degree of injury has been shown to result in long term behavioral and memory disorders in humans and in animal models [165]. Since many defects in memory and emotional function have been linked to neuropathology in the hippocampus, the expression of select biomarkers in this brain region was evaluated using western blotting. This information is presented in **Chapter 5**.

Additionally, inclusion of a detailed evaluation of the changes in expression of a possible novel marker of injury or healing (PSA-NCAM) was performed. Even though many of the functions of PSA-NCAM have yet to be revealed, it appears to be a critical

component of migration, axon guidance, and cell survival. The second goal of this project was to evaluate PSA-NCAM expression level changes throughout the brain following graded-CCI, and these results are presented in **Chapter 3**. Western blotting and immunohistochemistry were used to assemble this data. **Figure 8** presents an overview of the experimental plan executed to accomplish this goal. Identification of the time course, degree, and regional distribution of change may eventually be used to identify therapeutic windows, vulnerable periods, and clarify PSA-NCAM's role in post-TBI pathology and recovery.

Chapter 5 serves as an appendix presenting various data collected regarding the effects of DZ administration on the short- and long-term expression of various biomarkers following TBI. For example, a comparison of the results from the administration of DZ given before or following TBI on brain edema at 24 hours was evaluated. Evaluation of the effects of these single DZ doses on the expression of various biological markers in the hippocampi at 24 hours using immunoblotting is presented. Regarding the long term outcomes of post-TBI DZ administration, detailed in Chapter 2, DZ's effect on lesion volume at three weeks is presented. Finally, immunoblotting was used to evaluate the expression levels of various markers in the hippocampi at three weeks post-injury.

In all, the following **hypotheses** were tested:

1.) Compared to vehicle treated animals, post moderate-CCI administration of DZ will result in improved short-and long-term motor, memory, and behavioral outcomes. Additionally, post injury DZ administration will have a beneficial effect on the expression levels of biomarkers representing a neuroprotective

role while decreasing protein levels of biomarkers indicative of neuropathology.

2.) Graded-CCI will result in widespread short and long-term effects on the expression levels of PSA-NCAM throughout the brain.

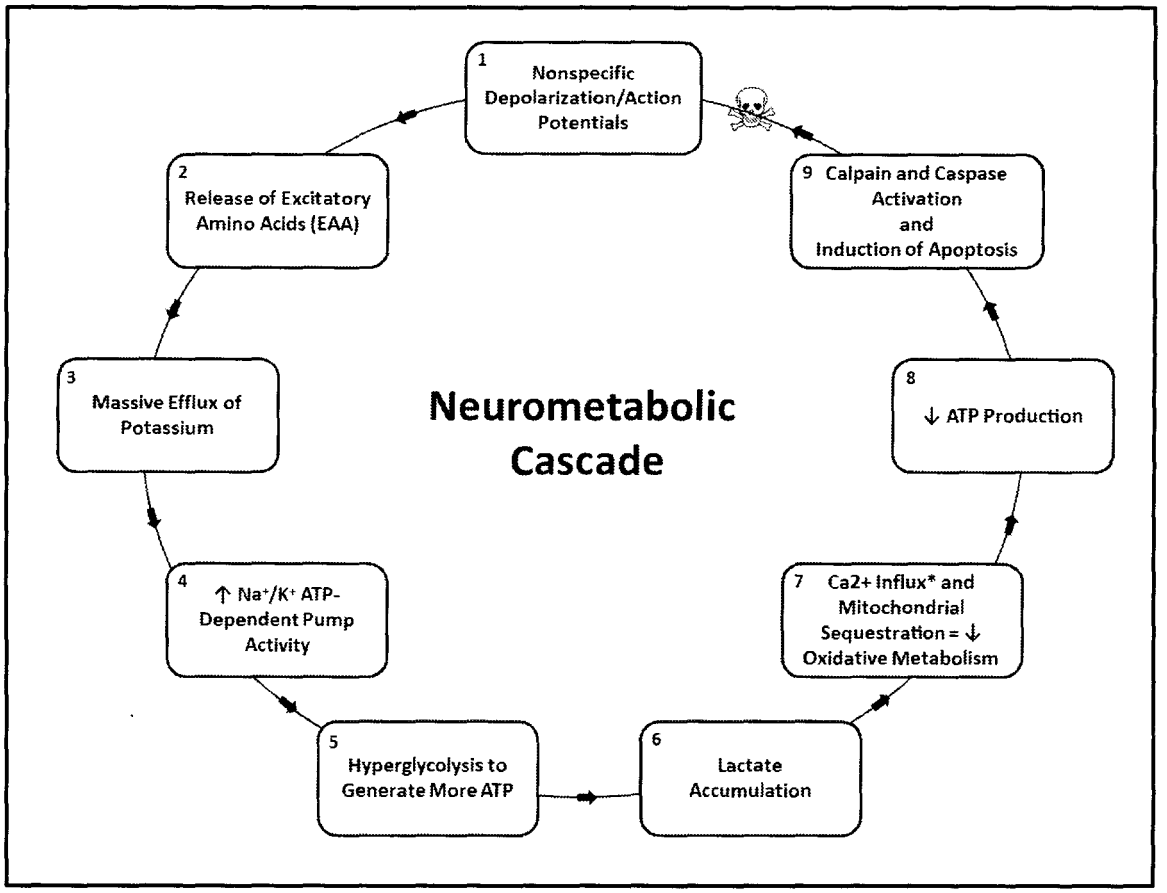

Figure 1. 'Neurometabolic Cascade" following TBI.

Flow-chart depiction of pathologic metabolic processes that occur pursuant to a primary TBI event. Beginning at the 12 o'clock position and moving counter-clockwise: (1) Stress on neuronal membranes initiates unregulated ion flux that depolarizes the membrane and produces action potentials; (2) Release of neurotransmitters (particularly excitatory amino acids such as glutamate) occurs on a massive scale; (3) Considerable efflux of potassium ions occurs; (4) Na^+/K^+ ATP-dependent pumps function at maximum capacity in an attempt to maintain ionic balance, thus depleting energy stores; (5) Accelerated glycolysis occurs in an attempt to meet the increased energy requirement; (6) Lactate production increases as a result of amplified glycolysis and decreased lactate metabolism; (7) Significant influx of Ca^{2+} through N-methyl-D-aspartate receptors (NMDRs) results in intracellular accumulation and mitochondrial sequestration of Ca^{2+} which interferes with oxidative metabolism; (8) Decreased ATP production results in energy

failure; (9) increased Ca^{2+} levels and oxidative stress cause cytosolic spillage of multiple mitochondrial intermembrane space (IMS) proteins that initiate cell death via several catabolic processes (i.e., calpains (non-caspase cysteine proteases) and caspases (cysteine-dependent aspartate-directed proteases) [254]. Note: * also designates Ca^{2+} influx into the axon due to disruption of the axolemma that occurs with traumatic axonal injury. This influx results in neurofilament compaction by phosphorylation or calpain mediated sidearm proteolysis. Microtubule disassembly limits transport of organelles. If injury is significant enough, then axonal swelling and eventual secondary axotomy will result. Figure is modified recreation of figures contained in [30] and [27].

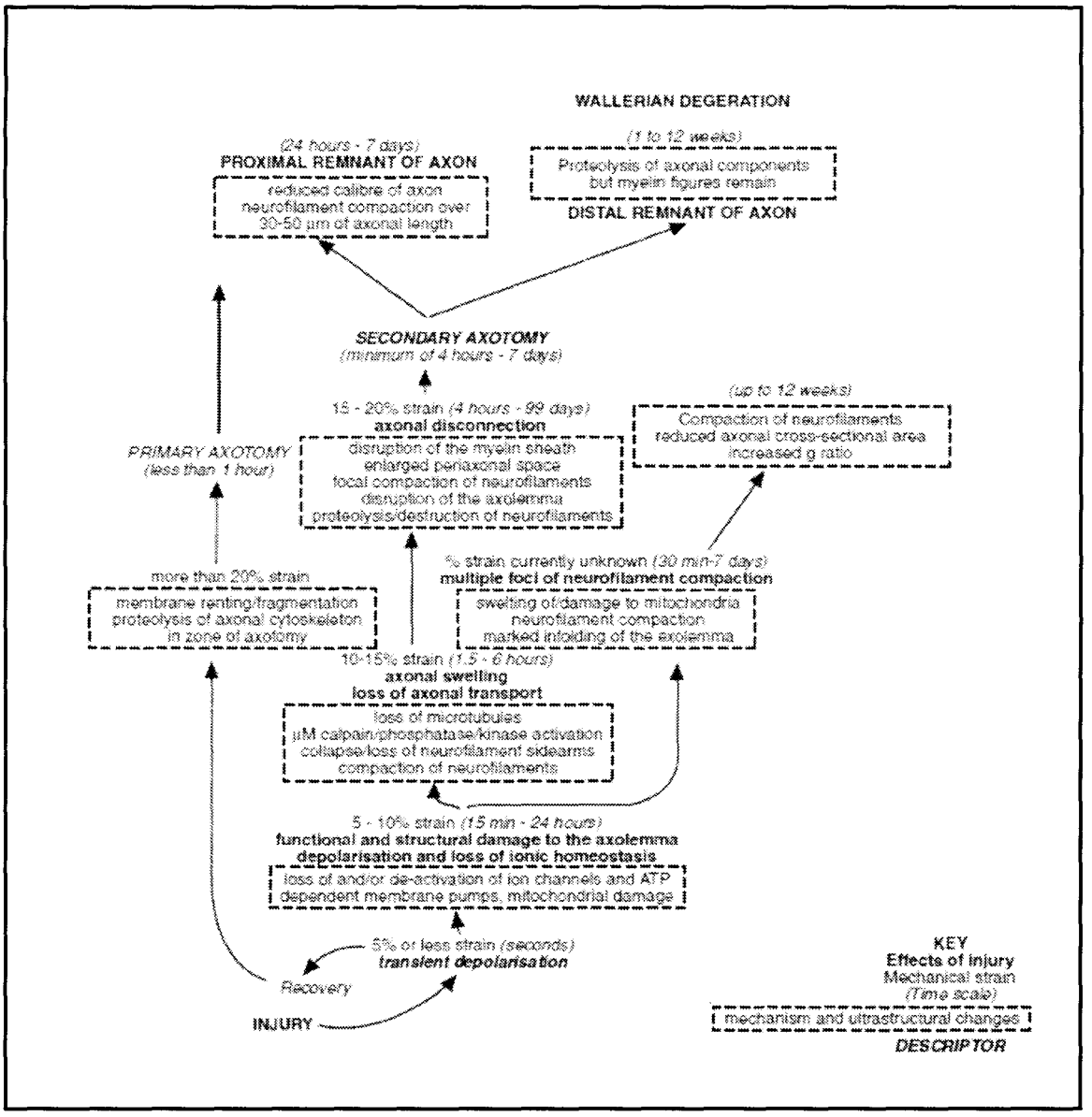

Figure 2. Time scale and processes involved in axonal injury following TBI.

The cartoon depicts the effects of varying degrees of strain energy on axon function and structural alterations and when these effects can be seen. Key located in lower right corner: Descriptor (i.e., injury, secondary axotomy); Broken line box lists mechanism of pathology and ultrastructural changes observed; % strain refers to the percent elongation; Effects of injury are listed in bold type near strain and time scale. Modified by author from [41], with permission.

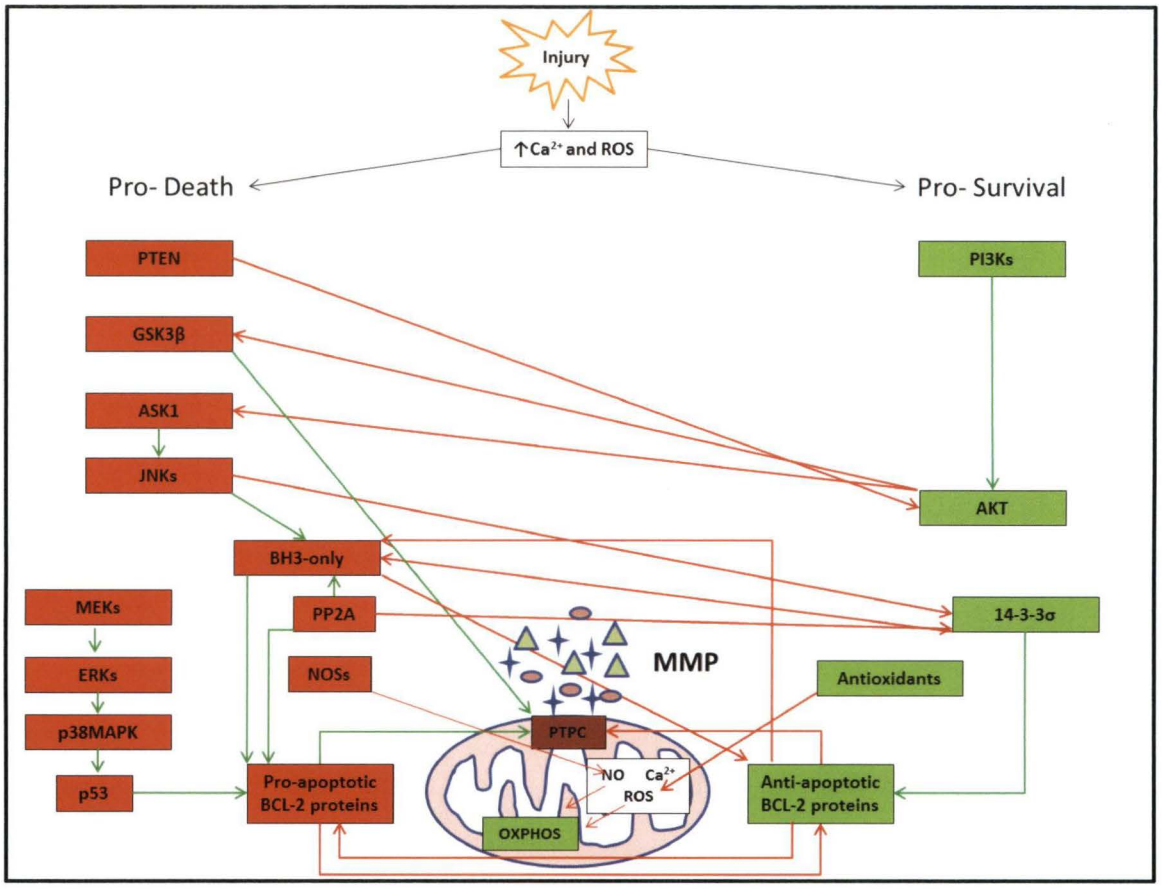

Figure 3. Signaling events that regulate mitochondrial membrane permeabilization (MMP).

Following mechanical or ischemic injury, neurons react with an increased production of reactive oxygen species (ROS) and a massive increase in cytosolic Ca^{2+}. Pro-survival and pro-death signaling cascades are activated. If pro-death signaling predominates, then MMP will result in mitochondrial transmembrane potential dissipation, uncoupling of the repiratory chain, production of ROS, and leakage of mitochondrial intermembrane space proteins (i.e., cyt *c* and AIF) into the cytoplasm. Legend: Red boxes = recognized instigators of MMP event; Green boxes = pro-survival factors that inhibit initiators of death and MMP; Red arrows = inhibitory/blocking effect; Green arrow = cooperative effect. Abbreviations: AKT, also called protein kinase B ; ASK1, apoptosis signal regulating kinase 1; BH3, BCL-2 homology domain 3; ERKs, extracellular signal-regulated kinase family; GSK3β glycogen synthase kinase 3β; JNKs, Jun N-terminal kinases; MEKs, mitogen-activated protein kinase (MAPK) family; NO, nitric

oxide; NOSs, NO synthases; OXPHOS, oxidative phosphorylation complexes; p38MAPK, p38 mitogen-activated protein kinase; p53, tumor protein 53; PI3Ks, Phosphatidylinositol 3-kinases; PP2A, protein phosphatase 2A; PTEN, phosphatase and tensin homologue deleted on chromosome 10; PTPC, permeability transition pore complex. Modified representation of graphic found in [90].

Mitochondrial Protein	Result of Protein Release
Apoptosis Inducing Factor (AIF)	• Translocates from cytosol to nuclear compartment producing chromatin condensation and DNA fragmentation [94].
Cytochrome c (*Cyt c*)	• Release from respiratory chain results in O_2 and H_2O_2 bursts and impaired energy production [255]. • Interacts with adaptor protein apoptotic peptidase activating factor 1 (Apaf1)Apaf-1 and procaspase 9 to assemble the apoptosome, which activates caspase 9 and other executioner caspases [90, 95]. • Increases cytosolic Ca^{2+} by translocating to endoplasmic reticulum and enhancing inositol (1,4,5) trisphosphate receptor (InsP(3)R)-mediated calcium release [256].
Endonuclease G (Endo G)	• Translocates from cytosol to nuclear compartment producing caspase-independent DNA fragmentation [94].
Heat-shock proteins (HSPs)	• Removal from mitochondrial matrix leads to poor structural integrity of mitochondria and the impairment of ATP generation [95]. • Removal from mitochondrial matrix leads to increased cyt *c* release and caspase-3 activity [257].
Omi stress-regulated endoprotease/high temperature requirement protein A 2 (Omi/HtrA2)	• Binds and cleaves inhibitor of apotosis proteins (IAPs) and results in enhanced caspase activation and caspase-dependent and independent mechanisms of cell death [95, 258, 259].
Pro-caspases	• Kept inactive in inner mitochondrial space via S-nitrosylation and are released for proteolytic processing in the cytosol [95].
Second direct activator of caspases/direct IAP-binding protein with a low pI. (Smac/DIABLO)	• Indirectly increases caspase activation by binding to inhibitor of apoptosis proteins [260].

Table 1. Effects of mitochondrial proteins on cell death mechanisms.

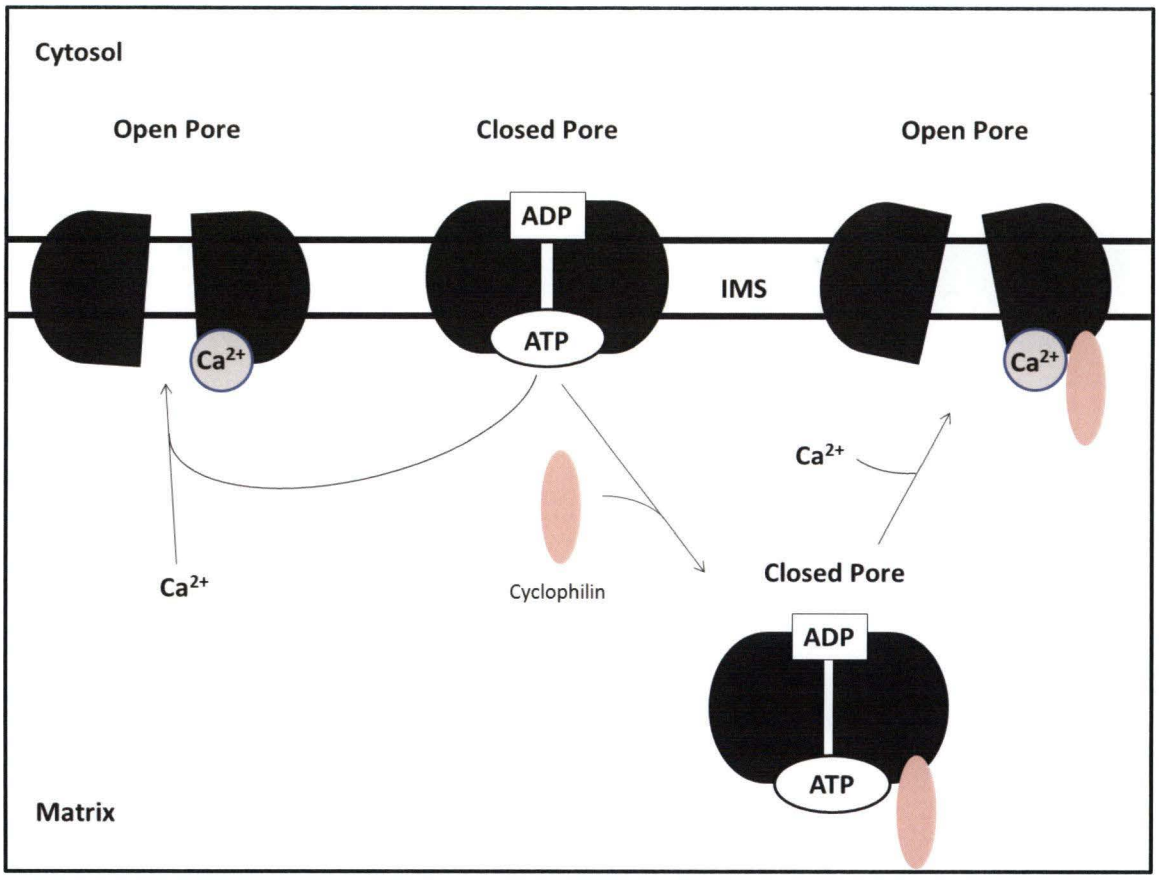

Figure 4. Mechanism of action of the PTPC.

Increased Ca^{2+} in the mitochondrial matrix will trigger PTPC opening (left). Adenine nucleotide binding to the adenine nucleotide translocase (ANT) is enhanced by $\Delta\psi m$ and consequently desensitizes the PTPC to Ca^{2+} induced opening (middle). Cyclophilin D binding to the ANT is enhanced by oxidative stress and induces conformational changes that sensitize the PTPC to Ca^{2+}. Adapted from [261] with permission.

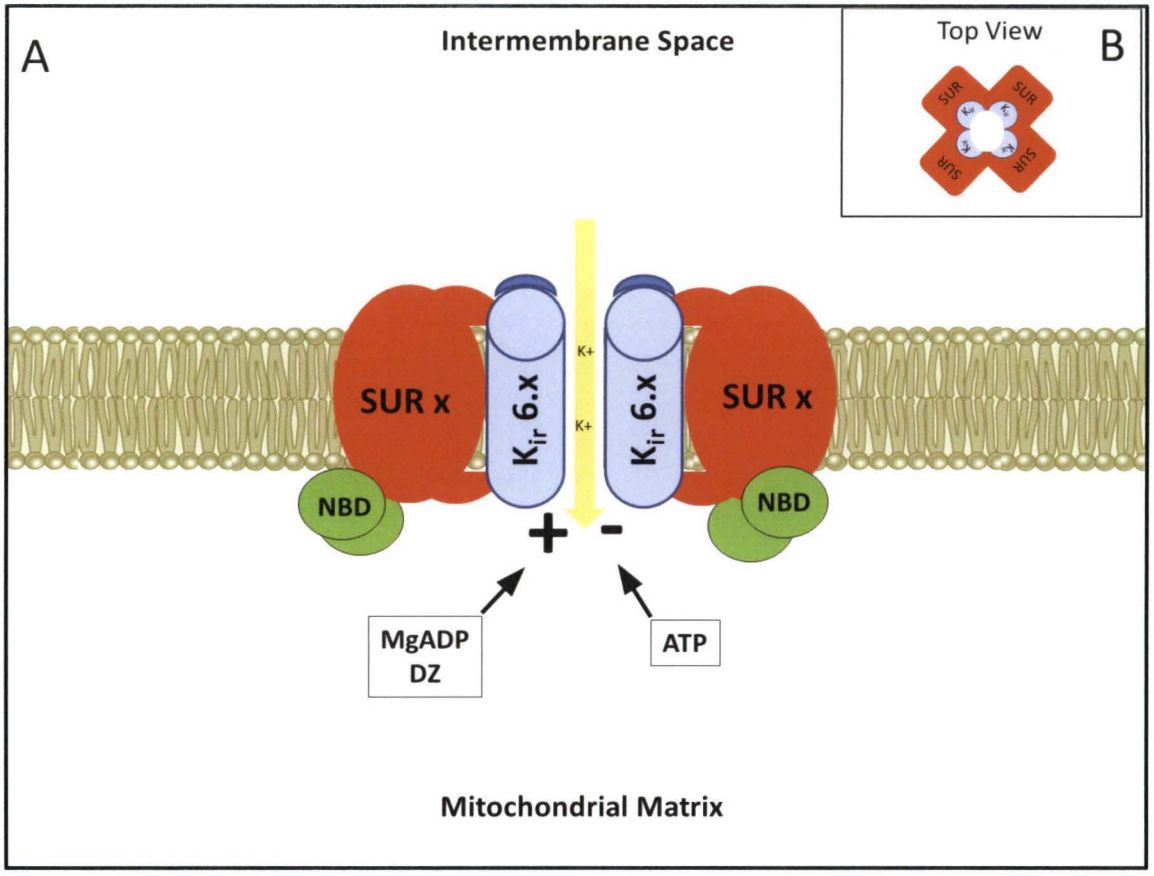

Figure 5. The mitochondrial ATP-sensitive (mK_{ATP}) channel.

The mK_{ATP} channel possesses different functional and pharmacological properties than sarcolemmal K_{ATP} channels. (A) The mK_{ATP} is believed to be an octameric structure composed of four pore-forming inward-rectifying subunits (K_{ir} 6.1 or 6.2) and four sulfonylurea receptor (SUR) subunits that interact with the K_{ir} units. Each SUR subunit also possesses two nucleotide binding domain (NBD). Low energy states activate the channel via binding of MgADP to the NBD. High energy states lead to closure of the channel via interaction of ATP with the K_{ir}. (B) Top view demonstrating the octameric structure of the mK_{ATP}.

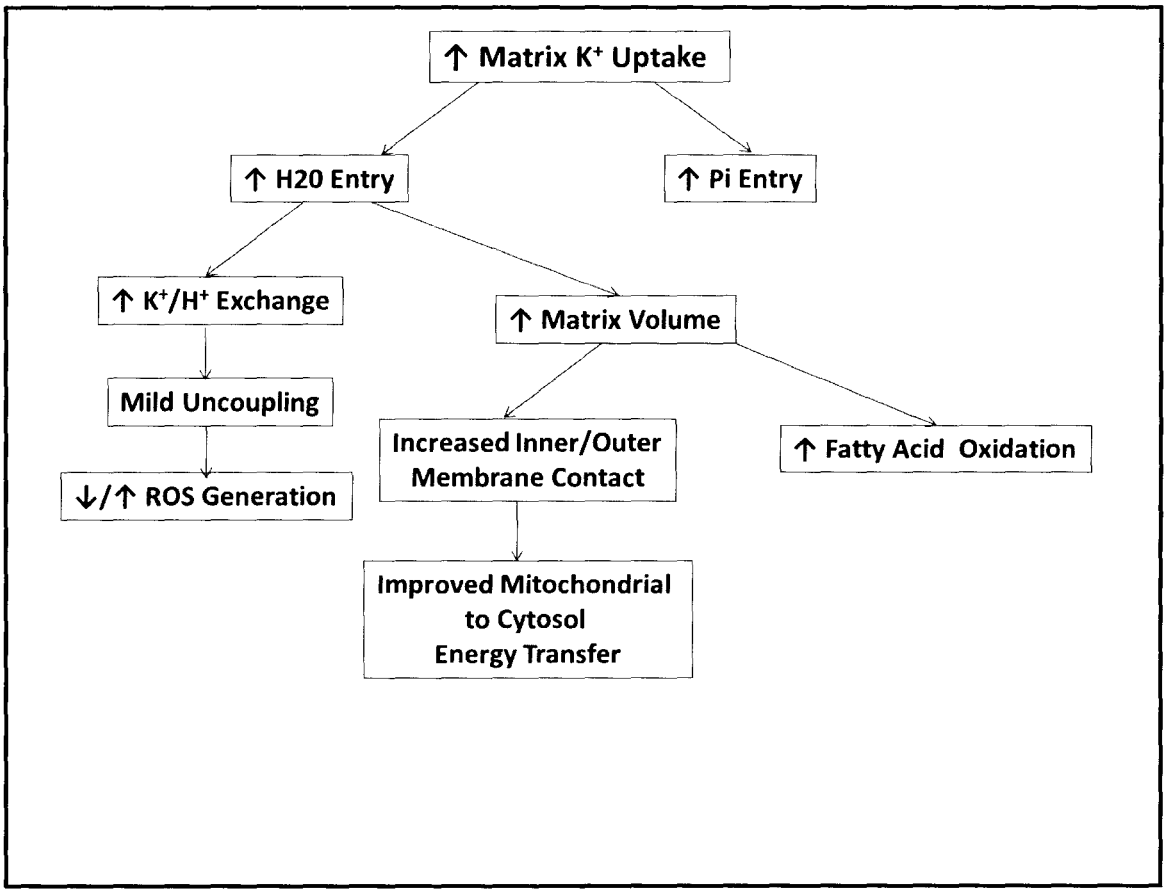

Figure 6. Effects of increased matrix K^+ uptake.

Schematic of the effects of K^+ influx into the mitochondrial matrix. Note that ROS generation effects appear to be influenced by mitochondrial bioenergetics state. Modified from [262].

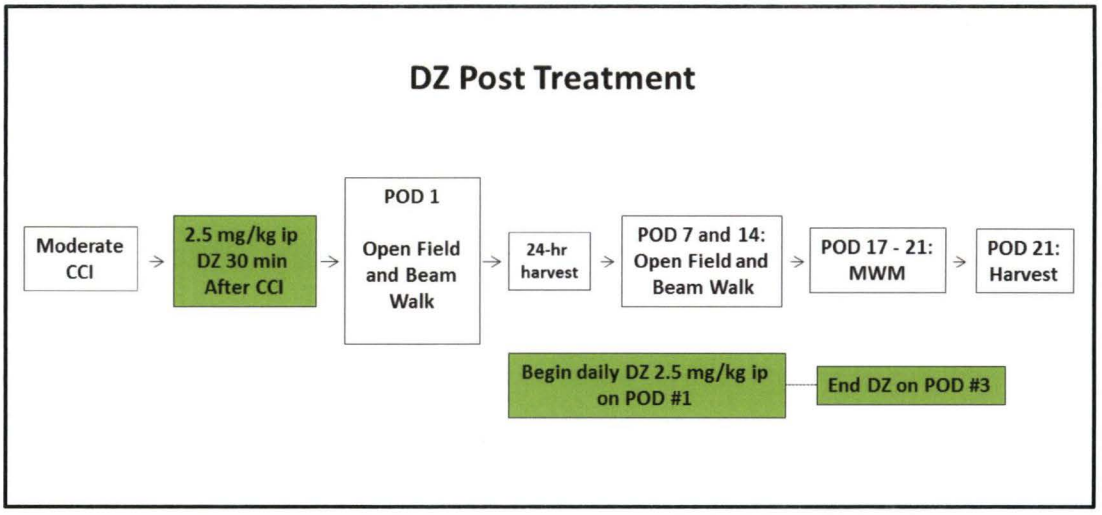

Figure 7. Overview of the behavioral testing schedule after CCI and DZ treatment.

Moderate CCI was defined as a 1mm impact depth at 5 m/sec with a 0.1 sec dwell time using a 3 mm impactor tip positioned over the left parietal cortex. Animals were harvested on post-operative day one (POD 1) to assess for acute motor, memory, behavioral, and select biomarker alterations. Animals assessed at three weeks received a 3-day course of DZ and underwent motor and behavioral evaluations on POD 7 and 14 and memory evaluation on POD 17-21. Additionally, long term changes of select markers in the hippocampi were evaluated using immunoblotting following sacrifice on POD 21. Ip, intra peritoneal; MWM, Morris water maze.

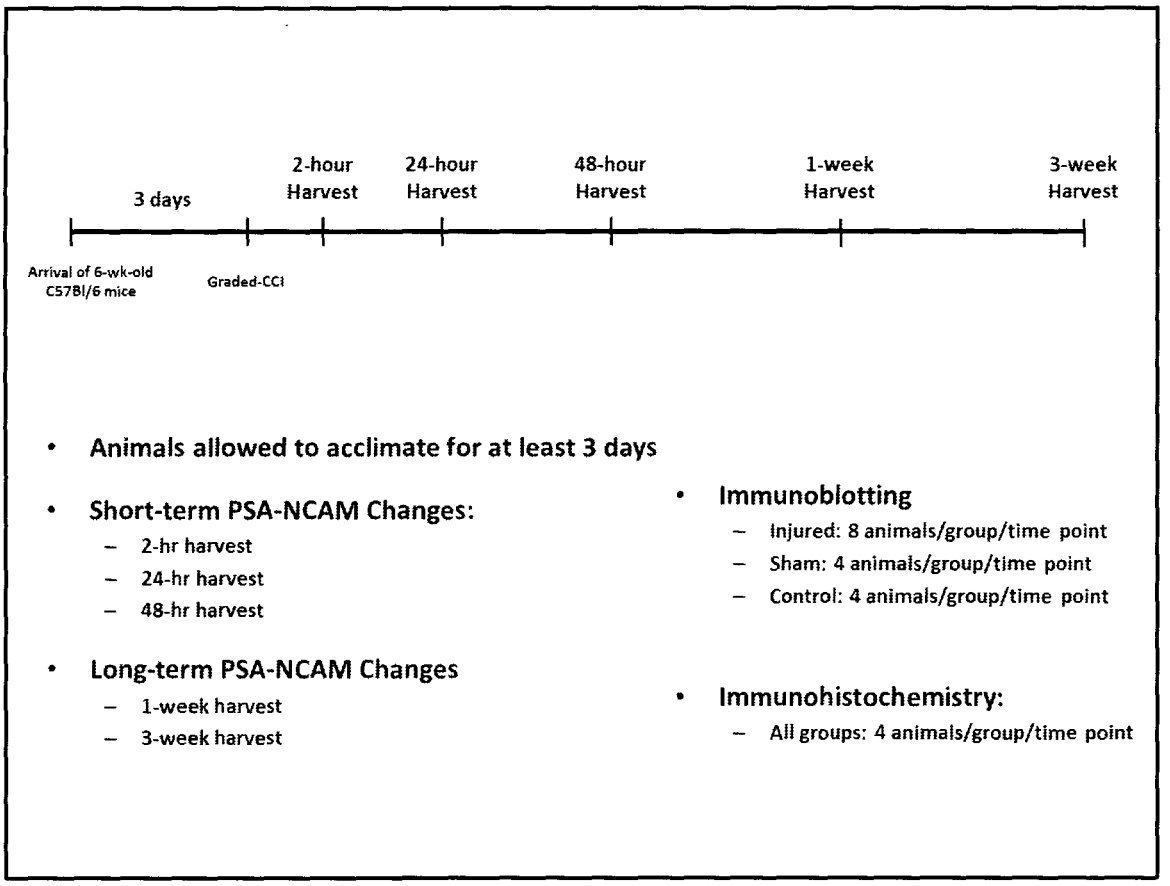

Figure 8. Overview of graded-CCI PSA-NCAM expression level investigation.

Following an acclimation period, mice were subjected to sham, moderate (1 mm depth of impact), or severe (2.5 mm depth of impact) CCI. Short-term changes in PSA-NCAM levels were evaluated at 2, 24, and 48-hours. Long-term changes were evaluated at 1 and 3-weeks. The number of animals evaluated by immunoblotting and immunohistochemistry at each time point is given. Refer to Chapter 2 (**Mouse brain PSA-NCAM levels are altered by graded controlled cortical impact injury**) for details of procedures implemented.

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Chapter 2

Short and long-term motor and behavioral effects of diazoxide and dimethyl sulfoxide administration in the mouse after traumatic brain injury

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Abstract:

Traumatic brain injury (TBI) is a worldwide phenomenon that affects all ages and socioeconomic classes and results in varying degrees of immediate and delayed motor, cognitive, and emotional deficiencies. A plethora of pharmacologic interventions that target recognized initiators and propagators of pathology are being investigated in an attempt to ameliorate secondary injury processes that follow primary injury. Diazoxide (DZ), a K_{ATP} channel activator, has been shown to provide potent short and long-term protective effects in a variety of *in vitro* and *in vivo* cerebral ischemia models. However, the effects of diazoxide on behavioral outcome following TBI have not been investigated. This study examined the effects of DZ on motor and behavioral recovery following TBI. TBI was induced in male C57Bl/6J mice by controlled cortical impact (CCI) and followed by intraperitoneal administration of equal volumes of either NaCl, dimethyl sufoxide (DMSO), or 2.5 mg/kg DZ in DMSO at 30-minutes post-injury and daily for three days. Open field and beam-walk performances were used to assess motor and behavioral function at 24-hours, 7-days, and 14-days following injury. Spatial learning and memory was assessed 3 weeks following injury using the Morris water maze. Injured mice were significantly impaired on the beam-walk and Morris water maze tasks, and were hyperactive and anxious in an open field environment. Injured mice treated with DZ performed better on the beam-walk task 14 days after the injury, however, both DMSO and DZ increase the amount of time it took the animals to perform the task on post-injury day one. There was no therapeutic effect of the treatment or vehicle on open field behavior or learning and memory function in the Morris water maze, but mice treated with either DMSO or DZ swam slower in the water maze on the final day of training.

In summary, moderate-CCI produces significant long-term impairment of motor, memory, and behavioral performance measures. The overall therapeutic effect of the dosing scheme used in this work was minimal, but some interesting findings were revealed. First, DZ appears to provide some degree of long-term motor function preservation following injury. Second, injured animals given DMSO with or without DZ displayed slowed ambulation and swim speed when compared to NaCl treated CCI animals.

Keywords: Diazoxide, TBI, DMSO, Open Field, Morris Water Maze

1. Introduction

Traumatic brain injury (TBI) is a leading cause of death and long-term disability that places an enormous burden on individuals and society regardless of age, gender, or socioeconomic class (Langlois et al., 2006). In addition to being the leading cause of death and physical disability in civilian victims aged 5 to 44 years (Zupan et al., 2011), it is believed that the incidence of TBI among soldiers wounded in combat situations may be as high as 22% (Martin et al., 2008). These sobering statistics translate into a loss of lifetime productivity costing over 360 billion dollars in the United States alone (Corso et al., 2006). Apart from the physical disabilities resulting from injury, TBI is recognized to be a strong epigenetic risk factor for long-term psychiatric and degenerative disorders such as Alzheimer's' disease (Sivanandam and Thakur, 2012). Remarkably, it is estimated that 40% of TBI victims suffer from two or more long-term neuropsychiatric disorders following injury (Vaishnavi et al., 2009).With an appreciation of the impact that TBI has on humankind, it cannot be overstated that aggressive efforts focused on not only preventing but ameliorating the effects of all levels of TBI must continue in earnest.

Due to significant heterogeneity among TBI victims and the complexity of the secondary injury cascades that follow the primary event, promising pre-clinical drug therapy results have thus far been inconclusive or even harmful when evaluated in clinical trials (Lei et al., 2012). One theme that has emerged from both pre-clinical and clinical trials is the need to evaluate either combinatorial pharmacologic therapy and/or assess the ability of agents that possess multiple mechanisms of action in order to counter the progression of early and late neuropathology resulting from TBI (Maas et al., 2010).

A survey of research in the field of ischemic brain injury reveals a possible candidate: diazoxide (DZ).

Diazoxide, a benzothiadiazine derivative, has been used clinically for over 30-years as a therapy for symptomatic hypoglycemia or clinical hypertension (Arnoux et al., 2011; He et al., 2008) and as a result, the chemical, safety, and side-effect profile is well established. Regarding potential neuroprotective attributes, the primary mechanism of action appears to be the ability of DZ to act as a putative mitochondrial ATP-sensitive potassium channel (mK_{ATP}) opener. Preclinical efforts have demonstrated significant *in vivo* and *in vitro* neuroprotective effects in brain tissue when administered before and after (Abe et al., 2010; Adamczyk et al., 2010; Domoki et al., 2005; Farkas et al., 2005a; Farkas et al., 2006; Farkas et al., 2004; Garcia de Arriba et al., 1999; Kis et al., 2003; Liu et al., 2002; Nagy et al., 2004; O'Sullivan et al., 2007; Rajapakse et al., 2003; Robin et al., 2011; Shake et al., 2001; Shimizu et al., 2002; Zhang et al., 2010) exposure to oxygen-glucose deprivation (OGD) conditions. Of particular clinical relevance, Farkas et al. noted that three months following permanent bilateral common carotid artery occlusion, improved spatial learning performance was observed during Morris water maze (MWM) assessment of rats that were post treated with 5 mg/kg DZ in dimethyl sulfoxide (DMSO) i.p. immediately following surgery and for four consecutive days versus non-treated injured animals. Because preemptive administration of therapeutic agents before TBI is impractical, the potential ability of post-injury DZ (and DMSO, see below) administration to improve performance on hippocampus-dependent memory tasks is a remarkable discovery.

Because DZ does not dissolve well when mixed with inorganic solvents, dimethyl sulfoxide (DMSO) was used as the solvent for drug administration in the present study. Dimethyl sulfoxide [$(CH_3)_2SO$] is an amphipathic solvent that was discovered in the 19th century and is widely used as a reagent in bioscience research and therapeutically in human and veterinary medicine (Santos et al., 2003). With over 10,000 articles dedicated to investigating the biological actions of DMSO (Jacob and de la Torre, 2009), it has been referred to as "a relatively simple compound that has stimulated much scientific controversy" (Ali, 2001). A wide range of DMSO percent concentrations have demonstrated neuroprotective effects in human and animal subjects in the face of assorted insults, including various TBI models (see (Jacob and de la Torre, 2009) for review). However, recent experimental data has revealed that not only does DMSO possess several favorable attributes but that it may project far reaching effects on metabolism and cell dynamics that could produce undesired or deleterious effects on experimental outcome (Nasrallah et al., 2008).

The objective of the work presented in this paper was to evaluate the short- and long-term effects of post-TBI DZ administration on behavioral outcomes in mice that received moderate controlled cortical impact (CCI) injury. For the purposes of this study, moderate injury was defined as a lack of noticeable hemorrhage or significant physical disruption in the underlying hippocampus when examined at 24-hr and 3-weeks, respectively. To the best of our knowledge, assessment of the effects of DZ administration on behavioral outcomes following TBI has not been undertaken prior to our work. An interesting and unexpected finding regarding the effect of DMSO vehicle

on behavioral outcome may serve as evidence that a drug vehicle may indeed produce undesirable effects on test subject outcome.

2. Materials and Methods

2.1. Animals

A total of 122 six-to-nine-week old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) weighing 20–29 g at the time of surgical procedures were group-housed in an animal colony at a constant temperature (23 $\pm$ 2°C) with a 12-hour light/dark cycle and food and water *ad libitum* for at least three-days prior to surgery. Mice were assigned to groups designated NaCl control, sham, or injury (n=8, 16, 16, respectively), DMSO control, sham, or injury (n=8, 16, 16), or DZ control, sham, or injury (n=10, 16, 16). Twenty additional animals were processed 24 hours or three weeks after injury for assessment of hemorrhage and structural alterations using whole brain and coronal section analysis. All experimental procedures used in this investigation were approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC).

2.2. Surgery

Anesthetic induction was performed via spontaneous ventilation using 3% isoflurane in 100% oxygen (1.0 L per minute flow rate) in a rodent volatile anesthesia box. After application of protective ointment (Lacri-Lube®) to the eyes, the head was shaved using electric clippers, placed in a standard rodent stereotaxic frame and positioned using atraumatic ear and incisor bars (Stoelting, Wood Dale, IL). The skin was prepped with 70% isopropyl alcohol and betadine ointment in sequence and 0.1 ml of

0.025% bupivacaine was injected subcutaneously into the planned incision site. An isothermal heating pad with feedback controller (Stoelting, Wood Dale, IL) was used to maintain rectal temperature at 37°C and isoflurane (1.5-2%) during the procedure maintained an adequate level of anesthesia. Employing a previously described technique (Brody et al., 2007; Budinich et al., 2012), unilateral CCI to the left cerebral hemisphere was performed. The CCI device (Impact One^{TM}, Leica, Wetzlar, Germany) consisted of a computer-controlled, electromagnetically-driven impactor fitted with a 3.0-mm-diameter steel tip mounted on a stereotaxic micromanipulator. A center of impact was reached after the impactor was moved 1.5 mm anteriorly and 1.2 mm to the left. Following positioning of the center of the impactor tip (3.0 mm anterior to lambda and 2.7 mm left of midline), moderate injury was produced with the following parameters: 1.0 mm depth of impact at 5 meters/sec with a dwell time of 0.1 seconds. Following injury, the skull fragment was carefully replaced, and the incision was closed with silk sutures. Sham mice underwent all of the described surgical procedures but did not receive moderate CCI. All surgical procedures were performed in an aseptic manner. At the conclusion of the procedure, all animals received a subcutaneous injection of 0.5 ml of 37°C 0.9% sodium chloride to combat dehydration. Mice were placed in a heated cage to maintain body temperature until fully awake and were then returned to their home cage.

2.3. Drugs

All mice were administered intraperitoneal (i.p.) sodium chloride, 2.5 mg/kg DZ in 10-15% DMSO (Sigma Chemical Co., St.Louis, MO), or a 10-15% DMSO(Sigma Chemical Co., St. Louis, MO) solution 30 minutes following craniectomy, CCI, or at a designated time for controls and at 24-hr increments for three days using a standardized

technique (Arras et al., 2001). In order to ensure that DZ remained in suspension, a fresh stock solution of DZ dissolved in full strength DMSO with a final concentration of 5 mg/ml was mixed at each scheduled dosing time. Due to syringe calibration limitations, administration volumes were calculated in two-gram increments (i.e., 25 g mouse received 26 µl of the stock solution diluted to 260 µl with NaCl) in order to ensure that a 10-15% DMSO final solution was administered in the DZ and DMSO groups.

2.4. Histologic assessment of injury

At the conclusion of behavioral testing three-weeks following injury, all mice were administered deep anesthesia (60 mg/kg ketamine with 60 mg/kg xylazine, i.p.) and were processed for immunohistochemical analysis as previously described (DiLeonardi et al., 2009). A frozen sliding microtome was used to acquire 30-µm-thick coronal sections from the olfactory bulbs to the rostral cerebellum, and all slices were stored in cryoprotectant (Watson et al., 1986) at -20°C until processed for assessment using standard hematoxylin and eosin (H&E) staining technique.

2.5. Motor and behavioral training and evaluation

2.5.1. Beam walking assay

The method instituted to evaluate motor deficits resulting from injury and the response to drug therapy was a modification of a previously published protocol (Stanley et al., 2005). All mice were trained to complete the beam walk task over three days prior to sham or CCI procedures. Each mouse was placed on a start platform and then walked across a wooden beam (80 cm long, 6 mm wide, and 30 cm above a padded surface) toward a goal box containing bedding. A basal level of competence at this task was established on the final day of training. Acceptable performance was defined as the

ability to take 50 steps with fewer than 10 foot slips in less than 240 seconds. All mice were judged to be competent on the final training day and subsequently evaluated on post injury days 1, 7, and 14 (POD 1, 7, and 14).

2.5.2. Open field evaluation

Evaluation of anxiety, exploratory behavior, and spontaneous locomotor activity occurred in a 40 cm x 40 cm open field apparatus with opaque black plastic walls (Stoelting, Wood Dale, IL). Each mouse was placed in the arena for 10 minutes on Day 0 (baseline evaluation before TBI surgery), and on POD 1, 7, and 14. Mice were placed in the center of the apparatus and monitored via an overhead camera linked to ANY-maze behavioral tracking software (Stoelting, Wood Dale, IL). The following parameters were recorded: total distance traveled, total number of mobile episodes (with immobility defined by Any-Maze as 65% of the animal remaining still for a minimum duration of two seconds), and distance the mouse traveled in the center of the field (defined as greater than 10 cm from a wall) expressed as a percent of the total distance.

2.5.3. Morris water maze (MWM) evaluation

The water maze apparatus used consisted of a modified version originally described by Morris (Morris, 1984). The circular pool was 122 cm in diameter, filled with tap water maintained at 24±1°C. A transparent platform (11 cm diameter) was submerged approximately 1.0 cm below the surface 15 cm from the wall of the pool. Multiple large visual cues were secured to the walls of the room in positions that could be viewed by the mice in the pool. Four starting positions were designated around the perimeter, and the surface of the pool was divided into four equal quadrants. All activity

was monitored by an overhead camera linked to a computer with ANY-maze behavioral tracking software.

All mice underwent a three phase evaluation process: orientation, training, and a probe trial. On post-procedure day 17 (orientation), the mice were acclimated to the testing room for 30 minutes. Following this period, the mouse was gently placed on the submerged platform located in the northwest (NW) quadrant and allowed to remain or swim for 60 seconds. Each mouse was then removed from the pool, dried with a towel, and placed in a warmed cage and exposed to a heat lamp to prevent hypothermia. On days 18 – 21 (training), the mice were allowed to orient to the room and then underwent four 60 second trials separated by 1-2 minutes per trial. The mice were inserted facing the pool wall of a predetermined alternating quadrant for each trial. Mice were allowed 60 seconds per trial to find the platform using the visual cues posted on the walls. If the platform was reached before 60 seconds the mouse was allowed to remain in place for 15 seconds or until the 60 second trial was complete, whichever was first. If the mouse did not find the platform within 60 seconds, then he was placed on the platform and allowed to remain for 15 seconds. All mice were towel dried and placed in a heated cage after each trial. Training trial parameters that were evaluated included latency to platform, speed of travel, and total distance traveled.

A single probe trial took place one hour following the final training session on day 21. The platform was removed and the mice were placed into the pool quadrant opposite from the previous location of the platform and allowed 60 seconds to search for the platform. Probe trial parameters that were evaluated included time spent in the NW quadrant and the number of times the animal crossed the exact location of the platform.

2.6. Statistical analysis

All recorded motor and behavioral measures were categorized and saved in Microsoft Excel, and the effects of injury and drug treatment were evaluated using SPSS (Version 20, IBM SPSS Statistics). A mixed-design analysis of variance (ANOVA) was performed with the Injury factor (Naïve, Sham, CCI) and Drug treatments (NaCl, DMSO or DMSO + DZ treatment) considered between-subject variables, and test day being the within-subject variable. Significance was stated with probability values of $p<0.05$ based upon the computed Wilk's lamba F-statistic. However, initial tests of the assumptions related to the Days after Injury factor (the Mauchly Test for Sphericity on this repeated measures variable), indicated the data did not meet assumptions of equal variance. Since our main interest was the impact of TBI and drug treatments on behavioral performance, ANOVA tests of the data from each test day was evaluated separately. In this case, the design was a 2-way ANOVA with the factors, Injury and Drug Treatment. Almost all tests met assumptions of normality and homogeneity of variances. When a main effect was significant, pairwise comparisons (Least Significant Difference Test; SPSS) were performed. In cases where this assumption was not met (Levene's Test of Equality of Error Variance; SPSS), the data was transformed to ln or square root values and in all cases homogeneity tests were met.

3. Results

3.1 Extent of injury

The goal of inflicting this degree of injury was to avoid gross physical damage and hemorrhage to the underlying hippocampus in an attempt to emulate the diagnostic findings seen in human mild and moderate TBI. In agreement with previous work in

which we evaluated injury severity at 24 hr and three weeks following moderate CCI (Budinich et al., 2012), H&E evaluation revealed damage to the left cortex while the underlying hippocampus was spared from noticeable physical disruption (Figure 1).

3.2 Beam walking assay

Beam walk performance was used to evaluate the impact of TBI and drug treatments on vestibulomotor function. Foot fault measures in each mouse were recorded on the day before surgery (Baseline) and on post-operative day (POD) 1, 7, and 14. The Baseline performance of each mouse was then used to compute a relative percentage of their step performance on the days after injury. ANOVAs of the factors, Injury x Drug, were performed to test the mean percentage of steps animals performed without slipping, as well as the time required for a mouse to reach the end of the beam. CCI had a major impact on beam walk step performance and latency to cross the beam. The CCI groups performed significantly more poorly than the Control and Sham groups on the average number of correct steps in crossing the beam (Figure 2A) and required a longer time to cross (Figure 2B), while the Control and Sham Groups of mice exhibited essentially no impairment.

To determine the impact of drug treatments on performance in the CCI groups, a Drug x Day ANOVA was used to assess performance. While all groups of CCI animals exhibited impaired stepping across the beam on the day after CCI (Figure 2C), on Days 7 and 14 after injury all groups progressively improved stepping across the beam from Day 1 to Day 7, to Day 14 after injury. There were no significant differences between the effects of drug treatments for the CCI mice on Days 1 or 7 after injury, however, on Day 14 after injury DZ treatment had significantly improved the stepping capability of CCI-

injured mice compared to animals that received saline (Figure 2C). In terms of the time required to cross the beam, on Day 1 after injury CCI mice required a significantly longer amount of time to cross the beam if they had received DMSO or DMSO + DZ (Figure 2D). On Days 7 and 14, when animals were no longer receiving these compounds on the days they were tested, there were no differences in the time required to cross the beam as a function of drug treatment.

3.3 Open field test

The open field test was used to evaluate motor activity and the effects of CCI and drug treatments. No significant effect on open field performance was observed as a function of drug treatments. However, on all test days (Figure 3A) CCI-injured mice exhibited a significant increase in mobile activity. This was accompanied by them exhibiting less time (in terms of percentage of total activity) in the center of the open field (Figure 3B). In addition, on test days 1 and 2 weeks after CCI, these animals exhibited significantly fewer episodes of movement onsets (3C). Taken together, CCI induced a state of increased mobility where the mice spent a greater amount of time moving, especially in the periphery of the arena. Once they were mobile, however, they did not engage in stopping, as was observed in the Control and Sham-treated animals.

3.4. Morris water maze

Over the four trials in the acquisition phase of the Morris water maze, the CCI-injured mice required a significantly greater amount of time to find the platform (Figure 4A) and swam a longer distance (Figure 4B) compared to the Control or Sham groups. During the probe trial, CCI-injured mice (Figure 4C) spent significantly less time in the quadrant that was the former location of the platform, compared to the other treatment

groups, and these mice had significantly fewer instances (Figure 4D) where they swam directly over the site where the platform had previously been located. While swim speed was not affected on the first three trail days by either former injury or drug treatments, on the last day of training swim speed was significantly faster by mice in the CCI group compared to the Control or Sham-treated animals (Figure 5A). The groups were found to also differ as a function of prior drug treatment (Figure 5B). Mice that had received NaCl treatments weeks earlier swam significantly faster than mice that had received DMSO or DMSO + DZ.

4. Discussion

Traumatic brain injury may be broadly defined as an "alteration in brain function, or other evidence of brain pathology, caused by an external force" (Menon et al., 2010). The acute and chronic sequelae associated with TBI syndrome present as a group of varied physical and psychological symptoms that reflect the complexity of the pathological processes that underlie this often devastating injury. To date, there is no pharmacologic intervention evaluated clinically that has consistently improved physical, behavioral, or memory function (Stein, 2012). Several authors believe that the failure to translate promising preclinical results to the clinical arena is due to too narrow of a focus on individual targets of pathology and therapy (Margulies and Hicks, 2009). Furthermore, the ability of a drug to ameliorate neuropathology when administered *after* the injury has occurred will prove to be a necessary step in advancing the search for a viable TBI pharmacologic intervention.

Our study investigated the effects of post-TBI DZ administration on behavioral and memory outcomes because it has previously been shown to afford protective effects

in ischemic injury scenarios (Lenzser et al., 2005; O'Sullivan et al., 2007; Zhang et al., 2010), and its putative mechanisms of action may affect multiple pathologic pathways (Drose et al., 2006; Hanley and Daut, 2005; Nagy et al., 2004; Zhang et al., 2011), thus making it a possible pleiotropic drug candidate for TBI therapy. Additionally, DMSO, the vehicle used to deliver DZ, has previously been shown to possess therapeutic potential in the TBI setting (de la Torre et al., 1975; Karaca et al., 1991). In spite of the existing evidence, our study reveals two somewhat unexpected findings. First, compared to NaCl, the administration of DZ following moderate-CCI does not measurably improve short or long-term locomotor function, anxiety measures, or memory performance in the mouse. Second, post-injury DMSO administration exerts a significant long-term effect on measures of motor function.

Our definition of moderate TBI predominantly displaying cortex injury while excluding noted injury to the ipsilateral hippocampus was chosen to evaluate the memory and behavioral deficiencies resulting from TBI in which the hippocampus is generally spared from direct impactor injury. Previous investigators have demonstrated significant spatial learning deficits and anxiety related behaviors in rodents receiving "mild" to "intermediate" injury in which there is no or limited injury to the hippocampus (Dawish et al., 2012; Schwarzbold et al., 2010). It was our intention to inflict injury sufficient enough to produce behavioral defects while avoiding widespread destruction because a severe injury would have likely produced such profound disability that the likelihood of discerning a detectible effect from our treatment would have been difficult.

4.1. Actions and benefits of DZ treatment after brain insult

Diazoxide has been in clinical use for over 30 years for conditions ranging from hypertension to chronic symptomatic hypoglycemia; however, the beneficial, potentially harmful, and miscellaneous outcomes regarding interaction with nervous, cardiac and vascular, pancreatic, and liver tissue have also been examined (**Table 1**). Existing *in vivo* evidence regarding the protective effects of DZ in neuropathology has focused on pre-injury administration in the face of ischemic insult. We focused on post-TBI administration due to the direct clinical applicability of post-injury administration and because the effects of DZ in this scenario had not been previously evaluated.

Although the precise mechanism(s) by which DZ provides neuroprotection have not been fully explicated, they are believed to be related to cellular and subcellular modifications centered around mitochondrial function that occur during pre and post-ischemic conditioning (Lehotsky et al., 2009; Samavati et al., 2002). It is generally accepted that a cornerstone action responsible for the neuroprotective properties of DZ is the highly selective opening of the mitochondrial ATP-sensitive potassium channel (mK_{ATP}) located on the inner mitochondrial membrane (Busija et al., 2004; Kis et al., 2003). The fact that the administration of the mK_{ATP} blocker 5-hydroxydecanoate (5-HD) abolishes the protective action of DZ supports this assertion (Kowaltowski et al., 2006; Nakagawa et al., 2005). Activation of the mK_{ATP} channel during oxygen-glucose deprivation conditions produces several results including: prevention of mitochondrial permeability transition, preservation of the mitochondrial membrane potential ($\Delta\Psi$) (Kowaltowski et al., 2006), attenuation of ATP depletion (Belisle and Kowaltowski, 2002), reduced swelling and accumulation of Ca^{2+} in mitochondria (Domoki et al., 2004; Murata et al., 2001), anti-apoptotic effects via increased mitochondrial Bcl-2 levels and

decreased Bax translocation (Liu et al., 2002), decreased cytochrome *c* release and caspase-3 activation (He et al., 2008), and decreased microglial production of proinflammatory mediators such as tumor necrosis factor alpha (TNF-α) and inducible isoform of nitric oxide synthase (iNOS) (Liu et al., 2006). On the other hand, conflicting evidence exists regarding the implications and role of DZ-mediated mK_{ATP}. DZ has been reported to increase (Andrukhiv et al., 2006) or decrease (Facundo et al., 2007) production and release of mitochondrial reactive oxygen species (ROS). As research in this field continues, alternate mechanisms of action and dose related effects, such as stimulation of ROS production via mK_{ATP}-independent succinate dehydrogenase inhibition (Busija et al., 2008) or activation of protective MAPK and PKC signaling cascades (Gao et al., 2008) will be explored.

The dose of 2.5 mg/kg DZ was chosen for our investigation based on prior studies that demonstrated *in vivo* neuroprotective effects associated with DZ doses ranging from 0.5–10 mg/kg administered in both ischemic preconditioning and postconditioning paradigms (Farkas et al., 2004; Farkas et al., 2005b; Shake et al., 2001). Additionally, a three-day dosing scheme was chosen in order to ensure that DZ (MW = 230.7 kD) would enter the brain parenchyma through the injured blood-brain barrier. Conflicting evidence regarding the ability of DZ to cross an intact BBB in appreciable amounts exists (Bantel et al., 2009; Kishore et al., 2011; McCrimmon et al., 2005). Previous investigators have shown that following severe blast injury, and IgG immunoreactivity indicates that BBB permeability returns to baseline levels within 72 hrs following injury (Baskaya et al., 1997; Chodobski et al., 2011; Readnower et al., 2010). Although larger doses of DZ were considered, preliminary work performed by us revealed an undesired hyperglycemic

response (300-600 mg/dl) that continued for two-hours following dosing when 5 or 10 mg/kg doses were administered. It has long been understood that acute hyperglycemia in proximity to TBI is associated with poor neurological outcome and increased mortality (Griesdale et al., 2009; Jeremitsky et al., 2005; Kinoshita et al., 2002). Interestingly, this long-held belief has come into question because recent novel evidence reveals that acute and chronic hyperglycemia in the rodent experiencing moderate TBI may not adversely affect behavioral function, lesion volume, or brain edema (Hill et al., 2010).

It is notable that the existing evidence supporting the neuroprotective properties of DZ is considerable. When administered at times ranging from several days to 30 min before transient or permanent ischemic insult in the rodent, administration of DZ preserved the cerebral arteriolar response to hypercapnea (Domoki et al., 2005), reduced CA 1 neuron cell death and cerebral infarct volume (Watanabe et al., 2008), and improved spatial memory performance in the MWM (Farkas et al., 2006). Remarkably, postischemic administration of DZ has also been shown to offer significant immediate and long-term protection. For example, i.p. administration of DZ in 0.25 ml of DMSO to Wistar rats immediately after and for 5 days following permanent occlusion of the common carotid arteries (2 VO) resulted in a significant reduction in neuron loss in the dentate gyrus, prevention of microglial activation in the hippocampus, and improved MWM performance similar to that of sham animals when tested 13 weeks following injury (Farkas et al., 2004). Of note, the investigators also found that DMSO alone decreased the severity of hippocampal lesions following ischemia and improved MWM performance, though not to the level of DZ + DMSO. Using the same injury model and dosing scheme, the same investigators further demonstrated the ability of DZ and DMSO

to moderate white matter neuroinflammation by reducing astrocyte proliferation and microglial activation in the corpus callosum and optic tract (Farkas et al., 2005a). Acknowledging the apparent protective effects of DMSO, the investigators dissolved DZ in an NaOH solution and discovered that DZ alone reduced the degree of long-term microglial activation following 2VO but did not lessen the severity of memory impairment seen when compared to untreated 2 VO rats (Farkas et al., 2005b). More recently, investigators evaluated the ability of immediate post ischemic administration of DZ to decrease neuropathology in the rodent (Adamczyk et al., 2010). The investigators determined that the administration of DZ 30 min before reperfusion following one hour of transient ischemia reduced the volume of cerebral infarction, improved motor function, and improved performance on neurologic disability assessment.

4.2 Actions and benefits of DMSO treatment after brain insult

DMSO has been used extensively as a solvent in biomedical experiments and also as a therapeutic agent in human and animal conditions ranging from osteoarthritis to TBI, and many of its biological effects has been extensively documented (see **Table 2** and Santos et al., 2003 for review). Subsequent to the first use of DMSO in a mammalian system in 1959 (Brayton, 1986), intense interest in DMSO as a neuroprotectant followed the discovery that, among other things, DMSO and its metabolite dimethylsulfide can trap the hydroxide radical and oxygen free radicals, respectively (Hill et al., 1983). Additional research has revealed the ability of DMSO to decrease edema and elevated intracranial pressure following TBI (Ikeda and Long, 1990), maintain cerebral blood flow during ischemic episodes(de la Torre, 1991), and alter cell membrane properties to enhance survival in the face of injury (Notman et al., 2006; Shi et al., 2001).

Furthermore, investigators have combined DMSO with other compounds, such as fructose 1,6-diphosphate (FDP) (de la Torre, 1995) or nimodipine (Greiner et al., 2000) and discovered pronounced protective effects in ischemic and closed head injury. For instance, Greiner et al. exposed adult guinea pig hippocampal slices to ischemic conditions and determined that the latency of anoxic terminal negativity was increased when the slices were incubated in a nimodipine and DMSO solution for 30 min prior to the induction of anoxic conditions. Unfortunately, an interspecies lack of response was discovered when the same protocol was utilized in human temporal lobe tissue without a noted improvement in ischemic tolerance (Greiner et al., 2003).

Other contrasting effects of DMSO have been discovered. For example, using hot-plate and tail-flick evaluation in rats, previous investigators exposed a long-lasting analgesic effect following i.p. injection of high doses of DMSO (5.5 g/kg of 50% DMSO solution) (Haigler and Spring, 1983). This analgesia was comparable to a 10 mg/kg dose of morphine sulfate; however, the DMSO effect outlasted that of morphine, and the effects of the DMSO were local as well as systemic. Conversely, a 10 day schedule of i.p. injection of 3.6 and 7.2% DMSO in Sprague-Dawley rats produced allodynia and lowered mechanical nociceptive threshold compared to baseline results without altering motor function (Authier et al., 2002). A similar dosing scheme resulted in sciatic nerve myelin disruption and decreased tail nerve conduction velocity that persisted for 15 days after ending the dosing schedule and recovered by post-DMSO administration day 45 (Cavaletti et al., 2000).

One theme that emerges when the existing work concerning DMSO is surveyed is that the line between beneficial and deleterious effects depends on the concentration and

dose of DMSO that is administered. In our study, 22–33 mg of a 10-15% DMSO solution was administered at 24 hour intervals over four days. Subject to mouse weight, a cumulative dose of 88–132 mg, or 4.4 g/kg, was administered. We believe that this dose was the ideal compromise between ensuring DZ solubility, optimizing the total of i.p. volume injected, and reducing the total DMSO dose administered to mice. The reported LD_{50} of DMSO in the mouse varies widely depending on the route of administration. For example, i.p. LD_{50} is 14.7-17.7 g/kg (Smith et al., 1967), while oral is 7.9 g/kg and intravenous is 2.5 g/kg (Lewis, 1996). Additionally, the range of DMSO concentrations and doses administered in prior rodent studies is quite broad.

More recent investigations have taken advantage of technological advances to further define the effects of DMSO on the CNS. For example, acknowledging that DMSO blocks $Na^+$and K^+channels, may alter the properties of T and L-type Ca^{2+} channels, and decreases N-methyl-d-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), and γ-aminobutyric acid (GABA) receptor activity, investigators examined the effects of low and high dose i.p. DMSO on seizure activity in WAG/Rij rats (Kovacs et al., 2011). A single dose of 1.65 mg/kg was noted to decrease the number of spike wave discharges (SWDs) while doses of 825.3 mg/kg and above increased the number of SWDs during the 5 hour post-injection period. Additional clarification regarding the pharmacokinetics and pharmacodynamics of DMSO is also emerging. Specifically, although the half-life of DMSO in the rodent brain is 10-12 hours, and accumulation of the drug in the brain has not been noted previously (Kaye et al., 1983), a recent study revealed that guinea pig brain cortical slices incubated in a DMSO-containing solution accumulated DMSO in a non-linear, saturable manner

(Nasrallah et al., 2008). The potential for accumulation of DMSO in brain tissue after single, and particularly multiple doses, could accentuate the beneficial and detrimental effects of the drug on the brain.

4.3. Behavioral effects of DMSO and DZ after CCI

Considering the existing evidence regarding DZ and DMSO effects, the three behavioral/motor assessment tools utilized to evaluate post-CCI deficits and recovery utilized in our study were chosen because of a well-established history of ease of use, sensitivity, and specificity. The beam walk assay has an over 20 year history of efficacy in the assessment of injury severity, lateralization, and motor pathway integrity by measuring balance and coordination via foot slips and time required to complete the task (Fujimoto et al., 2004). As described in previous studies, CCI related motor deficits are evident upon beam walk assessment for more than 4 weeks following injury (Fujimoto et al., 2004). Not unexpectedly, we noted a significant decrease in the number of steps and increase in duration to task completion in all injured groups compared to sham and control animals at each time point following CCI (Figures 2A & 2B). Performance on the beam walk task requires fine motor coordination and may be considerably anxiogenic (Stanley et al., 2005). Further evaluation of performance between injured animals revealed that CCI mice who received DZ completed a greater number of steps during task completion on post-operative day (POD) 14 than animals that received NaCl. This apparent beneficial result from DZ administration in the face of TBI is the first of its kind and offers a positive direction for future study, which will be discussed below. Inclusion of additional motor evaluation tools such as accelerated rotarod or the adhesive removal test (Bouet et al., 2009) should be considered in future studies. Even though the strengths

and weaknesses of these tools are debated (Piot-Grosjean et al., 2001), the combination of multiple tests may allow for enhanced sensitivity in detecting early and late motor function recovery among groups (Baquet et al., 2005; Fujimoto et al., 2004).

The increased time required by injured animals that received DMSO or DZ dissolved in DMSO to complete the beam walk task on POD 1, when compared to injured mice that received NaCl was an interesting finding. Notably, this effect on beam walk speed was not observed in Control and Sham animals. The reason(s) for this differential effect is not known, but a phenomenon similar to previously described myelin disruption and decrease in motor nerve conduction velocities seen following i.p. injection of DMSO in rats (Cavaletti et al., 2000) may have occurred in our animals; however, we did not monitor these parameters. As previously described (Authier et al., 2002), another possible cause for decreased speed of beam walk completion could be allodynia resulting from DMSO injection. In this case, motor function would be preserved, but foot or limb pain would adversely affect ambulation. Further research investigating the effect of DMSO on tactile sensation and nerve conduction efficiency in large myelinated motor neuron axons is warranted. Perhaps in the presence of brain injury, the sedative effects of DMSO may be enhanced or the central processing of afferent stimulus (allodynia) may be affected. The addition of DZ appears to have reduced the effect of DMSO on speed; however, it was not a significant improvement.

Results from the open field apparatus are challenging to interpret. The most obvious finding is that drug treatment did not have an appreciable effect on any of the parameters monitored. The increased total distance traveled in all CCI animals may reflect a post-injury hyperactivity disorder linked to impaired memory function. A

hyperactive phenotype, characterized by increased locomotion in the home cage or a novel environment resulting from lesions to the cerebral cortex, is not unexpected because lesions to a complex network connecting the olfactory and entorhinal cortices can increase locomotor activity (see (Viggiano, 2008) for review). The increased mean distance traveled on POD 1, 7, and 14 (Fig. 3A) as well as the decreased number of mobile episodes (mice moved longer distances between stops) noted on POD 7 and 14 (Fig. 3C) in injured mice concur with a diagnosis of hyperactivity. Researchers involved in ischemic and traumatic brain injury projects have suggested that this hyperactivity is linked to impaired spatial memory function, and the inability to reliably build and retrieve a spatial map, rather than motor disorders (Li et al., 2006; Schwarzbold et al., 2010).

Anxiety is one of the most prevalent neurobehavioral pathologies that manifests following TBI in rodents (Chauhan et al., 2010). An increased total distance traveled or time in the center of an open field apparatus is believed to be directly correlated with decreased anxiety (Lau et al., 2008; Matsuo et al., 2010; Prut and Belzung, 2003). Our injured animals spent considerably less time in the center of the open field apparatus than Control and Sham mice on POD 1 & 7 and less than Sham animals on POD 14 (Fig. 3C). We have no explanation for this observation. Notably, this anxiety related behavior has typically been associated with the chronic phase following TBI (Washington et al., 2012). In retrospect, use of the elevated plus maze may have provided us with a more definitive ability to probe anxiolytic and anxiogenic behavior in our test subjects. Interestingly, recent investigation of all degrees of CCI in mice revealed a decrease (Washington et al., 2012) or lack of effect (Zohar et al., 2011) on anxiety-associated behavior.

Because neither DMSO nor DMSO/DZ had a significant effect on improving performance on measures of working memory and memory retrieval during the acquisition phase (latency to platform and distance swam (Fig. 4A&B)) or probe trial (NW quadrant time and platform crossings (Fig. 4C&D)) of MWM evaluation, the dosing schedule examined in this study cannot be said to reduce the severity of memory disabilities resulting from moderate-CCI. Existing evidence supports a strong connection between poor MWM memory performance and increased post-TBI travel distance in the open field test (D'Hooge and De Deyn, 2001). We believe that the lack of a detectible beneficial influence on the hyperactive phenotype and memory function in DMSO/DZ treated animals in our study serves as evidence that the chosen dose and dosing schedule instituted in our work was not effective in decreasing the severity of memory and behavioral deficits following CCI. Further work is required to increase the understanding of the complex relationship between post-TBI behavioral alterations and memory function.

4.4. Functional implications of DZ and DMSO for TBI

The results of our work show that DZ administered using the current schedule and dosing scheme provides minimal benefit to post-TBI function. On one hand, the marginal improvement seen in the number of steps taken by the CCI+DZ animals on beam walk evaluation versus other injured animals coupled with the lack of a detectable positive behavioral or memory response was disappointing. However, the general lack of a therapeutic response and the unanticipated effects of DMSO provide insights that may be used when conducting future research.

First, although evidence regarding the efficacy of pharmacologic preconditioning using DZ in cerebral ischemia is fairly convincing, the relative paucity of post-ischemic DZ studies demonstrates that this therapeutic approach is in its infancy. Additionally, the pathology following TBI may be more heterogeneous than that following ischemic injury. For example, physical shear forces creating widespread axonal injury, rapid development of excitotoxicity, and systemic inflammatory responses may produce a complex, distinctly different secondary injury sequence than that seen with ischemic injury. Also, ischemic injury initiates an "active" apoptotic pathway that is amenable to therapy with DZ. On the other hand, TBI may involve mixed "passive" necrotic and "active" apoptotic pathology. As a result, perhaps increasing the dose of DZ and moving the administration time of the drug to within 5-10 minutes following injury may improve the chances for a successful intervention. The window for ischemic postconditioning mechanisms, and presumptively those of DZ, to exert protective effects appears to range from minutes (Zhao et al., 2012) to days (Burda et al., 2006; Ren et al., 2008; Sun et al., 2012) following ischemic cerebral injury. However, the role and efficacy of these mechanisms in mitigating post-TBI pathology is unknown. Combining DZ with another drug believed to address a different injury pathway (i.e., Mg^{2+} or NMDA receptor antagonists) may also improve the chance of success. Furthermore, recent evidence demonstrated that long-term administration of DZ (10 mg/kg/day for 8 weeks) reduced amyloid-β and tau pathologies and improved memory performance measures in a mouse model of Alzheimer's Disease (Liu et al., 2010). The ability to administer moderate doses of DZ for extended periods without noticeable detrimental effect offers promise for future

efforts aimed at combating the long-term evolving pathology that may exist following TBI.

Second, the fact that the vehicle used in our study produced effects independent of DZ was an interesting, although not unexpected, finding. Even though the preponderance of research regarding DMSO and ischemic or traumatic brain injury supports a beneficial effect of administration, we failed to detect favorable effects on any of the parameters that were measured. This is not to say that a detailed examination of changes in specific molecular markers of injury and regeneration would not reveal an effect of DZ, but the "common final pathway" of injury – the TBI phenotype – was not improved. In our study, it appears that DZ and DMSO do not exert additive or synergistic effects. Future investigators would be wise to consider use of a vehicle and route of administration that minimizes the introduction of confounding effects that make interpretation of the therapeutic intervention difficult. In closing, our work did not reveal a definitive improvement in post-CCI short or long-term memory, behavioral, or motor function in the mouse. However, future investigation into the effects of an extended DZ dosing scheme on post-TBI outcome is warranted.

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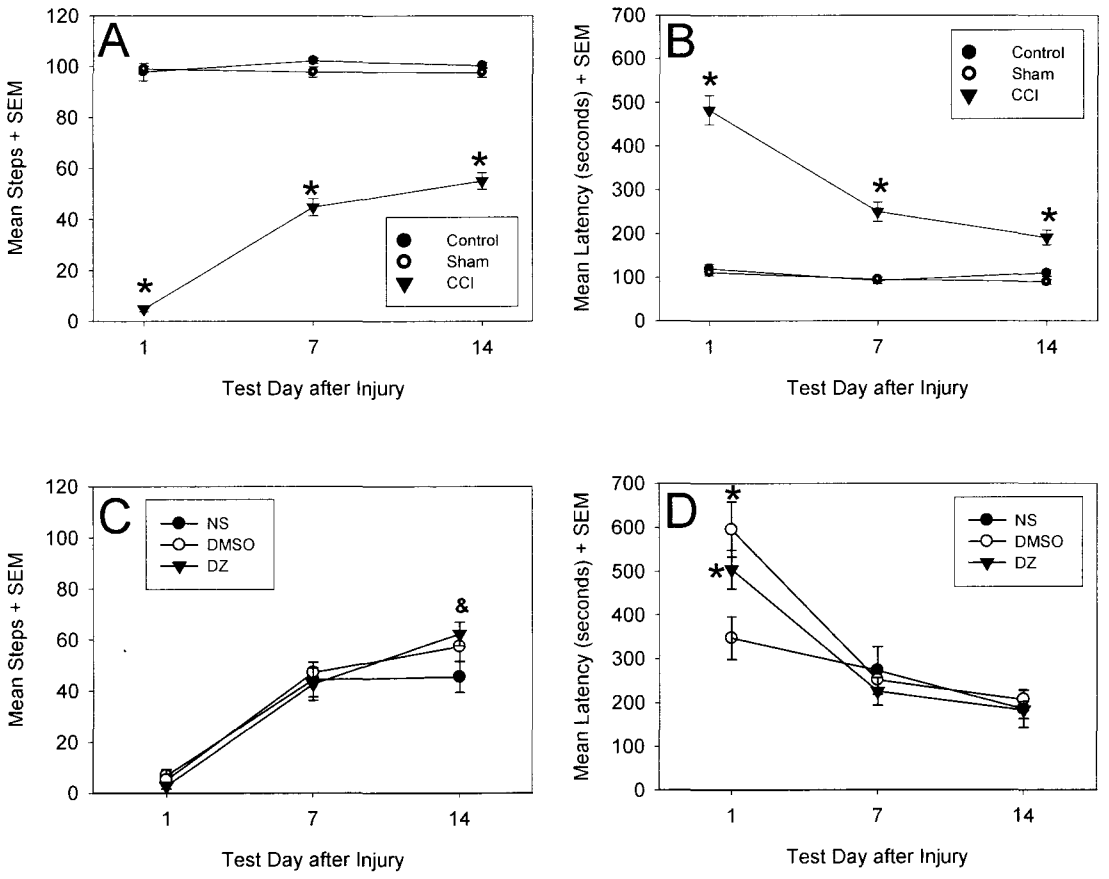

Figure 2: Beam Walk Performance.

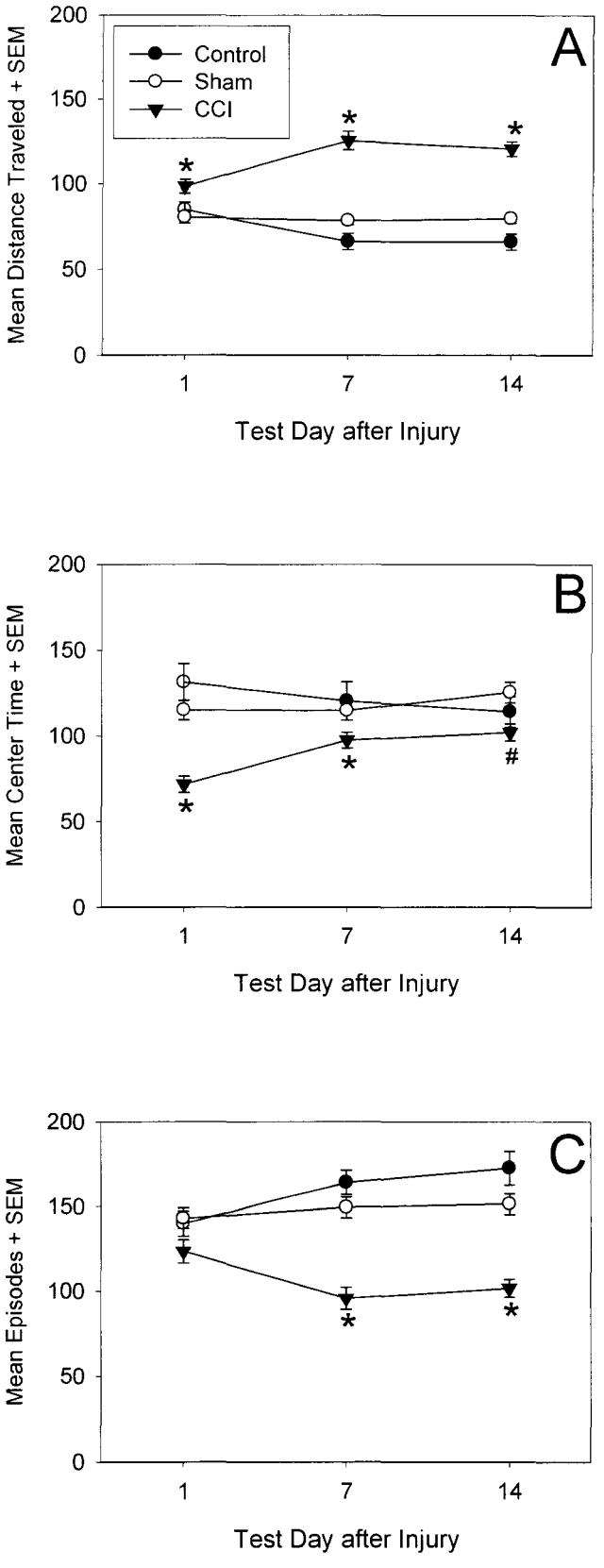

Figure 3. Open field test performance after TBI and drugs treatments.

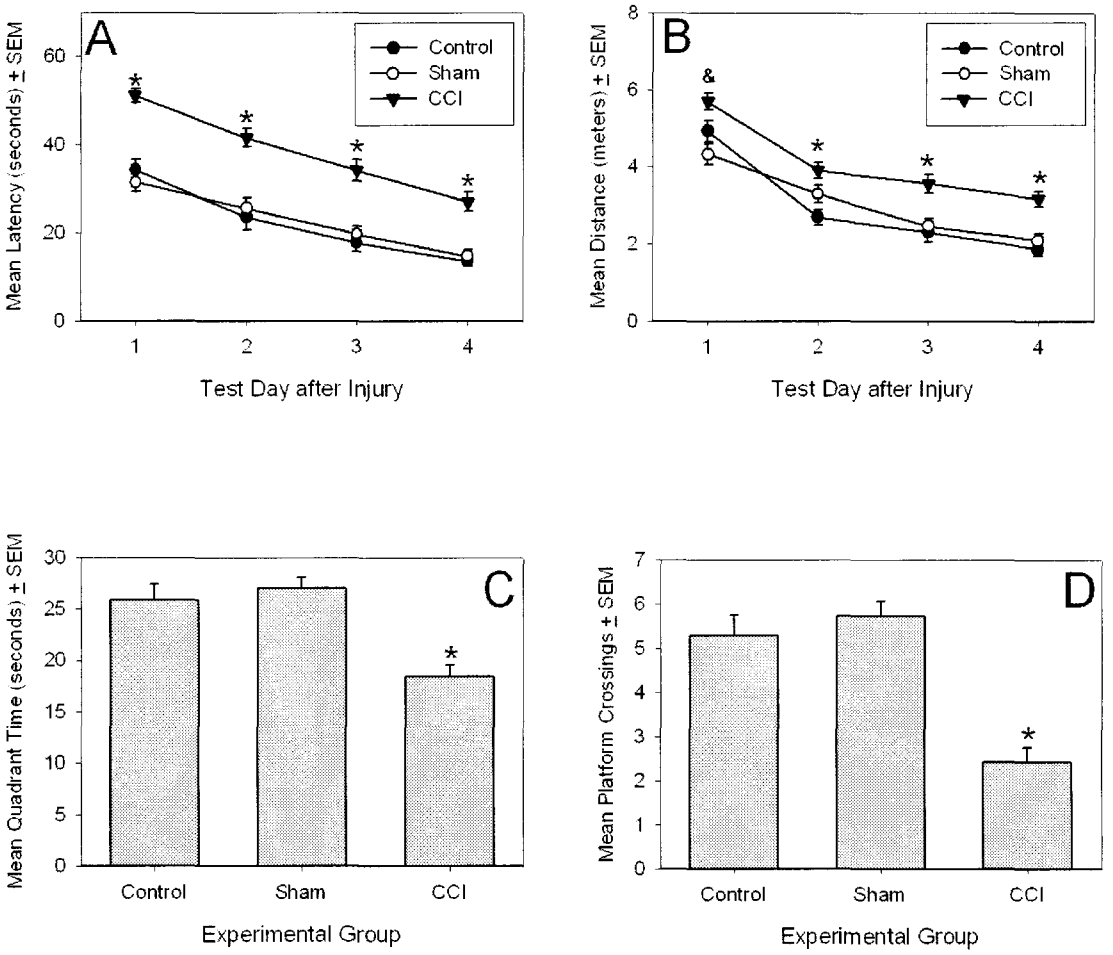

Figure 4.Morris water maze performance.

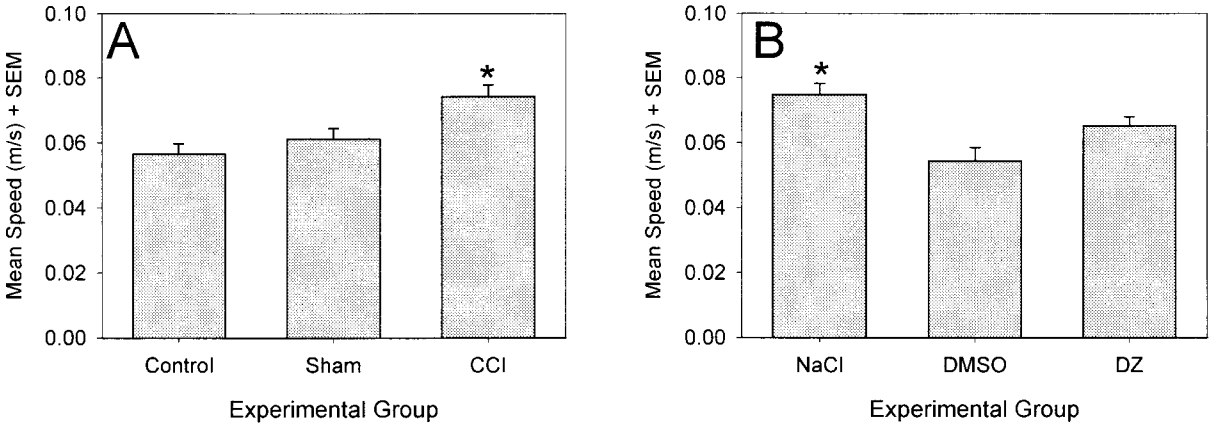

Figure 5. Morris water maze speed (m/s) on Trial 4 of the Acquisition Phase.

Diazoxide (DZ)	
Beneficial	**References**
"Preconditioning" and "Post conditioning" protection against ischemic injury	(Facundo et al., 2007; Farkas et al., 2006; Farkas et al., 2005b; Liu et al., 2002; Nagy et al., 2004; Robin et al., 2011; Wang et al., 2011; Watanabe et al., 2008; Zarch et al., 2009)
Increased cerebral blood flow (CBF) and vascular responsiveness	(Dieguez et al., 1980; Domoki et al., 2005)
Decrease oxidative and Aβ toxicity	(Goodman and Mattson, 1996; Liu et al., 2010)
Anti-inflammatory	(Virgili et al., 2011)
Anti-apoptotic	(He et al., 2008; Liu et al., 2002; Wang et al., 2011; Zarch et al., 2009)
Deleterious	**References**
Undesired Hyper/hypotension	(Virgili et al., 2011; Wang et al., 2004)
Hyperglycemia	(Arnoux et al., 2011)

Table 1: Recognized biological effects of DZ relevant to TBI.

Varied *in vivo* and *in vitro* evidence demonstrates the diverse effects of DZ on the nervous system.

Dimethylsulfoxide (DMSO)	
Beneficial	**References**
Improved neurologic outcome following brain insult	(Albin et al., 1983; Bardutzky et al., 2005; de la Torre, 1995; de la Torre et al., 1975; Farkas et al., 2005a; Karaca et al., 2002)
Sedative and antipsychotic effects	(Smith, 1992)
Reduced cerebral edema and improved blood-brain barrier integrity	(Camp et al., 1981; de la Torre et al., 1975; Hill et al., 1983; Ikeda and Long, 1990; Nagel et al., 2007)
Reduced intracranial pressure (ICP)	(Karaca et al., 1991; Kulah et al., 1990; Wolf and Simon, 1983)
Increased cerebral blood flow (CBF)	(Bardutzky et al., 2005; Karaca et al., 2002)
Free radical scavenger	(Regoli and Winston, 1999; Repine et al., 1981)
Reduced excitotoxicity	(Hulsmann et al., 1999; Lu and Mattson, 2001)
Deleterious	**References**
Fluid and electrolyte abnormality (hypernatremia), hemolysis	(Haigler and Spring, 1983; Marshall et al., 1984)
Encephalopathy, seizures, and cerebral infarct	(Abdelkefi et al., 2009; Bond et al., 1989; Hoyt et al., 2000; Yellowlees et al., 1980)
Pro-apoptotic effect	(Hanslick et al., 2009; Liu et al., 2001)
Miscellaneous	**References**
Increased exploratory behavior and locomotion	(Matheus et al., 1997)
Analgesic effects and/or modulation of other analgesic agents; allodynia; decreased nerve conduction velocity	(Authier et al., 2002; Fossum et al., 2008; Haigler and Spring, 1983)
Increases cell membrane permeability and influences cellular lipoprotein dynamics	(Gurtovenko and Anwar, 2007; Notman et al., 2006; Santos et al., 2003)

Increase cellular metabolism	(Nasrallah et al., 2008; Tsvyetlynska et al., 2005)
Alters activity of CYP isoenzymes	(Yoon et al., 2006)

Table 2: Recognized biological effects of DMSO relevant to TBI.

Varied *in vivo* and *in vitro* evidence demonstrates the diverse effects of DMSO on the nervous system.

Legends

Figure 1. Extent of injury three-weeks following moderate-CCI. Representative samples of H&E stain at 24-hrs (**A**; n=16) and 3-weeks (**B**; n=16) following moderate-CCI (n = number of animals at each timepoint). The extent of injury was limited to the ipsilateral cortex while the underlying hippocampus appears to have been spared from impactor induced hemorrhage and gross physical injury (see text for detail).

Figure 2. Beam Walk Performance. **(A)** Foot fault performance of Control, Sham, and CCI treatment groups. On all days, the CCI group exhibited the poorest performance, while the Control and Sham Groups exhibited beam walk performance that matched their pre-injury performance level (* $p<0.05$). **(B)** The CCI mice required a significantly longer amount of time to cross the beam, compared to the Control and Sham-treated mice (* $p<0.05$). **(C)** An ANOVA was performed to determine the impact of drug treatment on animals that sustained CCI. Step performance was greatly impaired on Day 1 after injury in all CCI groups whether they received NS, DMSO, or DMSO + DZ. Improvement was seen on Day 7 and Day 14, and on Day 14, DMSO + DZ treatment had significantly improved beam walk performance compared to NS treatment (& $p=0.044$). **(D)** The latency to cross the beam was impaired on Day 1, especially in the two groups that received DMSO or DMSO + DZ (* $p<0.02$), while there was no difference as a function of drug treatments (Days 7 and 14) when animals were no longer receiving DMSO.

Figure 3. Open field test performance after TBI and drug treatments. On Days 1, 7, and 14 after surgery, the CCI animals exhibited significant increases in open field mobility in terms of distance they traveled in the arena (3A), but they spent significantly less time exploring the center of the arena (3B) and on Days 7 and 14 they exhibited fewer episodes of initiation of moving in the open field (*$p<0.05$ comparing the CCI group to the Control and Sham groups on each day). On POD 14, the episodes measure for the Sham Group was also significantly less than the Control Group.

Figure 4. Morris water maze performance after TBI and drug treatments. During the acquisition phase of the Morris water maze task, the CCI-injured mice required a significantly longer amount of time (**A**) to reach the platform, compared to the Control and Sham-treated animals (* $p<0.001$). During these trials (**B**), the CCI animals likewise swam a greater distance exploring the maze before reaching the platform (& indicates $p<0.05$ comparing the Sham group to the CCI group, and $p=0.053$ compared to the Control group). During the probe trial, when the platform was removed from the tank, CCI-treated mice (**C**) spent significantly less time in the quadrant where the platform formerly was placed (* $p<0.001$), and they exhibited significantly fewer crossings (**D**) over the site where the platform formerly had been positioned (* $p<0.001$). Although not indicated in 4A, over the test days the Control group's latency was significantly longer on Day 1 compared to subsequent days, the Sham group's latency to find the platform significantly improved on Days 3 and 4, compared to the first day, and mice in the CCI group exhibited significant improvement in finding the platform on each day of testing (i.e., latency for

Day 1>2>3>4). Likewise, although not indicated in 4B, the Control and Sham group's swim distances were shorter on Days 2, 3, and 4, compared to Day 1, and the swim distance on Day 1 for the CCI animals was greater than on Days 2, 3, and 4, while the latter three days were not different.

Figure 5. Morris water maze swim speed. ANOVA indicated there was no significant difference in the speed of swimming during the first three days of training on the Morris water maze task (data not shown). On the 4th day of training, the groups exhibited an overall significant difference for the Injury factor, where the CCI-injured mice swam faster than the Control and Sham-operated mice (**A**), and the groups also differed with respect to drug treatments (**B**), where animals that had received NaCl vehicle, swam significantly faster than mice that had received DMSO or DMSO + DZ three weeks prior to testing (* $p<0.001$ comparing the group associated with the asterisk to the other two treatment conditions).

Chapter 3

Mouse brain PSA-NCAM levels are altered by graded controlled cortical impact injury

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Abstract:

Traumatic brain injury (TBI) is a worldwide endemic that results in unacceptably high morbidity and mortality. Secondary injury processes following primary injury are composed of intricate interactions between assorted molecules that ultimately dictate the degree of longer term neurological deficits. One comparatively unexplored molecule that may contribute to exacerbation of injury or enhancement of recovery is the posttranslationally modified polysialic acid form of neural cell adhesion molecule, PSA-NCAM. This molecule is a critical modulator of central nervous system plasticity and reorganization after injury. In this study, we used controlled cortical impact (CCI) to produce moderate or severe TBI in the mouse. Immunoblotting and immunohistochemical analysis were used to track the early (2, 24, and 48 hour) and late (1 and 3 week) time course and location of changes in the levels of PSA-NCAM after TBI. Variable and heterogeneous short and long-term increases or decreases in expression were found. In general, alterations in PSA-NCAM levels were seen in the cerebral cortex immediately after injury, these reductions persisted in brain regions distal to the primary injury site, especially after severe injury. This information provides a starting point to dissect the role of PSA-NCAM in TBI-related pathology and recovery.

1. Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability throughout the world. In the United States alone, the Centers for Disease Control (CDC) report that of the nearly 1.7 million people who suffer TBI each year, over 50,000 will die [1]. A substantial number of those who survive may live with long-term TBI related morbidities such as dementia, emotional and memory disorders, and musculoskeletal complaints [2-4]. Determining the range of outcomes following TBI is a challenge. Not only is there a remarkable degree of variability depending on the severity of injury but also establishing reliable and predictive diagnostic criteria and assessment tools used to evaluate treatment effectiveness has been formidable [5, 6]. It is obvious that TBI continues to be a significant worldwide problem demanding effective therapy that affects high yield molecular targets to decrease morbidity and mortality—a demand that has, to this point, not been met.

Studies aimed at identifying molecular targets that may improve survivability and decrease disability following TBI are manifold. However, in spite of decades of research and promising results in pre-clinical animal studies, no single effective therapy has successfully and consistently transferred to human clinical trials [7, 8]. It is now understood that multiple pathways converge and diverge in an intricate balance that either preserves normal function or spirals into pathology. Following the immediate and irreversible primary injury that results from the mechanical stress produced by the energy of a traumatic event, delayed and multifactorial secondary injury processes are initiated. These processes include excitotoxicity, oxidative stress, mitochondrial dysfunction, edema, inflammation, and cell death [9, 10]. Considering the heterogeneity of secondary

injury processes, it can be appreciated that the levels and functional capacity of receptors and other proteins and their respective ligands and downstream signaling cascades may be adversely affected.

One molecule that may serve as a harbinger of the complex balance between central nervous system (CNS) post-injury amelioration and exacerbation is neural cell adhesion molecule (NCAM). Described over 30-years ago, NCAM was the first cell adhesion molecule (CAM) identified [11]. NCAMs are cell surface glycoprotein members of the immunoglobulin superfamily of CAMs that are expressed in abundance in neural tissue as well as the lung, kidney, skeletal and heart muscle, and various cancers [12, 13]. Alternate splicing of at least 26 exons contained in the single NCAM gene produces three major isoforms of NCAM: NCAM-180, NCAM-140, and NCAM-120 [14]. These isoforms are categorized based upon their apparent molecular weight in kDa, and each is attached to the cell membrane via a class 1 transmembrane protein (NCAM 180 and 140) or glycosoylphosphatidylinositol (GPI) anchor (NCAM 120) [15]. Regarding neural tissue expression, the general temporal and cell-type location of each isoform has been characterized. NCAM-180 expression is limited to neurons, NCAM-140 is expressed in neurons and glia, and NCAM-120 is primarily expressed in glia [16, 17].

Of paramount importance in maintaining the differentiation, migration, and anchoring function of NCAM during development and neurogenesis and cell survival functions in the face of insult during adulthood is the degree of expression of the posttranslationally glycosylated NCAM isoforms manifesting the large, negatively charged α2,8-linked N-acetylneuraminic homopolymer named polysialic acid (PSA-

NCAM). The necessity of polysialyation for efficient function of select NCAM isoforms has recently been appreciated (see Gascon et al. [[18]] for review). In addition to the indispensable role of PSA-NCAM in maintaining synaptic plasticity and health, recent evidence has also demonstrated the fundamental role of PSA-NCAM in preventing neurodegeneration in the face of ongoing insult such as epileptogenic central nervous system disorders [19]. Additionally, repair and recovery following TBI may be critically dependent on the generation, survival, migration, and integration of neurons from postnatal neurogenic niches into damaged circuitry in order to restore function. PSA-NCAM has been shown to be required for the survival of newly generated neurons *in vitro* [20, 21] and *in vivo* [22]. Efficient migration of neuroblasts also appears to be reliant on PSA-NCAM expression [23]. And finally, the presence or absence of PSA-NCAM at the appropriate time following generation is believed to allow for the proper differentiation of neuronal precursors at the destined endpoint of migration [23, 24].

Considering the essential contributions of PSA-NCAM to efficient CNS function, it may be presumed that, at least in part, alterations in expression levels and function are involved in the complex pathology that follows TBI. To date, the short and long-term alterations in expression levels of PSA-NCAM following graded-controlled cortical impact (CCI) in the mouse have not been characterized. The current study examines the immediate (two, 24, and 48 hour) and long-term (one and three week) change in the expression levels of PSA-NCAM in eight distinct regions of the mouse brain following graded CCI that results in either moderate or severe injury. An understanding of the time course and regional distribution of PSA-NCAM alterations following TBI may contribute to the knowledge base that is being assembled to identify novel therapeutic targets.

2. Materials and Methods

2.1 Experimental Groups

All experimental procedures used in this investigation were approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC). Six to nine-week old male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) weighing 20–29 g were housed in an animal colony at a constant temperature (23 $\pm$ 2°C) with a 12-hour light/dark cycle and food and water *ad libitum* for at least three-days prior to surgery. Mice were assigned to immunohistological (IHC) or western blot analysis groups that were sacrificed at either two, 24, or 48 hour, or one or three week post injury time points. Either four or ten animals per injury severity group per time point were assigned for IHC or western blot analysis, respectively. Twenty additional animals were processed at 24 hours or three weeks for assessment of hemorrhage and structural alterations using whole brain and coronal section analysis.

2.2 Surgery

Induction of mice was performed via spontaneous ventilation using 3% isoflurane in 100% oxygen (1.0 L per minute flow rate) for 3-minutes in a rodent volatile anesthesia box. After the application of protective ointment (Lacri-Lube®) to the eyes, the head was shaved using electric clippers. Following hair removal, the head of the animal was placed in a standard stereotaxic frame and positioned using ear and incisor bars (Stoelting, Wood Dale, IL) and the skin was prepped with betadine ointment. Following skin preparation, 0.1 ml of 0.025% bupivacaine was injected subcutaneously into the planned incision site. Rectal temperature was maintained at 37°C with an isothermal heating pad and feedback controller (Stoelting, Wood Dale, IL). Anesthesia was maintained with 1.5–2%

isoflurane. Unilateral CCI to the left cerebral hemisphere was performed using a modified technique previously described [25]. In brief, a 10-mm midline incision was made to expose the bregma and lambda sutures. Skin and fascia were reflected, and a 5-mm craniectomy was performed over the left parietotemporal cortex using a 0.75-mm bit attached to an electric rotary drill. A 4x microscope (Zeiss, Thornwood, NY) was used to visualize the skull during craniectomy, and intermittent saline irrigation was performed to reduce intensity of heat generated during drilling. Care was taken to avoid mechanical injury to the dura mater, and dural integrity was examined prior to impactor positioning. The impactor was positioned at a 15 degree angle to account for curvature of the skull, and the right edge of the impactor was aligned with the midline suture while the posterior edge was aligned with the horizontal portion of the lambda. The position of impact was reached after the impactor was moved 1.5 mm anteriorly and 1.2 mm to the left, thus producing a center of impact 3.0 mm anterior to the lambda and 2.7 mm left of midline. The CCI device (Impact One^{TM}, Leica, Wetzlar, Germany) consisted of a computer-controlled, electromagnetically-driven impactor fitted with a 3.0-mm-diameter steel tip mounted on a stereotaxic micromanipulator. The impactor tip was positioned by bringing it into contact with the exposed dura and retracted prior to setting an impact depth of 1.0 or 2.5 mm for moderate or severe injury, respectively. The goal of moderate injury was to avoid gross hemorrhage in the underlying hippocampus in an attempt to assess the effects of a possible secondary injury response on PSA-NCAM levels in regions removed from direct injury. Speed of impact was set to 5 meters/sec, and dwell time was 0.1 seconds. Following injury, the skull fragment was carefully replaced, and the incision was closed using interrupted 4.0 silk sutures. Sham mice were subjected to all of the described

surgical procedures but did not receive CCI. All surgical procedures were performed in a sterile manner. All animals received a subcutaneous injection of 0.5 ml of 37°C 0.9% sodium chloride at the conclusion of the procedure to combat dehydration. Mice were placed in a heated cage to maintain body temperature until fully awake, after which they were returned to their home cage.

2.3 Tissue Processing

Processing for Hemorrhage Evaluation and Tissue Disruption

Twenty-four hours following graded-CCI, four sham, eight moderate, and four severe injury mice were administered deep anesthesia (60 mg/kg ketamine with 60 mg/kg xylazine, i.p.). The animals were perfused intracardially with heparinized (1,000 u/L) 0.9% sodium chloride followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Following decapitation, two sham, six moderate, and two severe injury brains were harvested for whole brain and coronal video microscopy (Dazor Speck Finder, St. Louis, MO). Additionally, one moderate and one severe injury animal was sacrificed at three weeks. Whole brain and 1 mm coronal sections through the epicenter of the impact site were imaged to assess the extent of hemorrhage and gross tissue disruption in the brain. The remaining brains were postfixed in the skull overnight, and then removed from the skull and sequentially cryoprotected in 20 and 30% sucrose in phosphate buffer saline (PBS) until the brains sank. A frozen sliding microtome was used to acquire 30-μm-thick coronal sections from the olfactory bulbs to the rostral cerebellum. Brain slices were stored in cryoprotectant [26] at -20°C until processed for assessment using standard hematoxylin and eosin (H&E) staining technique.

Immunoblot Assessment

Western blot analysis was performed as previously described [27, 28]. Briefly, at the time of sacrifice, each brain designated for western blot analysis was dissected on a chilled stage into eight regions (left and right cerebral cortex, hippocampus, and temporal lobe; the diencephalon and cerebellum were dissected as whole units) via a modified approach previously detailed [29]. The tissue was immediately transferred to storage in liquid nitrogen until protein extraction was performed. For protein extraction, the frozen tissue was transferred to centrifuge tubes containing ice-cold lysis buffer (50mM Tris HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 0.5% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.1% sodium deoxycholate) with Complete Mini protease inhibitor tablets (Roche, Indianapolis, IN). Brief sonication was performed to ensure dismembranation, and the samples were tumbled at 4°C for one hour. Samples were centrifuged at 12000 rpm for 20 min at 4°C to separate the supernatant from the pellet. The supernatant was removed and protein concentrations were determined with a BCA kit (Pierce, Rockford, IL). Each sample protein concentration was normalized to either 3 or 5 μg/μl (determined by brain region). Equivalent amounts of protein (27–30 μg) per sample were migrated through SDS-PAGE on 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA). Proteins were electrophoretically transferred to a nitrocellulose membrane (Invitrogen, Carlsbad, CA) that was immersed for one hour at 22°C with 5% nonfat dried milk, and then probed with primary antibodies overnight at 4°C to evaluate levels of PSA-NCAM after CCI. An affinity-purified mouse monoclonal antibody raised against α-2-8 linked neuramic acid (PSA) (1:2000; clone 2-2B, Millipore, Billerica, MA) was used. Membranes were then exposed to a donkey anti-

mouse HRP-conjugated secondary antibody (1:5000; Vector Laboratories, Burlingame, CA) for 1.5 hours at 22°C. Detection of antibody binding was performed using enhanced chemiluminescence (ECL; Millipore, Billerica, MA) and captured with the Fuji LAS-3000 imaging system (Fujifilm, Valhalla, NY). To ensure equal loading of protein, membranes were stripped and re-probed with an anti-β-actin antibody (1:10000; clone AC-15, Sigma-Aldrich, St. Louis, MO) and an HRP-conjugated anti-mouse secondary antibody (1:20000; Vector Laboratories, Burlingame, CA). Expression of protein was quantified by normalizing the integrated density value (IDV) of the PSA-NCAM band to the IDV of the actin bands using Quantity One® image analysis software (Bio-Rad, Hercules, CA).

Immunohistochemistry Assessment

At the designated harvest times, additional mice were euthanized (60 mg/kg ketamine with 60 mg/kg xylazine, i.p.) and brains were processed for immunohistochemical analysis using a modified technique previously described [30]. All animals were sacrificed in the early afternoon (1200–1500 hrs) in order to account for the rhythmic variation in PSA-NCAM level expression in the hypothalamus (see below). In brief, mice were perfused intracardially with heparinized (1,000 u/L) 0.9% sodium chloride followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Following decapitation, the brains were postfixed in the skull overnight, and then removed from the skull and sequentially cryoprotected in 20 and 30% sucrose in phosphate buffer saline (PBS) until the brains sank. A sliding microtome was used to acquire frozen 30-μm-thick coronal sections from approximately 1.54 mm anterior to 5.02 mm posterior to the bregma. Brain slices were stored in cryoprotectant at -20°C until needed [26].

For PSA-NCAM immunohistochemical evaluation, with diaminobenzidine as the chromogen, a modified technique previously described was utilized [30, 31]. Briefly, intermittent sections spanning the above noted distance were washed in 0.01 M PBS for three 10-minute cycles on an orbital shaker to remove residual cryoprotectant. Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in 0.01 M PBS for 30 minutes followed by a one hour incubation in 5% normal goat serum (NGS) with 0.1% Triton X-100 to block nonspecific binding sites. Sections were then incubated with antibody to PSA-NCAM (1:1000; clone 2-2B, mouse monoclonal, Millipore, Billerica, MA) overnight at 4°C in 0.01M PBS with 5% NGS. Following three 10 min wash cycles in 0.01M PBS the slices were incubated with biotinylated goat anti-rat or anti-mouse antibody (1:300; Jackson ImmunoResearch) in 2% NGS and 0.05% Triton X-100 in 0.01 M PBS for 30 minutes. Following further washes, incubation in the avidin-biotin-horseradish peroxidase (HRP) complex with DAB (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) was performed for two minutes. Sections were washed, mounted on gelatin coated slides, cleared with xylenes, and coverslipped for examination via light microscopy (Zeiss Axiovert 200, New York, NY). Images were captured with a digital camera and documented using the Axiovision 4.2 software (Zeiss) package. Additionally, images were scanned and examined using the Nanozoomer microscopy apparatus and software suite (Hamamatsu, Bridgewater, NJ). As a negative control, primary antibody was omitted and sections were incubated with secondary antibody and the ABC reagent prior to exposure to DAB. No immunoreactivity was noted in control tissue in which the secondary antibody was withheld. Immunohistochemistry findings using DAB were evaluated by two blinded

investigators. Unaltered images of the regions of interest were presented as pairs using the Microsoft Powerpoint© slideshow format. Each evaluator was asked to determine which image appeared to present with the more intense degree of staining.

2.4 Statistical Analysis

Quantitative western blot measurements of PSA-NCAM were normalized by dividing optical density values by corresponding measures of β-actin derived from the same samples in each blot. All data are expressed as the mean value ± standard error (SEM) for PSA-NCAM/ β-actin where data across specimens were normalized to sham mean density = 1. Statistical analyses were performed using GraphPad Prism (v.5; La Jolla, CA) statistical package. Injury-induced alterations in PSA-NCAM levels in immunoblot samples were compared to sham animals using a one-way ANOVA followed by Tukey's HSD test if ANOVA analysis rejected the null hypothesis. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1 Extent of Injury

Examination of tissue samples obtained 24 hours after injury revealed a progressively larger hemorrhagic lesion of the brain in the graded-CCI animals that correlated well with the depth of impact. Sham animals demonstrated no obvious injury (Figs. 1A and B). Five of the six moderate injury (1 mm depth of impact) animals examined 24 hours after CCI had evident hemorrhagic lesions of the cortex with extension to the corpus callosum (Figs. 1C and D). There appeared to be no apparent injury to the hippocampus, and H & E staining supported this finding (Fig. 1G). The severe injury specimens (2.5 mm CCI depth) revealed widespread hemorrhage and

structural damage to the ipsilateral cortex, hippocampus, and thalamus (Fig. 1E and F; H&E Fig. 1H), that partially extended to the contralateral hemisphere. Evaluation of severe injury specimens obtained three weeks after CCI revealed extensive cavitation of the ipsilateral hemisphere with significant thalamic deformation (Fig. 1I; H&E Fig. 1K). Moderate injury samples had significantly less evidence of gross anatomical distortion (Fig. 1J); however, expansion of the dorsal ipsilateral hippocampus into the region previously occupied by the cortex was noted (Fig. 1L).

3.2 Quantitative Immunoblot Findings

Short Term Alterations in PSA-NCAM Levels after TBI

Western blot analysis of acute PSA-NCAM level alterations following moderate TBI revealed heterogeneity as a function of brain region (Fig. 2A). Immediately following injury, the left cortex (the site of direct primary injury) as well as the contralateral right cortex exhibited decreased PSA-NCAM levels, while the other six brain regions examined did not have significant changes relative to the sham-treated group. At the 24 hour post-injury time point, the left cerebral cortex region displayed increased levels after moderate injury and reduced PSA-NCAM levels following severe CCI. Additionally, the right cortex responded to moderate injury with decreased PSA-NCAM levels while an increase in PSA-NCAM levels in response to severe injury was seen in the cerebellum. A more global response was seen 48 hours following CCI. Moderate injury induced an increase in PSA-NCAM expression in the diencephalon and a decreased expression in the right hippocampus and cerebellum. Severe injury produced a decrease in PSA-NCAM expression in the right cortex and hippocampus and an increase in PSA-NCAM expression in the left temporal lobe 48 hrs after CCI

Long Term Alterations in PSA-NCAM Levels after TBI

PSA-NCAM levels were evaluated one and three weeks after CCI (Fig. 2B). Seven days after CCI, PSA-NCAM changes were notable in some brain regions quite distal to the primary impact site. Levels in the left hippocampus, underlying the impact site, were elevated while more distal brain regions (the left and right temporal lobe, diencephalon, and cerebellum) exhibited significantly reduced levels; particularly after severe CCI. Western blot results for one week samples are presented in Fig. 3. Reduced levels persisted in the left temporal lobe, three weeks after injury, and were still evident in the diencephalon; even after moderate CCI. Severe injury resulted in decreased PSA-NCAM expression in the cerebellum at three weeks.

3.3 Qualitative Immunohistochemistry Findings

Qualitative evaluation of tissue slices was undertaken to further examine PSA-NCAM changes seen with western blotting. The approach was to carefully survey all regions of brain sections that were obtained at the indicated time points after injury that we believed would produce large enough alterations in expression as to be detected using microscopy. In spite of attempts to process tissue sections identically, variability within groups was observed, so that qualitative findings intermittently supported the trends revealed with immunoblotting. For example, the two hour samples from immunoblot analysis indicated that PSA-NCAM expression decreased in the right cortex of both injury groups at this time point, but a noticeable difference between groups was not reliably observed using immunohistochemical evaluation (data not shown). Likewise, PSA-NCAM expression in the medial prefrontal cortex did not appear to be different between groups. These observations are partially anticipated as the general PSA-NCAM

expression pattern in most of the cortex does not show a distinct structural distribution (with exceptions; see below for temporal lobe PSA-NCAM staining) and appears as faint labeling of neuropil or immunonegative [32]. In agreement with immunoblot findings, assessment of right sham and severe injury hippocampi revealed a distinct decrease in PSA-NCAM staining of the soma and processes of cells located in the subgranular zone of the dentate gyrus of severely injured animals (Fig. 4). Assessment of the left cortex at one week confirmed the increased PSA-NCAM expression levels seen in moderate injury animals (Fig. 5). Of note, immunohistochemical evaluation revealed that expression of PSA-NCAM on cells displaying morphological characteristics of reactive astrocytes accounts for a significant proportion of the increase. Additionally, evaluation of the left cortex and temporal lobes one week following graded-CCI also confirmed immunoblot data; an obvious decrease of PSA-NCAM labeling of the soma and apical dendrites (Fig. 6) of what are most likely layer II semilunar cells of the olfactory cortex [33].

4. Discussion

4.1 Alterations in PSA-NCAM after TBI

Originally thought to be only expressed in the CNS during embryonic development [34], PSA-NCAM manifestation in distinct regions of the adult CNS illustrates an important lifelong role of the molecule in maintaining nervous system plasticity. Of note, PSA-NCAM is constitutively expressed in some brain regions related to neuroendocrine and behavioral functions [35, 36]. Recent findings also indicate that NCAM function can be dramatically altered by multiple factors, and that the biosynthesis of PSA-NCAM is intimately related to increases in cell axon activity and alterations in cell input [37].

The qualitative and quantitative distribution of PSA-NCAM in the uninjured adult CNS has been examined and characterized in previous studies [32], but no reports describe the fate of PSA-NCAM in the mechanically contused brain. The results of the present study show that graded-CCI induces short and long-term increases and decreases in PSA-NCAM levels in a range of brain areas. Furthermore, the alterations in these levels were heterogeneous and complex events over time with distinct patterns of change across anatomically separated brain regions. These alterations may have important implications for understanding neural plasticity changes and repair responses following brain injury, including proximal and distal interactions between various brain regions as a function of injury severity.

Following injury in the parietal cortex, levels exhibited fluctuations that included an initial increase in PSA-NCAM levels in the region of primary impact in the left parietal cortex in moderately-injured animals, while levels were depressed when the injury was severe. Correspondingly, in the contralateral hemisphere, PSA-NCAM reductions in the corresponding parietal cortex remained for a longer time after moderate injury and were persistently depressed after severe CCI. Perhaps the final outcome following CCI is telling. At three weeks after CCI, levels of PSA-NCAM were elevated in the left parietal lobe that sustained primary injury, while levels were reduced in the right parietal cortex and the diencephalon. In the left temporal lobe, levels were not reduced during the initial two days after injury, but then were depressed by the first and third week post-injury. However, in the contralateral temporal lobe, PSA-NCAM levels were unaffected by the injury. These data may belie the overall direct versus indirect connections of the injured parietal cortex with proximal and more remote structures.

Specifically, by three weeks after injury there was a "local" increase in PSA-NCAM levels as a result of reorganization at the primary site of injury, while regions with more direct and robust connections to the injured site (the thalamus, the contralateral parietal region, and the ipsilateral temporal lobe) exhibited diminished levels. Conversely, the more distal, contralateral temporal lobe appears to have been unaffected in terms of PSA-NCAM levels. Although we were unable to fully corroborate the PSA-NCAM changes seen in the hippocampus via western blotting efforts with the results obtained using IHC, these alterations too may have implications in long term recovery. For example, the immunoblot data indicated an increase in PSA-NCAM levels at one week in either the ipsilateral or contralateral hippocampi following severe or moderate TBI, respectively. Even though variability in the intensity of staining prevented us from isolating the location and degree of these changes, these alterations may indicate the response of a group of immature neurons in the subgranular zone reacting to injury [38]. This increase in PSA may promote efficient migration of these neurons to the site of injury and reintegration of into hippocampal circuits [39]. Additionally, the increased PSA expression may also be occurring on reactive astrocytes as a component of the formation of a glial scar.

While the present results are one of the first to examine PSA-NCAM alterations after controlled cortical impact, the role of PSA-NCAM in pro-survival pathways initiated during CNS excitotoxicity insult has been investigated [19]. In this study, endoneuraminidase (endoN) removal of PSA from NCAM following hippocampal injection of kainic acid (KA) in mice was used to determine that PSA-NCAM serves as an indispensable co-receptor of GDNF family receptor α1 (GFRα1) to mediate pro-

survival Ret-independent glial cell-derived neurotrophic factor (GDNF) initiated Fyn-FAK-MAP kinase pathway activation. It was determined that retrograde neurodegeneration was reduced in the presence of PSA-NCAM in the contralateral hippocampus, thus demonstrating the ability of PSA-NCAM to affect survival in the KA-treated hippocampus via GDNF signaling across the dorsal hippocampal commissure [19]. Additionally, an interesting neuroprotective relationship between heat shock protein 70 (HSP-70) and PSA-NCAM has been revealed [38, 40]. PSA-NCAM has also been shown to protect hippocampal neurons from glutamate-induced excitotoxic death most likely by inhibition of NR2B subunit-containing NMDA receptors [41]. These investigations serve as evidence supporting the importance of PSA-NCAM on the cell survival response following injury. Post-injury alterations in PSA-NCAM expression demonstrated in our study makes it difficult to confidently explain the significance of an alteration in an isolated brain region at a specific time point. However, an early decrease in PSA-NCAM expression may perpetuate a feed-forward injury cycle that could amplify long-term neuropathology. Conversely, the increased PSA-NCAM at the primary injury site noted in our study at one and three weeks may reflect an attempt to enhance progenitor differentiation and neuronal survival or act as a compensatory mechanism in response to probable TBI-induced BDNF dysfunction [42-44].

4.2 Therapeutic Potential

PSA-NCAM's involvement in cell signaling is both far reaching and essential for proper nervous system function. Figure 7 presents an overview of the currently understood signaling pathways initiated by NCAM and PSA-NCAM. Neurotrophin activity (i.e., brain-derived neurotrophic factor (BDNF)) ensuring appropriate neuron

differentiation, plasticity, and ultimately survival and death appears to require PSA expression [18]. The interaction of PSA –NCAM with tyrosine kinase (Trk) and tumor necrosis factor (TNF) receptors is the most likely mechanism of action [46, 47]. Broadly speaking, the interaction between PSA-NCAM and various cell adhesion molecules, proteoglycans, and cell surface receptors allows it to serve as a modulator of cell-cell and cell-extracellular matrix interactions in order to allow for preservation and migration of neuronal and glial progenitors, modulation of synaptic plasticity, and the refinement of survival and maintenance roles throughout the cell life cycle [45].

Do the alterations in PSA-NCAM expression following TBI present as possible targets of therapy? The ability of PSA to modulate cell interactions in order to promote plasticity, precursor cell migration, and axonal defasiculation and targeting, raises the possibility of using PSA gain-of-function to augment the regenerative response of the brain to injury. Our study reveals several potential therapeutic treatment windows for PSA therapy to promote function of endogenous recovery mechanisms while combating the post injury inhibitory environment in the brain. For example, PSA-NCAM expression was significantly decreased three weeks following injury in multiple brain regions including the cerebral and temporal cortices, hippocampus, diencephalon, and cerebellum following moderate or severe injury. In some cases, this decrease followed an earlier period of increased expression of PSA-NCAM (Fig. 2A and B). Multiple studies reinforce the idea of overexpressing PSA in astrocytes or Schwann cells to produce a permissive environment for axonal regeneration following brain injury. For example, to maintain and increase PSA expression in the cerebellum of L1/GAP-43 transgenic mice, a lentiviral vector carrying polysialytransferase (ST8SiaIV) cDNA was injected into a

cerebellar stab wound or used to transfect Schwann cells that were subsequently transplanted into the site of injury [48]. As a result of these interventions, Purkinje cell axonal sprouting was enhanced, and the density of sprouting in the PSA-enhanced Schwann cell graft was nine times that that seen in non-PSA vector transfected grafts at two months. The *overexpression* of PSA appears to be critical to this process because in the cerebellum of wild-type mice PSA is present, and late axonal sprouting occurs, at the injury site 12 months after axotomy [49]. Perhaps the anti-adhesive properties of an immediate increase in PSA expression in the wound following injury could combat the inhibitory effect of chondroitin sulfate proteoglycans (CSPGs) that are expressed rapidly following injury [50]. *In vitro* and *in vivo* studies have noted that ectopic expression of PSA in Schwann cells has been shown to improve initial post-injury migration and enhance earlier and more effective remyelination when compared to endogenous Schwann cells [51, 52]. Because PSA seems to be a permissive molecule that does not override normal cell interactions, it does not appear to have a deleterious effect on uninjured tissue function [53]. Additionally, increased PSA expression in the cortex and hippocampus could enhance the migration of progenitors from the SVZ and SGZ to the site of injury [54]. All of the above evidence provides an encouraging foundation to begin exploring NCAM and PSA-NCAM CNS repair efforts with such efforts as mimetic compounds and molecules that alter biosynthesis of these versatile and potentially healing adhesion molecules.

4.3 Summary

Traumatic brain injury is a devastating and life altering event that is sharply increasing in incidence throughout the world, particularly among military members, as

well as in civilian populations in developing countries where there is a recent increase in motor vehicle use [55]. An ever expanding understanding of the mechanisms responsible for the acute and chronic pathology following TBI has allowed for epidemiological associations to be made between TBI and the development of chronic neurodegenerative disease. In particular, an appreciation of the complex workings of the secondary injury cascade has led to the exploration of the role of several molecules as contributors to pathological processes or potential therapeutic targets. A particular class of adhesion molecule that has received little attention regarding a potential role in TBI is PSA-NCAM.

In this study we examined changes in PSA-NCAM expression following graded-CCI in the mouse. Severe and moderate injury produced immediate as well as long term alterations in PSA-NCAM expression both proximal and distal to the impact site. Alterations in these species of adhesion molecule have been shown to result in acute and long lasting alterations in neuron migration, neurite formation and axon fasciculation, synapse development and function, memory function, and emotional status. The significant expression level changes seen in our study may contribute to dysfunction and/or healing following injury.

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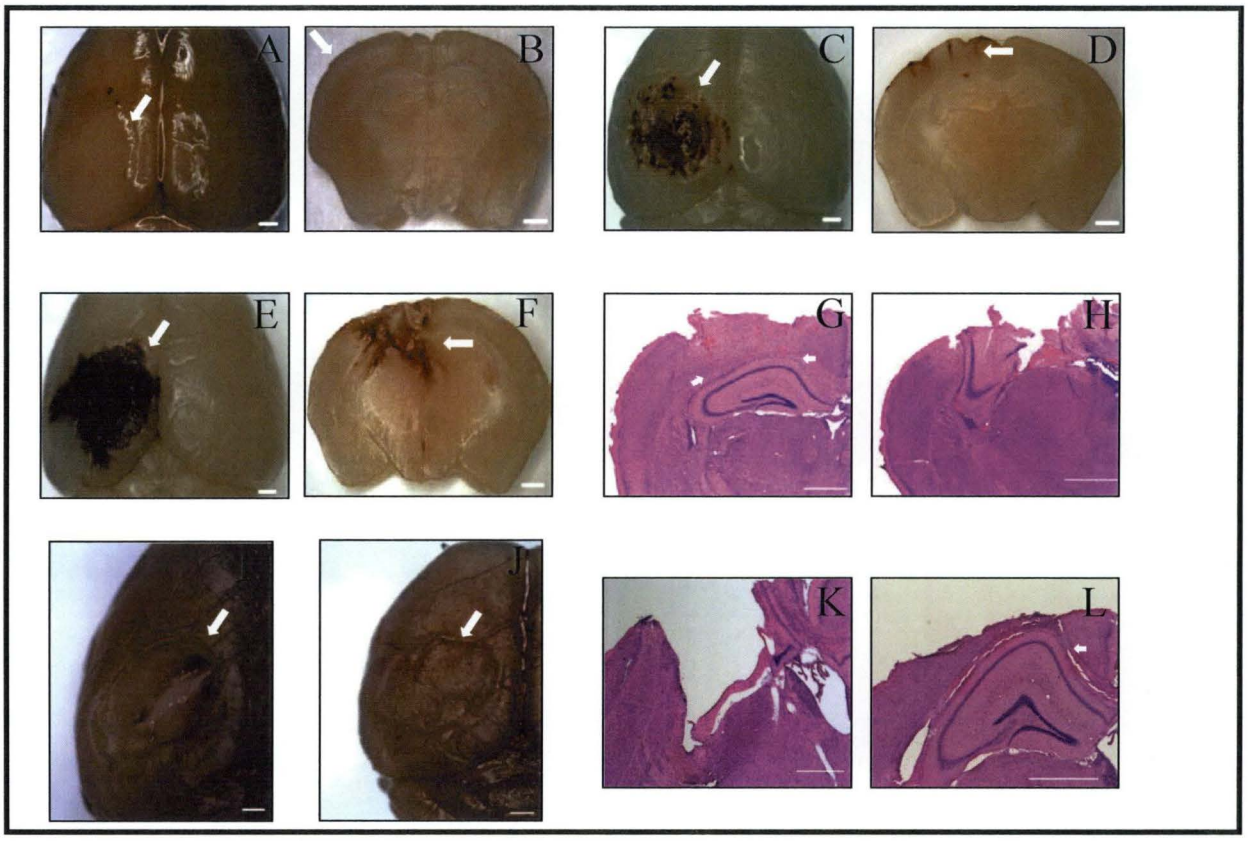
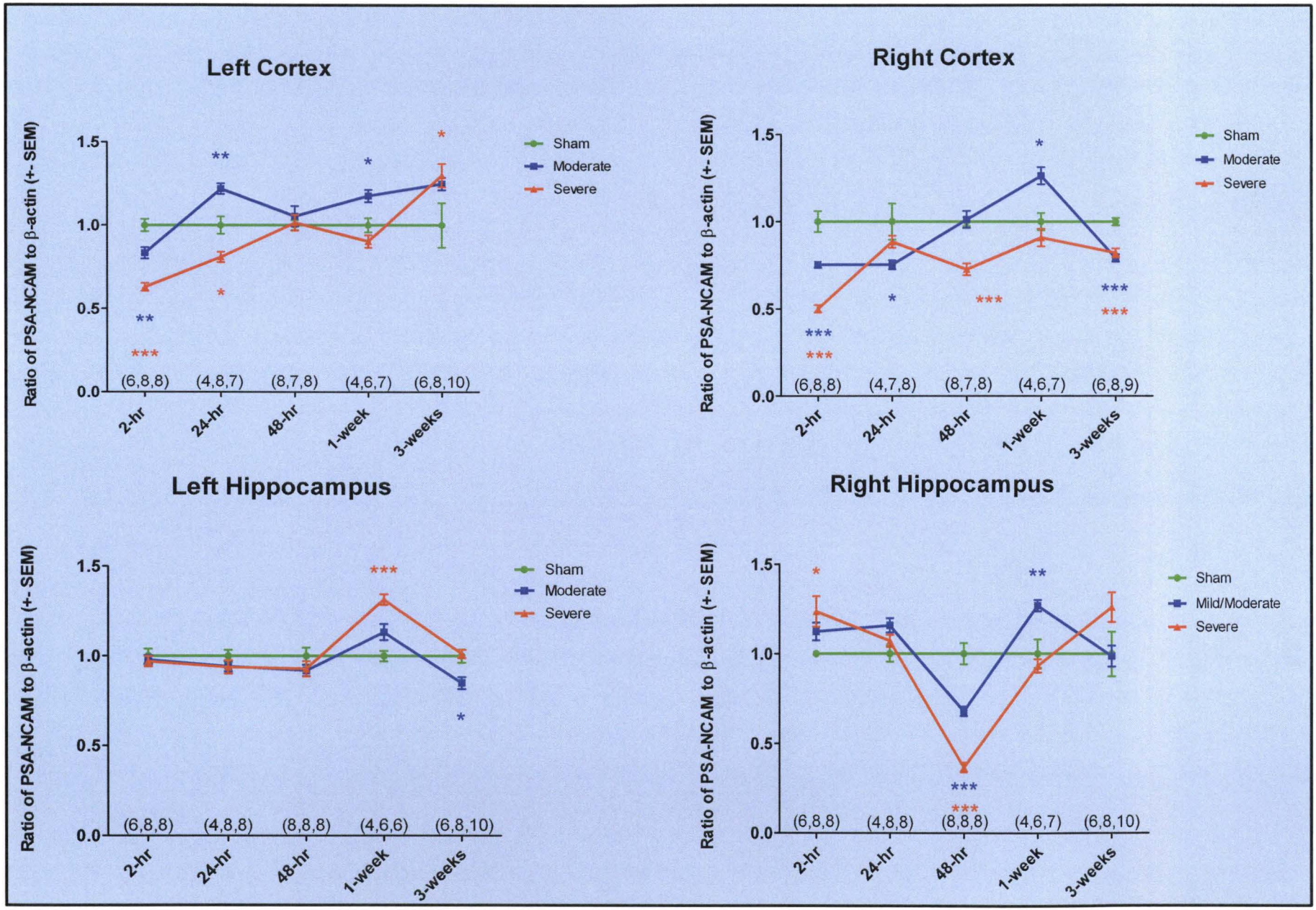


Figure 1. Grading injury severity.

Whole brain and coronal sections displaying injury severity at 24 hours in sham (A, B), moderate (C,D), and severe (E, F) injury groups. Coronal H&E sections revealed no apparent hemorrhage in the underlying hippocampus in the moderate injury animals 24 hours following injury (G). Severe injury revealed extensive hippocampal and thalamic injury at the 24 hour collection point (H). Lesions on the cerebral surface were evident three weeks following severe injury (I). Moderate injury whole brain samples displayed no apparent surface defect three weeks following injury (J). Coronal sections from brain samples obtained 3 weeks after CCI revealed graded substantial cortical and hippocampal damage (K) while moderate CCI resulted in loss of cortical tissue and

deformity and expansion of hippocampal neuropil (L). Arrows denote region of interest. Coronal sections approximate bregma -1.70. Scale bar equals 1 mm.


Left Cortex
Ratio of PSA-NCAM to β-actin (+- SEM)
1.5
1.0
0.5
0.0
Sham
Moderate
Severe
(6,8,8)
(4,8,7)
(8,7,8)
(4,6,7)
(6,8,10)
2-hr
24-hr
48-hr
1-week
3-weeks
Right Cortex
Ratio of PSA-NCAM to β-actin (+- SEM)
Sham
Moderate
Severe
(6,8,8)
(4,7,8)
(8,7,8)
(4,6,7)
(6,8,9)
2-hr
24-hr
48-hr
1-week
3-weeks
Left Hippocampus
Ratio of PSA-NCAM to β-actin (+- SEM)
Sham
Moderate
Severe
(6,8,8)
(4,8,8)
(8,8,8)
(4,6,6)
(6,8,10)
2-hr
24-hr
48-hr
1-week
3-weeks
Right Hippocampus
Ratio of PSA-NCAM to β-actin (+- SEM)
Sham
Mild/Moderate
Severe
(6,8,8)
(4,8,8)
(8,8,8)
(4,6,7)
(6,8,10)
2-hr
24-hr
48-hr
1-week
3-weeks

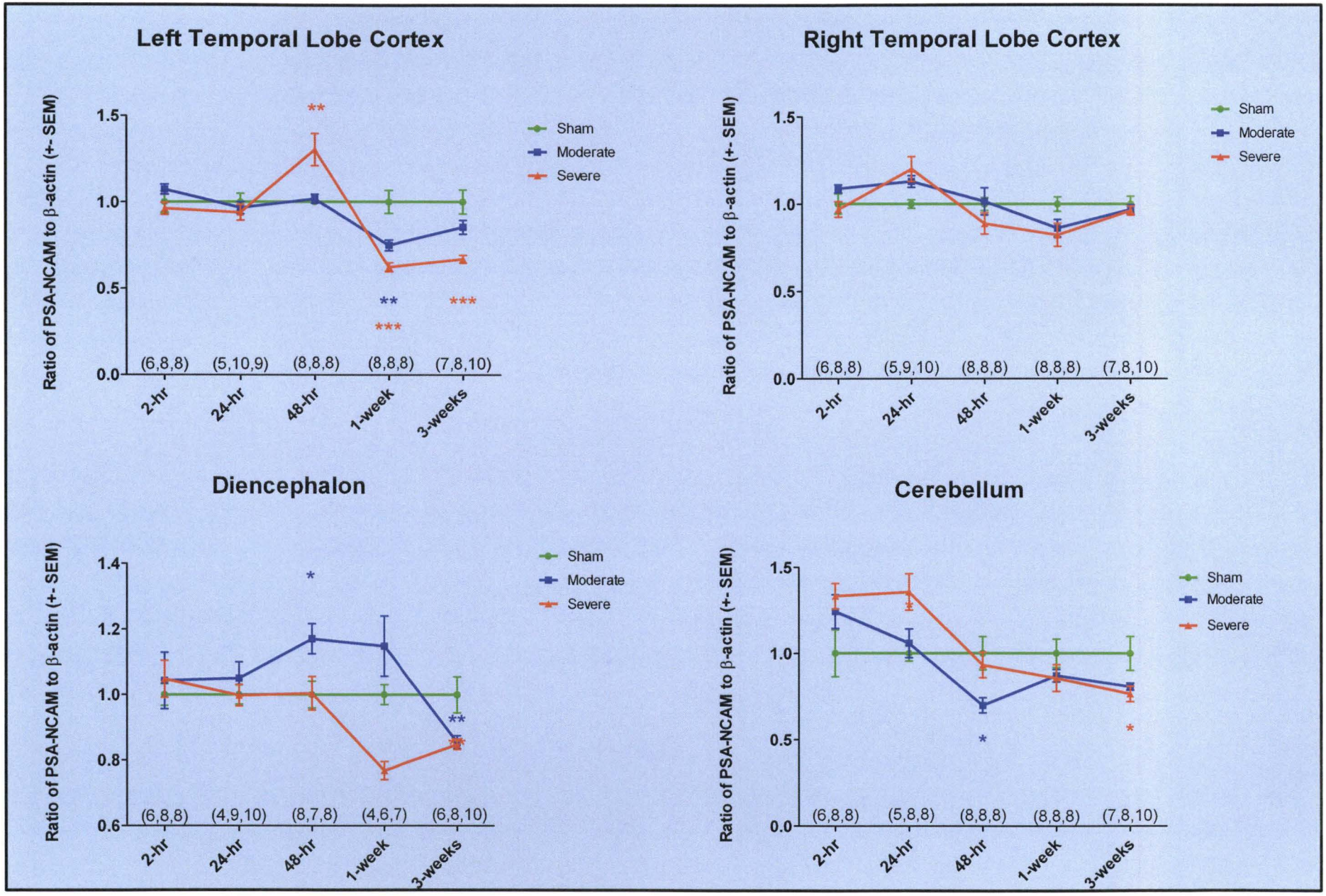

Left Temporal Lobe Cortex
Ratio of PSA-NCAM to β-actin (+- SEM)
1.5
1.0
0.5
0.0
**
**
***
***
(6,8,8)
(5,10,9)
(8,8,8)
(8,8,8)
(7,8,10)
2-hr
24-hr
48-hr
1-week
3-weeks
Sham
Moderate
Severe
Right Temporal Lobe Cortex
Ratio of PSA-NCAM to β-actin (+- SEM)
1.5
1.0
0.5
0.0
(6,8,8)
(5,9,10)
(8,8,8)
(8,8,8)
(7,8,10)
2-hr
24-hr
48-hr
1-week
3-weeks
Sham
Moderate
Severe
Diencephalon
Ratio of PSA-NCAM to β-actin (+- SEM)
1.4
1.2
1.0
0.8
0.6
*
**
(6,8,8)
(4,9,10)
(8,7,8)
(4,6,7)
(6,8,10)
2-hr
24-hr
48-hr
1-week
3-weeks
Sham
Moderate
Severe
Cerebellum
Ratio of PSA-NCAM to β-actin (+- SEM)
1.5
1.0
0.5
0.0
*
*
(6,8,8)
(5,8,8)
(8,8,8)
(8,8,8)
(7,8,10)
2-hr
24-hr
48-hr
1-week
3-weeks
Sham
Moderate
Severe

Figure 2. Quantitative analysis of PSA-NCAM levels following graded-CCI.

Short and long-term changes in expression levels of PSA-NCAM in the eight brain regions evaluated using western blotting. Graphs depict mean PSA-NCAM density differences ± SEM of injury groups compared to the sham group (adjusted to mean = 1). Star color indicates statistical significance of difference between injury and sham values via one-way ANOVA and *post hoc* Tukey's test (*= $p<0.05$, ** = $p<0.01$, *** = $p<0.001$). Animal numbers per group (Sham, Moderate, Severe) for each brain region are indicated in parentheses above the abscissa.

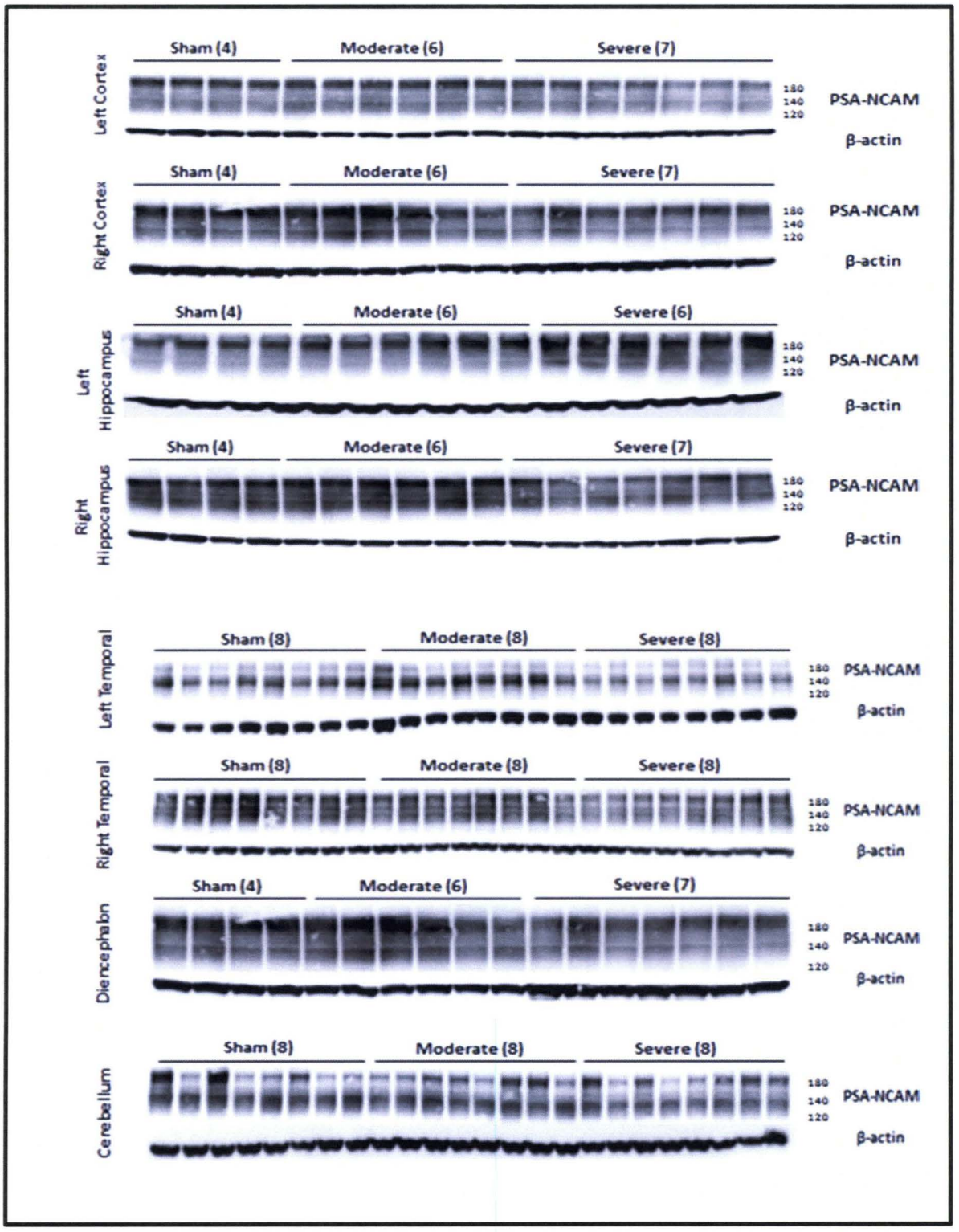

Sham (4)
Moderate (6)
Severe (7)
Left Cortex
180
140
120
PSA-NCAM
β-actin
Sham (4)
Moderate (6)
Severe (7)
Right Cortex
180
140
120
PSA-NCAM
β-actin
Sham (4)
Moderate (6)
Severe (6)
Left Hippocampus
180
140
120
PSA-NCAM
β-actin
Sham (4)
Moderate (6)
Severe (7)
Right Hippocampus
180
140
120
PSA-NCAM
β-actin
Sham (8)
Moderate (8)
Severe (8)
Left Temporal
180
140
120
PSA-NCAM
β-actin
Sham (8)
Moderate (8)
Severe (8)
Right Temporal
180
140
120
PSA-NCAM
β-actin
Sham (4)
Moderate (6)
Severe (7)
Diencephalon
180
140
120
PSA-NCAM
β-actin
Sham (8)
Moderate (8)
Severe (8)
Cerebellum
180
140
120
PSA-NCAM
β-actin

Figure 3. Immunoblot results for PSA-NCAM one week after CCI.

One week western blot results of the eight brain regions examined following sham, moderate, or severe injury illustrate a heterogeneous response to injury. PSA-NCAM blotting is noted to span from approximately 120 – 250 KDa. The appearance of bands of different intensity is the result of variable degrees of polysialylation of NCAM 180, 140, and 120 (approximate weight noted on right side of image). The results represented in this figure were used to construct the one week histogram in Fig. 2

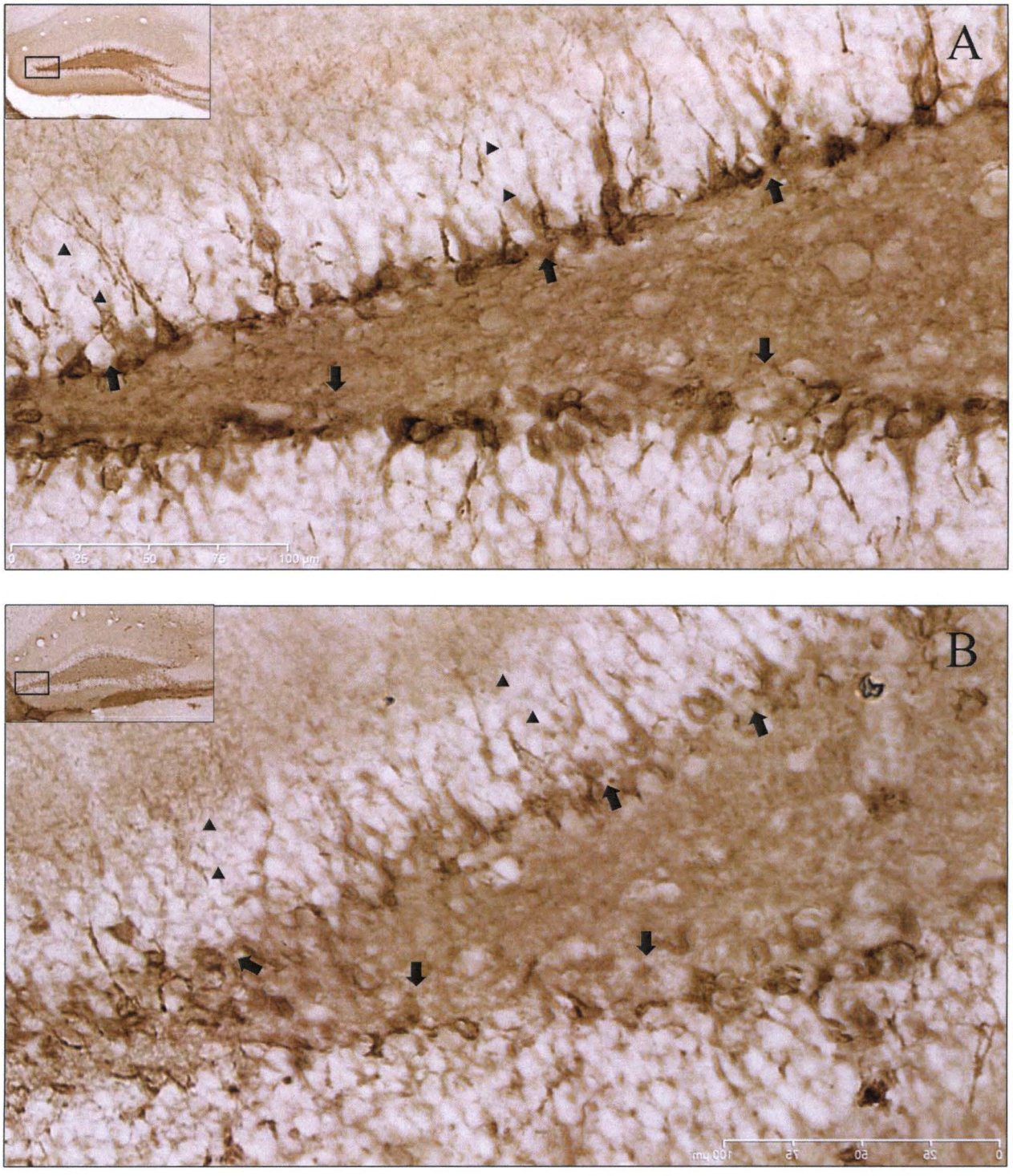


Figure 4. Comparison of PSA-NCAM levels in right dentate gyrus of sham and severe injury animals 48 hours following surgery.

PSA-NCAM staining of subgranular zone cells in the contralateral hippocampus reveals more intense expression in the soma (arrows) and processes (arrow heads) of sham (A) vs. severe (B) injury animals. Images are 40(x) magnification; scale bar = 100μm.

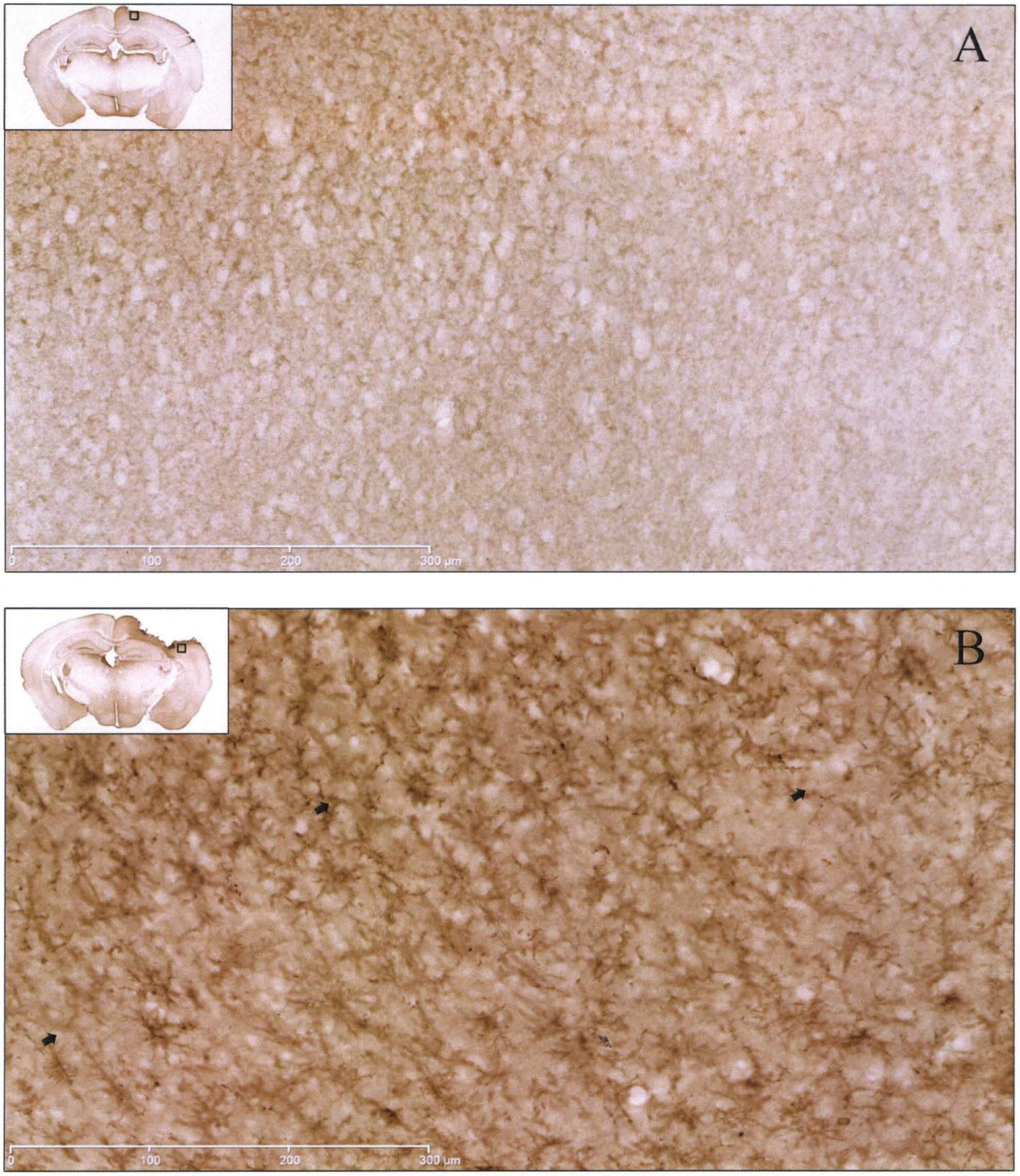


Figure 5. Immunohistochemical evidence of PSA-NCAM expression alterations in the left cortex one week following injury.

(A) Sham animals demonstrate subtle, uniform PSA-NCAM staining in the left cortex one week following craniectomy. (B) PSA-NCAM expression in the ipsilateral cortex of moderate injury animals is significantly increased with particularly dark staining of cells that display the appearance of reactive astrocytes (arrows). Images are 20(x) magnification; scale bar = 300μm.

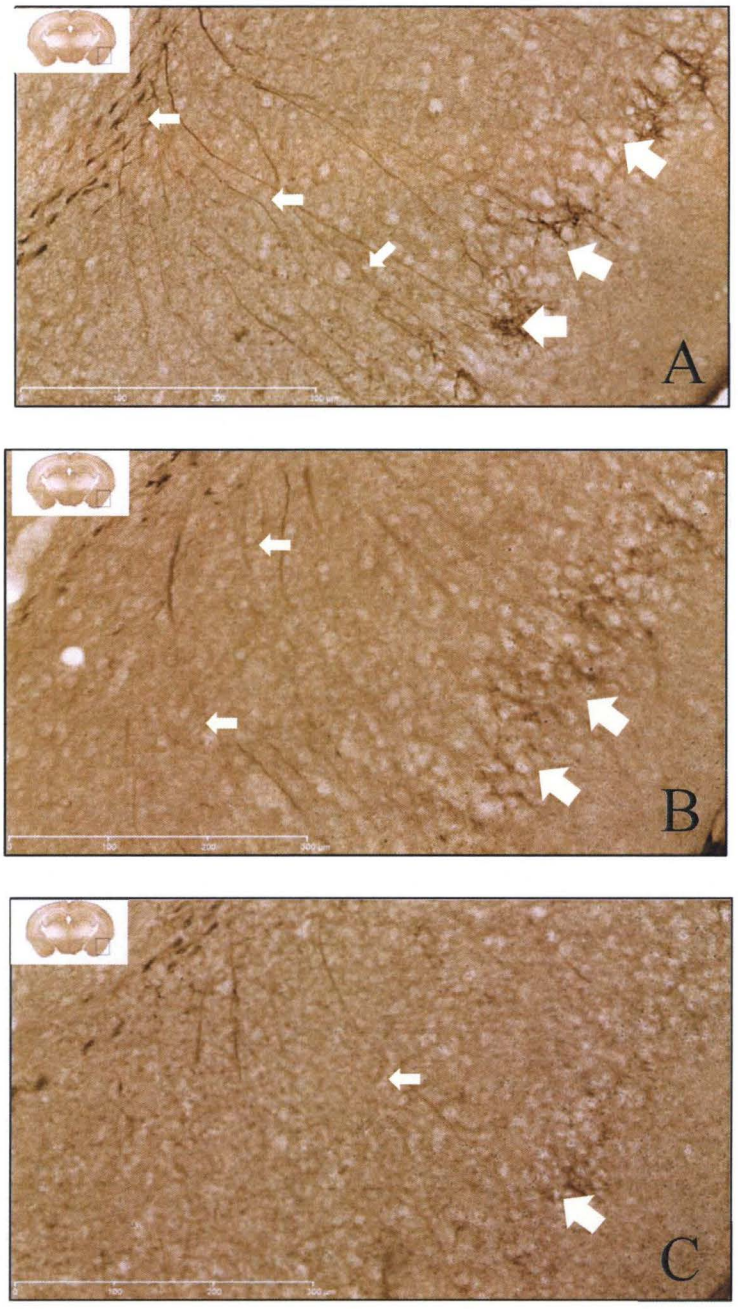


Figure 6. PSA-NCAM staining in left temporal lobe one-week following graded-CCI.

Diaminobenzidine staining for PSA-NCAM in sham (A), moderate (B), and severe (C) injury animals one week after CCI demonstrates decreased staining of the soma (large arrows) and apical dendrites (small arrows) of neurons in the piriform cortex.

Insert denotes approximate region of interest at approximately bregma -1.70 mm. Images are 20(x) magnification; scale bar = 300μm.

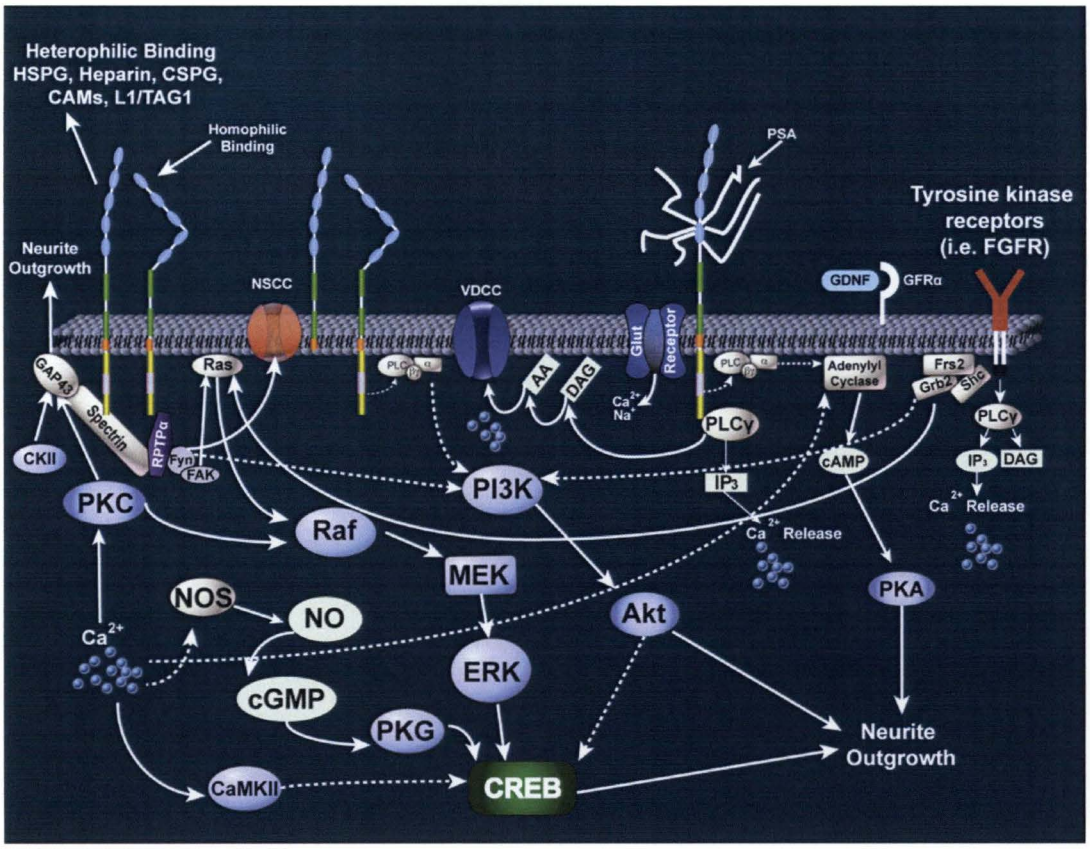


Figure 7. NCAM and PSA-NCAM signaling pathways.

Currently accepted signaling pathways believed to be involved in neurite outgrowth; the most studied and understood result of NCAM activation. NCAM-180 and 140 are represented by five Ig like domains and two fibronectin III domains extracellularly and an intracellular segment of varying length. NCAM 120 is attached to the membrane via a GPI anchor. Color designations: tyrosine kinases = red; other protein kinases = blue; non-proteins = silver. Abbreviations: AA, arachidonic acid; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element–binding protein; CaMK, Ca^{2+}-calmodulin-dependent kinase; cGMP, cyclic guanosine monophosphate; CKII, casein kinase II; DAG, diacylglycerol; ERK, extracellular regulated kinase; FAK, focal adhesion kinase; FGFR, fibroblast growth factor receptor; Frs2, FGFR substrate; GAP-43, growth-associated protein 43; NO, nitric oxide; NOS, NO synthase; NSCC,

nonspecific cation channel; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PLC, phospholipase C; RPTP, receptor protein tyrosine phosphatase; VDCC, voltage-dependent Ca^{2+}-channel. Broken lines indicate putative intracellular interactions. Illustration adapted with permission from [[18, 56]].

Chapter 4

Discussion and Conclusions

Objectives

The economic and emotional burden of TBI is substantial and results in over 50 thousand deaths and over $60 billion of direct medical and indirect loss of productivity costs in the United States alone [1]. The estimated lifetime cost of each TBI case in the U.S. is estimated to be $200,000 [2]. It is imperative that novel research be conducted to address the acute and long-term disability associated with TBI. The first objective of the work presented here was to evaluate the neuroprotective attributes of DZ when administered following TBI by investigating the short and long term effects on motor, behavioral, and memory functional outcomes. As discussed, a significant neuroprotective effect of both pre and post injury DZ administration in OGD models has been demonstrated by previous investigators [3-15]. Motor, behavioral, and memory parameters were evaluated in our work because multiple evaluation tools exist to assess such parameters and abnormalities in these parameters are what is used to define the post-TBI phenotype [16]. In addition, an extension of the first objective of this work was to evaluate the effect of a single dose of DZ administered 30 minutes before or after CCI on select biomarker expression 24 hours following injury. This portion of the study serves as an effort to validate the feasibility of using DZ as an immediate pre or post conditioning agent. The effect of post injury administration of DZ on biomarker expression 3 weeks following injury was also explored. Following an exhaustive review of the existing

literature, we believe that our work is the first directed at evaluating the neuroprotective effect of DZ in the TBI setting.

The search for novel biomarkers that reflect the state of injury or restorative efforts following TBI continues in order to allow for an improved understanding of the pathology underlying injury as well as providing new targets for therapeutic intervention. PSA-NCAM is recognized as an important molecule during ontogeny as well as one that participates in the maintenance of synaptic health, neuronal migration, and cell survival throughout life. Even though PSA-NCAM does possess an array of important functions, alterations in acute and long term expression levels following TBI have not been investigated. The second objective of this work was to chronicle the early and long term changes in PSA-NCAM expression throughout the brain following graded-CCI in the mouse. It is our hope that this information may be used to further define the complex biological mechanism underlying TBI as well as reveal possible therapeutic windows after injury.

Post-TBI DZ Effects on Memory, Motor, and Behavior

The first hypothesis that was tested was that post injury administration of DZ would provide an immediate and lasting beneficial effect on motor, behavioral, and memory outcomes. Post injury administration of a therapeutic intervention offers several benefits. First, due to the unpredictability of TBI, effective pretreatment would require administration of a drug on a continual basis, and this would expose the person to possible side effects of the drug. For example, chronic use of DZ may place a person at increased risk for minor adverse reactions such as dizziness and pruritus or severe effects

such as ketoacidosis, hyperosmolar coma, and death [17]. Second, the clinical applicability of post injury administration of DZ is obvious as it mimics the treatment sequence that is experienced in real world trauma situations. As a whole, the post-TBI DZ administration paradigm that was instituted in this work did not appear to lessen the severity of motor, behavioral, or memory deficits displayed by the mouse following moderate-TBI; however, interesting findings regarding the effect of DMSO on behavior and motor function were seen.

CCI was chosen as the method to inflict TBI because this technique of direct brain deformation offers the ability to control velocity, depth of impact, and dwell time [18]. It produces focal and widespread acute and chronic axonal injury and is considered a valuable tool for developing novel therapeutic approaches for brain injuries [19]. Even though CCI is widely considered an acceptable model for duplicating human TBI injuries including concussion, contusion, and axonal injury, the high degree of variability of animal response to injury means that even minor changes in impact parameters may alter injury severity from minor to fatal [18]. Over 400 mice were examined during this project and there were only two-procedure related deaths. Both of the deaths occurred during the initial investigator training stage and were most likely due to poor placement of the CCI apparatus ear bars and subsequent animal asphyxiation.

Beam Walk Performance

Motor deficiencies on the beam walk test are known to persist for one month or longer in mice following TBI [20, 21]. Our results indicate that a significant injury effect on foot fault number did persist for two weeks and that DZ+DMSO and DMSO alone did

not improve performance regarding this variable. Results for beam walk duration were difficult to interpret. The noted decrease in time required for DMSO animals across all groups to cross the beam on POD 7 may indicate an extended anxiolytic effect of DMSO; however, this result would have to be repeated in further testing scenarios before it could be stated with confidence that this effect does exist. The lack of fine motor skill benefit or noted improvement in center distance during open field testing (see below) tend to indicate that the decreased crossing time noted on beam walk may be an anomaly.

Initially used by Feeney et al. in 1982 [22] to assess recovery of motor cortex function following injury, the beam walk assay has been widely used to successfully assess vestibular, sensorimotor function, and fine motor coordination in mutant mice with movement disorders and following various forms of CNS injury [23]. Assessment of these parameters is a critical end-point in evaluating the efficacy of neuroprotective therapy associated with TBI. In retrospect, the inability to detect any significant difference in motor performance between groups may indicate a lack of sensitivity of the beam walk assay in detecting subtle improvements. A larger sample size may have improved the ability of this test, and all tests used during conduct of this work, to detect differences in sensorimotor function between groups. The length of injury effect on beam walk performance may extend from as little as two weeks to more than four weeks in mice following CCI [24]; therefore, inclusion of additional sensorimotor evaluation tools during this period may have improved our ability to detect differences between groups. For example, the neurological severity score (NSS) for mice (reviewed in [25]) could have been integrated into the evaluation sequence at 1, 4, 24, and 72 hours as well as weekly until animal sacrifice. The ability to assess paresis, seeking behavior, balancing

and beam walk performance may have provided additional information that could have been used to compose a more detailed and complete picture of neurological deficiency and recovery. Recently developed tests to assess sensorimotor function could have been included in our study as well. Bouet et al. [26] describe use of the the adhesive removal test as a sensitive method to assess sensorimotor deficits in mice following cerebral ischemia by measuring time-to-contact (mouth sensitivity) and time-to-removal (correct dexterity) of adhesive tape applied to the forepaw. Application of this procedure had not been perfected at our institution when our project began; however, other investigators have since implemented the process and demonstrated relative ease of use and good sensitivity to sensorimotor function status.

Open Field Evaluation

Our discoveries regarding hyperactivity following moderate-CCI are in line with recent findings by Schwarzbold et al. [27]. Regardless of treatment, CCI animals in our study exhibited hyperactive motor patterns on POD 7 and 14 on all measures: increased total distance traveled, center distance traveled, and traveling speed. As discussed in Chapter 2, and in agreement with previous investigations [27, 28], we believe that the injury inflicted upon the mice produces a deficit in spatial learning and working memory rather than an effect on motor activity, per se. It is hypothesized that this "hyperactive syndrome" results from the loss of inhibition from cortical to posterior and subcortical regions [27, 29]. Perhaps the extensive cortical damage sustained by our animals (see **Fig. 1** in Chapter 2) was severe enough that the recognized neuroprotective effect of both DZ and DMSO were not sufficient to promote the necessary conditions required to

preserve neuronal integrity and maintain or reestablish functional cortical connections. As a result, the normal inhibitory output to deeper cortical structures may have been absent.

Additionally, a general depressant effect of DMSO with or without DZ was noted on measures of mobility in the open field across all injury categories on POD 1 and 14. This effect was displayed as decreased total distance travelled and number of mobile episodes. As discussed in Chapter 2, this may indicate an undesirable side effect of DMSO administration. Tests to evaluate nerve conduction velocity, myelin integrity, analgesic, or allodynia effects were not conducted during our project, but previous investigators have noted these potential side effects of i.p. DMSO administration [30-32]. Further discussion of DMSO use is detailed below.

Evaluation of center distance and percent of distance traveled in open field center, a possible measure of anxiety [33], may indicate that the injured animals experience an early anxiety (POD 1) that is absent at POD 7 and 14. The early increased anxiety response agrees with existing studies. Chauhan et al. [34] noted increased anxiety in mice subjected to moderate-CCI injury using the elevated plus maze system 24 and 48 hours after injury in male C57BL/6 mice. If our drug intervention did have an axiolytic effect within the first 24 hours of injury, it was not detected using the evaluation tools that were employed. In retrospect, although detection of locomotor differences between groups was accomplished during our work with the use of the open field apparatus, inclusion of tests developed to specifically explore anxiety-like behavior (i.e., elevated plus-maze test) or depressive-like behavior (i.e., forced swimming test) may have allowed for an increased ability to detect subtle early and late "emotional" differences between groups.

Morris Water Maze Performance

As stated by Eric Kandel, "Learning is the process by which we acquire knowledge about the world and memory is the process by which that knowledge of the world is encoded, stored, and later retrieved" [35]. Regarding the current rodent model, a defect or interruption in any of these processes will manifest as deficits in performance on tests measuring learning and memory function. The Morris Water Maze was utilized to test hippocampal-dependent spatial learning and memory function. As would be expected, injury produced impaired learning and memory performance as evidenced by less time in the NW quadrant, fewer platform crossings, and decreased swim speed during the probe trial. Neither DZ+DMSO nor DMSO alone had identifiable beneficial or detrimental effects on probe trial performance.

Investigations into the mechanisms of pathology has shown there are associations between TBI, even mTBI, and chronic degenerative neurologic conditions such as Alzheimer's Disease. There is gathering evidence that TBI is most likely a significant contributing factor to long term memory dysfunction; the ability to hold information in mind and manipulate it in order to address incoming material. Injury to the frontal lobes or the extensive system of circuitry connecting the frontal lobes to subcortical structures such as the thalamus, limbic system, and cerebellum underlies deficits in working memory and executive function [36]. The link to chronic degenerative disorders is striking. For example, an examination of a database of veterans who visited a Veterans Administration hospital over a six year period revealed that the rate of diagnosed dementia in patients who had a history of TBI was over twice that of patients who did not suffer TBI (15.3 vs. 6.8%) [37]. Significant links between TBI and neuropsychiatric

disorders also exists. Indeed, a 2008 report in the *New England Journal of Medicine* demonstrated that the incidence of posttraumatic stress disorder (PTSD) in military members who suffered TBI is much greater than those who have not [38]. Compared to the 9% incidence of PTSD found in deployed service members who did not suffer injury, those TBI victims who lost consciousness had a 44% incidence of PTSD, while those who reported "seeing stars" had an incidence of 27%. The authors also recognize that the heterogeneity of symptoms associated with TBI and PTSD produces overlap in symptoms (insomnia, irritability/anger, trouble concentrating, depression, and hyper arousal), so that a causal role to TBI is not a clearly defined process. Considering the complexity of the post injury recovery period, preclinical work aimed at defining and categorizing learning and memory deficits that manifest as a result of TBI is critical.

As discussed in Chapter 2, the MWM is considered the "gold standard" for evaluating spatial learning and memory retrieval; however, weaknesses in the testing paradigm have been exposed. For example, Washington et al. [16] noted that MWM performance instituting the standard 4-day training protocol utilized in our study was considered an excellent tool for distinguishing sham from severe-CCI animals, but moderate-CCI injury animal performance on the probe trial fluctuated between mild and severe injury groups. In an attempt to rectify this issue, the authors instituted an additional platform reversal step in which, following completion of the initial probe trial, the platform was moved to the quadrant opposite the original location and the mice were trained for four days and re-tested on the fifth day (Days 22-26 post injury). The addition of this step to their testing regimen allowed for clear identification of sham, mild, moderate, and severe animal performance.

A survey of the literature reveals that there is an increasing knowledge base regarding the contributions of different brain regions to spatial and contextual learning. For example, using neurotoxic lesions of the hippocampus, perirhinal, postrhinal, and/or entorhinal cortices, Burwell et al. [39] determined that profound and variable effects on contextual fear conditioning and contextual discrimination occurred with the rhinal lesions, while only hippocampal lesions affected spatial navigation. Our MWM paradigm may have over trained the animals, and inclusion of the platform reversal step described above or a rapid place learning test [40] may have allowed us to detect more subtle effects of injury and treatment on performance. Evidence exists that if injured animals are given a sufficient number of training trials over several days, stable place information will be acquired and consolidated by the neocortex alone even with significant hippocampal injury [40, 41]. Inclusion of additional tests that discriminate between allocentric learning (i.e., hippocampus based learning using external cues) and egocentric learning (i.e., caudate nucleus- and striatum-based learning based on self-generated movements) [42] may further differentiate which memory system is affected by injury and the more effective therapeutic interventions [43]. Of note, the general lack of gross hemorrhage and physical injury to the ipsilateral hippocampus in our animals provides further evidence in agreement with existing studies that significant hippocampal pathology resulting in considerable spatial learning deficits exists following mild or moderate injury in which the HC seems to be grossly spared from harm.

PSA-NCAM Expression Following Graded-CCI

Following an extensive review of the literature, we believe that our work evaluating the acute and long-term changes in PSA-NCAM expression following TBI is the first of its kind. The essential role of PSA-NCAM in synaptic plasticity, memory formation and storage, emotional function, and progenitor survival in neurogenic niches has been better understood over recent years. It is our belief that the data presented in this document may be used by future investigators as a foundation for descriptive and interventional investigations aimed at unraveling the pathological processes responsible for post TBI dysfunction as well as developing potential therapeutic interventions to address those processes.

It has been documented that changes in PSA-NCAM expression levels are seen in the dentate gyrus, amygdala, and the entorhinal cortices and have been associated with learning and memory consolidation [44]. In some regions of the brain, the role of PSA-NCAM in memory formation is well understood. It is thought that PSA-NCAM's ability to modify adhesive forces at synapses in the hippocampus permits plasticity via efficient synapse selection and function during spatial memory formation [45]. On the other hand, PSA-NCAM's role in other brain regions, such as the amygdala, is less well understood, although at least one study relates this molecule with emotional processing. As shown by Markram et al.[46], decreased PSA-NCAM in the amygdala in proximity to a stressful event results in enhanced fear extinction. Alterations of PSA-NCAM levels in this region may result in significant psychological implications regarding recall of traumatic events. Unfortunately, the amygdala normally has sparse and ill-defined PSA-NCAM staining throughout. As a result, we were unable to corroborate the immunoblot results showing decreased PSA-NCAM expression in the left temporal lobe at 1 and 3 weeks following

severe-CCI injury (see Fig. 2 in Chapter 3) with changes in the amygdala. On the other hand, the noted decrease in PSA-NCAM staining of the semilunar cells of the primary olfactory (piriform) cortex was noted during immunohistochemical evaluation (see Fig. 6 in Chapter 3) and may have profound effects on the ability of post injury rodents to establish "odor images" because these cells receive strong afferent input from the mitral/tufted cells in the olfactory bulb [47].

The results of our efforts regarding PSA- NCAM expression following graded-CCI may serve as the foundation for future investigators' efforts focused on exploring the behavioral implications and possible therapeutic potential of these changes. For example, institution of a similar injury model could be used to evaluate the behavioral repercussion of PSA-NCAM changes in various regions of interest in the brain. As an example, the above mentioned amygdala nuclei PSA-NCAM expression changes that are associated with fear memory formation, storage and extinction. A fear conditioning paradigm such as auditory fear conditioning could be used to assess if enhanced fear extinction occurs coincident with observed decreased levels of PSA-NCAM in the amygdala following graded-CCI. This data could then be used to understand and further dissect the molecular alterations linked to PTSD and other emotional difficulties following TBI.

Additional untoward consequences of a decrease in PSA expression on NCAM could also be appreciated. Specifically, newborn progenitor cells in the subgranular zone of the dentate gyrus express high levels of PSA-NCAM, and this is required for the proper maturation and migration of these progenitors. Burgess et al. [48] demonstrated that removal of PSA from NCAM using endoN halts migration and promotes neuronal differentiation of the progenitor cells in the SGZ. This effect was realized following 48

hours of PSA-NCAM deficiency in the hippocampus. Though not directly transferrable to TBI, this finding may have implications regarding post TBI memory and behavioral recovery. For example, the significant decrease in PSA-NCAM expression in the right hippocampus seen in our study 48 hours following injury may not only decrease the ability of progenitor cells to mount a response to the insults associated with the secondary injury cascade, but it may also induce precocious differentiation of progenitors in the SGZ and thus limit the ability of the neurogenic zone to produce neurons that could eventually be integrated into a damaged hippocampal circuit. A similar situation may have existed in the subventricular zone in our model at one & three weeks. However, even though immunoblot results indicate that there were significant decreases in the PSA-NCAM expression in tissue samples that may have included the subventricular zone of the lateral ventricles; we were unable to verify that finding using qualitative evaluation of immunohistochemistry samples due to significant variability of staining area and intensity (not shown).

Data from our study may also help future investigators understand other components of the neuropsychiatric phenotypes that develop following TBI. For example, the amount of PSA expressed on NCAM in the suprachiasmic nucleus (SCN) changes very rapidly and appears to be an indispensable component of the regulation of circadian rhythms as evidenced by the fact that enzymatic removal of PSA from the SCN results in a loss of photic and non-photic regulation of the circadian clock [49]. A TBI induced significant, long-term decrease in PSA-NCAM expression in the SCN may result in disruption of the sleep cycle, chronic fatigue, or stress. Chronic stress, in turn, may lead to an increase or decrease in the levels of PSA-NCAM expression in various brain

regions. Pham et al. [50] noted that a biphasic increase in PSA expression in the hippocampus was observed after three and six weeks of restraint stress in rats that led to a *decrease* in granule cell proliferation and volume. The authors hypothesized that this phenomenon may be responsible for stress induced hippocampal shrinkage and memory dysfunction associated with chronic stress. Though a hypothetical scenario, the sequence of events presented may illustrate one possible component of a complex feed forward chronic pathological process that can be linked to TBI.

The realm of study regarding the synthesis of PSA and how these systems may be affected by TBI has not been explored, but should also be pursued in the future. For instance, the temporal and spatial distribution of the polysialyltransferases (PST) responsible for the posttranslational modification of NCAM may be affected by TBI and result in distinct effects on regional brain function. It has been found that deletion of the ST8SiaII/STX-gene in mice results in decreased PSA-NCAM in the hippocampus and abnormal mossy fiber sprouting in the CA3 region that results in ectopic synapse formation and detrimental behavioral effects [51]. Angata et al. [51] demonstrated an abnormal response to cued and contextual fear-conditioning was seen in mice with ST8SiaII/STX deficiency. Of note, PSA-NCAM expression was normal in the amygdala, hypothalamus, and piriform cortex of these mice

Regarding potential therapeutic interventions centered on PSA-NCAM application to injured tissue, no existing evidence has revealed an *in vitro* or *in vivo* deleterious effect of PST overexpression [49]. To date, promising results related to transfection of astrocytes and Schwann cells with PST constructs have been seen. The overexpression of PST by astrocytes and Schwann cells in spinal cord lesions improved

the ability of regenerating axons to extend neuritis and as improved the extracellular environment for growth and myelination, respectively [49, 52]. The permissive environment produced by PST overexpressing astrocytes along a lesion path in injured mouse brain has also been shown to enhance the migration and recruitment of SVZ neuronal precursors in the injury site [53]. One theme that appears to emerge from evidence regarding PSA-NCAM's function in the CNS is that an appropriate spatial and temporal distribution of the molecule in select well defined regions is required to promote efficient synaptic, and perhaps whole cell function, and the possibility of influencing this expression holds great promise for future therapy.

Other Biological Markers of Injury and Healing

Evaluation of the effect of DZ administration on alterations in the expression levels of select early and late markers of injury or healing was performed. Due to the existing evidence regarding preconditioning and postconditioning in the ischemic setting, we did employ a limited amount of evaluation of the effects of pre- and post-injury DZ administration on biomarker expression 24 hours following moderate CCI. All 24 hour marker evaluation was performed on animals that received a single dose of i.p. DZ (2.5 mg/kg), DMSO, or NaCl 30 minutes before or after injury. Additionally, the long-term effects of post injury DZ administration (2.5 mg/kg i.p. 30 minutes after and every 24 hours for three days following injury) on the expression levels of select markers were evaluated 21 days after TBI. These results are presented in **Chapter 5**.

Additional Comments and Future Directions

In addition to the suggested modifications of specific tests and recommendations for future research offered above, additional comments regarding the present study and

future directions is warranted. In retrospect, several areas of our study could have been modified to better clarify and define the findings presented. Perhaps the most obvious modification is the exclusion of DMSO as the vehicle used to dissolve DZ for i.p. administration. The manifold pharmacologic actions of DMSO that have been discussed may have exerted such far reaching effects on multiple pathological pathways that the beneficial effects of DZ administration were obscured. Additionally, DMSO administration could have enhanced injury severity or induced changes in peripheral nerve or motor function that complicated interpretation of behavioral outcomes. Regardless, existing literature appears to be divided into two camps: one that praises the beneficial effects of DMSO on pathology as diverse as liver ischemia [54], CNS injury, and enhancement of antibacterial agent effects on antibiotic resistant strains of bacteria [55]; and another that appreciates the ability of the drug to accumulate in tissue [56] and exert far reaching and unintended effects as an undesirable feature of the agent. Ideally, future investigations should attempt to administer DZ in a medium that allows for accurate and reliable dosing and absorption without possessing significant pharmacological action.

An interesting prospect for future investigation would be an evaluation of extended post-TBI oral administration of DZ on long-term outcome. This is a particularly enticing prospect considering the results of a recently published investigation examining the effect of long-term DZ oral administration on chronic neuropathology and function associated with Alzheimer's disease. As briefly discussed in Chapter 2, Liu et al. [57] evaluated the effects of administering 8 months of oral DZ treatment (10 mg/kg/day) in drinking water to 3xTgAD mice. These mice exhibit severe frontotemporal lobe dementia

due to Aβ and tau pathologies resulting from a double mutation of amyloid precursor protein ($APP_{KM670/671NL}$) and a tau mutation (tau_{p301L}). Results demonstrated that DZ improved neuronal bioenergetics *in vitro* as evidenced by plasma membrane hyperpolarization and mitochondrial membrane depolarization and a decrease in NMDA-induced elevation of intracellular Ca^{2+} levels. The *in vivo* results were equally impressive with increased levels of SNAP-25 and synaptophysin in cortical neurons, decreased hippocampal and cortical accumulation of Aβ and hyperphosphorylated tau, increased CBF, and MWM performance showing reduced goal latencies and swim path lengths. Evidence of this type serves as confirmation that long-term administration of high doses of DZ are well tolerated and have profound positive effects on neuropathology. Oral administration would avert the potential problems presented by DMSO administration and allow for an assessment of the effects of large, extended doses of DZ on improving the post-TBI phenotype. Finally, due to the complexity of secondary injury pathways, combinatorial therapy with another agent that attenuates a different major contributing pathway of pathology (i.e., NMDA antagonists or Mg^{2+}) should be considered.

Conclusions

Traumatic brain injury is an exceptionally complex pathology whose impact is only beginning to be fully understood and appreciated. The work presented in this volume covers a diverse range of measures and outcomes in an attempt to serve as an initial screening tool and starting point for future investigations concerned with evaluating the neuroprotective effects of DZ in the TBI setting. Evaluation of the effects of post-TBI DZ administration on motor, memory, and behavioral parameters was

performed in an attempt to assess the effects of this clinically applicable dosing scheme on the observed phenotype. Evaluation of the effect of DZ administration on expression levels of various biomarkers served as not only a proof of concept experiment but also as a high throughput screening tool that can be used to focus additional future efforts on specific molecular pathways and disease processes that are influenced by the drug. The subtle effects of DZ on some performance measures (i.e., number of correct steps recorded on beam walk) and biomarkers warrants further investigation of the effects of different DZ dosing schemes on TBI outcome. The identification of significant early and late changes in the levels of PSA-NCAM expression following TBI serves as new evidence that this molecule may be used as another marker to further characterize and classify the pathological processes that contribute to injury following TBI. This information may also serve as a beginning effort in identifying PSA-NCAM as a potential therapeutic target to ameliorate injury and promote healing.

The tools and techniques utilized during this investigation are believed to have been acceptable for identifying significant changes in post-TBI in biomarker levels or behavioral, motor, and memory performance. However, modifications in some of the testing parameters or molecular biology techniques that were instituted may have allowed for identification of subtle and elusive changes. In all, the data presented here serves as a worthy launching point for future efforts such as evaluating the long–term administration effects of high dose DZ or combinatorial therapy on post-TBI outcome.

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Chapter 5

Appendix

Pretreatment 24-hour western blot results

Due to the extensive evidence in existence regarding the neuroprotective effects of DZ when administered as an immediate and delayed preconditioning therapeutic [1, 2], a portion of our project was dedicated to establishing proof of concept by tracking select biomarkers in the left and right hippocampi (LHC and RHC, respectively) 24 hours after moderate CCI. Due to time constraints and resource management efforts, the hippocampus was the sole structure examined during this phase of the project. Often, even when this structure is not directly injured by CCI, it has been shown to have a vital role in the observed alterations in memory and behavioral performance following TBI. Please refer to Chapter 3 for the technique utilized to prepare samples for immunoblotting. Any variation from the description offered there is noted below.

Initial evaluation of 24-hour markers was conducted by comparing brain samples from CCI+DMSO and CCI+DZ animals to samples from Sham+DMSO animals (n = 8 animals/group). In this way, significant change in marker levels related to CCI and drug treatment would be revealed against any effects that the DMSO vehicle administration may have induced in sham animals. If significant change was noted, then immunoblotting was repeated, including the addition of CCI+NaCl tissue (as displayed in the figures contained within this chapter; n = 6 animals/group), unless otherwise noted due to time

constraints. If results were not reproducible, then that marker was included in the no significant change category. Additionally, if the degree of change in marker expression was less than or equal to 10% of that seen in Sham animals, then it was considered to be a change within the margin of error and was not included for further evaluation. Of the antigens tested in the hippocampus samples, there were no significant changes found for a significant number of the selected markers as a function of treatments with DZ. These markers (listed in **Table 1**) will not be discussed and the focus will be related to proteins where changes were observed.

Alpha spectrin (α-spectrin) (Figure 1)

α-spectrin (Enzo, mouse monoclonal, utilized at a dilution of 1:5000) was used to evaluate variations in protein expression levels. The results regarding α-spectrin are unclear. α-spectrin serves as a major substrate for the calpain and caspase-3 cysteine proteases [3]. The decreased full length α-spectrin level seen with DZ administration in both HC was perplexing. There was no significant change in either HC across all groups regarding caspase- (~150 kDa) or calpain- (~120 kDa) dependent SBDPs. The lack of change in SBDPs with these changes indicates that the decrease is most likely not a result of caspase or calpain related degradation. The increased full length molecule levels expressed with CCI+NaCl and CCI+DMSO in the RHC may reflect a healing cytoskeleton response that is absent in the CCI+DZ group. Further work will be required to interpret the significance of these findings. Levels of SBDPs in the CSF are being investigated as a marker of the severity of TBI [3], so future studies could attempt to assess if immunoblot results from brain homogenate and CSF agree.

Apoptosis-inducing factor (AIF) (Figure 2)

AIF antibody (Millipore, rabbit polyclonal, c.f., **Table 2**) was used to evaluate variations in protein expression levels. Interestingly, AIF levels in the left hippocampus (LHC) of the CCI group that received NaCl (CCI+NaCl) were approximately 30% lower than the sham animals that received DMSO. CCI animals that received DMSO (CCI+DMSO) had an approximately 50% greater level of AIF than the CCI+NaCl animals. Finally, the CCI animals that received DZ (CCI+DZ) had AIF levels comparable to CCI+NaCl animals. Expression levels in the right hippocampus (RHC) for CCI+ DZ treated animals was 20-30% lower than all other groups. Although more research is required to understand the implications of the noted changes, two interpretations may be made from these preliminary results. First, DMSO alone may have a deleterious effect on the hippocampus by increasing AIF expression and possibly induce a degree of caspase independent apoptosis. Second, DZ may have the ability to counteract this effect of DMSO. Additionally, DZ appears to have the ability to decrease AIF expression in the HC contralateral from the impact site, thus demonstrating the far reaching effects of injury and the protective effects of DZ administration in this situation.

Caspase-3 (Figure 3)

The results regarding caspase-3 immunoblot data (Cell Signaling, rabbit polyclonal, utilized at a dilution of 1:1000) are also vague. Though statistically significant, the decrease in caspase-3 levels in the LHC for CCI+DZ animals is no greater than 10–15%. Therefore, it may be a result influenced by experimental error. Interestingly, the RHC expression levels may indicate, as in the case of AIF, that DMSO

alone has a deleterious effect on the health status of the HC as evidenced by the fact that all injury groups expressed less caspase-3 than Sham+DMSO. As is the case with AIF expression, further work must be done to interpret these results. For example, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) may be used to determine if an increase in TUNEL-positive cells in the HC occurs in conjunction with the caspase-3 labeling. Interestingly, we were not able to detect elevated levels of cleaved caspase-3 in any region of the brain at any time following CCI; however, this is in agreement with previous investigator's findings [4, 5].

Iba-1 (Figures 4 and 5)

Ample evidence exists that implicates the central role of activated microglia in inflammation-propagated pathology following TBI. In fact, a frequent indicator of cellular pathology noted in all grades of human TBI pathology is the postmortem identification of microglial clusters, particularly in white matter tracts [6]. Of note, this persistent activation is often in locations remote from the focal area of injury, and increased microglial activation in the thalamus of humans is associated with the degree of cognitive impairment [7]. The pathological implications of microglial activation includes impairment of hippocampal neurogenesis [8], increased BBB permeability as a result of tight junction restructuring and epithelial dysfunction due to microglial released TNFα, IL-1β, and ROS [9], and accelerated apoptotic cell death via an aberration in the CXC-chemokine stromal cell-derived factor 1 (SDF-1)/CXCR4 signaling pathway that results in excessive glutamate release from astrocytes [10]. Whitney et al., [11] presents a comprehensive review of the varying roles of inflammation in brain injury.

Immunoblot analysis (Wako, rabbit polyclonal, utilized at a dilution of 1:1000) of pretreated animals at 24 hours following injury revealed no significant increase in Iba-1 levels in the L HC; however an interesting finding was seen in the R HC. A significant increase in Iba-1 expression in Sham+DMSO and CCI+DMSO versus CCI+NaCl was observed. Although additional study is required, the precise mechanism and implications of this finding are not known. It may signify that microglial activation results from the administration of DMSO alone. This novel finding should be confirmed in future studies. Existing evidence regarding the effect of DZ and DMSO on microglial activation following chronic cerebral ischemic injury is mixed. In one case [12], a five day course of post-ischemic DZ (5 mg/kg), but not DMSO (0.25 ml), administration in Wistar rats led to decreased microglial activation (particularly in the corpus callosum and optic tract) thirteen weeks after injury. A low dose DZ paradigm utilized by the same investigators showed that DZ (0.5 mg/kg) in NaOH administered for five days has similar results on microglial activation [13]. On the other hand, in a repeat study using the 0.5 mg.kg dosing schedule, the investigators noted a significant increase in hippocampal microglia activation in the ischemic animals post treated with DZ [14].

In order to further evaluate the immunoblot findings, IHC analysis of Iba-1 expression was performed. All hippocampal tissue samples were processed and prepaired using a Vector Labs DAB Substrate Kit as detailed in **Chapter 3**. In order to enhance image contrast, nickel was used as the chromogen. This portion of the evaluation was conducted by five blinded evaluators. Prior to the formal evaluation period, all blinded investigators were given a picture of a representative sample of Iba-1 expression on an arbitrary 1 to 4 scale with 1 representing the lowest expression level and 4 representing

the highest level. Evaluators were instructed to grade the picture according to Iba-1 cell number and morphology. A ten picture trial evaluation run was performed to ensure 80% agreement among investigator evaluations.

Regarding the evaluation, there were no significant differences in Iba-1 expression between animals within an injury group (Sham NaCl, DMSO, or DZ and CCI NaCl, DMSO, and DZ). In the L HC, a significant difference/increase in Iba-1 positive cell morphology and number was noted between Sham and CCI groups. On the other hand, the R HC revealed a significant increase in Iba-1 staining when comparing Sham to CCI animals, and a more pronounced (though not statistically significant) decrease in Iba-1 expression than that seen in the L HC when DMSO+CCI and DZ+CCI animals were compared to the NaCl+CCI animals. These data were contradictory to what was observed in the western blot findings and indicate the significant variability in results that may be introduced when analyzing tissue using different techniques.

MAP-2 (Figure 6)

Early and variable changes in MAP-2 expression following CCI have been observed by previous investigators [15, 16]. Using immunohistochemical techniques, Saatman et al. [15] observed a general decrease in MAP-2 expression in the ipsilateral HC for 48 hours following either 0.5 or 1.0 mm depth of impact CCI in mice; however, some regions such as the granule cell layer of the dentate gyrus displayed increased expression. The authors also noted that the changes were restricted to the injured hippocampus. Immunoblot analysis (Millipore, rabbit polyclonal, c.f., **Table 2)** of our samples at 24 hours revealed no significant decrease in MAP-2 in the ipsilateral (left)

hippocampus, but we did observe an increase in MAP-2 in the R HC CCI+DMSO vs CCI+NaCl animals. The reason for a lack of noted change in the injured HC may be due to the variability of MAP-2 changes in the different hippocampal layers essentially cancelling each other out. This hypothesis could be confirmed with IHC analysis in future studies. The finding in the R HC is puzzling. Even though the significance of increased MAP-2 expression is not known, previous researchers have hypothesized that it may signify transient neuronal dysfunction or reactivity rather than a healing response [15].

Tissue inhibitor of metalloproteinase 1 (TIMP 1) (Figure 7)

TIMP 1 (Abcam, rat monoclonal utilized at a dilution of 1:1000) is an endogenous inhibitor of metalloproteinases that has a preference to inhibit MMP-9 although it may inhibit all of the MMPs [17]. Matrix metalloproteinases are zinc-dependent endopeptidases that are involved in tissue remodeling following trauma or stroke via degradation of most constituents of the extracellular matrix [18]. Expression of TIMP 1 has been shown to increase following ischemic brain injury [19], and this is believed to be a form of endogenous neuroprotection against hypoxic injury. In mice, MMP and TIMP 1 expression rise in response to CCI for at least 14 days post injury, and this increase as well as the ratio of this increase is more pronounced in aged (21–24 mos/old) mice [20]. If the increase in TIMP 1 is greater than that of the pathological increase in MMP, then BBB integrity may be maintained and neurons protected via improvement of the extracellular milieu and inhibition of excitotoxic cell death [19]. Unfortunately, it appears that with injury in the absence of pharmacologic intervention, the levels of MMP increase more quickly and significantly than those of TIMP 1 [19, 20].

Our findings regarding TIMP 1 expression 24 hours following injury are notable. As would be expected, compared to Sham animals, Timp 1 levels in the ipsilateral HC of injured animals is increased 24 hours following injury. Remarkably, the increase seen in CCI+DZ animals, though not statistically significant, suggests there was a robust change in expression that was not seen in brain samples from CCI+NaCl and CCI+DMSO animals. The degree of variability noted most likely reflects a subtle inconsistency seen in the degree of injury inflicted to the ipsilateral hippocampus. Factors such as a difference in the tension applied to the mouse head with the ear bars may cause protrusion of the brain from the skull and will affect the depth of impactor penetration [21]. Future work regarding TIMP 1 expression should include zymography analysis and measurement of MMP levels in an effort to further quantify the effect that DZ may have on the final ratio of TIMP 1 to MMP and thus reveal the balance of restorative to destructive factors following TBI.

Post treatment 24-hour western blot results

Due to time constraints, limited immunoblot analysis of post treated animals was performed. Unchanged marker levels in the left and right HC include those listed in **Table 1** and apoptosis inducing factor (AIF).

Tissue inhibitor of metalloproteinase 1 (TIMP 1) (Figure 8)

The results of post treatment DZ on TIMP 1 expression were difficult to interpret. Again, the R HC levels of TIMP 1 were unaltered following injury, and the L HC demonstrated a robust increase in expression in all injury groups. Although not statistically significant, it appears that TIMP 1 protein changes in samples from the

DMSO+CCI and DZ+CCI groups suggest a trend of decreased TIMP 1 expression at 24 hours following injury. As noted in the pretreatment groups, additional work will be required to determine if this trend may be significant and if it ultimately produces a detrimental effect on outcome measures.

Pretreatment and Posttreatment BBB Integrity and Brain Edema (Figures 9-11)

As discussed in **Chapter 1**, the significance of the contribution of edema to poor outcome following TBI is profound. Evidence exists that highlights the ability of preconditioning to preserve both cerebral and general vascular endothelial cell function in the face of injury [22-24]. Additionally, there is limited evidence supporting the ability of DZ to preserve the permeability characteristics of the brain endothelium following injury. Lenzer et al. [25] demonstrated that three days of pretreatment with 6, 20, or 40 mg/kg/day DZ resulted in a significant reduction in edema and markers of increased BBB permeability following 30 minutes of global cerebral hypoperfusion. For example, water content in the cerebral cortex 24 hours following reperfusion revealed that the cortical percent water content in the 20 mg/kg/day was 80.75 ± 0.2% vs. 81.25 ± 0.3% (*$P < 0.05$) in the vehicle treated injured animals. Earlier markers of BBB integrity (i.e., Evans Blue extravasation into the cortex 30 minutes after initiating reperfusion) were also comparably decreased vs. vehicle-injured animals. To our knowledge, the effect of pre or post-injury administration of DZ on brain edema formation following TBI has not previously been evaluated.

To evaluate the effect of pre and post-injury DZ administration on BBB permeability and edema formation, two techniques were utilized. First, we utilized IHC

techniques to examine the degree of IgG immunoreactivity in brain slices 24 hours following moderate-CCI. Using the surgical techniques described in **Chapter 2**, animals were administered either NaCl, DMSO, or DMSO+DZ 2.5 mg/kg as previously described either 30 minutes before or 30 minutes after CCI injury was induced. A modified technique previously described was used to evaluate IgG extravasation [15]. In brief, free floating sections retrieved from approximately the center of impact were chosen for evaluation. Endogenous peroxidases were quenched using 3% hydrogen peroxide in 50% methanol for 30 min, and non-specific binding sites were blocked for one hour using 10% normal goat serum (NGS). The tissue was then incubated with biotinylated goat anti-mouse IgG antibody (1:1,000; Jackson ImmunoResearch, West Grove, PA) at room temperature for one hour. Detection of staining was performed with Vectastain Elite ABC amplification (Vector Labs), and visualization was done using a nickel-diaminobenzidine chromogen. Omission of primary antibody served as a negative control.

Analysis was performed as described by Onyszchuk et al., [26]. In brief, images were scanned and captured using the Nanozoomer microscopy apparatus and software suite (Hamamatsu, Bridgewater, NJ). Identical white balance and exposure settings were used to capture all images. These images were then evaluated for anti-IgG staining in the injured cortex with the NIH ImageJ software, using a thresholding technique. The threshold was set at a level just above that which would be counted as background. The optical density was then recorded for identical 0.44 mm^2 boxes positioned 100 um from the medial and lateral edges of the impact lesion. Comparable areas were also measured in the sham animals.

The findings for assignment of IgG extravasation are presented in **Figure 9.** As can be seen, it appears that pre-treatment with DMSO or DZ does not appreciably affect the amount of IgG that moves into the brain parenchyma when compared to animals given NaCl (**Figure 9A**). On the other hand, our results demonstrate a significant decrease in IgG staining density in animals that are given post-CCI DMSO or DZ (**Figure 9B**). It does not appear that DZ at the given dose provides additional protection beyond that which may be provided by DMSO. Several caveats regarding these results must be mentiond. First, IgG is a large molecule (150 kDa), so these results only indicate the presence or absence of significant BBB disruption. Additional studies should include the use of smaller, variable weight markers that may be used to further quantify and classify the degree of BBB violation. Additionally, other more remote time points should be examined in order to better characterize the time course of increased BBB permeability. The post administration results are perplexing because existing evidence indicates that DMSO alone may either acutely and reversibly open the BBB [27] or reduce edema when given in high doses (2 g/kg) [28]. Additional research, perhaps including large and/or multiple doses of DZ, should be pursued.

Cerebral edema 24 hours following CCI was evaluated using a wet-dry weight technique previously described [29]. In brief, immediately following euthanasia, the brains were quickly removed and cut using a razor blade. One 4 mm coronal section that encompassed the lesion was collected. Each section was then rapidly dissected into left and right cortex, left and right hippocampi, and diencephalon on an ice cold glass plate (see **Fig. 11**). All tissue sections were then rapidly transferred onto preweighed 2.5 cm^2 aluminum foil squares and then weighed on a precision analytical scale. The tissue and

foil were then dried in a general purpose incubator at 100°C for 48 hr, and reweighed for the dry weight. The percent brain water was calculated as the difference between wet and dry weights divided by the wet weight.

Figure 10 reveals the composite edema percentages for Sham and CCI animals with and without drug treatment. These values were obtained by combining the individual values noted for each of the five sections evaluated per animal. No significant difference was noted between any of the groups.

Figure 11 presents the edema values for each of the dissected brain regions. As would be expected, a significant increase in brain water content was observed in the left cortex when CCI+NaCl tissue was compared to Sham tissue. Interestingly, a similarly significant increase was noted in Pre-CCI DZ and Post-CCI DMSO tissue. On the other hand, the right cortex tissue indicated lower water content in Pre-CCI DMSO and Post-CCI DZ animals when compared to Sham. Likewise, right cortex of Post-CCI DZ animals contained less water than CCI+NaCl and Pre-CCI DZ cortex samples. Edema values in the left hippocampus demonstrate a significantly greater amount of edema in the Pre-CCI DZ animals vs. Sham, Pre-CCI DMSO, and Post-CCI DZ mice. No significant differences were noted in the right hippocampus. Finally, Pre-CCI DZ animals had greater edema content in the diencephalon than Sham and Post-CCI DZ animals.

The results of our edema evaluation should be viewed with caution. Although all steps were taken to ensure precise dissection and identical tissue handling techniques, investigator error may have been introduced into the process. Because the percentage water change that occurs with injury is under 5%, even minor errors in technique will

have profound effects on the final results. We believe that evaluation of edema content in five relatively easy to identify and dissect regions of the brain allows for improved ability to detect regional changes in edema formation. In total, edema evaluation at 24 hours reveals interesting trends. Perhaps the most obvious trend is an apparent trend toward worsening of edema in Pre-CCI DZ animals (right cortex, left hippocampus, and diencephalon). Although we did not observe a hyperglycemic response following administration of 2.5 mg/kg DZ during our pilot study evaluating DZ effects on serum glucose levels or during random sampling of test subjects during this portion of the project, perhaps an unidentified increase in serum glucose may have occurred in some animals. With injury to the BBB, this would allow for the entry of glucose into the brain parenchyma and possibly induce formation of edema via an osmotic gradient. Additional studies should be conducted to confirm or question this finding.

Post-treatment 3-week western blot results

Select biomarkers were chosen in order to evaluate long term changes that occur in the hippocampi following moderate-CCI injury. **Table 2** lists biomarkers that were found to be significantly altered as a result of injury or drug intervention. Two markers, GFAP and PSA-NCAM, displayed significant long term changes in protein levels.

Glial fibrillary acidic protein (GFAP) (Figure 12)

GFAP levels in the L HC were significantly altered as a result of injury and drug treatment. Sham levels were less than any of the three injury groups. Additionally, brain samples of hippocampi from animals in the CCI+DMSO and CCI+DZ treatment groups displayed higher levels of GFAP than what was seen in CCI+NaCl samples, but did not

differ from each other. GFAP levels in the R HC were not significantly altered 3 weeks following injury.

The increased GFAP expression in the ipsilateral hippocampus is not unexpected, and this increase (including serum and CSF levels) has been related to outcome [30-32]. Existing evidence regarding the effects of post-injury DZ administration is limited and only relates to ischemic injury. Farkas et al. [13] did not observe significant long-term astrocyte proliferation (13 weeks after injury) in the hippocampus as a result of either hypoperfusion or DZ treatment. Of note, DMSO was not used as a solvent and the DZ treatment (0.5 mg/kg DZ in 0.1M NaOH following surgery and for five days) did significantly decrease microglial activation. The lack of a difference in GFAP response between DMSO and DZ groups serves as evidence that DMSO can have substantial biological effects; however, the implications of these effects are unknown in this setting and require further investigation.

PSA-NCAM (Figure 13)

The long-term changes in PSA-NCAM seen in the injured hippocampus are curious. By three weeks following injury, Sham+DMSO, CCI+NaCl, and CCI+DZ animals have equivalent levels of GFAP expression, and this level is considerably less than that seen in CCI+DMSO animals. Using IHC evaluation, we were unable to reliably isolate the location(s) within the HC where the changes occurred. The possible reasons for this increase including, a reparative response of SGZ progenitors and/or the CA1 to CA3 pathway or a chronic inflammatory state accentuated by DMSO administration in

the face of injury. Again, if future investigators utilize DMSO as a vehicle for drug delivery, the biological actions of the substance should be considered.

Post-Tx Lesion Volume

Quantification of cortical tissue loss was performed 3 weeks following injury using a modification of previous techniques [15, 26, 33]. In brief, following preparation for immunohistochemistry (described in **Chapter 2**), brains were sectioned on a frozen sliding microtome. Beginning at bregma 0.0, 20 μm slices were cut and every 25th slice was assessed. In this way, a representative slice for every 0.5 mm of anterior to rostral distance was preserved to encompass the lesion. As a result, nine slices were used to evaluate lesion volume. Slices were mounted onto gelatin-coated slides and stained with Cresyl Violet (0.5%), dehydrated in graded ethanol solutions, cleared with xylenes, and coverslipped according to a previously published protocol [34]. All slices were scanned and captured using the Nanozoomer microscopy apparatus and software suite (Hamamatsu, Bridgewater, NJ). The cavity area was measured in nine sections spaced 0.5 mm apart. Careful freehand tracing was utilized to approximate the lesion area in each slice. The total cavity volume was calculated using the following formula [26, 35]:

$$A_1\ (0.5X_1) + A_2(0.5X_1+0.5X_2) + A_{n-1}(0.5X_{n-1} + 0.5X_n) + A_n(0.5X_n)$$

where A_n is the area of the cavity for section (n), and X_n is the distance between sections (n) and (n-1).

Figure 14 summarizes our findings regarding lesion volume measurements. A statistically significant effect of DMSO or DMSO+DZ was not seen. In multiple previous studies, cognitive and motor behaviors have been correlated well with lesion volume

[36]. Our findings should be interpreted with care. First, although we performed all steps to ensure accurate spacing of brain slices throughout the lesion, small differences that occur in mounting and cutting the brain may lead to inaccurate calculations. Additionally, the inherent variation that exists with CCI injury may have introduced significant variability in injury severity. The presence of what may be considered a trend toward decreased lesion volume serves as a guide for future investigation. For example, institution of a multi-week DZ (or combinatorial) dosing scheme may prove to be of benefit in decreasing lesion size and improving behavioral measures.

Table 1. Pretreatment biomarkers that did not display a significant change in expression level resulting from drug treatment when evaluated using immunoblot analysis.

Marker	Manufacturer	Host	Primary Concentration
β-Amyloid Precursor Protein (β-APP)	Invitrogen	Rabbit	1:250
B-Cell Lymphoma 2 (Bcl-2)	Cell Signaling	Rabbit	1:1000
Bcl-2–Associated X Protein (Bax)	Cell Signaling	Rabbit	1:1000
Brain-Derived Neurotrophic Factor (BDNF)	Abcam	Rabbit	1:400
Glial Fibrillary Acidic Protein (GFAP)	Thermo	Mouse	1:5000
Growth Associated Protein 43 (GAP-43)	Abcam	Rabbit	1:5000
Heat Shock Protein 25 (HSP 25)	Enzo	Rabbit	1:2000
Heat Shock Protein 70 (HSP 70)	Enzo	Mouse	1:2000
Heme Oxygenase-1 (HO-1)	Enzo	Rabbit	1:1000
Postsynaptic Density Protein 95 (PSD-95)	Millipore	Rabbit	1:5000
PSA-NCAM	Millipore	Mouse	1:1000

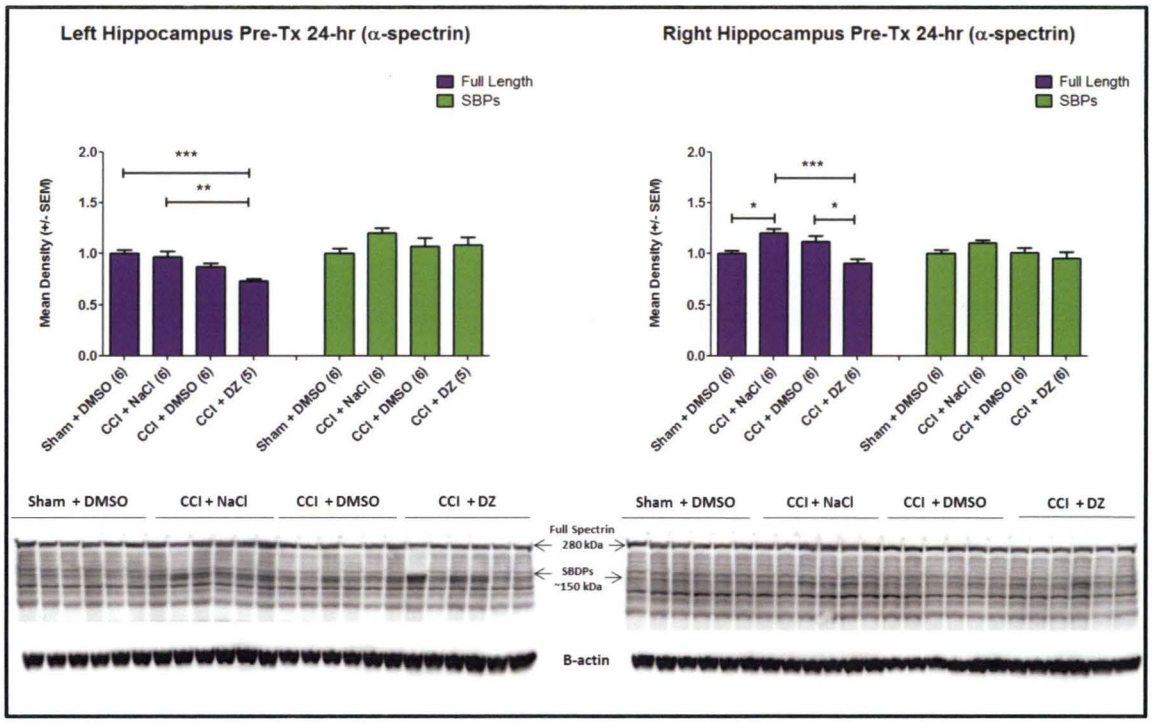

Figure 1. Left and right hippocampus α-spectrin protein levels 24 hours following DZ pretreatment and CCI. All data evaluation was performed using one-way ANOVA and *post-hoc* Tukey's HSD, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

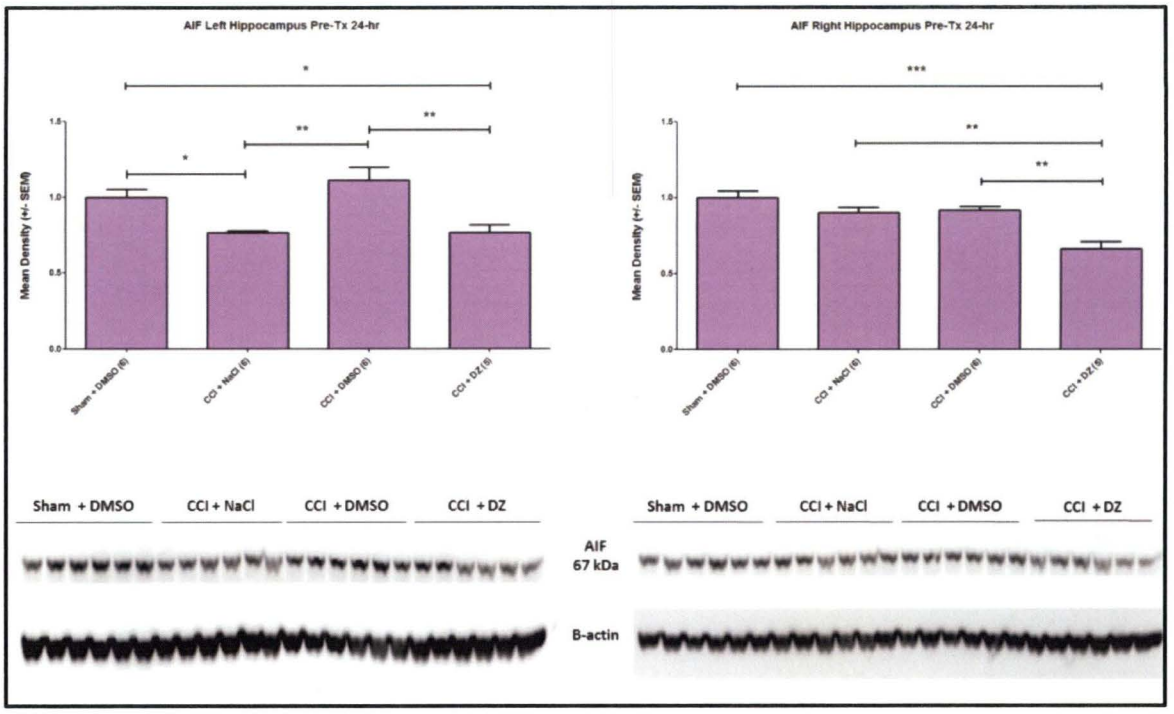

Figure 2. Left and right hippocampus AIF protein levels 24 hours following DZ pretreatment and CCI. All data evaluation was performed using one-way ANOVA and *post-hoc* Tukey's HSD, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

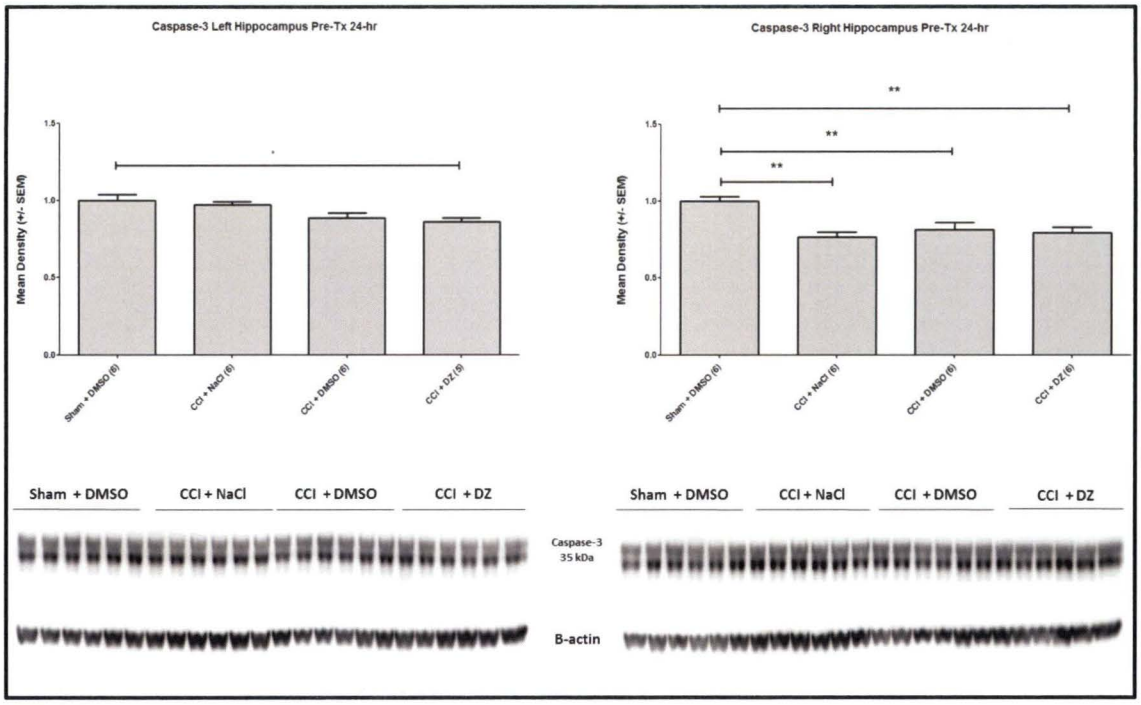

Figure 3. Left and right hippocampus caspase-3 protein levels 24 hours following DZ pretreatment and CCI. All data evaluation was performed using one-way ANOVA and *post-hoc* Tukey's HSD, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

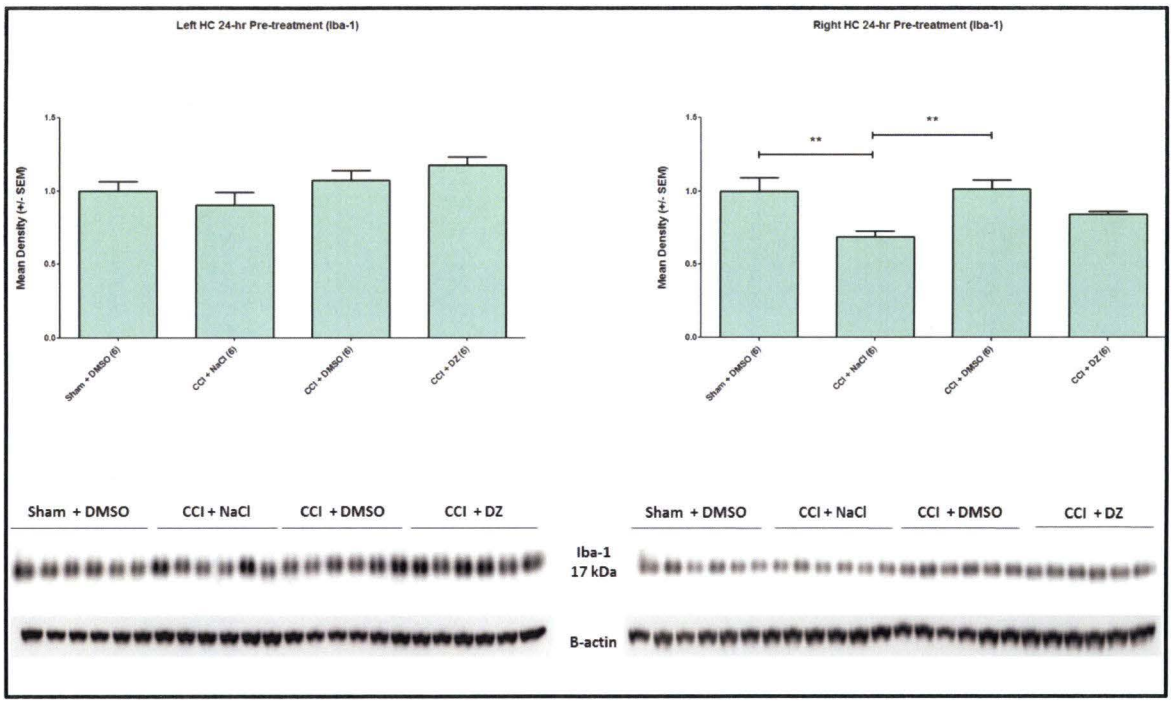

Figure 4. Left and right hippocampus Iba-1 protein levels 24 hours following DZ pretreatment and CCI. All data evaluation was performed using one-way ANOVA and *post-hoc* Tukey's HSD, ** = $p < 0.01$.

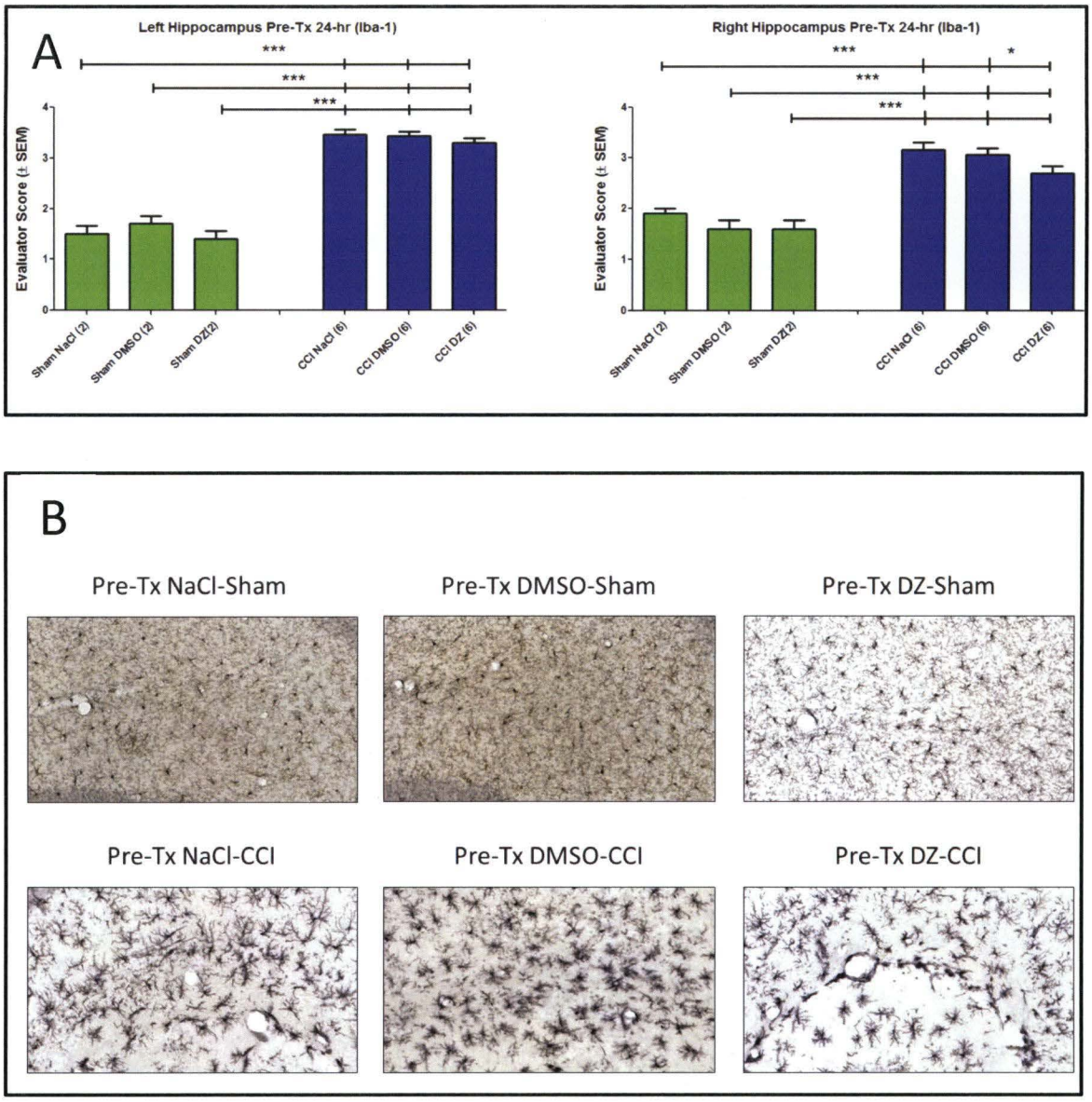

Figure 5. Qualitative analysis of left and right hippocampus Iba-1 protein levels 24 hours following DZ pretreatment and CCI. (A) Results from blinded evaluator analysis. See text for details. (B) Pictures of representative samples of the left hippocampus 24 hours post-injury. The above pictures are all 20(x) magnification. Numbers in parentheses indicate the number of animals per group. All data evaluation was performed using one-way ANOVA and *post-hoc* Tukey's HSD, * = $p < 0.05$; *** = $p < 0.001$.

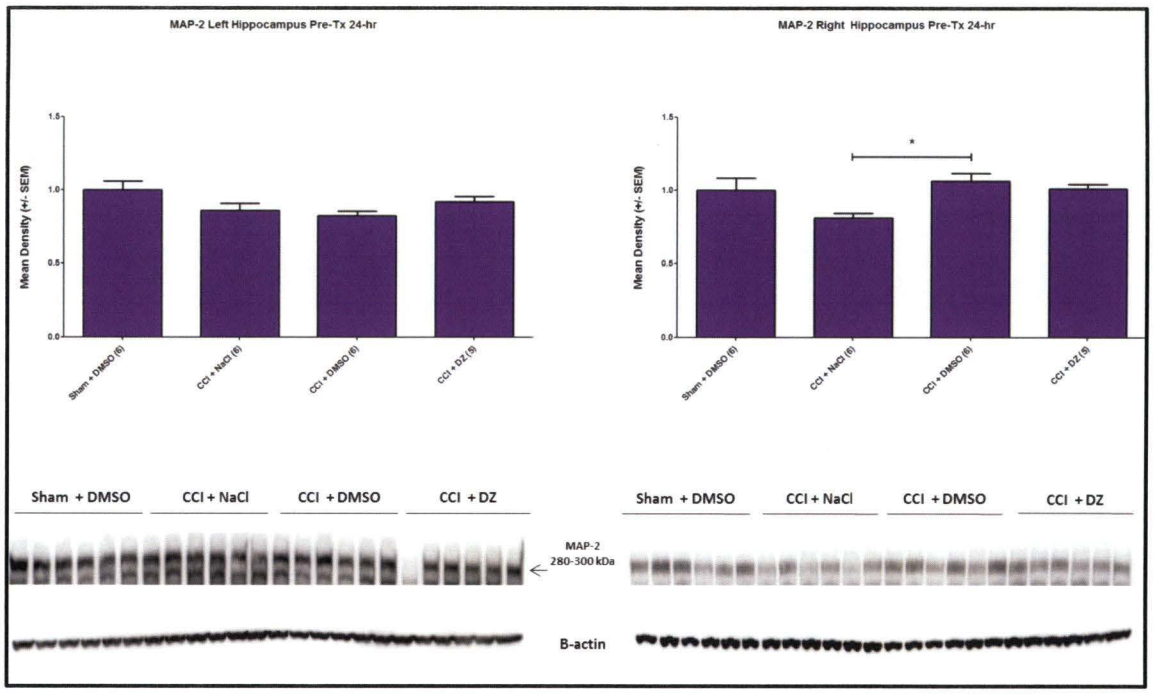

Figure 6. Left and right hippocampus MAP-2 protein levels 24 hours following DZ pretreatment and CCI. All data evaluation was performed using one-way ANOVA and *post-hoc* Tukey's HSD, * = $p < 0.05$.

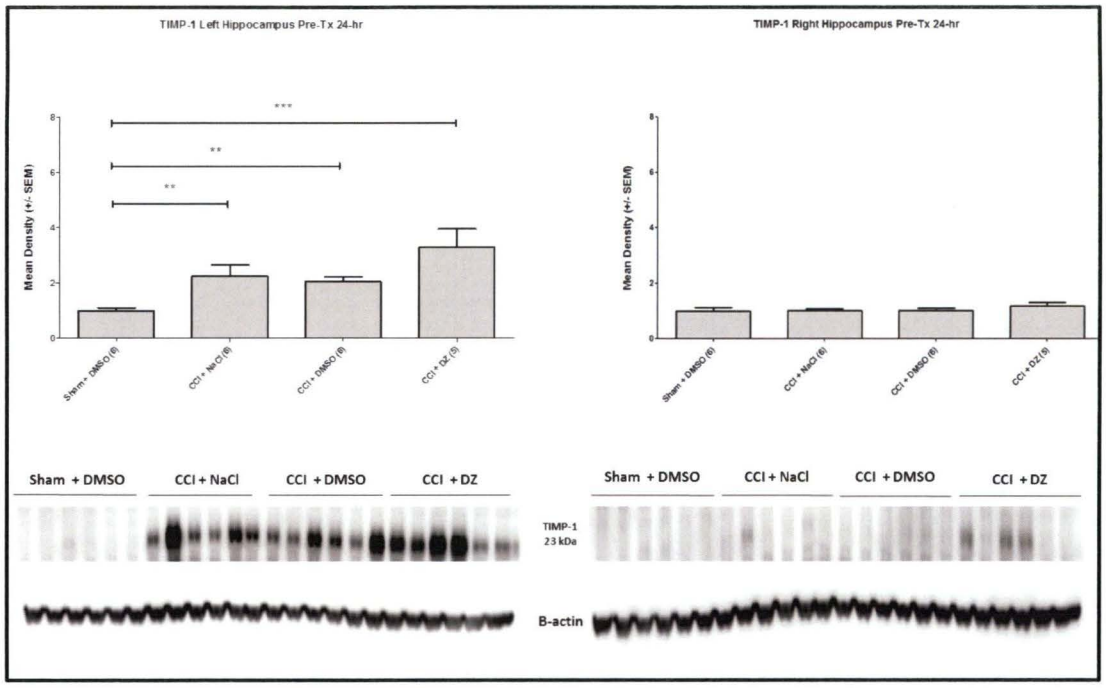

Figure 7. Left and right hippocampus TIMP 1 protein levels 24 hours following DZ pretreatment and CCI. Following natural log transformation, the data passed Bartlett's test for equal variance. All data evaluation was performed using one-way ANOVA and *post-hoc* Tukey's HSD, ** = $p < 0.01$; *** = $p < 0.001$.

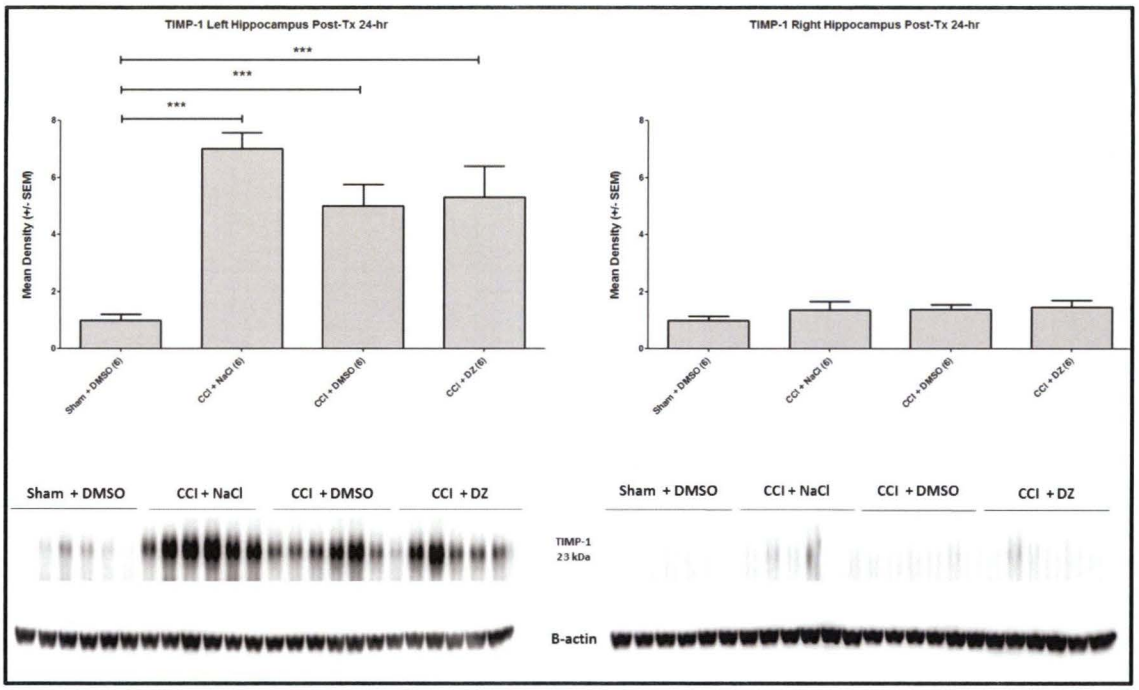

Figure 8. Left and right hippocampus TIMP 1 protein levels 24 hours following DZ post treatment and CCI. Following natural log transformation, the data passed Bartlett's test for equal variance. All data evaluation was performed using one-way ANOVA and *post-hoc* Tukey's HSD, *** = $p < 0.001$.

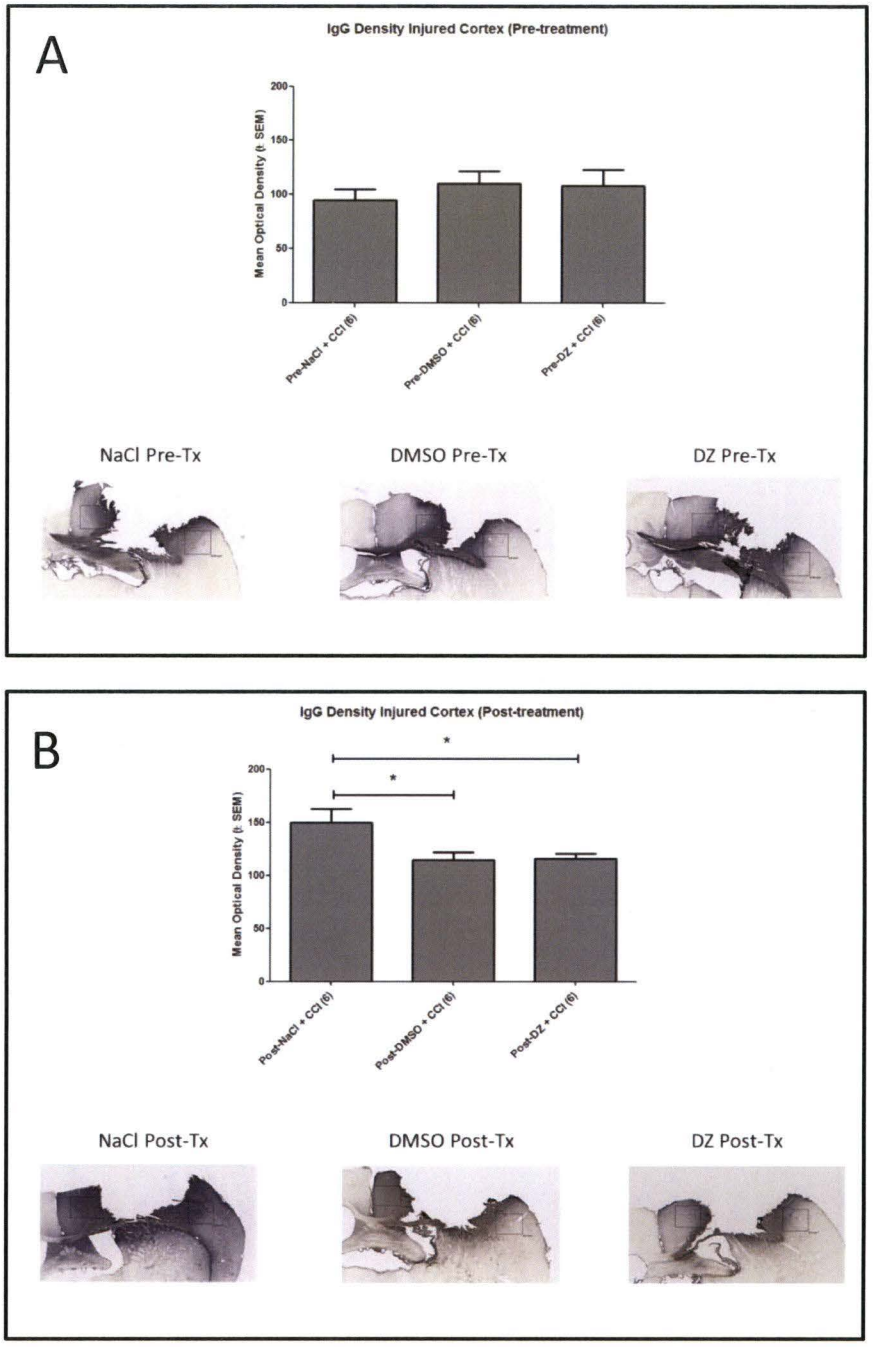

Figure 9. Optical density of IgG staining in the injured cortex 24 hours after CCI. (A) IgG density measurements 24 hours after CCI in pre-treated animals. Pictures are representative samples of each category. (B) IgG density measurements 24 hours after CCI in post-treated animals. Pictures are representative samples of each category. (n) =

number of animals per group. All data evaluation was performed using one-way ANOVA and *post-hoc* Tukey's HSD, * = $p < 0.05$.

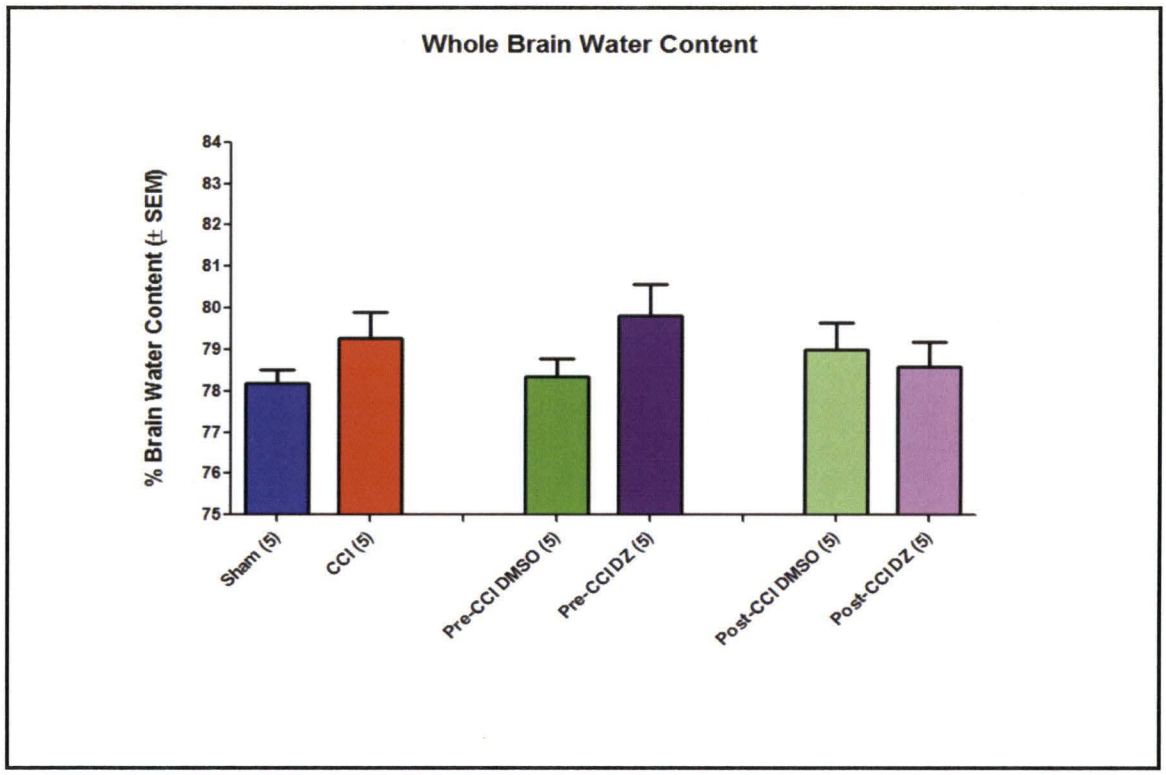

Figure 10. Brain water content 24 hours after CCI.

Composite percent brain water content 24 hours following procedure in Sham, CCI, and CCI animals that received DMSO and DZ+DMSO. See text for details. (n) = number of animals per group. All data evaluation was performed using one-way ANOVA.

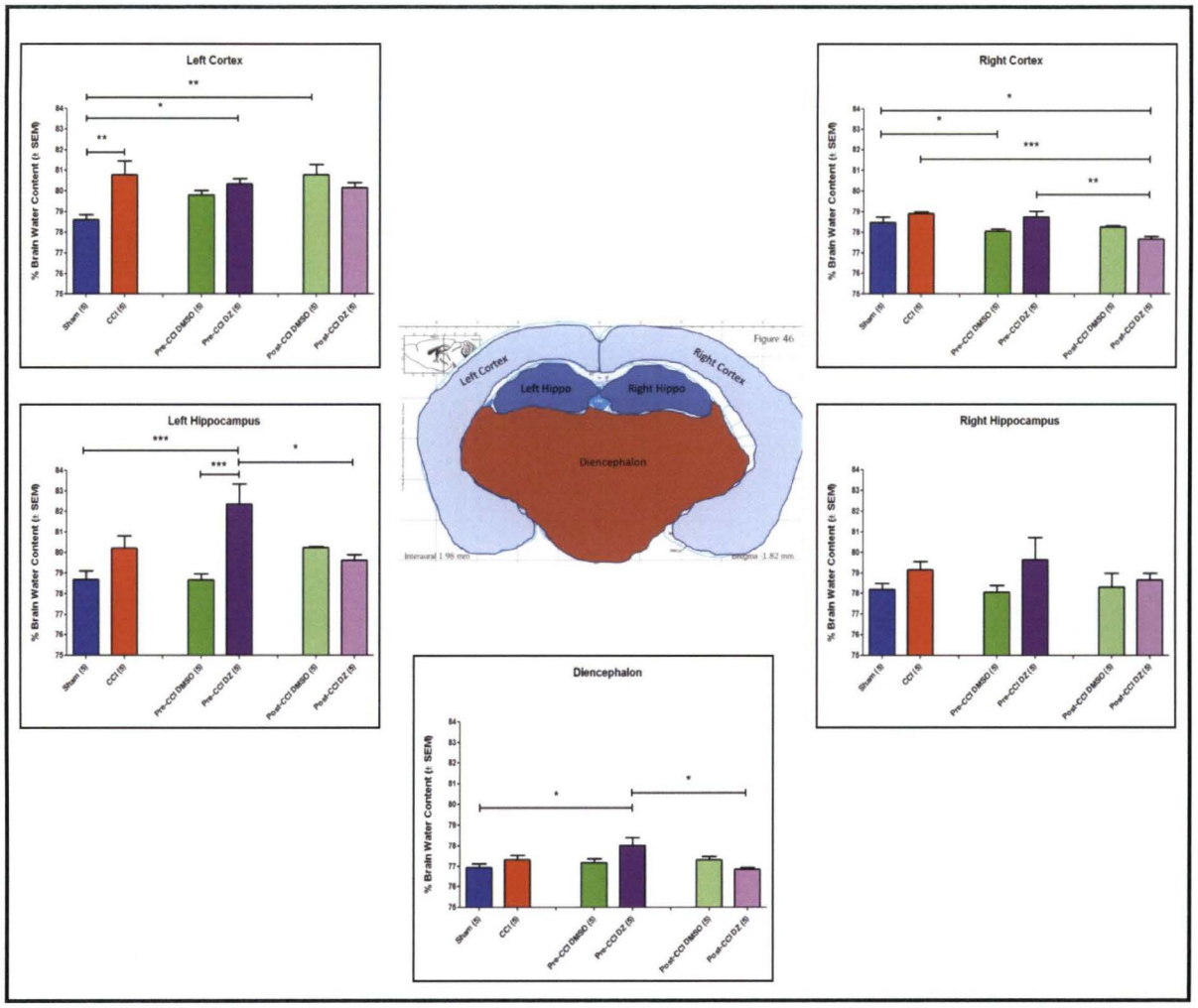

Figure 11. Regional brain water content 24 hours after CCI.

Percent brain water content in each of the regions dissected. Center figure denotes regions dissected. See text for details. (n) = number of animals per group. All data evaluation was performed using one-way ANOVA and *post-hoc* Tukey's HSD, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Coronal section image modified from [37] with permission.

Table 2. Post-treatment biomarkers that did not display a significant change in expression level as a result of drug treatment when evaluated using immunoblot analysis.

Marker	Manufacturer	Host	Primary Concentration
Apoptosis Inducing Factor (AIF)	Millipore	Rabbit	1:1000
β-Amyloid Precursor Protein (β-APP)	Invitrogen	Rabbit	1:250
Caspase-3 (Casp 3)	Cell Signaling	Rabbit	1:1000
Growth Associated Protein 43 (GAP-43)	Abcam	Rabbit	1:5000
Iba-1	Wako	Rabbit	1:1000
Microtubule-Associated Protein (MAP-2)	Millipore	Rabbit	1:1000
Postsynaptic Density Protein 95 (PSD-95)	Millipore	Rabbit	1:5000
Tau	Sigma	Mouse	1:10,000

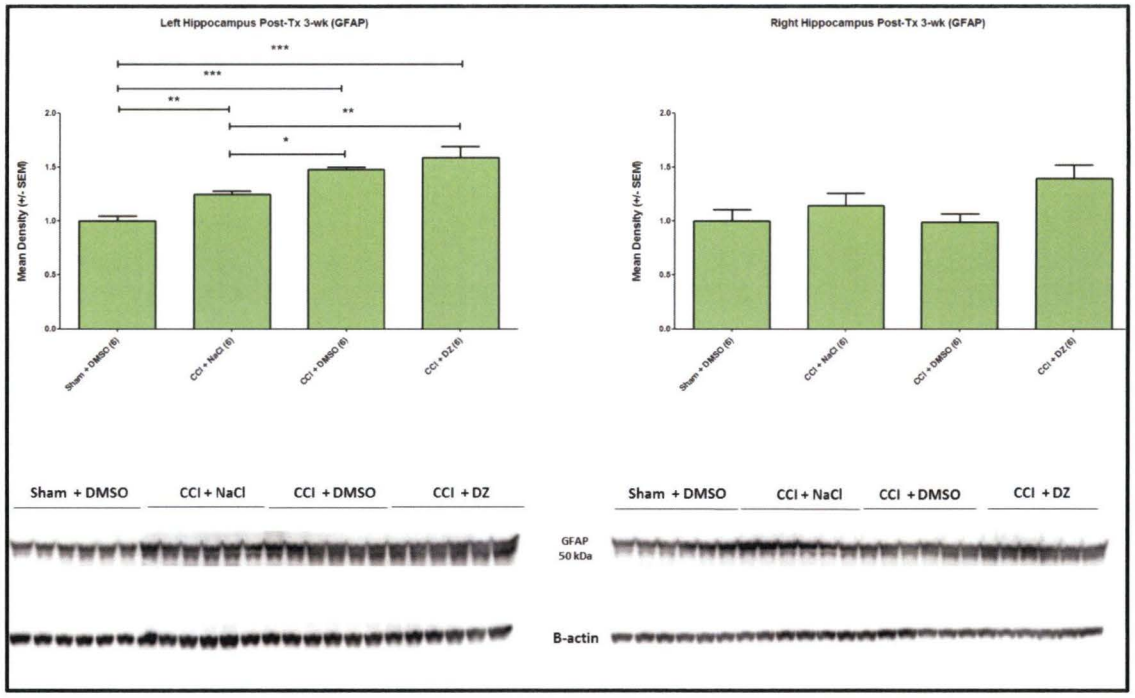

Figure 12. Left and right hippocampus GFAP protein levels 3-weeks following CCI and DZ post-treatment. All data evaluation was performed using one-way ANOVA and *post-hoc* Tukey's HSD, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

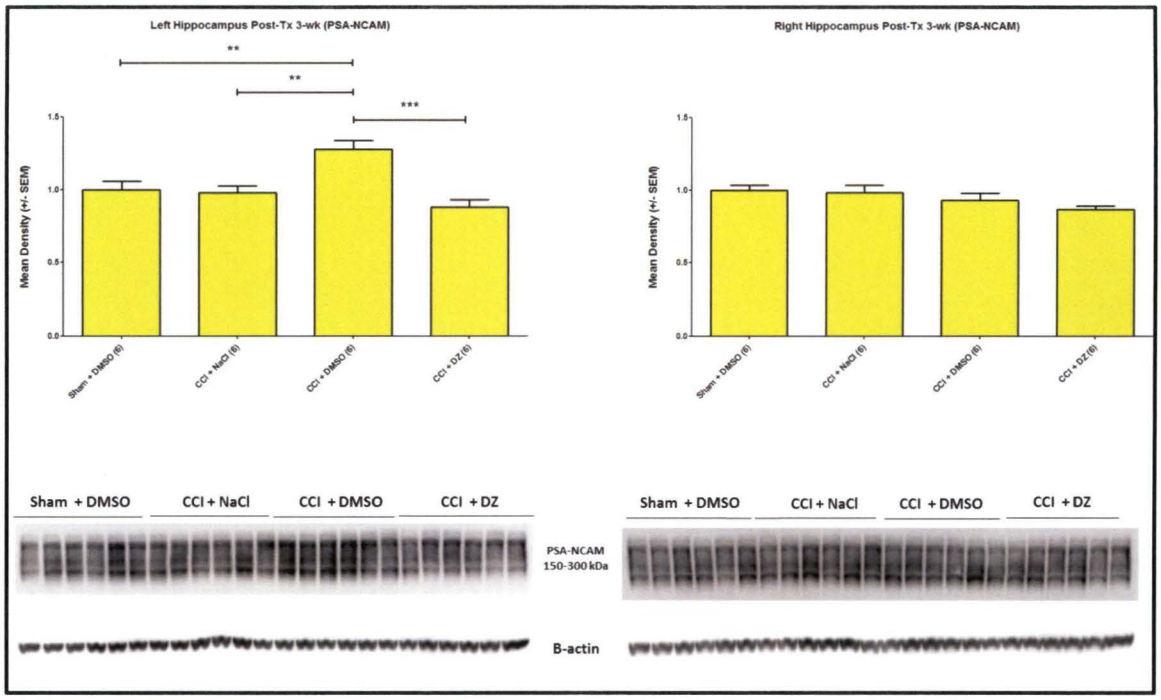

Figure 13. Left and right hippocampus PSA-NCAM protein levels 3-weeks following CCI and DZ post-treatment. All data evaluation was performed using one-way ANOVA and *post-hoc* Tukey's HSD, ** = $p < 0.01$.

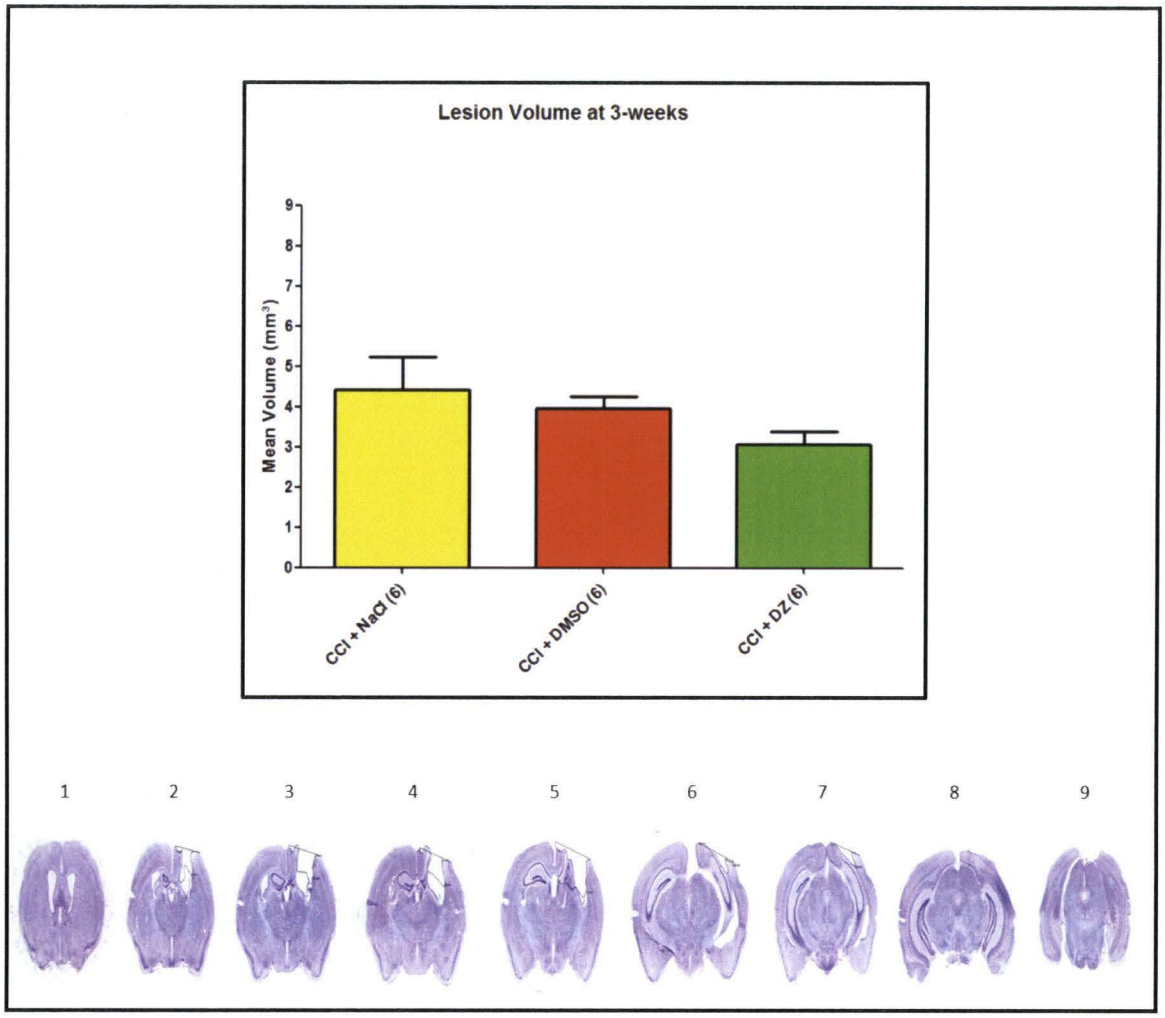

Fig 14. Lesion volume 3 weeks following injury.

Histogram representing results of analysis of lesion volume 3 weeks following injury. Cresyl violet coronal sections represent a typical series of sequential section through the lesion. CCI+NaCl: 4.421 ± 0.8092 mm^3; CCI+DMSO: 3.968 ± 0.2904 mm^3; CCI+DZ: 3.085 ± 0.3176 mm^3. (n) = number of animals per group. All data evaluation was performed using one-way ANOVA.

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