NEUROPROTECTIVE EFFECTS OF POSTINJURY LITHIUM TREATMENT: DETERMINING THE OPTIMAL DOSING PARADIGM AND ASSESSING POTENTIAL MECHANISMS OF ACTION

Katharine Eakin
Virginia Commonwealth University

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NEUROPROTECTIVE EFFECTS OF POSTINJURY LITHIUM TREATMENT:
DETERMINING THE OPTIMAL DOSING PARADIGM AND ASSESSING
POTENTIAL MECHANISMS OF ACTION

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University

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Richmond, VA
May, 2010
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It has been a long journey to get here…it is said that the journey is more important than the destination. While I do not disagree with this sentiment, I find tremendous joy in reaching the end of this long road. I could not have made this trek without the unconditional love and support of my family, friends, colleagues, and mentors. I am grateful to each of you. I thank my mentor, Bob Hamm, who opened the door for me to achieve my dream of becoming a doctor. Thank you for your guidance, tutelage, and most of all, patience. To my committee members, Joseph Porter, Aron Lichtman, Linda Phillips, Thomas Reeves, and Dong Sun, I thank you for consistently encouraging me and generously sharing your considerable expertise.

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<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>2DG</td>
<td>[11C] raclopride and 2-deoxyglucose</td>
</tr>
<tr>
<td>5-HT</td>
<td>serotonin</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormones</td>
</tr>
<tr>
<td>ADHD</td>
<td>attention deficit hyperactivity disorder</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptosis-protease-activating factor 1</td>
</tr>
<tr>
<td>atm</td>
<td>atmospheres of pressure</td>
</tr>
<tr>
<td>BAD</td>
<td>bipolar affective disorder</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma/leukemia-2 protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine (5-bromo-2-deoxyuridine)</td>
</tr>
<tr>
<td>BW</td>
<td>beam walk</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>calcium</td>
</tr>
</tbody>
</table>
CA1    Cornu Ammonis, area 1
CA3    Cornu Ammonis, area 3
CaM    calcium-calmodulin
cAMP   cyclic adenosine monophosphate
CCI    controlled cortical impact
CDC    Centers for Disease Control and Prevention
CDP-DG cytidine disphosphate – diacyl glycerol
CGC    cerebellar granule cells
cm     centimeter
CNS    central nervous system
CO₂    carbon dioxide
COMT   catechol-O-methyltransferase
CPP    cerebral perfusion pressure
CREB   cyclic adenosine monophosphate response element binding protein
CSF    cerebral spinal fluid
DA     dopamine
DAG    diacylglycerol
DAI    diffuse axonal injury
DAT    dopamine transporter
DG     dentate gyrus
DISC   death-inducing signaling complex
DLPFC  dorsolateral prefrontal cortex
DNA    deoxyribonucleic acid
DSM-IV  Diagnostic and Statistical Manual of Mental Disorders, 4th Edition
EAA  excitatory amino acid
ED  Emergency Department
EPI  epinephrine
ERK  extracellular regulated kinase
$F$  F statistic
FADD  Fas-associated death domain protein
FPI  fluid percussion injury
$g$  gram
GABA  $\gamma$-aminobutyric acid
GAD-67  glutamic acid decarboxylase 67
GAP-43  growth associated protein-43
Glu  glutamate
Grp78  glucose-regulated protein, 78-kilodalton
GSK-3  glycogen synthase kinase-3
GSK-3\(\alpha\)  glycogen synthase kinase-3 alpha
GSK-3\(\beta\)  glycogen synthase kinase-3 beta
hr  hour
HCl  hydrogen chloride
HII  hypoxic-ischemic injury
HIV  human immunodeficiency virus
HSF-1  heat-shock factor-1
HSP70  heat-shock protein 70
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ip</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>iv</td>
<td>intravenous</td>
</tr>
<tr>
<td>IC50</td>
<td>median inhibition concentration</td>
</tr>
<tr>
<td>ICP</td>
<td>intracranial pressure</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate early genes</td>
</tr>
<tr>
<td>IP</td>
<td>inositol monophosphate</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>KA</td>
<td>kainic acid</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>LiCl</td>
<td>lithium chloride</td>
</tr>
<tr>
<td>LSD</td>
<td>least significant difference</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>M</td>
<td>mean</td>
</tr>
<tr>
<td>mABF</td>
<td>mean arterial blood flow</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCAO</td>
<td>middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>mEq</td>
<td>milliequivalent</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>magnesium ion</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
</tbody>
</table>
mm  millimeter
mM  millimolar
MM  MagicMark
mmol  millimole
MPFC  medial prefrontal cortex
MRI  magnetic resonance imaging
ms  milliseconds
MWM  Morris water maze
N  total number of subjects
n  number of subjects in a group
NCL  normal cell lysate
NE  norepinephrine
NF-κB  nuclear factor-kappa B
ng  nanogram
NINDS  National Institute of Neurological Disorders and Stroke
nM  nanomole
NMDA  N-methyl-D-aspartate
NR1  NMDA receptor subunit 1
NR2  NMDA receptor subunit 2
NR2A  NMDA receptor subunit 2A
NT  neurotransmitter
p  level of alpha
pGSK-3β  phosphorylated glycogen synthase kinase-3 beta
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>po</td>
<td>orally (per os)</td>
</tr>
<tr>
<td>PAP</td>
<td>3’ (2’) phosphoadenosine 5’-phosphate</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>pH</td>
<td>partial pressure hydrogen</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PID</td>
<td>postinjury day</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidylinositol biphosphate</td>
</tr>
<tr>
<td>PIP₃</td>
<td>phosphatidylinositol-3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A (a.k.a. cAMP-dependent protein kinase)</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B (a.k.a. Akt)</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PSD-95</td>
<td>postsynaptic density protein 95-kDa</td>
</tr>
<tr>
<td>PTSD</td>
<td>post-traumatic stress disorder</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RDF</td>
<td>remote functional depression</td>
</tr>
<tr>
<td>RnPIP</td>
<td>rat PAP phosphatase</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Src</td>
<td>sarcoma family of protein tyrosine kinases</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SPECT</td>
<td>single photon emission computed tomography</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>$t$</td>
<td>Student’s $t$-test distribution, sample value of the $t$-test statistic</td>
</tr>
<tr>
<td>TAI</td>
<td>traumatic axonal injury</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>tris-buffered saline – tween</td>
</tr>
<tr>
<td>TrkA</td>
<td>tropomyosin-related kinase A</td>
</tr>
<tr>
<td>TrkB</td>
<td>tropomyosin-related kinase B</td>
</tr>
<tr>
<td>TTBI</td>
<td>time to become immobile</td>
</tr>
<tr>
<td>TTI</td>
<td>total time immobile</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase nick-end labeling</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>$\mu$l</td>
<td>microliter</td>
</tr>
<tr>
<td>$\mu$m</td>
<td>micron</td>
</tr>
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Abstract

NEUROPROTECTIVE EFFECTS OF POSTINJURY LITHIUM TREATMENT: DETERMINING THE OPTIMAL DOSING PARADIGM AND ASSESSING POTENTIAL MECHANISMS OF ACTION

By Katharine Coryell Eakin, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2010

Major Director: Robert J. Hamm, Ph.D., Professor Emeritus
Department of Psychology

Traumatic brain injury (TBI) has a dramatic impact on our society in terms of mortality, morbidity, and inherently high financial costs. Formidable research efforts are being addressed to the identification of neuroprotective agents capable of ameliorating the neurological outcome after TBI. Preclinical studies have recently demonstrated lithium to be a promising neuroprotective agent for both acute ischemic brain injury and chronic neurodegenerative disease. In light of these encouraging data, we designed a lateral fluid-percussion injury (FPI) study aimed at investigating the role of early post-traumatic administration of lithium as a strategy for reducing TBI-induced motor and cognitive deficits. The optimal dose of this agent and the time window for its administration have been determined on the basis of data derived from the assessment of motor and cognitive functioning in experimental animals, as well as from
the stereological quantification of neuronal survival (PID 7) within the CA3 and hilar regions of the hippocampus ipsilateral to the FPI. In addition, we attempted to elucidate the mechanisms underlying the neuroprotective properties of this drug via western blot analysis of levels of the pro-apoptotic marker caspase-3 (PID 1, 7) and two neuroplasticity markers, growth associated protein-43 (GAP-43) and brain-derived neurotrophic factor (BDNF) (PID 1, 7, 21).

Our findings indicate that low-dose lithium chloride (0.125 or 0.25 mmol/kg), given either 30 min or 8 hr after lateral FPI significantly ameliorates injury-induced cognitive and motor impairment. Specifically, cell survival in the CA3 region of the hippocampus of the injured lithium-treated animals (but not in the hilus) was significantly increased compared to injured vehicle-treated animals. Western blot analyses revealed a significant increase in GAP-43 levels on PID 7 in injured animals when treated with lithium, indicating a possible mechanism for lithium-induced neuroprotection. In contrast, BDNF levels were relatively unchanged until PID 21, and caspase-3 activation was not observed at all, suggesting that these proteins play less significant roles in the observed neuroprotective effects of lithium treatment after lateral FPI. Early administration of lithium, within 8 hours after TBI, holds promise as an effective therapy to ameliorate postinjury neurobehavioral deficits and warrants further investigation in clinical TBI studies.

**Keywords:** lithium, traumatic brain injury, TBI, cognitive function, vestibulomotor function, depression, forced swim test, beam walk, neuroprotection, hippocampus, CA3, hilus, BDNF, GAP-43, caspase-3, preclinical, translational research, Morris water maze, MWM.
Neuroprotective Effects of Postinjury Lithium Treatment: Determining the Optimal Dosing Paradigm and Assessing Potential Mechanisms of Action

Traumatic Brain Injury

Epidemiology. TBI represents a major public health problem in the United States. TBI is one of the leading causes of preventable mortality and morbidity among children, young adults and the elderly (Langlois, Rutland-Brown, & Thomas, 2004; Sosin, Sniezek, & Thurman, 1996; Thurman et al., 1999).

According to the Centers for Disease Control and Prevention (CDC), approximately 1.4 million Americans sustain a TBI each year. To put the magnitude of this number into perspective, there are more new cases of TBI each year than breast cancer (212,920) (American Cancer Society) and HIV/AIDS (43,700) combined. Of these 1.4 million, approximately 1.1 million will receive care from an emergency department (ED), 230,000 will require hospitalization, and 50,000 will die. The most common causes of TBI are falls (28%), motor vehicle accidents (20%), “struck by/against” events (19%), and assault (11%) (Langlois et al., 2004).

Incidence of TBI is highest in children (0-4 years), young adults (15-19 years), and older adults (>75 years). In almost every age group, males are about 1.5 times more likely than females to incur TBI, possibly due to a higher incidence of motor vehicle accidents, use of firearms, and risk-taking behavior among males (Langlois et al., 2004). Additionally, in the young adult population (15-24 years), mortality rates for males are 3.6 times higher than for females.

TBI survivors may suffer enduring physical, emotional, cognitive and behavioral changes that significantly interfere with daily functioning. Recent epidemiologic data from the CDC
indicate that 80,000-90,000 individuals become disabled from TBI each year. A conservative estimate is that 5.3 million Americans currently live with long-term disabilities due to TBI, accounting for 2% of the total population (Langlois et al., 2004; Thurman, Alverson, Dunn, Guerrero, & Sniezek, 1999). Functional impairment may also occur following mild brain injury, and it is estimated that 25% to 35% of individuals will continue to present cognitive and emotional difficulties when re-evaluated at 3 and 6 months from the time of injury.

In addition to the physical, psychosocial and vocational disabilities caused by TBI, the related long-term morbidity may place a dramatic financial burden on the individual, his or her family, workplace, and society. The direct and indirect costs associated with TBI in the U.S. are estimated to be $60 billion annually (Finkelstein E, Corso P, Miller T and associates. The Incidence and Economic Burden of Injuries in the United States. New York (NY): Oxford University Press; 2006.), and the lifetime cost for one person surviving a severe TBI can reach $4 million (Langlois et al., 2004).

**Biomechanics of TBI.** TBI can result from rapid acceleration or deceleration of the head and neck, blunt trauma to the head caused by an object striking the head, or an object penetrating the skull (McIntosh et al., 1996; Povlishock & Christman, 1994). Holbourn (1943) was the first to describe the effects of shearing strains, specifically rotational acceleration forces, as a primary cause of predictable injury in the brain. The effect of acceleration or deceleration forces on the brain is varied and largely depends on the presence of rotational forces applied to the head and neck. Individuals who sustain a sagittal (front to back) injury have the greatest likelihood of recovery, lateral (side to side) injuries have the worst percentage for recovery, and oblique injury outcomes are somewhere in between (McIntosh et al., 1996). These forces are sufficient to produce brain injury without any accompanying contact injury.
The mechanical forces that produce TBI can be attributed to either static or dynamic loading. In order to assess the severity of a TBI resulting from dynamic loading, it is important to account for inertial, acceleration and impact forces. The greater the force acting on the head and neck, the more damage is inflicted (Gaetz, 2004; Ommaya & Gennarelli, 1974). By understanding the biomechanical events associated with TBI, researchers are better able to replicate injuries in animal models, and this might ultimately lead to the development of new treatments for TBI.

**Static loading.** Static loading occurs when forces are applied to an unmoving head over an extended time course (greater than 200 ms) and generally produces multiple, comminuted, or eggshell fractures of the skull. This type of mechanical force does not normally produce the characteristic symptoms commonly associated with TBI, and neurological signs such as coma are not generally seen unless the force is sufficient to cause deformation of the skull and brain tissue (Graham, McIntosh, Maxwell, & Nicoll, 2000).

**Dynamic loading.** Dynamic loading is the mechanical force that is most commonly associated with the typical sequelae of TBI. Dynamic loading generally occurs in a much faster timeframe compared to static loading (less than 50 ms). Dynamic loading can be further classified as either impact or impulsive (Graham et al., 2000; McIntosh et al., 1996).

**Impact loading.** Impact loading occurs when a blunt object strikes the head, typically producing both contact- and inertial-related injuries. The amount of damage produced is directly related to the amount of force applied to the skull. Contact forces can generate stress waves that radiate through the skull and can cause additional skull fractures distant from the point of impact (Graham et al., 2000; McIntosh et al., 1996).
**Impulsive loading.** Impulsive loading occurs when the head is set into motion or when a moving head is suddenly stopped, either without striking anything or by contact with an object. This type of injury can be produced when the head moves indirectly as a result of impact to another part of the body (McIntosh et al., 1996). Primary damage to the brain parenchyma is caused by non-uniform distribution of pressure and strain (Graham et al., 2000; McIntosh et al., 1996). Biological tissue is more resilient to slower strains as compared to fast strains (Graham et al., 2000).

**Primary and secondary brain injury.** Historically, the pathophysiology of TBI has been characterized by two distinct phases: primary and secondary injury. Primary injury consists of the immediate craniocerebral damage (i.e., injury to the skull, brain or both) that occurs at the moment of the initial impact, and is classically distinguished into two components, focal (cerebral contusion, laceration, rupture of intracranial vessels) and diffuse (traumatic axonal injury (TAI), diffuse microvascular injury) (Graham et al., 2000; McIntosh et al., 1996; Povlishock & Becker, 1985), although both may coexist to varying degrees (Graham et al., 2000; Povlishock & Katz, 2005).

Secondary injury can be described as a dynamic process of neuronal dysfunction eventually culminating in cell death, triggered by the activation of a cascade of complex and interrelated neurochemical events that initiate in the immediate post-traumatic period. This process, which evolves in the hours and days following the initial traumatic event, is responsible for progressive neurological impairment and may significantly alter prognosis. Therefore, an understanding of the biomolecular mechanisms underlying this secondary process of neuronal demise and the development of effective therapeutic strategies capable of arresting it has become
a focus of intense research activity over the last two decades, both in the clinical and preclinical setting.

One of the most important concepts emerging from this research is that a lack of oxygen supply to the brain (i.e., hypoxia) is the key factor initiating the cascade associated with the development of the secondary brain injury. Therefore, prevention or prompt recognition and treatment of factors such as hypotension and increased intracranial pressure (ICP) that lead to decreased oxygen delivery to the injured brain appear to be crucial to a good neurological outcome. Increase in intracranial pressure is related to several factors such as brain edema (both cytotoxic and vasogenic), hydrocephalus, and intracranial mass (brain contusion or intraparenchymal hematoma). Hypoxic events lead to the production of free radicals, disruption of intracellular calcium, release of cytochrome c from mitochondria, and initiation of apoptotic cascades (Gaetz, 2004; McIntosh et al., 1996; Merenda & Bullock, 2006).

**Focal injury.** Primary focal injury such as a missile (e.g., gunshot wound) or penetrating wound is characterized by the presence of contusions or direct disruption of brain tissue and can include hemorrhage and hematomas in the epidural, subdural, subarachnoid, and intraparenchymal areas (Gaetz, 2004; Gennarelli, 1993). Secondary damage from focal injuries includes delayed neuronal injury to neighboring regions, microvascular injury, focal ischemic-hypoxic injury, herniation, and regional and diffuse hypometabolism. Focal injuries produce regions of significantly reduced cerebral blood flow creating ischemic conditions that promote inflammation and cytotoxicity in addition to neuronal necrosis (Bullock, Maxwell, Graham, Teasdale, & Adams, 1991; Gaetz, 2004).

**Contusions.** The presence of a contusion is extremely common in patients with focal TBI; however, TBI can occur without contusion. Contusions cannot be used as an explanation
for loss of consciousness at the time of injury or as a factor in the maintenance of a comatose state, but they are linked to focal seizures and/or functional deficits in the language centers of the brain (Povlishock & Christman, 1994; Ribas & Jane, 1992). In most cases, contusions are the byproduct of hemorrhagic lesions within the gray matter or at the gray-white interface and contribute to neuronal damage and ischemia (Povlishock & Katz, 2005). Contusions are most commonly located on the frontal and temporal poles, the lateral and inferior surfaces of the frontal and temporal lobes, and above the Sylvian fissure (Gaetz, 2004; Gennarelli & Graham, 1998; Gurdjian, 1976; McIntosh et al., 1996; Povlishock & Christman, 1994). They are commonly seen at the apex of gyri and can appear as punctate hemorrhages or streaks of hemorrhage usually accompanied by progressive bleeding into adjoining white matter (Gennarelli & Graham, 1998).

There are several types of contusions, including coup contusions that occur directly beneath skull fractures, contracoup contusions that occur some distance (not always directly opposite) from the fracture, and gliding contusions. Gliding contusions are most likely to be associated with diffuse brain injury and are produced by cortical gray matter moving in opposition to the underlying white matter causing shearing strains that damage the penetrating vessels located at the gray/white interface, producing hemorrhagic lesions in the parasagittal cortex (Adams, Doyle, Graham, Lawrence, & McLellan, 1986; Povlishock & Christman, 1994). In addition, nonhemorrhagic contusions may be identified using MRI. These lesions are not associated with hemorrhage but are located on the cortical surface where one would expect to see “traditional” contusions. Another type of nonhemorrhagic contusion is identified within the subcortical white matter, leaving the superficial cortex unharmed. These lesions are associated with shearing forces and diffuse axonal injury (Povlishock & Christman, 1994).
**Hematomas.** There are several kinds of hematomas, including intraparenchymal hematoma, epidural hematoma, and acute subdural hematoma (Gaetz, 2004; Gennarelli & Graham, 1998; Povlishock & Christman, 1994). One possible explanation for the formation of hematomas is the rupturing of cerebral arterioles caused by the shearing and tensile forces generated by the injury (Povlishock & Christman, 1994). Intraparenchymal hematomas are located deep within the parenchyma and are associated with rupture of a blood vessel. Epidural hematomas are most commonly associated with a skull fracture. Acute subdural hematomas are caused by the rupturing of bridging veins beneath the dura or cortical arteries (Gennarelli & Graham, 1998). Cerebral hematomas are usually formed at the time of injury; however, there is evidence that delayed hematoma formation may occur in patients with injuries ranging from mild to severe (Povlishock & Christman, 1994; Soloniuk, Pitts, Lovely, & Bartkowski, 1986). Hematoma and contusion are associated with secondary ischemic damage and subsequent necrosis from mass effect from blood that affects the adjacent tissue (Gennarelli & Graham, 1998).

**Diffuse injury.** Primary diffuse injury consists of diffuse axonal injury (DAI) and petechial white matter hemorrhage. Secondary diffuse injury is associated with delayed neuronal injury, microvascular injury, diffuse hypoxic-ischemic injury (HII), and diffuse hypometabolism (Povlishock & Katz, 2005). In addition to secondary injuries, delayed pathology resulting from brain injury can lead to DAI, which is characterized by axonal swelling and degradation followed by axonal separation from its downstream segment and characteristic formation of a retraction bulb (Povlishock & Christman, 1995). Diffuse cell death is among the sequelae commonly observed following TBI and can be attributed to one of two morphologically distinct processes: apoptotic and necrotic.
Necrosis. Necrotic cell death occurs following degradation of the cell membrane and disruption of ionic homeostasis leading to the rapid destruction of the cytoskeleton and cytoplasmic components (Povlishock & Katz, 2005). Rapid cell death is linked to the activation of the cysteine proteases calpain and caspase, causing degeneration and increased porosity of the plasma membrane and leakage of cell contents into the extracellular space (Rowe & Chuang, 2004).

Apoptosis. Apoptosis is programmed cell death from internal mechanisms. Unlike necrosis, apoptosis does not require disruption of the cell membrane. Apoptotic events are mediated by internucleosomal DNA strand breaks with nuclear condensation that cause the cell to slowly die (Povlishock & Katz, 2005). Apoptotic cells are characterized by dense packing of cellular material into apoptotic bodies that are subsequently phagocytized (Rowe & Chuang, 2004). Trauma-induced apoptosis has many mitigating factors, including excessive neuroexcitation via excitatory amino acids (EAA) (e.g., glutamate, aspartate, NMDA, AMPA), decreased ATP level, radical-mediated injury, ionic imbalance (e.g., dysregulation of calcium homeostasis), aberrant proteolytic enzyme activity, dysregulation of pro-apoptotic genes (e.g., Bcl-2), and traumatic axonal injury (Ansari, Roberts, & Scheff, 2008; Povlishock & Katz, 2005; Raghupathi, 2004; Raghupathi, Graham, & McIntosh, 2000; Yakovlev & Faden, 2004).

Traumatic axonal injury / diffuse axonal injury. DAI was first described by Strich in 1956. Originally, the pathogenesis was assumed to be tearing of axons throughout the brain caused by shearing forces generated at the time of injury. This hypothesis was based on the observation that the injured axons appear reactive and swollen when visualized postmortem using histological techniques (Adams, Graham, Murray, & Scott, 1982; Strich S.J., 1956). However, subsequent research has shown that DAI is not the result of immediate shearing of
axons, since reactive axons are undetectable using histological techniques unless the patient survives for a minimum of 12 hours postinjury (Pilz, 1983). Instead, DAI is a dynamic and evolving process that can continue for months postinjury. DAI is characterized by disruption of the neurofilament and cytoskeletal network allowing accumulation of typically excluded ions and neurofilament compaction, in addition to damaged mitochondria within the axon. All of this leads to Wallerian degeneration, the reactive swelling and detachment of the axon segment downstream from the point of injury forming characteristic retraction bulbs. Damaged axons can be found throughout the subcortical white matter, basal ganglia, diencephalon, and all areas of the brainstem. Interestingly, these damaged neurons are typically observed next to healthy axons and can also be found running along healthy microvessels. However, damaged axons can also be found in foci of petechial hemorrhaging or in the area surrounding a hematoma (Povlishock & Christman, 1994). Other aspects commonly associated with DAI are edema, petechial hemorrhages, non-hemorrhagic macroscopic white matter lesions, and small subarachnoid and intraventricular hemorrhages (Povlishock & Katz, 2005).

**Generalized changes.** In addition to the characteristic changes of focal and diffuse brain injury, there a number of generalized changes that occur following TBI, including alterations in the permeability of the blood brain barrier (BBB), neurotransmitter responses, carbon dioxide (CO₂) levels, and cerebral blood flow (Povlishock & Christman, 1994).

Findings from clinical studies have found elevated neurotransmitter levels in the cerebral spinal fluid (CSF) of brain-injured patients. Studies have also shown that increases in the levels of EAAs such as glutamate and aspartate in the extracellular fluid surrounding the contusion area can remain elevated for as many as four days after injury (Povlishock & Christman, 1994). EAA release is determined by the severity of the initial injury combined with any secondary events
that may have occurred prior to stabilization at a hospital (Zauner & Bullock, 1995). Most of the research supporting the presence of generalized changes comes from preclinical studies and will be discussed in greater detail in the experimental TBI section.

**Neurotransmitters involved in TBI pathology.** Following TBI, multiple neurotransmitter systems are affected and changes can persist for years following the insult. A brief discussion of each of the neurotransmitters involved in TBI pathology follows, as well as a description of the effects of lithium treatment. The neurotransmitters that are affected by both TBI and lithium include: serotonin (5-HT), glutamate, γ-aminobutyric acid (GABA), norepinephrine (NE), dopamine (DA), and acetylcholine (ACh) (Faden, Demediuk, Panter, & Vink, 1989; Fonseca, Sierra, Geraldes, Cerdan, & Castro, 2009; Hamill, Woolf, McDonald, Lee, & Kelly, 1987; Hayes, Jenkins, & Lyeth, 1992; O'Dell, Gibson, Wilson, DeFord, & Hamm, 2000; Rowe & Chuang, 2004; Shaldubina, Agam, & Belmaker, 2001; Verbois, Sullivan, Scheff, & Pauly, 2000).

**Serotonin.** 5-HT is released by presynaptic serotonergic neurons and activates specific postsynaptic receptors, mostly affecting the 5-HT$_2$ subgroup of 5-HT receptors. 5-HT also activates presynaptic autoreceptors. These subgroups of receptors are known as 5-HT$_{1A}$ or 5-HT$_{1B}$ and they decrease the amount of 5-HT released per nerve impulse via an inhibitory feedback loop. 5-HT$_{1A}$ autoreceptors are localized on the soma and dendrites of serotonergic neurons and 5-HT$_{1B}$ autoreceptors are located on the terminal boutons of presynaptic serotonergic neurons. Levels of this neurotransmitter can be modulated by preventing the breakdown of 5-HT via monoamine oxidase inhibition or by preventing the reuptake of 5-HT from the synaptic cleft via blockade of autoreceptors on the presynaptic neuron. The discovery of the therapeutic benefits of 5-HT modulation in the treatment of mental illness has lead to
intensive study and subsequent discovery of its involvement in brain activity for sleep regulation, sexual activity, depression, and anxiety, as well as the perception of hunger and satiety (Zafonte, Cullen, & Lexell, 2002).

There is substantial evidence that 5-HT plays a significant role in the pathogenesis of TBI. Pharmacological treatments that target 5-HT have been shown to significantly improve outcome following TBI. Reduction in the metabolites of 5-HT have been found in the CSF of patients with TBI (Markianos, Seretis, Kotsou, & Christopoulos, 1996; Zafonte et al., 2002). Major depression is one of the most prevalent co-morbid conditions reported by individuals following TBI (Rosenthal, Christensen, & Ross, 1998). A study by Kreutzer, Seel, and Gourley (2001) investigating the prevalence of depression following TBI reported that 42% of individuals met the American Psychiatric Association’s requirements for diagnosis with depression, as outlined in the *Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV, 1994)* (4th ed.; *DSM–IV*; American Psychiatric Association, 1994). In clinical studies, selective serotonergic reuptake inhibitors (SSRI) were administered to patients diagnosed with depression resulting from a TBI, and improvements were found in neuropsychological assessment of psychomotor speed, recent verbal memory, recent visual memory, and general cognitive efficiency (Fann, Uomoto, & Katon, 2001; Zafonte et al., 2002), as well as normalization of mood swings (e.g., crying or laughing spells) (Nahas, Arlinghaus, Kotrla, Clearman, & George, 1998; Zafonte et al., 2002).

**Glutamate.** Glutamate is the primary excitatory neurotransmitter in the brain. It is important in synaptic plasticity, neuronal migration during development, neuronal viability, and long-term potentiation (LTP) (Lu, Roder, Davidow, & Salter, 1998; Schumann, Alexandrovich, Biegon, & Yaka, 2008). In the acute phase following TBI, increases in extracellular glutamate
have been observed both in animal models and in human patients (Bullock et al., 1995; Schumann et al., 2008). Release of glutamate activates ionotropic and metabotropic receptors, particularly the ionotropic N-methyl-D-aspartate (NMDA) receptor with its NR2A and NR2B subunits. In non-injured brains, the NMDA receptor is crucial for the initiation of LTP in both the hippocampus and the cortex (Lu et al., 1998; Rosenblum, Dudai, & Richter-Levin, 1996; Schumann et al., 2008). Injury-induced hyperactivation of NMDA receptors allows calcium levels to reach toxic levels, and calcium is among the key mediators of cell death initiated by secondary injury.

**γ-Aminobutyric acid (GABA).** GABA is the primary inhibitory neurotransmitter in the brain and is responsible for maintaining control over excitatory feedback loops. Following TBI, modulation of GABA receptors can produce markedly different outcomes depending on the time point and duration of postinjury treatment. In a rat weight-drop model of diffuse brain injury, levels of GABA metabolites were significantly increased at 24 and 48 hours postinjury (Pascual et al., 2007), corresponding to the timeframe of the hypofunctional state in the biphasic hypothesis of secondary brain injury. The drug diazepam, a benzodiazepine and indirect GABA<sub>A</sub> agonist, has been found to significantly impair motor recovery following daily short-term administration beginning 12 hours post-lesion (Schallert, Hernandez, & Barth, 1986). However, administration of diazepam either 15 min prior to or 15 min after central fluid percussion injury was found to reduce mortality rates and improve performance in the Morris water maze (MWM) compared to untreated injured animals (O’Dell, Gibson, Wilson, DeFord, & Hamm, 2000). Likewise, administration of the GABA<sub>A</sub> antagonist bicuculline 15 min after TBI significantly increased the latency to reach the goal platform as compared to injured saline-
treated animals (O'Dell et al., 2000). These studies show that increasing the activity of inhibitory pathways may be neuroprotective in the acute phase of secondary injury.

**Norepinephrine.** Catecholamines are important in the normal functioning of the prefrontal cortex (PFC), particularly in the case of working memory (Goldman-Rakic, Muly, III, & Williams, 2000). In clinical studies, serum levels of NE have been correlated with injury severity, with higher concentration of NE corresponding to a greater severity of injury (Clifton, Ziegler, & Grossman, 1981; Hamill et al., 1987). NE levels were found to be increased in the hypothalamus at 1 hr and 1 week after fluid-percussion injury (FPI) (McIntosh, Yu, & Gennarelli, 1994). Additionally, using the CCI impact model, NE levels were increased in the medial PFC (MPFC) at 7 and 14 days postinjury (Kobori, Clifton, & Dash, 2006).

**Dopamine.** DA is a key mediator in neurological functioning following TBI. DA agonists administered in the hypofunctional chronic phase postinjury have been shown to improve cognitive outcome following TBI in experimental and clinical settings (Gualtieri, 1988; Kline, Yan, Bao, Marion, & Dixon, 2000; Whyte, Vaccaro, Grieb-Neff, & Hart, 2002). Both the D1-R and D2-R subtypes are involved in memory dysfunction following brain injury. Long-term memory dysfunction has been attributed to depleted dopamine levels in the hippocampus (Tang, Noda, Hasegawa, & Nabeshima, 1997). Catecholamines in the PFC are necessary for regulating aspects of cognitive functioning such as working memory and attention (Goldman-Rakic et al., 2000). The PFC is known to be particularly vulnerable to TBI and injury-induced disturbances to the DA system are related to impaired cognitive functioning (Massucci, Kline, Ma, Zafonte, & Dixon, 2004; Mattson & Levin, 1990; McDowell, Whyte, & D'Esposito, 1997; Sawaguchi & Goldman-Rakic, 1991).
Acetylcholine. The medial PFC and hippocampus are innervated by the cholinergic basal forebrain complex and are involved in cortical arousal, consciousness, memory and learning (Brand, Groenewald, Stein, Wegener, & Harvey, 2008; Picciotto, Brunzell, & Caldarone, 2002; Sarter & Bruno, 2000). The involvement of ACh in TBI has been well established. Regional and temporal changes in ACh levels and its synthesizing enzyme choline acetyltransferase (ChAT) have been reported following TBI (Donat et al., 2008; Saija et al., 1988). Fluctuations in ACh levels of up to 50% have been reported immediately postinjury. In the hippocampus there is a significant decline in cholinergic activation at 1 hour, persisting for up to 21 days postinjury (Verbois et al., 2000).

Human TBI outcome. Individuals who have sustained a TBI are likely to suffer cognitive and/or behavioral impairments following the injury. In minor head injuries, accounting for 75% of the total number of TBI’s per year, the most common clinical syndrome seen after injury is brain concussion. Many patients with mild TBI develop what is known as post-concussion syndrome, marked by disabilities due to cognitive impairment or psychological sequelae for variable periods of time. TBI patients who suffer a moderate to severe injury usually experience unconsciousness and/or post-traumatic amnesia (PTA) following TBI (Povlishock & Katz, 2005). PTA is commonly observed following moderate and severe injury, but individuals with mild injury may also experience PTA. PTA is associated with two types of memory impairment: retrograde and anterograde amnesia. Permanent memory loss is associated with severe head injuries.

Retrograde amnesia. Retrograde amnesia is the inability to recall events prior to the traumatic event. Most retrograde amnesia cases follow Ribot’s law, which states that the closer temporally a memory is to the traumatic event, the more susceptible it is to disruption by TBI.
Recovery from retrograde memory loss begins with more remote memories and typically only the moments immediately prior to injury remain lost. Usually the period of permanent memory loss can be measured in minutes, although in rare cases memory loss of 2 or more days prior to the traumatic event can occur (Capruso & Levin, 1992).

**Anterograde amnesia.** Anterograde amnesia is the inability to form new memories following the traumatic event. In severe head injury, typically following recovery from coma, individuals may experience a confusional state accompanied by anterograde amnesia. The hallmark of anterograde amnesia is disorientation. Individuals with this type of memory loss can also exhibit other symptoms of confusion such as deficits in attention, agitation, combativeness, hypokinetic or hyperkinetic behavior, inappropriateness, disinhibition, and severe perseveration. Recovery from anterograde amnesia typically follows a set sequence: first, orientation to person; second, orientation to place; and third, orientation to time (Capruso & Levin, 1992).

The frontal cortex is highly susceptible to TBI-induced pathology. Executive functioning (e.g., information processing, memory, and attention), controlled by the dorsolateral PFC (DLPFC) is frequently impaired, even following mild injury (Lipton et al., 2009). In a recent study, diffusion-tensor imaging was used to evaluate the relationship between post-TBI structural changes and performance on neuropsychological assessment in the continuous performance and executive maze tasks. Patients with mTBI performed worse compared to healthy controls in overall performance on both tests. Additionally, lower fractional anisotrophy, an indicator of axonal injury, was predictive of poorer performance on measures of executive functioning (increased errors of omission in the continuous performance task and increased number of trials and errors in the executive maze) in mTBI patients (Lipton et al., 2009). The frontal cortex oversees and regulates many important aspects of life such as motivation for self-initiated
behavior and the regulation, initiation, and planning of behavior. Long-term attentional deficits after TBI are reported to be a significant problem by both individuals with TBI and their families as a significant problem. TBI patients have difficulty in tasks requiring vigilance and focused attention. They generally can perform untimed tasks of immediate attentional span (e.g., digit span) as well as uninjured individuals, but if a task requires switching focus, decision making, or speed of processing, TBI patients are often significantly impaired.

**Psychological disease.** An individual’s outcome following TBI is related to several factors, including age, preinjury abilities, personality, and injury severity (Capruso & Levin, 1992). Acute confusional states are most commonly observed during the first few weeks following a TBI (Jorge, 2005). Post-traumatic agitation is another common symptom and typically resolves within 10 days (Jorge, 2005; Kadyan et al., 2004). Patients frequently report slowed or diminished cognitive abilities after TBI. Memory is the most susceptible of cognitive functions following TBI. TBI can also bring about psychiatric conditions, and the frequency of mood disorders such as major depression, bipolar disorder, and anxiety are significantly greater in TBI populations. Post-traumatic stress disorder (PTSD) is commonly associated with TBI as well, and there is a correlation between the presence of neuropsychiatric disorders and difficulty with psychosocial adjustment and ability to return to work following TBI (Jorge & Robinson, 2002). Given the profound impact of TBI on the individual as well as his or her family, co-workers and friends, it is important to evaluate therapeutic measures that may be able to prevent these cognitive sequelae from occurring.

**Experimental Traumatic Brain Injury.** Experimental brain injury models must generate similar injuries to those observed following human TBI. Regardless of the physiological, behavioral, or anatomical outcome measure, it is important that the results be
reproducible and quantifiable, clinically relevant, and produce a continuum of injury severities (Lighthall, Dixon, & Anderson, 1989). No single model can replicate the complex mechanisms that occur following human TBI, but several preclinical models of TBI have been developed and implemented to properly characterize its underlying pathology.

Models and mechanics of experimental TBI. There are four models that have been used to investigate the effects of TBI: physical, computational, cell culture, and animal. All of these models have provided important data for the understanding and treatment of TBI. However, to date, only animal models are able to fully represent how a living organism responds to trauma. There are a number of animal models that reliably reproduce the sequelae associated with human TBI. Animal models of injury include dynamic closed head injury, penetration, ablation, lesioning, and quasistatic injury. Since the present study utilizes a closed head model of head injury, other models of TBI will not be discussed further. One aspect that must be taken into consideration when evaluating experimental data is the time course of events following trauma. The pathophysiological mechanisms that occur following experimental injury in animal models occur over a faster timeframe than that which is observed following human TBI (Zauner & Bullock, 1995). This is also important when evaluating the effects of pharmacological treatments in animal models of injury, such as the rate of metabolism of the selected drug.

Fluid-percussion injury. FPI is the most commonly used rodent model of TBI. The model requires a small-diameter (4.8 mm) craniotomy to expose the dura mater, and the injury is produced by applying a brief fluid pulse directly to the surface of the dura. The injury can be delivered either centrally or laterally. Central FPI delivers the fluid pulse along the central suture midway between bregma and lambda. Lateral FPI delivers the injury to the parietal lobe midway between the coronal and lambdoid sutures. The FPI model is able to replicate the cognitive and
histological changes seen in human head injury (Dixon et al., 1987). In rodent models, FPI has been shown to produce cognitive deficits that can last for weeks or months postinjury (Hamm, Lyeth, Jenkins, O'Dell, & Pike, 1993). Other aspects of human TBI are also generated following FPI, such as hemorrhage at the gray/white interface, acute hypertension, bradycardia, increased plasma glucose levels, and suppression of electroencephalogram amplitude that is related to the magnitude of the head injury (Cortez, McIntosh, & Noble, 1989; Dixon, Lighthall, & Anderson, 1988).

Both central and lateral FPI models are capable of producing cognitive deficits via damage to the hippocampus, a region of the brain known to be selectively vulnerable in human TBI. The two models differ in the type of damage inflicted on the hippocampus. Central FP does not produce the same magnitude of cell loss that is typically seen following lateral FP, but it does produce hippocampal damage (Hamm et al., 1993; Lyeth et al., 1990). The memory impairment observed following central FPI is not believed to be due to cell death but instead due to neuronal dysfunction in the hippocampus (Hayes et al., 1992; Lyeth et al., 1990). Lateral FPI is known to cause significant cell death in the CA3 region of the hippocampus as well as bilateral cell loss in the hilus (Cortez et al., 1989; Hicks, Smith, Lowenstein, Saint, & McIntosh, 1993; Smith, Okiyama, Thomas, Claussen, & McIntosh, 1991). Memory dysfunction observed following lateral FPI is directly related to the amount of cell death in the dentate hilar region (Smith, Lowenstein, Gennarelli, & McIntosh, 1994).

The lateral FPI model of TBI was selected for use in the present study because it reliably produces aspects of both focal (cortical contusion) and diffuse (impaired functioning of subcortical structures) brain injury, resulting in motor and cognitive deficits typical of human TBI (Hallam et al., 2004; Hicks, Soares, Smith, & McIntosh, 1996; Nahas et al., 1998; Nolan et
al., 1997). Lateral FPI produces alterations in regional cerebral blood flow, BBB permeability (Cortez et al., 1989; Yamakami & McIntosh, 1991), metabolic functioning, and electroencephalographic activity (Hovda et al., 1995; Kokiko-Cochran, Michaels, & Hamm, 2008; McIntosh et al., 1989), all of which are known to occur following human head trauma. Neurochemical changes detected in human patients have been reported in animal models using lateral FPI, including neurotoxicity from glutamate over-expression, alteration in the expression of neurotrophic factors and stress-induced proteins, and compromised integrity of cytoskeletal architecture (Di, Gordon, & Bullock, 1999; Hallam et al., 2004; Hicks, Martin, Zhang, & Seroogy, 1999; Huh, Raghupathi, Launer, Helfaer, & Saatman, 2003; Raghupathi, McIntosh, & Smith, 1995). Because it is able to closely approximate the injuries observed following human TBI, the lateral FPI model was selected for use in the present study.

Controlled cortical impact. Controlled cortical impact (CCI) uses either a pneumatic or magnetically driven piston to strike exposed brain tissue. The advantage of this model is that the biomechanical events contributing to the injury can be precisely quantified, and force, velocity, and tissue deformation can be correlated to the amount of tissue damage and/or functional impairment. The CCI model has been shown to produce cognitive deficits similar to those observed following human TBI (Hamm et al., 1992). Moreover, CCI is able to simulate the neuropathology of severe human head injury more effectively than FP injury (Dixon, Clifton, Lighthall, Yaghmai, & Hayes, 1991). Hoffman and colleagues developed a bilateral model of frontal cortical contusion that was able to reproduce deficits typically observed after frontal lobe damage in humans (Hoffman, Fulop, & Stein, 1994). This model uses a pneumatically-controlled cortical impactor to create bilateral contusions of the medial prefrontal cortex in male Sprague-Dawley rats. Both CCI models were able to produce cognitive deficits as measured by
the Morris water maze (MWM) in addition to producing neurological, histological, and physiological deficits (Hamm et al., 1992; Hoffman et al., 1994). CCI can also cause direct hemorrhage within the cortical gray matter and produce significant edema and damage to the BBB (Beaumont et al., 2000).

**Weight drop model.** First described by Foda and Marmarou (1994), this model induces diffuse TBI using a free-falling brass weight dropped from a predetermined distance onto a stainless steel disk cemented onto the animal’s skull. The stainless steel disk prevents skull fractures, allowing for greater impact-acceleration levels and increased range of brain injury severity (Foda & Marmarou, 1994). Postinjury apnea, convulsions, subarachnoid hemorrhage, and intraventricular hemorrhage are common after this type of injury. In severely injured animals, there is also evidence of petechial hemorrhage. Foda and Marmarou (1994) used this model to show that brainstem damage is not a necessary component of severe head injury as previously thought. Irrespective of the severity of the brain injury (mild, moderate, severe), this model produces microscopic damage to neurons, axons, astrocytes, and small blood vessels. However, the extent of these changes is directly proportional to the severity of the TBI.

**Pathobiology of experimental TBI.**

**Focal.** The FPI (Dixon et al., 1987) and CCI (Lighthall et al., 1989) mechanisms of injury are capable of eliciting focal contusion and hemorrhage in various animal models including primates, rodent and non-rodent models. In the most severe injuries, hemorrhage can lead to further destruction of the cortical gray matter followed by the formation of a cystic cavity surrounded by glial cells. Precontusional changes observed in animal models of TBI correlate with nonhemorrhagic contusions observed in human TBI. Hemorrhage in injured cortical areas
can expand over time and produce a larger hemorrhagic mass that can facilitate secondary ischemia and infarction (Povlishock & Christman, 1994).

Intraparenchymal hemorrhage can be generated following FPI and is typically seen only following severe injury. As in human TBI, the presence of a contusion is not always associated with mortality, nor does the presence of a contusion necessarily have a direct correlation with behavioral pathologies. Only when the contusion spans a large area of nervous tissue and/or involves a discrete functional area does it have a direct relationship to behavioral outcome (Povlishock & Christman, 1994).

**Diffuse.** Not all animal models of TBI are able to mimic the pathology of diffuse axonal injury. FPI and CCI models are only able to produce focally confined axonal damage, but these models have nevertheless been used to obtain the majority of data regarding traumatically-induced DAI (Povlishock & Katz, 2005). The model that most closely replicates the pathology associated with DAI was described in a study by Gennarelli et al. (1982). In this study, nonhuman primates sustained DAI as a result of rapid acceleration of the head in one of three directions (sagittal, oblique, or lateral) without impact (Gennarelli et al., 1982). Due to the difficulty and expense associated with nonhuman primate studies, new models utilizing optic nerve stretch have been developed to study DAI (Maxwell, Povlishock, & Graham, 1997).

The nature of DAI was not fully investigated until adequate animal models were developed. Povlishock and colleagues used anterograde tracers in the major conducting pathways prior to applying varying levels of experimental injury to determine if the axons were disconnecting at the time of injury or if there was another process occurring within the axon that was facilitating axonal degradation. These experiments showed that there was a change in axon length within 1-2 hours postinjury and an accumulation of the anterogradely transported tracer,
which caused local swelling of the axon. Within 3- to 6-hours postinjury, the axonal swelling increased to form a retraction bulb and ultimately resulted in axonal separation (Cheng & Povlishock, 1988; Povlishock & Becker, 1985; Povlishock & Kontos, 1985). This demonstrated that axonal injury is not caused by tearing of the axon by external forces at the time of injury, but rather is the result of injury-induced changes within the axon. In their 2005 review article, Povlishock and Katz noted that while previous research had focused on investigating the effects of injury on large caliber myelinated axons, more recent findings have shed light on the importance of injury to myelinated and unmyelinated fine caliber fibers. This aspect of injury may be more important to outcome following TBI (Reeves, Phillips, & Povlishock, 2005).

**Generalized.** FPI produces an immediate increase in extracellular release of neurotransmitters, including catecholamines, acetylcholine, and glutamate (Faden et al., 1989; Hayes et al., 1992; Zauner & Bullock, 1995). Shearing injuries can initiate widespread changes in neurotransmitter functioning and ionic homeostasis. These changes set off widespread depolarization of cells allowing influx of sodium and calcium ions into the cell and efflux of potassium ions to the extracellular space (Katayama, Becker, Tamura, & Hovda, 1990). These cellular changes are related to neuronal and glial swelling that can lead to edema and increases in intracranial pressure (ICP) (Zauner & Bullock, 1995). A hallmark feature of experimental brain injury is excessive release of EAA’s (Katayama et al., 1990; McIntosh et al., 1989). Although there are several excitatory neurotransmitters that are associated with TBI-induced neurotoxicity, glutamate appears to be the most important (Rothman & Olney, 1986). Increased release of EAA’s such as glutamate and aspartate from the hippocampus is seen following moderate to severe TBI, and amount of neurotransmitter release seems to correlate with injury severity (Hayes & Dixon, 1994). Animal studies have confirmed that multiple agonist-receptor
interactions are involved in TBI pathologies, and this is the basis of the strategy of treatment with receptor antagonists as a method of neuroprotection after TBI. Povlishock and Christman (1994) discuss several studies that have used EAA antagonists to elicit a neuroprotective effect. When multiple EAA antagonists are combined they offer even greater neuroprotection than when used individually (Jenkins et al., 1988).

In brain tissues affected by moderately reduced regional cerebral blood flow (rCBF), glutamate excitotoxicity may be responsible for secondary ischemic damage. Hypoxia-related neuronal depolarization is caused by increased extracellular levels of glutamate due to increased release and decreased reuptake of glutamate. High levels of glutamate can cause depolarization of cell membranes and activation of voltage-gated calcium channels that in turn activates the release of more glutamate via a positive feedback loop, resulting in glutamate neurotoxicity and ultimately cell death (Gennarelli, 1993). Other amino acid neurotransmitters such as glycine are reported to be involved in seizure activity and toxicity from secondary damage (Nilsson et al., 1994). Glutamate antagonists have been found to be effective in reducing intracranial pressure produced by edema (Schroder, Muizelaar, Bullock, Salvant, & Povlishock, 1995).

Edema is the accumulation of serous fluid within a body cavity or tissue and is a significant factor related to secondary injury. Edema can be caused by a multitude of events and is the endpoint of several pathological processes. There are two primary types of edema: vasogenic and cytotoxic. Vasogenic edema is a common element in clinical and experimental TBI. The breakdown of the BBB occurs at the tight junctions of endothelial cells, resulting in vascular permeability to water and plasma proteins (Tanno, Nockels, Pitts, & Noble, 1992b) allowing passage of neurotoxic vascular components into the parenchyma (McIntosh et al., 1996). Cytotoxic edema is produced by acute ischemic events and characterized by swelling of
neurons, glia, and endothelial cells. Lack of oxygen prevents adenosine triphosphate (ATP)-
dependent sodium and potassium ion transport. ATP levels can be disrupted by ischemic
reduction in cerebral blood flow or mitochondrial dysfunction. Sodium accumulates within cells,
disrupting osmotic equilibrium and allowing excessive amounts of water into the cell.
Intracellular calcium levels are also increased and lead to the activation of phospholipases and
the subsequent release of arachidonic acid followed by the release of oxygen-derived free
radicals and infarction (Kandel, 2000).

Disruption of the BBB may be exacerbated by the occurrence of a secondary insult such
as hypoxia or ischemia (Tanno, Nockels, Pitts, & Noble, 1992a; Tanno et al., 1992b). Mild and
moderate focal TBI have been linked to alterations in the permeability of the BBB that has been
shown to persist for up to 15 hours postinjury (Cortez et al., 1989). In another study, lateral FPI
was combined with a secondary ischemic insult, and the combined injuries were associated with
transient breakdowns of the BBB that spanned from 1-72 hr post-TBI. The re-establishment of
the BBB occurred in stages; brain regions farther away from the injury site recovered more
quickly, some within 1 hr, while the region directly below the injury site demonstrated BBB
disruption that lasted up to 72 hr after injury (Tanno et al., 1992a; Tanno et al., 1992b).
Hypertensive responses following moderate or severe injury are also known to disrupt the BBB
(Hayes & Dixon, 1994).

Vascular abnormalities in preclinical research are very similar to those seen following
human TBI and include impairment or loss of autoregulation (Lewelt, Jenkins, & Miller, 1980),
impaired physiologic cerebral vascular responsiveness to changes in arterial blood gases (Wei,
Dietrich, Povlishock, Navari, & Kontos, 1980), and altered cerebral blood flow (DeWitt et al.,
1986; Povlishock & Christman, 1994). Abnormally low levels of carbon dioxide in the blood
stream have been found following experimental TBI. It is hypothesized that shear and tensile strains may produce functional and structural changes in cerebral blood vessels (Povlishock & Christman, 1994).

**Biphasic Hypothesis**

Remote Functional Depression (RFD) is a hypothesis proposed by Feeney (1991) to explain the apparent biphasic condition associated with the brain’s response to trauma-induced injury. RFD is derived from the idea of diaschisis, first proposed by von Monakow in 1914 which postulates that a focal injury to one area of the brain can produce damage to a morphologically separate area via common neural pathways. Von Monakow attributed the remote damage to a loss of excitatory input from the injured area. It was speculated that spontaneous recovery from this state was due to the resolution of the dysfunctional signaling pathway (von Monakow, 1914, translated to English in 1969).

The biphasic model of brain injury deals with the sequelae associated with secondary injuries and is divided into acute and chronic phases. In the acute phase, marked cerebral hypermetabolism leads to increases in the extracellular release of several neurotransmitters leading to neurotoxicity. The chronic phase is characterized by a hypofunctional state with delayed cell loss in selectively vulnerable brain regions, leading to cerebral atrophy on neuroimaging and decreased cerebral metabolism. These are associated with neuronal loss and inflammation that is observed via magnetic resonance spectroscopy (MRS) and which can persist for months following TBI in humans. Experimental therapies to treat TBI are designed to target either the acute or chronic phase. It is within these two time points that treatments can be implemented. Typically, treatments that are effective in the excitotoxic acute phase are ineffective when administered during the hypofunctional chronic phase, and vice versa. The
time-sensitive nature of treatment intervention can make the translation from acute treatments in preclinical models to clinical use extremely difficult.

In the proposed study, postinjury treatment with lithium is theorized to be beneficial in ameliorating the pathobiology associated with both the acute and chronic phases following TBI. Due to the known mechanisms of lithium’s action, it is hypothesized that lithium will be able to attenuate the excitotoxic processes associated with the acute postinjury phase and prevent apoptotic events as well as enhance neuronal regeneration in the chronic postinjury phase (Ren, Senatorov, Chen, & Chuang, 2003).

**Lithium**

**Background.** Lithium is the third element on the periodic table and a member of the alkali metal group. Lithium has many remarkable therapeutic applications, most notably its ability to treat both the manic and depressive phases of bipolar affective disorder (BAD) (Shaldubina et al., 2001). This drug has been also used to alleviate aggressive behavior, migraines, cluster headaches, the inappropriate secretion of anti-diuretic hormone, and chemotherapy-induced leucopoenia (Shaldubina et al., 2001). More recently, the evidence that this simple cation may exert beneficial effects in various models of brain injury has brought renewed interest in the therapeutic potential of this drug. The following sections will outline the general effects of the clinically relevant doses of lithium in the brain that are related to lithium’s effectiveness as a therapy for BAD and factors related to learning and memory, followed by an in-depth review of lithium’s role in neuroprotection, neuroplasticity, and neurogenesis.

Lithium has a multitude of effects in the brain, not all of which are relevant to this thesis. In the following section, a short synopsis of some of the prominent aforementioned effects produced by clinically relevant doses of lithium will be discussed. These lithium-induced effects
include inositol monophosphatase (IMPase), glucose metabolism, enzymes, and the neurotransmitters 5-HT, ACh, and NE.

**Inositol monophosphatase (IMPase).** Lithium is speculated to inhibit IMPase and thereby hinder the functioning of the cycle of the second-messenger phosphatidylinositol (PI) (Shaldubina et al., 2001). Allison and Stewart (1971) showed that lithium decreases free inositol concentration and increased inositol monophosphate (IP) concentration in the brain (Allison & Stewart, 1971; Shaldubina et al., 2001). Allison and Bilsner, using a rat model, found that therapeutic doses of lithium inhibited brain inositol monophosphatase, providing an explanation for the reduction in inositol and accumulation of inositol monophosphate (Allison & Bilsner, 1976). Lithium inhibits the dephosphorylation of IP by inositol monophosphatase, thus preventing the release of inositol. Other mood stabilizers (e.g., valproic acid and carbamazepine) also inhibit inositol. A common mechanism of action for these drugs is the depletion of inositol, thereby suggesting that it may have a role in the clinical effectiveness of these therapies (Teo et al., 2009).

**Glucose metabolism.** Regional cerebral metabolic rate for glucose (rCMRglc) is a neuroimaging technique that can be utilized to determine areas of brain activity *in vivo* (Basselin, Chang, & Rapoport, 2006). It was determined that chronic (6-week) lithium treatment, within the therapeutic range used to treat BAD, produced an increase in regional brain glucose metabolism in 30 of 81 brain regions examined. The increased activity was observed in auditory and visual areas as well as in the frontal cortex, amygdala, hippocampus, nucleus accumbens, caudate-putamen, interpeduncular nucleus, and substantia nigra (Basselin et al., 2006).

**Serotonin.** Lithium treatment downregulates the expression of 5-HT₁B autoreceptors and thereby increases 5-HT levels in the brain (Odagaki, Koyama, Matsubara, Matsubara, &
In vitro studies report that administration of therapeutic levels of lithium caused specific inhibition of 5-HT$_{1B}$ receptor binding in membrane preparations (Massot et al., 1999; Shaldubina et al., 2001). In support of this effect, in vivo studies revealed that lithium is able to prevent behaviors mediated by 5-HT$_{1B}$ (Massot et al., 1999; Shaldubina et al., 2001). Lithium-induced actions at 5-HT neurons were correlated with management of hyperactive behavior in rats (Grahame-Smith & Green, 1974; Redrobe & Bourin, 1999). Short-term, two-day, treatment with lithium was found to increase 5-HT levels without altering 5-HT firing rate or alteration in the number of spontaneously active neurons, which indicates that short-term lithium treatment inhibits presynaptic 5-HT receptors (Blier & De Montigny, 1985; Redrobe & Bourin, 1999).

Lithium’s observed effect of inhibiting 5-HT$_{1B}$ may have beneficial effects in learning and memory. Research indicates that antagonists of 5-HT$_{1B}$ receptors can prevent memory impairment and facilitate learning during tasks that require a high cognitive demand (Zafonte et al., 2002). In support of this finding, agonists of 5-HT$_{1B}$ receptors produced deficits in learning (Zafonte et al., 2002). Additionally, lithium’s actions may help to reduce aggressive behavior in TBI patients. However, due to potential lithium-induced toxicity, it is recommended that less toxic treatment options be considered first (Levy et al., 2005).

**Acetylcholine.** Some of lithium’s effects on memory may be related to its effects on ACh. ACh is known to be involved in memory formation in both humans and animals (Hogg, Raggenbass, & Bertrand, 2003; McNamara & Skelton, 1993). Lithium was found to increase the synthesis and release of ACh in the hippocampus, mediated by 5-HT$_{1A}$ receptor-mediated pathways (Fujii, Nakai, Nakajima, & Kawashima, 2000). A time- and concentration-dependent increase in the expression of the muscarinic ACh receptor was reported following lithium
administration. Additionally, lithium prevented agonist-induced downregulation of the muscarinic receptor (Liles & Nathanson, 1988). ChAT activity was also reported to be influenced by lithium administration (Giovanni, Scali, Prosperi, Bellucci et al., 2002). Lithium was found to reduce memory deficits related to the drug, H-89, a PKAII agonist, in part by increasing the activation of cholinergic markers (Sharifzadeh et al., 2007).

**Norepinephrine.** Lithium is reported to enhance the reuptake of NE in both synaptosomes and brain slices. The ability of lithium to decrease the activity of NE is reported to likely contribute to the acute antimanic and prophylactic effects of the drug. In a study by Katz and Kopin (1969), lithium administration decreased the amount of the [³H]NT released following electrical stimulation of brain slices but did not affect the spontaneous release of NE. Another study reported that lithium treatment reduced yohimbine-induced increases in NE levels in the cerebral cortex, possibly by reducing NE release (Swann, 1988).

**Enzymes.** Numerous enzymes are affected by lithium; however, only few are significantly inhibited at therapeutic serum concentrations. Clinically relevant serum levels of lithium (0.5 – 1.2 mM) are known to have direct effects on the metabolic enzyme phosphoglucomutase and on a superfamily of structurally related phosphomonoesterases. Of this family, the enzymes IMPase, polyphosphate 1-phosphatase (IPPase), fructose 1,6-biophosphatase (FBPase), and bisphosphate nucleotidase (BPNase) have been shown to have a magnesium ion binding site within the active site of the enzyme (Atack, Broughton, & Pollack, 1995b; Gould, Quiroz, Singh, Zarate, & Manji, 2004; Harwood & Agam, 2003). Lithium has an ionic radius similar to magnesium and as such can substitute for magnesium through uncompetitive inhibition, binding to the active site and preventing product release and trapping products in the active site (Atack, Broughton, & Pollack, 1995a; Harwood & Agam, 2003).
**Pharmacokinetics of lithium.** Lithium has the narrowest therapeutic window of any drug routinely prescribed in psychiatric medicine (Kilts, 1998). The therapeutic range for human serum concentrations of lithium was determined to be 0.6 – 1.2 mEq/L (Feldman, Meyer, & Quenzer, 1997; Kilts, 1998; Komoroski & Pearce, 2004). Serum levels that fall below 0.6 mEq/L have been associated with increased rates of relapse of symptoms and poorer psychosocial functioning in BAD patients (Gelenberg et al., 1989; Kilts, 1998; Solomon et al., 1996). Serum levels above 1.5 mEq/L are associated with adverse side effects and levels above 3.5 mEq/L are associated with life-threatening lithium intoxication. It is very important for individuals prescribed lithium to have routine monitoring of serum lithium concentrations to prevent aversive effects. If serum levels go beyond 2.0 mEq/L, lithium is known to have toxic effects that include vomiting, diarrhea, nephrogenic diabetes insipidus (including polyuria and polydipsia), bone loss, hypercalcaemia, ataxia, tremor, fatigue, irritability, seizures, coma, and death (Feldman et al., 1997; Schou, 2001). Due to the side effects of lithium it is estimated that over one third of patients are poorly able to tolerate its effects. Lithium also has reported teratogenic effects. In humans, there is an increased risk for cardiovascular malformations, particularly Ebstein’s anomaly, a congenital heart defect, when lithium is taken during the first trimester of pregnancy (De Santis et al., 2008).

Lithium is the smallest of the alkali cations, with an ionic radius of 0.60 Å. The structural similarity of lithium to sodium (0.95 Å) and potassium (1.4 Å), all members of group 1A in the periodic table, allows lithium to substitute for these cations in various cellular processes. However, the differences in ionic radii alter the effectiveness of lithium to carry out these processes to the same degree as the other cations (Ehrlich & Diamond, 1980). For example, in the brain, lithium can replace sodium in extracellular fluid and is easily drawn into
neurons during an action potential. However, it is not easily removed by the sodium-potassium pump, and thereby prevents potassium from reentering the cell. The resulting imbalance in electrolytes prevents the cell from repolarizing properly and inhibits conduction and thereby neuronal excitability (Grafe, Reddy, Emmert, & ten, 1983). Lithium can also substitute for the divalent cations, calcium and magnesium. Calcium and lithium have a similar charge density, and magnesium and lithium have a very similar ionic radius, 0.65 and 0.60 Å respectively. It is the similarity between lithium and the afore mentioned divalent cations that is physiologically relevant because of their regulatory actions in cellular functioning (e.g., enzymes, kinetics, and synaptic events) (Ehrlich & Diamond, 1980).

Lithium negligibly binds to plasma proteins and it is not metabolized or biotransformed in any appreciable way. Lithium is excreted by the kidneys in its intact form and is reabsorbed in the proximal renal tubules (Feldman et al., 1997; Janicak & Munson, 1998; Kiltz, 1998). Lithium has high energy of hydration and its hydrated ionic radius (310 pm) is similar to sodium with its hydration coat (340 pm). Because of this ionic radius, lithium can substitute for sodium in many of the active sodium transport mechanisms. In periods of dehydration or sodium depletion, both sodium and lithium retention is increased, necessitating adequate hydration prior to initiating lithium treatment to prevent excessive build-up of lithium in the body (Janicak & Munson, 1998).

As previously mentioned, the similarities between sodium and lithium allow for lithium to replace it in sodium transport mechanisms, such as: the sodium-potassium pump, sodium-lithium countertransport, bicarbonate-sensitive pathway, voltage-dependent sodium channel, and leak (i.e., passive diffusion). However, under physiological conditions, the affinity of lithium relative to other cations in the various transport mechanisms precludes the likelihood for certain
mechanisms to be important in the transport of lithium across a particular membrane. For example, the transport of lithium across the membrane of a red blood cell (RBC) occurs through leak and bicarbonate-sensitive pathways, whereas sodium-lithium countertransport is important for the removal of lithium from the cell. The influx of lithium into excitable cells (i.e., nerve and muscle cells) is primarily through via voltage-dependent sodium channels and to a lesser extent, passive diffusion. Similar to RBCs, the removal of lithium from excitable cells is largely via sodium-lithium countertransport (Ehrlich & Diamond, 1980).

Lithium enters the brain via the blood-CSF barrier (Ehrlich & Wright, 1982). The authors reported that lithium is passively transported across tight junctions formed by the epithelial cells of the choroid plexus via sodium-lithium countertransport (Ehrlich & Wright, 1982). In this mechanism, one lithium ion is transported out of the cell in exchange for one external sodium ion, which goes into the cell. Once across the choroid plexus, lithium enters the brain through the CSF, which is continuous with the interstitial fluid that surrounds the cells of the brain.

In humans, lithium (typically lithium carbonate) is easily absorbed and reaches peak plasma levels in 1 – 2 hours for standard release formulations and 4 – 5 hours for sustained release formulations (Grandjean & Aubry, 2009). The effect of lithium administration on serum levels can vary greatly among human subjects depending on factors like weight and age. The half-life of lithium is 18 – 20 hours in healthy young adults and up to 36 hours in elderly patients (Janicak & Munson, 1998). The elimination half-life of lithium is between 18 – 36 hours. In humans, the difference between brain and plasma lithium concentrations is significant. Brain concentrations are approximately half of serum levels (Komoroski & Pearce, 2004; Pearce, Lyon, & Komoroski, 2004). In one study, it was reported that brain concentrations of lithium are
better correlated with effective treatment of mania as compared to serum concentrations (Kilts, 1998). Similarly, (Gould et al., 2008), using a mouse model of depression, found that the observed antidepressant-like effects of lithium were due to its action in the brain, and not to any peripheral effects of the drug (Gould et al., 2008). However, MRI studies show that among animals there is less variability between brain and serum concentrations of lithium (Komoroski & Pearce, 2004; Pearce et al., 2004). Indeed, in rodents, following long-term treatment with lithium, whole brain and serum lithium concentrations were found to be equivalent (Ghoshdastidar, Dutta, & Poddar, 1989; Gould et al., 2008; Gould, O'Donnell, Picchini, & Manji, 2007).

The distribution and retention of lithium in the blood and brain of male mice was evaluated following single-dose and chronic (21 day) lithium treatment. A single-dose of lithium (0.5 mmol/kg) produced peak plasma levels at 8 hours post-injection and peak brain concentration at 12 hours post-injection. Chronic daily administration of this dose showed no change in overall lithium concentration in the whole brain or the following brain regions: cerebral cortex, striatum, hippocampus, diencephalon, brain stem and cerebellum (Messiha, 1976). Lambert and colleagues also reported steady brain levels of lithium following both acute and chronic lithium dosing (Lambert, McGirr, Ely, Kilts, & Kuhar, 1999).

Serum lithium levels in rats were assessed after acute (single-dose) and chronic (30 day) dosing with 2.0 mmol/kg lithium chloride, administered via intraperitoneal injection (Tsaltas, Kyriazi, Poulopoulos, Kontis, & Mailllis, 2007). The serum concentrations were evaluated to ensure that daily lithium administration did not result in accumulation of the drug, which might cause increased serum levels over days and possibly affect the health of the animals. Serum concentrations were assessed at 1, 12, or 24 hours following the last lithium injection. The
findings from this study show that there was no difference in the serum lithium levels of chronically or acutely treated rats at any of the time points. Thus, the authors concluded that daily intraperitoneal injections of lithium are not likely to produce excess lithium accumulation over time (Tsaltas et al., 2007). However, there was a significant difference in the serum levels of lithium over the three post-injection time points (1, 12, and 24 hours), irrespective of the duration of treatment. The 2.0 mmol/kg (84.7 mg/kg, ip) dose of lithium produced peak serum concentrations (1.7 ± 0.4 mEq/L) at 1 hour post-injection. Serum concentrations decreased at 12 hours (0.5 ± 0.4 mEq/L) and were almost absent by 24 hours (0.4 ± 0.4 mEq/L) post-injection (Tsaltas et al., 2007). In another study, single dose of 5.0 mmol/kg administered to rats produced a maximum brain concentration of 1.5 mmol/kg between 9 and 24 hours, and the half-life was determined to be 12 hours (Wraae, 1978). These animal findings are in accord with the decline in serum concentrations observed in human pharmacokinetic studies (Tsaltas et al., 2007).

**Lithium is neuroprotective.** There is compelling evidence to state that lithium is neuroprotective. Supporting data comes from *in vitro, in vivo* and clinical studies employing vastly diverse methodologies and parameters (Rowe & Chuang, 2004).

**In vitro.** In studies using both rodent and human cell lines, (e.g., cerebellar granule cells (CGC), cerebral cortical cells, hippocampal neurons, PC12 cells, and neuroblastoma cells) lithium has been found to be neuroprotective against a variety of insults (e.g., β-amyloid peptide (Aβ), potassium deprivation, staurosporine and heat shock, ouabain, β-bungarotoxin, and glutamate excitotoxicity (Alvarez et al., 1999; Bijur, De Sarno, & Jope, 2000; Bournat, Brown, & Soler, 2000; Hennion, el-Masri, Huff, & el-Mailakh, 2002; Mora et al., 2001; Nonaka, Hough, & Chuang, 1998; Nonaka, Katsube, & Chuang, 1998; Rowe & Chuang, 2004; Wei et al., 2000).
In an animal model of Alzheimer’s disease, primary cultures of rat cortical neurons were treated with Aβ, known to induce cell death in cultured neurons. Lithium treatment (10 mM and 2 mM), was found to prevent Aβ-induced tau hyperphosphorylation in addition to providing significant neuroprotection to cortical neurons (Alvarez et al., 1999). These effects are hypothesized to involve lithium-induced inhibition of GSK-3β, a kinase known to phosphorylate a wide range of substrates including tau. The 10 mM dose of lithium was protective against Aβ; however, this dose resulted in lithium-induced toxicity and cell death after 4 days of treatment. The lower dose of lithium did not have the same toxic effects. Therefore, it was speculated by the authors that low doses of lithium, similar to those used in the treatment of BAD, may be beneficial in treating Alzheimer’s disease, as well as other neurodegenerative disorders (Alvarez et al., 1999).

When cells in culture are deprived of potassium, the result is increased activated caspase-3 levels, in addition to promoting the phosphorylation of PKB and GSK-3. Granule cells subjected to potassium deprivation, in the presence of 5 mM lithium chloride, were completely protected against potassium-induced apoptosis. Additionally, the protective effect was observed for up to 48 hours after lithium treatment. These findings indicate that the neuroprotective actions of lithium are effective for a period of time after lithium treatment has ended (D'Mello, Anelli, & Calissano, 1994). In another study, Mora and colleagues also examined the effects of potassium deprivation in cerebellar granule cells, with the aim to elucidate a potential mechanism of action for lithium-induced neuroprotection (Mora, Gonzalez-Polo, Fuentes, Soler, & Centeno, 1999). Caspase-3 is an effector caspase and has been established as a biochemical marker of apoptosis (Bijur et al., 2000). In CGC’s, 5 mM lithium, co-incubated with low potassium, significantly decreased the levels of activated caspase-3 protein. Lithium was found
to prevent the cleavage of procaspase-3, and not the activity of the active form of capase-3 (Mora et al., 2001). The authors speculate that the phosphorylation state of PKB and GSK-3β are related to lithium’s effects.

Staurosporine is used to experimentally induce apoptosis in all cell types and thereby increase activation of caspases-3 and -9 (Bijur et al., 2000). Human neuroblastoma SH-SY5Y cells were subjected to staurosporine or heat shock in the presence of GSK-3β over-expression. GSK-3β alone was not sufficient to induce apoptosis. However, over-expression did confer greater vulnerability to caspases-3 activation and apoptosis when combined with staurosporine or heat shock (Bijur et al., 2000). Lithium treatment was able to significantly reduce the apoptotic effect. It was proposed that lithium’s activation of the phosphoinositide 3-kinase/Akt (PI3k/Akt) pathway may partially contribute to these protective effects via PI3-K inhibition of GSK-3β. Additionally, lithium-induced inhibition of GSK-3β prevents degradation of β-catenin. Decreased β-catenin has been linked to increased cell death (Bijur & Jope, 2000). Contrary to these findings, another study, using CGCs exposed to staurosporine, and then treated with lithium, failed to find neuroprotection (Nonaka et al., 1998). Because lithium’s neuroprotective effects against other insults in cerebellar granule cells has been well documented in the literature, these findings indicate that lithium neuroprotection may be specific to particular initiators of apoptosis, i.e., GSK-3β (Bijur & Jope, 2000; Nonaka et al., 1998).

Oubain is a toxic agent used to experimentally mimic the neurochemical changes observed following manic episodes in individuals with BAD. Mania is associated with increased sodium retention, increases in intracellular sodium and calcium, and decreased activity of the sodium-potassium pump (Hennion et al., 2002). Low dose lithium treatment for 72 hours prior to ouabain exposure was protective against ouabain-induced toxicity. However, if lithium was
administered 24 or 48 hours prior to exposure, the protective effect was not observed. This finding indicates that for some of the beneficial effects of lithium, a loading period is required. Indeed, in the treatment of BAD, the mood stabilizing effects are not observed for a period of time following initiation of treatment (Hennion et al., 2002).

Cultured cerebellar granule cells exposed to the neurotoxin β-bungarotoxin, a NMDA receptor and L-type calcium channel agonist, results in increased neuronal calcium influx. Lithium pre-treatment, ranging from 2 – 7 days, was found to be dose and time-dependent. Maximum effectiveness was observed following pretreatment with 1.2 mM lithium for 3 days. This dose and duration of pre-treatment attenuated the β-bungarotoxin-induced increase in reactive oxygen species (ROS) and superoxide anion. However, pre-incubation for 6 – 7 days using the same dose (1.2 mM) of lithium resulted in suboptimal levels of neuroprotection. A higher concentration of lithium (3.0 mM) was also found ineffective in preventing β-bungarotoxin neurotoxicity, in addition to being slightly toxic to the cells (Tseng & Lin-Shiau, 2002). The dose of lithium found to be most effective against this neurotoxin is in accordance with the therapeutic serum levels of lithium used in human patients (between 0.5 and 1.2 mM) (Hopkins & Gelenberg, 2000; Tseng & Lin-Shiau, 2002).

Several studies have reported on lithium’s neuroprotective effects against glutamate-induced neurotoxicity in cultured CGCs (Chen & Chuang, 1999; Kopnisky, Chalecka-Franaszek, Gonzalez-Zulueta, & Chuang, 2003; Manji, Moore, & Chen, 1999; Nonaka et al., 1998). Glutamate excitotoxicity leads to initiation of pro-apoptotic pathways and is known to play a role in TBI pathology. Cerebellar granule cells pre-treated (7 – 9 days) with lithium (0.5 – 5.0 mM) showed significant protection against glutamate-induced neurotoxicity by modulation of NMDA receptor mediated calcium influx (Nonaka et al., 1998). Lithium is able to protect against not
only NMDA receptors mediated calcium influx, but also suppresses apoptotic mechanisms initiated by excitotoxic calcium influx.

Lithium’s involvement in preventing apoptosis was assessed via examination of gene expression and protein levels of pro-apoptotic (p53 and Bax), and cytoprotective (Bcl-2) (Chen & Chuang, 1999; Manji et al., 1999). CGCs pre-treated with lithium (3 mM) for 7-days prevented glutamate-induced increases in p53 and Bax mRNA levels, and at the same time, sustained lithium-induced increases in Bcl-2 mRNA (Chen & Chuang, 1999). Indeed, lithium also increased the ratio of Bcl-2 protein levels over Bax protein. These findings are further supported by both in vitro and in vivo studies, where chronic lithium treatment increased the expression of Bcl-2 and decreased the expression of p53 and Bax (Manji et al., 1999). Bax and Bcl-2 are both involved in modulating cytochrome c release by mitochondria. Pre-treatment with lithium for 7 days was found to block glutamate-induced cytochrome c release by mitochondria, thereby preventing the release of cytochrome c into the cytosol (Chen & Chuang, 1999).

Cyclic AMP-responsive element binding protein (CREB) is a signaling system involved in cell survival and increases in CREB are associated with synaptic plasticity and neuronal reorganization in hippocampal mossy fiber cells following TBI (Finkbeiner, 2000; Hu et al., 2004). Additional supporting evidence for its beneficial effects in cell survival is from data showing phosphorylated CREB (pCREB) directly regulates the CRE-dependent transcription of the BDNF and Bcl-2 (Kopnisky et al., 2003; Tao, Finkbeiner, Arnold, Shaywitz, & Greenberg, 1998). CGCs exposed to glutamate for 8 days revealed a dose- and time-dependent change in pCREB at Ser133. Decreases in pCREB due to glutamate exposure were completely blocked by treatment with MK-801 (a potent NMDA receptor antagonist), indicating that the glutamate-
induced changes in pCREB are related to the activation of NMDA receptors (Kopnisky et al., 2003). Lithium pre-treatment for 7 days prior to glutamate exposure suppressed glutamate-induced decreases in pCREB, whereas lithium treatment for 30 minutes, 4 hour and 24 hours did not affect pCREB levels. Increased protein phosphatase-1 (PP-1) activity is reported to be involved in glutamate-induced decreases in pCREB. It was found that lithium treatment prevented glutamate-induced increases in PP-1 activity (Kopnisky et al., 2003). In addition to PP1, the MEK/ERK pathway also regulates pCREB and is increased following lithium administration (Kopnisky et al., 2003).

Cerebral cortical neurons exposed to toxic levels of glutamate can be protected by NMDA receptor antagonists (e.g., MK-801). However, non-NMDA receptor antagonists were not effective. Again, this indicates that glutamate toxicity in these neurons is mediated by NMDA receptors (Hashimoto, Hough, Nakazawa, Yamamoto, & Chuang, 2002). Lithium treatment, at therapeutic levels, generates plasma levels within the range of 0.6 – 1.2 mM (Hashimoto et al., 2002; Hopkins & Gelenberg, 2000). Doses of lithium between 0.1 mM – 1.0 mM showed significant neuroprotective effects when administered 6 days prior to excitotoxicity. These data indicate that lithium at therapeutic and sub-therapeutic concentrations is neuroprotective in cerebral cortical neurons (Hashimoto et al., 2002). Additionally, the duration of lithium pre-treatment was critical for its neuroprotective effects. The minimum duration for pre-treatment to achieve neuroprotection was at least 2 days. Maximum neuroprotection was attained after 5 – 6 days (Hashimoto et al., 2002).

**In vivo.** In vivo studies have also demonstrated the neuroprotective effects of lithium in several animal models (e.g., transient and focal ischemia-induced brain damage, striatal infusion of quinolinic acid to produce an excitotoxic model of Huntington’s disease, excitotoxic
lesions of the cholinergic system, kainic-acid-induced brain lesions, and irradiation) (Inouye et al., 1995; Rowe & Chuang, 2004). Manji, Moore, & Chen, (1999), reported that in both in vitro and in vivo studies, chronic lithium treatment increased the expression of the anti-apoptotic protein Bel-2 and decreased the expression of pro-apoptotic proteins p53 and Bax.

Compelling evidence for lithium’s efficacy as a treatment against secondary injuries associated with TBI comes from an experimental model of stroke that produces transient focal cerebral ischemia via middle cerebral artery occlusion (MCAO) (Ren et al., 2003; Xu, Culman, Blume, Brecht, & Gohlke, 2003; Yan, Wang, Hou, Ji, & Zhou, 2007). MCAO is characterized by ischemia-induced infarct, damage to the basal ganglia (i.e., striatum, subthalamic nucleus, and substantia nigra), in addition to sensory, motor and behavioral deficits (Borlongan, Cahill, & Sanberg, 1995).

Heat shock proteins (HSP) (e.g., HSP-70 & HSP-71) are molecular chaperones that protect the cell from thermal and oxidative stress. Additionally, HSPs protect neurons by antagonizing apoptosis-inducing factor (AIF) (Ravagnan et al., 2001). HSP-70 is a stress-induced protein that acts to protect unfolded or malfolded proteins by binding to them, ensuring proper folding and preventing intracellular protein aggregation (Hendrick & Hartl, 1993; Ren et al., 2003). This cellular protector also participates in the disposal of damaged or defective proteins. Heat shock factor-1 (HSF1) is a cytoplasmic transcription factor that when activated is converted to a nuclear protein that regulates gene transcription of HSPs. HSF-1 is negatively regulated by serial phosphorylation at Ser307 by MAPK, allowing GSK-3β to phosphorylate the adjacent Ser303 (Chu, Soncin, Price, Stevenson, & Calderwood, 1996).

Lithium treatment following transient MCAO has been shown to significantly increase HSF-1 and HSP-70 levels as compared to saline controls (Ren et al., 2003). The upregulation of
HSF-1 and HSP-70 induced by lithium occurs rapidly and is temporally allied to lithium’s neuroprotective effects against MCAO / reperfusion-induced brain injury (Ren et al., 2003). Lithium-induced neuroprotective effects resulting from increased HSP-70 are thought to be due to either direct inhibition of GSK-3β or activation of the phosphoinositide 3-kinase/Akt signaling pathway. Both are related to disinhibition of HSF-1, thereby allowing increased transcription and expression of HSP-70. Indeed, over-expression of HSP-70 was found to protect neurons from ischemic brain damage (Hoehn et al., 2001; Ren et al., 2003). Additionally, activation of HSP-70 inhibited ischemia-induced activation of NF-κB (a pro-apoptotic factor) and subsequent increases in apoptotic cell death (Feinstein et al., 1996; Ren et al., 2003; Schneider et al., 1999). The study by Ren (2003), showed that increases in HSF-70 following MCAO were observed only in the ipsilateral side. The contralateral side revealed increases in HSF-1 binding, but not in HSP-70. Sham animals exhibited the same effects observed in the contralateral hemisphere. These data indicated that a further stimulus may be necessary to induce HSP-70 transcription (Pirkkala, Nykanen, & Sistonen, 2001; Ren et al., 2003).

Chronic (2 week) treatment with lithium was evaluated for its neuroprotective effects in a rodent model of stroke (Xu et al., 2003). Ischemic brain damage was produced by MCAO lasting 90 min followed by reperfusion. Rats were administered 1 mmol/kg lithium (sc) each day, for 12 days prior to the ischemic insult, with the dose of lithium on day 12 administered 2 hours prior to MCAO. Treatment with lithium was continued for two additional days following the insult. During these days, two hours post-injection, animals underwent neurological assessment. Data from these assessments show that lithium-treated rats had significantly better scores on measures of neurological function, derived from two previous studies, Garcia et al. (1995) and Bederson et al. (1986). The neurological functions assessed were: spontaneous
activity, symmetry in moving all four limbs, forepaw outstretching, climbing, body proprioception, response to vibrissae touch (Garcia, Wagner, Liu, & Hu, 1995), forelimb flexion, decreased resistance to lateral push, and circling behavior (Bederson et al., 1986). In addition to improvements in the neurological functioning score, Xu and colleagues (2003) found significant reductions in total infarct volume (32.7%) as compared to vehicle-treated injured rats. Edema formation, however, was not affected by lithium treatment. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), an assay used to detect DNA fragmentation resulting from apoptotic cascades, revealed a significant reduction in TUNEL-positive cells of lithium-treated injured rats as compared to vehicle-treated injured rats. Analysis of the apoptotic marker, caspase-3, revealed significantly fewer caspases-3 positive-cells in the ischemic penumbra of the occluded area of lithium treated rats (Xu et al., 2003). Levels of the AP-1 transcription factor c-Jun were increased in the ipsilateral cortex following ischemic injury resulting from MCAO. Proteins modulated by AP-1 are generally increased in surviving cortical regions. Lithium has been shown to indirectly reduce AP-1 levels via inhibition of NMDA receptor-mediated increases in pro-apoptotic p38 and JNK (Rowe & Chuang, 2004). Indeed, Xu and colleagues found that c-Jun levels were significantly lower in the ischemic penumbra of lithium-treated rats. The authors purport the observed neuroprotective effects of lithium are due to reduced apoptotic events in the cortex, as determined by inhibition of caspase-3 and decreased fragmentation of DNA. Additionally, the lithium-induced alteration in c-Jun levels suggests that reduced AP-1 protein expression may be involved in the neuroprotective effects of lithium in ischemic brain injury (Xu et al., 2003).

Extracellular signal-regulating kinase 1 and 2 (ERK1/ERK2) is a member of the mitogen-activated protein kinase superfamily and is activated via phosphorylation on threonine and
tyrosine residues as a reaction to extracellular stimuli including growth factors. Neurotransmitters and neurotrophins can also be activated by brain injury. ERK1/ERK2 is known to be involved in cell growth, proliferation, differentiation, apoptosis, synaptic plasticity, and learning and memory via involvement in LTP (Atkins, Selcher, Petraitis, Trzaskos, & Sweatt, 1998; Liou, Clark, Henshall, Yin, & Chen, 2003; Ménard et al., 2002; Schaeffer & Weber, 1999). Lithium’s influence on signaling cascades, specifically, mitogen-activated protein kinase (MAPK) and ERK1/ERK2, was evaluated following transient global cerebral ischemia (Yan, Hou, Wu, Liu, & Zhou, 2007). At 6 hours postinjury, lithium treatment was found to up-regulate ERK1/ERK2 in the CA3 and DG of the hippocampus in both the sham and ischemic-injured groups. Cognitive assessment using the MWM (PID 19-21) revealed a trend towards shorter latencies for the lithium-treated ischemic rats as compared to ischemic rats (Yan et al., 2007).

Another study by Yan and colleagues (2007), evaluated preinjury lithium treatment on several behavioral outcome measures (beam balance, elevated plus maze, open field task, and MWM) following transient global ischemia (Yan et al., 2007). Rats were pre-treated with lithium (1.0 mmol/kg, ip) daily for 2 weeks prior to ischemic brain injury, with treatment continuing for the duration of the behavioral assessments (i.e., 9 days post-insult). Beam balance, elevated plus maze, and open field tasks were administered on PID 3. The MWM task was performed on PID 7 – 9. Performance in the beam balance task was significantly improved on PID 7 only. The behavioral assessments revealed that untreated ischemic rats were more likely to engage in hyperactive behavior, as measured by the open field task, and exhibited greater risk taking behavior, as measured by increased open arm exploration in the elevated plus maze. The MWM protocol employed two sets of four trials on the first day of testing, followed
by a single set of four trials for the remaining two days. On the first day of testing the ischemic rats and the lithium-treated ischemic rats did not differ significantly; however, the lithium-treated rats did show a trend toward shorter latencies. On the second and third days tested in the MWM, the lithium-treated ischemic rats performed significantly better as compared to the ischemic rats (Yan et al., 2007). Histological analysis and quantification of pyramidal neurons within CA1 of the hippocampus was performed 9 days after reperfusion. Significant cell death was observed within the CA1 region of ischemic rats. Lithium treatment significantly reduced cell death, with no significant difference observed between lithium-treated and sham rats (Yan et al., 2007).

Clinical studies. Clinical research provides the final pieces of supporting evidence for lithium’s neuroprotective ability. However, in clinical studies it is difficult to determine to what extent the beneficial effects of lithium are due to neuroprotection or to neurotrophic effects (Rowe & Chuang, 2004). Neuroimaging techniques and post-mortem morphology studies have revealed decreases in brain volume and cell number in patients with mood disorders (Drevets et al., 1999). These changes could be due to developmental abnormalities, disease progression, or alterations in neurochemistry resulting from changes in neurotransmitter levels in chronic affective disorders (Duman, Heninger, & Nestler, 1997).

MRI studies have shown increased gray matter volume within several brain structures of individuals diagnosed with BAD and treated with lithium as compared to individuals with BAD not undergoing lithium treatment (Bearden et al., 2007a; Bearden et al., 2007b; Manji, Moore, & Chen, 2000a; Monkul et al., 2007; Moore, Bebchuk, Wilds, Chen, & Manji, 2000). Levels of N-acetyl-aspartate (NAA), a marker of neuronal viability, were increased following 4 weeks of lithium treatment. It is difficult to determine if these effects are due to neuroprotective or neurotrophic effects.
Moore and colleagues (2000), using three-dimension magnetic resonance imaging (3D-MRI) and quantitative brain-tissue segmentation, investigated the neuroprotective/neurotrophic effects of therapeutic levels of chronic lithium treatment on grey matter volume (Moore et al., 2000). Patients with BAD were given lithium for 4 weeks. A baseline MRI scan was performed while each individual was “medication free” and had been off medication for at least 2 weeks prior to the initiation of the study. These individuals were subsequently treated with lithium for 4 weeks and upon conclusion of the treatment, a second MRI was performed. These scans were then compared with scans taken following 4-weeks of lithium treatment. Analysis of the MRI’s showed a significant increase (3% or approximately 24 cm³) in total grey matter volume in 8 out of 10 patients. There was no significant change in either regional cerebral water content or brain white matter volume. The authors postulate that the changes in grey matter volume were likely due to neurotrophic effects and not to cell swelling or alterations in contrast medium related to the lithium treatment (Moore et al., 2000).

NAA, an accepted neuronal marker, is localized primarily within the cytosol of mature neurons and not found in mature glial cells, CSF, or blood (Manji, Moore, Rajkowska, & Chen, 2000; Tsai & Coyle, 1995). NAA is synthesized in the mitochondria and is related to mitochondrial oxygen and ATP consumption. It is generally accepted as a gauge for neuronal viability and function (Manji et al., 2000a; Tsai & Coyle, 1995). Individuals with BAD have been found to have decreased levels of NAA bilaterally in the hippocampus and DLPFC (Bertolino et al., 1999; Winsberg et al., 2000). Moore and colleagues conducted another study using quantitative proton magnetic resonance spectroscopy in BAD patients undergoing lithium treatment (Moore et al., 2000). Using an experimental design similar to the previously mentioned (2000) study by Moore, Bebchuk, Wilds, and co-workers, the levels of NAA were
examined following 4-weeks of lithium treatment (Moore et al., 2000). Total levels of NAA were found to be significantly increased in all brain regions. Based on the findings, the authors concluded that chronic lithium treatment may increase neuronal viability and function in the human brain (Moore et al., 2000).

**Neuroprotective mechanisms.** The mechanisms of action for lithium’s neuroprotective effects are mediated by several pathways and likely via multiple levels of the same pathway. This section will detail the two pathways most accepted in the literature as being involved in lithium-induced neuroprotection. These are lithium’s induction of the cell survival signaling pathway and lithium’s inhibition of NMDA receptor-mediated calcium influx. Within the cell survival signaling pathway, lithium’s effects will be discussed in relation to upstream, central, and downstream factors. Upstream factors include BDNF and activated pathways (PI3K/Akt and MEK/ERK). Centrally, lithium acts on GSK-3β and downstream factors include effector systems (β-catenin, HSF-1, AP-1, and CREB), the Bel-2 family and caspases (Rowe & Chuang, 2004).

**Upstream survival events.** Lithium’s role in neuroprotection begins at the cell surface-trophic factor receptors. BDNF, a neurotrophin involved in neuronal development, survival, and plasticity, is increased following lithium administration. BDNF activates the trophic receptor, TrkB, inducing phosphorylation of its Tyr490. Once activated the TrkB receptor has several receptor systems, including phospholipase Cγ (PLCγ) signaling, MEK/ERK signaling, and PI3K/Akt signaling (Huang & Reichardt, 2001).

The importance of BDNF in lithium-induced neuroprotection is supported by two important pieces of evidence. First, *in vitro* and *in vivo* studies have showed that treatment with lithium increases BDNF levels. Second, lithium-induced neuroprotection is dependent on
activation of BNDF. Indeed, in a study where TrkB activation was inhibited by either the TrkB antagonist K252a or in BDNF knockout mice, lithium-induced neuroprotection from glutamate toxicity was blocked (Hashimoto et al., 2002). Once BDNF binds to the TrkB receptor, the PI3K/Akt and MEK/ERK pathways are activated. Both of these pathways are vital for lithium’s observed neuroprotective effects.

Via the PI3K/Akt pathway, the activation of TrkB results in the phosphorylation of the adaptor protein She that recruits Grb-2 to form a protein complex that initiates further downstream signaling events (Liou et al., 2003; Pearson et al., 2001). From this point the pathway can go in one of two directions, both leading to the activation of PI3K. Once PI3K is activated, it produces 3-phosphoinositides that in turn recruit phospholipid-dependent kinases (PDKs) that phosphorylate Ser473 and/or Thr308 to activate Akt-1. Akt-1, also known as protein kinase B, is a serine/threonine protein kinase that regulates anti-apoptotic activity on multiple targets including GSK-3β, BAD (part of Bcl-2 family), CREB, some members of the forkhead family, and procaspase-9. In vitro studies demonstrate that overexpression of Akt protects neurons subjected to serum or growth factor withdrawal (Chen, Yuan, Jiang, Huang, & Manji, 1998; Kermer, Klocker, Labes, & Bahr, 2000; Springer, Nottingham, McEwen, Azbill, & Jin, 2001). The involvement of PI3K in lithium-induced Akt-1 upregulation was demonstrated in vitro by inhibiting PI3K, thereby preventing lithium-induced increases in Akt-1 (Chalecka-Franaszek & Chuang, 1999; Kumari, Liu, Nguyen, Zhang, & D'Mello, 2001). Glutamate excitotoxicity has been reported to decrease levels of Akt-1. This is mediated by long-term lithium treatment. Additionally, long-term lithium treatment was found to increase recovery of Akt-1 phosphorylation and activity (Chalecka-Franaszek & Chuang, 1999). However, these findings do not universally apply to all cell types. Using certain cell lines, some studies were unable to
detect changes in Akt-1, specifically, phosphor-Ser-473-Akt, following lithium treatment (De Sarno, Li, & Jope, 2002; Zhang, Phiel, Spece, Gurvich, & Klein, 2003).

MAP kinases are another important pathway involved in preventing apoptosis and promoting neuroprotection (Rowe & Chuang, 2004). Some of the prominent members of the MAP kinase family include c-Jun, N-terminal kinase (JNK), p38 kinase, and ERK. The involvement of ERK in neuroprotection has been well documented in the literature (Chang et al., 2003; Segal & Greenberg, 1996). The ERK pathway begins with the phosphorylation of the Trk receptor by BDNF, leading to activation of Ras (Yan, Roy, Apolloni, Lane, & Hancock, 1998). Ras binds to Raf proteins (Raf-1 and B-Raf) that subsequently activate MEK, resulting in phosphorylation of ERK. ERK has direct and indirect effects on effector systems, such as NF-κB and ribosomal S6 kinase (RSK). These effector systems subsequently regulate CREB and GSK-3β. The MEK/ERK pathway also has deleterious downstream effects on the Bcl-2 family, caspases, and AP-1 transcription factor (Rowe & Chuang, 2004; Steelman et al., 2004). Using an in vivo model, it was reported that the MEK/ERK pathway was responsible for the neuroprotective effects of chronic lithium treatment (Einat et al., 2003). However, others have reported that lithium blocks the MEK/ERK pathway, although differences in results appear to be cell-type-dependent (Pardo, Andreolotti, Ramos, Picatoste, & Claro, 2003). Together these findings indicate that lithium-induced neuroprotection is complex and not solely related to any one particular target of the drug (Rowe & Chuang, 2004).

The stress-induced kinases p38 and JNK are typically involved in promoting apoptosis. Activation of these two pathways begins with a cellular stressor that activates GTPases (Rho, Rac, Cdc42). The GTPases activate sets comprised of a MAP kinase kinase kinase (MEKK1/2/3/4), a MAP kinase kinase (M KK3/4/6/7), and a MAP kinase (JNK or p38) (Mielke
MAP kinase translocates to the nucleus and phosphorylates, thereby activating transcription factors (e.g., AP-1 and p53) (Sionov & Haupt, 1999; Whitmarsh & Davis, 1996). Support for lithium’s involvement in this pathway comes from neuroprotection studies where cell damage induced by glutamate-toxicity or deprivation of trophic factors was prevented by lithium (Chen et al., 2003; Hongisto et al., 2003; Rowe & Chuang, 2004).

The central factor. Glycogen synthase kinase-3 is a serine/threonine protein kinase that is highly abundant systemically as well as in the brain. There are at least 2 isoforms of GSK-3. They are GSK-3α and GSK-3β. GSK-3 is involved in the regulation of neuronal plasticity, gene expression, and cell survival, and may be involved in the pathology of neuropsychiatric and neurodegenerative diseases (Li, Bijur, & Jope, 2002; Martinez, Castro, Dorronsoro, & Alonso, 2002). There are several kinases that regulate GSK-3β activity including Akt, protein kinase A (PKA), protein kinase C (PKC), MAP kinases and the Wnt signaling pathway. GSK-3 acts on diverse substrates, including more than 40 proteins that are involved in cellular metabolic processes, signaling, and structural functions (Jope & Johnson, 2004; Martinez et al., 2002). Among these diverse substrates are several signaling proteins (e.g., AP-1, CREB, activated T-cells, HSF-1, β-catenin, NF-κB, c-Jun, and Myc) (Davies, Jiang, & Mason, 2001; Li et al., 2000; Martinez et al., 2002; Rothwarf & Karin, 1999; Rowe & Chuang, 2004; Yoganathan et al., 2000).

There are three potential mechanisms for lithium-induced regulation of GSK-3β: directly, indirectly, and autoregulation. It is important to note that the neuroprotection afforded by lithium treatment is likely related to action via all three mechanisms. Additionally, GSK-3α is also affected by lithium and may have relevance to lithium-induced neuroprotection (Rowe & Herdegen, 2000; Tibbles & Woodgett, 1999).
However, lithium’s effects on GSK-3β have been more thoroughly characterized and therefore will be the focus of the following discussion.

Direct inhibition of GSK-3β. Direct inhibition of GSK-3β by lithium is thought to be through competitive binding to magnesium channels. It was theorized that the direct inhibition of GSK-3β may account for the observed neuroprotective effects in animal models of stroke. However, lithium’s inhibition of GSK-3β occurs quickly. Only one study found that a single dose of lithium was effective in reducing neurobehavioral deficits, when administered 3 hours after the onset of MCAO (lasting 24 hours) (Ren et al., 2003). This is in contrast to several studies that have shown that chronic treatment is necessary for neuroprotection (Bauer, Alda, Priller, & Young, 2003; Rowe & Chuang, 2004; Xu et al., 2003). Additionally, the IC_{50} for direct inhibition of GSK-3β is between 1.5 and 2.0 mM lithium. This range is higher than doses typically found in neuroprotective studies. Thus, it was reported by Rowe and Chuang in their 2004 review article, that neuroprotection induced by lithium treatment can occur with less than 50% inhibition of pro-apoptotic GSK-3β.

Indirect inhibition of GSK-3β. Indirectly, it is speculated that lithium regulates the phosphorylation status of GSK-3β by increasing Ser9 phosphorylation (Klein & Melton, 1996; Li et al., 2002). The activity of GSK-3β is regulated by the phosphorylation states of Ser9 and Tyr216. Phosphorylation of Ser9 decreases GSK-3β activity and phosphorylation of Tyr216 increases activity. Another potential avenue for lithium’s action is through the survival signaling pathway. On this path, lithium treatment stimulates BDNF, which in turn promotes the PI3K/Akt and MEK/ERK pathways, leading to inhibition of GSK-3β (Li et al., 2002).

Autoregulation of GSK-3β. The third potential mechanism of action for lithium-induced inhibition of GSK-3β is through autoregulation (Rowe & Chuang, 2004). The activity level and
phosphorylation state of GSK-3β can be altered via autoregulation. This is mediated by the protein phosphatase-1 / inhibitor-2 (PP-1/I-2) complex (Zhang et al., 2003). In one direction, increased activation of GSK-3β phosphorylates I-2, and activates PP-1. Activated PP-1 dephosphorylates GSK-3β at Ser9, thereby increasing the activation of GSK-3β, which starts the cycle again. Working in the opposite direction, inhibition of GSK-3β inhibits PP-1 by I-2. This results in an increase in the phosphorylation of Ser9, thereby decreasing the activation of GSK-3β. Lithium-induced inhibition of GSK-3β inhibits PP-1, thereby inhibiting Ser9 dephosphorylation, further reducing GSK-3β activation (Rowe & Chuang, 2004; Zhang et al., 2003).

_Downstream factors._ There are numerous downstream factors that may be affected by lithium treatment. However, for the purposes of this dissertation, rather than detail all of these factors, only those that are well established in the literature as being involved in lithium’s neuroprotective effects will be discussed. These factors include: β-catenin, HSF-1, AP-1, and CREB (Rowe & Chuang, 2004).

_β-Catenin._ β-Catenin is a proto-oncogene produced by the canonical Wnt pathway (Yu & Malenka, 2003). Briefly, Wnt proteins bind to extracellular receptors that are part of the family known as Frizzleds. Frizzleds subsequently activate the intracellular protein disheveled-1, which in turn inhibits GSK-3β (Moon, Bowerman, Boutros, & Perrimon, 2002). Active GSK-3β forms part of a β-catenin destruction complex. This complex phosphorylates β-catenin, resulting in its breakdown. Therefore, inhibition of GSK-3β leads to increased levels of β-catenin. Increases in β-catenin promote interactions with the transcription factor T-cell factor/lymphoid enhancer factor (Tcf-Lef) forming the Tcf/Lef-β-catenin complex. This complex moves into the nucleus and promotes the transcription of several genes, many of which
are involved in preventing apoptosis (Huelsken & Behrens, 2002; Moon et al., 2002; Rowe & Chuang, 2004; Seidensticker & Behrens, 2000; Yu & Malenka, 2003). Due to lithium-induced inhibition of GSK-3β and subsequent activation of the Wnt pathway, lithium is expected to influence β-catenin levels. Indeed, both in vitro and in vivo studies have found that lithium treatment increases β-catenin levels (Gould, Chen, & Manji, 2004; Rowe & Chuang, 2004; Stambolic, Ruel, & Woodgett, 1996).

**HSF-1.** As described previously in this thesis, HSF-1 is a transcription factor that when activated initiates the chaperone protein HSP-70. HSF-1 can be suppressed by serial phosphorylation. ERK phosphorylates HSF-1 at Ser307, which then allows GSK-3β to phosphorylate HSF-1 at Ser303 (Chu et al., 1996). This dual activation hinders the binding of HSF-1 to DNA, as well as its ability to facilitate transcription (Bijur et al., 2000; Rowe & Chuang, 2004; Xavier et al., 2000). HSP-70 is known to exert neuroprotective effects related to its interaction stabilizing Akt, thus impeding Apaf-1 by mediating cytochrome c release and caspase activation, in addition to inhibiting activation of JNK (Beere et al., 2000; Rowe & Chuang, 2004).

**AP-1.** The transcription factor AP-1 can be either neuroprotective or neurodegenerative. It is a dimeric complex that includes members of the Jun, Fos, CREB, and ATF families. The action of AP-1 is dependent on the molecules that form the AP-1 complex and the genes targeted by those complexes. Stress factors and cellular signals influence what role AP-1 plays in either neuroprotection or apoptosis. In general, the signals that activate AP-1 subsequently activate MAP kinases (e.g., JNK and p38), resulting in AP-1 binding to an assortment of genes (Rowe & Chuang, 2004; Shaulian & Karin, 2002; Whitmarsh & Davis, 1996). AP-1 can be modulated by secondary factors such as GSK-3β. Increases in GSK-3β cause phosphorylation of c-Jun and
impairs AP-1 binding activity (Boyle et al., 1991; Rowe & Chuang, 2004). Lithium has been found to both inhibit and induce AP-1 activity. Specifically, lithium inhibits the stimulus-induced activation of AP-1 and concurrently increases the basal activity of AP-1 (Asghari, Wang, Reiach, & Young, 1998; Hongisto et al., 2003; Ozaki & Chuang, 1997; Rowe & Chuang, 2004).

**CREB.** The transcription factor, cyclic adenosine monophosphate response element binding protein (CREB), is functionally related to learning and memory formation (Silva, Kogan, Frankland, & Kida, 1998). CREB is a nuclear protein that regulates genes that contain a CRE promoter region. Increases in calcium levels or cyclic adenosine monophosphate (cAMP) can initiate the phosphorylation and activation of CREB. CREB-dependent transcription is necessary for the cellular events involved in long-term memory formation (Silva et al., 1998). CREB also plays a role in apoptosis. The sensitivity of CREB is modulated by many pathways including BDNF, MEK/ERK, and GSK-3β (Bonni et al., 1999; Hansen, Rehfeld, & Nielsen, 2004; Kopnisky et al., 2003; Rowe & Chuang, 2004). CREB-induced neuroprotective effects are dependent on the induction and increased expression of anti-apoptotic factors such as Bcl-2 and BDNF. As stated above, CREB can be induced by BDNF. The induction of CREB by BDNF initiates a feedback loop whereby increases in CREB upregulate BDNF expression which then increases CREB (Kopnisky et al., 2003; Rowe & Chuang, 2004).

**Glutamate and NMDA receptors.** Dixon and Hokin (1998), in an effort to determine the mechanism of action for lithium, speculated that lithium was exerting therapeutic effects via glutamate. They discovered that acute treatment with high levels of lithium (20 – 25 mM) increased glutamate levels by blocking the reuptake of glutamate by the presynaptic neuron. Due to the toxic effects of lithium associated with dosages higher than 1.2 mM, it is likely that
the elevation in glutamate is related to lithium toxicity. Alternately, chronic lithium administration in mice at therapeutic levels resulted in a significant increase in glutamate reuptake. The reduction of glutamate in the synaptic cleft is hypothesized to be involved in the anti-manic effects of the drug (Shaldubina et al., 2001). Increased reuptake of glutamate may also be involved in lithium’s neuroprotective effects.

The NMDA receptor is an ionotropic glutamate receptor involved in numerous essential processes including development, neuroplasticity, and excitotoxicity. When activated under normal conditions, the NMDA-receptor allows calcium and sodium into the cell. When over-stimulated, the calcium influx is detrimental, as it is the primary element in glutamate excitotoxicity (Rowe & Chuang, 2004; Sattler & Tymianski, 2001). Excessive calcium accumulates in the mitochondria and disrupts the mitochondrial membrane potential, initiating apoptotic pathways (Rowe & Chuang, 2004; Stout, Raphael, Kanterewicz, Klann, & Reynolds, 1998). It is generally supported that inhibiting the overflow of calcium into the cell would mediate the excitotoxic effects and protect the cell (Hashimoto et al., 2002; Rowe & Chuang, 2004). NMDA receptor-mediated neurotoxicity is also dependent on the MAP kinases. Glutamate-induced excitotoxicity activates apoptotic-inducing factors such as JNK, p38, p53, and AP-1. Pre-treatment with lithium provides neuroprotection from these potentially harmful factors (Chen et al., 2003).

The NMDA receptor is comprised of heteromeric subunits, NMDA receptor 1 (NR1) and NMDA receptor 2 (NR2). NMDA receptors must contain at least one NR1 and two or more NR2 subunits A – D (NR2A – D) (Rowe & Chuang, 2004). The NR1 is required for receptor function and the NR2 regulates the receptor function. Activation of the NMDA receptor allows the influx of calcium into the cell. The activation state of the receptor is controlled by the
phosphorylation state of the NR2A and NR2B subunits. Growing evidence supports the regulation of NMDA receptors by the sarcoma family of protein tyrosine kinases (Src) (Salter, 1998). Src is found throughout the CNS, particularly in neurons, and increases in Src activation are correlated with increased LTP in the CA1 of the hippocampus (Lu et al., 1998). Based on these findings, overstimulation of Src-mediated receptor tyrosine phosphorylation may be responsible for the over-activation of NMDA receptors, as in the case of glutamate-induced toxicity (Rowe & Chuang, 2004; Salter, 1998).

Calcium-induced damage is one of the primary instigators of cytochrome c release and apoptosis. An analysis of calcium levels, via uptake studies and Fura-2 calcium imaging, revealed that glutamate-induced toxicity increased NMDA receptor-mediated calcium influx. Lithium pre-treatment was able to prevent this insult-dependent increase in calcium and this was correlated with a significant decrease in NR2B phosphorylation by tyrosine (Hashimoto et al., 2002). In a rat model, ischemic injury was produced using permanent occlusion of both vertebral arteries (Ma & Zhang, 2003). This injury type resulted in increased NMDA receptor activation due to increased phosphorylation of the NR2A subunit. Injury-induced elevations in the level of postsynaptic density protein 95 kDa (PSD-95) (NMDA receptor scaffolding protein) was found to modulate the interaction of Src and Fyn with the NR2A subunit, resulting in increased phosphorylation of NR2A. Pre-treatment with lithium for 7 days resulted in complete inhibition of the interactions of Src and Fyn, members of the Src family and regulated by PSD-95, with NR2A. Interestingly, no difference was observed in overall protein expression of Src, Fyn, NR2A, or PSD-95. This indicates that some of lithium’s neuroprotective effects are related to preventing the modulation of Sch, Fyn, and NR2A by PSD-95 (Ma & Zhang, 2003).
**Lithium-induced neurogenesis and plasticity.** Lithium’s neuroprotective properties have been well established in the literature and there is compelling evidence that chronic lithium treatment also enhances hippocampal neurogenesis (Chen, Rajkowska, Du, Seraji-Bozorgzad, & Manji, 2000; Yan et al., 2007). Support for lithium-induced neurogenesis comes from *in vitro* and *in vivo* studies, which show lithium promotes differentiation of hippocampal neural progenitor cells into neurons (Chen et al., 2000; Kim et al., 2004; Yan et al., 2007). Some of the mechanisms that are reported to be involved in lithium-induced neuroprotection are thought to also be involved in promoting neurogenesis, such as Bcl-2 and BDNF (Chen et al., 1999; Fukumoto, Morinobu, Okamoto, Kagaya, & Yamawaki, 2001; Hashimoto et al., 2002; Wei et al., 2001).

Following lithium treatment, bcl-2 levels are markedly increased in the CNS, particularly within the hippocampus (Chen et al., 1999). Bcl-2 has been found to exert neurotrophic effects, thereby enhancing axonal regeneration, promoting neurite outgrowth, and increasing axonal growth rate (Chen, Schneider, Martinou, & Tonegawa, 1997). Based on findings from human MRI studies showing increased brain volume following lithium treatment, Chen et al., 2000, examined the effects of lithium treatment on the levels of bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) expression, a measure of proliferating cells, in the hippocampus of C56BL/6 mice. The mice were given lithium chow for 3 – 4 weeks, resulting in an average serum level of 0.97 ± 0.20 mM lithium. BrdU was administered for the last 12 days of lithium treatment. Neurons were also stained with Neuronal Nuclei (NeuN), an antibody that recognizes the vertebrate nervous system and binds to neuron-specific nuclear protein (Mullen, Buck, & Smith, 1992). Chen, (2000) found a significant increase in BrdU positive neurons in the hippocampus, specifically in the dentate gyrus (DG) of lithium-treated animals as compared to
controls. However, there was no difference in NeuN expression between the lithium (66%) and control (68%) animals (Chen et al., 2000).

Vazey and Connor (2009) investigated the effect of lithium on the priming and differentiating of neural progenitor cells (NPC) derived from the subventricular zone (SVZ). Cells were primed using 3 mM lithium for 14 days. After the last day of priming, the neurospheres were plated. Four weeks later, the lithium-treated cells showed a significant increase in neuronal differentiation as compared to non-primed cultures. Using calbindin and glutamic acid decarboxylase 67 (GAD-67) as indicators, it was determined that lithium chloride significantly increased the generation of mature, region-specific neurons. The authors suggest that lithium has a role in increasing the ratio of cells expressing neuronal markers while concurrently decreasing glial progeny (Vazey & Connor, 2009).

Su, Chu, and Wu (2007) examined the effect of lithium on survival, proliferation and differentiation of spinal cord-derived neural progenitor cells (NPC) in vitro and after transplantation into the spinal cord of uninjured animals. NPCs from transgenic Sprague-Dawley rats, expressing green fluorescent expressing protein (GFP), were injected into the dorsal funiculi of female Sprague Dawley rats. Half of the animals that received the transplanted cells also received daily treatment with lithium chloride (85 mg/kg, ip), the others received saline. At 2 and 4 weeks post-transplantation, lithium-treated animals had significantly more GFP expressing cells and increased neuronal differentiated cells compared to saline controls. The authors cite several possible mechanisms for the observed increase in cell survival including inhibition of GSK-3β and activation of TrkB receptors via upregulation of BDNF (Su et al., 2007).
**Lithium and learning.** There are conflicting reports regarding lithium’s effects on learning and memory in both clinical and animal studies. Many older clinical studies have reported that lithium impairs long-term and short-term verbal memory recall (Kropf & Muller-Oerlinghausen, 1979; Kusumo & Vaughan, 1977; Shaw, Stokes, Mann, & Manevitz, 1987). However, some studies have found lithium treatment either had no effect on learning and memory (Squire, Judd, Janowsky, & Huey, 1980), or that it increased recall on tests that assess immediate and delayed memory function (Person-Data Test and 30 Face Test) (Smigan & Perris, 1983). Pachet and Wisniewski (2003) reviewed these studies and determined that the correlation between lithium treatment and verbal memory impairment was comparatively weak and they indicated that additional research was needed to verify the findings of the previous studies. Some of the concerns brought up in the article were discrepancies in testing procedures, a lack of standardized testing, and insufficient control for comorbid illnesses.

In a recent study, Senturk et al. (2007) found that euthymic BAD patients on monotherapy with lithium had significantly impaired immediate verbal memory recall assessed by the Logical Memory Subscale of the Wechsler Memory Scale I (WMS). Similar to previous studies, other areas of cognitive functioning were not affected by lithium treatment. Cognitive functioning such as psychomotor speed, working memory, and visuospatial abilities, were assessed using subsets of the Wechsler Adult Intelligence Scale Revised (WAIS-R) and revealed no significant impairments relative to control subjects (Senturk et al., 2007).

Preclinical studies have also reported inconsistent effects of lithium treatment on memory tasks. The study by Al Banchaabouchi, de Ortíz, Menéndez, Ren, and Maldonado-Vlaar (2004) investigated the effect of chronic lithium treatment, within the therapeutic range, on performance in memory-dependent behavioral tasks. Male Long Evans rats were administered lithium
carbonate chow for 4 weeks and were subsequently assessed for object recognition memory and spatial discrimination learning ability, using the novel object recognition task and hole-board spatial discrimination task respectively. No significant difference between groups was observed in the novel object recognition task. However, lithium-treated animals showed delayed acquisition in the hole-board task, but only on the first 2 days (out of 3) of testing. Two sessions were performed on each of the three testing days. Similar to the acquisition data, lithium-treated rats made more reference errors and had significantly longer search times as compared to non-lithium-treated rats in only the first two out of six test sessions. These data indicate that treatment with lithium may selectively impair initial performance in spatial discrimination learning tasks (Al Banchaabouchi, de Ortíz, Menéndez, Ren, & Maldonado-Vlaar, 2004).

Lithium’s effects on spatial memory and working memory under chronic stress conditions were evaluated using the MWM and a passive avoidance task respectively (Vasconcellos, Tabajara, Ferrari, Rocha, & Dalmaz, 2003). The animals were randomly assigned to one of four treatment groups (control, lithium, stress, stress + lithium), and were trained to locate a hidden platform in the MWM over a five-day period (4 trials per day). In the training sessions, no significant difference was detected between the treatment groups. In the recall test (probe trial), the hidden platform was removed and the swim behavior of the animals was recorded. The authors found that lithium-treated rats showed a trend towards shorter latencies to reach the location of the hidden platform. Additionally, lithium-treated rats had significantly more crossings in the area of the hidden platform (Vasconcellos et al., 2003). These data suggest that lithium may diminish stress-induced impairments in spatial memory tasks.

Eight-days later, these same animals were used in a working memory version of the MWM. In this task, the daily location of the platform was changed; however, during the daily sessions the
location of the platform was fixed. Lithium-treated animals did not exhibit significant
differences in performance in the reference memory task, indicating that under chronic stress,
working memory is unaffected by the selected doses of lithium (Vasconcellos et al., 2003). The
improvement observed in the reference memory portion of the MWM, a task dependent on the
hippocampus, and the lack of effect found in the working memory portion of the MWM, a task
reported to rely more heavily on the medial PFC region (O'Dell et al., 2000), suggest that the
beneficial cognitive enhancing effects of chronic lithium treatment are associated with
hippocampal functioning.

Data from Tsaltas et al., 2007, provide additional support for lithium-induced
improvements in spatial memory. Chronic lithium treatment was found to significantly enhance
spatial working memory in the T-maze, using a delayed non-matching to place rule. The authors
reported that lithium treatment did not affect spatial reference memory because there were no
differences in the acquisition of this task as compared to control animals. Lithium treatment
enhanced performance in the T-maze, as compared to saline-treated rats, when moderate
demands were placed on memory (i.e., test intervals > 30 s and < 60 s). However, when a
greater demand was placed on working memory, a test interval of 60 s, the lithium-treated
animals performed similar to saline-treated controls. The effect of lithium treatment on long-
term retention was assessed using a single trial step-through passive avoidance task. Lithium-
treated rats demonstrated longer retention of the passive avoidance task as compared to saline-
treated rats. Based on these data, it was suggested that lithium treatment potentiated the long-
term retention of a weak conditioning trace (Tsaltas et al., 2007).

These studies combined suggest that the previously held belief that lithium impairs
memory functioning may be incorrect (Tsaltas et al., 2007; Vasconcellos et al., 2003). Due to
the lack of consensus between studies investigating the effects of lithium treatment and cognitive function, and the new data emerging on the neuroprotective effects of lithium, it is necessary to further evaluate the effects of lithium as they relate to cognitive performance.

**Study Rationale**

Data from both *in vitro* and *in vivo* studies in animal models of various neurodegenerative diseases and human patients with BAD indicate that chronic administration of lithium inhibits NMDA-receptor-mediated calcium influx and induces both up-regulation of numerous survival signaling pathways (such as Bcl-2, CREB, BDNF, PI3K/Akt and MEK/ERK pathways, Hsp70, and β-catenin), and down-regulation of pro-apoptotic activities (e.g., p53, Bax, caspase, cytochrome c release, beta-amyloid peptide production, and tau hyperphosphorylation), thereby limiting neuronal cell death cascades (Rowe & Chuang, 2004; Wada, Yokoo, Yanagita, & Kobayashi, 2005). To our knowledge, however, no data are available describing the mechanism of action of this drug in animal models of TBI. The purpose of the present thesis is, therefore, to provide additional rigorous evidence in support of the beneficial effects of lithium in the treatment of TBI, to define the best dosing paradigm, and to shed more light on the potential mechanisms of action for the observed effects in the TBI setting.

**Hypotheses**

The general hypothesis is that postinjury lithium treatment will significantly improve outcome following TBI. Specifically, lithium will reduce TBI-induced deficits in vestibulomotor, cognitive, and behavioral functioning. It is also hypothesized that lithium treatment will reduce TBI-induced cell death in the hippocampus. Lastly, that lithium treatment will result in reduced activation of caspase-3 and increased expression of BDNF and growth associated protein-43 (GAP-43) within the hippocampus.
Specific Aims

The first aim was to determine the optimal dose of lithium that, when administered following TBI, would significantly reduce injury-associated cognitive deficits. The second aim was to evaluate the effects of lithium treatment as a therapy to ameliorate depressive-like behavior in rats following TBI. The third aim was to use the most effective dose of lithium and determine the therapeutic time window for administering that dose while still maintaining the observed beneficial effects on cognition. The fourth aim was to evaluate the neuroprotective properties of lithium as they relate to cell survival in the CA3 and hilar subregions of the hippocampus. The fifth aim was to evaluate the effect of lithium on selected markers known to be affected by TBI that are involved in neurogenesis, neuroprotection, and apoptosis.

General Methods

The methodologies contained within this section are common to all the experiments described in this thesis. Methodologies specific to the individual experiments (Experiments 1 – 4) are detailed in the corresponding chapters.

Subjects

Adult male Sprague Dawley rats (Hilltop Lab Animals, Inc., Scottsdale, PA), weighing between 300 – 370 grams were used in the present thesis. Animals were individually housed in a vivarium, maintained at 22 °C, on a 12 hour light/dark cycle. All animals were given ad libitum access to food and water. The procedures used in the present thesis followed the guidelines established in the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services) and were approved by the Institutional Animal Care and Use Committee (IACUC).
Experimental TBI

**Surgical preparation.** Each rat was initially anesthetized, in a Plexiglas bell jar, by inhalation of 4% isoflurane gas, in a carrier gas of 70% nitrous oxide: 30% oxygen, for 4 min. The surgical site was shaved and the animal was placed in a stereotaxic device, fitted with a nosecone to deliver the gas anesthesia during the procedure. The optimal depth of anesthesia was achieved with 2% isoflurane, in the same carrier gas mixture used previously. The surgical site was prepped using gauze sponges soaked in betadine and ethanol. The scalp was sagittally incised and a 4.8 mm diameter burr hole was performed over the right cerebral hemisphere between the lambda and bregma craniometrical points. Two small nickel-plated screws were placed 1 mm rostral to bregma on the ipsilateral side of the burr hole, and another 1 mm lateral to the central suture, midway between bregma and lambda, on the contralateral side to the burr hole. A Luer-Lok syringe hub was secured on the skull at the site of the burr hole first with α-cyanoacrylate, then with dental acrylic applied around the syringe hub and two screws were added for additional stability. The scalp was sutured and bacitracin applied to the surgical site. Each subject was monitored for full recovery from anesthesia before being returned to their home cage.

**Fluid-percussion injury device.** The fluid-percussion injury (FPI) device used to produce experimental TBI was identical to that described in detail by Dixon et al., 1987. Figure 1 shows an image of the injury device. The device consists of a 60-cm-long and 4.5 cm diameter Plexiglas cylinder with a rubber-covered O ring-fitted Plexiglas piston at one end and, on the opposite end of the cylinder, a 2-cm-long metal housing mounted with an extracranial pressure transducer (Entran Devices, Inc., Model EPN-0300*-100A). This metal housing was attached to a 5-mm transfer tube with a 2.6 mm inner diameter that ends with a male Luer taper fitting. At
the time of injury, an injection port with a Luer taper was used to connect the male end of the transfer tube and the surgically implanted female Luer-Lok fitting. The entire system was then filled with distilled water. The injury was produced by releasing a metal pendulum positioned to strike the piston of the injury device. A small volume of distilled water was injected into the closed cranial cavity to produce a brief displacement and deformation of brain tissue. The magnitude of injury was controlled by varying the height from which the pendulum was released.

![Image of fluid percussion injury (FPI) device.](image)

**Lateral fluid-percussion injury.** Twenty-four hours after surgical preparation, at the time of injury, the rats were anesthetized by breathing 4.0% isoflurane gas, in a carrier gas of 70% nitrous oxide to 30% oxygen, for 4 min. The surgical site was exposed and the animal was connected to the FPI device. The force of the injury administered was between 2.0 to 2.2 atmospheres of pressure (atm), which is equivalent to a moderate-level severity brain injury. The
atm were recorded by the in-line transducer connected to a storage oscilloscope (Tektronix 5111; Beaverton, OR). Sham-injured controls received the same surgical preparation, anesthesia, and connection to the injury device, however, no injury was delivered. All animals were immediately ventilated with room air until spontaneous breathing was resumed.

**Postinjury neurological assessment.** The injury resulted in loss of consciousness and temporary suppression of the animal’s reflexes. Immediately following the injury, the animal was placed supine and suppression of the righting reflex was assessed. The amount of time required for the animal to regain this reflex after TBI is a good predictor of injury severity (Gennarelli, 1993; Hallam et al., 2004; Hamm, 2001; Schmidt & Grady, 1995; Thompson et al., 2005).

**Drug Preparation and Administration**

Lithium chloride (Sigma, St. Louis, MO) was dissolved in 0.9% sterile saline to make 1.00, 0.50, 0.25, and 0.125 mmol/kg solutions. Lithium was administered via intraperitoneal injection, in a volume of 1 ml/kg. Vehicle treated animals were administered 1 mg/kg, 0.9% sterile saline.

**Statistical Analysis**

Injury severity was evaluated by analysis of the suppression of the righting reflex. A one-way ANOVA was used to determine if the level of injury was comparable among the groups that received a TBI. Where appropriate, a Fisher least significant difference (LSD) post hoc analysis was used to compare individual group differences.

**Experiment 1**

**Study Rationale**

Cognitive impairments in memory and attention can occur following even mild TBI and
can persist for years after the traumatic event (Draper & Ponsford, 2008; Kashluba, Hanks, Casey, & Millis, 2008; McDonald, Flashman, & Saykin, 2002). It is widely believed that these sequelae are largely attributable to the damage caused by secondary injury events, including ischemia, excitotoxicity, excess calcium release, and mitochondrial dysfunction that ultimately may lead to neuronal injury or death. Findings from preclinical studies show that these disruptions to cognitive and cellular functioning can be attenuated by the administration of neuroprotective agents and drugs used in clinical settings for the treatment of memory and attention deficits (Marklund, Bakshi, Castelbuono, Conte, & McIntosh, 2006). Thus, pharmacotherapies that positively modulate neuroprotective mechanisms and/or have cognitive enhancing effects are sought after as potential therapies for brain-injured patients.

One such drug is lithium, a therapeutic agent extensively used for over 50 years in the treatment of BAD. The renewed interest in lithium is due to findings from preclinical studies that have demonstrated both neuroprotective and cognitive enhancing effects following lithium treatment. Indeed, several studies using animal models of ischemic injury and stroke have demonstrated that lithium treatment improved cognitive outcome and prevented neuronal cell loss (Kopnisky et al., 2003; Ren et al., 2003; Yan et al., 2007; Yan et al., 2007). Furthermore, some recent studies have reported that lithium treatment, in non-brain-injured rodents, was found to enhance cognitive function (Nocjar, Hammond, & Shim, 2007; Tsaltas et al., 2007; Vasconcellos et al., 2003).

Similar to what has been reported in clinical studies, the data from animal studies lack a consensus regarding the benefit to vs. impairment of cognitive functioning following lithium treatment. Despite the discord among preclinical studies, there are sufficient data to warrant further examination of lithium-induced cognitive enhancement. Studies that support this
assertion are briefly described here. Lithium treatment was found to block drug-induced impairment of spatial memory retention by the protein kinase AII (PKAII) inhibitor H-89, thus reducing the swim latencies in the MWM (Sharifzadeh et al., 2007). Rats exposed to chronic stress demonstrate impaired performance in tasks that require reference memory abilities. Lithium treatment negated the effect of stress on spatial learning and significantly improved MWM performance (Vasconcellos et al., 2003). Furthermore, lithium administration to naïve rats was found to improve performance in neurobehavioral tasks, such as delayed non-matching to place in the T-maze, spatial memory retention in the MWM, and passive avoidance assessments (Tsaitas et al., 2007; Vasconcellos et al., 2003).

Irrelevant of the severity level of TBI (mild, moderate, severe), individuals who have sustained a brain injury are more likely to present with psychiatric and mood disorders, including depression, anxiety, psychosis, and behavioral problems (e.g., poor motivation, agitation, aggression, disinhibition) (Fleminger, 2008; Jones et al., 2008; Jorge & Starkstein, 2005; Rogers & Read, 2007). The most commonly reported psychiatric illness following TBI is depression. Based on the cumulative effects of depression combined with the other sequelae associated with human TBI, it is important to evaluate potential therapies that target the psychological, in addition to the biological, components of brain injury.

Lithium is effective as a prophylaxis and a treatment for the manic and depressive episodes associated with BAD, and is highly effective as an adjunct antidepressant in treatment-refractory patients (Bauer et al., 2003; Gould et al., 2008; O'Donnell & Gould, 2007; Schou, 2001). Eroğlu and Hizal (1987), using Wistar rats, showed that chronic lithium treatment, via intraperitoneal injection, was associated with antidepressant-like effects in the forced swim test (FST). Chronic treatment with lithium chow was found to significantly reduce the time...
immobile in a mouse model of the FST (Gould et al., 2008; O'Brien et al., 2004; Shaldubina et al., 2006).

There are several lines of evidence from preclinical studies that indicate lithium treatment has neuroprotective effects against insult-induced neuronal injury and that it may have memory-enhancing effects. Lithium has also been found to reduce depressive-like behavior in rodents. The physiological and behavioral response to lithium treatment, as observed in preclinical studies, appears to affect key aspects of the sequelae associated with TBI (i.e., neuronal cell loss, cognitive impairment, and neuropsychiatric illness). Based on these reported findings, it seems a logical progression to investigate the potential benefit of post-TBI lithium treatment.

Surprisingly, there is a paucity of research combining lithium treatment and TBI.

**Treatment paradigm.** A predominant feature of lithium-induced neuroprotection studies is the usage of a preinjury treatment paradigm (Chuang, 2004; Chuang, 2005; Rowe & Chuang, 2004; Wada et al., 2005; Xu et al., 2003; Yan et al., 2007). To our knowledge, there has been only one *in vivo* study (Ren et al., 2003) that has evaluated the effect of post-insult lithium treatment. Animals were subjected to MCAO for 1 hr followed by reperfusion for 23 hours. This injury produced an extensive infarction in the cerebral cortex. Single doses of lithium chloride (between 0.50 – 3.00 mEq/kg, sc) administered immediately after initiation of MCAO significantly reduced the infarct volume when assessed at 24 hours post-insult (Ren et al., 2003). A single dose of lithium, 1.00 mEq/kg, delayed up to three hours after the onset of MCAO, was associated with a significant reduction in ischemia-induced neurological deficits at 24 hours post-insult. Neurological deficits were assessed using tests for motor (flexion of hind limb and forelimb, head movement, ability to walk in a straight line, circling to paralytic side, and falling down on paralytic side), sensation (visual and proprioceptive tests), and reflex response (pinna,
and startle reflex) (Ren et al., 2003). Lithium treatment, administered immediately after the onset of MCAO and continued daily for 7 days, was correlated with a significant reduction in ischemia-induced neurological deficits on post-insult days 1 – 7. Additionally, the benefits associated with 7-day-lithium treatment were still observed up to 14 days post-insult. These data, taken together, support the use of an early postinjury time point for initiating lithium treatment, that lithium must be administered over the course of several days and that the beneficial effects of lithium treatment can continue beyond the duration of treatment.

It is well known that time is a critical factor when treating an individual with a TBI. Secondary injury cascades are initiated immediately after sustaining a TBI and the resulting neuronal death or dysfunction and can last from hours to weeks postinjury. To mitigate these harmful effects, it is generally accepted that pharmacological interventions that target neuroprotective mechanisms must be administered early after injury since treatment delays may render the drug ineffective due to irreparable damage. Additionally, the innate mechanisms that support neuronal plasticity and recovery are initiated within the first week after a TBI. Drugs, such as lithium, that have been shown to affect neuronal plasticity, may require early initiation of treatment to facilitate and maximize recovery.

Experimental TBI studies that have focused on the mechanisms associated with the acute phase of secondary brain injury typically administer a pharmacological treatment immediately prior to (30 – 15 min) or immediately following (10 – 30 min) injury. The time point selected for administering the initial dose of lithium in Experiment 1, 30 min post-TBI or sham-injury, is an approximate midpoint between the furthest time point successfully used in an animal model of ischemia (2 hr post-MCAO) (Ren et al., 2003) and preinjury drug administration time points.
**Dose selection.** The doses selected for use in the present experiment (1.00, 0.50, 0.25, and 0.125 mmol/kg, lithium chloride) were chosen based on previous *in vitro* and *in vivo* studies. These studies reported lithium-induced therapeutic and neuroprotective effects were observed following treatment with doses of lithium ranging between 0.5 – 1.0 mEq/kg (mEq/kg equivalent to mmol/kg) (Nonaka & Chuang, 1998; Ren et al., 2003; Xu et al., 2003; Yan et al., 2007). To put these doses of lithium into perspective, the intraperitoneal dose of lithium chloride used in rodent taste aversion studies is approximately 3.0 mmol/kg (127.20 mg/kg) (Masaki & Nakajima, 2006;Nachman & Ashe, 1973; Nolan et al., 1997).

**Neurobehavioral outcome assessment.** In the present study, the neuroprotective effects of lithium were evaluated based on the performance of the animals on neurobehavioral outcome measures: i.e., beam walk (BW), MWM, and FST. In order to best determine the dose/doses of lithium that would provide neuroprotective effects in the injured brain, a dose response was carried out using the animal stroke literature as a guide in selecting the dose range.

Vestibulomotor functioning was evaluated based on performance in the BW task. This task was selected because it is a reliable, sensitive, and valid measure of motor deficits following TBI (Hamm, 2001). The postinjury time points that we selected for assessment on the BW (PID 1, 3, 7) were based on previous data from our laboratory showing that motor deficits produced by lateral FPI are most pronounced between PID 1 – 3 and typically resolve by PID 7.

The MWM was selected as the primary cognitive outcome measure because it has been extensively used with the lateral FPI model and it is known to be sensitive to hippocampal damage, a brain region known to be vulnerable in human TBI. The MWM was selected to test the ability of the rat to utilize reference memory for spatial learning and memory (Morris, Garrud, Rawlins, & O'Keefe, 1982). Performance in the MWM is directly correlated with the
functionality of the hippocampus (Morris et al., 1982). Similar to the BW, the MWM is relatively quick and easy to learn for rats and food does not have to be withheld for the acquisition of this task. The timeframe for assessment in the MWM (PID 10 – 14) was selected because any motor deficits produced by the injury have subsided and TBI-induced cognitive deficits are more pronounced, thus making any drug effects more apparent (Hamm et al., 1993).

The FST is a well-established behavioral model used to assess the efficacy of antidepressant medications and as a measure of depressive-like behavior (Castagne, Porsolt, & Moser, 2009; Jones et al., 2008; Lahmame, Grigoriadis, De Souza, & Armario, 1997; Porsolt, Anton, Blavet, & Jalfre, 1978; Porsolt, Le, & Jalfre, 1977). Based on clinical data pertaining to the incidence of depression among TBI patients and the potentially debilitating effects of this illness, the FST was included in the present thesis to evaluate the impact of lithium on depressive-like behavior following TBI. Lithium, on its own, has been shown to significantly reduce the time spent immobile in the FST in both rats (Eroglu & Hizal, 1987; Tomasiewicz, Mague, Cohen, & Carlezon, Jr., 2006) and mice (Gould et al., 2007; O'Brien et al., 2004; Shaldubina et al., 2006). In the study by Eroğul and Hizal (1987), male Wistar rats were administered daily injections of lithium chloride (0.5, 2.0, & 5.0 mEq/kg, ip) over a 19 day period. On day 18 (day 1 of the FST), the animals were placed in a cylinder, filled with water from which they could not escape, for the 15 minute (exposure) session. During the 5 minute (recall) test session, on day 19 of lithium treatment (day 2 of the FST), the lithium-treated groups spent significantly less time immobile as compared to saline-treated controls. These data indicate that lithium reduced the depressive-like behavioral response to the FST (Eroglu & Hizal, 1987). In studies using rodent TBI models, only a handful have used the FST as an outcome measure and there is a lack of consistency in these findings. Some studies report that there is no
difference between brain-injured and sham-injured rats while others have found significant behavioral differences after TBI, as well as dysregulation of the neuroendocrine stress response system (Jones et al., 2008; Milman, Rosenberg, Weizman, & Pick, 2005; Tweedie et al., 2007; Yucel et al., 2008).

**Hypotheses**

It is our hypothesis that treatment with lithium will reduce the cognitive and motor deficits associated with lateral FPI. Research supporting the neuroprotective effects of lithium treatment in animal models of brain injury and neurodegenerative diseases found that serum concentrations of lithium at or below the levels used clinically in human patients provided the greatest neuroprotection. All the doses of lithium selected for use in the present study fall within this desired range. Therefore, we theorize that all the selected doses of lithium will ameliorate postinjury deficits. We extrapolated, from a thorough review of the literature, that lower doses of lithium, within the desired therapeutic range, provided the most benefit. Therefore, we hypothesized that the middle and low doses of lithium (0.50, 0.25, 0.125 mmol/kg) would be more effective (i.e., neuroprotective) as compared to the high dose (1.00 mmol/kg).

The findings from other TBI-focused laboratories, where the FST was employed as a measure of depressive-like behavior following TBI, support the use of this assessment in rodent models of brain injury. It is hypothesized that TBI will increase the amount of time spent immobile in the vehicle-treated rats as compared to the other treatment groups. Based on lithium’s mood stabilizing effects, it our hypothesis that animals receiving lithium treatment (both injured and sham) will exhibit less time spent immobile in the FST as compared to the TBI-Vehicle group and Sham-Vehicle group.
Specific Aims

The first aim of this study was to identify the dose of lithium associated with the greatest reduction in TBI-induced deficits, as measured by BW and MWM performance. The second aim of this study was to evaluate the efficacy of lithium treatment in preventing or reducing depressive-like behavior after brain injury, as assessed by the FST.

Methods

Information pertaining to the subjects, surgical preparation, experimental brain injury, neurological evaluation, and drug preparation used in this experiment are identical to what was described in Chapter 2, General Methods.

Experimental design. Animals underwent surgical preparation and lateral FPI or sham-injury. Injured animals were randomly assigned to one of five treatment groups, 1.00, 0.50, 0.25, and 0.125 mmol/kg lithium, or vehicle (0.9% sterile saline). Sham-injury animals were randomly assigned to one of two treatment groups, 1.0 mmol/kg lithium or vehicle. Table 1 illustrates the treatment group design for Experiment 1. Once-daily lithium- or vehicle-treatment was initiated 30 min postinjury and continued through the last day of behavioral assessment in the FST (PID 19). All drug vials were coded to prevent experimenter bias. Animals were injected at approximately the same time each day. On the days when the animals were behaviorally assessed, the start time for the testing was approximately 30 – 60 minutes after lithium or vehicle was administered.

Animals were assessed on the BW on PID 1, 3, and 7. MWM assessment took place on PID 11 – 15. Hidden platform testing in the MWM occurred on PID 11-14. The probe trial and visible platform assessments were performed on PID 15. The FST was performed on PID 18 and
19. The first session was a 15 min training session followed 24 hours later by the 5 min test session. Figure 2 illustrates the treatment plan for animals in Experiment 1.

Table 1

*Experimental group design for beam walk and MWM portion of Experiment 1*

<table>
<thead>
<tr>
<th>Treatment Group&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>TBI-Vehicle&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TBI + 1.00&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>TBI + 0.50&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>TBI + 0.25&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TBI + 0.125&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sham + 1.00&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sham-Vehicle&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

*Note.*<sup> </sup><sup>a</sup>x = 10 for each group. <sup>b</sup>Vehicle (0.9% sterile saline) injection volume 1 ml/kg. <sup>c</sup>Dosage of lithium as mmol/kg, dose volume 1 ml/kg
Figure 2. **Timeline for Experiment 1.** This figure provides a detailed timeline for the neurobehavioral assessments and dosing paradigm for all treatment groups in Experiment 1.

**Drug preparation.** Lithium chloride (Sigma, St. Louis, MO) was dissolved in 0.9% sterile saline to make 1.00, 0.50, 0.25, and 0.125 mmol/kg solutions. Lithium was administered via intraperitoneal injection, in a volume of 1 ml/kg. Vehicle treated animals were administered 1 mg/kg, 0.9% sterile saline, also called normal saline.

**Lithium dose response.** The doses of lithium selected for the present study correspond to the following doses in mg/kg: 1.00 mmol/kg (42.350 mg/kg), 0.50 mmol/kg (21.175 mg/kg), 0.25 mmol/kg (10.587 mg/kg), and 0.125 mmol/kg (5.293 mg/kg). These doses were selected based on previous *in vivo* studies that reported therapeutic and neuroprotective effects following lithium treatment that produced serum levels within the therapeutic range used clinically to treat BAD (0.60 – 1.20 mmol/kg) or below (Allagui et al., 2008; Nonaka & Chuang, 1998; Omata et al., 2003; Ozaki & Chuang, 1997; Xu et al., 2003; Yan et al., 2007). Significant amelioration of insult-induced deficits on behavioral and histological measures were reported following daily (23
day) treatment with lithium chloride, 1.00 mmol/kg via intraperitoneal injection (the highest dose selected for the present study), that produced serum levels within the therapeutic range, 0.97 ± 0.31 mM (Yan et al., 2007).

**Neurobehavioral assessment.** Three neurobehavioral outcome assessment measures were selected for use in the present study: BW, MWM, and the FST. Vestibulomotor functioning was evaluated using the BW. Cognitive ability was assessed using the MWM. Finally, depressive-like behavior was determined by performance in the FST.

**Beam walk task.** The vestibulomotor functioning of each rat was assessed using the BW, similar to that detailed by Feeney and colleagues (1982) (Feeney, Gonzalez, & Law, 1982; Dixon, Lyeth, Povlishock, Findling, Hamm, et al., 1987). The rat must escape a bright light and loud noise via walking along an elevated 100 cm long, 2.5 cm wide wooden beam to enter a darkened goal box at the opposite end of the beam. The latency for the rat to reach the goal box (not to exceed 60 s) was recorded and averaged over three trials for each assessment day. If the rat fell off the beam, 60 s was recorded as the time for that trial. On the training day there was no limit on the number of trials allowed for each rat. Each rat was trained on the BW until they met criterion, traversing from one end of the beam to the goal box on three consecutive trials in less than 10 s per trial. Rats were trained on the BW one day prior to injury or sham-injury. All subsequent assessments following the training day consisted of three trials per day.

**Morris water maze.** The MWM is a large circular tank (180 cm diameter by 45 cm high) filled to a depth of 30 cm with thermostatically controlled warm water maintained between 25 – 28 °C. Figure 3 is an image of the MWM and tracking system. For assessment, rats were given four trials per day for 4 consecutive days on PID 11-14. For each daily block of four trials, the rats were placed in the tank facing the wall at one of the four designated entry points. The order
of the entry points was randomized to minimize practice effects. Each rat started the trials once from each of the four cardinal directions (north, east, south, and west) in random order. The hidden goal platform was positioned 45 cm from the outside wall and was not moved during the experiment. Rats were given a maximum of 120 s to find the hidden platform. If the rat failed to find the platform within the allotted time, it was placed on the platform by the experimenter. Upon completion of each trial, all rats remained on the platform for 30 s before being placed in a heated incubator between trials. The inter-trial interval for all the trials was approximately 10 min.

The Videomex Water Maze Program (Columbus Instruments, Columbus, OH) was used to track the movement of an animal during each trial in the MWM and calculated several pieces of data useful in interpreting the maze performance of the test subjects, such as goal latency, cumulative distance, and proximity. Goal latency is the amount of time, in s, required for each animal to locate the goal platform and total distance traveled is recorded in meters per second (m/s). The swim speed is calculated by dividing the average distance traveled by the animal by the average latency to reach the goal platform. This outcome measure can indicate whether or not the latency to reach the platform is affected by injury-induced motor disturbances and/or drug-induced alterations in motor performance. For this experiment, the swim speed of different groups was compared to ensure that differences in performance were not due to motor function.
Gallagher, Burwell, and Burchinal (1993) first described a method of calculating the proximity based on the relative position of the animal to the center point of the goal platform. The distance of the subject from the platform center is calculated each second, and at the end of the trail, is summed. An “ideal” straight line, determined by the program, based on the entry position of the animal relative to the platform, is subtracted from the actual path swum by the animal. The resulting value is the cumulative index, a scalar value that represents how much the subject deviated from the “ideal” path to the goal and how close the animal was to the goal platform. The benefit of the cumulative index is that it is relatively insensitive to the starting position of the animal and distance to the goal platform. By dividing the cumulative value by the
latency to reach the goal platform, a proximity average value is produced. The proximity average was originally developed as a means of direct comparison between the performance of young, faster swimmers, and older, slower swimmers. Although the cumulative and proximity values are nominally presented in centimeters, they are calculated based on a scalar index and as such do not represent actual distance values. The lower the proximity score, the better the animal matched the “ideal” path to the goal. Fast swimmers that swam directly to the target area and then spent time exploring in the target area will commonly have negative values (Gallagher, Burwell, & Burchinal, 1993).

**Probe trial.** Assessment using the probe trial was carried out on PID 15 (i.e., after the last day of hidden platform testing). The probe trial consisted of a single 60-s trial in the MWM, with the hidden platform removed. Animals were randomly assigned a start position (one of the four cardinal points) and were allowed to swim freely for 60 s prior to removal from the pool by the investigator. The data collected by the Videomex tracking system was used to determine the proximity score. Lower proximity scores indicated that the animal took a more direct path to the platform. This test was always performed prior to the visible platform test.

**Visible platform.** The visible platform test (PID 15) was used to assess the visual acuity of each rat. The water level in the MWM was drained to 1 cm below the top of the previously hidden platform, and a 10 cm painted black pole was attached to the platform to make the platform more visible. For each of the 8 trials, the platform was moved to a new, randomly selected, location, which was different from the hidden platform location. The point of entry (one of the four cardinal directions) for each rat was randomly selected for each trial. To adequately perform this task, each rat had to locate the platform within 15 s after placement in the pool.
**Forced swim test.** The FST procedure detailed in the 1977 article by Porsolt and colleagues, modified by Lahmame et al., (1997), was used in the present study. The FST is a two-day procedure that took place on PID 18 and 19 with one trial per day. Animals were assessed between 11:00 and 16:00 hours. Each rat was placed in a clear Plexiglas cylinder (height, 45 cm, internal diameter, 25 cm) containing water maintained at a temperature of 25°C and filled to a level of 30 cm. At this water level the rats cannot touch the bottom or escape. The water was changed following each trial and the Plexiglas cylinder was wiped down using a 70% ethanol and 30% water solution. For the first exposure (PID 18), the rats were placed in the water for 15 min. Twenty-four hours later, during the second exposure (PID 19), the animals were placed in the water for a 5 minute test session. After completing the test period the animals were placed in an incubator and allowed to dry before being returned to their home cage. Both sessions were videotaped using a camera mounted on a metal pole lateral to the Plexiglas cylinder, such that the full length of the rat’s body could be seen on the tape. The 15 minute (exposure session) was used to train the rats that there was no escape (i.e., a model of learned helplessness and depressive-like behavior). Both test sessions were recorded on videotape, however, only the 5 min (recall session) tape was later evaluated by two reviewers blinded to the treatment conditions. The recall testing session revealed how the animals responded to a situation where there is no escape. Based on previous studies, the time to become immobile (TTBI) and the total time immobile (TTI) were selected as outcome measures for the present study. Immobility was operationally defined as when the rat remained motionless, or performed minimal movements, without breaking the surface of the water and not moving all four limbs to maintain their head/nose above the water (Lahmame et al., 1997).
**Statistical analysis.** Injury severity, as assessed by the suppression of the righting reflex, was analyzed as described in the general methods section. Statistical analyses specific to this experiment are described in the following paragraphs.

The mean daily latency for each group to traverse the beam and reach the goal box were calculated for PID 0, 1, 3, and 7. These data were then compared using a split-plot analysis of variance and a Fisher LSD post hoc analysis was performed to compare specific groups of interest. Data from the training session on the BW was not used for any statistical analysis.

The mean latency to locate the goal platform and proximity score for each treatment group was computed daily during the hidden platform portion of the MWM testing (PID 11–14). The average goal latencies and proximity scores were then analyzed using a separate split-plot analysis of variance. The swim speed was averaged for each day in the MWM, and a one-way ANOVA was performed to determine any relationship between the groups. The probe trial data and visible platform data (PID 15) were also analyzed using a one-way ANOVA. Where appropriate, a Fisher LSD post hoc test was performed to compare specific groups of interest.

The TTBI and the TTI from the 5 min test session on PID 19 were evaluated using a one-way ANOVA and an independent samples $t$-test respectively. Where appropriate a Fisher LSD post hoc was performed to compare groups of interest.

**Results**

**Neurological outcome.** There was no significant difference in the suppression of the righting reflex among injured groups. A one-way ANOVA was used to compare the mean duration of the suppression of the righting reflex, TBI-Vehicle, $M = 428.00$ s, TBI-1.00, $M = 422.00$ s, TBI-0.50, $M = 560.00$ s, TBI-0.25, $M = 457.70$ s, TBI-0.125, $M = 424.80$, $F(4, 45) = 1.965$, $p = .116$. The sham groups regained their righting reflex in less than 2 min from the time
they were removed from the bell jar used to anesthetize the animals. Based on our experience in
the laboratory, this is the same amount of time an animal requires to regain the righting reflex
after anesthesia alone.

**Beam walk.** A split plot [7 group x 4 day] ANOVA revealed a significant group effect,
\[ F(6, 63) = 3.585, p = .004. \] A Fisher LSD post hoc analysis revealed a significant reduction in
motor deficits in the TBI-1.00 \( (p = .021), \) TBI-0.25 \( (p = .035), \) Sham-Vehicle \( (p = .001), \) and
Sham-1.00 \( (p = .003) \) as compared to TBI-Vehicle. Although the TBI-0.125 group did not
significantly differ from the TBI-Vehicle group, there was a trend toward significantly shorter
latencies \( (p = .057) \). No significant difference was detected in the BW performance of the TBI-1.00,
TBI-0.25, TBI-0.125, Sham-Vehicle, and Sham-1.00 groups. No significant difference was
observed between the TBI-Vehicle and TBI-0.50 groups \( (p = .719), \) the two groups with the
longest latencies to reach the goal box. Figure 4 illustrates the primary groups of interest based
on performance, TBI-Vehicle, TBI-1.00, TBI-0.25, TBI-0.125, and Sham-Vehicle. A graph
comparing the average latencies to reach the goal box across all treatment groups in the BW task
is provided in Figure A1 in Appendix A.
Figure 4. Comparison of pre- and post-TBI motor performance among treatment groups of interest in Experiment 1. These data are expressed as the mean latency across days ± SEM, n = 10/group. A split-plot ANOVA showed that there was a significant effect of group. Post hoc analysis of the data using the Fisher LSD test showed that the TBI-1.00 and TBI-0.25 groups had significantly shorter latencies to reach the goal box as compared to the TBI-Vehicle group. The difference between the TBI-Vehicle and TBI-0.125 groups was not statistically significant; however, there was a trend indicating shorter latencies for the TBI-0.125 group. The three injured lithium-treated groups had statistically similar latencies to reach the goal box. All the doses of lithium are mmol/kg. Vehicle treated animals received 0.9% sterile saline. All treatments were administered in a volume of 1 mg/kg. Specific statistical information is provided in the text. Comparisons are made to the Sham-Vehicle group. *** p ≤ .001.
Morris water Maze.

Hidden platform. The hidden platform portion of the MWM revealed a significant group effect, $F(6, 63) = 9.483, p < .001$. A Fisher LSD post hoc test determined that both the 0.25 and 0.125 mmol/kg doses of lithium were equally effective in reducing TBI-induced deficits in the MWM as compared to the TBI-Vehicle group. These data are presented in Figure 5. The 0.50 and 1.00 mmol/kg doses of lithium did not significantly improve maze performance as compared to the TBI-Vehicle treated group; however, the 1.00 mmol/kg dose did approach the level of significance ($p = .053$). None of the treatment groups, including the Sham-1.00 group, performed as well as the Sham-Vehicle group. See Figure A2 in Appendix A, for a comparison of MWM performance across all the treatment groups.

Swim speed. A one-way ANOVA revealed that there was a significant difference in the average swim speed of the treatment groups, $F(6, 63) = 4.896, p < .001$. A Fisher LSD post hoc analysis revealed that all the TBI-Lithium groups (1.00, 0.50, 0.25, 0.125 mmol/kg lithium) swam significantly faster as compared to the Sham-Vehicle, Sham-1.00, and TBI-Vehicle groups. These data are illustrated in Figure 6. There was no significant difference in the swim speeds across the TBI-Lithium groups. Additionally, there was no significant difference in the swim speeds of the two sham- and injured-vehicle-treated groups.
Figure 5. Comparison of MWM latency data among selected groups in Experiment 1. This graph shows the daily average latency to reach the goal platform for each treatment group \((n = 10/\text{group})\), ± SEM. A split-plot ANOVA and Fisher LSD post hoc revealed that the TBI-Vehicle group had significantly longer latencies to reach the goal platform as compared to the TBI-0.125, TBI-0.25, Sham-Vehicle, and Sham-1.00 groups. None of the groups performed as well as the Sham-Vehicle group. All doses of lithium are mmol/kg. (*) indicate comparisons made to the Sham-Vehicle group. (†) represent comparisons made to the Sham-1.00 group. (‡) represents comparisons made to the TBI-Vehicle group. * \(p < .05\). **, ## \(p < .01\). ***, ††† \(p < .001\).
Figure 6. Comparison of the overall average swim speed for each treatment group in Experiment 1. Data are expressed as the mean ± SEM. $n = 10$ group ($N = 70$). A one-way ANOVA showed a significant difference in the overall swim speeds, $F(6, 63) = 4.896$, $p < .001$. A Fisher LSD post hoc analysis showed that all of the TBI-Lithium-treated groups swam significantly faster as compared to both sham groups and the TBI-Vehicle-treated group. There was no significant difference in the swim speeds of the Sham-Vehicle, Sham-1.00, and TBI-Vehicle groups ($p > .05$). No differences were observed in the swim speeds of the TBI-Lithium-treated groups ($p > .05$). (*) indicates comparisons made to the Sham-Vehicle group. (†) indicates significant differences in treatment groups as compared to Sham-1.00. (#) indicates comparisons made to TBI-Vehicle. *, †, # $p < .05$. **, ††, ## $p < .01$. 

![Average Swim Speed Graph](image-url)
**Proximity score hidden platform.** The average daily proximity score was calculated for each treatment group (n = 10/group). Lower scores indicated a more direct path to the goal platform. A split-plot ANOVA revealed a significant main effect of treatment group, $F(6, 63) = 4.459, p = .001$. A Fisher LSD post hoc analysis showed that only the Sham-Vehicle ($p < .001$) and Sham-1.00 ($p = .003$) had significantly lower proximity scores as compared to the TBI-Vehicle group. There was no significant difference among the proximity scores of the injured-vehicle and -lithium groups. Figure 7 illustrates data from the two most effective doses of lithium, as determined by the analysis of the MWM latency data (i.e., TBI-0.25 and TBI-0.125), in addition to the TBI-Vehicle and both sham groups. The scores for the Sham-Vehicle and Sham-1.00 groups were statistically similar ($p = .235$). Although the Sham-Vehicle group had significantly lower proximity scores as compared to the injured lithium-treated groups, the scores from the two most effective doses of lithium, TBI-0.25 and TBI-0.125, did not statistically differ from the proximity score of the Sham-1.00, $p = .061$ and .185 respectively. A comparison of proximity scores during MWM across all treatment groups is provided in Figures A3 and A4 of Appendix A.

**Probe trial.** The average proximity score for each treatment group during the probe trial (PID 15) was analyzed using a one-way ANOVA. These data are illustrated in Figure 8. There was a significant main effect of group, $F(6, 59) = 3.407, p = .006$. A Fisher LSD post hoc analysis determined that the Sham-Vehicle group had a significantly lower proximity score as compared to all the other treatment groups, $p < .01$. No other differences were found.
Figure 7. Comparison of the proximity scores for the groups of interest across days during hidden platform testing in MWM. This graph shows the daily average proximity score for the treatment groups of interest (n = 10/group) during MWM testing, ± SEM. All doses of lithium are in mmol/kg. A repeated measure ANOVA revealed a significant difference among the average daily proximity scores of the treatment groups. A Fisher LSD post hoc found that the two sham groups had significantly lower proximity scores (indicating better performance) as compared to the TBI-Vehicle group. The two injured groups that had significantly shorter latencies to reach the goal platform as compared to the TBI-Vehicle group during the hidden platform testing, TBI-0.25 and TBI-0.125, had significantly higher proximity scores as compared to the Sham-Vehicle group (i.e., poorer performance). However, these two injured groups did not significantly differ from the Sham-1.00 group, suggesting that these groups took a more direct path to the goal platform. There was no appreciable difference in the proximity scores of the TBI-Vehicle group and the TBI lithium-treated groups. (*) indicate comparisons to the Sham-Vehicle group. (†) indicate comparisons made to the Sham-1.00 group. **, ††p < .01. ***p < .001.
Figure 8. Analysis of the proximity scores for the groups of interest during the probe trial in Experiment 1. The data are expressed as the mean ± SEM, (n = 10/group). An ANOVA found significant differences between the treatment groups. A Fisher LSD post hoc revealed that the Sham-Vehicle group had a significantly lower proximity score as compared to all other treatment groups. * p < .05. ** p < .01. *** p < .001.
**Visible platform.** The mean latency, in seconds, for each group \( (n = 10/\text{group}) \) to locate the visible platform revealed the following data, expressed as the group mean \((\pm SEM)\): TBI-Vehicle, \( M = 10.87 \text{ s (± 1.07)} \); TBI-1.00, \( M = 12.58 \text{ s (± 1.18)} \); TBI-0.50, \( M = 13.79 \text{ s (± 2.49)} \); TBI-0.25, \( M = 10.85 \text{ s (± 1.51)} \); TBI-0.125, \( M = 14.08 \text{ s (± 2.14)} \); Sham-Vehicle, \( M = 10.04 \text{ s (± 2.93)} \); Sham-1.00, \( M = 9.88 \text{ s (± 1.13)} \). A one-way ANOVA revealed that there were no significant differences between any of the treatment groups in the average latency to locate the visible platform, \( F(6, 63) = 0.836, p = .547 \).

**Forced swim test.** The mean latency, in seconds, for animals in the TBI-Vehicle \( (n = 10) \), TBI-0.25 \( (n = 9) \), and Sham-Vehicle \( (n = 10) \) groups to become immobile (i.e., TTBI) are illustrated in Figure 9. These data were analyzed using a one-way ANOVA, and no significant difference was detected in the TTBI, \( F(2, 26) = 0.610, p = .551 \). Because the TTBI data was not significant, we analyzed only the TBI-Vehicle and Sham-Vehicle groups for the TTI data. An independent samples \( t \)-test was used to evaluate any difference in the TTI between the TBI-Vehicle \( (n = 10) \), \( M = 165.41 \text{ s, } SD = 43.78 \) and Sham-Vehicle \( (n = 9) \), \( M = 176.30 \text{ s, } SD = 36.81 \) groups; \( t(17) = -.576, p = .567 \). Similar to what was found with the TTBI data, the FST was not able to detect differences between injured and sham animals.
Figure 9. Mean latency for animals to become immobile in the forced swim test. The time to become immobile (TTBI) was assessed on the second day in the FST (PID 19). Data are expressed as the mean TTBI ± SEM. A one-way ANOVA showed that there was no significant difference in the TTBI between the TBI-Vehicle \( (n = 10) \), TBI-0.25 \( (n = 9) \), and Sham-Vehicle \( (n = 10) \) groups. Within the parameters of the present study, the FST lacks the sensitivity to detect differences between sham and injured animals.
Discussion

The aim of the present study was to determine the best dose or doses of lithium for reducing TBI-induced motor and cognitive (spatial navigation and depressive-like behavior) deficits. A dose response was used to determine the most effective doses of lithium as a therapy to ameliorate motor and cognitive deficits associated with moderate-severity TBI. Previous studies have shown lithium to be an effective therapy when administered prior to a traumatic insult \textit{in vitro} and when administered pre- and post-MCAO \textit{in vivo}. The results from the present study support the use of low doses of lithium as a postinjury therapy to ameliorate TBI-induced cognitive and vestibulomotor deficits.

**Beam walk task.** The effects of the different doses of lithium on vestibulomotor functioning indicate that the doses of lithium corresponding to the upper and lower end of the clinically relevant therapeutic range were the most beneficial. Injured animals treated with 1.00, 0.25, and 0.125 mmol/kg, doses of lithium showed marked improvement in their latency to reach the goal box on PID 1 and 3, as compared to animals that received the 0.50 mmol/kg dose. These findings partially fit with the working hypothesis of this study that the middle and lower doses of lithium would be more effective as compared to the high dose. Although the benefit associated with the higher dose was unexpected, it is not uncommon for high and low doses of drugs, particularly those used in brain injury studies, to have positive effects on performance whereas doses falling within the middle range of this scale are associated with a lack of improvement. Indeed, the review article by Calabrese (2008) examined multiple ischemic brain-injury and TBI studies, which varied greatly in their methodologies, and found over 30 drugs that were reported to have this same pattern of effective recovery of motor function with high and low doses of a drug, that was not observed following administration of mid-range doses. As
expected, by PID 7 all the injured groups had a return to near preinjury levels of performance in the BW task.

**Morris water maze.**

*Hidden platform.* The results from the hidden platform portion of the MWM task showed that lowest doses of lithium tested (0.25 and 0.125 mmol/kg) were the most effective in reducing TBI-induced cognitive deficits. It was speculated that the 0.50 mmol/kg dose would have also provided neuroprotection, however, this was not the case.

To ensure that the differences we found in the MWM performance between the treatment groups was not affected by motor deficits, the average swim speed per group was calculated. Although there were small differences in the overall swim speeds of the treatment groups, there was no significant difference in the swim speeds of the sham and injured vehicle-treated animals. Neither injury nor lithium treatment alone were sufficient to alter the average swimming speed of the groups. However, when TBI and lithium treatment were combined, the average swim speed of these groups (i.e., TBI-1.00, TBI-0.50, TBI-0.25, TBI-0.125) significantly increased. Additionally, there was no appreciable difference in the swim speeds of the injured lithium-treated groups. Based on these data, we hypothesize that there may be a combined effect of injury and lithium treatment on motor function and that an aspect of this effect is manifest as increase in swim speed. The differences in the group swim speeds could be a confounding variable, however, it is unlikely that the group differences in swim speed significantly affected the latencies to locate the hidden platform. All the injured lithium-treated groups swam faster than the TBI-Vehicle group. The groups treated with the lower doses (0.25 & 0.125 mmol/kg) had significantly shorter latencies to locate the hidden platform than the TBI-Vehicle, whereas the higher doses (1.00 & 0.50 mmol/kg) had similar latencies to reach the goal platform as
compared to the TBI-Vehicle group. A factor that could have contributed to the differences in swim speed is the mass of the animals. However, an analysis of the average daily mass for each treatment group did not reveal any significant between-group differences. The finding that the mass of the animals was comparable over the course of the study supports the theory that the differences in swim speed were due to drug effects rather than weight loss or gain.

The proximity score has been reported to be a better indication of spatial learning than goal latency when there are differences in overall swim speeds (Gallagher et al., 1993). Analysis of the proximity score data from the hidden platform testing in the MWM showed that none of the injured lithium-treated groups significantly differed from TBI-Vehicle. However, relative to the other TBI groups, the TBI-0.25 and TBI-0.125 were the only ones that performed as well as the Sham-1.00 group (i.e., had lower proximity scores). These data parallel the latency data in the MWM, which showed chronic postinjury treatment with the two lowest doses of lithium (0.25 and 0.125 mmol/kg) was associated with shorter latencies to the goal platform that were statistically similar to those observed in the Sham-1.00 group. The higher doses of lithium (1.00 and 0.50 mmol/kg) were not able to significantly reduce MWM latency relative to the low doses of lithium and both sham groups.

Despite the differences in swim speeds, because all of the injured lithium-treated animals swam significantly faster than sham groups, it is not likely that the swim speed is a confounding variable. The similarity in the proximity scores of the TBI-0.25, and TBI-0.125, and Sham-1.00 groups, despite having significantly different swim speeds, provides further support for the use of the selected dose of lithium (0.25 mmol/kg) in the subsequent experiments in this dissertation.

**Probe trial.** The probe trial was administered on PID 15 (day 5 in the MWM). This test was incorporated to measure the cumulative learning of each rat for the location of the hidden
platform. The proximity score from this assessment was compared across groups and was expected to support the data from the hidden platform testing portion of the MWM. However, the only difference that was detected was a significantly lower proximity score in the Sham-Vehicle group, indicating improved performance, as compared to all other treatment groups. These data could indicate that the injured lithium-treated groups did not effectively learn the location of the hidden platform. Additionally, the Sham-1.00 group had significantly higher proximity scores, indicating poorer performance, as compared to the Sham-Vehicle group, suggesting that lithium itself may have deleterious effects on MWM learning. Based on the similarities in performance between the Sham-Vehicle and Sham-1.00 groups on the BW and hidden platform portion of the MWM (i.e., latency, proximity, and swim speed), it is unusual that the probe trial data indicated that these groups were significantly different and that the Sham-1.00 group performance was statistically similar to all the injured groups. An alternate explanation for the observed results in this task is that the probe trial lacks the sensitivity to detect the injury- and drug-induced effects on MWM learning as compared to the latency and proximity outcome measures from the hidden platform test.

Visible platform. The visible platform test was used to ensure adequate visual acuity as well as motivation to locate the platform. Analysis of these data showed that all the animals met the criterion established for the visible platform task (i.e., they were able to locate the hidden platform within an average of 15 s over 8 trials). There was no significant difference between any of the treatment groups in the average latency to locate the visible platform, thereby excluding the possibility that the differences in overall performance between the treatment groups was attributable to visual or motivational differences.
Forced swim test. The FST was used to assess depressive-like behavior following TBI. However, in the present study we did not detect any differences in either the TTBI or the TTI between the TBI-Vehicle and Sham-Vehicle groups. Because there was no discernable effect of injury, further use of this test with the different doses of lithium was not carried out. The lack of an injury effect could be attributed to the animal’s prior experience in the MWM. Based on this assumption, the prior exposure to a swimming task may have desensitized the animals to the stress and subsequent “learned helplessness” associated with longer periods of immobility and shorter latencies to become immobile. Behavioral assessment of depressive-like behavior using the FST may be best suited for animals not previously subjected to assessment in the MWM.

Experiment 2

Rationale

It is well established that the therapeutic time window is critical when evaluating the effectiveness of a treatment. According to the biphasic model of TBI, there is a sharp increase in neuronal and metabolic activity followed by a steep decline in overall brain functioning that can persist for some time postinjury. Drug treatments typically target either the acute or chronic phases. Indeed, agents that are beneficial when administered during the acute postinjury phase (e.g., mu-opioid receptor agonists and glutamate antagonists) may be ineffective or even impair recovery when administered during the chronic postinjury phase (Hamm, O'Dell, Pike, & Lyeth, 1993; Lyeth, Jiang, Gong, Hamm, & Young, 1995). During the chronic postinjury phase, marked by a hypofunctional state, drugs that have excitatory effects are associated with improved outcome.

The findings from Experiment 1 showed that the lowest doses of lithium, administered 30 min postinjury, were effective in reducing cognitive and motor deficits associated with TBI.
This finding is encouraging and it may provide further insight into mechanisms of action for lithium as well as secondary injury processes occurring after lateral FPI. However, a 30 minute therapeutic time window does not translate well to clinical populations. The primary difficulty in translating successful therapies from the bench to the bedside is ensuring that the initiation of treatment is equivalent (Marklund et al., 2006). Therapies that are effective in the acute postinjury phase, in preclinical models, must be administered within a brief time period in clinical trials. This can present a challenge for investigators due to the time lag between the traumatic event, the arrival of the individual to a medical center, and obtaining informed consent from the patient or a family member. In the present study, we determined which of two postinjury time points (8 and 24 hr) is most effective in ameliorating the sequelae associated with the lateral FPI model of TBI, and provide insight into the therapeutic time window for initiating lithium treatment. The 8 and 24 hr postinjury time points were selected based on the clinical relevance, because a longer therapeutic time window will offer the best chance for translating these findings to clinical practice. Furthermore, delaying treatment may provide insights into the mechanisms of action through which lithium may exert its neuroprotective effects.

The data collected from the 8 and 24 hr treatment delays was analyzed with the data obtained from the 30 min treatment delay group that was treated with the optimal dose of lithium (0.25 mmol/kg) from Experiment 1 (i.e., the TBI-0.25 group). The data from the TBI-0.25 group, along with the two treatment delays from the present study, were used to establish the furthest time point from injury that chronic lithium treatment can be administered while still offering significant protection of vestibulomotor and cognitive function.
Hypotheses

It was hypothesized that the optimal dose (0.25 mmol/kg, ip), selected from Experiment 1, would be effective in reducing TBI-induced vestibulomotor and cognitive deficits at both treatment delay time points (8 and 24 hr).

Specific Aims

The aim of the present study was to evaluate the effect of delayed postinjury lithium treatment on cognitive and vestibulomotor function. Using the optimal dose from Experiment 1, we aimed to define a therapeutic time window for lithium administration.

Methods

The subjects, surgical preparation for lateral FPI, lateral FPI, postinjury neurological assessment, drug preparation, and neurobehavioral outcome assessment for BW and MWM, are identical to those described in Experiment 1. Due to the lack of a detectable difference between sham and injured animals in the FST, this test was not administered. The treatment timeline used for this study is detailed in Figure 10.

Experimental design.

Drug treatment. Animals received daily (PID 0-19) intraperitoneal injections of either lithium chloride or vehicle (0.9% sterile saline). Daily treatment was initiated at either 8 or 24 hours postinjury. Sham animals were randomly assigned to one of two treatment groups, lithium-treated (0.25 mmol/kg, ip) with treatment initiated 24 hours post-sham-injury (to test for any effects of the optimal dose of lithium in uninjured rats), or vehicle-treated (1 ml/kg, ip). See Table 2 for treatment group design.

Statistical analysis. In Experiment 1, the selected dose of lithium (0.25 mmol/kg, ip) was found to be effective in reducing TBI-induced cognitive and motor deficits when administered
30 min after injury. To determine the relative efficacy of the two time delays in the present study, the BW and MWM data from the optimal dose in Experiment 1 were included in the data analysis of the present study.

Data from the MWM was analyzed for goal latency using a split-plot ANOVA [6 (Group) x 4 (Day)] comparing injured animals and sham-injured animals. A mean daily latency to find the goal platform during MWM testing on day’s 11 – 14 postinjury was computed for each group. Proximity scores and swim speed were averaged individually for each day in the MWM, and a one-way ANOVA was performed for each outcome measure to determine the relationship between the groups. For each of the other tasks involving the MWM (i.e., probe trial and visible platform), a one-way ANOVA was used to determine any relationship between the groups. Where appropriate, a post hoc analysis was performed to compare specific groups of interest.

Figure 10. Timeline of events in Experiment 2. This figure shows the schedule for administration of both the drug-treatment and neurobehavioral assessments used in Experiment 2. Abbreviations: beam walk test (BW), Morris water maze (MWM), postinjury day (PID)
Table 2.

*Experiment 2: Treatment Groups*

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBI-Vehicle&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>Sham-Vehicle&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>TBI-30 min&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>TBI-8 hr&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>TBI-24 hr&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td>Sham-24 hr&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9</td>
</tr>
</tbody>
</table>

*Note.*<sup>a</sup>Vehicle (0.9% sterile saline, dose volume: 1 ml/kg), 30 min postinjury or sham.<sup>b</sup>24 or 8 hr treatment delay (dosage: 0.25 mmol/kg lithium, dose volume: 1 ml/kg).<sup>c</sup>Data from the TBI-30 min group are from the TBI-0.25 Lithium group in *Experiment 1.*

**Results**

**Neurological assessment.** The suppression of the righting reflex was assessed immediately after the delivery of the TBI. The mean latency (in seconds) necessary for each animal to recover the righting reflex was calculated for each treatment group that received a TBI. These data are as follows (± SEM): TBI-Vehicle, $M = 403.30 \text{ s} (± 24.95) \ (n = 10)$; TBI-30 min, $M = 430.20 \text{ s} (± 12.73) \ (n = 10)$; TBI-8 hr, $M = 430.20 \text{ s} (± 12.73) \ (n = 10)$; TBI-24 hr, $M = 413.67 \text{ s} (± 21.49) \ (n = 9)$. A one-way ANOVA failed to detect any significant differences in the suppression of the righting reflex among the injured groups, $F(3, 35) = 0.859, p = .471$. This finding indicates that all the injured animals had equivalent injury severities.

**Animal mass.** The mass of the animals was compared over the 21 days from the surgical preparation for the injury (PID -1) to the final day of lithium treatment (PID 19). The ANOVA
did not find any significant difference in the average mass of the treatment groups, \( F(5, 47) = 1.814, p = .128 \). The Sham-Vehicle and TBI-30 min data are the same that was used in the animal mass data analysis in Experiment 1.

**Beam walk.** Data from the BW assessment are presented in Figures 11-12. A split-plot ANOVA \( [6 \text{ (Group)} \times 4 \text{ (Day)}] \) revealed significant group differences in the mean latencies to reach the goal box, \( F(5, 52) = 5.012, p = .001 \). A Fisher LSD post hoc analysis showed that the Sham-Vehicle \( (p < .001) \), Sham-24 hr \( (p < .001) \), TBI-8 hr \( (p = .019) \), and TBI-30 min \( (p = .004) \) groups had significantly shorter latencies to reach the goal box as compared to the TBI-Vehicle group. Additionally, the average BW latencies for these four groups were not significantly different from each other \( (p = .125) \). No significant difference was observed between the TBI-Vehicle and TBI-24 hr group \( (p = .403) \). While the TBI-8 hr group was significantly different from the TBI-Vehicle, it did not significantly differ from the TBI-24 hr group \( (p = .139) \).

**Morris Water Maze.**

*Hidden platform.* The mean latency for each group to locate the hidden platform was compared across days. A split-plot ANOVA \( [6 \text{ (group)} \times 4 \text{ (day)}] \) showed that there was a significant difference in the latency to reach the goal platform between treatment groups, \( F(5, 52) = 8.042, p < .001 \). A Fisher LSD post hoc analysis found that the TBI-Vehicle group had significantly longer latencies to reach the goal platform as compared to the Sham-Vehicle \( (p < .001) \), Sham-24 hr \( (p < .001) \), TBI-30 min \( (p = .002) \), and TBI-8 hr groups \( (p = .002) \). No difference was detected between TBI-24 hr and TBI-Vehicle groups, \( (p = .319) \). The TBI-30 min \( (p = .040) \) and TBI-8 hr \( (p = .037) \) groups had significantly shorter latencies to reach the hidden platform as compared to the TBI-24 hr group. No significant difference was observed between the TBI-30 min and TBI-8 hr groups \( (p = .967) \). Animals in the Sham-Vehicle group
performed significantly better as compared to animals that received a TBI. No significant
difference in performance was detected between the Sham-Vehicle and Sham-24 hr groups (\(p = .352\)). MWM data for the 24 hr and 8 hr treatment-delay groups are presented in Figure 13 and 14 respectively.

Swim speed. The mean swim speed across days in the MWM was calculated for each
treatment group. These data are presented in Figure 15. A one-way ANOVA found a significant
difference between the swim speeds, \(F(5, 52) = 2.981, p = .019\). A Fisher LSD post hoc analysis
revealed that there was no appreciable difference in the average swim speeds between the Sham-
Vehicle, TBI-Vehicle, Sham-24 hr, and TBI-24 hr groups. Additionally, both of the 24 hr
treatment delay groups (TBI-24 hr and Sham-24 hr) had statistically similar swim speeds as
compared to the TBI-8 hr or TBI-30 min groups. However, the TBI-8 hr and the TBI-30 min
groups swam significantly faster (\(p = .008\) and \(.002\) respectively) in the MWM as compared to
the Sham-Vehicle group (\(p = .571\)), but only the TBI-30 min group swam significantly faster
than the TBI-Vehicle group, \(p = .015\). The average speed of the TBI-8 hr group was faster than
the TBI-Vehicle group and there was a trend toward a significant difference (\(p = .057\)) in speeds
between the two groups.

Proximity to hidden platform. The proximity score data from the hidden platform testing
was compared using a split-plot [6 (group) x 4 (day)] ANOVA and revealed a significant effect
of group, \(F(5, 52) = 5.379, p < .001\). A Fisher LSD post hoc analysis found that the TBI-30 min
\((p = .031)\) and TBI-8 hr \((p = .03)\) had significantly lower proximity scores, indicating a more
direct route to the goal platform, as compared to the TBI-Vehicle group. However, the proximity
scores of the TBI-30 min and TBI-8 hr were significantly higher than the Sham-Vehicle \((p = .034\) and \(.036\) respectively) but did not differ from the Sham-24 hr group \((p = .122\) and \(.128\)
respectively). Both sham groups had significantly lower proximity scores \( (p < .001) \) as compared to the TBI-Vehicle group. The TBI-24 hr group significantly differed \( (p < .01) \) only from the sham groups. The proximity data for the 24 hr and 8 hr treatment-delay groups are presented in Figure 16 and 17 respectively.

**Probe trial.** The probe trial (60 s) was conducted in the MWM on PID 15, 24 h after the last trial of the hidden platform testing. This test was performed with the platform removed from the MWM. The mean proximity score was calculated for each treatment group using the data collected by the VideoMex tracking software. The proximity score was determined by comparing the swim path of the animal and the most efficient path to the goal, as determined by the VideoMex program. In this analysis, a lower score is indicative of better learning for the location of the hidden platform. Data from the 24 hr and 8 hr treatment-delay groups are presented in Figure 18 and 19 respectively. A one-way ANOVA showed that there was a significant effect of treatment group on the proximity score, \( F(5, 45) = 2.959, p = .022 \). A Fisher LSD post hoc analysis revealed that the Sham-24 hr \( (p = .009) \) and Sham-Vehicle \( (p = .039) \) were the only groups with significantly lower scores as compared to the TBI-Vehicle group. The Sham-24 hr group also had significantly lower proximity scores as compared to the TBI-30 min \( (p = .023) \), TBI-24 hr \( (p = .006) \); however, no difference was observed between Sham-24 hr and TBI-8 hr \( (p = .257) \). Sham-Vehicle differed only from TBI-24 hr \( (p = .030) \). No difference was found in the proximity score between the Sham-Vehicle and TBI-30 min \( (p = .07) \) or TBI-8 hr \( (p = .314) \) groups.

**Visible platform.** The mean latency, in seconds, for each treatment group to locate the visible platform was calculated based on the performance of each animal over 8 trials (PID 15). The group means \( (\pm SEM) \) are as follows: TBI-Vehicle, \( M = 10.54 \text{ s} \ (\pm 1.56) \); TBI-30 min, \( M = \)
10.85 s (± 1.51); TBI-8 hr, $M = 11.35$ s (± 1.54); TBI-24 hr, $M = 12.99$ s (± 1.06); Sham-Vehicle, $M = 13.03$ s (± 2.71); Sham-24 hr, $M = 8.88$ s (± 0.56). A one-way ANOVA failed to detect any statistically significant differences among the group means, $F(5, 52) = 0.862, p = .513$.

![Graph](image)

**Figure 11. Comparison of 24 hr treatment delay on beam walk performance.** These data represent the mean latency to reach the goal box, ± SEM. TBI-Vehicle ($n = 10$), TBI-24 hr ($n = 9$), Sham-24 hr ($n = 9$), Sham-Vehicle ($n = 10$). An ANOVA followed by a Fisher LSD post hoc test determined that the TBI-24 hr and TBI-Vehicle group performed significantly worse on the beam walk task as compared to both sham groups. There was no difference in performance detected between the two injured groups and no significant difference between the two sham groups. (*) indicates comparisons made to the Sham-Vehicle group. (†) indicate significant differences in treatment groups as compared to Sham-1.00. †††, *** $p < .001$. 

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Figure 12. Comparison of 8 hr treatment delay on beam walk performance. These data represent the mean latency to reach the goal box, ± SEM. TBI-Vehicle (n = 10), TBI-30 min (n = 10), TBI-8 hr (n = 10), and Sham-Vehicle (n = 10). The TBI-30 min group data is identical to the TBI-0.25 from Experiment 1 and is included in the data analysis as a basis for comparing the effectiveness of the selected dose of lithium when the initiation of treatment is delayed. The TBI-8 hr and TBI-30 min had significantly shorter latencies to reach the goal box as compared to the TBI-Vehicle and TBI-24 hr groups. (*) indicate comparisons made to the Sham-Vehicle group. (##) indicate comparisons made to TBI-Vehicle. * p < .05. ## p < .01. *** p < .001.
Figure 13. Effect of 24 hr treatment delay on MWM performance. Data are the mean latency for the selected treatment groups, TBI-Vehicle (n = 10), TBI-24 hr (n = 9), Sham-Vehicle (n = 10), and Sham-24 hr (n = 9), ± SEM. There was a significant main effect for group. A Fisher LSD showed that the TBI-24 hr and TBI-Vehicle groups had significantly longer latencies to reach the goal as compared to both sham groups. There was no significant difference between the latencies of the two sham groups or the two injured groups. (*) indicate comparisons made to the Sham-Vehicle group. (†) indicate significant differences in treatment groups as compared to Sham-1.00. †††, *** p < .001.
Figure 14. Effect of 8 hr treatment delay on MWM performance. Data represent the mean latency for the selected groups ($n = 10$/group) to reach the hidden platform, ± SEM. The dose of lithium used is 0.25 mmol/kg (ip). The TBI-30 min group data is the TBI-0.25 from Experiment 1 and is included in the data analysis as a basis for comparing the effectiveness of the selected dose of lithium when the initiation of treatment is delayed. An ANOVA showed significant differences between the treatment groups. A Fisher LSD post hoc analysis found that the TBI-30 min and TBI-8 hr groups had significantly shorter latencies to reach the hidden platform as compared to the TBI-Vehicle group ($p = .002$). The Sham-Vehicle group performed significantly better in this task as compared to the TBI-Vehicle ($p < .001$), TBI-30 min ($p = .032$), and TBI-8 hr ($p = .035$) groups. (*) indicate comparisons made to the Sham-Vehicle group. (##) indicate comparisons made to the TBI-Vehicle group. * $p < .05$. ## $p < .01$. *** $p < .001$. 

![Graph showing latency to goal platform over days tested in the MWM](image-url)
Figure 15. Average swim speed for each treatment group in Experiment 2. Data represent the mean swim speed for each treatment group across days in the MWM during hidden platform testing, ± SEM. TBI-Vehicle (n = 10), TBI-24 hr (n = 9), TBI-8 hr (n = 10), TBI-30 min (n = 10), Sham-Vehicle (n = 10), Sham-24 hr (n = 9). The TBI-30 min group data is the TBI-0.25 from Experiment 1 and is included in the data analysis as a basis for comparing the effectiveness of the selected dose of lithium when the initiation of treatment is delayed. The swim speed is calculated by dividing the distance traveled by the latency to reach the goal platform (cm/s). A significant difference in swim speed was found for the TBI-30 min and TBI-8 hr groups as compared to all other treatment groups. Initial comparisons were made to the Sham-Vehicle group (*). Additional significant between-group comparisons are indicated by braces. *p < .05. **p < .01.
Figure 16. Comparison of 24 hr treatment delay on proximity to the goal platform during MWM testing. Data represent the proximity score obtained during hidden platform testing in the MWM for the TBI-Vehicle ($n = 10$), TBI-24 hr ($n = 9$), Sham-Vehicle ($n = 10$), and Sham-24 hr ($n = 9$) groups, ± SEM. Lower scores indicate the animal took a more direct path the hidden platform (i.e., improved performance). Both sham groups had significantly lower proximity scores relative to the two injured groups. No statistically significant difference was observed between the proximity scores of the two sham groups or the two injured groups. (*) indicate comparisons made to the Sham-Vehicle group. (†) indicate significant differences as compared to Sham-1.00. ††$p < .01$. †††,$**p < .001$. 
Figure 17. Comparison of 8 hr treatment delay on proximity to the goal platform during MWM testing. Data represent the mean proximity score for each treatment group (n = 10/group), ± SEM. The TBI-30 min group data is the TBI-0.25 from Experiment 1 and is included in the data analysis as a basis for comparing the effectiveness of the selected dose of lithium when the initiation of treatment is delayed. An ANOVA, followed by a Fisher LSD post hoc analysis found that the Sham-Vehicle group performed significantly better (i.e., lower scores) as compared to the selected injured groups. (*) indicate comparisons made to the Sham-Vehicle group. (#) indicate significant differences as compared to TBI-Vehicle. *: #p < .05. †: †p < .01. ††: ††p < .001.
Figure 18. Comparison of 24 hr treatment delay on the proximity score from the probe trial on PID 15. The bars represent the mean proximity score for each treatment group, TBI-Vehicle ($n = 10$), TBI-24 hr ($n = 9$), Sham-Vehicle ($n = 10$), Sham-24 hr ($n = 9$), ± SEM. Proximity score is based on a 60 s probe trial (no platform in MWM) on postinjury day (PID) 15. A Fisher LSD post hoc analysis revealed that the Sham-Vehicle ($p = .039$) and Sham-24 hr ($p = .009$) groups had significantly lower scores (i.e., took the most direct route and remained closest to the location of the hidden goal platform) as compared to the TBI-Vehicle group. (*) indicate direct comparisons made to the Sham-Vehicle group. Other statistically significant group differences are indicated by braces. **$p < .01$. 

![Graph showing comparison of proximity scores](image_url)
Figure 19. Comparison of 8 hr treatment delay on proximity score from the probe trial on PID 15. The TBI-30 min group data is from the TBI-0.25 group in Experiment 1. The mean proximity score for each treatment group, obtained from the 60 s probe trial (no platform in MWM) on postinjury day (PID) 15. The bars represent the mean proximity score for each treatment group \((n = 10/\text{group})\), ± SEM. The TBI-30 min group data is the TBI-0.25 from Experiment 1 and is included in the data analysis as a basis for comparing the effectiveness of the selected dose of lithium when the initiation of treatment is delayed. An ANOVA followed by a Fisher LSD post hoc analysis revealed that the Sham-Vehicle and TBI-8 hr groups had significantly lower proximity scores (i.e., took the most direct route and remained closest to the location of the hidden goal platform) as compared to the TBI-Vehicle group. The proximity score for the TBI-30 min group was significantly higher as compared to Sham-Vehicle and did not statistically differ from TBI-Vehicle. (*) indicate direct comparisons made to the Sham-Vehicle group. Other significant group differences are indicated with braces. *p < .05. **p < .01.
Discussion

In the present study we examined the therapeutic time window for administering the selected dose of lithium from Experiment 1. Lithium treatment was delayed for either 8 or 24 hours postinjury. We selected the 8 and 24 hr time delays for initiating treatment because they are clinically relevant, i.e., they would allow sufficient time for the treatment to be administered, taking into account time for transportation and obtaining informed consent. The current investigations into the therapeutic window for the optimal dose of lithium, selected from Experiment 1, revealed that lithium treatment can be delayed up to 8 hours postinjury and still provide significant neuroprotection as evidenced by improved motor and cognitive performance postinjury.

**Beam walk.** The TBI-8 hr group showed a significant reduction in the latency to traverse the beam in the days following the TBI and performed similarly to the TBI-30 min group from Experiment 1. The beneficial effects observed following the 8 hr delay did not carry over for the TBI-24 hr group. In fact, the latter group performed comparably to the TBI-Vehicle treated group. These data indicate that lithium may mediate posttraumatic vestibulomotor impairment.

**Morris Water Maze.**

**Hidden platform.** Analysis of the hidden platform data revealed similar findings to those observed in the BW. The TBI-8 hr group exhibited significantly shorter latencies to reach the goal platform as compared to the TBI-Vehicle group. Delaying treatment with lithium for up to 8 hr postinjury appears to maintain the cognitive protective effects observed after the 30 min treatment delay in Experiment 1. All benefit associated with this dose of lithium (0.25 mmol/kg, ip) was lost when treatment was delayed for 24 hr. In fact, a comparison of latency between TBI-Vehicle and TBI-24 hr groups was statistically indistinguishable. Although the 24 hr
treatment delay, using the selected dose of lithium, was not protective, the findings from the 8 hr
treatment delay are very encouraging.

Similar to the findings from the previous study, there was no difference in swim speed
between the Sham-Lithium, Sham-Vehicle and TBI-Vehicle groups. However, the hypothesized
interaction between TBI and lithium on swimming speed did not carry over into this experiment.
There was no appreciable difference in the swim speed of the TBI-24 hr relative to any treatment
group. The TBI-8 hr group swam significantly faster than the Sham-Vehicle group but did not
statistically differ from the swim speed of the TBI-Vehicle group. At this time, it is unclear what
is producing these group differences in swim speed.

Dissimilarities in group swim speeds can represent a confounding variable, leading
Gallagher et al. (1993) to develop the proximity score, which is a measure of spatial memory that
is not affected by swim speed or entry position in the maze. This score provided additional
information about the spatial distribution of the rat’s search, and was designed to supplement
existing measures frequently used in assessing MWM performance (e.g., latency, path length,
swim speed) (Gallagher et al., 1993). The results from our analysis of the daily proximity score
data from the hidden platform assessment are congruent with the corresponding latency data.
The proximity score data indicates that both the TBI-30 min and TBI-8 hr groups had learned,
more efficiently, the location of the hidden platform as compared to the TBI-Vehicle group.
Although the TBI-30 min and TBI-8 hr groups did not perform as well as Sham-Vehicle, there
was no difference in performance as compared to the Sham-24 hr group.

**Probe trial.** The single probe trial was performed on PID 15, after the last day of hidden
platform testing. This assessment shows the spatial bias of the animals search pattern (Gallagher
et al., 1993) and was included to assess the cumulative learning of each rat for the location of the
hidden platform. The only difference that we observed was between the Sham-Vehicle group and the other treatment groups. The homogeneity of the scores from the other treatment groups suggests that this measure is not sensitive enough to detect small between-group differences.

**Visible platform.** The visible platform was used to assess the visual acuity of the animals and ensured that any differences detected in the latency to reach the goal platform were not due to the inability of the rat to see the external maze cues. There were no differences observed in the latency to locate the visual platform across treatment groups.

**Experiment 3**

**Study Rationale**

The hippocampus, a brain structure critical for learning and memory, is known to be vulnerable to TBI (Dixon et al., 1987; Hicks et al., 1996; Lowenstein, Thomas, Smith, & McIntosh, 1992). Lateral fluid percussion injury has been shown to produce cell loss within the CA3, hilus, and DG of the hippocampus (Dietrich, Alonso, & Halley, 1994; Hicks et al., 1996; McIntosh et al., 1989). The time course and regional variability for neuronal loss in the hippocampus was evaluated following lateral FPI in rats (Sato, Chang, Igarashi, & Noble, 2001). Injured animals were evaluated at the following time points postinjury: 3 hr, 1, 3, 7 and 28 days. Affected cells were identified using Fluoro-Jade, a marker of irreversible cell damage that stains the axon, cell body, and processes of apoptotic and necrotic cells (Hornfelt, Edstrom, & Ekstrom, 1999; Schmued, Albertson, & Slikker, Jr., 1997); silver staining, used to detect degenerating neurons; and terminal deoxynucleotidyl transferase nick-end labeling (TUNEL), used to identify irreversibly damaged neurons by labeling fragmented DNA. Quantification of cell loss was evaluated from three adjacent slices directly beneath the site of injury. Pyramidal neurons in the hippocampus, CA1, CA2, and CA3, exhibited Fluoro-Jade staining at 3 hours, and on PID 1
through 7, with the most intense staining observed on PID 1 in the CA1 region. The relative number of Fluoro-Jade positive structures on PID 7 was significantly reduced as compared to PID 1 and was nonexistent on PID 14. Brain slices adjacent to those used for Fluoro-Jade were processed using silver stain and TUNEL in the brains of animals from the 3 hour and 1 day time points. These adjacent slices exhibited silver staining and TUNEL-positive cells that corresponded to the same regions that were marked by the Fluoro-Jade (Sato et al., 2001). The study by Sato did not perform histological examination of the dentate hilar region of the hippocampus; however, in a different study that also used the lateral FPI model, a marked increase in Fluoro-Jade B positive cells was detected in the ipsilateral hilus at 6 and 12 hours postinjury. Analysis of this region at 24 hours postinjury found a significant decline in Fluoro-Jade B positive cells, suggesting that by 24 hours significant cell death has occurred (Lee & Agoston, 2009).

The Giemsa stain is a hematoxylin and eosin stain that is useful for histology because of the high-quality staining of chromatin and the nuclear membrane, the metachromasia of some cellular components, and the different qualities of cytoplasmic staining depending on the cell type (Barcia, 2007). This stain is composed of methylene blue, azure B, and eosin. Methylene blue and azure B, basic dyes, bind to acidic cellular components (e.g., DNA, RNA, neutrophil-specific granule matrices, platelets, and ribosome-rich cytoplasm). Eosin, the acidic part of the stain, binds to basic cellular components (e.g., hemoglobin, eosinophilic granular contents, and basic cellular proteins) (Woronzoff-Dashkoff, 2002). Differentiation of neuronal types within the hippocampus is determined by color. Neurons within the CA3 and hilus stain violet in color and neurons within the DG stain a blue or dark blue color.
Previous studies that have examined post-TBI cell survival in the hippocampus found that 7 days postinjury was sufficient for significant cell loss to have occurred. Following this time point there is little evidence for further significant cell death (Sato et al., 2001). The analysis of neuronal survival within the CA3 and hilus may correlate with alterations in MWM performance following lateral FPI. To evaluate this potential interaction, animals were treated daily for 7 days postinjury. To address this question, hippocampal neurons within the CA3 and hilar regions were counted using unbiased stereological methods, optical dissector and fractionators sampling (West, Slomianka, & Gundersen, 1991).

**Hypotheses**

Injured vehicle-treated rats will have significantly fewer healthy neurons in both the CA3 and hilus, as compared to sham animals. It is also hypothesized that injured lithium-treated rats will have more surviving neurons in these subregions of the hippocampus as compared to injured vehicle-treated rats.

**Specific Aims**

The aim of the present study is to evaluate the effect of lithium treatment on cell survival in the ipsilateral CA3 and hilar regions of the hippocampus.

**Methods**

Animals underwent surgical preparation identical to that described in Chapter 2, General Methods. Twenty-four hours after surgical preparation, on the day of injury, animals were randomly assigned to one of three treatment groups: TBI-Vehicle \( (n = 4) \), TBI-Lithium \( (n = 4) \), and Sham-Vehicle \( (n = 4) \). Postinjury neurological assessment and preparation/administration of the 0.25 mmol/kg dose of lithium was identical to that described in Experiment 1. Treatment with either lithium chloride (0.25 mmol/kg) or 0.9% saline was initiated 30 min post- TBI or
sham injury. Both the lithium and saline were administered via intraperitoneal injection, in a volume of 1 ml/kg. Injections with lithium or saline were continued daily for 7 days. The same animals were used to obtain the cell counts from the CA3 and hilus.

**Tissue preparation.** On PID 7, animals were sacrificed by an overdose of sodium pentobarbital (200 mg/kg, ip) to effect and subsequently underwent transcardial perfusion with 500 ml of 0.9% saline, followed by 500 ml of 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS). After completing the perfusion process the brain was excised and placed in 4% paraformaldehyde for 24 – 48 hr and then stored in PBS solution. Coronal slices of brain tissue, 60 μm thick, were cut using a vibratome (Leica VT1000s). Sections of cortex were collected from bregma -2.30 mm through -4.16 mm, based on stereotaxic coordinates obtained from a rat atlas (Paxinos & Watson, 1982), including the area beneath the injury site. The tissue was mounted on microscope slides (Superfrost/Plus) and allowed to air-dry overnight prior to Giemsa staining.

**Giemsa cell staining.** 10% Giemsa solution was prepared fresh and heated in an oven to 60 °C. Slides were placed in a staining dish placed indirectly under tepid running tap water and allowed to soak for 3 min. The slides were then placed into the pre-heated Giemsa staining solution, in a 60 °C oven, for 30 min. The slides were then removed from the oven and soaked in tepid indirect running tap water for 2 min. The slides were then differentiated in 0.5% Acetic acid for 1 - 2 min, at which point the intensity of the stain was assessed by visual inspection under a microscope. Once the desired stain intensity was reached, the slides were quickly rinsed in dH₂O and placed in 95% alcohol for 1 min. This was followed by two, 1 min immersions in 100% alcohol and then two 5 min immersions in Xylene. Separate glass staining dishes were used for each solution. Slides were then coverslipped using permount and allowed to dry.
Hippocampal cell counts. The number of neurons in the hippocampal subregions, CA3 and hilus, was determined by using unbiased stereological methods, optical dissector and fractionators sampling, with the Olympus image system CAST program. Every third coronal hippocampal section for a total of 10 sections per brain was examined. This ensured that 120 μm would separate the slices used for counting and thereby prevented counting the same cell twice. The dissector was set to count cells within the range of -3 μm to -15 μm and the step size across each section was fixed at 175 μm. The sampling areas were outlined under a 10X objective while cell counting was done under a 64X oil immersion lens. Giemsa stained neurons (dark blue/violet) in the CA3 and hilus region were counted. Neurons were identified by their distinct morphological features; only those cells with a clearly defined nucleolus within a defined nuclear membrane were included in the counts. Additionally, a cell was counted only when the nucleolus of that cell first came into focus within the specified depth (12 μm) of the dissector frame, and part or all of the soma was within the frame of the counting box but not touching the bottom or left sides of the frame and/or the extensions of these lines. Due to the scarcity of neurons within the hilar region, all cells with a neuronal phenotype were counted to ensure accurate cell counts. Alternately, within the CA3 region, due to the high density of cells, counting frames, randomly selected by the CAST software, representing 12% of the sampling area samples was used in establishing the cell counts for this region. The 12% parameter used for the CA3 region yielded cell counts above 150 cells per brain in injured vehicle-treated rats, the group that is expected to have the greatest hippocampal cell loss. Based on the article by West and colleagues, counting 100 – 200 cells per brain ensures the accuracy of the results (West et al., 1991).
**CA3.** The CA3 region of the hippocampus, or regio inferior, consists of roughly half of the pyramidal cell layer, the other half is the CA1, or regio superior. The CA3 region contains pyramidal cells that are larger in size as compared to those found in the CA1. The border between CA2 and CA3 served as one of the anatomical markers that were used when delineating the CA3 region of interest. CA2 is the narrow zone of pyramidal neurons that forms the transition between CA3 and CA1, distinguishable by the mixing of neurons with large and small cell bodies. The other boundary was represented by an imaginary plane, perpendicular to the suprapyramidal and infrapyramidal blades of the DG. This imaginary plane marks the transition of CA3 neurons from a tightly packed grouping of cell bodies, roughly 4 – 5 cells deep, to a distinctly less organized, loosely packed formation (West et al., 1991).

**Hilus.** The hilus was defined as the crescent-shaped area between the C-shaped blades of the DG, excluding the loosely packed cells of the CA3 that extend into this area. This region contains large, lightly packed polymorphic cells with a uniform distribution (West et al., 1991). There are several neuronal types found within the hilus, including mossy cells, granule cells, basket cells, multipolar cells and fusiform cells. Only those cells with a pyramidal shape were included in the cell counts in this study (i.e., no granular cells).

**Statistical analysis.** A one-way ANOVA was performed to determine the relationship between treatment groups and neuronal survival within the CA3 and hilus. Where appropriate, a Fisher LSD test was performed to compare specific groups of interest.

**Results**

**CA3 cell counts.** The number of healthy cells in the CA3 was calculated using unbiased stereological methods. These data are presented in Figure 20. A one-way ANOVA revealed a significant difference between treatment groups $F(2,9) = 11.727, p .003$. A Fisher LSD post hoc
analysis revealed a significant effect of injury. Animals in the TBI-Vehicle group had significantly greater ($p = .001$) cell loss in the CA3 region as compared to animals in the Sham-Vehicle group. Daily treatment with lithium following TBI significantly ameliorated injury-induced cell loss in the CA3 region of the hippocampus ($p = .022$). Additionally, there was no significant difference detected between the Sham-Vehicle and TBI-Lithium groups ($p = .07$).

**Hilus cell counts.** The graph in Figure 21 shows the group differences in cell survival in the ipsilateral hilus. The comparison of the mean cell counts ($\pm SEM$) for each group revealed a large difference between the Sham-Vehicle, $M = 5782 (\pm 917)$ and injury groups, TBI-Vehicle, $M = 2275 (\pm 191)$ and TBI-Lithium, $M = 3462 (\pm 386)$. A one-way ANOVA revealed a significant difference in the hilar cell counts of the treatment groups, $F(2, 9) = 9.302, p = .006$. A Fisher LSD post hoc analysis showed that the Sham-Vehicle group had significantly more cells in the ipsilateral hilus as compared to both the TBI-Vehicle ($p = .002$) and TBI-Lithium ($p = .021$) groups. No significant difference in hilar cell counts was detected between the TBI-Vehicle and TBI-Lithium groups ($p = .185$).
Figure 20. Effect of post-TBI daily lithium treatment on cell survival in the ipsilateral CA3 subregion of the hippocampus. The number of surviving cells was quantified using stereological technique 7 days after injury or sham in the ipsilateral hippocampus. Groups were treated with either vehicle (0.9% sterile saline) or lithium (0.25 mmol·kg·day), initiated 30 min postinjury (n = 4/group). Data are expressed as mean, ± SEM. A one-way ANOVA indicated a significant group difference (p < .05) when comparing the average cell counts for each group. Further comparisons using a Fisher LSD post hoc analysis revealed significantly more healthy neurons in the CA3 region of TBI-Lithium and Sham-Lithium groups as compared to the number of healthy CA3 neurons in the TBI-Vehicle group. No significant difference in cell survival was observed between the TBI-Lithium and Sham-Vehicle groups. (*) indicate significant group differences relative to Sham-Vehicle, additional between group comparisons are indicated by braces. *p < .05. **p < .01.
Figure 21. Effect of post-TBI daily lithium treatment on cell survival in the ipsilateral hilar subregion of the hippocampus. The number of surviving cells in the hilus was assessed 7 days after injury or sham, using stereological technique. Treatment groups (n = 4/group) received daily injections with either vehicle (0.9% sterile saline, ip) or lithium (0.25 mmol·kg·day, ip), initiated 30 min postinjury. Data are expressed as mean, ± SEM, of the total cell counts (x1000) for the ipsilateral hilar region. A one-way ANOVA showed an effect of group. A Fisher LSD post hoc analysis determined that there was a significant effect of injury on neuronal survival in the ipsilateral hilus. The Sham-Vehicle group had a significantly larger number of neurons within the hilus as compared to the TBI-Vehicle and TBI-Lithium groups. There was no appreciable difference in number of cells within the ipsilateral hilar regions of the two injury groups. (*) indicate comparisons made to the Sham-Vehicle group. *p < .05. **p < .01.
Discussion

The CA3 and hilus are known to be selectively vulnerable to lateral FPI and are also brain regions that are commonly affected following TBI in humans. Neuroprotective therapies that can prevent secondary injury-induced cell loss or facilitate the repair of injured neurons are hypothesized to mitigate vestibulomotor and cognitive sequelae associated with TBI. The reported protective effect of lithium treatment against the induction of apoptosis \textit{in vitro} as well as in models of acute brain injury (e.g., MCAO, transient global ischemia) (Nonaka et al., 1998; Ren et al., 2003; Rowe & Chuang, 2004) was the basis for our interest in the potential benefits to cell survival following lithium administration in our injury model.

Cell loss in the CA3 and hilus regions of the hippocampus following lateral FPI has been correlated with poorer performance in cognitive tasks such as the MWM. In accordance with the findings of previous studies, the CA3 and hilus of animals in the TBI-Vehicle group showed significant cell loss as compared to animals in the Sham-Vehicle group (Cortez et al., 1989; Hicks et al., 1993; Sato et al., 2001; Smith et al., 1991). It was hypothesized that lithium treatment would enhance cell survival in the CA3 and hilus regions of the hippocampus. The data obtained from the ipsilateral CA3 region cell counts support the working hypothesis. Not only did lithium treatment significantly increase cell survival as compared to the TBI-Vehicle group, there was no significant difference found between the Sham-Vehicle and TBI-Lithium groups. Based on these findings, we expected the same neuroprotective effects observed in the CA3 would also be seen in the hilus. We were surprised to find that lithium treatment did not show any benefit to cell survival in the hilus. Both the TBI-Vehicle and, to a lesser extent, TBI-Lithium groups had significant cell loss as compared to the Sham-Vehicle group. Taken
together, these data indicate that 7-day lithium treatment, initiated 30 min post-TBI, offers significant protection to cells in the CA3 region but not the hilus.

A possible explanation for this observation is that the cells within the hilus are more vulnerable to TBI-induced pathology as compared to those in the CA3 region, thus requiring greater neuroprotection than that provided by the selected dose of lithium. This assumption is supported by data from Lowenstein and colleagues, who found that, following lateral FPI, a significant reduction in the number of healthy cells occurs bilaterally within the hilus, while no significant changes are detectable in the number of surviving neurons within the CA3 region of the hippocampus (Lowenstein et al., 1992), although this region has been shown in previous research (Cortez et al., 1989; Hicks et al., 1993; Sato et al., 2001; Smith et al., 1991) as well as in the present study, to be also selectively vulnerable to FPI. Additional insight into the regional pattern of neuroprotection that we have observed is discussed in the article by Toth and colleagues (1997), which identifies the existence of a temporal pattern of cell death in the hilus following lateral FPI. In particular, these authors have reported that the primary damage to neurons within the hilus occur at the moment of the fluid pulse and not as a result of delayed, secondary injury processes. The authors showed this by means of the Gallyas silver stain, used to detect injury to neuronal processes in a rapid fashion after an insult (Toth, Hollrigel, Gorcs, & Soltesz, 1997). This silver stain accumulates in injured neurons presumably as a result of the interaction of silver with charged monomers derived from the breakdown of polymeric cytoskeletal elements, in a fashion similar to the intensification process occurring during photography (Gallyas, Zoltay, & Dames, 1992; Toth et al., 1997). Thus, it is possible that the damage to cells within the hilus occurs prior to the initiation of treatment with lithium.
In a recent clinical study, brain tissue samples from 21 individuals, obtained surgically during temporal lobectomies to alleviate temporal lobe epilepsy that developed after these individuals sustained head trauma, were used for histopathological analysis of neuronal organization and number in addition to other neurological inflammatory changes, and chronic gliosis (Swartz et al., 2006). The individuals included in this study had no other risk factors for epilepsy, defined by the authors as “family history, perinatal distress, prolonged febrile convulsion, history of significant substance abuse, history of CNS infection, and history of other CNS pathology”. Of the 21 neocortical specimens, 15 included hippocampal tissue and, of these, 11 were processed with cresyl violet (a stain used to identify nissl substance) and NeuN (used to identify neurons). All 11 brains showed some degree of cell loss in the hilus, however, only those samples with moderate to severe cell loss in the hilus, based on visual pathologic examination, also had damage to other hippocampal regions (Swartz et al., 2006). Although the samples from this clinical study were obtained from individuals with epilepsy, a neurological disease that is associated with cell loss in the hippocampus even when it is not preceded by brain injury, it does demonstrate an ordered progression of cell death in the hippocampus that starts with the hilus.

**Experiment 4**

**Study Rationale**

At this time, the exact mechanisms of action through which lithium treatment protects the brain are not clear. One reason for this is the widespread effect of lithium on multiple neuroprotective targets and pathways, sometimes acting at several points within the same pathway (Rowe & Chuang, 2004). Based on the known actions of lithium in the brain, it is possible that lithium may protect against both the acute and chronic phases of secondary brain
injury. The neuroprotective effects of this drug against excitotoxic insults and cell death, detailed in the previous sections of this dissertation, suggest to us that lithium treatment may reduce neuronal damage by acting on targets associated with the acute (excitotoxic) phase of secondary injury. Additionally, lithium treatment is reported to promote neuronal reorganization and plasticity, suggesting that this drug could facilitate functional recovery during the chronic (hypofunctional) postinjury phase.

Long-term treatment with lithium has been shown to protect neurons against apoptosis, prolong cell survival, and promote regeneration of axons in the mammalian brain (Chen et al., 2000; Shaldubina et al., 2001). To elucidate further the potential mechanisms involved in lithium-induced neuroprotection, we selected three markers: GAP-43, a measure of postinjury structural reorganization; BDNF, an indicator of neuronal plasticity and neuroprotection; and caspase-3, to evaluate apoptotic cell death. These markers are described in detail in the following sections.

**GAP-43.** GAP-43 levels are widely accepted as a good marker for synaptic plasticity in the adult CNS (Benowitz & Routtenberg, 1997; Christman, Salvant, Jr., Walker, & Povlishock, 1997; Emery et al., 2000; Hulsebosch, DeWitt, Jenkins, & Prough, 1998). This phosphoprotein, specific to the central nervous system, is a primary constituent of growth cones that head the extension of growing axons. GAP-43 is also known as protein F1, neuromodulin, neural phosphoprotein B-50, axonal membrane protein GAP-43, calmodulin-binding protein P-57, nerve growth-related peptide GAP43, neuron growth-associated protein 43, and PP46 (Kosik et al., 1988). For the purposes of this thesis, and from this point onward, it will be referred to as GAP-43. GAP-43 expression and axonal transport are only found in young animals and in neurons undergoing reorganization (e.g., following brain injury) (Karns, Ng, Freeman, &
Fishman, 1987; Skene, 1989). The function of GAP-43 is to regulate the interactions between the membrane skeleton/cytoskeleton components and plasma membrane (Meiri & Gordon-Weeks, 1990; Meiri, Pfenninger, & Willard, 1986). The activity of this protein is shaped by extracellular signals such as phosphorylation (e.g., PKC), calmodulin (CaM) binding, and calcium-dependent proteolysis (Apel, Byford, Au, Walsh, & Storm, 1990; Apel & Storm, 1992). When GAP-43 is phosphorylated it is associated with growing axons whereas unphosphorylated GAP-43 is associated with retracting axons. Previous literature showed increases in GAP-43 levels as early as 48 hours following TBI (Emery et al., 2000; Hulsebosch et al., 1998) and continuing for up to 28 days postinjury (Christman et al., 1997; Hulsebosch et al., 1998).

Several studies, using a variety of injury models, have reported increased GAP-43 messenger and protein expression. The involvement of GAP-43 in structural remodeling, neurogenesis, and induction of LTP have lead to a general consensus that increased expression postinjury is indicative of a period of enhanced plasticity (Christman et al., 1997; Emery, Royo, Fischer, Saatman, & McIntosh, 2003; Hulsebosch et al., 1998). Following axotomy in the CNS, GAP-43 is upregulated and rapidly transports to distal parts of the axon (Tetzlaff, Alexander, Miller, & Bisby, 1991). In a previous study, using the central FPI model, GAP-43 protein expression was significantly increased in the hippocampus and cortex of injured animals (Hulsebosch et al., 1998). Another study, using the same injury model in cats, found that injured axons expressed increased levels of GAP-43 protein for up to 28 days postinjury (Christman et al., 1997). Following lateral FPI, in a rat model, GAP-43 levels were increased in the hippocampus of injured animals as compared to sham animals. This increased expression was observed at 48 and 72 hr postinjury in several hippocampal subregions, but decreased over time (Emery et al., 2000). These aforementioned effects of FPI-induced injury on GAP-43 expression
were observed following moderate-severity brain injuries. However, following severe brain injury, produced by CCI, there was no change in GAP-43 protein expression in either the hippocampus or cortex (Thompson, Gibson, Thompson, Deng, & Hall, 2006). These data taken together suggest that there is a negative correlation between injury severity and postinjury GAP-43 expression. This indicates that the degree to which the injured brain is able to modulate mechanisms involved in neuroplasticity is linked to the severity of the injury.

**BDNF.** Brain derived neurotrophic factor (BDNF) is a neurotrophic factor that is important in the survival, growth, and plasticity of several neuronal types, including dorsal root ganglion, hippocampal, and cortical neurons (Binder & Scharfman, 2004; Fukumoto et al., 2001; Huang & Reichardt, 2001; Mai, Jope, & Li, 2002). Strong support exists for the role of BDNF in LTP in multiple brain regions, including the hippocampus (Binder & Scharfman, 2004; Mai et al., 2002). The effects of BDNF are mediated by the TrkB receptor, part of a family of tyrosine kinases that act as receptors for several other neurotrophins. Activation of the TrkB receptor is important for initiating multiple signaling cascades such as the PI3K/Akt and MEK/ERK pathways, regulating transcription factors, and controlling gene expression. This receptor is also necessary for the induction of LTP in the hippocampus. (Barnabe-Heider & Miller, 2003; Binder & Scharfman, 2004).

To determine a potential mechanism of action for lithium’s reported neuroprotective effects, Fukumoto et al. (2001) investigated the effect of lithium treatment on several neurotrophic factors, including BDNF. Male Wistar rats were fed a 2% lithium-carbonate diet for 1, 7, 14, and 28 days. BDNF levels in the hippocampus, temporal cortex, and frontal cortex were measured by ELISA. There were no differences in BDNF levels on treatment day 1 between the lithium-chow and regular-chow fed rats. However, 14 and 28 days of treatment...
significantly increased BDNF expression in both the hippocampus and temporal cortex. In the PFC of lithium-treated rats, expression was significantly increased at 14 days, with a trend towards significance on day 28 (Fukumoto et al., 2001). In contrast, a study by Angelucci and colleagues (2003) determined that chronic (6 week) lithium treatment did not significantly increase BDNF levels in the hippocampus as determined by ELISA (Angelucci, Aloe, Jiménez-Vasquez, & Mathé, 2003). However, there were several differences in the methodologies employed in the Angelucci study as compared to the Fukumoto study. These differences include the estimated serum concentration of lithium (0.5 mEq/L vs. 1.02 ± 0.04 and 0.80 ± 0.02 mEq/L), duration of treatment (6 weeks vs. 2 and 4 weeks), and strain of rat used (Flinders Sensitive Line and Flinders Resistant Line vs. Wistar) (Angelucci et al., 2003; Fukumoto et al., 2001).

Lithium alone has been shown to increase the expression of BDNF in the hippocampus and cortex of rats (Fukumoto et al., 2001) as well as the overall brain expression of BDNF mRNA (Nibuya, Morinobu, & Duman, 1995). Increases in BDNF expression have been observed in specific regions of the hippocampus as early as 30 min following mild- and moderate-severity FPI (Hicks et al., 1999; Hicks, Zhang, Dhillon, Prasad, & Seroogy, 1998; Truettner et al., 1999). A significant increase in BDNF mRNA expression in the DG of the hippocampus was reported at 1 hr and remained elevated for up to 72 hr following lateral FPI. Within the CA3 region, significant increases in BDNF mRNA expression was observed between 1 – 6 hours postinjury. However, at 24 hr postinjury, expression levels had returned to sham values. These alterations in BDNF expression indicate that these increases are related to neuroprotection and plasticity (Hicks, Numan, Dhillon, Prasad, & Seroogy, 1997; Hicks et al., 1998).
A recent clinical study using serum acquired by immunoaffinity capillary electrophoresis, reported a negative correlation between neurotrophin levels (BDNF, CNTF, NT-3, NT-4, beta-NGF) and severity of head injury. Specifically, the serum concentration of BDNF among patients with severe brain injuries was 93% lower as compared to the BDNF concentration found in mild brain-injured patients (Kalish & Phillips, 2009). BDNF expression has been also associated with improved outcome following spinal cord injury when infused continuously after treatment with the drug methylprednisolone (Kim & Jahng, 2004).

**Caspase-3.** Cysteinyl aspartate-specific proteinase-3 (caspase-3) was selected as a marker for apoptosis. Several studies have investigated the neuroprotective effects of lithium against a variety of insults and have correlated activated caspase-3 inhibition with improved outcome (Xu et al., 2003). Additionally, the inhibition of pro-apoptotic caspases following TBI is speculated to improve outcome by preventing secondary injury-induced cell loss.

Caspases are part of a family of cysteine proteases that are involved in the initiation of apoptosis. So far, there are 14 identified mammalian caspases (Nicholson & Thornberry, 1997). They are synthesized as pro-enzymes that subsequently undergo proteolysis and activation by other caspases in a sequential fashion. Based on function, caspases can be categorized into three classes. The first two are initiator and effector caspases and are involved in apoptosis. The third class is related to cytokine maturation. Only the initiator and effector caspases (i.e., those involved in apoptosis) will be discussed further.

Initiator caspases include caspase-2, -9, -8, -10, and CED-3. These caspases have a long prodomain (e.g., DED or caspase recruitment domain) that allows the interaction between the caspases and the complexes that activate them. This interaction results in the cleavage and thereby activation of the initiator caspase, allowing it to activate an effector caspase. Effector
caspases include caspase-3, -6 and -7. These caspases have a short prodomain that is removed by cleavage following activation, leaving a large and small subunit (Lawen, 2003). All cleavage occurs after aspartate (Asp) residues. Effector caspases are also known as executioner caspases because once they are activated, cell death is imminent.

The proform, or inactive form of caspase-3, is a 32-kDa protein synthesized in the cytoplasm until it is cleaved, thereby activating the 17-kDa active form of caspase-3. Based on previous findings from Tseng and Lin-Shiau (2002), 24 hr pre-incubation with lithium did not afford any neuroprotection to cultured neurons. Additionally, following a 3 day pre-incubation period, lithium was able to only partially protect neurons; significant neuroprotection was not observed until 6 or more days of pre-incubation (Tseng & Lin-Shiau, 2002). However, Ren et al. (2003), using an in vivo model of ischemia (MCAO), found that a single dose of lithium effectively reduced neurobehavioral deficits and infarct size 24 hr post-insult. Neurobehavioral deficits were assessed using ten different tests for motor, sensation, and reflex abnormalities. This study also reported that daily treatment with lithium for 7 days, initiated 3 hours post-insult, was able to minimize caspase-3 activation and DNA damage, as determined by a significant reduction in TUNEL staining (Ren et al., 2003).

**Hypotheses**

**GAP-43.** The TBI-Lithium group will have elevated GAP-43 levels as compared to TBI-Vehicle and Sham-Vehicle groups. Both injured groups will have increased GAP-43 protein as compared to the Sham-Vehicle group on PID 7. The relative abundance of GAP-43 protein in the TBI-Lithium group will be higher as compared to the TBI-Vehicle group on PID 1 and 7. By PID 21 we expect that all treatment groups will have similar levels of GAP-43 protein.
**BDNF.** It is hypothesized that relative abundance of BDNF protein within the ipsilateral hippocampus of animals in the TBI-Lithium group will be significantly increased as compared to the other treatment groups on PID 1. The TBI-Lithium group will have higher levels of BDNF protein as compared to both the TBI-Vehicle and Sham-Vehicle groups on PID 21.

**Caspase-3.** In Experiment 3, we found significant cell loss in the CA3 region of the hippocampus among animals in the TBI-Vehicle group as compared to both the TBI-Lithium and Sham-Vehicle groups. Based on these data, in addition to the reported antiapoptotic effects of pre-insult lithium treatment in other models of brain injury, we hypothesize that lithium treatment after lateral FPI will significantly ameliorate TBI-induced increases in apoptosis via the inhibition of activated caspase-3, on PID 1 and 7. We also theorize that animals in the TBI-Lithium group will have significantly higher levels of proform caspase-3 expression as compared to TBI-Vehicle. Additionally, animals in the TBI-Vehicle group, as compared to Sham-Vehicle, are expected to have significantly greater levels of activated caspase-3 protein with a concomitant reduction in the expression of proform caspase-3 at both postinjury time points. We do not expect to find significant differences between the TBI-Lithium and Sham-Vehicle groups.

**Specific Aims**

The aim of this experiment was to evaluate, in the ipsilateral hippocampus, potential mechanisms of action for the observed neuroprotective effects of lithium treatment. Expression levels of GAP-43 protein were used to assess structural remodeling on PID 1, 7, and 21. Neuronal plasticity was assessed by analysis of BDNF protein expression on PID 1, 7, and 21. Lastly, caspase-3 levels were evaluated to determine the impact of postinjury lithium treatment on apoptosis.
Methods

Four animals per treatment group (i.e., TBI-Vehicle, TBI-Lithium, Sham-Vehicle) per time point (i.e., PID 1, 7, 21) were used for the protein analysis using a western blot technique \((N = 36)\). Each rat was anesthetized using 4\% isoflurane gas, in a carrier gas of 70\% (nitrous oxide):30\% (oxygen), for 4 min. The anesthetized animal was immediately decapitated and the brain quickly removed from the skull. The hippocampus was bilaterally dissected on an ice-cold glass plate and was quickly transferred to a centrifuge tube containing 125\(\mu\)l RIPA lysis buffer (1x RIPA, Upstate; 1 tablet Complete Protease Inhibitor, Roche). Samples were homogenized and centrifuged at 14,000g for 20 min at 4 °C. Aliquots of the supernatant were transferred into tubes and stored at -80 °C.

**Western blot.** Total protein concentrations in each sample were assessed using a FLUOStar microplate reader and BCA Protein Assay Kit (Pierce, 23227). Samples were diluted 1:20 and the final amount of protein loaded into each lane was 20\(\mu\)g. Sample buffer (7.5 \(\mu\)l) and reducing agent (1.5 \(\mu\)l) were added to each tube prior to heating the samples for 5 min at 95 °C. After heating, the samples were loaded onto SDS-PAGE precast gels (12\% Bis-Tris Criterion XT, Bio-Rad), and were run using 1x MES Running Buffer (Bio-Rad) at 150 V for 1 hour. Once competed the separated gels were then transferred to PVDF membranes (Bio-Rad, 100V, 1 hour) using Transfer Buffer (1x Tris/Glycine Buffer [Bio-Rad] + methanol). After the transfer was complete, the gels were removed and stained with Coumassie Brilliant Blue to ensure complete transfer to the membranes. The PVDF membranes were washed two times in nanopure water and once in phosphate-buffered saline before blocking in 0.5\% non-fat dry milk + 0.05\% Tween 20 (milk TBS-T), for one hour on a shaker. After blocking, the PVDF membranes were incubated at 4 °C, overnight in milk TBS-T and the appropriate dilution of primary antibody.
The following antibodies and dilutions were used: mouse monoclonal anti-GAP-43 (1:1000) (Invitrogen, 7B10), rabbit polyclonal BDNF (1:300) (Santa Cruz Biotechnology, Inc., sc-546), and rabbit monoclonal caspase-3 (1:1000) (Cell Signaling, MA, 9665). Mouse monoclonal anti-β-actin (1:1000) (Sigma Aldrich, A1978) was used as a control to ensure equal loading of the protein. Membranes were washed six times in milk TBS-T, and incubated for 1 hour in milk TBS-T with the appropriate dilution of the secondary antibody. Goat anti-mouse secondary antibody conjugated to horseradish peroxidase (1:20,000, Rockland) was used for GAP-43 and β-actin; goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:20,000, Rockland) was used for BDNF and caspase-3. Each membrane was thoroughly washed in TBS-T and developed for 5 min with Dura SuperSignal (Pierce). The blots were then imaged using GeneSnap and GeneTools software (Syngene). Negative controls were used to verify the absence of nonspecific binding of the secondary antibody. These blots received identical treatment with the exception of omitting the primary antibody, see Figure 18.

**Western strip and re-probe.** The GAP-43 blots corresponding to PID 1 and 7 were stripped and re-probed using the caspase-3 antibody. These blots were selected for re-probing because the bands of interest for the two antibodies, GAP-43 (45-kDa) and caspase-3 (17-kDa & 32-kDa), do not overlap. Additionally, the specificity of the GAP-43 antibody (a solitary band at 45-kDa) removed any question regarding the authenticity of the bands observed after using the caspase-3 specific antibody. All of the blots were stripped and re-probed for β-actin, used as a load control. Each PVDF blot was stripped and re-probed no more than three times, including β-actin.

**Positive control.** A positive control for activated caspase-3 was used to confirm the ability of the selected antibody to detect both the 32-kDa, proform and 17-kDa, activated form of
the pro-apoptotic marker caspase-3. Samples containing staurosporine in cell lysate were obtained from Cell Signaling Technologies, MA. Three levels (low, medium, high) of staurosporine + cell lysate and cell lysate alone were loaded onto a SDS-PAGE precast gel (12 % Bis-Tris Criterion XT, Bio-Rad) along with 3 samples each from TBI-Vehicle, TBI-Lithium, and Sham-Vehicle groups from PID 1, selected for comparison. All samples were prepared using the same protocol detailed in the western blot section of the methods. The PID 1 time point was selected based on the findings from previous studies that showed the highest levels of activated caspase-3 expression in the hippocampus occurred between 24 – 48 hr post-TBI (Conti, Raghupathi, Trojanowski, & McIntosh, 1998; Knoblach et al., 2002; Newcomb, Zhao, Pike, & Hayes, 1999).

**Statistical analysis.** The relative abundance of the proteins of interest for TBI-Vehicle, TBI-Lithium, and Sham-Vehicle treatment groups were standardized to, and reported as, the percent of Sham-Vehicle. The raw value from the relative optical density from each subject was divided by the average optical density of the corresponding Sham-Vehicle group and converted into a percent. Each antibody, at each postinjury time point, was evaluated separately using a one-way ANOVA. Additional comparisons were made to assess the change in protein expression over time for each treatment group. A Fisher LSD post hoc analysis was performed where appropriate.

**Results**

The following sections detail the results obtained from the quantification and statistical analysis of the abundance of GAP-43, BDNF, caspase-3, and β-actin (used as a loading control) protein in the ipsilateral hippocampus at the specified postinjury time points. Figures 22 and 23 show images of the blots used for the negative control. Figure 24 shows representative samples
from each blot. The primary aim of this experiment is to evaluate if postinjury lithium-treatment alters the relative abundance of the specified proteins of interest as compared to the TBI-Vehicle and Sham-Vehicle groups. Although we did run and analyze samples taken from animals in the Sham-Lithium group, to evaluate the actions of lithium alone on these proteins, these data do not directly pertain to the specific aims of this experiment and as such the analysis of the Sham-Lithium group data will not be discussed in this chapter. A separate analysis of the relative abundance of GAP-43, BDNF, and caspase-3 between the two sham-injured groups are presented in Tables C1 – C3. The comparison of the two sham groups revealed that lithium-treatment alone may have an effect on β-actin, a commonly used loading control. These data are provided in Figure C1 in Appendix C. Additional analyses using an ANOVA and Fisher LSD post hoc test where appropriate were performed on all four treatment groups (TBI-Vehicle, TBI-Lithium, Sham-Vehicle, Sham-Lithium). These analyses compare the raw data (relative optical density) and the raw data normalized to the load control β-actin, see Tables C4 – C6 in Appendix C.
Figure 22. Negative control for secondary antibody used with GAP-43 and caspase-3. Protein from one animal per treatment group was run twice on a gel. The resulting PVDF membrane was cut into strips, lanes 2 – 5 were incubated with the primary antibody (1°) followed by the secondary antibody. This strip, labeled as +1°, shows that the primary antibody is able to detect the 45 kilodalton (45 kDa) band corresponding to the molecular weight of GAP-43 protein. The strip that contains lanes 7 – 9 was incubated with the secondary antibody only. No bands were detected in lanes 7 or 8, showing that there was no nonspecific binding associated with the secondary antibody. Lanes 5 and 9 contain the standard MagicMark (MM), the molecular weights corresponding to these bands are presented on the right of the minus primary (-1°) blot. These blots clearly show that there was no nonspecific binding associated with the secondary antibody.
Figure 23. Negative control for secondary antibody used with BDNF. Protein from one animal/treatment group was run twice on a gel. The resulting PVDF membrane was cut into strips, lanes 2 – 5 were incubated with the primary antibody (1°) followed by the secondary antibody. This strip, labeled as +1°, shows that the primary antibody is able to detect the 14 kDa band corresponding to the molecular weight of BDNF protein. The strip that contains lanes 7 – 10 was incubated with the secondary antibody only. No bands were detected in lanes 7 – 9, showing that there was no nonspecific binding associated with the secondary antibody. Lanes 5 and 10 contain the standard MagicMark (MM), the molecular weights corresponding to these bands are presented on the right of the minus primary (-1°) blot. These blots clearly show that there was no nonspecific binding associated with the secondary antibody.
Figure 24. Representative images from western blot analysis of GAP-43, BDNF, caspase-3. This figure illustrates the relative abundance of protein for GAP-43, BDNF, and caspase-3 for each treatment group, at each analyzed time point. The molecular weight of each protein is indicated to the right of the images. There was no significant difference in the relative abundance of β-Actin (43 kDa), used as a loading control, across any of the blots.

**GAP-43.** This section contains the results from the statistical analysis of the between-group differences in the relative abundance of GAP-43 protein in the ipsilateral hippocampus for each postinjury time point (PID 1, 7, 21). Additionally, the temporal changes in GAP-43 protein expression were assessed for each treatment group.

**PID 1 (GAP-43).** Figure 25 illustrates the relative abundance of GAP-43 protein levels in the ipsilateral hippocampus on PID 1 and analyzed as the percent of Sham-Vehicle. An one-way ANOVA revealed no significant difference in the relative abundance of GAP-43 protein on PID 1, \( F(2, 9) = 2.047, p = .185 \).
**PID 7 (GAP-43).** The relative abundance of GAP-43 protein on PID 7 among the three treatment groups of interest are presented in Figure 26. A one-way ANOVA showed a significant difference in GAP-43 protein levels on PID 7, $F(2, 8) = 6.440, p = .022$. A Fisher LSD post hoc analysis revealed significantly greater abundance of GAP-43 protein in the TBI-Lithium ($p = .019$) and Sham-Vehicle ($p = .013$) groups as compared to the TBI-Vehicle group. There was no statistically significant difference between the TBI-Lithium and Sham-Vehicle groups.

**PID 21 (GAP-43).** A one-way ANOVA was used to compare GAP-43 expression on PID 21 and revealed significant between-group differences, $F(2, 9) = 7.731, p = .011$. Further analysis using the Fisher LSD post hoc test showed that the TBI-Lithium group had appreciably lower GAP-43 protein levels as compared to the Sham-Vehicle ($p = .004$) and TBI-Vehicle ($p = .037$) groups. There was no significant difference between the TBI-Vehicle and Sham-Vehicle groups. These data are presented in Figure 27.

**Temporal changes in the relative expression of GAP-43.**

**TBI-Vehicle.** The change in GAP-43 expression for each treatment group over time revealed that there was no significant difference between groups in GAP-43 expression across the postinjury time points, $F(2, 9) = 0.743, p = .503$.

**TBI-Lithium.** The analysis of the relative abundance of GAP-43 protein across days, within the TBI-Lithium group, revealed that lithium treatment significantly altered the temporal expression of this protein, $F(2, 8) = 12.875, p = .003$. A Fisher LSD post hoc analysis found that GAP-43 protein expression on PID 1 was significantly elevated as compared to PID 7 ($p = .05$) and PID 21 ($p = .001$). Additionally, GAP-43 protein levels on PID 7 were significantly
increased as compared to PID 21 (p = .044). The temporal changes in the relative expression of GAP-43 across groups are illustrated in Figure 28.

![Western blot quantification of relative abundance of GAP-43 protein in ipsilateral hippocampus on PID 1](image)

**Figure 25. Western blot quantification of relative abundance of GAP-43 protein in ipsilateral hippocampus on PID 1.** The raw data are expressed as the mean percent of Sham-Vehicle, ± SEM, (n = 4/group). An one-way ANOVA failed to detect any significant between-group difference, $F(2, 9) = 2.047$, $p = .185$. We did notice a trend towards elevated GAP-43 levels in the TBI-Lithium group as compared to the TBI-Vehicle group.
Figure 26. Relative abundance of GAP-43 protein in the ipsilateral hippocampus on postinjury day (PID) 7. TBI-Vehicle (n = 4), TBI-Lithium (n = 3), Sham-Vehicle (n = 4). The raw data are expressed as the mean percent of Sham-Vehicle, ± SEM. An ANOVA was used to compare the raw data and revealed a significant difference between treatment groups. GAP-43 protein levels in the TBI-Vehicle group were significantly lower as compared to the TBI-Lithium (p = .019) and Sham-Lithium groups (p = .013). No difference was found between the Sham-Vehicle and TBI-Lithium groups.
Figure 27. Relative abundance of GAP-43 protein in the ipsilateral hippocampus on PID 21. The raw data are expressed as the mean percent of Sham-Vehicle, ± SEM, (n = 4/group). An ANOVA revealed a significant effect of group. A Fisher LSD post hoc analysis showed that the TBI-Lithium group had significantly lower GAP-43 expression as compared to both the Sham-Vehicle and TBI-Vehicle groups. No difference was detected between the TBI-Vehicle and Sham-Vehicle groups. Initial comparisons were made to Sham-Vehicle, indicated by an asterisk(s); subsequent between-group comparisons are indicated by a brace. *p < .05. **p < .01.
Figure 28. Changes in the relative abundance of GAP-43 protein across days. The graphs show the average optical density for each group compared relative to Sham-Vehicle, ± SEM. Separate ANOVAs were performed to compare changes in GAP-43 expression within the individual treatment groups across time, PID 1, 7, 21. There was no significant change in GAP-43 protein levels in the TBI-Vehicle animals across the three time points, \( p > .05 \). The TBI-Lithium group was the only group to show significant changes in GAP-43 expression over time, \( p = .003 \). A Fisher LSD post hoc showed that GAP-43 levels on PID 1 were significantly higher as compared to levels on PID 21 (\( p = .001 \)) and showed a trend suggesting higher levels of GAP-43 as compared to PID 21 (\( p = .068 \)). GAP-43 expression on PID 7 was significantly higher as compared to PID 21 (\( p = .033 \)). Initial comparisons were made to PID 1; subsequent between-group differences are indicated by a brace. Trends in the data are indicated by a brace with a dashed line. *\( p < .05 \). ***\( p < .001 \).
This section contains the results from the statistical analysis of the between-group differences in the relative abundance of BDNF protein in the ipsilateral hippocampus for each postinjury time point (PID 1, 7, 21). Additionally, the temporal changes in BDNF protein expression were assessed for each treatment group.

**PID 1 (BDNF).** Figure 29 shows the raw data from the analysis of BDNF protein levels on PID 1. The relative abundance of BDNF protein was assessed in the ipsilateral hippocampus on PID 1 and analyzed as the percent of Sham-Vehicle. A one-way ANOVA showed there was no significant difference in BDNF expression across groups on PID 1, $F(2, 9) = 3.012, p = .10$.

**PID 7 (BDNF).** The average percent change in BDNF protein levels on PID 7 was analyzed using a one-way ANOVA. There was no significant difference in the relative abundance of BDNF protein detected among the groups, $F(2, 9) = 1.766, p = .225$. However, there was a trend toward elevated BDNF levels in the TBI-Lithium group as compared to the TBI-Vehicle and Sham-Vehicle. These data are shown in Figure 30.

**PID 21 (BDNF).** The relative abundance of BDNF protein in the ipsilateral hippocampus on PID 21 was evaluated across treatment groups. These data are presented in Figure 31. A one-way ANOVA revealed a significant difference in BDNF levels between the treatment groups, $F(2, 9) = 17.579, p = .001$. A Fisher LSD post hoc analysis of the data revealed a significant decrease in the amount of BDNF protein detected in the TBI-Vehicle ($p < .001$) and the TBI-Lithium ($p = .004$) groups relative to Sham-Vehicle. Additionally, we observed a trend suggesting an increase in BDNF protein in the TBI-Lithium group as compared to the TBI-Vehicle group ($p = .081$).

**Temporal changes in the relative expression of BDNF.** The temporal changes in BDNF protein expression were evaluated for each of the treatment groups across the postinjury time
points (PID 1, 7, 21). Figure 32 illustrates the raw data for each treatment group at each time point, expressed as the percent change relative to the Sham-Vehicle. In the following paragraphs, only the data from the TBI-Vehicle and TBI-Lithium groups will be discussed. The reason for this is that the Sham-Vehicle group is the basis of comparison for all the treatment groups, including itself. The statistical value of the $F$ test is always equal to zero, and the $p$ value is always 1.00.

**TBI-Vehicle.** BDNF protein levels from each postinjury time point were analyzed using a one-way ANOVA. A significant effect of time was observed, $F(2, 9) = 24.402, p < .001$. A Fisher LSD post hoc analysis revealed that BDNF expression on PID 1 and PID 7 was significantly higher as compared to expression levels on PID 21, $p = .001$ and $p < .001$ respectively. There was no significant difference in the relative abundance of BDNF protein between PID 1 and PID 7, $p = .170$.

**TBI-Lithium.** The TBI-Lithium group showed significant changes in protein levels over the three postinjury time points, $F(2, 9) = 5.874, p = .023$. A Fisher LSD post hoc test revealed that BDNF expression was significantly higher on PID 1 ($p = .01$) and PID 7 ($p = .033$) as compared to PID 21. No statistically significant difference was found between the PID 1 and PID 7 time points ($p = .474$).
Figure 29. Comparison of the relative abundance of BDNF protein levels in the ipsilateral hippocampus on PID 1. These data are expressed as the percent of Sham-Vehicle ± SEM, (n = 4/group). No significant difference in BDNF protein amount was detected among the treatment groups. However, there appears to be an increase in BDNF protein levels in the TBI-Lithium group relative to the other groups.
Figure 30. Comparison of the relative abundance of BDNF protein on PID 7. These data are expressed as the percent of Sham-Vehicle ± SEM. No significant difference in BDNF protein amount was detected among the treatment groups.
Figure 31. Comparison of the relative abundance of BDNF protein on PID 21. Values are expressed as percent of Sham-Vehicle ± SEM, (n = 4/group). A significant difference was found between the Sham-Vehicle group and both injured groups. A trend was observed suggesting that the relative abundance of BDNF in the TBI-Lithium group was higher as compared to TBI-Vehicle. Initial comparisons were made to Sham-Vehicle. Trends in the data are indicated by a brace with a dashed line. **p < .01. ***p < .001.
Figure 32. Comparison of BDNF levels among individual treatment groups across time. Values are expressed as percent of Sham-Vehicle ± SEM. Individual ANOVAs were performed for each treatment group. A Fisher LSD post hoc analysis found significant decreases in BDNF protein levels on PID 21 in both the TBI-Vehicle and TBI-Lithium groups relative to the levels on PID 1 and 7. Significant between-group comparisons are indicated using braces. *p < .05. **p ≤ .01. ***p ≤ .001.
**Caspase-3.** Data from the statistical analysis of the relative abundance of the proform of caspase-3 protein levels, at the two specified postinjury time points (PID 1, 7), are presented in the following paragraphs. TBI-induced increases in activated caspase-3 protein levels have been reported during the early postinjury phase (within 1 week of injury); therefore, we did not examine the levels of this protein (activated or proform) on PID 21. The temporal changes in the detected levels of proform caspase-3 within the TBI-Vehicle and TBI-Lithium groups are also illustrated. We were not able to detect any activated caspase-3 in any of our samples.

**Caspase-3 (PID 1).** The proform of caspase-3 protein was evaluated across the treatment groups as the percent of Sham-Vehicle. A one-way ANOVA revealed that there was no significant difference in proform caspase-3 protein expression between the treatment groups, $F(2, 9) = 1.929, p = .201$. These data are presented in Figure 33.

**Caspase-3 (PID 7).** We further investigated the potential neuroprotective effects of post-TBI lithium treatment on apoptotic activity by analysis of the relative abundance of proform caspase-3 protein in the ipsilateral hippocampus on PID 7. There was no significant difference in proform caspase-3 expression across groups on PID 7, $F(2, 8) = 1.594, p = .261$. These data are illustrated in Figure 34.

**Temporal change in relative amount of proform caspase-3.** The raw data showing the relative change of proform caspase-3 levels for each treatment group are displayed in Figure 35. These data show an apparent injury-induced increase in the detected levels of proform caspase-3 protein within the ipsilateral hippocampus from PID 1 to 7. There was no evidence of activated caspase-3 in our samples. However, the trend towards elevated proform caspase-3 over time in the TBI-Vehicle group suggests that activated caspase-3 plays a role during early postinjury time points.
**TBI-Vehicle.** The difference in proform caspase-3 levels for the TBI-Vehicle groups on PID 1 and 7 were compared using an independent samples t-test, \( t(6) = -2.234, p = .067 \). Although there was no significant difference detected between the two time points, there appears to be a trend indicating increased proform caspase-3 levels on PID 7.

**TBI-Lithium.** The TBI-Lithium group did not exhibit significant alterations in the relative abundance of proform caspase-3 protein between PID 1 and PID 7, \( t(5) = -1.428, p = .213 \).

![Figure 33. Comparison of proform caspase-3 levels on PID 1 across treatment groups.](image)
Data from each treatment group (\( n = 4 \)/group) are expressed as the percent of Sham-Vehicle ± SEM. An ANOVA failed to detect any significant differences between the treatment groups. However, the TBI-Lithium group appears to have an increase in the detected levels of proform caspase-3 protein as compared to the TBI-Vehicle group.
Figure 34. Comparison of proform caspase-3 levels on PID 7 across treatment groups. Data are expressed as the percent of Sham-Vehicle ± SEM. TBI-Vehicle (n = 4), TBI-Lithium (n = 3), Sham-Vehicle (n = 4). No significant difference was detected in the levels of proform caspase-3 across all treatment groups.
Figure 35. Changes in proform caspase-3 protein levels over time. Data are presented as the mean, ± SEM. Analysis of the raw data showed a trend for increased proform caspase-3 on PID 7 as compared to PID 1 in the TBI-Vehicle group. No significant difference was found between PID 1 and 7 in the TBI-Lithium group. Trends in the data are indicated by a brace with a dashed line.
It was hypothesized that lithium was exerting neuroprotective effects by preventing the activation of caspase-3. However, on the blots from PID 1 and 7 there was an absence of the 17-kDa band corresponding to the activated caspase-3 protein. The positive control, using pre-prepared samples (Cell Signaling Technology, Inc., Boston, MA) clearly showed a band corresponding to the 17-kDa, activated caspase-3 protein, in the lanes containing the normal cell lysate (NCL) + staurosporine only, see Figure 36. These data confirm that the absence of a protein band for activated caspase-3 on the PID 1 and 7 blots is legitimately due to the absence of detectable levels of this protein in our experimental samples, thus confirming the accuracy of our findings from the both the PID 1 and 7 blots.

Figure 36. Image of positive control immunoblot used to detect activated caspase-3. Image taken of the western blot that was used to evaluate the specificity of the caspase-3 antibody for activated caspase-3 (17 kDa). The numbers on the right side correspond to the molecular weights of the MagicMark (MM) protein standard. Normal cell lysate (NCL) was used as a control (lane 7) and no activated caspase-3 was detected in this lane. Low (L), medium (M) and high (H) indicate the relative level of apoptosis-inducing staurosporine + NCL. Higher levels of staurosporine resulted in increased levels of activated caspase-3, as evidenced by the dark band corresponding to the 17 kDa activated caspase-3, in lanes 1, 3, and to a lesser extent lane 5. The analysis of the positive control confirms the lack of activated caspase-3 in our samples.
Discussion

We evaluated potential mechanisms of action for the observed lithium-induced neuroprotective effects found in Experiments 1 – 3. Previously, we showed that chronic, postinjury treatment with the selected dose of lithium chloride (0.25 mmol/kg, ip) was effective in reducing TBI-induced cognitive and motor deficits. In addition, this dose was found to significantly enhance neuronal survival in the ipsilateral CA3 region of the hippocampus following lateral FPI. In the present study, the western blot technique was used to measure the levels of GAP-43, indicative of axonal sprouting, to characterize the effect of lateral FPI and postinjury lithium-treatment on this marker of plasticity. Additionally, the abundance of BDNF protein, a neurotrophin that is reported to be involved in neuronal plasticity and neuroprotection, was evaluated. The pro-apoptotic marker, caspase-3 was used to evaluate the effect of our injury model and postinjury lithium treatment on TBI-induced apoptosis.

GAP-43. The relative optical density, used to quantify the abundance of GAP-43 protein in the ipsilateral hippocampus, showed that there was no significant difference in the GAP-43 protein levels across the treatment groups on PID 1. This finding is in accordance with the studies by Emery et al. (2000) and Hulsebosch et al. (1998), which reported that, 24 hr following FPI, GAP-43 levels in the hippocampus were not significantly different from sham controls. However, in another study, GAP-43 protein levels were significantly elevated in the ipsilateral hippocampus at both 24 and 48 hr post-TBI (Thompson et al., 2006). The apparent disparity between the findings from these studies are likely due to experimental differences; Hulsebosch (1998) and Emery (2000) used the central and lateral FPI model respectively, whereas Thompson (2006) used CCI. Additionally, there were differences in the postinjury time points selected for evaluating GAP-43. With these factors taken together, their data collectively show increased
levels of GAP-43 between 24 – 36 hr postinjury that decrease to baseline values or lower by 7 days postinjury (Emery et al., 2000; Hulsebosch et al., 1998; Thompson et al., 2006). This is similar to what we observed in our study.

The analysis of the relative optical density of GAP-43 protein on PID 7 indicate that the Sham-Vehicle and TBI-Lithium groups had statistically similar levels of GAP-43 protein and that both groups had significantly higher levels of GAP-43 as compared to the TBI-Vehicle group. Our finding that the TBI-Vehicle group had significantly lower levels of GAP-43 is contrary to Emory et al. (2000), who reported no significant difference in GAP-43 protein levels between injured- and sham-vehicle treated animals. Additionally, the in vitro study by Chen et al. (2003) investigated the effects of 1 mmol/L lithium treatment on that healthy (i.e., uninjured) hippocampal cells, treated for 7-days with lithium. They found that lithium-treatment resulted in a slight increase in GAP-43 expression; however, it did not reach the level of significance (Chen, Wang, Sun, & Young, 2003).

We hypothesized that on PID 7, GAP-43 protein levels would be increased in both TBI groups relative to Sham-Vehicle and that the TBI-Lithium group would have increased GAP-43 protein levels relative to the TBI-Vehicle group. Our data, however, support a very different outcome. Animals in the TBI-Vehicle group showed a significant decline in the relative abundance of GAP-43 relative to TBI-Lithium and Sham-Vehicle groups. Furthermore, post-TBI lithium treatment was able to completely reverse the injury-induced decrease in GAP-43 protein, returning it to sham levels. These data provide support for the role of GAP-43 as a potential mechanism of action for lithium-induced neuroprotection in our injury model. The significant increase in the relative abundance of GAP-43 protein levels on PID 7 in the TBI-Lithium group relative to TBI-Vehicle could suggest that postinjury lithium-treatment supports
neurite outgrowth, remodeling, and reorganization. Additional studies will be needed to evaluate this hypothesis.

The final postinjury time point (PID 21) that we assessed for evidence of lithium-induced axonal sprouting and remodeling by analysis of GAP-43 protein expression revealed a significant decrease in GAP-43 levels in the TBI-Lithium group relative to the other treatment groups. This finding was unexpected, but might indicate decline in structural remodeling several weeks after injury in animals treated with lithium.

No changes were detected in the total amount of GAP-43 protein in the TBI-Vehicle group across the three postinjury time points. However, the effect of postinjury lithium-treatment did reveal a significant change in protein expression across the three time points. The highest levels were observed on PID 1 and declined in a stepwise fashion over the remaining two time points. These findings are contrary to previous studies that indicated that lithium treatment had no significant effect on GAP-43 expression at 7-days in hippocampal neurons (Chen et al., 2003). Taken together, these data suggest that post-TBI treatment with lithium may facilitate structural remodeling in the ipsilateral hippocampus via modulation of growth cone activity.

**BDNF.** This neurotrophic factor was selected as a measure of neuroprotection and neuroplasticity. During development of the nervous system, BDNF plays an important role in neuronal survival and differentiation. In the adult brain, it is associated with neurogenesis, neuroprotection, and plasticity (Gustafsson, Lindvall, & Kokaia, 2003). Downstream effects of BDNF activation, such as those on synapsin 1 and CREB are linked to the regulation of synaptic vesicles and, in so doing, neurotransmitter release and the induction of LTP respectively are also linked to cognitive enhancing processes (Griesbach, Hovda, & Gomez-Pinilla, 2009; Silva et al., 1998). Several studies have reported that BDNF alone administered prior to brain injury or
insult is protective against injury-induced neuronal cell loss and cognitive deficits (Almli et al., 2000). Additionally, a recent study by Griesbach (2009) showed that following lateral FPI, exercise-induced increases in BDNF expression were correlated with markedly improved performance in the MWM over injured-vehicle treated animals. The improvement in MWM performance was blocked in animals treated with TrkB-IgG, an immunoadhesin chimera of the TrkB receptor that prevented activation of this receptor by BDNF (Griesbach et al., 2009). Our hypothesis that BDNF might underlie the protective effect of lithium was based on the observation chronic lithium administration is associated with significant increases in BDNF expression in the rat hippocampus (Fukumoto et al., 2001).

Both injury- and lithium-treatment have each been shown to increase levels of BDNF mRNA and protein in several brain regions, including the hippocampus (Hicks et al., 1996; Hicks et al., 1999). In a previous study, using the lateral FPI model, significant increases in BDNF mRNA levels were detected bilaterally in the DG (1 hr – 3 day) and CA3 (1 – 6 hr) regions of the hippocampus (Hicks et al., 1997). Injury-induced changes in BDNF mRNA occur within hours of the injury (Hicks et al., 1999) whereas it takes several days to detect significant changes in hippocampal BDNF protein levels (Griesbach, Hovda, Molteni, & Gomez-Pinilla, 2002). Our data show that BDNF protein levels on PID 1 were not significantly altered following lateral FPI. The delay between the expression of BDNF mRNA and the appearance of BDNF protein may account for the lack of a significant increase in the relative abundance of BDNF protein in the TBI-Vehicle and TBI-Lithium groups on PID 1.

On PID 7, both the TBI-groups showed a modest but non-significant increase in the relative abundance of BDNF protein compared with Sham-Vehicle. The delayed increase in BDNF protein, relative to the early increases in BDNF mRNA seen during the early postinjury
period (Hicks et al., 1997), in both TBI groups could be indicative of the innate plasticity response of the brain to injury. Following brain injury induced by intracerebroventricular administration of kainic acid, Shetty, Rao, Hattiangady, Zaman and Shetty (2004) reported an injury-induced increase in BDNF levels in the hippocampus 4-days postinjury.

In the present study, we found that the relative abundance of BDNF protein in the ipsilateral hippocampus was significantly reduced in both injured groups relative to sham. Our hypothesis that BDNF protein levels in the TBI-Vehicle group would be significantly decreased relative to Sham-Vehicle on PID 21 is supported by our findings. Further support for the observed TBI-induced reductions in BDNF protein on PID 21 comes from a previous study that showed BDNF mRNA levels were significantly reduced in the ipsilateral hippocampus on PID 21 following CCI (Griesbach, Sutton, Hovda, Ying, & Gomez-Pinilla, 2009). Fukumoto (2001), had reported that chronic lithium-treatment, for 14 and 28 days, resulted in a significant increase in hippocampal BDNF expression. We hypothesized that increased BDNF levels associated with chronic lithium treatment (Fukumoto et al., 2001) would outweigh the TBI-induced deficits in BDNF expression (Griesbach et al., 2009). Based on our data, chronic post-TBI lithium was associated with a trend suggesting increased BDNF protein levels over TBI-Vehicle. However, the amount of BDNF protein detected in the TBI-Lithium group was significantly less than Sham-Vehicle. It is interesting that on PID 7 and PID 21, both TBI groups showed similar trends in the relative abundance of BDNF protein that was detected. Specifically, in both injured groups the levels of this protein were elevated on PID 7 and significantly decreased on PID 21. The parallel effects on these days could reflect neurochemical alterations in this neurotrophic factor that are purely the result of injury-induced processes and not an effect of lithium treatment. However, due to the sample size in our treatment groups and the large volume of
tissue (whole hippocampus) used for the western blot analysis, subtle changes in BDNF protein levels may have been masked. Additional studies with increased sample sizes in addition to immunohistochemistry techniques would be needed to confirm the observed effects of post-TBI lithium administration on BDNF levels in the hippocampus.

**Caspase-3.** One of the reported mechanisms through which lithium exerts neuroprotective effects is the prevention of apoptosis by blocking the activation of caspase-3. However, there was no detectable expression of the activated form of caspase-3 on either PID 1 or 7. Expression of the proform of caspase-3 was detectable in all the treatment groups at both postinjury time points; however, there was no significant difference in proform caspase-3 levels between any of the treatment groups on either PID 1 or PID 7. These findings do not support our hypothesis that activated caspase-3 expression will be increased on PID 1 and 7 in the TBI-Vehicle groups.

The presence of apoptotic cells following TBI was reported in the DG and CA3 regions of the hippocampus 24 hr after mild or moderate severity lateral FPI, in vehicle-treated rats (Rink et al., 1995). Additionally, significant increases in caspase-3 have been reported following CCI in rats (Clark et al., 2000; Springer, 2002; Tweedie et al., 2007). In contrast to these studies, the 17-kDa band, corresponding to the activated caspase-3 protein, was absent in our TBI groups. To rule out any technical problems, we used a positive control that contained normal cell lysate, with and without staurosporine, and ran them on a separate blot with selected samples from PID 1. Analysis of these data confirmed the presence of activated caspase-3 in the positive control samples only, thus supporting our initial findings that there was no activated caspase-3 in our experimental samples.
The findings from the present study do not support the hypothesis that activated caspase-3 would be elevated in the ipsilateral hippocampus following injury. A possible explanation is that activated caspase-3 levels had peaked and declined by the time the animals were sacrificed. Indeed, Rink et al. (1995) reported that cells undergoing necrosis and apoptosis can be eliminated within a few hours. Conti et al. (1998) reported prominent, but non-significant, increase in the number of cells with apoptotic morphologies in the ipsilateral hippocampus at 12 and 48 hr following lateral FPI. The increase in apoptosis reported by Conti (1998) temporally coincides with the decrease in proform caspase-3 on PID 1 observed in the TBI-Vehicle group, relative to the TBI-Lithium and Sham-Vehicle groups, in the present study. The decrease in proform caspase-3 could be attributed to its conversion to an activated state. Alternately, it is possible that the cell death in the ipsilateral hilus and CA3 regions of the hippocampus, observed in injured rats from Experiment 3, was primarily due to necrosis.

Another possibility is that the hippocampal subregions affected by apoptosis at the selected postinjury time points were disproportionately small, and when compared to the entire hippocampus, the presence of this protein was masked. It was reported that following lateral FPI, the presence of apoptotic cells was not localized to any one area of the hippocampus. Instead, these cells were scattered throughout the structure (Conti et al., 1998). Furthermore, Conti (1998) identified larger numbers of cells with apoptotic appearance in the cortex. It is possible that the lithium-induced neuroprotective effects that were observed in Experiments 1 & 2 were produced, in part, by lithium’s effects in other brain regions. Further study is needed to evaluate the effects of post-TBI lithium-treatment on other brain regions, particularly those regions important in learning and memory that are also known to be vulnerable to TBI-induced pathology (e.g., prelimbic cortex).
**General Discussion**

Our experiments have provided strong evidence to support the neuroprotective effects of lithium and its use as a therapeutic agent in the treatment of the sequelae associated with TBI. The present study indicates that (i) low doses of lithium chloride initiated 30 min after lateral FPI and administered daily are effective in reducing TBI-induced deficits in vestibulomotor and cognitive function; (ii) treatment with the selected dose of lithium can be delayed up to 8 hr post-TBI and still provide similar beneficial effects associated with the 30 min treatment; (iii) chronic low-dose lithium is associated with significant cell survival in the CA3 but not in the hilar region of the hippocampus of the injured rats.

**Dose of lithium and time window for neuroprotection after TBI**

Previous research in animal models of stroke have shown that pre- or post-injury administration of lithium, at doses equal to or below those used in the clinical setting for the treatment of BAD, achieves neuroprotection (Nonaka & Chuang, 1998; Ren et al., 2003; Xu et al., 2003; Yan et al., 2007; Yan et al., 2007). The current study, which assessed the effect of postinjury administration of lithium in a rat model of lateral FPI, provides further evidence in support of the neuroprotective role of this agent. Among the four doses of lithium that we tested (1.00, 0.50, 0.25, 0.125 mmol/kg, ip), only the 0.125 and 0.25 mmol/kg doses, administered 30 min after injury, significantly ameliorated TBI-induced cognitive deficits, as assessed by performance in the MWM. To date, only one other study, employing a rat model of ischemic injury, has reported on the beneficial effects of postinjury administration of lithium (Ren et al., 2003). The aforementioned study focused on the lithium-promoted recovery of neurological functioning, assessed by performance on tests evaluating motor, sensory, and reflex abilities. The authors of the study found that lithium treatment with doses ranging from 0.50 – 3.00
mmol/kg, initiated up to 3 hr after the onset of MCAO, significantly improved injury/reperfusion-induced neurological deficits, evaluated at 24 hr, 7 and 14 days post-ischemic injury (Ren et al., 2003). Similarly, in our study we detected improvements in the motor performance on the BW task (PID 1, 3, 7) of our injured-lithium treated animals. However, we observed that the 1.00, 0.25 and 0.125 mmol/kg doses improved BW performance whereas the 0.50 mmol/kg dose did not. This phenomenon of varying physiologic response to different dosages of a neuroprotective agent has been previously described in various models of brain injury (Calabrese, 2008). Dawson et al. (2001) found that the most effective dose for reducing infarct volume in a MCAO mouse model for ischemic injury did not always improve motor performance, and in some cases significantly impaired it (Dawson, Wadsworth, & Palmer, 2001).

In Experiment 1, the two most effective doses were 0.25 and 0.125 mmol/kg. Although both doses improved MWM performance in TBI-Lithium animals over the TBI-Vehicle treated group, the 0.25 mmol/kg dose was selected for use in Experiment 2, as the degree of cognitive improvement achieved with this dose was higher (although not significantly) than that observed with the lower 0.125 mmol/kg dose.

Based on the results from Experiment 2, lithium appears to provide neuroprotection even when its administration is delayed up to 8 hr (but not 24 hr), postinjury. This finding makes lithium a good candidate for evaluation in TBI clinical trials, as the elapsed time between the occurrence of a traumatic event and the administration of a test drug in humans is estimated to be 4 hours, which takes into account the arrival of the patient at the hospital and the subsequent acquisition of an informed consent for study enrollment.
Mechanism of the neuroprotective effects of lithium

*In vitro* studies have shown that lithium is effective in preventing cell death induced by a variety of cellular insults (Chen et al., 2003; Hennion et al., 2002; Hongisto et al., 2003; Nonaka et al., 1998; Rametti, Esclaire, Yardin, Cogne, & Terro, 2008; Senatorov, Ren, Kanai, Wei, & Chuang, 2004). Similarly, cell death reduction with lithium treatment has been documented by *in vivo* studies showing decreased infarct volume following ischemic brain injury (Ren et al., 2003; Yan et al., 2007; Yan et al., 2007), and reduced TUNEL staining following exposure to neurotoxins (Ghribi, Herman, Spaulding, & Savory, 2002) or radiation (Yazlovitskaya et al., 2006).

In the present study, we sought to investigate potential mechanisms of action for the observed lithium-induced neuroprotective effects observed in *Experiments 1 – 3*. Specifically, we assessed the neuroprotective properties of lithium by means of western blot analysis of the relative abundance of the proteins GAP-43 and BDNF, selected as measures of postinjury structural reorganization and plasticity (Hashimoto et al., 2002), and by measurement of proform and activated caspase 3, markers of apoptosis.

BDNF expression has been associated with improved outcome in TBI (Griesbach et al., 2009) and previous research has shown that lithium alone increases the expression of BDNF in the hippocampus and cortex of rats, as well as the overall brain expression of BDNF mRNA. In particular, lithium-induced neuroprotection has been correlated, in several models of injury, with increased expression of BDNF and its primary receptor TrkB, and a study by Hashimoto et al. (2002) provides strong evidence in support of this interaction. These authors demonstrated that the protective effects of lithium-treatment against glutamate induced neurotoxicity in cortical cells could be blocked by treatment with either an inhibitor of the TrkB receptor or a neutralizing
antibody specific to BDNF. Additionally, cortical neurons from BDNF knockout mice were not protected by lithium, thus further supporting the important role of BDNF-TrkB interactions in lithium-induced neuroprotection (Hashimoto et al., 2002).

Results from our Experiment 4 indicate that the amount of BDNF protein, assessed by western blot analysis, was appreciably higher in TBI-Lithium animals on PID 1. This increase, however, was only transient, and on PID 21 BDNF levels were significantly decreased as compared to Sham-Vehicle. This finding was unexpected, given the plethora of data in the literature pointing at the significance of the BDNF-TrkB interaction in mediating the neuroprotective effects associated with lithium (Chuang, 2004; Chuang, 2005; D'Mello et al., 1994; Ghribi et al., 2002; Manji & Duman, 2001; Mora et al., 1999; Xu et al., 2003). It must be noted, however, that our study is the first to investigate the effects of this drug in an experimental model of TBI.

We also performed analysis of GAP-43, a neural-specific calmodulin binding protein, which is a major protein kinase C substrate found in developing and regenerating neurons. We found that on PID 1, the relative abundance of GAP-43 was noticeably elevated in the TBI-Lithium group as compared to the TBI-Vehicle group. This suggests that post-TBI lithium-treatment not only protects against the observed injury-induced decrease in GAP-43, it elevates levels of this protein relative to Sham-Vehicle. The small sample size and greater variability in the relative abundance of GAP-43 protein levels among animals in the TBI-Vehicle and Sham-Vehicle groups on PID 1 could have masked the effect of postinjury lithium treatment on GAP-43. In addition to increasing the sample size, immunohistochemical analysis the levels of GAP-43 protein in specific regions of the hippocampus or quantification of mRNA using ELISA.
would provide additional, more specific, information regarding the effects of post-TBI lithium administration on GAP-43 levels in the hippocampus.

On PID 7 we found that the relative abundance of GAP-43 was significantly decreased in the TBI-Vehicle group as compared to both the Sham-Vehicle and TBI-Lithium groups. This finding suggests that, relative to the Sham-Vehicle group, the abundance of GAP-43 protein in animals in the TBI-Vehicle is lessened on PID 1 and significantly reduced on PID 7. Additionally, these data indicate that, in the injured brain, lithium-treatment continues to protect against TBI-induced decline in GAP-43 for up to 7 days postinjury, maintaining the relative abundance of this protein at Sham-Vehicle levels. However, by PID 21, we observed that the levels of GAP-43 in the TBI-lithium group significantly rebounded to Sham-Vehicle levels. This is in contrast to the significant decrease in the relative abundance of GAP-43 protein in the TBI-Lithium group as compared to both the Sham-Vehicle and TBI-Vehicle groups. Based on the study by Emery et al. (2000) the postinjury timeframe associated with increased GAP-43 levels is between 48 -72 hrs; later postinjury assessment time points including 1, 2, and 4 weeks postinjury were not associated with changes in GAP-43 abundance relative to sham controls (Emery et al., 2000; Hulsebosch et al., 1998). We interpret these data to indicate that the most likely timeframe for postinjury lithium-induced effects on neuronal remodeling most likely occur within the first 7-days after injury.

It is believed that the inhibition of post-TBI activation of pro-apoptotic caspases improves outcome by preventing secondary injury-induced cell loss. In agreement with this, several studies have linked the neuroprotective effects of chronic lithium treatment with the inhibition of apoptotic pathways. Specifically, it has been shown that lithium treatment prevents injury-triggered pro-apoptotic events such as cytochrome $c$ translocation, decreases in anti-
apoptotic Bel-2, and increases in pro-apoptotic Bax, GSK-3β, and tau (Chen et al., 1999; D'Mello et al., 1994; Ghribi, Herman, & Savory, 2003; Li et al., 2002; Manji et al., 1999; Manji, Moore, & Chen, 2000b; Mora et al., 1999; Rametti et al., 2008; Xu et al., 2003). Nevertheless, the results of our western blot analysis indicate that only the proform of caspase-3 was present at PID 1 and 7, with no significant between-group differences in protein expression. Activated caspase-3 was not present in any of the experimental groups.

Taken together, our findings indicate that postinjury lithium treatment is significantly effective in reducing injury-induced motor and cognitive deficits, and that this treatment is effective in reducing post injury cognitive and motor dysfunction even when delayed up to 8-hours following TBI. Treatment with this drug is also associated with a marked reduction in cell death in the CA3 region of the hippocampus, a structure that is critical to memory formation and retrieval. Although lithium has a very narrow therapeutic range, the dose that was most effective in our study is well within that limit. These encouraging findings support further testing of lithium in clinical TBI studies.
List of References
List of References


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induced by the protein kinase AII inhibitor H-89 in rats. *Pharmacology, 80*(2-3), 158-165. doi:10.1159/000103265


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Appendix A

Figure A1. *Comparison of motor performance pre- and post-TBI.* Lines represent the mean latency for each treatment group (n = 10/group) across days, ± SEM. A split-plot ANOVA showed that there was a significant effect of group. A Fisher LSD analysis revealed that the two sham groups had significantly shorter latencies to reach the goal box as compared to the TBI-0.50 and TBI-Vehicle groups. There was no significant difference between the sham groups and the TBI-1.00, TBI-0.25, and TBI-0.125 groups. All the doses of lithium are mmol/kg. Vehicle treated animals received 0.9% sterile saline. All treatments were administered in a volume of 1 mg/kg. (*) indicate significant differences as compared to Sham-Vehicle. (†) indicate comparisons made to the Sham-1.00 group. *p ≤ .05. **†† p ≤ .01. ***p ≤ .001. Specific statistical information is provided in the text.
Figure A2. MWM data from Experiment 1. This graph shows the daily average latency to reach the goal platform for each treatment group \((n = 10/group)\), ± SEM. A split-plot ANOVA and Fisher LSD post hoc revealed that the TBI-Vehicle group had significantly longer latencies to reach the goal platform as compared to the TBI-0.125, TBI-0.25, Sham-Vehicle, and Sham-1.00 groups. None of the groups performed as well as the Sham-Vehicle group. All doses of lithium are in mmol/kg. (*) indicate comparisons to Sham-Vehicle. (†) indicate comparisons to Sham-1.00. (‡) show comparisons made to TBI-30 min. (*) indicate comparisons to TBI-8 hr. *\(p < .05\). **, ††, ‡‡, •• \(p < .01\). ***, †††, ‡‡, ••• \(p < .001\).
Figure A3. Proximity score from MWM hidden platform testing in Experiment 1. This graph shows the daily average proximity score for all treatment groups ($n = 10$/group) during MWM testing, ± SEM. All doses of lithium are in mmol/kg. Vertical lines represent SEM. A repeated measure ANOVA revealed a significant difference among the average daily proximity scores of the treatment groups. A Fisher LSD post hoc found that the two sham groups had significantly lower proximity scores (indicating better performance) as compared to the TBI-Vehicle group. The two injured groups that had significantly shorter latencies to reach the goal platform as compared to the TBI-Vehicle group during the hidden platform testing, TBI-0.25 and TBI-0.125, had significantly higher proximity scores as compared to the Sham-Vehicle group, however, they did not significantly differ from the Sham-1.00 group. There was no appreciable difference in the proximity scores of the TBI-Vehicle group and the TBI lithium-treated groups. (*) indicate comparisons to the Sham-Vehicle group. (†) indicate comparisons made to the Sham-1.00 group. **, †† $p < .01$. *** $p < .001$. 
Figure A4. Analysis of the proximity score for each group during the probe trial in Experiment 1. The data are expressed as the mean, ± SEM. An ANOVA found significant differences between the treatment groups. A Fisher LSD post hoc revealed that the Sham-Vehicle group had a significantly lower proximity score as compared to all other treatment groups. *p < .05. **p < .01. ***p < .001.
Figure B1. Comparison of performance on beam walk task across groups in Experiment 2. These data represent the mean latency to reach the goal box, ± SEM. TBI-Vehicle \((n = 10)\), TBI-30 min \((n = 10)\), TBI-8 hr \((n = 10)\), TBI-24 hr \((n = 9)\), Sham-24 hr \((n = 9)\), Sham-Vehicle \((n = 10)\). The TBI-30 min group data is the TBI-0.25 from Experiment 1 and is included in the data analysis as a basis for comparing the effectiveness of the selected dose of lithium when the initiation of treatment is delayed. The TBI-8 hr and TBI-30 min had significantly shorter latencies to reach the goal box as compared to the TBI-Vehicle and TBI-24 hr groups. (*) indicate comparisons to Sham-Vehicle. (†) indicate comparisons to Sham-1.00. (‡) compare to TBI-30 min. (•) indicate comparisons to the TBI-8 hr. ‡• \(p < .05\). **, ††, ‡‡ \(p < .01\). ***, †††, ‡‡‡ \(p < .001\).
Figure B2. Comparison of MWM performance across groups in Experiment 2. Data are the mean latency for each treatment group, TBI-Vehicle (n = 10), TBI-24 hr (n = 9), TBI-8 hr (n = 10), TBI-30 min (n = 10), Sham-Vehicle (n = 10), Sham-24 hr (n = 9), ± SEM. The TBI-30 min group data is identical to the TBI-0.25 from Experiment 1 and is included in the data analysis as a basis for comparing the effectiveness of the selected dose of lithium when the initiation of treatment is delayed. An ANOVA, followed by a Fisher LSD post hoc analysis, showed that the Sham-Vehicle, Sham-24 hr, TBI-8 hr and TBI-30 min groups had significantly faster latencies to reach the goal as compared to the TBI-Vehicle and TBI-24 hr groups. The TBI-8 hr and TBI-30 min groups had longer latencies to the goal platform as compared to the Sham-Vehicle group. There was no significant difference in MWM performance between the two sham groups. (*) indicate comparisons to Sham-Vehicle. (†) indicate comparisons to Sham-24 hr. (‡) denote comparisons to the TBI-30 min group. (*) indicate comparisons to the TBI-8 hr. *: †: ‡: *p < .05. **: ††: ‡‡: **p < .01. ***: †††: ‡‡‡: ***p < .001.
Figure B3. Proximity score during hidden platform testing in the MWM from Experiment 2. The average proximity score for each treatment group TBI-Vehicle \((n = 10)\), TBI-24 hr \((n = 9)\), TBI-8 hr \((n = 10)\), TBI-30 min \((n = 10)\), Sham-Vehicle \((n = 10)\), Sham-24 hr \((n = 9)\) over the four days in the MWM was compared using a split-plot \([6 \text{ (group)} \times 4 \text{ (day)}]\) ANOVA. A Fisher LSD was used to compare individual differences among the groups. The TBI-30 min group data is the TBI-0.25 from Experiment 1 and is included in the data analysis as a basis for comparing the effectiveness of the selected dose of lithium when the initiation of treatment is delayed. The TBI-30 min \((p = .031)\), TBI-8 hr \((p = .03)\), Sham-Vehicle and Sham-24 hr \((p < .001)\) groups had significantly lower proximity scores, indicating a more direct route to the hidden platform location, as compared to the TBI-Vehicle group. The TBI-24 hr group had significantly higher proximity scores as compared to the Sham-Vehicle \((p = .001)\) and Sham-24 hr \((p = .008)\) groups. The proximity scores for the TBI-30 min and TBI-8 hr groups were significantly higher than Sham-Vehicle \((p = .034 \text{ and } .036 \text{ respectively})\) but did not differ from the Sham-24 hr group \((p = .122 \text{ and } .128 \text{ respectively})\). (*) indicate comparisons made to the Sham-Vehicle group. (†) indicate comparisons made to the Sham-24 hr group. (#) indicate comparisons made to the TBI-Vehicle group. *, # \(p < .05\). †† \(p < .01\). ††† \(p < .001\).
Figure B4. Proximity score from probe trial in Experiment 2. The mean proximity score for each treatment group, obtained from the 60 s probe trial (no platform in MWM) on postinjury day (PID) 15. The TBI-30 min group data is the TBI-0.25 from Experiment 1 and is included in the data analysis as a basis for comparing the effectiveness of the selected dose of lithium when the initiation of treatment is delayed. The bars represent the mean ± SEM. A Fisher LSD post hoc analysis revealed that the Sham-Vehicle ($p = .039$) and Sham-24 hr ($p = .009$) groups had significantly lower scores (i.e., took the most direct route and remained closest to the previous location of the hidden goal platform) as compared to the TBI-Vehicle group. Neither the TBI-30 min ($p = .07$) nor the TBI-8 hr ($p = .314$) significantly differed from the Sham-Vehicle group. (*) indicate comparisons made to Sham-Vehicle. (†) indicate comparisons made to the Sham-24 hr group, * † $p < .05$. †† $p < .01$.\[0.5cm\]
Appendix C

The Sham-Lithium group was included in the western blots to determine if lithium alone had any effect on the relative abundance levels of the proteins of interest, GAP-43, BDNF, and caspase-3. The raw values from the relative optical density from each subject were divided by the corresponding value from the analysis the β-actin, and the resulting value was converted to the percent of Sham-Vehicle.

Figure C1. Comparison of β-actin levels between sham groups. Lithium is associated with a relative abundance of β-actin at multiple time points after injury. Data are expressed as mean ± SEM. The analysis of the β-actin protein levels were not consistent across all treatment groups and blots, indicating that there were differences in the total concentration of proteins loaded on the gel. However, β-actin, a cytoskeletal protein, appears to be differentially affected by lithium-treatment and injury. The levels of β-actin protein in the Sham-Lithium group show a significant increase in this protein as compared to other treatment groups, suggesting that lithium treatment may affect β-actin. *p < .05. ***p < .001.
Table C1.

Comparison of GAP-43 levels between Sham-Vehicle and Sham-Lithium groups in the ipsilateral hippocampus for each postinjury time point

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<td>±SEM</td>
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<td>M %</td>
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Note. An independent samples t-test was used to compare the raw data (relative optical density) and data normalized to β-actin between Sham-Vehicle and Sham-Lithium groups at each postinjury time point. All data are expressed as the % Sham-Vehicle. PID = postinjury day.
Table C2.

Comparison of BDNF levels between Sham-Vehicle and Sham-Lithium groups in the ipsilateral hippocampus at each postinjury time point

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Note. An independent samples t-test was used to compare the raw data (relative optical density) and data normalized to β-actin between Sham-Vehicle and Sham-Lithium groups at each postinjury time point. All data are expressed as the % Sham-Vehicle. PID = postinjury day.
Table C3.

Comparison of proform caspase-3 levels between Sham-Vehicle and Sham-Lithium groups in the ipsilateral hippocampus at each postinjury time point.

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Note. An independent samples t-test was used to compare the raw data (relative optical density) and data normalized to β-actin between Sham-Vehicle and Sham-Lithium groups on PID 1 & 7. All data are expressed as the % Sham-Vehicle. PID = postinjury day.
Table C4.

*Analysis of the relative abundance of GAP-43 protein, raw data and normalized to β-actin, on PID 1, 7, & 21*

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*Note.* A separate ANOVA was performed for the raw data (relative optical density) and data normalized to β-actin for each postinjury time point. Data are expressed as the % Sham-Vehicle. PID = postinjury day. Subscripts indicate trends in the data based on individual group comparisons using the Fisher LSD post hoc test. 

- **a** = increase in GAP-43 levels relative to TBI-Vehicle.
- **b** = increase GAP-43 levels relative to Sham-Vehicle.
- **c** = decrease in GAP-43 levels relative to both sham groups.
- **(*)** indicate comparisons made to Sham-Vehicle.
- **(†)** indicate comparisons made to Sham-Lithium. 

\*p < .05. \***p** < .01.
Table C5.

*Analysis of the relative abundance of BDNF protein, raw data and normalized to β-actin, on PID 1, 7, & 21*

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<td>132.40</td>
<td>26.55</td>
</tr>
<tr>
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<td>4</td>
<td>122.34†</td>
<td>7.77</td>
<td>4</td>
<td>131.47††</td>
<td>12.84</td>
</tr>
<tr>
<td>PID 7 normalized</td>
<td>4</td>
<td>129.69††</td>
<td>10.92</td>
<td>4</td>
<td>123.60††</td>
<td>12.69</td>
</tr>
<tr>
<td>PID 21</td>
<td>4</td>
<td>57.29**††</td>
<td>3.30</td>
<td>4</td>
<td>71.67†</td>
<td>7.69</td>
</tr>
<tr>
<td>PID 21 normalized</td>
<td>4</td>
<td>61.83_b</td>
<td>4.60</td>
<td>4</td>
<td>75.67_b</td>
<td>10.41</td>
</tr>
</tbody>
</table>

*Note.* A separate ANOVA was performed for the raw data and data normalized to β-actin for each postinjury time point. Data are expressed as the % Sham-Vehicle. PID = postinjury day. Subscripts indicate trends in the data based on individual group comparisons using the Fisher LSD post hoc test. a = .053. b = lower BDNF protein levels relative to both sham groups. (†) indicate comparisons made to Sham-Lithium. († †) indicate comparisons made to the Sham-Lithium group. *p < .05. ** † † p < .01.
Table C6.

Analysis of the relative abundance of proform caspase-3 protein levels, raw data and normalized to β-actin, on PID 1 & 7

<table>
<thead>
<tr>
<th>PID</th>
<th>Treatment group</th>
<th>n</th>
<th>M %</th>
<th>±SEM</th>
<th>n</th>
<th>M %</th>
<th>±SEM</th>
<th>n</th>
<th>M %</th>
<th>±SEM</th>
<th>F</th>
<th>p</th>
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<td>100</td>
<td>12.32</td>
<td>1.567</td>
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<td>10.94</td>
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<td>80.06</td>
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<td>100</td>
<td>12.78</td>
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<td>17.69</td>
<td>4</td>
<td>104.83</td>
<td>17.69</td>
<td>2.436</td>
<td>.120</td>
</tr>
</tbody>
</table>

Note. A separate ANOVA was performed for the raw data (relative optical density) and data normalized to β-actin at both postinjury time points. Data are expressed as the % Sham-Vehicle. PID = postinjury day.
Vita

Katharine Coryell Eakin was born on September 22, 1977, in Washington D.C., and is an American citizen. She graduated from James Madison High School in Vienna, VA in 1995. In 1999, she graduated Cum Laude with a Bachelor of Science in Psychology from Virginia Commonwealth University (VCU), Richmond, Virginia. She has been a member of Psi Chi, National Honor Society in Psychology, since 1997. In 2001 she matriculated into the Ph.D. program in Biopsychology at VCU, Richmond Virginia. While attending VCU she was a teaching assistant for two introductory psychology courses from August, 2001 through May, 2003. She taught two undergraduate courses, VCU 101, Introduction to the University, in the fall semesters and VCU 102, Turning Point, Discovering a New MINDSET, in the spring semesters. She taught these courses from January, 2004 through May, 2006. In 2008, she taught the laboratory section of Statistics, PSYC 214. She is a member of the National Neurotrauma Society (NNS) and Women in Neurotrauma Research (WiNTR). She has presented posters at the 20th Annual National Neurotrauma Society Symposium and 6th Annual International Neurotrauma Symposium (INTS), in Tampa, FL in October, 2002; the 23rd National Neurotrauma Society Symposium, Washington, D.C., November 2005; the 24th NNS in St. Louis, MO July 2006; the 25th NNS in Kansas City, MO, July 2007; the 26th NNS in Orlando, FL, July 2008; and 2nd joint INTS/NNS and 27th NNS in Santa Barbara, CA, September 2009.