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Exploring the Impact of Decreased AMPK Pathway Activity on Brain Injury Outcome

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A

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by

Julia Lee Hill B.A.

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DEDICATION

This work is dedicated to my father, Jerel J. Hill, who has always encouraged me to pursue my passions (whether or not they were of interest to anyone else). I didn't follow in your exact footsteps and become a lawyer, but I often pretend that I am working on an opening or closing argument when I write and think of you. I hope this work makes you proud.

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EXPLORING THE IMPACT OF DECREASED AMPK PATHWAY ACTIVITY ON BRAIN INJURY OUTCOME

ABSTRACT

Julia Lee Hill, B.A.

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Each year, 150 million people sustain a Traumatic Brain Injury (TBI). TBI results in life-long cognitive impairments for many survivors. One observed pathological alteration following TBI are changes in glucose metabolism. Altered glucose uptake occurs in the periphery as well as in the nervous system, with an acute increase in glucose uptake, followed by a prolonged metabolic suppression. Chronic, persistent suppression of brain glucose uptake occurs in TBI patients experiencing memory loss. Abberant post-injury activation of energy-sensing signaling cascades could result in perturbed cellular metabolism. AMP-activated kinase (AMPK) is a kinase that senses low ATP levels, and promotes efficient cell energy usage. AMPK promotes energy production through increasing glucose uptake via glucose transporter 4 (GLUT4). When AMPK is activated, it phosphorylates Akt Substrate of 160 kDa (AS160), a Rab GTPase activating protein that controls Glut4 translocation. Additionally, AMPK negatively regulates energy-consumption by inhibiting protein synthesis via the mechanistic Target of Rapamycin (mTOR) pathway.

Given that metabolic suppression has been observed post-injury, we hypothesized that activity of the AMPK pathway is transiently decreased. As AMPK activation increases energy efficiency of the cell, we proposed that increasing AMPK activity to combat the post-injury energy crisis would improve cognitive outcome. Additionally, we expected that inhibiting AMPK targets would be detrimental. We first investigated the role of an existing state of hyperglycemia on TBI outcome, as hyperglycemia correlates with increased mortality and decreased cognitive outcome in clinical studies. Inducing hyperglycemia had no effect on outcome; however, we discovered that AMPK and AS160 phosphorylation were altered post-injury. We conducted

work to characterize this period of AMPK suppression and found that AMPK phosphorylation was significantly decreased in the hippocampus and cortex between 24 hours and 3 days post-injury, and phosphorylation of its downstream targets was consistently altered. Based on this period of observed decreased AMPK activity, we administered an AMPK activator post-injury, and this improved cognitive outcome. Finally, to examine whether AMPK-regulated target Glut4 is involved in post-injury glucose metabolism, we applied an inhibitor and found this treatment impaired post-injury cognitive function. This work is significant, as AMPK activation may represent a new TBI therapeutic target.

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LIST OF ABBREVIATIONS

2-DG	2-Deoxyglucose
AICAR	5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
AS160	Akt Substrate of 160 Kilodaltons
AD	Alzheimer's Disease
AMPK	AMP-Activated Kinase
BBB	Blood-Brain Barrier
CCI	Controlled Cortical Impact
EAA	Excitatory Amino Acid
GLUT	Glucose Transporter
ICV	Intracerebroventricular
kDa	Kilodalton
mTOR	Mechanistic Target of Rapamycin
mRNA	Messsenger RNA
NMDA	N-methyl-D-aspartate
PET	Positron Emission Tomography
PPP	Pentose Phosphate Pathway
S6	Protein S6
RabGAP	Rab GTPase-Activating Protein
STZ	Streptozotocin
T2D	Type 2 Diabetes
TBI	Traumatic Brain Injury

CHAPTER I. GENERAL INTRODUCTION

TBI Epidemiology, Pathology, and Cognitive Dysfunction

Traumatic Brain Injury: A major public health concern

Traumatic brain injury (TBI) is defined as an alteration in brain function resulting from transfer of energy from an external source (1). It is estimated that 1.4 million people sustain a TBI annually in the U.S., and that over 5 million individuals require daily assistance for TBI-related issues (2, 3). In addition, trauma impacts a relatively young population, as it is the leading cause of death in individuals under the age of 45 (4). Awareness of TBI has increased in recent years, as individuals engaged in armed combat are at high risk for sustaining brain injuries (5). Domestically, the leading causes of TBI-related emergency room visits are falls, car crashes, and assault (6). Patients with TBI require life-long assistance. This is especially significant as TBI impacts an individual's subsequent long-term quality of life and ability to maintain employment. The annual U.S. TBI-related economic burden is estimated at 56 billion dollars (7).

The two temporally distinct stages of TBI pathology

Tissue-damage as a result of TBI progresses in two stages: primary pathology, which occurs as a direct result of the injury, and secondary cellular and biochemical changes that continue long after the initial impact (8). Because brain injury can occur from a multitude of traumatic events, the location and severity of affected regions can vary greatly between individuals. As a result of these factors, there is a wide variation in the level of damage sustained and the magnitude of the underlying pathology in TBI patients. However, the damage resulting from brain injury is generally progressive, and presents a large time window for potential therapeutic intervention. Therefore, an understanding of the cellular and molecular changes contributing to TBI damage may lead to development of mechanism-based treatment.

TBI-induced primary, or focal, damage occurs as a result of biomechanical force exerted on the tissue at the time of trauma (9). Examples of primary damage include brain contusion, vascular damage, and hemorrhaging. Movement of the brain within the skull, and

collision with bony ridges, results in contact damage. The skull is designed with rough contours to hold the brain in place during normal movement; however during sudden impact this results in injury. The temporal lobe is in close proximity to the middle cranial fossa and the bony sphenoid ridge and for this reason trauma routinely results in direct biomechanical tissue damage to the temporal lobe (10). This damage may not be as readily reversible as later events, as it leads directly to cell death and tissue necrosis (11). In addition, primary damage, as detected by CT or MRI, is not found to correlate directly with post-injury neuropsychological issues (12).

A multitude of secondary, prolonged biochemical changes occur in response to the initial biomechanical damage (13). Delayed changes at the cellular level are known as diffuse, secondary damage. This type of damage is not necessarily localized to the injury site, and continues for hours to weeks following the trauma. The event hypothesized to initiate much of the secondary cellular damage is an indiscriminant release of the excitatory amino acids, including the neurotransmitter glutamate. Glutamate release results in a significant increase in excitatory transmission, and increased cytosolic calcium as a result. A number of cellular pathologies occur in conjunction with increased calcium, including blood-brain barrier (BBB) disruption, cellular edema, inflammation, and perturbed signal transduction, (11, 14-16) that can give rise to lasting changes in neuronal function. Clinically, these changes can result in altered synaptic transmission, neurologic dysfunction, coma, and death (17).

Progressive secondary pathology has been found to correlate with post-injury cognitive dysfunction. Atrophy of the hippocampus several years following neurological insult has been demonstrated to correlate with an inability to form new memories (18). One recognized contributing factor that results in progressive tissue damage is Diffuse Axonal Injury (DAI). White matter tracts are deformed following TBI, leading to disruption of axonal transport and swelling of the axons. Complicating matters further, this progressive pathology is difficult to assess clinically, as the pathology is microscopic and often not observed by CT scan (19). In experimental animal models of TBI, pathological changes such as ventricular enlargement and

hippocampal shrinkage that correlate with cognitive changes have been observed for up to a year post-injury (20). Metabolic disturbance, including mitochondrial disruption and other alterations of energetic homeostasis, have been hypothesized to contribute to this process (21, 22).

TBI results in cognitive dysfunction and memory loss in patients

Despite the heterogeneous nature of injury, TBI results in characteristic behavioral dysfunction. This is in part because certain cognitive areas are vulnerable to TBI-induced secondary damage, including white matter of the frontal and temporal lobes (23). For this reason, the main behavioral domains affected by trauma include affective changes, executive functioning, attention, and cognition/memory (8, 23). Associated neuropsychological sequelae include affective changes include aggressiveness and an increase in impulsivity (24).

Cognitive dysfunction is one of the most common post-injury complaints (25, 26). Cognition represents the capacity to acquire and retain knowledge, and the ability to apply this information in the correct context. TBI patients exhibit slowed learning rate of new material, reduced processing speed, decreased ability to utilize semantic knowledge, and higher rate of forgetting than controls (27, 28). Decreased cognitive capacities in TBI sufferers are found despite intelligence quotients comparable to non-injured controls (29, 30).

One of the primary cognitive pathologies associated with TBI is memory loss (27). Patients often have difficulty acquiring and retaining new information learned post-injury (24). This results in difficulties in problem-solving and learning which can impact an individual's ability to return to work, personal interactions, and overall quality of life (31, 32). In general, TBI patients demonstrate significant cognitive improvement up to six months post-injury, with subtle improvement continuing up to a year (33). In addition to being the most common post-injury cognitive issue, memory loss is also often the most chronic deficit. Once the window for spontaneous recovery has passed, patients suffering from severe TBI have been observed to have episodic memory deficits up to 6-10 years post-injury (26, 29, 34). For example, Zec et al.

(2001) found that in a various tasks involving verbal or visual recall of presented items, severe closed head injury victims on average 10 years post-injury were able to recall significantly less. Thus, understanding the pathology underlying the damage which leads to memory loss and treatment development could potentially lead to significant improvement in quality of life for TBI sufferers.

Experimental work- modeling brain injury and aberrant glucose uptake as a secondary pathology

Experimentally modeling traumatic brain injury

Experimental animal models of TBI have been utilized to elucidate the mechanisms underlying injury pathology and develop therapeutic treatment strategies. Different injury models have been developed to specifically examine these different processes. As described by Cernak (2005), the fundamental requirements for an animal model of TBI, dictate that the injury method be controlled and reproducible, resultant pathology mimic the human conditions, dysfunctional outcome occur as a direct result of the mechanical trauma, and the injury magnitude should directly relate to injury severity. The most common rodent category of injury models is impact injury, including weight drop, fluid percussion injury (FPI), and controlled cortical impact (CCI). Impact injury entails delivery of a direct trauma to the brain through a weight or impactor device.

In the work for this thesis project, controlled cortical impact (CCI) injury was used to experimentally model brain trauma (35-37). CCI utilizes a compressed-air, piston driven device to deform the cortex at a designated depth and velocity (38, 39). This model of injury is advantageous because the impact is more controlled than FPI or weight-drop (40). The control over injury parameters that the CCI method leads it to be especially advantageous for work with mice, as greater precision is necessary on such a small scale (41). The resultant injury has been shown to induce pathological alterations including blood-brain barrier breakdown, reactive astrogliosis, cell loss, and aberrant signaling cascade activation (38, 41-43).

CCI injury also results in cognitive deficits that are qualitatively similar to the memory loss seen in TBI patients (43, 44). Memory deficits were first demonstrated in the radial arm maze task in animals with mild-moderate levels of experimental injury resulting in little to no hippocampal cell loss (44). The Morris water maze is routinely used to assess spatial memory deficits in injured animals. In this task, animals are trained to find the location of a hidden platform submerged in water using spatial cues external to the maze (45). Hamm et al. (1992) first demonstrated that animals with a moderate CCI injury take longer to locate the hidden platform in the Morris water maze task, and that this persisted for at least one month post-injury. Subsequent work demonstrated a deficit in memory retention utilizing the CCI paradigm with mice (41). The Dash laboratory has subsequently routinely found deficits in both injured mice and rats in various versions of the Morris water maze task (35-37, 46, 47).

The implications of high blood glucose following injury

High peripheral blood glucose at the time of injury or within the first 24 hours post-injury (>200 mg/dL; baseline is 100 mg/dL) has been correlated with increased mortality in severe TBI patients (48, 49). Within recent years, a large prospective clinical study with a general population of trauma patients investigated the benefit of strict blood glucose maintenance (50). This study found that critically injured patients with lower blood glucose had decreased mortality rates and shortened length of hospital stay. However, subsequent clinical studies restricted to head-injured trauma patients found that strict maintenance of low blood glucose was not beneficial, as this may result in increased episodes of hypoglycemia without any improvement in functional outcome (50-52). In addition, during the early post injury period persistently low extracellular glucose in the brain has been correlated with worsened cognitive outcome in TBI patients (53, 54). In the first 24 hours post-injury, insulin treatment of injured animals that was designed to decrease blood glucose post-injury resulted in an increase in mortality (55). Thus, whether the presence of high blood glucose exacerbates secondary pathology and decreases outcome on other measures such as mortality or cognitive capacity

following injury is an important question as decreasing glucose supply to the brain may be equally detrimental.

Rather than high blood glucose exacerbating TBI pathology, hyperglycemia may be a marker of an underlying injury-related dysfunction of cellular metabolism. Peripheral insulin resistance has been observed in severely head-injured rats, and this was associated with a decrease in messenger RNA (mRNA) for glucose transporter 4 (GLUT4) in adipose tissue (56). Systemic insulin resistance has also been demonstrated following experimental stroke, and this was correlated with behavioral dysfunction (57). A correlation between decreased outcome and suppression of glucose metabolism has also been supported by the clinical literature. A recent retrospective study demonstrated a significant increase in mortality for diabetic patients versus those without diabetes (58). Furthermore, they found that patients with insulin-dependent diabetes had a higher rate of mortality than those who were not insulin-dependent, suggesting that the degree of perturbed metabolism may be an important determinant of outcome. With over 40% of the U.S. population being diabetic or pre-diabetic, the relationship between high blood glucose and TBI outcome, and what level of glycemic regulation is beneficial post-injury is an important issue that requires further investigation (59, 60).

Alterations in brain glucose uptake following TBI

In addition to the observations that hyperglycemia is detrimental to TBI outcome, a bi-phasic change in brain glucose uptake following TBI has been well-characterized through microdialysis and autoradiography of glucose metabolites. The most common method of measuring changes in cellular glucose uptake in rodents is 2-deoxglucose-D[^{14}C] glucose (2-DG) autoradiography (61). This technique utilizes a modified, radiolabelled form of glucose. Upon the initial cellular reaction of glucose metabolism, phosphorylation by the enzyme hexokinase, 2-DG becomes trapped inside the cell as it is an unsuitable substrate for further metabolism. Two factors- knowledge of the time between 2-DG delivery, and the optical density of accumulated 2-DG staining in a specific cell layer- enable for the calculation of the rate of

glucose metabolism in a given region of interest. The amount of ^{14}C accumulation in a given region serves as a quantitative measure of glucose uptake and utilization.

In animal models of TBI, there is an acute, transient increase in brain glucose uptake occurring in the first few hours post-injury; this is followed by a prolonged suppression in glucose metabolism lasting up to five days post-injury (14, 15, 22, 62). Disturbed metabolism occurs in regions critical to cognition, such as the cortex and the hippocampus (63-66). This increased glucose uptake as detected by 2-DG imaging in closed head injury, where there is no disturbance of the blood brain barrier (BBB) (65). This period of increased glucose uptake, referred to as “hypermetabolism”, has been observed to occur in the absence of ischemia and prior to later-onset dysfunctional oxidative phosphorylation (15, 67). Qualitatively similar changes are seen in experimental and clinical settings, although the time course of metabolic alteration is extended in humans (14). Although it is possible that glucose uptake could be confounded by additional variables such as reduced blood flow or BBB alteration, these findings demonstrate that metabolic alterations in brain metabolism occur in the absence of these factors, across all injury magnitudes.

Experimental examinations of the acute increase in glycolysis have led to the understanding that glucose hypermetabolism is a unique period of metabolic dysfunction occurring in response as a result of tissue damage and excitotoxicity. Increased post-injury brain glucose uptake is hypothesized to be triggered by neuronal membrane disruption and glutamate release. High levels of excitatory amino acids such as glutamate are released into the synapse as a result of mechanical injury to neurons, resulting in an overstimulation of N-methyl-D-aspartate (NMDA) receptors (68). This leads to calcium accumulation within the cell, and ionic imbalance. Increased glucose uptake is triggered by the cell’s need for ATP produced by glycolysis to operate ionic pumps and restore imbalance (69). Increased glucose uptake is accompanied by an increase in lactate, which accumulates as a result of this increased neuronal depolarization (70). In the first 24 hours post-injury, glucose is also metabolized at elevated levels through pathways other than glycolysis, including the pentose-phosphate

pathway (PPP) (71, 72). The PPP pathway is important in production of reducing equivalents such as NADPH, and also synthesizing ribose-5-phosphate, a molecule necessary for synthesis of nucleic acids for DNA synthesis and repair (73). This early post-injury increase in glucose utilization can be blocked by administration of excitatory amino acid (EAA) antagonists (74). Despite identification of this period in experimental models, no functional improvement in outcome has been found in clinical trials where TBI patients received NMDA antagonist therapy (75-77). One difficulty with this treatment is that the excitotoxic event occurs early following injury, and it may not be feasible to treat patients at the time when treatment would be most efficacious. Thus, although this period may be an early post-injury pathological event, targeting it for clinical purposes may not be ideal.

Following this immediate period of increased brain glucose metabolism, there is a prolonged suppression of glucose uptake. This period is characterized by a concomitant decrease in both glucose uptake and mitochondrial functioning (22, 78, 79). In injured animals, recovery from metabolic suppression has been correlated with the rate of recovery from behavioral dysfunction (80). Rats trained pre-injury in the water maze task were found to return to baseline performance 3 days post-injury, and this correlated with recovery from suppressed glucose metabolism as measured by serial PET imaging. In addition, it has been shown that a secondary insult leads to a prolonged metabolic suppression as measured by decreased ATP levels, and this is correlated with an increase in hippocampal cell death (81).

In addition to the experimental evidence, there is correlative evidence for a link between the extent of metabolic disturbance and functional outcome in the clinical literature. In TBI patients, PET studies demonstrated that suppression of brain glucose uptake occurs across a broad range of injury levels, and spontaneously resolves one month post-injury (82). Neuroimaging supports a correlation between regional alterations in metabolic activity and worsened cognition. In an initial study with severely head-injured patients followed in the first year post-injury, PET imaging demonstrated a positive correlation between decreased glucose metabolism with performance on both tasks of executive functioning and memory (83).

Subsequent work has supported this observation of decreased metabolism in relation to decreased TBI behavioral outcome, regardless of injury severity (82, 84-87). Thus, metabolic suppression has been correlated with post-injury cognitive outcome. Due to these correlative observations, therapeutic treatments to target metabolism have become of great interest in TBI research.

Alternative fuel source treatments improve TBI outcome

As altered metabolism has been identified as an important secondary TBI pathology, several groups have investigated the effect of providing alternative brain metabolites on brain injury outcome in experimental animal models (55, 88-91). For the most part, these treatments are designed to circumvent the identified dysfunction in glucose metabolism either by providing a metabolite alternative to glucose, such as ketone bodies, or by molecules which can be immediately used or converted for the purpose of anaerobic glycolysis, including pyruvate and lactate. The intake of a ketogenic diet immediately post-injury has been found effective in reducing contusion volume; although this effect is limited in adult animals due to the brain's decreased ability to metabolize ketones with age (89). The authors also note that the ketogenic diet induces mild hypoglycemia, so it is difficult to determine whether this treatment would have an unexamined impact upon metabolic signaling pathways. Indeed, subsequent work has demonstrated that fasting in the immediate post-injury period is neuroprotective (55).

Alessandri et al. (2012) demonstrated that brain infusion of lactate immediately following moderate injury results in a contusion volume reduction. Animals treated with lactate were protected from a CCI-related decrease in cerebral blood flow, and this did not alter extracellular glutamate levels. However, this effect may be injury-level dependent, as at a more severe level of injury lactate administration does not prevent TBI-related decreases in energy metabolites (92). Lactate may also not be an ideal source as its conversion to pyruvate by lactate dehydrogenase requires oxidizing co-enzyme nicotine adenine dinucleotide (NAD^+), levels of which are known to be decreased post-injury (93). Thus, sodium pyruvate treatment

has been examined both in the context of acute and chronic post-injury treatment (90, 91, 94, 95). Intraperitoneal injection of sodium pyruvate immediately prior to behavioral training has been found to improve working memory outcome (90). Yet, acute treatment in the first 24 hours post-injury has produced mixed results- it did not improve memory (95), and a decrease in contusion volume two weeks post-injury required three hourly injections immediately after injury (91). Although such efforts clearly offer promise in that they are able to attenuate some TBI-related dysfunction, they do not attempt to address or alleviate the underlying, well-recognized alterations that occur to glucose uptake following injury.

Characterization of brain glucose uptake

In order to understand how glucose uptake may be altered following injury, it is important to first understand the general mechanisms of brain glucose uptake. Glucose transporters (GLUT) are facilitative, 12 trans-membrane proteins which regulate the uptake of glucose into cells throughout the periphery and into the brain (96, 97). These transporters have an extracellular and cytoplasmic binding sites for glucose, and do not have the capacity for simultaneous occupation (98). According to the classical neuroenergetics model, under normal conditions, glucose is transported through the blood brain barrier through Glucose transporter 1 (GLUT1, 55 kilodaltons, kDa) in microvascular endothelial cells (See Figure 1.1, p.14)(99). Once glucose enters the interstitial space, it can be taken up by astrocytes by a lower molecular weight isoform of GLUT1 (45 kDa) or into neurons via glucose transporter 3 or glucose transporter 4 (GLUT3, GLUT4) (96). GLUT3 and GLUT4 have a high affinity for glucose, which ensures that glucose uptake is maximal even during low concentrations in the brain (100). Additional transporters have been identified as well, with limited patterns of expression- including GLUT2, GLUT5, and GLUT8 (96).

In neurons, GLUT3 is known to be the predominant glucose uptake mechanism (101, 102). Immunohistochemical co-labeling of GLUT3 with cell surface markers has revealed that it is basally expressed at neuronal plasma membranes (102, 103). Expression of GLUT3 in other

tissues is limited to areas with high glucose metabolism, including sperm and red blood cells (104). A role for GLUT3 in synaptic activity is suggested by the fact that its expression is up-regulated during neuronal maturation and synaptogenesis (105). Despite the fact that GLUT3 is predominantly expressed in the neuropil, the neuronal cell body has a similar metabolic rate. A study in which immunohistochemistry was combined with autoradiography demonstrated that GLUT3 expression patterns do not correlate with brain glucose uptake in a linear manner, and this disparity was especially clear in the hippocampus (106). For this reason it has been hypothesized that additional transporter isoforms may be responsible for glucose uptake in the somatodendritic portions of the cell (96). In areas of brain GLUT4 immunoreactivity, GLUT3 has demonstrated an overlapping expression profile with GLUT4 (107).

GLUT4 has been most frequently studied in muscle and adipose tissue due to its ability to rapidly amplify glucose uptake into these tissues (108). Unlike other glucose tranpsorters, GLUT4 is stored within the cell and stimulated by intracellular signaling cascades to translocate to the plasma membrane. This has led to the hypothesis that GLUT4-expressing cells in the brain have high energy demands that would necessitate such an intracellular store could supply during low-energy conditions (109). GLUT4 is abundant in neuronal cell bodies and processes, and this is known to correlate with the distribution of brain insulin receptors (110-112). Under basal conditions, GLUT4 is retained almost exclusively in intracellular compartments in GLUT4 Storage Vesicles (GSVs) (108, 113-116). Similar to localization observed in peripheral tissues, electron microscopy work has demonstrated that GLUT4 is maintained in an intracellular location in the brain, with the majority being expressed in the endoplasmic reticulum or Golgi bodies (117). In humans, PET imaging has demonstrated that physiological levels of insulin increase brain glucose uptake (15%), and augmentation of glucose uptake is most prominent in cortical regions (118). Additionally, disruption of insulin signaling in the brain in rodents results in nervous system dysfunction including decreased glucose uptake, increased lactate production, decreased ATP production, as well as deficient learning and memory (119-121). This evidence suggests that GLUT4 is capable of providing

supplementary glucose uptake in highly metabolic areas of the brain that impact cognitive function.

There is selective neuronal expression of GLUT4 in cortex, thalamus, cerebellum, and in the hippocampus (96, 107, 110, 122, 123). Specific areas of high expression include the cerebellum, pyramidal cells in CA1-CA3 of the hippocampus, the granule cell layer of the dentate, as well as layers 3, 5, and 6 of motor cortex. GLUT4 also labels dendritic spines within motor cortex and the hippocampus, forming asymmetric synapses (109, 117). Interestingly, Messari et al. (1998) noted that in cortical and hippocampal dendrites that have multiple contacts, only certain synapses expressed GLUT4. Post-transcriptional regulation of GLUT4 may also play a role in glucose metabolism in the brain, as there are areas where there are high levels of GLUT4 mRNA expression in accompanied by comparably less protein expression (123). These observations have led to the suggestion that GLUT4 expression may be dependent upon neural activity and demand (109, 112, 123). Similar neural demand-dependent regulation has also been suggested for other glucose transporters. For instance, there is an intracellular pool of GLUT1 in the endothelial cells of the BBB, and exposure of animals to chronic hypoglycemia or toxins leads to a significant increase in GLUT1 luminal expression (124, 125). In the forebrain, GLUT4 is expressed in cholinergic and parvalbumin-containing GABA-ergic neurons, which are known to have high energy demands (107). Sporadic Alzheimer's disease affects cholinergic forebrain neurons, and decreased glucose metabolism has been found to occur early in the stages of this disorder (126, 127). In summary, GLUT4 is a transporter linked to energy metabolism with an expression pattern and manner of regulation suggesting that it may have a role in cognitive functioning.

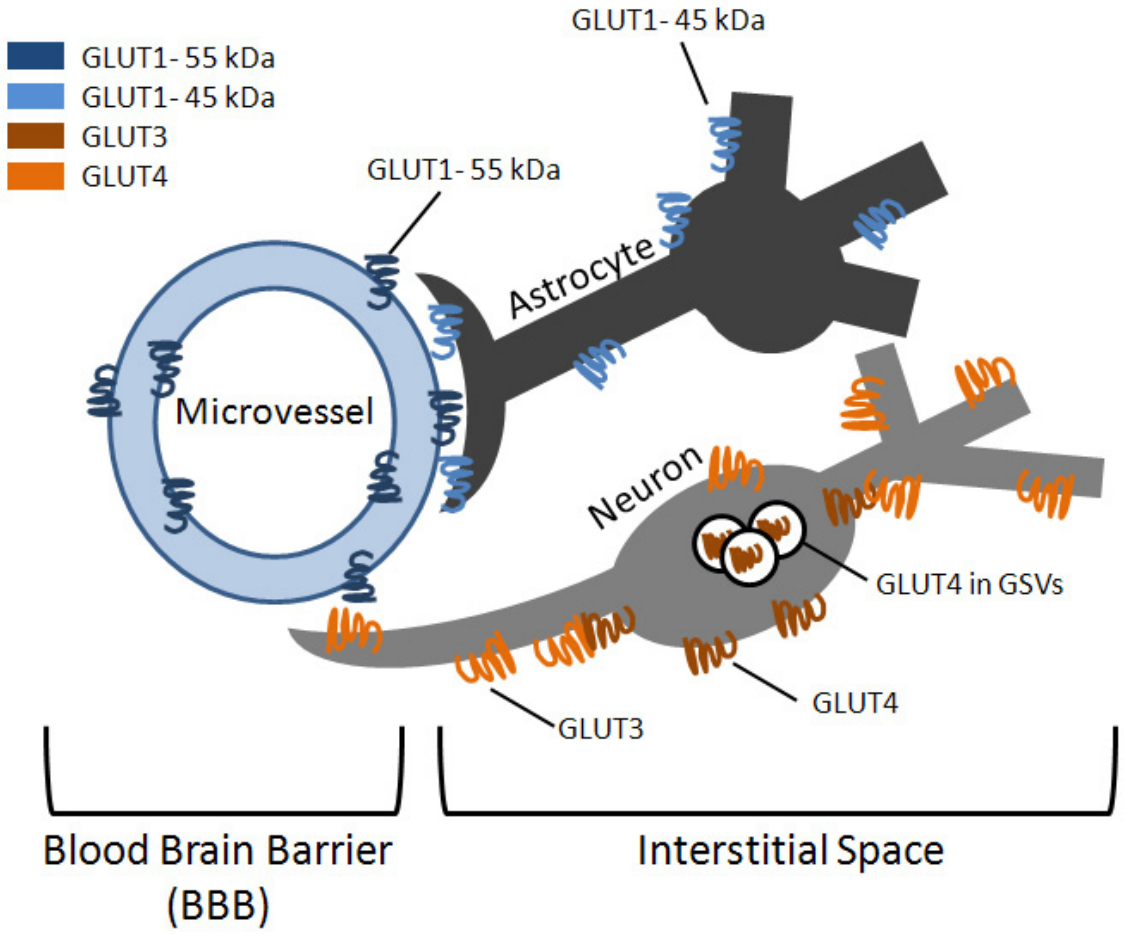

Figure 1.1 Mechanisms of brain glucose uptake. Glucose transporter 1 (GLUT1) is expressed in the luminal and abluminal surface of endothelial cells at the blood-brain barrier (BBB). Upon entry into the brain through GLUT1 in the BBB, glucose can be taken up by astrocytes through the lower molecular weight GLUT1 isoform, or into neurons through glucose transporter 3 or 4 (GLUT3, GLUT4). GLUT3 is expressed predominantly in the neuropil, while GLUT4 is expressed at the neuronal cell body. GLUT4 is located both in the plasma membrane and in GLUT4 Storage Vesicles (GSVs) in the cytoplasm.

Investigating the relationship between glucose uptake and neuropathology

Despite the extensive characterization of the bi-phasic changes in brain glucose uptake by imaging and autoradiography methods, changes in glucose transporter localization and expression have not been thoroughly examined after brain injury. Resected tissue from TBI patients 7-8 hours following trauma has demonstrated that there is a significant increase in GLUT1 expression at the blood-brain barrier (BBB) (128). This tissue would have been acquired during the described period of hyperglycolysis, and PET imaging has also

demonstrated increased brain glucose metabolism in severely injured patients at this time (129). Specific to neuronal glucose transport, Hamlin et al. (2001) found that in rats subjected to diffuse injury, GLUT3 was increased in the cortex 4-48 hours post-injury. Alterations in glucose uptake via GLUT4 has not yet been examined following TBI. However, GLUT4 has previously been demonstrated to participate in the neural response to excitotoxic insult (130). Western blotting of hippocampi that were processed by subcellular fractionation from animals dissected 4 hours following ischemic injury demonstrated that there is increased GLUT4 in the plasma membrane fraction. If an NMDA antagonist was administered, this increase in plasma membrane GLUT4 was attenuated.

Several diseases have been identified that link glucose metabolism and decreased cognition. In a recent study with Alzheimer's disease (AD) patients, it was shown that a decline in memory over time was associated with hypometabolism at baseline examination (131). Deep brain stimulation in AD patients for one year was shown to lead to an increased glucose metabolism, and a slowed cognitive decline (132). Intranasal insulin has also recently been tested as an AD therapy, and it is found to slow cognitive decline and progression of hypometabolism (133). Type 2 Diabetes (T2D) occurs as a result of decreased glucose uptake due to insulin resistance, and T2D cognitive decline has been thoroughly documented (134). The most well-established T2D-related cognitive deficit is dysfunctional learning and memory (135). Gold et al (2007) demonstrated that patients with controlled diabetes have reduced hippocampal volume, accompanied by declarative memory deficits (136). An animal model of T2D has also directly demonstrated long-term memory impairment in animals, and this was associated with decreased GLUT4 in the hippocampal plasma membrane (137). Thus, aberrant neuronal glucose uptake via GLUT4 has been implicated in the pathology of CNS disorder, although this has not yet been examined in the context of TBI.

AMP-activated Kinase (AMPK) and its role in the nervous system

AMP-activated Kinase (AMPK): a metabolism-regulating cascade present in the brain that is responsive to cell stress

One possible mechanistic explanation for changes in brain glucose uptake affecting TBI outcome is altered signaling of cascades that are responsive to cellular energy levels. A kinase proposed to link cellular metabolism and cognition is the energy-sensing AMP-activated protein kinase (AMPK) (138, 139). AMPK is an evolutionarily conserved serine/threonine kinase activated under low-energy conditions by phosphorylation at a single site (Threonine 172) by upstream kinases, Liver Kinase B-1 (LKB1) and Calcium/calmodulin-dependent protein kinase kinase-beta (CAMKKβ) (140) (Fig 1.2, next page). Activation under conditions of cell stress or low energy leads AMPK to promote processes resulting in the production of ATP (141, 142). AMPK is a heterotrimeric protein composed of an α catalytic subunit, and β and γ regulatory subunits. Upstream kinases bind to the α subunit of AMPK, whereas AMP and ATP compete for binding in the γ subunit (140). The γ subunit contains two domains, each of which binds an adenosine molecule (143). Binding of AMP to these sites leads to 1) allosteric AMPK activation, 2) conformational change which makes it a better substrate for upstream kinases, 3) inhibition of phosphatase activity (144). AMPK is predisposed to activation under low energy conditions because the ATP:AMP ratio is very high. Thus, processes that lead to a small decrease in ATP result in large increases in AMP concentration within the cell (145). The combination of AMP binding and phosphorylation by upstream kinases leads to a 1,000 fold increase in the activity of AMPK (146).

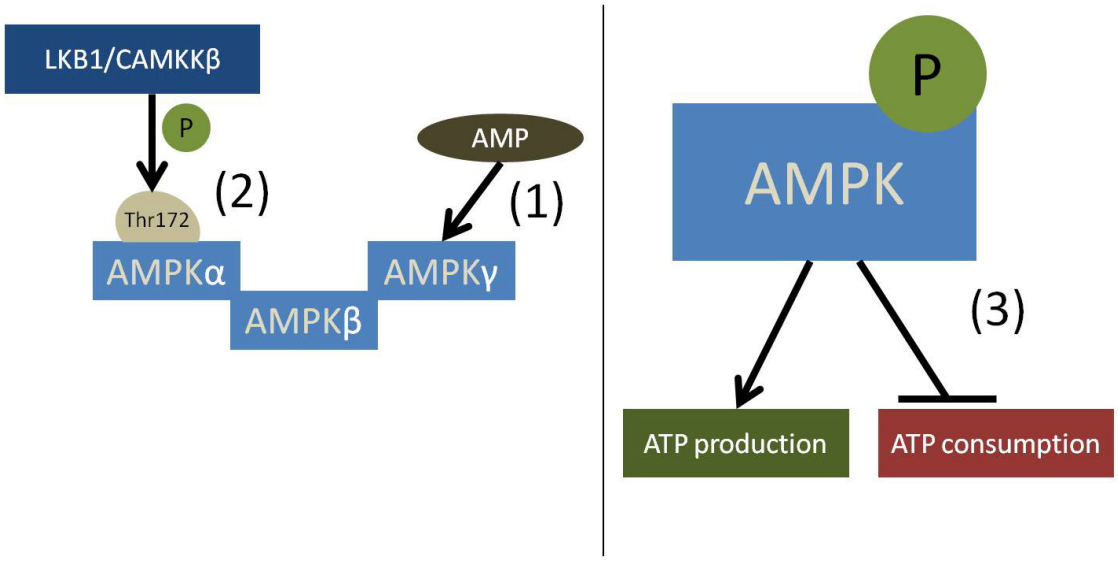

Figure 1.2 Mechanisms of AMPK activation. (1) AMPK is displayed in its heterotrimeric subunit form here. During low-energy conditions, AMPK is allosterically activated by binding of AMP to its γ subunit. (2) This induces a conformational change, making it a favorable substrate for phosphorylation on the Threonine 172 (Thr172) of the α subunit by LKB1 or CAMKKβ. (3) The net effect of AMPK phosphorylation is that activated AMPK promotes catabolic processes which promote ATP production, while simultaneously inhibiting anabolic processes requiring ATP consumption.

AMPK is expressed throughout the nervous system- particularly in areas known to have the highest rates of glucose consumption (147, 148). The areas of highest AMPK expression have been found to include cortex, hippocampus, cerebellum, spinal cord, and cranial nerves (147). In neurons, the α2 subunit appears to be expressed in the nucleus, while all other subunits are predominantly expressed in the cytoplasm (147, 149). Levels of the α2 catalytic subunit are much higher in embryonic development than the expression seen in the adult nervous system, and this was especially noted in pyramidal cells of the hippocampus and cortex (149). Specifically, AMPK expression is upregulated between embryonic days 10 and 14 during neuronal development, which has led to the hypothesis that it may be important to neurogenesis (147, 149). There is only low basal AMPK expression in astrocytes, suggesting that the role of AMPK is predominantly to restore neuronal energy levels (147).

AMPK phosphorylation promotes energy-producing processes, and inhibits anabolic processes. AMPK exerts its effects through both short-term and long-term mechanisms to

control the cell's energy metabolism. Activation of AMPK immediately promotes catabolic processes by phosphorylation of regulatory enzymes involved in glycolysis and other ATP-producing processes. AMPK activation also increases energy production in the long-term through increasing expression of transcription co-activators, which leads to increased expression of genes that promote catabolic activity within the cell (138, 150). For catabolic processes, some of the well-characterized targets of AMPK include promoting fatty acid uptake for oxidation via Acetyl CoA Carboxylase-2 (ACC-2), mitochondrial biogenesis via peroxisome proliferator activated receptor-γ coactivator 1-α (PGC1-α), and glucose uptake via Akt Substrate of 160 kDA (AS160) (151-153). The activation of various substrates (enzymes and transcription factors) is designed to increase ATP production within the cell. As mentioned, it also inhibits anabolic processes that consume energy, including protein synthesis via inhibition of mechanistic target of rapamycin (mTOR), regulation of cholesterol synthesis by inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, and many other enzymes involved in lipogenesis and gluconeogenesis (150). Thus, AMPK activation generally results in energy production and inhibits ATP-consuming, energetically costly processes.

The functions of AMPK in the brain have been studied extensively and include neuronal glucose transport, mitochondrial biogenesis, and regulation of neurodevelopment and neuronal viability (154). Surface expression of glucose transporters in cerebellar neurons is increased following excitotoxic insult, and this phenomenon is regulated by AMPK (155). Silencing of the AMPK catalytic subunit led to the attenuation of this transporter translocation, resulted in decreased mitochondrial functioning, and led to increased neuronal death. Additionally, ischemia results in a transient, 2-fold increase of hippocampal GLUT4 plasma membrane expression. This event is also correlated with an increase in AMPK activity (130). Although it has not yet been examined in the context of the nervous system, AMPK has also been shown to regulate transcription of GLUT4 (156, 157). Thus, it could potentially regulate both glucose uptake in the short term through transporter translocation, as well as perhaps long-term through controlling total levels of the protein.

In addition to its ability to augment glucose uptake, AMPK also enhances mitochondrial functioning and biogenesis. *In vitro* work has shown that treatment with AMPK activators leads to increased neuronal mRNA expression of mitochondrial proteins and transcription factors (158). This may also be beneficial following injury, as mitochondrial dysfunction has been observed post-TBI (78, 159). AMPK activators increase neuronal survival in response to glucose deprivation and other forms of cellular stress (149, 160). AMPK is vital for nervous system development, as deletion of the β subunit results in neuronal loss as well as progenitor cell abnormalities (161). A role for AMPK in neurodegenerative diseases such as Alzheimer's Disease and Huntington's Disease is also beginning to be uncovered (162, 163). Therefore, sufficient levels of AMPK activity appear to be important in proper nervous system functioning, as decreased AMPK expression results in abnormalities of neurodevelopment, neural metabolism, and leads to an increase in cell death and neurodegeneration.

The functional role of AMPK subunits has been further determined through genetic manipulation in rodents and in fruit flies. In both species, complete knockout of both α1 and α2 isoforms of the catalytic subunit is embryonic lethal (164, 165). Knockout of the α2 subunit alone results in mice that are relatively normal, except for exhibiting significant glucose intolerance (166). Deletion of the β subunit leads to significant atrophy of neuronal tissues, specifically a near complete-loss of the dentate gyrus, suggesting that AMPK activation affects neural stem cells during development (161). Finally, deletion of the γ subunit in fruit flies results in a reduced ability to synthesize cholesterol, accompanied by neurodegeneration (167). These findings demonstrate that activity of the catalytic subunit is essential for survival, and partial deletion of other subunits result in deficits related to glucose metabolism and functioning of the central nervous system.

Activated AMPK regulates glucose uptake through phosphorylation of Akt Substrate of 160 kDa (AS160)

Despite evidence that AMPK regulates glucose transporter surface expression in the brain, the mechanisms by which it governs this process are unclear. In peripheral tissues, it has been shown that AMPK can regulate glucose transport via its phosphorylation of Akt Substrate of 160 kDa (AS160), also known as TBC1D4 (168). Relevant to the current project, AMPK-regulated AS160 phosphorylation occurs in tissues during times of high-energy demand, such as during exercise-induced muscle contraction (169). AS160 is a Rab Guanosine Triphosphate-ase Activating Protein (RabGAP) that participates in the intracellular sequestering of GLUT4 by converting Rab to its inactive, GDP-bound state (170). Fusion of GLUT4 storage vesicles (GSVs) with the plasma membrane requires Rab proteins to be in their active, GTP-bound state. AS160 is considered to be the protein which links the extracellular signaling to the intracellular trafficking events associated with GLUT4 due to its interaction with Rab small G proteins (108). Thus AS160 could be an important part of the brain's response to low energy conditions, and its regulation may be perturbed under pathological conditions.

Structurally AS160 contains two N-terminus phosphotyrosine binding domains (PTB), and a C-terminus RabGAP domain. The identified regulators of AS160 include Akt, AMPK, and PKC. AS160 possesses 8 phosphorylation sites, with unique patterns of phosphorylation by its upstream kinases (171). Downstream, AS160 is known to target Rabs 2A, 8A, 8B, 10, 11 and 14 in a tissue-specific manner (172-174). In its unphosphorylated state, AS160 participates in GTP hydrolysis, leading its target Rabs to remain inactive. AS160 phosphorylation following insulin/AMPK stimulation turns off its GAP activity, allowing Rab targets take the active GTP form, and dissociation of AS160 from GLUT4 occurs. This results in translocation of GLUT4 to the plasma membrane (174) (Figure 1.3, next page). When active AMPK directly phosphorylates AS160, there is an increased trafficking of GLUT4 to the cell surface in skeletal muscle (175). Conversely, a decrease in AS160 phosphorylation would imply less GLUT4 in the membrane, and attenuated glucose uptake. Study of AMPK-null mice has also shown that

there is decreased basal phosphorylation of AS160 (175). Animals with decreased AS160 phosphorylation demonstrate a prolonged amount of time necessary to return to baseline blood glucose in response to glucose challenge, demonstrating the functional impact of this protein (176). It has also been shown that dietary treatments which increase AS160 phosphorylation improve glucose uptake in rats maintained on a high fat diet (177). Finally, AS160 has been shown to have clinical relevance, as AS160 phosphorylation is decreased in patients with Type 2 Diabetes in response to insulin stimulation when compared to non-diabetic controls (178).

Following identification of AS160 protein, Kane et al. (2002) performed immunoblotting with tissues from several different organs and concluded that the highest areas of expression were the brain and the pancreas. This finding has further been confirmed as knockout AS160 animals have demonstrated a significant decrease in expression of total AS160 in the brain (179). Therefore, although AS160 has not been directly examined in relation to the nervous system functioning, it is expressed in the brain.

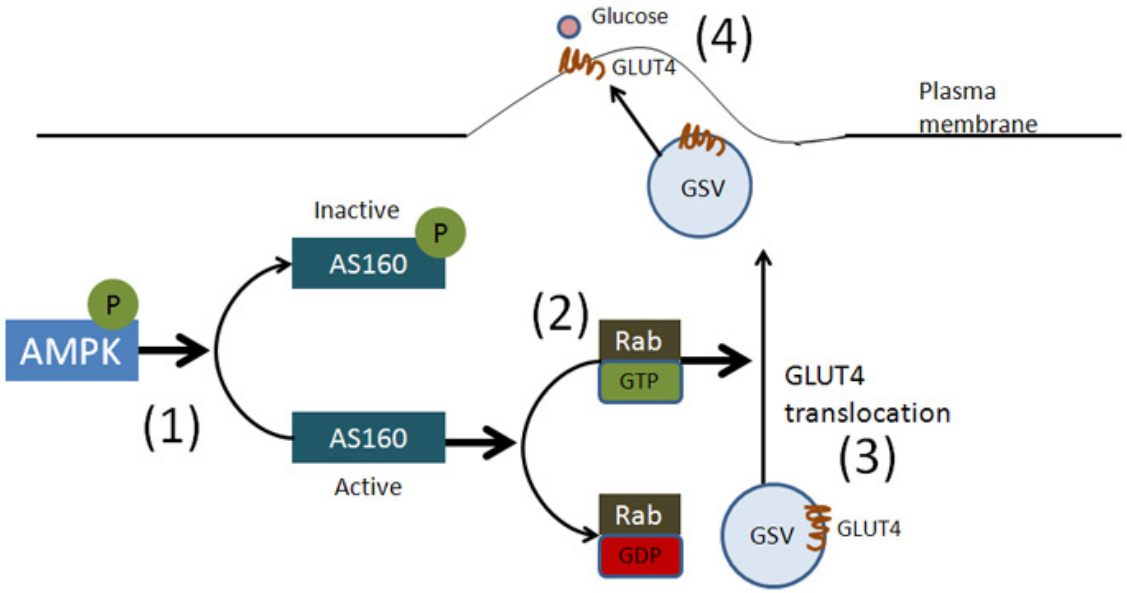

Figure 1.3 AMPK regulation of glucose transport by AS160 phosphorylation. (1) When AMPK is active and phosphorylated, it phosphorylates and inactivates AS160. When AS160 is not phosphorylated, it promotes the hydrolysis of GTP so that target Rabs (2A, 8A, 8B, 10, 11 and 14) remain in the inactive, GDP-bound state. (2) When AS160 is phosphorylated, Rabs are in the active, GTP-bound state. (3) Activated Rab leads to the trafficking of Glucose transporter 4 (GLUT4), maintained intracellular in GLUT4 Storage Vesicles (GSVs), to the plasma membrane. (4) After fusion with the plasma membrane, GLUT4 can uptake glucose.

Activated AMPK inhibits protein synthesis via its regulation of the mechanistic target of rapamycin pathway (mTOR)

AMPK inhibits anabolic processes through multiple targets, one of which is the mechanistic target of rapamycin (mTOR) (Fig 1.4, next page) (180). mTOR is an evolutionarily conserved serine/threonine kinase that regulates many energy-dependent processes, such as cell survival and cell growth. AMPK specifically regulates activation of mTOR complex 1 (mTORC1), a multiprotein complex of mTOR and other proteins that acts as a cellular energy gauge, responding to input from both nutrients and growth factors (142, 181). mTORC1 is regulated by the tumor suppressor genes TSC1 (hamartin) and TSC2 (tuberin) (182, 183). AMPK directly phosphorylates mTORC1-regulators raptor (184) (not shown here) and TSC2 (185, 186). Similar to AMPK regulation of AS160, phosphorylated TSC2 exerts GAP activity acts upon the small GTPase Rheb (186). Thus, active TSC2 leads to a decrease in the ratio of GTP-bound versus GDP-bound Rheb. When Rheb is in its active, GTP-bound form, it physically interacts with mammalian target of rapamycin (mTOR) and promotes its activation by phosphorylation (186, 187). It has been demonstrated that application of an AMPK activator inhibits mTORC1 in a TSC2-dependent manner (188). Other growth factors, such as Akt, also phosphorylate TSC2 at other residues, although in these cases phosphorylation of TSC2 leads it to become inactive (189).

The rate-limiting step of protein synthesis is the initiation of translation, and mTORC1 is known to regulate this process mainly through protein S6 and eukaryotic initiation factor 4Ebinding protein-1 (4E-BP1, not discussed here) (190). Activated mTORC1 promotes protein synthesis by phosphorylating S6 Kinase at (S6K), leading to the phosphorylation of its downstream target, protein S6 (S6) (184, 191, 192). Protein S6 is part of the ribosomal complex, and its phosphorylation leads to increased translation (193). It has previously been shown that mTOR activity is increased following TBI, with prolonged and robust increases noted in phosphorylation of protein S6 (194, 195). Treatment with rapamycin aimed to

decrease activity of the mTOR pathway, either alone or in combination with an Akt inhibitor, has been shown to improve post-injury outcome in experimental models (194, 196). Increased mTOR activity is consistent with a suppression of AMPK activity, and the current experimental literature suggests that inhibition of this pathway in an AMPK-dependent manner may be beneficial for injury outcome.

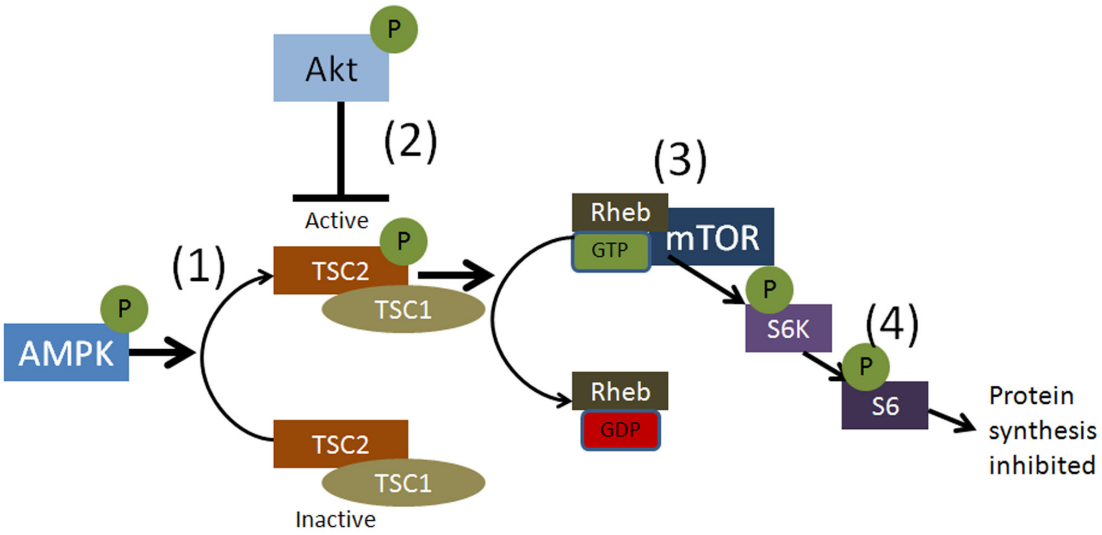

Figure 1.4 AMPK phosphorylation inhibits protein synthesis by its regulation of mTOR. (1) When AMPK is active and phosphorylated, it phosphorylates and activates TSC2. (2) Active Akt also phosphorylates TSC2 at a separate residue, however this event results in its inhibition. (3) When TSC2 is active, Rheb is in the inactive, GDP-bound state. When Rheb is active and GTP bound, it activates mTOR. (4) Active mTOR phosphorylates S6 Kinase (S6K) S6K, which phosphorylates protein S6. Thus, the end product of mTOR activation is protein synthesis, and this process is inhibited when AMPK is negatively regulated under low-energy conditions.

AMPK is a potential therapeutic target for improving TBI outcome

Increased AMPK activation under low-energy conditions serves as a signal to the body that less energy resources are available, and ATP must be conserved. Decreased cellular energy supply can be destructive to the central nervous system, specifically resulting in oxidative stress, cell death, and excitotoxicity (197). Increasing activation of metabolic cascades that enhance the efficiency of ATP use and production, such as AMPK, could improve TBI outcome. Treatment with AMPK activators would be clinically translatable, as there are FDA-approved drugs that activate AMPK, including Metformin, the most widely prescribed Type 2 Diabetes drug. In addition to AMPK-activating drugs, there are many natural supplements which have been identified as AMPK activators that could eventually be investigated for their long-term application following head injury (198).

Enhancing the efficiency of ATP production could attenuate cellular pathology related to metabolic dysfunction, as well as improve performance in cognitive tasks that are energetically demanding. Treatment with AMPK activators has shown to improve long-term memory in uninjured animals, specifically in tasks with high cognitive demands (199-201). In the Morris water maze task, AMPK activation improves memory recall in probe trials 24 hours post-training (200, 201). One study demonstrated improved spatial memory following chronic treatment with Metformin (202, 203). There is also evidence that short-term treatment with AMPK activators following trauma may improve long-term outcome. Treatment with Metformin following ischemia has been shown to decrease contusion volume and improve motor skills (204). Administration of newly discovered AMPK-activating drug FM19G11 to animals for one week following spinal cord injury increases GLUT4 translocation to the plasma membrane, and this treatment has been shown to improve functional recovery one month post-trauma (205). In animals experiencing an energy crisis following injury, AMPK activation could increase the supply of ATP to distressed tissue as well as minimizing unnecessary ATP expenditures by the cell.

In response to TBI, AMPK activation is *decreased* at seven days following moderate injury. Dietary supplementation with AMPK activators for several weeks prior to injury can reverse this deficit and can also increase expression of proteins necessary for synaptic plasticity (206). Whether direct AMPK activation can improve cognitive outcome has not been tested. Furthermore although pre-injury drug treatment to augment AMPK has been examined, no post-injury AMPK activation treatment has been attempted. Theoretically, under conditions of decreased AMPK phosphorylation following TBI, there may be decreased GLUT4 transport due to decreased phosphorylation of AS160 (Figure 1.5, next page). There would also be decreased inhibition of the mTOR pathway, resulting in increased phosphorylation of its downstream targets. As metabolic recovery has been associated with improvement in behavioral performance following injury, we reasoned that enhancing activity of the AMPK pathway would lessen metabolic dysfunction following injury, and improve long-term memory. The work presented in this thesis suggests that there is decreased AMPK activity post-injury, and that preventing this decreased period of AMPK activity is beneficial to post-injury outcome.

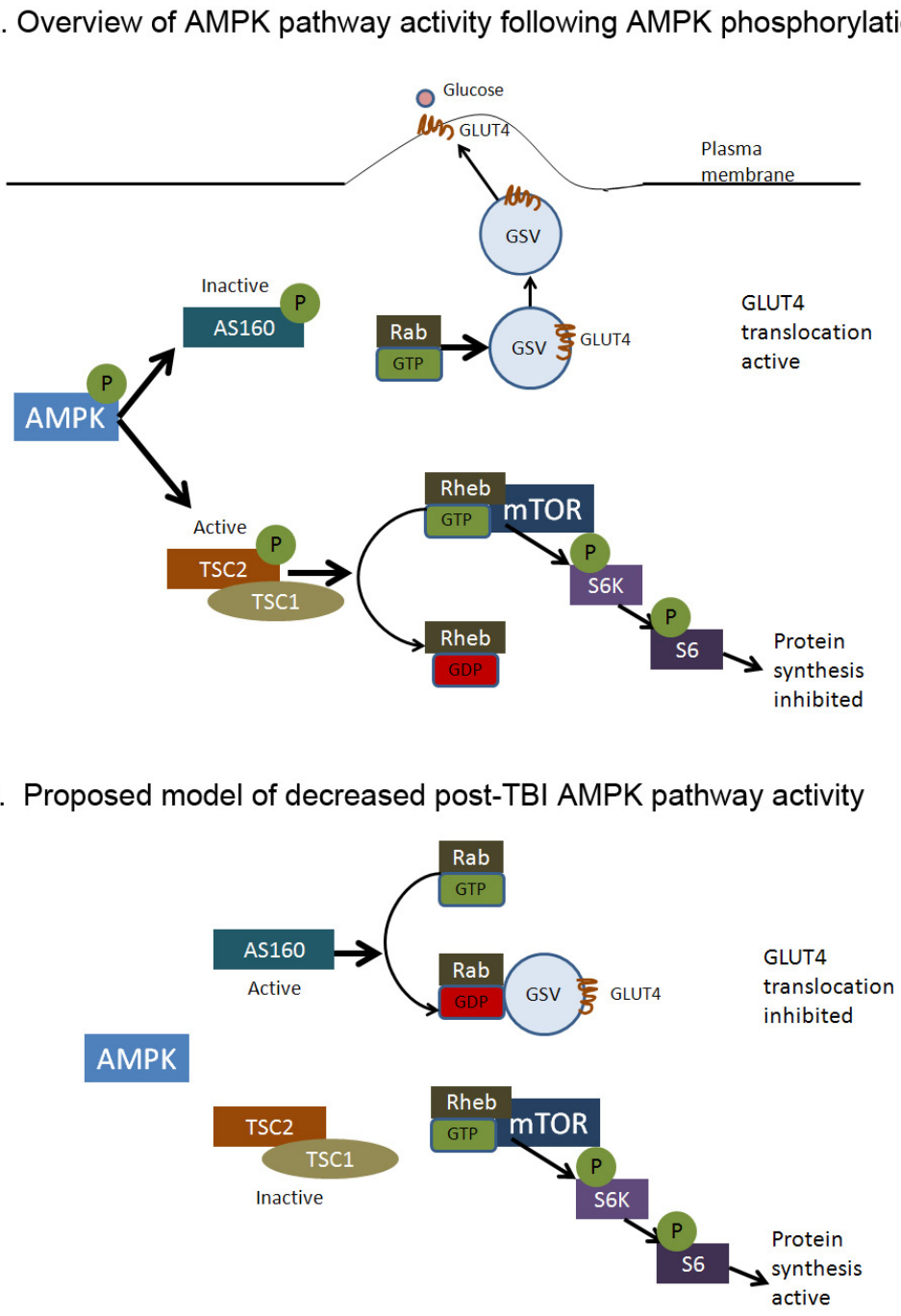

Figure 1.5 Overview of hypothesized alterations in AMPK signaling post-TBI. (A) Overview of AMPK pathway under active, phosphorylated conditions. AS160 is phosphorylated, and Rab is in its active, GTP bound form. This promotes translocation of GLUT4 to the plasma membrane. AMPK also phosphorylates and activates TSC2, leading Rheb to be inactive. As a results, there is less phosphorylation of mTOR pathway components. (B) We hypothesize that there is a decrease in AMPK phosphorylation post-injury. AS160 remains unphosphorylated. AS160 GTP-ase activity will be active, and Rab will be in the inactive, GDP-bound state. As a result, GLUT4 will remain intracellular. Inactive AMPK will lead mTOR-regulator TSC2 to also be less active. Rheb will be activated, and as a result mTOR activity, specifically phosphorylation of protein S6, will be increased post-injury.

Central Hypothesis and Specific Aims:

We hypothesize that activity of the AMPK pathway is transiently decreased in the brain post-injury, and that increasing AMPK activity following TBI would improve cognitive outcome. Additionally, inhibiting AMPK target GLUT4 in the brain post-injury would negatively impact post-injury cognitive outcome.

Aim 1. To examine the impact of artificially increased blood glucose on TBI cognitive and pathological outcome, and to determine whether this treatment impacts central activation of AMPK. High blood glucose in the acute post-injury period is correlated to increased mortality in the clinical literature. If an increased concentration of glucose in the blood at the time of a moderate injury has a direct, negative effect on pathological or cognitive outcome has not been thoroughly examined. Based on the clinical literature, we hypothesized that increasing blood glucose at the time of injury would be detrimental to post-injury outcome. To investigate this, we artificially induced hyperglycemia by systemic injection of glucose in mice 15 minutes pre-injury and behaviorally tested the recovery of motor skills and of water maze performance (Hill et al 2010). Following the behavioral experiment, we examined contusion volume in a subset of animals and found no difference between groups. There was also no effect of hyperglycemia on cerebral edema, as measured in a separate set of animals. Finally, a biochemical examination was completed in animals with artificially induced hyperglycemia in which phosphorylation of AMPK and AS160 phosphorylation in treated animals was compared to those receiving vehicle. We demonstrated that AMPK and AS160 phosphorylation decreases post-injury in vehicle-treated animals, and glucose administration did not alter phosphorylation of these proteins.

Aim 2. To determine the phosphorylation levels of AMPK and its targets AS160 and protein S6 following injury, and examine tissue expression of AS160 in areas related to cognition. It has previously been shown that AMPK is decreased post-injury, the time course of this alteration has not been investigated. Further, although AMPK has been shown to regulate GLUT4 trafficking in the nervous system, expression of AMPK target protein AS160 in the brain has also not been examined. We hypothesized that there would be a transient decrease in AMPK activity, and that this would occur during the observed period of suppressed glucose uptake. We investigated the phosphorylation of AMPK, AS160, and protein S6 via western blotting using hippocampal and cortical samples from multiple post-injury time points. We found that there was a transient decrease of AMPK, and that phosphorylation of AMPK targets AS160 and protein S6 were altered in a manner consistent with this metabolic suppression. We then conducted immunohistochemistry to examine the location of AS160, and found it to be expressed in neuronal cell layers throughout the hippocampus and cortex, in a manner consistent with its role regulating GLUT4 translocation. Finally, observational immunohistochemistry work conducted with tissues collected at 24 hours and 3 days post-injury suggests that AS160 phosphorylation and GLUT4 expression are decreased following TBI, consistent with the western blotting results.

Aim 3. To investigate whether peripheral drug application of an AMPK activator will lead to increased AMPK phosphorylation following injury, and if this treatment will be associated with increased cognitive outcome. The time course data results from Aim 2 show that TBI causes a reduction in AMPK activity. In order to examine if this decrease is correlated with memory impairments, I tested if post-injury administration of AMPK activator 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR) reverses the decrease in post-injury AMPK phosphorylation, and if this improves hippocampal dependent learning and memory. Animals were tested for motor skills recovery, water maze performance, and context

discrimination. This experiment demonstrates that increasing AMPK activity during the reported period of reduced glucose metabolism can be used to improve cognitive outcome following TBI.

Aim 4. To explore whether brain-specific inhibition of AMPK target GLUT4 is detrimental to post-injury cognitive outcome. Evidence suggests that glucose uptake via GLUT4 is activated following neural insult, and that expression of this transporter plays an important role in learning and memory. Given that we found AMPK activation to improve cognitive outcome, we hypothesized that direct brain inhibition of AMPK target GLUT4 by intracerebroventricular injection immediately prior to injury result in decreased cognitive outcome. Animals were tested using the exact paradigm as outlined in Aim 3. We found that GLUT4 led to impaired memory, and histopathological examination in these animals leads to the suggestion that GLUT4 inhibition generally exacerbates TBI pathology.

Project Significance:

Brain injury is an issue impacting a large number of individuals worldwide and often resulting in life-long cognitive deficits. Despite the fact that secondary damage that contributes to this cognitive dysfunction occurs long after the initial impact, there are no treatments to improve TBI functional outcome. A secondary pathology widely observed following and correlated to cognitive outcome post-injury is dysfunctional metabolism. The efficacy and safety of peripheral insulin administration to lower blood glucose improve this perturbed metabolic state has been questioned in recent years, given that the brain is an obligate consumer of glucose. Beyond TBI, aberrant brain metabolism has been linked to a cognitive disorder in a number of diseases, including Alzheimer's disease and Type 2 Diabetes. The role of signaling cascades such as AMP-activated Kinase (AMPK), and its targets related to glucose uptake, including Akt Substrate of 160 kDa (AS160), and Glucose transporter 4 (GLUT4) have only been preliminarily examined. Additionally, it has been shown that activation of the negatively-regulated AMPK target, the mechanistic Target of Rapamycin (mTOR) pathway is increased following injury, and that combination treatments to inhibit this event improve cognitive outcome. Therefore, a greater understanding of how AMPK signaling is altered in a pathological state such as TBI and contributes to functional outcome may represent a new therapeutic option for injured patients, as well as opening a new avenue of research for other neurodegenerative disorders which affect a large portion of the population.

CHAPTER II. THE EFFECT OF HIGH BLOOD GLUCOSE ON TBI OUTCOME

Introduction:

In patients with severe TBI, hyperglycemia in the first 24 hours post-injury is correlated with increased mortality and decreased long-term cognitive outcome (48, 49, 58, 207-209). A blood glucose above 200 mg/dL in the first 24 hours post-injury is associated with 3.6 fold increase in the mortality rate (209). Beyond its impact on mortality, hyperglycemia is associated with decreased long-term neurological outcome in TBI survivors (48, 49, 208, 210). Despite the fact that hyperglycemia is negatively correlated with outcome, no benefit has been demonstrated for strict maintenance of blood glucose in head-injured patients. Studies have found that strict maintenance of blood glucose in TBI patients results in increased episodes of hypoglycemia without improvement in cognitive outcome, and increased markers of cellular stress (51, 52, 183, 211). This suggests that although hyperglycemia may be correlated to worsened outcome, the presence of high levels of glucose may not directly exacerbate secondary TBI pathologies.

In other experimental models of neural insult such as stroke, it has been shown that hyperglycemia worsens secondary pathology (212-215). The effect of glucose pre-treatment on increased mortality and tissue damage in these animals is attributed to lactacidosis as a result of increased anaerobic glycolysis (216). In a rat model of severe TBI, it was demonstrated that intraperitoneal administration of glucose 20 min prior to, but not 20 min post, cortical impact injury led to an increase in post-injury contusion volume (217). A similar effect has been seen with glucose treatment 5 minutes post-injury, and this was histopathologically correlated to an increase in inflammatory processes (217, 218). Other groups have found no effect of high blood glucose in the immediate post-injury period on pathology or functional outcome, and hyperglycemia did not result in alterations of tissue pH and lactacidosis as was seen with this treatment in ischemia (219). Thus, whether increasing blood glucose directly impacts TBI outcome, especially at a moderate level of injury, remains unclear.

One possibility is that high blood glucose does not directly contribute to TBI pathology, that it is instead simply a symptom of an underlying metabolic suppression. There is evidence

from the experimental literature that this may be a factor contributing to post-injury hyperglycemia. Following mid-cerebral artery occlusion (MCAO), mice have an acute increase in their fasting blood glucose levels up to 24 hours post-stroke, suggesting transient glucose intolerance. This period of metabolic suppression was also associated with behavioral disturbance (57). Similarly, in severely brain injured rats there is also a decrease in peripheral insulin sensitivity, and this is accompanied by decreased mRNA expression of glucose transporter-4 (GLUT4) compared to sham animals in adipose tissue (56).

In this experiment, we investigated the impact of a transient increase in blood glucose on TBI outcome in a model of moderate injury. In this work, we found a transient episode of hyperglycemia did not impact behavioral outcome, contusion volume, or cerebral edema. As described in Chapter 1, it is known that the activity of secondary signaling cascades regulate glucose uptake and may contribute to this metabolic suppression, with AMP-activated kinase (AMPK) being a specific cascade of interest. We examined the activation of AMPK and its target which controls glucose uptake, Akt Substrate of 160 kDa (AS160), in brain tissues of animals treated in a manner consistent with the behavioral study to examine the state of AMPK signaling post-injury. The results presented here demonstrate that there was a decrease in phosphorylation of the AS160 following CCI, and that this was not altered by administration of glucose.

Methods:

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Julia Hill, J., J. Zhao, and P. K. Dash. 2010. High blood glucose does not adversely affect outcome in moderately brain-injured rodents. Journal of neurotrauma 27:1439-1448. Figure numbers altered for reproduction. Reprinted with permission from Lieber Publishing.

> Materials: Glucose was purchased from Sigma. Antibodies for phosphorylated AMPK (Threonine 172), total AMPK, protein S6, phosphorylated protein S6 (Serine 235/236), and phosphorylated AS160 (Serine 588 and Threonine 642) were all purchased from Cell Signaling Technology (Danvers, MA). The antibody for total AS160 was purchased from Sigma (St. Louis, MO), and GLUT4 was purchased from Pierce Biotechnology (Rockford, IL).
>
> Animals: Adult male C57/BL6 mice weighing 25-35 grams and Sprague Dawley rats weighing 350-400 grams were purchased from Charles River Laboratories (Wilmington, MA, USA). Animals were group housed and maintained on a 12-h

light/dark cycle with *ad libitum* access to food and water. Male Sprague-Dawley rats (275-300g) were purchased from Charles River Laboratories (Wilmington, MA) and housed two per cage. All experimental procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the recommendations provided in the *Guide for the Care and Use of Laboratory Animals*.

Controlled cortical impact injury: Mice were initially anesthetized with 5% isoflurane and 1:1mixture of N2O/O2. While being maintained under anesthesia (2% isoflurane and 1:1 mixture of N2O/O2), mice were placed in a stereotaxic frame and a 5 mm craniotomy (halfway between bregma and lambda, 0.5 mm lateral to midline) was performed. A heating pad was used to maintain body temperature at 37 °C. Using a 3 mm diameter impact tip and an electromagnetic cortical impact device, a single impact was delivered to the parietal association cortex at an angle of 10° from the vertical plane. The injury depth for mice was 1.6 mm at a velocity of 4.0 m/s. For all rat experiments, the injury was performed using a pneumatic injury device, with a depth of 1.5 mm at a velocity of 4.9 m/s. The diameter of the impact tip for the pneumatic device was 5 mm. After injury, the scalp was be sutured closed with stainless steel surgical staples. The duration of suppression of righting response was then measured. The righting response was defined as the animal's ability to right itself three times consecutively after being placed on its back. After the completion of assessment of righting response, the animals were given time to recuperate in a warming chamber before being returned to their home cages. Animals were weighed daily after the injury for the first 3 days, then weekly thereafter.

Blood glucose testing: For blood draws, mice were placed in a restraining device, and approximately 0.6 µL of blood was extracted from the tail vein with a 25-gauge needle. An Aviva Accu-check monitor (Roche Diagnostics) was used in both experiments to analyze blood glucose levels from each sample.

Acute hyperglycemia: A previous TBI study has shown that administration of a dose of glucose 2.2 g/kg prior to injury results in significant hyperglycemia at the time of injury (217). Guided by this study, we employed the 2.2 g/kg dose of glucose 15 minutes prior to controlled cortical impact injury. Animals were administered glucose in saline, or saline-only intraperitoneally.

Western blotting: Animals were administered 2.2g/kg of glucose 15 minutes prior to injury, and then cortical and hippocampal tissue was collected from all groups and snap-frozen post-injury. Protein concentration was measured using a Bradford assay with BSA as the standard. Samples loaded with equal amounts of protein were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Billerica, MA), followed by 1 hour of blocking in Tris-buffered saline with Triton X-100(TBST) plus 5% BSA. Membranes were incubated in primary antibody overnight at 4 degrees. After extensive washing in TBST, immunoreactivity was assessed by an alkaline phosphatase–conjugated secondary antibody and a CDP-star

chemiluminescent substrate (New England Biolabs, Ipswich, MA). The optical density of the immunoreactive bands was measured using ImageJ (Available from the NIH). All proteins examined, phosphorylated and total, were corrected by their percent expression of β-actin from the same membrane.

Motor skills testing: Vestibulomotor and motor skills were tested using the beam balance and foot fault tasks, respectively (220-223). These tests were conducted on days 2, 3, 4, and 8 post-injury. Three testing trials were given daily and averaged for each animal. Animals were placed on a cylindrical metallic beam (diameter= 1cm) and the time spent balancing was recorded. Paw placement was evaluated by placing the animal on a wire grid (opening size of 1x1cm) and counting the number of foot faults out of a total of 50 steps (224). A foot fault was defined as when a front paw misses and appears below the plane of the grid. Paw placement was repeated three times and averaged for each animal. Non-injured animals were tested in the manner described prior to the beginning of the study to establish baseline values for each measure.

Morris water maze task: Mice were trained in the standard Morris water maze task, as previously described (225-228). Over 8 days, animals were trained to find the location of a stationary, hidden platform with four separate trials per day. In each trial, the mouse was placed into the tank facing the wall, and then allowed to search for the submerged platform for 60 seconds. If the animal did not find the platform during this time period, it was led to the location by the investigator. The animal was then required to stay on the platform for 30 seconds before removal from the tank. During the four minute inter-trial interval period, animals were placed in a 37˚C warming cage. Probe trials were conducted 30 minutes and 24 hours after the completion of training. In probe trials, the hidden platform was removed from the tank and animals were allowed to search for it for 60 seconds. The search path was monitored using a tracking device connected to a video camera (Ethovision, Noldus). The data was analyzed for latency to the first platform crossing, distance travelled to platform, swimming speed, quadrant preference, and number of platform crossings. Quadrant preference analysis, which is used to analyze a group's ability to remember the general spatial location of the platform, the tank was divided in the tracking program into four zones of equal area. Within each group, the data was then analyzed to determine the amount of time spent searching for the platform in each quadrant.

Contusion volume measurement: Following the completion of the behavioral studies, approximately 32 days following the injury, mice were deeply anesthetized with sodium pentobarbital (100mg/kg) and then transcardially perfused with 100 mL of phosphate buffered saline (PBS), followed by 100 mL of 4% paraformaldehyde. Brains were removed, postfixed overnight in perfusant, and then cryoprotected in a 30% sucrose solution. Contusion volume was estimated by previously described methods (229). In brief, cryosections (40 micron thickness) spanning the rostral-caudal extent of the injured cortex were selected and stained with cresyl violet. The area of cortical tissue loss for each section was carefully outlined using *Image J,* from the National Institutes of Health. Contusion volume was calculated using the equation $A_1(0.5X_1) + A_2(0.5X_1 + 0.5X_2) + A_{n-1}(0.5\ X_{n-1} + 0.5X_n) + A_n(0.5X_n)$, where A is the area ($mm^2$) of the contusion for each slice, and X is the distance (mm) between two sequential slices. Group differences were then assessed.

Cerebral edema measurement: The animals were decapitated, and the brain removed as quickly as possible in order to avoid water loss (230). The cerebellum was removed, and then the brain was bisected into the ipsilateral and contralateral hemispheres, relative to the site of injury. These were quickly weighed 3 times each in order to yield an average wet weight, and then placed in an oven. 3 days later, the brains were re-weighed in order to produce a dry weight. 24 hours after this the brains were weighed again in order to ensure that the dry weight was stable. The percent tissue water was calculated using the following formula: [(wet wt – dry wt)/wet wt x100]. This measure was calculated both for the ipsilateral and contralateral hemispheres.

Statistics: In all experiments, data collected from the same animal with one or more factors, such blood glucose level analysis, latency to platform, probe trial quadrant preference, was subjected to repeated measures (RM) ANOVA. Data comparing only one factor between groups, including probe trial data analysis, and contusion volume, was subject to t-tests. In cases in which data did not pass the Shapiro-Wilk normality test, a non-parametric rank-sum test (ANOVA or t-test, depending on data to be analyzed) was performed. These cases are noted in the text.

Results:

Acute elevation of blood glucose does not affect recovery of vestibulomotor or motor skill performance*:* To investigate if acutely elevated glucose at the time of injury affected subsequent measures of motor performance and cognition, we administered either 2.2 g/kg glucose (n=10) or vehicle (n=11) (i.p.) 15 min prior to TBI. A schematic of the experimental timeline is presented in 2.1A. As expected, administration of this dose led to a significant increase of blood glucose in the treated mice (2-way RM ANOVA, time-group interaction, p=.027; Fig 2.1B). No significant difference was found between glucose and vehicle animals in rate of recovery of vestibulmotor faculties, as judged by beam balance (Fig 2.1C). Similarly, foot fault testing demonstrated that there was no difference between the two groups in motor skill recovery (Fig 2.1D). In both cases, performance of animals returned to pre-injury levels by 8 days post-injury.

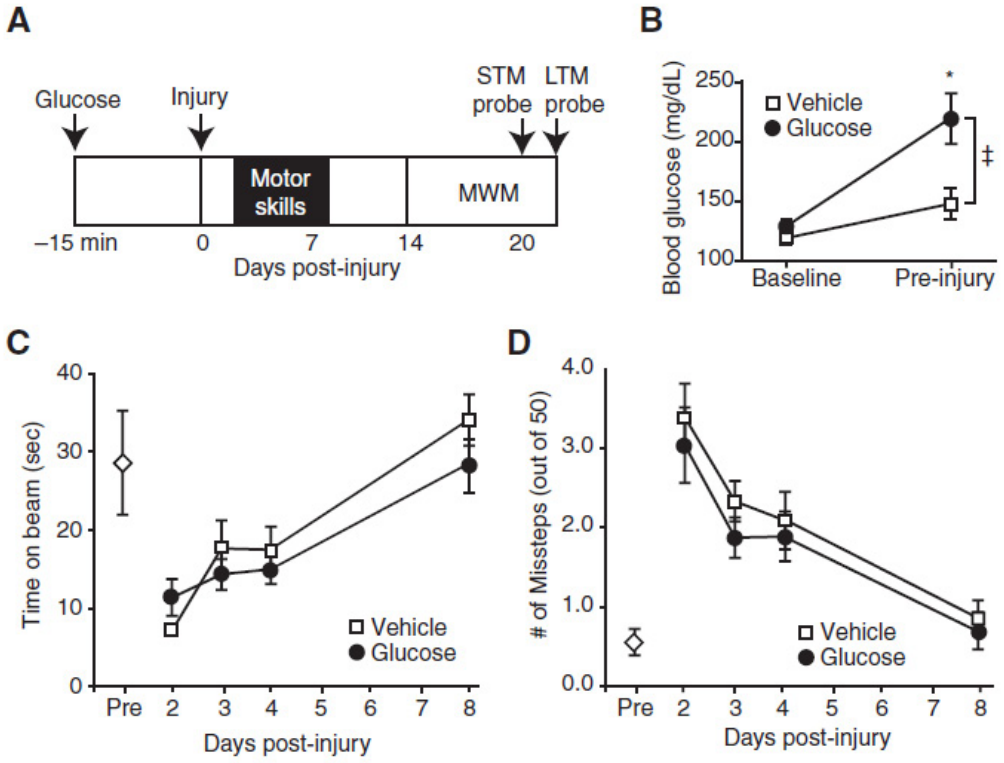

Figure 2.1. Hyperglycemia at time of injury does not affect recovery of motor skills. (A) Schematic diagram showing the paradigm for glucose injection, injury, motor assessment, and behavioral testing.(B) Intraperitoneal injection of glucose led to a significant increase in blood glucose immediately prior to injury. Post-injury, there is no difference between vehicle and glucose animals in performance of (C) the balance beam task, or in (D) the foot fault task. For figures 3.1-3.3, vehicle, n=11; glucose, n=10. Open diamond= Task performance pre-injury. Data are presented as mean±SEM. *p<0.05.

Acute elevation of blood glucose does not impair neurocognitive outcome: The effect of acute hyperglycemia at the time of injury on learning and memory was assessed 14 days post-TBI. Animals that received glucose 15 minutes pre-injury did not show any significant difference in their performance in the Morris water maze, as evidenced by similar latency to platform in both groups across all days of training (2-way RM ANOVA, p=0.724; Fig. 2.2A). This was further confirmed by similar latency to platform in both the short-term and long-term probe trials, designed to test short-term (STM) and long-term memory (LTM), respectively (Fig 2.2B).

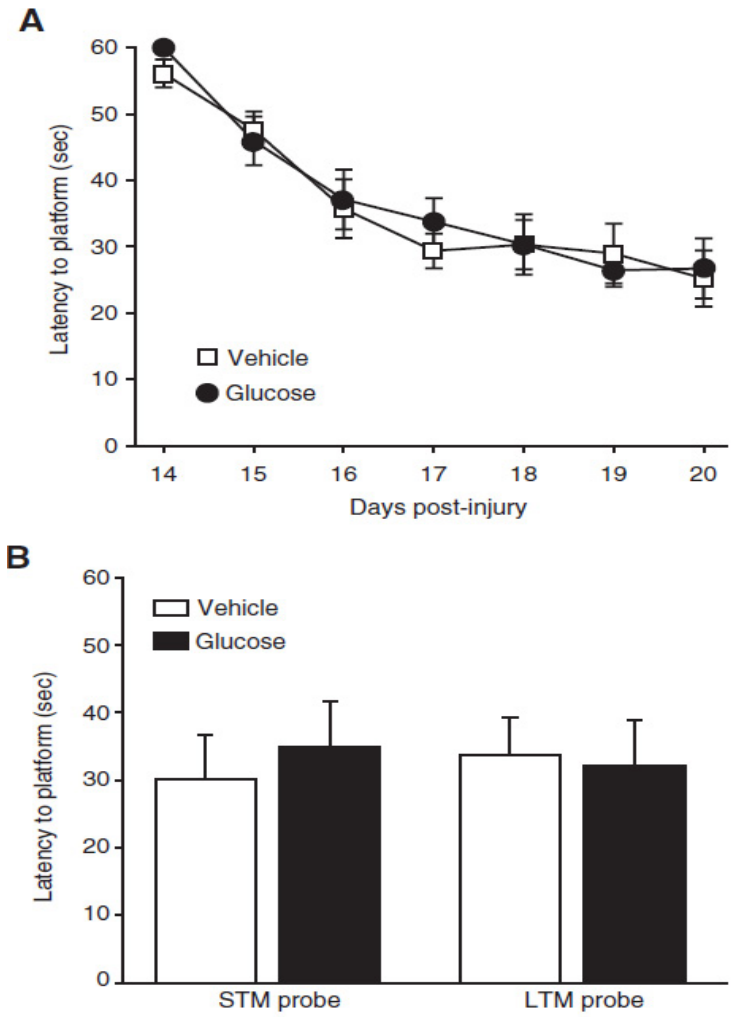

Figure 2.2. Pre-injury acute elevation of blood glucose does not result in overt differences in behavioral performance. Treatment with glucose did not result in a change in behavior in either the (A) acquisition of MWM, or (B) changes in latency to platform during short-term (STM) 30 minutes following the completion of training and long-term memory (LTM) 24 hours after the completion of testing. Data are presented as mean ± SEM. *p<0.05.

Next, we analyzed the quadrant preference of the animals during both LTM and STM probe trial performance (STM data, Fig 2.3A). Neither group showed a significant quadrant preference during the LTM probe trials (vehicle, p=0.138; glucose, p=0.530). However, both groups showed an increased quadrant preference during the STM probe trials (ANOVA 1-way RM rank-sum, both groups p<.001). A post-hoc Holm-Sidak pairwise comparison revealed that the glucose animals spent significantly more time in the platform-containing quadrant than any other quadrant, while the vehicle animals preferred the platform-containing quadrant and an adjacent quadrant equally. Despite this difference in preference, there was no significant difference in the number of platform crossings (t-test, p=0.569; Fig 2.3B). There was also no significant difference in swim speed during the STM probe (t-test, p=0.482; Fig 2.3C).

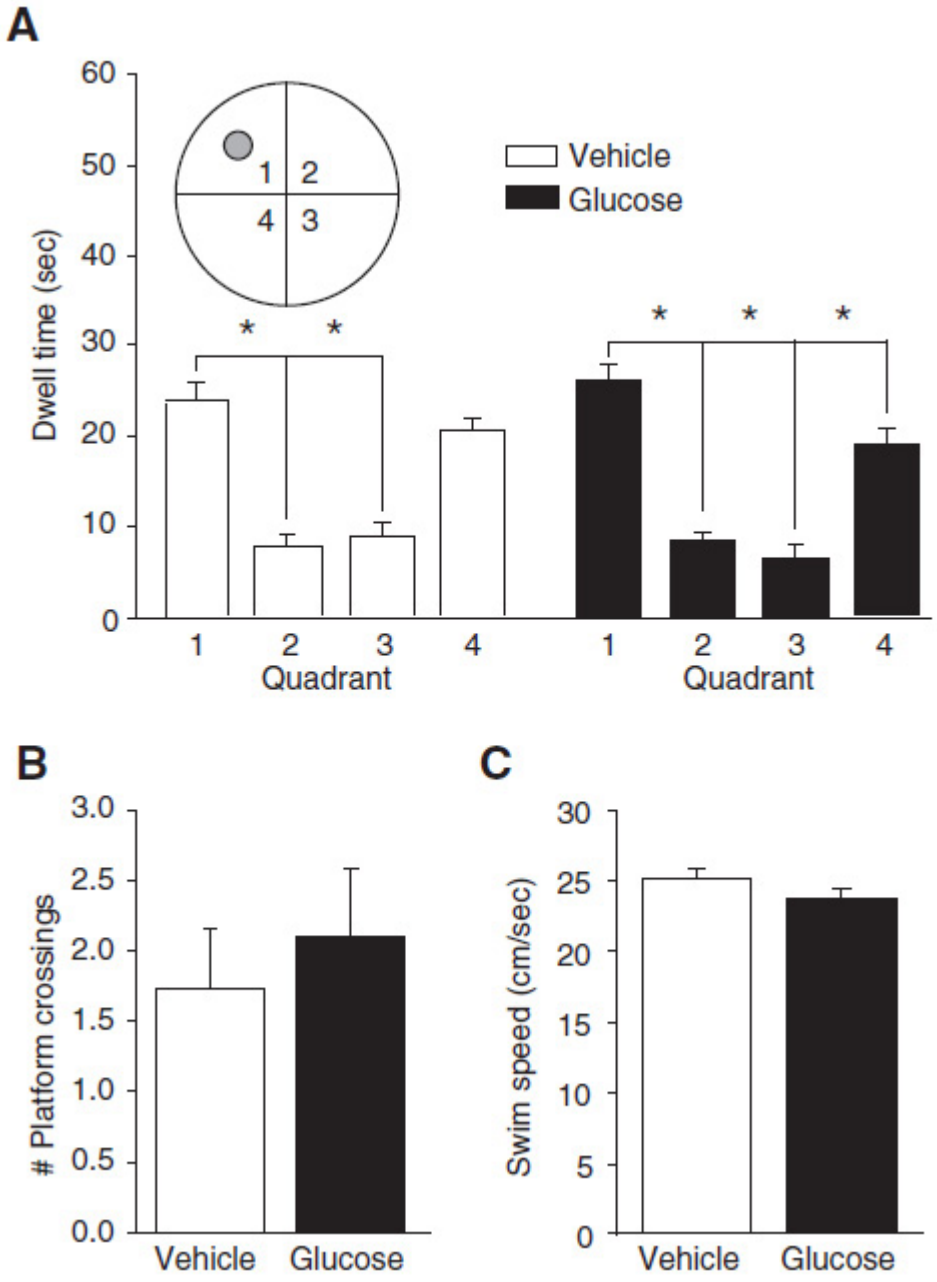

Figure 2.3. Pre-injury administration of glucose leads animals to demonstrate an increased ability to remember general spatial location during STM probe trials. (A) Glucose animals spent significantly more time in the quadrant-containing platform than any other quadrant (Q1), while the vehicle animals spent increased time in both Q1 and Q4. Inset, schematic depiction of quadrants. Grey circle= platform location. (B) Despite a difference in quadrant preference, glucose administration does not significantly alter the number of platform crossings during the STM probe trial. Data presented as mean ± SEM. *p<0.05.

Increased pre-injury blood glucose does not affect contusion volume: To examine if acute hyperglycemia exacerbates brain damage, cortical contusion volume of each injured animal was measured as described in the methods section (vehicle, n=4; glucose, n=5). As evidenced by photographs of animals representative of each group, injury resulted in no gross differences in contusion volume (Fig 2.4A). Further histological measurement of contusion volume shown in Fig. 2.4B demonstrated that acute hyperglycemia did not significantly increase contusion volume (t=test, vehicle=5.3 ± 0.88 mm^3, glucose=5.4 ± 0.63 mm^3; p=0.937).

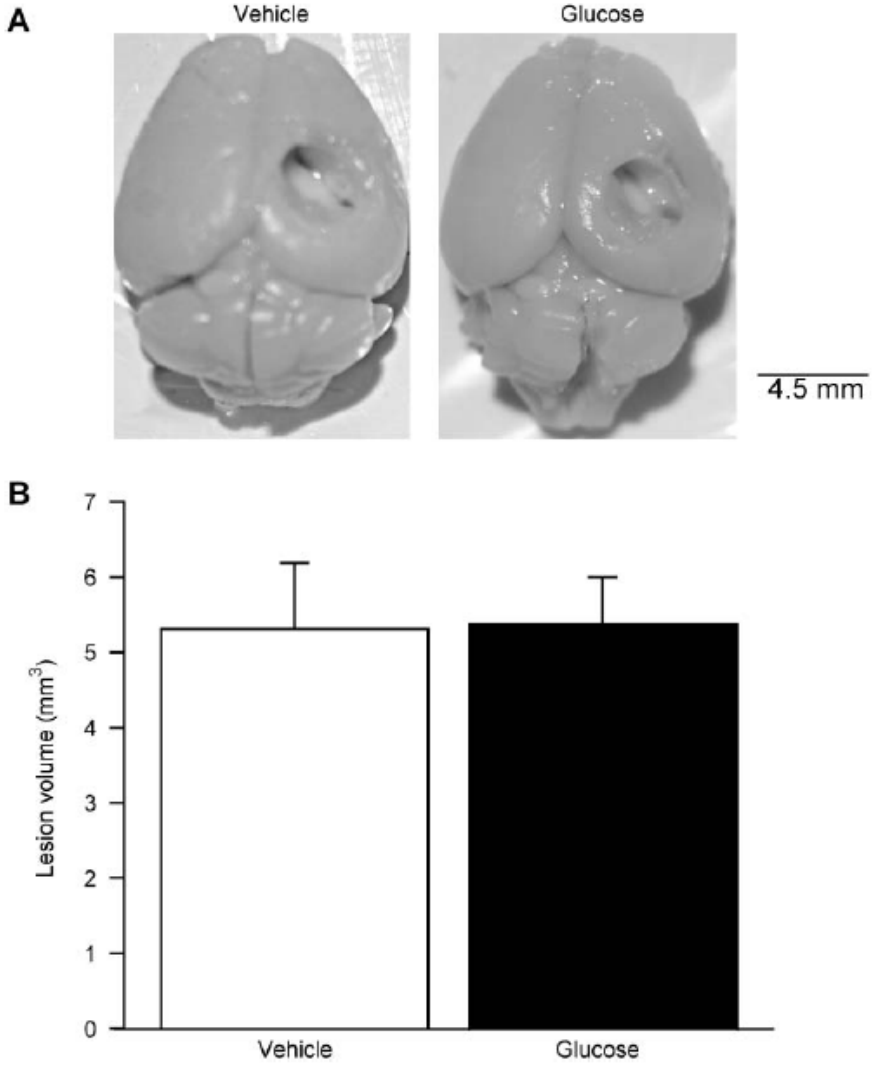

Figure 2.4. Contusion volume is not significantly affected by administration of 2.2 g/kg of glucose 15 minutes pre-injury. (A) Representative photographs of animals from each group demonstrate no visible difference in contusion volume. Scale bar represents 2.5 mm. (B) Histological measurement of contusion volumes in animals further confirms that there hyperglycemia at the time of injury did not have a significant effect. Vehicle n=4; glucose, n=5. Data are presented as mean±SEM.

Acute hyperglycemia at the time of injury does not affect cerebral edema: In the clinical literature, evidence has suggested a correlation between maximum ICP and post-operative levels of blood glucose in patients undergoing trauma-related neurosurgery (49). A primary cause for TBI-associated mortality is cerebral edema, which can give rise to elevated intracranial pressure (ICP) (231). We therefore investigated whether there was a relationship between edema and an acute episode of hyperglycemia in rats through intraperitoneal administration of 2.2 g/kg glucose or vehicle 15 minutes prior to injury (n=8/group), followed by sacrifice and measurement of brain water content 48 hours post-injury (Fig 2.5A). Again, this dose of glucose resulted in an increase in blood glucose immediately prior to injury in the glucose-treated animals, which was significantly different from the vehicle group (2-way RM ANOVA, group-time interaction, $p<.001$) (Figure 2.5B). At the time of sacrifice two days post-injury, blood glucose levels in both groups were not significantly different than they were at pre-injury baseline. We found that significantly increased blood glucose at the time of injury did not have an influence on cerebral edema at 48 hours post-injury (rank-sum t-test, ipsilateral, $p=0.597$; contralateral, $p=0.941$; Fig 2.5C). The dashed line in Fig 5C illustrates the mean percent brain water content present in uninjured animals.

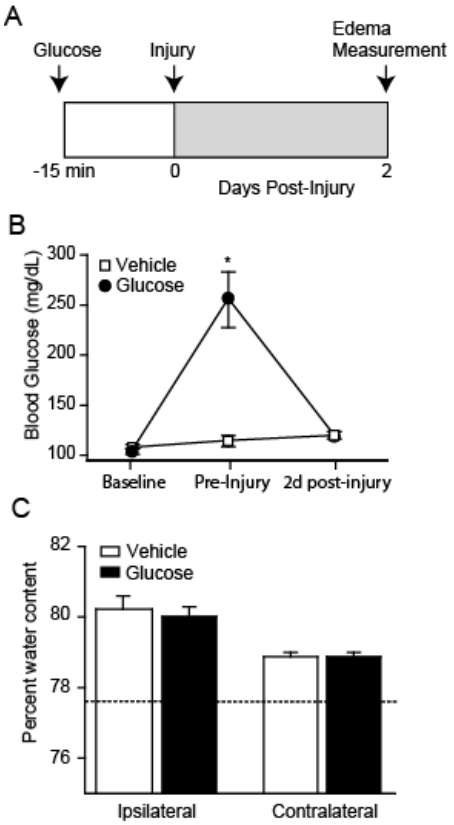

Figure 2.5. Cerebral edema is not significantly affected by administration of 2.2 g/kg of glucose 15 minutes pre-injury. (A) Schematic timeline of edema experiment. (B) Administration of this dose of glucose to rats significantly raises blood glucose prior to injury. 2 days later, this level has returned to normal. (C) 48 hours post-injury, there is not a significant difference in the level of cerebral edema between animals with elevated glucose at the time of injury and injured vehicle animals. Dotted line represents average level of brain water content previously observed in uninjured animals. N=8/group. Data are presented as mean±SEM.

Preceding indented text and figures from: Julia Hill, J., J. Zhao, and P. K. Dash. 2010. High blood glucose does not adversely affect outcome in moderately brain-injured rodents. Journal of neurotrauma 27:1439-1448. Figure numbers altered for reproduction. Reprinted with permission from Lieber Publishing.

Increasing blood glucose does not impact central AMPK phosphorylation 3 hours post-injury:

To investigate the biochemical changes induced by glucose injection, animals were administered 2.2 g/kg glucose or saline intraperitoneally 15 minutes prior to injury and then cortical tissue near the injury site was dissected at 3 hours post-injury for western blotting assessment (Sham, n=5; Vehicle, n=3; Glucose n=3). Given that glucose is known to affect AMPK activity, and that systemic increase of AMPK is beneficial following ischemia, we examined whether our treatment induced changes in AMPK phosphorylation. Increasing glucose may not have an impact upon outcome, if metabolic signaling through AMPK is suppressed.

(Fig 2.6).

To eliminate multiple comparison variables, we first compared the sham, uninjured animals to the injured animals dissected at 3 hours that received saline vehicle by t-test. As can be seen in Fig 2.6A and 2.6C there is a general trend towards a decrease in phosphorylation of AMPK and AS160 in the cortex of injured mice 3 hours post-TBI, and this is not altered by pre-injury glucose injection (Fig 2.6B, D). The decrease in the vehicle-treated, injured mice of AS160 phosphorylation reaches significance when compared to the sham group (Fig 2.6C; $p<0.001$). However, when the vehicle-treated and the glucose-treated groups are compared (Fig 2.6D), there is no difference between these groups. There was no difference in total AMPK or AS160 across groups. The fact that there is no discernible difference between the injured groups suggests that the addition of glucose 15 minutes pre-injury had no impact on central activation of the AMPK pathway post-injury.

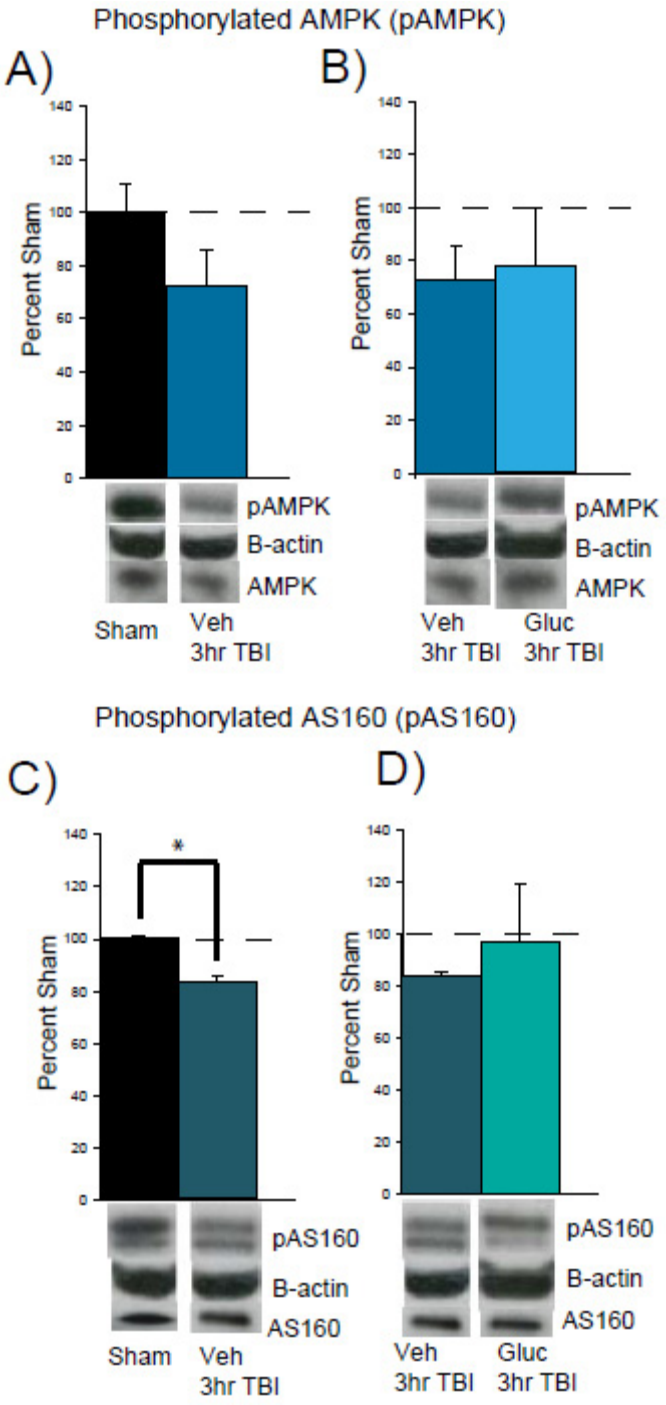

Figure 2.6 Brain AMPK and AS160 phosphorylation are not altered by peripheral glucose injection. (A)There is a general trend towards decreased AMPK phosphorylation in the ipsilateral cortex if injured mice 3 hours post-injury. (B) There is no difference between injured, vehicle-treated and injured, glucose-treated animals in pAMPK levels. (C) There is a significant decrease in pAS160 in injured mice. (D) This is not altered by pre-injury glucose administration. N/Group, Sham=5, Vehicle=3, Glucose=3. Data are presented as mean±SEM. *p<0.05. Dashed line is representative of the sham value.

Discussion:

In these experiments, we investigated the effect of hyperglycemia at the time of injury upon subsequent TBI outcome in moderately injured animals, based on the clinical observations of a correlation between high blood glucose and worsened TBI outcome. We draw two major conclusions from this work: a transient, artificial increase in blood glucose does not have an impact on behavioral or pathological outcome in moderately injured animals, and that there is a decrease in AMPK pathway activity post-injury, and suppression of this cascade is not altered by glucose treatment.

In the behavioral study, we found that inducing high blood glucose 15 minutes prior to injury had no impact on post-injury task performance or secondary pathology. This is in contrast to previous reports, in which the same dose of glucose was associated with an increase in contusion volume of injured animals (217, 218). Previous studies that demonstrated an increase in contusion volume in hyperglycemic animals did not report whether glucose-treated animals displayed altered motor or cognitive outcome. Additionally, it was previously reported that acute hyperglycemia does not impact motor outcome in moderately injured animals (219). Behavioral testing demonstrated that animals with increased blood glucose at the time of injury were not impaired, and may have experienced mild cognitive improvement. Thus, our negative results for the effect of hyperglycemia on TBI outcome are consistent with the previous literature.

We demonstrated that although there was a general trend towards a decrease in AMPK phosphorylation post-injury upon preliminary examination of cortical samples by western blotting, treatment with glucose did not appear to alter the activity of this kinase. Additionally, we found that AS160 phosphorylation was significantly decreased following injury, and was the level of pAS160 present in the cortex was not altered by pre-injury glucose treatment. These findings suggest that cellular metabolism is perturbed following TBI, and that simply increasing the supply of glucose is not sufficient to affect activation of energy-sensing cascades and alter functional outcome.

Is high blood glucose a direct effector of cellular pathology, or is it a marker of the underlying alterations in brain metabolism? A state of decreased expression of GLUT4 and insulin resistance that would result in high blood glucose has been observed in severely injured animals in the acute post-injury (56). In addition to increasing GLUT4 trafficking, insulin also increases activation of the energy-consuming mTOR cascade, which is already hyper-activated in the brain following injury (195, 232, 233). Insulin injection has also been found to increase mortality in CCI-injured animals when administered in the first 24 hours post-injury (55), and in our work we have found that Insulin administration leads to an increase in post-injury cerebral edema (35). Furthermore, treatment with glucagon, associated with a significant *increase* in blood glucose concentrations, has been shown to reduce contusion volume following injury by 60% (234). The existence of an insulin-resistant state provides further motivation for investigating whether alternative treatments, such as AMPK activation. Treatment with AMPK activators would increase cellular glucose uptake and also inhibit mTOR activation, and this may improve TBI outcome.

The experiment we performed models a transient period of high blood glucose at the time of injury, whereas the effects of persistent hyperglycemia on TBI outcome may more closely model the clinical situation. We attempted to address the impact of chronic high blood glucose by intraperitoneal injection of a drug the drug Streptozotocin (STZ), which leads to the destruction of insulin-producing beta cells in the pancreas (35). Although we did not conduct behavioral work due to the lethargy occurring in these animals, we did find that there was a decrease in cerebral edema 48 hours post-injury in STZ-treated animals with high blood glucose, and that insulin led to an increase in edema in these animals. This was in contrast to a previous study of the impact of STZ treatment on cerebral edema, which found that such treatment led to an increase in edema (235). One possible reason for this could be the length of hyperglycemia. In that study, animals maintained with high blood glucose for four weeks prior to injury; whereas ours were only hyperglycemic for one week. The issue of how chronically increased blood glucose impacts TBI pathology is difficult to study in normal

animals, given how quickly glucose is metabolized. The impact of persistent hyperglycemia on TBI outcome could be further examined through implantation of an osmotic pump that delivered glucose for a prolonged time period in injured animals.

It is possible that if a more challenging behavioral paradigm had been used to assess cognitive outcome, differences as a result of glucose treatment may have been more apparent. Glucose-treated animals did demonstrate a slight improvement in quadrant preference in the short-term memory probe following standard water maze training. In addition, these animals were subsequently trained in a working memory version of the water maze task. In this task mice are trained to find a location of a hidden platform during a “location” trial, and then after removal of the tank for 5 sec they must re-locate the same location in a “match” trial. This is repeated with four separate platform locations in a day. Following trial 1 each day, there was a trend for glucose-treated animals to take longer in the “location” trial of the task (unpublished observation). Whether this is suggestive of a difference in their behavioral outcome is something that could only be addressed with additional experimentation.

In summary, in this work we found that artificially increasing high blood glucose in mice pre-injury did not impact TBI outcome, either cognitively or pathologically. The results obtained from follow-up biochemical work suggested that there was an underlying suppression of AMPK pathway-related activity, and we decided to examine the nature of this alteration in subsequent work.

CHAPTER III. THE EFFECT OF BRAIN INJURY ON AMPK PATHWAY ACTIVATION

Introduction:

The maintenance of proper energy stores is critical to brain functioning, and there are many secondary signaling cascades that sense energy availability in the brain (154, 197, 233, 236). The progressive secondary damage and dysfunction that occurs following brain injury is partially mediated by changes at the protein level by aberrant signaling cascade activation (11, 22). It has been established that a neurometabolic response occurs as a result of the release of excitatory amino acid release post-injury, and subsequent calcium overload, even following mild injury (22). These changes occur within the first hours post-injury and result in an increase in brain glucose uptake. This response originates as an attempt to restore ionic homeostasis (237). A more prolonged, delayed suppression of metabolism also occurs post-injury (14, 15, 60, 61). This metabolic suppression has been correlated to worsened cognition in experimental and clinical research (80, 82, 84-87). Whether there are changes in secondary signaling energy-sensing cascades correlated with this metabolic suppression has received less investigation. As decreased brain metabolism appears to play a role in TBI outcome, we sought to investigate activity of the AMP-activated Kinase (AMPK) and its downstream targets post-injury.

AMPK is a cellular-energy sensing serine/threonine kinase which is normally activated by phosphorylation at a single site (Threonine 172) under conditions of cell stress (140). Upon activation, AMPK acts to increase activity of catabolic processes to produce ATP, while simultaneously inhibiting processes that consume energy. This cascade is expressed throughout the nervous system, with specific expression in neurons (147, 154). AMPK is known to regulate neuronal glucose transport, specifically in response to excitotoxic insults (130, 155). Additionally, treatment which increases the translocation of Glut-4 to the plasma membrane in an AMPK-dependent manner has been shown to elicit functional improvement following spinal cord injury (205). AMPK phosphorylation is *decreased* seven days post-TBI in the hippocampus, although the activity of this kinase at other points has not been investigated (206). Since the metabolic suppression seen post-injury is transient, we were interested in

examining the time course of AMPK phosphorylation across time post-injury to determine if there was a similar pattern (14). If similar alterations in AMPK phosphorylation are seen across other injury-affected regions, such as the cortex, has not been investigated.

AMPK is capable of regulating translocation of glucose transporter 4 (GLUT4), and preliminary evidence suggests AMPK-mediated increases in GLUT4 translocation could occur in the brain in response to cellular stress (130). Evidence of a role of GLUT4 in response to neural stress includes the observation that application of potassium chloride to hippocampal slice preparations led to increased pyramidal neuron glucose uptake, and this was effect was attenuated by a drug inhibitor of GLUT4 (238). One target of AMPK regulating glucose transport in the periphery is Akt Substrate of 160 kDa (AS160), a protein with Rab GTP-ase activating (RabGAP) activity that is key in controlling translocation of GLUT4 (170). AS160 phosphorylation by AMPK inhibits its GTP-ase activity, allowing Rab targets take the active GTP form, and dissociation of AS160 from GLUT4 occurs. In the periphery, AMPK phosphorylation of AS160 occurs in tissues undergoing conditions of high energy demand, such as contracting muscle tissue (169). We were interested in further studying AS160 phosphorylation, as the brain experiences increased glucose demand post-injury, and AMPK regulation of GLUT4 trafficking has been implicated in response to neural insult.

AMPK is also known to negatively regulate activation of the mTOR pathway, through direct phosphorylation of Raptor and TSC2 (142, 184, 186). Consistent with the hypothesis that AMPK activity is decreased following injury, it has now been shown in two separate studies that activity of the mTOR pathway dramatically increases post-injury (194, 195). Chen et al. (2007) found that though the upstream components of the mTOR cascade demonstrated a modest, transient increase in phosphorylation post-injury, protein S6 was significantly elevated, with increased phosphorylation persisting in the injured cortex up to 72 hours post-injury. Treatment with mTOR inhibitor rapamycin only improves cognitive outcome when administered in combination with an Akt inhibitor, suggesting that targeting the upstream regulators of mTOR may be beneficial in improving TBI outcome (194).

Based on the previous research, we hypothesized that TBI would be associated with a transient decrease in phosphorylation of AMPK and its target AS160, and there would be an associated increase in phosphorylation of mTOR-regulated protein S6. In addition, we hypothesized that by performing an immunohistochemical assessment, we would demonstrate that AS160 protein would be present in the brain and demonstrate similar regional expression to GLUT4. Finally, we hypothesized that performing immunohistochemistry with brain tissue from animals 24 hours and 3 days following TBI would further show that phosphorylation of these proteins were visibly reduced post-injury. These findings would be consistent with observations of metabolic suppression following TBI, and would represent a potential link between decreased metabolism post-injury and worsened functional injury outcome.

Methods:

Materials: Antibodies for phosphorylated AMPK (Threonine 172), total AMPK, protein S6, phosphorylated protein S6 (Serine 235/236), and phosphorylated AS160 (Serine 588 and Threonine 642) were all purchased from Cell Signaling Technology (Danvers, MA). The antibody for total AS160 was purchased from Sigma (St. Louis, MO), and GLUT4 was purchased from Pierce Biotechnology (Rockford, IL).

Controlled Cortical Impact injury: All animal use complied with NIH's Guide for the Care and Use of Laboratory Animals.

For western blotting using time course samples, previously prepared rat hippocampus and cortical samples were used. These animals received a moderate, unilateral injury, as described (239). Male rats were deeply anesthetized induced with 5% isoflurane and 1:1 O_2:N_2O for 4 minutes, and maintained at 1:1 O_2:N_2O through a nose cone for the duration of the surgery. A midline incision is made and the soft tissue reflected to expose the skull. A single craniotomy is made over the right parietal cortex. To produce moderate injury, a single impact was delivered to right parietal cortex using a 5 mm diameter impact tip and an electric driven,

stroke-constrained piston. The injury depth was 2.7 mm deformation, the velocity of 6.0 m/sec. Animals receiving sham surgery underwent the same procedure excluding the craniotomy and cortical impact. All animals recovered in a warm chamber following the surgery, prior to being returned to their home cages.

For immunohistochemistry, mice were CCI injured. The mouse CCI injury was carried out essentially as described by our and other laboratories (35, 36, 41). These animals were injured as described above for rats, except for the use of a 3 mm diameter impact tip for the injury, and a reduction in depth to 1.0 mm, and a decrease in velocity to 5.0 m/s. This magnitude was based on previous work in which the magnitude of tissue loss was determined. Specifically, the goal was to reduce the injury to a depth at which there was cortical tissue loss but the ipsilateral hippocampus remained relatively intact. The decision was also informed by the published literature for mouse CCI (41, 240, 241).

Western blotting: Protein concentration was measured using a Bradford assay with BSA as the standard. Samples loaded with equal amounts of protein were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Billerica, MA), followed by 1 hour of blocking in Tris-buffered saline with Triton X-100 (TBST) plus 5% BSA. Membranes were incubated in primary antibody overnight at 4 degrees. After extensive washing in TBST, immunoreactivity was assessed by an alkaline phosphatase–conjugated secondary antibody and a CDP-star chemiluminescent substrate (New England Biolabs, Ipswich, MA). The optical density of the immunoreactive bands was measured using ImageJ (Available from the NIH). All proteins examined, phosphorylated and total, were corrected by their percent expression of β-actin from the same membrane.

Immunohistochemistry: At 24 hours and 3 days post-injury, brains from injured mice were removed and then coronally segmented into smaller portions to increase surface area exposure prior to fixation. These segments were then post-fixed overnight in a 15% picric acid, 4%

paraformaldehyde solution with PBS. Brains were then cryo-protected for 24 hours in PBS with 30% sucrose. Samples were then frozen in TCS, sectioned on a cryostat set to -20˚C, at a thickness of 20 microns, and stored in 24-well plates. Free-floating sections of interest were selected, washed with PBS and then placed in the relevant primary antibody in PBS containing 2% BSA and 2.5% normal goat serum. After extensive washing, immunoreactivity was detected using species-specific fluorescent (Alexa-Fluor) secondary antibody. For confocal microscopy, a *z*-series of optical sections were captured using a Zeiss LSM 510 confocal microscope using a 63× oil-immersion lens. For presentation purposes, all images were color-corrected and cropped in Adobe Photoshop.

Statistics: For western blotting analysis, optical density of immunoreactive bands for the protein of interest was summed for all animals at each time point. All post-injury time points were then compared to the sham using a 1-way ANOVA. Post-hoc Holm Sidak t-test comparisons were then performed to examine the level of significance of protein expression at specific post-injury time points in comparison to the average sham value.

Results:

Phosphorylation of AMPK is decreased in both rodent hippocampus and cortex following traumatic brain injury: To understand the changes in glucose-dependent cellular signaling cascades which take place following TBI, we conducted western blotting with an antibody specific to the phosphorylated activation site of AMPK (pAMPK). As shown in Figure 3.1, AMPK phosphorylation is decreased following brain injury. Representative images from a single animal in each group are displayed for both the cortex and hippocampus, ipsilateral to the injury site. A 1-way ANOVA performed upon the cortical data yielded AMPK phosphorylation to be significantly decreased at both 24 hours and 3 days post-injury [$F(5, 21)=2.834$; $p=0.042$] (Fig 3.1A). AMPK phosphorylation was found to be significantly decreased 24 hours post-TBI [$F(5, 22)=3.113$; $p=0.028$] (Fig 3.1B).

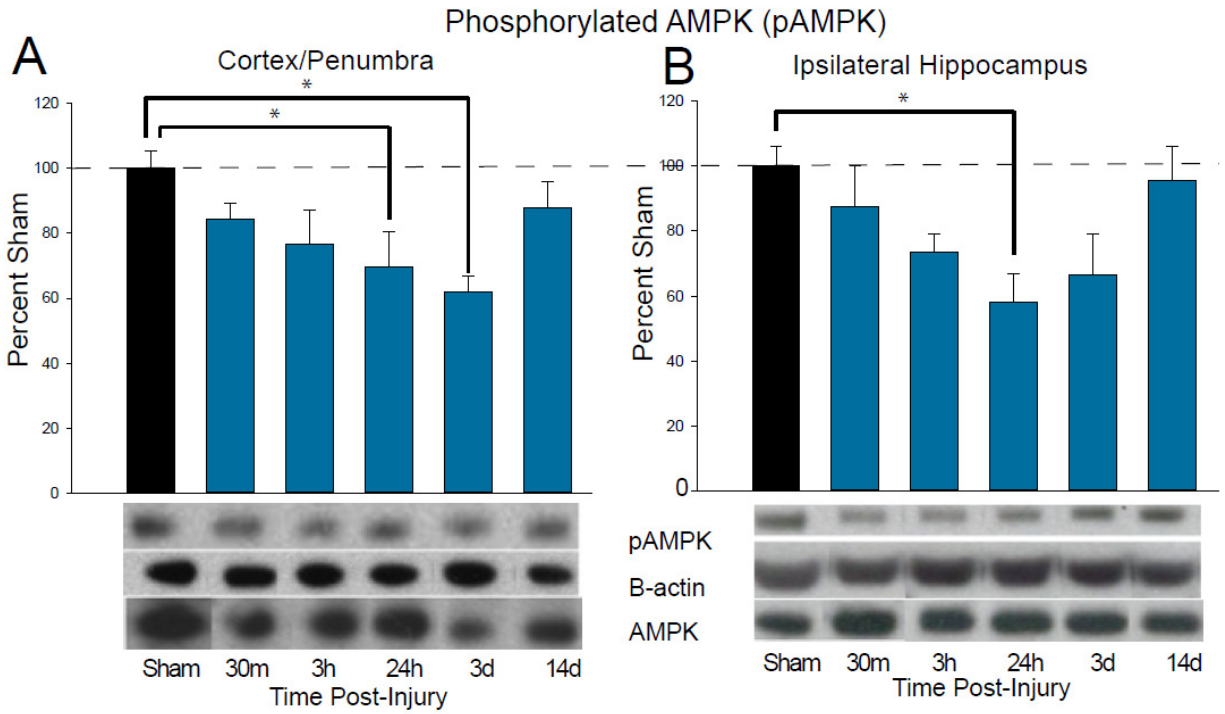

Figure 3.1 Phosphorylation of AMPK decreases following traumatic brain injury. (A) AMPK phosphorylation decreases both in the (A) cortex and (B) the hippocampus of injured animals. Total levels of AMPK protein do not change significantly in either tissue. Data are presented as mean ± SEM. *$p<0.05$. Dashed line is representative of average sham value.

Phosphorylation of AMPK downstream targets is altered in a manner consistent with decreased AMPK activity: We examined the phosphorylation state of S6 and AS160 following injury using Western blot analyses in order to assess the impact of AMPK activation on its downstream targets. Active AMPK directly phosphorylates AS160, and this leads to increased GLUT4 trafficking to the cell surface. We used an antibody that identifies phosphorylation of AS160 at serine 588, as this has been shown to be the phosphorylation event most increased by AICAR (171). The results are shown in Figure 3.2. Although it did not reach significance, there was a general trend towards a decrease in AS160 expression. This was more apparent in the hippocampus than in the cortex (Fig 3.2B, A). Total levels of AS160 did not change significantly over time.

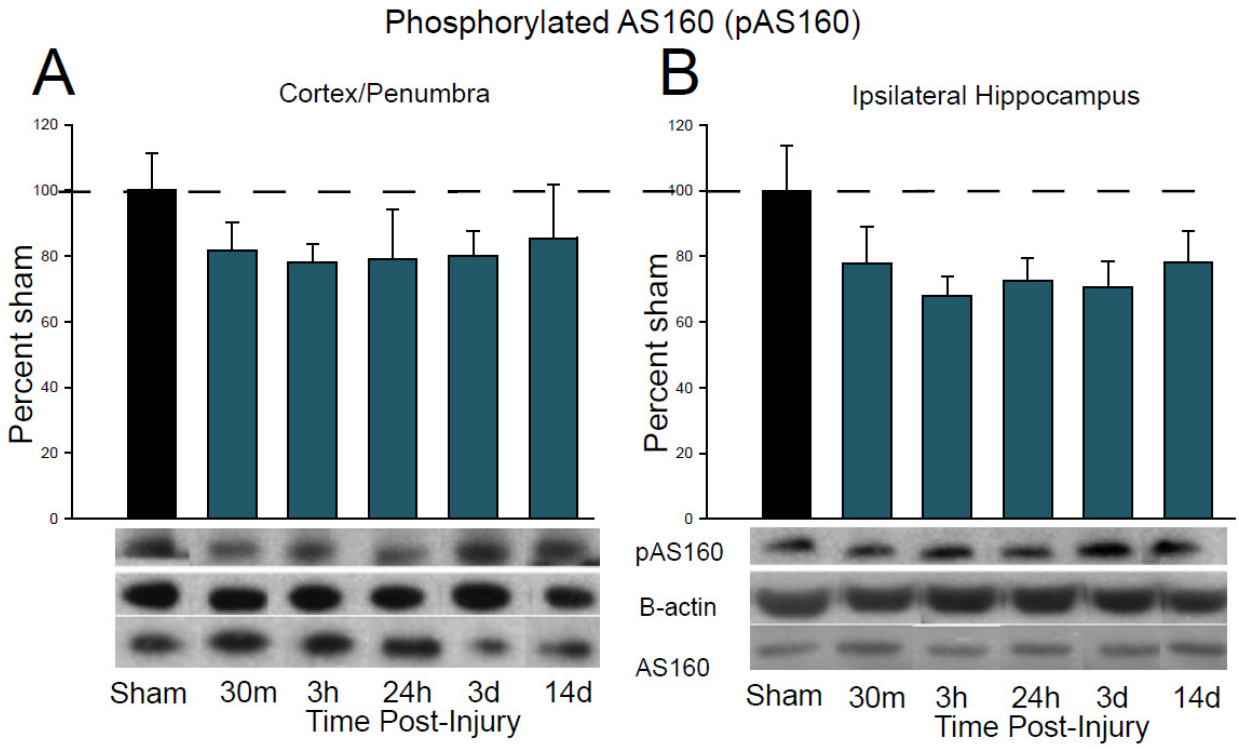

Figure 3.2 Phosphorylation of AS160 decreases following traumatic brain injury. AS160 phosphorylation appears to decrease in both (A) the cortex and (B) the hippocampus of injured animals. Total levels of AS160 protein do not change in either tissue. Data are presented as mean ± SEM. Dashed line is representative of average sham value.

When AMPK is less active, mTOR activity is increased, and it promotes protein translation through phosphorylation of its downstream target, protein S6 (pS6) (184, 191, 192). Phosphorylation of protein S6 is significantly increased in the cortex and the hippocampus following TBI (Fig 3.3A, B). The increase in S6 phosphorylation is pronounced in the cortical time course, where all time points up to two weeks are significantly different from the sham [$F(5, 21)= 13.299$; $p<0.001$] (Fig 3.3A). Similar to the pAMPK time course data, the points with the lowest p values compared to sham are 24 hours ($p=0.010$) and 3 days ($p=0.013$). In the hippocampus, S6 is phosphorylation is increased in comparison to sham [$F(5,22)=4.379$; $p=0.006$], with 3 hours ($p<0.001$) and 24 hours ($p=0.009$) reaching significance (Fig 3.3B).

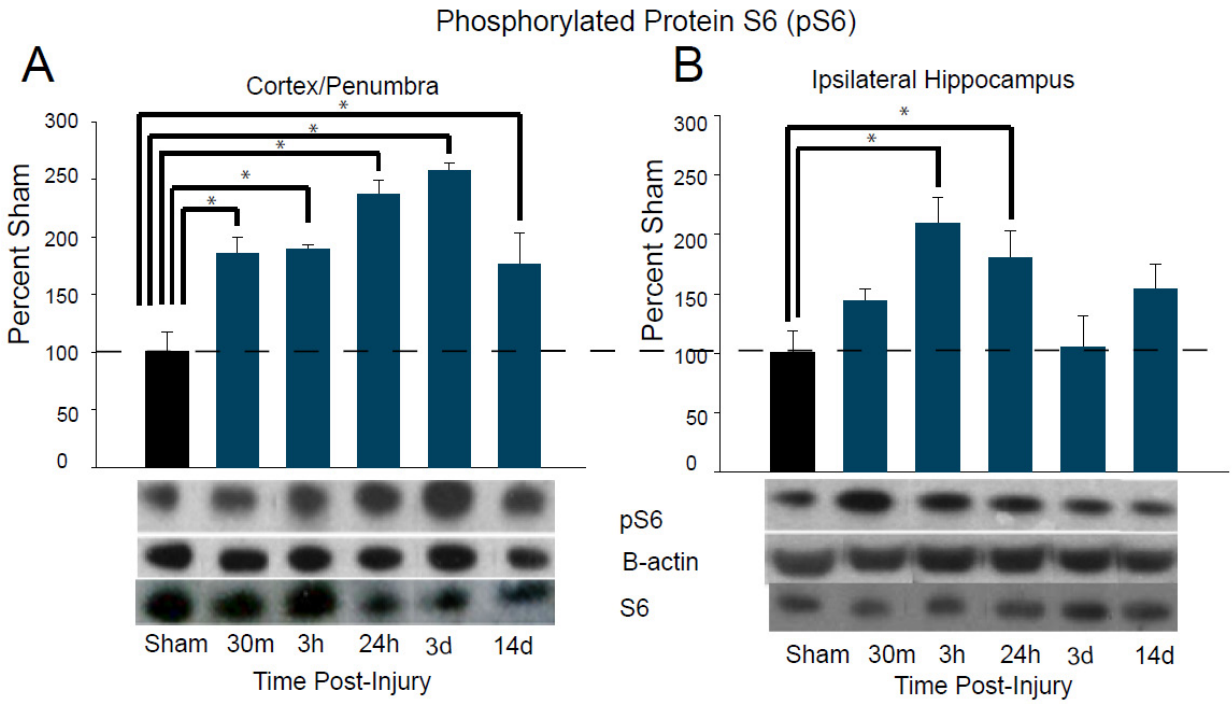

Figure 3.3 Phosphorylation of protein S6 is increased post-injury. Following TBI, there is a significant increase in phosphorylated protein S6 in both (A) the cortex and (B) the hippocampus. Total levels of s6 protein do not change in either tissue. Data are presented as mean ± SEM. *p<0.05. Dashed line is representative of average sham value.

Total AS160 and phosphorylated AS160 (Thr642) are present in mouse hippocampus and cortex: We performed immunohistochemistry with samples from naïve mice to determine whether AS160 was detectable in the brain by this method and also to determine what area it was localized to. Specifically, we focused on expression in the hippocampus and cortex. As shown in Figure 3.4 and 3.5, AS160 and AS160 phosphorylated at Threonine 642 are abundantly expressed in these regions, specifically in neuronal cell layers (3.4 A-E, 3.5 A-F). It appears to have expression in the cell soma and the proximal processes of pyramidal neurons throughout the cortex, CA1, and CA3.

The most robust expression of phosphorylated AS160 (pAS160) was found in the cortex and in the CA1 region of the hippocampus (3.5 B,C), with less expression in the dentate gyrus (3.5 E). The pAS160 expression occurring in the dentate appears to be in the subgranular zone, the site of hippocampal neurogenesis. As AS160 has multiple phosphorylation sites, it is also possible that other phosphorylation sites are more active in this dentate gyrus (171). Our work demonstrates that GLUT4 and pAS160 exhibit similar regional expression, as evidenced by confocal microscopy (3.5F).

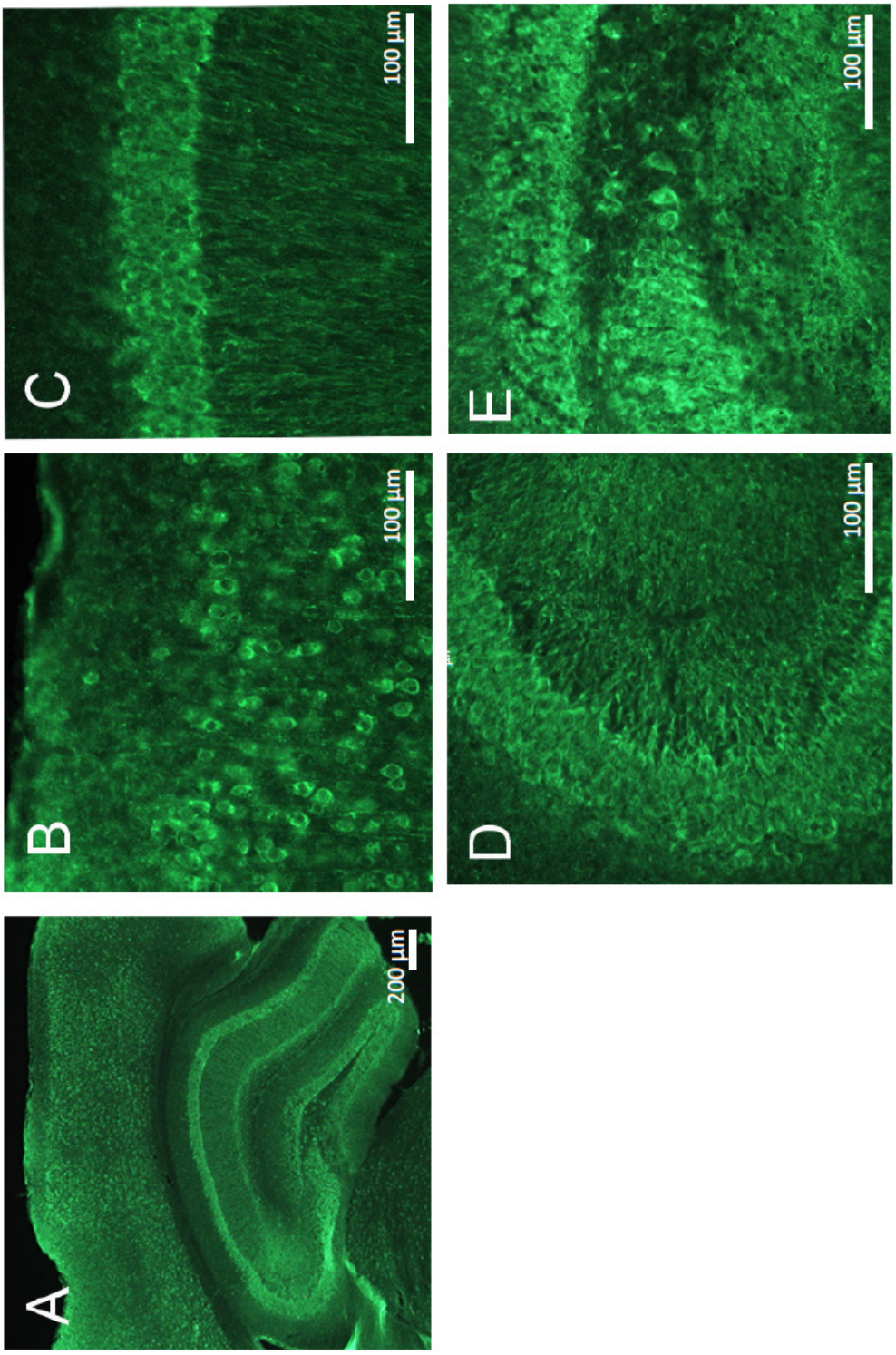

Figure 3.4. Total AS160 is expressed in the hippocampus and cortex of naive mice. (A) Lower magnification image of AS160 demonstrates its widespread expression in both the cortex and hippocampus. (B-E) Images of AS160 photographed at higher magnification- (B) in cortex, (C) CA1 region, (D) CA3 region, (E) Dentate gyrus.

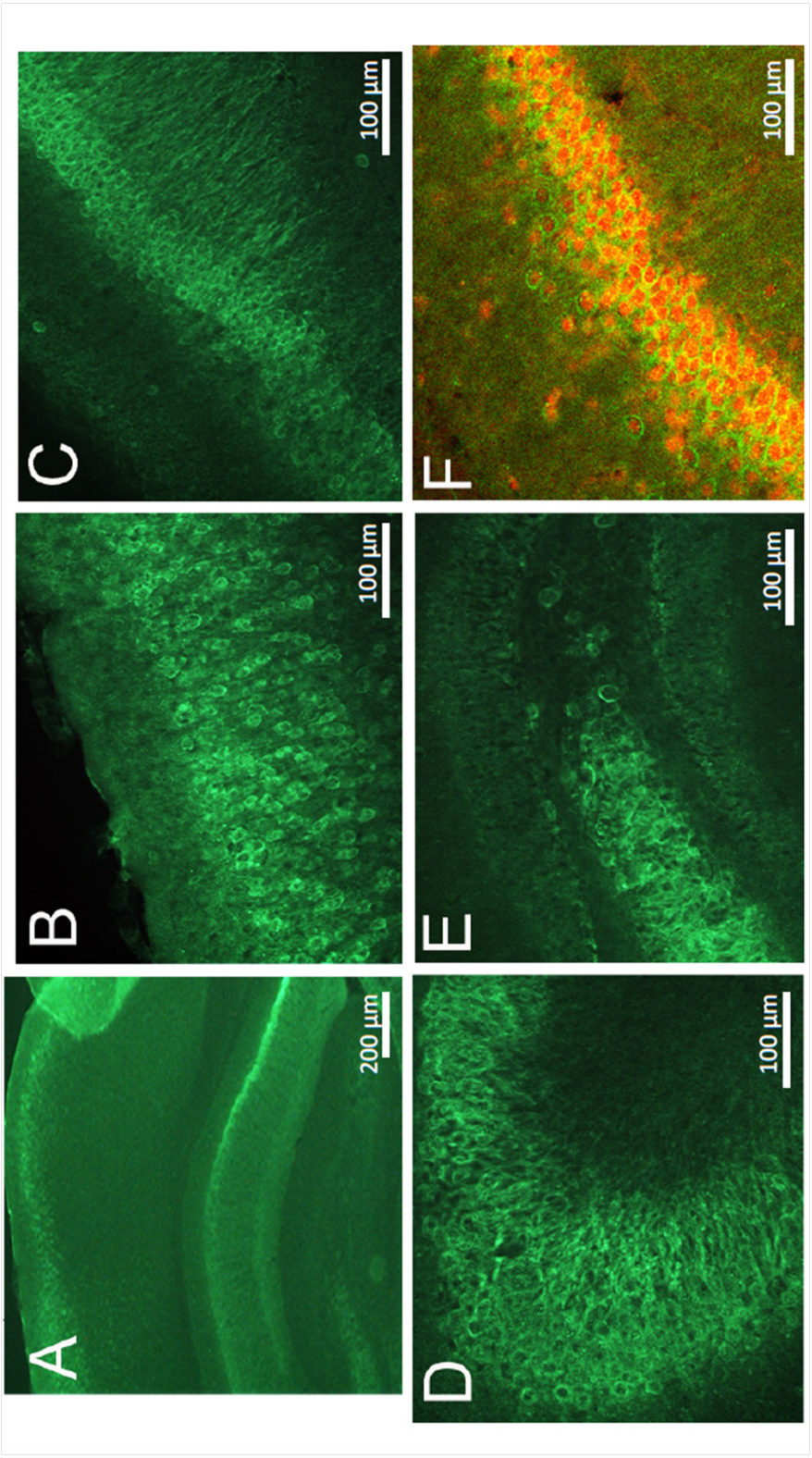

Figure 3.5. Phosphorylated AS160 (pAS160) is expressed in the hippocampus and cortex of naive mice. (A) Lower magnification image of pAS160 expression. (B-E) Images of pAS160 at photographed at a higher magnification- (B) in cortex, (C) CA1 region, (D) CA3 region, (E) Dentate. (F) Image of CA1 region taken with a confocal microscope shows that pAS160 (green) and Glucose transporter 4 (GLUT4, red) demonstrate overlapping expression profiles.

The phosphorylation of AMPK target AS160, and presence of glucose transporter 4 are reduced following TBI: We observed a reduction in AMPK-related activity through western blotting analysis of TBI time course samples. To support this conclusion, we examined the phosphorylation levels of downstream targets of AMPK activity, Akt substrate of 160 kDa (pAS160) and glucose transporter 4 (GLUT4) by immunohistochemistry at 24 hours and 3 days following TBI in moderately injured mice (n=3/group). 24 hours post-injury, phosphorylation of direct AMPK target AS160 appears decreased in the cortex and in CA1 following injury. Total levels of GLUT4 appear to be visibly decreased in cortex, CA1, and in the dentate gyrus 3 days post-injury. These changes are consistent with an upstream decrease in AMPK phosphorylation.

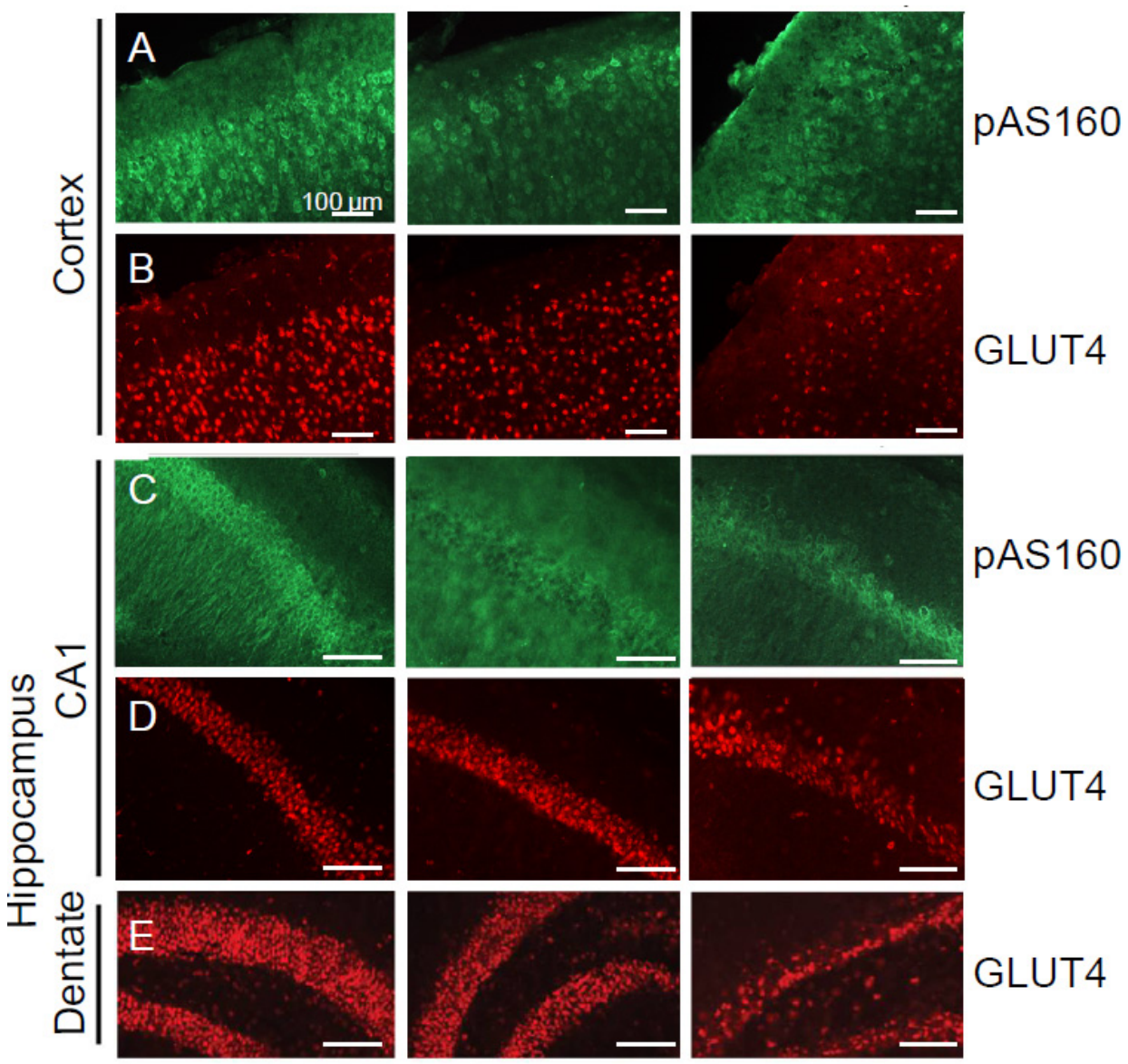

Figure 3.6 Levels of phosphorylated AS160 (pAS160) and glucose transporter 4 (GLUT4) decrease in the ipsilateral hippocampus and cortex post-injury. Each time point is organized into a column, with images captured from a representative animal from each group. (A, C) pAS160 appears decreased 24 hours post-injury, with some restoration of signal by 3 days post-injury in (A) the cortex and in (C) hippocampal region CA1. (B, D, E) GLUT4 expression appears to be decreased by 3 days post-injury in (B) the cortex, and hippocampal regions (D) CA1 and (E) the dentate. Scale bar represents 100 µm in all images presented.

Discussion

Based on the biochemical results obtained in Chapter 2 that suggested a deficiency in AMPK pathway post-injury, we sought to characterize activity of the AMPK pathway following TBI. Additionally, we conducted an immunohistochemical examination of the regional expression of AS160 in the brain in areas, as AMPK phosphorylation of AS160 increases glucose uptake via GLUT4 in other tissues in response to rapid increases in energy demand.

We draw three major conclusions from this work. The western blotting results demonstrate that activity of the AMPK pathway is decreased following injury. This is consistent with the previous TBI research, in which a decrease in AMPK activity has been shown (206, 242) and an increase in mTOR-related activity, including protein S6 phosphorylation, was demonstrated (194, 195). Consistent with previous western blotting analysis suggesting that protein AS160 was expressed in the brain (168, 179), we have shown that this protein is expressed in neuronal cell layers in the cortex and hippocampus, and has expression which overlaps with its target protein, GLUT4. The expression profile of AS160 is similar to what has previously been reported for hippocampal expression of insulin-regulated aminopeptidase (IRAP), a molecule which regulates GLUT4 trafficking in the same manner as AS160 (238). Finally, we utilized immunohistochemistry to examine the phosphorylation of AS160 and expression of GLUT4 at time points of phosphorylated AMPK decrease identified by western and found consistent alterations in these proteins. Interestingly, GLUT4 total expression appeared decreased 3 days following injury. As AMPK is capable of regulating GLUT4 total levels, this is something that may be worth investigating in subsequent work (156, 157). The fact that GLUT4 experienced a delayed suppression in comparison to pAS160 could be due to the difference in the event examined (phosphorylation versus total protein reduction) or due to the fact that AMPK suppression could control immediate and prolonged factors related to glucose metabolism.

The previous work conducted to examine post-TBI alterations in AMPK activity has been focused upon the impact of AMPK upon oxidative stress, and how dietary

supplementation prior to injury may improve AMPK phosphorylation. Sharma et al. (2009) demonstrated a similar decrease in the pAMPK/AMPK ratio in the hippocampus 7 days following moderate fluid percussion injury. In addition to a decrease in AMPK phosphorylation, this group also found a decrease in expression of mitochondrial proteins associated with increasing mitochondrial mass and the uncoupling of electron transport from protein synthesis [49, 52]. Despite hypothesizing that these changes may impact cognitive function, behavioral outcome was not tested in these animals. Their observation of decreased pAMPK is a later time point than that observed here; however, this could be due to a difference in injury model or severity. Overall, our results in combination with the past TBI research suggests that AMPK activity is decreased following injury, and that this is correlated with a decrease in other protein markers which could contribute to TBI pathology and impact cognitive outcome.

The mechanism by which this decrease in AMPK activation occurs is unclear. A transient alteration in the AMP:ATP ratio has been observed in a number of injury paradigms (243-247). However, decreased ATP is generally short-lived post-injury, whereas glucose metabolism suppression is more prolonged. In addition, decreased glucose uptake has been observed without a concurrent alteration in ATP levels (243).. An additional possibility is that AMPK phosphorylation decreases due to disturbance of its upstream activator, LKB1. LKB1 becomes active upon forming a complex (collectively called AMPK kinase or AMPKK) with two additional proteins, pseudokinase STE-related adaptor alpha (STRADα) and mouse protein 25 alpha (MO25α) (248). Recently, it has been demonstrated that there is decreased LKB1 signaling in glioma cells *in vitro* due to downregulated MO25α protein expression, and that this is associated with increased cell death when glioma cells are switched to low glucose conditions (249). This down-regulation of MO25α can occur as a result of increased expression of microRNA 451 (miR-451), a microRNA that is regulated by glucose. Our laboratory has previously shown that miR-451 is upregulated in the rat hippocampus at 3 and 24 hours following TBI (250), a time period in which an acute increase in glycolysis has been demonstrated.

We recognize that beyond AMPK, there are many alterations in energy cascade activation that may play a role in secondary TBI pathology. Additive effect of other energy-related cascades could be one reason for the difference in the time course activation between AMPK and protein s6, this is something which was observed by Chen et al. (2007) in relation to a different time course between activation of mTOR and protein s6. Other kinases, such as Akt (Protein Kinase B) which is activated by phosphorylation by the PI3 kinase, and it in turn is capable of regulating AS160 and s6 phosphorylation. Akt is known to have increased activation in neurons 4 hours post-injury (194, 251). However, intracerebroventricular treatment with rapamycin and an Akt inhibitor has an additive effect, suggesting that activation of mTOR is occurring through multiple mechanisms (194). Furthermore, peripheral administration of Simvastatin, which increases Akt, has been shown to improve spatial memory following TBI (252). Additionally, Simvastatin in arteries is known to lead to increase AMPK phosphorylation as well (253). Thus, the interplay between the cascades regulating energy metabolism is complex, and should be subject to additional study.

A limitation of this project was the use of rat CCI for western blotting and mouse CCI for immunohistochemistry. Mouse immunohistochemistry was used as the subsequent behavioral experiments were planned to be executed using mice. The time course samples were previously generated in our lab, and it was not feasible within the scope of my project to generate a new time course from injured mice. Furthermore, rat tissue was also used for this project and qualitatively similar results were observed but not reported here. The observation of similar alterations in AMPK post-injury in mice and rats strengthens the argument that such metabolic changes are an intrinsic and potentially important part of the TBI response.

An additional variable is that antibodies targeted to different AS160 phosphorylation sites were used in the western blotting (Ser588) and immunohistochemistry (Thr642) experiments. Phosphorylation of both of these sites in response to AICAR treatment has been found with western blotting analysis, with Ser588 exhibiting the highest levels of phosphorylation by this method (171). In addition, the results from both experiments suggest a

general decrease in AS160 phosphorylation following injury. As the activation of AMPK appears to have implications for neurogenesis and GLUT4 is expressed in the dentate (123), it would be interesting in further work to examine whether there is more robust expression of other phosphorylated AS160 sites in this region.

In conclusion, we have identified a transient period of suppressed AMPK-related activity that could correlate to the previously identified period of post-injury suppressed brain glucose uptake (14, 15, 22, 62). We identified changes in AS160 phosphorylation and GLUT4 immunoreactivity that are further consistent with altered glucose uptake via this pathway. GLUT4 has been hypothesized to increase glucose uptake during periods of cellular distress (109), and there is preliminary evidence to suggest proper functioning of this transporter may influence cognitive functioning (254). Additionally, multiple studies with naïve animals have now demonstrated that AMPK activators are capable of improving cognitive functioning (199-201). For these reasons, we conducted further work as outlined in Chapters 4 and 5 to examine how altering activity of the AMPK pathway would influence TBI functional outcome.

CHAPTER IV. THE IMPACT OF RESTORING AMPK ACTIVITY ON TBI OUTCOME

Introduction:

Across all Traumatic Brain Injury (TBI) magnitudes, energetic dysfunction has been well characterized as a secondary pathology (14, 22, 60, 62). The brain is extremely sensitive to shifts in cellular metabolism, as neural tissue has a high metabolic rate and low energy stores. In both the clinical and experimental literature a prolonged period of decreased brain oxidative and glucose metabolism has been observed. Disturbed cellular metabolism occurs in regions critical to cognition, such as the hippocampus (63-65). Further, a link has been demonstrated between recovery from metabolic suppression and behavioral performance in injured animals (80), suggesting that the energy crisis may influence cognitive dysfunction.

Energy-sensing signaling cascades, including AMP-activated kinase (AMPK), are known to control both metabolism and cognition (139, 199, 201). AMPK is a serine/threonine kinase activated by phosphorylation at a single site (threonine 172) by its upstream kinases under low-energy conditions (140). AMPK activation with the drug AICAR (5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide) has been shown to have neuroprotective effects (149, 155, 199, 200). Upon entry into cells, phosphorylation of AICAR results in the production of the AMP analog ZMP. AICAR results in allosteric activation of AMPK; thus it does not increase the AMP:ATP ratio (255). AICAR has been demonstrated to increase survival of hippocampal neurons in response to various cell stresses i*n vitro* (149). Application of AICAR increased survival of pyramidal neurons following glucose deprivation; this effect was attenuated by application of antisense oligonucleotides which block the catalytic subunit of AMPK (149). Further *in vitro* work in cerebellar neurons has shown that AICAR application following glutamate excitotoxicity increases trafficking of Glucose transporter 3 (GLUT3), and that inhibition of GLUT3 led to a significant increase in cell death (155).

AMPK represents a strong potential therapeutic target for TBI patients, as its activation would directly promote ATP production, glycolysis, and mitochondrial biogenesis. In response to TBI, AMPK activation has been found to be *decreased* at seven days following moderate injury, and others have found that dietary supplement for several weeks prior to injury can

reverse this deficit and increase expression of proteins necessary for synaptic plasticity (206). In addition to the described effects at the cellular level, AICAR has established in vivo effects upon spatial memory in naïve animals (199, 200). Similarly, chronic application of the AMPK-activating drug Metformin was also recently observed to improve long-term spatial memory (201). Increasing activation of AMPK, which promotes the conservation and increased production of ATP, may be beneficial in TBI-injured animals experiencing a brain energy crisis, and lead to improved behavioral performance.

The time course of altered AMPK phosphorylation and whether enhancing AMPK activity can improve cognitive outcome following traumatic brain injury has not been tested. As metabolic recovery has been associated with improvement in behavioral performance following injury, we reasoned that enhancing activity of the AMPK pathway through administration of the drug AICAR would lessen metabolic dysfunction following injury, and improve long-term memory. We designed a unique battery of tests to examine the impact of AMPK augmentation on cognitive outcome, including the first use of contextual fear discrimination in CCI-injured animals. We found that significantly increasing AMPK activity through AICAR administration improved spatial recall in two versions of the Morris water maze task, as well as in context discrimination. These findings are significant, as AMPK could represent a potential therapeutic target for improving cognitive outcome in TBI patients.

Methods:

Materials:

5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR) was purchased from Toronto Research Chemicals (Ontario, Canada). Antibodies for phosphorylated AMPK (Threonine 172) and total AMPK were purchased from Cell Signaling Technology (Danvers, MA).

Animals: Adult male C57/BL6 mice weighing 25-35 grams were obtained from Charles River Laboratories (Wilmington, MA, USA). Animals were single-housed due to cage fighting, and

maintained on a 12-h light/dark cycle with *ad libitum* access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the recommendations provided in the *Guide for the Care and Use of Laboratory Animals*.

Drug preparation and treatment: 500 mg/kg of AICAR (Toronto Research Chemicals) was prepared in a 50 mg/mL saline solution or vehicle was administered to animals intraperitoneally 15 minutes, 24 hours, and 48 hours following injury in both the biochemical and in the behavioral study.

Controlled Cortical Impact injury : All animal use complied with NIH's Guide for the Care and Use of Laboratory Animals. For all experiments, mice were moderately injured using an electromagnetic cortical impact device. Mice were initially anesthetized with 5% isoflurane and 1:1mixture of N2O/O2, and then maintained under anesthesia for the duration of the procedure (2% isoflurane and 1:1 mixture of N2O/O2). Mice were placed in a stereotaxic frame and a 5 mm craniotomy (halfway between bregma and lambda, 0.5 mm lateral to midline) was performed. A heating pad was used to maintain body temperature at 37 °C. Using a 3 mm diameter impact tip and an electromagnetic cortical impact device, a single impact was delivered to the parietal association cortex at an angle of 10° from the vertical plane. The injury depth for mice was 1.0 mm at a velocity of 5.0 m/s.

The scalp was sutured closed after injury with stainless steel surgical staples. The length of time for which the righting response was suppressed then measured. The righting response was defined as the animal's ability to right itself three times consecutively after being placed on its back. Animals were given time to recuperate in a warming chamber after completion of the acute neurological assessment.

Western blotting: Animals were treated with AICAR as described in the drug preparation and treatment section. They were then sacrificed 1.5 hours following the final injection (49.5 hours post-injury), and then cortical and hippocampal tissue was collected from all groups and snap-frozen. Protein concentration was measured using a Bradford assay with BSA as the standard. Samples loaded with equal amounts of protein were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Billerica, MA), followed by 1 hour of blocking in Tris-buffered saline with Triton X-100(TBST) plus 5% BSA. Membranes were incubated in primary antibody overnight at 4 degrees. After extensive washing in TBST, immunoreactivity were assessed by an alkaline phosphatase–conjugated secondary antibody and a CDP-star chemiluminescent substrate (New England Biolabs, Ipswich, MA). The optical density of the immunoreactive bands was measured using ImageJ (Available from the NIH). All proteins examined, phosphorylated and total, were corrected by their percent expression of β-actin from the same membrane.

Motor skills testing: Vestibulomotor and motor skills were tested using the beam balance and foot fault tasks (220-223). These tests were conducted on days 1, 2, 3 and 6 post-injury. Three testing trials were given daily for each task and averaged for each animal. To test for vestibulomotor recovery the beam balance test was used. Animals were placed on a suspended cylindrical metallic beam (diameter= 1cm) and the time the mouse was able to maintain balance out of 60 seconds was recorded. Motor skill recovery as assessed by paw placement was evaluated by placing the animal on a wire grid (opening size of 1x1cm) and counting the number of foot faults out of a total of 50 steps. A foot fault was defined as when a front paw misses and appears below the plane of the grid.

Morris water maze task:

Abrreviated water maze training: 7 days post-injury, animals were trained to find a stationary, hidden platform using the Morris water maze in a single day. Each animal received ten trials,

with a 4 minute ITI. In each trial, the mouse was placed into the tank facing the wall, and then allowed to search for the submerged platform for 60 seconds. If the animal did not find the platform during this time period, it was led to the location by the investigator. The animal was then required to stay on the platform for 30 seconds before removal from the tank. During the four minute inter-trial interval (ITI) period, animals were placed in a 37˚C warming cage. A probe trial was conducted 24 hours after the completion of training. In probe trials, the hidden platform was removed from the tank and animals were allowed to search for it for 60 seconds. The search path was monitored using a tracking device connected to a video camera (Ethovision, Noldus). The data was analyzed for latency to the first platform crossing and swimming speed. The data was examined further using concentric circle analysis.

Concentric circle analysis: To examine approach of areas surrounding the platform, rings of increasing diameter are drawn around the platform (256). The data for concentric circles was analyzed for latency to first platform crossing, duration of time in the platform area, and the number of platform crossings.

Standard water maze training: Mice were trained in the standard Morris water maze task, as previously described (225-228). This occurred one week after mass water maze training (14 days post-injury), in a second room with a new platform location. All other aspects of the testing were identical to the mass training protocol, including the 4 minute ITI. Over 3 days, animals were trained to find the location of a stationary, hidden platform with four separate trials per day. Following the last day of training, the animals received a probe trial at 24 hours. The data was analyzed for latency to first platform crossing, duration of time in the platform area, and the number of platform crossings. Velocity was also examined as a control measure.

<u>Contextual discrimination:</u> The ability to differentiate between two similar contexts using a holistic spatial representation has been shown to be hippocampal-dependent. 3 weeks post-

injury, animals were trained in a context discrimination version of fear conditioning (257). In this task, animals are exposed to two boxes with both similar and distinct spatial cues each day- a "shock" cage where a brief shock is administered, and a "safe cage". Animals were pre-exposed (without shock) to two contexts sharing certain features (horizontal grid floor, background noise, animal handling to and from the room) while differing in others (differently spaced grids, scent, distal cues and floor shape). Animals were given 2 trials, one in each chamber, each day. In the shock chamber, animals were placed for 3 min and a 2 sec, 0.75 mA shock given at 148 sec. In the safe chamber, animals were placed for 3 min and no shock was given. Discrimination of the two contexts was assessed by comparing the time spent freezing (monitored in 2 sec intervals) in each chamber during the time before the shock was given. On subsequent training days, freezing behavior is scored within each context. The animal's freezing in each box is used to indicate their memory for that specific context. Within each group, order of exposure is counterbalanced, such that daily half of the group is exposed to the shock chamber first, and the other half is exposed to the safe chamber first.

Statistics: In all experiments, data collected from the same animal with one or more factors, such as water maze training performance across multiple trials and contextual discrimination across days, was subjected to repeated measures (RM) ANOVA. Concentric circle analysis was conducted using a 2-way RM ANOVA, with experimental group and level of circle as the variables. The ability to perform context discrimination was also analyzed with a 2-way RM ANOVA, with Day and Context (shock or non-shock) as the two variables. Data comparing only one factor between groups, including western blotting data and probe trial latency to the platform, duration, and crossings, were subjected to a Student's t-test.

Results:

AICAR administration does not affect recovery of vestibulomotor or motor skills post-injury: To examine whether application of a known AMPK activator could improve post-injury outcome, 500 mg/kg AICAR was administered intraperitoneally at 15 minutes, 24 hours, and 48 hours following controlled cortical impact injury (N for each group, Vehicle=8, AICAR=9). Timeline of this experiment is outlined in Figure 4.1A. Historical sham data is displayed for comparison in the behavioral results, but was not included for statistical purposes. Time points for AICAR administration were chosen based on the changes seen in AMPK phosphorylation via the TBI time course samples. To assess the biochemical effect of this treatment regimen, a separate group of animals were injured and sacrificed 1.5 hours following their third dose of vehicle or AICAR (49.5 hours post-injury). AICAR administration resulted in a significant increase in phosphorylated AMPK in the ipsilateral cortex as compared to vehicle-treated, injured animals (Fig 4.1B, t-test, $p=0.028$). No significant difference was found in recovery of vestibulomotor skills or in motor skills, as judged by the balance beam and foot fault tasks, respectively (Fig 4.1C-D).

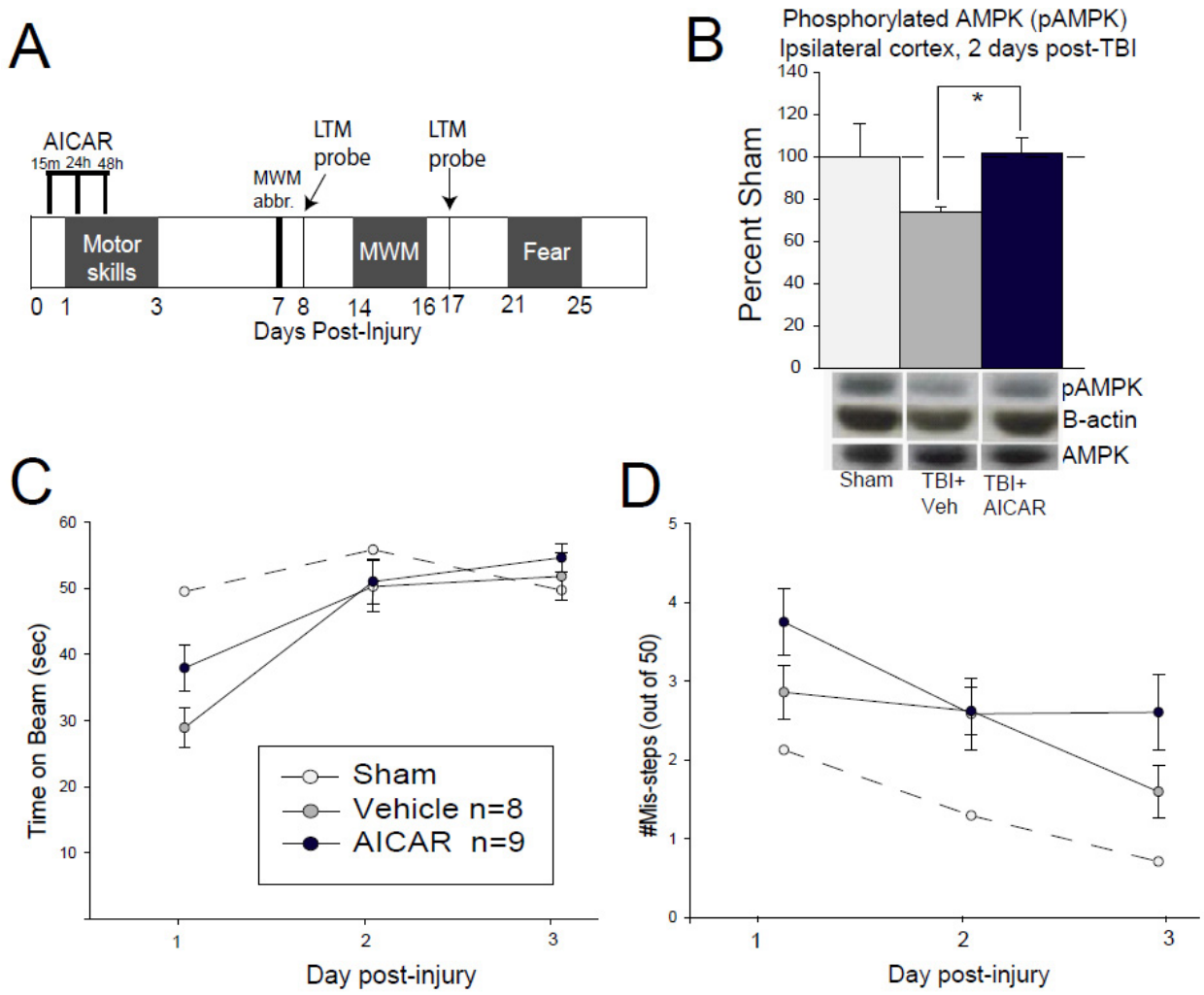

Fig 4.1 AICAR behavioral study design, biochemistry and motor skills results. Study Design (A). Animals were administered either 500 mg/kg or AICAR post-injury, once daily at 15 min, 24 hr, and 48 hr. Recovery of motor skills was assessed Days 1-3 post-injury, and then the mice were subjected to a battery of cognitive tests. One week post-injury they were trained in an abbreviated MWM paradigm (MWM abbr), then re-trained at 2 weeks for three days. Both training sessions were followed by a long-term memory (LTM) probe at 24 hours. Three weeks post-injury animals were trained in the contextual discrimination task. (B) Three days of AICAR treatment significantly increases AMPK phosphorylation in the cortex of injured animals. N=3/group. (C-D) Motor skills testing: there was no difference between AICAR and vehicle-treated animals in the balance beam task (C), or number of mis-steps with the left foot in the foot fault task (D). For figures 4.1(C-D)-4.4, vehicle, n=8; AICAR, n=9. Data are presented as mean±SEM. *p<0.05.

AICAR treatment enhances long-term memory following 1-day abbreviated water maze training: To investigate whether using an AMPK activator improves cognitive outcome, animals were trained in the abbreviated Morris water maze task on Day 7. During this training, mice received 10 trials with a 4 minute ITI. There was no difference between groups in latency to platform during training trials (Fig 4.2A). 24 hours following training in the probe trial, there was no overall difference in latency to the platform (Fig 4.2B). Conducting concentric circle analysis to more closely examine how animals in each group are approaching the platform revealed a significant difference between groups, with AICAR animals reaching the area near the platform faster than vehicle-treated animals (Fig 4.2C; 2-way RM ANOVA: $F(1, 45)= 11.668$; $p=0.004$). There was no difference between groups in swim speed during the probe trial (Fig 4.2D).

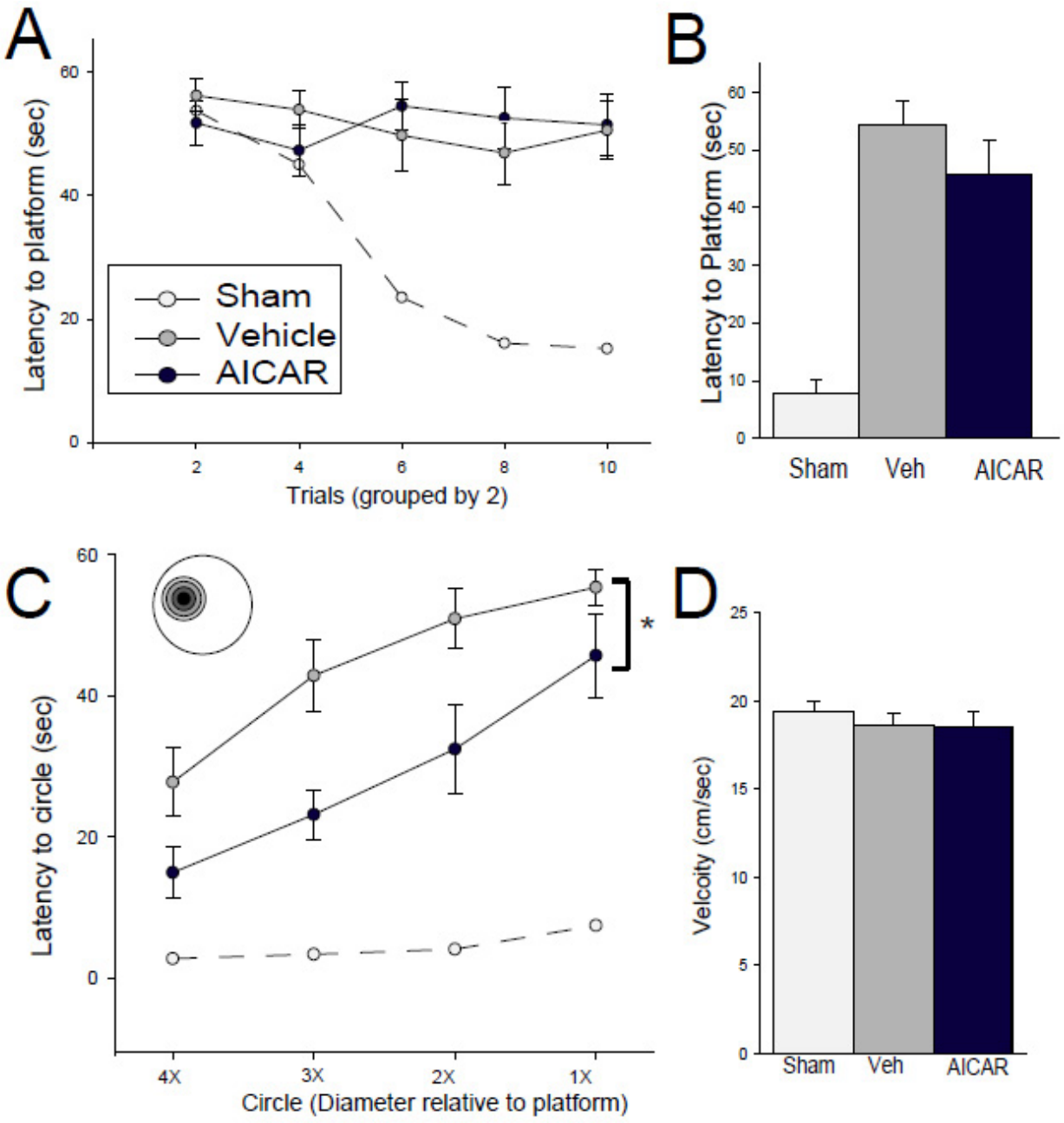

Figure 4.2 AICAR-treated mice demonstrated enhanced probe trial performance in the MWM 1-day abbreviated paradigm. (A) AICAR treatment has no impact on latency to the platform during training, or (B) on latency to the platform during the 24 hr probe. (C) AICAR animals approach area near platform significantly faster as shown by decreased latency to rings surrounding the platform. (D) There was not a difference between the injured groups and sham controls in swim speed. Data are presented as mean±SEM. *$p<0.05$.

AICAR treatment improves performance across days during standard water maze training, and long-term memory 24 hours post-training: Abbreviated training with AICAR treated animals suggested that animals may have an improvement in long-term memory. To explore this difference further, mice were re-trained 14 days post-TBI in a second tank with a new platform location using a standard water maze paradigm. The results were similar to the 1-day training paradigm, with no observable difference between the vehicle and AICAR-treated animals across training days (Fig 4.3A). However, when probed at 24 hours, the AICAR animals demonstrated significantly improved memory for the platform location (Fig 4.3B-E). Overall, this was shown by decreased latency to platform (Fig 4.3C; t-test, $p=0.041$), increased platform crossings (Fig 4.3D; $p=0.040$), and increased duration in the platform area (Fig 4.3E; t-test, $p=0.040$).

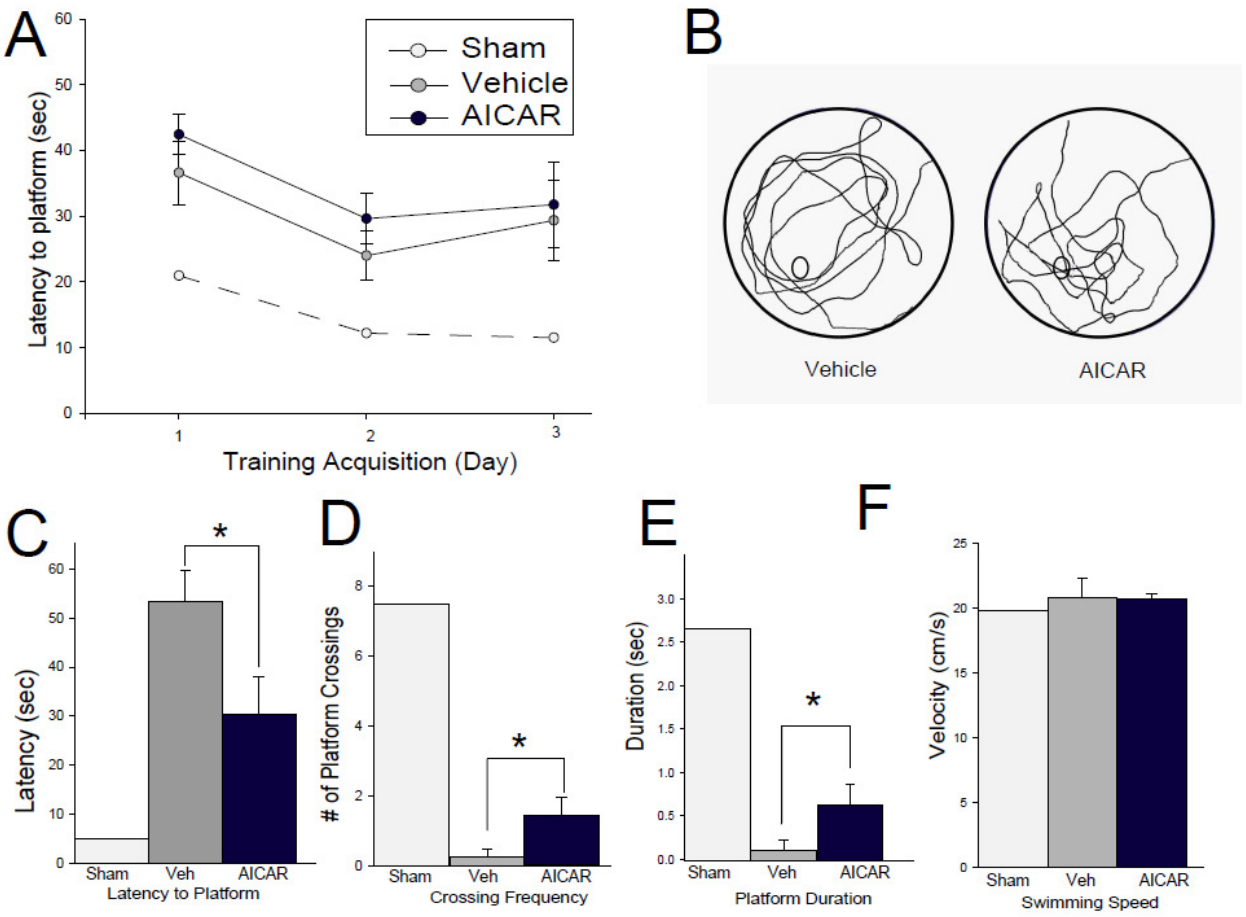

Figure 4.3 AICAR improves long-term memory following standard water maze training. (A) AICAR administered post-injury had no impact on task acquisition. (B-F) Data from the 24 hr LTM probe trial demonstrates that AICAR animals have improved long-term memory in comparison to vehicle-treated, injured animals. (B) Representative traces of the search path from both injured groups. In searching for the platform, AICAR animals demonstrated (C) decreased latency, (D) increased crossings, and (E) increased duration in the platform area. (F) There was not a significant difference between both injured groups and controls in swim speed. Data are presented as mean±SEM. *p<0.05.

Treatment with AMPK-activator AICAR improves contextual discrimination:

Beginning 3 weeks post-injury, animals were trained in a context discrimination version of fear conditioning. Animals are exposed daily to two spatially similar contexts, one in which they receive a foot shock, and one in which they do not (Testing environment displayed in Fig 4.4A). The amount of freezing behavior of the animal, indicated by a complete cessation of movement, in each of these contexts is observed and utilized as an index of learning and memory. Non-injured animals can accomplish this task on the first day of training (Fig 4.4 B). Animals treated with AICAR (Fig 4.4C) are able to discriminate a day earlier than vehicle-treated, injured animals (Fig 4.4D), and they show improvement across days [Day-box interaction, (2-way RM ANOVA: $F(2, 16)=9.928$; $p=0.002$.]. Vehicle animals are able to discriminate between the shock and safe context after 3 days of training Day 3, post-hoc t-test, $p=0.03$]; however, they did not show improvement over days [2 way RM ANOVA, day-box interaction, $F(2,14)=0.624$; $p=.550$].

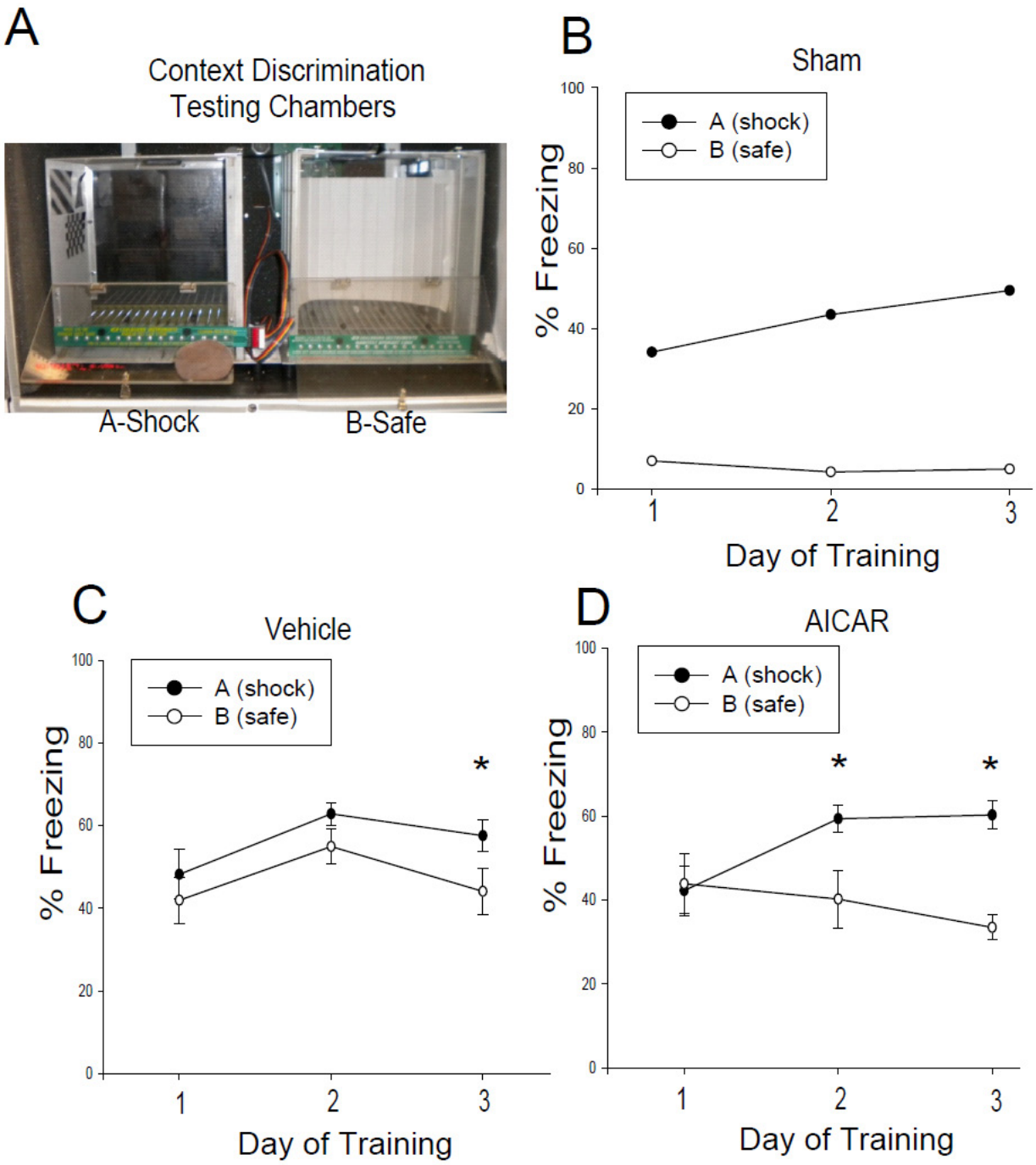

Figure 4.4 AICAR treated mice demonstrate improved contextual discrimination. (A) Context discrimination testing environment. During training animals are exposed daily to two separate contexts, a chamber in which they receive a foot-shock, “A”, and a safe chamber with no foot-shock, “B”. (B) Sham animals are capable of performing context discrimination on the first day of training. (C) Vehicle-treated, injured mice are able to discriminate between two contexts after three days of training. (D) AICAR-treated, injured mice were able to discriminate context a day earlier than vehicle-treated, injured animals. They also showed improvement in this task across days. Data are presented as mean±SEM. *p<0.05.

Discussion:

In these studies, we have investigated whether application of AICAR could increase AMPK phosphorylation post-injury, and the impact of increasing AMPK activity using this treatment on TBI cognitive outcome. We draw two major conclusions from this work. First, the biochemical results highlight that AMPK activators can be used to increase AMPK phosphorylation during the post-injury period of AMPK phosphorylation decrease discussed in Chapter 2. Secondly, the behavioral results demonstrate that across three separate behavioral tasks, AMPK-activator AICAR improved long-term memory in moderately brain-injured animals.

Using our unique behavioral testing paradigm, we were able to demonstrate the cognitive improvements associated with increased AMPK activity in injured animals. We did not find that AICAR treatment had an effect upon gross motor skills, although this is something which may be necessary to examine with more sensitive tasks. This work also has significant clinical implications, as it demonstrates that post-injury treatment to improve AMPK activity has the potential to improve cognitive outcome. Despite the fact that the treatment period was limited to 3 days post-injury, the cognitive improvement lasted an entire month post-injury, suggesting that transiently treating dysfunctional metabolism may have a long-lasting impact following injury. In addition, this is the first study which has demonstrated an impact of AMPK activation in the contextual fear discrimination task.

We previously conducted a study in which the FDA-approved AMPK activator, Metformin, was administered to moderately injured rats at 30 min, 24 hr, and 48 hr post-injury. These animals were trained in the standard water maze 2 weeks post injury. We found that after 9 days of task acquisition, these animals demonstrated a significant improvement in their performance during a 30 minute short-term memory probe (See Appendix, Fig A.1). Unfortunately at this time, we were not yet using context discrimination in the lab. The dose was also lower than what has been shown effective for improvement of spatial memory (201). However, this provides preliminary evidence that Metformin, an AMPK activator which increases GLUT4 expression, may have beneficial effects on TBI cognitive outcome.

With a similar AICAR drug treatment regimen to our own using naïve animals, in which two weeks passed between treatment and behavioral testing, acute administration of AICAR resulted in improved long-term memory in the water maze (200). In this study, animals treated for 7 days with the same dose of AICAR (500 mg/kg) did not perform differently during two different training paradigms, but they had increased preference for the platform-containing quadrant in the following probe trials (200). In addition, animals treated with AMPK-activator Metformin demonstrated signs of improved long-term memory in the water maze (201). Again, there was no difference between groups during acquisition. When animals were re-trained with a new platform location and subjected to an additional probe test at 24 hours, it was found that Metformin-treated animals spent significantly longer in the target quadrant. We did examine this parameter in the current study; however, there was no difference between groups. Injured animals have difficulty establishing quadrant preference. Our results are consistent with these findings, with no training differences between groups, but a subsequent improvement in long-term recall.

The mechanism by which AMPK leads to behavioral improvement should be subject to further investigation. A role for AMPK in long-term memory has previously been suggested by our lab (258) and others (139), specifically through its role in regulating mTOR activity and resulting dendritic protein synthesis (259). Due to the fact that AMPK inhibits mTOR and protein synthesis, *in vitro* application of AMPK activators immediately prior to long-term potentiation (LTP) results in a blockade of LTP due to inhibition of synthesis of new proteins (139). The Dash lab has demonstrated that hippocampal infusion of glucose following water maze training improves long-term memory, and that this effect is mediated through its activation of the TSC2-mTOR pathway (258). These studies were conducted in the context of activation of the AMPK and mTOR pathways in the immediate period of examination, and how a period of altered phosphorylation would affect subsequent behavioral performance in the context of injury is less clear. In their investigation of mTOR hyper-activity following injury, Chen et al. (2007) hypothesized that increased activation of this pathway may participate in the

aberrant neuroplasticity in the hippocampus. Thus, one possibility is that the alterations occurring as a result of decreased AMPK activity in the early post-injury period could contribute to subtle structural damage in areas contributing to spatial memory maintenance.

Previous behavioral studies in which naïve animals have exhibited improved performance from AMPK activators have implicated increased neurogenesis in the hippocampus (199-201). These works involved a gap in time between daily drug administration and subsequent behavioral testing. In addition, the ability to perform context discrimination task where we saw improvement in AICAR-treated mice has been shown to rely upon adult hippocampal neurogenesis (260, 261). Metformin was found capable of enhancing hippocampal neurogenesis after a prolonged treatment regimen (201). Relevant to the idea AMPK activation influences neurogenesis specific to its effects upon glucose metabolism, human pluripotent stem cells and somatic tissues being redirected to a pluripotent state are known to have an increased reliance upon glycolysis (262). It is hypothesized that these tissues have a unique combination of anabolic and catabolic requirements, including a need for fast ATP generation and a supply of reducing cofactors, which can be met by up-regulation of glycolysis and the associated pentose phosphate pathway (PPP). Increased flux of glucose through the PPP pathway has previously been demonstrated 24 hours post-injury (74, 75). The Dash lab has demonstrated that neurogenesis is increased following injury 24 hours and 3 days post-injury (263), and others have shown that genetic deletion of the progenitor cell population following injury results in an impairment of water maze task acquisition and probe trial performance (264). Whether AICAR exerts its functional effect through its influence on glucose metabolism and hippocampal neurogenesis post-injury is something which should be examined further.

AICAR may exert its effects through influencing post-injury neuronal survival, although evidence for the effect of this cascade on cell viability is conflicting. There is work to suggest that AMPK could control neuronal survival following metabolic insult such as glucose deprivation *in vitro* (149, 155). AICAR applied to hippocampal neurons during glucose

deprivation has been shown to increase the number of surviving neurons (153). Application of AICAR to cerebellar granule neurons leads to an increase in surface expression of Glucose transporter 3 (GLUT3), and silencing of GLUT3 leads to a significant increase in excitotoxic insult-related cell death (159). Despite these positive findings, there has also been in vivo work which has demonstrated that high levels of AMPK activation lead to increase cell and tissue loss. Phosphorylation of AMPK is elevated in the brain following stroke, and it has been shown in that model that pharmacological inhibition or genetic deletion of the α2 subunit of AMPK leads to reduced tissue loss (265, 266). Further, application of a high-dose AICAR to animals prior to behavioral training have been shown to lead to decreased cognitive performance and increased apoptosis by up-regulation of proapoptotic Bcl-2 associated protein (BAX) (199). Thus, while work in peripheral tissues has demonstrated a clear link between AMPK and cell death or autophagy, this link is less clear in the brain (138, 154). In the present work, there also did not appear to be a large amount of hippocampal cell death in mice at this injury level, so this hypothesis was not examined further.

The effect of AMPK activation may also relate to its modification of excitatory transmission. It was first demonstrated many years ago that AICAR is capable of inhibiting seizures in a dose-dependent manner (267). Kuramoto et al. (2007) demonstrated that phosphorylated AMPK interacts with $GABA_B$ receptors, resulting in the activation of inwardly rectifying K^+ channels. In this paper, it was demonstrated that AMPK phosphorylated $GABA_B$ receptors in the hippocampus, that this phosphorylation was increased in vivo following ischemia, and that preventing this phosphorylation in culture resulted in a significant increase in neuronal death post-anoxia (268). One study following mild TBI in which patients were subjected to extensive neuropsychological, electrophysiological, and structural imaging has recently shown that there is decreased cortical inhibition two weeks post-injury, which returns to normal by six weeks (269). It was hypothesized that this effect was mediated by $GABA_B$ receptors. Therefore, it is possible that AICAR could be altering cortical excitation in a beneficial manner following injury.

In summary, our work demonstrates that augmentation of AMPK phosphorylation could represent a potential therapeutic treatment option to improve post-injury outcome. This is a significant finding, as there are FDA-approved AMPK activators, such as the anti-diabetic treatment drug Metformin, which could potentially be administered to TBI patients. Although indirect evidence has implicated that altered activity or presence of GLUT4 may be part of the mechanism of AMPK-associated improvement, this has not been directly tested. For this reason, we decided to directly examine the effect of inhibiting glucose uptake via GLUT4 in the brain on TBI outcome as outlined in the following chapter.

CHAPTER V. THE INFLUENCE OF BRAIN-SPECIFIC INHIBITION OF AMPK TARGET GLUCOSE TRANSPORTER-4 ON TBI OUTCOME

Introduction:

Metabolic suppression following head injury has been extensively documented (14, 15, 22, 62). It is important that brain glucose uptake exceeds utilization following TBI, since an altered metabolic state can render cells vulnerable to increased damage and cell death (81). Despite the knowledge that glucose metabolism is altered post-injury, the underlying mechanism responsible for this change remains unclear. In peripheral tissue, it is known that translocation of glucose transporter 4 (GLUT4) to the cell surface results in fast amplification of glucose uptake (270, 271). Although it has been hypothesized that GLUT4 serves a similar energetic purpose within the brain (109), this has not been directly tested. Neurologically stressful events, such as ischemia, have been demonstrated to result in an acute increase in plasma membrane expression of hippocampal GLUT4, and it is suggested that this occurs in a AMPK-regulated manner (130). As demonstrated by the western blotting and immunohistochemistry results presented in Chapter 3, there is evidence to suggest that AMPK phosphorylation and GLUT4 activity is decreased following brain injury. The work demonstrated in Chapter 4 highlights that increasing post-injury AMPK phosphorylation through drug administration of AICAR results in improved functional outcome. Whether further suppression of AMPK-regulated GLUT4 would be detrimental to outcome has not been examined.

There is experimental evidence demonstrating that chronically decreased GLUT4 expression in the brain can impact behavior. The Zucker fatty rat, a genetic animal model of Type 2 Diabetes, has shown that affected animals have impaired memory. This was demonstrated by worsened performance compared to controls in a task with long inter-trial intervals (137). These rats had decreased GLUT4 expression in hippocampal plasma membrane fractions, as identified by western blotting. Many groups have also demonstrated that animals with chemically-induced diabetes are cognitively impaired in tasks of spatial memory (119, 120, 272, 273). Yet, results from these genetic manipulations cannot be used to directly assess the role of GLUT4 in cognition, as these animals also have hyperinsulinemia and other metabolic alterations. If direct inhibition of glucose uptake via GLUT4 would influence

behavior has not been examined. Furthermore, despite the existing hypothesis that activation of GLUT4 is important in response to neural stress, how manipulating GLUT4 levels following trauma affects outcome has only been recently evaluated (205).

Indinavir sulfate is a drug which has been recently identified as an inhibitor of GLUT4. This drug is a potent inhibitor of the HIV protease, thus attenuating replication and spread of the HIV virus (274). When orally administered to HIV patients, Indinavir is known to have a number of associated systemic toxicities, including jaundice and kidney impairment (275). Protease inhibitors including Indinavir have also been observed to induce metabolic disturbances, with one study reporting a rate of Type 2 Diabetes 4 times greater in the HIV treated patients (276, 277). In both healthy human volunteers and in rodents, application of protease inhibitors has been shown to result in decreased glucose uptake into tissues (278, 279). Further *in vitro* testing has demonstrated that the drug Indinavir is particularly adept at specifically inhibiting GLUT4 uptake (280-284). In contrast, the other two predominant glucose uptake via the other brain transporters, GLUT1 and GLUT3, are not significantly inhibited by physiologically relevant concentrations of Indinavir (282). This drug does not alter insulin receptor activation or trafficking of GLUT4 (280, 283). It is now believed to operate by competitively inhibiting the cytoplasmic binding site of GLUT4 (284). Glucose transporters have binding sites on either side of the lipid bilayer which cannot be simultaneously occupied. Thus, if the cytoplasmic site of GLUT4 is occupied by Indinavir, this would prevent GLUT4-mediated entry of extracellular glucose into the cell (98). Significant to the current work, this drug has been shown to inhibit an increase in glucose uptake in hippocampal slice preparations following excitotoxic insult (238). Thus, Indinavir appears to specifically inhibit GLUT4, and this has previously been shown to alter hippocampal glucose uptake *in vitro*.

We hypothesized that further inhibiting of glucose uptake via GLUT4 present at the cell surface by Indinavir would be detrimental to TBI outcome. Here, we present evidence that application of Indinavir 15 minutes pre-injury did not affect motor skills but led to significant cognitive impairment. Specifically, it led to impairment in animals to improve their behavioral

performance across days when compared to injured, saline-administered mice. This is demonstrated by decreased performance of Indinavir mice during training in the standard Morris water maze task, as well as a complete impairment in their ability to contextual fear discrimination after three days of training.

Methods:

Materials: Indinavir Sulfate was purchased from Toronto Research Chemicals (Ontario, Canada).

Animals: Adult male C57/BL6 mice weighing 25-35 grams were obtained from Charles River Laboratories (Wilmington, MA, USA). Animals were single-housed due to cage fighting, and maintained on a 12-h light/dark cycle with *ad libitum* access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the recommendations provided in the *Guide for the Care and Use of Laboratory Animals*.

Drug preparation and treatment: A 50 millimolar stock solution of Indinavir in saline was prepared. A concentration of 100 micromolar Indinavir is known to inhibit glucose transporter 4 *in vitro*, and has been shown to inhibit glucose uptake in the hippocampus (238, 282). Based on this, we decided to deliver a concentration of 1.5 millimolar, assuming dilution to 100 micromolar within the brain upon delivery.

Surgical Procedures

Intracerebroventricular (i.c.v.) Administration of Indinavir: Male mice deeply anesthetized induced with 5% isoflurane and 1:1 O_2:N_2O for 4 minutes and 30 seconds, and maintained at 1:1 O_2:N_2O through a nose cone for the duration of the surgery. A midline incision was made and the soft tissue reflected to expose the skull. 15 minutes prior to injury, drug delivery into the

lateral ventricle was performed as described by our lab previously for mice (285). A small burr hole was made ipsilateral to the injury site, and 1 uL of Indinavir was infused into the lateral ventricle (1.0 mm lateral, 0.40 mm rostral to bregma). The needle was lowered 2.25 mm from the skull surface. The drug was delivered at a rate of 0.3 microliters for a minute, and then the needle was held in place for 1 minute following completion of drug infusion. Animals were maintained under anesthesia until CCI injury.

Controlled Cortical Impact (CCI) Injury: CCI injury was performed using the electromagnetic controlled cortical impact device and performed essentially the same as described for the AICAR-related studies in chapter 4. 15 minutes after completion of i.c.v Indinavir administration, a 5 mm craniotomy (halfway between bregma and lambda, 0.5 mm lateral to midline) was performed over right parietal cortex. In this study, injury magnitude was reduced in anticipation of worsened cognitive outcome in Indinavir-treated animals. The depth of impact was 1.0 mm, and the velocity was 4.0 m/s. All animals recovered in a warm chamber following the surgery, prior to being returned to their home cages.

Motor skills testing: Vestibulomotor and motor skills were tested using the beam balance and foot fault tasks (220-223). These tests were conducted on days 1, 2, 3 and 6 post-injury. Three testing trials were given daily for each task and averaged for each animal. To test for vestibulomotor recovery the beam balance test was used. Animals were placed on a suspended cylindrical metallic beam (diameter= 1cm) and the time the mouse was able to maintain balance out of 60 seconds was recorded. Motor skill recovery as assessed by paw placement was evaluated by placing the animal on a wire grid (opening size of 1x1cm) and counting the number of foot faults out of a total of 50 steps. A foot fault was defined as when a front paw misses and appears below the plane of the grid.

Morris water maze task:

Abbreviated maze training: 7 days post-injury, animals were trained in to find a stationary, hidden platform using the Morris water maze in a single day. Each animal received ten trials, with a 4 minute ITI. In each trial, the mouse was placed into the tank facing the wall, and then allowed to search for the submerged platform for 60 seconds. If the animal did not find the platform during this time period, it was led to the location by the investigator. The animal was then required to stay on the platform for 30 seconds before removal from the tank. During the four minute inter-trial interval (ITI) period, animals were placed in a 37˚C warming cage. A probe trial was conducted 24 hours after the completion of training. In probe trials, the hidden platform was removed from the tank and animals were allowed to search for it for 60 seconds. The search path was monitored using a tracking device connected to a video camera (Ethovision, Noldus). The data was analyzed for latency to the first platform crossing and swimming speed.

Standard water maze training: Mice were trained in the standard Morris water maze task, as previously described (225-228). Training occurred one week after mass water maze training (14 days post-injury), in a second room with a new platform location. All other aspects of the testing were identical to the mass training protocol, including the 4 minute ITI. Over 3 days, animals were trained to find the location of a stationary, hidden platform with four separate trials per day. Following the last day of training, the animals received a probe trial at 24 hours. The data was analyzed for latency to first platform crossing, duration of time in the platform area, and the number of platform crossings. Velocity was also examined as a control measure.

Concentric circle analysis: To examine approach of areas surrounding the platform, rings of increasing diameter are drawn around the platform (256). The data for concentric circles was analyzed for latency to first platform crossing, duration of time in the platform area, and the number of platform crossings.

Contextual discrimination: The ability to differentiate between two similar contexts using a holistic spatial representation has been shown to be hippocampal-dependent. 3 weeks post-injury, animals were trained in a context discrimination version of fear conditioning (257). In this task, animals are exposed to two boxes with both similar and distinct spatial cues each day- a "shock" cage where a brief shock is administered, and a "safe cage". Animals were pre-exposed (without shock) to two contexts sharing certain features (horizontal grid floor, background noise, animal handling to and from the room) while differing in others (differently spaced grids, scent, distal cues and floor shape). Animals were given 2 trials, one in each chamber, each day. In the shock chamber, animals were placed for 3 min and a 2 sec, 0.75 mA shock given at 148 sec. In the safe chamber, animals were placed for 3 min and no shock was given. Discrimination of the two contexts was assessed by comparing the time spent freezing (monitored in 2 sec intervals) in each chamber during the time before the shock was given. On subsequent training days, freezing behavior is scored within each context. The animal's freezing in each box is used to indicate their memory for that specific context. Within each group, order of exposure is counterbalanced, such that daily half of the group is exposed to the shock chamber first, and the other half is exposed to the safe chamber first.

Tissue processing and immunohistochemistry: After completion of the behavioral study, animals were euthanized and prepared for histological assessment of brain tissue. Mice were deeply anesthetized through intraperitoneal injection of sodium pentobarbitol, and then perfused with saline followed by 4% paraformaldehyde in PBS. After perfusion, brains were then removed and cryo-protected for 24 hours in PBS with 30% sucrose. Samples were then frozen in TCS, sectioned on a cryostat set to -20˚C at a thickness of 20 microns, and stored in 24-well plates. Free-floating sections of interest were selected, washed with PBS and then placed in the relevant primary antibody in PBS containing 2% BSA and 2.5% normal goat serum. After extensive washing, immunoreactivity was detected using species-specific

fluorescent (Alexa-Fluor) secondary antibody. For presentation purposes, the images were color-corrected and cropped in Adobe Photoshop.

Statistics: In all experiments, data collected from the same animal with one or more factors, such as water maze training performance across multiple trials and contextual discrimination across days, was subjected to repeated measures (RM) ANOVA. Concentric circle analysis was conducted using a 2-way RM ANOVA, with experimental group and level of circle as the variables. The ability to perform context discrimination was also analyzed with a 2-way RM ANOVA, with Day and Context (shock or non-shock) as the two variables. Data comparing only one factor between groups, including probe trial latency to the platform, duration, and crossings, were subjected to a Student's t-test.

Results:

Inhibition of GLUT4 does not result in worsened recovery of vestibulomotor or motor skill performance: To determine whether glucose uptake through GLUT4 can contribute to TBI recovery, we administered 1.5 mM Indinavir into the lateral ventricle (1 uL) ipsilateral to the injury site 15 minutes prior to TBI (Indinavir=8; Vehicle=10). Compared to the behavioral experiment outlined in Chapter 4, the injury magnitude was decreased in anticipation of worsened behavioral performance in Indinavir-treated animals. Timeline of this experiment is outlined in Figure 5.1A. No significant difference was found in recovery of vestibulomotor skills or in motor skills, as judged by the balance beam (Fig 5.1B) and foot fault tasks (Fig 5.1C), respectively. Representative sham data is included in graphs for comparative purposes, but was not included in the statistical analysis.

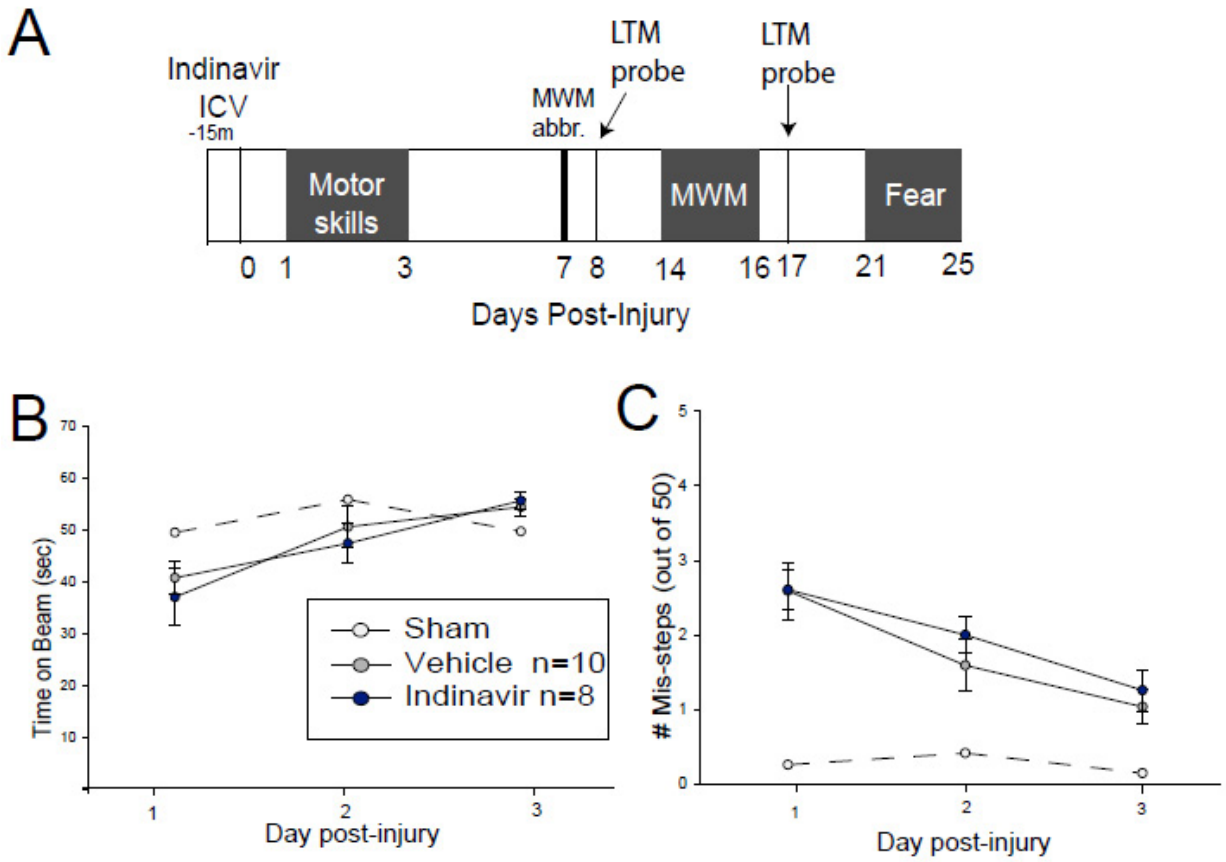

Figure 5.1 Indinavir behavioral study design and motor skills recovery data. (A) Study Design. 15 minutes prior to injury, animals were ICV injected with either Indinavir or Saline vehicle. Recovery of motor skills was assessed Days 1-3 post-injury, and then the mice were subjected to a battery of cognitive tests. One week post-injury they were trained in an abbreviated MWM paradigm, then re-trained at 2 weeks for three days. Both training sessions were followed by a long-term memory (LTM) probe at 24 hours. Three weeks post-injury animals were trained in the contextual discrimination task. (B-C) Motor skills testing: there was no difference between AICAR and vehicle-treated animals in (B) the balance beam task, or (C) number of mis-steps with the left foot in the foot fault task. For figures 5.1-5.4, vehicle, n=10; Indinavir, n=8. Data are presented as mean±SEM. *p<0.05.

Indinavir treatment does not significantly impair performance following abbreviated water maze training: To assess the impact of GLUT4 inhibition via Indinavir, animals were first subject to 1 day of abbreviated training in the Morris water maze on Day 7. Two animals were removed from further behavioral testing from the Indinavir group because they were incapable (or unwilling) to swim. During this training, mice received 10 trials with a 4 minute ITI. There was no difference between groups in latency to platform during training trials (Fig 5.2A). 24 hours

following training, latency to platform during the probe trial is not significantly different (t-test, p=0.438) (Fig 5.2B). There was no difference in swim speed (Fig 5.2C).

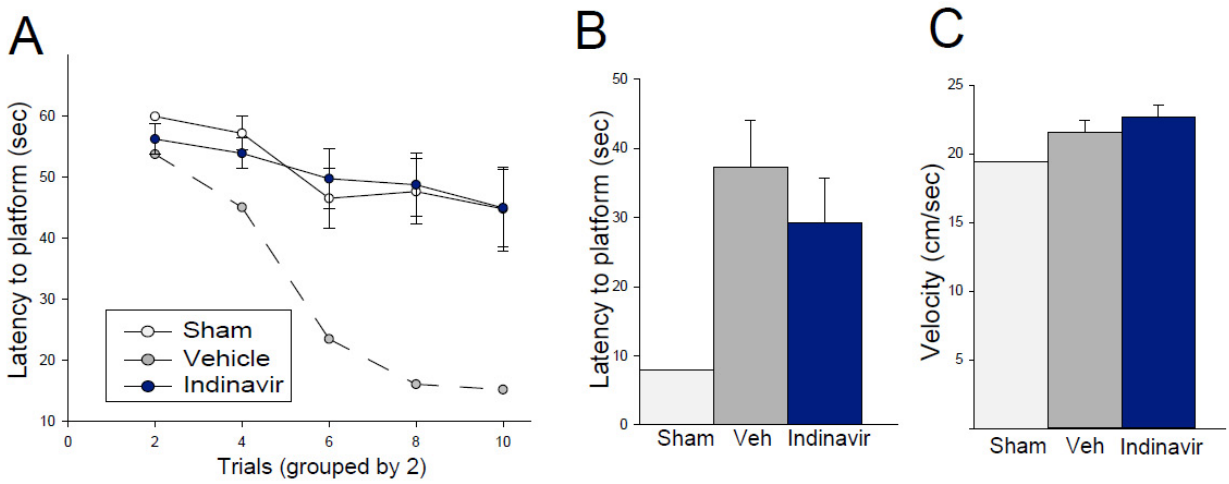

Figure 5.2 Indinavir treatment has no impact on performance in abbreviated water maze paradigm. Mice receiving a pre-injury intracerebroventricular injection of Indinavir did not perform significantly different than vehicle-treated animals in the 1 day paradigm of the water maze. (A) Treatment did not affect task acquisition. There was also no difference in probe trial performance between groups, as demonstrated (B) by latency to platform and (C) no difference in swim speed. Data are presented as mean±SEM.

Indinavir-treated mice demonstrate impaired memory during standard water maze training, and general memory impairments 24 hours post-training: To more thoroughly examine whether long-term memory is impaired across days in animals exposed to acute inhibition of GLUT4, mice were re-trained 14 days post-TBI in a second tank using a standard water maze paradigm. Animals received 4 trials per a day, with a 4 minute ITI. There was a significant difference in training trial performance between the two groups over 3 days (group-day interaction, 2-way RM ANOVA, $F(1,2)=4.243$; $p=0.02$) (Fig 5.3A). Vehicle-treated, injured mice demonstrated a decrease in latency across days of training, whereas the latency of Indinavir-treated animals did not significantly decrease across days.

In the probe trial 24 hours post-injury, there was a general trend towards vehicle animals having a significantly shorter latency to the platform during the probe trial (t-test, p=0.08) (Fig 5.3B). There was not a significant difference in swim speed (Fig 5.3C). Quantification of parameters in the surrounding platform area by examining behavior in

concentric circles of increasing diameter centered on the platform revealed that Indinavir animals had significant impairments. Specifically, vehicle-treated animals had significantly decreased latency to the immediate vicinity of the platform (2-way RM ANOVA: $F_{(1,18)}$=18.503; p<0.001) (Fig 5.3D). Vehicle mice also had a significant increase in the frequency of crossing the concentric circle area (2-way RM ANOVA: $F_{(1,18)}$=9.970; p=0.005) (Fig 5.3E), and spent significantly more time in the general vicinity of the platform location than Indinavir-treated animals (2-way RM ANOVA: $F_{(1,18)}$=12.21; p=0.003). (Fig 5.3F)

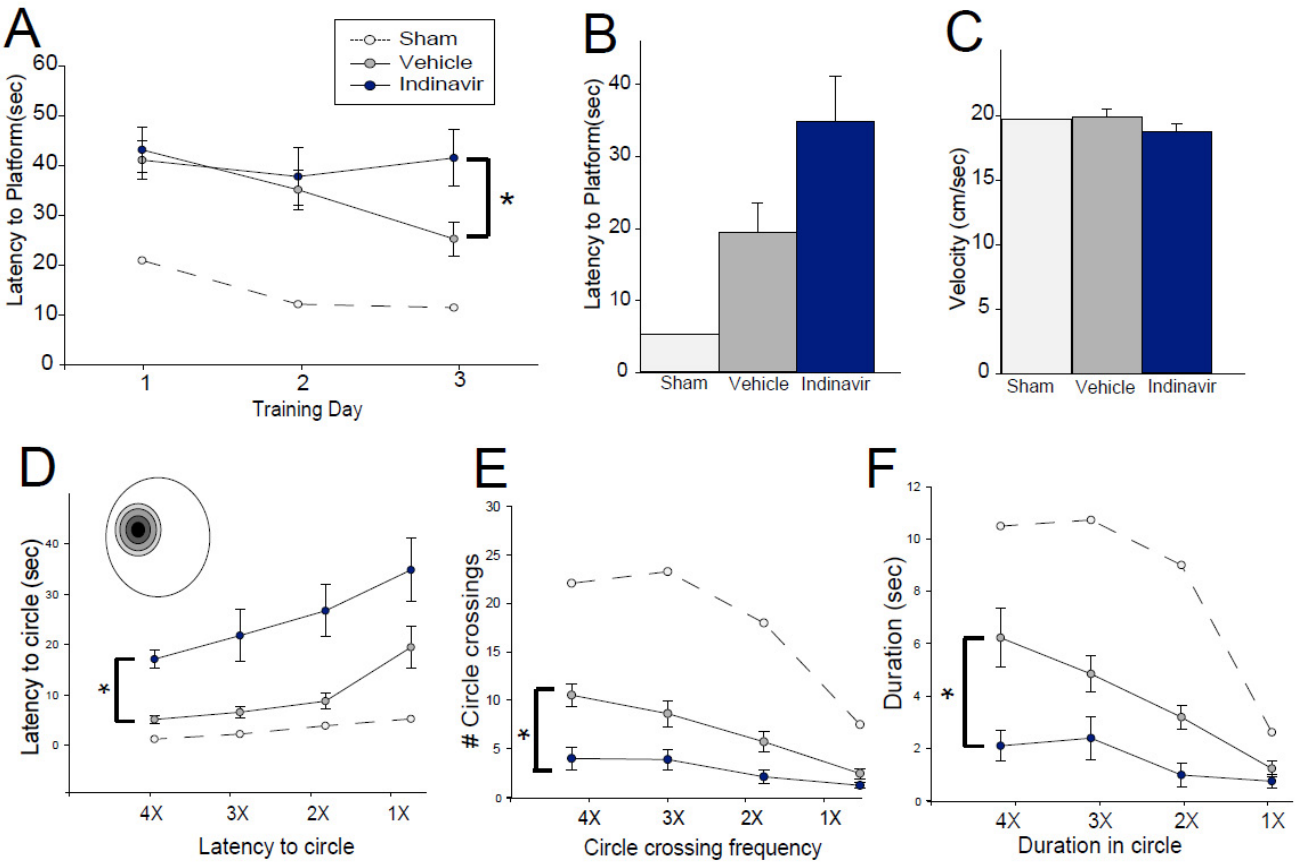

Figure 5.3 Indinavir mice are impaired in learning and memory in the standard water maze paradigm. (A) Mice treated with Indinavir 15 min pre-injury are significantly impaired in their acquisition of the standard water maze task across days compared to saline-treated, injured animals. (B-F): 24 hr probe-trial data. (B) There was a trend towards higher latency to the platform in Indinavir-treated animals (p=0.08). (C)There was no difference in swim speed. There were significant differences in their approach to the platform as examined by concentric circle analysis. In comparison to vehicle-treated mice, Indinavir mice demonstrated (D) increased latency, (E) decreased frequency of crossing, and (F) decreased duration in the areas near the platform. Data are presented as mean±SEM. *p<0.05.

Indinavir treatment results in inability to perform context discrimination task: Beginning 3 weeks post-injury, animals were trained in a context discrimination version of fear conditioning (257). In this task, animals are exposed to two boxes with both common and distinct spatial cues each day- a “shock” cage where a brief shock is administered, and a “safe cage”. This task is difficult for animals with hippocampal damage because it requires the ability to use the entire context to discriminate, rather than reliance upon a single cue. The ability of the animal to differentiate between the shock and safe cages is used as an index of learning and memory. Un-injured sham animals are able to discriminate on the first training day (5.4A). Injured vehicle-treated animals learn to discriminate between shock and non-shock context by day 2, with improvement over days (day-box interaction, 2-way RM ANOVA: $F(2,20)=4.691$; $p=0.021$)(Fig. 5.4B) The ability to discriminate between the shock and safe environment does not improve in the Indinavir animals, even after multiple days of training (day-box interaction, 2-way RM ANOVA: $F(2,16)=0.804$; $p=0.465$) (Fig 5.4C).

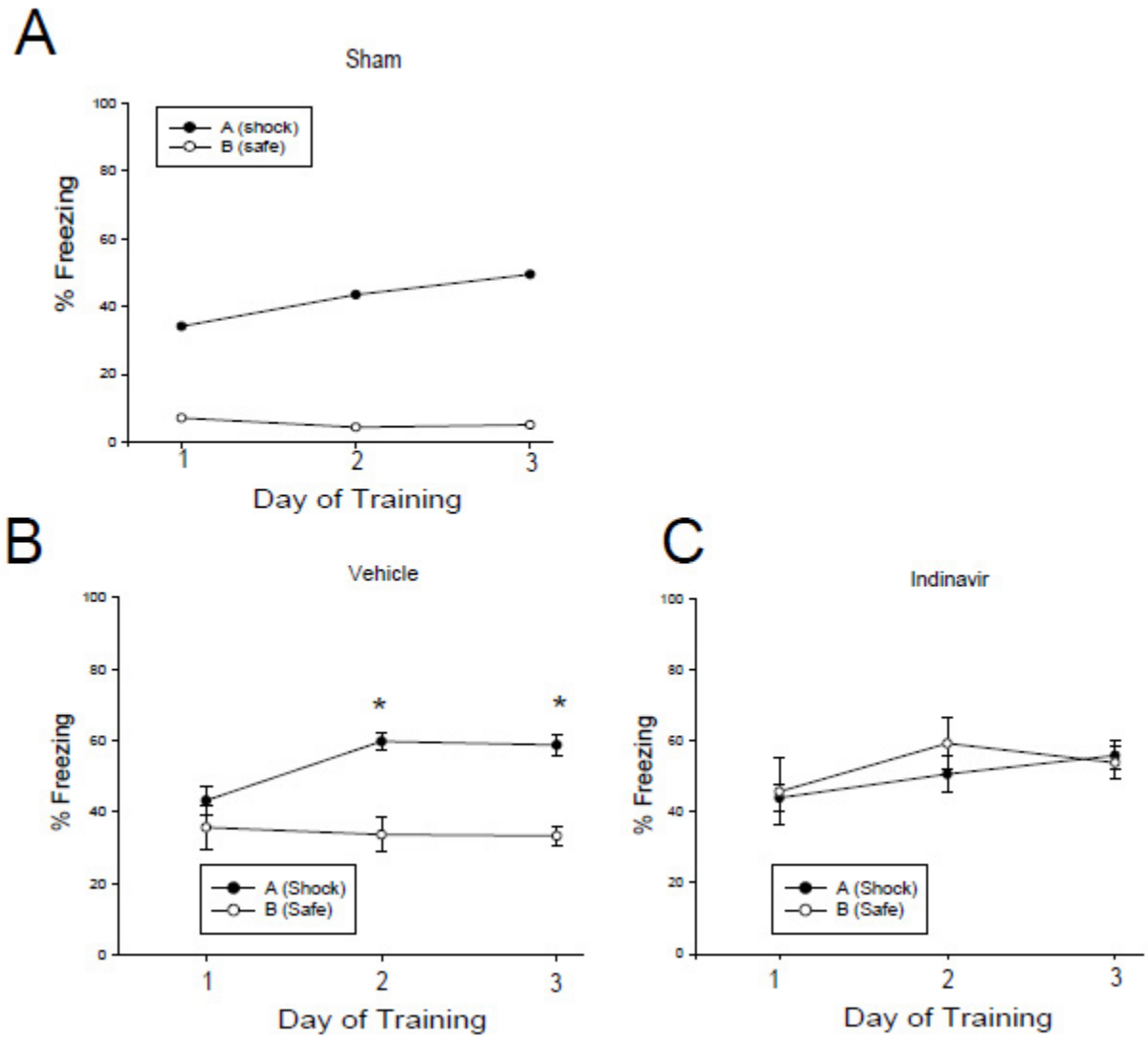

Figure 5.4 Indinavir context discrimination results. (A) Sham animals are able to discriminate between shock and non-shock contexts on Day 1 of training. (B) Vehicle-treated mice are capable of performing context discrimination by Day 2, while (C) Indinavir-treated animals are unable to discriminate even after 3 days of training. Data are presented as mean±SEM. *p<0.05.

Indinavir treatment worsens TBI histopathology: Following completion of the study, the brains of animals behaviorally tested were processed for further histopathological analysis. Preliminary examination suggests that there may be an increase in the cavity size in the Indinavir animals (Fig 5.5A). In addition, immunohistochemical analysis of Glial Fibrillary Acidic Protein (GFAP), and intermediate filament marker present in astrocytes which demonstrates altered morphology following neural insult, was completed in a small group of animals. There appeared to be an increase of GFAP in the dentate gyrus of animals administered Indinavir (Fig 5.5B). This finding was observed in other areas as well, including hippocampal CA3 and the thalamus.

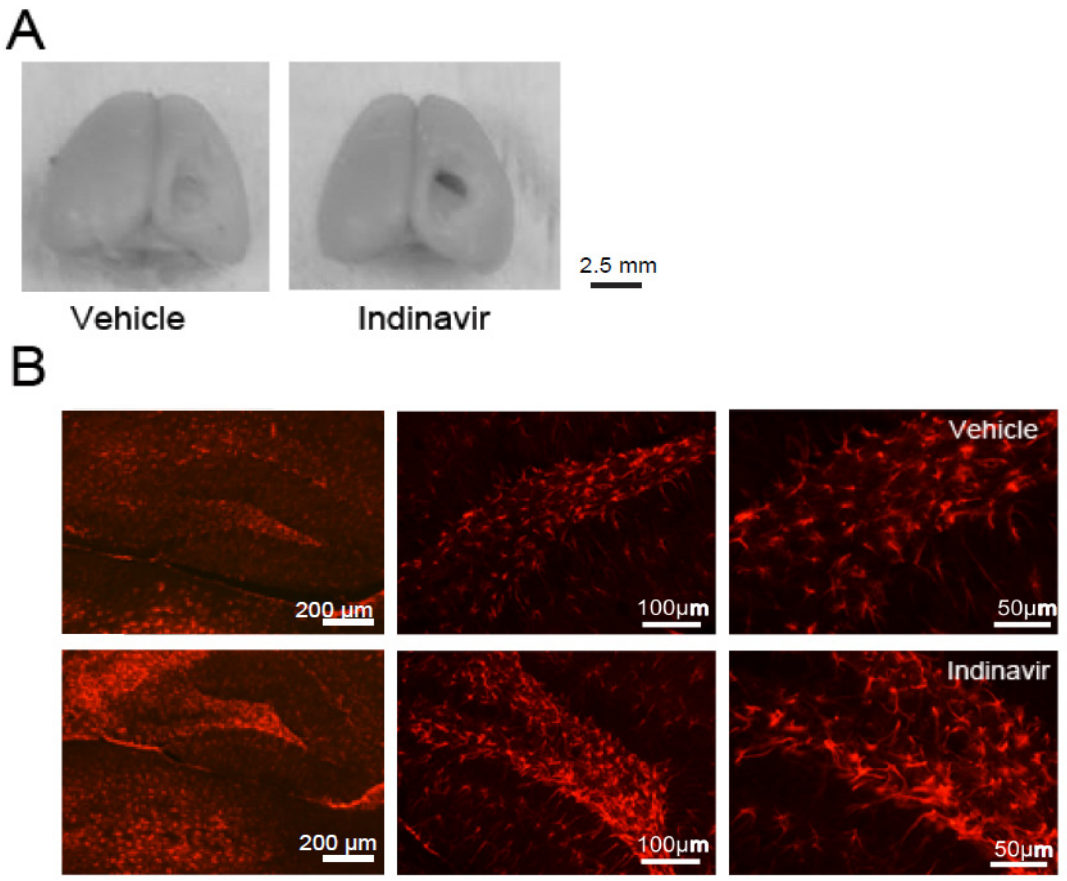

Figure 5.5 Indinavir treatment appears to influence underlying TBI histopathology. (A) Representative whole brain images from each group in the behavioral study prior to processing for immunohistochemistry. (B) Representative immunohistochemistry images of Glial Fibrillary Acidic Protein (GFAP) expression in the dentate gyrus. A section from one animal is presented in increasing magnification in each row. Vehicle-treated animals (top row) appear to have less GFAP expression in the dentate than those treated with Indinavir (bottom row).

Discussion:

In this work, we sought to examine whether further suppression of glucose transport via GLUT4 contributes to post-injury outcome. We found that animals treated 15 minutes prior to injury with the Glut-4 inhibitor Indinavir at a dose demonstrated in cell culture to inhibit glucose uptake have impaired long-term memory (280-283). A second finding was that animals tested in the behavioral study appeared to have increased contusion volume upon gross examination, and an increase immunoreactivity of Glial Fibrillary Acidic Protein (GFAP) in the hippocampus. These findings support the idea that glucose uptake following injury is capable of influencing cognitive outcome, and that GLUT4 may be important in post-injury recovery of metabolism.

In both the standard water maze task and in context discrimination training, Indinavir-treated mice showed limited performance improvement across days. In the standard water maze task, while the vehicle TBI group decreased their average initial latency by 20 seconds by the third training day, the Indinavir animals received the same group average on day 3 as they did upon the first day of training. In addition, despite three days of training in the context discrimination task, Indinavir mice were completely unable to discriminate between two similar contexts. This is an intriguing result, given that the injury magnitude was reduced from the AICAR experiment outlined in Chapter 4 in anticipation of this behavioral worsening. Further, these results are noteworthy in comparison to the results presented in Chapter 4, which demonstrated that increasing AMPK activation led to opposite results, including an improvement in probe trial performance following standard water maze and also in context discrimination. We would argue that Indinavir did not result in a general “sickness” of the animal, as motor skills recovery was unimpaired. It has previously been demonstrated in slice preparations that the cerebellum does not experience facilitative glucose uptake as the hippocampus does, thus motor function may be less affected by GLUT4 inhibition (286). In addition, there was no difference in performance between groups in the initial abbreviated training task or in recall during the subsequent probe trial one week post-injury.

Although it has been previously suggested that GLUT4 may play a role in amplifying glucose transport and maintaining homeostasis in the brain under stress conditions (109), this has not been directly tested. The gross observation that Indinavir-treated animals appear to have increased contusion volume, and an increase in GFAP immunoreactivity support the idea that deprivation of the energy source provided by GLUT4 transport may generally worsen TBI-related pathology. Following neural insult, one reason for increased expression of the intermediate filament protein GFAP is formation of a glial scar, separating lesioned areas from healthy tissue (286). Thus, increased GFAP reactivity can be one indication in an increase in tissue damage related to the inhibition of glucose transport via GLUT4 inhibited by Indinavir. In support of this interpretation, it has previously shown that application of Metformin, which increases GLUT4 translocation, decreases contusion volume following ischemia (204). These pieces of evidence support the idea that inhibiting GLUT4 is detrimental to post-injury pathology, although the exact mechanism should be subject to further study.

One previous study has examined the impact of Indinavir on water maze performance in rodents (287), in the context of its effect on experimental models of dementia. These authors hypothesized that because Indinavir inhibits the HIV protease, it may also be capable of targeting beta-amyloid converting enzyme (BACE). In this study, daily Indinavir alone was found to have no effect on behavior. When administered following injection of chemical agents used to model Alzheimer's disease, it was found to attenuate the memory deficits. However, the effect of Indinavir on β-amyloid was not directly measured. Further, the difference between this study and our own may be due to many other factors, such as the route and length of time of Indinavir administration. In addition, the class of drugs which Indinavir belongs has been associated with neurocognitive impairments in the HIV patient population. These deficits have been shown to improve upon treatment cessation (288). Thus, there is clinical evidence to suggest that Indinavir and other antiretrovirals may contribute to cognitive impairment.

As antiretroviral therapy has been hypothesized to contribute to this impairment, there has recently been research upon possible toxic effects of Indinavir on brain tissues *in vitro*,

including its effect upon brain endothelial cells and cultured astrocytes (289, 290). The consensus from these works is that Indinavir leads to an increase in reactive oxygen species, and this is correlated with a decrease in the antioxidant glutathione, and a decrease in the mitochondrial membrane potential. These effects are well-characterized results of glucose intolerance and are also correlated with diabetic neuropathy (291). Additionally, gene silencing of glucose transporter 3 (GLUT3) has been shown to affect the mitochondrial membrane potential in this manner, and increase death following excitotoxicity (155). Indinavir less directly affected cell viability than other antiretrovirals in astrocytes, arguing against direct toxicity in this population (290). These findings, coupled with the fact that Indinavir inhibits glucose uptake in hippocampal slices (238), provide support that this drug exerts a physiological effect on brain metabolism, specifically in a manner consistent with inhibition of glucose uptake.

Studies have investigated the impact of manipulating brain glucose availability on cognitive performance in non-diabetic animals. Glucose in the extracellular fluid has been demonstrated to decrease in a region-specific manner during and following cognitive testing (292). During hippocampal-dependent tasks this dip is seen in that region, and systemic treatment of glucose prior to behavioral testing enhances task performance and eliminates the observed decrease in extracellular glucose and improves behavioral performance. Furthermore, intracerebroventricular injection of glucose following behavioral training has been shown to enhance memory, demonstrating that memory enhancement is not strictly due to peripheral effects of glucose treatment (293, 294). Our lab furthered this finding by demonstrating that hippocampal glucose infusion leads to spatial memory improvements when animals were treated immediately following water maze training, and that this treatment was associated with increased activation of the TSC2-mTOR pathway (258). Specific to insulin signaling and GLUT4, intrahippocampal administration of peptides targeting endogenous insulin leads prior to training leads to worsened cognitive performance (295). Thus, there is evidence that glucose directly influences cognition in normal animals, and that this is in part regulated by insulin-mediated glucose uptake.

The impact of decreased brain glucose uptake on cognition has also been investigated in animal models of diabetes. This is often investigated through injection of Streptozotocin (STZ). When delivered directly into the brain via intracerebroventricular injection, STZ has been shown to decrease brain glucose uptake and cognitive performance without altering peripheral blood glucose (119, 120, 272, 273). STZ treatment has also been shown to correlate with decreased neurogenesis (296-298). Memory performance has also been examined in Zucker fatty rats, used to model Type 2 Diabetes (T2D) (137). These animals have decreased GLUT4 plasma membrane expression in the hippocampus as compared to their unaffected littermates, and are impaired at longer interval delays in performance of a go/no-go delayed alternation task. This is significant, as the animals were able to learn the task rule and perform similarly to controls at a shorter delay, suggesting their deficit is hippocampal-dependent memory, rather than working memory. Others have also shown that animals with high fat or high fructose diets have impaired hippocampal insulin signaling and impaired spatial memory (295, 299). Although this preliminary evidence suggests the importance of GLUT4-mediated transport in the brain, the results are difficult to interpret due to the other effects of the described manipulations. Future work to directly manipulate GLUT4 expression in the brain of non-diabetic animals (rather than relying upon insulin activators or inhibitors) is necessary for the role of glucose metabolism in cognition to be clarified.

The works described above have largely focused on manipulations that would potentially impact GLUT4 in the immediate period of behavioral testing. What makes our study unique is that the drug application was limited to the period of trauma, and that behavioral testing was not begun until one week following treatment. As Indinavir appears to act upon GLUT4 translocated to the cell surface, its inhibition of glucose uptake occurs farther downstream than if AS160 was pharmacologically inhibited. As we demonstrated in Chapter 3, AMPK phosphorylation is significantly decreased following injury, but not completely inhibited. (See Figure 3.1) Likewise, we have shown that phosphorylated AS160 and GLUT4 are still present in the hippocampus and cortex post-TBI with our immunohistochemical studies (See

Figure 3.5). Therefore, we would hypothesize that the GLUT4 inhibition occurring as a result of Indinavir treatment in this study resulted in a further suppression of glucose uptake via GLUT4 post-injury, and subsequent dysfunction of the affected cells during the behavioral testing post-treatment.

It would be interesting to examine whether directly increasing glucose uptake, either via augmenting AS160 phosphorylation or via increasing GLUT4 translocation, would lead to improvement of TBI outcome. As discussed in Chapter 4, I previously conducted a behavioral study with injured rats in which they were intraperitoneally administered Metformin, which increases GLUT4 trafficking, for three days following TBI (See Appendix Fig A.1). In that study, I found an improvement in memory following treatment. Although the results are not directly comparable, it suggests a specific role for GLUT4 in the modulation of memory and post-injury outcome. There is also recent precedence for this in a trauma model, as drug treatment shown to increase AMPK activation and GLUT4 translocation for one week following spinal cord injury improved hindlimb locomotion recovery four weeks post-injury (205). The mechanism by which short-term alterations in GLUT4 impact long-term injury outcome should be subject to further investigation.

One weakness of this experiment was that the impact of Indinavir on the AMPK pathway was not directly assessed. As it acts downstream upon AMPK-regulated GLUT4, whether it would have a feedback effect on this pathway is difficult to predict. This drug has been studied extensively in cell culture (280, 282, 283) and in animals in relation to its efficacy for the treatment of HIV and its impact on glucose intolerance (300, 301), but less testing has been devoted to the impact of Indinavir on signaling cascades. The only published study to date investigating the effect of Indinavir upon the hippocampus *in vivo* demonstrated that prolonged oral administration to rodents led to a decrease in aromatase 1 week following ischemia, and this was believed to be regulated by expression of transcription factor sterol regulatory element binding protein-1 (SREBP-1) (302). This is perhaps not surprising, as long-term Indinavir treatment is found to result in hypercholestorolaemia in HIV patients, and

SREBP-1 regulates cholesterol transport (276, 303). Again, this study differs from the work outlined in our study as it provided chronic Indinavir treatment. In relation to the effect of more acute Indinavir treatment on AMPK, acute Indinavir exposure in muscle tissue resulted in a decrease in protein synthesis, which was accompanied by a decrease in protein s6 phosphorylation (304). This may suggest a feedback mechanism by which inhibition of glucose uptake via GLUT4 leads to a subsequent increase in AMPK activation and inhibition of mTOR. Biochemistry work was conducted to attempt to examine the effect of Indinavir on the brain by western blotting using the same treatment paradigm, and no effect was found on AMPK pathway activation from samples collected 3 hours post-injury. Whether this is an issue of time point or the fact that Indinavir does not cause a feedback activation of AMPK is something which would require further investigation.

Although the effect of Indinavir on glucose uptake has been previously demonstrated *in vitro* in hippocampal slices (238), it is something that should be addressed through follow-up experiments using 2-DG autoradiography (61). This technique would allow for quantification of regional glucose uptake in subfields within the hippocampus and cortex, as it measures the amount of radio-labeled, non-metabolizable glucose accumulated within cells. Use of autoradiography would require implantation of a catheter either prior to or at the time of injury for injection of the radio-labeled substance. It has been shown that differences in glucose uptake using 2-DG can be seen after a survival period as short as five minutes (305); therefore, it may be possible to sensitively detect the effect of Indinavir on glucose uptake post-injury in subsequent work. If Indinavir were administered as described here and then glucose uptake was measured following injury, we would hypothesize that this would result in decrease in brain glucose uptake in injured animals treated with Indinavir as compared to injured, vehicle-treated animals.

In summary, our behavioral results indicate that animals that experience GLUT4 inhibition immediately post-injury have impaired long-term memory when tested at later time points. The exact mechanisms by which GLUT4 exerts this effect are as of yet unclear. The

histopathological work presented here and the previously observed correlations between neuropathology and GLUT4 suggest that expression of this transporter may be important in restoring neural homeostasis following insult, in areas critical to cognition. As activation of its regulator, AMPK, results in both short-term and long-term effects upon metabolism, perhaps inhibiting GLUT4 also has different temporal effects. Substantial work upon the role of glucose, insulin signaling, and GLUT4 in the hippocampus and its influence upon memory processes highlights the consistency of the findings presented here to the larger body of literature. The development of more specific means of targeting AMPK-regulated glucose transport via GLUT4 could potentially serve to increase the current understanding of how glucose regulates memory processes, as well as serve as therapeutic treatments for trauma and neurodegenerative disorders.

CHAPTER VI. Conclusions and Future Directions

Summary of findings

The central hypothesis of this thesis work was that activity of the AMPK pathway would be transiently decreased following TBI, and that enhancing AMPK activity following injury would increase cognitive outcome. Further, we hypothesized that inhibition of AMPK target GLUT4 would be detrimental to animals following moderate injury. We have examined alterations of AMPK signaling and the relevance of this cascade to TBI cognitive outcome through a combination of biochemical and behavioral experiments with moderately injured animals. We conclude that there is an energy crisis following injury that influences cognitive outcome, and that signaling of the AMPK pathway, which controls ATP production and consumption, is an important regulator of metabolic suppression. Enhancing AMPK activation, which would lead to more efficient energy use, improved outcome in injured animals that appear to be experiencing a post-injury energy crisis. A brief summary of these findings is presented in the following paragraphs.

As presented in Chapter 2, in our initial work to determine whether increasing peripheral glucose levels has an effect on cognitive outcome in moderate TBI, either a dose of glucose or saline vehicle was administered to C57 mice 15 minutes prior to CCI injury (35). Behavior was then compared in tasks testing recovery of motor skills, and spatial learning and memory. Despite producing a significant increase in blood glucose in our animals, acute hyperglycemia did not worsen outcome on either of these measures. In addition, there was evidence that glucose-treated animals had improved short-term memory in the Morris water maze task. In contrast to previous reports, there was no increase in cortical tissue loss in hyperglycemic animals (217, 218). This demonstrates that an increased peripheral blood glucose level in the immediate post-injury period may not directly lead to worsened outcome, as had previously been hypothesized. This is an important issue, because although tight blood glucose regulation in critically injured patients may be beneficial, the special reliance of the brain upon glucose may make this treatment strategy questionable for head-injured individuals. Subsequent biochemistry performed with an additional set of glucose-treated animals demonstrated that

there was a trend towards decrease in AMPK and AS160 phosphorylation in the cortex of injured mice compared to uninjured shams 3 hours post-injury, which was not further altered by glucose administration. This work led us to the hypothesis that rather than excessive glucose contributing to TBI pathology, it may be a symptom of aberrant activation of energy-signaling cascades.

An examination of AMPK-related activity was conducted, as described in chapter 3. We first observed a decrease in AMPK-related phosphorylation, and altered phosphorylation of its targets AS160 and protein S6 in multiple brain regions following traumatic brain injury through western blotting and immunohistochemistry. This work builds upon the existing literature for these proteins, and supports the idea of disturbance at the signaling level occurring in conjunction with the observed post-injury period of metabolic suppression. In this project, we examined for the first time the distribution and expression of AS160 in the hippocampus and cortex. We demonstrated that this protein is present in neuronal cell layers, co-localizes with GLUT4, and that its phosphorylation decreases following injury, consistent with decreased AMPK activity. This is an important finding, as AMPK is known to regulate expression and translocation of glucose transporters in the brain, but the cellular mechanism for this has not been examined.

In chapters 4 and 5, our drug manipulation studies targeting AMPK and its downstream target GLUT4 found that altering AMPK-related activity impacts cognitive outcome. Augmenting phosphorylation of the AMPK cascade improved cognitive outcome; while as further impairment of its target, GLUT4, decreased cognitive outcome. To examine whether targeting AMPK activation could represent a therapeutic treatment to improve TBI outcome, we administered the drug AICAR at the post-injury time points at which AMPK phosphorylation was found to decrease. Mice treated with AICAR demonstrated improved long-term memory, in two paradigms of the water maze as well as in the contextual fear conditioning paradigm. These results were consistent with previous results suggesting spatial memory improvement of uninjured animals treated with AICAR (199-201). This finding has translational significance for

two main reasons. First, the mice in this study were treated for a short duration post-injury, and yet the gains seen in performance lasted for at least a month following TBI. In addition, there are FDA-approved activators of AMPK. For these reasons, augmenting AMPK activation may represent a non-invasive, novel treatment strategy for TBI patients.

To investigate the role of brain GLUT4 in metabolic recovery, we directly applied Indinavir, a drug shown to selectively inhibit GLUT4, via intracerebroventricular (ICV) injection 15 minutes pre-injury. Behavioral outcome was assessed using motor skills, Morris water maze, and contextual fear conditioning. We found that animals treated with Indinavir exhibited worsened behavioral outcome, with decreased long-term memory in both the water maze and fear conditioning. Interestingly, despite the fact that there are high levels of expression of GLUT4 in motor cortex and the cerebellum, there was no effect of this drug on motor skills recovery. We propose that inhibiting glucose uptake via GLUT4 is detrimental to TBI outcome, and that this transporter is an important part of the pathological mechanism which links glucose metabolism suppression and decreased cognitive outcome post-injury.

Analysis of the effect of injury magnitude upon cognitive outcome

In anticipation of the inhibition of GLUT4 worsening behavior, there was a reduction in the injury magnitude for the Indinavir experiment outlined in Chapter 5 in comparison to that presented in Chapter 4 with AICAR. The depth of the injury was identical, but the velocity was lowered by 1 m/s (from 1mm, 5 m/s to 1mm, 4 m/s). A 20% reduction in the injury velocity resulted in some behavioral improvement. On preliminary examination, there was no change in performance between studies in performance in the abbreviated water maze (data not shown). This may speak to the general difficulty of this task for injured animals. In the standard water maze, there was also no difference in task acquisition (Fig 6.1A, next page). However, the vehicle-treated animals with the 4 m/s injury velocity from the Indinavir study were significantly improved in their latency to the platform (t-test, $p=0.003$; Fig 6.1B). Animals injured at the higher 5 m/s velocity are able to discriminate between the shock and safe context after 3 days

of training [Day 3, post-hoc t-test, p=0.03]; however, they did not show improvement over days [2 way RM ANOVA, day-box interaction, F(2,14)=0.624; p=.550](Fig 6.1C). Animals injured at the lower 4 m/s magnitude were able to discriminate between shock and non-shock context by day 2, with improvement over days (day-box interaction, 2-way RM ANOVA: F(2,20)=4.691; *p*=0.021)(Fig. 6.1D) This suggests that the behavioral tasks used are sensitive to changes in injury severity. In addition, the fact that these two measures were both affected by drug treatment lends further support to the idea that they had an impact upon the underlying pathology. It is worth noting that while both vehicle groups were eventually able to perform the context discrimination task, this was something that the Indinavir-treated animals were not able to do, despite the reduced injury magnitude and three days of testing.

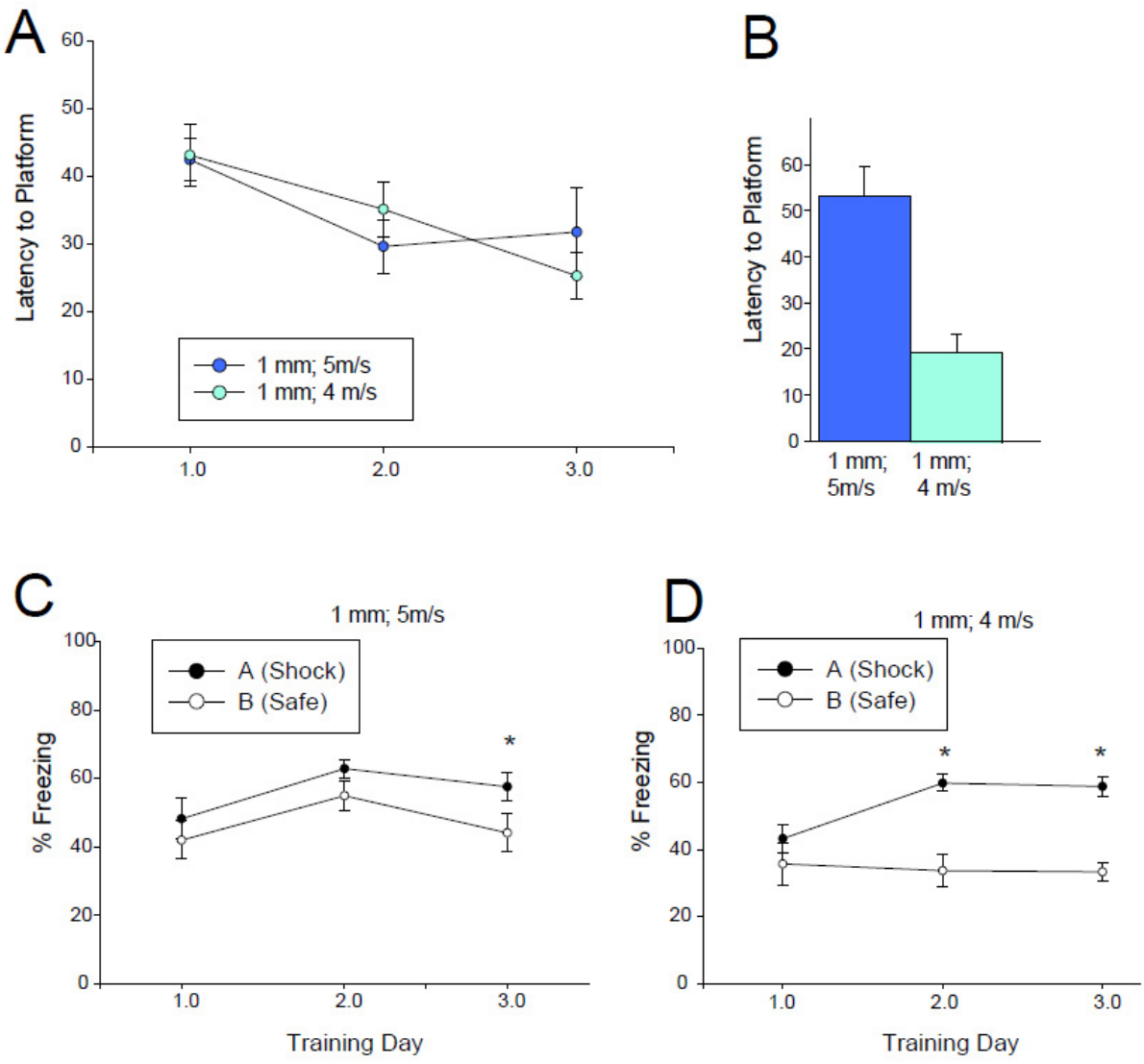

Figure 6.1 Injury magnitude impacts water maze and context discrimination performance. (A) A decrease in the injury velocity did not affect training in the standard water maze, but it resulted in a (B) significant difference in 24 hr probe trial latency. (C) Animals injured at the 5 m/s velocity require an extra day of training to discriminate, compared to (D) those injured at 4 m/s. Data are presented as mean±SEM. *p<0.05. 5 m/s injury level n=8, 4 m/s n=10.

Proposed Future Directions

We feel that there are several exciting potential avenues of investigation for this project in the future. The first would be to examine the effect of deletion of AMPK on injury outcome, either through the use of knockout animals or through application of siRNA technology. This would allow for direct assessment of decreased signaling through this pathway both on measures such as neurogenesis and cell death as well as on cognitive outcome. In development, systemic deletion of the β subunit results in atrophy of the dentate gyrus and other areas, increased neuronal apoptosis, increased seizure activity, and progenitor cell abnormalities (161). Other tissues did not seem affected by cell loss, suggesting that AMPK activity is extremely important in the brain for initial brain development, a finding that may be relevant to neuronal regeneration following injury. The same behavioral paradigm as described in Chapters 4 and 5 could easily be adapted to assessment of outcome in animals lacking protein expression of AMPK. We would hypothesize that animals with AMPK deletion would have significantly worse outcome following TBI.

It will also be important to further examine the role of AMPK in post-injury neurogenesis. Several papers have now shown that AMPK activation has an impact on neurogenesis, in both the developing brain and in relation to enhanced cognition in adult animals (161, 199-201). Furthermore, both drug and genetic manipulations to induce diabetes which would potentially disrupt GLUT4 in the brain lead to decreased neurogenesis (296-298). It has now also been shown that following spinal cord injury, application of a drug which increases GLUT4 translocation in an AMPK-regulated manner leads to induction of ependymal stem cell proliferation (205). As mentioned in chapter 4, it has previously been demonstrated that neurogenesis takes place following injury and that this is critical to spatial memory (263, 264). This was difficult to assess immunohistochemically following our behavioral study, as the animals were over a month post-injury. However, an acute experiment could be performed, in which animals would be given repeat injections of bromodeoxyuridine (BrdU) during the same time period of AICAR injection, and then sacrificed at an acute time point. BrdU becomes

incorporated into newly synthesized DNA and has become a standard technique for the assessment of neurogenesis as differences between groups can be detected using immunohistochemistry (306). The studies that have demonstrated an increase in neurogenesis related to AMPK activation have utilized this technique (199-201).

How exactly AMPK activation would affect neurogenesis following TBI is an especially intriguing question, given that neurogenesis is already increased following injury. Blaiss et al. (2011) observed that there is a decrease in early-stage progenitor cells post-injury, and hypothesized that injury may speed depletion of the progenitor cell pool in the hippocampus. Due to the fact that AMPK manipulation has been shown to have an impact on progenitor cell population, perhaps AMPK activation post-injury would make animals more resistant to progenitor cell depletion. In conjunction with the acute examination of AMPK activation on neurogenesis, a more long-term study on neurogenesis using animals with GFP-expression in the Type 1 progenitor cell population could be completed to explore this possibility.

Whether the observed alterations in AMPK activity take place in the clinical setting should be examined. Previous work has examined alterations in the expression of various glucose transporters post-injury in resected patient tissue using immunohistochemistry (128). This technique could be utilized to examine activity of AMPK proteins in tissue from injured patients as well. As resection typically occurs in the acute post-injury period, it may be difficult to observe a metabolic suppression as evidenced by decreased AMPK phosphorylation in this tissue. Examining whether treatment with an AMPK activator is capable of normalizing the decrease in the cerebral metabolic rate of glucose typically observed in injured patients by PET imaging may be more informative in determining the regulatory role of AMPK in post-injury glucose uptake (82). We would hypothesize that treatment with an AMPK activator of TBI patients with metabolic suppression would result in augmentation of brain glucose uptake. Additionally, the Glasgow Outcome Scale as well as other more sensitive measures of cognitive outcome could be serially administered to these patients to demonstrate that enhancing AMPK activation improves post-injury functional outcome.

More generally, the work presented here lends support to the idea that GLUT4 is implicated in neurological disorders accompanied by memory loss, and this is something that should be further examined. Decreased glucose metabolism has been implicated as part of the Alzheimer's disease pathology (127). Multiple studies have demonstrated that treatments that result in a slowed decline of memory loss are also associated with improvements in cerebral metabolism (132, 133). Given that the only technique available to confirm the diagnosis of this disorder is post-mortem examination, innovation of other techniques to assess the development and progression of this disorder would be highly beneficial. Additionally, over 40% of the U.S. population is reported to be diabetic or pre-diabetic (59). Given that metabolic disturbance impacts such a large proportion of the general population, considerably more research should be devoted to understanding how these issues impact neurological functioning. Further imaging work and examination of the effect of AMPK activators on these populations should be undertaken. Beyond drugs, there are many natural supplements, "nutraceuticals" that increase AMPK activation that could be investigated for their preventative benefits- including curcurmin, berberine, green tea extracts, and capsaicin (198).

APPENDIX

Metformin significantly improves cognitive outcome in moderately injured rats

Study design: Administration of the drug Metformin (dimethylbiguanide) has been shown to result in AMPK activation by inhibiting Complex I of the respiratory chain (307, 308). This results in inhibition of ATP synthesis and an increase in cellular AMP levels. Metformin is used extensively in the treatment of Type 2 Diabetes, and its pharmacokinetics and dosing have been well-established in the experimental literature (308-310). Its biochemical and behavioral impact on the nervous system has also been the subject of recent investigation (204, 311). Following mid-cerebral artery occlusion, three doses of 250 mg/kg Metformin was found to improve observed glucose intolerance, decrease contusion volume, and improve behavioral outcome (204). Given this evidence, and the observed period of AMPK phosphorylation decrease as presented in Chapter 3 (See Figure 3.1), we designed a behavioral study to test whether Metformin could improve cognitive outcome in moderately injured animals.

Methods: Metformin was purchased from Sigma Aldrich. Adult male rats were moderately injured as described in Chapter 2, using the electromagnetic controlled cortical impact injury device at a magnitude of 2.7 mm and 6 m/s. Animals were administered 250 mg/kg Metformin in saline or vehicle (Vehicle, n=10; Metformin, n=9) once daily for 3 days, beginning 30 minutes post-injury. Blood glucose readings were taken from the lateral saphenous vein at baseline and analyzed by an Aviva Accuchek blood glucose monitor from Roche (Indianapolis, Indiana), prior to the initial injection, and at 24 and 48 hours post-injury (no difference between groups, data not shown). Motor skills were assessed days 1-3 post-injury utilizing the foot fault and beam balance tasks (no difference between groups, data not shown). Rats were then trained in the standard hidden platform version of the Morris water maze task on day 14 post-injury, receiving 4 trials per a day for 9 days. Memory for platform location was assessed by removal of the platform and allowing animals to search for 60 seconds. This was tested 30 minutes

(Short-term memory, STM) and 48 hours (Long-term memory, LTM) following completion of training. All probe trial data presented was tested for statistical analysis using a student's t-test.

Results: Metformin improves short-term memory recall following water maze training

The study design is outlined in Figure A.1A. In the water maze task, there was no difference between groups in their performance during training, suggesting no difference in acquisition (Fig A.1B). However, the Metformin-treated animals demonstrated significant improvement in the short-term memory probe 30 minutes following the last training trial (Fig A.1C-E). Metformin animals reached the platform faster (Fig A.1C, $p=0.040$), demonstrated an increased number of platform crossings (Fig A.1D, $p=0.010$), and an increased amount of time in the platform area ($p=0.030$). There was no difference in the subsequent long-term memory probe at 48 hours (Latency to platform: Metformin, 39 sec, Vehicle, 30 sec; $p=.534$).

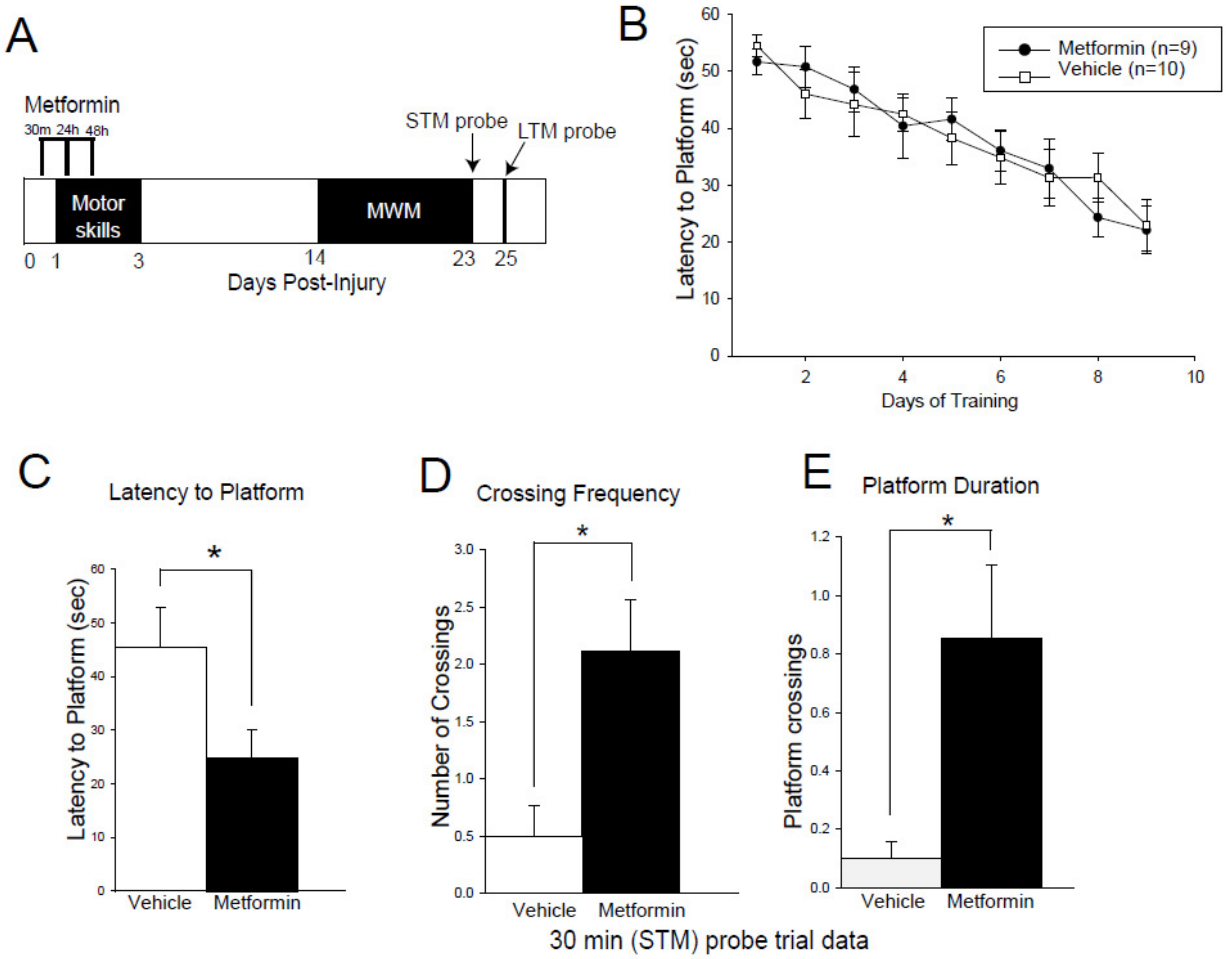

Figure A.1 Metformin improves performance during short-term memory probe in moderately injured rats. (A) Behavioral study design. (B) Metformin-treated animals perform equivalent to vehicle-treated animals in task acquisition. (C-E) Short-term memory probe trial results demonstrate injured, metformin treated rats have significantly better memory for the previous platform location. This is shown by (C) a decrease in latency to platform, (D) an increased number of platform crossings, and (E) an increase in the time spent in the platform location. Data are presented as mean±SEM. *p<0.05.

Discussion:

In this experiment, we found that peripheral application of the drug Metformin led to a modest improvement in short-term memory following TBI. When combined with the data presented in Chapter 4 for AICAR, these results strongly suggest that AMPK activation can be beneficial for improving TBI cognitive outcome. Additional tasks were examined in this study, including delayed fear conditioning, the working memory version of the morris water maze, and novel object recognition. No significant difference was found on any of these tasks. It is unfortunate that at the time of this experiment, the behavioral testing paradigm presented in Chapters 4 and 5 had not yet been adopted. We have found contextual discrimination to be a more sensitive

indicator of memory loss in injured animals. It could also be an issue of duration of treatment, as the previous study which demonstrated spatial memory improvements as a result of Metformin administered twice the dose administered in this study, for a duration of 40 days (201). However, the results presented here suggest that the FDA-approved drug Metformin may have the potential to improve post-injury outcome, and this should be further investigated.

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VITA

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