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Abnormalities of Glutamate Neurotransmission Following Traumatic Brain Injury

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ABSTRACT

This project aims to investigate abnormalities in glutamate neurotransmission in the rat cortex following traumatic brain injury (TBI). The overarching hypothesis is that excessive glutamate release originating at the site of injury begins an excitotoxic cascade leading to increased intracellular calcium and cell death. Glutamate, the primary excitatory neurotransmitter in the mammalian central nervous system, facilitates learning, memory, and other cognitive functions via activation of metabotropic and ionotropic glutamate receptors. A family of Na^+- dependent excitatory amino acid transporters (EAATs) on the plasma membranes of neurons and glial cells facilitate the rapid removal of glutamate from the synaptic cleft, maintaining basal levels of synaptic glutamate. EAATs are also essential for the maintenance of extracellular glutamate concentrations below toxic levels. High levels of extracellular glutamate can initiate calcium-mediated signaling cascades, likely through activation of extracellular glutamate receptors. Alterations of extracellular glutamate reuptake following TBI may be a critical component in this excitotoxic cascade and presents a possible target for therapeutic intervention.Herein we demonstrated that TBI resulted in decreased EAAT-mediated glutamate reuptake capacity, and possible remolding of astrocytic processes due to injury

induced signaling abnormalities. This was evaluated by measuring [3H]-glutamate reuptake and glutamate transporter protein expression profiles. The synaptosomal isolation protocol provides a functional characterization of glutamate clearance following TBI that may allow for the comparison of novel and existing therapeutic drugs.

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INTRODUCTION

Traumatic Brain Injury (TBI) is defined as damage to the brain resulting in functional alterations following the application of an external mechanical force. These forces can be either blunt or penetrating and functional alterations manifest as perceptual defects, confusion, alterations in consciousness, motor control deficits, coma, or death.[1] TBI is a leading cause of morbidity and mortality worldwide with an annual incidence rate of around 200 per 100,000 people and an estimated 57 million people have been hospitalized with at least one TBI.[2] Injury is most likely during three distinct age periods: early childhood, late adolescence and late adulthood.[3] Within the United States, TBI is the primary cause of mortality in individuals younger than 45. The majority of TBIs are the result of automobile accidents, followed by falls and recreational injuries.[3] Additionally, members of the active duty military are at elevated risk of TBI with an estimated instance of TBI among wounded soldiers to be as high as 22%.[4] Due to the number of individuals affected and the lasting deficits faced by those surviving TBI, research into the mechanisms propagating the functional alterations and the development of treatment strategies to cope with them is crucial.

Pathology

While perhaps not intuitively understood as such, TBI can best be characterized as an ongoing disease state resultant from both direct and indirect damage with two distinct pathological phases.[1] The primary injury occurs at the time of impact as a result of the disruptive mechanical forces applied to the skull and brain. Primary injury forces can lead to skull deformation and fracturing, both at the site of impact and elsewhere; hematomas, contusions, brain swelling, hypoxia or a combination thereof. TBI can additionally be categorized as focal or diffuse.[5] Focal injuries occur at the sites where the brain impacts the skull and are responsible for two thirds of deaths resulting from TBI.[6] The classical presentations of focal injuries are contusions resulting from brain acceleration which disrupts the blood-brain barrier. In fracture contusions there is a fracturing of the skull at the site of impact and a surface contusion forms beneath the site of the fracture. Coup and contre-coup contusions occur when the skull is not fractured but the force is sufficient to accelerate the brain within the intact skull resulting in surface contusions both at the site of injury, and the area of the brain directly opposite from the impacting force.[7] Additionally, gliding contusions can occur due to rotational forces acting on the brain which separates it from the overlaying dura leading to bilateral focal hemorrhage.[5] Another hallmark of focal injuries include a number of vascular injuries such as epidural hematomas, subdural hematoma, and ischemia.[7] Diffuse injuries are largely the result of axonal damage due to excessive shear strain generated by angular motion. This diffuse axonal injury (DAI, also known as traumatic axonal injury) results in widespread psychological dysfunction with little to no macroscopic damage, making clinical detection of diffuse injuries difficult.[8] Diffuse injuries represent the most

common cause of persistent neurological dysfunction resultant from TBI and account for around one third of all TBI related deaths.[6] The pathology thought to be responsible for the axonal damage includes mechanical shearing of the axons from the cell body, unregulated ion flux into and out of the cell leading to fluid imbalance as well as cell swelling, and inhibited cell transport leading to necrosis and apoptosis.[7]

The secondary injury processes are the result of a myriad of cellular responses by the brain as it responds to and accommodates the injury.[7] Cells in the brain that did not experience any direct mechanical damage during a TBI event nevertheless exhibit metabolic disturbances following injury, a phenomenon known as the secondary injury phase. In contrast to primary injuries, secondary injuries take hours or days to manifest. Portions of the aforementioned diffuse axonal injury may be considered secondary, although there is growing research that indicates that secondary injuries include a vast number of intracellular and molecular event that lead to a cascade of harmful intracranial and systemic effects.[1] These metabolic effects include unregulated neurotransmitter and ion release, cell swelling, free radical production and oxidative stress, mitochondrial dysfunction, inhibited ATP production, inflammation, and inhibited gene transcription.[1,8-12] These secondary events serve to further exacerbate the effects of the primary injury, increasing blood-brain barrier damage, edema, ischemia and hypoxia and ultimately augment the cell death process.[1,8]

Glutamate in TBI

The pathophysiological characteristic of the secondary injury phase appear to be initiated by a sudden, sharp rise in the concentration of extracellular glutamate.[11] While

mechanical disruption of the cell's plasma membrane can induce the release of intracellular ions such as potassium into the extracellular space, TBI alone can also lead to indiscriminate electrical discharge, which may also precipitate the release of neurotransmitters like glutamate from the presynaptic cell.[11]Astrocyte death and loss of expression or function of glutamate transportershave also been implicated in the pathology of glutamate release following TBI.[13,14] Glutamate acts on both ionotropic and metabotropic receptors on the post-synaptic cell and elicits cell depolarization and subsequent action potential firing. Metabotropic glutamate receptors mediate changes in intracellular ion concentration by means of a second messenger system that ultimately leads to release of calcium from intracellular storage sites. Glutamate binding to ionotropic receptors like the N-methyl-D-aspartate (NMDA) receptor alters membrane permeability to a number of ions including sodium, potassium and calcium.[15] Elevated extracellular glutamate levels, such as that produced by direct injections of glutamate and related analogues into nervous tissue or by prolonged activation of the glutamate receptors, are extremely toxic to cells due to cellular swelling produced by the influx of cations and subsequent cell degradation.[16,17] Following induction of severe TBI in rat cortical tissue, microdialysisstudies have shown elevations of extracellular levels of glutamate nine-fold above those of non-injured control rats.[18] This massive release of glutamate appears to have two primary pathogenic effects. The initial pathological event involves morphological changes in cell size due to glutamate activity on ionotropic receptors for sodium leading to increases in intracellular levels of this ion and subsequent swelling by osmotic pressure. The second pathological result of extracellular glutamate involves the actions of the neurotransmitter on ionotropic receptors for calcium.[15] The

increases in both of these ions appear to be largely the result of glutamate acting on the NMDA receptor as blockage of the NMDA receptor attenuates some of the neurotoxic effects of TBI, especially by influencing calcium homeostasis.[18]

Choi and colleagues have conducted experiments to discriminate the neurotoxic effects of the sodium and calcium on neuronal survival by examining cell cultures exposed to toxic levels of glutamate in isolated extracellular environments.[19] In an extracellular environment that mimicked baseline physiological concentrations of both sodium and calcium, cells exhibited immediate morphological changes followed later by significant cell degradation and death. Removal of both ions from the extracellular environment showed large protective effects on the cells, even at prolonged glutamate exposure. However separation of the two ions indicated their unique roles in the excitotoxic cascade. These results indicate that the acute event precipitated by sodium appears to be transient and largely non-toxic to cells. Cultures in a calcium free extracellular environment exhibited morphological changes including swelling and granulation to a greater degree than those in the baseline experiment, but were largely spared from cell death and returned to previous size within an hour.[19] Cultures in an environment where choline was substituted for sodium and calcium ions remained at physiological concentrations showed markedly less swelling and other morphological changes than those at baseline levels, but exhibited roughly the same amount of cell death as cultures with both ions at physiological levels.[19] It thus, appears that glutamate induced changes in calcium ion concentrations play a key role in subsequent cell death via overwhelming the calcium regulatory mechanisms and activation of downstream signaling pathways.[9] Prolonged increases in intracellular calcium have a number of

downstream effects including disruptions in mitochondrial production of ATP and activation of various proteases and kinases including nitric oxide synthase. The stimulation of these enzymes has widespread effects including generation of reactive oxygen species, disruption of cytoskeletal architecture and induction of genetic components responsible for apoptosis.[15]

While calcium plays a key role in cellular death following TBI, glutamate release is the initiating event. The extracellular concentration of glutamate is maintained by a family of sodium dependent excitatory amino acid transporters (EAATs), which are selectively expressed throughout the mammalian brain on both neurons and glia. EAATs 1 and 2 are expressed primarily in astrocytes while the remaining transporters generally reside on neurons.[20] The astrocytic glutamate transporters are crucial for the proper maintenance of extracellular glutamate levels; antagonists for them can lead to toxic levels of glutamate and subsequent cell death similar to that exhibited in TBI.[21] Of the astrocyte transporters, EAAT2 is responsible for approximately 90% of the clearance of glutamate from the synapse.[22] EAAT2-mediated glutamate reuptake may be significantly reduced following TBI in rat cortical tissue, as both mRNA levels and protein expression of EAAT2 are reduced following controlled cortical impact injury (CCI).[23] Studies using the fluid percussive injury (FPI) model of TBI have mixed results in regards to protein expression levels of EAATs. Goodrich and colleagues demonstrateda decrease in EAAT2 which persist up to7 days post-injury, while others have reported no change at 24 hours, and an increase in hippocampal EAAT2 expression following FPI.[24,25]However, knockdown of EAAT expression with antisense oligonucleotides has demonstrated that the transporters are crucial to maintaining concentrations below toxic levels. Knockout of

either EAAT1 or EAAT2 in non-TBI mice can produce excitotoxic levels of glutamate similar to that experienced following TBI and induce neuronal degeneration and loss of motor neuron viability.[26] Similarly, the introduction of knockdown antisense oligonucleotides for EAAT2 significantly increased hippocampal cell death compared to sense nucleotides and sham operated controls in the CCI injury model.[23] Other CCI studies have found comparable decreases in EAAT1 and EAAT2 as well as de novo expression in ramified microglia. This finding may be a compensatory reaction to the increased extracellular glutamate levels and loss of transporter function following TBI.[13] These studies demonstrate the crucial nature of astrocytic EAATs, especially EAAT2, in the maintenance of extracellular glutamate within physiological norms and illustrate how the pathology of TBI can be exacerbated when function or expression of these transporters is compromised.

While astrocytic EAATs are responsible for the majority of the removal of glutamate from the extracellular space, the neuronal EAATs are also affected by TBI. Increases in expression of EAAT4 have been reported in hippocampal astrocytes 3 to 7 days following lateral FPI.[27] This finding is atypical in that EAAT4 expression is typically neuronal, has a much higher affinity for glutamate, and has a unique chloride conductance that is not coupled to its glutamate transport function. The chloride ion conductance has a role in decreasing cellular excitability via influx of the ion resulting in cell hyperpolarization.[28] Thus, , the increased expression of the typically neuronal EAAT4 in hippocampal astrocytes may represent an endogenous neuroprotective attempt to mitigate high extracellular glutamate levels following the loss of the primary removal mechanism.[27] Other approaches that offset the loss of EAAT function following TBI

have involved the use of therapeutic drugs that upregulate expression of the transporters. Goodrich and colleagues usedintraperitoneal injections of the β-lactam antibody ceftriaxone to reverse the loss of EAAT2 expression in the ipsilateral cortex of FPI mice by 7 days post-injury, resulting in decreased astrocytic degeneration (as measured by GFAP expression).[25]

While the excitotoxic effects of are deleterious to cells in the short term, the effects of the neurotransmitter might be attenuated were they not exacerbated by dysfunction of the transporters responsible for the clearance of the molecule. As a consequence of either decreased levels of EAAT expression or function, the secondary injury phase is enhanced and the number of pathological results increases. This leads to sustained dysfunction of the cell, including ion imbalances, particularly in calcium homeostasis, which leads to signaling abnormalities that can ultimately cause cell death.

Signaling Pathways Regulating EAATs

The expression of EAATs appears to be modified on every level possible, from DNA transcriptional regulation, to mRNA splicing, protein synthesis, and post-translational modification.[29] A primary post-translational modification mechanism involves the recruitment of protein kinases – molecules with the ability to catalyze the transfer of the terminal phosphate from ATP onto a protein substrate. With more than 500 kinase molecules coded for in the human genome, nearly every post-translational modification cascade is mediated by a protein kinase molecule.[30] Kinases can be activated by soluble factors, small intracellular signaling molecules such as cAMP, or by other kinases. The effects of the addition of a phosphate to a particular amino acid residue

on a protein are diverse; however kinase pathways represent a primary means of intracellular signal amplification. One such example of signal amplification can be found in the regulation of astrocytic expression of EAAT2 by small soluble molecules given off by neurons. Astrocytes cultured in the absence of neurons preferentially express EAAT1 with very little expression of EAAT2, while neuronal co-culturing with astrocytes increases expression of EAAT1 and induces the expression of EAAT2.[29] This induction of EAAT2 in the presence of neurons is the result of a kinase mediated signaling cascade initiated by soluble factors given off by the neurons into the surrounding extracellular space; as evidenced by the de novo expression of EAAT2 in pure cortical astrocyte cultures following the introduction of neuron-conditioned media (NCM).[31]

An elucidation for the role of kinase molecules involved in the NCM induced increases in EAAT2 expression come from studies where the addition of dibutyryl-cAMP (dbcAMP), epidermal growth factor (EGF), or pituitary adenylate cyclase-activating polypeptide (PACAP) mimics the effects of NCM alone.[32]These effects seem to be dependent on the activation of phosphatidylinositol 3-kinase (PI-3K) and nuclear transcription factor-κB (NF-κB), the latter of which has been demonstrated to directly regulate the glutamate transporter gene.[33] A key mediator between these two signaling molecules is the protein kinase Akt. Akt is activated by a number of growth factors and frequently functions downstream of PI-3K where it has the ability to increase NF-κB activity by decreasing the activity of the protein responsible for NF-κB's sequestration in the cytoplasm.[34] Li and colleagues have demonstrated the importance of the Akt pathway in EAAT2 induction by using lentiviral vectors to create astrocytic cultures that express dominant-negative or constitutively active variants of Akt. The dominant-negative strain

decreased the effects of EGF on EAAT2 expression while the constitutively active stain demonstrated a dose and time dependent increase in EAAT2 protein expression, mRNA levels, and transport activity.[35] They conclude that Akt can regulate the expression of EAAT2 by increasing its rate of transcription.[35]

Another method for kinase-mediated EAAT induction involves preferentially targeting the receptor tyrosine kinase (RTK) pathway. Gegelashivili and colleagues have shown that inhibition of RTK by the cell permeable Typhostin A23 blocks the induction of EAAT2 in the presence of NCM.[36] The RTK pathway appears to represent a primary starting point for a second messenger cascade, which converges on the MAP kinases p42 and p44, and ultimately results in increased expression of EAAT2. Binding of RTK by growth factors results in recruitment of the GTP-binding protein Ras and the stimulation of MAPK kinase kinases like Raf, which activates MAPK kinases MEK1 and 2, that in turn activate p42/p44 MAPK.[37] A crucial prerequisite of EAAT2 induction involves this phosphorylation of p42/p44 MAPKs. The double phosphorylation of p42/p44 MAPKs at threonine-202 and tyrosine-204 correlates with expression of EAAT2 in the presence of NCM.[36] Phosphorylated p42/44 MAPKs can translocate to the nucleus and regulate gene transcription through activation of transcription factors like CREB.[37] Growth factors can bypass the RTK-p42/44 MAPK pathway and still influence the expression of EAAT2 by directly activating transcription factors such as CREM, CREB, and ATF-1, although the induction of EAAT2 via these pathways is weaker than through the RTK controlled pathways.[36]Additionally, the growth factor activation of PI-3K previously mentioned can lead to the activation not only of Akt, but also result in increased phosphorylation of

p42/44 MAPKs demonstrating a convergence point in these two EAAT2 regulating pathways.[37]

Further evidence supporting the importance of the p42/44 MAPK signaling pathway in the expression and regulation of EAAT2 comes from studies examining pure cultured cortical astrocytes exposed to varying concentrations of glutamate. Western blot analysis indicated that at increased concentrations of extracellular glutamate there were increased levels of phosphorylated p42/44.[37] The changes in phosphorylated proteins occurred in a time and concentration dependent manner and did not induce changes in the total amount of cellular p42/44.[37] Glutamate receptor agonists did not mimic the effects of glutamate-induced increases in phospho-p42/44, nor did glutamate receptor antagonists block them. However the effects of glutamate could be reproduced by molecules that could be transported into the cell by glutamate transporters; thus introducing aspartate or the transportable uptake inhibitors DL-*threo*-β-hyroxyaspartate (THA) and L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC) into the extracellular environment lead to increases in phosphorylated p42/44 almost as strongly as glutamate itself. Together these results indicate that EAATs are capable of modifying their own expression in response to extracellular glutamate independently of the activity of glutamate receptors. Thus the reuptake transporters are able to relay signals about the extracellular concentration of glutamate and activate second messenger systems leading to the recruitment of the p42/44 MAPK pathway and ultimately resulting in increases in the number of the transporters on the cell membrane.[37]

While the previous signaling molecules are predominantly involved in increases in EAAT expression and presence in the plasma membrane, glutamate transporters

expression can also be regulated either through sequestration in intracellular storage sites or ubiquitin-mediated degradation. Of primary importance in selective EAAT downregulation is the activity of the signaling molecule protein kinase C (PKC). PKC has differential effects on the EAAT subtypes; in mixed neuronal and astrocyte cell cultures, the activation of PKC with phorbol ester caused a rapid (within minutes) decrease in cell-surface expression of EAAT2 [38] and in increase surface expression in the neuronal glutamate transporter EAAT3.[39] These differential effects are thought to represent a switching mechanism from astrocytic to neuronal glutamate uptake, however as astrocyte transport represent the primary reuptake mechanism, the effect in a mixed cell culture would be overall reduced reuptake of glutamate and elevated extracellular levels of the neurotransmitter.[39] The deletion of amino acids 475-517 on EAAT2 abolishes the effects of phorbol ester-induced internalization, demonstrating a possible site of PKC phosphorylation on the transporter.[38] The decrease in cell-surface expression did not correspond with a reduction in total cellular levels of EAAT2, suggesting the immediate effect of PCK phosphorylation of EAAT2 involves internalization of the transporter to an intracellular sequestration site.[39] Internalization can be blocked in astrocyte cultures expressing a dominant-negative variant of clathrin[40] or by inhibition of the ubiquitin enzyme E1[41], suggesting an ubiquitin-dependent, clathrin-mediated endocytic mechanism of sequestration.

In contrast to short-term activation of PKC, long-term exposure to phorbol ester was accompanied by an overall decrease in total cellular EAAT2 expression.[40] This decrease was attenuated by lysosomal inhibitors, suggesting a cellular mechanism by which PKC can reduce EAAT2 levels under physiological or pathological

conditions.[40]Indeed the PKC induced lysosomal degradation of EAAT2 could partially account for the rapid reduction in protein expression levels following TBI. Studies in astrocyte cultures have indicated that the half-life of EAAT2 is longer than 24 hours[42]; therefore the observed decreases in EAAT2 expression in the hours immediately following TBI[43] cannot be accounted for solely by halting transcription of the transporter. Along with the aforementioned degradation of EAAT2 by caspase-3,[14] the activity of PKC could possibly represent a mechanism by which the secondary injury phase of TBI induces dysfunction in glutamate reuptake transporters and exacerbate the deleterious effects of elevated extracellular glutamate.

Cell Death and Signaling Abnormalities in TBI

In addition to modification of EAAT expression, TBI induces widespread cell degradation and neuronal death across cortical regions. The secondary injury phase of TBI induces these changes through a number of metabolic and genetic mechanisms including changes in molecular kinase signaling pathways. Many of these kinase signaling pathways are associated with programmed cell death and non-programmed cellular necrosis. Following injury to a cell that is severe enough to cause it to lose viability, the cell is either degraded via apoptotic or necrotic pathways. Apoptosis is a programmed cell suicide mechanism that is especially pronounced during development of neuronal tissue and allows for the elimination of unnecessary cells and the strengthening of plasticity.[44] Apoptotic hallmarks include fragmentation and condensation of the genetic material of a cell, “blebbing” of the membrane, and shrinkage of the entire cell into a membrane-bound vesicle that can be engulfed by neighboring cells.[45] In contrast, necrotic cell death represents unregulated destruction of a cell due to disruption of the

plasma membrane, ionic imbalance, cell swelling, and eventual lysis. This results in the expulsion of intracellular components into the extracellular space triggering an immune and inflammatory response to the site of injury, damaging neighboring cells.[46]

Necrotic and apoptotic neurons have been shown to exist both within the focal site of injury and diffuse areas post-trauma in animal models of TBI, with the ratio of necrotic to apoptotic neurons varying by region.[47]Nicotera and colleagues have shown that the excessive influx of glutamate and activation of NMDA receptor subtypes characteristic of the secondary injury phase of TBI can induce both necrosis and apoptosis.[48] Injections of pathological levels of glutamate lead to early collapse of the mitochondrial membrane potential and degradation of the cell due to depleted energy reserves. Cells able to survive the initial glutamate excitotoxicity recovered their mitochondrial membrane potential and later underwent apoptosis.[48] Thus the idea that a continuum exists between the two forms of degeneration following TBI has recently emerged in which the predominate mechanism is a result of the location and severity of the injury as well as molecular differences between cells including the predominant receptor types, energy availability, and differential activation of kinase signaling pathways.[12]

Because cell death is an actively mediated event coordinated by a number of kinase based cellular signaling pathways, a better understanding of the molecules involved in TBI induced apoptosis and necrosis could elucidate mechanistic targets for therapeutic intervention. As Choi and colleagues have demonstrated, calcium plays a key role in neuronal death following TBI.[19] There are a number of immediate consequences of excessive influx of calcium into the intracellular space that result in a disruption in the

balance between pro- and anti-cell death kinase signaling pathways in favor of those leading to cell death.[12] These including recruitment of mitogen activated protein kinases (MAPK), the activation of the death promoting members of the Bcl-2 gene family (e.g. Bax, Bad, etc.), and c-Jun N terminal kinase (JNK), along with altered levels of Akt (also known as protein kinase B) and p53 expression and direct activation of protease enzymes including calpains and caspases.[12,48] Again the notion of a continuum between apoptosis and necrosis must be emphasized as many of the molecules mentioned above are involved in both programmed and non-programmed cell death and the determination of which pathway is ultimately implemented can depend on a number of factors including concentrations of intracellular calcium, amount of DNA damage and the levels of reactive oxidative species.[49]

Calcium entry to the cell directly activates a family of nonlysosomal, cysteine proteases, calpains, which bind calcium and begin to enzymatically cleave the αII-spectrin protein, a key component of the cytoskeletal architecture resulting in cellular degeneration.[50] Prolonged calpain levels have been indicated in necrotic degeneration associated with TBI.[51]Calpains are one of the earliest mediators of cellular death in the TBI pathology with elevated levels of the proteins demonstrated in both cortices as early as 15 minutes after CCI, indicating that caplains may underpin a an early, pan-hemispheric response to TBI.[52] Other proteolytic enzymes activated by TBI include the caspases, a family of 14 cysteine proteases discovered to be related to gene products required for cellular apoptosis in the nematode.[45]Caspases function as either initiators, which respond to apoptotic cellular signaling, or effectors, which directly participate in cellular degradation largely through the cleavage of multiple proteins responsible for

proper cellular functioning.[45]Caspase levels increase following TBI while caspase inhibitors have been demonstrated to reduce apoptosis and neurological deficits following lateral FPI.[12] Additional reports have indicated that a specific effector caspase, caspase-3, is responsible for degradation of the principle glutamate transporter, EAAT2, by cleavage near the C-terminal domain at aspartate-505 resulting in significant loss of function.[14] This loss of transporter function can further exacerbate the excitotoxic effects of elevated glutamate levels in the extracellular space and serve to prolong the secondary phase of TBI, leading to even more neuronal cell death.

While activation of calpains and caspases represent immediate reactions to apoptotic and necrotic signaling, delayed responses to the TBI event include changes in genes involved in cell death and survival, including the blc-2 gene family which codes for both pro-apoptotic proteins bax and bad and the pro-survival protein blc-2.[53] Following CCI, increased mRNA levels of the anti-apoptotic gene blc-2 are present in surviving neurons as early as 6 hours after injury,[54] while transgenic mice that overexpress the blc-2 gene exhibit less neuronal degeneration after the injury.[55] Additionally in regions exhibiting increased loss of neurons, mRNA levels of blc-2 were markedly decreased, while those of the pro-apoptotic bax gene were significantly increased,[53] demonstrating the role of the blc-2 family and the ratio of the proteins that they code for are key in determining the fate of neurons following TBI. Part of the determination of this ratio involves post-translational modification of several downstream proteins by akt to promote cell survival.[56] Recent work demonstrated that akt has the ability to phosphorylate bad at serine-136 both in vitro and in vivo, inhibiting its ability to participate in the apoptotic pathway.[57]Akt is itself regulated by phosphorylation at serine-

473 and threonine-308. Decreased levels of phospho-akt have been shown one hour after CCI in the injured cortex, correlating with an increase in cell death, however in surviving neurons, phosphorylation of akt was rapidly accelerated after 4 hours post injury to encourage cell survival.[58]Phospho-akt has a number of downstream effectors, including forkhead family transcription factors, and cAMP response element binding protein (CREB), activation of these and other proteins by akt shifts the balance of pro- and anti-apoptotic enzymes in favor of cell survival.[56]

Pro-apoptotic signaling pathways are also activated in response to the myriad of toxic stimuli activated during the secondary injury phase of TBI. One signaling molecule appearing to have primary importance for apoptosis in neuronal tissues is the c-jun n-terminal kinase (JNK). JNKs are phosphorylated and activated in response to DNA damage, axonal injury, and the formation of reactive oxygen species, all of which are present in the secondary injury phase.[1] JNKs appear to represent a convergence point for a number of signaling molecules including mitogen-activated protein kinases (MKKs) and mixed-lineage kinases (MLKs).[59] Additionally the activation of Akt appears to inhibit the activity of JNK, reducing its apoptotic influence while inhibition of JNK using geldanamycin reduces its activity and provides neuroprotection both in vitro and in vivo.[60] Another pro-apoptotic protein, p53 also appears to increase its activity following DNA damage in TBI models.[12] Elevated levels of p53 mRNA and associated protein levels have been reported in experimental models of TBI.[61]

These studies demonstrate that the secondary injury phase of TBI induces important changes in signaling molecules responsible for cell death and survival. As these changes in molecular signaling take place over an extended period following the

injury, they represent potent targets for potential therapeutic intervention in human TBI where the therapeutic window is reduced to the secondary injury phase. However a final note must be made on the consequences of apoptosis. While neuronal death of either form following TBI is associated with behavioral and cognitive defects in humans and animal models, apoptosis represents a controlled means of degeneration and results in less immune activation than necrosis. Thus potential drugs targeting the apoptotic pathways must take into account the potential protective effects of the process on the overall health of cortical tissues.

Animal Models of TBI

Given the widespread epidemiology of TBI, its deleterious effects, and the ethical inability to invasively study the progression of the injury in humans, various animal models have evolved to serve as a controlled means for characterization and evaluation of the pathological progression of TBI. Because of their small size and cost-effectiveness, rodents are the primary animals used for this characterization.[1] Due to the heterogeneous nature of TBI in humans, a number of models of injury in rodents have been developed that model various aspects of human TBI allowing for comparison of injury type, location, region and severity.[62] Following the induction of TBI, rodents can be evaluated both physically and behaviorally by behavioral testing methods including the Morris water maze for memory deficits, an elevated beam paradigm designed to determine possible motor damage and a vast number of cellular and histopathological evaluations can be conducted both before and after animal euthanasia. Comparisons of the various models of injury can then be carried out to determine the strengths and weaknesses of the method of injury production. Four primary injury models in rodents have emerged as the

most widely used: Fluid Percussion Injury (FPI), Controlled Cortical Impact (CCI), Weight-drop Impact Acceleration (WDIA), and Blast Injury, each of which have unique strengths and weaknesses (Table 1).[1]

A Comparison of Common Models				
Model	**Type of Injury**	**Strengths**	**Weaknesses**	**Species**
Weight Drop Models				
Marmarou's	Mainly Diffuse	Biomechanics similar to human TBI. Well Characterized	Reproducibility. High Mortality Rate	Rat, mouse
Fluid Percussion Injury Models (FPI)				
Central	Mixed	Reproducibility. Clinical Relevance	Need for craniotomy. High Mortality rate	Cat, dog, rabbit, sheep, swine, rat
Lateral	Mixed	Reproducibility. Clinical relevance. Hemispheric Differences	Need for craniotomy. High Mortality rate	Cat, dog, rabbit, sheep, swine, rat
Other Models				
Controlled Cortical Impact Injury (CCI)	Mainly Focal	Reproducibility (btw Animals). Low Mortality	Need for craniotomy. Clinical Relevance. Reproducibility (btw labs)	Ferret, mouse, rat, swine, monkey
Blast Injury	Mainly Diffuse	Biomechanics similar to military TBI	Needs Standardization	Rat, swine, mouse

Table 1. Comparison of Common Animal Models of TBI

The FPI model of injury has become the most widely used and well-characterized model of TBI to date.[63] In FPI the rodent's skull is exposed and following trephination, attached to a fluid reservoir (usually saline). The injury is then produced by the striking of a pendulum to the back of the fluid reservoir which generates a rapid pulse of the fluid onto the intact dural surface of the brain, resulting in temporary displacement and mechanical deformation of the ipsilateral hemisphere.[1] Adjusting the pendulum height, which correlates to the acceleration force generated by the fluid, can modify the severity of the injury. Additionally, adjusting the position of the craniotomy in relation to the

sagittal suture can provide midline, parasagittal, or lateral models of injury; with lateral positioning being the most common.[1] FPI cannot replicate the skull fracturing and contusions across multiple gyri that is often associated with moderate to severe TBI in humans. However, the model does produce both focal and diffuse injury including subdural hematoma, intracranial hemorrhage, brain swelling, and axonal shearing, all of which are hallmarks of TBI pathology in humans.[64] Following FPI the injury site is marked by progressive loss of neurons and conversion to a glial lined cavity that does not readily spread to contralateral brain regions.[1] Lateral FPI results in a largely unilateral injury pattern within the ipsilateral hemisphere with cortical, thalamic, and hippocampal regions exhibiting the highest degree of histological markers of neuronal degradation.[63] Additionally the lateral FPI reliably produces the cognitive and behavioral deficits associated with TBI in humans.[65] The lateral FPI has become the most commonly used method due to its high degree of reproducibility and the ease with which injury severity can be adjusted, however the need for a craniotomy and the high mortality rate due to compromises in brainstem functioning represent key weaknesses of the model.[66] Despite these limitations, FPI is still highly useful in the study of neuronal death mechanisms following TBI.[1]

CCI models of injury use a pneumatic impactor device to drive a rigid rod into the intact dural surface of the exposed rat brain.[67] The model induces deformation of the cortex around the injury site and generates widespread degradation to cortical, thalamic, and hippocampal brain regions that results in cortical tissue loss, axonal shearing, and contusion.[67] A primary advantage of this model is the ease with which mechanistic factors such as velocity, and depth of injury can be controlled.[1] Adjustments in injury

severity and cortical displacement correlate with both histopathological markers of neuronal dysfunction and behavioral deficits, thus the model can be easily adjusted to fit experimental parameters.[68] CCI represents a more focal injury compared to FPI, which can have implications in behavioral and anatomical characterizations.[66] However the low mortality rate associated with this method and the reproducible pathology make CCI a useful model for biomechanical studies of TBI.[1]

The WDIA model induces trauma by via a free falling guided weight striking a metal disk cemented to the skull of a rodent.[69] Differences in injury severity can be achieved by variations in the mass of the weight and the distance that it falls.[1] The impacting force generates rapid acceleration of the brain within the skull and results in diffuse brain injury including petechial hemorrhage and edema in regions from the cortex to the brainstem without fracturing the skull.[62] WDIA produces characteristic pathological features including widespread and bilateral axonal and neuronal damage and extensive DAI as well as similar behavioral and cognitive defects found in FPI and CCI models.[1] While cost effective and useful in evoking DAI, this model of has significant drawbacks due to the relative variability in injury severity and the possibility of a rebound injury when the weight strikes the disc making this method difficult to consistently reproduce.[66]

One of the primary dangers faced by military personnel in modern warfare involves explosive blast forces such as those generated by an improvised explosive device.[4] Even individuals who do not experience any external injuries subsequent to an explosion can experience TBI as a result the forces generated by the blast.[4] In order to understand the mechanisms involved in the propagation of the injury from an explosive

force, animal models have been developed that seek to recreate the blast injury. These models place the rodent in a shock tube and use compression forces to simulate non-impact blast injuries.[4] Blast injuries exhibit a unique pathology that is distinct from that produced by other models. Hallmarks include widespread cerebral oedema, hyperemia and delayed vasospasm as well as widespread DAI.[1] Blast injuries also lead to behavioral and cognitive deficits similar to other models of TBI.[1] Due to the nature of current military deployments, research into the nature of blast mediated TBI represents a high priority area of investigation.

In summary, the lateral FPI model of TBI is the most widely used due to its reproducibility and unilaterality, allowing for the comparison of hemispheres within animals.[1]A comparison of the FPI and WDAI, the second most widely used model for TBI induction, has elucidated key differences in both the pathology and behavioral deficits produced by the two models. Hallam and colleagues compared rats subjected to lateral FPI against rats in the WDAI model on their performance in a Morris water maze, a radial beam walk, and differences in Fluoro-Jade (FJ) staining, a marker of histological degradation.[62] Rats in the FPI group had significantly longer motor defects (6 weeks compared to 14 days) and performed significantly worse on the memory based Morris water maze paradigm. FJ staining, which is indicative of neuronal degeneration, also illustrated key differences in the pathology of injury. FPI rats showed neuron loss throughout the cortex, thalamus, hippocampus, cerebellum and brainstem, while WDAI rats had the most significant losses in the optic tract, olfactory tract, corpus callosum, caudate putamen, and brainstem. These differences in pathology could account for the behavioral and cognitive differences between the two groups and give support for the

continued use of the FPI as a model for mild, moderate and severe TBI.[62] FPI also reliably replicates neurotoxic levels of glutamate that are responsible for the secondary injury phase of TBI, making it useful for the study of the cellular mechanisms underlying subsequent cortical tissue degradation.[1] Increased levels of glutamate in this model have been indicated by microelectrode arrays [70] and enzyme electrode bioassay.[71] Additionally, immunoblot studies of the transporters responsible for the clearance of glutamate indicates loss of some splice variants of these molecules following lateral FPI.[24] Together these results underline the rationality of using the FPI model for the study of the secondary injury phase of TBI.

Despite widespread acknowledgement of the harmful effects of the secondary injury phase of TBI, efforts aimed at producing effective therapeutic interventions remain in their early stages. In the following study, we hypothesize that TBI will result in induce alterations in glutamate homeostasis, leading to an inability to properly maintain extracellular glutamate. We hypothesize that this homeostatic imbalance will be the result of decreased transporter function, decreased transporter expression, or both. We aim to provide a tool for the evaluation of the functional status of glutamate transporters following FPI and to provide evidence regarding possible changes in expression profiles of the transporters following TBI.

TBI INDUCES ALTERATIONS IN CORTICAL GLUTAMATE HOMEOSTASIS WITHOUT A REDUCTION IN GLT-1 EXPRESSION

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Abstract

Traumatic brain injury (TBI) is a major cause of morbidity and mortality worldwide and often leads to chronic cognitive and behavioral impairments. Massive increases in extracellular concentrations of glutamate are thought to play an initiating and propagating role in the cellular derangement that persists for hours or days after the initial insult. These effects are exacerbated by abnormalities in the tightly controlled neuron-astrocyte-coupled regulation of synaptic and extrasynaptic glutamate levels. We hypothesize that the primary mechanism for removal of glutamate from the extracellular space, glutamate reuptake by glutamate transporter-1 (GLT-1), is altered after TBI. To evaluate this hypothesis we initiated TBI in adult male rats using the lateral fluid percussion injury model. At 24 hours post-TBI, expression of GLT-1 protein in the ipsilateral cortex and hippocampus was assessed by immunoblotting and glutamate uptake was assessed in synaptosomes isolated from the ipsilateral cortex. In the ipsilateral cortex we found that there were no significant differences in GLT-1 protein expression between injured and sham operated rats in both cortical homogenates and synaptosomes. However in the hippocampus, GLT-1 protein expression was increased at 24 hours post-injury. We also found that synaptosomal glutamate uptake in the cortex was decreased in the injured group as compared to the sham. Taken together, these data indicate that TBI induces a reduction in cortical glutamate uptake without alteration of GLT-1 expression. These results demonstrate that reduction in the functional capacity of glutamate transporters following injury is not always a direct result of decreased protein expression and that therapeutic modulation of glutamate uptake may be beneficial following TBI.

Introduction

Traumatic Brain Injury (TBI) represents a persistent major health risk with an annual incidence rate of around 200 per 100,000 people and results in an estimated 10 million hospitalizations or deaths per year.[1] TBI can lead to lasting cognitive and behavioral impairments and is the leading cause of death resulting from injury in individuals under the age of 45.[2] While the trauma itself is a onetime event, TBI can be understood as an ongoing pathology consisting of two distinct phases. The initial stage, primary injury, is a direct result of the mechanical forces applied to the brain that induce hemorrhage, contusion, and axonal shearing. The second stage, secondary injury, is characterized as a prolonged, diffuse pathophysiological sequence of events which include released excitatory neurotransmitters, free radical production, mitochondrial damage, changes in protein expression and eventually cell death.[3] The primary injury is ex post facto non-treatable; therefore therapeutic targets focus on the diminution of the secondary injury.

The metabolic and cellular derangements that are characteristic of the secondary injury phase are largely initiated by massive and indiscriminant release of glutamate into the extracellular space.[4] While glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system, it's interstitial concentrations must be actively maintained as it can be toxic to neurons even at low extracellular concentrations.[5] Various microdialysis studies have demonstrated that within minutes of sustaining a TBI, extracellular glutamate levels rise sharply in a force dependent manner[6], with some reports indicating a 9-fold increase in extracellular glutamate levels following a severe lateral fluid percussion injury (LFPI).[7] These excitotoxic levels of extracellular glutamate

arise from a number of factors. The mechanical force associated with the primary injury can result in both direct disruption of the cell's plasma membrane and lead to indiscriminate electrical discharge, either of which may precipitate the release of intracellular ions and glutamate into the extracellular space.[4,8] The ionic imbalance and widespread cellular derangement associated with the secondary injury phase results in astrocyte cell death, caspase-mediated degradation of glutamate transporters, and reversal of sodium-dependent glutamate transport; all of which have been implicated in furthering the pathological sequelae associated with TBI.[8-11]

Under physiological conditions low extracellular concentrations of glutamate are maintained by a family of sodium dependent glutamate transporters which are selectively expressed throughout the mammalian brain on both neurons and glia.[12] The glutamate and aspartate transporter (GLAST/EAAT1) and the glutamate transporter-1 (GLT-1/EAAT2) are expressed primarily in astrocytes while the remaining transporters reside primarily on neurons.[13] Of the astrocyte transporters, GLT-1 is responsible for approximately 90% of the clearance of glutamate from the synapse.[12] The astrocytic glutamate transporters are crucial for the proper maintenance of extracellular glutamate levels; antagonists for them can lead to toxic levels of glutamate and subsequent cell death.[14] Similarly, the use of antisense oligonucleotides to knockout expression of either GLAST or GLT-1 in non-TBI rats can produce excitotoxic levels of glutamate similar to that experienced following TBI and induce neuronal degeneration, loss of motor neuron viability and significantly increased hippocampal cell death.[15,16] While the immediate rise in extracellular glutamate quickly dissipates,[17] the long term ability for transporters to clear glutamate from the extracellular space may be compromised following TBI. This

is evidenced by alterations in synaptic homeostasis including a reduced ability to reuptake glutamate following controlled cortical impact (CCI).[18] In the immediate hours following CCI both mRNA levels and protein expression of GLT-1 are significantly reduced in the rat cortex.[16]

These findings prompted us to examine the effects of TBI on glutamate clearance by expounding on previous utilized tools in order to establish a functional characterization of the ability of neuronal and glial cells to reuptake glutamate following lateral fluid percussive injury. The LFPI model is a well characterized and commonly accepted rodent model of TBI which produces persistent motor and memory deficits as well as neuronal loss exhibited throughout the injured hemisphere including the cortex, thalamus, and hippocampus.[19-22]Synaptosome isolation and glutamate reuptake monitoring in rat cortical tissue offers a tool that will allow for the comparison of the functional effects of TBI on glutamate reuptake ability. Additionally, comparison of changes in protein expression between cortical, hippocampal and synaptosomal tissue will provide information regarding possible changes in localization of the transporters mediating glutamate reuptake following TBI. Finally, synaptosomal isolation represents a method for the evaluation of possible therapeutic drug targets in a biologically significant context.

Methods

Animals

Adult male Sprague-Dawley rats (348 ± 40 g, age 8-9 weeks, at time of surgery; Charles River Laboratories International, Inc.) were housed two per cage on a twelve

hour light/dark cycle in a temperature- (22° C) and humidity-controlled facility and allowed standard rat chow and water *ad libitum*. All animal care and experimental procedure complied with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. For generation of tissue, animals were divided into three groups. Uninjured control animals received craniectomy only (Sham, n=5) or no surgery (Naive, n=5). The injured animals received vehicle-treated TBI using a lateral fluid percussive injury model as described below (TBI, n=5). Five animals from each condition were humanely euthanized at 24-hours post treatment (24-hr, n=15) and tissue was extracted for synaptosomal isolation, protein quantification and electron microscopy.

Surgical Procedure

Surgical procedure was carried out as previously described.[21,23] Briefly, animals were anesthetized with 4% isoflurane gas in an O_2 carrier for 4 minutes, followed by intraperitoneal injection of 100/10 mg/kg mixture of ketamine/xylazine; anesthesia was maintained via ventilation with 1.5-3% isoflurane gas for the duration of surgery. Normothermia was maintained throughout surgery by keeping the animals on a water-jacketed heating pad. After securing the animal in a stereotaxic frame, a midline scalp incision was made and the skin and fascia reflected to expose the bregma, lambda, and sagittal sutures, as well as the lateral ridge. A 4.8 mm craniectomy was trephined over the right parietal cortex, midway between bregma and lambda, tangential to the sagittal suture. A rigid plastic injury tube (modified female Luer-lock 20G needle hub) was bonded to the skull with cyanoacrylate adhesive over the open crainectomy with the dura intact and a stabilizing screw was placed in a burr hole drilled rostral to bregma on the

ipsilateral side. The injury tube and stabilizing screw were secured with dental acrylic. The scalp was then sutured and the animal was placed in a warmed recovery cage.

Induction of later fluid percussion injury

Experimental TBI was using a fluid percussive device (VCU Biomedical Engineering, Richmond, VA) as previously described.[21] The device consists of a Plexiglas cylinder (60 cm in length and 4.5 cm in diameter) filled with sterile water. A piston is mounted on O-rings at one end and an extra-cranial pressure transducer (Entran Devices, Inc., EPN-0300A, Fairfield, NJ) connected to a storage oscilloscope (Tektronix, TDS 310, Beaverton, OF) is attached to the opposite end. A 5 mm tube (internal diameter 2.6 mm) ending in a male Luer-lock is fitted at the end. The animal was anesthetized with 4% isoflurane gas for 4 min, and moderate TBI was induced by rapidly injecting a small volume of sterile saline into the closed cranial cavity over the right, ipsilateral, hemisphere with the fluid percussion device. Immediately after the impact, the animal was removed from the device, monitored for duration of apnea and unconsciousness, and re-sutured while receiving supplemental oxygen ventilation. The magnitude of the pressure pulse was measured by a pressure transducer, stored on an oscilloscope, and later converted to atmospheres (ATM). The pressure pulse was monitored and controlled in order to deliver and equivalent impact to each animal.Brains were extracted and dissected into 20 separate areas of interest and immediately flash frozen on dry ice. Tissue was kept at -80°C until needed.

Synaptosomal isolation and [3H]-glutamate uptake

150 to 200mg of frozen cortical tissue (-80 °C) from the area of injury was transferred to 5mL of ice-cold HEPES-buffered sucrose solution (HBSS) at pH 7.4 in a glass homogenizer (Kontes Glass Co, #21, Vineland, NJ) and dounced (15 strokes) to yield a homogenate. The homogenate was transferred to microcentrifuge tubes and spun at 800g (3,000rpm) in a refrigerated centrifuge (Eppendorf, 5415R, Hamburg, Germany) for 10 min at 4°C. The supernate was transferred to new microcentrifuge tubes and spun at 10,000g (10,400rpm) in a refrigerated centrifuge for 15 min at 4°C. The resulting pellet was resuspended in 2mL of HBSS and layered onto 3mL of 1.2M sucrose columns. The sucrose columns were placed in swinging buckets (Beckman Coulter, SW60Ti, Pasadena, CA) and the corresponding rotor was placed in an ultracentrifuge (Beckman Coulter, L8-60M, Pasadena, California) and spun at 230,000g (41,000rpm) for 45 min at 4°C. The supernate band was collected from above the sucrose gradient and placed in 1.5mL of either standard glutamate uptake buffer (NaCl: 144mM, KCl: 2.5mM, CaCl2: 1.2mM, MgCl2: 1.2mM, K2HPO4: 1.3mM, Glucose: 10mM, HEPES: 10mM, and Tris: 5mM), or a sodium-free glutamate uptake buffer where choline chloride (144mM) was substituted for sodium chloride to determine non-specific binding of glutamate. The band layer was then pipetted onto a 2.5mL column of 0.8M sucrose and spun at 230,000g (41,000 rpm) for 45 min at 4°C. The pellet was collected and suspended in 100µL of appropriate glutamate uptake buffer. Total protein concentration was determined for each sample with a BCA assay.

Sample tubes were prepared with 20µg of re-suspended synaptosomes, and placed into tubes containing either: standard glutamate uptake buffer at 37°C (37°, n=3), standard glutamate uptake buffer at 0°C (0°, n=3), Na^+-Free glutamate uptake buffer at

37°C (Na^+, n=3) or standard glutamate uptake buffer with added 5M L-trans-pyrrolidine-2,4-dicarboxylic acid at 37°C (PDC, n=3). The tubes were then pre-incubated for 30 minutes at their respective temperatures. 10µM of unlabeled glutamic acid and 2µCi of [3H]-Glutamate were then added to the samples such that the final volume each tube reached 500µL. Uptake was allowed to occur at respective temperatures for 10 minutes. The sample tubes were then filtered through a cell harvester (Brandel, Gaithersburg, MD) with 0.9% cold saline solution and trapped on Whatman GF/C filters (GE Healthcare, Buckinghamshire, UK). The filters were then collected into scintillation vials, suspended in 5 ml of Ultima-Gold™ scintillation fluid (Perkin Elmer Inc., Waltham, MA) and counted on a scintillation counter (Beckman Coulter, LS 6500, Pasadena, CA).

Western blot analysis

10 or 20 µg of total protein and 6 µg of sample buffer (Invitrogen, Caralsbad, CA) were loaded into a pre-cast SDS gel (Mini-PROTEAN® TGX™ Any-kD™ Bio-rad, Hercules, CA) and run at 180V for one hour then transferred to polyvinylidene fluoride membranes (Bio-rad) at 16V for 30 minutes. Membranes were blocked overnight in blocking buffer (Li-Cor, Lincoln, NE) and then incubated in primary antibody (Abs; guinea-pig anti-GLT-1, AB1783 Milipore, Billerica, MA at a concentration of 1:5000; mouse anti-valosin-containing protein, AB11433 Abcam, Cambridge, MA at a concentration of 1:2500; rabbit anti-synaptophysin, AB23745 Abcam, at a concentration of 1:50000) overnight at 4°C and then washed three times with tris-buffered saline (TBS). Secondary antibodies with fluorescent reporters (Li-cor) raised against the appropriate species were all used at 1:5000, and blots were imaged on a LI-COR Odyssey Imager.

Electron Microscopy

Synaptosomal fragments were fixed in 1/2 Karnovsky fixative (2.5% paraformaldehyde and 2.0% glutaraldehyde in 0.1M Cacodylate buffer) and then post fixed in 1% Osmium Tetroxide in 0.1M Caco buffer. Samples were then dehydrated in a graded series of ethanol and infiltrated and embedded in Embed-812 embedding resin. Following embedding ultrathin sections were collected on copper mesh grids, post-stained with uranyl acetate and lead citrate, and examined using an FEI Tecnai T-12 electron microscope. All imaging was performed at the UAB Electron Microscopy Core (Birmingham, Al).

Statistics

All statistical tests were done with GraphPad Prism. Data was analyzed with a one-way ANOVA or student's t-test. Post-hoc analysis was performed with Turkey's multiple comparison test when necessary and are reported as mean value ± standard error of the mean (SEM)(*: $p<0.05$, **$p<0.01$).

Results

TBI results in significant loss of consciousness in adult male Sprague-Dawley Rats

The duration of transient unconsciousness following LFPI is an indication of injury severity.[21,24] Transient unconsciousness is measured by the duration of suppression of the righting reflex. Student's t-test indicated a significant effect for condition ($t(8) = 5.096$, $p < 0.01$), with rats subjected to TBI remaining unconscious for significantly longer (10:08 ±1:08 minutes), than did their sham-operated counterparts (4:18 ± 0:17

minutes). This indicates that, in animals receiving TBI, a portion of their unconsciousness is due to the induction of the injury and not a result of anesthesia alone (Fig 1).

Isolation yields synaptosome fragments containing the synapse marker synaptophysin

The synaptosome isolation protocol was adapted from previous studies utilizing human cortical tissue[25]. To determine the cellular makeup of these synaptic fragments, the pellets were imaged using Electron Microscopy (EM). EM imaging demonstrated that the synaptosome pellet contains synaptic fragments which are filled with vesicles and putative areas of pre- and post-synaptic density joining (Fig. 2 A-B). Western blot analysis on these synaptic fragments indicated the presence of the synaptic marker synaptophysin (Fig. 2C-D). Following normalization to the loading control valosin-containing protein (VCP), a one-way ANOVA demonstratedno detectible differences between groups, indicating statistically equivalent expression of the protein across the three experimental conditions ($F(2,11) = 0.439$, $p = 0.66$).

Synaptosomal glutamate uptake is temperature, sodium, and transporter dependent

To evaluate GLT-1 dependent glutamate reuptake, we developed a glutamate assay with the ability to monitor the amount of [H^3]-glutamate a given sample was able to transport. This resulted in synaptosomes isolated from the cortex of naive (i.e. non-injured, non-operated) rats with the ability to uptake radiolabeled [H^3]-glutamate in a physiologically relevant capacity (Fig. 3). Results from a one-way ANOVA indicated a significant effect of condition on uptake ability ($F(3,16) = 10.90$, $p = 0.0004$; $n = 5$ rats per condition). Synaptosomesanalyzed under physiological conditions (i.e. physiological extracellular ion concentration and temperature) were able to transport more [H^3]-

glutamate than synaptosomes brought up under other conditions. Tukey post-hoc comparison demonstrated that inhibition of reuptake is possible by physiological disruptions such as exposing the synaptosomes to freezing conditions, or by bringing them up in a sodium free environment, resulting in decreases of [H^3]-glutamate reuptake ability by 66 and 65% respectively relative to the 37° group. Additionally, post-hoc comparison indicated that [H^3]-glutamate reuptake is also dependent on the presence of functional glutamate transporters as 58% inhibition of reuptake was possible by the addition of 5M L-trans-Pyrrolidine-2,4-dicarboxylic acid (PDC), an inhibitor of both GLT-1 and GLAST.[26]

TBI results in decreased glutamate reuptake ability

To determine the effect of injury on the functional ability of synaptosomes to effectively clear glutamate from the extracellular space, we next compared the glutamate reuptake capacity of synaptosomes from injured rats to that of sham surgery controls. Twenty-four hours following induction of TBI, glutamate reuptake was examined in synaptosomal fragments isolated from the injured frontal and parietal lobes and from corresponding regions of sham operated controls. [H^3]-glutamate reuptake at physiological conditions (i.e. isotonic solutions with standard ion concentrations at 37°C) was then compared between the two groups (Fig 4). Student's t-test demonstrated synaptosomes isolated from the injured hemisphere of TBI rats were able to uptake 39% less [H^3]-glutamate than sham surgery controls ($t(8) = 3.43$, $p < 0.01$; $n = 5$ rats per condition). Thus, TBI induces a significant deficit in the ability of glutamate transporters to effectively reuptake extracellular glutamate

TBI results in differential expression patterns of GLT-1

We next investigated whether the decrease in reuptake capacity was a result of loss of transporter protein expression. We used Western blot analysis to determine mean GLT-1 expression in the cortex, synaptosomal fragments and the hippocampus of both injured and sham surgery rats. Student's t-test indicated no difference in GLT-1 expression in the cortices of sham surgery or injured rats (Fig. 5A). Likewise, there was no difference in synaptosomal expression of GLT-1 between treatment conditions (Fig. 5B). In contrast, GLT-1 expression in the hippocampus of injured rats increased significantly in comparison to their sham surgery controls (Fig. 5C). A student's t-test indicated 180% more expression of GLT-1 in the injured hippocampus of TBI rats than in uninjured sham controls ($t(8)= 2.302$, $p < 0.05$; n= 5 rats per condition). These data indicate that there are not differences in GLT-1 expression in the cortex at 24 hours after TBI. When considered with the glutamate uptake in the cortex (Fig. 4), these data suggest that cortical deficits in glutamate reuptake capacity are not the result of simple decreases in expression of the transporter.

Discussion

These results uniquely point to a deficit in the functionality of glutamate transporters as a result of TBI that is likely not due to decreases in their expression at 24 hours following moderate injury. The synaptosomal fragments isolated twenty-four hours after brain injury exhibited impairment in [H^3]-glutamate reuptake, indicating a breakdown in the ability of GLT-1 to effectively remove the neurotransmitter from the extracellular space. However western blot analysis demonstrated no differences in total

levels of cortical or synaptosomal expression of the transporter, thus the differences in reuptake capacity cannot be attributed solely to a reduction in the amount of GLT-1 expressed. TBI initiates a multifactorial response from the brain as it accommodates to the injury. Some of these responses result in alterations in gene transcription for a number of proteins, while others activate post-translational mechanisms that may alter either expression or function of molecular systems.[3] Part of this change likely involves compensatory reactions by the brain to offset deleterious effects of the injury. We tentatively speculate that we have identified one such mechanism as exhibited by the increases in hippocampal GLT-1 expression, perhaps demonstrative of an endogenous reaction aimed at decreasing the excitotoxic effects of elevated extracellular glutamate in a region further removed from the site of injury.

Possible Mechanisms of Altered Glutamate Uptake

There are a number of possible mechanisms to account for decreases in function of GLT-1 that are not coupled with a loss of GLT-1 protein expression. For example, one early consequence of TBI is changes in key ion concentrations including sodium and potassium.[4,27] Resting state astrocytes have a high potassium conductance as well as highly negative membrane potentials. These characteristics provide for efficient glutamate reuptake and are dependent to a large extent on the inwardly rectifying KIR4.1 channel, the most common CNS potassium channel.[28] Disruptions in KIR4.1 expression via RNA interference or transgenic manipulation result in astrocytes with a decreased ability to uptake glutamate.[29,30] Expression of KIR4.1 is reduced by up to 80% 7 days following spinal cord injury and levels of the transporter may be reduced for up to 4 weeks following injury.[31] Furthermore, decreases in gene transcription for KIR4.1 were

found as early as 4 hours following TBI in adult mice,[32] indicating an acute manifestation of KIR4.1 down-regulation may be possible and likely contributes to a breakdown of the potassium potential necessary for the proper function of GLT-1.

Another molecular change associated with TBI involves the activity and levels of key intracellular signaling molecules including growth factors. Astrocyte cell culture studies have indicated that growth factors are induced in response to axonal injury.[33,34] Several, including transforming growth factor-α (TGF-α), epidermal growth factor (EGF), and fibroblast growth factor-2 (FGF2) have been shown to promote GLT-1 expression in astrocyte cell cultures.[35] However, any putative growth factor induced stimulation of GLT-1 expression following TBI may be counteracted by the simultaneous, injury-induced, increase in endothelin expression. Multiple isoforms of endothelins are known to both down-regulate GLT-1 expression by themselves and to block the effect of growth factors on transporter induction.[36] It appears that TBI may result in a continuum between factors promoting increased expression of GLT-1 and thus reducing the excitotoxic rise in extracellular glutamate, and aberrant pathological signaling pathways responsible for both the loss of function and expression of the transporter.

The temporal nature of the balance between GLT-1 promoting and GLT-1 diminishing pathways is highlighted by astrocyte cell cultures examining the effects of PKC. Long term activation of protein kinase C (PKC) by phorbol esters in cultured cortical astrocytes result in overall decreases in cellular GLT-1; a decrease which can be attenuated by lysosomal inhibitors, indicating a possible cellular mechanism by which PKC can reduce GLT-1 levels under physiological or pathological conditions[37]. Mutation

of serine residue 114 to asparagine abolished phorbol ester-stimulated transport in these cultures, suggesting a putative phosphorylation site.[37] In contrast to long term activation of PKC, shorter term activity of the kinase results in sequestration of the transporter to an intracellular storage site by a clathrin-mediated endocytotic mechanism.[37] This intracellular sequestration of GLT-1 diminishes cell-surface expression, and thus the functional removal of glutamate from the extracellular space, but does not lead to an overall reduction in transporter expression. Similarly, caspase-3 has been demonstrated to cleave GLT-1 at aspartate 505, a highly conserved cytosolic C-terminal site present on mouse, rodent, and human isoforms of the transporter.[10] Cleavage of the transporter at this site is dependent on caspase-3 concentration and results in a significant loss of function of the transporter; however the truncated form is still detectible on immunoblots.[10]

Additional support for the notion that a time-dependent continuum between pathways promoting GLT-1 expression and those responsible for its decrease come from post-mortem human studies of excitatory amino acid transporter 2 (EAAT2, the human homologue of GLT-1). Ikematsu and colleagues identified a highly extensive EAAT2 staining pattern in the ipsilateral cortex of short term survival cases (1 – 24 hours after injury), indicating an increase in EAAT2 expression in the astrocytes in this region.[38] They suggest that this increase in expression may be a compensatory reaction to the elevated extracellular glutamate levels following TBI and may partially account for the rapid reduction in extracellular concentration of glutamate as the brain attempts to mitigate the excitotoxic effects of the injury. They also report a decrease in EAAT2 staining in long term survival cases, which may be a reaction either to decreases in

extracellular glutamate concentrations, or a result of the activity of intracellular signaling pathways promoting GLT-1 degradation. It is worth noting that, short term TBI survival is correlated with injury severity,[39] thus the severity of the injury may influence the GLT-1 homeostasis. Therefore there is ample evidence that a time and severity-dependent balance may exist regarding the relative mechanistic contribution of the GLT-1 promoting and GLT-1 diminishing intracellular signaling pathways.

Role of GLT-1 Splice Variants and Injury Model

Further complicating the quantification of GLT-1 expression following TBI, isoforms of the transporter are known to exist in at least three separate splice variants with differing C-terminal domains. The C-terminal of GLT-1 is an important functional domain, thus while the splice variants are highly homologous, and may not be distinguishable without custom made antibodies, they demonstrate varied responses to insult and injury. In contrast to their findings for the originally cloned form of the transporter (referred to in this paragraph as GLT-1α) Yi and colleagues have reported decreases in cortical protein expression of the splice variant GLT-1v twenty-four hours following TBI.[40] They reported no changes in hippocampal or thalamic expression of the variant at the same time period.[40] Severe hypoxia results in early loss of GLT-1α n the pig hippocampus, which is followed by the aberrant induction of a GLT-1 splice variant in neuronal cell types.[41] Detection of an alternately spliced variant of GLAST in neuronal cell populations following hypoxia is an early and highly sensitive marker for neurons at risk of cell death.[42] Similarly, engineered expression of the normally astrocytic EAAT2 on neuronal cell types has been correlated with an increased vulnerability to excitotoxicity in hippocampal slice cultures.[43] Induction of alternatively spliced forms of

EAAT2 in neuronal cell populations are found in neurodegenerative diseases including ALS and Alzheimer's disease,[44,45] although transcripts for these splice variants can also be found in control populations,[46] thus a causal implication for the role of splice variants in the progression of these diseases remains to be elucidated, however it has been suggested that normal expression of glutamate transporters may be negatively modulated by co-expression of splice variants.[47]

While multiple studies have demonstrated altered expression of glutamate transporters following TBI, these effects vary based on the aforementioned factors as well as the animal model used to induce the injury.[45] CCI models of injury generally demonstrate a clear and consistent down-regulation of cortical and hippocampal expression of the transporter beginning 4-6 hours after injury and persisting for up to 72 hours.[9,16,48]However, studies using LFPI have reported mixed effects of the model on glutamate transporter levels. While Goodrich and colleagues report decreases in GLT-1 expression by 29% in the ipsilateral cortex for up to 7 days post-injury,[49] others have shown no change in cortical expression of the transporter in the first 24 hours and increases in hippocampal expression across the same time period.[40] The discrepancies between the models may be a result of differences in severity of the injury, the focal nature of the CCI model, the recruitment of different second messenger signals, or a combination of factors.[3,8]Studies in astrocyte cultures have found that the half-life of GLT-1 is longer than 24 hours[50]; therefore the observed decreases in protein expression in the hours immediately following CCI[48] cannot be accounted for solely by diminishing transcription of the transporter.

Sullivan and colleagues have previously demonstrated decreasedsynaptosomal glutamate uptake in TBI, however their study utilized the CCI injury model, thus it would be difficult to conclude that any decreases in uptake could not be attributed to loss of expression of the transporters.[18] As such the results of our experiment data uniquely suggest that decreases in protein expression are not necessary for the concomitant manifestation of functional deficits. The use of synaptosomes to monitor glutamate reuptake represents a unique method for the functional comparison not only of injury models and severity, but potential therapeutic treatment strategies. The use of synaptosomes that function in a biologically significant capacity, and which demonstrate measurable deficits in functionality following TBI, has the potential to be a potent therapeutic tool in TBI research for the evaluation of the effectiveness of future treatment strategies.

References

1 Langlois, J. A., Rutland-Brown, W. & Wald, M. M. The epidemiology and impact of traumatic brain injury: a brief overview. *The Journal of head trauma rehabilitation***21**, 375-378 (2006).

2 Bruns, J., Jr. & Hauser, W. A. The epidemiology of traumatic brain injury: a review. *Epilepsia***44 Suppl 10**, 2-10 (2003).

3 Xiong, Y., Mahmood, A. & Chopp, M. Animal models of traumatic brain injury. *Nature reviews. Neuroscience***14**, 128-142, doi:10.1038/nrn3407 (2013).

4 Katayama, Y., Becker, D. P., Tamura, T. & Hovda, D. A. Massive increases in extracellular potassium and the indiscriminate release of glutamate following concussive brain injury. *Journal of neurosurgery***73**, 889-900, doi:10.3171/jns.1990.73.6.0889 (1990).

5 Olney, J. W. Excitotoxicity: an overview. *Canada diseases weekly report = Rapport hebdomadaire des maladies au Canada***16 Suppl 1E**, 47-57; discussion 57-48 (1990).

6 Nilsson, P., Hillered, L., Ponten, U. & Ungerstedt, U. Changes in cortical extracellular levels of energy-related metabolites and amino acids following concussive brain injury in rats. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism***10**, 631-637, doi:10.1038/jcbfm.1990.115 (1990).

7 Faden, A. I., Demediuk, P., Panter, S. S. & Vink, R. The role of excitatory amino acids and NMDA receptors in traumatic brain injury. *Science***244**, 798-800 (1989).

8 Yi, J. H. & Hazell, A. S. Excitotoxic mechanisms and the role of astrocytic glutamate transporters in traumatic brain injury. *Neurochemistry international***48**, 394-403, doi:10.1016/j.neuint.2005.12.001 (2006).

9 van Landeghem, F. K. *et al.* Early expression of glutamate transporter proteins in ramified microglia after controlled cortical impact injury in the rat. *Glia***35**, 167-179 (2001).

10 Boston-Howes, W. *et al.* Caspase-3 cleaves and inactivates the glutamate transporter EAAT2. *The Journal of biological chemistry***281**, 14076-14084, doi:10.1074/jbc.M600653200 (2006).

11 Allen, N. J., Karadottir, R. & Attwell, D. Reversal or reduction of glutamate and GABA transport in CNS pathology and therapy. *Pflugers Archiv : European journal of physiology***449**, 132-142, doi:10.1007/s00424-004-1318-x (2004).

12 Kanai, Y. & Hediger, M. A. The glutamate and neutral amino acid transporter family: physiological and pharmacological implications. *European journal of pharmacology***479**, 237-247 (2003).

13 Shigeri, Y., Seal, R. P. & Shimamoto, K. Molecular pharmacology of glutamate transporters, EAATs and VGLUTs. *Brain research. Brain research reviews***45**, 250-265, doi:10.1016/j.brainresrev.2004.04.004 (2004).

14 Velasco, I., Tapia, R. & Massieu, L. Inhibition of glutamate uptake induces progressive accumulation of extracellular glutamate and neuronal damage in rat cortical cultures. *Journal of neuroscience research***44**, 551-561, doi:10.1002/(SICI)1097-4547(19960615)44:6<551::AID-JNR5>3.0.CO;2-A (1996).

15 Rothstein, J. D. *et al.* Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron***16**, 675-686 (1996).

16 Rao, V. L., Dogan, A., Bowen, K. K., Todd, K. G. & Dempsey, R. J. Antisense knockdown of the glial glutamate transporter GLT-1 exacerbates hippocampal neuronal damage following traumatic injury to rat brain. *The European journal of neuroscience***13**, 119-128 (2001).

17 Palmer, A. M. *et al.* Traumatic brain injury-induced excitotoxicity assessed in a controlled cortical impact model. *Journal of neurochemistry***61**, 2015-2024 (1993).

18 Sullivan, P. G., Keller, J. N., Mattson, M. P. & Scheff, S. W. Traumatic brain injury alters synaptic homeostasis: implications for impaired mitochondrial and transport function. *Journal of neurotrauma***15**, 789-798 (1998).

19 Hallam, T. M. *et al.* Comparison of behavioral deficits and acute neuronal degeneration in rat lateral fluid percussion and weight-drop brain injury models. *Journal of neurotrauma***21**, 521-539, doi:10.1089/089771504774129865 (2004).

20 Thompson, H. J. *et al.* Lateral fluid percussion brain injury: a 15-year review and evaluation. *Journal of neurotrauma***22**, 42-75, doi:10.1089/neu.2005.22.42 (2005).

21 McIntosh, T. K. *et al.* Traumatic brain injury in the rat: characterization of a lateral fluid-percussion model. *Neuroscience***28**, 233-244 (1989).

22 Cernak, I. Animal models of head trauma. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics***2**, 410-422, doi:10.1602/neurorx.2.3.410 (2005).

23 Day, N. L., Floyd, C. L., D'Alessandro, T. L., Hubbard, W. J. & Chaudry, I. H. 17beta-estradiol confers protection after traumatic brain injury in the rat and involves activation of g protein-coupled estrogen receptor 1. *Journal of neurotrauma***30**, 1531-1541, doi:10.1089/neu.2013.2854 (2013).

24 Morehead, M. *et al.* Histopathologic consequences of moderate concussion in an animal model: correlations with duration of unconsciousness. *Journal of neurotrauma***11**, 657-667 (1994).

25 Whittaker, V. P. Synaptosome preparations. *Journal of neurochemistry***50**, 324-325 (1988).

26 Koch, H. P. *et al.* Differentiation of substrate and nonsubstrate inhibitors of the high-affinity, sodium-dependent glutamate transporters. *Molecular pharmacology***56**, 1095-1104 (1999).

27 Choi, D. W. Ionic dependence of glutamate neurotoxicity. *The Journal of neuroscience : the official journal of the Society for Neuroscience***7**, 369-379 (1987).
28 Olsen, M. Examining potassium channel function in astrocytes. *Methods in molecular biology***814**, 265-281, doi:10.1007/978-1-61779-452-0_18 (2012).
29 Olsen, M. L., Higashimori, H., Campbell, S. L., Hablitz, J. J. & Sontheimer, H. Functional expression of Kir4.1 channels in spinal cord astrocytes. *Glia***53**, 516-528, doi:10.1002/glia.20312 (2006).
30 Djukic, B., Casper, K. B., Philpot, B. D., Chin, L. S. & McCarthy, K. D. Conditional knock-out of Kir4.1 leads to glial membrane depolarization, inhibition of potassium and glutamate uptake, and enhanced short-term synaptic potentiation. *The Journal of neuroscience : the official journal of the Society for Neuroscience***27**, 11354-11365, doi:10.1523/JNEUROSCI.0723-07.2007 (2007).
31 Olsen, M. L., Campbell, S. C., McFerrin, M. B., Floyd, C. L. & Sontheimer, H. Spinal cord injury causes a wide-spread, persistent loss of Kir4.1 and glutamate transporter 1: benefit of 17 beta-oestradiol treatment. *Brain : a journal of neurology***133**, 1013-1025, doi:10.1093/brain/awq049 (2010).
32 Gupta, R. K. & Prasad, S. Early down regulation of the glial Kir4.1 and GLT-1 expression in pericontusional cortex of the old male mice subjected to traumatic brain injury. *Biogerontology***14**, 531-541, doi:10.1007/s10522-013-9459-y (2013).
33 Lisovoski, F. *et al.* Transforming growth factor alpha expression as a response of murine motor neurons to axonal injury and mutation-induced degeneration. *Journal of neuropathology and experimental neurology***56**, 459-471 (1997).
34 Junier, M. P. What role(s) for TGFalpha in the central nervous system? *Progress in neurobiology***62**, 443-473 (2000).
35 Figiel, M., Maucher, T., Rozyczka, J., Bayatti, N. & Engele, J. Regulation of glial glutamate transporter expression by growth factors. *Experimental neurology***183**, 124-135 (2003).
36 Rozyczka, J., Figiel, M. & Engele, J. Endothelins negatively regulate glial glutamate transporter expression. *Brain pathology***14**, 406-414 (2004).
37 Susarla, B. T. & Robinson, M. B. Internalization and degradation of the glutamate transporter GLT-1 in response to phorbol ester. *Neurochemistry international***52**, 709-722, doi:10.1016/j.neuint.2007.08.020 (2008).
38 Ikematsu, K., Tsuda, R., Kondo, T. & Nakasono, I. The expression of excitatory amino acid transporter 2 in traumatic brain injury. *Forensic science international***130**, 83-89 (2002).
39 Raj, R. *et al.* Predicting six-month mortality of patients with traumatic brain injury: usefulness of common intensive care severity scores. *Critical care***18**, R60, doi:10.1186/cc13814 (2014).
40 Yi, J. H., Pow, D. V. & Hazell, A. S. Early loss of the glutamate transporter splice-variant GLT-1v in rat cerebral cortex following lateral fluid-percussion injury. *Glia***49**, 121-133, doi:10.1002/glia.20099 (2005).
41 Pow, D. V. *et al.* Loss of glial glutamate transporters and induction of neuronal expression of GLT-1B in the hypoxic neonatal pig brain. *Brain research. Developmental brain research***153**, 1-11, doi:10.1016/j.devbrainres.2004.06.019 (2004).

42 Sullivan, S. M., Macnab, L. T., Bjorkman, S. T., Colditz, P. B. & Pow, D. V. GLAST1b, the exon-9 skipping form of the glutamate-aspartate transporter EAAT1 is a sensitive marker of neuronal dysfunction in the hypoxic brain. *Neuroscience***149**, 434-445, doi:10.1016/j.neuroscience.2007.08.011 (2007).
43 Selkirk, J. V. *et al.* Over-expression of the human EAAT2 glutamate transporter within neurons of mouse organotypic hippocampal slice cultures leads to increased vulnerability of CA1 pyramidal cells. *The European journal of neuroscience***21**, 2291-2296, doi:10.1111/j.1460-9568.2005.04059.x (2005).
44 Pow, D. V. & Cook, D. G. Neuronal expression of splice variants of "glial" glutamate transporters in brains afflicted by Alzheimer's disease: unmasking an intrinsic neuronal property. *Neurochemical research***34**, 1748-1757, doi:10.1007/s11064-009-9957-0 (2009).
45 Lauriat, T. L. & McInnes, L. A. EAAT2 regulation and splicing: relevance to psychiatric and neurological disorders. *Molecular psychiatry***12**, 1065-1078, doi:10.1038/sj.mp.4002065 (2007).
46 Honig, L. S., Chambliss, D. D., Bigio, E. H., Carroll, S. L. & Elliott, J. L. Glutamate transporter EAAT2 splice variants occur not only in ALS, but also in AD and controls. *Neurology***55**, 1082-1088 (2000).
47 Lin, C. L. *et al.* Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. *Neuron***20**, 589-602 (1998).
48 Rao, V. L., Baskaya, M. K., Dogan, A., Rothstein, J. D. & Dempsey, R. J. Traumatic brain injury down-regulates glial glutamate transporter (GLT-1 and GLAST) proteins in rat brain. *Journal of neurochemistry***70**, 2020-2027 (1998).
49 Goodrich, G. S. *et al.* Ceftriaxone Treatment after Traumatic Brain Injury Restores Expression of the Glutamate Transporter, GLT-1, Reduces Regional Gliosis, and Reduces Post-Traumatic Seizures in the Rat. *Journal of neurotrauma***30**, 1434-1441, doi:10.1089/neu.2012.2712 (2013).
50 Zelenaia, O. A. & Robinson, M. B. Degradation of glial glutamate transporter mRNAs is selectively blocked by inhibition of cellular transcription. *Journal of neurochemistry***75**, 2252-2258 (2000).

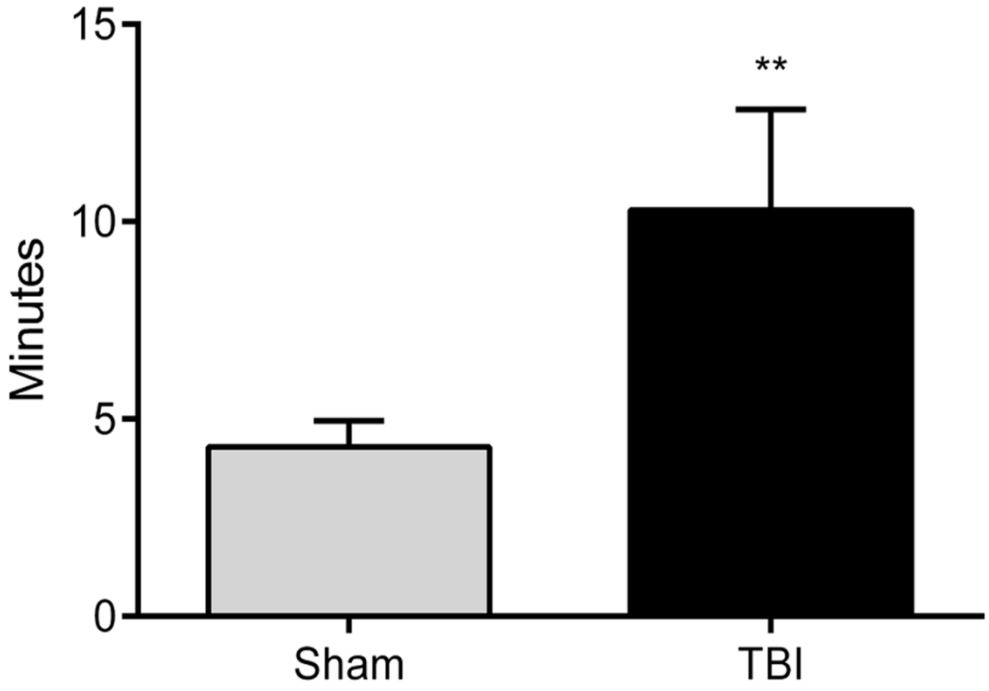

Fig 1. Return of righting reflex differs between sham surgery and TBI rats. Students t-test deomnstrated that a return of righting reflex, a signifier of transient unconsciousness, takes significantly longer in rats experiencing moderate LFPI when compared to sham operated controls. ** $p < 0.01$; $n = 5$ rats per condition. TBI; Traumatic Brain Injury.

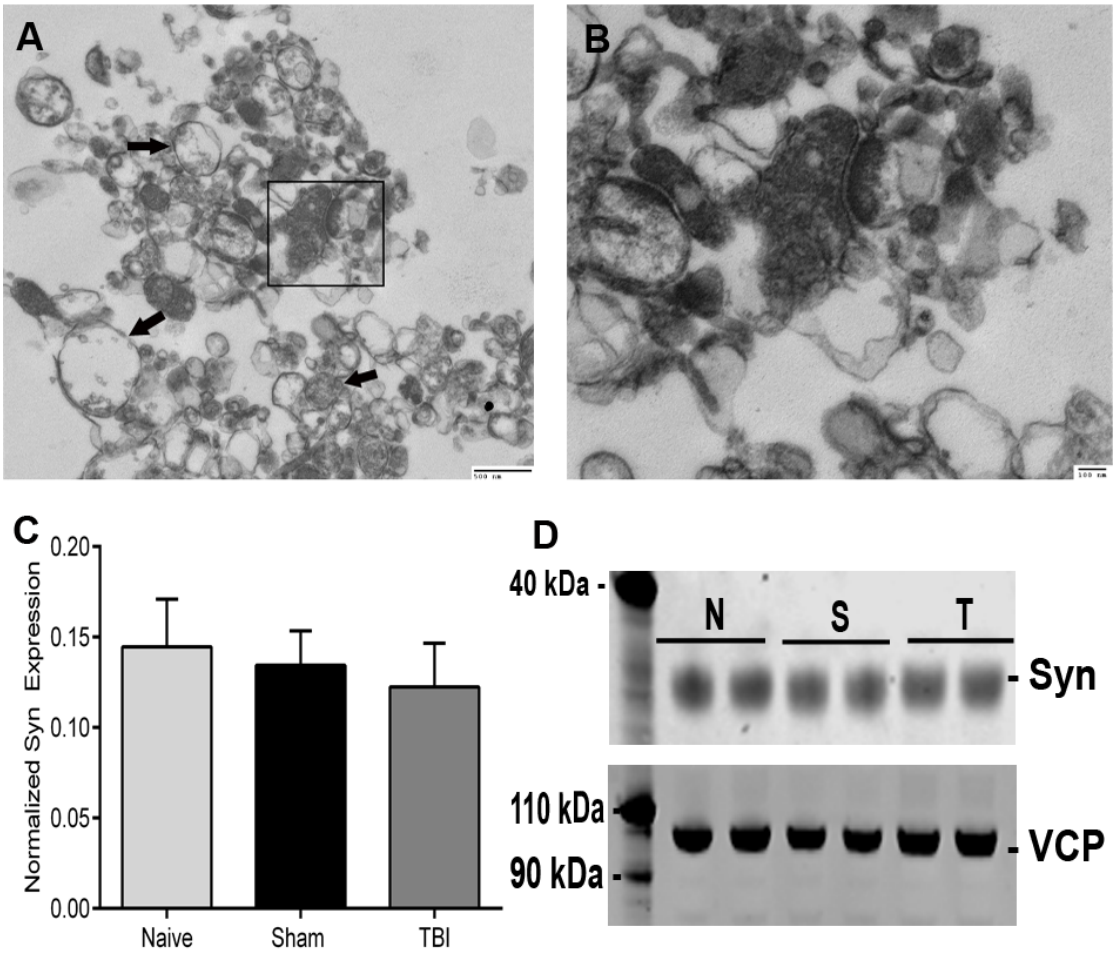

Fig 2. Isolation yields synaptosomes enriched for synaptophysin. (A) 6500x magnificationof synaptic fragments (arrows) and regions of putative pre- and post-synaptic density joining (box) (B) 15000x magnification of boxed area. (C). Bars indicate synaptophysin expression in synaptosomes isolated from 3 sample groups 24 hours after induction of LFPI, sham surgery, or non-operated, non-injured controls. A one-way ANOVA revealed no significant difference between synaptophysin expression across the groups (D). Representative western blots of synaptophysin expression 24 hours after TBI. N, naive; S, sham surgery; T, TBI. Syn, synaptophysin; VCP, valosin-containing protein

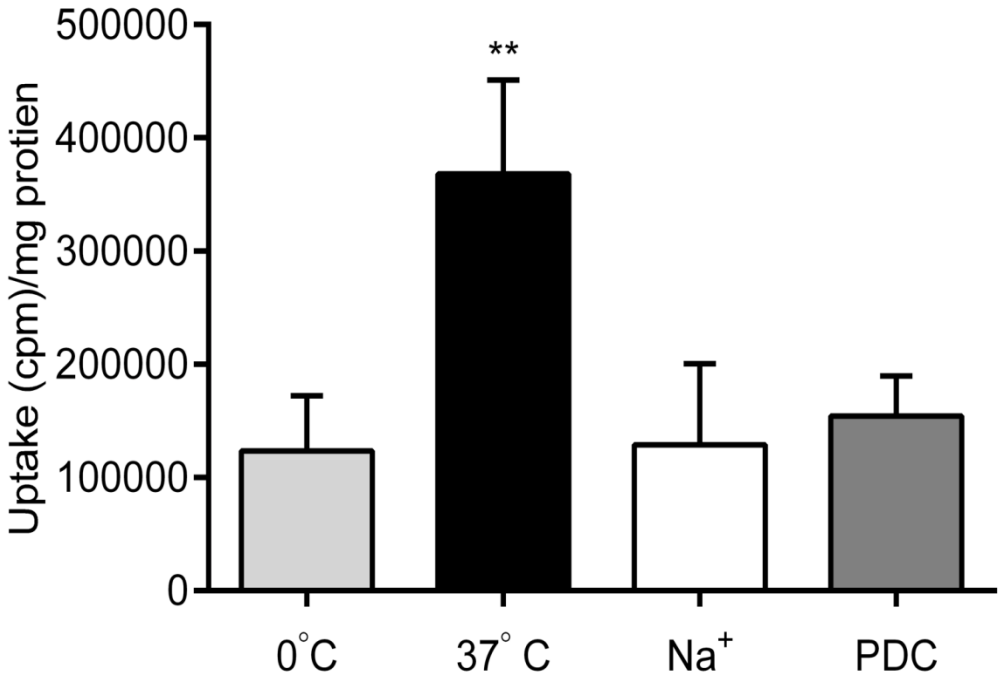

Fig 3. Synaptosomal glutamate uptake is temperature, sodium, and transporter dependent. Synaptosomes isolated from the frontal and parietal cortex of naive rats demonstrate an ability to reuptake radiolabled [H^3]-glutamate at physiological temperatures. Tukey post-hoc tests reveal inhibition of synaptosomal glutamate reuptake is possible by exposing the synaptosomes to freezing temperatures (0°C), bringing them up in a sodium free environment (Na^+), or by the addition of 5M PDC; resulting in decreases in uptake by 66, 65, and 58% respectively. Error bars indicate standard error of the mean. **$p<0.01$. PDC, L-trans-pyrrolidine-2,4-dicarboxylic acid; Na^+, sodium-free environment.

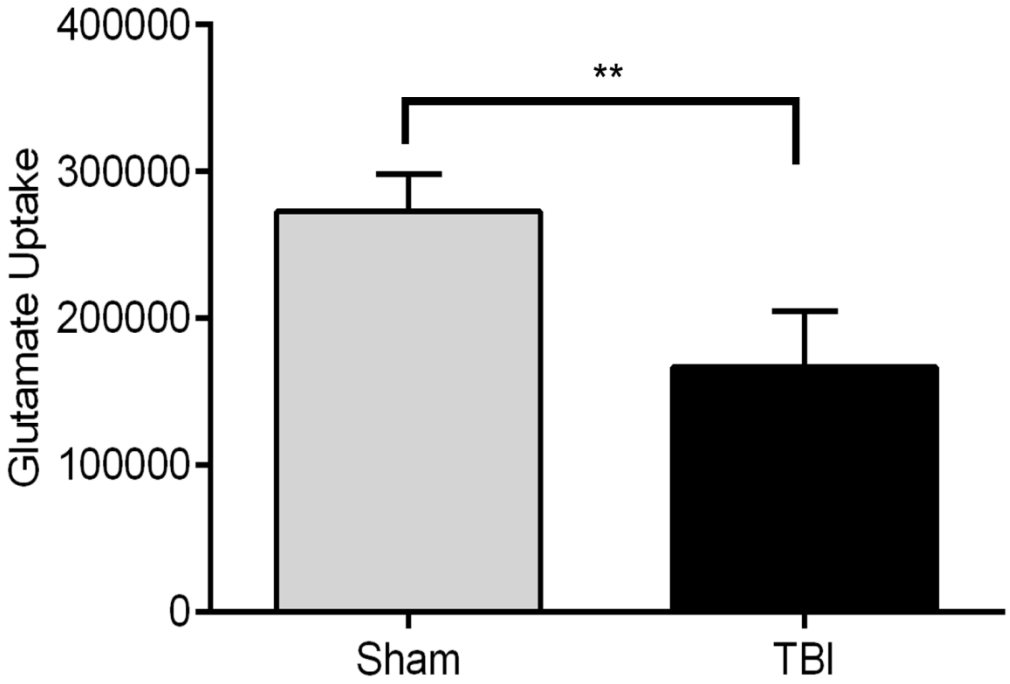

Fig 4. TBI results in decreased glutamate reuptake ability. Student's t-test demonstrated synaptosomes isolated from the injured cortical hemisphere 24 hours following LFPI have decreased reuptake capacity ($t(8) = 3.43$ $p < 0.01$). Synaptosomes from the injured cortex were ability to uptake 39% less radiolabled [H^3]-glutamate than synaptosomes isolated from sham operated controls (n = 5 rats per condition) **$p < 0.01$

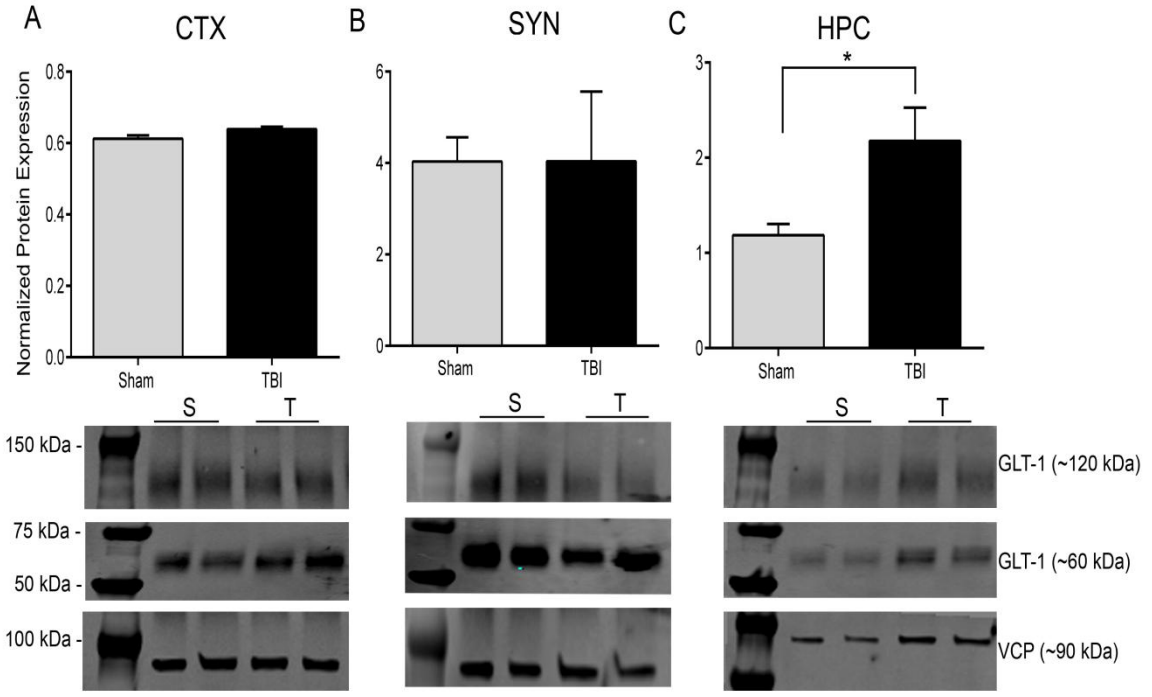

Fig 5. TBI results in differential expression of GLT-1. Bars indicate mean GLT-1 expression 24 hours following induction of LFPI, with representative western blots below. (A) Student's t-test revealed no differenence in cortical GLT-1 expression; (B) likewise, western blots of synaptosomes isolated from cortical homogenate demonstrated no difference in GLT-1 expression. (C) However a student's t-test revealed hippocampal levels of GLT-1 were increased by 180% in TBI rats when compared to sham opperated controls ($t(8) = 2.302$, $p < 0.05$). Error bars indicate standard error of the mean. GLT-1 levels expressed as a combination of kDa ranges as a percent of VCP expression. GLT-1, Glutamate transporter 1; VCP, Valosin-containing protein; S, sham surgery; T, TBI.

SUMMARY AND FUTURE DIRECTION

This project contributes to the field of TBI research in a number of distinct and important ways. It provides an important characterization of the effects of the LFPI model on glutamate transporter expression at a clinically relevant time point. By replicating the findings of Yi and colleagues regarding the increase in hippocampal GLT-1 expression 24 hours following injury, which is not accompanied by a decrease in cortical expression of the transporter,[24] we have helped to underline differences between LFPI and other animal models of injury induction.We anticipate that this information will be used to understand why other models are able to reliably demonstrate decreases in transporter expression when measured in the same regions, injury severity, and time period. Any potential explanation for these discrepancies would highlight the molecular underpinnings of each model and would allow investigators to choose the appropriate model for their study needs.In order to further the information regarding the effects of LFPI on glutamate homeostasis, we nextaddressed whether there was a decrease in transporter function despite adequate levels of transporter expression. Our results agree with previous researchinto intracellular signaling pathways regulating glutamate transporters, which demonstrate that the effects of TBI can lead to deficits in transporter function which did not depend on decreases in expression.

The method for this evaluation also represents a contribution to the field of TBI research. We adapted methods that yieldsynaptic fragments from rat cortex that are able to uptake [H^3]-glutamate in a physiologically relevant capacity and which depend on the presence of functional glutamate transporters. Next, we demonstrated that these synaptosomes are a sensitive marker for TBI induced alterations in glutamate

homeostasis. TBI leads to decreases in the ability of synaptosomal fragments to properly reuptake glutamate following injury. Given that we demonstrated no decreases in transporter expression in either the region the synaptosomes were isolated from, nor in the synaptosomes themselves, this deficit in reuptake capacityappears to be independent of transporter expression. The tools and results developed in this study may prove useful for the evaluation of potential therapeutic drugs. Synaptosomes provide a unique ability to compare the functional aspect of glutamate transporters in a biologically relevant capacity, which may be applied towards assessing the efficacy of different drug treatment strategies.

Current therapeutic techniques aimed at mitigating the effects of TBI on glutamate homeostasis, including recent reports that β-lactam antibiotics may be neuroprotective in vivo,[25] are often aimed at increasing expression of the transporter. As our results have indicated, decreases in transporter function are not always concomitant with decreases in transporter expression. While increases in transporter expression could lead to increased clearance of glutamate from the extracellular space, these conclusions could be strengthened by comparison of synaptosomal uptake ability across treatment groups.Furthermore, synaptosomal glutamate reuptake monitoring could widen the therapeutic drug base, as there could be possible treatment strategies that do not necessarily increase glutamate transporter expression following TBI, yet may still increase their function. Additionally, post-mortem human studies have indicated that in the hours immediately following severe TBI, there are increases in EAAT2 expression. However, it remains to be seen whether this increase in expression imparts any functional benefit towards decreasing the secondary injury pathology. As the synaptosomes isolated

during our experiment function in very similar fashion to those isolated from post-mortem human tissue (unpublished correspondence with Dr. McCullumsmith, 2013), the functionality of post-mortem human glutamate transporters could easily be evaluated by the application of synaptosomal glutamate uptake monitoring techniques.

While human synaptosomal glutamate uptake monitoring following TBI represents one possible future application of theses studies, there are several other areas of immediate interest. Perhaps the most apparent would be the isolation of synaptosomes from the hippocampus of the same injured and control rats in order to evaluate the effects of TBI on the ability of these synaptosomes to properly regulate extracellular glutamate concentrations. As the hippocampus is further removed from the site of injury, and shows increased expression of glutamate transporters following injury, a functional comparison could demonstrate whether this increase in expression is truly compensatory in nature. Another future direction of this study is the application of kinome profiling array technology to injured and control tissue. This technology allows for quantification of the activity level of hundreds of known kinases present in a sample. By correlating those substrates that become phosphorylated and 'working backwards' potential intracellular signaling pathways can be identified and then confirmed. Some of these pathways may underlie the discrepancies between models and brain regions on glutamate transporter expression. Eventually, following pathway confirmation, small molecularly specific, targeted drug designs could be applied in order to mitigate pathological signaling pathways.

As there are differences within models on outcome measures, including glutamate homeostasis, the application of synaptosmal glutamate monitoring and kinome profiling

technology to tissue isolated from other injury types, could allow for the pinpointing of which intracellular signaling pathways control not only glutamate homeostasis, but any number of cellular responses, under the pathological conditions of TBI. For instance, while further characterization and standardization is needed, the blast injury model represents a potentially rewarding area of research into glutamate homeostasis following injury. As the blast injury impact physiology differs from traditional models of head trauma, a comparison between models on regionalexpression and function of glutamate transporters could elucidate which intracellular signaling mechanisms are unique to which model, and perhaps explain the link between blast induced injuries and associated mood disorders like post-traumatic stress disorder.

We anticipate that results from the project outlined herein will serve as a useful beginning that will lead to the comparison of existing, and eventually novel, therapeutic strategies on glutamate homeostasis following TBI.

GENERAL LIST OF REFERENCES

1 Xiong, Y., Mahmood, A. & Chopp, M. Animal models of traumatic brain injury. *Nature reviews. Neuroscience***14**, 128-142, doi:10.1038/nrn3407 (2013).

2 Langlois, J. A., Rutland-Brown, W. & Wald, M. M. The epidemiology and impact of traumatic brain injury: a brief overview. *The Journal of head trauma rehabilitation***21**, 375-378 (2006).

3 Bruns, J., Jr. & Hauser, W. A. The epidemiology of traumatic brain injury: a review. *Epilepsia***44 Suppl 10**, 2-10 (2003).

4 Martin, E. M., Lu, W. C., Helmick, K., French, L. & Warden, D. L. Traumatic brain injuries sustained in the Afghanistan and Iraq wars. *Journal of trauma nursing : the official journal of the Society of Trauma Nurses***15**, 94-99; quiz 100-101, doi:10.1097/01.JTN.0000337149.29549.28 (2008).

5 Adams, J. H., Graham, D. I., Gennarelli, T. A. & Maxwell, W. L. Diffuse axonal injury in non-missile head injury. *Journal of neurology, neurosurgery, and psychiatry***54**, 481-483 (1991).

6 Gennarelli, T. A. *et al.* Influence of the type of intracranial lesion on outcome from severe head injury. *Journal of neurosurgery***56**, 26-32, doi:10.3171/jns.1982.56.1.0026 (1982).

7 Davis, A. E. Mechanisms of traumatic brain injury: biomechanical, structural and cellular considerations. *Critical care nursing quarterly***23**, 1-13 (2000).

8 Gaetz, M. The neurophysiology of brain injury. *Clinical neurophysiology : official journal of the International Federation of Clinical Neurophysiology***115**, 4-18 (2004).

9 Arundine, M. & Tymianski, M. Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. *Cellular and molecular life sciences : CMLS***61**, 657-668, doi:10.1007/s00018-003-3319-x (2004).

10 McIntosh, T. K. Novel pharmacologic therapies in the treatment of experimental traumatic brain injury: a review. *Journal of neurotrauma***10**, 215-261 (1993).

11 Katayama, Y., Becker, D. P., Tamura, T. & Hovda, D. A. Massive increases in extracellular potassium and the indiscriminate release of glutamate following concussive brain injury. *Journal of neurosurgery***73**, 889-900, doi:10.3171/jns.1990.73.6.0889 (1990).

12 Raghupathi, R. Cell death mechanisms following traumatic brain injury. *Brain pathology***14**, 215-222 (2004).

13 van Landeghem, F. K. *et al.* Early expression of glutamate transporter proteins in ramified microglia after controlled cortical impact injury in the rat. *Glia***35**, 167-179 (2001).

14 Boston-Howes, W. *et al.* Caspase-3 cleaves and inactivates the glutamate transporter EAAT2. *The Journal of biological chemistry***281**, 14076-14084, doi:10.1074/jbc.M600653200 (2006).
15 Arundine, M. & Tymianski, M. Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. *Cell calcium***34**, 325-337 (2003).
16 Olney, J. W. Excitotoxicity: an overview. *Canada diseases weekly report = Rapport hebdomadaire des maladies au Canada***16 Suppl 1E**, 47-57; discussion 57-48 (1990).
17 Liu, Z. *et al.* Seizure-induced glutamate release in mature and immature animals: an in vivo microdialysis study. *Neuroreport***8**, 2019-2023 (1997).
18 Faden, A. I., Demediuk, P., Panter, S. S. & Vink, R. The role of excitatory amino acids and NMDA receptors in traumatic brain injury. *Science***244**, 798-800 (1989).
19 Choi, D. W. Ionic dependence of glutamate neurotoxicity. *The Journal of neuroscience : the official journal of the Society for Neuroscience***7**, 369-379 (1987).
20 Shigeri, Y., Seal, R. P. & Shimamoto, K. Molecular pharmacology of glutamate transporters, EAATs and VGLUTs. *Brain research. Brain research reviews***45**, 250-265, doi:10.1016/j.brainresrev.2004.04.004 (2004).
21 Velasco, I., Tapia, R. & Massieu, L. Inhibition of glutamate uptake induces progressive accumulation of extracellular glutamate and neuronal damage in rat cortical cultures. *Journal of neuroscience research***44**, 551-561, doi:10.1002/(SICI)1097-4547(19960615)44:6<551::AID-JNR5>3.0.CO;2-A (1996).
22 Kanai, Y. & Hediger, M. A. The glutamate and neutral amino acid transporter family: physiological and pharmacological implications. *European journal of pharmacology***479**, 237-247 (2003).
23 Rao, V. L., Dogan, A., Bowen, K. K., Todd, K. G. & Dempsey, R. J. Antisense knockdown of the glial glutamate transporter GLT-1 exacerbates hippocampal neuronal damage following traumatic injury to rat brain. *The European journal of neuroscience***13**, 119-128 (2001).
24 Yi, J. H., Pow, D. V. & Hazell, A. S. Early loss of the glutamate transporter splice-variant GLT-1v in rat cerebral cortex following lateral fluid-percussion injury. *Glia***49**, 121-133, doi:10.1002/glia.20099 (2005).
25 Goodrich, G. S. *et al.* Ceftriaxone Treatment after Traumatic Brain Injury Restores Expression of the Glutamate Transporter, GLT-1, Reduces Regional Gliosis, and Reduces Post-Traumatic Seizures in the Rat. *Journal of neurotrauma***30**, 1434-1441, doi:10.1089/neu.2012.2712 (2013).
26 Rothstein, J. D. *et al.* Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron***16**, 675-686 (1996).
27 Yi, J. H., Herrero, R., Chen, G. & Hazell, A. S. Glutamate transporter EAAT4 is increased in hippocampal astrocytes following lateral fluid-percussion injury in the rat. *Brain research***1154**, 200-205, doi:10.1016/j.brainres.2007.04.011 (2007).

28 Fairman, W. A., Vandenberg, R. J., Arriza, J. L., Kavanaugh, M. P. & Amara, S. G. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. *Nature***375**, 599-603, doi:10.1038/375599a0 (1995).
29 Danbolt, N. C. Glutamate uptake. *Progress in neurobiology***65**, 1-105 (2001).
30 Zhang, J., Yang, P. L. & Gray, N. S. Targeting cancer with small molecule kinase inhibitors. *Nature reviews. Cancer***9**, 28-39, doi:10.1038/nrc2559 (2009).
31 Gegelashvili, G., Danbolt, N. C. & Schousboe, A. Neuronal soluble factors differentially regulate the expression of the GLT1 and GLAST glutamate transporters in cultured astroglia. *Journal of neurochemistry***69**, 2612-2615 (1997).
32 Swanson, R. A. *et al.* Neuronal regulation of glutamate transporter subtype expression in astrocytes. *The Journal of neuroscience : the official journal of the Society for Neuroscience***17**, 932-940 (1997).
33 Sitcheran, R., Gupta, P., Fisher, P. B. & Baldwin, A. S. Positive and negative regulation of EAAT2 by NF-kappaB: a role for N-myc in TNFalpha-controlled repression. *The EMBO journal***24**, 510-520, doi:10.1038/sj.emboj.7600555 (2005).
34 Kane, L. P., Shapiro, V. S., Stokoe, D. & Weiss, A. Induction of NF-kappaB by the Akt/PKB kinase. *Current biology : CB***9**, 601-604 (1999).
35 Li, L. B. *et al.* Regulation of astrocytic glutamate transporter expression by Akt: evidence for a selective transcriptional effect on the GLT-1/EAAT2 subtype. *Journal of neurochemistry***97**, 759-771, doi:10.1111/j.1471-4159.2006.03743.x (2006).
36 Gegelashvili, G., Dehnes, Y., Danbolt, N. C. & Schousboe, A. The high-affinity glutamate transporters GLT1, GLAST, and EAAT4 are regulated via different signalling mechanisms. *Neurochemistry international***37**, 163-170 (2000).
37 Abe, K. & Saito, H. Possible linkage between glutamate transporter and mitogen-activated protein kinase cascade in cultured rat cortical astrocytes. *Journal of neurochemistry***76**, 217-223 (2001).
38 Kalandadze, A., Wu, Y. & Robinson, M. B. Protein kinase C activation decreases cell surface expression of the GLT-1 subtype of glutamate transporter. Requirement of a carboxyl-terminal domain and partial dependence on serine 486. *The Journal of biological chemistry***277**, 45741-45750, doi:10.1074/jbc.M203771200 (2002).
39 Gonzalez, M. I. & Robinson, M. B. Protein kinase C-dependent remodeling of glutamate transporter function. *Molecular interventions***4**, 48-58, doi:10.1124/mi.4.1.48 (2004).
40 Susarla, B. T. & Robinson, M. B. Internalization and degradation of the glutamate transporter GLT-1 in response to phorbol ester. *Neurochemistry international***52**, 709-722, doi:10.1016/j.neuint.2007.08.020 (2008).
41 Martinez-Villarreal, J., Garcia Tardon, N., Ibanez, I., Gimenez, C. & Zafra, F. Cell surface turnover of the glutamate transporter GLT-1 is mediated by ubiquitination/deubiquitination. *Glia***60**, 1356-1365, doi:10.1002/glia.22354 (2012).

42 Zelenaia, O. A. & Robinson, M. B. Degradation of glial glutamate transporter mRNAs is selectively blocked by inhibition of cellular transcription. *Journal of neurochemistry***75**, 2252-2258 (2000).
43 Rao, V. L., Baskaya, M. K., Dogan, A., Rothstein, J. D. & Dempsey, R. J. Traumatic brain injury down-regulates glial glutamate transporter (GLT-1 and GLAST) proteins in rat brain. *Journal of neurochemistry***70**, 2020-2027 (1998).
44 Oppenheim, R. W. Cell death during development of the nervous system. *Annual review of neuroscience***14**, 453-501, doi:10.1146/annurev.ne.14.030191.002321 (1991).
45 Thornberry, N. A. The caspase family of cysteine proteases. *British medical bulletin***53**, 478-490 (1997).
46 Ghirnikar, R. S., Lee, Y. L. & Eng, L. F. Inflammation in traumatic brain injury: role of cytokines and chemokines. *Neurochemical research***23**, 329-340 (1998).
47 Hicks, R., Soares, H., Smith, D. & McIntosh, T. Temporal and spatial characterization of neuronal injury following lateral fluid-percussion brain injury in the rat. *Acta neuropathologica***91**, 236-246 (1996).
48 Nicotera, P., Leist, M. & Ferrando-May, E. Apoptosis and necrosis: different execution of the same death. *Biochemical Society symposium***66**, 69-73 (1999).
49 Gwag, B. J. *et al.* Calcium ionophores can induce either apoptosis or necrosis in cultured cortical neurons. *Neuroscience***90**, 1339-1348 (1999).
50 Saatman, K. E., Creed, J. & Raghupathi, R. Calpain as a therapeutic target in traumatic brain injury. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics***7**, 31-42, doi:10.1016/j.nurt.2009.11.002 (2010).
51 Wang, K. K. Calpain and caspase: can you tell the difference? *Trends in neurosciences***23**, 20-26 (2000).
52 Zhao, X. *et al.* Subcellular localization and duration of mu-calpain and m-calpain activity after traumatic brain injury in the rat: a casein zymography study. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism***18**, 161-167, doi:10.1097/00004647-199802000-00006 (1998).
53 Strauss, K. I., Narayan, R. K. & Raghupathi, R. Common patterns of bcl-2 family gene expression in two traumatic brain injury models. *Neurotoxicity research***6**, 333-342 (2004).
54 Clark, R. S. *et al.* Apoptosis-suppressor gene bcl-2 expression after traumatic brain injury in rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience***17**, 9172-9182 (1997).
55 Raghupathi, R. *et al.* BCL-2 overexpression attenuates cortical cell loss after traumatic brain injury in transgenic mice. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism***18**, 1259-1269, doi:10.1097/00004647-199811000-00013 (1998).
56 Zhang, X. *et al.* Increased phosphorylation of protein kinase B and related substrates after traumatic brain injury in humans and rats. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism***26**, 915-926, doi:10.1038/sj.jcbfm.9600238 (2006).

57 Datta, R., Kojima, H., Yoshida, K. & Kufe, D. Caspase-3-mediated cleavage of protein kinase C theta in induction of apoptosis. *The Journal of biological chemistry***272**, 20317-20320 (1997).
58 Noshita, N., Lewen, A., Sugawara, T. & Chan, P. H. Akt phosphorylation and neuronal survival after traumatic brain injury in mice. *Neurobiology of disease***9**, 294-304, doi:10.1006/nbdi.2002.0482 (2002).
59 Xu, A. *et al.* Phosphorylation of nuclear phospholipase C beta1 by extracellular signal-regulated kinase mediates the mitogenic action of insulin-like growth factor I. *Molecular and cellular biology***21**, 2981-2990, doi:10.1128/MCB.21.9.2981-2990.2001 (2001).
60 Wen, X. R. *et al.* Dual inhibitory roles of geldanamycin on the c-Jun NH2-terminal kinase 3 signal pathway through suppressing the expression of mixed-lineage kinase 3 and attenuating the activation of apoptosis signal-regulating kinase 1 via facilitating the activation of Akt in ischemic brain injury. *Neuroscience***156**, 483-497, doi:10.1016/j.neuroscience.2008.08.006 (2008).
61 Napieralski, J. A., Raghupathi, R. & McIntosh, T. K. The tumor-suppressor gene, p53, is induced in injured brain regions following experimental traumatic brain injury. *Brain research. Molecular brain research***71**, 78-86 (1999).
62 Hallam, T. M. *et al.* Comparison of behavioral deficits and acute neuronal degeneration in rat lateral fluid percussion and weight-drop brain injury models. *Journal of neurotrauma***21**, 521-539, doi:10.1089/089771504774129865 (2004).
63 Thompson, H. J. *et al.* Lateral fluid percussion brain injury: a 15-year review and evaluation. *Journal of neurotrauma***22**, 42-75, doi:10.1089/neu.2005.22.42 (2005).
64 Graham, S. H., Chen, J. & Clark, R. S. Bcl-2 family gene products in cerebral ischemia and traumatic brain injury. *Journal of neurotrauma***17**, 831-841 (2000).
65 McIntosh, T. K. *et al.* Traumatic brain injury in the rat: characterization of a lateral fluid-percussion model. *Neuroscience***28**, 233-244 (1989).
66 Cernak, I. Animal models of head trauma. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics***2**, 410-422, doi:10.1602/neurorx.2.3.410 (2005).
67 Hall, E. D. *et al.* Spatial and temporal characteristics of neurodegeneration after controlled cortical impact in mice: more than a focal brain injury. *Journal of neurotrauma***22**, 252-265, doi:10.1089/neu.2005.22.252 (2005).
68 Goodman, J. C., Cherian, L., Bryan, R. M., Jr. & Robertson, C. S. Lateral cortical impact injury in rats: pathologic effects of varying cortical compression and impact velocity. *Journal of neurotrauma***11**, 587-597 (1994).
69 Marmarou, A. A review of progress in understanding the pathophysiology and treatment of brain edema. *Neurosurgical focus***22**, E1 (2007).
70 Hinzman, J. M. *et al.* Diffuse brain injury elevates tonic glutamate levels and potassium-evoked glutamate release in discrete brain regions at two days post-injury: an enzyme-based microelectrode array study. *Journal of neurotrauma***27**, 889-899, doi:10.1089/neu.2009.1238 (2010).
71 Matsushita, Y., Shima, K., Nawashiro, H. & Wada, K. Real-time monitoring of glutamate following fluid percussion brain injury with hypoxia in the rat. *Journal of neurotrauma***17**, 143-153 (2000).

APPENDIX

IACUC FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: January 24, 2014

TO: CANDACE L. FLOYD, Ph.D.
SRC -547
(205) 996-6892

FROM:

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Abnormalities of Glutamate Neurotransmission Following Traumatic Brain Injury
Sponsor: Internal
Animal Project_Number: 140110040

As of January 24, 2014 the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and number of animals:

Species	Use Category	Number In Category
Rats	B	99

Animal use must be renewed by January 23, 2015. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 140110040 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee (IACUC)
CH19 Suite 403
933 19th Street South
(205) 934-7692
FAX (205) 934-1188

Mailing Address:
CH19 Suite 403
1530 3rd Ave S
Birmingham, AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

Notice of Approval for Protocol Modification

DATE: September 5, 2013

TO: CANDACE L. FLOYD, Ph.D.
SRC -547
(205) 996-6892

FROM:

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Opioid Abuse After Traumatic Brain Injury (TBI)
Sponsor: Department of Defense
Animal Project_Number: 130609429

On September 5, 2013, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the modification as described: Personnel: Christopher Dorsett The sponsor for this project may require notification of modification(s) approved by the IACUC but not included in the original grant proposal/experimental plan; please inform the sponsor if necessary.

The following species and numbers of animals reflect this modification.

Species	Use Category	Number In Category
Rats	A	Zero - Procedural modification only

The IACUC is required to conduct continuing review of approved studies. This study is scheduled for annual review on of before June 6, 2014. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files.

Refer to Animal Protocol Number (APN) 130609429 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

Notice of Approval for Protocol Modification

DATE: September 5, 2013

TO: CANDACE L. FLOYD, Ph.D.
SRC -547
(205) 996-6892

FROM:

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: A Novel Therapeutic for CNS Injury: Metalloporphyrin Catalytic SOD Mimetic
Sponsor: Internal
Animal Project_Number: 121109500

On September 5, 2013, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the modification as described: Personnel: Christopher Dorsett The sponsor for this project may require notification of modification(s) approved by the IACUC but not included in the original grant proposal/experimental plan; please inform the sponsor if necessary.

The following species and numbers of animals reflect this modification.

Species	Use Category	Number In Category
Rats	B	Zero - Procedural modification only

The IACUC is required to conduct continuing review of approved studies. This study is scheduled for annual review on of before October 17, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files.

Refer to Animal Protocol Number (APN) 121109500 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

Notice of Approval for Protocol Modification

DATE: September 5, 2013

TO: CANDACE L. FLOYD, Ph.D.
SRC -547
(205) 996-6892

FROM:

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Sexual Differences in Recovery After Spinal Cord Injury in a Porcine Model
Sponsor: Internal
Animal Project_Number: 130709922

On September 5, 2013, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the modification as described: Personnel: Christopher Dorsett The sponsor for this project may require notification of modification(s) approved by the IACUC but not included in the original grant proposal/experimental plan; please inform the sponsor if necessary.

The following species and numbers of animals reflect this modification.

Species	Use Category	Number In Category
Pigs	B	Zero - Procedural modification only

The IACUC is required to conduct continuing review of approved studies. This study is scheduled for annual review on of before July 25, 2014. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files.

Refer to Animal Protocol Number (APN) 130709922 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

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