THE ROLE OF MEMBRANE TRANSPORTERS IN TRAUMATIC BRAIN INJURY: INTERVENTIONAL AND GENETIC INVESTIGATIONS

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Traumatic brain injury (TBI) is a leading cause of death and disability in children and young adults in the US. The neurovascular unit conceptual frame work emphasizes the dynamic interplay between neurons, endothelial cells and glial cells in understanding the pathophysiology of TBI. Membrane transporters, as mediators of the movement of numerous endogenous and exogenous molecules within the neurovascular unit, are critical components of the functional neurovascular unit in TBI. The aim of this thesis is to understand the role of membrane transporters in the pathogenesis and pharmacotherapy of TBI through interventional and genetic approaches.

In the first part of the thesis, we investigated the utility of inhibition of transporters with probenecid, as a therapeutic strategy to simultaneously increase the systemic and brain concentration of the anti-oxidant drug, n-acetylcysteine, and the endogenous anti-oxidant, glutathione, in TBI. This approach counters oxidative stress, a major injury mechanism in TBI. Preclinical pharmacokinetic study showed that probenecid increases plasma and brain levels of n-acetylcysteine by inhibiting OAT1 and OAT3 transporters. In rat model of pediatric TBI, n-acetylcysteine showed potential in attenuating TBI-induced learning and memory deficits.

Probenecid caused transient motor function impairment. A combination of the two resulted in smaller cortical tissue volume loss. Optimization of dosage regimen for both drugs to enhance the effects of n-acetylcysteine and minimize the side effects of probenecid is warranted. Metabolomic and pathway analyses of cerebrospinal fluid of TBI patients treated with placebo or a combination of probenecid and n-acetylcysteine showed that the combination therapy enriched glutathione mediated anti-oxidative stress pathways.

In the second part of this thesis, we examined the association of genetic alterations in monocarboxylate transporters – responsible for shuttling lactate within the neurovascular unit – with clinical outcomes. In discovery and replication cohorts, patients with one or two alternate alleles at SLC16A7 rs10506399 showed favorable outcomes. The alternate allele at the SNP was associated with increased expression of SLC16A7 which suggests that increased uptake of lactate by neurons may be beneficial in TBI.

Collectively, this work has provided interventional and genetic evidence that transporters are important component of the injury mechanism and attractive therapeutic targets in TBI.

Keywords: Traumatic Brain Injury, Membrane Transporters, Oxidative Stress, Lactate, Metabolomics, N-acetylcysteine, Probenecid, Monocarboxylates, Neurovascular Unit.

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PREFACE

I am dedicating this dissertation to my sons, Noah and Caleb, who give meaning to every endeavor, and to my wife, Haikel, whose unwavering love, dedication, encouragement and sacrifice made this accomplishment possible.

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[Great are the works of the Lord, studied by all who delight in them. Psalm 111:2]

ABBREVIATIONS

ABC	ATP-Binding Cassette
AD	Alzheimer's Disease
ANLS	Astrocyte-Neuron Lactate Shuttle
CCI	Controlled Cortical Impact
CNS	Central Nervous System
CSF	Cerebro-Spinal Fluid
ENT	Equilibrative Nucleoside Transporter
FDR	False Discovery Rate
FPI	Fluid Percussion Injury
GCS	Glasgow Coma Scale
GOS	Glasgow Outcome Scale
GSH	Glutathione
ICU	Intensive Care Unit
MAF	Minor Allele Frequency
MCT	Monocarboxylate Transporter
MRP	Multidrug Resistance-Associated Protein
NAC	N-Acetylcysteine
OAT	Organic Anion Transporter
OATP	Organic-Anion-Transporting Polypeptide
PCA	Principal Component Analysis
PB	Probenecid
QTOFMS	Quadrupole Time-Of-Flight Mass Spectrometry
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
SLC	Solute Carrier
SNP	Single Nucleotide Polymorphism
TBI	Traumatic Brain Injury
UPLC	Ultra-Performance Liquid Chromatography
WT	Wild Type

1. Introduction

1.1. Review of the Role of Membrane Transporters in TBI

1.1.1. Traumatic Brain Injury – Background

Traumatic brain injury (TBI) is a major public health problem in the US with annual incidence of approximately 1.7 million, of which 50,000 injuries result in death [1], [2]. It is a leading cause of death and injury in children and older adolescents. It is estimated that about 2% of the US population has motor, cognitive and/or psychosocial impairments caused by a TBI [3, 4] and TBI has been associated with increased risk of neurodegenerative diseases such as Alzheimer's disease later in life [5]. Although several pharmacological agents have been studied in TBI clinical trials, none of them have shown positive results in terms of survival or relevant clinical outcomes [6] [7]; [8]). Nevertheless, significant progress has been made in understanding and describing the numerous cellular and molecular processes involved in the pathogenesis of TBI. This includes the potentially reversible and/or preventable secondary injury mechanisms such as excitotoxicity, oxidative stress, and inflammation that follow the irreversible primary injury phase of TBI which consists of shearing and tearing of the brain tissue [9].

1.1.1.1. The neurovascular unit, TBI and membrane transporters

Recently, there has been a growing interest in considering the neurovascular unit – originally developed as a framework for stroke – in TBI to expand on the current understanding of pathological mechanisms brain injuries [10]. This framework, which is hoped to accelerate the discovery of new therapeutic drugs, emphasizes the dynamic interaction among the different cells that constitute the neurovascular unit, i.e., neurons, endothelial cells and glial cells. A central component of this paradigm is the communication between the different cells (and compartments that house the cells) that is mediated by proteins, lipids, hormones, amino acids, and neurotransmitters [10]. Since many of these signaling molecules are polar in physiologic conditions, they require transporters – a wide variety of transmembrane proteins which regulate the trafficking of molecules across the lipid bilayer of cellular membranes – to enter intracellular environment or cross compartmental barriers [11-16]. This makes membrane transporters important contributors to the dynamic interplay within the neurovascular unit to regain homeostasis after TBI. Furthermore, lack of understanding of the nature and extent of the distribution of drugs into the brain and within the neurovascular unit has been emphasized as an unaddressed area of TBI research and has been consistently linked with the failure of clinical trials of drugs in TBI [17]. Transporters regulate the distribution of drugs into and within the brain, and could potentially determine the outcome of therapeutic intervention in TBI.

Transporters within the neurovascular unit are important targets for devising new therapies, optimizing existing therapies and helping understand the toxicities of certain drugs. For example, inducing the expression and/or activity of transporters that are involved in the clearance of

certain injury mediators can attenuate brain injury as attempted with inducers of glutamate transporters [18]. Given the well-documented role of transporters in restricting the distribution of drugs, modulating the relevant transporters could enhance the permeation of drugs to the brain to clinically relevant levels [12]. Reversibly, understanding how drugs commonly used in the management of acute brain injuries affect the function of transporters could determine how and to what extent those drugs should be utilized clinically [13, 19]. In a similar way as drug-drug interactions, transporters mediated drug-metabolite interactions, and drug-nutrient interactions, could provide new targets for intervention and/or optimize the way we use existing therapies [13].

In this chapter role of membrane transporters as regulators of the movement of injury mediators, signaling molecules and therapeutic drugs between cells and across biological barriers in the context of TBI will be discussed. Also, therapeutic strategies in TBI that target membrane transporters will be reviewed.

1.1.2. Membrane Transporters in CNS – Background

Membrane transporters are divided into two major superfamilies: (1) the adenosine triphosphate-binding cassette (ABC) transporters which utilize the energy in the form of adenosine triphosphate (ATP) to traffic various substrates across membranes, and (2) the solute carrier (SLC) transporters which rely on electrochemical gradient (passive gradient or gradient created

by secondary-active transporters) or ionic gradients to facilitate translocation of substrates across barriers [20, 21]. Because of regulatory requirements and the critical role transporters play in determining the pharmacokinetics of drug molecules and its implication in the efficacy and safety of drugs, this aspect of transporters has been a subject of intense research [13, 22]). The endogenous role of transporters in mediating physiological and pathological processes has remained relatively understudied. However, a growing body of evidence is revealing the importance of transporters in regulating the movement of endogenous molecules including metabolites, nutrients, signaling molecules, and biosynthesis and degradation by-products between cells, tissues and body fluid compartments [23] [24, 25] [21] [13, 26]. Transporters have been described as forming a "remote signaling and sensing system" to regulate the communication between organs, tissues and cells [11] [19, 26] [15]. This role makes transporters significant players not only in maintaining homeostasis but also in the initiation, progression, and resolution of pathological processes. For example, it is estimated that one in four of the SLC transporters is implicated in Mendelian diseases [27]. Numerous studies including several genome wide association studies have demonstrated the association of genetic variants in ABC and SLC transporters with complex human diseases including diabetes, neurodegenerative diseases, liver diseases, cardiovascular diseases and cancer [23] [20, 28] [27] [29] [30, 31] [32]. There are also several examples of FDA-approved drugs and drugs in development that target transporters directly or indirectly [23]. Interactions with transporters has also been described as the mechanism behind the toxicity of some commonly used drugs such as statins. In some cases, transporter-mediated toxicity has led to the discontinuation of clinical development of drugs [33] Zhang [34].

The CNS is very sensitive to fluctuations in the levels of nutrients, neurotransmitters, and toxins. Hence, the interaction of the CNS with blood and other peripheral fluids is tightly regulated by highly dynamic barriers, namely, the blood-brain barrier (BBB), the blood-cerebrospinal fluid barrier (Blood-CSF barrier) and the arachnoid epithelium [35] [36]. Similar barriers also exist within the CNS including the ependymal barrier as well as the cellular membrane barriers of the neurons and glia [37] [12] [38]. In addition to the physical and cellular structures, the prominent feature of all these barriers are numerous efflux and uptake transporters regulating the movement of mediators of physiologic and pathologic processes as well as drugs within the CNS, and between the CNS and the periphery. The significance of transporters on these barriers in health, disease, and pharmacotherapy of the CNS is very well established, years of study have been dedicated to understanding and characterizing the expression, distribution, localization, regulation and nature of substrates of transporters in the CNS. These have been reviewed extensively [12, 14, 24, 30, 39-43].

A number of recent publications have also reviewed the role of transporters in health and disease including in chronic neurodegenerative diseases such as epilepsy, Alzheimer's Disease (AD), Amyotrophic lateral sclerosis (ALS), Parkinson's Disease (PD), stroke and focal ischemia injury, brain tumors and multiple sclerosis (MS), [44] [45] [30]. The role of membrane transporters in TBI, on the other hand, has not been explored.

1.1.3. Membrane Transporters in TBI

Despite the general lack of systematic study of membrane transporters in TBI or other acute brain injuries, there are several instances where transporters were investigated as central components of an important mechanism or pathway in TBI, which will be reviewed below.

1.1.4. SLC Transporters in TBI

Perhaps the most studied class of SLC transports in TBI are a group of glutamate transporters and monocarboxylates transporters. Glutamate transporters will be discussed here and MCT transporters will be discussed separately in section 1.1.4.2. Additional SLC transporters such as members of the OAT and OATP family of transporters that has not been studied in TBI yet, but has been investigated in another type of brain injuries that share a similar mechanism with TBI will also be discussed.

1.1.4.1. Glutamate Transporters

Glutamate transporters are responsible for the trafficking of glutamate, the most predominant excitatory neurotransmitter in the mammalian brain, from the extracellular space into cells. Although essential to basic brain functions such as cognition, memory, and learning [46, 47], when present in excessive amount glutamate can be become neurotoxic due to overstimulation of

post-synaptic neurons [48]. Because of this, glutamate levels are tightly regulated through close coordination between its release and clearance, which depends on glutamate transporters [49].

In TBI, it has been shown, both in preclinical models and in humans, that there is a massive release of glutamate into the synapses immediately after injury [50, 51]. This increase in extracellular glutamate causes excessive stimulation of N-methyl-D-aspartate (NMDA) glutamate receptor and triggers several cellular processes such as Ca^{++} and Na^{+} overload, activation of phospholipases, endonucleases and proteases, and generation of reactive oxidative species that eventually lead to neuronal injury and death [52-54]. This glutamate-mediated excitotoxicity is a well-established injury mechanism in TBI that can lead to neurodegeneration and negative functional outcomes. Owing to their prominence in modulating of extracellular concentration of glutamate, glutamate transporters have been a subject of significant investigation in TBI [18].

In the mammalian brain, glutamate transporters comprise of five, sodium-dependent and high-affinity, subtypes [55]: Excitatory amino acid transporter (EAAT)-1 (also called glutamate-aspartate transporter or GLAST), EAAT2 (also called GLT-1), EAAT3/EAAC1, EAAT4, and EAAT5. EAAT2, expressed throughout the brain and spinal cord, is responsible for greater than 90% of total glutamate uptake which makes it the most important glutamate transporters [56, 57]. At the cellular level, EAAT2 is expressed mainly in pre-synaptic processes of astrocytes and to a lower extent, in neurons and oligodendrocytes [58, 59]. EAAT1's expression is limited to

astrocytes with the highest localization in the cerebellum [56, 57, 60]. It has also been detected in the spinal cord, cortex and hippocampus. EAAT3 is the neuronal glutamate transporters, specifically expressed in neuronal cell bodies and dendrites [61]. It is detected through the brain including cortex, hippocampus, cerebellum and basal ganglia [62, 63]. Recently, Akanuma *et al.* have reported that EAAT1 and EAAT3 were expressed in the ependymal and choroid plexus epithelial cells respectively [64]. They both face the CSF and appear to be involved in the elimination of glutamate from the CSF into the blood vessels. EAAT4 is mainly expressed in the soma and dendrites of cerebellar Purkinje cells with limited expression in hippocampus and neocortex. EAAT5 is expressed in the rod photoreceptor and bipolar cells in the retina [65]. Other sub-family of solute carriers that transport glutamate include SLC17A6, SLC17A7 and SLC17A8 which encode the vesicular glutamate transporters (vGLUTs), vGLUT2, vGLUT1 and vGLUT3 respectively [66]. These transporters are technically not a type of membrane transporters but are involved in trafficking glutamate intracellularly and they are known to be expressed only in neurons [67].

With a few exceptions, studies examining the role of glutamate transporters in TBI are mainly focused on EAAT2. In a controlled cortical impact, rat injury model of TBI, Rao *et al.*, found that the protein expression of GLT-1 and GLAST was significantly decreased 6-72 h after injury and was accompanied by reduced D-[3H]Aspartate binding activity [68]. Another study found that, compared to sham-injured rats, in fluid percussion injury rat model of TBI, the Vmax of GLT-1 in the cortex and hippocampus was significantly reduced as early as 5 minutes and up to 2 hours post injury [69]. Rao *et al.*, in CCI TBI model in rats, demonstrated that the RNA and

protein expression of GLT1 was decreased 24-72 hours in the ipsilateral hippocampus as compared to the contralateral hippocampus or sham-injured rats. Additionally, administration of antisense oligodeoxynucleotides specific for GLT-1 resulted in exacerbated hippocampal neuronal death and increased mortality in the rat CCI model as compared to control rats infused with random oligodeoxynucleotides [70]. Landeghem *et al.*, reported that in the CCI rat model of TBI, both GLAST and GLT-1 were down-regulated after injury with lowest expressions 24 to 72 h post injury [71]. This was accompanied by increase glutamate levels in CSF that reached a maximum value at 48 h post injury. In a rat fluid percussion injury (FPI) model of TBI, Yi *et al.*, found that GLT1v, but not GLT1a, GLAST or EAAC-1 were acutely downregulate following injury [72].

Two studies in human have examined the role of glutamate transporters in TBI using genomic approaches. Ritter *et al.* found that genetic variations in SLC1A1, but not SLC1A6 were associated with reduced time to first seizure and increased seizure risk up to 3-years post injury in human TBI patients [73]. Specifically, rs10974620 in SLC1A1 was associated with increased posttraumatic seizures risk up to 3 years post injury, and rs7858819 associated with increased early and later posttraumatic seizures risk from day 2 to 3 years post-injury. In the second human study, Madura *et al.* found that genetic polymorphism at rs7417284 in the promoter region of the SLC17A7 gene was associated with severity and duration of sport-related concussion [74]. Specifically, those carrying the minor allele were 6.33-times more likely to experience prolonged recovery rates.

Because the critical role of glutamate membrane transporters in countering the effect of the excessive release of glutamate, glutamate transporters, especially EAAT2, have been investigated as a target for therapeutic intervention. This approach has also gained traction as a preferred approach since antagonists to glutamate receptors have shown unacceptable toxicity [18, 75].

Approaches targeting glutamate transporters can be divided into those that intend to increase the expression of EAAT2 and those that increase the catalytic activity; the eventual purpose in both cases is to increase the clearance of glutamate from the extracellular synaptic environment. An extensive review of the molecules targeting EAAT2 for therapeutic purpose for numerous neurological conditions such as TBI, hypoxic-ischemic brain injury, spinal cord injury, ALS, Alzheimer's Disease, neuropathic pain has previously been reviewed [18]. Here those molecules studied in TBI preclinical models will be summarized.

Ceftriaxone, a β-lactam antimicrobial, is a transcriptional inducer of EAAT2 expression and has been investigated preclinically in diverse animal models of glutamate excitotoxity and clinically in ALS and stroke patients [76-79]. In the rat lateral FPI TBI model, Goodrich *et al.* found that ceftriaxone prevented the injury-related down expression of GLT1 that was observed in the placebo-treated group [80]. Ceftriaxone also mitigates the injury-associated regional astrogliosis and reduced cumulative post-traumatic seizure duration. Two studies conducted in the lateral CCI model of TBI in rats reported that ceftriaxone reduced glutamate levels in the brain,

attenuated cerebral edema and neuronal death, and improved cognitive function (Wei J. *et al.*, 2012 and Cui C. *et al.*, 2014).

Pituitary adenylate cyclase-activating polypeptide (PACAP), an endogenous peptide with neuromodulatory activity, has demonstrated GLT1 expression enhancing properties. In a weight-drop model of TBI in rats, exogenously administered PACAP significantly improved motor and cognitive dysfunction, attenuated neuronal apoptosis, and decreased brain edema [81]. Additionally, in a rat model of diffuse axonal injury induced by impact acceleration, PACAP was reported to reduce the density of damaged, beta-amyloid precursor protein-immunoreactive axons in the corticospinal tract. However, it worth mentioning that the beneficial effects of PACAP in TBI may be, at least in part, mediated by other mechanisms [82].

There are two molecules with GLT1 activity modulating capacity that have been studied in TBI: riluzole and MS-153. In a FPI model of TBI in rats, riluzole was shown to improve motor [83], and behavioral functions [84]. Similarly, in a FPI model of TBI in rats, MS-153 was shown to decrease neurodegeneration, loss of microtubule-associated protein 2 and NeuN (+) immunoreactivities, and attenuate calpain activation in both the cortex and the hippocampus at 24 h after the injury [69]. As with PACAP, the beneficial effects of both riluzole and MS-153 are at least in part attributed to mechanisms other than GLT1 activity such as anti-inflammatory activities and inhibition of voltage-gated Ca channels respectively.

In summary, the functions of glutamate transporters in clearing potentially toxic glutamate accumulation away from neurons into astrocytes and across the ependymal barrier into the CSF and blood demonstrate the critical role membrane transporters play in coordinating the functional relationship among different cells within the neurovascular unit. The importance of this function is further highlighted by the negative consequence of reduced function of these transporters in TBI. The efforts to bolster the capacity of glutamate transporters in TBI as a therapeutic strategy has been explored with some success in preclinical TBI models. Translation of these therapeutic agents into the clinic has not been successful yet as none have been advanced to studies in TBI patients. Nevertheless, two recent studies have reported the discovery of potent activators of EAAT2 raising the possibility of targeting EAAT2 effectively in TBI and other neurological disorders [85, 86].

1.1.4.2. MCT Transporters

The human SLC16 gene family of transporters, also known as the monocarboxylate (MCT) family, comprises of 14 members identified based on sequence homology. Only the first four – MCT1 (SLC16A1), MCT2 (SLC16A7), MCT3 (SLC16A8) and MCT4 (SLC16A3) – are demonstrated to transport monocarboxylates including L-lactate, pyruvate, and ketone bodies [87, 88]. The transport of monocarboxylates follows a symport mechanism involving an exchange with a proton with an equimolar stoichiometry. The only other two de-orphaned members of the family are SLC16A2 which encodes for the thyroid hormone transporter MCT8

[89] and SLC16A10 encoding MCT10 which transporters aromatic amino acids [90]. In this dissertation, only MC1, 2 and 4 will be discussed, based on currently available evidence of the involvement of transporter and their substrates in acute brain injury.

Expression of MCT transporters: The study of distribution pattern of MCT transporters through the brain has been complicated by cross-reactivity of antibodies, the gap in RNA-protein correlation and interspecies differences. However, a large body of evidence, mainly from rodents, has established the distinctive expression of the different MCT isoforms in the cells and regions of the brain [91, 92]. MCT1 is expressed throughout the brain and specifically on endothelial cells, astrocytes and oligodendrocytes [91]. MCT4 exhibits widespread brain expression in the brain but it shows the strongest expression in the cortex, hippocampus, striatum, and cerebellum. At the cellular level, MCT4 expression is restricted to astrocytes. Similarly, MCT2 is expressed throughout the brain with the strongest localization in the cortex, hippocampus, and cerebellum. MCT2 is the main isotype expressed in neurons with some studies suggesting its level of expression is associated with synapses[92]. MCT3 is expressed in the retinal pigment epithelium and the choroid plexus [87].

MCT Transporters Kinetics: Like their expression pattern, MCT1-4 show distinctive kinetic properties and substrate specificities which, together with the expression pattern, are highly indicative of the specific role each isotype plays within a particular tissue [91, 92]. MCT2 shows the highest affinity for both lactate and pyruvate with Km value of about 0.7mM and 0.08mM

respectively. This explains, in part, the expression of MCT2 in neuronal cells with its specific role being to facilitate uptake of monocarboxylate for consumption in these oxidative cells. MCT4, on the other hand, has the lowest affinity with Km of about 35mM for lactate and an even greater Km of 150mM for pyruvate. In agreement with this property, MCT4 is expressed in astrocytes (and white muscle cells) where its role is to traffic lactate (but not pyruvate) from the glycolytic cells to the neurons. The affinity of MCT1 for lactate and pyruvate is 3.5mM and 1mM respectively which reflects its ubiquitous expression and its function as the routine transporter.

Monocarboxylate as an energy source for the brain: Monocarboxylates including pyruvate, lactate and ketone bodies are important intermediaries in brain energy metabolism processes. In the developing brain, monocarboxylates contribute substantially to the energy needs of the developing brain. For example, during the preweaning period, acetoacetate and 3-hydroxybutyrate, two ketones derived from fatty acids in maternal milk, account up to 30% of the total cerebral energy consumption [93]. Monocarboxylates also play a key role in maintaining homeostasis when the energy requirement of the brain changes. Under normal physiological conditions, the brain uses glucose as its main energy source through glycolysis where glucose is metabolized to pyruvate [94]. In aerobic conditions, the pyruvate from glycolysis is oxidized and enters the TCA cycle eventually leading to the production of ATP in the mitochondria. During extreme but normal physiological conditions such as during extended starvation, intense exercise or during periods of high neuronal activity such as long-term memory formation, the brain, specifically neurons can utilize lactate and other monocarboxylates as

energy source [91, 95-97]. In such situations, lactate could be derived from peripheral tissues, mainly the liver or from the brain itself, specifically from the glycolytic astrocytes. The lactate specific mechanism in the brain is known as astrocyte-neuron lactate shuttle (ANLS). The ANLS involves a sequence of events where synaptically released glutamate initiates neuronal activity followed by increased rate of glucose uptake and glycolysis by astrocytes and subsequent release of lactate that can be used as energy substrate by neurons via oxidative-derived ATP production [98, 99]. Although controversies regarding the specific source of lactate and the magnitude of its significance exist, substantial evidence by numerous investigators support the main features of the model and the ANLS remains an important paradigm in understanding the brain neuroenergetics and role of monocarboxylates [94, 100, 101].

As in extreme physiological conditions, monocarboxylates assume a critical role in pathological conditions as such cerebral ischemia and acute brain injuries [101, 102]. Brain energy utilization dysfunction is one of the hallmarks of acute brain injuries and is associated with poor prognosis [103, 104]. Monocarboxylates, and lactate in particular counter the consequences of the imbalance between increased energy demand and inadequate supply due to ischemia – a phenomenon known as metabolic decoupling – which is a distinctive characteristics of acute brain injuries [105-110]. Like in ANLS, there is unresolved controversy regarding the therapeutic efficacy of exogenously administered lactic acid in acute brain injuries in general and in TBI in particular. In rodent models of TBI and stroke, exogenous administration of lactate was demonstrated to improve histological and cognitive outcomes [111, 112]. However, Lama *et al.*, using a modified weight drop model of severe TBI and magnetic resonance spectroscopy,

showed excessive build-up of lactic acid in injured brain tissue with potentially detrimental effects [113]. Two studies by Glenn *et al.*, using isotope tracers of glucose and lactate, and sampling blood from the brain through arterial and jugular bulb catheters, provided evidence that large amounts of lactate are transferred from systemic circulation to the brain and the systemic derived lactate was directly consumed by injured human brain [105, 106]. However, citing technical shortcomings of human studies using microdialysis, limitations of in-vivo and in-vitro TBI models, stoichiometry considerations and lack of biological plausibility, some have questioned the notion that lactate could be preferentially and beneficially used as a fuel in TBI [114]. However, a recent clinical trial of human TBI patients monitored with cerebral microdialysis, showed that exogenous hypertonic solution of lactic acid administered as a short infusion resulted in increased levels of glucose in the dialysate indicating a clinically beneficial glucose sparing-effect [115]. Additional beneficial effects of hypertonic lactate including decreased intracranial pressure and dampened glutamate excitotoxicity were also reported. Another study has also reported that exogenous lactate infusion improved neurocognitive function in mild traumatic brain injury patients [116].

MCTs in brain injuries: Because all the monocarboxylates exist as anionic species in the body, they require transporters to effectively cross biological barriers making MCT transporters key determinants of the trafficking monocarboxylates from the tissues/cells they are produced to where they are consumed. However, relatively little attention has been paid to the role of MCT transporters in either the controversy around the specific role of monocarboxylates in normal and extreme physiological conditions or acute brain injury. Most of MCTs related research has

focused on the changes in expression of the different MCT isotypes or the effect of small molecule inhibitors or genetic manipulation in in-vivo and in-vitro models of diverse acute brain injury. These studies, described below, provide evidence on how the monocarboxylates and MCTs are essential components of the brain's adaptation mechanism to altered energy sources and requirements in TBI and other brain injuries.

Rosafio *et al.* observed a region-specific and cell-specific changes in the brain expression of MCT1, 2 and 4 transporters the mouse model of stroke at 1 hour and 24 hours following transient occlusion of the left middle cerebral artery [117]. At 1 hour, all three MCTs showed increased expression in the striatum and surrounding cortex of the ipsilateral section that persisted for 24 h or more. Specifically, they demonstrated that neurons, which normally express MCT2 only, show strong expression of MCT1 at both time points, and MCT4 at 24 h time point. Interestingly, expression of MCT transporters was also observed in areas of the brain distant from the injury site, i.e., in the hippocampus (MCT1 and MCT2 in the ipsilateral side and MCT2 in the contralateral side), contralateral side striatum (MCT1), and contralateral side cortex (MCT1, MCT2). Zhang *et al.* studied the effects of cerebral ischemia by using permanent middle cerebral artery occlusion in a spontaneously hypertensive rat model on the expression of MCT transporters [118]. In the pre-infarct area, MCT expression was greatly increased in astrocytes (mRNA and proteins), endothelial cells (mRNA and protein) and neurons (mRNA). They also showed that in the short period of time before cell death occurs, increases in MCT1, MCT2, and MCT4 expression were observed in cells within the infarct and bordering the scar, although which specific cells have not been identified.

Moreira *et al.* investigated the expression of MCT1 and MCT2 following a focal ischemia episode created by unilateral extradural compression of rats, a model of TBI [119]. MCT1 mRNA expression was increased in ipsilateral and contralateral cortex and ipsilateral hippocampus while MCT2 mRNA expression was enhanced in the contralateral hippocampus. They also observed strong MCT1 and MCT2 protein expression was found in perilesional macrophages/microglia and in an isolectin B4+/S100β+ cell population in the corpus callosum. Gao *et al.* evaluated the protein expression of MCT4 in primary astrocyte-neuron co-cultures in an oxygen deprivation (OD) protocol that mimics hypoxic conditions [120]. They found the MCT4 expression increased significantly in OD cells. They also found that astrocyte-neuron co-cultures can tolerate 24h of OD treatment but RNAi-mediated suppression of MCT4 results in decreased neuronal survival without affecting astrocytes.

Wang *et al.* examined the expression of MCT1 and 2 in a rodent model of sleep apnea and stroke [121]. They also investigated how MCT2 overexpression, siRNA based knock-down or inhibition with small molecule affect middle cerebral artery occlusion induced infarct size. Expression of MCT1 mRNA (in cortex and hippocampus) and protein (in cortex) were not altered due hypoxia. RT-qPCR, western blot and immunohistochemistry showed an overall lowered expression of MCT2 overtime in cortex and hippocampus after intermittent exposure to hypoxia. Administration of both, an inhibitor of MCT2, CN-4, and siRNA against MCT2 resulted in increased infarct size. Conversely, overexpression of hMCT2 resulted in smaller infarct size as compared to wild-type controls whether MCAO was preceded by IH or not.

In two very similar studies, Schurr *et al.* and Izumi *et al.* showed, using hippocampal slices and oxygen deprivation, and CN-4 (inhibitor of MCT transport), endogenously produced monocarboxylates, through MCT transporters, are critical at regaining synaptic function and morphological integrity even in the presence of glucose [122, 123].

In an in-vivo study of cardiac-arrest-induced transient global cerebral ischemia rat mode, Schurr *et al.* demonstrated that administering the MCT inhibitor, 4-CIN, resulted in a significantly greater degree of delayed neuronal damage in the hippocampus [124]. Prins *et al.*, in a CCI model of TBI in pediatric rats (35 days postnatal) and adults, reported increased protein expression of MCT2 in the ipsilateral cerebral vasculature [125]. Expression of MCT2 was greater in the postnatal day 35 rats as compared to adults.

A definitive conclusion regarding the specific ways monocarboxylates and MCTs affect neuronal activity and survival, and functional outcomes may not be possible based on these studies because of disparities in models of injury employed, modalities of intervention, regions of the brain examined, non-selectivity of pharmacologic inhibitors utilized, and time periods of interventions and outcomes measured. Nevertheless, these studies provide strong evidence that monocarboxylates are critical to brain function and that the brain adaptation mechanism to acute injuries involves a significant role for monocarboxylates and their transporters. This points toward a greater need in developing a deeper understanding and more research of the role of MCT transporters in acuter injuries directly in humans. This could provide mechanist insight into

resolving the long-running dispute on whether lactate is a realistic energy substrate or even beneficial for neurons in the setting of acute brain injuries. Leveraging the naturally occurring generic variability in SLC16A1, SLC16A3, and SLC16A7, the effect of altered function of these transporters in clinical outcomes in human TBI patients could be examined.

1.1.4.3. Other SLC Transporters

To date, there are not many SLC transporters extensively studied in TBI other than MCTs and glutamate transporters. There are, however, a few SLC transporters that are worth mentioning here because 1) they are studied in acute brain injuries that share some pathology mechanism as TBI; 2) they transporter substrates that are known to be involved in TBI or 3) they are mediate the transporter of drugs that are commonly administered to TBI patients.

1.1.4.3.1. Prostaglandin Transporters

Prostaglandins [prostacyclin I_2 (PGI_2), prostaglandin E_2 (PGE_2), prostaglandin D_2 and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)] are a group of bioactive lipids derived from arachidonic acid through the activity of cyclooxygenase enzymes. In the brain, prostaglandins are released by neurons and glia in response to various insults such hypoxia and neuroinflammation in disease conditions such as TBI, stroke and neurodegenerative diseases [126]. The effect of prostaglandins in the CNS includes regulation of cerebral blood flow, synaptic transmission, and gene expression

[127]. The biological effects include body temperature regulation, pain perception, body temperature and sleep/wake cycle [127, 128].

Crucial to the specificity of each prostaglandin are transporters that determine the distribution, compartmentalization and signal termination of prostaglandins [129]. Several transports are known to mediate the trafficking of prostaglandins including SLCO21A (also known as prostaglandin transporter), SLCO1A2, SLCO1B1, SLCO2A1, SLCO2B1, SLCO3A1, SLCO4A1, SLC22A8, and SLC22A6 [129, 130]. Among the ABC transporters, ABCC4 and ABCC1 are known to transporter prostaglandins and glutathione-conjugates of prostaglandin respectively [131, 132].

Several lines of evidence have established the critical role of prostaglandins in TBI in mediating neuroinflammation, body temperate dysregulation, and memory loss [126, 133]. The role of prostaglandin transporters in these processes is not studied well. In one study, Scafidi *et al*. have studied the changes in expression of SLCO21A in the developing brain of mice in response to hypoxia [128]. They observed decreased expression of SLCO21A in neurons compared to normoxic conditions while the effect on astrocytes was the reverse of what was observed in neurons. Whether these findings imply that prostaglandins could be participating in the pathogenesis of brain injuries or help the brain counteract effects of injuries remains to be elucidated.

1.1.4.3.2. Nucleoside Transporters

There are two families of transporters that are responsible for trafficking nucleosides, nucleosides analogs and nucleoside like drugs, the concentrative nucleoside transporters (CNTs) of the SLC28 family and the equilibrative nucleoside transporters (ENTs) of the SLC29 family [134]. By mediating the uptake of nucleosides, these transporters play a key role in salvage pathways of nucleotide biosynthesis. Critically, these transporters determine the intracellular and extracellular levels of purine and pyrimidine nucleosides, cyclic nucleosides, adenosine and other endogenous and exogenous nucleosides. Several studies have examined the changes in levels and effects of different types of nucleosides after TBI [135]. Adenosine, in particular, is extensively studied in TBI owing to its anticonvulsant effects [136]. Diamon *et al.* studied the association of a variant of the gene SLC19A1, as part of the adenosine signaling regulatory cycle, with epileptogenesis or post-traumatic epilepsy risk [137]. These were based on the role of SCL29A1 in maintaining the intracellular and extracellular levels of adenosine. The study reported a significant association of epileptogenesis or post-traumatic epilepsy risk with other genes of the adenosine signalizing regulatory cycle, not SLC29A1, although the negative finding with regards to SLC29A1 could be attributed to the insufficient power of the study among other things. Given the increasing interest in understanding the role of nucleosides in TBI [135], focus on SLC28 and SLC29 families of transporters would ensure a better understanding of the brain distribution and levels of nucleosides.

1.1.4.3.3. Multidrug SLC Transporters

From drug transport perspective, there are several families of SLC transporters with individual members expresses on the BBB and/or blood—CSF barrier and play an active role in mediating the uptake and efflux of drugs with a variety of structures and chemical properties. These include organic anion transporting polypeptides (OATPs or SLCO), organic cation transporters (OCTs or SLC22A), organic cation transporter novel type (OCTN or SLC22A), concentrative nucleoside transporter (CNT or SLC28A), equilibrative nucleoside transporters (ENTs or SLC29A), multidrug and toxin extruder (MATE or SLC47A), and peptide transporters (PEPT or SLC15A) [14, 41, 130]. It is very important to determine if a particular TBI drug candidate's access to the brain is dependent on or restricted by transporters. It is also important to understand the effect of TBI disease mechanism on the activity and expression of these transporters. For example, In a hypoxia/reoxygenation (H/R) stress model of injury on the rat brain microvessels, Thompson *et al.*, showed that H/R resulted in increased expression of Oatp1a4 [138]. Uptake of atorvastatin, a statin and Oatp1a4 substrate, which has been investigated for a number of brain injury conditions due to its neuroprotective effect, was also increased. This suggests one explanation for the mixed results observed with statins in acute brain injuries could be due to their limited distribution to the brain and that modulating OATP1A4 transporter could facilitate greater brain penetration.

Drug-drug interactions mediated by these transporters is also possible and could contribute towards pharmacotherapy failure or disease progression. Many drugs commonly administrated to TBI patients including antibiotics such as cephalosporins, and diuretics such as furosemide are known to competitively or non-competitively inhibited these transporters [14, 130, 139].

To conclude, SLC transporters, remain relatively understudied in human disease, especially TBI [23]. However, their importance in maintaining brain homeostasis and, modulating the levels numerous injury mediators and therapeutic drugs is well demonstrated by the examples provided above. More studies of SLC transporters and their substrates are needed to fully understand the magnitude and extent of their involvement in pathogenesis and recovery after TBI, as well as develop therapies that target SLC transports and their substrates.

1.1.5. ABC Transporters in TBI

The ABC superfamily of transporters consists of 49 total transporters divided into 7 families, designated as A-G, based on sequence homology and functional similarities [21]. The ABCE/F subfamily, which lack the transmembrane domain and are involved in protein synthesis and expression, are not subject of this review and will not be discussed further. The other five families of ABC transporters are involved in trafficking diverse molecules including lipids, amino acids, steroid hormones, peptides and protein degradation products, hydrophobic molecules, and conjugates of numerous xenobiotic and endogenous molecules, drugs and their metabolites, environmental toxins and their metabolites. At the cellular level, ABC transporters, with some notable exceptions, are predominantly expressed on the cell membranes and to a lesser extent in the membrane of intracellular organelles such as endoplasmic reticulum and the Golgi apparatus. Except for ABCD4 (expressed on mitochondria membrane), all members of the ABCD subfamily of transporters are localized exclusively in the membrane of the peroxisome

[140]. Four members of the ABCB subfamily of transporters, specifically, ABCB6, ABCB7, ABCB8, and ABCB10, are localized in the mitochondrial membrane. Certain member of the ACB superfamily of transporters such as ABCB1, ABCC1, 2, 3, and 4, and ABCG2 show strong expression in excretory or barrier members including the intestine, kidney, BBB, and blood-testis barrier membranes. They function as part of regulatory system that selectively allow access to certain molecules while rejecting out others.

In the CNS, ABC transporters 'garnish' the various cellular and compartmental barriers to ensure that various endogenous and exogenous molecules are maintained at optimal levels intracellularly or extracellularly to maintain homeostasis. The localization, substrates, functional activity, regulation and roles in several cellular processes are extensively studied and reviewed here [12, 141]. Also, the role of ABC transporters in several monogenic CNS diseases and complex chronic and/or neurodegenerative disease such as ALS, AD, PD an epilepsy are well documented and reviewed here [24, 25, 141]. Additionally, the role of ABC transporters in determining the distribution of numerous drugs and environmental molecules is well established and reviewed here [24, 25]. The role of these transporters in TBI and other acute brain injuries, however, is starting to gain attention recently and will be reviewed below.

1.1.5.1. ABCA Transporters

ABCA family of transporters consists of 12 members, ABCA1-10, 12, and 13 [16]. All members except ABCA4 and ABCA13 show some degree of expression in the CNS [141]. ABCA transporters mediate the trafficking of cholesterol, phospholipid, apolipoproteins and several other physiologic lipid compounds [142]. Mutations in four members of the family are known to cause monogenic diseases in humans: ABCA1 (Tangier disease), ABCA3 (neonatal surfactant deficiency), ABCA4 (autosomal recessive macular dystrophies) and ABCA12 (hereditary keratinization disorders) [142]. All of these disorders are related to dysregulation of lipid homeostasis. Additionally, ABCA is involved in complex diseases where lipids are part of the central mechanism including Alzheimer's disease [16].

Thus far, only one study has studied the specific role of ABCA family of transporters in TBI. In a mice model of TBI [143]. Loane *et al.*, showed accumulation of Aβ after TBI. The accumulation of amyloid beta (Aβ) was attenuated by administration of liver X receptor (LXR) agonist that induced the expression of ABCA1 thereby enhancing the clearance of Aβ. This was accompanied by improved functional recovery.

Furthermore, the substrates of the ABCA family transporters, specifically, apolipoprotein E (apoE) and cholesterol-by products are implicated in the pathogenesis of TBI especially in transitioning from acute injury to chronic neurodegenerative disease. Evidence from genetic-association studies, animal models and in isolated neuronal and astrocytic systems have shown

that ABCA1, plays a critical role in facilitating cholesterol and phospholipid loading onto apolipoproteins [16]. Specifically, in the CNS, ABCA1 mediates the lipidation of ApoE (the most abundant lipoprotein in the CNS), trafficking of cholesterol to and from neurons, astrocytes and endothelial cells, and Aβ deposition. Separately, the apoE ε4 variant which is a strong genetic risk factor for AD, dramatically increased the risk of developing AD in TBI patients [144]. It is also associated with worse outcomes including mortality and worse functional outcomes in severe, moderate and repetitive TBI through a mechanism that is thought to involve dysregulation lipid homeostasis [145, 146] [147]. Given this strong, mechanistically definable, interaction between ABCA1 (and other members of the ABCA family), ApoE, and AD on one hand, and association of ApoE, TBI and AD, on the other hand, close examination of ABCA family transporters as a possible link between TBI and AD is warranted.

1.1.5.2. ABCB Transporters

This subfamily of transporters has 11 members, ABCB1-11 [148]. ABCB1 (also known as P-glycoprotein, P-gp, or MDR1) is perhaps the most studied of any transporters in CNS or otherwise, owing to its prominence as the most quintessential transporter involved in effluxing numerous drugs and other xenobiotic molecules from cells and compartments thereby significantly altering their pharmacokinetic properties including permeation to the CNS [141, 149]. A major endogenous substrate of ABCB1 is Aβ and decreased the clearance of Aβ due to downregulation of ABCB1 has been implicated in AD [150, 151]. More recently, it has been

shown to mediate the transport of several endogenous molecules including endogenous opioid peptide although the implication in health and disease of these findings is yet to be studied [152]. Other members of ABCB family include ABCB11 (which is the bile salt export pump or BSEP), the mitochondrial transporters (ABCB6, ABCB7, ABCB8 and ABCB10), ABCB4 and ABCB2. To date, only mRNA expression of ABCB2-11 has been detected in various brain structures and the discussion here will be limited to ABCB1.

In a single-center study in human TBI patients, Cousar *et al.* found that ABCB1 genotype was associated with functional outcome recovery. The specific mechanism of the effect remains unknown, but it could be by affecting the transport of drugs or endogenous substrates [153]. In another study in brain tissue from human TBI patients, Willyerd *et al.*, demonstrated that although the expression of ABCB1 doesn't seem to be altered, they observed possible proteolysis of ABCB1 into peptide fragments with the implication that degraded ABCB1 could lose its function [154].

ABCB1 is well-known for its effects on the brain permeation of therapeutic drugs, however, this aspect is not yet explored during TBI. However, in a closely related brain injury – focal cerebral ischemia – Spudich *et al.* demonstrated that inhibiting ABCB1 increases the accumulation of two neuroprotective drugs in the brain [155]. This study, in addition to the numerous studies conducted in various CNS diseases, imply that due to the profound effect of ABCB1 on drug

distribution into the brain, whether drugs administrated in TBI are substrates of ABCB1 should be considered.

1.1.5.3. ABCC Transporters

The ABCC family of transporters consists of 13 members, 9 of which are transporters (ABCC1-6 and ABCC10-12), one is a truncated protein with no transport capacity (ABCC13), one is an ion channel (cystic fibrosis transmembrane regulator gene), and two are cell surface receptors (SUR1 and SUR2) [156]. In the CNS, ABCC1-5 are the best-characterized members of the family. ABCC7, 8, 10, 11, and 12, while their expression and localization in the CNS are known, their specific functions remain unknown [141, 157]. Substrates of ABCC transporters include endogenous and exogenous organic anions including a large number of drugs and their metabolites. Often, ABCC transporters work in conjunction with OAT transporters to mediate a vectorial transport process of organic anions.

ABCC1, 2, 3 generally show preference to conjugates of lipophilic compounds including glucuronate, sulfate, glutathione conjugates, and bile acid conjugates [156, 157]. ABCC4 and 5 substrates include cAMP, cGMP, nucleotide analogs, and glutamate conjugates [157, 158]. ABCC6 transports small peptides and GSH conjugates. ABCC1, 2 and 4 are also reported to transport reduced GSH [157] [159].

Expression and localization of the two structurally closely related ABCC transproters, ABCC1, and ABCC4, are well-established [141, 156]. They are reported to be expressed in BBB, choroid plexus, microglia, and astrocytes. ABCC2 is enriched brain capillary endothelial cells and choroid plexus. Only limited data exists suggesting possible expression of ABCC3 in BBB, CP. ABCC5 show a high level of expression in the BBB, neurons, and glia [158].

Studies of ABCC transporters in TBI: A study by Willyerd *et al.* examined the changes in protein expression of ABCC1 following TBI in brain tissue of TBI patients [154]. The study reported an increased expression of ABCC1 compared to non-TBI controls that could possibly alter the levels of endogenous and exogenous substrates of ABCC1. In the second study, Cousar *et al.* studied the association of SNPs within ABCC1 and ABCC2 genes with neurological outcomes in human TBI patients [153]. They reported that ABCC1, but not ABCC2, genotype was associated with neurological functional recovery after TBI.

Studies are underway to examine the role of MRP4 in TBI through its role in effluxing 2',3'-cAMP from intracellular space – where it opens mitochondrial permeability transition pores – to extracellular space where it gets converted to the neuroprotective adenosine by CNPase [135].

SUR1, encoded by ABCC8, is nonselective cation channel. Although not strictly transporters of 'small molecules' as the definition of transporters is applied for the purposes of this dissertation, it plays a significant role in the pathogenesis of TBI and other acute brain injuries. Its expression

in the neurons, astrocytes and/or endothelial cells is upregulated in TBI, stroke, spinal cord injury and SAH. Mechanistic studies in animal models of acute CNS injuries and human genetic association studies have confirmed the role of ABCC8 in edema formation [160-164]. Clinical trials with glyburide, an inhibitor of SUR1, are underway in stroke and TBI to prevent edema formation [165, 166].

The fact that there are only a few studies examining the role of ABCC transporters in TBI indicate the area is highly underappreciate for its role in TBI given the main endogenous substrates of these transporters such as GSH (and its conjugates such as 4-HNE), cyclic nucleotides, and prostaglandins are of paramount importance in the pathogenesis of neuronal death and injury in TBI. GSH is an important intracellular anti-oxidant and plays a key role in countering major injury mechanisms in TBI. In the brain ABCC transporters, in coordination with EAAC3 and cystine/glutamate exchanger, facilitate the biosynthesis, distribution, compartmentalization and recycling of GSH, GSSH (oxidized form of GSH), and GSH conjugates with toxic reactive molecules such as 4-HNE [158, 167, 168]. Furthermore, ABCC1 and ABCC4, along with ABCB1 and ABCG2, are the major determinants of the distribution of many drugs into the brain [169] further highlighting the relevance of this transporter family in TBI.

1.1.5.4. ABCD Transporters

The ABCD subfamily of transporters consists of four members, three of which (ABCB1, 2 and 3) are expressed on the peroxisomal membrane while the fourth one, ABCD4, is localized on the endoplasmic reticulum membrane [140, 170, 171]. Although functional redundancy among the three perixosomal transporters has been reported, each has the main responsibility for transporting a specific type of fatty acids: ABCD1 is the main transporter for very-long-chain fatty acids-CoA (VLCFA-CoA), ABCD2 transporters C24:6w3, ABCD3 transporters docosahexaenoic acid (DHC)-CoA, trihydroxycoprostanoyl (THC)-CoA, dicarboxylic acid (DCA)-CoA and pristanoyl-CoA [170].

Peroxisomes, in coordination with mitochondria, are responsible for chain shortening of very long-chain fatty acids followed by β-oxidation, the major mechanism for degradation of fatty acids [171]. A vital step in this process is the trafficking of fatty acid substrates for beta-oxidation into the peroxisome and ABCD transporters play a critical role [172]. In fact, mutations in the ABCD1 gene result in X-linked adrenoleukodystrophy (X-ALD) which results in impaired peroxisomal β-oxidation and accumulation of VLCFA in tissues, especially the brain and the adrenal glands [173].

To date, ABCD transporters have not been investigated in acute brain injuries. Lipid β-oxidation, on the other hand, is known to contribute to neuronal damage and death in TBI and other brain

injuries [174]. Hence, the close examination of ABCD transporters in lipid β-oxidation mediated neuronal injury and death is warranted.

Additionally, polyunsaturated fatty acids (PUFAs), specifically, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and α-linolenic acid (ALA) have been reported to improve outcomes in preclinical TBI model when administered exogenously.[175]. PUFA are known to interact with ABCD transporters and predicted as substrates [176], indicating the need to investigate whether ABCD transporters are directly involved in the metabolism and therapeutic efficacy of exogenously administered and therapeutic DHA, EPA, and ALA.

1.1.5.5. ABCG Transporters

To date, there are five members of the ABCG subfamily identified in humans: ABCG1, 2, 4, 5, and 8 [148, 177, 178]. ABCG1,4,5, and 8 are involved in the transport of cholesterol, plant sterols and various precursors intermediaries of sterol biosynthesis, as well as some synthetic and semisynthetic sterols[177, 179]. ABCG1 and 4 are known to play a significant role in the lipid transport and metabolism in macrophages. Loss of function mutations in ABCG5 or 8 are identified as causative in sitosterolemia, a rare autosomal-recessive lipid metabolism disorder [178]. In CNS, the mRNA or protein of these transporters are reported to be expressed in various cell types and barriers, however, the specific functions are not yet elucidated.

ABCG2, first identified as multidrug resistance protein in human breast cancer cell lines – hence its other name ‘breast cancer resistance protein ‘(BCRP) [180] - is the best-studied member of the family. In the CNS it has been identified in BBB, choroid plexus, pericytes, astrocytes, microglia and neuronal progenitor and stem cells [141]. Most of the studies have been focused on the BBB and choroid plexuses, where ABCG2 along with PGP and MRPs is known to play a critical role in restricting access of anticancer and other drugs from permeating into the brain.

With regards to its endogenous substrates and physiological role, it was through genetic studies that its function as urate transporters was established. Reduced function mutations in ABCG2 were associated with increased serum uric acid levels and gout [181]. Subsequently, more endogenous and exogenous substrates of ABCG2 has been identified including porphyrins, amyloid beta and numerous therapeutic drugs [182-184]. It has also been implicated in a number of pathological conductions including PD and AD in the CNS [182, 183, 185].

A recent study has provided insight as to what the role ABCG2 could play in TBI. In the study, Adams *et al.*, reported that a genetic variant of ABCG2, ABCG2 c.421C>A, was associated with Glasgow Outcome Scale (GOS) a measure of functional outcome recovery, after severe TBI in an age-dependent manner [186]. Patients with the variant allele at ABCG2 c.421C>A have decreased protein expression of ABCG2 and associated increased blood uric acid levels. Since uric acid is a potent antioxidant, the favorable outcome in patients with the variant allele was attributed to possible increased levels of uric acid in the brain. However, other functions of

ABCG2 such as clearance of the toxic porphyrins [184, 187, 188], and its general role as the gate-keeper against toxic substances of stem cells, could not be overruled.

1.1.5.6. ABC Transporters and Pharmacoresistance

Resistance to pharmacotherapy mediated by transporters, especially efflux transporters, is a well-established phenomenon. While this phenomenon is a well-recognized hurdle in the treatment of brain cancer and HIV infection, lately, it has been gaining recognition in other brain disorders such as epilepsy, depression, schizophrenia, and ALS [25, 30]. For example, in ALS, Trotti D, Pasinelli P and colleagues have shown that there is an increase in both expression and activity of Pgp and BCRP in the BBB which results in progressive resistance to the CNS penetration of rizuloze, the only drug currently approved for ALS [189]. They also demonstrated that administrating elacridar, an inhibitor of Pgp and BCRP, improved efficacy of riluzole in ALS [190]. In acute brain injuries, a study by Spudich *et al.* showed that Inhibition of ABCB1 resulted in the accumulation of neuroprotective drugs after focal cerebral ischemia suggesting the possibility of phamacoresitance in acute brain injuries [155]. Similarly, Ibbotson *et al.* demonstrated that in a rat model of hypoxia-reoxygenation stress, there is an increased expression of Abcc1, Abcc2 and Abcc4 transporters in the BBB that is mediated by Nrf2 signaling [191]. Also, Wang *et al.* showed that activating Nrf2 through the administration of sulforaphane increases the expression of P-gp, Bcrp, and Mrp2 in rat brain capillaries which was

accompanied by decreased uptake of verapamil a P-gp substrate [192]. Interestingly, Nrf2 is activated in TBI in response to oxidative stress.

Although disruption in BBB observed in TBI could negate the impact of the increased expression of efflux transporters following TBI, it is important to consider that in many patients the disruption may be limited to small area. Also, often injury to brain tissue and cells occurs further away from the point of impact where there is no breakage of BBB. Additionally, the function of efflux transporter localized on the membrane of neurons, astrocytes, and other brain cells could be as important in conferring resistant to pharmacotherapy as those expressed on BBB. Hence, due consideration needs to be paid to transporters mediated pharmacoresistance in TBI.

In summary, ABC transporters, like the SLC transporters, are underexplored in TBI. As stated above, one or more mechanistic link exists between the function of each of the ABC transporters, and disease pathogenesis and outcomes in TBI, suggesting potentially powerful impact ABC transporters could have on outcomes following TBI. To date, studies of ABC transporters in TBI are limited to a few genetic association studies in humans and ever fewer mechanist studies in animal models. This calls for more rigorous and systematic investigation ABC transporters in TBI.

1.1.6. Conclusion – TBI and Membrane Transporters

The summary of membrane transporters investigated for their role in TBI is presented in Table 1. The role of membrane transporters in TBI remains under-studied. The limited number of studies that have investigated the role of membrane transporters in TBI have provided important insight into the functions of transporters within the neurovascular unit in TBI. In general, there are four ways transporters could impact outcomes in TBI. First, immediately after TBI, transporters are involved in helping the brain manage the aftermath of the injury, for example, by clearing injury mediators. This is exemplified by glutamate transporters that remove excess glutamate from synapsis to prevent excitotoxicity, and by MRP transporters that efflux out potentially damaging by-products such as 4-HNE after they are conjugated with GSH. Additionally, in the immediate time after injury, transporters also play a critical role in directing the movement of substrates that would be needed in higher amounts in neurons in the context of injury. For example, MCT1, 2 and 4 work in close coordination to direct lactate from the periphery or astrocytes to neurons to be used as energy source. Second, transporters play a significant role in the transition of TBI from acute brain injury to a chronic neurological disease. For example, patients with reduced function in ABCA1 due to genetic variants or due to decreased expression as a result of disease or injury could be at higher risk to develop TBI-related AD. Third, transporters dynamically control the uptake of many drugs aimed at the brain and their distribution within the brain. Understanding the nature of drugs or candidate drugs in development with respect whether it is a substrate or inhibitor of transporters becomes highly relevant as a drug that can't distribute to its target in sufficient quantities won't be effective. Forth, transporters determine the levels of potential small molecule biomarkers in the CSF or blood – two main compartments which allow

routine measurement of biomarkers – if the biomarker is transporter substrate. This could have a profound impact on how the levels of biomarkers in CSF or blood are interpreted relative to the levels in the brain. The same could also hold true where biomarkers are measured in the parenchyma of the brain either by dialysis or by homogenization in case of preclinical TBI studies.

It is hypothesized that systematic study of membrane transporters in the neurovascular unit in TBI will enable identifying targets for therapeutic intervention, provide insight into the details of pathological processes involved in TBI, and help determine the most promising drug candidates for development in TBI.

Table 1. Summary of Membrane Transporters investigated for their role in TBI

Transporter	TBI Model or Type of Clinical Study	Major Finding	Reference
EEAT (SLC1A)	CCI in rats	Decreased RNA protein expression of EEAT2 at 6-72 h after injury.	[68] [68][66]
	FPI in rats	Decreased Vmax of EEAT2 in cortex and hippocampus 5 minutes to 2h after injury.	[69]
	CCI in rats	Decreased RNA and protein expression of EEAT2 in hippocampus 24-72 h post injury. Administration of antisense oligodeoxynucleotides targeting EEAT2 resulted in exacerbated hippocampal neuronal death and increased mortality	[193]
	Genetic association, time to first seizure and seizure risks as outcomes	GG genotype at SLC1A1 rs10974620 was associated with increased posttraumatic seizures risk. TT genotype at SLC1A1 rs7858819 was associated with increased early and later posttraumatic seizures risk.	[73]
	FPI in rats	Ceftriaxone prevented the injury-related down expression of Slc1a3, and reduced cumulative post-traumatic seizure duration.	[80]
	CCI in rats	Ceftriaxone reduced glutamate levels, attenuated cerebral edema and neuronal death, and improved cognitive function.	[194, 195]
SLC17A7	Genetic association,	Patients carrying the minor allele at SLC17A7 rs7417284 were more likely to experience prolonged recovery.	[74]
SLC16A (MCT)	Unilateral extradural compression in rats	Increased MCT1 mRNA expression in ipsilateral and contralateral cortex, and ipsilateral hippocampus. Increased MCT2 mRNA expression in contralateral hippocampus.	[119]
	CCI in Rats	Increased protein expression of MCT2 in the ipsilateral cerebral vasculature.	[125]
	Transient occlusion of the left middle cerebral artery in mice	Region-specific and cell-specific changes in the brain expression of MCT1, MCT2 and MCT4.	[117]

SLC19A1 (ENT1)	Genetic association, post-traumatic epilepsy risk as outcome	Significant association of epiloptogenesis after TBI was found with other genes of the adenosine signaling regulatory cycle, but now with SL29A1.	[137]
SLCO1A4 (OATP1A4)	Hypoxia/Reoxygenation stress, Rat brain microvessels	Increased expression of Oatp1a4 and increased uptake of atorvastatin, an Oapt1a4 substrate	[138]
ABCA1	CCI in Mice	Administration of LXR agonist increased expression of ABCA1 leading to increased clearance of Aβ.	[143]
ABCB1 (Pgp MDR1,)	Genetic association, GOS score dichotomized to favorable v unfavorable outcomes	Patients homozygous for the T allele at ABCB1 rs1045642 were less likely to be have poor outcome versus those possessing the C allele	[153]
	Protein expression study of brain tissue from human TBI patients	No change in expression of ABCB1. Proteolysis of ABCB1 into peptide fragments.	[154]
	Focal ischemia injury in mice	Abcb1 was upregulated on capillary endothelium. Pharmacological inhibition or genetic knockout enhanced the accumulation and efficacy of neuroprotectants that are Abcb1 substrates.	[155]
ABCC (MRP)	Genetic association, GOS dichotomized to favorable v unfavorable outcomes	For ABCC1 rs4148382, patients homozygous for the G allele were less likely to be assigned poor outcome versus those possessing the A allele	[153]
	Protein expression study of brain tissue from human TBI patients	Expression of ABCC1 was increased in brain cortical tissue in TBI patients.	[154]
	Hypoxia/Reoxygenation stress, Rat	Increased expression of Abcc1, Abcc2, and Abcc4 in BBB mediated by Nrf2 signaling.	[191]
ABCG2 (BCRP)	Genetic association, GOS	ABCG2 c.421C>A was associated with GOS after severe TBI in age dependent manner.	[186]

CCI = Controlled Cortical Impact; FPI = Fluid Percussion Injury;

GOS = Glasgow Outcome Scale

1.2. Targeting Transporters to Attenuate Oxidative Stress in TBI

1.2.1. Oxidative Stress in TBI

Oxidative stress is one of the major and well-established injury mechanisms of TBI that causes neuronal damage and death [7, 196, 197]. The role of oxidative stress in the pathophysiology of TIB was first described 30 years ago in a fluid percussion model of TBI in cats where a rapid release of superoxide in the extracellular space during and at least 1h after the injury was observed [198, 199]. Subsequently, numerous studies, both in various animal models of TBI and human TBI patients, have confirmed that different type of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are released excessively during TBI [200, 201] [202] [203] [204, 205]. Oxidative stress occurs when the body's antioxidant defenses which is comprised of enzymes such superoxide dismutase and non-enzymes such as glutathione and ascorbic acid becomes overwhelmed by the excess reactive oxygen species (ROS) and reactive nitrogen species (RNS) creating an imbalanced redox state. The excess and highly reactive ROS and RNS then interact with proteins, lipids and DNA causing structural and functional alterations to these macromolecules eventually leading cell damage and death.

1.2.2. Targeting Transporters with Probenecid to Simultaneously Increase Brain Levels of N-acetylcysteine and Glutathione

Based on the demonstrated significance of oxidative stress in the pathophysiology of TBI, several approaches to counter oxidative stress as a therapeutic strategy have been proposed including attempts to replenish the components of the anti-oxidative stress reservoir [8, 196]. This is based on the observation that the endogenous cellular scavengers of ROS and RNS, specifically alpha-tocopherol, ascorbate, and GSH, while effective in their function, become depleted fairly quickly after TBI [201]. We will focus on GSH here.

GSH, a tripeptide of glutamic acid, cysteine and glycine, a critical role in maintaining the redox homeostasis in the brain cells (and virtually all body cells) [167]. There are at least two ways to maintain or supplement that brain GSH levels: 1) provide precursors that are critical for the biosynthesis of GSH. N-acetylcysteine which acts as a donor of cysteine -- the rate-limiting substrate in the biosynthesis of GSH -- is extensively used for this function in numerous disease conditions and will be used in that capacity in this dissertation. 2) preventing the efflux of GSH and its conjugates from the brain thereby increasing the overall GSH pool available for neurons and other brain cells. As mentioned in section 1.1.5.3 some ABCC transporters are involved in the efflux of GSH, hence inhibiting these transporters provids an opportunity to maintain brain GSH levels. In this dissertation, probenecid, an FDA approved drug, will be used to test this strategy.

These two approaches have several advantages that would allow them to synergistically enhance brain GSH level. First by inhibiting of organic anion transporters, probenecid could increase the exposure of NAC in plasma and importantly in the brain. Second, NAC, chemically a thiol, can act as an antioxidant by itself by reacting with and scavenging a number of radicals including •OH, •NO2, CO3•− and thiyl radicals [206]. Third, both NAC and probenecid are already FDA approved drugs with excellent safety profile, hence providing an opportunity for rapid translation to the clinic. Fourth, the combination therapy targets multiple processes vis-à-vis TBI and oxidative stress. Many of the agents targeting oxidative stress were single agents that target one aspect of oxidative stress, and although many of them show effectiveness in preclinical models of TBI, none prove to be effective in clinical trials. This is in part because oxidative stress involves multiple ROS and RNS that propagate oxidative damage via multiple pathways before eventually leading cell damage and death. As such, a single agent that targets a single point in the oxidative might not provide sufficient protection [7, 207]. To circumvent this problem, a combination therapy that targets multiple points in the oxidative stress cascade has been proposed to achieve an adequate level of neuroprotection (Hall, 2015).

Hypothesis: Probenecid, an inhibitor of OAT and MRP transporters, could simultaneously increase the brain levels of the co-administered antioxidant drug NAC, and the endogenous antioxidant GSH to enhance the antioxidative stress reservoir of the brain, and improve functional outcomes after TBI.

This hypothesis is tested in Chapters 2, 3, and 4. The aim of chapter 2 is to investigate whether probenecid when co-administered with NAC could increase the systemic and brain levels of

NAC by inhibiting OAT and/or MRP transporters. The aim of chapter 3 is to examine whether co-administration of probenecid and NAC would improve the motor and behavior functional outcome after TBI in a pediatric rat, CCI model of TBI. The aim of chapter 4 is to assess the capacity of the combination of NAC and probenecid to modulate GSH-associated anti-oxidative stress biochemical processes in the CSF of human TBI patients using a combination of metabolomics, and pathway and network analyses. Also, relevant biochemical processes linked to the pharmacological action of the combination therapy especially that of transporter inhibitor probenecid would be elucidated.

1.3. Examining the Role of SLC16A1, SLC16A3 and SLC16A7 transporters in Human TBI Patients with a Genetic Approach

The function of MCT transporters to direct the movement of lactate, pyruvate and ketone bodies between systemic circulation and brain, as well as within the neurovascular system is discussed in section 1.1.4.2. Also, the role of MCT transporters in traumatic brain injuries was discussed. It was indicated that due consideration of the role of MCT transporters within the neurovascular system could provide significant insight and help resolve the long-running controversy on whether lactic acid is shuttled to neurons to be utilized as an alternative/preferred energy source in TBI. In the brain, MCT1, 2 and 4 work in coordination – as reflected by their distinctive expression patterns and kinetic properties – to facilitate the movement of lactic acid from peripheral or astrocytic sources into the neurons especially in conditions such as TBI where the

normal energetics of the brain is disrupted. In Chapter 5 of this dissertation, using candidate-gene approach and focusing on well-studied SNPs, we aim to evaluate the association of genetic alteration in MCT1, 2 and 4 with neurological outcomes in TBI.

Hypothesis: TBI patients with variants in the genes SLC16A1, A3 or A7 that result in increased function or expression would show higher recovery rate in clinical outcomes.

2. Pharmacokinetic interaction between probenecid and n-acetylcysteine, in-vitro and in-vivo evidence.

2.1. Introduction

N-acetylcysteine is a thiol-containing, acetylated derivative of cysteine that has been in clinical use for over 50 years [206, 208, 209]. It acts as an antioxidant by directly scavenging reactive oxygen and nitrogen radicals via its thiol group, as well as by acting as a cysteine donor in the biosynthesis of glutathione, the most abundant intracellular antioxidant [206, 210, 211]. *N*-acetylcysteine is FDA-approved for prevention of liver toxicity due to acetaminophen/paracetamol overdose and as a mucolytic for patients such as those with cystic fibrosis or chronic bronchitis [208, 212-214]. It has also been investigated as a cytoprotective agent in several conditions including TBI [215], contrast-induced nephropathy, cancer chemotherapy, cardiovascular disease, diabetes, human immunodeficiency virus infection, neuropsychiatric disorders, and heavy metal toxicity (reviewed here, [208, 209, 211]. *N*-acetylcysteine is a hydrophilic molecule (log *D* = -5.4; [216]) that is approximately 30% renally eliminated [217]. Despite its extensive clinical and research use, an understanding of how *n*-

acetylcysteine crosses certain biological barriers and its interactions with membrane transporters remains incomplete. Additionally, controversy exists regarding the extent to which *n*-acetylcysteine can enter the central nervous system (CNS) through the blood-brain barrier (BBB) [206].

Our group is investigating the use of n-acetylcysteine in combination with probenecid, the prototypical organic anion transporter inhibitor, in the treatment of pediatric traumatic brain injury [218] . We proposed that a potentially synergistic interaction between n-acetylcysteine and probenecid exists to enhance antioxidant capacity of the brain after injury. While n-acetylcysteine acts as donor of cysteine, the rate limiting substrate in biosynthesis of glutathione, probenecid has been shown to prevent active efflux of glutathione and its conjugates by inhibiting MRP transporters [219]. Probenecid is known to increase the plasma and CNS exposure of a number of drugs such as acyclovir, zidovudine and bumetanide by inhibiting transport systems in renal tubules, BBB, and/or blood-cerebrospinal fluid barrier [220-223]. The objectives of the current study were to evaluate the effect of probenecid on the brain and plasma exposure of n-acetylcysteine, and to assess the mechanism through which their interaction may be mediated.

2.2. Material and Methods

2.2.1. Animals and treatment.

All studies were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Healthy, male Sprague-Dawley rats (n=48), were purchased from Harlan Laboratories (Indianapolis, Indiana, USA) and acclimated for one week prior to study initiation when rats were 17-18 days old. The animals were allowed free access to food and water and were housed on a 12 h light/dark cycle.

2.2.2. Drug administration and pharmacokinetic sampling.

Rats were administered a single dose of either 163 mg/kg of n-acetylcysteine (Sigma-Aldrich, St. Louis, MO, USA) alone or in combination with 150 mg/kg of probenecid (Sigma-Aldrich, St. Louis, MO, USA) via intraperitoneal injection. Blood was collected at 0.5, 1, 2, 4, 6 and 8 h after injection by cardiac puncture into heparinized tubes, centrifuged immediately to isolate plasma, and frozen at -80°C for drug analysis. Similarly, brain tissue was harvested following decapitation at each time point and brain hemispheres were isolated carefully to avoid contamination with blood. Hemispheres were then homogenized by sonication in brain homogenization buffer (containing 137mM of NaCl, 2.7mM of KCl, 10mM of Na2HPO4, 1.8mM KH2PO4 and 1mM of EDTA dissolved in H2O and adjusted to pH of 7.4) and an aliquot

of the homogenate was used for LC-MS/MS analysis. Four rats were studied per time point in each group. Concentration-time data is reported as mean ± SEM.

2.2.3. Drug analysis by LC-MS/MS.

Total n-acetylcysteine concentrations were quantified in plasma and brain tissue using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Deuterated internal standard (d3- n-acetylcysteine, Cambridge Isotopes (Andover, MA) was added to plasma (50 µL) or brain homogenate (50 µL) and samples were reduced by adding dithiothreitol, derivatized with n-ethylmaleimide (NEM), and proteins were precipitated with acetonitrile. The supernatant was dried out under nitrogen and reconstituted with water before injecting onto the UPLC-MS/MS system. Chromatographic separation was achieved using a porous graphitic carbon hypercarb column 3.0 µm (1.0 X 100 mm) and an acetonitrile-formic acid gradient on an Accela series UPLC system (Thermo, San Jose, CA) with an 8 min run time. MS/MS detection of NAC-NEM (m/z 433.1→304.2) was performed on a Thermo TSQ Quantum Ultra mass spectrometer with a heated electrospray source (Thermo, San Jose, CA). Calibration curves were linear from 100-10,000 ng/mL ($r^2 > 0.995$). Inter-day accuracy (% bias) and precision (RSD) determined at the concentrations of 300, 2000, and 7000 ng/mL ranged from -1.11% to 10.5% and 1.88% to 7.63%, respectively. All samples were diluted as necessary to be within the linear range of the assay.

2.2.4. Pharmacokinetic analysis.

Pharmacokinetic parameters were estimated using standard noncompartmental methods (WinNonlin Phoenix, Certara, St. Louis, MO). Area under the curve (AUC) profiles for plasma and brain concentrations were calculated using the trapezoidal rule. The AUC from the last measured time point to infinity (AUC0-inf) was estimated by dividing the last measured concentration by the elimination rate constant. Apparent plasma clearance was calculated by dividing the dose given by AUC0-inf.

2.2.5. Cell uptake studies.

Human embryonic kidney cell lines stably transfected with human OAT3 (HEK-OAT3), human OAT1 (HEK-OAT1), and the corresponding HEK-empty vector (HEK-EV, control cells) were kindly provided by Professor Kathleen Giacomini (University of California, San Francisco, CA, USA). Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose (4.5g/L) supplemented with L-glutamine (Life Technologies, Long Island, NY, USA), 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), Hygromycin-B (100ug/mL, Corning, Corning, NY) and penicillin-streptomycin (100 ug/mL, Mediatech Inc., Manassas, VA) at 37°C in a humidified atmosphere with 5% CO_2. Cells were plated in 6-well, poly-D-lysine coated tissue culture plates (Corning, Corning, NY, USA) 48-72 hours before the uptake study. On the day of the experiment, medium was removed and cells were washed twice with warm Dulbecco's Phosphate Buffered Saline containing calcium and magnesium (DPBS, Mediatech

Inc., Manassas, VA, USA). Cells were then incubated in a DPBS solution containing 5mM of the transporter activator glutaric acid (Acros Organics, Geel, Belgium) for 30 minutes at 37°C. This solution was then replaced with incubation buffer (containing 128mM NaCl, 4.73mM KCl, 1.25mM $CaCl_2$, 1.25mM $MgSO_4$ and 5mM HEPES in H_2O and adjusted to pH 7.4) for 10 minutes as previously described [224]. Uptake was initiated by adding ^{14}C–Acetyl-L-Cysteine (^{14}C-NAC; specific activity 0.0581 Ci/mmol or 0.054 Ci/mmol; Moravek Biochemicals, Brea, California) and unlabeled NAC (Sigma-Aldrich, St. Louis, MO, USA) followed by incubation at 37°C for the indicated period of time. Uptake studies were terminated by removing the incubation solution and washing cells with ice-cold DPBS three times. Cells were then lysed with a solution of 0.1N NaOH containing 0.1% sodium dodecyl sulfate. Lysate radioactivity was then measured using a LS 6500 Scintillation System (Beckman Coulter, Brea, CA). Protein concentration was also assayed using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) per the manufacturer's protocol. The resultant uptake rates were normalized for protein content to account for differences in cell number between plates. Inhibition experiments were performed as described above except that cells were pre-incubated with 200 μM of probenecid in incubation media for 5 minutes. For the concentration-dependent uptake study, varying amounts of unlabeled n-acetylcysteine were added to the loading buffer containing a fixed amount of radiolabeled n-acetylcysteine to give total NAC concentrations ranging from 1 μM to 10 mM. All experiments were conducted in triplicate and means ± SD were calculated. Uptake activity due to OAT1 and OAT3 was calculated by subtracting NAC uptake in HEK-EV cells from that of HEK-OAT1 and HEK-OAT3 at each of the corresponding concentrations. Michaelis-Menten kinetic analysis of the uptake data was conducted using Prism 5.04 (Graphpad Software, La

Jolla, CA). Data were first plotted and then the best-fit curve was fit using the following equation: $V = V_{max} \cdot [S]/(K_m + [S])$, where V is the rate of uptake activity, V_{max} is the maximum rate of uptake activity, [S] is the substrate concentration and K_m is the concentration of the substrate where rate of uptake activity is half of the maximum.

2.2.6. Vesicular uptake assay.

Human MRP1 and MRP4 membrane vesicles, negative control vesicles, and ABC Transport Assay Reagent Kit were purchased from Life Technologies (Carlsbad, CA, USA). A positive control substrate, 3H-estradiol-17β-D- glucuronide (E217βG; 41.4 Ci/mmol) was obtained from Perkin Elmer,Inc. (Boston, MA, USA). MultiScreenHTS 96-well glass fiber filter plates were purchased from EMD-Millipore Corporation (Billerica, MA, USA). ATP-dependent and independent uptake was measured using either 50 µg of control, MRP1, or MRP4 baculovirus-transfected Sf9 insect cells. Assays were performed at 37^0C in a final volume of 50 µl in a reaction mixture containing either 4 mM ATP or AMP, 2 mM glutathione, 10 µM cold E217βG, 200 nM 3H-E217βG (for MRP1) or 100 nM (for MRP4). The reaction was initiated by adding vesicles to reaction mix, incubating for 0.5, 1, 2.5, or 5 minutes, and stopped by adding 200 µl ice-cold buffer. Samples were added to glass fiber filter plates and rapid filtration was performed using a Bio-Rad vacuum filtration unit. Wells were washed 5 times, filters collected, and counted using the liquid scintillation counter. Additional experiments were performed using varying concentrations of the known MRP1 substrate E217βG (0.25-100 µM) for 0.5 minutes as

described above. Radioactivity counts in wells without membrane vesicles were subtracted to account for any 3H-E217βG bound to the filter. n-acetylcysteine assays were performed as above using MRP1 or MRP4 vesicles with ATP or AMP, with and without glutathione, using 15 µM cold NAC and 10 µM of radiolabeled n-acetylcysteine for a final concentration of 25 µM. Reactions were carried out for 3.5, 7, and 10 minutes.

2.2.7. Statistical analysis.

Between groups comparisons were made using unpaired Student's t tests. $p < 0.05$ was considered significant. All analyses were conducted using Graphpad Prism 5.04 (Graphpad Software, La Jolla, CA).

2.3. Results

2.3.1. Probenecid increased n-acetylcysteine plasma and brain exposure and decreased apparent plasma clearance.

The impact of probenecid co-administration on n-acetylcysteine plasma and brain levels was first investigated. N-acetylcysteine plasma concentrations were increased significantly in the presence of probenecid starting at 2 h after administration compared to when n-acetylcysteine was given

alone (Figure 1.A). Moreover, the n-acetylcysteine AUC was increased by 1.65 fold by probenecid co-administration (Figure 1.B). Probenecid reduced apparent plasma clearance of n-acetylcysteine by 65% (CL/F: 1113±29 vs 674±8.2 mL/h/kg; $p < 0.001$).

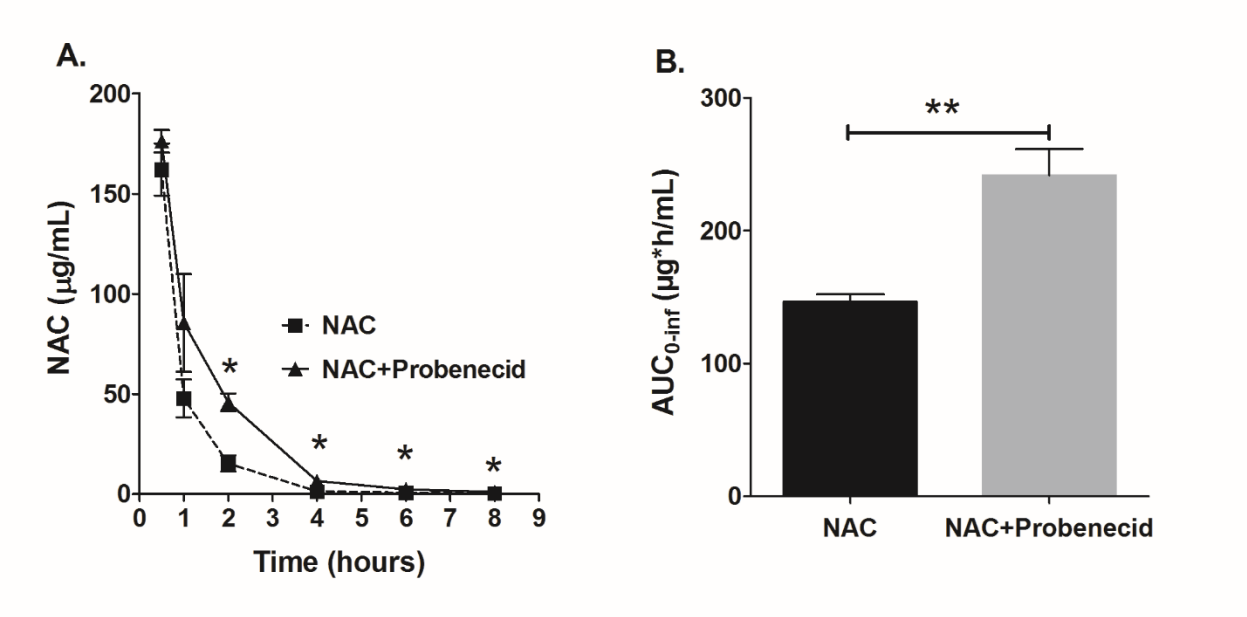

Figure 1. N-acetylcysteine plasma concentration-time profile (A) and total plasma exposure, AUC, (B) with or without co-administration of probenecid.

Sprague-Dawley rats were administered a dose of either n-acetylcysteine (163 mg/kg) alone or in combination with probenecid (150 mg/kg) intraperitoneally and serial n-acetylcysteine plasma concentrations were measured. (A) Probenecid increased n-acetylcysteine plasma concentration starting from the 1h up to the last sampling time point (mean ± SEM, n = 4 per time point; *, $p < 0.05$). (B) Probenecid increased n-acetylcysteine plasma AUC0-inf by 1.65-fold (mean ± SEM; $p < 0.01$).

Similarly, n-acetylcysteine brain concentration-time profile was significantly increased by probenecid (Figure 2.A). Differences were observed starting from 1 h up to the 4 h time point. While n-acetylcysteine levels remained measurable until the last sampling time point (8 h) in the group that received both n-acetylcysteine and probenecid, n-acetylcysteine levels were below the limit of quantification at the 6 h and 8 h time points in the group that received n-acetylcysteine only. Figure 2.B shows that n-acetylcysteine total brain exposure was significantly increased by 2.46 fold by probenecid co-administration. Interestingly, the proportional increase in n-acetylcysteine total brain exposure with co-administration of probenecid was higher than that observed in plasma (2.46 vs 1.65 fold increase, respectively).

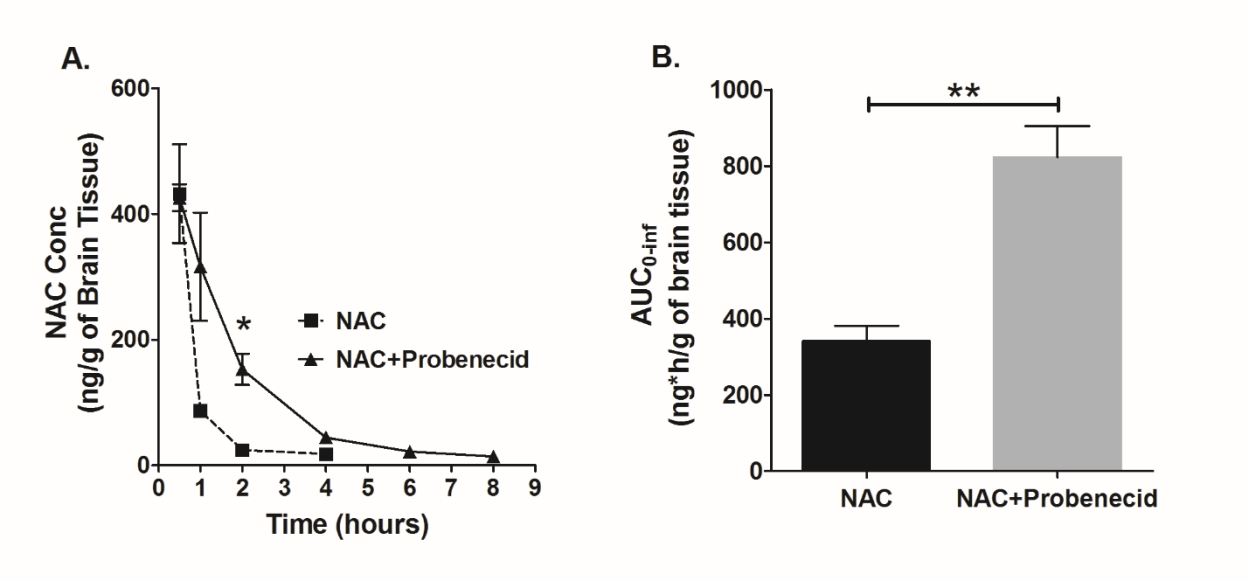

Figure 2. N-acetylcysteine brain concentration-time profile (A) and total brain exposure, AUC, (B) of N-acetylcystiene, with or without co-administration of probenecid.

Sprague-Dawley rats were administered a dose of either n-acetylcysteine (163 mg/kg) alone or in combination with probenecid (150 mg/kg) intraperitoneally and brain hemispheres were collected at several time points for n-acetylcysteine analysis. **(A)** Probenecid increased n-acetylcysteine brain concentrations starting from 1h up to the 4h time point. n-acetylcysteine levels were measurable until the last sampling time point (8h) in the group that received both n-acetylcysteine and probenecid, but were below limit of quantification at 6h and 8h in the group that received n-acetylcysteine only (mean ± SEM, $n = 4$ per time point, * $p < 0.05$). **(B)** Probenecid increased brain $AUC_{0\text{-}inf}$ of NAC 2.46-fold (mean ± SEM; $p < 0.01$).

2.3.2. N-acetylcysteine uptake by OAT3 and OAT1.

To understand the mechanism of interaction between n-acetylcysteine and probenecid, we examined the time- and concentration-dependent uptake of n-acetylcysteine by transporters known to be inhibited by probenecid. OAT3- and OAT1-mediated transport activities were evaluated using cell lines that stably express those transporters and their negative controls (cell lines stably transfected with the empty vector). Figure 3 shows that the uptake of n-acetylcysteine by OAT3- and OAT1-overexpressing cells is linear up to at least 15 and 42.5 (latest time point measured) minutes, respectively. At 25 μM n-acetylcysteine, update rates were 37.7±1.9 and 4.2±0.1 pmol/mg/min respectively. Subsequent concentration dependent uptake studies were then examined within the established linear range, 25 minutes for OAT1 and 10 minutes for OAT3.

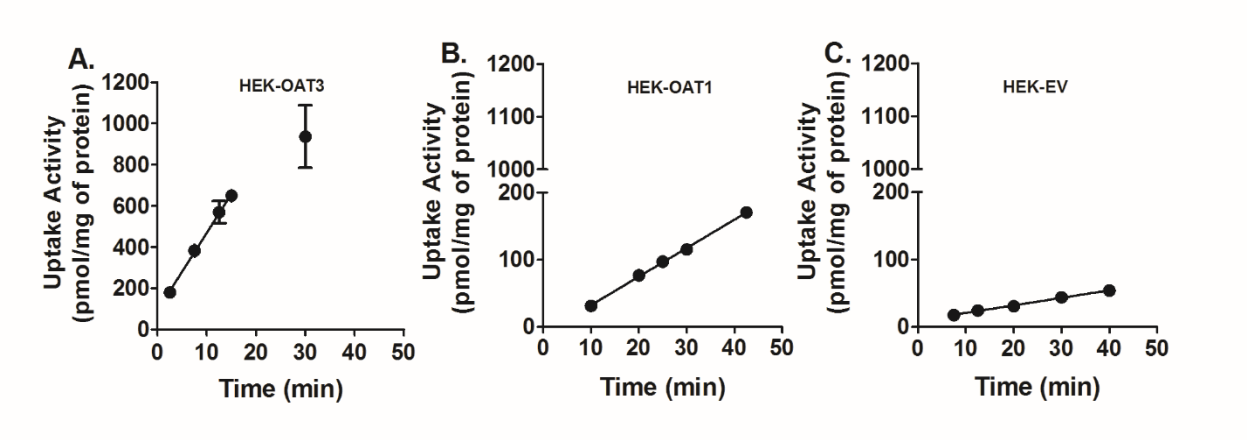

Figure 3. Time-dependent Uptake of n-acetylcysteine by OAT1- and OAT3-overexpressing cells

Human OAT1-, OAT3- and EV-HEK291 cells were incubated in transport buffer containing 25 µM of n-acetylcysteine traced with ^{14}C–Acetyl-L-Cysteine at 37°C for designated incubation times. N-acetylcysteine uptake was linear up to 15 minutes for HEK-OAT3 **(A)**, 42.5 minutes (latest time point measured) for HEK-OAT1 **(B)** and 40 minutes (latest time point measured) in control HEK-EV cells **(C)**. Each value represents the mean ± SD of three determinations.

N-acetylcysteine uptake by either of the transporter overexpressing cell lines was concentration-dependent, but did not reach saturation even at very high substrate concentration of 10 mM (Figure 4).

Figure 5 shows that probenecid significantly decreased n-acetylcysteine uptake by both OAT1 (59% decrease, 3.88±0.26 to 1.59±0.0.34 pmol/min/mg of protein, n = 3; $p < 0.01$) and OAT3

(93.9% decrease, 51.08±3.8 to 3.1±0.15 pmol/min/mg of protein, n = 3; $p < 0.001$). There was no difference in n-acetylcysteine uptake by control cells in the presence or absence of probenecid (n = 3; N.S.).

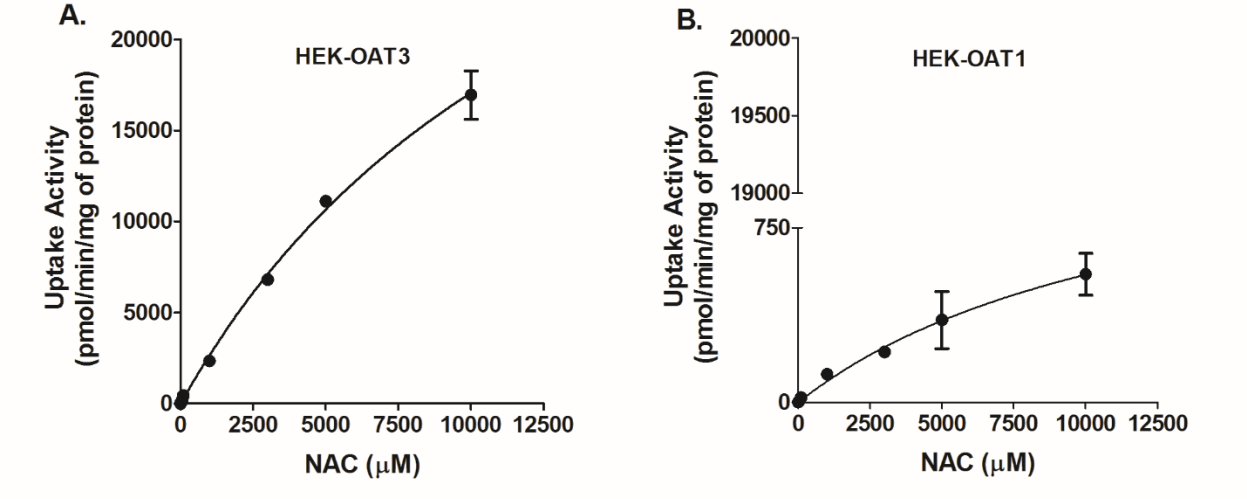

Figure 4. Concentration-dependent n-acetylcysteine uptake by OAT1- and OAT3-overexpressing cells

HEK-OAT3 **(A)** and HEK-OAT1 **(B)** cells were incubated in transfer buffer for 10 minutes (HEK-OAT3), 25 minutes (HEK-OAT1) with increasing concentrations of n-acetylcysteine traced with ^{14}C–Acetyl-L-Cysteine. Non-specific uptake was accounted for by subtracting uptake activity in HEK-EV cells from that of HEK-OAT1 or HEK-OAT3. The rate of uptake of n-acetylcysteine did not reach saturation even at high substrate concentrations. Each value represents the mean ± SD of three determinations.

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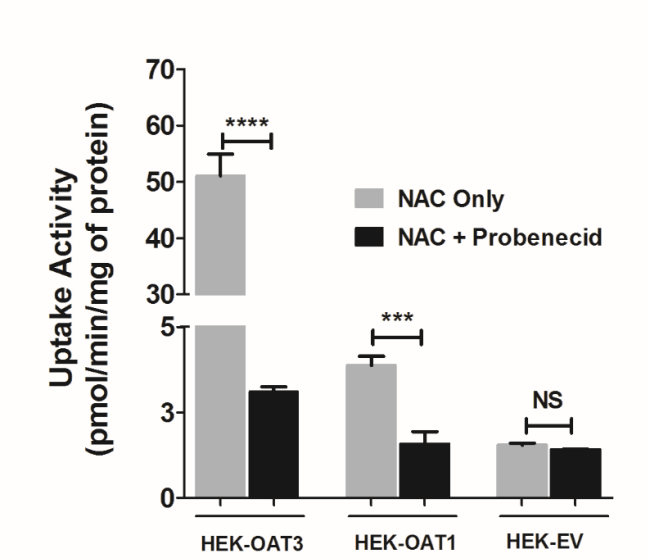

Figure 5. Inhibition of OAT1- and OAT3-mediated n-acetylcysteine uptake by probenecid

Uptake of 25 µM of n-acetylcysteine traced with ^{14}C–Acetyl-L-Cysteine was evaluated in human OAT1-, OAT3- and EV-HEK291 cells with or without 200 µM of probenecid for 10 minutes (HEK-OAT3), 25 minutes (HEK-OAT1) and 25 minutes (HEK-EV). N-acetylcysteine uptake activity was reduced in HEK-OAT1 and HEK-OAT3, but not HEK-EV cells following pre-incubation with probenecid. Each value represents the mean ± SD of three determinations. ** $p <$ 001. **** $p < 0.001$.

To evaluate whether n-acetylcysteine is a substrate for probenecid-sensitive efflux transporters that are expressed in the renal proximal tubules and in the brain, n-acetylcysteine uptake in MRP1- and MRP4-overexpressing membrane vesicles was evaluated. However, n-acetylcysteine transporter-medicated uptake by either of the transporters was not detected despite observing saturable uptake of $E_2$17βG, a well established substrate for both transporters as a positive control (Figure 6).

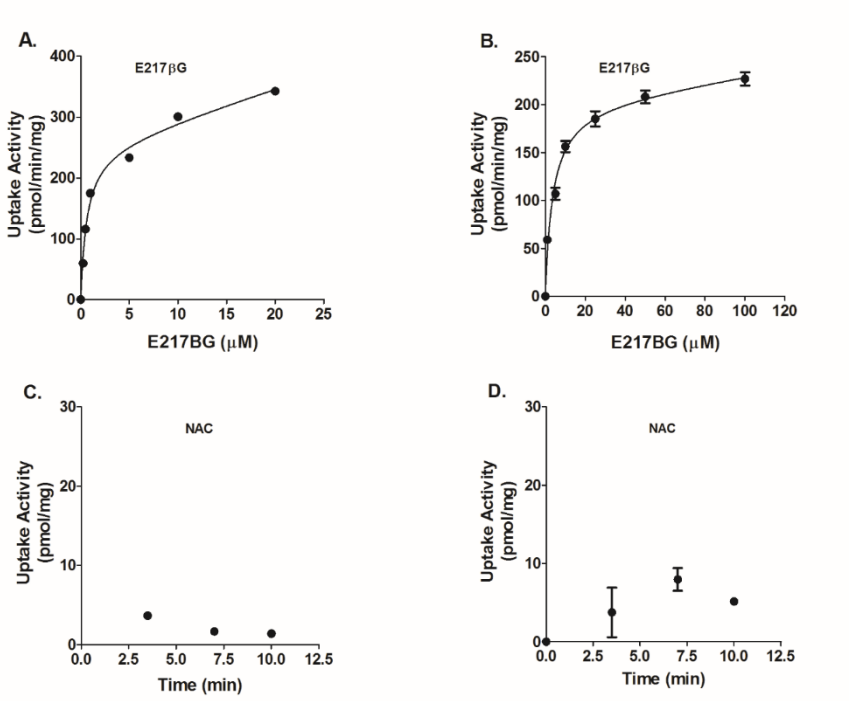

Figure 6. Effect of MRP1 or MRP4 on n-acetylcysteine uptake in membrane vesicles

Uptake was measured in the presence of ATP or AMP and data corrected by subtracting the amount of substrate bound to filters in the absence of protein vesicles. Final ATP-dependent uptake of $E_2$17βG by membrane vesicles expressing MRP1 **(A)** or MRP4 **(B)** was determined by substrating uptake in the presence of AMP from that in the presence of AMP . Membranes were incubated for 30 seconds at 37°C. Time-course of n-acetylcysteine uptake in presence of AMP or ATP by MRP1 **(C)** and MRP4 **(D)** membrane vesicles. Membranes were incubated with 25 µM n-acetylcysteine (15 µM cold + 10 µM ^{14}C- n-acetylcysteine). There was no statitically significant difference in the uptake rate (i.e., slope) between AMP- or ATP-dependnet uptake with either transporter. Each value represents the mean ± SD of two determinations.

2.4. Discussion

We report the novel finding that probenecid increases n-acetylcysteine plasma and brain exposure in vivo, which suggests that n-acetylcysteine is a likely putative transporter susbstrate. Further, we demostrated that NAC is an OAT1 and OAT3, but not a MRP1 and MRP4, substrate. N-acetylcysteine has been touted as a potential therapeutic agent for a number of ailments, including CNS injuries, but the process by which this hydrophilic molecule (log *D* = -5.4; [216]) crosses certain biological barriers is poorly understood.

N-acetylcysteine plasma exposure was significantly increased and its apparent clearance was signficantly decreased by probenecid co-administration in the rat. Probenecid is a well-established organic anion transport inhibitor and has been used both clinically and preclinically (in rats) to determine the role of OAT1 and OAT3 in the clearance of renally-excreted drugs. Human and rat forms of both OAT3 and OAT1 show overlapping substrate specificity and comparable level of activity [225, 226]. This is suggestive that these transporters mediate the uptake of n-acetylcysteine into the renal tubule for eventual secretion into the urine. In order to determine if n-acetylcysteine is substrate for OAT1 and/or OAT3, we conducted an *in vitro* uptake study of n-acetylcysteine with HEK-293 cell lines that overexpress human OAT1 or OAT3 transporters.

In vitro uptake and inhibition experiments confirm that n-acetylcysteine is a substrate for OAT1 and OAT3. However, OAT1 and OAT3 concentration-dependent uptake did not reach saturation even after incubation with 10 mM of n-acetylcysteine. One possible explanation for the apparent inconsistency between the lack of saturability and inhibition of uptake by probenecid is that n-acetylcysteine may exist in various chemical forms following admininistration. Once n-acetylcysteine enters the systemic circulation, it exists in a reduced form and in various oxidized forms that are products of redox reactions between n-acetylcysteine, cysteine, glutathione and proteins [227, 228]. Consistent with the possibility that some conjugate forms of n-acetylcysteine could be the substrates for OAT1 and OAT3, previous reports have demonstrated that endogenous and exogenous mercapturic acid conjugates of n-acetylcysteine as well as methylmercury conjugates of n-acetylcysteine are substrates of OAT1 in *Xenopus Laevis* oocytes that overexpress rat Oat1 [229, 230]. Further investigation is warranted in order to elucidate which among the different froms of n-acetylcysteine are substrates of OAT1 and OAT3. Furthermore, since probenecid is also known to inhibit organic anion transporting polypeptide(OATP) transporters, albeit less potently [139, 225, 231, 232], future studies should investigate whether NAC or any of its derivatives are substrates of the OATPs.

Adequate brain penetration is of paramount importance for treating CNS pathologies such as traumatic brain injury. Prior to our study, contradictory reports have been published regarding the capacity of NAC to cross the BBB. Following administration of a single dose of n-acetylcysteine IP or IV into the tail vein of mice, n-acetylcysteine levels in the brain and spinal cord are below the lower limit of quantification [233, 234]. However, Offen *et al.* administered

n-acetylcysteine and *N*-acetylcysteine amide both IP and orally and were able to detect *N*-acetylcysteine amide but not n-acetylcysteine in the brain of mice [235]. Similarly, low levels of n-acetylcysteine were measurable in the brain of mice after IP administration and were significantly increased with coadministration of lipopolysaccharide [236]. Route of administration also appears to impact n-acetylcysteine brain distribution. Farr *et al.* reported that n-acetylcysteine crosses BBB readily and accumulated in the brain after administration via jugular vein in mice [237] while Neuwelt *et al.* showed that n-acetylcysteine can cross the BBB when given into the carotid artery in rats [238].

In the current study, we show that NAC is detectable in the brain of juvenile rats after single IP administration using ultra sensitive methods; but that brain clearance of NAC is rapid with undetectable levels by 4 h. Importantly, we also report that NAC brain penetration can be enhanced with probenecid coadministration. NAC brain exposure increased by nearly 2.5 fold and an additional 46% over that expected by the increase in plasma exposure alone, suggesting a possible transporter effect at the BBB or blood-CSF barrier . In addition to renal tubules, OAT3 is expressed in the basolateral side of the endothelial cells on the BBB and epithelial cells of the choroid plexus. Uptake transporters such as OAT3 that are expressed in the basolateral side of the endothelial cells on the BBB and the apical side of the choroid plexus epithelial cells that constitute the blood-CSF barrier have been implicated as initiators of a vectorial transport process that transfers molecules out of the brain into the circulating blood [139]. Such a mechanism has been proposed in the clearance of PGE2, zidovudine and bumetanide from the brain tissue through the choroid plexus and/or BBB [221-223, 239]. Inhibitors of OAT3

decreased the clearance of PGE2, zidovudine, and bumetanide from the CSF and increased levels of these molecules in the brain. The enhanced n-acetylcysteine brain exposure following administration of OAT3 inhibitor probenecid could be explained by the same mechanism. Although we explored the possibility of involvement of MRP1 and MRP4 given their known expression in the brain and probenecid-sensitivity [240], our *in vitro* data suggest that n-acetylcysteine is not a substrate of these transporters.

Recently, clinical modulation of efflux transporters at the BBB as a therapeutic strategy to increase CNS drug levels has been ruled improbable [241]. The findings in this study are provocative in that they support the possibility of increasing the brain exposure of certain drugs by inhibiting uptake transporters at the BBB and/or choroid plexus using an FDA-approved drug with a favorable safety profile. A Phase I/II study is underway (ClinicalTrials.gov Identifier #NCT01322009) to test the safety of n-acetylcysteine and probenecid co-administration in children with TBI as a first step to evaluate this potential therapeutic strategy.

Limitations to the study should be noted. The n-acetylcysteine LC-MS/MS assay used employs sample reduction and derivitization to achieve high sensitivity to detect n-acetylcysteine in biological matrices. While this approach is ideal to measure overall exposure following n-acetylcysteine administration, it cannot distinguish potential downstream forms of n-acetylcysteine such as conjugates. Similarly, an inherant limitation to radiolabeled-based assays is they cannot differentiate between parent molecules such as n-acetylcysteine and conjugates or

degradation products. The work was also conducted in control animals and it is also possible or even likely that n-acetylcysteine pharmacokinetics and/or brain penetration may differ is disease conditions or following injury. Future work will focus on molecular speciation and potential varying interactions with OAT3, OAT1, and probenecid as well as n-acetylcysteine pharmacokinetics following traumatic brain injury.

In summary, we report that co-administration of probenecid reduces the clearance of n-acetylcysteine and increases both plasma and brain levels of n-acetylcysteine in the rat. *In vitro* data reveal that the mechanism of interaction between probenecid and n-acetylcysteine include transport by OAT1 and OAT3. To the best of our knowledge, this is the first study to report the interaction of n-acetylcysteine with OAT1 and OAT3 transporters, and the effect of probenecid on the n-acetylcysteine pharmacokinetics mediated through these transporters. With expanding interest in the use of n-acetylcysteine as an antioxidant therapy for several pathologies, including those within the CNS, these findings have particular relevance for understanding how n-acetylcysteine crosses certain biological barriers. Repurposing the FDA-approved transporter inhibitor, probenecid, is a promising therapeutic strategy to increase n-acetylcysteine exposure.

3. The effect of a combination of n-acetylcysteine and probenecid on functional outcomes in rat, traumatic brain injury model

3.1. Introduction

The objective of the current study is to assess the capacity of a combination of n-acetylcysteine and probenecid to improve histological and neurological functional outcomes in a rats subjected to controlled cortical injury (CCI), a well-established TBI model.

Oxidative stress is a well-established injury mechanism of TBI that cause neuronal damage and death (Bains & Hall, 2012; Hall, 2015, 2016). Numerous studies, both in various animal models of TBI and human TBI patients, have confirmed that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are released excessively during TBI (Awasthi, Church, Torbati, Carey, & Pryor, 1997; Bayir *et al.*., 2002; Deng, Thompson, Gao, & Hall, 2007; Hall & Braughler, 1993; Rodriguez-Rodriguez, Egea-Guerrero, Murillo-Cabezas, & Carrillo-Vico, 2014; Singh, Sullivan, Deng, Mbye, & Hall, 2006). A number therapies such as polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) and tirilazad have been developed to target oxidative stress, for TBI (Bains & Hall, 2012; Kochanek *et al.*., 2015) and have demonstrated effectiveness in

preclinical models of TBI, but none prove to be effective in clinical trials. This may be in part because oxidative stress involves multiple ROS and RNS that propagate oxidative damage via multiple pathways before eventually leading cell damage and death. As such a single agent that targets a single point in the oxidative might not provide sufficient protection (Hall, 2015; Margulies, 2009).

Combination therapy that targets multiple points in the oxidative stress cascade has been proposed to achieve adequate level of neuroprotection (Hall, 2015). In this study, we evaluate a combination of n-acetylcysteine and probenecid, both FDA-approved drugs, to synergistically counter the effects of oxidative stress following TBI through multiple mechanisms. First, n-acetylcysteine, as a thiol, can act as antioxidant by itself by reacting with and scavenging a number of radicals including •OH, •NO2, CO3•− and thiyl radicals (Samuni, Goldstein, Dean, & Berk, 2013). Second, n-acetylcysteine is an acetylated derivative of cysteine and acts a donor of cysteine, the rate limiting substrate in the biosynthesis of glutathione. Hence, n-acetylcysteine can play important role in replenishing intracellular glutathione which is depleted following TBI (Bayir *et al.*., 2002). Third, probenecid can preserve the intracellular pool of glutathione by virtue of its ability to inhibit multidrug resistance protein (MRP) transporters which are involved in active efflux of glutathione and its conjugated from cells (Versantvoort, Bagrij, Wright, & Twentyman, 1995). Finally, by inhibiting of organic anion transporter (OAT)-1 and OAT3, probenecid can increase the exposure of n-acetylcysteine in plasma and importantly in the brain [242]. This is particularly important since inadequate pharmacokinetics and brain distribution

characteristics of CNS targeted drugs has been described as one of the reasons for failed clinical trials.

Previously, we have demonstrated that a combination of probenecid and n-acetylcysteine increases n-acetylcysteine exposure and prevents loss of intracellular glutathione and inhibits neuronal death after mechanical stretch injury *in vitro* [242, 243].

3.2. Methods

3.2.1. Animals

Male pediatric Sprague-Dawley rats, postnatal day 17 (PND-17), 35-43g Harlan Laboratories (Indianapolis, Indiana, USA) were used for pharmacokinetics study (n=18) and for functional outcome and histological studies (n=66). Rat pups were initially housed with dams in a Plexiglas cages in a temperature (21±1°C) and light (on 0700 to 1900) controlled environment, acclimated for one week prior to study, and were allowed access to food and water ad libitum throughout the study. Pups were weaned on PND23 and subsequently pair-housed. All studies were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

3.2.2. Surgery

On the day of surgery, rats weighing 35 - 43g were randomly assigned to either controlled cortical impact (CCI) or sham injury groups. Surgical procedures were performed as previously published [1]. Briefly, rats were weighed, placed in an induction chamber, and exposed to 4% isoflurane in 2:1 N2O:O2, for approximately 1.5 minutes. Rats were then placed into a stereotaxic frame and maintained with a constant flow of inspired anesthetizing mixture of 2% isoflurane, 2:1 N2O: O2 carrier gases via nose cone. All rats were maintained at 37±0.5°C during surgery with a heating blanket. Utilizing aseptic techniques a ~9 mm craniotomy was made over the right hemisphere (between bregma/lambda and the sagittal suture/coronal ridge) with a hand-held high speed dental drill (Aseptico Torque Plus +, Woodinville WA). The impact tip (6mm, flat) was lowered and centered through the craniectomy such that it produced a brain injury of moderate severity (2.4 mm tissue deformation at 4m/sec). After the impact, anesthesia was discontinued, the incision was promptly sutured, and the rats placed in a temperature controlled recovery chamber (maintained at 37±0.5^{o}C) until the effects of anesthesia abated. Rectal temperatures were monitored for 2hrs post-surgery every 15minutes and then rats were returned to their dam and the colony. Sham-operated control rats, received all surgical manipulations; i.e. anesthesia and craniotomy, but did not receive an impact.

3.2.3. Animal pharmacokinetics study

15 minutes after CCI, rats were administered a single dose of 163 mg/kg of n-acetylcysteine (Sigma-Aldrich, St. Louis, MO, USA) in combination with 150 mg/kg of probenecid (Sigma-

Aldrich, St. Louis, MO, USA) or vehicle via intraperitoneal injection. Blood was collected 1 hour after injection by cardiac puncture into heparinized tubes, centrifuged immediately to isolate plasma, and frozen at -80°C for drug analysis. Brain tissue was harvested following decapitation 1 hour after injection and brain hemispheres were isolated. Hemispheres were then homogenized by sonication in brain homogenization buffer (containing 137mM of NaCl, 2.7mM of KCl, 10mM of Na_2HPO4, 1.8mM KH_2PO_4 and 1mM of EDTA dissolved in H2O and adjusted to pH of 7.4) and an aliquot of the homogenate was used for LC-MS/MS analysis. Six rats were studied in each group. The assays for probenecid and n-acetylcysteine assay were described in chapter 2.

3.2.4. Drug preparation and administration for functional outcomes study

Both NAC and probenecid dosing solutions were prepared in NaOH and adjusted to an approximate pH of 7.4 and sodium content of about 0.9%. According to *a priori* block randomization scheme, each of the CCI and sham groups were divided into four groups: CCI+NAC+Probenecid (*n*=11), CCI+NAC+vehicle1 (*n*=10), CCI+Probenecid+vehicle2 (*n*=10), CCI+vehicle1+vehicle2 (*n*=10), Sham+NAC+Probenecid (*n*=5), Sham+NAC+vehicle1 (*n*=5), Sham+Probenecid+vehicle2 (*n*=5) and Sham+vehicle1+vehicle2 (*n*=5). Drugs and/or vehicles were administered together intraperitoneally at 15 minutes, 1h, 12h, 24h, 36h and 48h after injury. Due to the short half-life of NAC, a dose of NAC (or vehicle 2) was also given at 4h. Doses of NAC and probenecid were 163 mg/kg and 150 mg/kg, respectively. Vehicle1 and vehicle2 were normal saline solutions. Equivalent dosing solution volumes (on a per kg weight

of rat basis) and sodium concentrations of all treatments were used to ensure equivalent fluid status and to maintain blinding.

3.2.5. Motor function test

Motor function was evaluated using the beam balance and inclined plane tests on days 1–5 after CCI or sham injury[2, 3], and similar to measures associated with adult motor function post-injury[4-9]; however, slightly modified to accommodate for reduced size of pediatric rat pups. The beam balance task consisted of three trials per day (days 1-5 post-injury), placing the rat on an elevated narrow beam (1.2 cm wide) and recording the time it remained on the beam, for a maximum of 60sec/trial, were recorded and use for statistical analysis. The incline plane task (days 1-5 post-injury) consisted of an adjustable angled surface, which began at 45o to horizontal and progressed at 5o increments to a maximum of 80o to horizontal. Three 10 sec trials per angle were measured (pass/fail). Three failures to complete 10 sec on a single angle resulted in the completion of the task for that day and marked as the maximum angle achieved. The maximum angle achieved each day was used for statistical analysis. Training was conducted for both the beam balance and incline plane task prior to surgery. Rat pups were able to achieve criterion, i.e. three consecutive 60 sec trials on the beam balance, and a maximum angle of 70o. Immediately prior to surgery baseline measures of motor ability were collected.

3.2.6. Spatial learning test

Spatial learning was assessed in a Morris water maze (MWM)[1, 2, 5, 6, 8, 10], which is sensitive to cognitive function/dysfunction after TBI[11-13]. Briefly, the maze consisted of a plastic pool (180 cm diameter, 60 cm high) filled with tap water (26±1°C) to a depth of 28 cm, and was situated in a room with visual cues that remained constant throughout the study. The platform was a clear acrylic glass stand (10 cm diameter, 26 cm high), that was positioned 26 cm from the maze wall in the southwest quadrant and held constant for each rat. Spatial learning acquisition began on postoperative day 11, and consisted of providing a block of four daily trials (4-min inter-trial interval) for 5 consecutive days (11−15) to locate the platform when it was submerged 2 cm below the water's surface (i.e., invisible to the rat). For each daily block of trials the rats were placed in the pool facing the wall at each of the four possible start locations (north, east, south, and west quadrants) in a randomized manner. Each trial lasted until the rat climbed onto the platform or until 120 sec had elapsed, whichever occurred first. Rats that failed to locate the goal within the allotted time were manually guided to it. All rats remained on the platform for 30 sec before being placed in a heated incubator between trials. The times of the four daily trials for each rat were averaged and used in the statistical analyses. The data were obtained using ANYMAZE (Stoelting, Wood Dale, IL).

3.2.7. Memory Retention Test

One day after the final acquisition training session in the MWM (i.e., day 16), all rats were given a single probe trial to measure memory retention. During this trial, the platform was removed from the pool, and the rats were placed in the maze from the location point most distal to the southwest quadrant (i.e., the ‘‘target quadrant’’ where the platform was previously located) and allowed to freely explore the pool for 30 sec and the percent time spent in the target quadrant was recorded. The data were obtained using ANYMAZE (Stoelting, Wood Dale, IL) to record the search areas relative to time, i.e. percent time in the target quadrant.

3.2.8. Histology, cortical lesion volume.

Cortical lesion volumes (mm3) were determined by first calculating the area of the lesion (mm2) by outlining the inferred area of missing cortical tissue for each section taken at 1-mm intervals (MCID software; Imagining Research, Ontario, Canada), and then multiplying the distance between each section (1 mm) by the sum of the lesion area obtained from each section.

3.2.9. Histology, hippocampal neurons survival

An observer blinded to experimental conditions analyzed one coronal section underlying the area of contusion (-3.5 mm posterior to Bregma) from each rat in all groups for determination of

treatment efficacy on selectively vulnerable hippocampal CA1 and CA3 neurons. To reduce counting errors associated with false positive identification of dying neurons, only morphologically intact neurons (i.e., those with a clearly defined cell body and nucleus) were counted using a Nikon Eclipse i90 microscope (Nikon Corporation, Tokyo, Japan) with a 40X objective. Thus, neurons partially seen due to the level of sectioning were not included. All data are reported as the percent of total neurons in the ipsilateral (injured) CA1 and CA3 regions relative to the contralateral hippocampus.

3.2.10. Statistical analysis

A two-tailed Student's t-test was used for two group comparison. Statistical significance among more than two groups was determined by analysis of variance (ANOVA), followed by the Tukey post hoc test for multiple comparisons. A p value less than 0.05 was considered as statistically significant.

3.3. Results

3.3.1. Probenecid increases the brain and plasma levels of NAC after CCI

First, the concentration of probenecid that can be achieved with the chosen dose and route of administration was assessed and compared to the Ki of probenecid for various transporters. One hour after administering a single dose of 150mg/kg probenecid via intraperitoneal route, concentrations of 51.23 ± 12.99 µg/g wet brain tissue and 52.69 ± 3.73 µg/g of wet brain tissue were achieved in the contralateral (uninjured) and ipsilateral (injured) hemispheres respectively, and the plasma level was 313.3 ± 12.99 µg/mL. Next, In order to determine if probenecid increases the plasma and brain levels of NAC in rats after CCI, NAC plasma and brain levels were measured 60 minutes after injection of NAC (and 75 minutes after CCI) in the presence or absence of probenecid. Figure 7A shows that co-administration of probenecid increased NAC plasma levels 1.76-fold (59.01 ± 7.5 to 10.41 ± 11.1 µg/mL; n = 5-6/group, $p < 0.0001$) at 1 h. When given alone, the concentration of NAC was higher in the ipsilateral (injured) hemisphere as compared to the contralateral (uninjured) hemisphere (496.6 ± 186.2 vs 83.9 ± 34 ng/g of wet brain tissue, n = 5-6/group, $p < 0.05$). Co-administration of probenecid significantly increased brain concentration of NAC by 2 fold in the ipsilateral hemisphere (496.6 ± 186.2 to 1004 ± 293.6 ng/g of wet brain tissue, n = 5-6/group, $p = 0.0088$) and by 3 fold in the contralateral hemisphere (83.9 ± 34 to 254.0 ± 22.76 ng/g of wet brain tissue; n = 5-6/group, $p < 0.0001$) (Figure 7B and 7C).

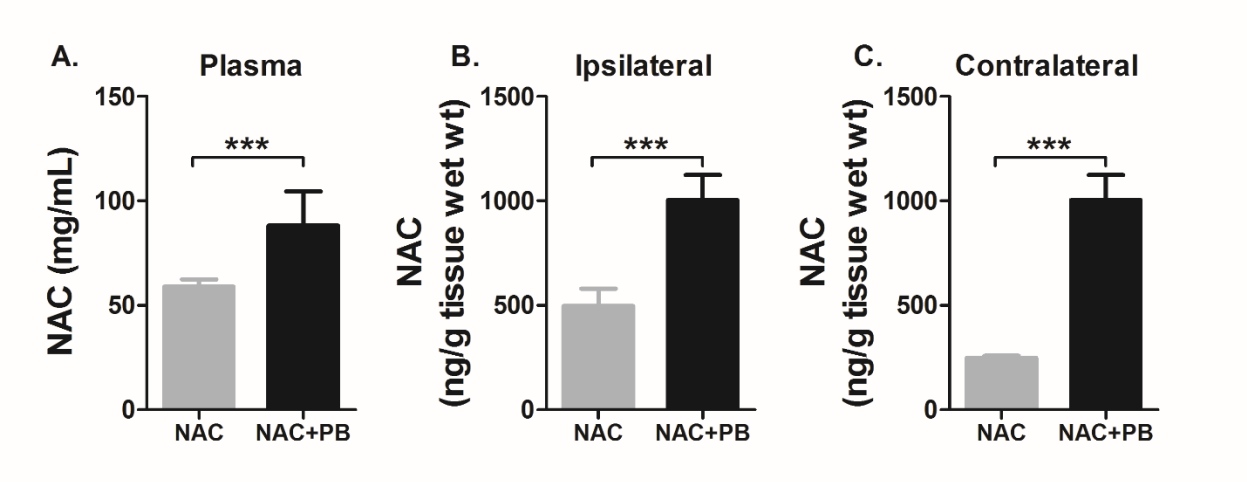

Figure 7. Brain and plasma exposure of NAC after controlled cortical impact (CCI) in the presence or absence of probenecid.

Following CCI, administration of probenecid (150mg/kg) resulted in increased plasma (1.76 fold, panel A) and brain levels (panel B, 2 fold in ipsilateral and 3 fold in contralateral hemispheres) of NAC as compared when NAC was administered alone (163mg/kg). n = 5-6/group, $p<0.0001$). NAC = n-acetylcysteine, PB=probenecid.

3.3.2. Motor performance

Prior to surgery, animals in all groups were able to balance on the beam for the allotted 60 sec on each of three trials (Figure 8A). Two-way, repeat measure ANOVA analysis of the sham groups only shows following surgery, there was a significant Treatment ($F_{3,16} = 4.30$; $p < 0.05$) and Time ($F_{5,80} = 21.26$; $p < 0.0001$) effects, and interaction between Treatment and Time ($F_{15,80} = 3.91$; $p < 0.0001$; Figure 8A). Bonferroni multiple comparison post tests reveal that at Day 1

two of the sham groups (i.e., Sham+NAC+Probenecid and Sham+Probenecid+vehicle2) showed slight but statistically significant impairment compared to each of the other sham groups, Sham+NAC+vehicle1 or Sham+vehicle1+vehicle2 on day 1 ($p < 0.001$ for all comparisons) . However, the impairment was resolved on day 2. Figure 8B shows that all TBI groups showed statistically significant impairment on days 1 and 2. However, two of the TBI groups, CCI+Probenecid+vehicle2 and CCI+NAC+Probenecid, showed worse impairment as compared to the CCI+vehicle1+vehicle2 group which was statistically significant on day 1 ($p < 0.001$ vs CCI+NAC+Probenecid; $p < 0.01$ vs CCI+Probenecid+vehicle2) but not on day2. All TBI groups showed slight impairment on day 3 which was not statistically significant, and their motor function returned to baseline starting on day 4.

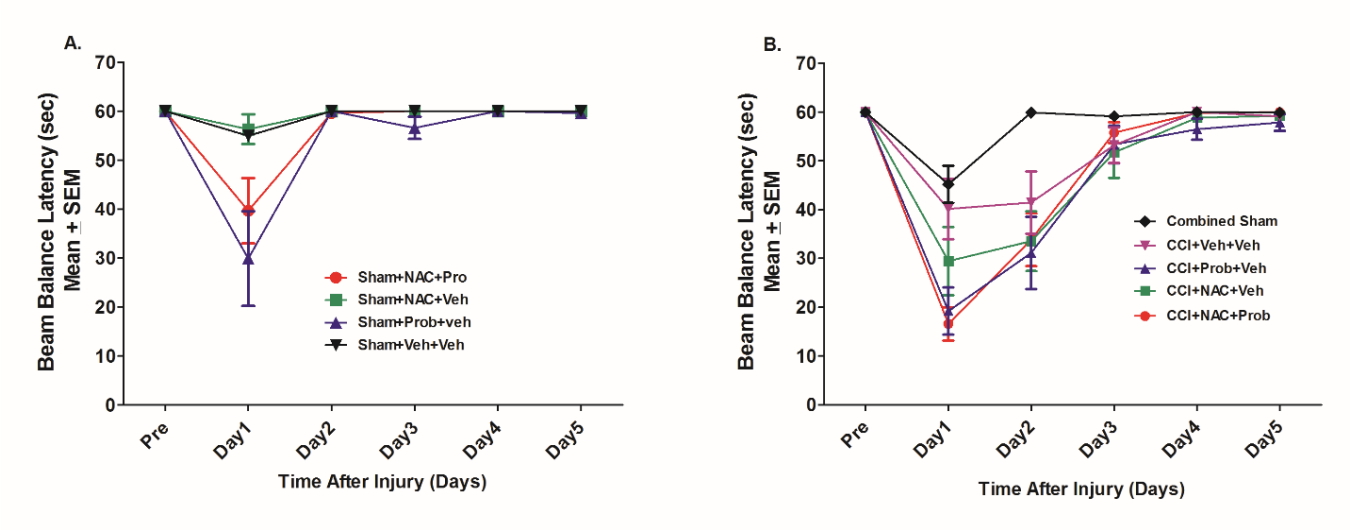

Figure 8. Assessment of the ability of rats to maintain balance on elevated narrow beam before and after CCI or Sham surgery, and drug treatment. 3-A shows sham groups only. 3-B shows all groups.

In the Shams only groups, the presence of probenecid lead to significant motor impairment ($p < 0.05$) in day that was resolved in day 2 (A). Compared to shams, all CCI groups showed significant impairment on days 1 and 2 (B). Two of the TBI groups, CCI+PB+vehicle2 and CCI+NAC+PB, showed more impairment as compared to the other TBI groups, which was statistically significant on day 1 ($p < 0.05$) but not on day2. CCI, n=10-11 per treatment group. Sham surgery, n=5 per treatment group. NAC = n-acetylcysteine, PB=probenecid.

3.3.3. Acquisition of spatial learning

Analysis of the water maze data revealed that all animals acquired spatial learning ability as it took them progressively shortened latencies to locate the hidden platform (Two-way, RM-ANOVA analysis, significant Day differences, $F_{5,280} = 64.36$, $p < 0.0001$; Figure 3.3). The analysis also shows that there is significant Group differences ($F_{4,56} = 12.07$, $p < 0.0001$; Figure 9). Bonferroni multiple comparison analysis revealed that the group difference was due to the Sham group learning to locate the hidden platform faster than each of the CCI groups except the CCI+NAC+vehicle group (for at least 2 days out of total 6 days of testing, $p < 0.05$). There was not Group x Day interaction ($F_{20,280} = 1.21$, $p > 0.05$). Analysis of the visible platform performance task reveals a significant group difference as the SHAM group took significantly less time to locate the platform than each of the CCI groups ($F_{4,57} = 7.204$, $p < 0.05$). The CCI groups didn't differ in terms of performance from one another

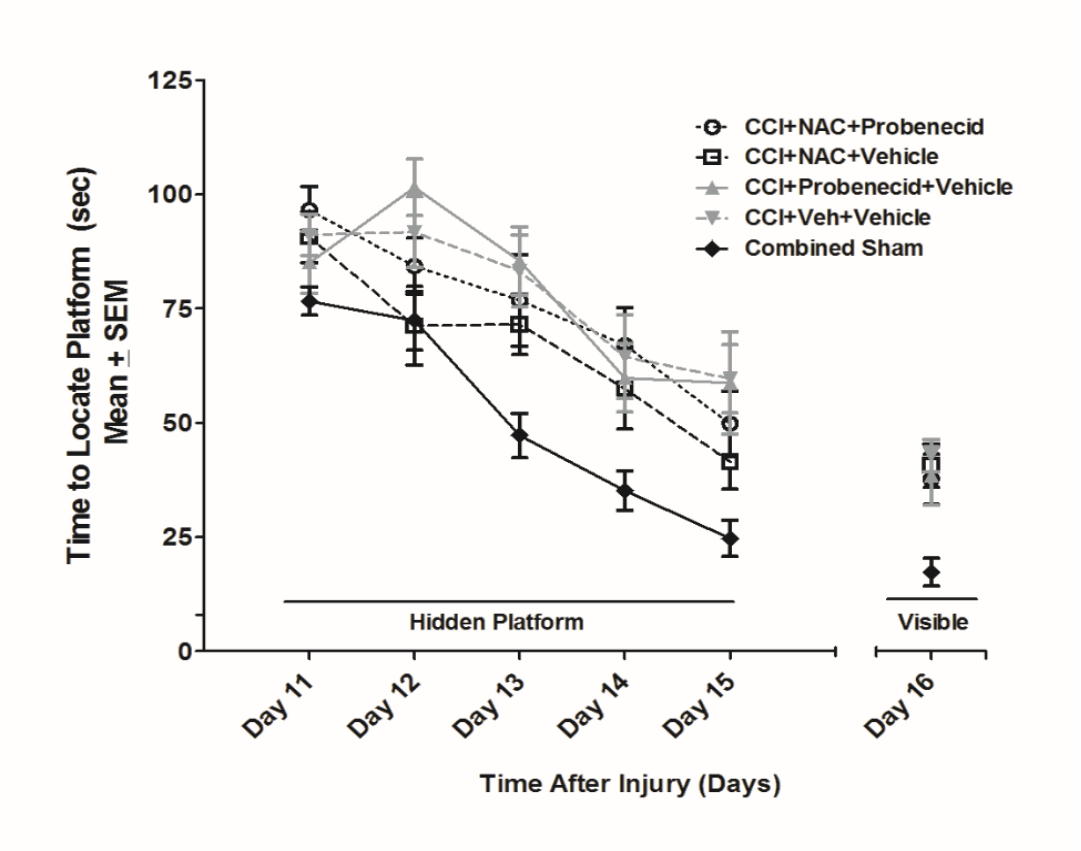

Figure 9. Evaluation of time to locate hidden and visible platform in a water maze.

Analysis of the MWM, hidden platform trial data shows a significant difference among groups ($F_{4,56} = 12.07$, $p < 0.0001$). Bonferroni multiple comparison analysis revealed that the group difference was due to the Sham group performing better than each of the CCI groups except the CCI+NAC+vehicle group ($p < 0.05$). CCI, n=10-11 per treatment group. Sham surgery, n=5 per treatment group. NAC = n-acetylcysteine, PB=probenecid.

3.3.4. Memory Retention.

Spatial memory retention was determined by single probe trial where the time the rats spent the target quadrant is measured. Analysis of the single probe trial data shows a significant difference among groups ($F_{4,57} = 3.269$, $p < 0.05$) which after post-hoc analysis was found to be due to the SHAM group performing better than the CCI+NAC+Probenecid ($p < 0.05$) and CCI+Probenecid+vehicle ($p < 0.05$). The CCI+NAC+vehicle group performed better than any of the other CCI groups, but the difference didn't reach significant level against any of the groups.

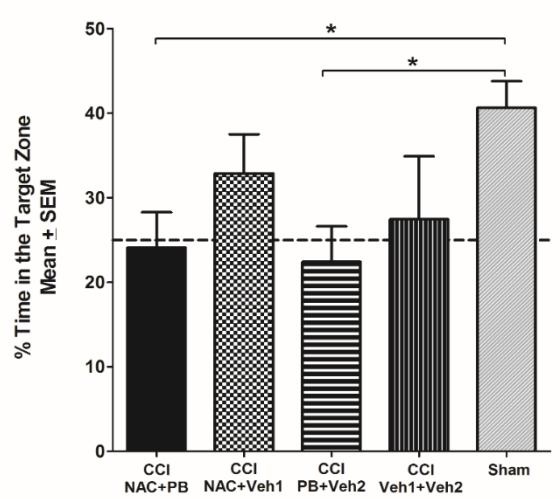

Figure 10. Evaluation of percentage of time spent in the quadrant where the platform was previously located.

Sham group performed better as compared to the CCI+NAC+PB ($p < 0.05$) and CCI+PB+vehicle ($p < 0.05$). In both MWM tests, the CCI+NAC+vehicle group performed better than any of the other CCI groups, but the difference did not reach significance after a single trial 16 days after CCI or Sham surgery. CCI, n=10-11 per treatment group. Sham surgery, n=5 per treatment group. NAC, N-acetylcysteine; PB, Probenecid.

3.3.5. Tissue loss volume.

Analysis of tissue volume loss after injury showed that probenecid and n-acetylcysteine, when given alone, didn't result in improvement of tissue loss as compared to those treated with vehicle only. When given in combination, probenecid and n-acetylcysteine, attenuated tissue loss in significant manner (Figure 11, 59±3.5 mm^3 vs 80±4.5 mm^3, $p < 0.05$).

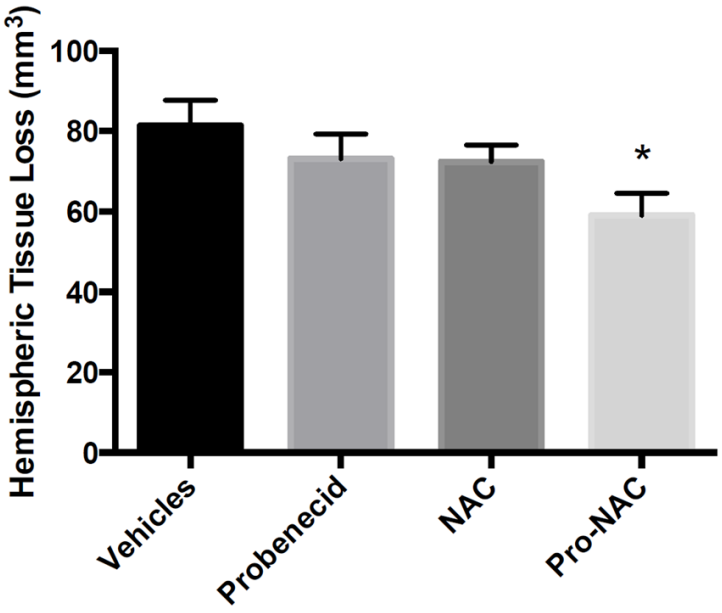

Figure 11. Tissue volume loss

Treatment with a combination of probenecid and n-acetylcysteine resulted in significant attenuation of tissue volume loss after CCI ($p<0.05$). CCI, n=10-11 per treatment group. Sham surgery, n=5 per treatment group. NAC = n-acetylcysteine, PB=probenecid.

3.3.6. Hippocampal neuronal survival.

As shown in Figure 12, as compared to sham groups, each of the injury groups demonstrate decreased neuronal survival in both CA1 and CA3 regions of the hippocampus ($p<0.05$). However, there was not difference among each of the injury groups indicating that treatment with either probenecid or n-acetylcysteine, alone or in combination, didn't improve neuronal survival in the hippocampus ($p>0.05$).

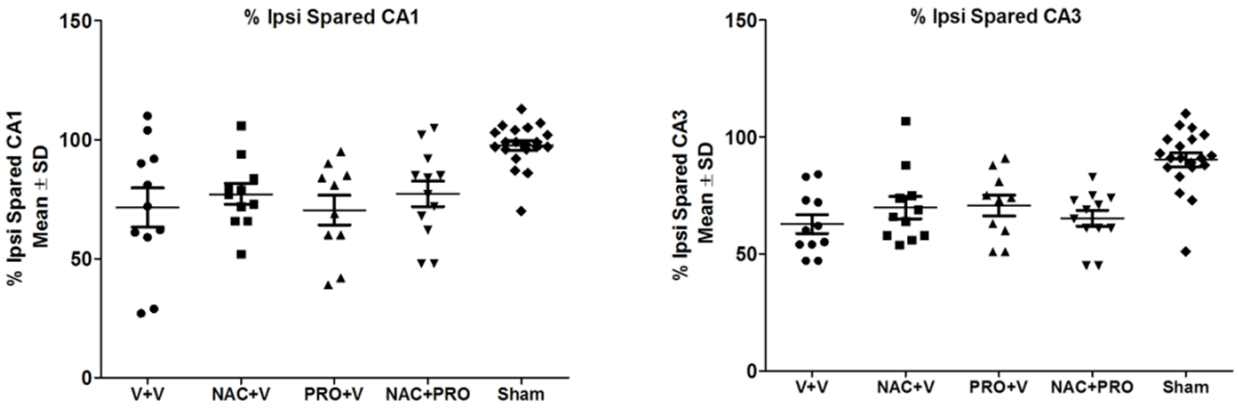

Figure 12. Hippocampal neuronal survival analysis

No treatment benefit was observed in hippocampal neuronal survival when probenecid or n-acetylcysteine were given alone or in combination ($p>0.05$). CCI, n=10-11 per treatment group. Sham surgery, n=5 per treatment group. NAC = n-acetylcysteine, PB=probenecid

3.4. Discussion

In this study we assessed the capacity of a combination therapy strategy involving the transporter inhibitor probenecid and the antioxidant n-acetylcysteine to attenuate oxidative stress thereby improving histological and functional outcomes in pediatric rat model of TBI.

Over the past thirty years, oxidative stress has been studied intensity as one of the main TBI injury mechanisms [198, 199]. It was a target of numerous therapeutic interventions, albeit with no clinical efficacy reported to date. Limited penetration of antioxidant drug candidates has been attributed as one of the reasons for failure of TBI clinical trials [7]. In this study, we demonstrated that probenecid can increase the delivery of n-acetylcysteine into the brain in CCI model of pediatric TBI rats. First, we showed that a single dose of 150mg/kg probenecid via intraperitoneal route can achieve concentrations of 51.23 ± 12.99 µg/g of wet brain tissue and 52.69 ± 3.73 µg/g of wet brain tissue in the contralateral and ipsilateral hemispheres respectively. The plasma level was 313.3 ± 12.99 µg/mL. The concentrations achieved in all cases were at least twice the Ki of probenecid for several transporters which ranges 0.5-30 µg/mL [232, 244-247]. Next, we showed that the n-acetylcysteine penetration into the brain was increased 2-3 fold by probenecid as early as 1h after injury. The findings in this chapter corroborate what we observed in naïve pediatric rats in the Chapter two of this dissertation. The mechanism behind the effect of probenecid on systemic and brain pharmacokinetics of n-acetylcysteine is discussed in detail in Chapter two. It involves inhibiting OAT1, OAT3 and/or MRP transporters in renal

tubules, BBB and choroid plexus. This finding highlights the therapeutic potential of inhibiting transporters in order to enhance brain penetration of candidate drugs for TBI.

Another major reason for failure of TBI clinical trials of drugs targeting oxidative stress is the notion that a single agent can't match the complex and multiple ways oxidative stress propagates before eventually leading to neuronal injury and death [7, 207, 218]. In this study, we utilize probenecid to, not only increase brain levels of n-acetylcysteine, but also to preserve the brain levels of the major endogenous antioxidant, glutathione, which is depleted following TBI [201, 248]. Since n-acetylcysteine is antioxidant on itself, and a precursor to glutathione, the combination of n-acetylcysteine and probenecid could have a synergistic effect in countering oxidative stress in TBI. We assessed this therapeutic strategy in rat CCI model of pediatric TBI.

In the motor function test, we observed that administration of probenecid, alone or in combination of probenecid, causes temporary impairment of motor function in both SHAM groups and CCI groups (Figure 8). The clinical relevance of this transient effect is unclear. On one hand, if the adverse effect of probenecid in limited to motor deficit, given most sever TBI patients are bed-ridden in the few days after injury, the clinical implication of this finding will also be limited. Admittedly, it is difficult to specifically measure motor function immediately after injury, however, in the phase-I safety clinical study of a combination of probenecid and n-acetylcysteine in pediatric severe TBI patients, no indications of motor deficit were observed [249]. On the other hand, if the transient motor deficit observed with administration of

probenecid is related to or is an indication of long-term functional outcomes, the clinical consequence will be significant. Either way, more preclinical study to establish the mechanism of this adverse effect of probenecid in TBI should be conducted.

In cognitive assessment, n-acetylcysteine, when given alone, attenuated both spatial learning and memory retention deficits but the improvements versus CCI groups (i.e., placebo group or the other treatment groups) did not reach statistical significance. However, the performance of the n-acetylcysteine treated group was also not statistically different from that of the SHAM injured group (Figure 9). Interestingly, the mean scores in both tests for probenecid only treated group was the worst compared to all the other groups. Also, the mitigating effects observed with n-acetylcysteine only group was not observed with the probenecid and n-acetylcysteine treated group although probenecid is expected to enhance the brain penetration of n-acetylcysteine. This suggests that the adverse effects of probenecid may be negating any potential beneficial effects of n-acetylcysteine.

A recent review has evaluated the efficacy of n-acetylcysteine and its analogs in clinical and preclinical studies of TBI [250]. The review concluded that the lack of well-designed and controlled clinical studies made the proper evaluation of effectiveness of n-acetylcysteine in human TBI patients impossible based off the currently completed studies. In animal models, however, the study which reviewed 20 preclinical studies, found overall strong effect of n-acetylcysteine and its analogues in improving neurological functional outcomes and attenuating

various inflammatory and oxidative stress markers in brain tissues. Differences in animal models, routes of administration, injury types and assessment methods make it difficult to assess dose-effect relations from the past experiments and our experiment. However, the overall strong signal of effectiveness n-acetylcysteine in TIB observed warrants continued examination of n-acetylcysteine alone or in combination in TBI with higher doses. Risk of adverse effects of higher doses of n-acetylcysteine also appear to be minimal since no safety concerns were raised by both clinical and preclinical studies [250].

Interestingly, although undesirable in the context of current objectives of the therapeutic intervention, the adverse effects observed with probenecid is highly suggestive of potentially important role that membrane transporters inhibited by probenecid, including OAT1, OAT3, OATP1B3 and MRPs [232, 244-247, 251], play in re-gaining homeostasis after brain injury.

In histological assessment, rats treated with n-acetylcysteine in combination of probenecid showed modest improvement in tissue volume loss that was statistically significant (Figure 11). No treatment benefit was observed in hippocampal neuronal survival either drug alone or in combination (Figure 12).

The main limitation of the study was that it didn't incorporate multiple doses of n-acetylcysteine and probenecid to establish dose-effect relationship for both drugs. With regards to n-acetylcysteine, higher or more frequent dose would have helped assess whether the spatial learning and memory deficit attenuation of observed with the given dose is a true effect, in that

these effects would have reached statistical significance. Similarly, study design with multiple doses of probenecid in both the pharmacokinetic and function outcomes studies would have allowed thorough evaluation of the capacity of probenecid to increase levels of n-acetylcysteine without adverse effects. Given the levels of probenecid achieved with the dose used in this study are significantly higher than the Ki of probenecid of most transporters [232, 244-247, 251], it is possible that significant increase of level of n-acetylcysteine could be achieved even with lower doses of probenecid.

In conclusion, in this study we found that, following experimental TBI, at the given dosage regime, n-acetylcysteine appears to attenuating spatial learning and memory retention deficits that didn't reach statistical significance. Probenecid seems to cause acute motor deficit, and the combination of n-acetylcysteine and probenecid improved tissue volume loss. However, probenecid increased plasma and brain levels of n-acetylcysteine significantly. Hence, a combination of n-acetylcysteine and probenecid with dose regimen optimization of both drugs to enhance the beneficial effects of NAC and minimize the adverse effects of PB should be pursued as therapeutic strategy in TBI.

4. Neuropharmacometabolomics of Treatment Response of a Combination of Probenecid and N-Acetylcysteine in Traumatic Brain Injury

4.1. Introduction

The biochemical milieu of the CNS is dynamic and tightly regulated, and can be explored in exquisite detail using metabolomics [252]. Recently, metabolomics has been used to evaluate biochemical responses and identify relevant metabolic pathways in CNS disease [253]. However, to-date the metabolomics profile after TBI—an important cause of mortality and morbidity in humans [254]—has not been investigated [255]. In TBI, the primary impact onto the brain and/or disruption of brain tissue is followed by a set of secondary biochemical processes including a surge in excitatory amino acids, glycolysis, oxidative stress, and mitochondrial dysfunction [256]. Given that CSF provides a window into the CNS, evaluation of the metabolomics profile in CSF after TBI and identification of prominent biochemical pathways altered in response to injury, could uncover new and validate suspected pathophysiologic mechanisms in humans, and define clinically relevant therapeutic targets. In addition to establishing the metabolomics signature of TBI, evaluation of CSF metabolites could also be used to determine in detail the holistic effects of pharmacotherapies, such as target engagement or potential toxicity.

While there are many regulators of the biochemical milieu of the brain at the BBB and blood-CSF barriers, membrane transporters such as ABC and SLC transporters such as organic anion transporters (OAT) are particularly important in terms of brain bioavailability of clinically used drugs, especially those that are lipophilic and do not readily cross the BBB. Probenecid is a promiscuous SLC and ABC transporter inhibitor that has been used to identify CSF elimination pathways for endogenous organic acids and monoamines such as homovanillic acid and serotonin [257], and exogenous drugs such as methotrexate [258]) and N-acetylcysteine [242].

We recently reported the repurposed use of probenecid and NAC in a randomized, placebo-controlled phase I trial (Pro-NAC I) after severe TBI in children [160, 249] to capitalize on this drug interaction. As part of the Pro-NAC I trial, CSF was collected for pharmacokinetic assessment of probenecid and NAC, providing an opportunity for a "pharmacometabolomics" interrogation of the CNS. Accordingly, the purpose of the present study was twofold, to evaluate metabolomic changes in human TBI compared with control subjects, and to evaluate pharmacometabolomic changes in TBI patients treated with probenecid and NAC compared with placebo.

4.2. Materials and Methods

4.2.1. Parent Study

Samples for this study represent CSF obtained from patients who participated in an Institutional Review Board approved, randomized, double-blind, Phase I study (Trial Registration NCT01322009) of the combination of probenecid and NAC (n = 7) versus placebo (n = 5) in children 2 to 18 years-of-age after severe TBI (Glasgow Coma Scale [GCS] score ≤ 8) recruited from November 2011-September 2013 at a single, tertiary children's hospital [160]. The study was approved by the University of Pittsburgh Institutional Review Board and informed consent was obtained from parents and/or legal guardians of all children enrolled in the study. Control CSF samples were obtained from five age-matched control subjects who underwent lumbar puncture to rule out meningitis.

4.2.2. Sample Collection

CSF Samples for this study were collected 41.7 hours (range 34-47.25) after injury and 24 hours after the first dose of probenecid (25 mg/kg) and NAC (140 mg/kg). Drugs or placebo were administered via naso/orogastric tube. CSF was immediately centrifuged at 3000 ×g for 10 min and supernatant was stored at -80°C for batch analysis.

4.2.3. UPLC-QTOFMS Analysis

CSF samples were prepared by first precipitating protein by addition of acetonitrile at a 3:1 ratio followed by centrifugation. Five µL of supernatant was injected into ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOFMS, Synapt G2-S Waters, Milford, MA). An Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm; Waters, Milford, MA) was used to separate metabolites. The flow rate of the mobile phase was 0.5 mL/min using a gradient ranging from 2% to 98% acetonitrile/water containing 0.1% formic acid over 12 min. The column temperature was maintained at 50°C. Time-of-flight mass spectrometry (TOFMS) was operated in positive and negative modes with electrospray ionization. The source and desolvation temperatures were set at 150°C and 500°C, respectively. Nitrogen was applied as cone and desolvation gas, and the gas flow rates were set as 50 L/h and 800 L/h, respectively. Capillary and cone voltages were set at 0.8 kV and 40 V, respectively. TOFMS was calibrated with sodium formate and monitored by the intermittent injection of lockspray leucine encephalin in real-time. Mass spectrometry (MS) data were acquired in centroid format over a range of 50-1000 Da with an acquisition time of 0.1 sec/scan.

4.2.4. Metabolomics Analysis

The XCMS-online metabolomics platform (Scripps Research Institute, CA) was used for data management and analysis including feature detection, retention time correction, alignment, annotation, principal component analysis (PCA), statistical analysis, and data visualization [259-

262]. Raw data files were initially converted to mzData files using ms2mz. mzData was uploaded to XCMS-online and the data was processed as a pair-wise experiment in positive and negative MS modes when comparing control subjects versus placebo-treated TBI groups, and when comparing placebo-treated TBI versus probenecid + NAC treated TBI groups. A multi-group experiment setup was also used in positive and negative MS modes where the three groups were compared simultaneously.

For data analysis with XCMS-online, the centWave method was used for feature detection with 15 ppm m/z tolerance, minimum peak width of 2 sec and maximum peak width of 25 sec. The Obiwarp function was used for retention time correction. Chromatogram alignment was performed with mzwid (mz width) = 0.1, minfrac (minimum fraction) = 0.05, and bw (bandwidth) = 2. Isotopes and adducts were annotated using CAMRRA with m/z absolute error of 0.015 and 5 ppm. Selection and filtering of metabolite features for inclusion in pathway/network analysis were done based on fold change ≥ 1.5 and p-value ≥ 0.01 based on unpaired parametric t-test (Welch t-test) when comparing only two groups, or one-way ANOVA with Tukey's post-hoc test when comparing the three groups together. Metabolites were identified by searching the Human Metabolome Database (HMDB) and with Δppm < 10.

4.2.5. Pathway and Network Analysis

Network and pathway analyses were performed using the software *Mummichog* (Emory University, GA) which is incorporated within the XCMS-online platform [261]. The significant

metabolite features in the pair-wise comparisons were used as input, and the total list of features were used as reference. For the pathway analysis, the *Mummichog* software tested the enrichment of input metabolites against random data resampled from the reference list (in the background of known human metabolic pathways) and provided adjusted p-values while accounting for the fact that single m/z values can map to different metabolites and different m/z features can represent a single metabolite. Network analysis was done in a similar way to pathway analysis except that compounds are placed within the human metabolic network model. Modules, which by definition show more internal connectivity than expected randomly in the whole network, are identified by their connectivity. Output data from the network analysis were imported to Cytoscape 3.4.0 [263] to generate network visualization. Excel 2013 (Microsoft Corporation, WA) was used to generate cloud plots of the impacted pathways. R-Studio 1.0.136 was used to generate volcano plots of metabolite features.

4.3. Results

4.3.1. Patient Characteristics

In the parent study 14 patients were randomized into either probenecid + NAC (n = 7) or placebo groups (n = 7) [160]. Residual CSF was available from 12 patients collected 24 hours after the first drug or placebo administration, seven in the probenecid + NAC group and five in the

placebo group. The demographic characteristics of the study participants are summarized in Table 4.1.

Table 2. Summary of Patient Characteristics

Patient Characteristics	**Control Subjects**	**TBI Placebo**	**TBI Probenecid+NAC**
N	5	5	7
Age (years), mean (SD)	10.0 (8.9)	7.5 (5.0)	8.6 (4.9)
Weight (Kg), mean (SD)	Unknown	26.4 (14.3)	37.9 (21.3)
Male sex (%)	2 (40%)	2 (40%)	6 (86%)
White race (%)	Unknown	5 (100%)	6 (86%)
Glasgow Coma Scale score, median [range]	N/A	6 [4-7]	6 [4-6]

4.3.2. Multi-group metabolomics analysis

Multi-group analysis was performed to compare the CSF metabolomics profiles of control subjects, TBI patients administered placebo, and TBI patients treated with the combination probenecid and NAC. Two-component PCA revealed clustering within each of the study groups (Figure 13), and separation between groups, demonstrating that the three groups have different metabolomics signatures.

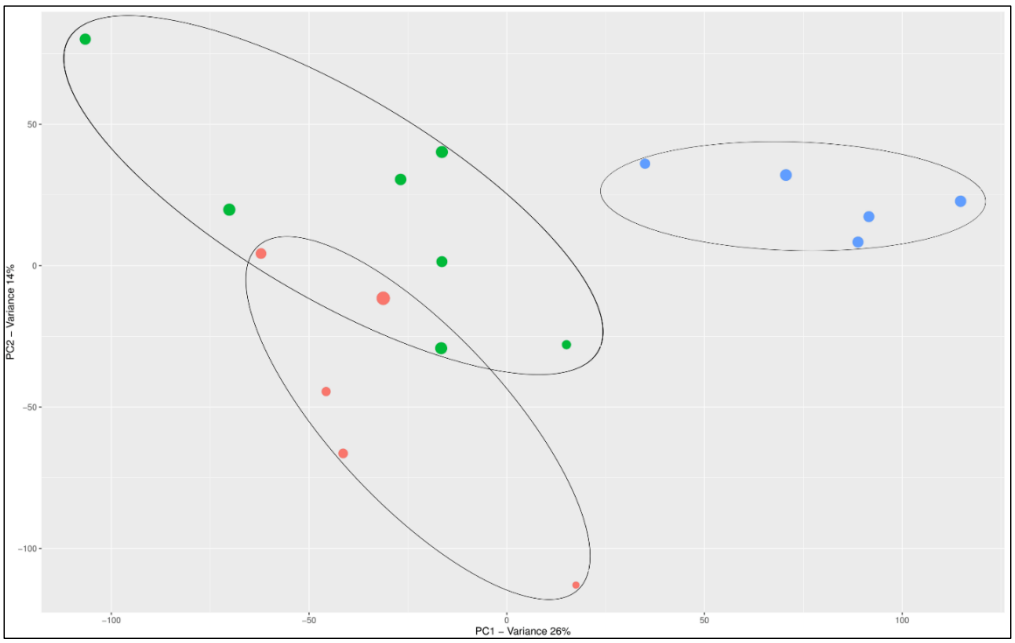

Figure 13. PCA analysis based on the first two components shows clustering of the three study groups based on their difference in metabolic profile.

Blue = control subjects, red = TBI treated with placebo, green = TBI treated with probenecid and NAC .The dot size indicated values of DModX which indicates the observed distance of a particular individual to the principal components model.

As a means of internal validation, we sought to identify drugs and their metabolites uniquely administered to one or more groups. We specifically evaluated probenecid, as a study drug received only in the probenecid + NAC TBI group, and phenytoin, which was administered to 11 of 12 TBI patients for seizure prophylaxis. Searching for either the parent drugs or metabolites of probenecid and phenytoin among the putative metabolites list shows matching features with expected differential abundance in the three different groups (Figure 14A and B). In addition, the peak area of probenecid determined by UPLC-QTOFMS correlated with previously quantified probenecid concentrations determined by UPLC-MS/MS [160, 249][158, 247](Clark, Empey et al. 2017, Jha, Puccio et al. 2017)[249]in individual patients treated with probenecid and NAC (Figure 14C; $r^2 = 0.76$, $p = 0.01$).

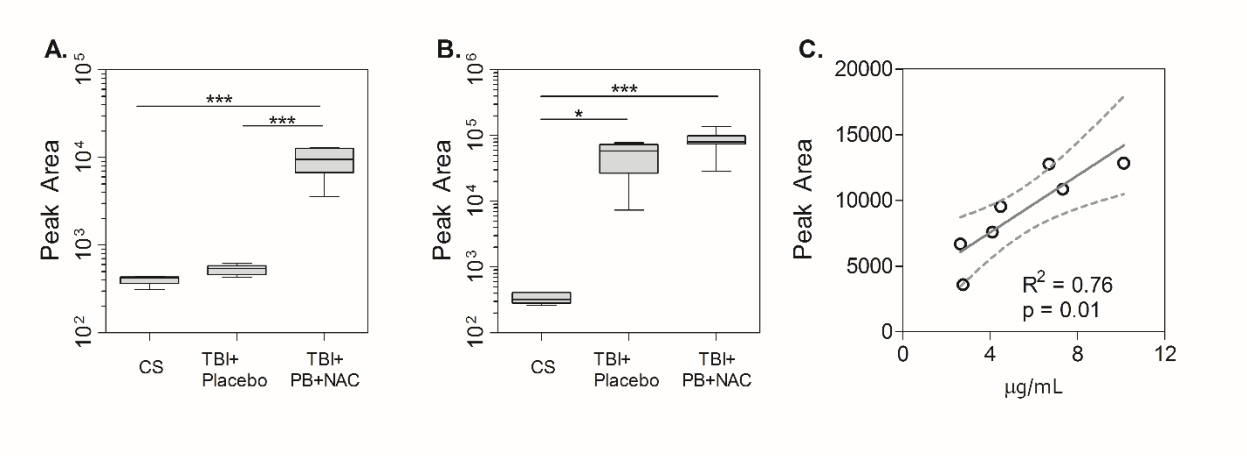

Figure 14. The abundance of probenecid and phenytoin in the three study groups.

(A) Shows that the feature in the mass spec, putatively identified as probenecid was increased in the treated TBI group as compared to either the placebo treated TBI group or HC group (***p>0.001, n=5-7). (B) Shows that phenytoin, administered as a standard of care after TBI to prevent seizures, increased in both TBI groups as compared to the healthy control group (***p>0.01, *p>0.01 n=5-7). PB = Probenecid, NAC=n-acetylcysteine, CS=Control Subjects. (C) Shows good correlation between peak area of probenecid determined by UPLC-QTOFMS and previously quantified probenecid concentrations determined by UPLC-MS/MS in individual patients treated with probenecid and NAC (R^2 = 0.76, p = 0.01).

4.3.3. Pair-wise analysis of control subjects vs. placebo treated TBI patients

To identify metabolites that are altered in response to TBI, CSF samples from placebo treated TBI patients and control subjects were run through UPLC-QTOF MS. Retention time, ion fragments, and abundance were entered into XCMS-online for pair-wise comparison. The m/z-retention time cloud plots in Figure 15 show 1010 features in the positive MS mode and 89 features in the negative MS mode that were significantly increased or decreased based on a p-

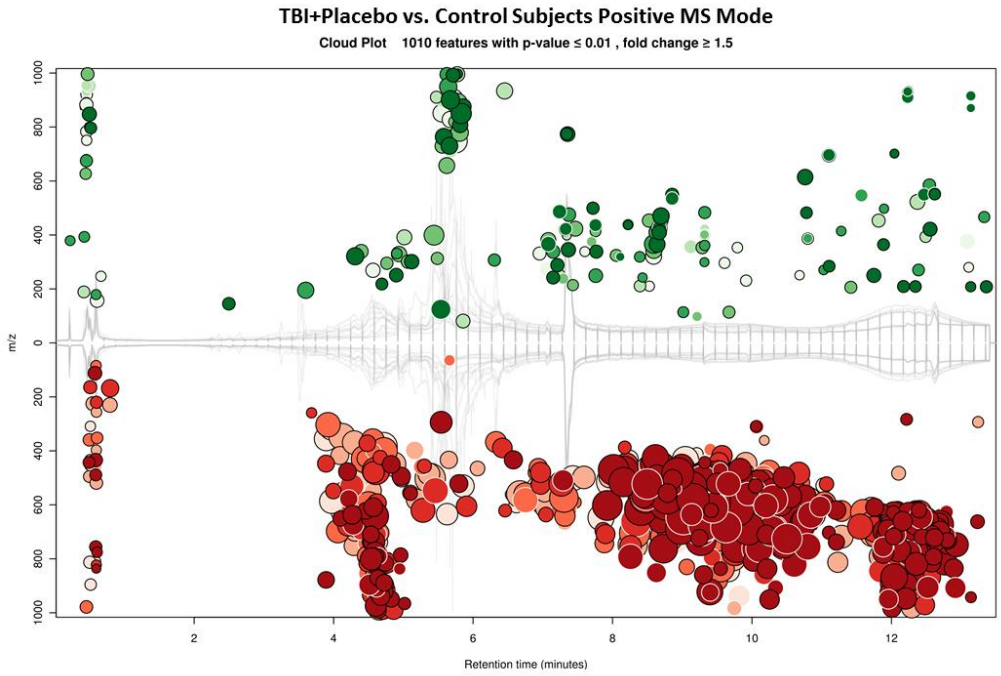

Figure 15. Cloud plots of features increased (green) or decreased (red) in TBI patients compared to HC in positive MS mode.

The size of the circle corresponds to the fold change of a particular feature between the two groups. The intensity of the color corresponds to the p-value with more intense shades representing a smaller p-value.

value of ≤ 0.01 and fold change of ≥ 1.5. Note that the majority of features appeared to decrease in TBI patients vs. control subjects (red vs. green circles in the cloud plot, respectively).

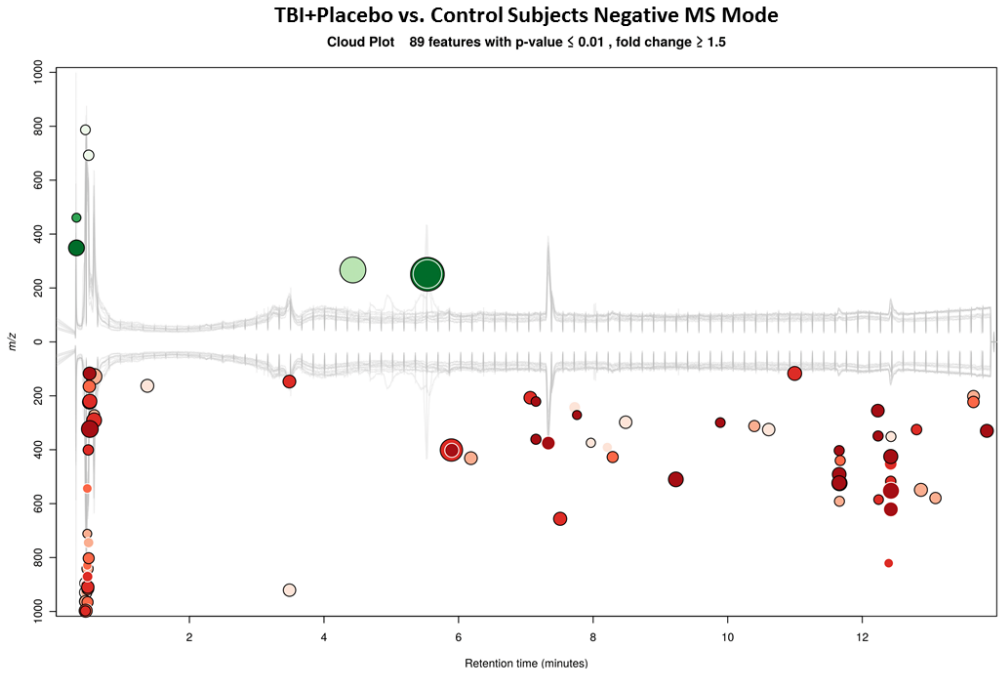

Figure 16. Cloud plots of features increased (green) or decreased (red) in TBI patients compared to HC in negative MS mode.

The size of the circle corresponds to the fold change of a particular feature between the two groups. The intensity of the color corresponds to the p-value with more intense shades representing a smaller p-value.

4.3.4. Pathway analysis of control subjects vs. placebo treated TBI patients.

To gain insight into the molecular mechanisms underlying the pathogenesis of TBI by identifying previously suspected or novel biological pathways altered after TBI, we conducted a

pathway analysis of CSF metabolites in placebo treated TBI patients compared with control subjects. Metabolites that were significantly changed in response to TBI were enriched in six pathways in the positive MS mode and 17 pathways in the negative MS mode. One pathway in the positive MS mode and six pathways in the negative MS mode showed ≥ 50% overlap of significantly altered putative metabolites with known components of human biological pathways (Figure 17 and Table 2).

Table 3. Metabolic pathways altered in TBI patients compared with control subjects

Pathway	Mode	Overlap size	Pathway size	Percentage overlap	Overlapping features
α-tocopherol degradation	+	3	6	50%	α-tocopherol, 13'-hydroxy-α-tocopherol 5'-α-carboxylmethylbutylhydroxychroman
Lactate fermentation (re-oxidation of cytosolic NADH)	-	2	2	100%	(S)-Lactate Pyruvate
Pyrimidine deoxyribonucleosides degradation	-	3	4	75%	2'-deoxyuridine, 2-deoxy-α-D-ribose 1-phosphate Thymidine
Methylglyoxal degradation VI	-	3	4	75%	(R)-Lactate Methylglyoxal Pyruvate
Spermine and spermidine degradation I	-	2	3	66.7%	3-acetamidopropanal N1-acetylspermidine
Methylglyoxal degradation I	-	3	5	60%	Glutathione Pyruvate Methygyloxal (R)-S-Lactoylglutathione (R)-Lactate
Cysteine biosynthesis/ homocysteine degradation (trans-sulfuration)	-	2	4	50%	2-oxobutanoate L-Homocysteine

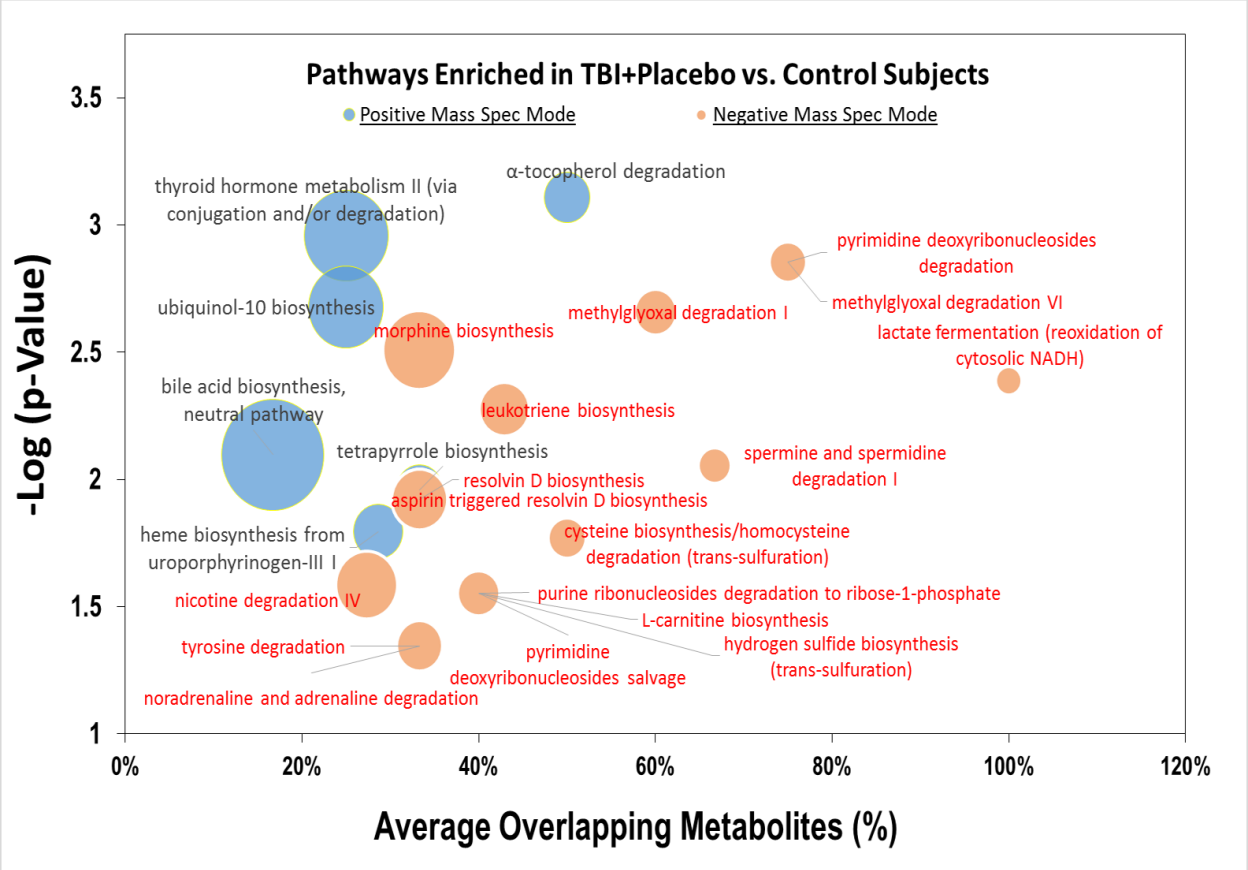

Figure 17. Cloud plot of affected pathways in placebo treated TBI patients as compared to control subjects.

Darker circles mean more than one pathways with the same average overlapping metabolites and p-value are represented. The radius of each circle represents the number of metabolites relative to the number of metabolites represented by other circles.

4.3.5. Individual metabolites altered in control subjects vs. placebo treated TBI patients

Figure 18 shows a volcano plot of metabolites in CSF in control subjects and TBI patients. Similar to the cloud plots in Figure 13, the majority of features appeared to decrease in TBI

patients vs. control subjects. Metabolites with maximum absolute intensity ≥ 2,500, -log(p) > 3, and $\log_2$(fold change) > 1 were selected for possible identification. Excluding metabolites with poor chromatographic resolution by manual inspection yielded 41 significant features. To identify putative metabolites that correspond to these features, their masses were searched in the HMDB based on a 10 ppm error margin [264-266]. Twelve of the searched features returned metabolites or putative metabolites that were of dietary or exogenous drug source. The rest of the features yielded one or more endogenous metabolites with a previously known or biologically plausible association with TBI. Putative metabolites in 19 of these features corresponded to long-chain fatty acids, which because of the known difficulty with distinguishing among analogues with the same m/z, were not further characterized. The final list of 10 features and their corresponding TBI-associated metabolites is shown in TableA1 in Appendix A. Of potential interest, are changes in multiple species of gangliosides, ceramide, and dipeptides in TBI patients relative to control subjects.

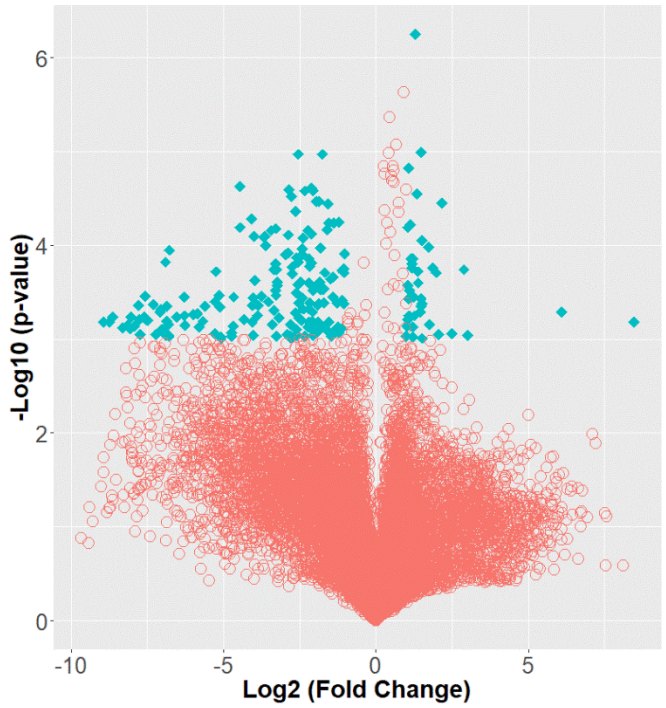

Figure 18. Volcano plot of metabolome profiles of the CSF of placebo treated TBI patients as compared to control subjects in both positive and negative mass-spectrometry modes. Metabolites that were upregulated or downregulated by ≥ two-fold with a p-value < 0.001 are shown in blue.

4.3.6. Pair-wise analysis of TBI patients receiving placebo vs. probenecid + NAC

To elucidate the impact of probenecid and NAC treatment on the CSF metabolomics profile after TBI, a pair-wise comparison of placebo-treated TBI patients and combination therapy treated TBI patients was performed. The cloud plot in Figure 19 and Figure 20 shows that using criteria of a p-value ≤ 0.01 and fold change ≥ 1.5, there are 153 features in the positive mode and 296

features in the negative mode that were significantly different between the two groups respectively. Seventy-four in the positive mode and four in the negative mode were down-regulated in the probenecid + NAC group, while 79 in the positive mode and 294 in the negative mode, were up-regulated.

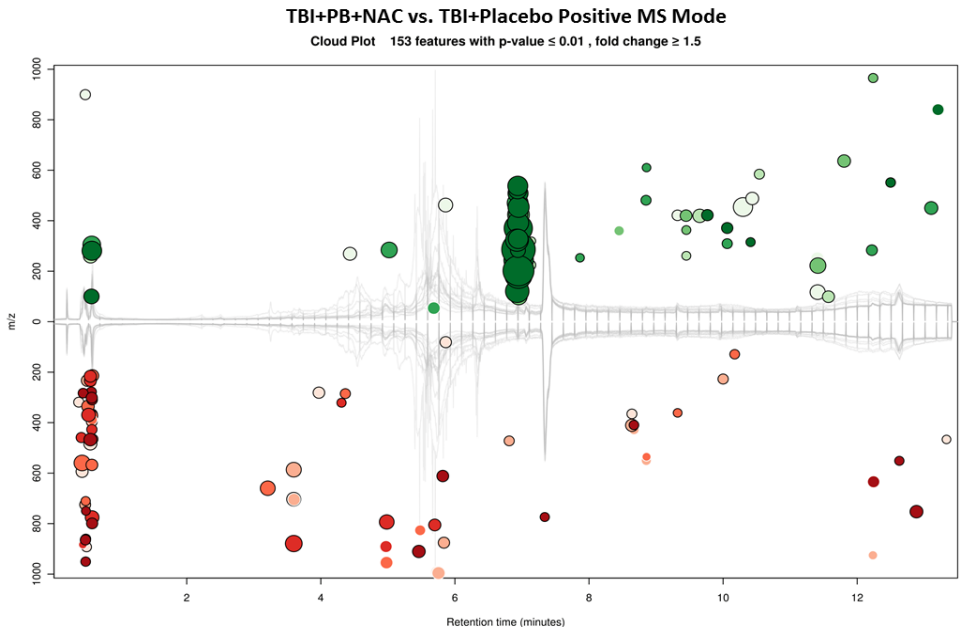

Figure 19. Cloud plots of features increased (green) or decreased (red) in TBI patients treated with probenecid+NAC as compared to placebo treated TBI patients in positive MS mode.

The size of the circle corresponds to the fold change of a particular feature between the two groups. The intensity of the color corresponds to the p-value with more intense shades representing a smaller p-value.

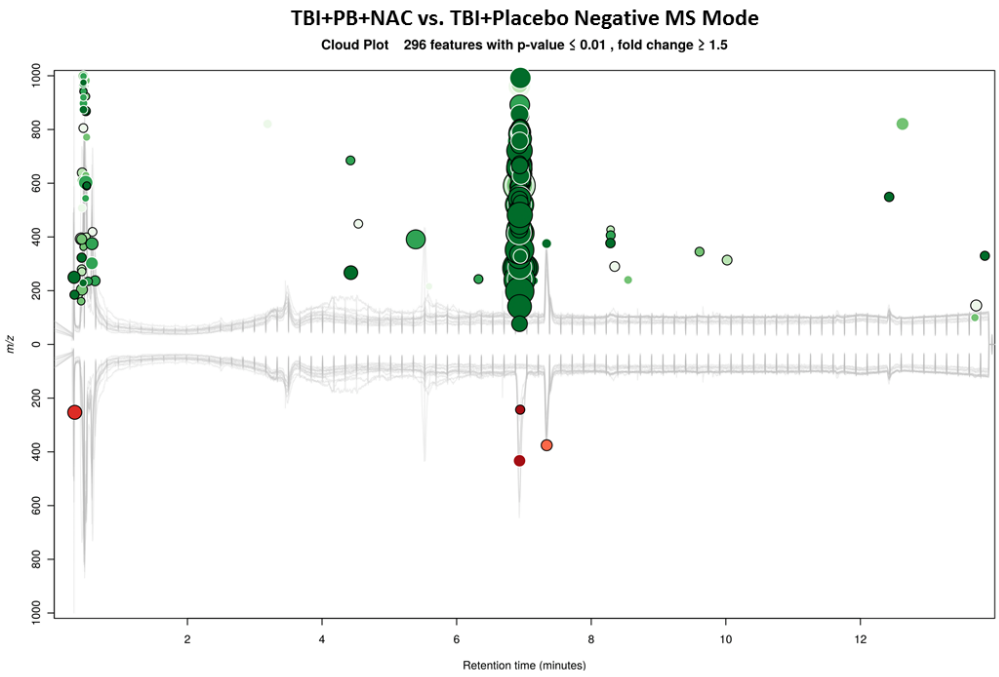

Figure 20. Cloud plots of features increased (green) or decreased (red) in TBI patients treated with probenecid+NAC as compared to placebo treated TBI patients in negative MS mode.

The size of the circle corresponds to the fold change of a particular feature between the two groups. The intensity of the color corresponds to the p-value with more intense shades representing a smaller p-value.

4.3.7. Pathway analysis of TBI patients receiving placebo vs. probenecid + NAC

Pathway analysis was performed to identify biochemical processes modified by the combination therapy of probenecid and NAC after TBI, which could yield insight into the mechanism of action of these repurposed drugs as well as any potential undesirable biochemical effects after TBI. In the positive MS mode, a total of 77 pathways were found to be enriched in the metabolites that were differentially dysregulated between the two groups, with 45 pathways showing ≥ 50% overlap between putative metabolites with a known component of the particular pathway (Figure 21 and TableA2 in Appendix A). In the negative MS mode, a total of 47 pathways were found to be significantly dysregulated, with eight pathways showing ≥ 50% overlap between putative metabolites (Figure 21 and TableA2 in Appendix A).

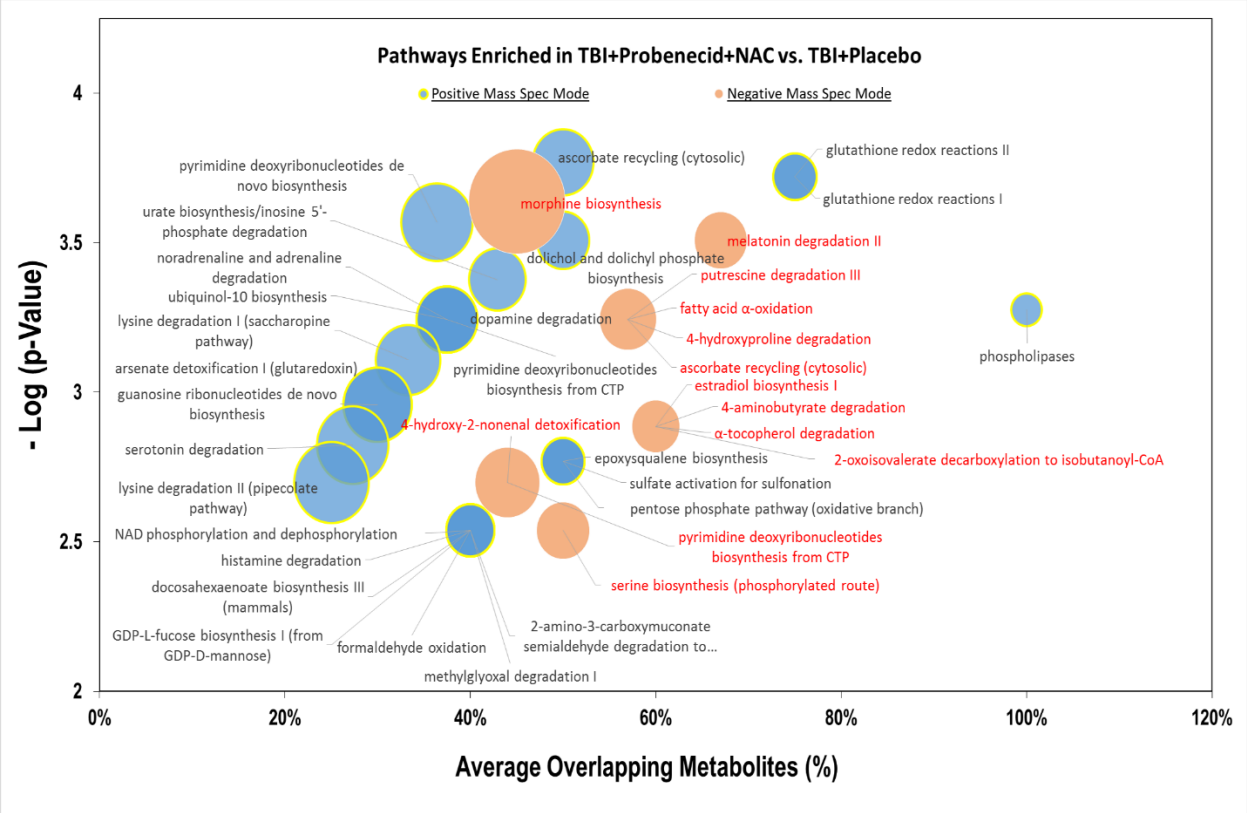

Figure 21. Cloud plot of select pathways affected by treatment with probenecid and NAC vs. placebo in TBI patients, identified by metabolomics analysis of CSF.

The radius of each circle increases with the number of metabolites relative to the number of metabolites represented by other circles.

4.3.8. Network analysis of TBI patients receiving placebo vs. probenecid + NAC

Network analysis identified five modules that are significantly affected by probenecid + NAC treatment in the positive MS mode and four modules in the negative MS mode. Modules represent internal connectivity more than what is expected randomly in the whole network. The complete list of modules and the putative metabolites that constitute the modules are in Table A4 (for positive MS mode) and Table A3 (for negative MS mode) in Appendix A. It is possible that a bigger module consist of smaller modules that are also identified independently. For example, in the negative MS mode analysis, modules 2 and 3 are identified independently but are also part of module 1. Similarly, in the positive MS mode, module 1 comprises of modules 2, 3, and 4.

Notable features of these modules include module 3 in the negative MS mode where glutathione and its conjugates are upregulated (Figure 22A). Similarly, module 5 in the positive MS mode (Figure 23B) shows glutathione in dynamic interaction among glutathione conjugates and reactive molecules detoxified by glutathione. Module 4 in the negative MS mode (Figure 22B) shows upregulation of sulfate donors in sulfate conjugation process and two metabolites with sulfate conjugation characteristics that are confirmed substrates of probenecid-inhibitable transporters [267]. A segment of module 1 in the positive MS mode (Figure 23D) shows a collective downregulation of prostaglandins which are also known to be substrates of a number of transporters inhibited by probenecid [268]. Module 2, which is also a component of the bigger module 1, in the positive MS mode is centered around 2-ketoglutarate which is closely related to the OATs, which are the main transporters inhibited by probenecid (Figure 23A). Also, some members of module 2 such as kynurenic acid, 3-hydroxykynurenine and dipeptides are substrates

of OAT transporters [269-272]. Another interesting feature of module 1 is the distinctive up-regulation of UDP-conjugated monosaccharides, i.e., nucleotide sugars, UDP-alpha-D-galactose and UDP-alpha-D-galactose, and the down-regulation of their interacting counterparts, acetylated sugar moieties (Figure 23C). The transporters responsible for the translocation of the

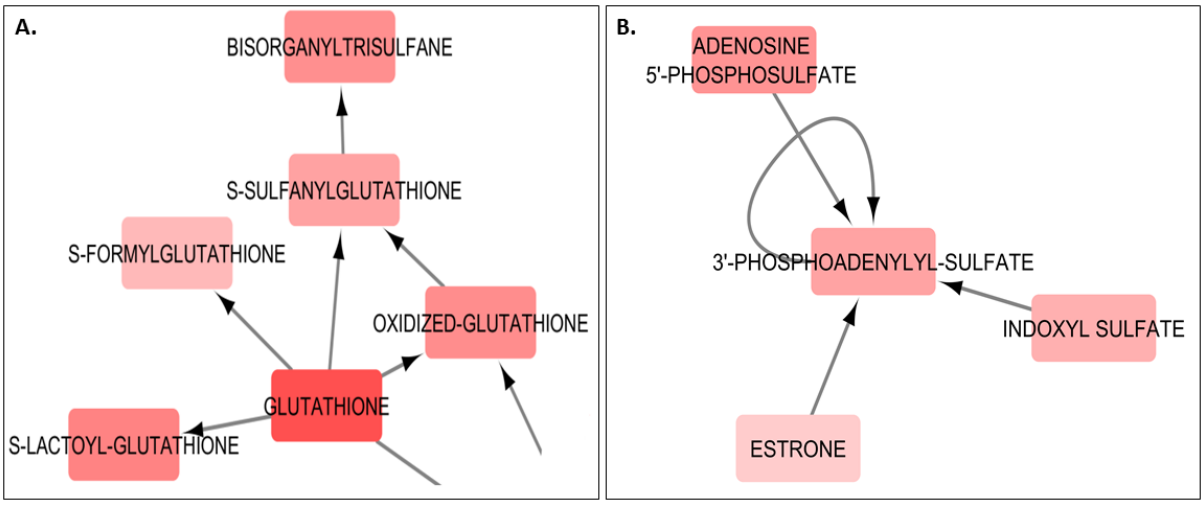

Figure 22. Network connectivity of representative modules in negative MS mode enriched in probencid+n-acetylcystiene treated TBI patients.

In module 4 (A), the module shows upregulation of glutathione and a number of its conjugates and connectivity between NADP-dependent pathways are highlighted. In module 3 (B), the module shows upregulation of sulfate donors in the sulfate conjugation process, and two sulfate-conjugated metabolites that are confirmed substrates of probenecid-inhibitable transporters. Red nodes are upregulated while the blue nodes are downregulated relative to other nodes within individual mode. The color intensity indicates the extent of upregulation or downregulation.

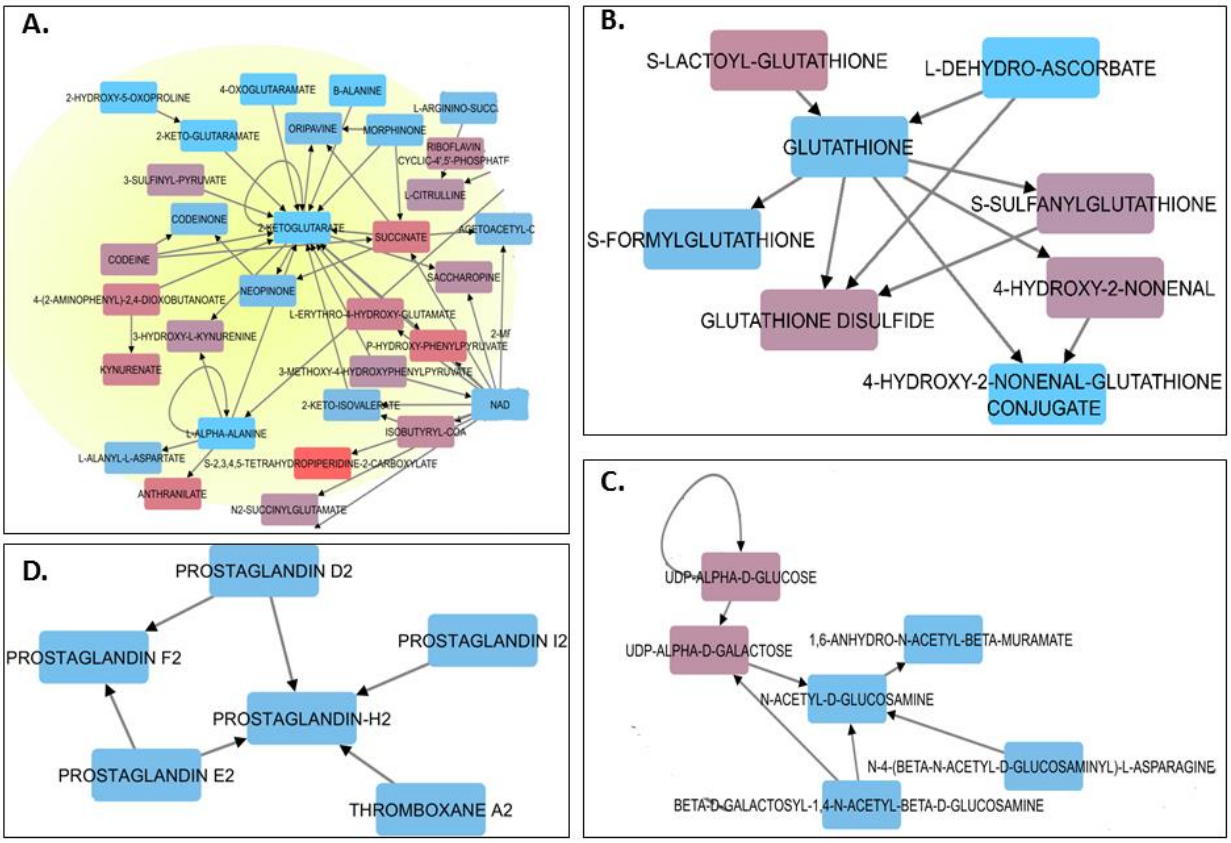

Figure 23. Network connectivity of representative modules in positive MS mode enriched in probencid+n-acetylcystiene treated TBI patients.

(A) Module 2, alterations in pathways centered around 2-ketoglutarete. (B) Module 5, glutathione in dynamic interaction among glutathione conjugates and reactive molecules detoxified by glutathione. (C) Segment of module 1, upregulation of UDP-conjugated monosaccharides and downregulation of their counterparts, acetylated sugar moieties. (D) Segment of module 1 showing collective downregulation of prostaglandins. Red nodes are upregulated while the blue nodes are downregulated relative to other nodes within individual mode. The color intensity indicates the extent of upregulation or downregulation.

4.3.9. Individual metabolites altered in TBI patients receiving placebo vs. probenecid + n-acetylcysteine

Figure 24 shows a volcano plot of metabolites in CSF from TBI patients that received placebo or probenecid + NAC. Similar to the cloud plots in Figure 6, the majority of features appeared to increase in TBI patients treated with probenecid and NAC vs. placebo. Metabolites with maximum absolute intensity $\geq$ 2,500, -log(p) > 2.3, and $\log_2$(fold change) > 1 were selected for possible identification. Excluding metabolites with poor chromatographic resolution by manual inspection yielded 67 significant features. To identify putative metabolites that correspond to these features, their masses were searched in the HMDB based on a 10 ppm error margin [264-266]. Forty-two of the searched features returned metabolites or putative metabolites that were of dietary or exogenous drug source, leaving 25 metabolites that may be affected by probenecid + NAC treatment after TBI (TablesA5 in Appendix A). Probenecid is one of the metabolites that are highly upregulated confirming the TBI group assignment and validating the XCMS-online platform's capacity to discriminate patients based on their metabolic signature. Glutathione was also increased in probenecid + NAC treated patients vs. placebo, consistent with drug combination target engagement.

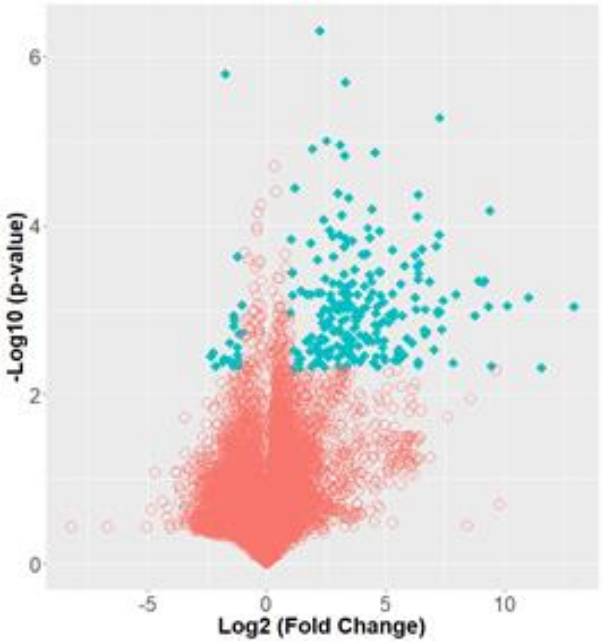

Figure 24. Volcano plot of metabolome profiles of the CSF of TBI patients treated with placebo or a combination of PB and NAC in both positive and negative MS modes. Metabolites that are upregulated or downregulated by two fold or more with a significance p value of 0.005 (blue dots) are selected for further investigation.

4.4. Discussion

To our knowledge, this is the first study to perform a comprehensive LC-MS based metabolomics analysis of TBI patients. This study is also the first clinical application of "neuropharmacometabolomics" after administration of potentially therapeutic drugs, including one with known impact on the biochemical milieu of the CSF, probenecid [257, 258]. PCA analysis shows that patients cluster into their respective groups (control subjects, TBI placebo, TBI probenecid + NAC) based on the unique signature of their metabolomics profile in the CSF

(Figure 1), validating the metabolomics approach. We focused on pathway and network analysis of the metabolomics data, leveraging recent advances that enable exploration of metabolomics data without exhaustive verification of individual metabolites [261]. Advantages of pathway and network analyses in terms of gaining insight into biological systems over analysis at the individual metabolite or gene level have been described previously [274], and include increasing statistical power by consolidating multiple metabolites into a smaller number of pathways and networks. Pathway and network analyses allow for interpretation of altered processes rather than metabolites, provide a framework for quantitative modeling based on experimental observations, and assist in identifying potential causal mechanisms. Pathway analysis revealed novel biochemical processes associated with TBI, while also confirming others with a previously known role. Treatment with probenecid and NAC resulted in a significant alteration in the metabolomic signature of TBI, with the majority of features showing relative increases compared with placebo (Figures 6 and 9). Importantly, relative glutathione levels were increased 665-fold (Table 5) and both pathway and network analyses showed that biochemical processes involving detoxification with glutathione and glutathione recycling were enriched in the probenecid + NAC treated group (Figures 7 and 8), verifying target engagement of at least one of the components of the combination therapy. Furthermore, a number of modules and pathways with components known to be substrates of transporters inhibited by probenecid were identified, providing insight into the pharmacological actions of probenecid. These data validate the anticipated effects of the probenecid and NAC treatment and demonstrate the value in pathway-based metabolomics analysis.

Comparing the TBI placebo-treated patients with the control subjects revealed several biochemical pathways that were activated in TBI. Previous studies have reported TBI-related catabolism of various types of small molecules such as amino acids, catecholamines, hormones, and polyamines. All or several components of a number of these molecular pathways were amplified in the TBI group. These include: tyrosine degradation, noradrenaline and adrenaline degradation, spermine and spermidine degradation I, nicotine degradation IV, thyroid hormone metabolism II via conjugation and/or degradation, and L-carnitine biosynthesis. Other pathways found to be enriched that are implicated in the pathophysiology of TBI include oxidative stress (alpha-tecopherol degradation, ubiquinol-10 biosynthesis), bioenergetics (lactate fermentation pathway), lipid peroxidation (leukotriene biosynthesis, resolvin-D biosynthesis), and heme metabolism (tetrapyrrole biosynthesis and heme biosynthesis from uroporphyrinogen-III I). Cystathionine-β-synthase, which catalyzes the first step in the transulfuration of homocysteine to cystathionine, plays a critical role in a variety of physiological processes of the CNS, has also been reported to mediate a number of pathological processes after TBI [275]. Furthermore, two pathways, cysteine biosynthesis/homocysteine degradation and hydrogen sulfide biosynthesis, were also found to be upregulated.

Some of the enriched pathways uncovered have not been associated with TBI previously, e.g. morphine biosynthesis. Endogenous morphine biosynthesis by human neuroblastoma cells has been reported [276, 277], and the activation of this pathway in TBI patients may indicate a role in pain modulation after injury. Two methylglyoxal-related pathways were found to be upregulated in the CSF of TBI patients, indicating potential involvement of methoglyoxal in

TBI. Methylglyoxal, a potentially toxic byproduct of glycolysis, threonine catabolism, and lipid peroxidation has been implicated in models of cerebral ischemia *in vivo* and *in vitro* [278, 279], but its involvement in TBI is undefined. Also in the present study, pathways related to purine and pyrimidine metabolism were enriched, namely pyrimidine deoxyribonucleoside salvage and degradation, and purine ribonucleoside degradation. Purine and pyrimidine nucleotides and nucleosides mediate activation of astrocytes and other glia, described before in relation to acute and chronic neuroinflammation [280].

Treatment with a combination of probenecid and NAC resulted in significant changes in the metabolomics signature of TBI, as detected in the CSF. Pathway and network analyses revealed over 80 pathways and nine modules, respectively, that were dysregulated in the treatment group vs. placebo. Interestingly, seven pathways that involve glutathione as a component of detoxification processes were enhanced in the treatment group: glutathione redox reactions I, glutathione redox reaction II, 4-hydroy-2-nonenal detoxification, methylglyoxal degradation I, ascorbate recycling, arsenate detoxification, and formaldehyde oxidation. These observations were congruent with the network analysis, with glutathione-centered modules prominently featuring in both negative and positive MS modes. These findings are important as they serve as internal validation for the metabolomics-based pathway and network, given that probenecid and NAC can increase intracellular glutathione via two distinct mechanisms, preventing cellular efflux of glutathione disulfide (GSSG) and glutathionylated xenobiotics and serving as cysteine donor for glutathione synthesis, respectively. Furthermore, NAC is a substrate for probenecid inhibitable OAT1 and OAT3 providing additional drug synergy [242]. Collectively, our findings

indicate that, as hypothesized, a combination of probenecid and NAC modulate levels of glutathione and its conjugates, strongly implicating target engagement.

Other prominent pathways changed by treatment represent biochemical processes such as purine and pyrimidine nucleotide and nucleoside metabolism; catabolism, salvage and biosynthesis of amino acids; cathecolamines; co-factors; hormones; peptides and polyamines; oxidation, degradation and biosynthesis of fatty acids and other lipids; and glycolysis and aerobic cellular respiration. This suggests that many substrates, intermediates, and end products of biochemical pathways and/or signaling molecules may be substrates of transporters inhibited by probenecid. Some components of the enriched pathways and modules such as urate (urea cycle and urate biosynthesis pathways [281], kynurenate and xanthurenate (tryptophan degradation via tryptamine), indoxyl sulfate (module 4 in negative MS mode) [269, 270], cyclic nucleotides (several pathways and modules) [282], and prostaglandins (module 1 in positive MS mode) [268] are trafficked by transporters that are inhibited by probenecid. Additionally, one of the biggest modules identified by network analysis is centered around 2-ketoglutarate. 2-Ketoglutarate is intimately related to the mechanism of transport of organic anions by the family of OAT transporters, acting as a gradient exchanger. As such, its intra and extracellular levels are expected to change in presence of modulators of OAT transporters [283].

Many alterations in the biochemical milieu observed in probenecid and NAC treated compared with placebo treated TBI patients can be explained by the known properties of probenecid. Some of these changes, such as increased abundance of kynurenine may be beneficial after TBI, as

kynurenine is considered neuroprotective and in fact had been studied in combination with probenecid to attenuate seizures, quinolinic acid-induced neurotoxicity, and neuropathic pain [284-286]. Other effects, such as increased abundance of indoxyl sulfate may represent undesirable effects – indoxyl sulfate, a uremic toxin, has been linked with CNS toxicity in patients with impaired kidney function [287].

We also sought to tentatively identify individual metabolites significantly altered in TBI patients vs. control subjects, as well as probenecid + NAC vs. placebo treated TBI patients. Initial screening yielded ten features in CSF that distinguished TBI patients from control subjects. Subsequent querying of the human metabolome database showed that gangliosides appear as putative metabolites in four out of ten features. This is consistent with a previously reported role for gangliosides in TBI [26, 288]. Comparing placebo and probenecid + NAC treated TBI patients, after initial screening and excluding non-endogenous metabolites, yielded 24 putative metabolites. Congruent with pathway and network analyses, glutathione and its conjugates were commonly featured. Other molecules included dipeptides, nucleotides, and glucuronide- or sulfate- conjugated molecules. Many of these are known to be transporter substrates.

Some limitations to the present study should be noted. First, the sample size is relatively small, although it does represent the first comprehensive metabolomics analysis of any kind in human TBI. Second, the MS assay was performed after sample preparation with acetonitrile. Thus, the analytical approach does not cover the entire spectrum of molecules, such as large proteins including those currently being evaluated as TBI biomarkers. Third, individual

metabolites/features (with the exception of probenecid) and pathways were not confirmed independently in this study. Future studies using MS/MS fragmentation patterns and comparisons to pure standards for individual metabolite validation, and stable isotope marking for pathway confirmation using clinical samples and preclinical models will be necessary.

In this, first of its kind, CNS metabolomics and pharmacometabolomics study, we demonstrate that global metabolomics signatures can differentiate control subjects, placebo treated TBI patients, and TBI patients treated with a combination of probenecid and NAC. Using advances in pathway and network analysis of metabolomics data, this study revealed pathways and networks that are dysregulated by TBI. Some of these pathways have previously documented involvement in TBI while others represent novel findings potentially bringing new insights into the pathogenesis of TBI. Similarly, treatment with a combination of probenecid and NAC resulted in enrichment of numerous pathways and networks including several glutathione mediated detoxification pathways supporting the *a priori* hypothesis for using this drug combination in patients with severe TBI. Several individual features, pathways, and networks with biologically plausible interactions with the combination therapy were also identified. Furthermore, several potential markers for probenecid and/or NAC therapeutic target engagement (efficacy and/or toxicity) were identified. Neuropharmacometabolomics may represent a powerful tool for developing a comprehensive understanding of the multiple biochemical processes and mechanisms involved in the pathogenesis of TBI, and in comprehending the effects of pharmacological interventions.

5. Association of SLC16A1, SLC16A3 and SLC16A7 genetic variants with long-term outcomes in TBI patients

5.1. Introduction

The metabolic crisis that occurs during the early phase traumatic brain injury and other acute brain injuries is one of the main determinants of the pathogenesis of TBI and is associated with unfavorable disease progression and long-term outcomes [105, 289-291]. The brain's adaptive response to the metabolic crisis includes increased production, mobilization and/or utilization of lactate [31, 106] . A number of preclinical and clinical studies in TBI have reported the trafficking of lactate from astrocytes and peripheral sources to neurons to be used as energy source [94, 104, 109, 110, 292]. [105, 106, 115] .This agrees with the astrocytic-neuronal lactate shuttle (ANLS) hypothesis which posits that synaptically released glutamate initiates neuronal activity that is followed by increased rate of glucose uptake and glycolysis by astrocytes and subsequent release of lactate that can be used as energy substrate by neurons via oxidative-derived ATP production [99] [98]. However, the ANLS itself and the notion that lactate could be traffic into neurons and used as energy source in TBI are not far from controversy. Citing

technical shortcoming of human studies using microdialysis, limitations of in-vivo and in-vitro TBI models, stoichiometry considerations and lack of biological plausibility, some have questioned the notion that lactate could be preferentially and beneficially used as a fuel in TBI [94, 100, 114, 293]. A study by Lama *et al.* reported that while lactic acid is continuously produced during TBI, but might be more of a toxic by product that results in neuronal death than a beneficial and neuroprotective energy source [113].

Little attention has been paid to the specific role of monocarboxylates transporters (MCTs) – responsible for trafficking lactic acid, which exist as ionic species in the body – in this controversy. In the brain, members of the SLC16A family of transporters, MCT1 (SLC16A1), 2 (SLC16A7) and 4 (SLC16A3) function in coordination – as reflected by their distinctive expression patterns and kinetic properties – to facilitate the movement of lactic acid from peripheral or astrocytic sources into the neurons [91, 92]. This is particularly important in conditions such as TBI, where the normal energetics of the brain is disrupted [103, 104, 291]. In fact, MCT1, 2, and 4 constitute central part of the ANLS [99]. As discussed in Chapter one of this dissertation, the study of role of MCT transporters in TBI and other acute brain injuries has been limited to observations of changes in mRNA and/or protein expression, and the interrogation of the consequences of largely non-selective MCT inhibitors in preclinical models of acute brain injuries. Nevertheless, despite their limitations, these studies provide evidence that MCT transporters are crucial components of the brains adaptation mechanism to altered energy requirement following acute brain injuries. In the current study, using candidate-gene approach and focusing on well-studies SNPs in the genome of SLC16A1, SLC16A7 and SLC12A3, we

aim to evaluate the association of genetic alterations in these transporters with neurological function outcomes in TBI patients. Previous studies have found association between the genetics of these transporters in other conditions such as injury-susceptibility in elite athletes, male infertility, juvenile idiopathic arthritis and cancer [294-297]. To the best of our knowledge, this is the first study to investigate the role of MCT transporters in human TBI patients and will help clarify whether endogenously generated or exogenously administered lactate would be beneficial in TBI.

5.2. Methods

5.2.1. Patients

This study was approved by the University of Pittsburgh Institutional Review Board and informed consent was obtained from the study subject's surrogate. The University of Pittsburgh Brain Trauma Research Center database was used to identify two independent cohorts, separated by date of enrollment, based on the following inclusion criteria: age 16 and older, GCS score of 8 or less, external ventricular drain (EVD) placement, and positive computed topography (CT) scan confirming TBI. Patients with penetrating trauma and previous neurological impairment were excluded. Study subjects were divided into discovery and replication cohorts based on data of injury with at least four years of separation. Glasgow Outcome Scale (GOS) scores ranging 1 to 5 were collected by qualified neuropsychologist at 3, 6, 12 and 24 months post injury. A score

of 1 corresponds to deceased, 2 corresponds to a persistent vegetative state, 3 corresponds to sever disability, 4 corresponds to minor disability, and 5 corresponds to little or no disability.

5.2.2. SNP selection

For the current study, attempts were made to identify well-characterized SNPs with published reports of association of these SNPs with functional outcome measures. For SLC16A1, rs1049434 was the only well-studied SNP which was previously associated with injury-susceptibility, accumulation of lactate in capillaries during high-intensity training and hyperinsulinism [295, 297, 298]. Other two SNPs, rs9429505 and rs7169 which are in perfect LD with each other and pin partial LD with rs1049434, were also reported to have association with hyperinsulinism. However, these SNPs were tagged as low-quality sites in the gnomAD browser. Thus, rs1049434 was selected for analysis in this study. For SLC16A7, rs10506399 and rs3763980, are the two well-studied SNPs associated with male-infertility and methotrexate response in juvenile idiopathic arthritis respectively [294, 296]. They are also in perfect LD with each other, so we chose rs10506399 for this study since it has a better call-rate in our genotyping assay. For SLC16A3, there are no reported SNPs associated with any functional outcome. Additionally, based on search results in the gnomAD browser, all SNPs with missense and loss of function characteristics have low MAFs that prohibit statistical analysis in this study's population. Hence, no SNP from SLC16A3 were included in this study.

5.2.3. Sample collection and genotyping

Blood or CSF samples were used to extract DNA using a commercially available kit per manufacturer's protocol (Qiaamp kit, Qiagen, Chatsworth, CA, USA). Genotyping was performed with a TaqMan-allelic discrimination assay for the discovery cohort (Applied Biosystems, Foster City, CA) and a mix of TaqMan + Illumina Human Core Exome v1.2 (Illumina, San Diego, CA) for the replication cohort. Genotype data quality were addressed by using technical replicates, and blinded double calls of raw data. Hardy-Weinberg equilibrium test was performed for both SNPs to assess genotype distributions in each cohort were within the expected proportions based on comparisons with published genotype frequencies for the non-Finnish European population from the Broad Institute's gnomAD Browser.

5.2.4. Statistical Analysis

Comparison of demographic data between discovery and replication cohorts were performed using two-tailed Student's t-test for continuous variables or chi-square test for categorical variables. Additive and common dominant genetic models of disease were considered for both SNPs, and the most significant genetic model was selected at the end. The outcome measure, GOS, was treated as multilevel ordinal variable. Analysis of association of the SNPs with GOS was performed using multilevel mixed effects ordered logistic regression approach in Stata 14.2 (Stata Corp LLC, College Station, TX, USA). All model runs were initially performed by forcing potentially clinically relevant covariates that include age, post-injury time period, GCS score,

sex, and race, but only those that shown statistically significant effect were kept in the final model.

5.3. Results

The demographic and clinical characteristics of the study subjects is summarized in Table 3. There were 285 and 179 subjects identified in the discovery and replication cohorts respectively. The characteristics of both cohorts are similar except for age, where the replication cohort subjects were significantly older than the discovery cohort. Adherence to Hardy-Weinberg equilibrium was confirmed for both SNPs in each cohort separately and in the combined population set ($p>0.05$ for all comparisons). The calculated minor allele frequencies (MAFs) for each SNP in each cohort and the combined cohort were not statistically significant different from MAF for non-Finnish European population based on the gnomAD Browser ($p>0.05$ for all comparisons)

Table 4. Summary of patient characteristics

	Cohort 1 (N=285)	Cohort 2 (N=179)	p-value
Age, Median, (IQR)	34 (23-47)	37 (23-54.5)	0.029
Sex, Female, n (%)	60 (21%)	42 (23%)	0.54
GCS, Median, (IQR)	6 (4-7)	7 (5-7)	0.25
Race, n (%)			
Caucasian	263 (92%)	164 (92%)	0.79
Others	22 (8%)	15 (8%)	
Genotype, n (%)			
SLC16A1, rs1049434, AA	76 (27%)	41 (23%)	
SLC16A1, rs1049434, TA	149 (52%)	90 (50%)	0.321
SLC16A1, rs1049434, TT	60 (21%)	48 (27%)	
SLC16A1, rs1049434, AA/AT	225 (79%)	131 (73%)	0.153
SLC16A1, rs1049434, TT/AT	209 (73%)	138 (77%)	0.388
SLC16A7, rs10506399, GG	154 (54%)	107 (60%)	
SLC16A7, rs10506399, AG	116 (41%)	60 (34%)	0.216
SLC16A7, rs10506399, AA	13 (5%)	12 (7%)	
SLC16A7, rs10506399, NA	2 (1%)	-	
Gntyp3 SLC16A7, rs10506399, GG/AG	270 (95%)	167 (93%)	0.329
Gntyp2 SLC16A7, rs10506399, AA/AG	129 (45%)	72 (40%)	0.258

The mixed-effects multi-level ordinal regression model was built with relevant clinical and demographic covariates that were also found be statistically significant factors in the model. Based on these criteria, the final model consists of age, time post-injury and GCS as covariates. Each of these covariates were tested with genotype for two-way interactions. The dominant genetic model and the additive genetic model were employed in for SLC16A7 and for SLC16A1

respectively in the final model as each resulted in more significant differences compared to the alternative disease model for respective gene.

The final models for both SNPs and both discovery and replication cohorts are summarized in Table 3 and the combine model is presented in Table 3. In case of *SLC16A7*, significant association was found between GOS and the rs10506399 genotype in both cohorts and the combined model ($p = 0.007$ [discovery], $p = 0.004$ [replication] and $p<0.0001$ [combined model]). However, this association was age dependent as significant association between GOS and the interaction term between age and rs10506399 genotype was also found in both cohorts and the combined model ($p = 0.003$ [discovery], $p = 0.001$ [replication] and $p<0.0001$ [combined model]).

Table 5. Mixed effects ordered logistic regression model, SLC16A7 and SLC16A1

SLC16A7				
	Discover Cohort (N=285)		**Replication Cohort (N=179)**	
Factor	**Coefficient [95% CI]**	**p-value**	**Coefficient [95% CI]**	**p-value**
Genotype*LnAge	-4.25 [-7.10, -1.40]	0.003	-6.23 [-10.00, -2.47]	0.001
Genotype	14.105 [3.78, 24.43]	0.007	19.75 [6.23, 33.26]	0.004
Lnage	-8.80 [-11.05, -6.55]	<0.0001	-7.51 [-9.90, -5.11]	<0.0001
GCS score	2.80 [2.22, 3.38]	<0.0001	1.98 [1.33, 2.62]	<0.0001
Post injury test period vs 1				
2	1.25 [0.73, 1.77]	<0.0001	1.08 [0.38, 1.77]	0.002
3	2.13 [1.56, 2.70]	<0.0001	2.31 [1.51, 3.12]	<0.0001
4	2.80 [2.22, 3.38]	<0.0001	1.85 [0.93, 2.78]	<0.0001
SLC16A1				
	Discover Cohort (N=285)		**Replication Cohort (N=179)**	
Factor	**Coefficient [95% CI]**	**p-value**	**Coefficient [95% CI]**	**p-value**
Genotype*Lnage	-2.30 [-4.47, -0.13]	0.038	N/A	
Genotype	6.19 [-1.70, 14.08]	0.124		
Lnage	-8.92 [-11.70, -6.13]	<0.0001		
GCS score	2.79 [2.21, 3.36]	<0.0001		
Post injury test period vs 1				
2	1.24 [0.72, 1.76]	<0.0001		
3	2.12 [1.55, 2.68]	<0.0001		
4	2.79 [2.16, 3.41]	<0.0001		

Table 6. Mixed effects ordered logistic regression model of the combined, discovery and replication cohorts

		Combined Cohort (N=464)	
Factor		**Coefficient [95% CI]**	**p-value**
Genotype*LnAge		-5.16 [-7.54, -2.79]	<0.0001
Genotype		16.74 [8.13, 25.35]	<0.0001
Lnage		-8.32 [-9.96, -6.69]	<0.0001
GCS score		2.49 [2.04, 2.93]	<0.0001
Post injury test period vs 1			
	2	1.18 [0.77, 1.60]	<0.0001
	3	2.18 [1.72, 2.64]	<0.0001
	4	2.53 [2.01, 3.04]	<0.0001

To facilitate the interpretation of the findings based on the model above, a more parsimonious logistic regression model was developed using the combined cohort and after categorizing the outcome, GOS, into two levels, unfavorable (for GOS scores of 1 and 2) and favorable (for GOS scores of 3, 4 or 5). The results of this model are presented in Table 6. This binary logistic model was then used to calculate the expected probabilities of favorable outcomes for either genotype of the SNP and at various values of age. A plot of predicted probabilities versus age for each genotype was generated and is shown in Figure 25**.** The figures show that the probability of favorable outcomes was significantly higher for patients which has one or both of the alternate alleles. But the beneficial effect of the alternate allele decreases as age increases.

Table 7. Binary logistic regression of the combined, discovery and replication cohorts

		Combined Cohort (N=464)	
Factor		**Coefficient [95% CI]**	**p-value**
Genotype*LnAge		-0.67 [-1.21, -0.12]	<0.01
Genotype		1.98 [0.084, 3.88]	<0.05
Lnage		-1.42 [-1.76, -1.08]	<0.0001
GCS score		0.4 [0.31, 0.49]	<0.0001
Post injury test period vs 1			
	2	0.58 [0.25, 0.91]	<0.001
	3	0.96 [0.62, 1.29]	<0.0001
	4	0.82 [0.47, 1.17]	<0.0001

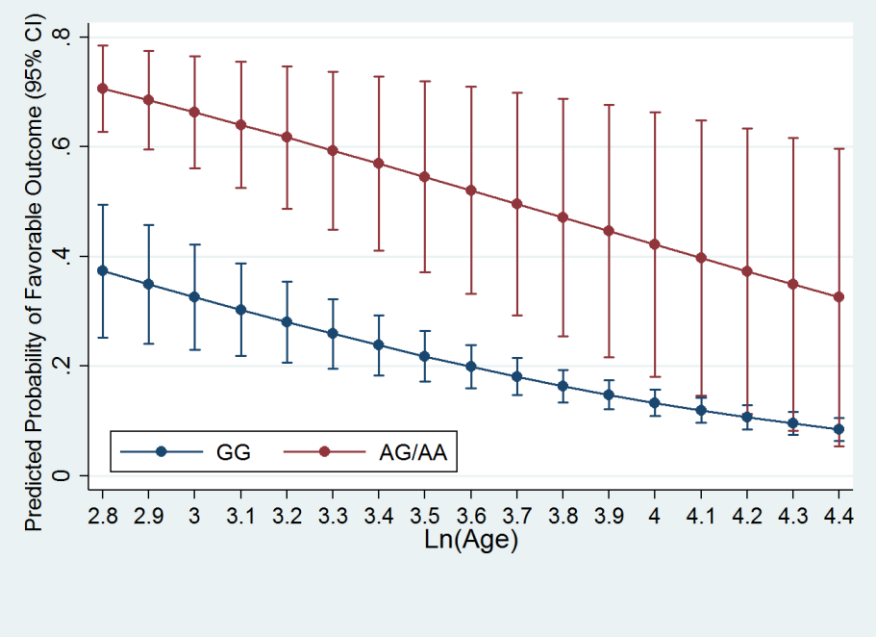

Figure 25. The margins of probability of favorable outcome for the genotypes at rs10506399 across age based on logistic regression.

Patients with that carry one or two 'A' allele have significantly better favorable outcome, but the effect diminishes with age.

To assess the functional consequence of the rs10506399 SNP, the Genotype-Tissue Expression (GTEx) Project portal was queried using the SNP ID rs10506399 [299, 300]. The search yielded currently available eQTL data for four tissues for this particular SNP. The expression of SLC16A7 by rs10506399 genotype in cerebellum tissue, shown in Figure 26, was statistically significant (p= 3.8x10-8, FDR>0.05) based on linear regression analysis. The effect size of the eQTLs – defined as the slope of the linear regression, comparing the alternate allele (A) to the reference allele (G) – was 0.710 indicating a strong effect of the genotype on expression.

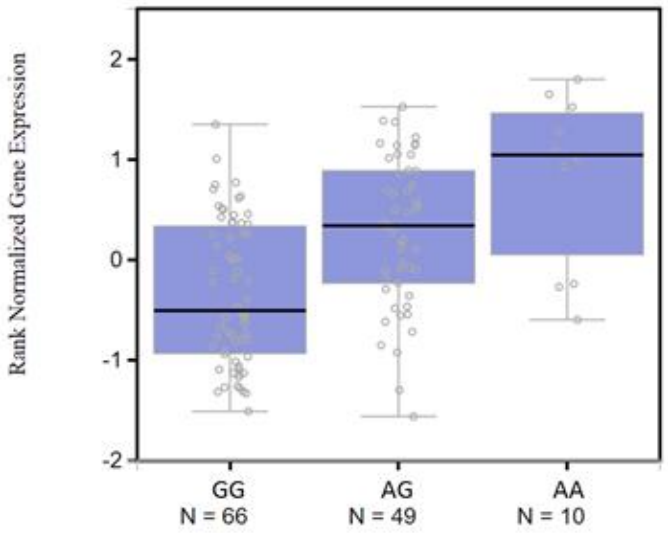

Figure 26. SLC16A7 gene expression by genotype of rs10506399.

The data was obtained from GTEx portal and represent the gene expression levels in brain cerebellulm tissue of 135 donor individuals. The presence of two minor allele 'A' is associated with highest expression level of SLC16A7.

In case of SLC16A1, the association between GOS and the rs1049434 genotype was not statistically significant in the discovery cohort ($p = 0.124$). According to our a priori rule, further analysis of this SNP was not conducted.

5.4. Discussion

In this chapter, we examined the role of MCT transporters in TBI patients using genetic association study. We report that patients who have one or both of the alternate allele at rs10506399 have a higher probability of favorable outcome after TBI. We also showed that the alternate allele at rs10506399 are associated with increased expression of SLC16A7 in the brain cerebellum hemisphere. Additionally, we found that s1049434, the most studied SLC16A1 SNP was not associated with outcomes in TBI. MCT transporters are responsible for trafficking of lactate and other monocarboxylates within the neurovascular unit in the brain [87]. The findings in this study suggest important role of these transporter in determining the signaling and metabolic function of lactate and possibly other monocarboxylates.

There is an ongoing controversy on whether lactic acid is shuttled to neurons to be utilized as an alternative or preferred energy source in TBI. The fact that there is increased lactate production following TBI is agreed up on consensus. However, the specific origin of the lactate and/or the relative contribution of several plausible sources, the fate of lactate after production (i.e.,

whether taken-up by neurons or trafficked out into blood stream), and whether neurons could effectively use lactate to generate ATP are all matters of considerable debate [92, 94, 100, 108, 301]. The debate about the role of lactate in TBI is focused on its movement within the neurovascular unit. Lactate exists as an ionic species at the physiological pH, hence it needs transporters to cross biological barriers, which makes MCT transporter critical regulators of the function of lactate. As discussed in Chapter one of this dissertation, numerous *in vitro* and *in vivo* studies have underlined the importance of MCT transporters in the setting of neuronal injury. Specifically, inhibition or down regulation of MCT transporters either using small molecules or siRNA leads to greater harm in various models of acute brain injury. Also, the expression level and pattern of this transporters change in response to injury. This study is the first one to assess the role of MCT transporters in human TBI patients. We sought to understand the role of brain MCT transporters, MCT1, MCT2 and MCT4 using genetic association study. To limit the chances of false positive, we only included SNPs that previously have been reported to be associated with functional outcomes, and those SNPs that have sufficient MAF to conduct statistical analysis. According to our criteria, no SNP in MCT4 were selected for analysis. One SNP each were selected for MCT1 (rs10494340 and MCT2 (rs10506399).

In case SLC16A7, significant association of was found between the SNP rs10506399 and the outcome of interest, GOS, in age dependent manner. MCT2 is deemed the neuronal MCT transporter because it is mainly expressed in neuronal cells [91, 92]. Previous studies have reported that the SNP rs10506399, which is located in the 3’ UTR region, is associated with

infertility in Asian men [296]. Also, rs3763980, which is in perfect LD with rs10506399 is associated with treatment response to methotrexate in juvenile idiopathic arthritis [294].

We also demonstrated that the SNP rs10506399 is associated with expression of SLC16A7. Specifically, the presence of one or more of the alternate allele was associates with increased expression of SLC16A7 with strong effect size in the cerebellum hemisphere of brain tissue. Since MCT2 is a high affinity transporters [92], changes in expression such as observed as a result of alternation in the SNP rs10506399 could have profound effect on its function, hence the observed association with clinical outcome in TBI. If the findings of this study would be confirmed, it would suggest that there is an increased uptake of lactate or other monocaboxylates by neurons duo to increased expression of MCT2 in the patients with the one or both of the alternate allele, and that this is beneficial in TBI.

With regards to MCT1, we didn't find statistically significant association of genotype at rs1049434 and GOS in the discovery cohort, and we didn't pursue it further in the replication cohort. The SNP rs1049434 was previously reported to be associated with susceptibility to muscle injury in elite soccer players with statistically significant barely below the 0.05 present threshold (p=0.048)[295]. Another study has also reported that there was 35-40% reduction of lactate transport rate in erythrocytes of individuals carrying the alternate allele 'T', but this observation was only in two individuals [302]. Search in the GTEx portal shows that there was no statistically significant association of the genotype at rs1049434 with expression of SLC16A1

in any of the brain tissues examined except the hypothalamus where a weak association with low effect size (p=0.04, normalized effect size=0.23) was identified (Figure 27). Put together, this findings suggest minimal functional consequence of the SNP rs1049434, particularly in the brain tissues.

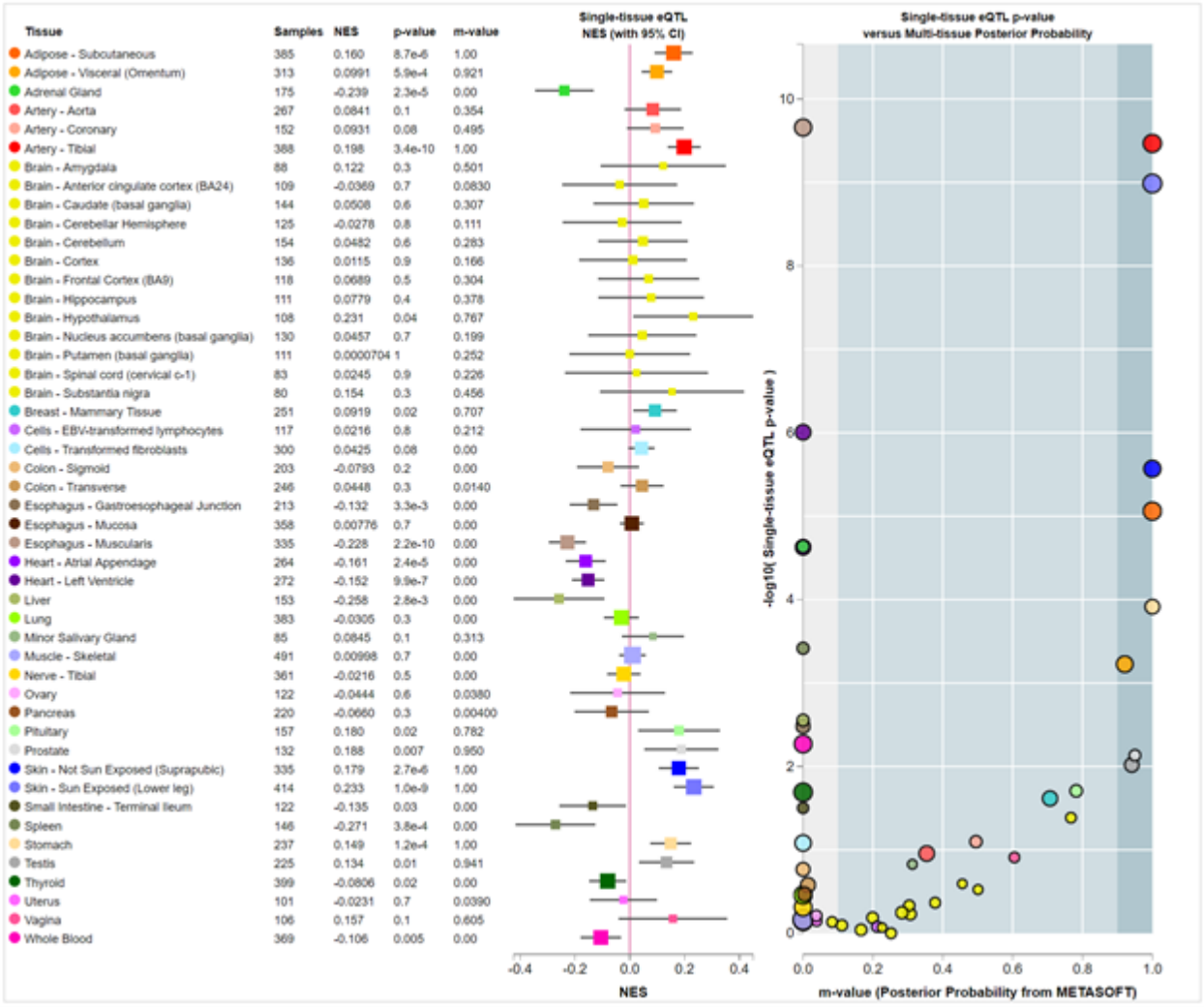

Figure 27. Multi-tissue eQTL comparisons of the SNP rs1049434.

There was no statistically significant association between genotype and SLC16A1 expression in all brain tissue (shown in yellow) except hypothalamus. The effect size (NES) of the association in hypothalamus was weak at only 0.23.

Some limitations of the study should be noted. This is a single center study that needs to be replicated with multi-center study. However, the replication of the findings, with patients that are separated from the discovery cohort by time, reduces some of the bias that could emanate from a single-center study. For this study, important clinical variables such as comorbidity and medication use were not available for analysis. This is particularly important for older patients, and possible explains why the influence of genotype diminished with age, which appears as age-genotype interaction in the model. Finally, although plausible explanation – change in expression of SLC16A7 – is provided, because this is a retrospective association study, causation could not be established. More clinical and molecular studies are needed to fully elucidate the relationship between SLC16AT rs10506399 genotype and GOS in TBI.

In conclusion, we report that in this genetic association study comprising of discovery and replication cohorts, the genotype at rs10506399 of SLC16A7 is associated with GOS scores in TBI patients. A genotype-age interaction was also detected indicating the effect of genotype was age dependent. Specifically, the beneficial effect having one or two alternative alleles at rs10506399 decreases as age increases. The findings in this study reveal potentially important role for MCT2 and its substrates, especially lactate, in regulating the brain energetics following TBI. In the light of current findings, patient stratification by MCT genotype at rs10506399 should be considered in clinical studies investigating the function, kinetics and clinical effect of endogenous and exogenous lactate.

6. Summary and Perspectives

6.1. Major Findings and Implications

6.1.1. Probenecid, transporter inhibitor, increases brain levels of antioxidant drug n-acetylcysteine

In this chapter, we sought to understand how the transporter inhibitor, probenecid, affects n-acetylcysteine pharmacokinetics and to evaluate the interaction of n-acetylcysteine with transporters. Juvenile Sprague-Dawley rats were administered n-acetylcysteine alone or in combination with probenecid intraperitoneally. Plasma samples and brain hemispheres were collected serially and n-acetylcysteine concentrations were measured. Transporter studies were conducted with human embryonic kidney (HEK)-293 cells that overexpress organic anion transporter (OAT)1 or OAT3 and with human multi-drug resistance-associated protein (MRP)1 and MRP4 membrane vesicles.

N-acetylcysteine area under the curve was increased in plasma (1.65-fold) and brain (2.41-fold) by probenecid co-administration. The apparent plasma clearance was decreased by 65%. Time- and concentration-dependent n-acetylcysteine uptake was inhibited by probenecid in OAT1 and OAT3, but not in MRP1 or MRP4 overexpression systems. Our results indicate for the first time that n-acetylcysteine is substrate for OAT1 and OAT3 and that probenecid increases n-acetylcysteine plasma and brain exposure *in vivo*. These data provide insight regarding how n-acetylcysteine crosses biological barriers and suggest a promising therapeutic strategy to increase n-acetylcysteine (and possibly other drugs) exposure in the brain (and plasma) by inhibiting membrane transporters.

6.1.2. The effect of a combination of n-acetylcysteine and probenecid on functional outcomes in rat, traumatic brain injury model

In this chapter we examined the capacity of a combination of n-acetylcysteine and probenecid to improve functional and histological outcomes in a preclinical model of pediatric TBI. This was based on the observation that a combination of n-acetylcysteine and probenecid can counter oxidative stress through multiple ways. While n-acetylcysteine donates cysteine, the rate limiting substrate in biosynthesis of glutathione, probenecid prevents active efflux of glutathione and its conjugates by inhibiting MRP transporters. Additionally, as described in Chapter 2, as transporter inhibitor probenecid increases plasma and brain levels of the antioxidant n-acetylcysteine.

Seventeen day old rats underwent either controlled cortical impact (CCI; *n* = 10-11 per treatment group) or sham surgery (n = 5 per treatment group) and divided into four treatment groups each; n-acetylcysteine (163m/Kg)+vehicle, probenecid (150mg/Kg)+vehicle, n-acetylcysteine +probenecid and vehicle+vehicle. Drugs or vehicle were administered twice a day for the 3 days with additional dose of n-acetylcysteine in the first day. Motor function was assessed using beam balance tests on day 1-5 following surgery. Spatial learning and memory retention were evaluated with Morris water maze on days 11-16. Rats were sacrificed on day 19 and brain tissue fixed and stained with H&E followed by assessment of tissue volume lost and hippocampal neuronal survival.

In the motor function test with beam balance, compared to shams, all CCI groups showed significant impairment on days 1, and 2. Two of the TBI groups, CCI+probenecid+vehicle2 and CCI+ n-acetylcysteine + probenecid, showed worse impairment as compared to the other TBI groups, which was statistically significant on day 1 but not on day2. In the MWM, hidden platform trial that tested acquisition of Spatial learning, all CCI groups performed worse that sham groups. Results of the MWM, single probe trial that tested spatial memory retention show that the sham group performed better than the CCI+ n-acetylcysteine+PB ($p < 0.05$) and CCI+ probenecid +vehicle ($p < 0.05$). In both MWM tests, the CCI+ n-acetylcysteine +vehicle group performed better than any of the other CCI groups, but the difference didn't reach significant level. No treatment benefit was observed in hippocampal neuronal survival analysis while the

CCI+ n-acetylcysteine +probenecid showed significant attenuation of tissue volume loss after CCI.

Following TBI, at the given dosage regime, n-acetylcysteine appears to attenuating spatial learning and memory retention deficits that didn't reach statistical significance; probenecid seems to cause acute motor deficit; and the combination of both improved tissue volume loss. Optimization of the dosage regime for both drugs is justified to enhance the beneficial effects of n-acetylcysteine and minimize the adverse effects of probenecid.

6.1.3. Neuropharmacometabolomics of treatment response of a combination of probenecid and n-acetylcysteine in traumatic brain injury

Metabolomics is a powerful tool for comprehensively profiling metabolites, altered biochemical processes, and discovery of perturbed systems due to injury and/or drug administration. In this chapter, we employed metabolomics along with pathway and network analyses to evaluate the CSF metabolome after severe TBI in children. The capacity of combination therapy with probenecid and n-acetylcysteine to modulate glutathione metabolism and related pathways after TBI—the purported mechanism of action—were also evaluated. CSF was obtained from 12 children enrolled in an IRB-approved, Phase I, randomized, placebo-controlled trial of a combination of probenecid and n-acetylcysteine after severe TBI (Trial Registration NCT01322009), 24 h after the first of dose of drugs (n = 7) or placebo (n = 5), and age-matched

control subjects (n = 5). After protein precipitation with acetonitrile, samples were injected into an UPLC-QTOFMS to analyze the CSF metabolome. Feature detection, retention time correction, alignment, annotation, and principal component analysis (PCA) and statistical analysis were conducted using XCMS-online. The software mummichog was used for pathway and network analyses. A two-component PCA revealed clustering of each of the groups, with distinct metabolomics signatures. Several novel pathways with plausible mechanistic involvement in TBI were identified. A combination of metabolomics and pathway/network analyses showed that seven glutathione-centered pathways and two networks were enriched in the CSF of TBI patients treated with probenecid and n-acetylcysteine vs. placebo treated patients. Several pathways/networks consisting of components that are known substrates of probenecid-inhibitable membrane transporters such as prostaglandins, kynurenate and urate were also enriched, providing additional mechanistic validation.

This first of its kind neuropharmacometabolomics assessment reveals alterations in known and previously unidentified metabolic derangements after TBI, and supports target engagement of the combination of probenecid and n-acetylcysteine in the treatment of severe TBI in children

6.1.4. Association of SLC16A1, SLC16A3 and SLC16A7 genetic variants with long-term outcomes in TBI patients

In Chapter 5, using candidate-gene approach and focusing on well-studied SNPs, we evaluated the association of genetic alteration in MCT1 (SLC16A1), MCT2 (SLC16A7) and MCT4 (SLC16A3) with neurological outcomes in TBI. These MCT transporters are responsible trafficking lactic acid within the neurovascular unit, and hence understanding their role could help resolve the controversy on whether lactate could be used as alternative or preferred energy source in TBI.

Adults (age 17-78 years) with sever TBI (n=275 in discovery cohort and 189 in replication cohort, Glasgow Coma Scale (GCS) score ≤ 8) were genotyped for the SNPs rs10506399 of SLC16A7 and rs1049434 of SLC16A1. No relevant SNP was identified for SLC16A3 as there were no previously reported association of any SNP in SLC16A3 with functional outcomes. Glasgow Outcome Scale (GOS) scores were evaluated at 3, 6, 12 and 24 months post injury. A mixed effects ordered logistic regression model with GCS score, age and time posit-injury incorporated as covariates was used to assess the association between GOS and genotype at each of the SNPs. The GTEx Project portal was searched to determine the consequence of the SNPs found to be significantly associated with GOS. Adherence to Hardy-Weignberg equilibrium was achieved with both SNPs in both cohorts and the combined cohort. The mixed-effect ordered logistic regression model analysis showed patients with one or both of the alternate variants in rs10506399 were associated with GOS ($p>0.0001$). The association was age dependent as the

probably of favorable outcome associated with the alternate allele diminished with age. The SNP rs1049434 was not associated with GOS in the discovery group and hence not investigated in the replication cohort. The genotype at rs10506399 was significant associated with expression of SLC16A7 with the alternate allele showing higher expression.

The findings in this study reveal potentially important role for MCT2 and its substrates, especially lactate, in regulating the brain energetics following TBI. In the light of current findings, patient stratification by MCT2 genotype at rs10506399 should be considered in clinical studies investigating the function, kinetics and clinical effect of endogenous and exogenous lactate.

6.2. Limitations and Future Directions

Overall, this dissertation has mainly focused on translational and clinical aspects of the role of membrane transporters in TBI. It is outside the scope of this dissertation, but some outstanding mechanistic details at the molecular level remain to be studied. Some limitations specific for each chapter are discussed below.

In Chapter two of this thesis, we showed that inhibition of OAT1 and OAT3 transporters with probenecid increased plasma and brain levels of n-acetylcysteine. The fact that the increased in levels in the presence of probenecid was higher in the brain than plasma is indicative of possible

effect on BBB and/or choroid plexus, our method was not sufficient to conclusively attribute the effect to inhibition of transporters on these barriers. Future studies, through tissue specific knockout models, should investigate the significant of inhibiting of transporters on BBB and choroid plexus in altering the levels of drugs and endogenous molecules.

In Chapter three, we showed that while n-acetylcysteine appear to have beneficial effects, probenecid was implicated in some side effects. The main limitation of the study was that it didn't incorporate multiple doses of n-acetylcysteine and probenecid to establish dose-effect relationship for both drugs. With regards to n-acetylcysteine, higher or more frequent dose would have helped assess whether the spatial learning and memory deficit attenuation of observed with the given dose is a true effect, in that these effects would have reached statistical significance. Similarly, study design with multiple doses of probenecid in both the pharmacokinetic and function outcomes studies would have allowed thorough evaluation of the capacity of probenecid to increase levels of n-acetylcysteine without adverse effects. Given the levels of probenecid achieved with the dose used in this study are significantly higher than the Ki of probenecid of most transporters [232, 244-247, 251], it is possible that significant increase of level of n-acetylcysteine could be achieved even with lower doses of probenecid.

In Chapter four, we reported that global metabolomic signatures can differentiate among control subjects, placebo treated TBI patients, and TBI patients treated with a combination of probenecid and n-acetylcysteine. We also reported discovery of system level and individual markers of TBI

and pharmacological action of a combination of probenecid and n-acetylcysteine in TBI. Some limitations to the present study should be noted. First, the sample size is relatively small, although it does represent the first comprehensive metabolomics analysis of any kind in human TBI. Second, the MS assay was performed after sample preparation with acetonitrile. Thus, the analytical approach does not cover the entire spectrum of molecules, such as large proteins including those currently being evaluated as TBI biomarkers. Third, individual metabolites/features (with the exception of probenecid) and pathways were not confirmed independently in this study. Future studies using MS/MS fragmentation patterns and comparisons to pure standards for individual metabolite validation, and stable isotope marking for pathway confirmation using clinical samples and preclinical models will be necessary.

In Chapter five, we showed that genetic variation in SLC16A7, specifically rs10506399, was associated with GOS in age dependent manner in TBI patients. Some limitations of the study should be noted. This is a single center study that needs to be replicated with multi-center study. However, the replication of the findings with patients that are separated from the discovery cohort by time, reduces some of the bias that could emanate from a single-center study. For this study, important clinical variables such as comorbidity and medication use were not available for analysis. This is particularly important for older patients, and possible explains why the influence of genotype diminished with age, which showed up as age-genotype interaction in the model. Finally, although plausible explanation – change in expression of SLC16A7 – is provided, because this is a retrospective association study, causation could not be established. More

clinical and molecular studies are needed to fully elucidate the relationship between SLC16AT rs10506399 genotype and GOS in TBI.

6.3. Commentary and Conclusion

In summary, in this dissertation work, we sought to understand the role of membrane transporters in TBI. Through *in vitro* and *in vivo* methods, we demonstrated that it is possible to inhibit membrane transporters, such as with probenecid, to enhance the brain penetration capacity of candidate drugs, such as n-acetylcysteine, both in the presence or absence of brain injury. A recent report had indicated that inhibiting ABC efflux transporters in the intact BBB is unlikely to results in clinical meaningful increase in brain levels of substrate drugs without causing significant systemic toxicity [241]. Our finding, along with other previous reports [221, 222, 225], suggest uptake transporters on BBB or choroid plexus could possibly targeted to cause increased brain penetration of certain drugs. In the setting of TBI, even a small, non-toxic increase in either systemic Cmax or AUC through transporters inhibition together with breakage in BBB, the smaller volume of the brain tissue (relative to blood) and inhibition of transporters in BBB or choroid plexus could result in meaningful increase in brain levels of drugs [303, 304]. These possibility indicate that transporter inhibition as useful therapeutic strategy to overcome pharmacokinetic related challenges in TBI drug development.

We also showed that membrane transporters, such as OAT1, OAT3 and MRP transporters, could be therapeutically targeted to increase the brain levels of potentially beneficial endogenous molecules, such as glutathione. In this study, relatively high dose of probenecid was used and probably lead to adverse effects, including transient motor deficit. This highlights the notoriety transporters for having overlapping substrates and poor selectivity of transporter inhibitors [169] and shows how critical appropriate dose selection is to achieve optimal effects. Recently, there has been concerted effort to try to understand transporters as a class, identify common features and interrelationships, develop and standardize tools and methods for investigating transports, and characterize their structure-activity relationship [23]. Such efforts could be key in overcoming the challenges of targeting transporters therapeutically, in TBI or otherwise, such as identifying the optimal doses, or discover specific inhibitors or alternative ways of targeting transporters.

Furthermore, we showed that inhibition of transporters could profoundly alter the metabolome and associated pathways and networks of the CSF. The study in Chapter 4 of this dissertation underscores the usefulness of metabolomics as a tool to understand the range of substrates and functions of transporters, and in identifying markers of efficacy and toxicity for drugs that target transporters, as we were able to do in this case for a combination of probenecid and n-acetylcysteine in TBI.

Finally, we reported that understanding the role of transporters could provide unique insight in revealing the role of endogenous and exogenous small molecules, such as lactate, in a particular cellular processes, such as brain energetics after TBI. The lack of specific transporter inhibitors makes genetic methods that take advantage of naturally existing genetic variations as valuable approach to study the function of membrane transporters in humans.

Altogether, this dissertation project has provided experimental and clinical evidence that membrane transporters, as mediators of the movement of small molecules within the neurovascular unit, significantly contribute to the pathogenesis and pharmacotherapy of TBI and hence deserve due consideration as components of injury mechanism and as therapeutic targets in TBI.

APPENDIX A. Extended Data, Metabolomics

Table A1. Tentative identification of metabolites in CSF that are altered by TBI compared to control subjects

METLIN Name	RT	Measured mass (m/z)	Compound ID	Compound Name	Molecular Formula	Mass Error (delta)	Fold Change
M252T6*	5.54	252.085	HMDB00953	Suberylglycine	$C_{10}H_{17}NO_5$	0.000339	+68
			HMDB00726	Isovalerylglutamic acid	$C_{10}H_{17}NO_5$	0.000339	
M549T9_2	8.85	549.1804	HMDB28737	Asparaginyl-methionine	$C_9H_{17}N_3O_4S$	0.003229	+2
			HMDB28968	Methionyl-asparagine	$C_9H_{17}N_3O_4S$	0.003229	
			HMDB41685	5'-hydroxy-o-desmethylangolensin	$C_{15}H_{14}O_5$	0.004877	
			HMDB41654	3'-hydroxy-o-desmethylangolensin	$C_{15}H_{14}O_5$	0.004877	
			HMDB41695	6'-hydroxy-o-desmethylangolensin	$C_{15}H_{14}O_5$	0.004877	
M125T6	5.54	124.5233	HMDB28840	Glycyl-glutamate	$C_7H_{11}N_2O_5$	0.000684	+7
			HMDB28819	Glutamyl-glycine	$C_7H_{11}N_2O_5$	0.000684	
			HMDB12259	Methylarsonite	CH_5AsO_2	0.000928	
M603T8	7.93	602.4463	HMDB11847	Ganglioside gd2 (d18:0/24:0)	$C_{85}H_{153}N_3O_{34}$	0.00226	+92
			HMDB12535	12S-HHT	$C_{17}H_{28}O_3$	0.004787	
M617T10_1	9.79	616.9443	HMDB04898	Ganglioside ga2 (d18:1/25:0)	$C_{63}H_{118}N_2O_{18}$	0.004768	+261
			HMDB04887	Trihexosylceramide (d18:1/25:0)	$C_{61}H_{115}NO_{18}$	0.004769	
M646T9	9.02	646.4535	HMDB37830	Xi-7-hydroxyhexadecanedioic acid	$C_{16}H_{30}O_5$	0.001029	-484
			HMDB37831	Xi-8-hydroxyhexadecanedioic acid	$C_{16}H_{30}O_5$	0.001029	
			HMDB11498	Lysope (0:0/24:1(15z))	$C_{29}H_{58}NO_7P$	0.00196	
			HMDB11528	Lysope (24:1(15z)/0:0)	$C_{29}H_{58}NO_7P$	0.00196	
			HMDB28714	Arginyl-lysine	$C_{12}H_{26}N_6O_3$	0.0064	
			HMDB28945	Lysyl-arginine	$C_{12}H_{26}N_6O_3$	0.0064	
M603T9_3	9.10	603.4283	HMDB04884	Trihexosylceramide (d18:1/26:1(17z))	$C_{62}H_{117}NO_{18}$	0.005783	-111
			HMDB04896	Ganglioside ga2 (d18:1/26:0)	$C_{64}H_{120}N_2O_{18}$	0.005784	
M624T10_2	10.16	623.9521	HMDB04896	Ganglioside ga2 (d18:1/26:0)	$C_{64}H_{120}N_2O_{18}$	0.004742	-115
			HMDB04884	Trihexosylceramide (d18:1/26:1(17z))	$C_{62}H_{117}NO_{18}$	0.004744	
M573T9_4	8.82	573.4075	HMDB02890	3-cis-hydroxy-b,e-caroten-3'-one	$C_{40}H_{54}O$	0.000816	-212
M974T5_2	4.60	974.2563	HMDB41729	(-)-epicatechin 7-o-glucuronide	$C_{21}H_{22}O_{12}$	0.000225	-10
			HMDB41728	(-)-epicatechin 3'-o-glucuronide	$C_{21}H_{22}O_{12}$	0.000225	
			HMDB60161	3(S)-Hydroxy-4(R),8-dimethyl-nonanoyl-CoA	$C_{32}H_{56}N_7O_{18}P_3S$	0.005544	
			HMDB02992	S-2-octenoyl CoA	$C_{29}H_{48}N_7O_{17}P_3S$	0.008093	
			HMDB03949	(2e)-octenoyl-CoA	$C_{29}H_{48}N_7O_{17}P_3S$	0.008093	
			ECMDB23397	trans-Oct-2-enoyl-CoA	$C_{29}H_{48}N_7O_{17}P_3S$	0.008093	

Table A2. Metabolic pathways altered in probenecid+n-acetylcystiene treated TBI patients compared to placebo treated TBI patients

Pathway	Mode	Pathway Size	Percentage Overlap	Overlapping Features
Melatonin degradation II	+	6	66.7%	5-methoxyindoleacetaldehyde 5-methoxytryptophol Melatonin acetate 5-methoxytryptamine 5-methoxyindole acetate
CMP phosphorylation	+	3	66.7%	ATP CMP CDP
N-acetylglucosamine degradation I	+	3	66.7%	β-D-fructofuranose 6-phosphate α-D-glucosamine 6-phosphate Acetate
Methylglyoxal degradation I	+	3	66.7%	Glutathione Pyruvate (R)-S-lactoylglutathione
Formaldehyde oxidation	+	3	66.7%	Glutathione S-formylglutathione S-hydroxymethylglutathione
Melatonin degradation III	+	3	66.7%	N-acetyl-5-methoxykynurenamine Melatonin, N^1-acetyl-N^2-formyl-5-methoxykynuramine
Geranylgeranyldiphosphate biosynthesis	+	3	66.7%	(2E,6E)-farnesyl diphosphate Geranylgeranyl diphosphate Isopentenyl diphosphate
Glutathione redox reactions II	+	3	66.7%	Glutathione Glutathione disulfide NADPH
Glutathione redox reactions I	+	3	66.7%	Glutathione Glutathione disulfide NADPH
α-tocopherol degradation	+	5	60.0%	13'-hydroxyα-tocopherol α-carboxyethylhydroxychroman 5'-α-carboxylmethylbutylhydroxychroman α-tocopherol NADPH
2-oxoisovalerate decarboxylation to isobutanoyl-CoA	+	5	60.0%	NAD^+ 3-methyl-2-oxobutanoate Coenzyme A Isobutanoyl-CoA
4-aminobutyrate degradation	+	5	60.0%	L-glutamate NAD^+ Succinate 2-oxoglutarate, Succinate semialdehyde
Estradiol biosynthesis I	+	5	60.0%	19-hydroxyandrostenedione 17-β-estradiol Estrone Androst-4-ene-3,17-dione 19-oxoandrostenedione
Fatty acid α-oxidation	+	7	57.1%	AMP ATP NAD^+ Succinate 2-oxoglutarate Coenzyme A
4-hydroxyproline degradation	+	7	57.1%	L-glutamate NAD^+ Pyruvate 2-oxoglutarate (3R,5S)-1-pyrroline-3-hydroxy-5-carboxylate L-4-hydroxyglutamate semialdehyde

				Erythro-4-hydroxy-L-glutamate
Ascorbate recycling (cytosolic)	+	7	57.1%	Glutathione Glutathione disulfide NAD^+ L-ascorbate, L-dehydro-ascorbate NADPH Monodehydroascorbate
Putrescine degradation III	+	7	57.1%	NAD^+ Coenzyme A Acetate N-acetylputrescine Acetyl-CoA 4-acetamidobutanoate 4-acetamidobutanal
Serine biosynthesis (phosphorylated route)	+	7	50%	L-glutamate NAD^+ 2-oxoglutarate 3-phospho-D-glycerate 3-phospho-L-serine 3-phospho-hydroxypyruvate
trans, trans-farnesyl diphosphate biosynthesis	+	6	50%	Dimethylallyl diphosphate Geranyl diphosphate (2E,6E)-farnesyl diphosphate Isopentenyl diphosphate
Fatty acid α-oxidation III	+	4	50%	AMP ATP NAD^+ Coenzyme A
Thyronamine and iodothyronamine metabolism	+	4	50%	3,3',5,5'-tetraiodothyronamine 3'-iodothyronamine Thyronamine 3-iodothyronamine
Asparagine degradation	+	4	50%	L-glutamate 2-oxoglutarate Oxaloacetate N^4-(beta-N-acetyl-D-glucosaminyl)-L-asparagine
Epoxysqualene biosynthesis	+	4	50%	Squalene (2E,6E)-farnesyl diphosphate (3S)-2,3-epoxy-2,3-dihydrosqualene NADPH
Thiamin salvage III	+	4	50%	AMP ATP Thiamin Thiamin diphosphate
Phospholipases	-	2	100%	Phosphocholine D-myo-inositol (1,4,5)-trisphosphate
Glutathione redox reactions II	-	4	75%	Glutathione Glutathione disulfide $NADP^+$ NADPH
Glutathione redox reactions I	-	4	75%	Glutathione Glutathione disulfide $NADP^+$ NADPH
Ascorbate recycling (cytosolic)	-	8	50%	Glutathione Glutathione disulfide NAD^+ NADH $NADP^+$ NADPH L-ascorbate

				L-dehydro-ascorbate
Dolichol and dolichyl phosphate biosynthesis	-	6	50%	CDP CTP $NADP^+$ NADPH (2E,6E)-farnesyl diphosphate, diphosphate
Sulfate activation for sulfonation	-	4	50%	Diphosphate Adenosine 5'-phosphosulfate 3'-phosphoadenylyl-sulfate ADP
Pentose phosphate pathway (oxidative branch)	-	4	50%	$NADP^+$ NADPH 6-phospho D-glucono-1,5-lactone β-D-glucose 6-phosphate
Epoxysqualene biosynthesis	-	4	50%	$NADP^+$ NADPH (2E,6E)-farnesyl diphosphate Diphosphate

Table A3. Top Network Modules, TBI vs TBI+PB+NAC, Negative MS mode

Module	Metabolites
Module 1, p=0.02738, 24 metabolites	glutathione, NAD+, leukotriene B4, 3-methoxy-4-hydroxyphenylglycol, NADP+, glutathione disulfide, riboflavin, S-adenosyl-L-homocysteine, quinine, bisorganyltrisulfane, XMP, urate, (R)-S-lactoylglutathione, albendazole s-oxide, 2-dihydro-NAD, 2-oxoadipate, S-sulfanylglutathione, 6-phospho D-glucono-1,5-lactone, 2-aminomuconate 6-semialdehyde, 6-dihydro-NAD, 6-dihydro-NADP, 2-dihydro-NADP, S-formylglutathione, GDP-4-dehydro-&alpha-D-rhamnose
Module 2, p=0.02835, 6 metabolites	R)-S-lactoylglutathione, glutathione, S-sulfanylglutathione, bisorganyltrisulfane, S-formylglutathione, glutathione disulfide
Module 3, p=0.02998, 9 metabolites	albendazole s-oxide, riboflavin, leukotriene B4, quinine, 6-phospho D-glucono-1,5-lactone, NADP+, 6-dihydro-NADP, 2-dihydro-NADP, GDP-4-dehydro-&alpha-D-rhamnose
Module 4, p=0.0441, 4 metabolites	adenosine 5'-phosphosulfate, indoxyl sulfate, estrone, 3'-phosphoadenylyl-sulfate

Table A4: Top Network Modules, TBI vs TBI+PB+NAC, Positive MS mode

Module	Metabolites
Module 1, p=0.00252, 86 metabolites	3-methoxy-4-hydroxyphenylpyruvate, NAD+, allopregnanolone, 4-oxoglutaramate, N4-(&beta-N-acetyl-D-glucosaminyl)-L-asparagine, acetate, erythro-4-hydroxy-L-glutamate, 2-hydroxy-5-oxoproline, 18-hydroxyoleate, 4-imidazoleacetate, octanoyl-CoA, L-erythro-5,6,7,8-tetrahydrobiopterin, &beta-D-galactosyl-1,4-N-acetyl-&beta-D-glucosamine, 5-methoxytryptamine, riboflavin cyclic-4',5'-phosphate, morphinone, codeine, isobutanoyl-CoA, molybdopterin adenine dinucleotide, biotin, &beta-alanine, nicotinamide, oleate, 3-sulfinopyruvate, AMP, biotin amide, (25R)-3&alpha,7&alpha,12&alpha-trihydroxy-5&beta-cholestan-26-al, N-acetyl-D-galactosamine 6-phosphate, prostaglandin E2, 2-oxoglutaramate, 2-phenylethanol, 3-methyl-2-oxobutanoate, (S)-2,3,4,5-tetrahydropiperidine-2-carboxylate, thiamin diphosphate, 4-methylumbelliferyl acetate, L-alanine, prostaglandin I2, succinate, anthranilate, 6-pyruvoyl tetrahydropterin, thromboxane A2, 2-methyl-3-hydroxybutyryl-CoA, UDP-&alpha-D-glucose, &alpha-linolenate, 4-acetamidobutanoate, 4-(2-aminophenyl)-2,4-dioxobutanoate, prostaglandin D2, prostaglandin-H2, androst-4-ene-3,17-dione, XMP, decanoate, L-citrulline, N2-succinylglutamate, 19-hydroxyandrostenedione, 3-[(3aS,4S,7aS)-7a-methyl-1,5-dioxo-octahydro-1H-inden-4-yl]propanoate, UDP-&alpha-D-galactose, (25R)-3&alpha,7&alpha,12&alpha-trihydroxy-5&beta-cholestan-26-oate, (25R)-3&alpha,7&alpha-dihydroxy-5-&beta-cholestanate, oripavine, neopinone, L-saccharopine, NMNH, 2-aminomuconate, N-acetyl-&alpha-L-aspartyl-L-glutamate, kynurenate, N-acetyl-&beta-D-glucosamine, xanthosine, 1,6-anhydro-N-acetyl-&beta-muramate, 3-phospho-D-glycerate, 4-hydroxyphenylpyruvate, D-galactosamine 6-phosphate, 3-hydroxy-L-kynurenine, codeinone, 2-methylacetoacetyl-CoA, N-acetyl-4-O-acetylneuraminate, 5-androstene-3,17-dione, acetoacetyl-CoA, 4-tyrosol, D-glycerate, L-arginino-succinate, 2-oxoglutarate, L-alanyl-L-aspartate, 2-phospho-D-glycerate, 3-oxo-2-(cis-2'-pentenyl)-cyclopentane-1-octanoate, N-acetyl-L-aspartate, prostaglandin F2&alpha

Module 2, p=0.02238, 24 metabolites	kynurenate, 3-methoxy-4-hydroxyphenylpyruvate, succinate, anthranilate, codeine, isobutanoyl-CoA, 4-oxoglutaramate, &beta-alanine, erythro-4-hydroxy-L-glutamate, 4-hydroxyphenylpyruvate, 3-hydroxy-L-kynurenine, 2-hydroxy-5-oxoproline, codeinone, 4-(2-aminophenyl)-2,4-dioxobutanoate, 3-sulfinopyruvate, 2-oxoglutarate, 2-oxoglutaramate, L-alanyl-L-aspartate, 3-methyl-2-oxobutanoate, morphinone, oripavine, neopinone, L-saccharopine, L-alanine
Module 3, p=0.02357, 21 metabolites	2-methyl-3-hydroxybutyryl-CoA, 3-phospho-D-glycerate, androst-4-ene-3,17-dione, 4-tyrosol, 4-imidazoleacetate, 2-phospho-D-glycerate, NAD+, (S)-2,3,4,5-tetrahydropiperidine-2-carboxylate, 4-acetamidobutanoate, 6-pyruvoyl tetrahydropterin, D-glycerate, N2-succinylglutamate, 19-hydroxyandrostenedione, 2-methylacetoacetyl-CoA, L-erythro-5,6,7,8-tetrahydrobiopterin, prostaglandin E2, 2-phenylethanol, allopregnanolone, 5-androstene-3,17-dione, 2-aminomuconate, nicotinamide
Module 4, p=0.02741, 22 metabolites	xanthosine, molybdopterin adenine dinucleotide, biotin, &alpha-linolenate, oleate, AMP, acetoacetyl-CoA, biotin amide, XMP, decanoate, (25R)-3&alpha,7&alpha,12&alpha-trihydroxy-5&beta-cholestan-26-al, L-citrulline, octanoyl-CoA, L-arginino-succinate, 18-hydroxyoleate, 3-[(3aS,4S,7aS)-7a-methyl-1,5-dioxo-octahydro-1H-inden-4-yl]propanoate, (25R)-3&alpha,7&alpha,12&alpha-trihydroxy-5&beta-cholestan-26-oate, (25R)-3&alpha,7&alpha-dihydroxy-5-&beta-cholestanate, riboflavin cyclic-4',5'-phosphate, thiamin diphosphate, 3-oxo-2-(cis-2'-pentenyl)-cyclopentane-1-octanoate, NMNH
Module 5, p=0.04775, 8 metabolites	L-dehydro-ascorbate, (R)-S-lactoylglutathione, glutathione, S-sulfanylglutathione, 4-hydroxy-2-nonenal, glutathione disulfide, S-formylglutathione, 4-hydroxy-2-nonenal-glutathione conjugate

Table A5. Tentative identification of metabolites in CSF altered by treatment with probenecid and NAC after TBI

*Negative MS mode, otherwise positive MS mode

METLIN Name	RT	Measured mass (m/z)	Compound ID	Compound Name	Molecular Formula	Mass Error (delta)	Fold Change
M352T7*	6.93	352.0831	HMDB00125	Glutathione	$C_{10}H_{17}N_3O_6S$	0.001093	+665
M353T7*	6.94	353.086	HMDB41723	Dihydroferulic acid 4-o-glucuronide	$C_{16}H_{20}O_{10}$	0.001257	+81
			HMDB01015	N-formyl-l-methionine	$C_6H_{11}NO_3S$	0.001348	
			HMDB59611	Thiomorpholine-3-carboxylic acid	$C_5H_9NO_2S$	0.001351	
M432T7*	6.94	432.0258	HMDB61355	([(2-azaniumylethoxy)(hydroxy)phosphoryl] oxy}({[5-(2-hydroxy-4-imino-1,4-dihydropyrimidin-1-yl)-1,3-oxathiolan-2-yl]methoxy))phosphinic acid	$C_{10}H_{19}N_4O_9P_2S$	0.001721	+18
M354T7*	6.93	354.082	HMDB10319	Inodxyl glucuronide	$C_{14}H_{15}NO_7$	0.001053	+75
			HMDB59804	Inodxyl glucuronide	$C_{14}H_{15}NO_7$	0.001053	
M521T7*	6.93	521.0661	HMDB12208	D-erythro-imidazole-glycerol-phosphate	$C_6H_{11}N_2O_6P$	0.003046	+81
M326T7*	6.931	326.0834	HMDB59609	Albendazole S-oxide	$C_{12}H_{15}N_3O_3S$	0.001787	+84
M242T7_1*	6.935	242.1028	HMDB61717	O-succinylcarnitine	$C_{11}H_{19}NO_6$	0.000047	+115
			HMDB13133	Methylmalonylcarnitine	$C_{11}H_{19}NO_6$	0.000047	
			HMDB12215	Dihydrozeatin	$C_{10}H_{15}N_5O$	0.000474	
M520T7*	6.935	520.0636	HMDB00652	Chondroitin 4-sulfate	$C_{14}H_{21}NO_{15}S$	0.002209	+552
M284T7_1*	6.934	284.0964	HMDB15166	Probenecid	$C_{13}H_{19}NO_4S$	0.000197	+7731
M659T7_2*	6.931	659.1668	HMDB41849	Carboxytolbutamide	$C_{12}H_{16}N_2O_5S$	0.003036	+156
M285T7_2*	7.01	285.099	HMDB61112	3-carboxy-4-methyl-5-propyl-2-furan-propanoic acid	$C_{12}H_{16}O_5$	0.001025	+430
			HMDB41756	Isopropyl 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoate	$C_{12}H_{16}O_5$	0.001025	
M653T7*	6.931	653.1501	HMDB60343	2-(s-glutathionyl)acetyl glutathione	$C_{22}H_{34}N_6O_{13}S_2$	0.005151	+162
M669T7*	6.935	669.1219	HMDB59645	beta-nicotinamide D-ribonucleotide	$C_{11}H_{16}N_2O_8P$	0.000322	+132
			HMDB06824	Dihydroneopterin phosphate	$C_9H_{14}N_5O_7P$	0.003007	
M268T4*	4.428	268.0796	HMDB02095	Malonylcarnitine	$C_{10}H_{17}NO_6$	0.000653	+5
M202T7	6.948	202.0173	HMDB01274	Thymidine diphospate	$C_{10}H_{16}N_2O_{11}P_2$	0.001442	+247
M183T7_1	6.948	183.0463	HMDB00095	Cytidine monophosphate	$C_9H_{14}N_3O_8P$	0.000175	+8
			HMDB11692	Cytidine 2'-phosphate	$C_9H_{14}N_3O_8P$	0.000175	
			HMDB60024	(2S,3S,4R,5R,6S)-6-(4-carboxy-2-methoxyphenoxy)-3,4-dihydroxy-5-methyloxane-2-carboxylic acid	$C_{15}H_{18}O_9$	0.000512	

M286T7_1	6.948	286.1114	HMDB15166	Probenecid	$C_{13}H_{19}NO_4S$	0.000645	+643
M288T7_1	6.948	288.1095	HMDB04328	Temurin	$C_9H_{12}N_4O_3$	0.002795	+52
M268T7_1	6.950	268.1004	HMDB29126	Valyl-glutamate	$C_{10}H_{17}N_2O_5$	0.002565	+17
			HMDB28832	Glutamyl-valine	$C_{10}H_{17}N_2O_5$	0.002565	
M121T7	6.929	121.0288	HMDB00594	Glutamylphenylalanine	$C_{14}H_{18}N_2O_5$	0.000942	+24
			HMDB28875	Hydroxyprolyl-tyrosine	$C_{14}H_{18}N_2O_5$	0.000942	
			HMDB29106	Tyrosyl-hydroxyproline	$C_{14}H_{18}N_2O_5$	0.000942	
M467T1_2	0.562	467.1365	HMDB10325	Ethyl glucuronide	$C_8H_{14}O_7$	0.000624	-3
M162T7	6.949	162.0554	HMDB03320	Indole-3-carboxylic acid	$C_9H_7NO_2$	0.000446	+11
			HMDB02285	2-indolecarboxylic acid	$C_9H_7NO_2$	0.000446	
			HMDB04077	4,6-dihydroxyquinoline	$C_9H_7NO_2$	0.000446	
			HMDB03426	Pantetheine	$C_{11}H_{22}N_2O_4S$	0.001168	
M216T1_2	0.562	216.0878	HMDB01557	Riboflavin reduced	$C_{15}H_{16}N_4O_6$	0.000485	-2
			HMDB12149	2-isopropyl-3-oxosuccinate	$C_7H_{10}O_5$	0.001154	
			HMDB61388	Dimethyl 2-oxoglutarate	$C_7H_{10}O_5$	0.001154	
			HMDB00125	Glutathione	$C_{10}H_{17}N_3O_6S$	0.0012	
M305T1_2	0.583	305.0236	HMDB06512	3-mercaptolactate-cysteine disulfide	$C_6H_{11}NO_5S_2$	0.000029	+6
M335T1_4	0.527	335.0941	HMDB00052	Argininosuccinic acid	$C_{10}H_{18}N_4O_6$	0.000306	+3
			HMDB02032	8-hydroxyguanine	$C_5H_5N_5O_2$	0.001825	
			HMDB00401	2,8-dihydroxyadenine	$C_5H_5N_5O_2$	0.001825	
			HMDB00544	5-hydroxymethyl-4-methyluracil	$C_6H_8N_2O_3$	0.002102	
			HMDB01014	4-imidazolone-5-propionic acid	$C_6H_8N_2O_3$	0.002102	
			HMDB02320	Imidazolelactic acid	$C_6H_8N_2O_3$	0.002102	
			HMDB23730	3-(Imidazol-5-yl)lactic acid	$C_6H_8N_2O_3$	0.002102	

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