Attributes of Astrocyte Response to Mechano-Stimulation by High-Rate Overpressure

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Abstract

Blast neurotrauma represents a significant mode of traumatic injury to the brain. The incidence of blast neurotrauma is particularly high amongst military combat personnel and can be debilitating and endure clinically for years after injury is sustained. Mechanically, blast represents a unique and complex loading paradigm associated with compressive shock waves that propagate out from an explosive event and interact with the head and other organs through high-rate loading. When subjected to such insult, brain cells undergo characteristic injury responses which include neuroinflammation, oxidative stress, edema and persistent glial activation. These features of the injury have emerged as important mediators of the chronic brain damage that results from blast.

Astrocytes have emerged as a potential therapeutic target because of their ubiquitous roles in brain homeostasis, tissue integrity and cognitive function. This glial subtype has a characteristic reactive response to mechanical trauma of various modes. In this work, custom *in vitro* injury devices were used to characterize functional models of astrocyte reactivity to high-rate insult to study mechano-stimulation mechanisms associated with the reactive phenotype. The working hypothesis was that high-rate overpressure exposure would cause metabolic aberrations, cell junction changes, and adhesion signal transduction activation, all of which would contribute to astrocyte response and reactivity. Astrocyte cultures were exposed to a 20 psi high-rate overpressure scheme using an underwater explosion-driven device.

Astrocytes experienced dynamic energetic fluctuations in response to overpressure which were followed by the assumption of a classically defined reactive phenotype. Results indicated specific roles for cationic transduction, cell junction dynamics (gap junction and anchoring junctions) and downstream signal transduction mechanisms associated with adhesion alterations in onset of the astrocyte reactive phenotype. Investigation into adhesion signaling regulation by focal adhesion kinase in 2D and 3D cultures was also explored to better understand cellular reactivity as a function of extracellular environment. Additionally, another underwater *in vitro* device was built to study combination effects from overpressure and fluid shear associated with insult.

Overall, the combined studies offer multiple mechanisms by which to explore molecular targets for harnessing astrocytes' potential for repair after traumatic injury to the brain.

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General Audience Abstract

Blast neurotrauma represents a significant mode of traumatic injury to the brain. The incidence of blast neurotrauma is particularly high amongst military combat personnel in which close to 80% of the injuries sustained in combat are attributed to explosive mechanisms. This injury, like other traumatic brain injuries, can be debilitating and result in altered quality of life for years after injury is sustained. There is a critical need to understand how brain cells are injured by and respond to blast loading in order to develop effective therapeutic strategies.

The following work approaches this problem through the use of cellular models of blast-type insult. Custom injury devices were used to develop models of brain cell reactive response to high-rate insult based on experimental simulations of blast neurotrauma. In particular, a sub-type of brain cells called astrocytes were studied. Astrocytes have emerged as a potential therapeutic target because of their ubiquitous roles in brain homeostasis, tissue integrity and cognitive function. The working hypothesis was that high-rate overpressure exposure would cause metabolic aberrations, cell junction changes, and adhesion signal transduction activation, all of which would contribute to astrocyte response and reactivity.

Astrocytes experienced dynamic energetic fluctuations in response to overpressure which were followed by the assumption of a classically defined reactive phenotype. Results indicated specific roles for cationic transduction, cell anchorage and downstream signaling mechanisms associated with adhesion alterations in onset of the astrocyte reactive phenotype. Investigation into adhesion signaling regulation by focal adhesion kinase in 2D and 3D cultures was also explored to better understand cellular reactivity as a function of extracellular environment. Additionally, another underwater cell injury device was built to study combination effects from overpressure and fluid shear associated with insult.

Overall, the combined studies offer multiple mechanisms by which to explore molecular targets for harnessing astrocytes' potential for repair after traumatic injury to the brain.

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List of Abbreviations

5mc – 5-methylcytosine

ANOVA – analysis of variance

AQP – aquaporin

BBB – blood brain barrier

BCA – bicinchoninic acid

CNS – central nervous system

CX—connexin

DMEM – Dulbecco's modified eagle medium

DTT – dithiothreitol

ECM – extracellular matrix

EMSA – electrophoretic mobility shift assay

ERK – extracellular signal-regulated kinase

FAD – flavin adenine dinucleotide

FAK – focal adhesion kinase

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GFAP – glial fibrillary acidic protein

GLT – glutamate transporter

HA – hyaluronic acid

HOS – high-rate overpressure simulator

ICAM – intercellular adhesion molecule

IKK – inhibitor of κB kinase

IL-1β – interleukin-1 beta

IL-6 – interleukin-6

ILK – integrin-linked kinase

IP3 – inositol 1,4,5-triphosphate

JNK – c-Jun N-terminal kinase

MAPK – mitogen-activated protein kinase

MEK1/2 – MAPK kinase

MEKK – MAPK kinase kinase

MMP – matrix metalloproteinase

MTT—3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADPH – nicotinamide adenine dinucleotide

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NOX – NADPH oxidase

OP – overpressure

PCNA – proliferating cell nuclear antigen

PTSD – post-traumatic stress disorder

ROS – reactive oxygen species

SHOS – shear-combination high-rate overpressure simulator

SOD – superoxide dismutase

TBI – traumatic brain injury

TNF-α – tumor necrosis factor-alpha

qPCR – real-time polymerase chain reaction

Chapter 1. Introduction

1.1. Significance

Traumatic brain injury (TBI) represents a substantial clinical burden in the United States, accounting for millions of new cases each year (Faul et al., 2010; Taylor et al., 2017). It is defined by a diverse cellular and molecular pathology that results from external physical force acting on the head and brain parenchyma. Common injury sources include falls, motor vehicle accidents, sports, and explosive (blast) exposure. Patients that sustain a TBI are at greater risk of developing neurodegenerative diseases, in addition to experiencing cognitive and psychological deficits following injury (Gavett et al., 2010). Few therapeutic strategies have proven successful in mitigating the long-term effects of TBI and none have reached status of being approved by the Food and Drug Administration. This is largely attributable to the heterogeneity of mechanical insults that cause TBI and a lack of mechanistic understanding of brain cell response to trauma. It is important to distinguish the injury hallmarks, progression and clinical outcomes associated with injury source and severity. In particular, recent efforts have focused on understanding the unique pathology associated with blast-type TBIs. As military conflicts have increased in the past two decades, an increasing number of combat personnel and civilians are inflicted with symptoms of blast neurotrauma (Owens et al., 2008). The mechanics of this injury are defined by short-duration, low-impulse shock waves that often result in mild severity injury and progressive brain dysfunction. Blast neurotrauma most commonly presents clinically as memory impairment and increased anxiety in both humans and experimental models (Koliatsos et al., 2011; Kamnaksh et al., 2012; Cho et al., 2013; Mac Donald et al., 2014).

TBI studies have traditionally focused on neuronal dysfunction. However, mechanical disruptions, such as TBI, lead to perturbations in cellular communication and neural networks involving glial cells. Thus, emphasis has shifted to the significance of glial cells, particularly astrocytes, and their role in the progression of TBI (Kumar and Loane, 2012; Burda et al., 2016; Karve et al., 2016). In the healthy brain, astrocytes serve roles in homeostatic and trophic support to brain parenchyma. They also serve primary structural roles and are critical in force transmissions that occur in brain tissue. In a pathological environment, astrocytes assume a reactive phenotype that is acutely neuroprotective but can also be detrimental and potentiate symptoms of TBI into the chronic stages. Reactivity typically involves aberrant metabolism, persistent phenotypic changes, and alterations to the cell adhesion and the microenvironment (Morganti-Kossmann et al., 2002; Buffo et al., 2010; Burda et al., 2016; Sajja et al., 2016). Evidence exists to suggest that many of the

molecular hallmarks of astrocyte reactivity are governed by mechanical perturbations. *In vitro* models of astrocyte injury have shown that in the absence of other brain cells, astrocytes assume a characteristic reactive response that may involve altered metabolism, proliferation, signaling, membrane/adhesion, and extracellular matrix (ECM) proteins. This suggests a role for mechanobiological pathways in their activation. Evidence from our lab suggests that astrocytes are reactive to shock wave insults, such as those in blast neurotrauma. Moreover, impaired neuronal-astrocytic signaling occur as a result of traumatic insult, lending to excitotoxicity, metabolic failure and neurodegeneration, all of which have implications for the memory deficits and behavioral outcomes of TBI (Pekny et al., 2014; Sajja et al., 2016). Astrocytes play a distinct role in TBI progression, and thus understanding their fundamental capacity and response to injury are important targets in the way of developing effective treatments.

In this regard, shock wave interactions with brain tissue is not well characterized. With emerging need to understand blast-related injuries, studies have established that astrocytes assume a reactive phenotype in response to blast exposure (Cernak et al., 2011; Sajja et al., 2014a; Sajja et al., 2015a; Bailey et al., 2016a). This reactive response can involve a number of mechanically-induced phenotypes which have been minimally explored within blast brain injury. Many studies investigating astrocyte reactivity to blast-type injuries have narrowly focused on the classical hallmark of increased glial fibrillary acidic protein (GFAP) expression. Characterization of GFAP is critical in understanding injury progression as evidence has emerged to suggest that these hallmarks are relevant in human patients that have sustained a blast neurotrauma. However, it is important to distinguish other patterns of astrocyte reactivity to isolated shock wave including aberrations in metabolism, cellular signaling capacity, and adhesion.

To do this, it is important to utilize relevant *in vitro* models to recapitulate the proper mechanics of blast injury and decipher the phenotypes which ensue. *In vitro* models allow the capability to study phenotypic alterations in a controlled environment in order to gain insight into how shock wave interacts with subpopulations of brain cells and how it imparts molecular changes which drive specific aspects of cellular reactivity. It is still unknown how astrocytes can alter their responses depending on injury type and severity, particularly in response to shock wave insult. Therefore, research efforts will focus on distinguishing mechanisms for pathways and molecular interactions in astrocyte reactivity to blast neurotrauma.

1.2 Objectives

The hypothesis for this work is that high-rate overpressure exposure induces acute astrocyte reactivity and dysfunction through mechanically-derived signals which impart unique metabolic, signaling and adhesive alterations in astrocytes. *In vitro* astrocyte models were used in the following specific aims to study this hypothesis.

Specific Aim 1. Evaluate acute metabolic aberrations in astrocytes following high-rate overpressure

Sub-aim 1.1. Examine transient alterations in bioenergetics capacity (ATP production) and mitochondrial integrity due to overpressure

Sub-aim 1.2. Determine fluctuations in amino acid metabolism following insult

Sub-aim 1.3. Assess the influence of the cationic channel blocker gadolinium on pro-oxidative phenotype in astrocytes

Specific Aim 2. Characterize a role for mechano-stimulation in proliferative and junctional signaling responses in astrocytes after overpressure insult

Sub-aim 2.1. Determine a temporal pattern for regulation of classical features of astrocyte reactivity

Sub-aim 2.2. Examine changes in cell junction regulation as it relates to phenotypic reactivity

Sub-aim 2.3. Assess the nuclear localization of signal transduction molecules and the transcriptional activity of p65 as related to expression changes of intercellular adhesion molecule 1 (ICAM-1)

Sub-aim 2.4. Investigate patterns of global DNA methylation at time points of cellular reactivity

Specific Aim 3. Develop model methodologies to study the influences of overpressure mechanics on astrocyte mechano-stimulation and reactivity following exposure

Sub-aim 3.1. Determine the role for focal adhesion kinase (FAK) in contributing to a reactive astrocyte phenotype and to alterations in cellular adhesion following overpressure

Sub-aim 3.2. Develop a computational model to study the mechanics of overpressure profiles on cellular substrates within a shear-combination high-rate overpressure simulator

Chapter 2. Background

2.1. Traumatic Brain Injury (TBI) Overview

TBI and its associated pathologies have confounded research efforts for many decades. Although various therapeutics have entered into clinical trials, no successful strategies are yet available. One of the greatest challenges associated with treating TBI is the range of heterogeneous outcomes. Although all broadly referred to as TBI, there are many injury modes and severities that produce highly varied cellular, molecular and clinical consequences. These include injuries ranging from concussion on the sports field to penetrating wounds to blast exposures. More recent research efforts for TBI have a growing appreciation for these heterogeneities and the need for increased understanding of molecular mechanisms associated with both neuronal and glial dysfunction.

2.1.1. Clinical Significance

TBI accounts for a significant clinical burden as the leading cause of death and disability in persons less than 45 years old. Not only does this include close to 2.5 million emergency department visits annually but also long-term disabilities in over three million individuals in the United States alone (Zaloshnja et al., 2008; Faul et al., 2010; Taylor et al., 2017). Mild TBI, in particular, is difficult to diagnose and treat effectively due to the diversity of injury modes and delayed manifestation of clinical symptoms. High morbidity rates accompany drastic changes in quality of life, including fluctuations in mood and cognition. Moreover, TBI is a significant co-factor for developing neurodegenerative diseases and post-traumatic stress disorder (PTSD) (Hoge et al., 2008; Gavett et al., 2010; Chauhan, 2014). Severe TBIs typically result in immediate and sustained loss of consciousness as well as significant changes in memory and mood. While immediate disturbances in motor function, memory and mood have been observed, sustained cognitive deficits are common and may include changes in social behavior, psychomotor processing, and memory (Levin et al., 1979; Mazaux et al., 1997; Konrad et al., 2010).

TBI is a complex and compounding injury with a dire need for therapeutic options. Importantly, close to 300,000 people are hospitalized and 56,000 deaths occur annually as a result of TBI (Faul et al., 2010). While this is a daunting statistic, it displays the need and possibility for a therapeutic window after initial insult. However, pharmacological treatment strategies have yielded very little success in long-term improvement of outcomes from TBI. Previous interventional strategies have included generalized therapies involving various anti-inflammatory molecules, neuro-receptor antagonists, and free radical scavengers (Ikonomidou and Turski, 2002; Narayan et al., 2002;

Bergold, 2016). These strategies have been largely ineffective due to a lack of understanding of the molecular mechanisms and cellular interactions in TBI pathology.

2.1.2. Primary and Secondary Injury

TBI is a progressive injury that can be the result of biochemical dysfunction due to lethal and sub-lethal damages to brain cells. This process is initiated by mechanical insult itself, also known as primary injury. Primary injuries are broadly structural in nature and may include axonal damage, vascular compromise, and hemorrhage (Gennarelli et al., 1982; Levi et al., 1990; DeWitt and Prough, 2003; Povlishock and Katz, 2005). Focal injuries more commonly result in contusions and hematoma, while diffuse injuries may involve axonal damage and vascular impairment (Meythaler et al., 2001; Smith et al., 2003; Povlishock and Katz, 2005; Farkas and Povlishock, 2007).

Following primary injury, brain cells attempt to repair but often experience residual damage from ensuing molecular cascades and cellular activation. This phase can involve a number of cellular stress mechanisms including but not limited to excitotoxicity, oxidative stress, neuroinflammation, blood brain barrier (BBB) impairment, cytotoxic edema, and glial reactivity (Kontos, 1989; Yi and Hazell, 2006; Werner and Engelhard, 2007; Laird et al., 2008; Shlosberg et al., 2010; Kumar and Loane, 2012). Similar to primary injury, the molecular cascades that ensue are highly injury and severity dependent. Excitotoxicity is a common feature of early TBI secondary damage (Yi and Hazell, 2006). It results from transient disruptions in cellular membranes with subsequent redistribution of ions. In particular, potassium tends to move extracellularly and may be both dependent on dysfunctional neuronal firing in mild-moderate injuries (Katayama et al., 1990). This response is accompanied by surges in neurotransmitters like glutamate. Calcium overload also tends to both contribute to and result from excitotoxicity. As intracellular calcium levels transiently increase after injury, mitochondria become impaired, eliciting metabolic aberrations and oxidative stress (Xiong et al., 1997; Peng and Jou, 2010). Mitochondrial dysfunction is a prominent feature of acute stage TBI pathophysiology and is closely linked with oxidative stress mechanisms (Xiong et al., 1997; Keller et al., 1998; Sullivan et al., 1999; Hiebert et al., 2015). Oxidative stress is the result of imbalanced pro-oxidative and antioxidative mechanisms that causes accumulation of reactive oxygen species (ROS). ROS accumulation then lends to cellular damage by lipid peroxidation and protein/DNA oxidation. ROS are byproducts of oxidative metabolism and may result from either inefficient mitochondrial processes or increased metabolic demand.

Interestingly, mitochondrial dysfunction and ROS accumulation are also linked to neuroinflammation. Inflammation after TBI involves two major groups of cells: infiltrating cells (neutrophils, macrophages) and resident cells (glial cells). Together neurogenic and classical inflammatory mechanisms coordinate necessary repair processes after TBI, however chronic inflammation is a major contributor to cellular dysfunction and apoptosis (Kumar and Loane, 2012; Corrigan et al., 2016).

Lastly, one of the most prominent injury features in both human TBI and experimental models is glial activation, or reactivity. Persistent glial activation can lead to prolonged BBB disruption, chronic neuroinflammation, spreading depolarization, neuronal toxicity and apoptosis (Buffo et al., 2010; Burda et al., 2016; Seidel et al., 2016). Glial cells are also important for modulating BBB permeability and water volume control. Therefore, dysfunctional glial can directly contribute to vasogenic and cytotoxic edema. However, acute glial responses also serve several neuroprotective functions, discussed later, and are necessary for initial recovery after injury. The highly involved nature of glia in accentuating and moderating a range of TBI pathologies make them a unique therapeutic target for a broad range of cellular and molecular stressors after injury.

2.2. Primary Blast-Induced Neurotrauma

Blast neurotrauma has emerged in the past several decades as one of the leading morbidity factors in military populations, accounting for close to 80% of reported mild TBIs (Hoge et al., 2008; Elder and Cristian, 2009). Blast injuries occur as a result of exposure to explosives, through both impending blast waves and shrapnel. Since the rise in improvised explosive device use in the early 2000s, incidence of blast neurotrauma has also increased (Wolf et al., 2009; Hoge et al., 2008; Elder and Cristian, 2009). Blast neurotrauma is now estimated to affect close to 20% of Veterans (Hoge et al., 2008). Often, patients that suffer from blast neurotrauma do not have outward signs of injury but experience prolonged disturbances in mood, anxiety and memory.

Blast injury encompasses several modes of injury. Primary blast injury refers to that which is imparted by shock wave interaction with the body's tissues. This mode of injury has been the major focus for brain injury research, however the mechanisms of injury are highly debated and not well understood. Secondary blast injury includes wounds due to debris and shrapnel from the explosive event. This injury commonly includes blunt and penetrating injuries. Tertiary blast injury is defined as acceleration/deceleration injuries associated with dynamic pressures from the

explosion. This may include coup/contre-coup injuries to the brain upon head impact with objects in the surrounding environment. Importantly, this injury mode causes shearing of the brain tissue beyond that caused by the shock wave itself. Lastly, quaternary injury is sustained as a result of heat exposure and is most commonly superficial burns.

Little is understood about the molecular and cellular pathology associated with primary blast, in part due to an incomplete understanding of how shock waves interact with brain cells and their environment. Similar to other diffuse TBIs, blast neurotrauma has several well-characterized injury hallmarks which include astrocyte reactivity (Cernak et al., 2011; Svetlov et al., 2012; Sajja et al., 2014a; Bailey et al., 2016a/b), oxidative stress (Cernak et al., 2001; Readnower et al., 2010; Abdul-Muneer et al., 2013; Cho et al., 2013), neuroinflammation (Readnower et al., 2010; Abdul-Muneer et al., 2013; Sajja et al., 2014a), and BBB compromise (Readnower et al., 2010; Yeoh et al., 2013). However, it remains unclear how shock wave creates acute sequelae that prime the brain for chronic dysfunction.

2.2.1. Blast and Shock Wave Physics

Blast waves are associated with explosive events. They are the result of rapid expansion of combustion material which causes supersonic flow of surrounding air. The outward airflow from the source is characterized by both static and dynamic pressures. The static pressure, or shock wave, that results in the far-field is known as a Friedlander waveform. This is often referred to as the overpressure or peak pressure associated with a blast wave. As shown in Figure 1, the Friedlander wave is composed of an instantaneous rise to an overpressure with a duration that is typically only a few milliseconds. The positive duration is followed by a negative phase, which is below ambient pressure, and then a return to ambient. Relevant wave characteristics are also highlighted in the figure below. The resulting dynamic pressure, also called blast wind, is defined as the kinetic energy associated with the air flow. The blast wind follows the static overpressure wave and causes acceleration/deceleration type injuries.

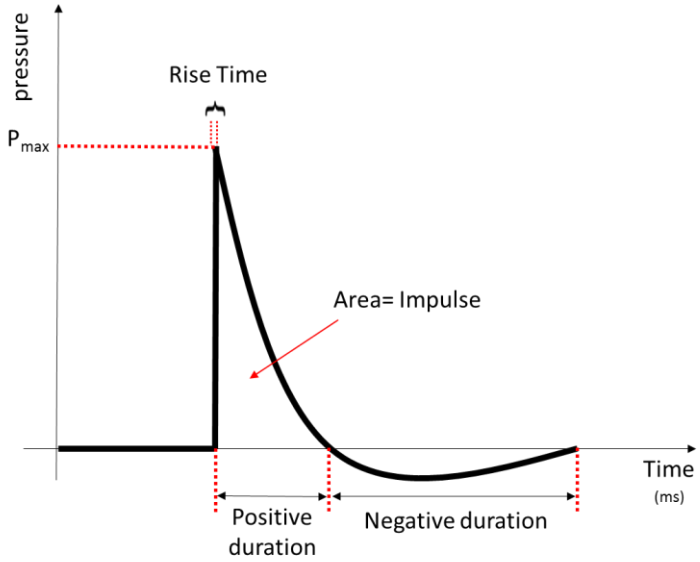

Figure 1. The Friedlander waveform represents an idealized shock wave from a far-field blast event.

It is important to consider the effect of the medium in which the blast wave travels. The Rankine-Hugoniot relationship defines the conditions for a shock front as is dependent on temperature and density of the fluid in which the shock wave travels (Needham, 2010). This has important implications for how shock waves travel from the air into the body, as sharp density gradients exist between these two mediums. In studies that isolate primary blast injuries, the dynamic pressure effect is minimized in an attempt to understand shock wave interactions with the body's tissues.

2.2.2. Potential Mechanisms of Shock Wave Interaction with Brain Tissue

Research efforts in animal and computational models have shown that blast waves are mechanically transmitted into the brain to cause pressure disturbances and tissue level damage (Leonardi et al., 2011; Ganpule et al., 2013; Wang et al., 2014). Yet despite significant research efforts, the mechanisms by which shock waves are transmitted into and through the brain parenchyma are still controversial. Several theories have been proposed and explored, but limitations to these studies remain. Although incompletely understood, it is possible that some or all of these mechanisms contribute to the complex interactions of blast waves with the brain.

The best supported theory for shock wave transmission is skull flexure. The theory is based on the viscoelastic properties of the skull. Upon shock wave impact, the skull is deformed and vibrates

generating large, localized pressure gradients throughout the brain parenchyma (Moss et al., 2009; Bolander et al., 2011; Hampton and VandeVord, 2012).

Other theories include shock wave entrance into the skull by orifices or by imparting significant vascular surges that increase intracranial pressure (Zou et al., 2013; Simard et al., 2014; Xydakis et al., 2015). Vascular surge theory implies that compression of air-filled organs can increase the venous pressure, which is ultimately results in elevations in intracranial pressure and damage associated with vascular compromise. The last theory relies on displacement of brain tissue relative to the skull by acceleration/deceleration action. It is important to note that this injury mode is dependent on dynamic pressure effects on the head and does not isolate shock wave interactions with the brain. Acceleration/deceleration in blast neurotrauma is similar to coupe/contrecoup injuries in impact-type TBIs.

2.2.3. Shock Wave as a Unique TBI Pathology

Even though mechanisms are poorly characterized for blast specifically, it is known that magnitude of the peak pressure, rise time, and duration all contribute to differential cellular outcomes (Ellis et al., 1995; LaPlaca et al., 2005; Cullen et al., 2007; Sajja et al., 2014a; Bailey et al., 2016a). Peak overpressure has been correlated to lethality in studies conducted on several mammal species (Damon et al., 1967). Severe injuries, leading to 50% mortality within one hour, were observed in rats exposed to a range from 30 to 50 psi (Cernak et al., 2001; Hubbard et al., 2014). Additionally, mild behavioral changes were observed in rats exposed to lower peak overpressures between 10-20 psi, indicating brain injury (Bauman et al., 1997; Cho et al., 2013; Sajja et al., 2014b). The clinical outcomes of mild TBI from exposure to high intensity overpressure are similar to those observed from impact-related closed head injuries, although it is unlikely that the underlying cellular and molecular mechanisms are the same (Cernak and Noble-Haeusslein, 2010). Moreover, it is essential to quantify thresholds of injury at very high rates in order to elucidate the pathological mechanisms of brain trauma in a broader set of injury scenarios. The prevalence of mild TBI has been a growing concern in recent years. Most notably athletic and military groups have seen increases in the diagnosis of mild TBI as a result of common exposure to injurious events. A recent study reported that 88% of military personnel treated at a medical unit in Iraq were injured by explosives (Murray et al., 2005). Reports have found that 30% of Veterans with brain injury suffered from several neurological conditions such as headaches, PTSD, and anxiety (Elder and

Cristian, 2009; Levin et al., 2010). There is a significant need to establish cellular mechanisms associated with these pathologies and distinguish a role for mechanics in initiating complex and prolonged tissue level damage.

2.3. Astrocyte Roles in Brain Function

Astrocytes represent the most abundant glial cell type in the brain accounting for about 30% of all glia. Although traditionally thought to only be support cells for neurons, astrocytes are quite heterogeneous and serve many parts in neural circuit function. Their spatial distribution and differential morphologies have some indication for their ubiquitous roles in the brain, yet astrocyte functions are still incompletely understood. Several morphologies are well established and lend to their particular purposes in the brain. Protoplasmic astrocytes, found in gray matter, have finer branches and irregular shape. These cells tend to be somewhat uniformly distributed and have distinct domains. Fibrous astrocytes extend long processes from their soma and are longitudinally aligned along white matter tracks. These two subtypes are distinct functionally and biochemically from one another and impart equally important roles in physiological processes (Oberheim et al., 2012).

The following subsections discuss some of the primary functions that astrocytes impart in the brain parenchyma. It should be noted that these functions are also important for consideration in pathological environments.

2.3.1. Synaptic Support

For a long time, astrocytes were considered secondary cells with passive roles in maintaining biochemical homeostasis of metabolites, ions and the neuronal microenvironment (Sofroniew and Vinters, 2010). It is now recognized that bi-directional signaling between neurons and astrocytes is necessary for information transmission. Astrocytes are one component of the tripartite synapse along with pre- and post-synaptic neurons (Figure 2). Synapse firing requires large shifts in intra- and extracellular ions, notably potassium, sodium and calcium. Upon firing, depolarization of the membrane necessitates rapid inflow of sodium, and neurotransmitter release is stimulated by an inflow of calcium. Eventually repolarization occurs as potassium equilibrates the membrane potential.

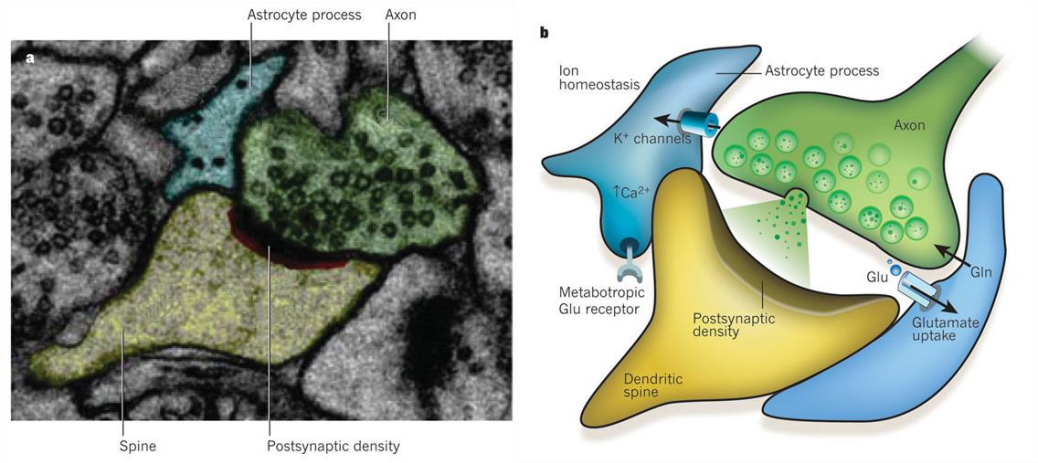

Figure 2. Tripartite synapse and its ion-driven signaling processes. *Adapted from Eroglu and Barres 2010.*

Astrocytes express subsets of ion channels with specificity for calcium, sodium and potassium ions. In particular, they are highly sensitive to extracellular potassium levels that fluctuate as part of neuronal activity at synapses (Amzica, 2002). High potassium permeability is due to the presence of $K_{ir}4.1$ channels on the astrocyte membrane (Olsen et al., 2006; Olsen and Sontheimer, 2008). It appears that potassium conductance plays a critical role in astrocyte depolarization (Djukic et al., 2007), while calcium and sodium channels have other functions in cellular signaling. Aside from ionic balance, astrocytes at synaptic clefts mediate neurotransmitter release. In order to enact these functions, subsets of astrocytes highly express receptors for neurotransmitters, among which the most well studied are glutamate transporters (GLT). Hippocampal, cortical and striatal astrocytes express GLT1, the primary receptor for glutamate. In pathological conditions neurotransmitter expression is sometimes altered which can exacerbate excitotoxicity in neurons and overall metabolic dysfunction (Su et al., 2003; Yi and Hazell, 2006).

Astrocytes form complex, functional networks throughout the brain that serve to actively transmit electrical stimuli and impart intercellular signals. One of the main mechanisms by which astrocytes receive and send signals is via calcium shuttling through ion channels and gap junctions. There are several types of calcium signaling that are known to occur in astrocytes, both neuron dependent and independent (Bazargani and Attwell, 2016). The first type involves large waves of calcium that propagate through astrocyte networks. It has been proposed that this type of signaling contributes to pathologies associated with spreading depolarization. Another signaling mechanism is a more localized, spontaneous signaling that originates in branches of astrocytes. This is not due

to neuronal firing but instead is dependent on receptor activation and signal transduction (inositol 1,4,5-triphosphate (IP3) activation). Importantly, this has particular ties to mitochondrial function.

Apart from ion channels, astrocyte communication with neurons is intricately coupled by a number of adhesion molecules, including gap junction proteins and integrins (Fields and Stevens-Graham, 2002; Hermosilla et al., 2008; Belousov et al., 2017). These adhesion molecules are used by astrocytes in range of neurocognitive processes. For instance, neuro-glial adhesion can have both stimulatory and inhibitory actions and allow for astrocytes to dynamically control axonal repair and synaptic plasticity (Rege and Hagood, 2006; Keasey et al., 2013). It is also important to note that evidence has shown that neurons can transmit signals but not receive them without physical contact from astrocytes (Barker et al., 2008). Knock-out experiments have shown that gap junctions are critical for intercellular communication in the hippocampus, implicating astrocyte networks in electrophysiological and behavioral processes (Theis et al., 2003). Lastly, astrocyte express functional molecules, such as D-serine, in response to neuronal coupling which are imperative for long-term potentiation and memory (Henneberger et al., 2010; Newman et al., 2011; Ota et al., 2013). Altogether, these multimodal processes support the fact that astrocyte signaling is important for memory and other neurocognitive functions (Min et al., 2012; Ota et al., 2013; Adamsky and Goshen, 2017).

2.3.2. Neurovascular Coupling and Interstitial Fluid Control

Astrocyte function extends beyond synaptic regulation to participation in neurovascular units which make up the BBB (Lécuyer et al., 2016). The BBB is an important modulator of nutrient supply to the brain and is dynamically controlled by endothelial junctions. Endfeet on astrocytes are important for ensheathing vessels and tightly monitoring the interstitial space (Figure 3). More recent studies have also come to appreciate the existence of a glymphatic system, in which astrocytes are able to shuttle extracellular waste products and water for clearance from the brain (Jessen et al., 2015). Astrocytes express a number of functional and structural molecules which allow for this coupling. Endfeet are enriched with hemichannel proteins so that passage of small molecules and secondary messengers can be exchanged between astrocytes and endothelial cells. Gap junctions established by hemichannel formation allow for both biochemical and electrical coupling which is critical in the function of both the tripartite synapse and the neurovascular unit. Connexin (CX) proteins are the most abundant class of gap junction proteins which have an array

of functions in brain function. In this case, CX43 and CX30 are expressed in perivascular astrocytic endfeet and allow for glucose and metabolites exchange (Giaume et al., 2010). Moreover, when CX43 and CX30 are selectively deleted the integrity of the BBB is compromised (Ezan et al., 2012). Astrocytes also secrete a number of vasoactive and inflammatory molecules to regulate BBB permeability for inflammatory cell infiltration (Abbott, 2000; Didier et al., 2003; Attwell et al., 2010).

Other important contributors to neurovascular coupling are potassium channels and glucose transporters. Similar to the tripartite synapse, a significant amount of molecular shuttling takes place around the vasculature for which these specialized transporters are critical for efficient exchange (Iadecola and Nedergaard, 2007). Astrocytes also maintain interstitial equilibrium by mobilizing water content. This is largely accomplished by aquaporin (AQP) proteins, which are generally expressed on endfeet. AQP4 is the primary water channel in the brain and in astrocyte endfeet. AQPs form into tetrameric units in which each part is an individual channel (Assentoft et al., 2015). It has been suggested to function in potassium clearance (around synapses) and interstitial volume control (around vessels) in both normal and pathologic environments. AQP4 has been implicated in both edema formation and alleviation in several brain pathologies (Papadopoulos et al., 2004; Bloch et al., 2005; Papadopoulos and Verkman, 2007; Yang et al., 2008). Additionally, AQP4 null mice seem to have slightly decreased potassium clearance but evidence is still contradictory (Binder et al., 2006; Strohschein et al., 2011; Haj-Yasein et al., 2015). This is at least in part due to the fact that AQP can be co-localized with specialized potassium channels. Overall, astrocyte expression of AQP4 is critical multiple aspects of their structural role in environmental maintenance.

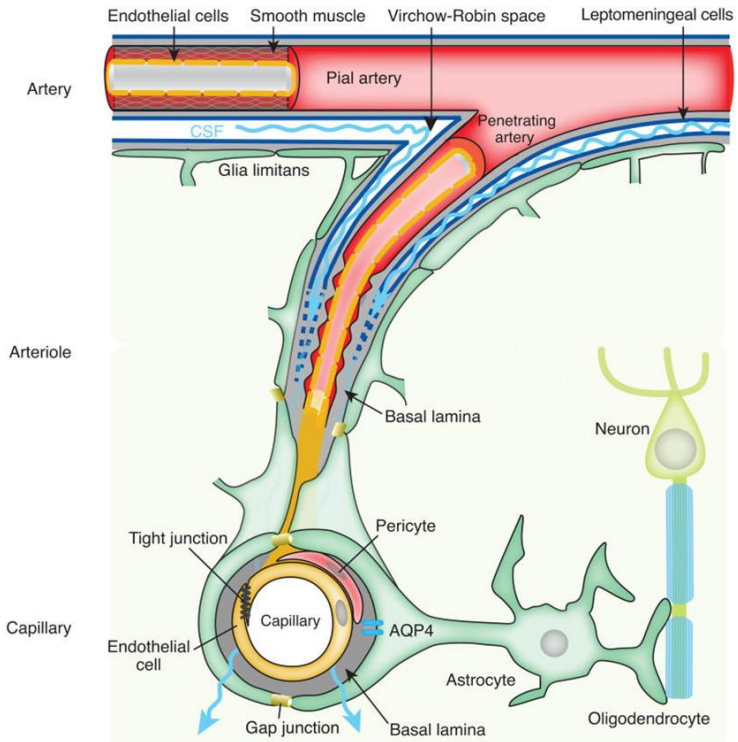

Figure 3. Structural composition of the neurovascular unit. *Adapted from Jessen et al. 2015.*

2.4. Astrocyte Reactivity in TBI

Astrocyte reactivity, a hallmark of impact and non-impact TBI alike, is typically a transient phenotypic response that involves hypertrophy, increased proliferation and dynamic alterations in structural proteins, gap junctional proteins, and inflammatory cytokines. Astrocytes are dual effectors in TBI sequelae, as much evidence exists in both the need for astrocytes in repair processes as well as for their hindrance on full recovery (Pekny et al., 2014; Ben Haim et al., 2015; Karve et al., 2016). Protective influences include resolving metabolic dysfunction, eliminating toxic waste, generating mechanical support for axonal regeneration, and mitigating oxidative stress/damage (Hamby and Sofroniew, 2010; Pekny et al., 2014; Anderson et al., 2016; Sajja et al., 2016). However, sustained activation can have detrimental effects on neuronal health and cognitive function (Morganti-Kossmann et al., 2002; Buffo et al., 2010; Burda et al., 2016; Sajja et al., 2016). In all, persistent glial activation can lead to prolonged BBB disruption, spreading depolarization, neuronal toxicity and apoptosis (Buffo et al., 2010; Burda et al., 2016; Seidel et al., 2016). Reactivity eventually results in later-stage proliferation and scar formation. Glial scars create a functional barrier around an inflammatory or necrotic core of tissue. This has significant implications for repair and regeneration of neural tissue. In the acute stages, astrocyte reactivity

also encompasses a heterogeneous combination of genetic, epigenetic and proteomic regulation that are injury-specific. Considerable evidence has emerged to suggest that astrocyte response to *in vitro* insult is highly dependent on injury severity, type and mechanics (Rzigalinski et al., 1997; Rzigalinski et al., 1998; LaPlaca et al., 2005; Cullen et al., 2007; Cullen et al., 2011). Extent of astrocyte reactivity to blast neurotrauma is also dependent on overpressure severity (Sajja et al., 2014a; Bailey et al., 2016a/b). Although astrocyte roles in TBI progression are somewhat well-defined, there is still a lack of understanding of the molecular effectors that cause phenotypic shifts in astrocytes from harmful to neuroprotective, especially within the context of various injury contexts, and even more so in blast neurotrauma. Herein, there is discussion of supportive and detrimental aspects of astrocyte reactivity that are manifested in TBI.

2.4.1. Protective Efforts by Astrocytes to Resolve Neuronal Dysfunction after TBI

Rapid astrocyte reactivity after primary damage to the brain involves recruitment of resident and infiltrating inflammatory cells for debris clearance. This is accomplished by extracellular ATP release as well as pro-inflammatory cytokine/chemokine secretion, all of which can be initiated within minutes after injury (Hide et al., 2000; Franke et al., 2012; Idzko et al., 2014; Colombo and Farina, 2016). Early inflammatory responses in astrocytes have proven essential in commencing repair processes (Myer et al., 2006; Anderson et al., 2016; Colombo and Farina, 2016). The protective phenotype involves a complex balance of inflammatory cytokines, chemokines and damage associated molecular patterns expressed by reactive astrocytes to enact BBB permeability, recruitment of phagocytic cells, and inflammatory modulation (Colombo and Farina, 2016).

Not only is early astrocyte response necessary for cellular debris clearance, but it also has a more complex role in processes such as improved synaptic plasticity and clinical outcomes (Tyzack et al., 2014; Anderson et al., 2016). Additionally, astrocytes provide the necessary scaffold by which axonal regrowth and synapse formation is accomplished. Astrocytes secrete thrombospondins, glypicans and extracellular matrix (ECM) components to provide adequate signals for reformation (Tyzack et al., 2014; Anderson et al., 2016). They also partake in synapse pruning and guidance processes (Chung et al., 2015; Nikolakopoulou et al., 2016).

As part of the secondary injury process, ionic balances and extracellular glutamate become very dysregulated. This causes acute excitotoxicity and further damage to neurons. As previously discussed, astrocytes are important for maintenance of extracellular glutamate homeostasis and

thus have an increased demand after TBI. However, expression of glutamate receptors has been shown to be downregulated after injury (Rao et al., 1998). Dysregulated ionic balances also lend to metabolic dysfunction and oxidative stress in both astrocytes and neurons. Neurons are particularly susceptible to oxidative damage because they rely heavily on oxidative metabolism. Astrocytes have the capacity to mount antioxidant responses for repair purposes. This can help restore metabolic function in the brain in addition to mitochondrial transfer and compensatory metabolic responses by astrocytes after neuronal damage (Bartnik-Olson et al., 2010; Hayakawa et al., 2016). As already implied, ATP production by astrocytes has a number of important roles in early repair processes. Astrocytes utilize CX hemichannels to mobilize ATP and utilize it for signaling purposes. Not only is it used to compensate for increased metabolic demand by neurons but also to regulate ion channel and AQP4 activity (Lee et al., 2008). In a related manner, astrocytes do have a protective effect on resolving BBB disruption after TBI (Bush et al., 1999). However, this process is dynamic and requires the synthesis of multiple classes of vasoactive molecules.

Studies across TBI modes have reported that ablation of reactive astrocytes leads to increased neurodegeneration, less debris clearance, chronic BBB dysfunction, and overall less functional recovery (Pekny et al., 1999; Myer et al., 2006; Liu et al., 2014). Interestingly, it has also been reported that increased inflammation (CD45+ cells) results from removing reactive astrocytes, suggesting a more complex role in inflammatory modulation, or anti-inflammatory balances (Myer et al., 2006). This is in agreement with astrocyte roles in modulating BBB permeability after TBI (Willis et al., 2004a; Willis et al., 2004b). Altogether, it is necessary to consider that neuroprotective mechanisms are different depending on the type and severity of injury. It is therefore critical to better understand the way in which astrocytes sense and respond to inflammatory and injurious stimuli within relevant injury regimes in order to harness their full therapeutic potential.

2.4.2. Astrocyte Potentiation of Harmful TBI Sequelae

Despite their necessary roles in repair, extensive and prolonged astrocyte reactivity exacerbates injury and inhibits functional recovery. Astrocytes have been strongly implicated in both cytotoxic and vasogenic edema responses after TBI, both of which increase intracranial pressure to harmful limits (Jayakumar et al., 2014). Increased AQP4 may be related to inflammatory recruitment in

astrocytes and thus may implicate one negative aspect to pro-inflammatory stimulation in acute TBI (Fukuda and Badaut, 2012). Moreover, sustained expression of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β), can be detrimental (Shohami et al., 1999; Colombo and Farina, 2016). However, this is still controversial. It is thought that the temporal and spatial coordination of these molecules must be considered in conjunction with each other. It is also important to note that TNF-α has a role in activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which has multiple known detrimental consequences after TBI, including persistent inflammation and activation of matrix metalloproteinases (MMPs) (Colombo and Farina, 2016). Most likely, differential injury mechanisms and time course of injury contribute to the functions of inflammatory mediation by astrocytes. Altered adhesion in neurons has been related to changes in astrocyte phenotype. Reactivity can alter cellular and extracellular stiffness which impedes neuronal recovery and causes pathogenic signaling processes (Miller et al., 2009; Hemphill et al., 2015). It is also noteworthy that studies have found that extensive astrogliosis, or scar formation, can enact inhibitory effects on axonal regrowth (Monnier et al., 2003; Sharma et al., 2012). Reactive astrocytes can upregulate their expression of chondroitin sulfate proteoglycans which have inhibitory effects (Sharma et al., 2012; Wang et al., 2012). In summary, many of the astrocyte-regulated responses have dynamic responses and require substantial modulation in order to be neuroprotective. In order to effectively harness the protective potential of astrocytes, much research is needed to understand how these molecules are regulated in reactive astrocytes and their complex temporal regulation as it relates to functional outcomes.

2.5. *In vitro* Models of Astrocytes in TBI

Because TBI pathologies encompass such a wide range of symptoms and outcomes, it is well appreciated that the cellular and molecular mechanisms that divulge into these outcomes are even more so conspicuous. Many universal treatment approaches which target over-arching anti-inflammatory molecules, neuro-receptor antagonists, and free radical scavengers have been met with little success (Ikonomidou and Turski, 2002; Narayan et al., 2002; Bergold, 2016). In reality, little is understood about the capacity for individual brain cell types to sense, respond and adapt to injury stimuli in a variety of injury contexts. There is a significant need to better understand direct cellular mechanisms associated with particular types of injury mechanics. Importantly, it is not well understood how damaging signals from mechanical insult are imparted to specific aspects of

astrocyte reactivity. This is especially true for blast-type TBIs in which shock waves have unique interactions with brain tissue. *In vitro* models offer valuable insight into these types of mechanistic questions. Below is a review of existing *in vitro* models of neurotrauma, both impact-type injuries as well as shock wave injuries, and what they have contributed thus far to understanding of astrocyte sensation and response to mechanical insults.

2.5.1. Stretch Injury and Other in vitro Models

In vitro experimental models of neurotrauma have existed for close to 100 years. They began as rudimentary studies of transection and regrowth of axons *in vitro* (Ramón y Cajal, 1928). Other early models consisted of explanted brain cells exposed to mechanical disruption by a stir bar moving at various speeds (Epstein, 1971). These models provided insight into the possibility for secondary injury to develop after initial injury. However, as instrumentation developed, emphasis has shifted to developing models in which mechanics can be appropriately measured within the system in order to create reproducible injury metrics. Several *in vitro* models have been designed and built to account for specific mechanical inputs. These include stretch-injury, barotrauma, acceleration/deceleration, and hydrodynamic models. Each of these offers unique advantages to studying particular types of cell injury responses to unique loading paradigms. Because stretch injury has been the most common model for studying astrocytes, contributions of this model will be reviewed.

Cell stretch and other shear strain models encompass a large area of *in vitro* neurotrauma research. Stretch injury has been utilized since the 1990s to better characterize pathophysiological mechanisms associated with direct mechanical insult to cells (Ellis et al., 1995; Rzigalinski et al., 1998; Morrison et al., 2006). This particular type of injury model has been broadly applied for mild to severe injuries across a range of durations typically on the scale of high rate (duration~50 ms) to quasi-static (1 s). Often this injury is created by applying compressed gas to cultures grown on deformable membranes. An important aspect for this type of model is the analysis of cellular strain that results from application of external shear stresses. Validation of micro-strains allows for direct thresholds of cellular injury to be measured and translated across models. Earlier studies of astrocytes under stretch analysis found that membrane deformation and cellular injury were proportional to both pressure duration and peak (Ellis et al., 1995; Morrison et al., 2006). Upon stretch injury astrocytes become susceptible to oxidative damage and experience altered signal

transduction consequences, including mitogen-activated protein kinase (MAPK) activation (Floyd et al., 2001; Neary et al., 2003; Neary et al., 2005). These studies became the forefront to understanding mechanical thresholds for astrocyte injury, activation and repair (Ellis et al., 1995; Lamb et al., 1997; Ahmed et al., 2000; Morrison et al., 2000; Floyd et al., 2004). Stretch models were also foundational in establishing a role for mechano-activation of calcium signaling in astrocytes and its role in progressive injury development. Early findings linked calcium perturbations with mitochondrial dysfunction, IP3 signaling and membrane distortions (Rzigalinski et al., 1997; Rzigalinski et al., 1998; Ahmed et al., 2000; Geddes and Cargill, 2001).

More recent evidences that have emerged from stretch injury models include exploration of mechanically-induced astrocyte reactivity, including phenotypic changes and adaptations in inflammation, adhesion and neurovascular coupling. Stretch-activated astrocytes assume classical phenotypic reactivity by increased expression of intermediate filament proteins, like glial fibrillary acidic protein (GFAP) (Cullen et al., 2007; Miller et al., 2009). In addition to protein alterations, many studies have shown that signal transduction pathways are activated in cultured astrocytes after mechanical insult (Wang et al., 2002; Neary et al., 2003; Neary et al., 2005; Ranaivo et al., 2011). Ranaivo et al report that mild stretch injury gives rise to IL-1β, MMP-2 and MMP-9 expression in astrocytes, likely by MAPK-mediated pathways (Wang et al., 2002; Ranaivo et al., 2011). Studies have explored altered adhesion and cellular stiffness following a range of stretch-induced strains (Miller et al., 2009; Sturdivant et al., 2016).

Other *in vitro* models exist which recapitulate particular aspects of central nervous system (CNS) trauma (Kumaria and Tolias, 2008; Morrison et al., 2011). Barotrauma devices are those which utilize the displacement of fluid to impart transient pressure at higher rate (durations on the order of tens of milliseconds) from modified weight drop or fluid percussion instruments (Shepard et al., 1991; VandeVord et al., 2008). Other hydrodynamic models offer another type of injury design in which cells are exposed to inertial loading which causes significant shear stresses and diffuse type injuries. These models tend to involve the application of fluid shear stress by jet stream (of air or media) or by rotating disk (Morrison et al., 2011).

Acceleration/deceleration models are designed to mimic shear injuries that occur diffusely following significant rotation of the head and brain. Shear injury is caused by tissue lag as the brain moves relative to the skull. Inertial loading of cultures has particular challenges associated

as it may be difficult to recapitulate tissue deformations derived from *in vivo* experiments. Yet valuable insight has stemmed from this type of model for strain rates on the order of [10-50]s^{-1} (Kumaria and Tolias, 2008).

Altogether, stretch injury and other *in vitro* models have proved very informative in understanding astrocyte reactivity and mechanical tolerance. They have also been valuable in creating tolerance criteria which can be translated whole brain analysis (Elkin and Morrison, 2007). Yet, there has been particular challenges in developing reliable methodologies for studying cellular response at higher deformation rates (Morrison et al., 2011). This is the limiting factor for utilizing these types of models for blast neurotrauma studies. Therefore, several blast-specific *in vitro* models have been developed to study shock wave interactions with brain cells and are discussed below.

2.5.2. In vitro Models of Primary Blast

As discussed previously, shock wave interactions with brain parenchyma are still not completely understood. Therefore, primary injury mechanisms and associated cellular responses have become much of the focus for blast-related research. Shock waves typically occur on the order of a few milliseconds and are characterized by an instantaneous rise in pressure, a positive duration, followed by a negative phase (Figure 1). *In vitro* models have attempted to recapitulate these mechanics in several different ways. Barotrauma devices have been used to replicate the high rate transient overpressures associated with blast waves (Leung and VandeVord, 2009). However, this model is still limited to longer durations than most shock waves (< 10ms). A different model of primary blast neurotrauma utilizes a pneumatic system to expose cultures to shock wave without and without shearing within a fluid-filled receiver section (Ravin et al., 2012; Hue et al., 2013; Ravin et al., 2016). Others have attempted to use air blast chambers or open-area explosives to expose submerged cell cultures to shock wave without receivers (Arun et al., 2013; Zander et al., 2015; Sawyer et al., 2017a). Although these models produce injury outcomes including delayed signal transduction activation, membrane permeability and glial activation, they must also consider that blast wave physics is compounded by boundaries and impendences differences in media. As a shock wave propagates from air into fluid this significantly alters the shock wave and may tend to over-exaggerate the injury. In order to account for this, relevant mechanical measurements with specimens are necessary, such as those collected by Sawyer et al (Sawyer et al., 2017a).

Because of the discrepancies in loading condition for *in vitro* blast studies, cellular and molecular responses remain largely confounded. A few studies have reported that significant cell death occurs in organotypic slices exposed to mild-moderate severity shock wave (Miller et al., 2015; Miller et al., 2017), while others report minimal cell death with sub-lethal cellular activation instead (VandeVord et al., 2008; Hue et al., 2013; Effgen et al., 2014; Hlavac et al., 2015). A likely contributing factor to this disparity is differences in measured mechanical outputs. More specifically, shock wave parameters, such as duration and peak overpressure, will be highly varied depending on the location relative to specimens. There are a number of methods for creating the transient overpressure as well. Therefore mechanics of the wave may be important to consider across models.

Despite current limitations, *in vitro* blast injury models have provided useful insight into cellular injury mechanisms. Primary *in vitro* blast has been shown to cause transient intracellular calcium signaling that is dependent on purinergic signaling (Ravin et al., 2016). Moreover, when transient pressure was combined with shear, calcium signaling had a more dramatic effect in human brain cells (Ravin et al., 2012). Arun et al reports mitochondrial dysfunction and fluctuations in ATP in neurons at six hours with resolved outcomes by 24 hours (Arun et al., 2013). In a different study using three-dimensional cultures of human-derived cells, Zander et al report increased ROS generation at 24 hours at more moderate to severe overpressures (Zander et al., 2017). Table 1 summarizes other studies which have reported that there is a mechanical basis for classical astrocyte reactivity and BBB dysfunction using *in vitro* blast neurotrauma models (VandeVord et al., 2008; Hue et al., 2013; Hlavac et al., 2015; Ravin et al., 2016; Canchi et al., 2017; Miller et al., 2017; Sawyer et al., 2017b). Still much work remains to be done to understand brain cell responses to shock wave insult. Importantly, there is a significant need to further develop relevant *in vitro* injury devices in order to study molecular mechanisms and therapeutic targets in a repeatable paradigm.

Table 1: Existing models for high-rate overpressure injury in astrocytes			
Study	**Injury Mechanism**	**Mechanics**	**Findings**
VandeVord et al 2008	Barochamber	~30 ms duration	pro-survival genes (IL-3, GDNF) and reactivity genes (GFAP, vimentin), 24-72 hours
Ravin et al 2016	Pneumatic device with air gun +shear	~0.5 ms duration, <1Pa of shear force	calcium transients prolonged in astrocytes compared to neurons, 10 minutes
Canchi et al 2017	Split Hopkinson pressure bar	60 psi, 1-2 ms duration	brain slices, altered morphology by GFAP, 1-4 hours
Miller et al 2017	Open-end helium shock tube	20-40 psi	brain slices, membrane leakage (propidium iodide staining), minimal apoptosis, no changes in GFAP, 2 hours
Sawyer et al 2018	Underwater explosion (pond)	45-200psi, 0.12-0.30 ms	no changes observed in GFAP expression, significant increase in phosphorylated protein kinase B (p-Akt)

2.6. Mechano-Stimulation in Controlling Cellular Function

Cells have the ability to convert external mechanical stimuli, or forces, into intracellular biochemical and signal transduction events. This process is accomplished by functional adhesions and cell junctions created between the cell membrane and the surrounding environment. Biological consequences of mechano-stimulation involve complex changes in gene expression accompanied by chemical alterations, both which contribute to altered cellular phenotypes. Cells also commonly use mechanotransduction and adhesion mechanisms to facilitate migration and polarity. Because mechano-activation is relevant for highly motile cell types and those which experience cyclic loading, like osteoblasts and cardiomyocytes, much of the mechanotransduction research has focused on disease and pathologies associated with the heart, bone and immune system (Jaalouk and Lammerding, 2009; Wang and Ha, 2013). However, more recent research efforts have extended to understanding mechano-sitmulation mechanisms associated with other pathologies, including cancer and TBI (Hemphill et al., 2011; Hemphill et al., 2015; Chin et al., 2016).

2.6.1. Adhesion Mediated Mechano-Stimulation Mechanisms

Mechano-stimulation can involve functional interactions between the cell membrane and the extracellular environment, either matrix material or surrounding cells. Matrix interactions are most often facilitated by the formation of focal adhesions. Focal adhesions are comprised of clusters of integrin proteins that are dynamically controlled for cell polarity, adhesion and migration. There are 24 known integrin subtypes which form heterodimers as functional adhesion units (Ning, 2017). Alpha and beta subunits dimerize to devise specificity of integrins for ECM components.

A single integrin dimer binds to ECM with a bond strength on the order of tens of pico-newtons (Ning, 2017). Talin and vinculin molecules act as intermediary components between integrin subunits and F-actin filaments, which transduce forces throughout the cytoplasm. Initiator proteins for signal transduction are closely associated with these structural molecules and include Src family proteins, focal adhesion kinase (FAK), and integrin-linked kinase (ILK). Altogether, these components compromise the functional unit of a focal adhesion and associated mechanotransduction signaling (Figure 4). Mechanical stimulation can cause reorganization/re-clustering of integrin proteins as well as activation of initiator proteins in mechanotransduction signaling.

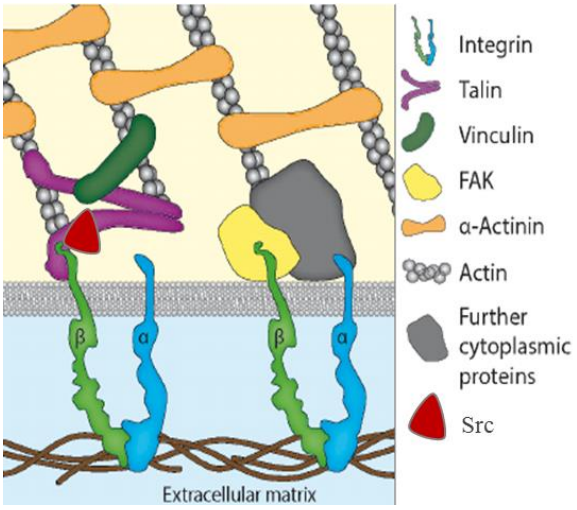

Figure 4. Focal adhesion unit containing the extracellular integrin protein and intracellular initiator molecules. *Adapted from Brüggemann et al. 2014.*

Preliminary experiments determined that focal adhesions are bi-directional, with force sensing occurring inwardly as intracellular signals (Choquet et al., 1997), but also with outward signals which govern cell motility (Pelham and Wang, 1997; Balaban et al., 2001). Interestingly, force transmission via integrin stimulation can be altered in multiple ways. The most obvious is that integrin conformation is changed as a result of matrix alteration and force interactions with adhesion structures. However, mechanical unfolding, or stretching, of matrix proteins can also effectively alter adhesion by shifting the binding sequences available to cells (Gao et al., 2003; Smith et al., 2007). Force transductions can also occur very rapidly over tens of microns because of the interconnections of focal adhesions and actin networks in cells (Hu et al., 2003). This has

interesting implications for studying mechanical injury to tissue as transient pressures may be directly influential in altering the state of matrix proteins.

Other types of adhesion and junctional molecules have been also been implicated as mechano-sensors in various cells. These include adherens junctions like cadherins, gap junctional proteins like connexins, and transmembrane receptors with specific extracellular targets (DuFort et al., 2011). Cadherins are calcium-dependent adhesion molecules and have various types within their family of proteins. They have been implicated as mechano-sensors of fluid shear stress in endothelial and matrix-sensing in mesenchymal cells (le Duc et al., 2010; Cosgrove et al., 2016). In the brain, N-cadherin is expressed by astrocytes and is integral in neurite outgrowth (Ferguson and Scherer, 2012). Astrocytes also form another type of adhesion, gap junctions, using hemichannel proteins. These channels are made up of CX proteins and form both within astrocyte networks and between astrocytes and other cells. Phosphorylation of CX proteins occurs as the result of mechanical damage to the brain, but also the protein levels are differentially regulated depending on injury severity (B. Chen et al., 2017). Lastly, several classes of ion channels are sensitive to mechano-stimulation. These are broadly referred to as stretch-activated channels and have been strongly implicated in pathologic calcium signaling and oxidative stress responses (Lansman et al., 1987; Ostrow et al., 2011). Although these adhesion molecules do not all form junctions with the extracellular environment, they are regulated by overlapping mechano-stimulation mechanisms and influence the same downstream signal transduction pathways that ultimately influence cellular phenotype.

2.6.2. Signal Transduction Pathways

Integrins and other mechanosensitive membrane proteins utilize common signal transduction pathways to impart their changes on cellular function and phenotype. Signals from focal adhesions begin with initiator proteins, including Src, FAK, and ILK. Src can be activated within 300ms following mechanical stimulation and can result from far-away (tens of microns) force application (Na et al., 2008). Subsequent activation can occur on the order of minutes later. Several studies have investigated the differential activation of FAK in response to various shear stress applications. In most cases, FAK activation occurs within seconds to minutes after shear exposure and seems to be sensitive to magnitude of insult (Li et al., 1997; Young et al., 2009). The signaling

cascade involves a number of Rho-family GTPases and subsequent coordination of cell survival pathways and activation of transcription factor pathways.

The most commonly studied of these signal transduction pathways are MAPK and NF-κB pathways across a range of cell types (Fanning et al., 2003; Jaalouk and Lammerding, 2009; Petzold et al., 2009; Shih et al., 2010; Thompson et al., 2012; Whitney et al., 2012). MAPK represents a family of proteins which are serine/threonine kinases. Upon integrin stimulation, Rac and Ras are activated by initiator proteins which signal to precursor molecules of the MAPK pathway, MAPK kinase (MEK1/2) and MAPK kinase kinase (MEKK). Downstream to these, are effector molecules of the MAPK family. These include extracellular signal-regulated kinase (ERK1/2), p38, and c-Jun N-terminal kinase (JNK). Altogether this family of proteins is involved in a wide range of cellular processes, including survival, proliferation, homeostasis, and metabolism (Johnson and Lapadat, 2002). ERK1/2 has a particularly important role in mitosis and differentiation. In the CNS, ERK1/2 mediates learning and memory processes. Specifically in astrocytes, ERK1/2 mediates sensitivity to mechanical perturbations and is important in regulating gene expression (Neary et al., 2003; McCoy and Sontheimer, 2010; Li et al., 2017). The p38 kinases are enzymes that were first discovered in inflammation stimulatory processes in neutrophils (Lee et al., 1994). Reactive astrocytes are dynamically regulated by p38 pathway activation, which has notable implications in inflammatory modulation by these cells (Lo et al., 2014; Li et al., 2017) but also in their reactive response to insults (Choudhury et al., 2014; Li et al., 2017). Lastly, JNKs regulate the DNA-binding protein c-Jun by influencing its transcriptional activity. This pathway has been implicated in response to environmental stresses and inhibition of apoptosis.

The NF-κB p65 pathway represents another ubiquitous signal transduction pathway highly conserved across cell types. Upon activation through downstream signaling events from integrins, inflammatory stimuli, or growth factor receptor ligation, the transcription factor p65 is released from its sequestering protein complex with inhibitor of κB kinase (IKK). The activation of p65 requires several actions, for which the first step is translocation after release from IKK. Following nuclear localization, the transcription factor must also bind to DNA sequences in order to finally impart transcriptional activity. NF-κB has genetic targets implicated in neurogenic inflammation as well as antioxidant potential and cellular adhesion (Kang and Hébert, 2011; Shih et al., 2015).

The p65 factor has a number of known relevant binding sites, including genes for junctional proteins like intercellular adhesion molecule 1 (ICAM-1) and also influences and is influenced by the function of multiple integrin proteins (Scatena et al., 1998; Ferri et al., 2003; Lawrence, 2009; Hoesel and Schmid, 2013). Importantly, the MAPK pathway can also regulate the activity of p65, especially in the context of cytokine stimulation (Saha et al., 2007). Moreover, several reports have found that fluid shear stress of various cell types can cause activation of this pathway (Bhullar et al., 1998). These overlapping signal transduction pathways offer a clear therapeutic avenue for treating disease and pathologies associated with mechano-activation of cells, however there remains much unknown in the spatial and temporal regulation of adhesion and other signals into coordination of signaling events.

2.6.3. Brain ECM, Astrocyte Adhesion and Mechano-Signaling in TBI

The brain ECM is largely composed of hyaluronic acid (HA), glycoproteins and proteoglycans containing lectin and HA-binding domains (Ruoslahti, 1996). The brain matrix is much softer than most other tissues in the body, with viscoelastic properties that make it particularly challenging to model shear strain associated with traumatic insult. Moreover, brain cells are individually susceptible to membrane distortions and instability at the microscale and thus have incongruent responses to traumatic insult (Singleton and Povlishock, 2004; Cullen et al., 2011). It is also important to consider that the ECM can be altered after brain injury, influencing the material properties of the tissue which could affect glial cell morphology and function after injury. In the injured brain, increased levels of basement membrane proteins laminin and fibronectin have been associated with aberrant expression in astrocytes (Liesi et al., 1984; Liesi, 1985; Stichel and Müller, 1994). These studies suggest that astrocyte-derived laminin is important for neurite outgrowth after injury. Studies have also reported that the conformation of these proteins are potentially altered following TBI insult, a likely contributor for altered adhesion and phenotypes in injured brain cells.

Astrocyte adhesion alterations have been implicated in migratory and proliferative phenotypes following injury. Moreover, modified integrin conformation or clustering may have implications for the morphological changes that occur in astrocytes after exposure to mechanical stimuli. Integrin signaling self-regulates expression of integrin subunits for migration. Integrin alpha-v/beta-8 is required for astrocyte migration in response to stimulus, while integrin beta-1 (β-1) is

necessary for morphology (Wu and Reddy, 2012). Integrin β-1 forms complexes for adhesions to several of the brain ECM components, including laminin, fibronectin, collagen and HA. A number of studies have investigated the role of integrin β-1 expression in glial cells following altered ECM or CNS injury (Nasu-Tada et al., 2005; Renault-Mihara et al., 2011). Integrin β-1 is critical for migration of glial cells to sites of injury and may also contribute to astrocyte differentiation (Pan et al., 2014). Moreover, expression of integrins is also critical for synaptic plasticity (Staubli et al., 1990) and neurovascular unit development (Proctor et al., 2005), both of which are important following trauma.

Additionally, much effort has been extended to understanding the pathologic role of stretch-activated ion channels and other intercellular junctional proteins in astrocyte reactivity. N-methyl-D-aspartic acid receptors and "BK" potassium channels are two such conduits which allow for mechano-activated influx of sodium and calcium ions. Hemichannels are also mechano-activated and can propagate biochemical signals between networks of astrocytes in response to mechano-stimulation (Stout et al., 2002). Functional blockage of mechano-activated channels inhibits calcium waves from mechanical insult (Maneshi et al., 2015), and may have important implications for resolving acute cellular stresses.

Integrins and mechano-sensitive ion channels are broadly influential on early cellular responses (Figure 5). However, perturbations in the activation of mechanotransduction signaling cascades have remained largely unexplored in shock wave injury. Exploration into these signaling pathways will provide insight into how astrocytes sense traumatic insult and impart specific reactive responses.

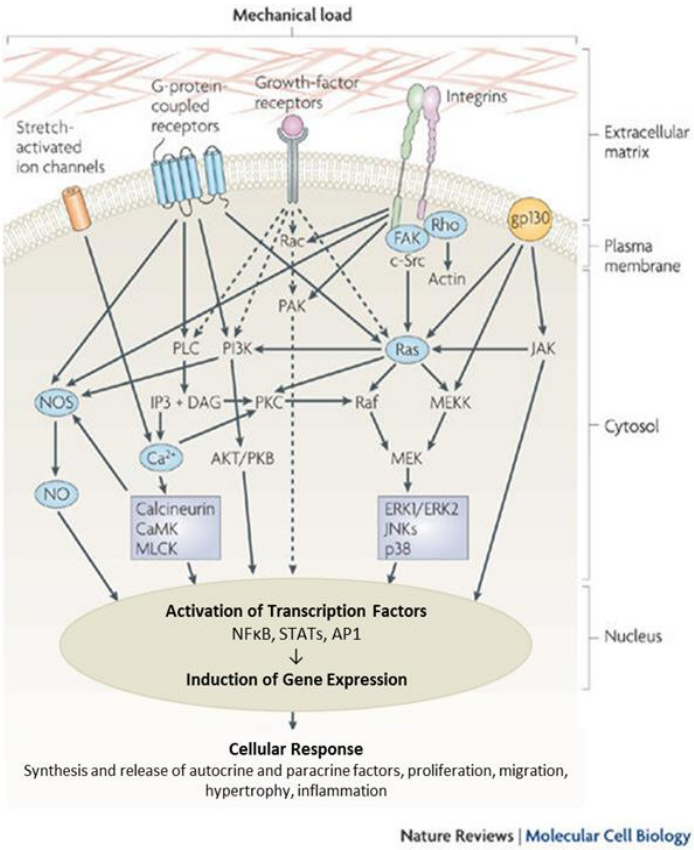

Figure 5. Pathways of mechano-stimulation and their contributions to healthy and pathogenic cellular responses.
Adapted from Jaalouk and Lammerding 2009.

Chapter 3. Investigation of dynamic metabolic state and related stress mechanisms as a primer for reactivity

3.1. Introduction

Metabolism forms the basis for all cellular functions and survival mechanisms. It is an important indicator of health and physiologic regulation. Both primary and secondary traumatic brain injury (TBI) insults can have a wide influence on metabolic processes in brain tissue. Many of these changes are related to acute fluctuations in the chemical environment of the brain due to mechanical compromise of cells and tissue structures. Despite the understanding of these sequelae, it is still not clear how particular cell types differentially respond to mechanical forces introduced by the TBI. These processes include but are not limited to energy production, cell death and proliferation, reactivity, morphology and function. For astrocytes in particular, this can have implications on their ability to maintain important structures like synapses and vascular networks as well as the chemical environment necessary for efficient neural communication.

3.1.1. Statement of Problem

The purpose of this study was to establish early patterns of metabolic dysfunction, specifically related to bioenergetics and amino acid metabolism, as well as understand how early ionic signaling may contribute to oxidative stress potential in cultured astrocytes exposed to high-rate overpressure. Oxidative stress is a prominent secondary feature of TBI (Cernak et al., 2001; Readnower et al., 2010; Abdul-Muneer et al., 2013; Cho et al., 2013) and may be primarily regulated by astrocytes. Additionally, metabolic impairment has the potential to influence cell health, function and phenotype, all of which will be important outcomes to assess in the process of astrocyte reactivity to mechano-stimulation by overpressure. Many of the early signaling events related to purinergic signaling, neurotransmitter release, and mechanical activation have important implications for astrocytes to be able to buffer ions efficiently (Rzigalinski et al., 1998; Hamilton and Attwell, 2010; Maneshi et al., 2015; Ravin et al., 2016). Purinergic signaling is that which is due to activation by purine nucleotides and nucleosides, like adenosine and ATP. It is an important mechanism in early TBI as ATP is released by resident cells like astrocytes to recruit inflammatory cells to a site of injury (Davalos et al., 2005; Burda et al., 2016; Karve et al., 2016; Shinozaki et al., 2017). Apart from purinergic signaling, it remains unclear how high-rate overpressure can induce metabolic change and oxidative stress potentiation in astrocyte networks. It was hypothesized that astrocytes would experience bioenergetic fluctuations in conjunction with altered amino acid metabolism, both of which have important implications for downstream phenotype effects. This contributes to an overarching goal which serves two purposes in

understanding TBI pathophysiology: the first being to establish early signaling events leading to downstream pro-oxidative potential and the second to study how astrocytes regulate fluctuations in metabolism because of their robust ability to compensate for changes.

3.1.2. Significance

Though much evidence for metabolic and oxidative stress has focused on neuronal vulnerability, it is also important to consider that astrocytes are susceptible to oxidative stress mechanisms as well (Kim et al., 2012; Cooney et al., 2013). Accumulation of reactive oxygen species (ROS) results in both selective and non-selective damage to mitochondrial, cellular proteins, and nucleic acids. Because astrocytes are integral regulators of anti-oxidant mechanisms, they represent an important component to early protection after TBI (Buffo et al., 2010). Astrocytes are very metabolically robust and able to handle large fluctuations in ionic and neurotransmitter profiles. Therefore, a better understanding of how they compensate for metabolic change and ultimately enact protective mechanisms is an important aspect toward therapeutically modulating early stage TBI. The purpose of this chapter was to establish metabolic pathways that may be altered in astrocyte autologous regulation following high-rate overpressure. Results of this work showed that functional inhibition of ionic channels during overpressure influenced oxidative stress potential, providing further understand the metabolic primers for astrocyte reactivity and oxidative stress in blast neurotrauma.

3.2. Literature Review

Astrocytes are central in metabolic and redox balances in the healthy brain. They contain molecular machinery for neurotransmitter metabolism, antioxidant potential, and neurovascular coupling. Astrocyte expression of specialized membrane proteins including neurotransmitter receptors, multiple types of ionic channels and widely interconnected gap junctional networks allows them to buffer the highly dynamic metabolic environment in the brain. Importantly, signaling from these sources can converge within astrocytes and largely involves calcium fluctuations and purinergic signaling between astrocytes and other cells (Hertz et al., 2007; Hamilton and Attwell, 2010; Agulhon et al., 2012).

Oxidative metabolism accounts for the majority of the energy production in the brain, and is dependent on mitochondrial respiration. Astrocytes also rely on glycolytic mechanisms to produce ATP while neurons do not. For this reason, astrocytes are more metabolically robust than neurons

and often must compensate for energy deprivation following central nervous system (CNS) insult (Bylicky et al., 2018). Despite their robustness, astrocytes are susceptible to metabolic stress damage (Jacobson and Duchen, 2002) and antioxidant signals (Olesen et al., 2008). Under metabolic tension, cells become susceptible to oxidative stress, and particularly the accumulation of secondary products associated with ROS. Oxidative stress is a well-documented hallmark of early TBI. Previous studies in rodents subject to blast neurotrauma have showed signs of oxidative stress in various brain regions beginning as early as four hours and was sustained multiple days following blast neurotrauma (Cho et al., 2013; Tumer et al., 2013; Sajja et al., 2014b; Sajja et al., 2015b; Arun et al., 2018). It is not well-known how high-rate overpressure contributes to metabolic and oxidative stresses in astrocytes or how they are able to mount protective affects against early fluctuations within their networks.

3.2.1. Oxidative Metabolism and Associated Stress

Although astrocytes are able to produce energy by multiple pathways, they, along with neurons, rely heavily on oxidative metabolism to meet the high energy demands for neural processing. Oxidative metabolism (respiration) refers to the set of processes by which cells convert carbohydrates to energy (ATP) using oxygen within the mitochondria. Two pathways must cooperate for oxidative metabolism: Krebs cycle and electron transport chain. Its equilibrium is centered on mitochondrial health which is directly affected by intracellular ionic concentrations, among which calcium is a significant contributor.

Calcium signaling is a pervasive intracellular process which controls numerous cellular functions, including cell proliferation, developmental processes and memory formation (Berridge et al., 2000). In an injury environment, intracellular calcium concentrations increase, and cells attempt to store the calcium in mitochondria, which can lead to metabolic dysfunction. Impaired mitochondrial function is linked to fluctuations in ATP production, oxidative stress and inflammatory responses by astrocytes and other cells. Calcium signaling is dramatically present in astrocytes exposed to various types of mechanical insult and appear to be dependent on mechanosensitive ion channel activity, ATP release, and purinergic receptor signaling (Paemeleire and Leybaert, 2000; Stout et al., 2002; Scemes and Giaume, 2006; Bowser and Khakh, 2007; Kuga et al., 2011). Fluctuations of intracellular calcium can occur within seconds after injury and extend to hours, depending on injury severity and type (Rzigalinski et al., 1998; Ahmed et al., 2000).

Studies have shown a role for calcium channels, voltage-gated and ligand-gated, in secondary astrocyte reactivity in following insults, including TBI. Cheli et al reported a relationship between astrocyte proliferation and L-type voltage-gated calcium channels following a scratch wound (Cheli et al., 2016). Another study showed the upregulation of a subtype of L-type voltage-gated calcium channel in reactive astrocytes across multiple pathologies including a mechanical lesion (Westenbroek et al., 1998). These studies suggest a long-term function of these channels but they also point to the potential for altered cationic signaling contributing to downstream reactivity in astrocytes. Still other studies have shown a more direct link between the extension of rapid calcium signals that extend within astrocyte networks and ultimately lead to the spread of damage signals to other cells like microglia. These are ligand-based channels that are often coupled with ATP release and signaling via purinergic receptors (Hertz et al., 2007; Sieger et al., 2012).

Despite this broad evidence, it is still unclear how calcium and other cationic signals during high-rate overpressure events may lead to downstream phenotypic shifts or cellular damage. A few studies have been conducted using *in vitro* models of blast neurotrauma (Alford et al., 2011; Ravin et al., 2012; Maneshi et al., 2015; Ravin et al., 2016). Maneshi et al showed a dependence of calcium signaling on the mechanical parameters of overpressure profile and notably the nonlinearity associated with high amplitude pressures. This suggests a need to better understand high-rate insults in terms of their potential to illicit pathological responses. Ravin et al reported that brain cells were less sensitive (in terms of calcium signaling) to peak overpressures when duration was less than one millisecond unless in the presence of shear. In the presence of overpressure (~0.2 ms duration) calcium fluctuations remained elevated at least out to 600 seconds post-exposure. The authors did not characterize responses for longer duration pulses.

Metabolic stresses also lend to harmful outcomes through oxidative stress mechanisms. Oxidative stress is a prominent feature in acute-stage TBI and may be implicated in astrocyte response to insult. When metabolic demands are higher, respiration can lead to an increase of free radicals for which the compensatory mechanisms cannot equilibrate. Free radical production is result of the electron transport chain that makes up energy production in mitochondria. Superoxide anion radicals are generated by the “leaky” energy chain and are very unstable. This process initiates damage to lipids and other molecules through interactions with the free radicals. Studies have shown that pro-oxidative machinery, including nicotinamide adenine dinucleotide (NADPH)

oxidases (NOX), are acutely upregulated after TBI and metabolic deprivation (Kim et al., 2012; Cooney et al., 2013). The NOX family is responsible for the production of free radicals upon the oxidation of biomolecules. In particular, NOX4 is an isoform which is expressed within the inner matrix of mitochondria and is constitutively activated. It is expressed in subtypes of brain cells, including astrocytes. Multiple studies have implicated increased NOX4 in astro-centric pathology associated with mechanical trauma (Zhang et al., 2017; Ma et al., 2018b). One study characterized increased NOX4 expression in the cortex of mice exposed to 20psi blast overpressure (Logsdon et al., 2017). The results were correlated with increased oxidation products at 72 hours post-exposure.

Under healthy conditions, cells have the machinery to mount an antioxidant response through molecules such as dehydrogenases and superoxide dismutase (SOD). As part of their protective response, astrocytes produce SOD and other antioxidant molecules in response to oxidative stress (Schreiner et al., 2015). SOD2 is mitochondrial-linked and has shown promise in eliminating harmful effects of oxidative stress in neurons (Xu et al., 2010). SOD2 production is increased at multiple time points in rats exposed to blast neurotrauma (Kobeissy et al., 2013; Sajja et al., 2014a), but the regulatory mechanisms for SOD2 production are unclear. It remains to be elucidated the sources of metabolic stress that ultimately lead to the protective SOD2 expression by astrocytes and other cells.

3.2.2. Amino Acid Metabolism

Amino acid metabolism has also been explored as a target for influencing cellular phenotypes, survival, and function after TBI. Most research related to this topic has focused on peripheral measurements of amino acids in TBI patients (Aquilani et al., 2000; Borsheim et al., 2007), but amino acid metabolism can also be altered specifically in brain cells after TBI. Glutamine is the most studied amino acid in TBI research (Scafidi et al., 2009; Bartnik-Olson et al., 2010; Guerriero et al., 2015; Yi et al., 2016). Glutamine is produced by conversion of glutamate, mostly accomplished through astrocytes. Because surges of glutamate typically occur in early TBI, glutamine fluctuations are commonly observed as protective response by astrocytes and are very dynamic in nature (Scafidi et al., 2009; Ben Haim et al., 2015; Guerriero et al., 2015). Methionine is an essential amino acid which has also been implicated in TBI, although has received less attention. Dash et al explored altered methionine metabolism in plasma from patients with graded severity TBI (Dash et al., 2016). Results indicated that intermediate processes in the methionine

methylation pathways were altered, which may suggest a potential for DNA methylation changes. Methionine is of particular interest for its role in epigenetic regulations. It is metabolized to create s-adenosylmethionine, an important mediator in DNA methylation equilibrium. This has important implications for downstream phenotype changes within the context of pathological stimuli. DNA methylation changes are associated with brain injury (Zhang et al., 2007; Haghighi et al., 2015), however are not well characterized at acute time points in astrocytes. Moreover, methionine metabolism contributes further to the redox state in cells. Under oxidative stress, derivatives of methionine metabolic pathway generate glutathione, which acts as a protective agent against oxidative damage. Therefore, the implications for altered amino acid metabolism in astrocytes are broad and likely influence their protective potential through dynamic genetic and epigenetic regulation.

3.3. Methods

3.3.1. Cell culture

Brain cortices were isolated from P2 Sprague-Dawley rat pups in accordance with protocols approved by Virginia Tech's University Institutional Animal Care and Use Committee. Cortical tissue was enzymatically digested in 0.05% trypsin for 5-10 minutes and cultured up to 14 days to allow populations to reach confluence before initial passage. Seven days after isolation, other resident cells were mechanically removed from cultures by shaking for 24-48 hours. Astrocytes were maintained in Dulbecco's modified eagle medium (DMEM/F12, Gibco cat# 11320) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Gibco cat# 15240-062). Cultures were routinely stained with anti-glial fibrillary acidic protein (GFAP, Abcam cat# ab7260) to ensure selection of a homogeneous population of astrocytes for this study (Figure 6). Prior to testing, astrocyte monolayers were seeded in standard six-well plates at a density of $1x10^5$ cells per well and were cultured for 6-7 days.

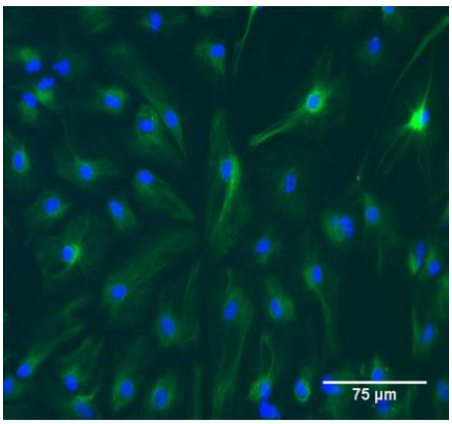

Figure 6. The study population was GFAP-positive primary rat astrocytes between passage zero and four*. In the image cells were immune-labelled with GFAP and tagged with FITC. Scale bar is 75 µm.*

3.3.2. High-rate overpressure simulator (HOS) for in vitro mechanical exposure

Upon reaching confluence, cell cultures were exposed to isolated, transient overpressure using a custom underwater HOS (Figure 7). The fluid-filled chamber was designed to mimic the intracranial pressure profiles associated with high-rate overpressure in a primary blast exposure (Leonardi et al., 2011). The HOS is a one-chamber conical device that creates repeatable short-duration waves through an exploding bridge wire technique. This mechanism operates by charging a closed electrical circuit which contains a small bridge component. The bridge consists of two angled plates over which a thin wire is tightly suspended. This portion of the circuit is submerged within the HOS as denoted in Figure 7. Upon discharging the attached capacitor, current flows through the circuit to the point of least resistance (at the bridge). The bridge wire is subsequently vaporized upon high current passage and produces a repeatable high-rate overpressure wave. For testing, the chamber was filled with warmed reverse osmosis water (at 37 ºC). Cell plates were filled completely with culture medium (no added serum) and sealed with sterile parafilm. The cells were then placed in a holder at a 90 degree angle, incident to wave propagation. The HOS was instrumented with a piezo transducer (Meggitt cat# 8350C or PCB cat# 113B21) located in the wall of the chamber directly adjacent to the cell cultures. Cells were exposed to an average positive overpressure of 17 psi (117.2 kPa) with an average one millisecond positive duration. This system was particularly advantageous for the study of high-rate overpressure, or blast, injuries because cells were exposed directly to the propagating pressure wave with little to no impedance change through various media. Sham samples paralleled each overpressure-exposed plate and underwent the same preparation and placement in the HOS, without exposure to overpressure.

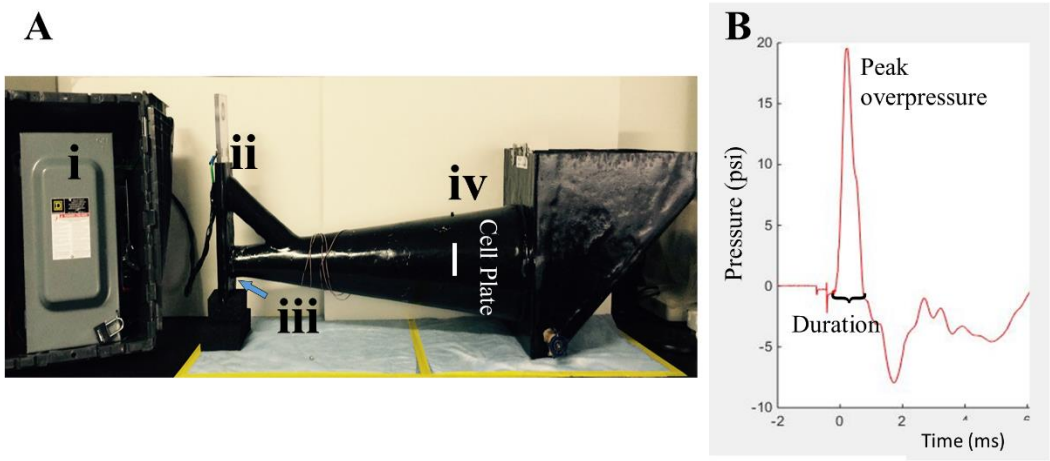

Figure 7. The HOS is a water-filled chamber which exposes in vitro samples to high-rate overpressure via an exploding bridge wire mechanism*. (A) The generator is composed of four main parts. (i) Capacitor (ii) Circuit mechanism (iii) Wire bridge (inside) (iv) Piezo-transducer* ***(B)*** *The simulated high-rate overpressure profile is based on intracranial pressure traces from in vivo blast testing*

3.3.3. ATP quantification

Quantification of endogenous ATP was conducted using a Calbiochem kit (Millipore, cat # 119107). This assay relies on bioluminescent conversion of luciferin and ATP to produce detectable light. Reagents were prepared by manufacturer's recommendation. In short, samples were lysed 2-24 hours post-overpressure exposure using the nucleotide releasing buffer (supplied) and transferred to a white-walled plate (Corning cat# 3990) in duplicates. One uL ATP monitoring enzyme was added to each sample and incubated for one minute. Luminescence was measured using a Molecular Devices LMax II 384 luminometer. Average luminescence values were normalized to the overall average sham.

3.3.4. Mitochondrial integrity

Mitochondrial integrity was measured using a live-cell cationic dye, JC-1. The molecule aggregates in functional mitochondria and fluoresces red while cytoplasmic JC-1 fluoresces green. Therefore, the ratio of red:green will be lower cells with compromised mitochondria. JC-1 dye was loaded into cells at a final concentration of 0.0005 mg/mL. Cells were incubated with stain for 15 minutes at 37°C before fluorescent images were obtained using a Zeiss Axiovert 40 CFL. Images were processed in MATLAB to obtain a pixel-to-pixel thresholded ratio between red and green images. Ratios were thresholded to include only positive signals relative to the green images and then averages were taken for the red: green ratio. Average ratios were normalized to sham.

3.3.5. Gadolinium treatment, calcium imaging and oxidative stress potential

In order to assess calcium and other cationic exchange in contributing to early oxidative stress potentiation in astrocytes after high-rate overpressure, samples were randomly assigned to one of four groups: overpressure, sham, overpressure + gadolinium, and sham + gadolinium. Gadolinium is a metal that interferes with lipid packing in cell membranes to cause non-specific cationic channel blockage. It has been used in a number of applications for understanding brain cell physiology, most notably to block calcium signaling. Samples were seeded in six-well plates and were assigned to one of three groups: calcium imaging (one well), staining (two wells), RNA/protein (three wells). Experiments were run similarly to that described in 3.3.2 with the exception of gadolinium (Sigma) supplementation into the media (10 µM) during the overpressure event and for three minutes of post-video analysis.

3.3.5.1. Calcium imaging

Flou-4AM (Invitrogen cat# F14201) was prepared at one µg/µL in DMSO-Pluronic F127 and then put in solution at 6.5 µg/mL in serum-free DMEM (Gibco cat# 31053-028). Following 30 minute incubation with Fluo-4AM at 37°C, samples were quenched for 20 minutes in serum-free DMEM before testing in the overpressure generator. Immediately after overpressure exposure, samples were quickly transferred to an EVOS fluorescent microscope. Calcium dynamics were captured by video for three minutes following overpressure exposure. For analysis, a specific time range was chosen in which the majority of samples overlapped in their time-after-exposure. This range was 180-240 seconds post-exposure. Video analysis was conducted using a ΔF/F function created in MATLAB for the time range specified. All values were normalized to the first second of data in order to understand how calcium was changing relative to the specific time interval analyzed. Calcium load was calculated as the area under the ΔF/F-time curve. Load could be both positive and negative because samples were normalized to 180-181 seconds post-overpressure instead of a baseline value.

3.3.5.2. Protein isolation

Proteins were extracted from the phenol phase of Trizol after RNA and DNA were removed. Samples extracted ethanol and propanol followed by three 20 minute washes with 0.3 M guanidine hydrochloride in 95% ethanol and one 20 minute wash in ethanol. Samples were re-suspended in 1:1 mixture of 1% sodium dodecyl sulfate solution and 8M urea in 1M Tris-HCl (pH=8.0) and protease inhibitor (Sigma-Aldrich cat# P8340) at 1:100. Protein samples were

homogenized for 10 seconds followed by a 10 minute incubation at 55°C to facilitate resuspension. This process was repeated two more times. Alternatively, some analyses involved protein isolation by a standard lysis protocol. Instead of Trizol, cells were lysed in a buffer containing 40 mM Tris-HCl (pH=7.5), 150 mM NaCl, 2.5 mM EDTA and 1% Triton X-100. After scraping cells from plate surface, samples were placed on ice and shaken vigorously (~600rpm) for 30 minutes. Following centrifugation at 16,000xg for 20 minutes, the solubilized proteins were transferred for further applications. Total protein samples were quantified by bicinchoninic acid (BCA) assay (Pierce cat# 23225) for use in western blotting experiments. Protein analysis of NOX4, SOD2 and proliferating cell nuclear antigen (PCNA) were conducted using automatic western techniques (Wes, Protein Simple). Standard settings were used for these analyses and primary antibodies are given in Table 2.

Table 2 | Antibodies used for western blotting in Chapter 3

Protein	**Abbreviation**	**Catalog #**
NADPH oxidase 4	NOX4	Abcam, Ab154244
Superoxide dismutase 2	SOD2	Novus Biologicals, NB100-1992
Proliferating cell nuclear antigen	PCNA	Cell Signaling, 13110S

3.3.6. Amino acid metabolism

Total amino metabolism was measured using a ninhydrin assay. Ninhydrin is a synthetic molecule that binds to primary amines to form a purple color (Ruhemann's purple). Cell media was removed from overpressure-exposed samples at four and 24 hours. Media from each sample was diluted to produce a five-point curve (between 1:5 and 1:50 dilutions). After diluting in PBS, samples were mixed with ninhydrin (Acros Organics cat# 165870250) in ethanol such that final concentration of ninhydrin was 1.75 ug/uL. Samples were heated at 100°C for 15 minutes. Following heat, samples were transferred to a 96-well plate in triplicate and the absorbance was measured at 570nm using a SpectraMax 190. A linear relationship was developed for each sample and the slope was used as the relative concentration of amino acids present in the culture media (Figure 8). Sample slopes were normalized to the average sham slope for each sample set. Because the media was standardized for each set of samples run, any changes measured were attributed to cellular metabolism differences.

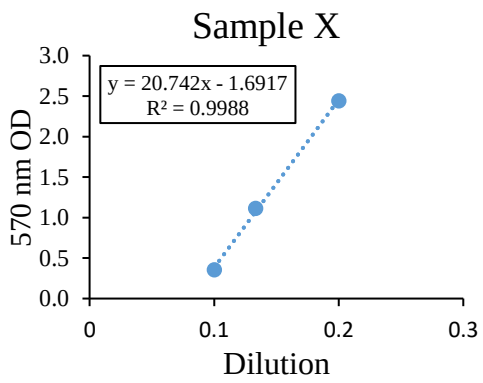

Figure 8. An example ninhydrin reaction curve for a given sample. *The slope was used as the best estimate for each sample.*

3.3.7. Statistics

Statistical comparisons were conducted between groups using JMP software (SAS) under Virginia Tech license. ANOVA was used to analyze significant differences amongst groups, followed by student's t-test for individual group comparisons. The assumptions for normality and homoscedasticity were confirmed by Shapiro-Wilk and Levene's tests, respectively. In the event that data was not normal, a logarithmic transformation was performed to conduct statistical comparisons. For sample sets with unequal variances, a Welch's t-test was performed. Statistical outliers were determined using residual analysis, and a p-value<0.05 was considered significant. Total number of replicates are denoted as the variable "n." Sample blocks were used for individual overpressure/sham tests and were from the same line of cells (from the same animal and passage number).

3.4. Results

3.4.1. Calcium oscillations following high-rate overpressure

Primary astrocyte cultures were exposed to a target peak overpressure of 20 psi. Pressure parameters for this study are summarized in Table 3.

Table 3 | Summary of overpressure mechanics on samples in Chapter 3

	Avg ± Std Dev
Peak Overpressure [psi]	19.3 ± 5.8
Positive Peak Duration [ms]	0.80 ± 0.33
Rise Time [ms]	0.34 ± 0.19

From fluorescent video analysis, calcium oscillations were observed and persisted at least several minutes, across the timeframe analyzed. The time period of 180-240 seconds post overpressure or sham treatment was selected for initial comparisons. Figure 9 shows a significantly higher calcium

load in the overpressure group (denoted as OP) compared to sham (p-value=0.0339). Calcium load was calculated as the area under the ΔF/F curve over the selected timeframe. A subset of this data was compared against equivalent samples which were incubated with gadolinium during both overpressure/sham exposure and video collection. The videos were all normalized to the same time period of 180-240 seconds after treatment and exposure. Moreover, the overpressure group tended to show a positive calcium load over this time period while the sham showed a negative load, suggesting the possibility for overpressure to induce periodic shifts in oscillations. Further studies will be necessary to confirm these trends as high variations accompany these measurements.

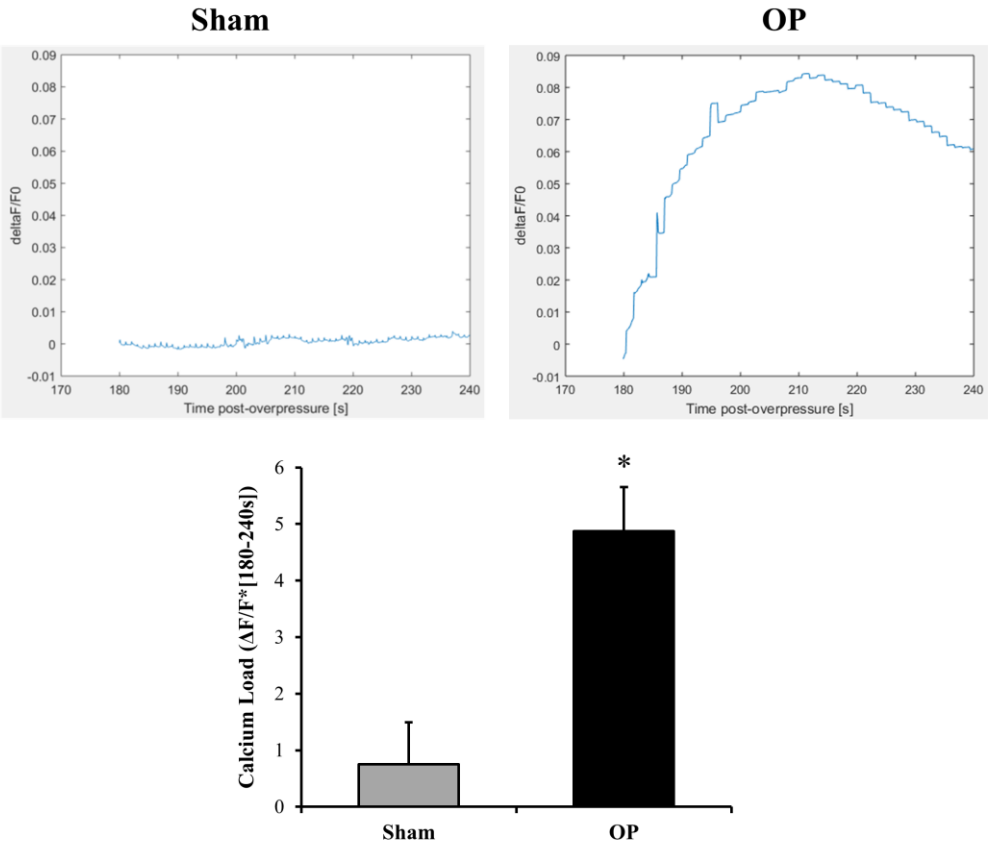

Figure 9. Calcium dynamics were quantified by Fluo-4AM video analysis following high-rate overpressure*. Calcium load was quantified over a 60 second period at 180-240 seconds post-overpressure. Representative profiles of ΔF/F are shown for sham and OP groups. Profiles are normalized to the first second of data (180-181 seconds post-overpressure). The OP group had a significantly higher calcium load over the time period from 180-240 seconds. Data are mean ±SEM *p-value<0.05 as compared to sham, n=3-4/group*

3.4.2. Early bioenergetic fluctuations following overpressure include dynamic changes in endogenous ATP

To assess changes in bioenergetic capacity, cells were assessed for endogenous ATP levels and mitochondrial integrity. The ATP profile between two and 24 hours post-overpressure is shown in Figure 10. ATP levels were dynamically regulated with a significant decrease four hours (p-value= 0.0158) compared to a significant increase at six hours compared to sham (p-value<0.0001). At four hours, this response was coupled with a slight, yet significant decrease in MTT activity. MTT assay assesses NADPH-dependent metabolism and may suggest a mechanism by which ATP production becomes dysfunctional. Otherwise, ATP fluctuations, especially in the presence of altered calcium signaling, can be the result of impaired mitochondria. In order to assess this hypothesis, JC-1 live cell staining was employed. Ratiometric fluorescent intensity measurements indicated no difference in crude mitochondrial membrane potential at four hours between groups (Figure 11).

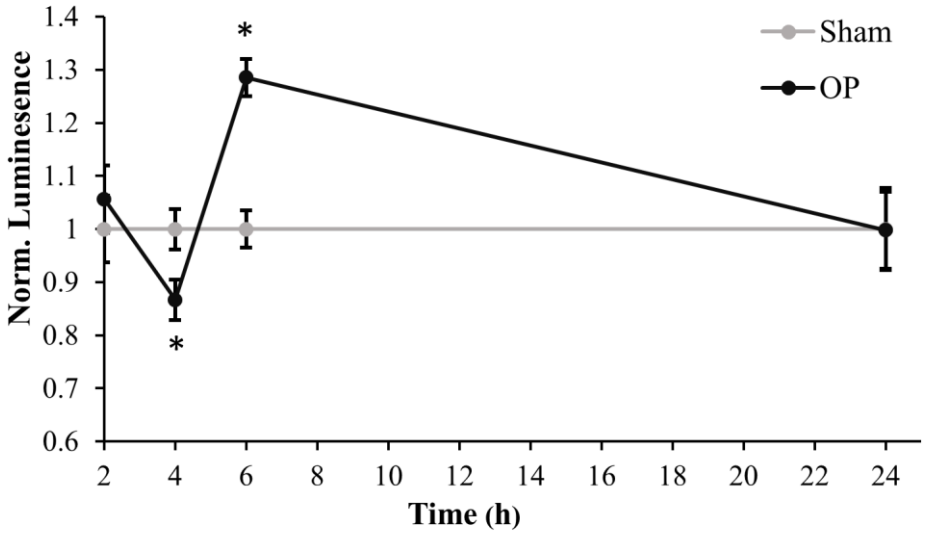

Figure 10. Dynamic profile of endogenous ATP as measured by a firefly luciferase assay. *Luminescence is normalized to sham at each time point. At four hours post-overpressure, astrocytes had decreased ATP levels while at six hours, endogenous ATP was increased in the overpressure group. Data are mean ±SEM *p-value<0.05 as compared to sham and individual time points (no sequential analysis conducted), n=11-15/group*

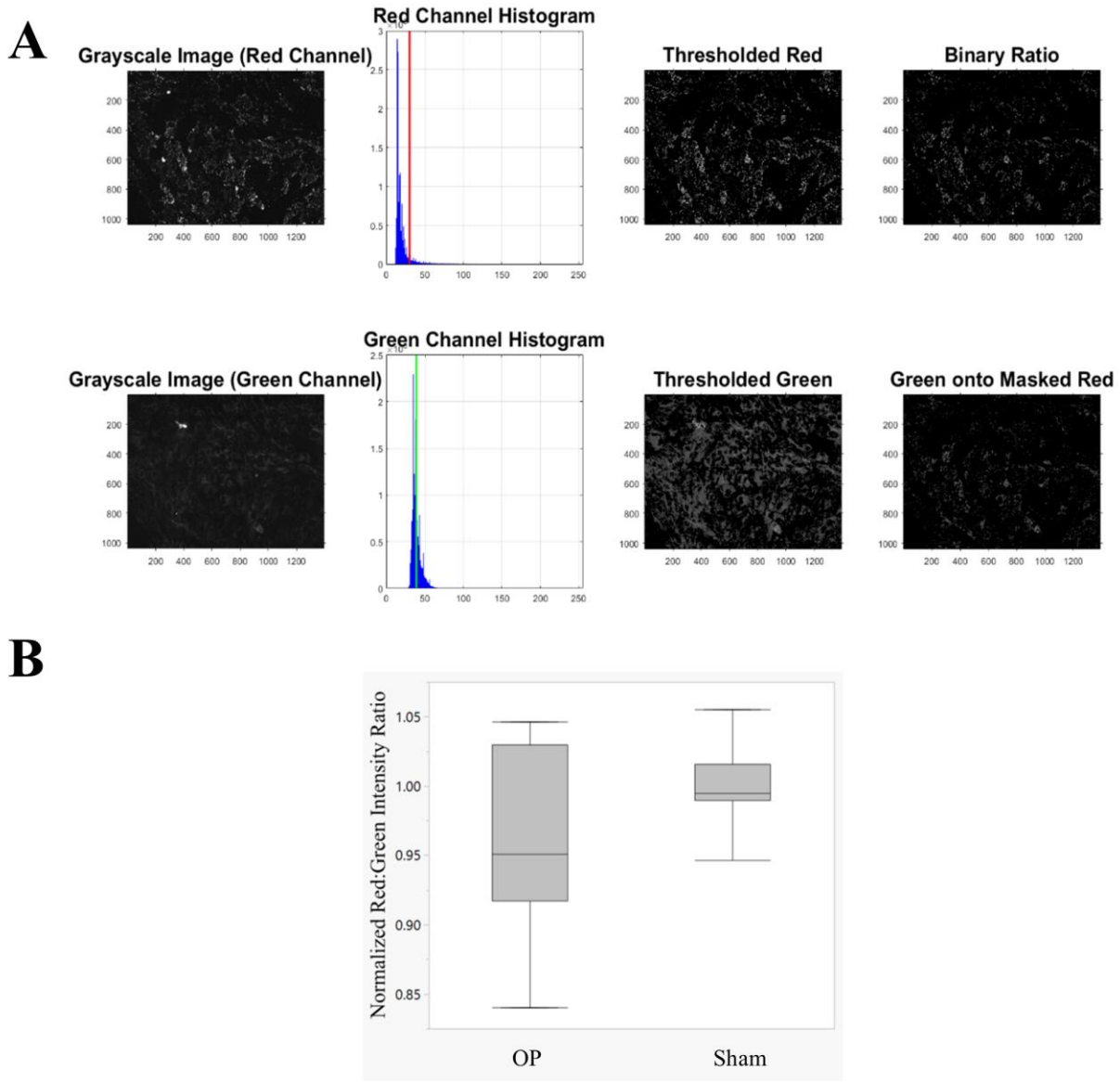

Figure 11. Mitochondrial membrane integrity was assessed by live-cell imagining using JC-1, a ratiometric dye. (A) *A custom MATLAB code was created to assess pixel-to-pixel ratios between the green and red fluorescent stains.* ***(B)*** *No significant differences in mitochondrial membrane integrity were observed at four hours post-overpressure using this technique. Data are displayed as box plots to show the distributions of normalized ratios between groups, n=13-14/group*

3.4.3. Pro-oxidative and proliferative (reparative) potential induced by overpressure was blocked by gadolinium treatment during exposure

In a subset of experiments from above, gadolinium treatment tends to ameliorate fluctuations in calcium across 180-240 seconds following high-rate overpressure (Figure 12). No statistics were run on these data given there were only three representations of the data available.

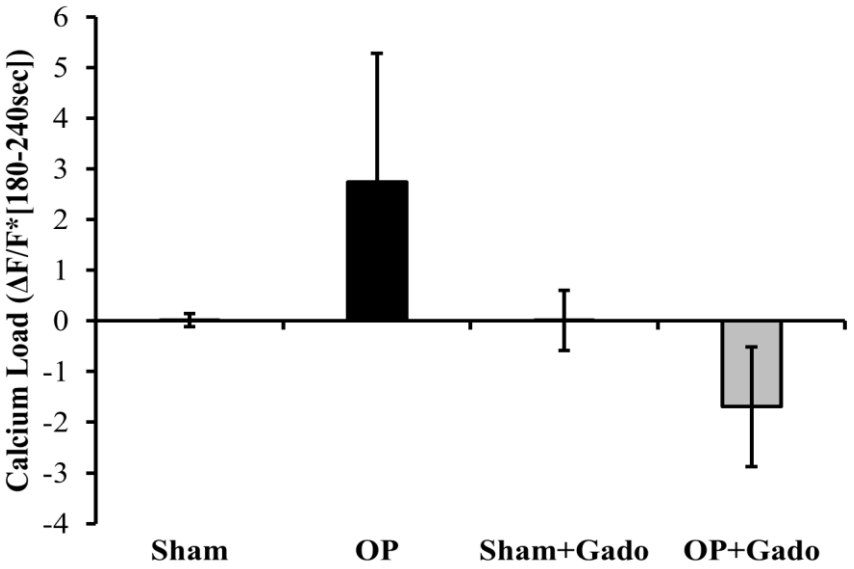

Figure 12. The application of a cationic channel blocker, gadolinium, influenced calcium fluctuations over a period of 180-240 seconds post-overpressure. *No statistics were conducted on these samples given low sample number. Data are shown as mean ±SEM *p-value<0.05 as compared to sham, n=3/group*

Additionally, Figure 13 shows NOX4 protein expression was significantly higher compared to sham at 24 hours post overpressure exposure (p-value= 0.021 for ANOVA), indicating a pro-oxidative potential in the astrocytes. This response was ameliorated when gadolinium treatment was applied during overpressure exposure. There were no significant differences in SOD2 production at either four or 24 hours post-exposure (Figure 13).

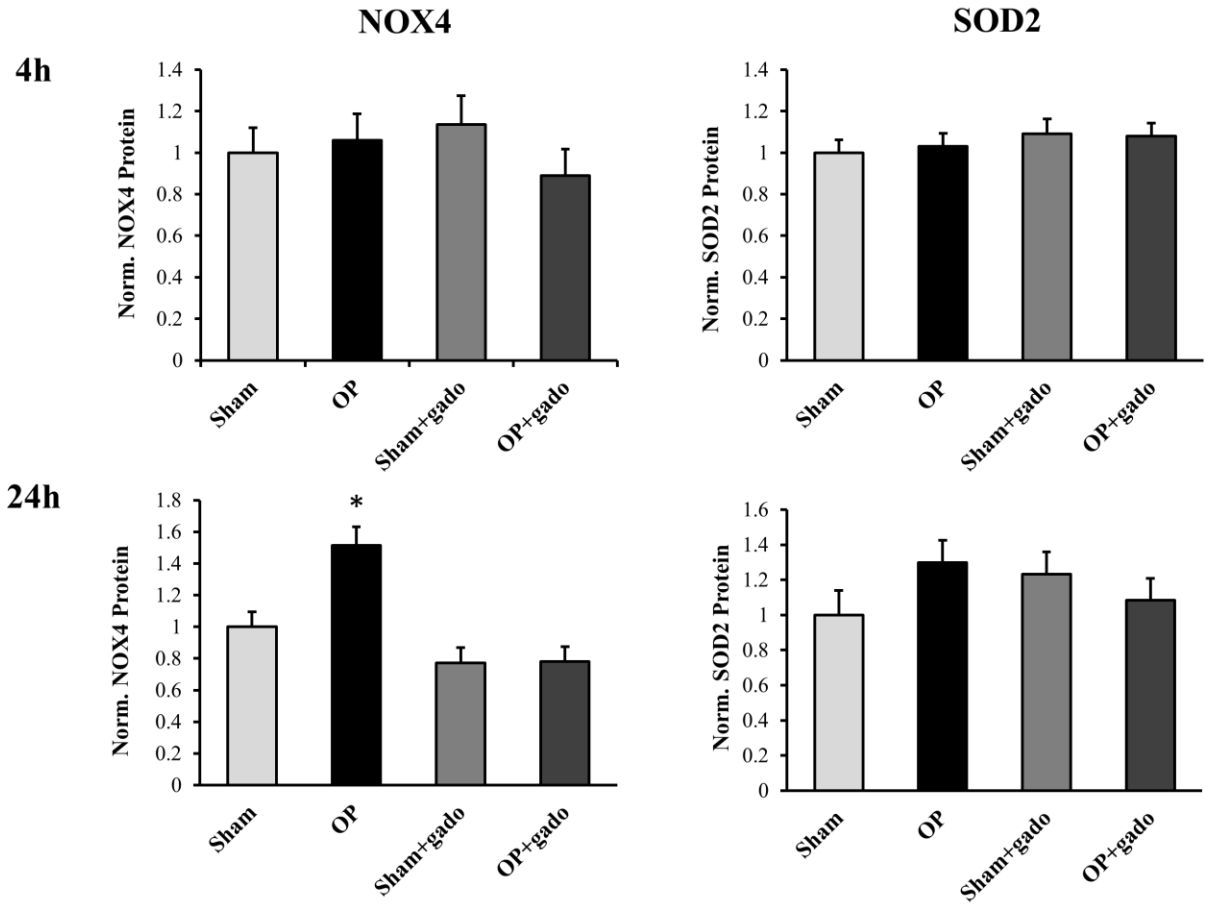

Figure 13. Overpressure-induced pro-oxidative effects were blocked by application of gadolinium during overpressure event. *At 24 hours post-overpressure, NOX4 was significantly elevated compared to sham as quantified by western blot. This response was mitigated with the application of gadolinium. No changes were observed at four hours and no changes occurred in SOD2 expression at either four or 24 hours post. Data are mean ±SEM *p-value<0.05 as compared to sham, n=6-9/group*

Apart from oxidative stress regulation, the influence of gadolinium was also assessed for PCNA upregulation in astrocytes at 24 hours post-overpressure. Figure 14 depicts a significant upregulation of PCNA at 24 hours which was mitigated by the application of gadolinium during the overpressure event (p-value<0.0001 compared to gadolinium exposed groups and p-value=0.0001 compared to sham).

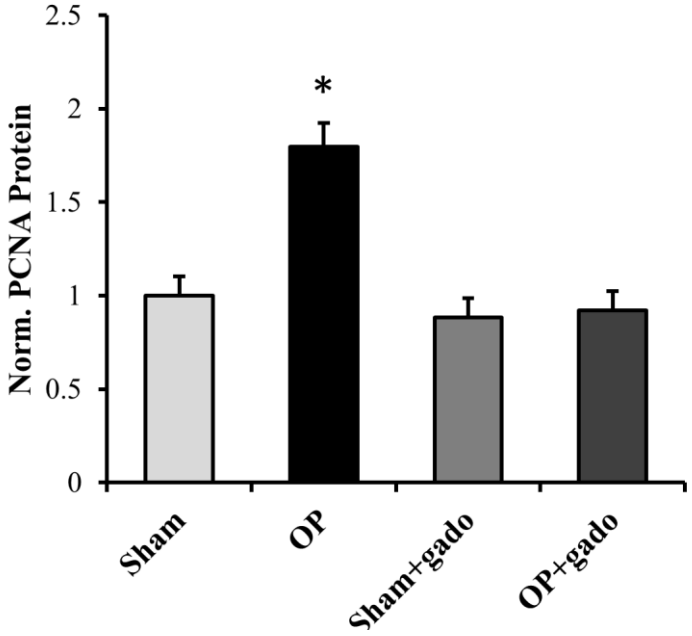

Figure 14. Western blot analysis of PCNA in overpressure and gadolinium treated astrocytes. *Gadolinium treatment reduces the upregulation of PCNA in response to overpressure at 24 hours. Data are mean ±SEM *p-value<0.05 as compared to sham; n=4-6/group*

3.4.5. Amino acid metabolism

A ninhydrin reaction was used to determine available amino acids in culture media from cells incubated for four and 24 hours post-overpressure exposure. Serial dilutions of samples were used to fit a line-of-best-fit to each sample as an estimation of free amino acid concentration. Results indicated a significant decrease in available amino acids in culture media (thus increased metabolism) at 24 hours as compared to sham (p-value= 0.0181, Figure 15) while no differences were observed at four hours.

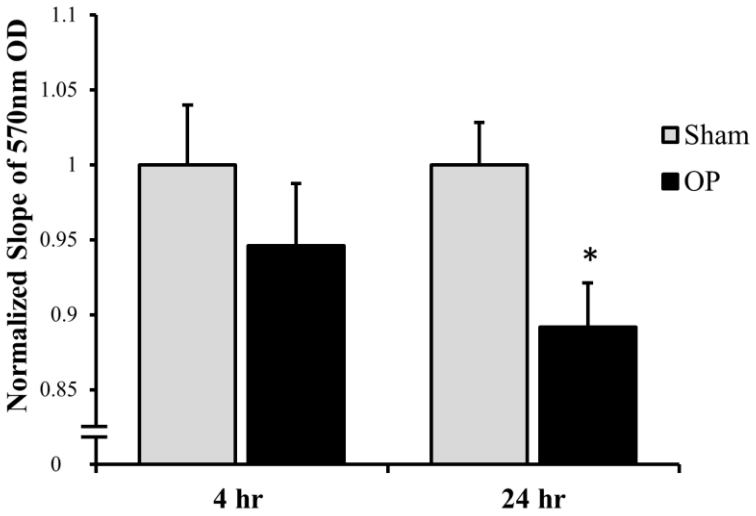

Figure 15. Amino acids metabolism was estimated from the amount of available free amino acids in astrocyte culture media at four and 24 hours. *A significant decrease in available amino acids at 24 hours suggested increased metabolism at this time point. Data are mean ±SEM *p-value<0.05 as compared to sham, n=12-13/group*

3.5. Discussion

Mechanical damage to brain tissue directly contributes to increased cellular debris and surges in both neurotransmitters and ions in the brain parenchyma immediately after injury. For astrocytes in particular, mechanical trauma is often associated with surges in intracellular calcium. Calcium is released from intracellular stores in astrocytes in response to excess glutamate and ATP (Bezzi et al., 1998; Kang et al., 1998; Fiacco and McCarthy, 2006; Fujii et al., 2017), both of which are associated with early TBI.

Under normal physiological circumstances, sources for calcium signaling in astrocytes include G-protein coupled receptor-activated IP3 signaling or voltage-gated channel fluxes (Agulhon et al., 2008). Under mechanical manipulation, stretch-activated ion channels can also be activated to allow for calcium changes in astrocytes (Sachs, 2010). Evidence exists for each of these types of channels in astrocyte signaling in TBI. Multiple groups have shown that high-rate overpressure scenarios lead to calcium perturbations in cultured brain cells (Ravin et al., 2012; Sieger et al., 2012; Maneshi et al., 2015; Ravin et al., 2016). However, there is not enough evidence to yet determine mechanisms and/or sources by which this phenomenon occurs. Ravin et al showed that rapid surges in calcium in astrocytes likely involves shearing effects on the cells. Maneshi et al reported thresholds for calcium signal activation in neurons depending on overpressure mechanics.

Similar to these studies, the results herein show that calcium oscillations were occurring several minutes after exposure to 20 psi overpressure.

Gadolinium was chosen to manipulate cationic signaling because of its ability to block multiple types of cationic channels. Maneshi et al have shown that gadolinium blocks channels in astrocytes specifically, having influence of calcium dynamics during mechano-stimulation (Maneshi et al., 2015). While it is likely that the highest surges in intracellular calcium would occur nearly instantaneously, this study showed that lasting perturbations may be involved in secondary signaling mechanisms in astrocytes. Results of this study suggested that cationic signaling during overpressure and for several minutes after influences the expression of NOX4 in astrocytes. NOX4 has been associated with multiple negative consequences of TBI, including protein peroxidation, neuronal survival and neurodegeneration (Ma et al., 2018a). Gadolinium application was able to ameliorate the upregulation of NOX4. Together these results support previous evidence that NOX4 regulation has a mechanical basis and may be related to early metabolic state. This study is the first to show that blocking cationic channel activity during high-rate overpressure. One study of CNS injury showed a protective effect from gadolinium in terms of reducing inflammation and cell death. Specifically, the study found a decrease in the number of apoptotic astrocytes after injury and overall improved outcomes in animals receiving gadolinium treatment (Fan et al., 2016). Another study showed that gadolinium is able to inhibit glutamate-induced ATP release from astrocytes (Zhao et al., 2017). It should be noted gadolinium has been reported to induce cell death and/or oxidative stress in neurons though (Xia et al., 2011). This suggests another mechanism by which astrocytic and neuronal metabolic regulation may be different. In this study, gadolinium was applied to astrocytes during and for approximately five minutes post-overpressure. This application proved to be protective in the case of pro-oxidative NOX4 expression. NOX4 is constitutively activated and thus is not influenced directly by calcium levels. The results in this study also indicate an indirect relationship between cationic signaling during overpressure and NOX4 production, as NOX4 was not significantly altered until 24 hours post.

In terms of antioxidant expression, SOD2 was not altered at either time point assessed. This result is consistent with stable mitochondrial integrity. SOD2 regulation is strongly associated with mitochondrial health and integrity. Targeting overexpression of antioxidant molecules, like SOD2, has shown promise in reducing neuronal susceptibility to damage (Xu et al., 2010; Baxter and

Hardingham, 2016). Following blast exposure, Arun et al have evaluated global mitochondrial function *in vivo* (Arun et al., 2013) to find acute perturbations (<24 hours), yet very little is understood about specific cellular responses and phenotypes that develop as a result. Therefore, it may be possible that SOD2 would be upregulated in the presence of damaged neurons but is not necessary at this stage after injury to defend against damage for astrocytes.

Despite no crude changes in mitochondrial integrity, MTT results and the endogenous ATP profile in astrocytes suggest a potential enzymatic imbalance. Together, these results support the fact that astrocytes are robust in their ability to combat dynamic chemical environments yet they do experience early metabolic fluctuations which may contribute to later pathology. This can be noted in the dynamic ATP response profile observed between four and six hours. The phenotype assumed at four hours post-exposure may indicate enzymatic dysfunctions associated with impaired mitochondrial function, specifically related to mitochondrial respiration and ATP production. Interestingly, constitutive activation of NOX4 seems to preferentially involve NADP+/NADPH and flavin adenine dinucleotide (FAD+)/FADH interactions and thus may provide an indication for specific enzymatic dysfunctions within the mitochondria (Jackson et al., 2010). The response may also include shuttling changes in the mitochondria complex proteins.

Energy expenditure is much higher in the injured brain due to loss of homeostasis and integrity of intracellular organelles. Previous studies conducted on brain cells have shown that neurons and glial cells may experience altered amino acid metabolism as result of acute injury (Scafidi et al., 2009; Bartnik-Olson et al., 2010; Guerriero et al., 2015; Dash et al., 2016). A few studies conducted with experimental CNS injury models have indicated altered amino acid metabolism in brain cells (Dash et al., 2016; Arun et al., 2018). Moreover, several studies indicate similar relative changes in amino acid metabolism as that found in astrocytes in this study. Amorini et al showed moderate changes in free amino acids in cerebral tissue from rats exposed to TBI. Increased localization of glutamate, glutamine and changes in methionine metabolism were observed by 24 hours post-injury (Amorini et al., 2017). In another study, Boyko et al showed chronic changes in amino acid profiles in tissue from rats with spinal cord injury were associated with nitric oxide production (Boyko et al., 2017). This draws a connection between amino acid metabolism and the oxidative environment within cells. Because amino acid metabolism can influence a number of cellular functions and processes, including DNA methylation, ATP release, inflammation, and

protection against oxidative stress, it is important to consider how individual cell types respond to injurious stimuli.

One limitation of this study is the exclusion of the crosstalk between astrocytes and other brain cell types. It is well known that astrocytes are intimately coupled for a metabolic purposes with neurons. The purpose of this study was to identify mechanisms by which astrocytes themselves respond to overpressure in an effort to understand their reparative nature, but it is possible that differential regulation of these responses may occur when in the presence of neurons. In particular, extracellular glutamate levels may be significantly increased in the presence of injured neurons and thus would influence not only calcium dynamics in astrocytes but also their metabolic profiles. Moreover, the mechanisms by which gadolinium influenced ionic signaling were not fully tested in this study. Although its function can be assumed from previous literature, the electrical potential of the cells could be more precisely analyzed in the absence and presence of gadolinium.

The purpose of this study was to identify cellular responses in astrocytes related to metabolic state which could be used to understand injury sequelae. While evidences point to robustness in astrocyte integrity, there are underlying biochemical changes and metabolic patterns that suggest potential pathways by which cellular stress and dysfunction can occur. These metabolic profiles have wide influence on cell state and may alter responses like neuroinflammation, epigenetic control and cell signaling, all of which could lead to further cellular dysfunction and reactivity in blast neurotrauma.

3.6. Conclusions

Many of the acute responses to metabolic and redox stress in astrocytes are reparative by nature, however, they serve as the premise by which pathological sequelae may develop. There is a need to better understand the progression of astrocyte reactivity in order to effectively harness the protective potential of reactive astrocytes while diminishing the harmful aspects. This study found that high-rate overpressure induced increased calcium load and bioenergetic fluctuations in astrocytes without significant loss of mitochondrial integrity. Additionally, by blocking immediate cationic exchange during high-rate overpressure, astrocytes did not assume a pro-oxidative phenotype as measured by NOX4 expression. These findings identify a particular source of metabolic imbalance that is specific to astrocytes.

3.7. Future Directions

An intriguing question that remains related to astrocyte pathophysiology in this context is how astrocytes in culture and *in vivo* transmit immediate calcium signals in response to high-rate overpressure. This study showed longer lasting calcium oscillations. In the future, patterns of oscillations should be further examined. Examination beginning immediately after overpressure will be an important to fully characterize this response. Various sources of calcium load including stretch-activated ion channels and various voltage-gated channels may play incongruent roles and be activated along different timescales. Previous studies have shown that voltage-gated channels are persistently upregulated in phenotypically reactive astrocytes and thus may represent a significant molecular target for astrocyte response to blast neurotrauma. These studies should also be conducted in mixed brain cells or organotypic slices in order to allow for the influence that neurons and other cells have on calcium signaling in astrocytes. Future work should also address the idea of enzymatic perturbations in astrocytes as a primer for pro-oxidative or protective changes. The NADP+/NAPDH and FAD+/FADH ratios that drive activation of NOX4 are an important consideration. Other pro-oxidant and antioxidants may be necessary to evaluate as responding elements in astrocyte response to high-rate overpressure as well. For example, the antioxidant Nrf2 may have a direct effect on NOX4 and ROS production (Kovac et al., 2015).

In terms of amino acid metabolism, high performance liquid chromatography could be used to study amino acid profiles after overpressure. This information can be used to better understand the influences of amino acid metabolism on aberrant cellular function. In particular, levels of methionine may be an important indicator of epigenetic state and therefore phenotypic regulation. Derivatives of the methionine metabolism pathway have also been shown to be altered in humans with TBI (Dash et al., 2016), but these pathways have not yet been explored in blast neurotrauma.

Chapter 4. Characterization of an *in vitro* model for primary astrocyte reactivity to isolated high-rate overpressure

4.1. Introduction

This chapter describes the development and exploration of an *in vitro* model which recapitulates molecular features of astrocyte reactivity related to isolated high-rate overpressure exposure. Multiple "classical" hallmarks of reactivity were characterized up to 72 hours post-exposure, including cellular repair and structural up-regulation. Following on the foundation of early metabolic and signaling events explored in Chapter 3, the purpose of this chapter was to evaluate regulation of other signaling mechanisms specifically related to cell junctions and adhesion which may coincide with and contribute to the assumption of the reactive phenotype. Cell junctions control various types of extracellular signals and transmit them within cells to enact phenotypic and functional changes. Therefore, they may critical regulators of astrocyte response to mechanical insult. The hypothesis for this study is that astrocytes will assume a progressive reactive phenotype in response to isolated high-rate overpressure, and this phenotype will involve dynamic expression of multiple cell junctional protein classes.

4.1.1. Statement of Problem

Glial reactivity is a ubiquitous feature of acute and chronic traumatic brain injury (TBI). It represents an important reparative process which is meant to control and modulate neurogenic inflammation, waste clearance and repair after injury. Astrocyte reactivity has specific roles in these processes and is well documented to support and facilitate return to proper function for the injured brain. Despite the necessity for astrocyte reactivity, it can become inadequately regulated and ultimately be detrimental. The mechanisms which regulate the assumption of astrocyte reactivity, particularly in response to high-rate overpressure exposure, are not well-known. Previous studies have eluded to a mechanical basis for disruption of astrocytes as well as their reactivity through the use of *in vitro* models (Hlavac et al., 2015; Ellis et al., 1995; Ahmed et al., 2000; LaPlaca et al., 2005; Cullen et al., 2007; VandeVord et al., 2008; Miller et al., 2009), yet in the case of blast-relevant mechanics, *in vitro* models for astrocyte reactivity are mostly recent in development (Maneshi et al., 2015; Ravin et al., 2016; Logan et al., 2017). Because astrocytes can become reactive to mechanical insult without other cell types present, there must be autologous signaling and regulation that occurs within their networks, ultimately leading to, at least in part, their reactive response. Many of these molecular sources for reactivity are not well characterized, particularly in high-rate mechanics. Moreover, there remains a large disparity in understanding thresholds of cellular responses in the context of high-rate mechanics like blast neurotrauma. There

is need for viable *in vitro* models to study astrocyte response to defined high-rate overpressure mechanics in order to develop novel molecular targets for modulating their response following neurotrauma.

4.1.2. Significance

Astrocytes have emerged as a promising therapeutic target in TBI because of their diverse roles in metabolic and ionic homeostasis, structural integrity and tissue repair (Buffo et al., 2010; Burda et al., 2016; Liddelow and Barres, 2017). This is especially true when considering their potential to communicate and adequately respond to injured neurons in a myriad of central nervous system (CNS) insults. Specifically, impaired neuronal-astrocytic signaling can lend to excitotoxicity, metabolic failure and neurodegeneration, all of which have implications for the memory deficits and behavioral outcomes of TBI (Pekny et al., 2014; Sajja et al., 2016). There is a need to understand dynamic changes in astrocyte network communication as a means for autologous phenotypic activation and regulation. Of particular interest in this study is deciphering changes in regulation and expression of multiple classes of cellular junction proteins as they may relate to classical features of astrocyte reactivity following high-rate overpressure. Cell junctions have the ability to influence a wide range of cellular responses from inflammation to proliferation and repair. A subset of junctional proteins also plays a critical role in cell stabilization and mechanical cues. Yet, their regulation has been largely uncharacterized in high-rate traumatic injuries, particularly in astrocytes. Because cell junctions have the ability to both regulate and respond to changes in cellular phenotype, they represent a significant aspect to fully understand astrocyte reactivity.

The purpose of this study was to characterize differential regulation of cell junction molecules and signal transduction activation in reactive astrocytes following high-rate overpressure exposure. Motivation for this work has arisen from studies on other cell types which have extensively shown signaling responses to transient and prolonged mechanical stress. Although biochemical signals also activate these pathways, several recent studies have explored how adhesion signaling and mechano-activation elicit cellular reactivity in various contexts (Alford et al., 2011; Nayak et al., 2013; Zhou et al., 2014; Zhu et al., 2016).

4.2. Literature Review

Current studies are focused on understanding astrocyte susceptibility to traumatic insult and how various cellular and molecular sources may be contributing to the extent of astrocyte activation. Moreover, mechanical thresholds and underlying mechanisms for brain injury in high-rate scenarios like blast neurotrauma remain undistinguished from other TBI modes with entirely different mechanics. Previous *in vitro* studies of astrocytes by our lab and others have suggested that astrocytes respond to isolated high-rate overpressure exposure and other mechanical insults by acute phenotypic reactivity (Rzigalinski et al., 1998; Neary et al., 2005; Cullen et al., 2007; VandeVord et al., 2008). Although these features are well-characterized in both acute and chronic stages of *in vitro* and *in vivo* TBI models, there is a significant lack in understanding how the reactive phenotype and astrocyte signaling potential are influenced by mechanical disruptions. Thus, there is a significant need to develop *in vitro* models which recapitulate the molecular hallmarks of astrocyte reactivity in order to study their underlying regulatory mechanisms.

4.2.1. Molecular Targets for Astrocyte Reactivity

"Classical" astrocyte reactivity is most ubiquitously characterized by altered expression of structural proteins and by increased proliferation (Buffo et al., 2010; Sirko et al., 2015). Structural integrity is mainly in the form of the intermediate filament proteins, such as glial fibrillary acidic protein (GFAP). Intermediate filaments make up a dynamic cytoskeletal network that is critical not only for cell structure but also for migration, shuttling, signal transduction and communication (Iwatsuki and Suda, 2010). There are multiple classes of intermediate filaments which can co-polymerize through the cytoskeleton and around organelles to perform their regulatory functions. The most commonly studied intermediate filament in astrocytes is GFAP. This protein has ten isoforms and is specific to astrocytes (and progenitors) in the brain. In a non-reactive state, as little as 8% of astrocytes may express GFAP (Rusnakova et al., 2013), however this intermediate filament has been widely associated with astrocyte pathophysiology and TBI progression. In GFAP knockout studies, it was determined that the CNS is more susceptible to mechanical damage in the absence of GFAP-expressing astrocytes (Nawashiro et al., 1998), providing evidence for GFAP in structural maintenance. In contrast, GFAP/vimentin-null mice showed increased neurogenesis after TBI, supporting the idea that neurogenesis is negatively regulated by astrocytes (Wilhelmsson et al., 2012).

Because GFAP upregulation is widely observed across TBI models, there must conserved molecular activation pathways for its regulation. Some of the pathways identified include mecahno-activated signal transduction, altered physical and extracellular matrix (ECM) cues, and dysfunctional cell-cell interactions, all of which are involved in the secondary injury phase of TBI. However, it is still unclear how these pathways interact and compete to produce both acute and chronic astrocyte activation.

Another ubiquitous feature of astrocyte reactivity following mechanical trauma to the brain is increased proliferation as a compensatory inflammatory response. Recent studies have suggested that astrocyte proliferation is a selective process and may be localized for various functions in repair. In particular, one study showed heterogeneous proliferative of astrocytes around vasculature following a penetrating TBI (Bardehle et al., 2013). More extensive proliferation is associated with later stage astrocyte reactivity in which cells replicate to form scar tissue (Sofroniew, 2009). Both cell-derived signals and mechanical stress are potential contributors to this feature. There is some evidence to suggest that pro-inflammatory cytokines can have a synergistic influence on astrocyte proliferation and influence early stages in particular (Selmaj et al., 1990; Levison et al., 2000). In addition, regulation pathways for proliferation by mechanical stress through ubiquitous signal transduction pathways have been identified in other cell types (Provenzano and Keely, 2011).

In this chapter, proliferating cell nuclear antigen (PCNA) expression was used as a molecular target for proliferative/reparative potential in astrocytes. PCNA is a DNA clamp which facilitates a number of processes involved in DNA replication and repair, including holding DNA polymerase in place for rapid synthesis (Kelman, 1997). It is used as a marker of proliferation potential, however, its upregulation does not strictly mean proliferation as cells can express PCNA strictly for repair purposes. Studies have reported the localized expression of PCNA in astrocytes and other brain cells as a result of experimental TBI (Johnson et al., 2004; Di Giovanni et al., 2005). Some studies have specified PCNA as a marker for proliferative, scar-forming astrocytes. Targeting cell cycle regulation by the drug flavopiridol (reduces cyclin D1) reduced PCNA expression in astrocytes and glial scar formation following TBI, thereby increasing neuroprotective effects (Di Giovanni et al., 2005). This molecular target may represent an inducible aspect of proliferation associated with the reactive phenotype.

Overall, there are many potential regulators of astrocyte phenotype following CNS injury. Among the initiating factors for reactivity, mechano-stimulation has been identified to induce aspects of the reactive phenotype, including both structural and proliferative mechanisms. The *in vitro* model presented in this chapter provides a mechanism to study mechano-regulation of classical reactivity targets involved with structural integrity and proliferation.

4.2.2. Cellular Adhesion Targets in Reactive Astrocytes

Aside from the classical molecular features of astrocyte reactivity, there are many other protein classes that have been explored in conjunction with the reactive phenotype. Of particular interest in this study is deciphering changes in regulation and expression of multiple classes of cellular junction proteins as they may relate to classical features of astrocyte reactivity following high-rate overpressure. More specifically, there is a need to understand dynamic changes in astrocyte network communication as a means for autologous phenotypic activation and regulation.

There are three major classes of cell junction proteins: gap junctions, anchoring junctions, and tight junctions (Figure 16). Gap junctions, notably connexins (CX), form dimers between cells to allow for passage of small molecules like ATP and other secondary messengers. Anchoring junctions form between cells and with the surrounding ECM. These proteins are necessary for maintaining cell shape and motility as well as structural integrity of tissue. Adaptor proteins such as vinculin act to connect the anchoring proteins to the cytoskeleton and create a force sensing mechanism for the whole cell. Lastly, tight junctions form between two cell membranes to prevent the passage of molecules between their spaces. Altogether, these classes of molecules have broad influence on cell function and phenotype and therefore may be important modulators of astrocyte reactivity and network function. Moreover, studies have suggested a role for mechanobiological cues and changes in the extracellular environment of injured brain in damage cascades (Liesi et al., 1984; Hemphill et al., 2011; Grevesse et al., 2015; Hemphill et al., 2015; Johnson et al., 2015). This has important implications for how brain cells may be differentially coupled to one another and the extracellular environment in turn regulating injury progression.

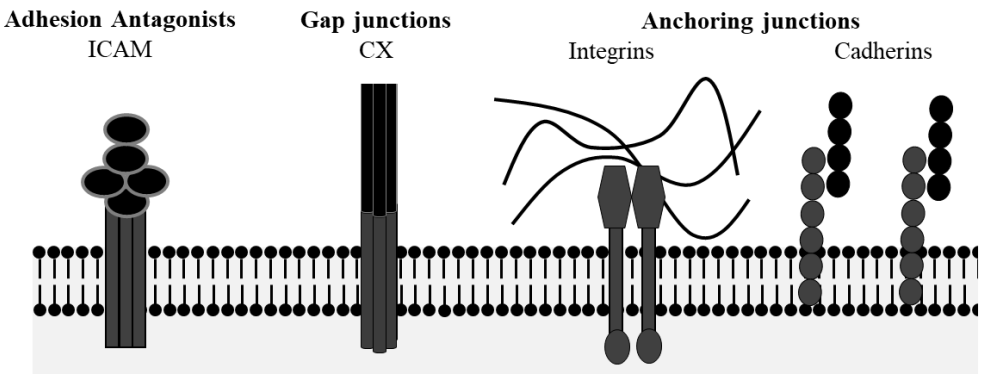

Figure 16. Major classes of cellular junction proteins

Moreover, alterations in junctional signaling have the potential to explain some of the sensitivity of astrocytes to isolated mechanical insult (Floyd et al., 2005; Ostrow and Sachs, 2005). Upon stimulation, adhesion signaling is activated via phosphorylation of a broad class of kinases, including focal adhesion kinase (FAK), which together direct numerous cell behaviors (Wozniak et al., 2004). Phosphorylation of FAK can be the result of mechanical stimulation or altered cell junction properties. In the brain, FAK affects glial cell morphology and adhesion in physiological and pathological environments (Hermosilla et al., 2008; Lafrenaye and Fuss, 2010; Kwiatkowska et al., 2011; Golubovskaya et al., 2013; J. Chen et al., 2017). One study indicated increased phosphorylated FAK (Y397) levels in reactive astrocytes following TBI (J. Chen et al., 2017), while another showed that inhibition of FAK phosphorylation closely coupled with STAT3 signaling and was integral from reactive astrocyte migration (Renault-Mihara et al., 2017).

Downstream of these initiator proteins, adhesion signals diverge on several major signaling pathways, including the mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways. Together, these transduction pathways have important influence on astrocyte phenotype after injury as they broadly modulate cell survival, activation, migration, and differentiation, among other functions. Sources for signal transduction mechanisms are well-documented in cells from other body tissues. These may include inflammatory stimuli, growth factors, and purines in addition to mechanical stimulation of integrins and other cell adhesion molecules. Signal transduction activation can also influence expression of adhesion proteins and therefore may be an important regulator of altered phenotype. It is well known that NF-κB p65 activation leads to the increased expression of intercellular adhesion molecule-1 (ICAM-1), an antagonist against tight junctions, which has multiple pro-

inflammatory roles in astrocytes following TBI. ICAM-1 overexpression has been associated with various TBI modes and has mostly been associated with detrimental consequences for recovery (Shibayama et al., 1996; Balabanov et al., 2001; Knoblach and Faden, 2002; Glushakova et al., 2014; Lutton et al., 2017) Specifically, ICAM-1 expression is associated with neutrophil infiltration and blood brain barrier (BBB) compromise and may contribute to early stages of neural dysfunction (Carlos et al., 1997). It is an important molecular target for TBI as it represents one mechanism by which neuroinflammation may become uncontrolled. Chronic elevation of ICAM-1 in the brain following TBI is concurrent with glial activation and other features of neuropathology (Glushakova et al., 2014). However, the role for ICAM-1 in initiating these features is of debate, as one study in an ICAM knockout mouse model showed no differences in histological or behavioral outcomes following controlled cortical impact (Whalen et al., 1999). Astrocytes have the ability to express ICAM proteins for inflammatory potentiation. In particular, astrocytic ICAM-1 has long been associated with cytokine expression and co-activation by metalloproteases and other pro-inflammatory molecules (Shibayama et al., 1996; Lyons and Benveniste, 1998; Lee et al., 2000; Kyrkanides et al., 2001). Therefore, astrocytes may represent an important source of ICAM-1 after brain injury which should be explored in more detail.

Astrocytes also utilize gap junctional proteins, like CXs, within their networks for mechanosensation and small molecule signaling. CX proteins form hemichannels which interact with other CX proteins on adjacent cells. Their selectivity results in size-exclusive passage of small signaling molecules and nucleotides. CX43 in particular has been widely studied in astrocyte physiology and pathophysiology. Knockout studies have associated CX43 with expression of GFAP in astrocytes whereby knockout significantly reduces GFAP expression and leads to pathological outcomes related to neuronal-glial coupling (Nakase et al., 2003; Prochnow, 2014). In CNS injury, CX43 has been debated as both protective and detrimental to outcomes (Rouach et al., 2000; Nakase et al., 2003; Theodoric et al., 2012; Sajja et al., 2016). CXs are important for redox balance, ionic exchange and ATP signaling. Earlier studies showed that CX43-mediated astrocyte communication was imperative to clearance of excess neurotransmitter release and neuroprotection (Rouach et al., 2000; Nakase et al., 2003), but recent studies have concluded that negative consequences involve increased cell death (Sajja et al., 2016). Moreover, several studies have implicated CX43 channels are a major conduit for ATP and glutamate release by astrocytes for inflammatory recruitment (Orellana et al., 2011; Theodoric et al., 2012).

Anchoring junctions represent the second class of adhesion proteins which form cell-cell or cell-ECM interactions. Cadherins make up a major family of anchoring junctions which form continuous networks with structural cytoskeletal networks like intermediate filaments (Pekny and Lane, 2007). Integrins are another family of anchoring junction and are heterodimeric complexes with alpha and beta subunits. This family of proteins is important for mechano-sensation both in response to shear and in cell motility/migration. They form the transmembrane connection in focal adhesions between the extracellular environment and intracellular cytoskeletal networks. Although minimally explored in TBI to date, integrins are imperative for cell phenotype and thus have significant potential to control the reactive astrocyte responses. A few studies have identified transient integrin expression in astrocytes as a response to inflammation or after CNS trauma (Pan et al., 2014; Lagos-Cabre et al., 2017). Integrin β-1 is of particular importance in astrocyte physiology as it controls morphology and differentiation of other cells in contact with astrocytes. For instance, astrocytic integrin β-1 regulated the extent of scarring and inhibition of neurogenesis following spinal cord injury (Pan et al., 2014). Integrin β-1 is also important for facilitating differentiation of oligodendrocytes (Corley et al., 2001) and lymphocyte invasion into the brain under multiple CNS pathologies (Bauer et al., 2009). Lastly, it is also well known that ECM alterations are a common feature of chronic TBI and may be directly influential on differential integrin expression in CNS cells.

In healthy cellular function, integrin stimulation contributes to a wide range of cellular processes including proliferation, migration and differentiation. However, upon mechanical stress, cells may utilize focal adhesions to sense and respond to stimuli. In addition to responding to mechanical stresses via adhesion signaling pathways, reactive astrocytes dynamically regulate their adhesion properties as they shift their phenotype in response to the surrounding environment. Dynamic regulation of intercellular proteins such as ICAM and cadherins is part of the focal adhesion signaling transduction pathway during normal and pathogenic migration (Weber et al., 2011; Wu and Reddy, 2012). In astrocyte reactivity, changes in adhesion can contribute to loss of neuronal communication, edema, and BBB compromise.

Altogether, the molecules discussed here play crucial roles not only in cell adhesion and phenotype but also important regulatory roles in secondary brain injury mechanisms such as neuroinflammation. Although not entirely encompassing of adhesion molecule classes, ICAMs,

CXs, and integrins have been highly motivated in the literature to either contribute to astrocyte reactive phenotype or serve a particular role in its function in TBI sequelae. Moreover, it is unclear the role that mechano-stimulation, specifically by high-rate overpressure, has on regulating the expression of these molecules in astrocytes. This chapter will evaluate the potential for reactive astrocytes to dynamically express these adhesion molecules and will thus implicate astrocyte-specific targets for high-rate injury studies in the future.

4.3. Methods

4.3.1. Cell culture and in vitro overpressure model

The *in vitro* overpressure model used in this study was described previously in 3.3.1 and 3.3.2. Briefly, primary rat astrocytes were extracted from Sprague-Dawley rat pups, and GFAP+ cell populations were selected for the experiments described herein. All experiments in Chapter 4 were conducted using cell monolayers cultured 6-7 days after passaging before being exposed to overpressure. The high-rate overpressure simulator (HOS) described in 3.3.2 was used to expose cells to a 20 psi high-rate overpressure.

4.3.2. RNA and total protein extraction

In order to analyze molecular alterations related to cellular reactivity, adhesion and signaling, astrocyte RNA and total protein were collected at time points between 24 and 72 hours after overpressure exposure. After removing culture medium, Trizol reagent (ThermoFisher cat# 15596018) was incubated on samples at room temperature. The remainder of the protocol was based on manufacturers' recommendations. Following phase separation using chloroform and centrifugation, RNA was isolated and precipitated using propanol, washed with ethanol, dried and resuspended in water. MinElute (Qiagen cat# 74106) spin columns were used under manufacturer's protocol to purify RNA samples. DNA contamination was removed using DNase treatment (Promega cat# M6101) at 37°C for 30 minutes. Samples were quantified using spectrophotometry, and those with 260/280 ratios between 1.8 and 2.0 were used for further analysis by polymerase chain reaction (PCR). Proteins were extracted from the phenol phase as described in 3.3.5.2.

4.3.3. Reverse transcription real-time PCR (qPCR)

Complementary DNA was synthesized from 1 µg of RNA by incubation with random hexamers and equimolar (10 mM) deoxynucleotide solution with dATP, dCTP, dGTP, and dTTP. Reverse

transcriptase M-MLV (Invitrogen Cat# 28025-013) was added to convert RNA to cDNA. A qPCR master mix was prepared using SYBR green, ultrapure water, and primers at a final concentration of 0.33 M forward and 0.33M reverse. Primers were designed using PrimerExpress and are listed in Table 4. Analysis of gene expression was conducted using a delta-Ct method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Results are shown as normalized gene expression relative to the sham average for each gene (i.e. sham=1).

Table 4 | Gene sequences from PrimerExpress used for qPCR analysis in Chapter 4

Gene	Abbreviation	Forward Sequence	Reverse Sequence
Connexin-43	CX43	TACAGCGCAGAGCAAAATCG	GGCGTGCGAGTTGGAGAT
Intercellular adhesion molecule	ICAM-1	GACAGTGCTGTACCATGATCAGAAT	CCCGCAATGATCAGTACCAA
Integrin beta-1	Int β1	GAAGAGTCTTGGGACGGATCTG	GCCAATGCGGAAGTCTGAA
Vinculin	Vinc	TCCTGCGCGGGATTACC	CAGACGTTCCAGAGAGGATTCC
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	TGGCCTTCCGTGTTCCTACC	AGCCCAGGATGCCCTTTAGTG

4.3.4. Western blotting

A capillary-based automatic western blotting system called Wes (Protein Simple) was used for relative protein quantification. Supplies for the assays were purchased from Protein Simple and include separation modules (cat# SW-004), anti-mouse detection modules (cat# DM-002) and anti-rabbit detection modules (cat# DM-001). Samples were prepared by following the manufacturer's protocol. This included a 10 minute reducing step at 95°C with dithiothreitol (DTT) (supplied). Primary antibodies used in to probe total protein samples are displayed in Table 5. Secondary antibodies and other reagents were all supplied through the manufacturer. Standard settings of 25 minutes for separation time (at 375 V), five minutes for blocking, 30 minutes for primary antibody incubation, and 30 minutes for secondary antibody incubation were maintained for all experiments. Prior to experimentation, each antibody was optimized with samples to ensure a dynamic linear range for signals. Electropherograms were used to quantify relative protein abundance. Target proteins were first normalized to their respective loading controls and then to the overall sham average. Results are displayed as normalized expression relative to sham averages.

Table 5 | Antibodies used for western blotting in Chapter 4

Protein	Abbreviation	Catalog #
Glial fibrillary acidic protein	GFAP	Abcam, Ab7260
Connexin-43	CX43	Novus Biologicals, NB100-91717
N-cadherin	N-cadherin	Novus Biologicals, NBP1-48309
Intercellular adhesion molecule 1	ICAM-1	Novus Biologicals, NBP2-22541
Proliferating cell nuclear antigen	PCNA	Cell Signaling, 13110
Integrin beta-1	Integrin β-1	Cell Signaling, D6S1W
Phosphorylated focal adhesion kinase (Y397)	p-FAK	Abcam, Ab81298
MAPK p38	p38	Cell Signaling, 8690
NF-κB p65	p65	Cell Signaling, 8242
Beta-actin	β-actin	Sigma-Aldrich, A5441
Gylceraldehyde 3-phosphate dehydrogenase	GAPDH	Novus Biologicals, NB600-502

4.3.5. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Similar to Chapter 3, astrocytes were assessed for metabolic activity by MTT assay. At 24 and 48 hours post-overpressure, cell media was changed and supplemented with tetrazolium dye (Sigma-Aldrich cat# M2128) at a final concentration of 0.25 mg/mL. Cultures were incubated at 37°C for three hours with the tetrazolium before being dissolved in dimethyl sulfoxide. Samples were then transferred in triplicate to a 96-well plate and absorbance was read at 570 nm and 650 nm (background). Percent activity was calculated based on average optical density measurements for the sham samples.

4.3.6. Nuclear protein extraction

Nuclear proteins were isolated using a commercially available kit from Epigentek (cat# OP-0002-1). Manufacturer's protocols were followed. Briefly, cells were trypsinized and centrifuged for five minutes at 1000rpm. Cell pellets were incubated with lysis buffer NE1 (kit component) for 10 minutes on ice. Following, samples were vortexed at 11,000xg for one minute, with the supernatant containing the cytoplasmic proteins. The nuclear protein pellet was then incubated with NE2 buffer (kit) and repeatedly vortexed to re-solubilize. Proteins were quantified using a micro-BCA assay (Pierce cat# 23235) for use in western blotting experiments.

4.3.7. Gene transfection and reporter assay

In order to assess overall NF-κB transcriptional activity, astrocyte cultures were transfected with a NF-κB response element vector using Lipofectamine 3000 (Thermo Fisher cat# 100022234). Lipid complexes were mixed with pNL3.2.NF-κB response element vector (Promega cat# N111A) and pGL4.53 vector (Promega cat# E501A) according to the recommended protocol. Briefly, Lipofectamine 3000 was prepared in Opti-MEM (Gibco cat# 31985-062) while DNA vectors were prepared separately in Opti-MEM before incubation at room temperature for 30 minutes. P3000

reagent was added to the DNA mixture right before combining with the Lipofectamine solution. The solution was diluted 5-fold in Opti-MEM such that finals concentrations of reagents were as follows: 3.75 uL/mL of Lipofectamine 3000, 1 ug/mL of NF-κB reporter vector, and 0.1 ug/mL of pGL4 control vector. After 15 minutes incubated at room temperature, one mL of solution was added to each well (six-well plate at four days post seeding, ~85% confluent). Cells were incubated with transfection solution for six hours at 37 °C before receiving a media change for a recovery period of 48 hours before exposure to overpressure. At 48 hours after overpressure, cells were lysed in 500 uL in passive lysis buffer for 15 minutes with shaking. Next, samples were transferred to microcentrifuge tubes and spun for one minute at 12,000xg. Serial dilutions of each sample were prepared in order to confirm linearity of signal (Figure 17). In order to detect luminescence of both the target and control vectors, a dual-luciferase kit was purchased from Promega. Reagents were prepared exactly according to manufacturer's instructions. Samples were first incubated with ONE-Glo EX reagent a 1:1 dilution for 10 minutes before luminescence was obtained via integrated signal over one second using a Molecular Devices LMax II 384 luminometer. This reaction was inhibited and replaced by NanoDLR Stop&Glo reagent added at 1:2 to the existing solution. This reagent measured relative activity of the target NF-κB vector. Following a 10 minute incubation, signal was again obtained as over a one second integral. Ratios of target to control luminescence were conducted prior to normalization between groups.

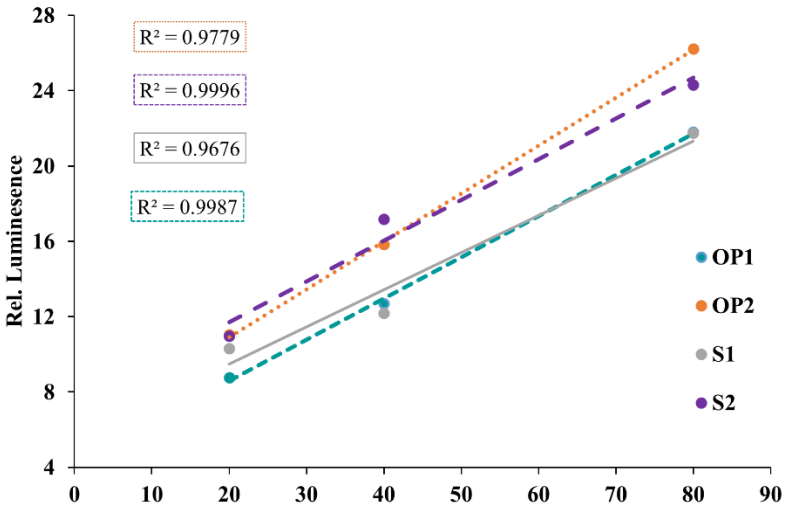

Figure 17. Optimization of reporter assay signal to determine linearity of signal. *The graph shows two sham (S) and two overpressure (OP) examples. The midpoint concentration was chosen for further analysis.*

4.3.8. Electrophoretic mobility shift assay (EMSA)

EMSA is a gel-based assay for the study of DNA-protein interactions. EMSA was used to assess specific activity of NF-κB within the nucleus for a known gene target. Nuclear proteins were extracted as described above from samples collected at 48 hours post-exposure or sham treatment. Oligonucleotides were constructed based on the NF-κB binding site at -218 on the rat ICAM-1 promoter. The 45-mer used for this study began at -236 and contained an NF-κB consensus sequence (5'-GGAAATTCC-3'). Biotinylated and unlabeled sequences were purchased from Integrated DNA Technologies. Reactions were carried out in accordance with manufacturer's protocol for LightShift Chemiluminescent EMSA kit (Pierce Cat# 20148). Sample preparation included recommended volumes of binding buffer, poly (dI·dC), 50% glycerol, 1% NP-40, and 100mM $MgCl_2$. Biotinylated DNA concentrations (200nmol) were optimized to ensure a linear region for reaction detection. Nuclear proteins were diluted to equal total concentration with a final reaction concentration of 120 ug/mL in DNA mixtures. Samples were run on 6% DNA retarding gels (Invitrogen Cat# EC6365BOX) and transferred onto 0.45 µm nylon membranes (Biodyne Cat# 77016) for detection on a FujiFilm LAS-3000 CCD camera. In order to determine specificity of binding, several experiments were conducted with unlabeled sequence in excess to ensure changes chemiluminescence.

4.3.9. 5-methylcytosine (5mc) fluorescent staining

Cell samples were fixed with ice-cold methanol for seven minutes at -20°C. Following fixation, samples were permeabilized for 15 minutes with PBS containing 0.5% Triton X-100, followed by two minutes with PBS containing 0.01% Tween-20. Hydrochloric acid was used to depurinate samples for 30 minutes. Next, samples were incubated with PBS containing 0.01% Tween-20 for 10 minutes and then were blocked with 10% bovine serum albumin for one hour. Overnight incubation with 5mc (Epigentek Cat# A-1014-100) was conducted at 4°C. Secondary antibody was incubated on the samples for 1.5 hours. Images were obtained using a standard fluorescent microscope (Zeiss) with a 20X objective.

4.3.10. Statistics

Statistical comparisons were conducted between groups using JMP software (SAS) under Virginia Tech license. ANOVA was used to analyze significant differences amongst groups, followed by student's t-test for individual group comparisons. The assumptions for normality and

homoscedasticity were confirmed by Shapiro-Wilk and Levene's tests, respectively. In the event that data was not normal, a logarithmic transformation was performed to conduct statistical comparisons. For sample sets with unequal variances, a Welch's t-test was performed. Statistical outliers were determined using residual analysis, and a p-value<0.05 was considered significant. Total number of replicates are denoted as the variable "n." Sample blocks were used for individual overpressure/sham tests and were from the same line of cells (from the same animal and passage number). Three to four sample blocks were used per time point depending on application.

4.4. Results

4.4.1. High-rate mechanical insult induces multiple hallmarks of astrocyte reactivity

The results herein focus astrocyte reactivity to the mechanical perturbation of a high-rate wave in the absence of other cellular signals. The first objective was to understand the extent and time course of reactivity induced by mechano-activation of astrocytes. Samples were exposed to overpressure parameters summarized in Table 6. The target overpressure mimics intracranial pressures traces based on a rodent model of blast neurotrauma (Leonardi et al., 2011; Sajja et al., 2014a; Bailey et al., 2016a).

Table 6 | Summary of overpressure mechanics on samples in Chapter 4

	Avg ± Std Dev
Peak Overpressure [psi]	19.7 ± 5.4
Positive Peak Duration [ms]	0.95 ± 0.27
Rise Time [ms]	0.47 ± 0.25

To assess the reactive phenotype, protein levels for PCNA and GFAP were at 24, 48 and 72 hours post-exposure by western blot analysis. Figure 18A shows that astrocytes assumed a proliferative/reparative phenotype as measured by PCNA at 24 hours post-exposure which was sustained throughout the final 72 hour time point assessed. Each overpressure-exposed group (denoted as OP) was statistically different from its respective sham at the same time point (p-values<0.05). It should be noted that despite increased PCNA, there were no detectable changes in MTT metabolism until 48 hours at which point there was a significant increase in enzymatic activity (p-value=0.0005 ; Figure 18B). GFAP expression had a delayed increase at 48 hours post high-rate overpressure exposure. These results are consistent with a previous study on gene expression of GFAP in C6 glioma cells exposed to the same mechanics (Hlavac et al., 2015). Increased GFAP in conjunction with proliferative/reparative potential will be referred to as "classical reactivity." These data establish a time course at which to evaluate the molecular

phenotype of mechanically-activated astrocytes using an *in vitro* high-rate overpressure model. This transition in phenotype which occurred between the 24 and 48 hour time points will be the focus for the subsequent analyses.

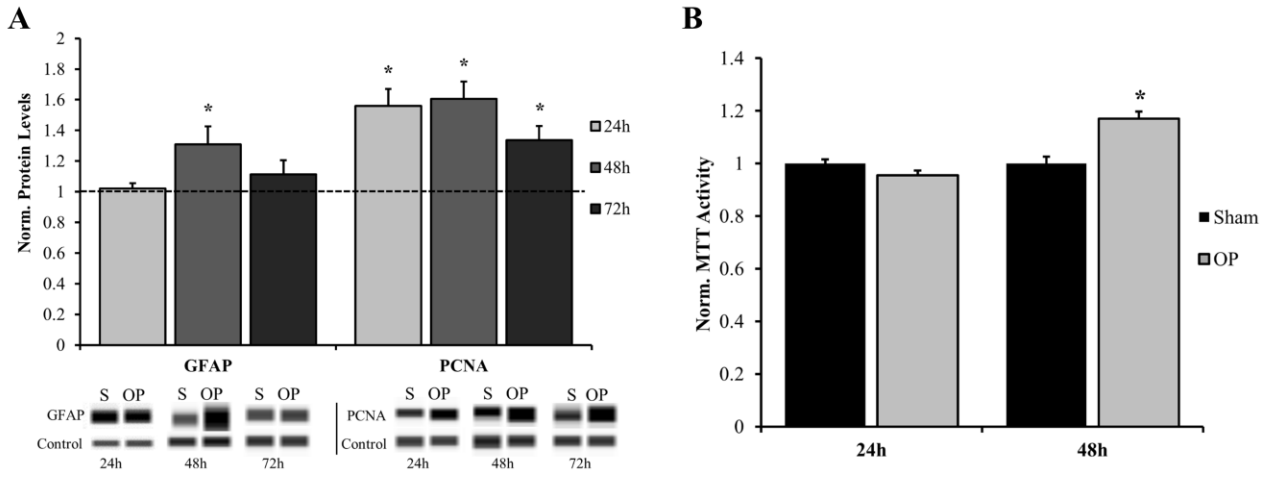

Figure 18. (A) Time course analysis for normalized protein expression of classical reactivity markers*. GFAP was significantly elevated in the overpressure group at 48 hours while PCNA was increased at 24, 48 and 72 hours compared to their respective shams.* ***(B) Normalized MTT metabolic activity.*** *A significant change was observed at 48 hours after overpressure exposure as compared to sham. *Indicates p-value<0.05, Data are represented as mean ±SEM, protein: n=8-13/group, MTT: n=8-9/group*

4.4.2. Cell junction molecules become dysregulated in reactive astrocytes

Several classes of junctional molecules were assessed for this study. Gene expression analysis was conducted for one gap junctional target (CX43), one anchoring junction target (integrin-β1) and one intermediate protein (vinculin). PCR results determined that gap-junctional CX43 was upregulated compared to sham at 24 hours (p-value=0.028), prior to the anchoring junction protein integrin-β1 (Figure 19A). As a gap junctional protein, CX43 mainly functions as a signaling protein for small molecule transport. Integrin proteins are associated with cell-matrix and cell-cell contact and are coupled with cytoskeletal elements. Integrin β-1 was upregulated as compared to sham in conjunction with structural reactivity at 48 hours (p-value=0.025). Conversely there was no significant difference in expression of the adaptor protein vinculin (p-value=0.092 at 48 hours). Subsequent protein analysis by western blot confirmed that increased gene expression of CX43 at 24 hours (p-value=0.035, Figure 19B) and integrin-β1 at 48 hours (p-value=0.029, Figure 19C) corresponded to increased protein translation after overpressure. Moreover, another anchoring junction protein, N-cadherin, was concurrently increased at 48 hours relative to sham as assessed by western blot (p-value= 0.047, Figure 19C).

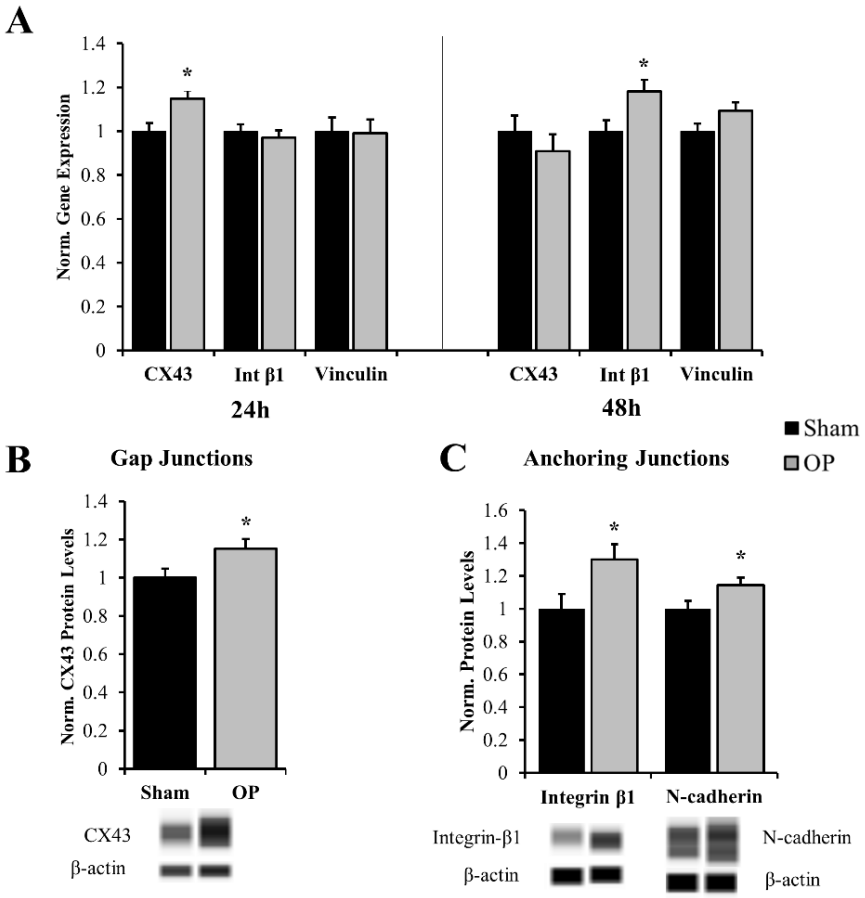

Figure 19. Gene and protein expression for cell junction targets. (A) *Gene expression analysis showed increased gap junction (CX43) regulation at 24 hours after high-rate overpressure and increased anchoring junction (integrin β-1) regulation at 48 hours as compared to sham. No changes were observed in vinculin mRNA expression.* ***(B)*** *Western blot results confirmed increased protein levels of CX43 at 24 hours after overpressure as compared to sham.* ***(C)*** *Anchoring junction proteins integrin β-1 and N-cadherin were significantly elevated as compared to sham at 48 hours after overpressure. *Indicates p-value<0.05, Data are represented as mean ±SEM, gene: n=7-9/group, protein: n=11-12/group*

4.4.3. Astrocytes undergo delayed signal transduction in response to high-rate insult

Activation of FAK by adhesion molecules and other membrane-bound proteins commonly involves phosphorylation of the molecule at tyrosine-397 (Hamadi et al., 2005). Results indicated that p-FAK levels were decreased at 24 hours (p-value=0.037) and increased at 48 hours (p-value=0.002) post-overpressure relative to sham (Figure 20A). Increased p-FAK corresponded to the time point where both features of classical reactivity were observed. These results implicate that this reactive profile may also be linked to dynamic changes at the membrane-level. Additionally, considering the downstream effects of FAK signaling will lead to further insights. Nuclear localization of both p38 and p65 followed a similar pattern as the FAK activation profile. A near-significant and significant decrease in localization was observed at 24 hours for p38 (p-value=0.056) and p65 (p-value=0.020), respectively (Figure 20B). This response was shifted to a

significant increase in nuclear localization for both p38 (p-value=0.015) and p65 (p-value=0.049) at 48 hours following insult. Together, these results indicate that signaling transduction may be augmented in conjunction with increased FAK activation. The results suggest a potential pathway to relate astrocyte phenotype to functional adhesion alterations.

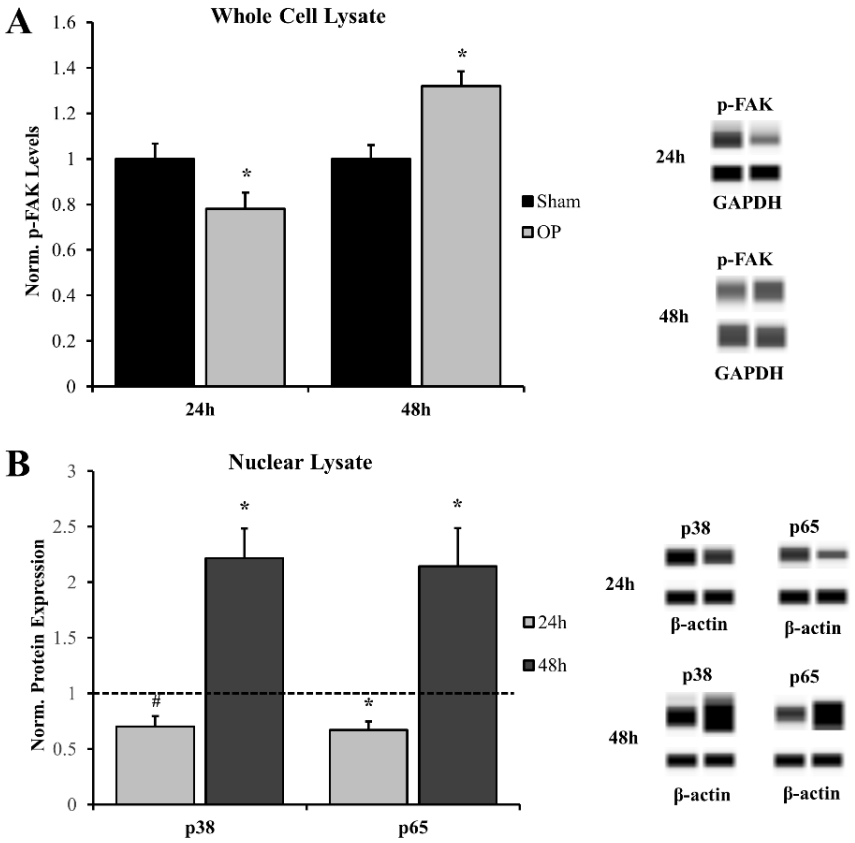

Figure 20. Western blot analysis for whole cell and nuclear signal transduction proteins. (A) *Phosphorylated FAK (Y397) was significantly decreased at 24 hours and significantly increased at 48 hours as compared to sham.* ***(B)*** *Nuclear localization of corresponding signal transduction molecules, p38 and p65, were decreased at 24 hours and increased at 48 hours as compared to sham. *Indicates p-value<0.05, #p-value=0.056, Data are represented as mean ±SEM, whole cell: n=9-10/group; nuclear: n=7-9/group*

4.4.4. NF-κB p65 has an increased binding propensity for the ICAM-1 gene in conjunction with upregulated ICAM-1 expression in reactive astrocytes

ICAM-1 is an antagonist for tight junction proteins and is expressed largely for inflammatory potentiation. Its transcription is regulated by the NF-κB pathway. To assess the potential for NF-κB localization to influence adhesion outcomes in reactive astrocytes, EMSAs were performed with nuclear extracts incubated with NF-κB-binding sequences on the ICAM-1 promoter. Optimization experiments were conducted with both biotinylated and unlabeled DNA to determine

binding specificity for the given sequence (Figure 21A). A significant increase in association of nuclear proteins with the NF-κB binding sequence was observed following mechanical insult as compared to sham at 48 hours (p-value=0.013, Figure 21B).

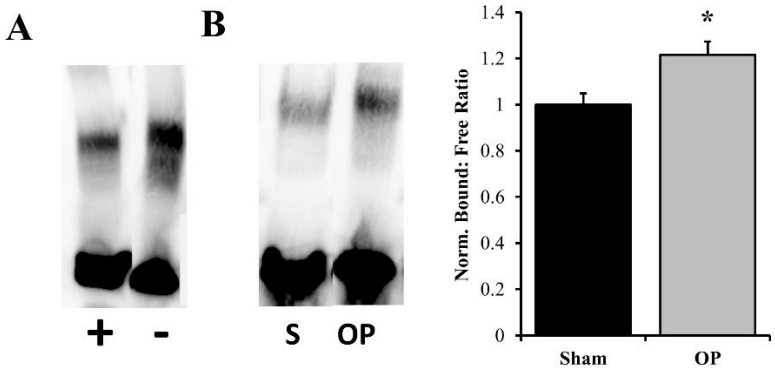

Figure 21. EMSA results for binding efficiency of nuclear proteins to ICAM-1 promoter sequence. (A) *Assay optimization was conducted for specificity of protein binding to the NF-κB binding sequence of interest. The same sample was incubated with and without unlabeled DNA sequence to ensure competitive binding. The first lane contains both labelled and unlabeled DNA while the second contains only labelled.* ***(B)*** *Representative DNA: protein shifts for sham and overpressure groups are shown. Quantification of bound to free DNA determined that overpressure induced a significantly higher association of nuclear proteins with the NF-κB binding site on the ICAM-1 promoter. *Indicates p-value<0.05, Data are represented as mean ±SEM, n=6-8/group*

This increased activity occurred despite no observed differences in the overall NF-κB reporter activity. This may be an issue of assay sensitivity, where the reporter is less sensitive than EMSA. Alternatively, it may suggest specificity for NF-κB activity in the nucleus to specific genetic elements related to reactivity (Figure 22).

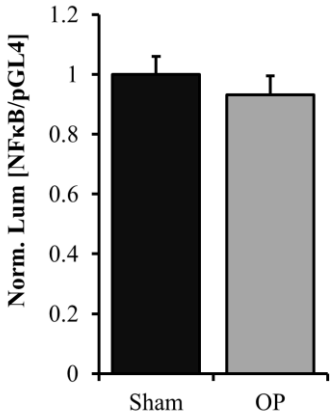

Figure 22. Normalized NF-κB reporter activity. *There was no difference in non-specific NF-κB transcriptional activity. Samples were first normalized to their pGL4 control vector and then normalized to average sham. Data are mean ±SEM, n=8-9/group*

Subsequent analyses of ICAM-1 gene and protein levels were conducted to determine expression patterns. ICAM-1 gene expression was significantly increased at 48 hours post-exposure as compared to sham (p-value=0.035, Figure 23A) in conjunction with EMSA results. Gene expression was not previously increased at the 24 hour time point, indicating a specificity for this time point and phenotype. The increased gene expression translated to increased protein expression at 48 hours as well (p-value=0.032, Figure 23B). These results suggest that the localized p65 had increased binding affinity for the ICAM-1 promoter in reactive astrocytes and therefore may be an important regulator of the adhesion alterations observed.

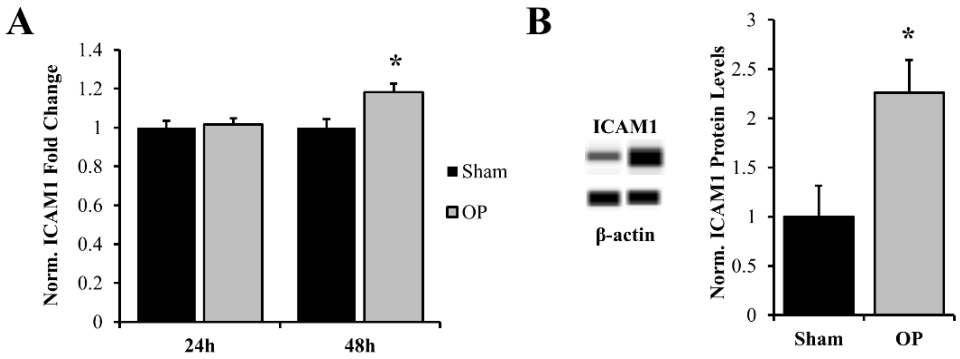

Figure 23. Normalized gene and protein expression of ICAM-1. (A) *qPCR results indicated no change in mRNA expression of ICAM-1 at 24 hours post-overpressure but a significant increase at 48 hours as compared to sham.* ***(B)*** *Subsequent western blot results confirmed elevated ICAM-1 protein levels at 48 hours compared to sham. *Indicates p-value<0.05, Data are represented as mean ±SEM, gene: n=7-9/group, protein: n=8-9/group*

4.4.5. Increased global DNA methylation status precedes delayed astrocyte reactivity

DNA methylation is an epigenetic process by which cellular transcription can be globally influenced. At 24 hours, significant hypermethylation was observed (increased by 5.04%), indicating potential gene repression (p-value=0.040, Figure 24). This response was ameliorated by 48 hours, at which point no significant differences were observed between groups. This methylation pattern corresponded to both the shifts in MAPK and NF-κB localization as well as cellular phenotype that occur across this timeframe.

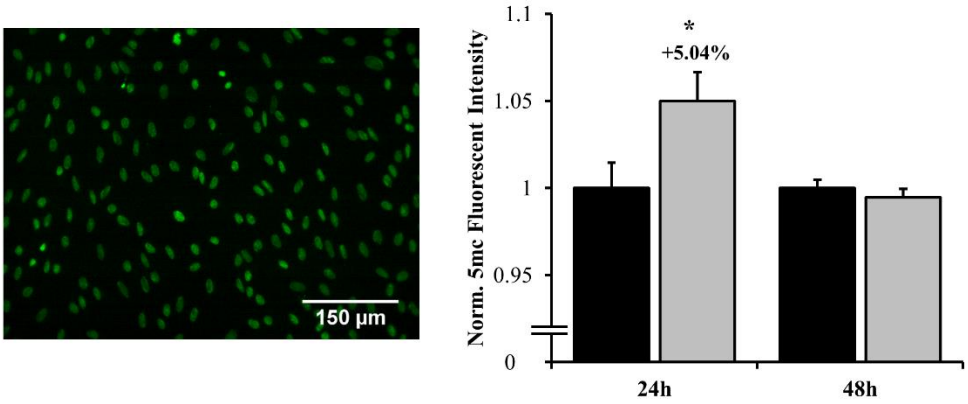

Figure 24. Global DNA methylation as assessed by fluorescent labelling of 5-methylcytosine*. Hypermethylation occurred at 24 hours post-overpressure exposure as compared to sham. No changes in methylation were observed at 48 hours. *Indicates p-value<0.05, Data are represented as mean ±SEM, n=10-12/group*

4.5. Discussion

From a therapeutic standpoint, astrocytes have become an important focus in neurotrauma research because of their ability to influence many of these aspects of secondary injury sequelae (Myer et al., 2006; Kimelberg and Nedergaard, 2010; Burda et al., 2016). Astrocyte reactivity has been well characterized following *in vivo* blast neurotrauma, and most notably involves classical reactivity with increased GFAP expression in astrocytes (Cernak et al., 2011; Svetlov et al., 2012; Sajja et al., 2014a; Bailey et al., 2016a; Goodrich et al., 2016). Studies *in vitro* have shown that even in the absence of other cell types, astrocytes assume an activated phenotype in response to varied mechanical perturbations (Floyd et al., 2004; Tran and Neary, 2006). While certain aspects of astrocyte reactivity are ubiquitous across injury modes, others are highly dependent on injury severity, insult mechanics, and location within the brain, among other factors. Therefore, it is possible that reactive phenotypic features are controlled by complex and interconnected injury mechanisms. There is a need for methodologies to study precise mechanisms of cellular responses, particularly to high-rate insults. While there remains limited data on the mechanics of blast neurotrauma, studies are emerging to suggest it is a multi-modal injury involving overpressure, compression and tension effects. The HOS model presented in this study was developed based on intracranial measurements in rodents exposed to blast (Leonardi et al., 2011). It provides a novel application for underwater explosion methodologies to specifically study intracranial overpressure effects on *in vitro* samples.

Moreover, results from the study indicate that HOS was capable of generating a reactive response in cultured astrocytes that is phenotypically comparable to *in vivo* findings. Primary astrocytes assumed a classically-reactive response to a one millisecond overpressure pulse and this phenotype corresponded to alterations in several classes of junctional molecules and mechano-activation through p-FAK. It can also be noted that PCNA was increased at 24 hours, prior to other signs of structural or adhesive reactivity. This may suggest differential regulation of proliferation/repair as compared to structural reactivity in the astrocytes. PCNA is an ideal target for understanding reparative potential as it directly recruits and interacts with many DNA replication proteins (Dieckman et al., 2012). It has been used previously as a marker for proliferating cells, including astrocytes (Kato et al., 2003; Di Giovanni et al., 2005). MTT results coincided PCNA expression as a significant increase in metabolic conversion was observed at 48 hours. Together, these results support the assumption that isolated high-rate insult has the potential to initiate increased, although mild, reparative potential and/or proliferation in astrocytes. It is believed that early stages of astrocyte proliferation can distinctly extend into a highly proliferative, scar-forming phenotype later after insult (Pekny and Nilsson, 2005; Buffo et al., 2008; Pekny and Pekna, 2014). Thus, better understanding of the onset of these reactive features is necessary to modulate astrocyte responses in TBI.

Additionally, molecular instigators of reactivity include broad signaling, adhesion and structural aberrations (Pekny and Nilsson, 2005; Sofroniew, 2009; Burda et al., 2016), which are largely uncharacterized in high-rate TBI. CX43 is one such junctional molecule which participates in normal and pathological ionic and metabolite buffering as well as secondary messenger passage. It is a mechanosensitive gap junction protein with a short half-life (Laing and Beyer, 1995; Beardslee et al., 1998; Salameh and Dhein, 2013), which may explain its early upregulation in exposed cells. CX43 is specifically expressed between astrocytes and is imperative for network communication. From *in vivo* experimentation, reactive astrocytes can display upregulated CX43 in a specific manner after TBI (Theodoric et al., 2012). Astrocytic CX43 has both protective and detrimental consequences after CNS insult. CX43 is mainly responsible for clearance of excitotoxic and damaging extracellular molecules after insult but also contributes to the spread of harmful signals (Frantseva et al., 2002; Ozog et al., 2002; Rovegno et al., 2015). Evidence for significant coupling of CX43 expression with GFAP+ astrocytes explains one potential mechanism by which reactivity may be linked to junctional regulation (Prochnow, 2014).

However, these protein networks are not physically connected and thus can respond independently in the case of injury. In general, CX43-mediated shuttling involves many types of signaling, including NF-κB activation and pro-inflammatory regulation in various pathologies (Brand-Schieber et al., 2005; Wu et al., 2013; Xie et al., 2013) as well as regulating proliferation in other cells. Evidence exists to suggest that CX43 can lead to increased cell death after trauma because of the spread of damaging molecules (Frantseva et al., 2002; Rovegno et al., 2015). Moreover, studies in cancer cells suggest that CX43 controls and inhibits proliferation (Song et al., 2010; Tittarelli et al., 2015). This may explain the delayed changes in metabolic profile observed by MTT, yet the regulation and function of CX43 in astrocytes remains to be fully elucidated. CX43 upregulation also preceded significant changes in NF-κB activation. Therefore, it is possible that some or all of the previously identified mechanisms influence early reactive astrocyte phenotype.

Cellular adhesion to the external environment and to surrounding cells is another autologous regulator of mechanical stimulation within networks of cells like astrocytes. Anchoring junctions are a major group of tethering proteins that interconnect the cytoskeleton to the extracellular space. The two classes of anchoring junctions assessed in this study include cadherins and integrins. Integrins proteins form heterodimers that bind to the extracellular matrix and other extracellular adhesion molecules (Barczyk et al., 2009). Integrins are dynamically regulated in cells to control alterations in migration, proliferation and adhesion (Schwartz and Assoian, 2001; Barczyk et al., 2009). They are important mechanosensors for normal physiological functions but have also been implicated in cellular outcomes of various CNS pathologies (Mattern et al., 2005; Hemphill et al., 2011; Wu and Reddy, 2012; Pan et al., 2014; Weaver et al., 2015). Integrins have been minimally explored in TBI and yet possess the potential to widely influence cellular phenotype. More specifically, integrin-β1 expression is required for astrocyte migration, stability and healing potential (Ogier et al., 2006; Peng et al., 2008; Zou et al., 2011; Wu and Reddy, 2012). A few studies have implied differential expression of integrin proteins by astrocytes following mechanical trauma (Renault-Mihara et al., 2011; Nathan and Li, 2017). Results of this study indicate a basis for mechano-regulation of anchoring junction proteins following isolated high-rate overpressure, thus implicating them in both simple and complex cellular architectures.

This study identified that integrin β-1 and N-cadherin were upregulated in conjunction with increased structural (intermediate filament) protein expression in astrocytes. This result is

supported by the fact that anchoring junctions form physical, mechano-sensing systems with intermediate filaments, which are necessary for stress modulation (Pekny and Lane, 2007). One study showed a functional relationship between integrin-β1expression in astrocytes and structural reactivity apart from proliferation (Robel et al., 2009). Other studies indicate that integrin-β1 also directly interacts with the intermediate filament vimentin and is important for mediating proliferation in conjunction with GFAP (Nishio et al., 2005; Triolo et al., 2006). There is evidence for a similar role for N-cadherin in modulating the reactive astrocyte response after traumatic insult as well (Kanemaru et al., 2013). Together, these results indicate multiple mechanisms by which astrocyte networks may become mechano-activated in response to their altered adhesion state. It should be noted that no changes were observed in gene expression of the intermediate adaptor protein vinculin. Vinculin is widely expressed as part of the intermediate complex between the cytoskeleton and anchoring junctions. In 2D cell culture, decreased vinculin expression would support development of the migratory phenotype in cells (Mierke et al., 2010). Studies have shown that expression profiles of integrin β-1 and intermediate filaments tend to be distinct in proliferating cells as compared to migrating (Nishio et al., 2005). Therefore, it is possible that these reactive cells are not assuming a migratory phenotype in response to autologous signals but instead require signals from other cells types in order to migrate to a source of injury.

The second portion of this study was directed at identifying autologous signal transduction activation in reactive astrocytes. Several initiating factors exist as inputs for signal transduction mechanisms in astrocytes. These include G-protein coupled receptors, inflammatory receptors, purinergic receptors and cell adhesion molecules themselves. Cellular signaling mechanisms that involve the first three classes of initiators are known to influence astrocyte outcomes and often involve neuroinflammation and signals from damaged neurons (Sofroniew, 2009; Buffo et al., 2010). Cell adhesion is not well characterized but may represent an important pathway by which autologous signaling in astrocyte networks contributes to phenotype. In the complex TBI pathophysiology it is likely that all of these initiators contribute to astrocyte responses. Connexins, cadherins and integrins can each directly or indirectly influence ubiquitous signal transduction like MAPK and NF-κB activity via initiator proteins such as FAK (Furnari et al., 2007; Chi et al., 2016; Mui et al., 2016). FAK is associated with initiation of adhesion signaling transduction pathways and can have a reciprocal influence on the expression and function of junctional proteins as well (Sawai et al., 2005; Yamada et al., 2005). This protein kinase is phosphorylated at tyrosine-397

when the pathway is activated by integrin and other cellular adhesion stimulation at the membrane level. In response to mechanical stress, phosphorylation of FAK can often be observed very rapidly as a reaction to cell shearing (Li et al., 1997; Liu et al., 2014). Additionally, multiple models of the CNS insult have implicated FAK in gliosis and chronic astrocyte pathology (Furnari et al., 2007; Malric et al., 2017; Wang et al., 2018).

Moreover, there is significant motivation to understand the molecular signature which relates initiation of dynamical adhesion signaling to downstream transduction and specific influences on gene transcription and phenotype (Figure 25). Phosphorylation of FAK can lend to subsequent activation of NF-κB and MAPK pathways, both of which influence shifts in cellular phenotype and may be important mediators in mechano-activation of astrocytes by high-rate insult. No previous studies have isolated this mechanism in astrocytes, however many have studied the mechano-regulation of NF-κB and MAPK in osteocytes, cardiomyocytes and other uniquely activated cells (Li et al., 1997; Wang and Thampatty, 2008; Young et al., 2010; Leychenko et al., 2011; Russell-Puleri et al., 2017). Results indicated increased phosphorylation of FAK in conjunction with significantly increased nuclear localization of transcription-related molecules, p38 and p65. Upon nuclear localization, MAPK p38 can activate transcription factors, such as NF-κB p65. NF-κB p65 is a transcription factor with known binding sites on multiple adhesion genes, including ICAM-1 (Melotti et al., 2001; Xia et al., 2001).

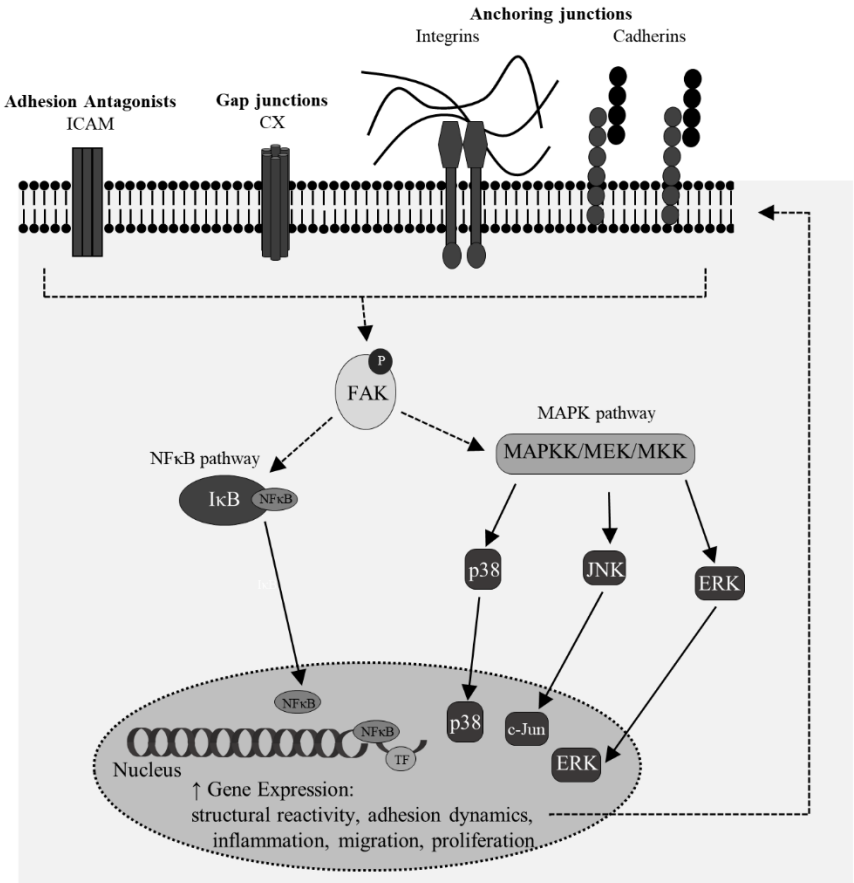

Figure 25. A simplistic overview of potential signaling feedback mechanisms for the regulation of junctional changes and structural reactivity in mechano-activated astrocytes. *Multiple of these targets coincided with features of classical reactivity observed in this model of high-rate overpressure exposure.*

The data showed that increased localization of p65 was accompanied by increased affinity of the transcription factor for ICAM-1 promoter, suggesting a potential pathway for regulation of adhesion molecules in reactive astrocytes. ICAM-1 is a glycoprotein that acts as an antagonist for another class of junction proteins, tight junctions. Astrocytes express ICAM-1 for pro-inflammatory potentiation as well as binding to integrin proteins on surrounding infiltrating cells (Weber et al., 1994; Lee and Benveniste, 1999). A recent study by Lutton et al showed that increased ICAM-1 expression in a mouse cortical impact model was associated with vascular endothelial cells and activated glial cells, likely astrocytes (Lutton et al., 2017). The study also inhibited ICAM-1 expression to decrease oxidative stress, blood brain barrier permeability and microglial co-activation. Other studies have reported ICAM-1 associated with region-specific glial activation in various CNS pathologies (Akiyama et al., 1993; Shrikant et al., 1994), however, this is the first report that links astrocytic ICAM-1 regulation to high-rate mechanical insult. The NF-

κB pathway is important in mediating neuroinflammation, structural phenotype and cell survival. Additionally, MAPK and NF-κB molecules influence the activation of other transcription factors in the nucleus for broader impacts on cellular phenotype. Co-activation of transcription factors may provide the molecular link between structural reactivity, proliferative phenotype and adhesion-mediated dynamics. Future studies should exploit these pathways to understand their specific contributions to outcomes related to astrocyte reactivity.

Lastly, signal transduction mechanisms can influence and are influenced by DNA methylation status. Hypermethylation status corresponds to patterns of gene repression, while the opposite is true for hypomethylation. Previously, DNA methylation changes have been observed in blast neurotrauma (Bailey et al., 2015; Haghighi et al., 2015). Results of this study suggest that DNA hypermethylation may be an important mediator in the delay of structural reactivity responses observed here and in *in vivo* studies of blast neurotrauma. It is also possible that CX43 overexpression and DNA methylation may co-exist to mediate proliferative responses and may be potentially linked to metabolic and redox state of the cells, but much more work is needed to understand this relationship. Future work will exploit methods to understand more specific DNA methylation patterns related to the features of reactivity obtained in this study.

It is necessary to consider certain limitations of this model. The presented *in vitro* model is a 2D, astrocyte-only system and therefore may not recapitulate certain aspects of cellular focal adhesion as found in the native brain tissue. Additionally, adhesion organization and activation of signaling molecules such as FAK are different in 3D as compared to 2D (Berrier and Yamada, 2007; Harunaga and Yamada, 2011). This relationship should be considered in future studies evaluating expression of these proteins by cells within a heterogeneous ECM. Cellular adhesion formation and migration is spatially dependent on extracellular architecture. However, the purpose of this study was to establish the ability of high-rate overpressure to induce both classical reactivity and dysregulated expression of adhesion molecules simultaneously. This study advances understanding of primary astrocyte response to high-rate insult and identifies multiple molecular targets for aberrant astrocyte adhesion. These results are the first step to informing directed experiments for manipulation of cellular adhesion, and particularly astrocyte mechano-activation in context of TBI. In addition, it creates a platform to further study adhesion dynamics in advanced 3D cell culture models.

4.6. Conclusion

The purpose of this study was to develop an *in vitro* model to characterize primary cell response to high-rate compressive overpressure. Altogether, the presented results have provided progress towards both fundamental understanding of initiating mechanisms for astrocyte reactivity but also into novel considerations for therapeutic modulation of astrocytes to improve TBI outcomes. This correlative study established specific profiles of adhesion/junctional protein regulation which correspond to aspects of astrocyte reactivity. Moreover, results have identified novel targets at multiple levels within signaling mechanisms for astrocyte mechano-activation. Future studies will specifically intervene in pathways associated with integrin and other cell adhesion molecules to determine their influences on astrocyte network function with the goal of interventional modulation of reactive astrocytes for improved brain repair.

4.7. Future Directions

This study establishes a temporal correlation between junctional proteins and features of classical reactivity. Further work will be necessary to understand the functionality and specificity of each of these junctional proteins in reactive astrocytes. This will be particularly necessary in considering sources for aberrant adhesion signal transduction and the resulting phenotypic results. This study only reflects the specific transcriptional regulation of ICAM-1 but there is potential for adhesion and junctional signaling to influence a multitude of functional molecules from proliferative to inflammatory potentiation. More work is required to identify the scope by which junctional and adhesion signaling may be regulating these processes within astrocyte networks. It will be necessary to assess these relationships within an *in vivo* model of astrocyte reactivity. ECM changes occur across TBI modes and experimental models and may contribute to differential junctional regulation in astrocytes (Liesi et al., 1984; George and Geller, 2018). Therefore, it is plausible that regulation of junctional molecules may be affected by overpressure exposure. Studying these responses *in vivo* would confirm results of this study and more thoroughly recapitulate signaling mechanisms, especially in the case of integrin expression and localization. In 2D, many of the functions for integrin proteins are conserved, including anchorage and polarity, but 3D studies will be necessary to assess focal adhesion formation (Yamada et al., 2003). The time course and persistence of astrocyte activation may be influenced by the presence of other cells and environmental stimuli (Sofroniew, 2009; Buffo et al., 2010). This study also exclude junctional proteins expressed between astrocytes another cell types. There are multiple specialized

neuronal-astrocytic junctions which have been reported to contribute to TBI-related cellular dysfunction (Sword et al., 2013). These molecules should be assessed in mixed cell cultures and *in vivo*.

Chapter 5. Evaluation of a FAK phosphorylation inhibitor in reactivity mechanisms associated with 2D and 3D astrocyte cultures

5.1. Introduction

Evidence has been provided for adhesion-based signaling in astrocytes reactive to high-rate overpressure. Multiple classes of junctional proteins were dysregulated in conjunction with focal adhesion signaling molecules that likely controlled altered signal transduction and transcriptional regulation.

The purpose of this study was to further establish the role for focal adhesion kinase (FAK) activation in the phenotypic changes that can contribute to astrocyte reactivity to high-rate overpressure. In addition, this chapter builds upon the 2D model used previously to create a multicomponent 3D model which better provides cells with active binding sites from brain extracellular matrix (ECM) components to more comprehensively study adhesion responses. This chapter will describe the model design and provide 2D and 3D comparisons for the regulation of several adhesion molecules under the influence of a FAK chemical inhibitor.

5.1.1. Statement of Problem

The limited existence of *in vitro* models for high-rate overpressure has led to many of the current studies being conducted on cell monolayers (2D). While these studies offer much information in regards to molecular mechanisms of injury, recent efforts have been afforded towards better recapitulating the *in vivo* environment through biomimetic scaffolds. This will be an important step in the direction of using *in vitro* models to translate mechanisms to *in vivo* targets. In particular, it is known that many aspects of adhesion properties in cells are different in 2D environments as compared to 3D. Despite this understanding, very little is known about the regulation and influences of FAK signals in the context of astrocyte reactivity to high-rate overpressure injuries. This chapter serves to evaluate some of the differences in the role of FAK activation in both environment types and determine important aspects of cellular adhesion, particularly to the ECM, that may be influencing how astrocytes ultimately become reactive in blast neurotrauma. Overall, this work will further fundamental mechanistic understanding of mechano-stimulation signal transduction in astrocytes as well as establish molecular targets in a 3D high-rate overpressure model of astrocyte reactivity.

5.1.2. Significance

Focal adhesions have a direct impact on cellular morphology and dynamic regulation of adhesion molecules. Focal adhesion signaling via FAK activation has been implicated in several pathologies

aside from traumatic brain injury (TBI), such as lung fibrosis and cancer, and has ubiquitous roles in mechano-stimulation and cell state. In disease states, inhibition of FAK activation has proved successful in decreasing proliferation in cancer cells and pathogenic differentiation (Bolós et al., 2010). Moreover, FAK activation has been associated with astrocyte reactivity and proliferation (Koyama et al., 2003), but has not been explored in the context of high-rate overpressure injury. The influences of specific mechano-stimulation pathways in reactive astrocytes remain unclear. Thus, adhesion properties will be investigated in the astrocyte reactive phenotype that results from high-rate overpressure in an effort to better understand those aspects of the phenotype. Previous results suggest a dynamic response in the FAK activation profile in progressive astrocyte reactivity to high-rate overpressure (Figure 20A). Because focal adhesion assembly and signaling activation are dependent on extracellular environment (Wozniak et al., 2004), this chapter will establish a role for FAK activation in adhesion regulation in 2D compared to 3D astrocyte cultures under the influence of high-rate overpressure. Together, these motivate increased exploration of mechanisms related to TBI pathophysiology.

5.2. Literature Review

The recruitment, formation and regulation of adhesion complexes like focal adhesions are one the central components to maintaining cell state. This chapter investigates the differential activation of FAK in 2D and 3D models of astrocyte reactivity to high-rate overpressure. Considerations for these studies must be made in regards to the influences that exist for cellular focal adhesion and its signaling components.

5.2.1. FAK Signaling Activation under Mechanical Stimulation

Focal adhesion formation and maintenance is a complex equilibrium which can self-regulate to create motile and proliferative phenotypes (Wozniak et al., 2004), both of which are important aspects of astrocyte reactivity. In general, focal adhesions are part of the cellular network responsible for maintaining mechanical integrity through somewhat continuous tensile networks (Burridge and Chrzanowska-Wodnicka, 1996; Wehrle-Haller and Imhof, 2002). Major components of these networks include extracellular and transmembrane integrins and other proteins, intermediate linker proteins, signal transduction molecules, like FAK and other tyrosine kinases, and cytoskeletal elements. Mechanical perturbations to these networks can be driven by external mechanical stimulus or through dynamic regulation of adhesion and motility. In

particular, FAK activation can be initiated by force transduction via adhesion recruitment or by mechano-activated molecules such as endothelin-1 (Cazaubon et al., 1997; Michael et al., 2009; Kong et al., 2013). FAK phosphorylation is most often a rapid response to mechanical, shearing disruption. Studies in various other cell types have found FAK phosphorylation within minutes after shear stress application (Li et al., 1997; Liu et al., 2014). Importantly, prolonged FAK activation is also possible in the presence of altered cellular adhesion. This may be due to cellular migration or changes in ECM composition or properties. FAK has been shown in multiple cell types to be involved in sensing ECM rigidity (Wang et al., 2001). Glioma studies have also shown a remarkable relationship prolonged FAK activation and tumor growth/ cell invasiveness. Inhibition of continuous FAK activation by a small molecule inhibitor TAE226 had a significant effect on glioma growth and invasiveness both *in vivo* and *in vitro* (Liu et al., 2007; Shi et al., 2007).

Because astrocytes are a major source for ECM proteins, they have been implicated in the ECM and adhesion alterations that occur after injury (Liesi et al., 1984; Wiese et al., 2012; Yao et al., 2014). Moreover, cell-matrix interactions and mechanotransduction mechanisms are important regulators of ECM gene expression in other cell types (Chiquet et al., 2009). Reactive astrocytes also secrete molecules for ECM removal. Matrix metalloproteinases (MMPs) are enzymes which in enact a range of functions in the ECM. Removal of debris and damaged material is important for neuronal repair. However, MMPs also perform a number of negative effects which include blood brain barrier (BBB) disruption, matrix degradation, and edema (Laird et al., 2008). Studies in cancer cells have shown the potential for altered extracellular environment to influence cell behavior through focal adhesion signaling (Tai et al., 2015). Because there are often extracellular changes that occur in TBI mechanisms (both mechanical and biochemical), this prompts the need to understand how focal adhesion signaling influences normal cell behavior and contributes to responses like cellular reactivity (Liesi et al., 1984; Tate et al., 2007; Hemphill et al., 2011). No studies have yet investigated the use of a FAK inhibitor in mature astrocytes for applications in adhesion dynamics and reactivity in high-rate overpressure.

5.2.2. Focal Adhesions in 2D and 3D Environments

It is known that most cells experience altered function, shape and response mechanisms depending on the stiffness of their extracellular substrate. This response however is not purely mechanical.

Studies on focal adhesion in 2D and 3D environments have revealed similarities and differences in cells' abilities to form functional connections with their environment depending also on the binding epitopes available to them. Integrins in particular localize and group differently depending on the ECM architecture surrounding the cell. Moreover, recruitment of focal adhesions involving protein clustering is unique between 2D and 3D environments (Cukierman et al., 2001). Despite differences in recruitment and maintenance of focal adhesions in 2D versus 3D, it has been suggested that 2D focal adhesions are an exaggerated form of focal adhesions that occur in 3D with respect to the same binding epitopes (Cukierman et al., 2002). However, it is important to note that studies have distinguished roles for phosphorylated FAK in cell maintenance in 2D matrices as compared to 3D (Figure 26) (Wozniak et al., 2004). This may mean differential activation of FAK depending on the extracellular environment but also may mean the activity of phosphorylated FAK is different.

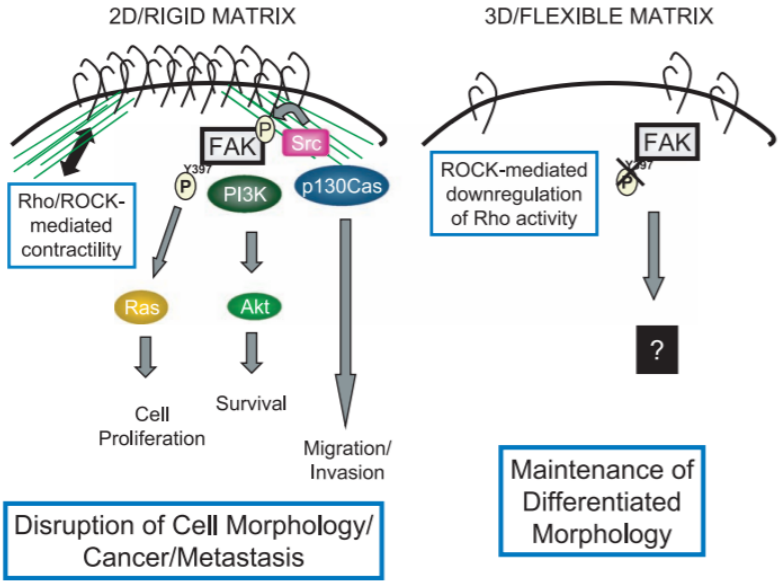

Figure 26. FAK phosphorylation has distinguishable roles in cell maintenance and function depending on the extracellular substrate to which the cell is bound. *Adapted from Wozniak et al. 2004.*

Within the context of brain mechanotransduction, studies have revealed the importance of cell binding sites on hyaluronic acid (HA) for astrocyte function and form (Konopka et al., 2016). In particular, fibrous astrocytes of the white matter use CD44 to bind to HA for modulation. Adult astrocytes in culture also express CD44 on cell bodies and processes (Moretto et al., 1993). CD44 is a glycoprotein with a hyaluronan receptor but it can also bind to various other fibrous ECM components and growth factors as well. CD44 is expressed in reactive astrocytes of various

pathological states and is thought to render a less productive phenotype in terms of extracellular clearance by astrocytes (Kang et al., 2008; Cotrina et al., 2014; Sosunov et al., 2014). It is also closely associated with calcium signaling and downstream FAK activation in neural cells (Ghosh et al., 2011).

5.3. Methods

5.3.1. 2D and 3D primary astrocyte cultures

Primary rat astrocytes were extracted from Sprague-Dawley rat pups as described in Section 3.3.1. Monolayers were grown under conditions specified previously and were seeded four days prior to overpressure exposure at a density of $1.5x10^5$ per well (six well plate). For the 3D studies, cultures were grown in a three-component hydrogel scaffold. The matrix contained 0.3% collagen, 0.15% HA and 0.15% Matrigel® (Placone et al., 2015). HA and Matrigel® were used for their material relevance and adhesion binding sites, and collagen was supplemented for its mechanically-tunable properties. HA is the major component of brain ECM, while collagen represents a smaller fraction (Ruoslahti, 1996). Moreover, HA has a distinct effect on cellular adhesion and mechanotransduction that will be important to recapitulate in this model (Chopra et al., 2014; Lam et al., 2014). Matrigel® contains a mixture of basement membrane proteins, another important aspect of astrocyte adhesion. Astrocytes form functional adhesions to vasculature and axonal tracts around which basement membrane proteins can be found. Several studies have optimized 3D matrices which allow for quiescent astrocyte phenotype before injury and were used to inform the hydrogel composition for this study (Placone et al., 2015; Kingsmore et al., 2016). Cells were seeded at $5x10^5$ per mL of hydrogel, with a total volume in each well of 1.25 mL.

5.3.2. High-rate overpressure and FAK inhibition

Samples were subjected to high-rate overpressure using the HOS described above in 3.3.2. Immediately following overpressure exposure, randomized samples were treated with TAE226 (Selleck Chemical cat# S2820). TAE226 chemically inhibits extracellular-induced phosphorylation of FAK at Y397. The chemical inhibitor was dissolved in dimethyl sulfoxide (DMSO) to make a 10 mM stock solution. The stock was then added to samples for a final loading concentration of 5 µM based on a study in glioma cells that showed dosage effects of TAE226 on cultured cells (Shi et al., 2007). TAE226 remained on the cells until harvest at 48 hours for 2D samples and 72 hours for 3D samples.

5.3.3. mRNA purification and qPCR

RNA was precipitated from Trizol and purified using a Qiagen MinElute kit per the manufacturer's instructions. Complementary DNA was synthesized using random hexamers as described in Section 4.3.3. PCR was conducted with a SYBR green master mix containing forward and reverse sequences (Table 7) at 5 µM each. Results reported in this chapter are based on delta-Ct calculations and are normalized to average sham to show relative fold change.

Table 7 | Gene sequences from PrimerExpress used for qPCR analysis in Chapter 5

Gene	Abbreviation	Forward Sequence	Reverse Sequence
Cluster of differentiation 44	CD44	TCCACCCCAACGCTATCTGT	TTGGATGCGAGGAGGATATACA
Integrin beta-1	Int β1	GAAGAGTCTTGGGACGGATCTG	GCCAATGCGGAAGTCTGAA
Matrix metalloproteinase 9	MMP-9	TCGAAGCGACCTCAAGTG	TTCGGTGTAGCTTTGGATCCA
Beta-actin	B-actin	ACCAGTTCGCCATGGATGAC	TGCCGGAGCCGTTGTC

5.3.4. Protein isolation and western blotting

Proteins were isolated from the phenol phase of Trizol-chloroform separation. The protocol for this process is described in Section 3.3.5.2. Total protein was quantified by bicinchoninic acid (BCA) assay to be used for western blotting. A capillary-based blotting system was used to detect and quantify protein targets (Sections 3.3.5.2 and 4.3.4). Standardized electropherograms were analyzed in Compass software (Protein Simple) to quantify proteins by peak area. Target protein area for glial fibrillary acidic protein (GFAP) was normalized to the loading control area glyceraldehyde triphosphate (GAPDH) before being normalized to the average sham value. Antibodies used for this study included anti-GFAP (Abcam cat# Ab7260) and GAPDH (Novus Biologicals cat# NB600-502). GAPDH showed prominent peaks for subunits between 37 and 60 kDa in some samples. In order to appropriately account for this, two peaks were summed for all samples in that group.

5.3.5. Statistics

Statistical comparisons were conducted between groups using JMP software (SAS) under Virginia Tech license. ANOVA was used to analyze significant differences amongst groups, followed by student's t-test for individual group comparisons. The assumptions for normality and homoscedasticity were confirmed by Shapiro-Wilk and Levene's tests, respectively. In the event that data was not normal, a logarithmic transformation was performed to conduct statistical comparisons. For sample sets with unequal variances, a Welch's t-test was performed. Statistical outliers were determined using residual analysis, and a p-value<0.05 was considered significant.

Total number of replicates are denoted as the variable "n." Sample blocks were used for individual overpressure/sham tests and were from the same line of cells (from the same animal and passage number).

5.4. Results

5.4.1. FAK phosphorylation inhibition results in a decrease in GFAP reactivity in 3D cultures following overpressure

At 72 hours post exposure, overpressure (denoted as OP) induced a significant upregulation of GFAP compared to sham (p-value=0.0016) in the 3D cultures as measured by western blot (Figure 27). The application of TAE226 (denoted as OP+TAE) for 72 hours significantly reduced this response after overpressure back to sham levels (p-value=0.0097).

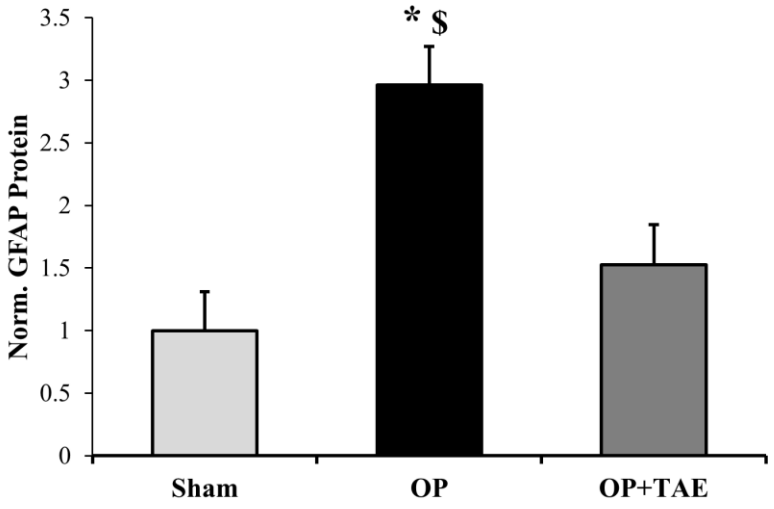

Figure 27. Normalized GFAP protein expression in 3D cultures at 72 hours post-overpressure*. TAE denotes the addition of FAK phosphorylation inhibitor (TAE226). The overpressure group had significantly increased GFAP expression compared to both sham and overpressure + TAE, which were not different from each other. Data are mean ±SEM *p-value<0.05 as compared to sham, $p-value<0.05 as compared to OP+TAE, n=4/group*

5.4.2. FAK inhibition causes decreased CD44 expression in cells exposed to overpressure

In a 3D environment, CD44 had an associated p-value of 0.095 towards downregulation in the overpressure group compared to sham while it was significantly decreased in the overpressure + inhibitor group compared to sham (p-value= 0.0062, Figure 28). This result corresponded with changes in GFAP expression above. Additionally, CD44 regulation occurred in the absence of significant changes in integrin β1 at 72 hours post overpressure between any of the groups.

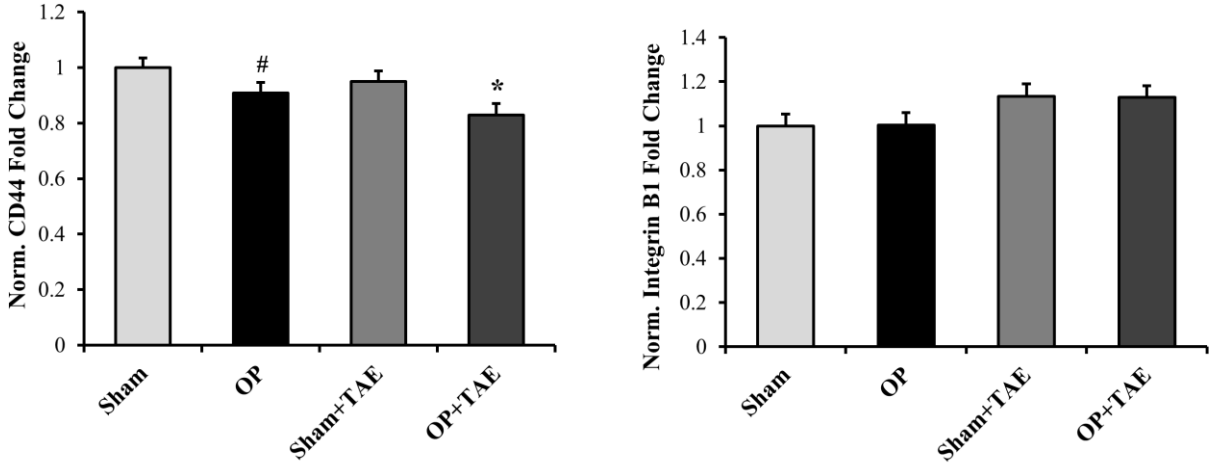

Figure 28. Gene expression analysis in 3D samples exposed to both overpressure and TAE226*. At 72 hours, CD44 was significantly decreased in the overpressure+TAE226 group compared to sham. No changes in gene expression for integrin β1 were observed in any groups. Data are mean ±SEM *p-value<0.05 as compared to sham, #p-value=0.095 as compared to sham, n=4-6/group*

5.4.3. FAK inhibition contributes to altered cadherin and MMP-9 expression in 2D cultures

PCR was conducted for three adhesion-linked genes: CD44, N-cadherin and MMP-9. CD44 was chosen for comparison to the 3D results above. In contrast to the 3D results, no groups were significantly different from one another at 48 hours in the 2D study (Figure 29A). N-cadherin was chosen as a cell-cell contact protein that was previously implicated in 2D astrocyte reactivity (at the protein level). N-cadherin expression was not significantly different between sham and overpressure but both groups were significantly different from the groups treated with the FAK inhibitor (p-value=0.0002 for Welch's t-test, Figure 29B). By contrast, MMP-9 was decreased in expression by the overpressure and inhibitor group as compared to all other groups (p-value=0.04 for Welch's t-test, Figure 29C),

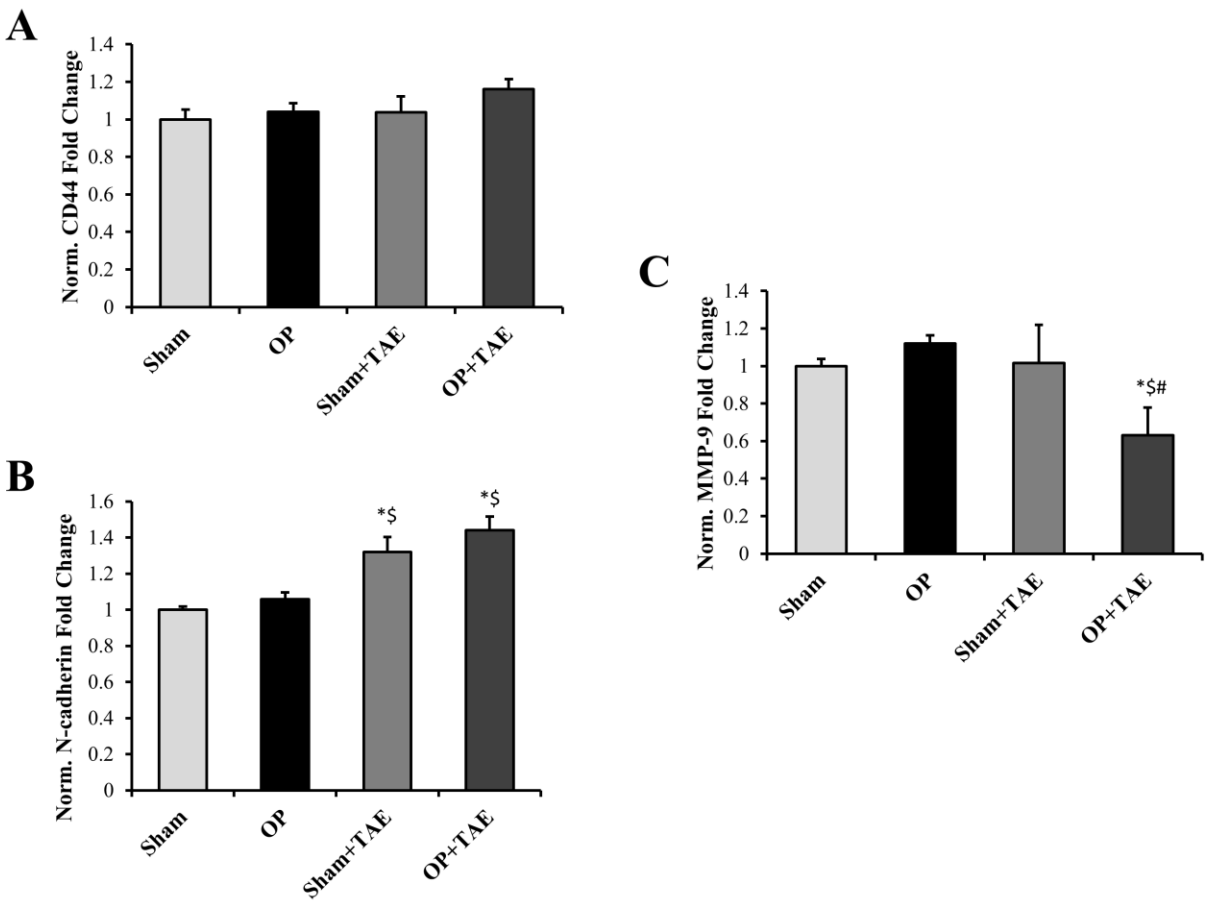

Figure 29. Adhesion gene expression analysis in 2D cultures exposed to overpressure and TAE226. *(A) No changes in CD44 gene regulation were observed at 48 hours. (B) TAE226 induced a significant increase in N-cadherin in both sham and overpressure as compared to both groups without inhibitor. (C) Overpressure and TAE226 together caused significant decrease in MMP-9 regulation as compared to all other groups. Data are mean ±SEM *p-value<0.05 as compared to sham, $^{\$}$p-value<0.05 as compared to OP, $^{\#}$p-value<0.05 as compared to Sham+TAE, n=6-9/group*

5.5. Discussion

FAK is a tyrosine kinase that is responsible for activation of many downstream effectors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) pathways (Wang et al., 2001; Benlimame et al., 2005). Because of its ubiquitous signal transduction potential, FAK is associated with multiple aspects of cellular phenotype and function, including proliferation, migration, invasion (Mitra and Schlaepfer, 2006). Integrins act as a major source for FAK activation but other cell surface receptors also contribute to phosphorylation of FAK. Similar to other adhesion mechanisms, FAK's role in cell maintenance is dependent on cell environment. In 2D cultures, FAK activation is often associated with migration, phenotypic changes and proliferation (Wozniak et al., 2004). Phosphorylation of FAK

tends to be higher when cells are growing on a rigid 2D substrate as compared to when they are in 3D. In fact, cells in 3D normally only have very low levels of phosphorylated FAK. However, FAK activation in cells with 3D attachments seems to appear in pathological contexts and often accomplishes similar outcomes but by different signaling mechanisms as compared to 2D. In cancer cells for example, FAK activation in 3D involves the suppression of apoptosis rather than an increase in proliferation as in 2D (Shibue and Weinberg, 2009; Walker et al., 2016). Both ultimately result in pathogenic morphogenesis but by different molecular mechanisms. Based on this idea, it is likely that divergence between 2D and 3D responses in FAK occurs a part of the activation in astrocyte reactivity.

One difference observed between the function of FAK inhibitor in 2D astrocytes as compared to 3D astrocyte has to do with CD44. CD44 gene expression was only significantly altered in the overpressure with FAK inhibitor group. Perhaps the most straightforward explanation for CD44 regulation differences between 2D and 3D was a lack of relevant binding sites for CD44 in 2D as a hyaluronan receptor. This result suggests two possible outcomes. The first is that blocking FAK phosphorylation after overpressure exposure leads to disruption of a regulatory pathway associated particularly with overpressure (as no differences were observed in the sham with drug group). CD44 upregulation is associated with Src kinase activation, a known molecular player in TBI mechanisms. The second notion from this result is that there is a potential link between downregulation of CD44 and the results observed related to decreased structural reactivity. CD44 is abundantly expressed by astrocyte precursor cells by is also associated with reactive astrocytes of multiple pathologies (Jones et al., 2000; Shin et al., 2005; Pollard et al., 2008; Haidet-Phillips et al., 2011). The cell surface receptor has been previously associated with astrocyte structural reactivity, particularly increased GFAP expression, via signaling pathways such as mTOR (Sosunov et al., 2013).

In the brain, FAK is expressed under highly aggressive forms of cancer, notably glioma cells. FAK is involved in vascular permeability in glioma cells, but interestingly, in FAK knockout animals, normal astrocytes are still able to form cell-cell junctions and reduce tumor size (Lee et al., 2010) while glioma cells experience disrupted ability for such function. In 2D, FAK is associated with increased invasive potential in cells, which also requires the disassembly of cell-binding proteins. Cadherins are dynamically regulated in cell adhesion processes and may serve roles in cell

proliferation and phenotype. Results of this study are limited to showing that inhibition of FAK phosphorylation in 2D cultures of astrocytes has a significant effect on the expression of N-cadherin. It is not yet known how this influence may cause or contribute to certain aspects of reactivity. Given that N-cadherin gene expression is not altered at this point in the overpressure group, it is possible that the effect of TAE226 on gene expression may lead to increased proliferative potential even in the overpressure group. This may also mean that FAK has a homeostatic role in astrocytes cultured in 2D and may be contributing to their return to structural equilibrium at 72 hours after overpressure exposure (Chapter 4). From the last chapter, phosphorylated FAK was decreased and then dynamically increased in cells as they shifted to a structurally reactive phenotype when exposed to overpressure in 2D. The increase in N-cadherin expression in the TAE226 group may mean that there will be increased structural reactivity as well in the absence of FAK phosphorylation.

It is generally considered that FAK activation signaling directly causes increased invasive and motile properties but adhesion regulation is quite complex. Cells also use these adhesion molecules to modulate their responses to the extracellular environment. One recent study on cell contact inhibition in the neural crest showed that blocking Src/FAK signaling led to increased cell junction formations, notably with vinculin (Roycroft et al., 2018). The study also showed a significant relationship between N-cadherin function and focal adhesion signaling in which N-cadherin regulates Src/FAK activation. Therefore, FAK has an important regulatory role in the prolonged adhesion properties of cells under normal and pathologic circumstances.

MMP-9 is a protease involved in the turnover of matrix material. Its upregulation is often associated with cell invasion and inflammatory processes (Rempe et al., 2016). Its upregulation is often associated with the breakdown of material as well as cell junctions in order for adjacent cells to be able to move. Because MMP-9 expression is typically coupled with the breakdown of material and junctions, it is consistent with expected results that MMP-9 showed a different expression profile as compared to N-cadherin.

Certain FAK activation effectors can be considered acquired as the result of a particular pathology or injury. For cells in 3D, FAK phosphorylation should generally be less than in 2D. This may explain some of the differences observed between the two models assessed. Despite this, FAK phosphorylation does play a role in structural reactivity of astrocytes in response to high-rate

overpressure. Moreover, the results in 3D are consistent with typical patterns of FAK inhibition in cells where an inhibition leads to decreased expression of cell surface attachment proteins (Sulzmaier et al., 2014). In this case, CD44 gene expression was significantly lower in the overpressure group that received TAE226. Despite no differences between sham and overpressure in CD44 expression, decreased expression in the TAE226 group may suggest one mechanism by which structural reactivity (i.e. GFAP) was significantly lowered by the drug.

Results of this study found that (1) regulation of adhesive proteins differs depending on the extracellular environment in which the cells are located and (2) FAK activation has a critical role in regulating multiple adhesion properties as well as phenotypic reactivity in astrocytes following high-rate overpressure. Previous work using the same overpressure model on C6 astroglioma cells, showed that cells in 3D culture experienced a significant upregulation between 48 and 72 hours. The same cells also had a significant change in expression of the intermediate focal adhesion protein vinculin between 48 and 72 hours (Hlavac et al., 2015). This suggests a differential time course and/or mechanism of structural and adhesive reactivity in brain cells after overpressure exposure depending on their environment, as cells in 2D experienced increased structural reactivity from overpressure at 48 hours instead (Chapter 4). These results are consistent with the notion that focal adhesions are exaggerated in 2D cultures and thus may accelerate reactivity of astrocytes (Wozniak et al., 2004). It is also necessary to consider that mechanics of the overpressure insult can vary depending on cell attachment to a 2D rigid substrate and a 3D deformable hydrogel and thus may influence differences observed as well (Rok et al., 2017). Focal and other junctional adhesions form mechanosensitive networks between the matrix and the cell cytoskeleton which have capacity to respond differently depending on the mechanical environment (Schwarz et al., 2006).

This study was limited in terms of the targets and effectors assessed in both 2D and 3D models. It is important to note the differences in regulation of cell junction molecules in particular between these two models and use this information to inform future studies in understanding the role for FAK signaling in astrocyte reactivity to high-rate overpressure. Further studies should be designed to understand how adhesion molecules may use FAK signaling to impart phenotypic changes in astrocytes following high-rate overpressure.

5.6. Conclusion

A 3D combination hydrogel was chosen to study the influences of matrix-based adhesion in astrocyte response to high-rate overpressure. First, FAK phosphorylation is a critical contributor and/or molecular effector in astrocyte structural reactivity in response to high-rate overpressure. At 72 hours post-overpressure insult, astrocytes showed increased structural reactivity which was ameliorated in the presence of the small molecule inhibitor against phosphorylation of FAK (TAE226). Although phosphorylated FAK exists in very low levels in normal tissue and 3D architectures, evidences exist to suggest that it has a role in the progression of various pathological states, notably cellular proliferation and morphology. This study confirmed a similar finding for FAK in astrocyte reactivity to high-rate overpressure in 3D. Further results showed that TAE226 caused decreased CD44 expression in astrocytes exposed to overpressure in comparison to all other groups indicating a potential role for CD44 in FAK-linked reactivity mechanisms. This was in contrast to a 2D astrocyte model at 48 hours post-overpressure. Cells on a rigid substrate had no changes in CD44 but did show changes in both N-cadherin (increased) and MMP-9 (decreased) in samples exposed to overpressure and receiving the inhibitor. Altogether, these results provide evidences for differential regulation of adhesion equilibrium in the context of FAK-induced astrocyte reactivity following overpressure.

5.7. Future Directions

In order to complete analysis for the comparisons of FAK phosphorylation inhibition between 2D and 3D cultures, the 2D samples should be assayed for GFAP and other reactivity protein levels. This will provide more insight for the potential reparative role of FAK in the cells exposed to high-rate overpressure in 2D. Additionally, this study only assessed gene expression changes for two adhesion molecules in the 3D group. Further analysis should be conducted to understand the regulation patterns of these adhesion molecules at the protein level. Because of the observed differences in CD44 gene expression which corresponded to decreased GFAP reactivity in the FAK inhibitor group, a next step in understanding this pathway would be to determine a potential mechanism for the regulation of GFAP and other reactivity elements in relation to the activation and turnover of CD44 in 3D adhesions. Other features of reactivity including invasive potential and cellular hypertrophy may be influenced by the addition of 3D adhesions as well (Burda et al., 2016). Lastly, integrin and FAK signaling mechanisms crosstalk heavily with GTPase RhoA pathways which have been implicated in other TBI mechanisms (Costa et al., 2013). This pathway

has not been well explored in blast neurotrauma, particularly for its role in activation of astrocytes and therefore it may provide an important mechanistic link to study in relation to FAK activation in 2D and 3D environments.

Chapter 6. Development of an *in vitro* device to evaluate the combination effect of high-rate overpressure with shear

6.1. Introduction

Much effort has been placed on understanding human tolerances to accelerations/decelerations associated with common traumatic brain injury (TBI) modes, such as those in motor vehicle crashes and sports. These efforts have resulted in the manufacturing of safer cars and sporting equipment through the use of standardized injury criteria. In the last two decades, blast neurotrauma has grown in prevalence and thus has become an equally important issue to understand and prevent. While blast mechanics and pathophysiology have become better defined, there remains challenges particularly associated with translating between experimental and computational models and even more so across biological scales. As previously discussed, the mechanics of shock wave entry into the brain parenchyma and the resulting mechanical insult are not well-defined. This presents challenges for studying relevant micro-level mechanics under this injury paradigm. However, studies have shown that shock wave overpressures result in both stress waves and shearing effects. Primary blast exposure thus causes both overpressure-dependent injury and shearing type injuries. There is a need for standardized models of high-rate overpressure, particularly at the cellular level to understand tissue tolerances to various aspects of the complex mechanics of blast exposure.

6.1.1. Statement of Problem

The advantage of using *in vitro* models to study thresholds of cellular responses to mechanical perturbations is the ability to tightly control and reproduce experimental conditions in order to afford cellular and molecular mechanisms which are consistently altered as a response. Existing *in vitro* models of TBI have largely focused on isolating high-strain mechanics associated with stretch or tearing injuries. Studies in blast neurotrauma have suggested rate-dependent responses in driving injurious mechanisms and resulting complex, high-rate pressure gradients and shear may be influencing cellular dysfunction (Ravin et al., 2012; Duckworth et al., 2013; Maneshi et al., 2015). There is a need for adaptable high-rate mechanical models to study micro-level responses in brain cells and their susceptibility to such injuries. This chapter introduces a novel high-rate overpressure simulator which allows for the application of both pressure gradients and fluid shear resulting from propagation of a high-rate compressive wave through a medium. Experimental and computational approaches were used to initially address the complex mechanics within the system in an effort to afford optimized testing conditions in the future.

6.1.2. Significance

Despite evolving methodologies for studying blast, the mechanics associated with shock wave interactions with brain tissue are still not well understood. Computational and experimental studies have identified multiple potential mechanisms which may interact to cause brain damage in blast neurotrauma (Leonardi et al., 2011; Ganpule et al., 2013; Wang et al., 2014; Fievisohn et al., 2018). There is a need for adaptable experimental systems which allow for the control and manipulation of micro-level mechanics to understand cellular tolerances to such unique loading paradigms as those associated with blast. The development of an *in vitro* model not only allows mechanical testing of cell tolerances to complex mechanics but also for the refinement of molecular mechanisms and therapeutic targets associated with the injury.

6.2. Literature Review

Primary blast neurotrauma consists of the interaction of a shock wave with the head, skull and brain parenchyma. A shock wave is a nearly infinite amplitude overpressure wave which is described by the idealized Friedlander waveform (shown in Figure 1). Although the mode of its transmission into the brain is still debated, studies have found that blast neurotrauma commonly involves shearing injuries (Duckworth et al., 2013). These shearing injury hallmarks include diffuse axonal injury, hemorrhage and edema. There is a need to understand micromechanics and cellular tolerances for such injuries under the complex conditions of shock waves interacting with cells in tissue.

6.2.1. Shearing Effects in Blast Neurotrauma

Shock wave exposure results in the propagation of shear and stress waves through the brain parenchyma. The mechanics of this phenomena are still not well characterized, but multiple strategies have been employed to study them (Fievisohn et al., 2018). The associated modes of injury from a shock wave resulting from blast are generally considered as compression, tension and shear. Compression results from the stress wave associated with positive pressure while tension can occur during the negative pressure phase. Tissue shear occurs as a result. Brain tissue responds as a nonlinear, viscoelastic material in response to mechanical manipulation. Mathematically, brain tissue is commonly modeled using hyperelastic constitutive models (Giordano et al., 2014; Budday et al., 2015). However, additional considerations must be made when modeling loading rates comparable to those in blast neurotrauma. Strain rates associated

with blast overpressure are on the order of 100-1000 s^{-1} therefore inertial loading must occur highly dynamically. Although not much work has been done to characterize cell and tissue micro-mechanical responses at such high rates, Maneshi et al have showed that high amplitude pressure application (2ms) causes highly viscous behavior of biological samples in terms of the magnitude of shear stress measured in the sample (Maneshi et al., 2015).

In an *in vitro* study of high-rate overpressure propagating through material with shock wave lithotripsy, the resulting strain in tissue associated with pressure propagation remained low (Howard and Sturtevant, 1997). Despite low strain, shock wave results in high strain rates while possible shear stress is due to wave scattering in nonhomogeneous regions of tissue. This is confirmed by increased neuropathology at interfaces of white and grey matter or in highly dense areas of tissue like the hippocampus following blast neurotrauma (Giaume et al., 2010; Sajja et al., 2014a; Shively et al., 2016). The propagation of shear waves in soft materials depends on directionality of the propagation, directionality of particle motion and the stress and strain within the material (i.e. dependent on material properties) (Namani and Bayly, 2009).

Resultant velocities are a function of both peak pressure and the acoustic impedance of the tissue. Shearing occurs in the direction of the shock wave propagation through the tissue (Bo et al., 2016). Moreover, there has been evidence for shearing effects in experimental TBI models (Sosa et al., 2013) as well as in soldiers returning from combat (Mac Donald et al., 2011; Duckworth et al., 2013). In particular, US military personnel exposed to blast overpressure have evidences for axonal injury (Mac Donald et al., 2011). Conversely, Wang et al developed a computational model in which they showed propagation of pressure directly through the skull and into the parenchyma being associated with stresses too low to cause axonal injury (Wang et al., 2014). Laksari et al created a computational model in which they showed that shock wave resulting in stress waves which propagate through the tissue with little shear effects (Laksari et al., 2015). Thus, there remains discontinuities between macroscale computational models of blast and the pathology observed in those exposed to blast.

6.2.2. Cellular Injury and Response to Shear

The mechanical interaction of injurious stimuli with soft brain tissue results in a divergent molecular signature associated with TBI. Many of the hallmarks of blast neurotrauma and impact TBI alike involve shearing type injuries to brain cells. Perhaps the most recognized of the shearing

effects from primary injury is diffuse axonal injury. This results from shearing that occurs in the heterogeneous white matter. Axonal tracts are long, dense structures that are susceptible to shearing mechanisms. Blast neurotrauma mechanisms can result in axonal shearing (Garman et al., 2011; Kuehn et al., 2011; Kallakuri et al., 2015). Multiple studies have shown that blast exposure results in cytoskeletal damage to brain cells (Säljö et al., 2000; Park et al., 2010; Valiyaveettil et al., 2014). Hemorrhage and edema can also be associated with more severe blast neurotraumas (Kuehn et al., 2011). Edema can result from mechanical damage to vascular structures (vasogenic) but can also result from cytotoxic-induced osmotic pressure within damaged tissue. Because astrocytes are imperative for waste clearance and volume control, their reactivity and dysfunction in TBI may exacerbate edema formation (Burda et al., 2016; Sajja et al., 2016). Moreover, vascular structures are areas for potential high shear during trauma and astrocytes may preferentially proliferate around them following insult (Bardehle et al., 2013).

6.3. Methods

6.3.1. Shear-combination high-rate overpressure simulator (SHOS)

A second *in vitro* overpressure device was designed by a similar principle as that described in Section 3.3.2. The SHOS works by an exploding bridge wire mechanism but exposes samples to overpressure in the same plane (parallel) to the direction of the pressure propagation rather than incident to it (as in the case of the HOS). The device is a two-chambered system separated by a thin silicone membrane. The chamber is equipped with a piezoelectric sensor (PCB cat# 113B21) for overpressure profile acquisition directly adjacent to the cells. This device has the capability to remove some of the imposing boundaries that exist in the HOS from rigid plates. The HOS was designed to study the effects of overpressure alone (on a minimally-deformable substrate) while the SHOS allows for samples to be exposed to shearing from the incident overpressure. The necessary first steps to optimize this system require methodologies for measuring the total pressure, static overpressure and associated fluid velocities within the chamber. This will inform future work to assess for shearing effects in *in vitro* samples.

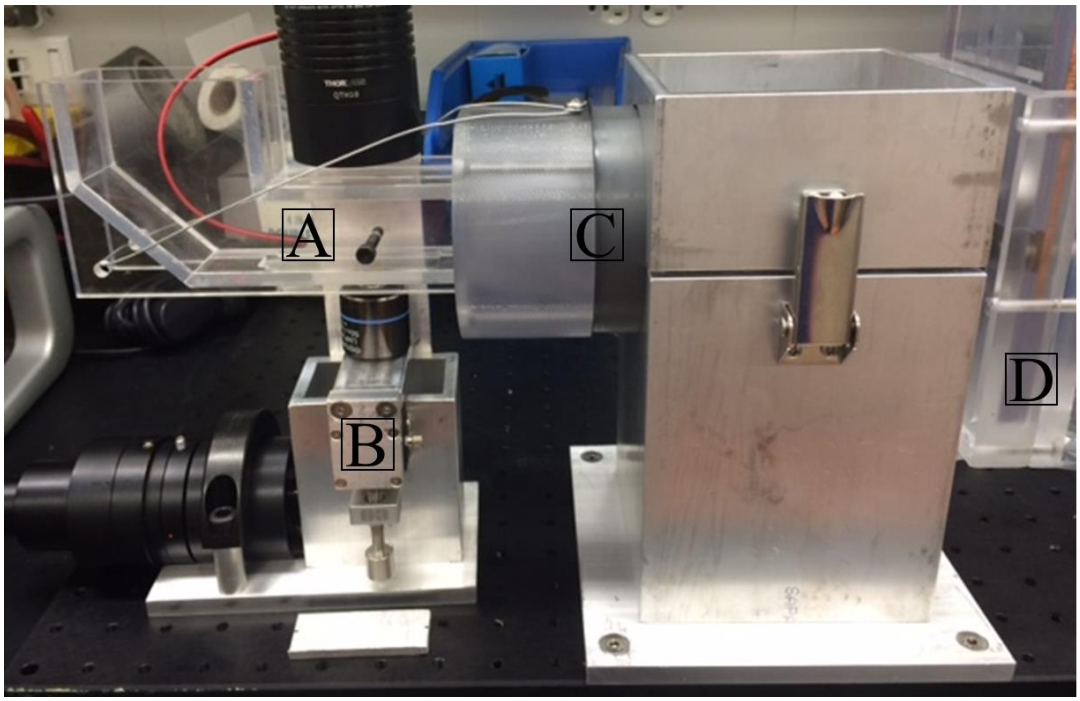

Figure 30. SHOS for cell culture exposure to overpressure combined with shear. *A) Test section where cell cultures are located B) Optical device for bright field imaging of cells during overpressure exposure C) Separation membrane D) Wire holder and wave propagation point*

6.3.2. Dynamic pressure and wave velocity approximation

There are two pressure phases associated with blast neurotrauma (i.e. shock waves) that are of interest in terms of their potential to cause damage. Static pressure, or primary shock, is comprised of the compression wave that results from the rapid expansion of fluid particles. The net displacement of the medium by the static pressure results in a dynamic pressure which propagates after the static overpressure and causes resultant fluid velocity. Total pressure is the summation of static overpressure with dynamic pressure. Given that the system of interest is underwater and results from a bridge explosion, the static overpressure is not infinite amplitude like an idealized shock wave, but it does have a rapid rise time followed by a short duration positive and negative overpressure. This profile mimics measurements made *in vivo* during blast exposure in rodents (Leonardi et al., 2011). As a first optimization for this model, static and total pressure were measured. Static overpressure was measured with a PCB cat# 113B21 piezo-sensor and total pressure was measured using a fiber optic pressure sensor (FISO cat# FOP-MIV-PK-C1-F1-M2-R3-ST). Wave speed could be estimated using the time of arrival between the set distance of the two sensors shown in Figure 31.

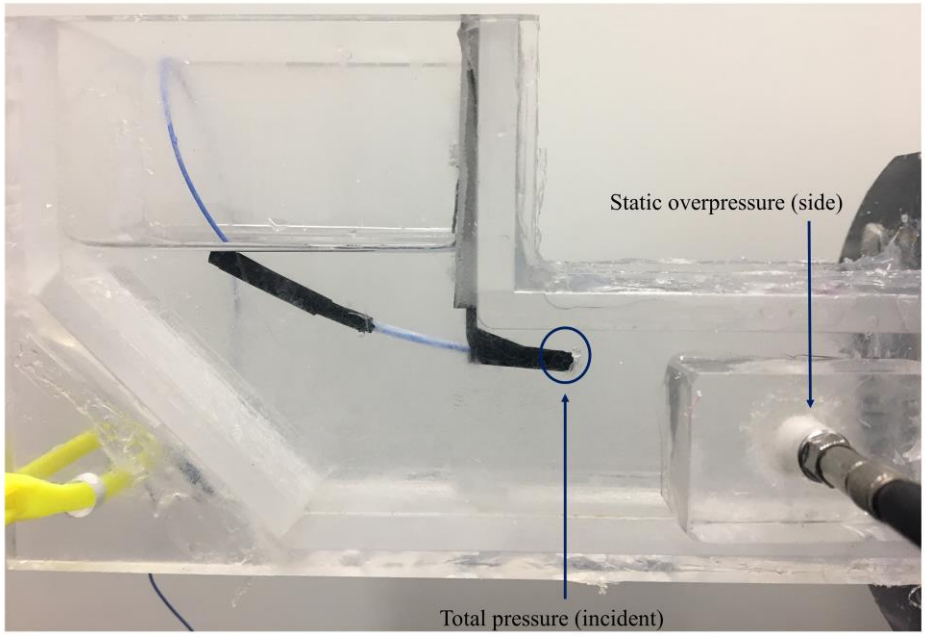

Figure 31. SHOS instrumented with static overpressure and total pressure sensors.

6.3.3. Computational model of SHOS fluid flow

Shearing effects can result from compression and tensional forces due to pressure gradients from overpressure but can also result in the presence of dynamic pressure effects (i.e. fluid velocity). Because this model is meant to directly expose cell samples to high-rate overpressure, there are fluid velocity (i.e. dynamic component) effects associated with the momentum of the water due to the compressible flow of the wave. A model was generated to assess for the resulting fluid flow using the computational fluid dynamics module in COMSOL Multiphysics 5.3. COMSOL was used to solve Navier-Stokes equations for the system described below. The region of interest for the study was set to a small length of the test section of the chamber. Based on estimates from experimental wave velocity in the chamber, flow was modeled as weakly compressible, laminar flow. An interpolation function was developed based on experimental static overpressure profiles and was applied as the inlet boundary condition for pressure. The outlet pressure boundary was set to the same profile but offset by the time it would take for the wave to travel through the section before exiting. The walls of the chamber were set as no slip boundaries. Mesh parameters were chosen by the COMSOL solver.

6.4. Results

6.4.1. Total pressure measurements and wave velocity in the SHOS

Figure 32 shows representation measurements of total and static overpressure in the SHOS. Only one simulation is shown as an example of data to be collected in this system. This example was used throughout the results in this study chapter. There was good agreement with the shapes of the pressure profiles and very little difference associated with total pressure as compared to static overpressure. The wave velocity was calculated from an average of time differences in static and total pressures along three points on the profile (Table 8). The average wave velocity will depend on peak overpressure and was 778 m/s for the profile shown in Figure 32 given a distance between sensors of 20 millimeters.

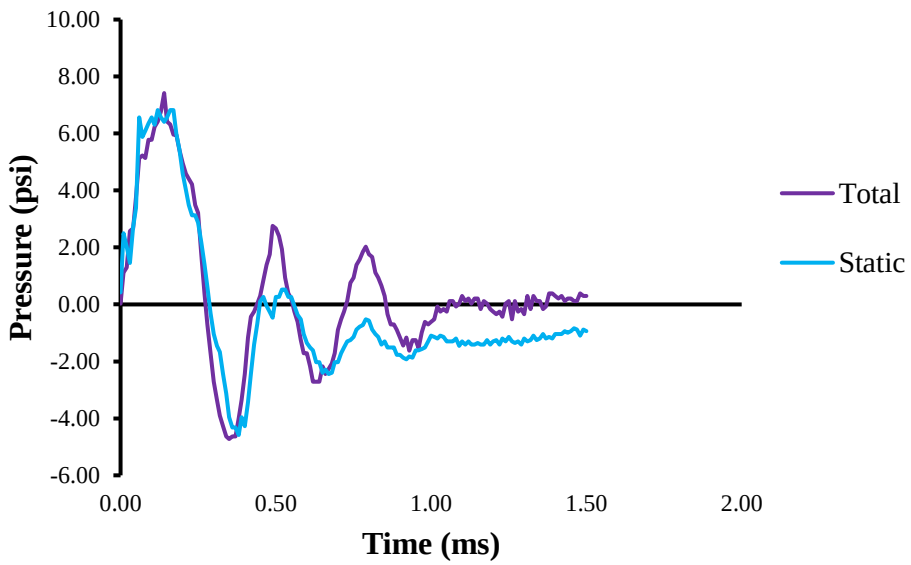

Figure 32. Representative total and static overpressures generated in the SHOS. *Differences between the two pressure measurements were very small, notably in regard to peak pressure.*

Table 8 | Wave velocity measurements for a 20 mm distance between sensors

Pressure Profile Position	Time Difference	Associated Wave Velocity
Start	0.02 ms	1000 m/s
First Peak	0.06 ms	333 m/s
Last Peak	0.02 ms	1000 m/s

6.4.2. Computational fluid velocity simulations

Simulations were conducted on a model of the SHOS test section in COMSOL using the Fluid Dynamics Laminar Flow module. Flow was modeled as weakly compressible given the calculated wave speed for the representative pressure waveform. Figure 33 shows pressure distributions over

the length of the test section with the largest gradient associated with the peak overpressure at 0.14 milliseconds. Additionally, results show that along the length of the test section, velocity remained constant for a given time step, suggesting uniform flow over the length. Negative pressures were associated with backward velocity fields as depicted in Figure 33, E-F. Moreover, velocity magnitudes corresponded with peaks on the total overpressure profile (Figure 34).

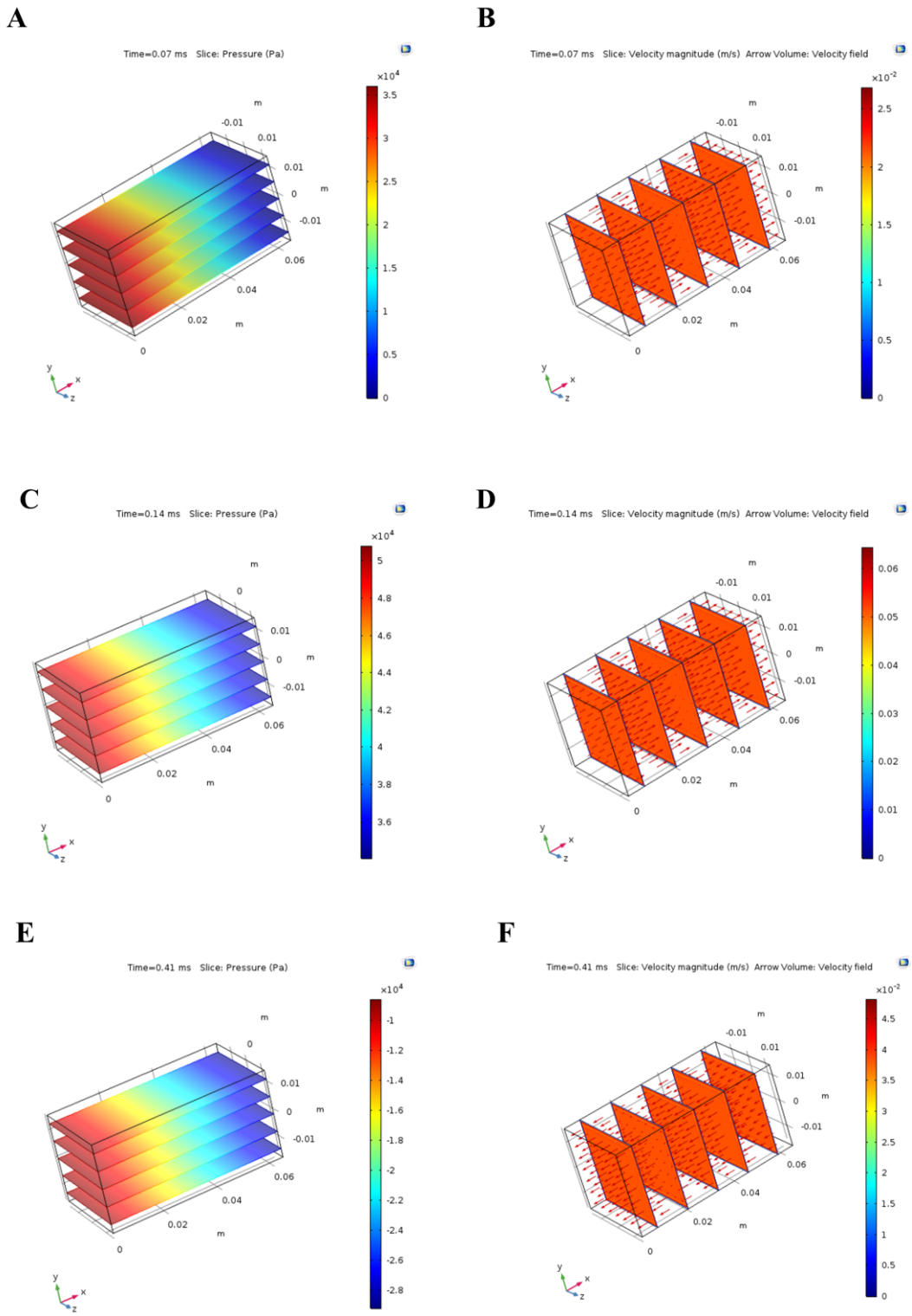

Figure 33. Pressure and velocity fields at three points in the overpressure profile*. (A) Pressure gradient along test section length at 0.07 ms (rise) (B) Forward velocity profiles along test section at 0.07 ms (C) Pressure gradient at 0.14 ms (peak overpressure) (D) Forward velocity field at 0.14 ms (E) Pressure gradient at 0.41 ms (negative peak overpressure) (F) Reverse velocity field at 0.41 ms*

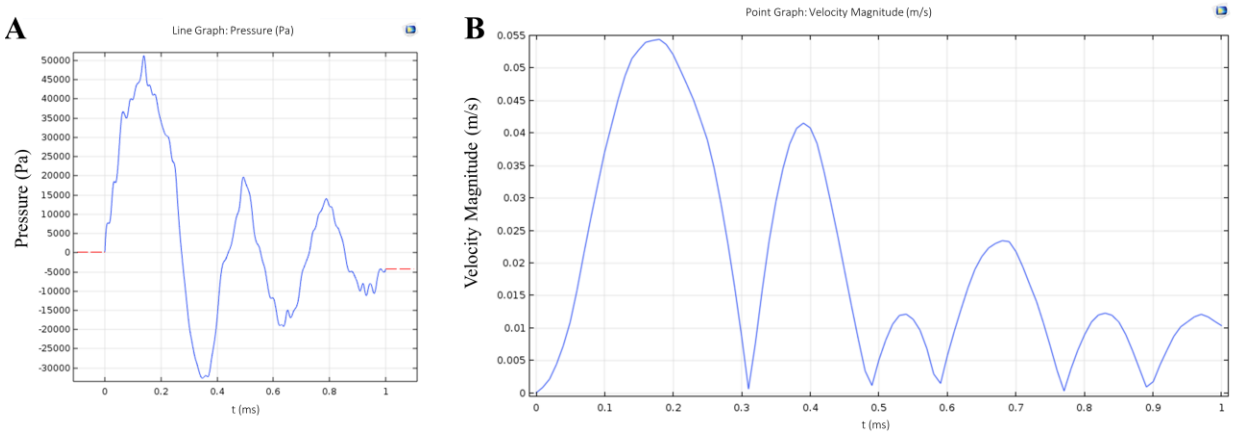

Figure 34. Magnitudes of fluid velocity at the center point of the test section temporally matched the total overpressure peaks applied at the boundary. *(A) Inlet total pressure condition for the model. (B) Velocity magnitudes at the center point (m/s) associated with the application of the inlet overpressure profile. Peak velocity occurred in accordance with the peak overpressure and was on the order of 0.05-0.06 m/s for this particular pressure scheme.*

6.5. Discussion

The combination of experimental and computational results in this study reveal that fluid velocities associated with high-rate overpressure propagation in the SHOS are not substantial at the macro-scale in terms of dynamic pressure effects but may be substantial to consider at a cellular scale. The experimental observation of high similarity of total pressure with static overpressure is concurrent with simulation results which show that resulting peak fluid velocities are on the order of 0.05-0.06 m/s for a ~52kPa (7.5 psi) overpressure profile. The fluid velocity associated with the representative overpressure profile above in Figure 32 would result in very low dynamic pressure of approximately 1.5 Pa (Needham et al., 2015). The fluid velocities in the given model are also close in magnitude to those found in a study conducted by Ravin et al in which similar application of a high-rate overpressure profile was associated with fluid velocities on the order of 0.001-0.003 m/s (Ravin et al., 2012). The impulse and peak overpressure used in their study were different from that analyzed here but the velocity profiles are multi-nodal are correspond closely in time the peak overpressure. Maneshi et al also used particle tracking methods in order to experimentally estimate fluid velocity in a high-rate overpressure system (Maneshi et al., 2015). Maneshi showed that shear stress was proportional to pressure amplitude across a range until reaching the order of two milliseconds. The authors suggest from their results that cellular and molecular responses to shear are highly nonlinear at high rates likely due to the fact that viscous effects dominant.

Both Ravin et al and Maneshi et al evaluated differences in calcium signaling associated with in vitro high-rate overpressure in the presence and absence of shearing mechanisms. Their combined results suggest that calcium signaling responses are dependent on mechanics, particularly the duration of the positive pressure, application of shear and the cell type under load. Similar to the SHOS, Ravin et al used a system to expose cells directly adjacent to the pressure report experimental velocities associated with high-rate overpressure.

There are certain limitations associated with this model in terms of recapitulating all of the features of a shock wave profile underwater. This type of high-amplitude pressure wave is highly complex as it is multidimensional and associated with compressible flow which leads to the generation of secondary pressure effects aside from the static overpressure wave propagation (Needham, 2010). The experimental results from this study suggest that dynamic pressure may not be a significant contributor but there are still limitations to estimating flow without the true propagation of the overpressure wave through the medium. Moreover, there are likely overestimations of the velocity trajectories in the computational model due to the application of an ideal outlet boundary condition (exactly matching the magnitudes of the input).

Overall, the model results show agreement between nodes of velocity magnitude and overpressure/under-pressure peaks. A stable velocity field is maintained across the test section under the given boundary conditions which suggests propulsion effects by pressure up to the outlet boundary. The outlet condition can be further optimized to achieve compressive-like gradients of pressure within the section of interest.

6.6. Conclusions

The SHOS creates similar high-rate overpressure waveforms as compared to the HOS in previous chapters with a shorter positive duration of pressure (~0.1-0.3 ms). Experimental methods were employed to estimate total pressure, static overpressure and wave velocity within the test section of the chamber. Experimental results were used to inform a computational study of resulting fluid velocity in the test section under total pressure conditions captured in the system. Experimental results were concurrent with computational analysis showing that fluid velocities are low in comparison to wave velocity but are relatively high in considering microscale shearing effects on cellular substrates. Further optimization of the SHOS design will allow for tunable *in vitro*

mechanical perturbations which are highly relevant to existing understanding of the complex mechanics associated with blast neurotrauma.

6.7. Future Directions

Experimental validation of fluid velocity measurements should be conducted within the chamber using the existing coupled optical component. Particle tracking can be employed for such measurements using bright-field high-speed video capture similar to techniques described by Ravin et al (2012). The computational model can also be refined to model as an acoustic (compressive) wave traveling through the entire chamber. Upon optimizing the wave propagation in the model, simulations of fluid-structure interactions would be the next step to analyze shear effects and strain energy in viscoelastic materials (Voyiadjis and Samadi-Dooki, 2018). There are material property differences and impedance changes that occur as pressure profiles travel from water to tissue or tissue surrogates. Computational measurements of shear stress can be coupled to experimental analyses of astrocytes grown in composite hydrogels in order to evaluate cellular tolerances for shear stress in high-rate overpressure. The model of total pressure, fluid velocity and viscoelastic shear in samples can be used for design modifications to the SHOS in relation to volumetric flow rate and shear application. Future studies should consider the evaluation of shear-induced signal transduction pathways and their role in adhesion dynamics and astrocyte reactivity in high-rate overpressure with associated shear. Such pathways may include integrin-linked kinase and mitogen-activated protein kinase p38 and extracellular signal-regulated kinases-1/2 (Neary et al., 2003; Li et al., 2017).

Chapter 7. Summary

7.1. Conclusions

Blast neurotrauma comprises a multifarious pathology that involves aberrant cellular function, neuroinflammation, metabolic and oxidative stress, and neurodegeneration. There is a dire need to therapeutically intervene after blast neurotrauma with pharmacological solutions which target the underlying cellular and molecular dysfunctions that make up secondary injury. Astrocytes represent a critical therapeutic target in the secondary window, as they mediate neuronal repair and vascular coupling. However, there is a significant lack of understanding the protective and detrimental aspects of astrocyte reactivity to blast exposure. *In vitro* models will provide an effective avenue for understanding particular characteristics of the reactive phenotype. The work herein describes the use of *in vitro* methodologies to advance understanding of molecular drivers of astrocyte reactivity and response to high-rate overpressure injuries. Overall, the results contribute to a greater goal in harnessing the therapeutic potential of astrocytes. Each of the following specific aims were addressed in this work:

7.1.1. Specific Aim 1

Chapter 3 summarizes the results related to Specific Aim 1 on metabolic perturbations in astrocytes following overpressure. Astrocytes in an isolated environment experienced early fluctuations in endogenous ATP not initially tied to any loss in mitochondrial membrane integrity. Astrocytes showed increased nicotinamide adenine dinucleotide (NADPH) oxidase 4 (NOX4) expression which followed this dynamic ATP response. NOX4 expression was coupled with increased proliferating cell nuclear antigen (PCNA) expression which may have been related to reparative mechanisms by these cells. Blockage of immediate cationic signaling resulted in decreases back to sham level of both NOX4 and PCNA indicating that early cationic signaling may play a role in both harmful and protective mechanisms in astrocytes following overpressure-induced trauma.

7.1.2. Specific Aim 2

Chapter 4 contains results for Specific Aim 2 related to proliferative and adhesive responses in astrocyte reactivity to high-rate overpressure. This work established an association of multiple cell junction protein classes with structural reactivity and proliferative potential in astrocytes. Additionally, mechano-stimulation signal transduction pathways involving focal adhesion kinase (FAK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and mitogen activated protein kinase (MAPK) were activated dynamically as astrocytes shifted to a structurally

reactive phenotype. Specifically, the affinity of the transcription factor NF-κB p65 was increased for ICAM-1. Altogether, results from this chapter suggested that altered adhesion may be critical in regulating and responding to reactive mechanisms following overpressure.

7.1.3. Specific Aim 3

Chapters 5 and 6 were conducted under hypotheses for Specific Aim 3. This aim was accomplished through the use of two *in vitro* high-rate overpressure devices. Chapter 5 focused understanding mechanistic differences in FAK regulation for astrocyte adhesion between 2D and 3D cell cultures under the context of high-rate overpressure. Results indicated that inhibition of FAK phosphorylation results in differential regulation of adhesion proteins depending on culture environment. Inhibition of FAK phosphorylation also mitigated structural reactivity in 3D cultures of astrocytes exposed to high-rate insult. This work provides fundamental understanding of astrocyte function and regulation in regards to mechano-stimulation as well technical considerations when translating to more complex *in vitro* or *in vivo* models.

7.1.4. Overall Conclusions

The purpose of this study was to develop and utilize *in vitro* models of high-rate overpressure insult to understand the driving factors for features of the astrocyte reactive phenotype. There is a body of literature which describes known molecular instigators of reactivity as summarized by Sofroniew (2009). While these features have been characterized under various contexts, the extent to which they contribute to astrocyte reactivity is still largely unknown. The combined work addresses distinct, yet converging features of cellular behavior that can influence astrocyte injury response within the context of high-rate insult. Together, the results provide multiple molecular pathways related to metabolic dysfunction, cell anchorage/adhesion and downstream signal transduction that coincide to influence specific features of astrocyte reactivity to high-rate overpressure. Chapter 4 showed that exposure to a high-rate overpressure scheme resulted in progressive and transient structural reactivity which was resolved within an isolated astrocyte network. This model provided a mechanism to study the onset of reactivity as driven by autologous signals within mechanically-stimulated astrocytes.

Metabolism acts as the driving force for all of the phenotypic shifts that occur in both normal and pathological cellular function. In particular, Chapter 3 established a model to study cationic transduction and its influences on cell behavior following high-rate overpressure. The model

results implicated a robust potential for astrocytes to respond to metabolic stresses, largely induced by early ionic fluctuations associated with mechanical perturbation. Cationic transduction, particularly of calcium and potassium, has been highly implicated in astrocyte dysfunction as a result of brain insult and trauma. Results showed application of gadolinium (a nonspecific cationic channel blocker) could influence a delayed pro-oxidative phenotype in astrocytes as measured by NOX4 production. Conversely, blocking cationic transduction with gadolinium also decreased production of PCNA, suggesting that immediate transduction may be an important mediator of astrocyte ability to repair and respond to damage after high-rate overpressure. It is possible that these responses are coordinated by immediate and lasting ionic transduction through mechano-sensitive other gated ionic channels.

Results between Chapters 3 and 4 converged a mechanism by which reactive potential may be influenced by both cationic transduction and dynamic phenotypic shifts. Astrocyte reactivity was associated with the dysregulation of cell junctional molecules and downstream signal transduction activation. The NF-κB and MAPK signaling pathways that were assessed in these studies are mediated by multiple extracellular inputs, including both ionic channels and adhesion complexes. Although they were explored only within context of altered adhesion and structural reactivity, it is possible the characteristic activation profiles for these two pathways is influenced by early metabolic changes as well. Future studies will be important to assess specific activation of these signal transduction pathways in association with cationic fluctuations and metabolic stress profiles as multiple inputs may be driving the assumption of the reactive phenotype.

Under the target overpressure scheme, astrocytes were able to robustly respond and survive metabolic disturbances but were driven to assume a progressively reactive phenotype despite no other inputs from non-astrocytic cells. In the future, it will be important to assess more complex extracellular and mechanical factors contributing to astrocyte reactivity responses, but this study has identified multiple features of reactivity which are at least in part governed by mecho-stimulation of astrocytic networks by high-rate overpressure.

7.2. Limitations

Experimental limitations were required for these studies and should be considered as the following:

1. **The studies described herein were limited to strictly *in vitro* approaches.**

   Cell models were used in the study to study fundamental molecular principles which govern how astrocytes respond to controlled mechanical perturbations. Astrocytes were cultured in an isolated environment apart from the influences of other cells that drive many of their regulatory mechanisms (Sajja et al., 2016, Sofroniew, 2009). Astrocytes, in particular, also have a baseline reactivity to being grown on rigid substrates which may make it more difficult to detect lower-level changes in reactivity. In vitro techniques also requires that cells be grown in artificial environments which removes some of their ability to respond to stimuli as if in their native state. Moreover, culturing cells in plastic dishes can lead to pressure reflections associated with the transmission of a compression wave through plastic interfaces (Del Razo and LeVeque, 2016). The potential mechanical effects in the high-rate overpressure simulator (HOS) models should be considered.

2. **There are challenges in experimentally and computationally modeling the mechanical environment associated with blast neurotrauma.**

   Shock waves are complex, multidimensional compressive waves which are highly susceptible to disturbances in flow. Their mechanical presence is also dependent on directionality of the wave and particle motion. Environmental factors like temperature and the interfaces through which the wave must propagate also must be considered. As such, it is still debated how shock waves enter the brain parenchyma and impart the mechanics that associated with pressure propagation through the tissue. Therefore, many challenges exist in modeling such phenomena particularly at the micro-scale. There is a need for *in vitro* models which recapitulate well-defined, yet adaptable mechanics in order to better understand the mechanisms which govern blast neurotrauma. This study utilized two devices to study overpressure and shearing effects that result from a high-rate compression wave, but the effects have not been completely defined either computationally or experimentally. Boundary conditions need to be optimized to the system to better recapitulate the momentum (and fluid velocities) associated with the compression wave in the shear-combination HOS (SHOS).

7.3. Future Work

The comprehensive results of this work establish multiple models of astrocyte reactivity to high-rate overpressure. Molecular mechanisms related to cellular adhesion, cell signaling and metabolism all converge to provide overlapping understanding of the "classical" features of astrocyte reactivity. Future studies should explore the underlying mechanisms associated particularly with cationic signaling and focal adhesion kinase as both of these had influences on cellular phenotype. Interactions effects between metabolic, ionic signaling and adhesion activation should also be considered as there exists many overlapping principles, especially in terms of calcium signaling.

More work is needed to develop and utilize the combination model for overpressure and shear mechanisms presented in Chapter 6. This model will have important applications for establishing mechanisms of high-rate overpressure on soft, viscoelastic materials. Ultimately, the goal is to translate these mechanisms to more biologically-complex models, including tissue explants and *in vivo* models of blast neurotrauma. Evidences herein and from the literature support the importance of adhesion and extracellular-driven mechano-stimulation mechanisms as significant contributors to astrocyte response under trauma. There are many therapeutic avenues to pursue from this route as a result.

7.4. Contributions

Table 9 | Related Publications

Year	Title	Journal
2015	Two and Three-Dimensional *in vitro* Models of Blast-Induced Neurotrauma	Biomedical Sciences and Instrumentation
2016	Role of Glia in Memory Deficits Following Traumatic Brain Injury: Biomarkers of Glia Dysfunction	Frontiers in Integrative Neuroscience
2018	Astrocyte Mechano-Activation by High-Rate Overpressure Involves Alterations in Structural and Junctional Proteins	Frontiers in Neurology, Neurotrauma *in review
2018	Cellular Mechanisms of Metabolic Stress in Brain Cell Response to High-Rate Overpressure	*In preparation*

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Figure 2 – [fair use]

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Figure 3 – [fair use]

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Figure 4 – [fair use]

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Figure 5 – [fair use]

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