TARGETING LEUKOTRIENE-MEDIATED NEUROINFLAMMATION IN
TRAUMATIC BRAIN INJURY

By

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Traumatic brain injury (TBI) is a major public health problem as it is a major cause of death and disability in all age groups. The pathophysiology of TBI is complex and heterogeneous. The primary injury at the time of trauma initiates a chain of events called secondary injury that extends beyond the initial site of injury and leads to additional neurological damage and impairments in cognitive function. TBI is an important risk factor for dementia, and accumulating evidence indicates that mild TBI (mTBI) can result in long-lasting pathological changes associated with neurologic and behavioral disabilities. However, the cellular mechanisms underlying these changes are largely unknown. The central hypothesis of the thesis is that leukotrienes, pro-inflammatory lipid mediators, activate neuroinflammatory signaling cascades, which ultimately contribute to detrimental secondary injury and associated cognitive deficits.

First, I investigated the role of leukotrienes in moderate to severe TBI using the fluid percussion injury model and the efficacy of blocking their production with a FLAP inhibitor. Administration of a FLAP inhibitor shortly after TBI significantly blocked injury-induced leukotriene synthesis, attenuated brain edema and blood-brain barrier disruption, and protected against impairments in synaptic plasticity and memory and learning. Next, I investigated the spatiotemporal nature of secondary injury and neuroinflammation after mTBI using a newly developed closed head injury (CHI) model. CHI resulted in a robust neuroinflammatory response that was initiated early and
persisted for at least one month in discrete brain regions. This neuroinflammatory response occurred in the absence of macroscopic lesions and blood-brain barrier disruption and was accompanied by neuronal and axonal degeneration. Acute administration of a FLAP inhibitor significantly blocked both acute and chronic neuroinflammation and the corresponding neurodegeneration. Last, I used a novel, unbiased proteomic approach to screen plasma at several time points after CHI and identified 7 putative biomarkers of mTBI. Taken together, these results indicate that the 5-lipoxygenase pathway of leukotriene biosynthesis initiates and maintains an endogenous immune response to brain injury that leads to delayed neurodegeneration and cognitive impairments. These findings have important implications for treating human TBI and provide support for FLAP inhibitors as a novel, targeted intervention.

The form and content of this abstract are approved. I recommend its publication.

Approved: Kim A. Heidenreich
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LIST OF ABBREVIATIONS

5-HpETE: 5-hydroxyperoxyeicosatetraenoic acid
5-LO: 5-Lipoxygenase
AA: Arachidonic Acid
ADC: apparent diffusion coefficient
AMPA: \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazole
BBB: Blood-brain barrier
BBBD: Blood-brain barrier disruption
BLT-1 or -2: Leukotriene \( B_4 \) receptor
CBF: Cerebral Blood Flow
CC: corpus callosum
CCI: controlled cortical impact
CHI: closed head injury
CNS: central nervous system
cPLA\( \alpha \): cytosolic phospholipase A\( \alpha \)
CSF: cerebrospinal fluid
CT: Computed Tomography
CTE: chronic traumatic encephalopathy
CTX: cortex
cysLTs: cysteiny1 leukotrienes (LTC\(_4\), LTD\(_4\), LTE\(_4\))
Cys-LT1, -LT2, -LT3: cysteiny1 leukotriene receptor
DAI: diffuse axonal injury
DAMP: danger-associated molecular patterns
DG: dentate gyrus
Dpi: days post-injury
DTI: diffusion tensor imaging
EB: Evans Blue
FA: fractional anisotropy
fEPSP: field excitatory post-synaptic potential
FJB: Fluoro-Jade B
FLAP: 5-Lipoxygenase Activating Protein
fMRI: functional MRI
FPI: fluid percussion injury
GCS: Glasgow Coma Scale
Gd: gadolinium-diethylenetriamine pentaacetic acid
GFAP: glial fibrillary acidic protein
GPCR: g-protein coupled receptor
H&E: hematoxylin & eosin
HFS: high-frequency stimulation
Hpi: hours post-injury
Iba-1: ionized calcium-binding adapter molecule 1
IP: intraperiotoneal
LTs: Leukotrienes (LTB\(_4\), LTC\(_4\), LTD\(_4\), LTE\(_4\))
LTP: long-term potentiation
mGluR: metabotropic glutamate receptor
MPO: myeloperoxidase
MRI: Magnetic Resonance Imaging
NLRs: nucleotide-binding oligomerization domain receptors
NMDA: N-methyl-D-aspartate
NSAID: non-steroidal anti-inflammatory drug
PET: positron emission tomography
PPR: paired-pulse ratio
PPRs: pathogen recognition receptors/ pattern recognition receptors
RAWM: radial arms water maze
ROI: region of interest
RP LC-MS/MS: reverse-phase liquid chromatography coupled to tandem mass spectrometry
SGZ: subgranular zone
SVZ: subventricular zone
TBI: Traumatic Brain Injury
UCH-L1: ubiquitin carboxyl-terminal hydrolase isoenzyme L1
CHAPTER I
INTRODUCTION

Traumatic Brain Injury Definition and Epidemiology

Acquired brain injury is defined as damage to the brain after birth, excluding neurodegenerative disorders, which can result from nontraumatic brain injury (e.g. stroke, brain tumor, infection, poisoning, or substance abuse) or from traumatic brain injury (e.g. physical trauma due to accidents, assaults, head injury). A traumatic brain injury (TBI) occurs when the brain is damaged due to an external mechanical force, such as the head striking or being struck by an object, acceleration-deceleration movements, a foreign body penetrating the brain, or a force generated by a blast or explosion (Maas et al., 2009; Menon et al., 2010; Prins et al., 2013a).

TBI is a serious worldwide public health and socio-economic problem as it is a leading cause of death and disability across all age groups. The incidence of TBI is increasing worldwide, due in part to the increasing use of motorized vehicles in developing countries (Roozenbeek et al., 2013). Of the 1.7 million people who suffer a TBI each year in the United States and seek medical treatment, 52,000 die as a result of their TBI and 730,000 develop long-term disability as a result of their injury (Faul et al., 2010; Rutland-Brown et al., 2003). An estimated 5.3 million people in the United States currently suffer from long-term disability associated with a TBI (Thurman et al., 1999). The direct costs of TBI have been estimated to be $13.1 billion per year with an additional $64.7 billion per year in indirect costs due to lost work and productivity (Ma et al., 2014).
The highest incidence of TBI occurs in young adults (15-24 years of age) and the elderly (>64 years of age). The most common cause of TBI is falls (40%), followed by unintentional blunt trauma (15%), motor vehicle accidents (14%) and assaults (10%). Combat-related TBI has emerged as the “signature injury” of the recent conflicts in Iraq and Afghanistan, affecting 20% of the more than 2.5 million service members deployed since 2003 to Operation Enduring Freedom, Operation Iraqi Freedom, and Operation New Dawn (Terrio et al., 2009). According to national and international studies, TBI occurs about twice as often in males compared to females (Faul et al., 2010; McKinlay et al., 2008).

The severity of TBI ranges from mild (80%) to severe (10%). However, medical care is rarely sought for mild TBI (mTBI), and for this reason TBI is considered a ‘silent epidemic’ because these cases go undiagnosed, undetected, and unreported. Including mTBI, the total incidence of TBI in the United States is estimated to be between 5 and 7 million per year (Langlois et al., 2006).

**Clinical Classification & Pathophysiology of TBI**

TBI is classified by mechanism (i.e. closed; penetrating; crash; blast), clinical symptom severity assessed by the Glasgow Coma Scale (GCS), and structural damage assessed by neuroimaging (i.e. Computed Tomography, CT; Magnetic Resonance Imaging, MRI). The Glasgow Coma Scale is an assessment of consciousness that is composed of three tests: eye, verbal, and motor responses. A patient is scored on their ability to perform in each of these categories, and the sum of the three scores classifies a TBI as mild (GCS 13-15), moderate (GCS 9-12), or severe (GCS < 9). Conventional CT scanning is the preferred neuroimaging method in the acute setting for patients with
moderate to severe GCS scores primarily to diagnose skull fractures, intracranial lesions and/or bleeding.

Depending on the nature and severity of the injury, TBI can result in any combination of physical, cognitive and emotional symptoms, some of which may appear immediately after the injury while others may not present for several days or weeks (Figure 1.1). The pathophysiology of TBI is a highly complex and heterogeneous disease process, not a single isolated event. The injury itself induces mechanical damage to the brain, referred to as the primary injury, which can manifest as skull fractures, intracranial hematomas, lacerations, contusions, or penetrating wounds. The primary injury activates several biochemical cascades that lead to the secondary injury days to weeks after the initial insult. The secondary injury expands beyond the initial site of damage and includes ischemia, edema, blood-brain barrier (BBB) disruption, neuroinflammation, and cell death. If unresolved these detrimental events can contribute to a chronic injury phase that includes behavioral and emotional changes, cognitive impairments, neurodegenerative disorders, and possibly death. (Figure 1.2).

The primary injury is the result of direct trauma independent of or in combination with acceleration, deceleration or rotational forces. A severe, penetrating TBI can directly shear blood vessels and axons and mechanically damage neurons and glia, leading to intracerebral bleeding (i.e. hemorrhage), brain tissue compression and bruising (i.e. contusions), and tears in the brain tissue (i.e. lacerations). However, non-penetrating TBI can still evoke mechanical damage to cells and axons, activating the same biochemical processes that contribute to the secondary injury phase of TBI.
Figure 1.1. Symptoms of TBI.
Figure 1.2. Pathophysiology of TBI.
The viscoelastic nature of the brain allows it to withstand strain that occurs during normal head movements. The threshold of elasticity, however, is exceeded in TBI when the brain undergoes rapid movement, inducing damage as a result of the shearing, tensile, and compressive forces. These forces can mechanically damage all cell types in the brain, but the stretching and/or shearing of blood vessels and axons is most common and can cause widespread damage in the brain.

The severity and extent of the primary injury determine the secondary injury. Mechanical damage to the cerebral tissue that occurs in focal and diffuse TBI can cause mechanoporation of cell membranes, which disrupts ionic gradients and leads to the efflux of potassium ($K^+$) and influx of sodium ($Na^+$) and calcium ($Ca^{2+}$) ions. This depolarization causes an uncontrolled, excessive release of excitatory neurotransmitters (i.e. glutamate), triggering a cascade of events called excitotoxicity (Prins et al., 2013b). Excess extracellular glutamate activates N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) ionotropic receptors as well as metabotropic glutamate receptors (mGluRs), leading to elevated intracellular $Ca^{2+}$ (Giza and Hovda, 2014). Excess intracellular $Ca^{2+}$ affects a wide array of cellular functions in neurons and glial cells. Mitochondria can sequester excess $Ca^{2+}$ in an attempt to restore homeostasis, but this impairs metabolic processes and leads to the production of reactive oxygen species, including the superoxide anion nitric oxide (Peng and Jou, 2010). Elevated $Ca^{2+}$ activates calcium-dependent enzymes, like calpains, caspases, and phosphatases, which damage voltage-gated ion channels, degrade the cytoskeleton, and can activate synthesis of pro-inflammatory lipid mediators and cell death signaling pathways. Compression/contusion of brain tissue and blood vessels during the primary injury can
cause reductions in local cerebral blood flow (CBF). This reduced CBF deprives neurons and glia of oxygen and necessary metabolic substrates. Because the brain does not have long-term energy stores ATP levels rapidly decline, causing cells to lose their ability to maintain their electrochemical gradients, further contributing to excess intracellular Ca\(^{2+}\) levels and excitotoxic processes (Giza and Hovda, 2001). The disruption in ionic concentration gradients leads to the net movement of water from the extracellular space into cells, which causes swelling of individual cells, or cytotoxic edema (Greve and Zink, 2009). This, however, causes the fluid in the extracellular space to become hypertonic compared to the blood, leading to the movement of water across the blood-brain barrier (BBB), which increases intracranial pressure.

The BBB is a dynamic interface between the blood circulation and the brain tissue that is responsible for protecting the brain from toxins and pathogens, regulating the passage of ions and molecules, maintaining brain homeostasis, and supporting neuronal function. Endothelial cells connected by tight junctions, pericytes, and astrocyte end feet make up the BBB. In many TBI cases, the primary injury disrupts BBB integrity and causes the extravasation of leukocytes, proteins, electrolytes and water. When peripheral immune cells invade the brain parenchyma they interact with endogenous immune cells in the brain (i.e. microglia and astrocytes) to exacerbate the neuroinflammatory response, leading to additional release of inflammatory signaling molecules and further disruption of the BBB to recruit additional immune cells to the site of injury. Tightly regulated ion, protein, and water gradients in the brain become disturbed when the BBB is disrupted. This drives water into the brain where it accumulates in extracellular spaces, leading to
vasogenic edema. (Abbott et al., 2010; Benarroch, 2012; Takeshita and Ransohoff, 2015; Wong et al., 2013).

The complex secondary injury signaling cascades together contribute to cell death and neuronal dysfunction, which can ultimately lead to neurodegeneration and impairments in cognition and executive function. Several studies indicate that a history of a single moderate to severe TBI is an important risk factor for the onset of dementia later in life (Graves et al., 1990; Lye et al., 2000; Mortimer et al., 1985; Salib et al., 1997). Also, accumulating evidence indicates that repeated mild TBI, common among athletes and military personnel, is highly correlated with Alzheimer’s-like dementia and pathology. The term chronic traumatic encephalopathy (CTE) is now widely used to describe this progressive neurodegeneration (McKee et al., 2013). It has been suggested that the effects of multiple mild TBIs are cumulative, especially when several occur over a short period of time. However, the mechanisms underlying the progression of post-traumatic dementia are largely unknown, and it is unclear whether this link between TBI and neurodegenerative pathology is dose-dependent with increasing injury severity and/or frequency-dependent (i.e. single vs. repetitive injury).

**Neuroinflammation**

Growing evidence supports the hypothesis that neuroinflammation is the pathological link between TBI and cognitive decline (Giunta et al., 2006; Bigler et al., 2013). A recent study demonstrated that both mild and severe experimental brain injury induce qualitatively similar inflammatory gene expression responses, suggesting that neuroinflammation is a universal response to TBI (Lagraoui et al., 2012). Neuroinflammation is the response of the central nervous system (CNS) to trauma and is
the result of innate immune activation. Although it is aimed at clearing debris, limiting further damage, and promoting recovery, if neuroinflammation goes unresolved it may exacerbate tissue damage and contribute to the detriment of TBI.

Vascular damage at the time of TBI can lead to the infiltration of peripheral immune cells. However, even in the absence of overt vascular damage, the mechanical damage to neurons and glia can elicit an inflammatory response. Although historically the CNS was considered to be ‘immunologically privileged’ due to the tight separation from the peripheral circulation by the BBB, it is now well-recognized that resident cells of the brain are important players in the neuroinflammatory response. Microglia, endogenous myeloid-derived cells, are considered the first responders to brain injury. Under non-pathological conditions, microglia are in a highly ramified, quiescent state and constantly survey the microenvironment for disruptions in homeostasis (Nimmerjahn et al., 2005). Microglia express pathogen recognition receptors (PRRs) that respond to molecules released by damaged cells, called damage-associated molecular patterns (DAMPs) that include heat shock proteins, histones, S100 proteins, DNA, ATP, and potentially many others (Kono and Rock, 2008). Injured neurons and glia release DAMPs, which bind to their respective PRRs on the microglial surface, initiating signaling cascades that cause microglia to transform into an ‘activated’ state. Activated microglia produce high levels of lipid inflammatory mediators, chemokines, inflammatory cytokines (e.g. interleukin (IL)-1β, tumor necrosis factor-a (TNFα), and interferon-γ (IFNγ)), and oxidative molecules (e.g. nitric oxide, reactive oxygen species). Activated microglia retract their processes and undergo a morphological transformation that enhances their migratory and phagocytic activities (Tambuyzer et al., 2009).
Activated microglia remove pathogens, cellular debris, and dying cells to protect neurons and promote tissue recovery and repair.

Astrocytes also respond readily to CNS damage and play an integral role in the innate neuroinflammatory response after TBI. Astrocytes are essential for maintaining homeostasis in the brain by regulating neuronal synapse formation, metabolic supply to neurons, ion and glutamate levels, and the BBB by direct contact of their end feet with endothelial cells around capillaries (Sofroniew and Vinters, 2010). Like microglia, astrocytes express a variety of PRRs that respond to DAMPS in the local environment and become reactive. Reactive astrocytes proliferate, accumulate intermediate filaments (i.e. glial fibrillary acidic protein (GFAP)), and take on a hypertrophic morphology. Reactive astrocytes release a variety of chemokines, cytokines, and neurotrophic factors that activate neighboring astrocytes and microglia to amplify the local immune response as well as modify BBB permeability to attract immune cells from the circulation (Farina et al., 2007). One important function of reactive astrocytes after trauma is their ability to migrate to the injury site and form a physical barrier between the lesioned tissue and the neighboring, healthy tissue. This glial scar plays an important role in demarcating damaged tissue to promote recovery and prevent further damage to surrounding tissue (Bush et al., 1999; Faulkner et al., 2004). Reactive gliosis can be beneficial by protecting cells against oxidative stress, facilitating BBB repair, stabilizing extracellular fluid solute balance, and limiting the spread of disease or damage.

The neuroinflammatory response is designed to minimize damage and restore homeostasis to the brain. However, activated microglia and reactive astrocytes have both reparative and deleterious roles after TBI. Although not well understood, there is a bi-
directional relationship between microglia and astrocytes that allows them to act in concert to regulate neuroinflammatory processes. These positive feedback loops, combined with biochemical and structural dysfunction of neurons, can trigger a vicious, self-perpetuating cycle that leads to prolonged microglial and astrocyte activation and an unresolved neuroinflammatory response. Chronic neuroinflammation is detrimental to normal brain function and can drive pathogenic processes and neurodegeneration.

Human and animal studies indicate that microglia are chronically activated after TBI and are associated with increased expression of pro-inflammatory cytokines (Bendlin et al., 2008; Bramlett and Dietrich, 2002; Holmin and Mathiesen, 1999; Maxwell et al., 2006; Smith et al. 1997). Post-mortem studies have detected activated microglia in the white matter up to 16 years after a TBI (Gentleman et al., 2004). Furthermore, it has been suggested that with aging, microglia become ‘primed’ to respond more rapidly and produce a more vigorous inflammatory response (Conde and Streit, 2006b; Godbout et al., 2005). It is now well established that amyloid-beta senile plaques, the hallmark pathological feature of Alzheimer’s disease, are associated with activated microglia and reactive astrocytes (Itagaki et al., 1989; Serrano-Pozo et al., 2011; Sheng et al., 1997). The injured brain undeniably produces an inflammatory response that involves complex signaling by microglia and astrocytes, and accumulating evidence lends support for the hypothesis that this chronic neuroinflammation is maladaptive and may represent an important, underlying link between TBI and progressive degeneration and cognitive impairments. However, little is known about the mediators and mechanisms of acute and chronic neuroinflammation after TBI. Lipid mediators have emerged as important signaling molecules in the initiation and maintenance of inflammatory processes.
Leukotriene-Mediated Inflammation

Bioactive lipids are important inter- and intracellular signaling molecules in the innate immune response. The 5-lipoxygenase (5-LO) pathway mediates the production of leukotrienes (LTs), a class of potent inflammatory lipid mediators made in leukocytes that contain a conjugated triene as part of their structure (Figure 1.3). The binding of DAMPS to their respective PRRs on the surface of innate immune cells (neutrophils, monocytes, basophils, eosinophils, and tissue specific macrophages i.e. brain microglia) increases the cytosolic calcium concentration, leading to the activation and translocation of cytosolic phospholipase A2α (cPLA2α) from the cytosol to the nuclear membrane where it cleaves arachidonic acid (AA) from glycerophospholipids (Leslie, 1990; Leslie, 2004). The release of AA is the first critical step in leukotriene formation. Like cPLA2, the enzyme 5-lipoxygenase (5-LO), translocates to the nuclear membrane in response to elevated intracellular calcium levels. There, it interacts with the 5-LO activating protein (FLAP) and oxidizes AA to its metabolites. The first enzymatic step is the abstraction of a hydrogen atom from C-7 of AA followed by the addition of molecular oxygen to form 5-hydroperoxyeicosatetraenoic acid (5-HpETE). The second enzymatic step is removal of a hydrogen atom from C-10, resulting in formation of the conjugated triene epoxide leukotriene A4 (LTA4) which is then enzymatically converted into either leukotriene B4 (LTB4) by LTA4 hydrolase or leukotriene C4 (LTC4) by LTC4 synthase. Metabolism of LTC4 occurs by sequential peptide cleavage reactions involving a γ-glutamyl transpeptidase that forms leukotriene D4 (LTD4) and a membrane-bound dipeptidase that converts LTD4 into leukotriene E4 (LTE4).
Figure 1.3. The 5-LO pathway of leukotriene biosynthesis.
Importantly, the synthesis of the cysteinyl leukotrienes (cysLTs: LTC₄, LTD₄, LTE₄) requires two cell types in a process called transcellular biosynthesis (Figure 1.4). As myeloid derived cells express 5-LO but not LTC₄-synthase, LTA₄ must be transported from the perinuclear region of the immune cell to another, non-immune cell that expresses LTC₄-synthase for cysLTs to be produced. For example, infiltrated neutrophils in the brain contain 5-LO and FLAP and are capable of making LTB₄ and the intermediate LTA₄. The LTA₄ is released from these cells and taken up by neighboring astrocytes that have LTC₄ synthase but silenced genes for 5-LO and FLAP. Astrocytes convert LTA₄ to LTC₄ and then subsequently in a step-wise fashion to LTD₄ and LTE₄ (Farias et al., 2007).

The biological activities of LTB₄ include neutrophil chemotaxis and sequential activation of downstream inflammatory responses. CysLTs are known to mediate vascular permeability, cytokine and chemokine production, and smooth muscle contractility. Leukotrienes exert their biological activities through g-protein coupled receptors (GPCRs); BLT-1 and BLT-2 receptors for LTB₄, and Cys-LT1, Cys-LT2, and Cys-LT3 receptors for the cysteinyl-leukotrienes. The rank order of potency for the Cys-LT1 receptor is LTD₄ >> LTC₄ > LTE₄ (Lynch et al., 1999) and for the Cys-LT2 receptor is LTD₄ = LTC₄ > LTE₄ (Nothacker et al., 2000). The recently discovered Cys-LT3 receptor (also referred to as GPR17) has the highest affinity for LTE₄ and also binds purinergic ligands (Maekawa et al., 2008). The Cys-LT1 receptor is primarily expressed in lung smooth muscle cells, interstitial lung macrophages, and spleen, and it is known to mediate airway inflammation and asthma. It is the molecular target of the receptor antagonists (i.e. montelukast, zafirlukast, and pranlukast) used for treating
Figure 1.4. Transcellular biosynthesis of leukotrienes.
asthma (Grossman et al., 1997; Reiss et al., 1998; Suissa et al., 1997). The Cys-LT2 receptor is predominately expressed in the heart, brain and adrenal glands. A selective Cys-LT2 receptor antagonist, HAMI3379, and a selective Cys-LT2 receptor agonist, N-methyl LTC$_4$ (NMLTC$_4$), have been recently reported (Wunder et al., 2010; Yan et al., 2011), which will help to identify the precise physiological and pathophysiological roles of the Cys-LT2 receptor. The receptor that binds both LTE$_4$ and purinergic ligands (Cys-LT3 or GPR17) is predominantly expressed in the brain and binds two receptor antagonists (AR-C69931MX and MR52179) selectively (Fumagalli et al., 2011; Ingall et al., 1999; Marteau et al., 2003).

The actions of cysteinyi leukotrienes have been studied primarily in the context of asthma. In the healthy brain, leukotriene levels are very low or absent. However, after acute injuries like focal ischemia (Ciceri et al., 2001; Dhillon et al., 1996) and TBI (Farias et al., 2009; Schuhmann et al., 2003), leukotrienes are markedly elevated in the brain. Our laboratory has shown that leukotrienes are produced rapidly after TBI by a transcellular process involving endogenous microglia or infiltrating neutrophils and non-immune brain cells (i.e. astrocytes, neurons). In rodents, leukotriene levels peak within 1-3 hours of injury and return to basal levels by 24 hour after injury (Farias et al., 2009; Voigt et al., 2012).

Consistent with the finding that leukotrienes are increased after TBI, leukotriene receptors (Cys-LT1 and Cys-LT2) are also up-regulated following brain injury (Fang et al., 2006; Hu et al., 2005; Zhang et al., 2004). The Cys-LT1 receptor has been reported to mediate increased permeability of the BBB, vasogenic brain edema, and astrocyte proliferation after brain ischemia, while the Cys-LT2 receptor is thought to regulate
cytotoxic brain edema after ischemic injury (Yu et al., 2005). In agreement with these findings, the Cys-LT1 receptor antagonist, pranlukast, was shown to decrease neutrophil infiltration, Immunoglobulin G (IgG) extravasation and lesion volumes when administered prior to ischemic brain injury (Chu et al., 2006). Additional studies are needed to understand the precise location and role of the leukotriene receptor subtypes in the brain.

**Thesis Aims**

Given that the primary injury of TBI is unavoidable and irreversible, the secondary injury phase provides a theoretical therapeutic window in which an intervention could effectively reduce the severity of secondary injury and prevent chronic impairments. The experiments in this thesis were designed to specifically investigate the hypothesis that leukotriene biosynthesis after TBI is initiated within minutes of the primary injury to activate neuroinflammatory signaling cascades, which ultimately contribute to the detriment of secondary injury and associated, chronic cognitive impairments. The goals of this thesis work are to further advance the understanding of neuroinflammation after TBI and to aid in the development of an effective treatment for attenuating secondary injury and preventing chronic impairments after TBI.
CHAPTER II

BLOCKING LEUKOTRIENE SYNTHESIS ATTENUATES THE PATHOPHYSIOLOGY OF TRAUMATIC BRAIN INJURY AND ASSOCIATED COGNITIVE DEFICITS

Abstract

Neuroinflammation is a component of secondary injury following traumatic brain injury (TBI) that can persist beyond the acute phase. Leukotrienes are potent, pro-inflammatory lipid mediators generated from membrane phospholipids. In the absence of injury, leukotrienes are undetectable in brain, but after trauma they are rapidly synthesized by a transcellular event involving infiltrating neutrophils and endogenous brain cells. Here, we investigate the efficacy of MK-886, an inhibitor of 5-lipoxygenase activating protein (FLAP), in blocking leukotriene synthesis, secondary brain damage, synaptic dysfunction, and cognitive impairments after TBI. Male Sprague Dawley rats (9-11 weeks) received either MK-886 or vehicle after they were subjected to unilateral moderate fluid percussion injury (FPI) to assess the potential clinical use of FLAP inhibitors for TBI. In most cases, MK-886 was also administered before FPI to determine the preventative potential of FLAP inhibitors. MK-886 given before or after injury significantly blocked the production of leukotrienes, measured by reverse-phase liquid chromatography coupled to tandem mass spectrometry (RP LC-MS/MS), and brain edema, measured by T2-weighted magnetic resonance imaging (MRI). MK-886 significantly attenuated blood-brain barrier disruption in the CA1 hippocampal region and deficits in long-term potentiation (LTP) at CA1 hippocampal synapses. The

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prevention of FPI-induced synaptic dysfunction by MK-886 was accompanied by fewer deficits in post-injury spatial learning and memory performance in the radial arms water maze (RAWM). These results indicate that leukotrienes contribute significantly to secondary brain injury and subsequent cognitive deficits. FLAP inhibitors represent a novel anti-inflammatory approach for treating human TBI that is feasible for both intervention and prevention of brain injury and neurologic deficits.

**Introduction**

Accumulating evidence of neurodegenerative pathology and progressive neurological dysfunction following repetitive concussion in high-impact sports (Jordan, 2013; McKee et al., 2013; Smith et al., 2013) and the rising number of TBI cases in war veterans exposed to explosive blasts (Taber et al., 2006; Warden, 2006) has increased public awareness of TBI. An estimated 1.7 million people in the United States suffer a TBI each year, but this estimate only includes injuries for which medical care is sought (Langlois et al., 2006; Faul et al., 2010). Because of this, TBI is considered a ‘silent epidemic’ as many mild TBI cases are unrecognized and unreported, and the magnitude of morbidity and mortality associated with these injuries has been largely underestimated. Advances in life support procedures have decreased the mortality rate of TBI, but many patients still face life-long physical and cognitive disabilities (Selassie et al., 2008). In the past several years, there has been increased interest in the diagnosis of mild TBI through advanced neuroimaging techniques and the use of plasma biomarkers. However, the development of drugs for blocking the detrimental consequences of TBI is lagging behind.
The pathophysiology of TBI is complex and heterogeneous. The primary injury at the time of trauma activates multiple pathways that lead to secondary injury days to weeks later (Gennarelli, 1993; Kochanek et al., 2009). The primary injury can present as any combination of skull fractures, intracranial hematomas, lacerations, contusions, and/or penetrating wounds. Secondary injury results from the activation of multiple pathways that lead to altered ionic balance, BBB permeability, edema, increased intracranial pressure, oxidative stress, neuronal cell death, and eventual neurologic impairment (Barkhoudarian et al., 2011). At the time of BBB disruption a neuroinflammatory response is activated that can persist for several weeks following TBI (Morganti-Kossman et al., 2007; Shlosberg et al., 2010). This disruption results from mechanical shearing of blood vessels at the time of injury and/or chemically mediated signaling cascades resulting in increased BBB permeability (Schmidt et al., 2005; Morganti-Kossman et al., 2007; Shlosberg et al., 2010). Infiltrating peripheral immune cells (i.e. leukocytes) activate resident astrocytes and microglia, which initiates pro-inflammatory signaling pathways that contribute to further BBB breakdown and brain edema (Streit et al., 2004; Schmidt et al., 2005; Morganti-Kossman et al., 2007).

Leukotrienes are potent bioactive lipids that are important mediators of inflammation (Murphy et al., 1979). Leukotriene biosynthesis is initiated by mechanical injury to cells or by calcium entry, which releases arachidonic acid (AA) from membrane glycerophospholipids (Folco et al., 2006). The enzymatic action of 5-LO and FLAP converts AA into leukotriene A₄ (LTA₄). LTA₄ is quickly converted to LTB₄ by LTA₄-hydrolase or to LTC₄ by LTC₄-synthase. LTC₄ can then be converted to LTD₄ and LTE₄, and these three LTs (LTC₄, LTD₄, LTE₄) are collectively known as the cysteinyli-
leukotrienes. The actions of cysteinyl leukotrienes have been studied primarily in the context of asthma where they are known to induce vascular permeability, extravasation of large molecules, stimulation of cytokine release, and contraction of bronchial smooth muscle (Boyce, 2007).

Leukotrienes are undetectable in the healthy brain (Farias et al., 2009). However, our laboratory has demonstrated that leukotrienes are rapidly produced after TBI by a transcellular mechanism involving infiltrating neutrophils and endogenous brain cells (Farias et al., 2007; Farias et al., 2009). To explore the role of leukotrienes in TBI and the clinical potential of using FLAP inhibitors, we investigated the efficacy of a commercially available FLAP inhibitor, MK-886, in reducing injury-induced leukotriene production, edema, BBB disruption, as well as hippocampal-related synaptic dysfunction and cognitive deficits. Our findings have important implications for treating human TBI and suggest that development of FLAP inhibitors for use in TBI is feasible for both intervention when administered shortly after TBI and prevention when administered to “at risk” individuals prior to potential brain trauma.

**Materials and Methods**

**Animals**

Adult male Sprague Dawley rats (9-11 weeks old, 250-300g; Harlan Laboratories) were housed individually in temperature- and light-controlled housing with free access to food and water ad libitum. All procedures as described were performed under protocols approved by the University of Colorado Institutional Animal Care and Use Committee and in compliance with National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*. A total of 134 animals were used in this study.
Lateral fluid percussion injury

Craniotomy and FPI were performed using a previously validated and published procedure (Farias et al., 2009; Frey et al., 2009). Briefly, animals were anesthetized with 3-5% isoflurane (Isosol, VEDCO Inc., St. Joseph, MO) via nose cone and mounted in a stereotaxic head frame. A 3 mm craniotomy was created and centered at 3 mm caudal to bregma and 3.5 mm left of the sagittal suture, keeping the exposed dura intact. One steel support screw was embedded in the skull on the contralateral side. A Luer-Lock hub (inside diameter 3.5 mm) was centered over the craniotomy and bonded to the skull with cyanoacrylate adhesive and capped. Dental acrylic (Snap, Parkell, Inc., Edgewood, NJ) was poured around the hub and screw. After the acrylic hardened, antibiotic ointment was applied around the cap, and animals were returned to their cages. The next day (15-20 hr later) animals were anesthetized with isoflurane in an induction chamber, immediately connected to the FPI apparatus, and received a 20 msec pulse of pressurized sterile saline (2.7 atm, moderate severity impact) on the intact dural surface before awakening from anesthesia. Sham-injured animals underwent craniotomy and were anesthetized and connected to the FPI apparatus, but they did not receive the fluid pulse. All animals received a subcutaneous injection of the analgesic, buprenorphine (0.05 mg/kg; Buprenex), prior to craniotomy, and subsequent injections every 12 hours for two days. Moistened food pellets were provided after injury, and all animals were monitored daily for well-being and weight changes.

Administration of MK-886 and vehicle

MK-886 was prepared at a concentration of 2.5 mg/ml, dissolved in DMSO and then diluted with 0.9% saline to 10% DMSO. Animals were briefly anesthetized with 3-
3.5% isoflurane and either MK-886 (6 mg/kg) or vehicle was administered intravenously (IV) by tail vein. All animals were allowed to wake before undergoing additional procedures.

**Measurement of leukotrienes in brain**

**Extraction of rat brain lipids**

Cortical and hippocampal regions from ipsilateral and contralateral hemispheres were collected in 4 ml of 80% methanol, homogenized with a Dounce homogenizer, and internal standards were added to the homogenates. Protein content was measured using BCA protein assay to normalize lipid levels to the amount of tissue. Samples were centrifuged and the supernatant was collected. Samples were diluted to a final methanol concentration of lower than 15% and then the lipids were extracted using a solid phase extraction cartridge (Strata C18-E, 100 mg/1 ml, Phenomenex, Torrence CA). The eluate (1 ml of methanol) was dried down and reconstituted in 70 µl of HPLC solvent A (8.3 mM acetic acid buffered to pH 5.7 with NH₄OH) + 20 ml of solvent B (acetonitrile/methanol, 65/35, v/v).

**RP LC-MS/MS**

An aliquot of each sample (35 µL) was injected into an HPLC system and subjected to reverse-phase chromatography using a C18 (Columbus 150 x 1 mm, 5 µm, Phenomenex) column eluted at a flow rate of 50 µL/min with a linear gradient from 25% to 100% of mobile phase B. Solvent B was increased from 25% to 85% by 24 min, to 100% by 26 min, and held at 100% for a further 12 min. The HPLC effluent was directly connected to the electrospray source of a triple quadrupole mass spectrometer (Sciex API 2000, PE- Sciex, Thornhill, Ontario, Canada) and mass spectrometric analyses were
performed in the negative ion mode using multiple reaction monitoring (MRM) of the specific transitions, $m/z \ 624 \rightarrow 272$ for LTC$_4$, $m/z \ 495 \rightarrow 177$ for LTD$_4$, $m/z \ 335 \rightarrow 195$ for LTB$_4$, $m/z \ 339 \rightarrow 197$ for d4-LTB$_4$, and $m/z \ 629 \rightarrow 277$ for d5-LTC$_4$. Quantitation was performed using a standard isotope dilution curve as previously described (Farias et al., 2007) with reference leukotriene standards and stable isotope analogs (Cayman Chemical, Ann Arbor, MI).

MRI

 Acquisition

All MRI studies were performed in the University of Colorado Animal Imaging Shared Resource (AISR) facility. Animals underwent MRI imaging at 72 hours after injury, using T2-weighted sequences. For all MRIs, the rats were anesthetized with 2.5% isoflurane. Scans were done using a 4.7 Tesla Bruker PharmaScan, and a quadrature birdcage coil (inner diameter 38 mm), tuned to the $^1$H frequency of 200.27 MHz, was used for RF transmission and reception. T2-weighted axial MR scans were acquired using a RARE (rapid acquisition with relaxation enhancement) sequence with the following parameters: FOV: 4.6cm; TE/TR: 32/5000 msec; slice thickness= 1.20 mm; interslice distance 1.20 mm (no gap); number of slices= 20; number of averages = 4 per phase encode step; matrix size= 128x256.

T2-weighted MRI analysis

For each rat, five slices (1.2 mm) spanning the entire area of injury were used to calculate FPI-related brain swelling. The diameter of the injured, ipsilateral hemisphere was measured from midline to the widest point of the cortex (Fiji/ImageJ, NIH). The difference between the ipsilateral (ipsi) and contralateral (contra) hemisphere diameters
was then calculated and normalized to the diameter of the contralateral hemisphere using the formula: {\((\text{diameter (Ipsi)} - \text{diameter (Contra)})/ \text{diameter (Contra)} \times 100\)}.

**Evans Blue administration and extravasation analysis**

One hour prior to FPI, animals received a 5ml intraperitoneal (IP) injection of EB solution (2% w/v in saline). Six hours post-FPI, animals were deeply anesthetized with sodium pentobarbital (50 mg/kg IP) and transcardially perfused with 200 ml ice-cold heparinized saline, followed by 100 ml freshly prepared 4% paraformaldehyde in PBS. Brains were removed and post-fixed in 4% paraformaldehyde/PBS for four hours at 4°C. Brains were then cryoprotected in 20% sucrose in PBS at 4°C, embedded in O.C.T. (Sakura Finetek USA Inc., Torrance, CA) and stored at -70°C. Whole brains were sectioned coronally at 30µm, and representative slices spanning the entire hippocampus at 270µm increments from each animal were mounted onto slides and cover-slipped with Fluoromount-G containing DAPI (SouthernBiotech, Birmingham, AL). Fluorescent images of whole brain sections were photographed using Surveyor by Objective Imaging software (Cambridge, UK) with a black and white Leica DFC 365FX camera on a Leica DM6000B microscope. A series of 10x images aligned in a grid was obtained using the multiscan setting. Images were stitched together in real time using the extended focus algorithm. Images of EB-positive hippocampal slices were captured using a Zeiss Axioplan2 microscope equipped with a HB0100w/2 lamp, a Photometrics CoolSnapfx camera (Roper Scientific), and IPLab software (BD Biosciences). Images from each slice were stitched together using Fiji/ImageJ (NIH), and EB-positive cells in the hippocampal cell layers were quantified using the cell counter tool.
Electrophysiology

Hippocampal slice preparation

Four days after FPI animals were sacrificed and the brains were rapidly removed and immersed in ice-cold, sucrose containing cutting buffer (in mM: 87 NaCl, 2.5 KCl, 7 MgCl₂, 0.5 CaCl₂, 1.25 NaH₂PO₄, 25 D-glucose, 35 sucrose, and 25 NaHCO₃) for 40-60 s to cool the interior of the brain. Transverse slices (400 µm thickness) were made using a McIlwain Tissue Chopper and the slices were stored individually for recovery (at least 60 min). After recovery, a single slice was transferred to a recording chamber and superfused with artificial cerebrospinal fluid (aCSF) at a bulk flow rate of 2-3 ml/min at 31°C. The aCSF contained the following (in mM): 126 NaCl, 3.0 KCl, 1.0 MgSO₄, 2.0 CaCl₂, 1.2 NaH₂PO₄, 11 D-glucose, and 25.9 NaHCO₃. A bipolar tungsten stimulating electrode was placed in the Schaffer collateral (SC) pathway to evoke synaptic field excitatory postsynaptic potentials (fEPSPs) recorded in the stratum radiatum using a nearby glass micropipette filled with aCSF.

Baseline recordings

Before each experimental run on a slice, an input-output curve was generated by increasing the stimulus voltage and recording the synaptic response until either a maximum was reached, or evidence of a population spike was observed on the fEPSP response. Also, a paired-pulse ratio (PPR) was run whereby pairs of stimuli were delivered to CA3 axons at an intrastimulus interval of 50 ms. PPR was quantitated as the amplitude of the second fEPSP / amplitude of the first fEPSP x 100.
Measurement of long-term potentiation

fEPSP responses were evoked with bipolar tungsten electrodes placed in the CA3 to CA1 dendritic field layer. Test stimuli were delivered once every 20 seconds with the stimulus intensity set to 40-50% of the maximum synaptic response. High-frequency stimulation (HFS) consisted of two trains of 100 Hz stimuli lasting 1 second each, with an inter-train interval of 20 seconds, at the control stimulus intensity. fEPSP recordings were made with a glass micropipette filled with aCSF and placed in the stratum radiatum approximately 200-300 µm from the cell body layer. This stimulation produced a potentiated response (LTP) that persisted for more than 60 minutes in control or sham animals. The slopes of fEPSPs were calculated as the slope measured between 10-30% from the origin of the initial negative deflection. Each time point shown is an average of six 20-second interval measurements.

Radial arms water maze testing

The RAWM consists of six 50 cm radial arms emanating from a circular area in a 160 cm diameter tank of 20.5°C water, surrounded by 4 walls, each with a unique pattern. An escape platform was situated at the end of one of the arms and submerged below the surface of black opaque water (non-toxic Dust Free Black Powder Paint, Rich Art). Rats were handled (2 min each) the day before craniotomy and three days after FPI. Training (Day 1; 4 days post-FPI) consisted of placing the animal in one of the arms and giving the animal a maximum of 60 seconds to find the platform in the goal arm. If the animal did not find the escape platform within 60 seconds, it was guided to the goal arm and allowed to stay on the platform for 15 seconds. Fifteen trials were administered with a five-minute inter-trial interval. The start arm for each trial was determined in a
pseudorandom fashion with three randomized sequences of the five non-goal arms. The start and goal arms were different for each rat, but equivocal relative to goal arm location, to avoid position and place preferences. Testing (Day 2) the following day consisted of 15 swim trials. The platform remained in the Day 1 goal arm for the first five trials and was then moved to a new arm for trials 6-15. The start arm for each trial was determined in a pseudorandom fashion so the animal did not start in the Day 1 goal arm until after all other arms (trial 10). Videos for each of the 30 trials per animal were analyzed using TopScan (Cleversys Inc.) tracking software for errors and perseverance duration. Errors are defined as entry into a non-goal arm or entry into goal arm without reaching the platform.

**Statistical analyses**

All data shown are mean +/- standard error of the mean unless otherwise noted. Results were analyzed in SPSS 20 (IBM) or Prism 5.0 (GraphPad). All analysis used two-tailed non-paired student’s t-tests for two groups, and one-way ANOVA for two or more groups followed by Tukey’s HSD for multiple comparisons unless otherwise noted. LTP I-O curves were analyzed with a two-way repeated measures ANOVA. RAWM day one learning curves were collapsed into groups of three swims and analyzed with a two-way repeated measures ANOVA followed by a one-way ANOVA of each collapsed time point. RAWM perseverance between trials one and three of the reversal task was expressed as a percentage of starting value (swim one=100%; swim three=swim three/swim one x 100) and analyzed with a within subjects two-way repeated measures ANOVA, followed by a two-tailed student’s paired t-test between the two trials. Alpha was set as p<0.05 to determine significance in all tests. Region specific outliers in the EB
study were identified by SPSS (>2 standard deviations from the mean) and eliminated from all analysis. In total three drug treated and one vehicle treated rat were removed.

Results

The effect of MK-886 on brain LTC₄ levels after FPI

Our previous results indicate that injury-induced leukotriene production is very rapid, peaking at 1-3 hours after FPI and declining to undetectable levels by 24 hours (Farias et al., 2009). To determine the efficacy of post-injury MK-886 administration in blocking leukotriene formation, rats were injected with a single dose of either MK-886 (6mg/kg in 0.9% sterile saline with 10% DMSO) or the same volume of vehicle 15 minutes after FPI. Ninety minutes after injury, the animals were euthanized and the levels of LTC₄ were measured in brain regions after extraction of lipids and analysis by RP LC-MS/MS (Figure 2.1A). The mean LTC₄ level (Figure 2.1B) in the ipsilateral hemisphere (23.41 +/- 1.98 pg/mg protein) was significantly higher (p <0.001, student’s t-test) than the contralateral hemisphere (7.92 +/- 1.02 pg/mg protein) of vehicle-treated animals. MK-886 reduced the levels of LTC₄ to below detectable threshold in both the ipsilateral and contralateral hemispheres (Figure 2.1B). To investigate the relative amount of LTC₄ production in injured ipsilateral cortex and hippocampus, these brain regions were dissected from injured rats prior to RP LC-MS/MS analysis. LTC₄ was detected in both the ipsilateral cortex (9.67 +/- 1.18 pg/mg protein) and hippocampus (6.05 +/- 3.70 pg/mg protein) of vehicle-treated rats. Similar to results in whole brain hemispheres, LTC₄ was undetectable in ipsilateral cortex and hippocampus of MK-886-treated rats (Figure 2.1C). These results demonstrate that the FLAP inhibitor, MK-886, effectively blocks leukotriene biosynthesis when administered after experimental TBI.
Figure 2.1. MK-886 blocks leukotriene synthesis after FPI. (A) Representative chromatograms from RP LC-MS/MS analysis of LTC₄ levels in the ipsilateral hemispheres of FPI-injured rat injected with either vehicle (left) or 6mg/kg MK-886 (right) 15 min after injury. (B) Quantitative analysis of LTC₄ levels in the ipsilateral (ipsi) and contralateral (contra) hemispheres in rats injected with either vehicle (-) or MK-886 (+) 15 min after injury. (C) Quantitative analysis of LTC₄ levels in ipsilateral cortex and ipsilateral hippocampus of rats treated with either vehicle (-) or MK-886 (+) 15 min after injury. Values are mean +/- SEM, n=4, n.d.= non-detectable. *p<0.05, different from ipsilateral hemisphere, student’s t-test. We acknowledge the assistance of Dr. Simona Zarini.
The effect of MK-886 on brain edema after FPI

T2-weighted MRI was used to investigate the effect of leukotrienes on TBI-related brain edema 72 hours after FPI. Animals were injected with MK-886 either 30 minutes before, 15 minutes after or 60 minutes after FPI. Additional groups of sham-injured and vehicle-injected rats were used as controls. The ipsilateral (left) and contralateral (right) brain hemispheres of sham rats showed no T2-weighted hyperintensity and were symmetrical in height and width (Figure 2.2A top panel). In contrast, the brains from FPI-injured rats consistently demonstrated T2-weighted hyperintensity and unilateral swelling in the ipsilateral hemisphere compared to the contralateral hemisphere (Figure 2.2A, bottom panel).

Ipsilateral hemispheric edema was quantified relative to the contralateral hemisphere (Figure 2.2B). Sham rats exhibited no difference between hemispheres in normalized brain swelling (0.00 +/- 0.03%). In contrast, injured rats given vehicle treatment had significantly more brain swelling than sham rats (vehicle=8.68 +/- 0.09%, p<0.001, one-way ANOVA followed by Tukey’s HSD) (Figure 2.2B). Rats receiving MK-886 either 30 minutes pre-injury, or 15 min post-injury had significantly lower swelling than vehicle-treated rats (30 pre=4.12 +/- 0.08%, p=0.004; 15 post=4.26 +/- 0.05%, p=0.028; Figure 2.2B). Rats injected with MK-886 60 min post-injury did not significantly differ from vehicle-treated rats (60 post=8.98 +/- 1.0%, p=0.999). These results demonstrate that blocking leukotriene production either before or shortly after TBI reduces the amount of injury-related brain swelling at 72 hours.
Figure 2.2. MK-886 attenuates edema after FPI. (A) Representative T2-weighted MRI images obtained 72 hours after FPI. The ipsilateral (left) and contralateral (right) hemispheres in the sham brain show no T2 pixel hyperintensity and are symmetric in shape and size. FPI brains demonstrate T2 pixel hyperintensity primarily in the ipsilateral cortex indicative of water content and unilateral swelling. (B) Quantitative MRI analysis of the mean normalized brain swelling was calculated from 5 continuous T2-MRI slices obtained from each animal using Fiji (NIH). Values are mean +/- SEM, Sham (n=4), Vehicle (n=8), MK-886 administered 30 min pre-injury (n=10), 15 min post-injury (n=5), and 60 min post-injury (n=8). Bar = 5mm. *p<0.05, different from vehicle FPI group, not significantly different from sham, one way ANOVA, followed by Tukey’s HSD.
The effect of MK-886 on BBB disruption after FPI

The effect of leukotrienes on TBI-related BBB disruption was assessed using EB fluorescence. EB (<1kDa) is an azo dye that has a strong affinity for serum albumin (67kDa) (Wolman et al., 1981). The resulting EB-albumin complex leaks into the parenchyma upon disruption of the BBB. We used fluorescence microscopy to image brain slices harvested five hours post-injury, taking advantage of EB’s intrinsic fluorescent properties (excitation 620nm, emission 680nm). This method is more sensitive than commonly used colorimetric readings of EB in brain homogenates (Uyama et al., 1988) and can be used to localize BBB permeability. As the EB-albumin complex is taken up by cells proximal to sites of permeability, this method allows for microscopic detection of BBB disruption (Loberg and Torvik, 1991). Consistent with this, the brightest EB signal was intracellular. Faint extracellular fluorescence was also observed surrounding EB-positive cell clusters. Not unexpectedly, the greatest density of EB-positive cells was in the ipsilateral cortex adjacent to the injury site and in the ipsilateral hippocampus, with some scattered cells in the thalamus and substantia nigra (Figure 2.3A). There was no EB detected in the corresponding contralateral brain regions. Since the hippocampus is relatively distant from the primary injury site and mediates memory and learning processes, we counted EB-positive cells in this area (Figure 2.3B). MK-886 administered 15 minutes after FPI significantly reduced the number of EB-positive cells in the CA1 region (FPI vehicle=148.75 +/- 45.65; FPI MK-886=69.25 +/- 36.82, p=0.035, student’s t-test) (Figure 2.3C,D) but did not statistically reduce EB-positive cells in CA2 (p=0.476) or CA3 (p=0.797). These results indicate that leukotrienes can mediate BBB permeability in selective brain regions vulnerable to injury.
Figure 2.3. MK-886 reduces BBB permeability in select regions of hippocampus. (A) Representative coronal cross-section of Evans Blue (EB, red) uptake in cells (DAPI, blue) in the ipsilateral hemisphere 5 hours after FPI. Bar = 500µm (B) Representative fluorescence images of EB uptake by hippocampal cell layers (DAPI) in the ipsilateral hippocampus 5 hours after FPI. Bar = 200µm (C) Higher magnification images of EB extravasation in the ipsilateral CA1 hippocampal cell layer in animals that received either vehicle or MK-886 15 minutes after FPI. Bar = 200µm (D) Quantitation of EB+ cells (EB-DAPI colocalization) in the hippocampal regions. Values are mean +/- SEM; n=4. *p<0.05, student’s t-test.
The effect of MK-886 on hippocampal synaptic plasticity after FPI

To examine the functional integrity of the hippocampus after FPI, electrophysiological measurements of LTP were recorded in hippocampal slices four days after FPI. LTP is a measure of synaptic plasticity and is thought to represent the molecular mechanisms underlying learning and memory. Synaptic fEPSP responses were evoked by stimulating the CA3 to CA1 Schaffer collateral pathway and recording from the CA1 dendritic field layer. There was no difference in the fEPSP input-output curves (F(4,381)=0.5329, p=0.992, two-way repeated measures ANOVA), nor in the paired pulse ratio measurements between sham and FPI-injured animals, (T(22)=1.883, p=0.073, student’s t-test), indicating similar levels of basal synaptic transmission for these groups (Figure 2.4A,B). This is consistent with our findings that there was no substantial hippocampal cell loss by H&E staining and no change in the levels of hippocampal neurofilament protein within one week of FPI (data not shown).

Hippocampal slices from sham animals exhibited robust LTP in response to high frequency stimulation (253.51 +/- 69.48%, 58-60 min, last 3 recorded time points). In contrast, hippocampal slices from uninjected FPI-injured animals failed to express LTP (135.06% +/- 42.62%, different from sham rats, p=0.007 one-way ANOVA of all groups included in Figure 2.5C followed by Tukey’s HSD) (Figure 2.4D). Similar to the uninjected FPI-injured animals, hippocampal slices from FPI-injured rats injected with vehicle also failed to exhibit LTP upon high frequency stimulation (Figure 2.5A). However, rats that received either an injection of MK-886 30 minutes before or 30 minutes after FPI demonstrated normal LTP. Both were significantly different from
Figure 2.4. FPI leads to marked deficits in hippocampal LTP. (A) Input-output (I-O) curves from sham (n=18) and FPI (n=18) rats prior to induction of LTP. Inset depicts representative signals obtained with successive increases in stimulation voltage (Scale bar x= 20 msec, y= 0.5 mV). There was no significant difference between injured and uninjured animals (two-way repeated measures ANOVA). (B) Paired-pulse responses (PPR) in slices from sham and FPI rats prior to LTP induction. Values are average % PPR +/- SEM (where % PPR = amplitude of peak 2/amplitude of peak 1 x100). Inset depicts representative signals from paired stimulations (50 ms interstimulus interval) including parameters (P1 and P2, peak amplitudes for the 1st and 2nd peaks, respectively) used to calculate % PPR (Scale bar x= 20 msec, y= 0.5 mV). PPR did not differ between injured and sham rats (student’s t-test). (C) LTP responses after high frequency stimulation at time 0 of two trains of 100 Hz (1 second each, train interval = 20s). Data are represented as a % of the control fEPSP slope. Each data point shown is an average of six 20-second interval measurements. Sham n=8, FPI n=9. Inset depicts a representative sham fEPSP. (D) Average LTP response in sham and FPI injured brain slices at 58-60 minutes after induction of LTP. Values are mean +/- SEM, Sham n=8, FPI n=8. *p<0.05, one-way ANOVA followed by Tukey’s HSD for all groups. We acknowledge the assistance of Dr. Ronald Freund.
Figure 2.5. MK-886 attenuates deficits in hippocampal long-term potentiation after FPI. (A) LTP measured over time in hippocampal slices from sham rats (black symbols, n=8) and FPI-injured rats injected with vehicle (grey symbols, n=7) or MK-886 (red symbols, n=6) 30 min prior to injury. (B) LTP measured in hippocampal slices from sham rats (black symbols, n=8) and FPI-injured rats injected with vehicle (grey symbols, n=7) or MK-886 30 minutes after FPI (green symbols, n=7) and 60 minutes after FPI (blue symbols, n=6). (C) Average LTP response (mean +/- SEM control slope) in all the groups measured at 58-60 minutes after induction of LTP. *p<0.05, **p<0.01, different from FPI vehicle, not significantly different from sham, one-way ANOVA followed by Tukey’s HSD. We acknowledge the assistance of Dr. Ronald Freund.
vehicle-injected rats (FPI vehicle= 130.86% +/- 55.63%, FPI MK-886 30 min pre-injury= 242.75 +/- 76.94%, p=0.032, FPI MK-886 30 min post-injury= 256.13 +/- 85.27%, p=0.007, one-way ANOVA followed by Tukey’s HSD) (Figure 2.5C). However, when MK-886 was delivered 60 min post-injury, the drug failed to prevent the LTP deficits observed in vehicle-treated rats (145.46 +/- 18.30, p=0.994). These results indicate that blocking early production of leukotrienes attenuates injury-induced deficits in rat hippocampal synaptic plasticity after injury and suggests a time window of less than one hour after injury for efficacy of MK-886 treatment in rodents.

The effect of MK-886 on memory and learning after FPI

To verify that injury-induced deficits in LTP reflect impairments in hippocampal-dependent spatial learning and memory, sham and FPI-injured animals treated with drug or vehicle were tested in a RAWM four and five days after FPI (Figure 2.6). The RAWM has an advantage over the Morris water maze in assessing cognitive impairments in rodents, in that the number of entries into an arm lacking the escape platform can be used as an assessment of learning, rather than latency to find the platform, thereby eliminating confounds induced by potential differences in swim speed among the experimental groups. Sham and FPI animals received an injection of either vehicle or MK-886 30 minutes post-injury. On the first day of behavioral testing, the animals completed 15 swim trials in which they used visual cues to navigate the maze to find a hidden escape platform in one of the arms (i.e. goal arm). There were no significant differences in initial task learning between the vehicle and MK-886 treated rats within the sham group or within the FPI group (F(3,155)=2.437, p=0.083, two-way repeated measures ANOVA)
Figure 2.6. MK-886 mitigates FPI-induced impairments in memory and learning in radial arms water maze. (A) Day 1 learning task. The average number of errors (mean +/- SEM) made during the indicated swims in sham rats treated with either vehicle (black circles, n=8) or MK-886 (open circles, n=8) and in FPI-injured rats treated with either vehicle (grey squares, n=9) or MK-886 (green squares, n=8) 30 minutes after injury. There were no significant differences in initial learning curves (one-way repeated measures ANOVA) and no differences between groups at any swim time point for each three-swim cluster (one-way ANOVA). (B) Day 2 reversal task perseverance. The change in perseverance (duration in previous goal arm) at swim 3 expressed as the percentage of perseverance in swim 1. *p<0.05, **p<0.01, within-subjects two-way repeated measures ANOVA followed by paired student’s t-test. (C) Day 2 post-perseverance reversal task performance. Errors (mean +/- SEM) made in swims 11-15 of the reversal task. *p<0.05, **p<0.01, different from FPI vehicle, no difference from either sham group, one-way ANOVA followed by Tukey’s HSD. We acknowledge the assistance of Dayton Goodell.
and no significant differences between groups for any cluster of 3 swims, (one-way ANOVA for each swim cluster) (Figure 2.6A). On the second day of behavioral testing, animals completed five swim trials in the maze with the goal arm in the same position as the previous day. The escape platform was then moved to a new location for ten more trials in order to assess the ability of the animals to learn and remember a new goal arm location (reversal task). In the first three trials of the reversal task, perseverance for the previous goal arm was measured as the percent of total swim duration spent in the arm where the platform used to be. On the first swim, there were no differences in perseverance between any of the groups (F(3,31)=0.713, p=0.522, one-way ANOVA). Both sham groups quickly learned between swims 1 and 3 that the platform was no longer in the previous goal arm. Likewise, FPI rats treated with MK-886 spent less time in the previous goal arm by swim 3 (overall interaction, injury (sham v. FPI) x drug (MK-886 v. vehicle) x trial (swim one to swim three), F(1,62)=5.564, p=0.025, within subjects two-way repeated measures ANOVA followed by student’s paired t-test, sham vehicle, p=0.001; sham MK-886, p=0.006; FPI MK-886, p=0.035). However, FPI rats that received a vehicle injection failed to learn that the platform location had changed between swims 1 and 3 (p=0.758) (Figure 2.6B). The last five swim trials of the reversal task (swims 11-15) were used for assessment of continued learning. Vehicle-treated FPI rats continued to make significantly more errors per swim than sham rats given either drug or vehicle (FPI vehicle= 2.33 +/- 1.21; sham vehicle= 0.755 +/- 0.705, p=0.003; sham MK-886= 0.850 +/- 0.583, p=0.005; one-way ANOVA followed by Tukey’s HSD) (Figure 2.6C). FPI rats given MK-886 did not differ from either sham group and were
significantly different from FPI-vehicle rats (1.26 +/- 0.700, p=0.044). These data indicate that MK-886 attenuates injury-induced deficits in spatial learning and memory.

Discussion

FLAP inhibitors target early leukotriene-mediated inflammatory events that are initiated and subsequently amplified by brain trauma. In our study MK-886 effectively reduced brain leukotrienes to levels below the detectable threshold of RP LC-MS/MS. Blocking this inflammatory cascade resulted in an attenuation of brain swelling, reduced BBB damage in area CA1 of the hippocampus, and restored both synaptic (LTP) and behavioral (RAWM) learning and memory to sham levels. Together, these results demonstrate that FLAP inhibitors represent a promising new approach toward mitigating brain damage and neurological deficits resulting from TBI. There is little published data on the role of leukotrienes in the brain due to the difficulty in measuring leukotrienes and a lack of verified antibodies. A few reports demonstrate efficacy of leukotriene receptor antagonists and FLAP inhibitors in reducing detrimental outcomes induced by head injury (Kiwak et al., 1985; Dhillon et al., 1996; Schuhmann et al., 2003; Farias et al., 2009; Voight et al., 2012) or brain ischemia (Minamisawa et al., 1988; Ciceri et al., 2001) when these drugs were administered prior to injury. We demonstrate, for the first time, protective effects of FLAP inhibitors on injury-related outcomes when administered both before and after injury.

FLAP was discovered in the late 1980s and early 1990s in screens for leukotriene inhibitors. Shortly after the discovery of FLAP, inhibitors including the indole MK-886, the quinoline BAY X1005, and the quinoline–indole MK-591 were developed and tested in human trials of asthma (Friedman et al., 1993; Diamant et al., 1995; Dahlen et al.,
All demonstrated good safety profiles and efficacy in blocking leukotrienes but were discontinued when the leukotriene receptor antagonists [zafirlukast (Accolate™), montelukast (Singulair™) and pranlukast (Onon™)] and the 5-LO inhibitor zileuton (Zyflo™) were brought to market and approved for treating asthma.

Consistent with our finding that leukotrienes are increased after TBI, leukotriene receptors (cys-LT1 and cys-LT2) are also up-regulated following brain injury (Zhang et al., 2004; Hu et al., 2005; Fang et al., 2006; Ding et al., 2007). The cys-LT1 receptor has been reported to mediate increased permeability of the BBB, vasogenic brain edema, and astrocyte proliferation after brain ischemia, while the cys-LT2 receptor is thought to regulate cytotoxic brain edema after ischemic injury (Wang et al., 2006). In agreement with these findings, the cys-LT1 receptor antagonist, pranlukast, was shown to decrease neutrophil infiltration, IgG extravasation and lesion volumes when administered prior to cold-induced brain injury (Qian et al., 2006) and ischemic brain injury (Yu et al., 2005; Chu et al., 2006). Another cys-LT1 receptor antagonist, montelukast, was reported to decrease BBB permeability and neutrophil infiltration after diffuse brain injury when administered pre-injury (Biber et al., 2009). A caveat of these studies is the lack of specific antibodies to leukotriene receptors and the cross-reactivity of the leukotriene antagonists. Although the receptor antagonists have been used successfully in treatment of asthma for almost 25 years, with the discovery of multiple leukotriene receptors (the cysLT-2 and cys-LT3 receptors were discovered after the cys-LT1 selective antagonists were developed) and increasing evidence that leukotrienes mediate other inflammatory conditions (Evans et al., 2008), there has been renewed interest in FLAP inhibitors. As FLAP inhibitors block the synthesis and action of all three cysteinyl-leukotrienes (LTC₄,
LTD$_4$, and LTE$_4$) as well as LTB$_4$, they are predicted to have greater efficacy compared to the existing receptor antagonists in blocking inflammatory disorders. Additional studies and more selective antibodies are required to understand the receptor-specific actions of leukotrienes.

In the present study, MK-886 administration attenuated EB extravasation detected by fluorescence microscopy in the brain parenchyma distant from the injury site. Other investigators reported that blocking leukotrienes prior to head injury either had no effect (Schuhmann et al., 2003) or mitigated (Qian et al., 2006; Ding et al., 2007; Biber et al., 2009) BBB permeability. An important factor that could explain these apparent discrepancies is the contribution of mechanical injury to blood vessels following a TBI. Mechanical injury to blood vessels could mask an effect of leukotrienes or other neurochemicals on regulating BBB permeability. Kenne et al. (2012) reported a similar conclusion regarding the potential masking of neurochemical regulation of BBB permeability by mechanical damage to blood vessels. In their study, neutrophil depletion (which reduces leukotriene production (Farias et al., 2009)), reduced vasogenic edema and tissue loss, but had no apparent effect on BBB disruption following CCI in mice (Kenne et al., 2012). Our finding that MK-886 selectively decreased EB accumulation in the CA1 region of the hippocampus, which is furthest away from the injury site, suggests neurochemical rather than mechanical BBB disruption in this region.

This study demonstrates for the first time that blocking the early production of leukotrienes after TBI leads to fewer deficits in hippocampal synaptic plasticity and hippocampal-mediated memory and learning. In our study, the deficits in hippocampal LTP following TBI were observed despite normal basal synaptic responses and no overt
hippocampal cell loss, suggesting that leukotrienes likely impair synaptic plasticity by altering synaptic signaling rather than as a by-product of cell loss. Neuronal mechanisms that could result in LTP deficits without loss of neurons involve changes in the morphology or density of hippocampal dendritic spines as well as changes in the levels or activity of postsynaptic density proteins known to regulate LTP. A calcineurin-dependent loss of dendritic spines in rat forebrain has been reported after fluid percussion injury that was followed by an eventual overgrowth of spines (Campbell et al., 2012a; Campbell et al., 2012b). Others have shown decreased hippocampal levels of calcium/calcmodulin dependent protein kinase II (CaMKII) and activated CaMKII, a protein kinase that regulates LTP by modulating the phosphorylation state and insertion of AMPA receptors in postsynaptic membranes after TBI (Atkins et al., 2006; Schwarzbach et al., 2006; Folkerts et al., 2007). A recent report by Hartig et al. (2013) shows CD43+ inflammatory cells in the dentate gyrus and CA3 region 24 hours after brain trauma, and MK-886 treatment reduced the number of inflammatory cells at this time point. Future studies involving more detailed investigation of mechanisms that regulate synaptic plasticity after TBI are needed to elucidate the role of leukotriene mediated inflammation in memory deficits. These studies will help provide important insight into the cognitive and executive disabilities that occur after all types of human brain injury including TBI.

Leukotrienes are produced rapidly after brain injury, peaking within 1-3 hours of injury, and returning to basal levels by 24 hour after injury (Farias et al., 2009; Voight et al., 2012). The early production of leukotrienes after head trauma dictates the therapeutic window for FLAP inhibition. In the current studies, MK-886 was efficacious in blocking multiple outcomes of TBI in rats when given 30 minutes after injury. This scenario for
intervention is not that different from the therapeutic window of tissue plasminogen activator (t-PA) used in stroke patients (Saver et al., 2013). Because several inflammatory pathways are amplified immediately after injury, early intervention is essential for an optimal therapeutic outcome. On the other hand, Schuhmann et al. (2003) reported a second phase of leukotriene production at 7 days after experimental TBI. It is intriguing to speculate that a second phase of leukotriene production after TBI could mediate reopening of the BBB and a chronic phase of inflammation. If proven true, the second phase of leukotriene production should also be amenable to FLAP inhibition.

In addition to using FLAP inhibitors shortly after brain injury to block or mitigate secondary injury, FLAP inhibitors have a potential preventative role in TBI. Individuals at high risk for head injury, including athletes in high contact sports and military personnel in combat scenarios, could be given FLAP inhibitors chronically or right before an event that predisposes them to risk of head trauma. Second generation FLAP inhibitors have longer half-lives and could theoretically be administered once daily for protection against head injury. FLAP inhibitors have several feasible routes of administration including oral, intravenous, intraperitoneal, and possibly nasal (currently under investigation) and thus far, have no reported toxicity or deleterious side effects in humans. Thus, this class of anti-inflammatory agents are promising new drug candidates for interventional therapy and prevention of functional brain deficits after TBI.
CHAPTER III

PROLONGED NEUROINFLAMMATION AFTER A SINGLE CLOSED HEAD INJURY IS MEDIATED BY THE 5-LIPOXYGENASE PATHWAY AND DOES NOT REQUIRE PERIPHERAL IMMUNE CELL INfiltrATION

Abstract

Traumatic brain injury (TBI) is a major public health concern. It is a major cause of death and disability in all age groups and there is growing evidence that even mild TBIs (mTBI) like concussions can result in long-lasting pathological changes to the brain associated with neurologic and behavioral disabilities. However, the cellular mechanisms underlying these changes are largely unknown. The current study sought to investigate the spatiotemporal neuroinflammatory response after mTBI and examine the hypothesis that the 5-lipoxygenase (5-LO) inflammatory pathway mediates the brain’s inflammatory response to injury and the associated degenerative pathology after mTBI. Results showed that mice subjected to a single closed head injury (CHI) model of mTBI had no macroscopic lesions by histologic staining and MRI and that the blood-brain barrier remained intact. However, CHI resulted in a marked increase in activated microglia and reactive astrocytes, which was evident within two hours of injury and persisted in select brain regions for at least 30 days. Neuronal and axonal damage was detected at 7 days post-injury in the same brain regions that showed elevated neuroinflammatory indicators. The acute and prolonged neuroinflammatory response to mTBI and the corresponding neuronal degeneration were blocked by administration of MK-591, an inhibitor of the 5-LO Activating Protein (FLAP), a key enzyme in the 5-LO pathway of leukotriene

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biosynthesis. These findings indicate that the 5-LO pathway initiates and maintains an endogenous immune response to brain injury that leads to delayed neuronal degeneration. Blockade of this pathway by FLAP inhibitors is a promising intervention to test in human clinical trials of mTBI.

Introduction

Traumatic brain injury (TBI) is a serious public health problem with 1.7 million reported cases per year in the United States (Faul et al., 2010). The incidence of TBI is greater than that of multiple sclerosis, spinal cord injury, HIV/AIDS, and breast cancer combined. The economic burden of TBI is estimated to be $13.1 billion in direct costs associated with treatment and an additional $64.7 billion in indirect costs due to missed work and reduced productivity (Ma et al., 2014). More than 75% of TBI cases are classified as mild (mTBI), which includes concussions and blast injuries (CDC, 2003; Faul et al., 2010). It is estimated that the majority of mTBI cases are undiagnosed or undetected, meaning that the number of mTBI cases is roughly three times that of the moderate to severe TBI cases that receive medical care. Most post-concussive symptoms (e.g. headache, fatigue, dizziness, sleep disturbances, cognitive impairments) resolve within a month after injury. However, some individuals have persistent symptoms that do not fully resolve and lead to long-term disabilities, a condition referred to as post-concussive syndrome. Additionally, recent studies suggest that repetitive mTBI leads to a neurodegenerative disorder called chronic traumatic encephalopathy (CTE) that is characterized by widespread neurofibrillary tangles and beta-amyloid plaques throughout the brain (Jordan et al., 2014; McKee et al., 2009; McKee et al., 2013).
The neuropathological mechanisms underlying the short- and long-term consequences of mTBI are largely unknown. However, it is generally believed that the initial impact, or primary injury, induces mechanical damage to neuronal axons and blood vessels, which activates a cascade of neurobiological events, leading to the secondary injury (Gennarelli et al., 1993; Prins et al., 2013a). Diffuse axonal injury, resulting from shearing forces at the time of injury, causes disruption of neuronal networks leading to deficits in executive function, memory, and behavior (Armstrong et al., 2015; Browne et al., 2011). Vascular damage at the time of TBI allows peripheral immune cells, which are normally kept out of the brain by tight endothelial junctions of the blood-brain barrier (BBB), to infiltrate the brain where they interact with endogenous cells (i.e. microglia and astrocytes) to promote the release of inflammatory cytokines and chemokines and mediate further disruption of the BBB (Alluri et al., 2015; Iadecola et al., 2007; Morganti-Kossman et al., 2007; Schmidt et al., 2005; Streit et al., 2004).

In the healthy brain microglia are in a ‘resting’ state with a ramified cellular morphology and are constantly surveying the surrounding environment for disruptions in homeostasis. Microglia are considered the first line of defense after TBI, becoming quickly activated in response to damage-associated molecular pattern (DAMP) molecules, like reactive oxygen species, ATP, and heat shock proteins (Colton, 2009; Hanisch et al., 2007). Upon activation microglia transform to a hypertrophic, amoeboid-like morphology that enhances their migratory and phagocytic activities (Hanisch et al., 2007). Activated microglia produce lipid mediators within minutes, and later cytokines and chemokines, which recruit other immune cells that enhance the inflammatory response (Gebicke-Haerter et al. 2001, Karve et al. 2015). Prolonged activation of
microglia leads to a dysregulated state that triggers a vicious, self-perpetuating cycle of damaging events, which drive pathogenic processes and degeneration (Loane et al., 2014; Perry et al., 2010; Perry et al., 2014; Tanaka et al., 2014).

At rest, astrocytes function to maintain physiological homeostasis by regulating synapse number and function, recycling neurotransmitters, and regulating cerebral blood flow through their astrocytic end feed that surround endothelial cells of capillaries in the brain (Iadecola et al., 2007; Pekny et al., 2014; Sofroniew et al., 2010). Astrogliosis is a universal response to tissue damage in the central nervous system, whereby astrocytes proliferate and take on a ‘reactive’ hypertrophic morphology that involves the upregulation of intermediate filament proteins like glial fibrillary acidic protein (GFAP) and vimentin (Pekny et al., 2014). Reactive astrocytes produce and secrete inflammatory mediators and growth factors that act in autocrine or paracrine fashion to coordinate an inflammatory response among the surrounding cells. A prominent feature of reactive astrocytes is their ability to proliferate and migrate toward lesioned tissue and form a glial scar that functions as a physical barrier between healthy and damaged tissue (Sofroniew et al., 2009). While the defensive response of astrocytes is conceivably aimed at limiting tissue damage and restoring brain homeostasis, persisting reactive astrogliosis can become maladaptive and may inhibit regenerative responses (Pekny et al., 2014).

We have recently discovered that leukotrienes (LTs: LTB$_4$, LTC$_4$, LTD$_4$, LTE$_4$), potent inflammatory lipid mediators derived from arachidonic acid (AA), are produced within minutes of a moderate-to-severe brain injury and contribute to injury-related BBB disruption, edema, and impairments in memory and learning (Corser-Jensen et al., 2014). LT$s$ are absent in healthy brain, but their biosynthesis is initiated after mechanical injury
by calcium entry into cells, which releases arachidonic acid (AA) from membrane glycerophospholipids. The enzymatic action of 5-lipoxygenase (5-LO) and the 5-LO Activating Protein (FLAP) converts AA into leukotriene A4 (LTA₄). LTA₄ is quickly converted to LTB₄ by LTA₄-hydrolase or to LTC₄ by LTC₄-synthase. LTC₄ can then be converted to LTD₄ and LTE₄, and these three LTs (LTC₄, LTD₄, LTE₄) are collectively known as the cysteinyll-leukotrienes. Until recently, the actions of cysteinyll leukotrienes have been studied primarily in the context of asthma, where their overproduction is a major cause of bronchial smooth muscle contractions, increased vascular permeability and subsequent extravasation of large molecules, leukocyte recruitment, and cytokine release (Boyce et al., 2007). Our studies have shown that leukotrienes contribute significantly to secondary brain injury and the subsequent cognitive deficits following moderate to severe experimental TBI, which are attenuated by administering a FLAP inhibitor to block leukotriene production.

The goals of the current study were (1) to investigate the spatial and temporal nature of neuroinflammation (i.e. activated microglia and reactive astrocytes) after a single mTBI and (2) to test the hypothesis that blocking leukotriene production with the FLAP inhibitor, MK-591, will interrupt both acute and prolonged inflammation after mTBI and promote restoration of brain function. Our findings have important implications for understanding the brain’s neuroinflammatory response to injury and provide further support for FLAP inhibitors as a targeted interventional therapy to block progressive damage after brain injury.
Materials and Methods

Animals

All experiments were performed on adult male wild type C57BL/6J mice (10-12 weeks old, 20-25g; Jackson Laboratories, Bar Harbor, ME) that were housed individually in temperature- and light-controlled housing with free access to food and water ad libitum. Mice were allowed to acclimate to the vivarium for one week prior to experimental procedures. All procedures as described were performed under protocols approved by the University of Colorado Institutional Animal Care and Use Committee and are in compliance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Lateral closed head injury

Experimental closed head injury was performed using the Impact One (Leica), an electromagnetically controlled stereotaxic cortical impact device for precise, graded experimental TBI. Mice were placed on a heating pad and anesthetized with 2.0 L/min and 3% isoflurane, which was maintained throughout the procedure. After confirmation of sufficient depth of anesthesia, the head was shaved and the skull was exposed by a 2 cm longitudinal midline scalp incision in order to have a direct impact of the tip onto the skull. Mice were then placed in a stereotaxic frame with an incisor bar at 0° and cup head holders. Next, the impact device, mounted on the left stereotaxic arm at an angle of 20° from vertical, was rotated into the field. The 5-mm blunt metal impact probe was centered between bregma and lambda on the left side of the sagittal suture and then lowered until the impact probe contacted the skull, detected by a contact sensor. From this zero depth position, the injury impact depth was determined by lowering the tip 3
mm for a mild injury. The tip was then retracted, and the injury was triggered using the myNeuroLab controller at an impact velocity of 5 m/s and dwell time of 100 msec.

Sham-operated animals underwent the same procedures and anesthesia duration but did not receive an impact. All animals received an intraperitoneal injection of the analgesic, buprenorphine (0.05 mg/kg; Buprenex), prior to injury and subsequent injections every 12 hours for three days post-injury.

Assessment of traumatic apnea, righting reflex, and motor function

After impact, the mice were allowed to regain a normal breathing pattern without assistance, measured as apnea time. Once a regular breathing pattern was achieved, the mice were sutured and placed on 100% oxygen until they regained a righting reflex and returned to an upright position, which was recorded as righting time. One hour after injury, animals were placed on a mesh grid that was inverted, and the duration to fall off the grid was timed and recorded as the latency to fall measurement of grip strength.

Blood collection and measurement of plasma UCHL-1 and GFAP

Animals were euthanized by 1.5% CO₂, and blood was collected by cardiac puncture (22G x 1). The needle was removed and blood was transferred to heparinized centrifuge tubes. Tubes were inverted three times and placed on ice for less than 2 hours until centrifugation. Plasma was prepared by centrifugation of whole blood (7000 rpm (1500 g) for 15 min) at 4°C and stored at -80°C. Levels of mouse plasma ubiquitin C-terminal hydrolase L1 (UCH-L1) were determined on protein-containing supernatants by sandwich UCH-L1 ELISA kit (Cloud-Clone Corp SEG945Hu) using the manufacturer’s protocol. Levels of mouse plasma glial fibrillary acidic protein (GFAP) were determined by sandwich GFAP ELISA kit (Millipore NS830) using standard procedures. Signals
were detected with a multiwell plate reader at 450 nm and reported as the mean +/- SEM of the results of two replica wells in picograms/mL plasma UCH-L1 and nanograms/mL plasma GFAP.

**T2-weighted magnetic resonance imaging (MRI)**

**Image acquisition**

All MRI studies were performed in the University of Colorado Animal Imaging Shared Resource (AISR) facility. Animals were anesthetized with 2% isoflurane and placed onto a mouse holder. A Bruker 4.7 Tesla/ 16-cm MRI/MRS PharmaScan (Bruker Medical, Billerica, MA) with a mouse volume transmitter/ receiver coil (36 mm diameter, tuned to 200 MHz for $^1$H) was used for all MRI scans. After obtaining localizer images, fast spin echo RARE (rapid acquisition with relaxation enhancement) T2 scans were obtained. The scan parameters were: FOV: 4.00 cm; slice thickness: 1.0 mm; inter-slice distance: 1.0 mm; TR/TE: 4000/80 ms; number of slices: 20; number of averages: 4; flip angle: 180 degrees; matrix size: 256x256; total acquisition time: 8min 32sec.

**Image analysis**

The calculation of edema was performed using Fiji (ImageJ) by manually placing three regions of interest (ROI; 500x500 µm) in the ipsilateral cortex above the hippocampus and one ROI in the background of the image (1x1 mm). The difference between the ipsilateral (ipsi) and background (bcg) average pixel intensity (PI) from all three ROIs was then calculated and normalized to the pixel intensity of the contralateral ROIs using the formula: $\{\frac{PI_{ipsi} - PI_{bcg}}{PI_{bcg}}\}$. 
Tracer injections and analysis of blood-brain barrier disruption

Blood-brain barrier disruption was assessed at the following post-injury time points: 0, 1, 4, 8, 24, 48hr. Animals were injected with a 1 mL intraperitoneal (IP) injection of Evans Blue solution (2% w/v in saline), which was allowed to circulate for one hour. At their designated time point, animals were deeply anesthetized with sodium pentobarbital (50 mg/kg IP) and transcardially perfused with 50 mL ice-cold heparinized saline, followed by 50 mL freshly prepared 4% paraformaldehyde in PBS. Brains were removed and post-fixed in 4% paraformaldehyde at 4°C. 6µm thick coronal brain sections were mounted on slides and coverslipped, and extravasated Evans Blue was assessed using fluorescence microscopy (Nikon Eclipse 80i Upright Microscope).

A separate cohort of mice was injected IP with two different tracers, 1kDa Alexa Fluor 647 Cadaverine (0.5 mg/20 gr, 100 µl; Invitrogen) and 70 kDa lysine fixable anionic fluorescein dextran (2.5 mg/20 gr, 100 µl; Invitrogen). Tracers were allowed to circulate for one hour prior to euthanization at the indicated post-injury timepoints. Mice were perfused and brains were processed in the same manner as for histology procedures. 6µm thick coronal brain sections were mounted on slides and coverslipped with ProLong Diamond Antifade Mountant (Life Technologies) and visualized by fluorescent microscopy.

Immunohistological staining and quantification

Staining procedures

Animals were deeply anesthetized with sodium pentobarbital (50mg/kg IP) and transcardially perfused with 50 ml ice-cold heparinized PBS, followed by 50 ml freshly prepared 4% paraformaldehyde in PBS. Brains were removed and post-fixed in 4%
paraformaldehyde at 4°C. Brain tissue was then cut into 4 coronal blocks and embedded in paraffin. 6µm thick sections were cut from each block and mounted on slides. One slide from each animal was stained with hematoxylin and eosin (H&E) according to standard procedures.

For analysis of blood vessel integrity, sections were de-paraffinized with decreasing concentrations of alcohol followed by antigen retrieval (15 min in steamer using 0.01M citric acid buffer). Sections were then incubated in 10% lamb serum with 0.01% triton-x for 1 hour followed with a 1 hour incubation in primary (2% lamb serum in PBS) solution containing antibody rabbit anti-Glut-1 (1:200, Thermo Scientific cat#: RB-9052) and mouse anti-GFAP (1:100, Cell Signaling Technology cat#: 3607). Following repeated washes in PBS, sections were incubated in the appropriate Alexafluor-conjugated secondaries (1:1000; Invitrogen) and co-labeled with DAPI to identify nuclei. For analysis of Glut-1 vessel density, stained sections were imaged using a Zeiss 780 LSM confocal microscope. Specifically, 10x magnification tile scans were obtained of the lesion site (demarcated with increased GFAP immunoreactivity) and a similar area of the cortex in the non-injured contralateral hemisphere of the same section. Sum of vessel lengths in the lesion site and contralateral, non-injury hemisphere were divided by the area analyzed to obtain a vessel density. Analysis was performed on 3 animals for each time point (n=3).

For myeloperoxidase (MPO) staining of neutrophils, sections were first de-paraffinized in xylene and hydrated through graded alcohol concentrations. Antigen retrieval was achieved by heating slides in a high pH 9.0 FLEX Tris/EDTA buffer (Dako K8004). A primary polyclonal rabbit antibody was used (Abcam αMyeloperoxidase
Ab9535), followed by EnVision+ Rabbit HRP (Dako K4011) and DAB (Dako 4007), and counterstained with hematoxylin (Dako S3301). For both Iba-1 and GFAP immunohistochemical staining of activated microglia and reactive astrocytes, respectively, sections were deparaffinized in xylene and hydrated through graded alcohol concentrations. Slides for Iba-1 were heated in a citrate buffer (Dako FLEX Citrate Low pH 6.1) and slides for GFAP staining were treated with proteinase K for antigen retrieval. Primary polyclonal rabbit antibodies were used for both Iba-1 (Biocare Medical CP290A, 1:200) and GFAP (Dako GFAP Z0334, 1:2000). Immunostaining procedures were performed on a DAKO Autostainer according to manufacturer’s instructions using the DAKO Envision+ Rabbit HRP (Dako K4003) and diaminobenzidine (DAB, Dako K3468). After immunostaining, sections were counterstained with hematoxylin and coverslipped. For Fluoro-Jade B (FJB) staining of degenerating neurons, sections were first deparaffinized in xylene and hydrated through graded alcohol concentrations and rinsed in water. They were then transferred to a solution of 0.06% potassium permanganate and then to a 0.0004% FJB working solution. After washing, the sections were air-dried and coverslipped.

**Histological analysis**

H&E-, MPO-, Iba-1- and GFAP-stained slides were digitally scanned using an Aperio ScanScope XT slide scanner (Aperio Technologies, Vista CA). GFAP- and Iba-1-positive cells were quantified using the positive pixel-count algorithm within the ImageScope software program (Aperio Technologies). Briefly, brown color of staining (DAB) is specified by range of hues and saturation. For pixels within a defined region of interest (ROI) that satisfy the color specification, the algorithm counts the number and
intensity sum of positive pixels in three intensity ranges: weak (220-175), moderate (175-100), and strong (100-0). Pixels that are stained but do not fall into the positive-color specification are considered negative stained pixels so that a total number of stained pixels in an ROI is determined. For analysis, we calculated pixel positivity as the ratio of strong positive pixels to the total number of pixels in the ROI. Pixel positivity was calculated for each animal on coronal brain sections in three defined ROIs: ipsilateral cortex (average of three 500x500 µm ROIs above hippocampus), ipsilateral corpus callosum (traced), and ipsilateral dentate gyrus (traced). Pixel positivity (PP) for drug effect was calculated as a percent above sham using the following formula \(((PP_{\text{CHI}} - PP_{\text{sham}}) * 100) – 100\).

**Drug administration**

MK-591 was prepared at a concentration of 5 mg/ml, dissolved in DMSO and then diluted with 0.9% saline to 20% DMSO. MK-591 was administered intraperitoneally 30 minutes prior to injury and every 24 hours for the next six days for a total of seven injections.

**Statistical analyses**

All data shown are presented as mean +/- standard error of the mean unless otherwise noted. All statistics were performed using Prism 6.0 (GraphPad). A p-value of <0.05 was considered significant in all tests. All analyses used a two-tailed, non-paired Student’s t-test for two groups, and Mann-Whitney test was used when normal distribution could not be assumed.
Results

Acute physiological responses

To evaluate the effects of mTBI on acute physiologic responses, apnea and righting times were compared between sham and CHI groups. Compared to sham mice that had no apnea, CHI mice experienced a significant period of apnea after TBI (106.1 +/- 11.39 sec; p<0.0001, Mann-Whitney test; Figure 3.1A). Additionally, the amount of time to elicit a righting reflex response was significantly longer in the CHI group than the sham group (Sham=68.5 +/- 7.2 sec, CHI=534.4 +/- 24.7 sec; p<0.0001, Mann-Whitney test), indicative of the duration of unconsciousness (Figure 3.1B). When assessed for grip strength by measuring the latency to fall from a suspended inverted grid one hour after injury, CHI mice had a significantly shorter latency (7.8 +/- 2.8 sec) compared to sham mice (72.2 +/- 27.7 sec), indicating they experienced acute motor deficits and were unable to hold on as long as the sham mice (p=0.033, Mann-Whitney test; Figure 3.1C).

Blood biomarkers of mTBI distinguish injured mice from sham controls

We measured levels of peripheral blood plasma ubiquitin carboxyl-terminal hydrolase isoenzyme L1 (UCH-L1) and glial fibrillary acidic protein (GFAP) as biomarkers of neuronal and astrocyte damage, respectively. UCH-L1 is a deubiquitinase enzyme highly and specifically expressed in neurons, and GFAP is an intermediate filament protein expressed exclusively in astrocytes. Damage to neurons and astrocytes results in increased levels of UCH-L1 and GFAP, respectively, in the cerebrospinal fluid and peripheral blood. After mTBI, UCH-L1 in the peripheral plasma of CHI mice was significantly elevated above sham levels as early as 30 minutes post-injury (sham=33.43 +/- 0.86 pg/mL, CHI=65.94 +/- 10.76 pg/mL; p=0.019, Student’s t-test) and remained
Figure 3.1. mTBI induces acute physiological impairments. (A) Apnea, recorded as the time after impact for mice to regain a normal breathing pattern. (B) Righting time, recorded as time for mice to regain righting reflex upon oxygen administration. (C) Latency to fall, measured 1h post-injury, as the amount of time the mouse remains suspended from a grid inverted over cage before falling. Values are mean +/- SEM, Sham n=29, CHI n=66. **p<0.01, ****p<0.0001, Mann-Whitney test.
significantly elevated at 1 hour post-injury (sham=25.58 +/- 8.99 pg/mL, CHI=69.14 +/- 11.71 pg/mL; p=0.02, Student’s t-test) before returning to sham levels by 2 hours post-injury (Figure 3.2A). Plasma GFAP levels peaked significantly above sham at 1 hour post-injury (sham=1.21 +/- 0.22 ng/mL, CHI=3.31 +/- 0.45 ng/mL; p=0.001, Student’s t-test) and returned to sham levels at 2 hours post-injury (Figure 3.2B). These results demonstrate that neuron and astrocyte damage both occur within minutes of the mTBI and that both UCH-L1 and GFAP levels in peripheral blood can distinguish injured from sham mice at early time points. These findings are in agreement with the clinical literature that has found similar results in human blood after TBI (Honda et al., 2010; Papa et al., 2010; Pelinka et al., 2004), indicating similarity between the CHI experimental model of mTBI and human brain injury.

**mTBI causes no gross brain pathology**

T2-weighted MRI was used to investigate edema and macrostructural damage at 7 and 30 days post-injury (dpi). The brains from CHI-injured mice were qualitatively indistinguishable from those of sham mice, symmetrical in height and width with no structural lesions (Figure 3.3A). Also, there were no obvious regions of T2-weighted hypointensity or hyperintensity, indicative of brain hemorrhage and edema, respectively (Figure 3.3A). To validate these observations, ipsilateral cortical edema was quantified as the average pixel intensity relative to the background of the image. There was no significant difference between the average pixel intensity of sham and CHI mice at either 7 days post-injury (p=0.45) or 30 days post-injury (p=0.87), indicating that mTBI does not result in detectable edema (Figure 3.3B). Similarly, on H&E-stained coronal sections, there was no superficial damage to the cerebral cortical surface, and no deep tissue
Figure 3.2. Plasma biomarkers of neuronal and astrocyte damage. (A) Plasma ubiquitin C-terminal hydrolase L1 (UCH-L1) levels as a function of time post-injury. (0.5h: sham n=3, CHI n=8; 1h: sham n=3, CHI n=6; 2h: sham n=5, CHI n=9; 4h: sham n=3, CHI n=5; 12h: sham n=4, CHI n=6; 24h: sham n=3, CHI n=5). (B) Plasma glial fibrillary acidic protein (GFAP) levels as a function of time post-injury. (0.5h: sham n=3, CHI n=8; 1h: sham n=6, CHI n=9; 2h: sham n=6, CHI n=10; 4h: sham n=3, CHI n=6; 12h: sham n=4, CHI n=7; 24h: sham n=4, CHI n=6; 48h: sham n=5, CHI n=4; 72h: sham n=3, CHI n=8). Values are mean +/- SEM; *p<0.05, **p<0.01; difference from sham, Student’s t-test.
Figure 3.3. CHI does not induce overt structural lesions. (A) Representative T2-weighted MRI images obtained at 7 and 30 days post-injury (dpi) in sham and CHI mice. Scale = 2mm. (B) Quantitative MRI analysis of edema in the ipsilateral cortex, calculated as the average pixel intensity from 3 ROIs (500µm²), normalized to the average background pixel intensity. Values are mean +/- SEM; 7dpi Sham n=5, CHI n=4; 30dpi Sham n=5, CHI n=4. (C) Representative H&E-stained brain sections from sham and CHI mice at 7dpi and 30dpi. Scale = 1mm.
lesions were observed in either sham or CHI groups at 7 post-injury (Figure 3.3C). The tissue integrity remained unchanged between 7 and 30 days post-injury (Figure 3.3C), indicating that there is no overt brain damage after mTBI at acute or chronic time points.

**CHI does not result in blood-brain barrier disruption or leukocyte invasion**

To assess the extent of blood-brain barrier (BBB) permeability after CHI, we examined extravasation of Evans Blue (EB; bound to serum albumin ~68 kDa), cadaverine (1 kDa), and fluorescein dextran (70 kDa) at several different time points post-injury. The extravasation of EB and fluorescein dextran is indicative of the passage of large molecules through the BBB, whereas extravasation of cadaverine is indicative of the entry of small molecules into the brain parenchyma. Tracers were detected by fluorescence microscopy. In addition to the brain parenchyma, we examined blood vessels and the choroid plexus. The choroid plexus is an epithelial structure with fenestrated capillaries that lacks tight junctions and therefore does not form a barrier to small molecules, so this region was used as a positive control. There was no evidence of tracer extravasation in any brain region at any of the time points indicating that the BBB was intact after CHI; however, tracer was detected in both intact blood vessels and the choroid plexus (Figure 3.4A,B). Furthermore, when brain sections were immunostained for the erythrocyte-type glucose transporter, Glut-1, to assess cerebrovascular integrity, stained blood vessels in the injured, ipsilateral cortex appeared to have similar density and vessel size as the uninjured, contralateral cortex at 7 dpi (Figure 3.4F,G). This was confirmed by quantitation of blood vessel density in ipsilateral and contralateral cortex (Figure 3.4H), demonstrating that the brain blood vessels are largely intact after CHI. In addition to the absence of BBB disruption, there was no detectable neutrophil infiltration
Figure 3.4. CHI does not induce BBB disruption or compromise blood vessels. (A) Representative image of Evans Blue tracer (70 kDa) within blood vessels and in choroid plexus (B) after CHI. (C) Representative image of 1 kDa tracer within blood vessels as well as the leptomeninges and choroid plexus (D) after CHI. (E) Representative image of myeloperoxidase (MPO) staining of neutrophils present in leptomeningeal layer and absent in brain. (F) Glut-1 staining of blood vessels (green) at cortical lesion site, co-stained for activated astrocytes (GFAP, red) and neurons (DAPI) at 7dpi. (G) Glut-1 blood vessel immunolabeling alone at 7dpi. (H) Quantification of Glut-1 blood vessel density in ipsilateral cortex at 7 dpi compared to uninjured contralateral cortex. Values are mean +/- SEM vessel length per mm$^2$. 7 dpi n=3. We acknowledge the assistance of Dr. Julie Siegenthaler.
in brain parenchyma after CHI, although there were a few positively-stained neutrophils in blood vessels of the leptomeningeal layer, as evidenced by immunostaining for myeloperoxidase (MPO, Figure 3.4E). Taken together, these results indicate that low impact CHI does not compromise the BBB or promote invasion of leukocytes from blood vessels into brain parenchyma.

**Prolonged neuroinflammatory response after CHI**

In order to determine the temporal and spatial nature of the neuroinflammatory response after CHI, the brains of sham and CHI mice were immunostained for ionized calcium-binding adapter molecule 1 (Iba-1) and glial fibrillary acid protein (GFAP) to quantify activated microglia and reactive astrocytes, respectively, two indicators of an active, endogenous neuroinflammatory response in the brain. Iba-1 and GFAP staining in CHI mice was elevated compared to sham mice in the ipsilateral hemisphere at both acute (7 dpi) and chronic (30 dpi) time points (Figure 3.5A,B). Staining was quantified in three regions of interest (ROIs) in the ipsilateral hemisphere: cerebral cortex, corpus callosum, and dentate gyrus at hyperacute (0-24 hpi), acute (7 dpi), and chronic (30 dpi) time points (Figure 3.5C). Within each ROI, pixels were categorized as negative (counterstain), weak (175-200), moderate (100-175), or strong (0-100) based on their intensity values (Figure 3.5C). The pixel positivity index for Iba-1 and GFAP staining quantitation was calculated as the proportion of strong positive pixels over the total number of pixels in the ROI.

In contrast to the absence of macroscopic brain pathology after CHI, there were significant microscopic changes in the brains of CHI mice. Examination of the hyperacute timecourse (2, 4, 12, and 24 hpi; Figure 3.6) revealed distinct patterns of
Figure 3.5. Overview of the distribution of GFAP and Iba-1 staining at acute and chronic time points. Representative H&E, GFAP, and Iba-1 stained coronal brain sections at 7 (A) and 30 (B) days post-injury (dpi). Scale = 2mm. (C) Left: Schematic depicting the regions of interest (ROIs) used for staining quantification of ipsilateral cortex (CTX), corpus callosum (CC), and dentate gyrus (DG). Right: Graphical depiction of pixel value assignment and pixel positivity analysis for staining quantitation.
Figure 3.6. Temporal and spatial patterns of Iba-1 and GFAP staining. (A) Timecourse of Iba-1 positivity in cortex, corpus callosum, and dentate gyrus after injury. 0hpi (sham baseline) n= 11, 2hpi n= 3, 4hpi n= 3, 12hpi n=3, 24hpi n=3. (B) Timecourse of GFAP positivity in cortex, corpus callosum, and dentate gyrus after injury. hpi: hours post-injury. Sham: baseline of pooled sham values from all time points and naïve. Values are mean +/- SEM fold-change relative to sham; *p<0.05, **p<0.0; Student’s t-test, difference from sham.
staining with Iba-1 and GFAP. Iba-1 staining showed a consistent biphasic temporal pattern in all three ROIs, peaking within the first few hours, returning to sham baseline by 12 hpi and increasing again at 24 hpi (Figure 3.6A). Similarly, GFAP staining was significantly elevated above sham at 2 hpi in all three ROIs and again at 24 hpi in the cortex (Figure 3.6B). These results indicate that microglia and astrocytes are activated within two hours of CHI in brain regions that control executive function as well as learning and memory and that their response to injury is not transient.

We further quantified the neuroinflammatory response at 7 and 30 dpi as these are clinically relevant for TBI management in the acute and chronic phases, respectively. At 7 dpi, Iba-1 staining was significantly higher in CHI mice compared to sham in the cortex (Sham=0.032 +/- 0.002, CHI=0.050 +/- 0.006; p=0.022, Mann-Whitney test, Figure 3.7A), corpus callosum (Sham=0.017 +/- 0.001, CHI=0.047 +/- 0.009; p=0.018, Mann-Whitney test, Figure 3.7B), and dentate gyrus (Sham=0.030 +/- 0.003, CHI=0.043 +/- 0.002; p=.005, Mann-Whitney test, Figure 3.7C). By 30 dpi the Iba-1 positivity returned to sham levels in cortex and dentate gyrus but remained significantly elevated in corpus callosum (CC: Sham=0.007 +/- 0.0008, CHI=0.011 +/- 0.001; p=0.031, Mann-Whitney test, Figure 3.7). Similar to Iba-1, GFAP positivity at 7 dpi was significantly higher in CHI compared to sham cortex (Sham=0.018 +/- 0.004, CHI=0.089 +/- 0.024; p=0.019, Mann-Whitney test, Figure 3.8A), corpus callosum (Sham=0.078 +/- 0.003, CHI=0.146 +/- 0.016; p=0.002, Mann-Whitney test, Figure 3.8B), and dentate gyrus (Sham=0.111 +/- 0.008, CHI=0.175 +/- 0.009; p=0.0003, Mann-Whitney test, Figure 3.8C). However, at 30 dpi, GFAP returned to sham levels in the dentate gyrus (Figure 3.8C) but remained significantly elevated in both cortex (Sham=0.018 +/- 0.002, CHI=0.080 +/- 0.013;
Figure 3.7. CHI induces acute microglial inflammatory response that persists in white matter. (A) Top: Representative images of the ipsilateral cortex of sham and CHI mice immunostained for activated microglia (Iba-1) at 7 and 30 days post-injury. Bottom: Quantitation of Iba-1 pixel positivity in cortex at 7 and 30 dpi. Scale = 100µm. (B) Top: Representative images of the ipsilateral corpus callosum of sham and CHI mice immunostained for Iba-1 at 7 and 30 days post-injury. Bottom: Quantitation of Iba-1 pixel positivity in corpus callosum at 7 and 30 dpi. Scale = 100µm. (C) Top: Representative images of the ipsilateral dentate gyrus of sham and CHI mice immunostained for Iba-1 at 7 and 30 days post-injury. Bottom: Quantitation of Iba-1 pixel positivity in dentate gyrus at 7 and 30 dpi. Scale = 200µm. dpi: days post-injury. Values are mean +/- SEM; 7 dpi sham n=7, 7 dpi CHI n=8, 30 dpi sham n=7, 30 dpi CHI n=11. *p<0.05, ** p<0.01, *** p<0.001. Mann-Whitney test.
Figure 3.8. CHI induces prolonged astrocyte neuroinflammatory response. (A) Top: Representative images of the ipsilateral cortex of sham and CHI mice immunostained for reactive astrocytes (GFAP) at 7 and 30 days post-injury. Bottom: Quantitation of GFAP pixel positivity in cortex at 7 and 30 dpi. Scale = 100µm. (B) Top: Representative images of the ipsilateral corpus callosum of sham and CHI mice immunostained for GFAP at 7 and 30 days post-injury. Bottom: Quantitation of Iba-1 pixel positivity in corpus callosum at 7 and 30 dpi. Scale = 100µm. (C) Top: Representative images of the ipsilateral dentate gyrus of sham and CHI mice immunostained for GFAP at 7 and 30 days post-injury. Bottom: Quantitation of GFAP pixel positivity in dentate gyrus at 7 and 30 dpi. dpi: days post-injury. Scale = 200µm. Values are mean +/- SEM; 7 dpi sham n=7, 7 dpi CHI n=8, 30 dpi sham n=7, 30 dpi CHI n=11. *p<0.05, **p<0.01, ***p<0.001, Mann-Whitney test.
p=0.002, Mann-Whitney test, Figure 3.8A) and corpus callosum (Sham=0.092 +/- 0.002, 
CHI=0.131 +/- 0.014; p=0.011, Mann-Whitney test, Figure 3.8B). These results indicate 
that the astroglial response in cortex and both the microglial and astrocyte inflammatory 
responses in corpus callosum persist for at least 30 days post-injury. These results also 
highlight the temporal and regional specificity of the neuroinflammatory response to 
mTBI.

The effect of MK-591 on neuroinflammation after CHI

To examine if FLAP inhibitors are capable of blocking both the acute and 
prolonged neuroinflammatory response to mTBI, sham and CHI mice received either 
vehicle or MK-591 30 min prior to injury and additional injections once daily for the 
following six days. Mice were transcardially perfused at either day 7 or day 30 post- 
injury, and brains were immunostained for GFAP and Iba-1. The brains of mice that were 
administered MK-591 had markedly less staining of GFAP and Iba-1 in all three regions 
compared to mice treated with vehicle at both 7 and 30 dpi (Figure 3.9A). Staining 
positivity values for CHI mice were normalized to their respective sham groups, 
expressed as % positivity above sham for both Iba-1 and GFAP positivity. Compared to 
mice treated with no drug (-MK591), those treated with MK-591 had significantly lower 
(70-80%) levels of Iba-1 staining at 7 dpi in cortex, corpus callosum, and dentate gyrus 
(CTX: -MK591=57.94 +/- 19.22%, +MK591=15.81 +/- 8.66%, p=0.042; CC: - 
MK591=171.6 +/- 56.56%, +MK591=34.61 +/- 11.25%, p=0.02; DG: -MK591=43.57 +/- 
7.82%, +MK591=15.18 +/- 8.06%, p=0.028; Figure 3.9B). Iba-1 remained significantly 
reduced at 30 days post-injury in both cortex and corpus callosum of mice treated with 
MK-591 compared to those treated with no drug (CTX: +MK591=6.32 +/- 12.5%,

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Figure 3.9. MK-591 blocks acute and chronic neuroinflammation. (A) Representative images of Iba-1 and GFAP stained coronal sections from all treatment groups: Sham: brains collected at 7 dpi; -MK591: CHI injured, received no drug, brains collected at 7dpi; +MK591 7dpi: CHI injured, received MK-591 for 7 days, brains collected at 7 dpi; +MK591 30dpi: CHI injured, received MK-591 for 7 days, brains collected at 30 dpi. (B) Quantitation of Iba-1 positivity in cortex, corpus callosum, and dentate gyrus of all CHI groups. (C) Quantitation of GFAP positivity in cortex, corpus callosum, and dentate gyrus of all CHI groups. Values are +/- SEM % above sham for each group; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; difference from -MK591 group, Mann-Whitney test.
p=0.034; CC: +MK591=28.42 +/- 9.264%, p=0.013; Figure 3.9B). CHI mice treated with MK-591 had significantly reduced (72-80%) levels of GFAP positivity at 7 dpi in cortex, corpus callosum, and dentate gyrus (CTX: -MK591=409.4 +/- 140.5%, +MK591=86.84 +/- 23.06%, p=0.015; CC: -MK591=88.40 +/- 20.98%, +MK591=22.20 +/- 6.39%, p=0.004; DG: -MK591=57.73 +/- 8.98%, +MK591=16.10 +/- 5.99%, p=0.014; Figure 3.9B). The significant reduction in GFAP positivity persisted for 30 dpi in corpus callosum and dentate gyrus of MK-591 treated mice compared to those treated with no drug (CC: +MK591= 8.38 +/- 6.39%, p<0.0001; DG: +MK591=0.537 +/- 4.93%, p=0.0002; Figure 3.9C). These data indicate that FLAP inhibitors block inflammation after TBI and that early administration of MK-591 is efficacious in blocking both acute and prolonged inflammation after a single mTBI.

The effect of MK-591 on neurodegeneration after CHI

In order to investigate the effect of MK-591 on neuronal degeneration induced by mTBI, we stained brain sections with Fluoro-Jade B (FJB) to label neurons and their axons undergoing degeneration. Although the mechanism by which FJB binds to damaged neuronal elements is unknown, it is widely accepted as a specific, high-affinity probe for damaged/degenerating neurons, dendrites, axons, and axonal terminals (Schmued and Hopkins, 2000). The time course of FJB staining after CHI revealed no significant staining in sham or injured mice within 24 hrs of injury, indicating that the degenerative condition takes time to develop (data not shown). In the sham group, no Fluoro-Jade B-positive (FJB+) cells were detected in the brains at 7dpi in the absence or presence of MK-591 (Figure 3.10A). In the CHI mice that did not receive MK-591
Figure 3.10. MK-591 prevents neuronal damage. Representative images of FluoroJade B stained coronal sections from (A) Sham, (B) -MK591 and (C) +MK591 7dpi treatment groups and representative ROI 10X images.
(-MK591) FJB+ cells were observed in a number of ipsilateral brain regions including: layer II/III of the cortex, axon tracts of the corpus callosum, CA1 of the hippocampus, and the subgranular zone of the dentate gyrus at 7dpi (Figure 3.10B). In the brains of mice that received MK-591 (+MK591) there were no FJB+ cells detected in any of these regions at 7dpi (Figure 3.10C). These data suggest that although mTBI did not cause significant neuronal cell loss in any brain region, it did result in damaged neurons and neuronal processes in the contused tissue of the ipsilateral cortex as well as brain regions more distal to the cortical injury site. MK-591, which mitigated microglial activation and astrocytosis, blocked the neuronal degeneration after CHI. At 30 dpi, there was no significant FJB staining in sham or injured mice (data not shown), suggesting that the damaged neurons and neuronal processes were either cleared from the brain by activated microglia with phagocytic activity or they had been repaired by 30 days.

Discussion

The present study establishes that neuroinflammation is an important, dynamic process after CHI that is initiated early and persists for at least a month in discrete brain regions. This neuroinflammatory process does not require disruption of the blood-brain barrier or participation of peripheral immune cells. The FLAP inhibitor, MK-591, shown previously to block injury-induced production of leukotrienes to levels below the limit of detection by tandem mass spectrometry, significantly reduced both the acute and prolonged inflammation and prevented neuronal degeneration in several critical brain regions, including cortical areas involved in executive function, white matter tracts of the corpus callosum, and the dentate gyrus of the hippocampus that mediates learning and memory. These data, taken together with previous reports (Schuhmann et al., 2003,
Farias et al., 2009, Voigt et al., 2012, Corser-Jensen et al., 2014), indicate that FLAP inhibitors represent promising therapeutic interventions for TBI.

Although our CHI rodent model does not mimic all of the features of human mTBI, particularly the predisposing factors and co-morbidities that exist in human brain injury, it does recapitulate many of the features of mTBI in humans. After CHI, the mice spontaneously recover a righting reflex without evidence of seizures, paralysis, skull fractures, or intracranial bleeding, and the brains of CHI-injured mice are indistinguishable from those of sham mice by MRI with no overt tissue lesions and no gross pathology. The absence of overt brain damage is similar to human concussions. Many individuals who sustain a concussion do not seek medical attention, and even those who do usually have normal CT and MRI scans, regardless of whether or not they are symptomatic at the time of the scan (Hofman et al., 2001; Hughes et al., 2004). While MRI is useful for ruling out intracranial bleeding and edema, it is not sensitive enough to detect the underlying brain pathology responsible for the variety of post-concussive symptoms. This has led to federal and private research initiatives to develop more sensitive imaging modalities like diffusion tensor imaging (DTI), functional MRI (fMRI), and positron emission tomography (PET) to diagnose concussions. However, neuroimaging is expensive and not convenient for detection of mTBI outside of a medical facility, limiting its feasibility for early detection in sports and military settings.

Cerebrospinal fluid (CSF) and blood-based biomarkers have emerged as an effective alternative for the diagnosis and management of mTBI. Currently, neurofilament (NF), S100β, UCH-L1, GFAP, and tau appear to be the best candidate biomarkers of mTBI, correlating strongly with other diagnostic measures (Jeter et al.,
2012; Kulbe and Geddes, 2015). In the present study, we measured levels of UCH-L1 and GFAP in the plasma at various time points after CHI using commercially available ELISA kits. UCH-L1 is an abundant protein in the soma of neurons and plays an important role in the removal of proteins that are in excess, oxidized, or misfolded, whereas GFAP is an intermediate filament protein in the cytoskeleton of astrocytes. In several clinical studies, UCH-L1 and GFAP have been detected in the blood within one hour of injury and their levels are associated with measures of injury severity and clinical outcome (Brophy et al., 2011; Papa et al. 2012; Papa et al. 2014). In the current study, both biomarkers peaked within the first 1-2 hours post-CHI and then gradually declined to baseline. The early appearance of UCH-L1 and GFAP in plasma is likely indicative of damage to neurons and astrocytes, respectively, at time of injury. As there was no breach of the BBB after CHI, these protein biomarkers are able to reach the peripheral circulatory system by mechanisms independent of BBBD. Recent studies suggest that the glymphatic system in the brain mediates the entry of brain-specific proteins into the peripheral circulation (Plog et al., 2015).

In many TBI cases, blood vessels are mechanically stretched and/or sheared along with neuronal axons as part of the primary injury. Damage to the BBB can result in the influx of peripheral blood cells into the brain where they interact with resident neurons and glia to mount a neuroinflammatory response. In this study, we sought to characterize the kinetics of BBB disruption after CHI to further investigate the involvement of the BBB and peripheral leukocytes in the post-traumatic neuroinflammatory response to mTBI. We injected fluorescent tracers of different molecular weights (1 kDa -70 kDa) and evaluated their extravasation at several different time points post-injury. While
tracers were detected in the leptomeningeal layer at the surface of the brain, within blood vessels throughout the brain, and in circumventricular regions that lack the tight junctions of the BBB, no extravasation was detected in the brain parenchyma at any of the time points. These findings reveal, unexpectedly, that the BBB was intact after CHI. Immunohistochemical staining of blood vessels confirmed this result, showing no difference in the cerebrovasculature between injured and uninjured hemispheres. Also unexpected was the absence of neutrophil infiltration after CHI. Previous studies by our laboratory and others have shown substantial neutrophil infiltration following fluid percussion injury, controlled cortical impact, and blast-injury (Clark et al., 1994; Tompkins et al., 2013; Utagawa et al., 2008). The likely reason for this disparity is the difference in severity of injury between the previous experimental TBI models and the current CHI model. The previous studies that demonstrated neutrophil infiltration after brain injury also showed significant cell death (lesion volumes), edema, and BBB disruption. As sensitive tracer studies have not been carried out in humans after concussion, it is unknown whether or not the BBB is compromised in most concussions. Almost all concussions involve diffuse axonal injury, however, blood vessels are much larger in diameter than neuronal axons and it’s conceivable that they may be spared of injury in mild concussions. Clearly, additional studies are required to understand the relationship between the biomechanical forces that impact the brain during concussion and the resulting damage to the cerebrovasculature. Moreover, it is likely that certain brain regions are more vulnerable to vascular injury just as they are to neuronal injury.

Despite the absence of overt neuropathology and compromise of the BBB, the brains of CHI mice demonstrated a robust neuroinflammatory response that persisted for
at least a month after mTBI, particularly in the cerebral cortex under the site of injury and the white matter tracts of the corpus callosum. These results indicate that the neuroinflammatory reaction to injury, also referred to as the innate immune response, can be mediated by endogenous microglia and astrocytes and does not require participation of the peripheral immune system. We believe that mechanical damage at the time of injury results in the release of DAMPs that bind to and activate microglia, which interact with neighboring astrocytes to produce pro-inflammatory lipid mediators (i.e. leukotrienes) and numerous pro-inflammatory cytokines and chemokines within the brain. The production of leukotrienes in response to brain injury is very rapid (within minutes) as the enzymatic machinery for leukotriene biosynthesis is already assembled and activated by calcium influx. The production of pro-inflammatory cytokines and chemokines follows as it may require transcription/translation events or assembly of the inflammasome events that take hours to complete. Meanwhile, additional microglia and astrocytes migrate to the area of injury leading to further release of pro-inflammatory mediators, creating a toxic environment that is believed to not only promote neuronal degeneration but also inhibit repair processes.

The regions of the brain that showed the greatest inflammatory response were the same regions that stained positive for Fluoro-Jade B, a fluorescent marker that binds to degenerating neurons and neuronal processes. The white matter tracts of the corpus callosum showed a pronounced neuroinflammatory response and damaged axons after CHI. Axons in the white matter appear to be especially vulnerable to mechanical injury and diffuse axonal injury (DAI) has emerged as one of the most common pathological features of mTBI. Structural imaging studies in humans using diffusion tensor imaging
(DTI) has been useful in detecting DAI (Bazarian et al., 2007, Shenton et al., 2012). Fractional anisotropy (FA), a degree of directionality of water diffusion, and apparent diffusion coefficient (ADC), the average rate of diffusion in all directions, calculated from DTI provide measures of structural integrity of the brain’s white matter. Normal myelinated axon tracts typically have low ADC and high FA values. After mTBI, increased ADC and lowered FA have been observed and a number of recent studies indicate that the severity of cognitive dysfunction after mTBI correlates with the extent of damage to the white matter (Miles et al., 2008, Niogi et al., 2008). DTI changes have also been reported in a study of athletes sustaining repeated subconcussive head injuries, indicating that even minimal trauma may result in long-term white matter alterations (Bazarian et al., 2012). In addition, recent evidence suggests that TBI may induce long-term progressive axonal pathology. Axonal degeneration has been found to continue years after injury in humans (Johnson et al., 2012) and appears to play a role in the development of AD-like pathological changes (Johnson et al., 2010).

The cellular mechanisms that mediate progressive axonal injury and synapse loss are largely unknown, but the precise overlap of areas of injury and neuroinflammatory markers indicate that the two events are closely linked. Recent studies have revealed that microglia and astrocytes play a direct role in synapse elimination during brain injury, neurodegenerative diseases, and aging, employing a mechanism similar to that of synaptic pruning during brain development. An astrocyte-secreted factor is believed to activate the classical complement cascade, which tags synapses that are weak and/or damaged. Activated microglia then recognize and eliminate these tagged, dysfunctional synaptic terminals by phagocytosis, leading to the loss of axons and ultimately neurons
by Wallerian degeneration. (Schafer et al., 2012; Stephan et al., 2012). It is likely that the damage to neuronal axons during the primary injury event sets in motion the neuroinflammatory response that is mediated by activated microglia and reactive astrocytes and numerous pro-inflammatory mediators secreted by these cells. The neuroinflammatory response is a double-edged sword that in some cases results in removal of axonal debris and resolution of brain injury, and in other cases, perhaps the majority, leads to a maladaptive state in which chronically activated microglia become dysregulated and indiscriminately prune neuronal processes leading to further degeneration and dysfunction.

Epidemiological studies and clinical observations have shown a positive association between head injury and increased risk of cognitive impairment, earlier onset of dementia, and increased Alzheimer’s disease-like neuropathology (Abner et al., 2014; Barnes et al., 2014; Johnson et al. 2010; Shively et al. 2012). Activated microglia and reactive astrocytes are increasingly recognized as a feature of TBI and Alzheimer’s disease in humans, suggesting that persistent inflammation and inflammation-mediated synapse loss may represent an underlying mechanistic link between TBI and neurodegenerative diseases. (Eikelenboom et al. 2010, Gentleman et al., 2004; Johnson et al., 2013; Pike et al., 1995; Ramlackhansingh et al., 2011).

As axonal injury, neurodegeneration, and neuroinflammation are all clearly linked, exploring therapeutic interventions for TBI should include anti-inflammatory approaches. Past studies examining the efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) have been unconvincing as some studies showed beneficial results and other did not (Chao et al., 2012; Girgis et al., 2013; Zangbar et al., 2015). NSAIDs target the
COX1 and COX2 arm of eicosanoid biosynthesis, thereby blocking prostaglandins and leaving other important inflammatory mediators (i.e. leukotrienes, cytokines, chemokines) untouched. In the current study, we investigated the other major arm of eicosanoid biosynthesis, the 5-LO/FLAP pathway of leukotriene production. The FLAP inhibitor, MK-591 significantly reduced the number of activated microglia and reactive astrocytes throughout the brain and prevented degeneration of neurons in the cortex, dentate gyrus, and axonal tracts in the corpus callosum at 7 days post-injury. Acute administration of MK-591 during the first week following injury also effectively prevented the chronic inflammation at 30 days post-injury, a time period that corresponds to 3-4 months in humans, indicating that the drug interrupted the inflammatory response.

In conclusion, neuroinflammation is a complex response to injury that attempts to clear dead or damaged cells and debris and return the affected tissues to a normal state. The current study shows that a single mild impact to the brain initiates an endogenous prolonged neuroinflammatory response, mediated by the 5-LO inflammatory pathway, that does not require infiltration of peripheral immune cells. Despite its critical host-protective function, acute inflammation is not sustainable over a prolonged period of time, giving rise to maladaptive features of chronic inflammation. Chronic inflammation is responsible for the pathogenesis of a wide variety of diseases (i.e. asthma, cardiovascular disease, rheumatoid arthritis, inflammatory bowel disease) that can be attributed to the failure of resolution of inflammation. It follows that progressive axonal injury and neurological deficits after TBI could be due to the inability to turn off or resolve neuroinflammation, and one could speculate that post-concussive syndrome results from failure to resolve inflammation. In order to minimize the progressive
cognitive and behavioral decline following brain injury, drugs must be developed to block either the initial inflammation and/or interrupt the existing chronic inflammatory cascades. FLAP inhibitors are promising anti-inflammatory candidates to test in clinical trials of TBI as well as a variety of other CNS disorders that involve chronic neuroinflammation.
CHAPTER IV

A TEMPORAL PROTEOMIC PROFILE OF EXPERIMENTAL MILD TRAUMATIC BRAIN INJURY

Abstract

There is a pressing need for biomarkers of mild traumatic brain injury (mTBI) that are reliable and easily measured. A number of recent human studies have identified a few candidate mTBI biomarkers using antibody-based assays, however, few studies have investigated the temporal nature of candidate biomarkers after injury. In this study, we used a modified aptamer-based proteomics technology, SOMAscan, to identify novel putative biomarkers and their temporal profile after experimental mTBI in mice. The mice were subjected to a Closed Head Injury (CHI) model of mTBI and at various times after injury, plasma was collected and analyzed by Somascan. Of the 1,129 proteins that were screened, 59 proteins showed significant differences between injured and naïve animals at 1 hour post-injury and 29 proteins showed significant differences between injured and naïve animals at 7 days post-injury. Proteins were classified into groups based on cellular processes known to be related to the pathophysiology of mTBI such as growth/protection, metabolism, inflammation, cell death, and general cell signaling. From this analysis, we identified 7 brain-specific or brain-enriched proteins that represent putative biomarkers of mTBI. One of these markers, glial fibrillary acidic protein (GFAP), has been previously identified in human studies. The other 6 putative mTBI biomarkers are novel and include neuroligin-4, pleiotrophin, ephrin-A5, PARK-7, GOT1, and MFRP. Our results demonstrate the ability of this novel, unbiased proteomic

3 This chapter has been submitted for publication in Journal of Neurotrauma.
screening tool, to discover new, measurable biomarkers that may elucidate underlying pathophysiological events, and improve the diagnosis and treatment of mTBI.

**Introduction**

The mechanisms of injury contributing to mild traumatic brain injury (mTBI) are complex. Injury can result from rapid acceleration/deceleration of the brain, a direct impact to the head, or an explosive blast, and it is estimated that 5-7 million people suffer a mTBI each year in the United States the majority of whom do not seek medical attention (Faul et al., 2010). Standard neuroimaging techniques like CT and MRI are unable to detect damage to the brain caused by a mTBI, further necessitating the implementation of biomarkers in the detection of mTBI to decrease the morbidity associated with these undiagnosed and undetected injuries.

Brain injury, like any other disease state, is accompanied by real-time changes in protein expression. Comparative investigation of proteomes from healthy and brain injured tissue or plasma can offer insight into the biological mechanisms of both the injury and the repair processes. Further, through identification of new biomarkers, this technique can assist in the diagnosis of disease, in estimating prognosis, and in monitoring recovery. To date, the discovery and validation of new mTBI biomarkers with diagnostic and/or clinical utility remain a considerable challenge for numerous reasons. The heterogeneity of injury mechanism, pre-analytical and analytical artifacts in experimental models, lack of adequate injury/disease controls, limits of detection, and clinical study design contribute to the lack of established mTBI biomarkers.

SomaLogic, Inc. recently developed a novel protein-capture proteomic assay, SOMAscan, for biomarker discovery that is capable of measuring 1,129-3,300 different
proteins in plasma and serum from sample volumes as small as 65 microliters. The proteomic platform has low limits of detection (median value of 38 fM), a wide dynamic range of 8 logs (femtomolar to micromolar), and a 5% median coefficient of variation which addresses the prevailing challenge of detecting low levels of brain-specific proteins in the plasma and serum after massive dilution in the circulation (Gold et al., 2010).

This technology utilizes SOMAmers (Slow Off-rate Modified Aptamers), a new class of protein binding aptamers which contain chemically modified nucleotides that bind to proteins with high affinity in complex matrices in the plasma or serum. The proteins identified by the SOMAmer technology are then transformed into unique DNA signatures that are quantified on commercial DNA microarray platforms or by qPCR. The assay utilizes the dual nature of SOMAmers as both protein affinity reagents with defined 3-dimensional structures and as unique nucleotide sequences recognizable by specific DNA hybridization probes. This technology has provided an efficient, highly multiplexed, proteomic array that has proven utility in identifying clinical biomarkers of various diseases (Ostroff et al., 2012; Mehan et al., 2012).

Genomic studies have contributed significantly to our understanding of basic biological and disease processes, however, the level of gene transcription provides only an estimate of its level of translation into protein. Conversely, proteomics offers the ability to quantify proteins, providing an immediate assessment of “real-time” health and disease status. Attempts at high-content proteomics began with 2-dimensional (2-D) gels and now employs mass spectrometry (Walther and Mann, 2010) and antibody-based technologies (Holm et al., 2012). Mass spectrometry can deliver specific analytical
capabilities, but many limitations remain including sensitivity (nM), specificity, reproducibility, throughput, and cost. Antibody-based methods are more sensitive than 2-D gels and mass spectrometry and can detect analytes in the sub-nanomolar range due to the high affinity of antibodies for their targets (nM to pM). However, the non-specific binding of antibodies to non-cognate proteins, other macromolecules, and surfaces requires a sandwich-type assays in which the second antibody enhances specificity through an independent binding event as in enzyme-linked immuno-sorbent assays (ELISAs). High sensitivity is attained by combining the specificity of two different antibodies to the same protein, requiring that both bind to elicit a signal (Lequinn, 2005). Although broadly used in single-analyte tests, ELISAs cannot be multiplexed beyond single digits largely because of the cross-reactivity and non-specific binding of secondary antibodies to surface-immobilized proteins. A recently reported proximity ligation assay that relies on antibody sandwich formation in solution followed by ligation of antibody-tethered nucleic acids and PCR amplification has been multiplexed with 6-8 analytes (Lundberg et al., 2011). Other promising strategies utilize mass spectrometry or antibody colocalization microarrays to enhance specific binding and decrease non-specific binding (Pla-Roca et al., 2012). Introducing additional stringency steps along with increasing signal amplification techniques may further enhance these methods. Despite the promising nature of these technologies, none of the current multiplex protein assays can compete with the large number of analytes that can be measured in a non-biased manner by SOMAscan.

This study utilized SOMAscan technology to investigate the temporal changes in the circulating mouse proteome after experimental mTBI. Because this type of study is
difficult to achieve in humans, improved experimental models of mTBI in mice have been developed to mimic human brain injury. We established a novel closed head injury (CHI) model in mice that mimics many of the neuropathological features of human mTBI, specifically, the long-lasting neuroinflammation and associated degeneration seen in human injury (Corser-Jensen et al., submitted). Plasma was collected from naïve mice and cohorts of CHI-injured mice at various times after injury and analyzed by SOMAscan. The resulting data were analyzed using non-parametric statistics to identify proteins that were differentially expressed between naïve and injured mice. Through this technique, we are better able to study molecular changes after brain injury on a temporal scale to direct time-dependent therapeutic administration as well as to identify putative mTBI biomarkers.

Methods

Animals

All experiments were performed on adult male wild type C57BL/6J mice (10-12 weeks old, 20-25g; Jackson Laboratories, Bar Harbor, ME) that were housed individually in temperature- and light- controlled housing with free access to food and water ad libitum. Mice were allowed to acclimate to the vivarium for one week prior to experimental procedures. All procedures as described were performed under protocols approved by the University of Colorado Institutional Animal Care and Use Committee and are in compliance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.
**Closed head injury model of mTBI**

Experimental closed head injury (CHI) was performed using the Impact One (Leica), an electromagnetically controlled stereotaxic cortical impact device for precise, graded experimental TBI. Prior to injury, animals received an intraperitoneal injection of the analgesic, buprenorphine (0.05 mg/kg; Buprenex). Mice were placed on a heating pad and anesthetized with 3% isoflurane (2.0 L/min), which was maintained throughout the procedure. After confirmation of sufficient depth of anesthesia, animals were shaved and the skull was exposed by a 2 cm longitudinal midline scalp incision in order to have a direct impact of the tip onto the skull. Mice were then placed in a stereotaxic frame with an incisor bar at 0° and cup head holders. Next, the impact device, mounted on the left stereotaxic arm at an angle of 20° from vertical, was rotated into the field. The 5-mm blunt metal impact probe was centered between bregma and lambda on the left side of the sagittal suture and then lowered until the impact probe contacts the skull, detected by a contact sensor. From this zero depth position, the injury impact depth was determined by lowering the tip to the desired impact of 3 mm. The tip was then retracted and the injury is triggered using the myNeuroLab controller at an impact velocity of 5 m/s and dwell time of 100 msec. After impact, the mice were allowed to regain a normal breathing pattern without assistance. Once a regular breathing pattern was achieved the mice were sutured and placed on 100% oxygen until they regained a righting reflex. Injured animals received an intraperitoneal injection of the analgesic, buprenorphine, prior to injury and subsequent injections every 12 hours for three days post-injury.
Sample collection and preparation

At various times after CHI (1hr, 4hr, 24hr, and 7days), naïve and CHI mice were euthanized by 1.5 % CO\(_2\) and blood samples were collected by cardiac puncture using a (22g x1) needle, and the blood was transferred to heparinized microcentrifuge tubes. The tubes were gently inverted 3 times and placed on ice for less than 1 hr until centrifugation (2000g for 15 minutes) at 4°C. The plasma supernatant (approximately 60 µl) was stored at -80°C until analysis.

SOMAscan procedures

Heparinized plasma samples were analyzed by SOMAscan as previously described (Gold et al., 2010; Gold et al. 2012; Rohloff et al., 2014). In brief, this assay uses SOMAmers to transform protein concentration into a corresponding DNA concentration through a series of steps involving affinity binding and capture of biotin onto streptavidin beads. Each sample was randomly assigned to plates within each assay run along with a set of calibration and normalization samples. No identifying information was available to the technicians operating the assay. The final DNA concentration is measured as relative fluorescence units (RFU) from the fluorescent SOMAmer hybridized to a complementary probe on an Agilent array. Intrarun normalization and interrun calibration were performed according to SOMAscan v3 assay data quality-control procedures as defined in the SomaLogic good laboratory practice quality system. The microarray slides were imaged with a microarray scanner (Agilent G2565CA Microarray Scanner System, Agilent Technologies) in the Cy3-channel at 5 mm resolution at 100% PMT setting and the XRD option enabled at 0.05. The resulting tiff images were processed using Agilent feature extraction software version 10.5.1.1.
Data analysis

We used a non-parametric Mann-Whitney rank sum test to test for significance in the differential expression of proteins between naïve and CHI-injured mice and also between sham and CHI-injured mice at particular times after injury. We performed a separate two-tailed Mann-Whitney test for each of the 1129 proteins measured by the SOMAscan platform. Multiple comparisons were corrected for by applying a false discovery rate (FDR) and enforcing a q-value threshold of 0.05 for all analytes. We then sorted the analytes expressing significance by their log ratios, comparing the naïve and CHI-injured samples. We chose to highlight the analytes with the highest log ratio within the set of significantly differentially expressed analytes.

Results

Identification of injury-induced proteomic changes

Plasma was collected from naïve mice and from mice subjected to a single closed head injury (CHI) at various time points (1hr, 4hr, 24hr, and 7days) post-injury, and SOMAscan proteomics technology was used to investigate injury-related changes in protein plasma levels over time. The SOMAmer reagents used in the SOMAscan assay were created for 1,129 protein targets that regulate diverse molecular and biological processes (Figure 4.1). These targets contribute to a variety of important biological processes including stress response, immune responses, cell motility, cell adhesion, cell differentiation, autophagy, and aging.

Research in our laboratory has revealed that mice subjected to CHI have a robust neuroinflammatory response in their brain tissue, as evidenced by elevated levels of activated microglia and reactive astrocytes despite negative neuroimaging scans and no
Figure 4.1. Index of all 1,129 screened protein targets by biological function.
detectable blood-brain barrier disruption (Corser-Jensen et al., submitted). Neuroinflammatory markers were detected as soon as 2 hours post-injury and were found to persist for at least 30 days in some brain regions. Along with the neuroinflammatory pathology following CHI, injured mice in this study showed notable proteomic changes at each of the time points studied when compared to naïve mice (Figure 4.2). After applying a median filter to exclude SOMAmers that did not vary across time, 443 SOMAmers were identified and differential expression was computed (described previously by Mehan et al., 2012). Separate Mann-Whitney r and sum tests were performed for each of these 443 Somamers and P values were subsequently corrected for multiple testing by applying the False Discovery Rate (FDR).

At two time points, 1 hour post injury and 7 days post injury, statistically significant differential expression was detected. At 1 hour post-injury, there were 59 proteins that were significantly different between naïve and CHI mice (Table 4.1), and at 7 days post-injury, 29 proteins differed significantly between naïve and CHI mice (Table 4.2). Interestingly, at 1 hour post-injury, 45 of the 59 proteins (76%) were increased in injured mice compared to naïve animals, whereas at 7 days post-injury, only one of the 29 total proteins was elevated in the injured cohort with the other 28 proteins comparatively lower than their naïve counterparts.

Categorization of differentially expressed proteins by biological function

To better understand the potential role of these proteins in the injury and repair processes that are known to occur after brain injury, we grouped the proteins into five biological processes: 1) growth/protection, 2) metabolism, 3) inflammation 4) cell death, and 5) cell signaling.
Figure 4.2. Fold change of protein expression in injured versus naïve plasma. We acknowledge the assistance of Dr. William Baumgartner.
**Table 4.1. Significantly changed proteins at 1 hour post-injury.** Value reflects fold change of the corresponding protein in CHI relative to naïve.

<table>
<thead>
<tr>
<th>Growth/Protection</th>
<th>Metabolism</th>
<th>Immune/Inflammation</th>
<th>Cell Damage/Cell Death</th>
<th>Cell Signaling</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td>Interleukin 2</td>
<td>Myoglobin 0.77</td>
<td>Calcineurin B 0.87</td>
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<td>Elafin 0.74</td>
<td></td>
<td>SLC25A18 0.75</td>
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<td>DKK1</td>
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<td>DERMI</td>
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<tr>
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<tr>
<td>WNT17A</td>
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<tr>
<td><strong>↓↓↓↓ (CHI &lt; Naïve)</strong></td>
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<tr>
<td>FYN</td>
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<tr>
<td>Galectin 3</td>
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<td>HSP 70</td>
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<tr>
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<tr>
<td>SRC</td>
<td>2.54</td>
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Table 4.2. Significantly changed proteins at 7 days post-injury. Value reflects fold change of the corresponding protein in CHI relative to naïve.

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<th>Growth/Protection</th>
<th>Metabolism</th>
<th>Immune/Inflammation</th>
<th>Cell Damage/Cell Death</th>
<th>Cell Signaling</th>
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<td>BGN 0.54</td>
<td>Diablo 0.75</td>
<td>DCTPP1 0.36</td>
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<td>KLK6 0.48</td>
<td>HSPD1 0.38</td>
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<td>HTRA2 0.59</td>
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↓↓↓↓ (CHI < Naïve)

↑↑↑↑ (CHI > Naïve)
At 1 hour post-injury many of the ‘growth/protection’ proteins that were elevated in injured mice are involved in cell migration and angiogenesis (e.g. FYN, Galectin-3, Integrin α1, METAP), cytoskeleton organization (e.g. NRCKB, NUDC3, Neuroligin-4, RBM39) and protection of the cell against oxidative injury and death (e.g. IGF-1, PARK7, PRDX5, PRDX6, Pleiotrophin, TS). Conversely, several proteins involved in cell fate, cell matrix assembly, and angiogenesis were found to be decreased in CHI mice at 1 hour post-injury.

At 7 days post-injury all proteins with growth/protection functions were decreased in CHI mice (e.g. GSTP1, METAP, PRDX1, PRDX5, PRDX6, VEGF) when compared to naïve. Notably, the temporal profile of two endocrine hormones, insulin-like growth factor 1 (IGF-1) and inhibin beta A chain (INHBA), were significantly down-regulated at 1 hour post-injury and their expression remained depressed at 7 days post-injury. This finding is particularly interesting in light of the functions of these two endocrine hormones. IGF-1, secreted by the pituitary gland, mediates the effects of growth hormone to regulate cell growth and development throughout the body. INHBA regulates follicle stimulating hormone (FSH) release from the pituitary gland to regulate gonadal function. As hypopituitarism and hypogonadism have both been reported after TBI in humans (Fernandez-Rodriguez et al., 2015; Silva et al., 2015; Barton et al., 2015), these findings warrant further exploration of these proteins as circulating diagnostic biomarkers of post-injury endocrine dysfunction.

We identified 11 proteins associated with metabolic functions, nine of which were significantly increased in CHI mice at 1 hour post-injury, and two that were down-regulated in CHI mice at 7 days-post injury. All 11 identified proteins are enzymes with
known roles in energy metabolism. The elevations noted at 1 hour post-injury may reflect a compensatory mechanism to accommodate for the increased energy demands associated with injury (Giza and Hovda, 2001; Prins et al. 2008).

Inflammation is a hallmark of tissue damage and we found a number of differentially expressed proteins associated with inflammatory processes (Table 4.1,4.2). The immune markers that were significantly elevated in CHI mice at 1 hour post-injury are primarily involved in chemokine and cytokine signaling as part of the innate immune response to tissue damage (e.g. GFAP, Gro b/g, HPGD, LYN, MAPK14). Conversely, two proteins, interleukin-2 (IL2) and Elafin, which have anti-inflammatory functions were both down-regulated at 1 hour post-injury. At 7 days post-injury, three pro-inflammatory proteins (BGN, KLK6, MIF) and one anti-inflammatory protein (PDCD1LG2) were down-regulated. Overall, these findings are consistent the view that inflammatory pathways are activated early and persist chronically after TBI. Although some pro-inflammatory signaling may be down-regulated at 7 days post-injury in an attempt to restore homeostasis, our histopathologic findings indicate that the robust neuroinflammatory response at 7 days after CHI can persist for at least 30 days post-injury in some brain regions (Corser-Jensen et al., submitted).

At 1 hour post-injury only one cell death/apoptotic marker (EIF4G2) is up-regulated. However, at 7 days post-injury 3 independent regulators of apoptotic cell death signaling (Diablo, HSPD1, HTRA2) were significantly down-regulated in the CHI mice. This is consistent with the dual nature of some these proteins, as they maintain both pro- and anti-apoptotic roles under different physiologic conditions.
A large number of proteins that function in intra- and extra-cellular signaling pathways were differentially expressed between injured and naïve at both time points. Many of these proteins have known functions in gene expression, signal transduction, nuclear translocation, and protein import. These maintenance proteins are primarily up-regulated at 1 hour post-injury and down-regulated at 7 days post-injury.

**Identification of brain-enriched putative biomarkers**

Although injury-induced changes in plasma protein expression offers insight into physiological processes after TBI, it is unclear whether these processes are occurring in the brain or systemically as a result of injury. An ideal mTBI biomarker is one that is specifically or highly expressed in the brain following injury with a biokinetic profile that would offer a clinically feasible window of detection. We identified seven proteins with high brain tissue expression that showed a large change in protein expression levels between naïve and injured animals and grouped these proteins based on temporal profiles (Figure 4.3). Ephrin-A5 and Parkinson disease protein 7 (PARK7) showed the same temporal pattern, with a peak at 1 hour post-injury and return to naïve levels at 4 hours post-injury (Figure 4.3A). Ephrin-A5 is a ligand for Eph receptor tyrosine kinase that regulates axonal and cerebrovascular development (Flanagan et al., 1998; Klein, 2004). A recent study found that Ephrin-A5 is up-regulated in reactive astrocytes after stroke and prevents axon regeneration in the injured tissue (Overman et al., 2012). PARK7 is a peptidase enzyme that protects cells against oxidative stress. Mutations in the PARK7 gene have been noted to cause autosomal recessive early-onset Parkinson’s disease (Bonifati et al., 2003). In the brain, PARK7 is expressed primarily by astrocytes rather than neurons (Bandopadhyay et al., 2004), and high levels of PARK7 have been detected
Figure 4.3. Temporal profiles of putative mTBI biomarkers.
in plasma and CSF in post-mortem patients after ischemic stroke (Allard et al., 2005). Thus, the elevation in Ephrin A5 and PARK7 levels likely reflect astrocyte activation following injury that initiates the neuroinflammatory response. The relatively short window of up-regulation of Ephrin A5 and PARK7 (less than 4 hours post injury) is similar to the temporal profile of other TBI biomarkers (i.e. UCHL-1) that have been developed for human diagnostics. This brief window of expression limits the utility of these biomarkers, as it requires that the blood must be drawn within this short time period.

Pleiotrophin and Neuroligin-4, X-linked (NLGNX) also show peak levels at 1 hour post-injury, however they do not return to naïve levels until 24 hours post-injury (Figure 4.3B). The peak level of pleiotrophin was 1.97 fold above naïve, and peak NLGNX level was 6.54 fold above naïve. Pleiotrophin is a secreted heparin-binding growth factor that stimulates angiogenesis and neurite outgrowth in the brain during development. Its expression has been shown to significantly increase in macrophages, endothelial cells, microglia and astrocytes after focal cerebral ischemia (Yeh et al., 1998; Miao et al., 2012). NLGNX is a neuronal cell surface protein that regulates synapse formation and stabilization. A mutation in the gene coding for NLGNX is associated with X-linked mental retardation and autism (Krueger et al., 2012; Laumonnier et al., 2004). Thus early elevations of pleiotrophin and NLGNX may serve as important biomarkers of glial activation and neuronal injury respectively, after TBI. Given their functional relevance, the high fold-changes in expression and the longer 24-hour detection window, Pleiotrophin and neuroligin-4 are new candidate biomarkers of human mTBI that merit further investigation.
Glial fibrillary acid protein (GFAP) is among the most widely studied biomarkers for TBI. Our results support the role of GFAP as a candidate biomarker, as GFAP levels in injured mice are increased above naïve for 7 days, with a peak at 4 hours post-injury (Figure 4.3C). GFAP is an intermediate filament protein exclusively expressed by astrocytes that regulates astrocyte morphology and mobility, astrocyte-neuron interaction, and glial scar formation. Increased GFAP expression is a known marker of neuroinflammation, a hallmark feature of TBI and other neurodegenerative disorders.

Our data demonstrated that Aspartate aminotransferase (GOT1) levels are slightly elevated above naïve at early time points, peaking at 24 hours post-injury and returning to baseline at 7 days post-injury (Figure 4.3C). GOT1 is involved in the regulation of glutamate levels and in scavenging for glutamate under hypoxic conditions to protect neurons (Rink et al., 2011). Given its lengthy course of elevation, GOT1 is another new putative mTBI biomarker that reflects metabolic dysfunction during the first 7 days post-injury.

Another unique temporal profile of a brain-enriched protein that was differentially expressed between injured and naïve mice is that of membrane frizzled-related protein (MFRP) which was decreased below naïve levels for the first 24 hours post-injury (Figure 4.3D). Although the molecular function of this protein is not well characterized, MFRP (which is predominantly expressed in the eye and the brain), is known to interact with the complement 1q tumor necrosis factor-related protein 5 (C1QTNF5), and induces retinal degeneration (Mandal et al., 2006).
Discussion

In this study, we sought to identify novel biomarkers of mTBI through the application of the new proteomic technology offered by SOMAscan in our experimental closed head injury (CHI) model of mild brain injury. Following mTBI, cerebral pathophysiology can be adversely affected for days in animals and weeks in humans. The neuropathological mechanisms underlying the short- and long-term consequences of mTBI are largely unknown however, it is generally believed that the injury itself (i.e. the primary injury) induces mechanical damage to neuronal axons and blood vessels which initiates a cascade of neurobiological events leading to secondary injury and, in some cases, long-term cognitive and behavioral impairment.

Standard neuroimaging lacks the necessary sensitivity to detect these changes after mTBI. Further, it is not a practical diagnostic tool in rural or underserved areas or in the acute, pre-hospital setting. Given the relative lack of diagnostic tools for mTBI, there is an urgent need for biomarkers to aid in diagnosis, prognosis, and treatment. Moreover, identification of these novel biomarkers may help to further elucidate the pathophysiology of mTBI.

The ‘ideal’ biomarker for mTBI is (1) unique to the central nervous system and must accurately reflect the extent of brain damage, (2) highly abundant and easily detectable over a wide time window, and (3) able to measure therapeutic efficacy. The majority of research on TBI biomarkers to date has used a hypothesis-driven approach of screening blood and CSF from humans after moderate to severe TBI for previously established markers associated with the pathophysiology of TBI. While this approach has yielded a few candidate biomarkers (i.e. GFAP, UCH-L1, S100B, NF) with high
sensitivity and specificity, it has not been successful in identifying biomarkers of mild injury. This challenge is magnified by the significant heterogeneity in pre-disposing factors and comorbidities associated with mild TBI, which makes it difficult to obtain sample groups and to identify appropriate controls (Berger, 2006; Mondello et al., 2014). As the majority of mTBIs go undiagnosed, it is also challenging to collect blood samples at specified time points post-injury.

In this study, we addressed many of these limitations through the use of an innovative, unbiased proteomics approach to identify candidate biomarkers of mTBI in mice that can be translated and validated in human mTBI. This approach yielded several novel findings. Firstly, we found that levels of two endocrine regulators, IGF-1 and INHBA, were suppressed for the entire 7 day duration of our study, demonstrating that mTBI impairs pituitary and endocrine function. Second, we validated the conclusion of several other studies that GFAP is a good candidate biomarker of mTBI, as it is both specific to the brain and was elevated in injured mice for 7 days after injury. Finally, we identified six new putative biomarkers of mTBI that provide information about the temporal nature of the injury process. Early elevations in Ephrin, PARK7, Pleiotrophin, and NLGNX indicate that neurons and glia are damaged and that the neuroinflammatory response is initiated within the first hour after mTBI, while the prolonged elevation in GFAP levels indicates that the neuroinflammatory response persists for several days post-injury. GOT1 has a temporal profile similar to that of GFAP, suggesting that the neuroinflammatory response to injury is accompanied by metabolic dysfunction and prolonged homeostatic imbalance. Although the function of MFRP is not well characterized, it is intriguing that it is down-regulated for several days post-injury.
Finally, the results of this study indicate that NLGNX is the most promising candidate biomarker of mTBI based on its relevant function in the brain, large fold change above naïve levels, and wide window of detection. Further studies should be conducted to assess its utility and significance in human trials.
CHAPTER V
DISCUSSION

The major findings in this dissertation provide evidence for the therapeutic efficacy of 5-lipoxygenase activating protein (FLAP) inhibitors in attenuating secondary injury and cognitive dysfunction after traumatic brain injury. In Chapter 2, I demonstrated that administration of the FLAP inhibitor, MK-886, after a moderate to severe TBI effectively blocked leukotriene biosynthesis, reduced injury related edema and blood-brain barrier disruption, and attenuated synaptic and behavioral cognitive function. In Chapter 3, I showed that neuroinflammation is initiated within hours of a mild TBI and persists chronically in some brain regions, and this occurs in the absence of positive neuroimaging and blood-brain barrier disruption. Acute administration of the FLAP inhibitor, MK-591, significantly attenuated acute neuroinflammation and corresponding neuronal degeneration and prevented these processes from continuing chronically. In Chapter 4, I detected proteins in the circulating plasma at various time points after mild TBI that reflect the temporal nature of physiological processes in response to brain injury. I identified seven proteins with high specificity in brain tissue and relevant biological functions that should be tested as biomarkers of mild TBI in humans.

Project Summary

Cysteinyl leukotrienes (CysLTs) are potent mediators of inflammation that belong to a family of lipids derived from membrane phospholipid arachidonic acid by the coordinated action of 5-lipoxygenase (5-LO) and the 5-lipoxygenase activating protein (FLAP). Our laboratory was among the first to demonstrate that CysLTs are produced in
the brain by a transcellular mechanism involving peripheral neutrophils and endogenous neurons and/or astrocytes (Farias et al., 2007). As CysLTs are undetectable in the healthy brain, the fluid percussion model (FPI) of experimental TBI was established in our lab to investigate the physiological relevance of CysLT production in the brain. Farias and colleagues (2009) found that CysLTs were detectable within 30 min after injury, peaked at 1-3 hr after injury, and declined to undetectable levels by 24 hr post-injury. Pretreatment with the FLAP inhibitor, MK-886, significantly reduced CysLT production and brain lesion volume. These experiments indicated that the injury-related production of CysLTs contributes to brain damage after TBI.

The first aim of my thesis was to determine the role of leukotrienes in the pathophysiology of TBI and test the hypothesis that blocking leukotriene biosynthesis will attenuate secondary injury and cognitive deficits after TBI. In these experiments, I used the FPI model of TBI and assessed edema, blood-brain barrier disruption (BBBD), synaptic plasticity, and learning and memory. Edema at 72 hrs post-injury was significantly reduced in rats that received MK-886 either 30 minutes before or 15 minutes after FPI. Disruption of the BBB was evident in the cortex and hippocampus of the hemisphere ipsilateral to the injury five hours post-injury, and administration of MK-886 15 minutes post-injury significantly reduced BBBD in the CA1 region of the hippocampus. These findings indicate that blocking the early production of leukotrienes after TBI effectively attenuates brain swelling and BBBD, two of the predominant features of secondary brain injury that are manifestations of an active inflammatory process in the brain. The effect of MK-886 on BBBD is particularly interesting because the CA1 of the hippocampus is relatively distant from the site of injury, suggesting that
increased BBB permeability in this region is due to neurochemical-mediated opening rather than mechanical disruption from the primary injury. I then investigated whether hippocampal function is impaired after FPI. We measured long-term potentiation (LTP) in hippocampal slices four days post-injury as a measure of synaptic plasticity underlying learning and memory function. We found that hippocampal slices from injured rats failed to exhibit LTP upon high frequency stimulation. However, rats that received MK-886 either 30 minutes before or 30 minutes after FPI demonstrated normal LTP, indicating that blocking early leukotriene production attenuates injury-induced deficits in cognition at a cellular level. To verify that injury-induced deficits in LTP reflect impairments in hippocampal-dependent learning and memory, sham and injured rats treated with either vehicle or MK-886 30 minutes post-injury were tested on a radial arms water maze, a spatial memory task that requires rats to find an escape platform submerged under the water in one of six radial arms. There were no differences between groups on the first phase of the test, indicating that they all successfully learned the task and were able to eventually locate the escape platform. However, when I switched the platform to a new arm the injured rats treated with vehicle were unable to locate and learn the new location while the injured rats treated with MK-886 performed just as well as both sham groups, successfully ‘un-learning’ the old platform location and ‘re-learning’ the new location. These results indicate that blocking early leukotriene production attenuates injury-induced deficits in hippocampal-mediated learning and memory.

The results from the first aim have elucidated the detrimental actions of leukotriene biosynthesis after TBI. Leukotrienes are produced rapidly after TBI and contribute to edema, BBBD, and impaired learning and memory function. Inhibiting
FLAP, and thereby blocking leukotriene production after FPI, effectively attenuated these post-injury processes. The early production of leukotrienes after TBI dictates the short therapeutic window for FLAP inhibition. However, the findings from this aim suggest that it is the early activation of neuroinflammatory pathway that sets in motion signaling cascades of secondary damage and that may ultimately lead to impaired neuronal signaling and cognitive function.

A single moderate to severe TBI is associated with increased risk of developing a progressive disorder of cognitive impairment, ultimately leading to dementia like Alzheimer’s disease (Graves et al., 1990; Molgaard et al., 1990; Mortimer et al., 1985; Plassman et al., 2000; Salib and Hillier, 1997). Due, in part, to the recent intense media attention, there is increasing awareness of the link between repetitive mild brain injury and long-term neurological impairment, like post-concussive syndrome, early onset Alzheimer’s disease, and chronic traumatic encephalopathy (CTE). An emerging hypothesis is that neuroinflammation underlies the neurodegenerative pathologies of both single and repetitive TBI. However, very little is known about neuroinflammation after mild TBI.

The second aim of my thesis was to, first, investigate the regional and temporal nature of neuroinflammation after mTBI, and, second, to test the hypothesis that blocking leukotriene production with the FLAP inhibitor, MK-591, will interrupt chronic inflammatory cascades and promote restoration of brain function. For these experiments, I had to establish the closed head injury (CHI) model of experimental TBI, a new experimental model in our lab. Although the ImpactOne device used for these experiments is commercially available, at the time we purchased the instrument there
were only three published manuscripts in which it was used for mTBI in mice. I spent a considerable amount of time researching and pilot testing which injury parameters to use, based on other investigators that have modified their controlled cortical impact (CCI) injury systems, a well-established experimental TBI model, to generate a closed head injury in mice. It was important that the head was stabilized, but somewhat mobile, during the injury as that is almost always the case in human TBI and is thought to be an important biomechanical component of the rotational and shearing forces that lead to diffuse axonal injury.

The first goal of this aim was to assess the severity of secondary injury edema and blood-brain barrier disruption as in Aim 1. Mice underwent MRI imaging at 2, 7, and 30 days post-injury, and the brains of injured mice looked indistinguishable from those of sham mice, compared to the rat brains after FPI that showed a clear lesion, tissue damage, ipsilateral hemispheric swelling and midline shift. Pixel intensity of the injured cortex was analyzed to investigate if there was detectable edema, and the quantitation revealed there were no differences between sham and injured brains at any of the time points. I hypothesized that BBBD after mTBI would not result from the mechanical shearing of blood vessels; rather, inflammation would activate signaling pathways to biochemically increase BBB permeability, leaving the BBB in a ‘leaky’ state. I undertook a more extensive approach than in Aim 1 to analyze the kinetics of BBBD by using three different injectable fluorescent tracers: one cohort of mice injected with Evans Blue (70 kDa) as in Aim 1 and a separate cohort injected with cadaverine (1 kDa), and dextran (70 kDa) tracers. Although the tracers could be visualized in the meninges, the blood vessels, and the choroid plexus, no intraparenchymal tracer extravasation was detected at any if
the time points (0, 1, 4, 8, 24, and 48 hours post-injury). This conclusion that CHI does not result in BBBD was supported by quantitation of blood vessel density and myeloperoxidase (MPO), which revealed that blood vessels remain intact after CHI and that there is no neutrophil infiltration after CHI, respectively.

In human TBI research there is an ongoing initiative to find a circulating biomarker in the serum or plasma that would aid in the diagnosis and management of mTBI. In this study, we measured levels of two of the top candidate human biomarkers, UCH-L1 and GFAP, indicators of neuron and astrocyte damage, respectively. I found that both markers were detected at high levels in the blood as early as 30 min – 1 hour post-injury and returned to baseline levels by 2 hours post-injury, indicating that damage to these cell types is occurring within minutes of TBI, despite no gross pathology or MRI abnormalities.

I next investigated neuroinflammation, characterized by activated microglia and reactive astrocytes, in the cortex, corpus callosum, and dentate gyrus at three different time frames: hyperacute (0-24 hrs post-injury), acute (7 days post-injury), and chronic (30 days post-injury). A significant increase in microglia and astrocyte activation was detected 2-4 hours post-injury and again at 24 hours post-injury in all regions of interest. At 7 days post-injury significant levels of activated microglia and reactive astrocytes were detected in cortex, corpus callosum, and dentate gyrus. The microglial response in corpus callosum, astrocyte response in corpus callosum, and astrocyte response in cortex persisted chronically for at least 30 days post-injury. Administration of the FLAP inhibitor, MK-591, once daily for 7 days after CHI significantly blocked the acute and chronic neuroinflammatory responses in all brain regions. Furthermore, I detected
neuronal degeneration in cortex, corpus callosum, and dentate gyrus at 7 days post-injury, which was blocked by MK-591.

The results of my second aim reveal several important findings. First, neuroinflammation is a key feature of mTBI that is activated within minutes and persists chronically in some brain regions, most notably, the white matter tracts. Second, this inflammatory response is not dependent on the recruitment of peripheral leukocytes and does not require disruption of the blood-brain barrier. Third, leukotrienes are required for the initiation and maintenance of the prolonged neuroinflammatory response after TBI as well as corresponding neuronal degeneration, and blocking injury-induced leukotriene biosynthesis prevents both neuroinflammation and neurodegeneration.

Taken together, the findings from both parts 1 and 2 of my thesis argue for the development of FLAP inhibitors for testing in a clinical trial of human TBI. After decades of clinical trials, there is currently no effective treatment for TBI. This may be attributed to several factors, one of which is the lack of quantitative outcome measures. To aid in the discovery and development of blood-based biomarkers of TBI, we collaborated with SomaLogic, Inc. for the third part of my thesis. This unbiased screen of 1129 proteins in blood samples from injured and uninjured mice at 4 different time points post-injury yielded 7 potential biomarkers that should be tested evaluated in human biomarker screens.

**Neuroinflammation in the Hippocampus**

One notable finding from these studies is that both microglia and astrocytes remained chronically activated in the white matter tracts of the corpus callosum after TBI, and FJB staining indicated that these tracts were damaged at 7 days post-injury.
(Chapter 3). Because there was no positive FJB staining at 30 days, it is unclear whether the damaged neuronal processes were repaired by 30 days or if they were cleared from the brain.

The sudden acceleration-deceleration and/or rotational forces of primary brain injury subject axons to shearing and torsional forces, culminating in diffuse axonal injury (DAI) that is considered a common feature across all severities of TBI. Disruption of the axon cytoskeleton perturbates ionic and metabolic equilibria and activates enzymes involved in cytoskeletal breakdown (Ma, 2013; Tang-Schomer et al., 2010). These processes cause compaction of neurofilaments, impairs axonal transport, and creates axonal swellings and bulbs, which ultimately leads to impaired synaptic transmission, axonal retraction and neurodegeneration, and widespread impairment of neural networks.

One hypothesis for the robust, chronic neuroinflammatory response in the white matter is that myelin debris from injured axons trigger an inflammatory response that causes further axonal injury. Accumulation of myelin debris is a hallmark of multiple sclerosis (MS), and histological examination of post-mortem human MS brain tissue shows that demyelinated axons are surrounded by myelin aggregates and macrophages/microglia with internalized myelin debris (Genain et al., 1999). Another emerging model is that astrocytes selectively tag weak or dysfunctional synapses for phagocytic elimination by microglia, in a mechanism similar to that of synapse elimination during development that utilizes immune system complement cascade signaling (Stephan et al., 2012). In a pilot experiment, we stained brains for complement component 3 (C3), a protein that is required for complement pathway signaling. I detected C3 staining solely in the ipsilateral corpus callosum of injured brains. It is
intriguing to speculate that leukotriene signaling in the white matter regulates neuroinflammation-induced neurodegeneration. The recently deorphanized P2Y-like receptor GPR17 is a cysteinyl leukotriene receptor, exclusively expressed in oligodendrocytes, that negatively regulates oligodendrocyte precursor differentiation and promotes demyelination (Chen et al., 2009; Ciani et al., 2006). Future studies should investigate the role of leukotrienes in diffuse axonal injury, synapse elimination, and neurodegeneration after TBI and explore whether FLAP inhibitors are efficacious in promoting functional repair and recovery of axons and neurons.

**Neuroinflammation and Neurogenesis**

The hippocampus is uniquely vulnerable to injury. In the adult brain, neurogenesis occurs predominantly in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. Newly born neurons in the SGZ integrate into the granule cell layer of the dentate gyrus and send axons into the CA3 region of the hippocampus. Numerous studies have reported that experimental TBI stimulates cell proliferation in the SGZ (Chirumamilla et al., 2002; Dash et al., 2001). However, only a small percentage of these newly born neurons survive and mature (Gao et al., 2008). It has been demonstrated that the inflammatory response encountered by newly born neurons is detrimental for their survival (Ekdahl et al., 2003; Monje et al., 2003). In our studies, activated microglia and reactive astrocytes were detected in the dentate gyrus as early as 2 hours post-injury as well as 7 days post-injury, and this inflammatory response at 7 days was accompanied by neuronal degeneration in the granule cell layer of the dentate gyrus (Chapter 3). In the adult mouse hippocampus it has been shown that newly born neurons take at least 3 months to fully mature and more
than 6 months to functionally integrate into the neuronal network (Toni et al., 2007; Zhao et al., 2006). Although the inflammatory response appears to have resolved by 30 days post-injury after CHI, inflammation-induced death of newborn immature neurons could impair learning and memory in the chronic phase of TBI. Four days after FPI, I detected significant deficits in hippocampal function, at both cellular and behavioral levels that were both attenuated with administration of MK-886 30 minutes after injury (Chapter 2). Future studies involving a more detailed investigation of the inflammatory mechanisms that regulate memory are needed to elucidate the role of injury-induced leukotriene production in cognitive deficits.

Conclusions

There is an undeniable link between brain injury and cognitive impairment, and growing evidence, including these studies, implicates an important role of neuroinflammation in this process. Inflammation is a survival mechanism to protect damaged tissue and is activated immediately upon injury, but if an inflammatory reaction proceeds unimpeded it can trigger chronic inflammation that is detrimental to normal physiological functioning. In the brain, inflammation is regulated by endogenous microglia and astrocytes and can invoke the peripheral immune system if the blood-brain barrier is disrupted. Pro-inflammatory leukotrienes are produced within minutes of a traumatic brain injury and activate inflammatory signaling pathways that induce secondary brain damage and cognitive impairments. There has been 100% failure to identify an effective treatment for TBI. However, a majority of the failed approaches were aimed at preventing neuronal cell death, a process that proceeds and is a result of neuroinflammation. The experiments in this thesis demonstrate that blocking the early
production of leukotrienes with acute administration of a FLAP inhibitor effectively
prevents both acute and chronic brain damage after TBI. FLAP inhibitors represent a
novel anti-inflammatory approach and should therefore be developed for interventional
therapy and prevention of brain deficits after TBI in humans.
REFERENCES


Sports have pervaded my life from a young age. My childhood was spent playing competitive sports and religiously following professional soccer, hockey and football. I have spent countless hours in doctors’ waiting rooms and on the couch icing various injuries, which have inadvertently taught me human anatomy, physiology and biomechanics. I became very familiar with the RICE (rest, ice, compression, elevation) protocol for reducing inflammation and recovering from an injury. However, when my brother, a competitive hockey player, suffered a series of four concussions in two years I became very interested in the notion that an injury to the brain is ‘invisible’ and there is not a standard protocol for recovering from a concussion. Even now, fifteen years later, these are both major problems that plague the neurotrauma research field. Sports-related concussion is a prime example of the challenges that exist in TBI research, and I have gained a comprehensive understanding of several of these issues through my clinical experience as part of the Colorado Clinical and Translational Science Institutes Pre-Doctoral Fellowship Program. My clinical mentor, Dr. Sourav Poddar, is the Head Medical Team Physician for the CU Buffaloes, Director of the Primary Care Sports Medicine Program at The University of Colorado, and a member of the NCAA Special Task Force on Sports-Related Concussion. This program afforded me the opportunity to perform baseline concussion testing with the football players at the beginning of their season and shadow him in the training room.

Student-athletes at CU undergo two different baseline concussion tests, the ImPACT (Immediate Post-Concussion Assessment and Cognitive Testing) test and the
SCAT3 (Sports Concussion Assessment Tool – 3rd Edition), which are then re-administered after a suspected concussion during the season. The ImPACT is a computerized test that begins with a health history and survey of concussion-related symptoms (e.g. headache, mental fogginess). Then participants are tested on visual and verbal memory, processing speed and reaction time. The SCAT3, on the other hand, is administered by qualified examiners includes medical history, symptom evaluation, cognitive assessment (orientation, immediate memory, concentration, and delayed recall), neck examination, balance examination, and coordination examination (Figure 1). In the case of a suspected concussion, additional sections are added to the SCAT3, which include concussion symptom questions, Glasgow Coma Score (GCS), and a Maddocks score of orientation.

I tested football players on the SCAT3 during their summer training camp prior to the start of the season. They were up early in the morning for their first practice, took a break for lunch and baseline concussion testing, and returned to the field for their afternoon practice. I had previously heard about athletes in the NFL admitting that they intentionally perform poorly on baseline tests so that if they get hit in a game and are re-evaluated, it will appear as if there was little deviation from baseline and they can go back on the field. One of the other examiners, their head athletic trainer, made it somewhat of a competition to try to avoid this problem during our testing session. For example, one of the tasks involves reading a list of words (i.e. elbow, apple, carpet, saddle, bubble) and asking the participant to repeat the words in any order. If it appeared that athlete was not trying very hard or was intentionally doing poorly, their performance
BACKGROUND

Name: 
Date: 
Examiner: 
Sport/team/school: 
Date/time of injury: 
Age: 
Gender: M F
Year of education completed: 

Dominant hand: right left neither

How many concussions do you think you have had in the past?
When was the most recent concussion?
When was your recovery from the most recent concussion?
Have you ever been hospitalized or had medical imaging done for a head injury?
Have you ever diagnosed with headaches or migraines?
Do you have learning disability, dyslexia, ADD/ADHD?
Have you ever been diagnosed with depression, anxiety or other psychiatric disorder?
Has anyone in your family ever diagnosed with any of these problems?
Are you on any medication? If yes, please list:

SCAT3 to be done in resting state. Rest 10 or more minutes post exercise.

SYMPTOM EVALUATION

How do you feel?

"...you should score yourself on the following symptoms..."

<table>
<thead>
<tr>
<th>Symptom</th>
<th>None</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Headache</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>&quot;Pressure in head&quot;</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Neck pain</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Nausea or vomiting</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Dizziness</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Blurred vision</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Balance problems</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Sensitivity to light</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Sensitivity to noise</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Feeling slowed down</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Feeling like &quot;as a fog&quot;</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>&quot;Don't feel right&quot;</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Difficulty concentrating</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Difficulty remembering</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Fatigue or low energy</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Confusion</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Drowsiness</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Trouble falling asleep</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>More emotional</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Irritability</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Soreness</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Nervous or anxious</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Total number of symptoms (Maximum possible 28)
Symptom severity score (Maximum possible 9)

Do the symptoms get worse with physical activity?
Y N
Do the symptoms get worse with mental activity?
Y N

self rated
self rated and clinician monitored

Clinician interviewed
Clinician rated with parent input

Overall rating: If you know the athlete well prior to the injury, how different is the athlete acting compared to his/her usual self?

Please rate the response:
no different very different unsure NA

Figure 1. The Sports Concussion Assessment Tool – 3rd Edition (SCAT3).
improved if we told them, “Your teammate before you was able to remember all six words. See if you can get all of them, too.” A majority of the athletes I tested performed very well, and I was pleasantly surprised to see that they took the testing seriously. In talking with the athletes, it seemed that this was primarily due to the fact that they respected their coaches, athletic trainers, and Dr. Poddar. If they were pulled out of a game for a concussion evaluation, they had the understanding that it was warranted and would not reflect badly on them. One player told me a story of a game in the previous season where he ran over to the sideline in the middle of the game to tell the trainers that one of his teammates needed to be evaluated for a concussion because he was not running the correct routes and seemed to have trouble walking and running a straight line. To me this was an indication that the players at CU are well-informed about concussions and take head injuries as seriously as any other injury, like a torn ACL or a pulled hamstring, which is not necessarily the mentality of all college and professional football players.

Shadowing Dr. Poddar in the training room gave me the first-hand exposure to the challenges of monitoring recovery from a concussion and managing their return activity. While athletes are primarily concerned with when they can return to their sport, recovery from a concussion also involves minimizing potential cognitive stressors, such as school work, reading, texting, and watching television. The period of time needed to avoid both physical and cognitive stressors is individualized, and the return-to-learn and return-to-play decisions are based on absence of concussion symptoms following cognitive and physical exposure, respectively. One athlete I observed in the training room with Dr. Poddar was a volleyball player who was hit in the head with the ball during practice and subsequently had concussion symptoms, like difficulty sleeping, headache, and difficulty
concentrating. I saw her two weeks post-injury and many of her symptoms still had not resolved. She had become very frustrated because she was taking a summer course and was unable to go to class, unable to practice with her volleyball team, and unable to even text her friends and watch movies at home. For me, this substantiated the need for both a TBI treatment other than rest and ibuprofen, as well as a more objective way to monitor recovery from a concussion other than prevalence of symptoms. It also highlighted the fact that even athletes in non-contact sports are at risk for concussions and that they can occur in practices as well as games. Another unique case I observed was that of a male cheerleader whom Dr. Poddar was seeing for knee pain. Prior to his appointment he explained to me that he is from Australia and grew up playing rugby. He was forced to give up his dream of playing professionally when, after his sixth concussion, his symptoms never fully resolved and he still has chronic headaches as a result. However, when I made a comment about him having so many concussions in a short period of time, his response was, “Well, I had six ‘official’ concussions, but I’ve had my bell rung hundreds of times.” This mis-conception about concussions is common in collegiate and professional sports, particularly in football where the culture encourages players to fight through any pain and go out of their way to not appear ‘weak’ to their teammates.

In 2013 I attended the Concussion Awareness Summit, a national conference with presentations by scientists, sports medicine physicians, and injury lawyers. The last talk of the day was a panel of professional NFL football players and NHL hockey players moderated by Leigh Steinberg, a sports agent who was the inspiration for the movie “Jerry Maguire”. It was fascinating to hear them speak openly about football, concussions, and the growing concerns about the prevalence of Chronic Traumatic
Encephalopathy (CTE) among NFL players. One retired NFL lineman admitted he learned what a concussion is nearly ten years after he retired, and he is among the players who have sued the NFL for concealing the dangers of concussion and rushing injured players back on the field. However, he also went on to say:

“You might be playing a game in Houston where it’s 100 degrees and 90% humidity—you have all your football pads and helmet on, you’re tired, and you’re dehydrated. When you make a tackle out on the field and get up feeling dizzy and seeing stars for a few seconds your first thought is not that you got a concussion, and the docs don’t really worry about it either. But now I’m learning here today that even your average tackle can be a subconcussive hit to your brain and you might not have any symptoms at all. Do I have CTE developing in my brain right now? I probably do and it keeps me up at night thinking about it.”

Although research is ongoing to characterize the natural history of the disease and detect it in a living brain, the diagnosis of CTE is currently only made post-mortem. Even if PET imaging, for example, could detect CTE in the brain there is still no intervention to slow or stop disease progression. The athlete panel was divided on whether or not they would want to know if they had CTE, some arguing that it would be helpful for their families to know and plan accordingly. Others argued that a CTE diagnosis would significantly impair their quality of life, especially without a clear understanding of when symptoms would appear and without any treatment options. When asked if the players will let their children play football the majority response was no, absolutely not. As a graduate student studying brain injury and a fan of football, hearing this panel discussion
and being able to talk to some of the players afterward was certainly a memorable and noteworthy experience.

The term translational research generally refers to the “bench-to-bedside” approach of harnessing knowledge from basic research to advance the prevention, diagnosis and treatment of disease and ultimately improve the health of patients. Although biomedical research makes important contributions to understanding the cellular, molecular and physiological mechanism underpinning human health and disease, it often falls short of bridging the gap between basic research and clinical practice. A translational approach is absolutely essential for TBI research, and sport-related concussion is a prime example. The average NFL lineman sustains between 900 and 1500 subconcussive hits each season. TBI is considered the strongest environmental risk factor for dementia, and there is growing awareness of the danger of repetitive concussive and subconcussive injuries to the brain. However, an estimated 75% of TBIs go undiagnosed and undetected, and there is currently no effective treatment for mitigating TBI symptoms and preventing chronic behavioral, emotional, and cognitive impairments.

Considering the fact that every single person is at risk for TBI and that every single TBI is unique, developing experimental animal models of TBI have helped to create a reproducible injury that recapitulates many of the features of human TBI. The advent of animal models of mild TBI has allowed laboratories like ours to begin to investigate mild TBI at a molecular and cellular level. It is the findings from these animal TBI studies that have advanced our understanding of the pathophysiology of TBI and have allowed for the development of novel detection and treatment approaches.
The translational and clinical experience has given me the unique opportunity to interact with current and retired athletes, athletic trainers and coaches, sports medicine experts, neurologists and neurosurgeons, pathologists, psychologists, lawyers and other scientists. The knowledge I gained from these experiences was invaluable in the design of all the experiments in my thesis. In setting up the CHI model of mild TBI in our lab and in choosing which post-injury outcomes and time points to investigate, I considered which parameters were most relevant to observations in human TBI and would generate results that would be most ‘translatable’ to improving detection, diagnosis, and treatment for human TBI. FLAP inhibitors and the novel biomarkers identified in my thesis will soon be tested in human studies, and I am hopeful that these studies will yield positive findings that can advance the detection and treatment of TBI.